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FUNCIÓN Y MECANISMO DE ACCIÓN DE LOS ACTIVADORES DEL PLASMINÓGENO EN CÁNCER

Mariano Hurtado Martínez
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Mariano Hurtado Martínez

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Este trabajo experimental ha sido realizado en la Unitat de Recerca Biomèdica de l'Institut de Recerca de l'Hospital Vall d'Hebron, bajo la dirección de la doctora Rosanna Paciucci Barzanti.

El interesado

Mariano Hurtado Martínez

La directora

Rosanna Paciucci Barzanti

El Tutor

Jaume Farrés Vicen

Han pasado casi diez años, casi diez años desde que el verano del 2000 me entrevisté con Rosanna en “la Planta 14” del hospital materno infantil, aún recuerdo lo nervioso que estaba y las ganas que tenía de saber cómo se trabajaría en un laboratorio, cuál sería el tema de investigación en el que me adentraría, que descubrimientos *importantísimos para la humanidad* llevaría a cabo…

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Mariano Hurtado Martínez

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ABREVIATURAS

α_2-AP	α_2-Antiplasmina
α_1-AT.	α_1-Antitripsina
α_2-M	α_2-Macroglobulina
uM	Micromolar
AIIt	Heterotetramero de anexina II y p11
aa	Aminoácido
EACA	Ácido aminocaproico
ADAMs	Proteína metaloproteinasa y desintegrina
AG 1296	Tirfostina inhibidora del PDGFR
AG 1478	Tirfostina inhibidora del EGFR
AHA	Ácido Aminobenzoil-gly-pro-D-ala-hidroxámico
AnnII	Anexina II
APC	Gen de la poliposis adenomatosa familiar
AR	Anfiregulina
ARF	Gen supresor de tumores
Arg	Arginina
AS	Antisense
Asp	Aspartato
ATD	Dominio amino terminal
ATF	Fragmento amino terminal
BMP	Proteína morfogénica del hueso
BSA	Albúmina de suero bovino
BTC	Betacelulina
C. Elegans	Caenorhabditis elegans
c-met	Receptor de HGF
C-ter (terminal)	Extremo carboxi terminal
Cbl	Proteína adaptadora homóloga de Cas NS-1
CCD	Dominio carboxilo terminal del "*core*"
CK	Citoqueratina
CKAP4	Proteína-4 asociada al citoesqueleto
CR1/CR2	Subdominios del EGFR ricos en cisteína
Crk	Proteína adaptadora
CRM 197	Toxina de la difteria mutada, inhibidora de hb-EGF
Cys	Cisteína
$D_{1,2,3}$	Dominios homólogos de uPAR ricos en cisteína
D. Melanogaster	Drosophila melanogaster
DA	Dominio de activación
DFP	Diisopropil fluorofosfato
Dok-R	Proteína adaptadora
DNA	Ácido desoxiribonucleico
DSP	Dominio serina proteasa
EGF	Factor de crecimiento epidérmico
EGFR	Receptor del factor de crecimiento epidérmico
EPI	Epigen
EPR	Epiregulina
Eps 15	Proteína adaptadora
ERK	Quinasa regulada por factores extracelulares
ETS	Factor de trascripción del virus de la eritroblastosis
F	Dominio "*Finger*"
FAK	Quinasa de adhesión focal

FAP	Poliposis adenomatosa familiar
FC	Factor de crecimiento
FDP	Producto de la degradación de la fibrina
FGFb	Factor de crecimiento fibroblástico básico
FGFR	Receptor del factor de crecimiento fibroblástico
Fn	Fibronectina
GDP	Proteína guanosina-5'-bifosfato
Glu-Plg	Plasminógeno con glutamato en el extremo N-terminal
GPCR	Receptor asociado a proteína G
GPI	Glicosilfosfatidilinositol
Grb	Proteína adaptadora
GTP	Proteína guanosina-5'-trifosfato
GTPasa	Enzimas hidrolíticas de la guanosina-5'-trifosfato
hb-EGF	*Heparin Binding* EGF
HC	Homocisteína
HER/c-erbB	Genes del EGFR humano
HGF	Factor de crecimiento hepático
His	Histidina
HLHb	Proteína hélice-"*loop*"-hélice básica
HMW-uPA	uPA de alto peso molecular
HNPCC	Cáncer colorectal hereditario sin poliposis
HP	Dominios homopexina
HPRG	Glicoproteína rica en residuos de prolina e histidina
HT29-M6	Células de cáncer de colon
IFN-α	Interferón autocrino alfa
IFN-β	Interferón autocrino beta
IGF	Factor de crecimiento tipo insulina
IGF-1R	Receptor del factor de crecimiento tipo insulina
Ile	Isoleucina
INK4A	Gen supresor de tumores
IPMN	Neoplasia papilar mucinosa intraductal
IR	Receptor de insulina
JAK2	Janus quinasa
K	Dominios "*kringle*"
Kb	Kilobases
kDa	Kilodalton
K_d	Constante de disociación
K_m	Constante de Michaelis
L1/ L2	Subdominios de EGFR ricos en leucina
LCKLSL	Hexapéptido formada por los aminoácidos: leucina, cisteína, lisina, leucina, serina, leucina
LGKLSL	Hexapéptido formada por los aminoácidos: leucina, glicina, lisina, leucina, serina, leucina
LKB1/STK1	Gen supresor de tumores
LMW-uPA	uPA de bajo peso molecular
Lp A	Lipoproteina a
LPR	Receptor de lipoproteina de baja densidad
Lys	Lisina
Lys-Plg	Plasminógeno con lisina en el extremo N-terminal
MAb	Anticuerpo monoclonal

MAPK	Proteína quinasa activadora de la mitosis
MCN	Neoplasia mucinosa enquistada
MEC (ECM)	Matriz extracelular
Met	Meteonina
ml	Mililitros
MLH1	Gen mutado en el HNPCC
MMP	Metaloproteinasa de matriz extracelular
MSH2/6	Gen mutado en el HNPCC
MSP	Proteína estimuladora de macrófagos
mRNA	Ácido ribonucleico mensajero
MT-MMP	Metaloproteinasa de matriz extracelular unida a la membrana
N-ter (terminal)	Extremo amino terminal
Nck	Proteína adaptadora
NES	Señal de exportación celular
NMDA	N-metil-D-aspartato
NMDAR	Receptor del N-metil-D-aspartato
ng	Nanogramo
nM	Nanomolar
p38	MAPK de 38 kDa que interviene en la proliferación celular
p53	Gen supresor de tumores
PAI	Inhibidor de los activadores del plasminógeno
PANC-1	Línea celular tumoral pancreática
PanIN	Neoplasia intraepitelial pancreática
PA	Activador del plasminógeno
PAR	Receptor activado por proteasa
PC	Péptido conector
PDCA	Adenocarcinoma ductal pancreático
PDGF	Factor de crecimiento plaquetario
PDGFR	Receptor del factor de crecimiento plaquetario
PEX	Dominio homopexina
PI3K	Fosfatidil-inositol 3-quinasa
PKB/AKT	Proteína quinasa B
PKC	Proteína quinasa C
PLC	Fosfolipasa C
Plg	Plasminógeno
Plm	Plasmina
PMA	Éster de forbol 12-miristato 13-acetato
PMS 1/2	Gen mutado en el HNPCC
PMSF	Fenilmetisulfonil fluoride
PN-1	Proteasa nexina 1
PPA	Péptido de preactivación
pro-MMPs	Cimógeno de las MMPs
pro-tPA	Cimógeno del tPA
pro-uPA	Cimógeno del uPA
pro-HGF	Cimógeno del HGF
PTB	Dominio de unión a fosfotirosinas
Pyk	Proteína tirosina quinasa rica en prolina
RA	Ácido retinoico
Raf	Factor activado por ras
Ras	Proteína de secuencia asociada al retrovirus

rfu	Unidades de fluorescencia relativa
RGD	Secuencia aminoacídica de arginina-glicina-aspartato
RNA	Ácido ribonucleico
rtPA	tPA recombinante
S100A4	Metastasina, expresada en diferentes tumores
S100A10/p11	Proteína dimérica de 11 kDa que forma el AIIt junto con AnnII
SC	Segmento de unión al colágeno
sc-tPA	tPA monocadena
sc-uPA	uPA monocadena
Scr	Proteínas ricas en cisteína
Ser	Serina
SF	Factor de *"scatter"*
SH	Dominios homologos de Scr
Shc	Proteína adaptadora que contiene el dominio SH
SK-PC-1	Línea celular tumoral pancreática
SMAD4/DPC4	Gen supresor de tumores
SNC	Sistema nervioso central
SomB	Dominio somatomedina B
SPD	Dominio serina proteasa
Src	Quinasa del virus transformante
suPAR	Forma soluble del uPAR
tc-tPA	tPA de doble cadena
tc-uPA	uPA de doble cadena
TGFα	Factor de crecimiento transformante tipo alfa
TGFβ	Factor de crecimiento transformante tipo beta
TGFBR2	Gen mutado en el HNPCC
TIMP	Inhibidor de metaloproteinasas
TKI	Inhibidor de tirosina quinasa
TM	Dominio transmembrana
TNFα	Factor tumoral de necrosis tipo alfa
tPA	Activador del plasminógeno tisular
tyr	Tirosina
uPA	Activador del plasminógeno tipo uroquinasa
uPAR	Receptor de uPA
UV	Ultravioleta
Val	Valina
VEGF	Factor de crecimiento del endotelio vascular
VEGFR	Receptor del factor de crecimiento del endotelio vascular
Vn	Vitronectina
WNT	Proteína Wingless

CAPÍTULO I: Introducción

El sistema de activación del plasminógeno (Plg) es el mecanismo de proteólisis extracelular más importante, y es fundamental en el mantenimiento del equilibrio hemostático (Dano et al., 1985). Este sistema está compuesto por la proenzima plasminógeno y su forma activa, plasmina (Plm), por serina proteasas activadoras del plasminógeno (PAs) y por receptores de estas proteasas que, junto con los inhibidores del sistema, regulan perfectamente la activación del plasminógeno. (Dano et al., 1985; Saksela and Rifkin, 1988; Vassalli et al., 1991, 1992) (**Figura 1 y 7**).

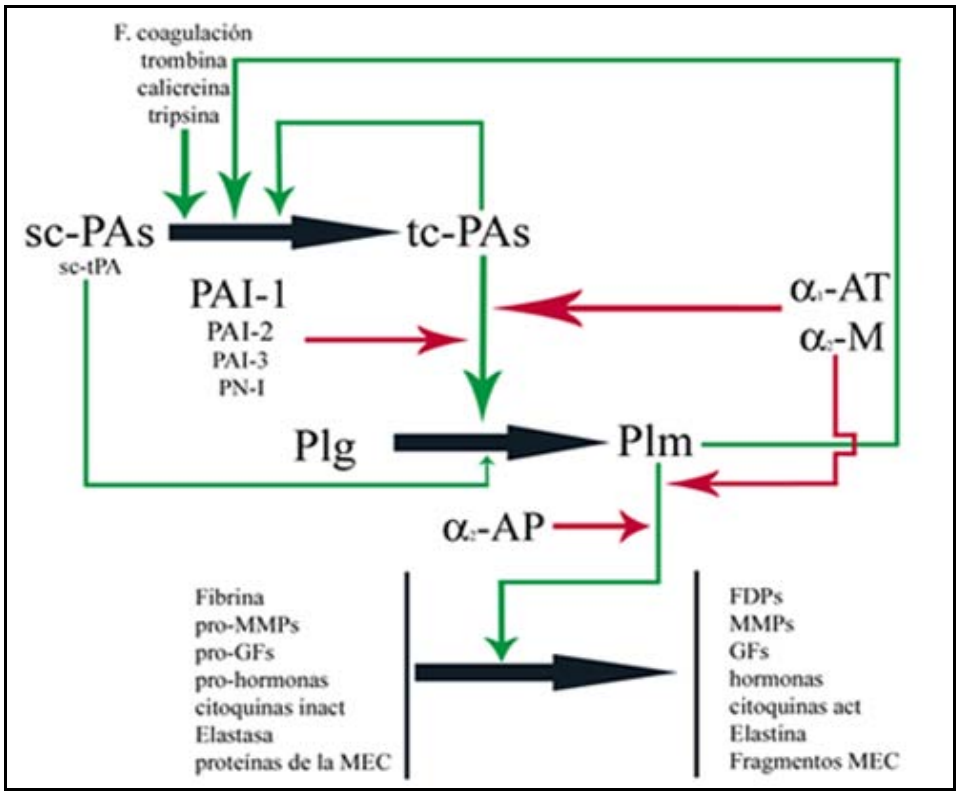

Figura 1. Esquema del sistema de activación del plasminógeno.
El Plg es activado a Plm por tPA (activador del plasminógeno tipo tisular) y uPA (activador del plasminógeno tipo uroquinasa, que a su vez son activados por Plm. La Plm lleva a cabo la activación de diferentes pro-enzimas y factores de crecimiento (FCs), así como la degradación de la matriz extracelular (MEC). El sistema está perfectamente regulado por los inhibidores de los PAs (PAIs), la proteasa nexina I (PN-I) y por la α_2–antiplasmina (α_2–AP) que inhibe la Plm. Los inhibidores inespecíficos α_1–antitripsina (α_1–AT) y α_2–macroglobulina (α_2–M) también intervienen en la regulación del sistema.

El plasminógeno es el cimógeno de la plasmina. Se sintetiza mayoritariamente en el hígado, se secreta al torrente sanguíneo y se distribuye a todos los fluidos y tejidos corporales (Raum et al., 1980; Saksela et al., 1985; Saksela and Rifkin, 1988; Dano et al., 1985). Su forma activa, la plasmina, tiene un papel central en la fibrinólisis. Esta serina proteasa tiene un amplio espectro de substratos, como la fibrina (Collen, 1980), componentes de la matriz extracelular (MEC) y varias pro-enzimas (Pasche et al., 1994;

Liotta et al., 1981; Saksela and Rifkin, 1988; Vassalli et al., 1991; Mignatti and Rifkin, 1993).

La activación del plasminógeno a plasmina es un mecanismo altamente regulado. Esta activación es ejercida por dos PAs, el activador del tipo tisular (tPA) y el activador del tipo uroquinasa (uPA) y es más eficiente cuando tanto estas serina proteasas, como el Plg están anclados a la superficie celular o a la fibrina (Hajjar et al., 1986, 1987; Hall et al., 1990; Ellis et al., 1991). Los dos activadores del plasminógeno son producto de genes diferentes, pero comparten propiedades estructurales y catalíticas. Algunas de las funciones de estas enzimas son complementarias, sin embargo, también intervienen en diferentes procesos del organismo (Collen, 1980; Dano et al., 1985; Carmeliet and Collen, 1995).

Para evitar una proteólisis excesiva y los consecuentes daños tisulares, es necesaria una regulación precisa, temporal y espacial del sistema. Esta regulación es llevada a cabo a través de la interacción de una gran variedad de mecanismos entre los que destacan: la biosíntesis, secreción y posterior activación de los PAs; el efecto de inhibidores específicos de plasmina y de los PAs; la unión de plasminógeno, PAs y sus inhibidores con proteínas de la MEC y/o receptores de la superficie celular; y, por último, una retroalimentación autocrina por la cual plasmina controla la activación de los PAs, de los factores de crecimiento (FCs) y de las MMPs en estado latente (**Figura 7**). Estos FCs intervienen en la expresión de los PAs y los PAIs (Inhibidores de los activadores del plasminógeno).

Los inhibidores que regulan el sistema del plasminógeno, actúan a dos niveles: sobre los PAs y sobre la Plm activa (**Figura 1**). Los inhibidores fisiológicos específicos de los PAs, son los PAI y la proteasa nexina-1 (PN-I) de la superfamilia de las serpinas (Collen, 1986; Sprengers and Kluft, 1987; Kruithof et al., 1995). Los principales inhibidores de la plasmina son la α_2-antiplasmina (α_2-AP) y la α_2-macroglobulina (α_2-M) (Vassalli et al., 1991; Gonias, 1992; Ellis and Dano, 1992; Plow et al., 1995).

Los receptores de uPA, tPA y plasminógeno-plasmina, también intervienen en la regulación de la actividad de este sistema focalizando la proteólisis de plasmina en el espacio pericelular (Appella et al., 1987; Cesarman et al., 1994; Hajjar et al., 1994), limitando la eficacia de los inhibidores del sistema (Pizzo, 1989; Hajjar, 1995; Cunningham et al., 1986; Orth et al., 1992) y participando en su eliminación fisiológica (Cubellis et al., 1990).

El sistema de activación del plasminógeno interviene en la trombólisis, la fibrinólisis y la degradación proteolítica pericelular de una gran cantidad de procesos fisiológicos y patológicos (Dano et al., 1985; Mignatti et al., 1986; Pollanen et al., 1991; Vassalli et al., 1991).

1 Componentes del sistema de activación del plasminógeno.

1.1 Plasminógeno-plasmina.

El plasminógeno es una glicoproteína de 92 kDa, que se sintetiza mayoritariamente en el hígado (Raum et al., 1980; Saksela et al., 1985; Saksela and Rifkin, 1988; Dano et al., 1985), aunque también se expresa en testis (Saksela and Vihko, 1986) y en células epidérmicas (Isseroff and Rifkin, 1983). El plasminógeno está distribuido de forma ubicua, su concentración en plasma es de 2 µM y extravascularmente también presenta niveles elevados (Collen et al., 1972; Lijnen and Collen, 1982; Dano et al., 1985).

El plasminógeno se secreta en forma de Glu-plasminógeno (Glu-Plg) como una única cadena de 791 aminoácidos (aa), 92 kDa, y con una actividad catalítica casi nula, entre 10^4 y 10^6 veces inferior a la de su forma activa, la plasmina (Andreasen et al., 2000). La molécula presenta 24 puentes disulfuro y está compuesta por la cadena pesada A, que contiene el extremo amino-terminal (N-terminal) y por la cadena ligera B situada en la región carboxilo-terminal (C-terminal). En la cadena A se encuentra el péptido de pre-activación del plasminógeno (residuo 1-77), seguido de cinco estructuras en tándem llamadas dominios "*kringle*", que contienen lugares de unión a lisina y del lugar de activación en el que los PAs proteolizan el cimógeno para dar lugar a la plasmina. En la cadena B se encuentra el dominio catalítico con la tríada característica de las serina proteasas (His_{602}, Asp_{645}, Ser_{740}) (Petersen et al., 1990; Ponting et al., 1992) (**Figura 2 y 5**). La plasmina corta el Glu-Plg entre los residuos Arg_{68}-Met_{69}, Lys_{77}-Lys_{78}, Lys_{78}-Val_{79}, y produce un plasminógeno con un residuo de lisina en el extremo N-terminal (Lys-Plg), que se activa más rápidamente a plasmina, tiene un peso molecular inferior (84 kDa) y una vida media más corta (Lijnen and Collen, 1982; Claeys and Vermylen, 1974; Miles et al., 2003).

Las dos formas de plasminógeno (Glu- o Lys-) son activadas proteolíticamente por los PAs en Arg_{560}-Val_{561} (Summaria et al., 1967; Summaria and Robbins, 1976; Collen, 1980; Castellino and Powell, 1981). La elastasa también puede proteolizar el

plasminógeno dando lugar a dos nuevas moléculas: la angiostatina, que contiene 4 dominios "*kringle*" y tiene un efecto antiangiogénico y el miniplasminógeno/miniplasmina, que contiene el dominio "*kringle*" 5 y la cadena B (Dong et al., 1997) (**Figura 3**).

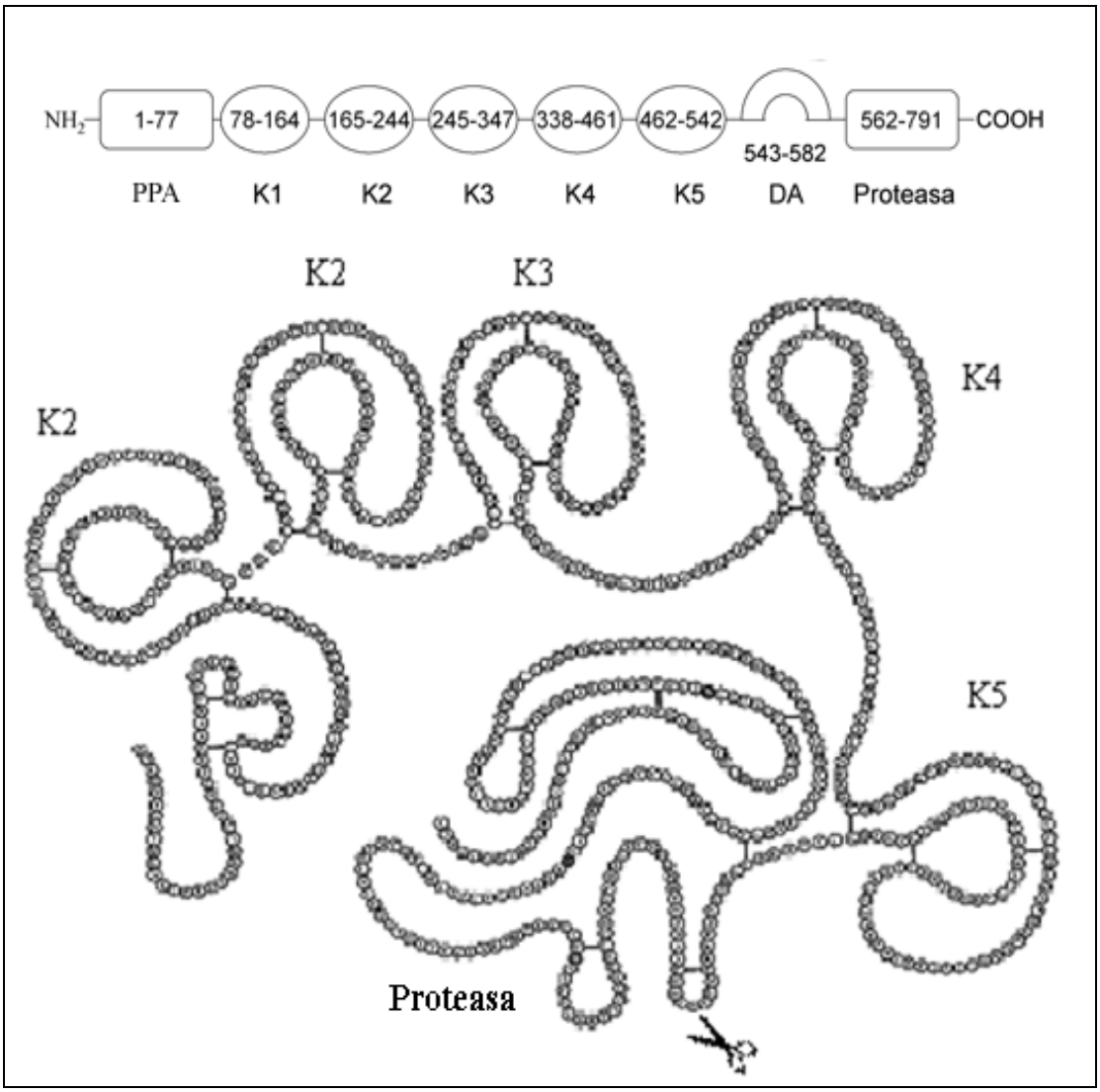

Figura 2. Estructura del plasminógeno.
En la figura se representa la estructura lineal y secundaria del plasminógeno formada por el péptido de pre-activación (PPA), seguido de los 5 dominios "kringle" (K_1-K_5), el dominio de activación (DA), en el que se produce el corte proteolítico que activa la plasmina, y que se solapa con el dominio con actividad serina proteasa.

La plasmina presenta la misma estructura que el plasminógeno, con la diferencia de que sus dos cadenas están unidas por dos puentes disulfuro (Lijnen, 2001) (**Figura 3**).

La plasmina proteoliza la fibrina originando los productos de degradación de la fibrina (FDPs), qué, a su vez, estimulan la acción de tPA, produciéndose así una

retroalimentación positiva que incrementa significativamente la fibrinólisis (Fleury et al., 1991; Wang et al., 1998; Nesheim et al., 1997). La plasmina presenta otros substratos como la glicoproteína IIIa, proteoglicanos, laminina, fibronectina, vitronectina, colágeno tipo IV y elastina (Pasche et al., 1994; Liotta et al., 1981; Saksela and Rifkin, 1988; Vassalli et al., 1991; Mignatti and Rifkin, 1993; Andreasen et al., 1997). La plasmina también puede proteolizar y activar una familia de endopeptidasas dependientes de Zn^{2+}, las metaloproteinasas de la matriz extracelular (MMPs) (He et al., 1989; Werb et al., 1997; Nagase, 1997; Mazzieri et al., 1997) (**Figura 1**).

La plasmina también puede proteolizar y activar factores de crecimiento asociados al crecimiento tumoral como el factor de crecimiento transformante tipo β (TGFβ) (Lyons et al., 1988; Odekon L. E., 1994) y el factor de crecimiento fibroblástico básico (FGFb) (Brunner et al., 1991) (**Figura 1**). Puede degradar receptores de membrana como el receptor de trombina (Turner et al., 1994) y el receptor tipo II TGFβ/betaglicano (LaMarre et al., 1994), además de otras proteínas como la proteína de unión al factor de crecimiento tipo insulina (IGF-1) (Campbell et al., 1992) y el interferón γ (Gonias et al., 1989 b).

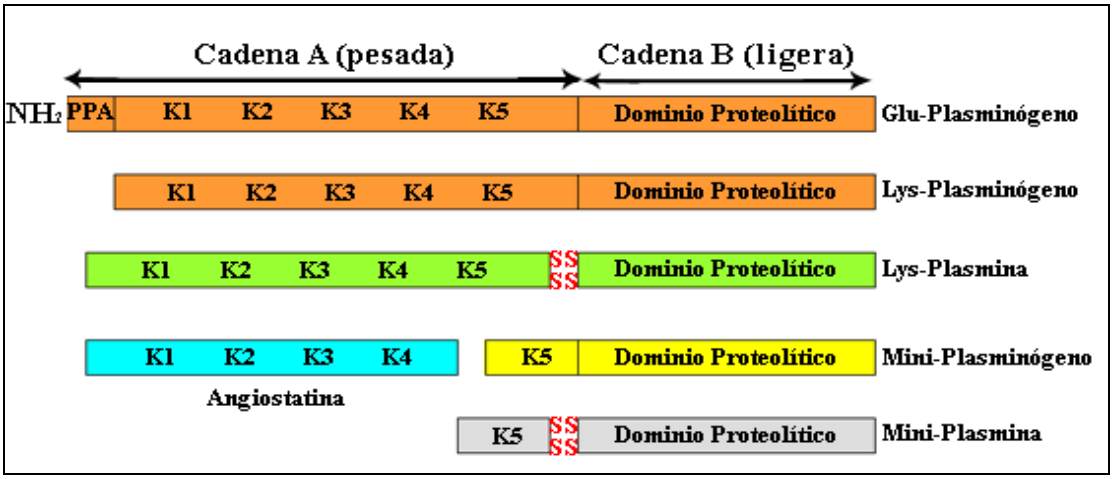

Figura 3. Productos de la proteólisis del plasminógeno.
El Lys-plasminógeno se genera a partir de la acción de la plasmina sobre el péptido de pre-activación (PPA) del Glu-plasminógeno. Los PAs catalizan la activación de Lys-plasminógeno y Mini-plsminógeno a Lys-plasmina y Mini-plasmina, respectivamente. Por último, la elastasa proteoliza el Lys-plasminógeno, creando 2 moléculas: la Angiostatina con 4 dominios "*kringle*" y el Mini-plasminógeno con el dominio "*kringle*" 5 y la cadena B del plasminógeno.

1.1.1 Receptores de plasminógeno y plasmina.

El Plg puede unirse a determinados componentes de la MEC como gangliósidos (Miles et al., 1989), glicosaminoglicanos (Stack et al., 1990) y proteínas como la

laminina (Salonen et al., 1984), la fibronectina (Fn) (Salonen et al., 1985), la trombospondina (Silverstein et al., 1985) y la vitronectina (Vn) (Preissner, 1990; Kost et al., 1992). El plasminógeno también puede unirse a proteínas disueltas en el plasma como la fibrina (Lucas et al., 1983), la glicoproteína rica en residuos de prolina e histidina (HPRG) (Lijnen et al., 1980; Silverstein et al., 1985; Borza and Morgan, 1997, 1998), la tetranectina (Clemmensen I., 1986) y la heparina (Soeda et al., 1989).

La mayoría de estas uniones no son covalentes e involucran los dominios "*kringle*", (mayoritariamente k1, k4 y k5), con residuos de lisina situados en el extremo C-terminal de la proteína a la que se une (Christensen et al., 1985, Miles et al., 1988; Fleury et al., 1991; Ponting et al., 1992). Estas interacciones pueden ser bloqueadas por análogos de lisina como el ácido ε-aminocaproico (εACA) (Urano et al., 1987; Hajjar et al., 1986; Gonias et al., 1989 a) o evitadas por el efecto de las carboxipeptidasas (Felez, 1998). El Plg también puede unirse a ciertas proteínas de manera independiente a los residuos de lisina, pero estas uniones no intervienen en la estimulación del Plg unido a las superficies celulares (Felez et al., 1996).

La unión de Plg a los diferentes componentes de la membrana celular es típicamente de baja afinidad pero de alta capacidad. Esta unión provoca un cambio conformacional en el Plg, que facilita su posterior activación (Markus et al., 1979; Urano et al., 1987; Mangel et al., 1990; Pointing et al., 1992). Esto sucede solo con el Glu-Plg ya que el Lys-Plg tiene una conformación que facilita de "*per se*" su activación.

Además de afectar a la cinética de activación del plasminógeno, su unión a proteínas de la membrana celular dificulta la acción de sus inhibidores (Ellis and Dano, 1992; Gonias 1992).

Se han descrito receptores específicos de la membrana celular para el Plg que promueven la acción de los PAs, disminuyendo la K_m de la reacción de activación (Hajjar et al., 1986; Plow et al., 1995). Esto se debe principalmente a la actuación de la superficie celular como soporte que favorece el contacto entre Plg y PAs, facilitando así su activación a Plm (Gonias et al., 2001). Una vez unida al receptor de membrana, la Plm queda protegida de la inactivación de sus inhibidores (Tapiovaara et al., 1996). La Plm se une a los mismos receptores que el Plg pero con mayor afinidad (Durliat et al., 1991; Burtin and Fondaneche, 1988). Estos receptores de Plg-Plm varían en función del tipo celular.

Se han descrito diferentes receptores de Plg:

- Anexina II (AnnII) expresado en la superficie celular de células endoteliales y macrófagos. Este receptor une las dos formas de plasminógeno (Lys- y Glu-) (Hajjar, 1995). La unión de Plg a AnnII necesita un corte proteolítico previo del receptor, entre los residuos Lys_{307}-Arg_{308}, creando así un extremo C-terminal con lisina que favorece su unión. Esta proteólisis es llevada a cabo por una serina proteasa tipo plasmina, (Hajjar et al., 1994; Cesarman et al., 1994). Tanto la Lipoproteína A (Lp A), con homología con los motivos "*kringle*" del plasminógeno, como el εACA inhiben esta unión de Plg a la AnnII (Hajjar, 1991) (**Figura 5**). AnnII funciona también como receptor para tPA, pero no para uPA, en células endoteliales y en células de cáncer de páncreas (Hajjar and Hamel, 1990; Hajjar, 1991). (*ver apartado 1.2.1.1*).
- S100A10 que junto con AnnII forma heterotetrámeros (AIIt) (Kassam et al., 1998; Fitzpatrick et al., 2000). Tiene una lisina en su extremo C-terminal a la que se une el Plg. Al igual que AnnII también puede unir tPA (*ver apartado 1.2.1.2*).
- α-enolasa se expresa en monocitos (Miles et al., 1991; Redlitz et al., 1995), en células de carcinoma humano (Lopez-Alemany et al., 1994), células neuronales (Nakajima et al., 1994), células hematopoyéticas, endoteliales y epiteliales (Pancholi, 2001; Lopez-Alemany et al., 2003). Esta enzima glicolítica interviene en varios procesos celulares como receptor del Plg entre los que destaca la miogénesis y la reparación muscular (Suelves et al., 2002; Lopez-Alemany et al., 2003, 2005). Se ha descrito que la unión del Plg a este receptor puede darse por la presencia de residuos de lisina en su extremo C-terminal (Miles et al., 1991) o por la unión del cimógeno a un motivo interno formado por nueve residuos (Ehinger et al., 2004).
- Anfoterina expresada en células de neuroblastoma y del sistema nervioso central (SNC). No presenta lisina en el extremo C-terminal, y a diferencia de la anexina II, la unión de esta proteína al plasminógeno no es reversible ni saturable. También posee regiones de unión a tPA (Parkkinen and Rauvala, 1991) (*ver apartado 1.2.1.4*).
- Citoqueratina 8 (CK8) expresada en hepatocitos (Hembrough et al., 1995), en células epiteliales de cáncer de mama (Correc et al., 1990; Hembrough et al.,

1995, 1996 a, b), y en células de carcinoma hepatocelular (Hembrough et al., 1996 a). Este miembro de la familia de los filamentos intermedios del citoesqueleto, presenta una lisina en el extremo C-terminal a la que puede unirse el plasminógeno. tPA también puede unirse a este receptor (*ver apartado 1.2.1.3*).

El Plg puede unirse a otras muchas proteínas como integrinas (Miles and Plow, 1985; Tarui et al., 2002), actina (Lind and Smith., 1991; Dudani and Ganz, 1996; Kwon et al., 2005) y glicoproteínas (Miles et al., 1986; Gonzales-Gronow et al., 1994). A pesar de ésto, sólo se consideran receptores que regulan la actividad de este cimógeno, las proteínas que unen Plg con alta afinidad, provocan el cambio conformacional de Glu- a Lys-Plg, protegen la Plm de sus inhibidores, y pueden unir los PAs.

Para la eliminación fisiológica, se han descrito otros receptores de la Plm, que actúan cuando ésta se une a un inhibidor. Un ejemplo de estos receptores son las serpinas (Inhibidores de serina proteasas) que unen el complejo Plm/α_2-AP eliminándolo (Pizzo, 1989); el receptor de lipoproteínas de baja densidad (LPR) que elimina el complejo Plm/α_2-M (Hajjar, 1995) y el complejo PAI-1/uPA/uPAR (receptor de uPA) (*ver apartado1.3.1*) y por último, el receptor de la nexina que elimina el complejo Plm/proteasa (Cunningham et al., 1986).

1.2 El activador tisular del plasminógeno

tPA es una glicoproteína de 68-70 kDa, producida por células endoteliales (Levin and Zoppo, 1994; Levin et al., 1997), por células de tejidos uterinos, células del sistema nervioso central, queratinocitos y melanocitos (Chen et al., 1993; Bizik, 1996; Teesalu, 2002). Esta proteína se encuentra en la mayoría de fluidos corporales (Rijken et al., 1979; Dano et al., 1985). Su concentración en plasma es de 5-7 ng/ml y está unida en un 95% a PAI-1 (Rijken et al., 1983 a, b). Esta serina proteasa también se produce en diferentes células neoplásicas, como las de melanoma, neuroblastoma, cáncer de ovario, de mama y de páncreas (Rijken and Collen, 1981; Neuman et al., 1989; Amin et al., 1987; Paciucci et al., 1998 a).

El gen de *tPA* está formado por 14 exones a lo largo de 20 Kb en el cromosoma 8 (Mullins and Rothrlich, 1983). Se han descrito dos orígenes de transcripción: un lugar de inicio mayoritario que es independiente de la caja TATA, y el minoritario, dependiente de ésta. La expresión del gen está regulada por diferentes factores de transcripción (Sp1, NF1, CREB), hormonas gonadotrópicas, ésteres de forbol, cAMP,

ácido retinoico (RA) y dexametasona (Irigoyen et al., 1999). Bulens y colaboradores identificaron una región "*enhancer*" situada 7.1 Kb "*upstream*" del promotor que se activa por glucocorticoides, progesterona, mineralocorticoides, andrógenos y RA (Bulens et al., 1995, 1997). Una vez sintetizado, este gen se transcribe dando lugar a un mRNA≅2.7 Kb que codificará tPA (Pennica et al., 1983; Fisher et al., 1985).

tPA se secreta como precursor monocadena (sc-tPA) de 527 aa, con diferentes grados de glucosilación (Rijken and Collen, 1981; Pennica et al., 1983; Wallen et al., 1983; Andreasen et al., 1984). Esta forma sc-tPA se activa por proteólisis limitada, entre los residuos Arg_{275}-Ile_{276}, dando lugar a la forma activa de tPA (tc-tPA), formada por dos cadenas unidas por un puente disulfuro (Ranby et al., 1982; Wallen et al., 1982; Rijken et al., 1982). Plasmina, calicreína tisular y el factor Xa de coagulación, pueden llevar a cabo esta proteólisis (Ichinoise et al., 1984). A diferencia de uPA, ambas formas de tPA tienen actividad catalítica, siendo tc-tPA entre 10-20 veces más activa que sc-tPA, por lo que tPA no es un verdadero cimógeno (Nienaber et al., 1992; Tachias and Madison, 1996).

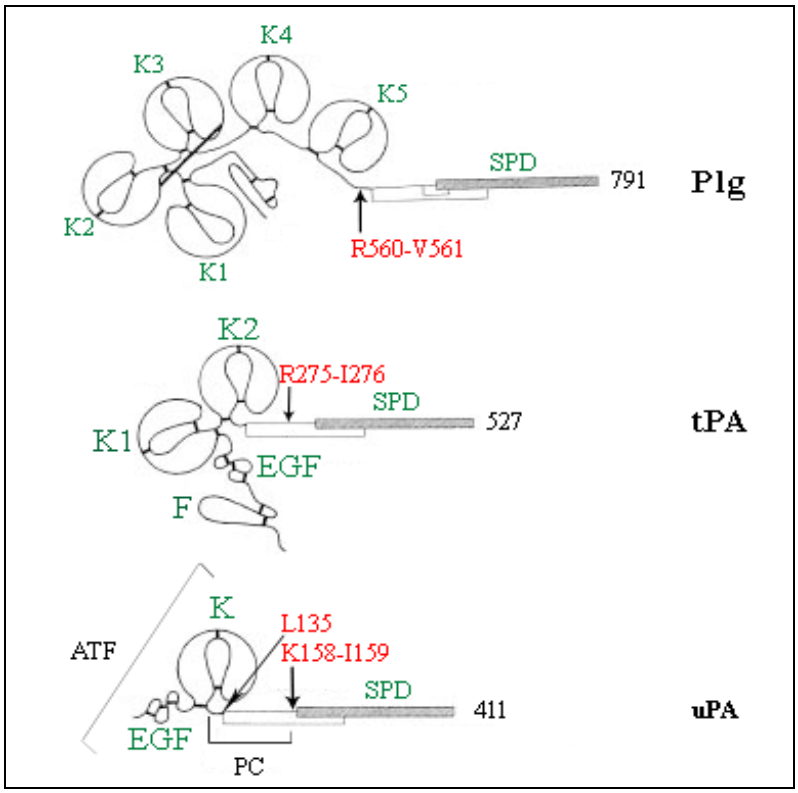

Figura 4. Dominios estructurales del Plasminógeno y los activadores del plasminógeno .
Tanto Plg como los dos PAs presentan dominios "kringle" (K). Los PAs presentan además un dominio EGF like (EGF), que junto con el "kringle" forma el ATF (fragmento amino terminal) en uPA. tPA presenta además un dominio "finger" (F), y uPA un péptido conector (PC).
En rojo se evidencian los residuos entre los cuáles se produce la activación proteolítica de estos cimógenos. La lisina 135 del uPA es el residuo en el que la plasmina corta el uPA (HMW-uPA) formándose el LMW-uPA. SPD (dominio serina proteasa)

La estructura de tPA es similar a la del plasminógeno. Se compone de dos cadenas, la cadena pesada A (36 kDa) situada en el extremo N-terminal y la cadena

ligera B (32 kDa) que contiene el extremo C-terminal. La cadena A contiene un dominio conocido como tipo fibronectina, o dominio "*finger*" de 47 residuos (4-50), seguido de un dominio homólogo al EGF (factor de crecimiento epidérmico) (residuos 50-87) y de dos dominios "*kringle*", similares a los del plasminógeno (residuos 87-262). La cadena B contiene el dominio serina proteasa de 252 residuos (276-527), en el que se encuentra la tríada catalítica His_{322}-Asp_{371}-Ser_{478} (Strassburger et al., 1983; Pennica et al., 1983) (**Figura 4**).

Los dominios de la cadena A modulan la actividad de esta serina proteasa. El dominio "*kringle*" 2 y en menor grado los dominios tipo EGF y "*finge*r" son responsables de la afinidad de tPA por la fibrina y de su ulterior activación (Van Zonneveld et al., 1986 a, b; Gething et al., 1988; de Vries et al., 1990; Bizik et al., 1997; Collen, 1999). Por otro lado, los dominios "*finger*" y tipo EGF son los responsables de la eliminación de tPA en hepatocitos (Kuiper et al., 1995).

La eficiencia catalítica de tPA incrementa cuando está unido a fibrina o a anexina II, esto se debe a que estas proteínas facilitan y estabilizan la unión entre tPA y Plg, formándose así un complejo ternario en el que la eficiencia catalítica de esta proteasa es mucho mayor que en solución (Hoylaerts et al., 1982; Hajjar et al., 1987). Otras moléculas de la MEC como colágeno tipo IV, laminina-1, fibronectina y trombospondina actúan como cofactores no esenciales de tPA incrementando su actividad catalítica (Salonen et al., 1984, 1985; Silverstein et al., 1986; Stack et al., 1990; Moser et al., 1993). El aumento pericelular de plasmina, por el incremento de la actividad de tPA focalizado por su unión a receptores de membrana, facilita la proteólisis de las proteínas de la MEC, necesaria para la migración y la invasión (Mignatti and Rifkin, 1993).

1.2.1 Receptores de tPA.

De manera similar al Plg, tPA interacciona con diferentes proteínas en la membrana celular. Se han encontrado receptores para tPA en hepatocitos (Bakhit et al., 1987), en células de hepatoma (Morton et al., 1990; Bu et al., 1992 b) en fibroblastos (Reilly et al., 1989), en plaquetas (Gao et al., 1990), en células de músculo liso (Werner et al., 1999), en células mamarias (Verrall and Seeds, 1989), en células endoteliales (Hajjar et al., 1987; Beebe, 1987), en células embrionarias (Carroll et al., 1993), monocíticas (Felez et al., 1991; Otter et al., 1991), epiteliales (Lind and Smith., 1991), así como en neuronas y en células de neuroblastoma (Parkkinen and Rauvala, 1991).

1.2.1.1 Anexina II.

En células endoteliales tPA se une de manera específica, saturable y de forma dependiente de Ca^{2+} a dos lugares: uno de alta afinidad, presente en muy baja cantidad, y otro de menor afinidad, pero altamente representado, que se ha considerado, el sitio mayoritario para la unión de tPA (Hajjar et al., 1987; Barnathan et al., 1988). Posteriormente, se identificó anexina II como proteína responsable de la unión reversible de tPA a estas células (Hajjar et al., 1994). La unión de tPA y Plg a anexina II (*ver apartado 1.1.1*), forma un complejo ternario que promueve la generación de plasmina activa en la superficie celular (Hajjar and Nachman, 1988; Hajjar and Hamel, 1990; Hajjar, 1991; Cesarman et al., 1994), análogamente a lo que sucede con la fibrina (Collen and Lijnen, 1994). La unión entre tPA y anexina II es Ca^{2+} dependiente y se da entre el dominio "*finger*" de tPA (Beebe et al., 1989) y el hexapéptido LCKLSL del extremo N-terminal de anexina II (Hajjar et al., 1996). El residuo esencial para esta interacción es la Cys_9, lo que explica que la homocisteína bloquee la interacción entre tPA y AnnII (Hajjar, 1993, Hajjar et al., 1998) (**Figura 5**). La función de AnnII como receptor para Plg-tPA se ha descrito también en células monocíticas (Falcone et al; 2001) y en células de adenocarcinoma pancreático (Vishwanatha et al., 1993; Díaz et al., 2004).

AnnII pertenece a una superfamilia de proteínas unidas a fosfolípidos aniónicos de forma calcio-dependiente (Swairjo and Seaton, 1994; Gerke y Moss, 2002). Se han descrito más de veinte proteínas de esta familia, presentes en todos los tipos celulares de mamífero, excepto en eritrocitos y ampliamente distribuidas en todos los organismos excepto virus, procariotas y levaduras. Esta familia de proteínas está implicada en diferentes funciones como la exocitosis, la endocitosis, la formación de canales iónicos, la inhibición de la actividad de la fosfolipasa A_2 y las interacciones célula-célula y célula-MEC. Algunos miembros de esta familia están implicados en procesos de diferenciación celular, proliferación y mitogénesis (Kwon et al., 2005).

Anexina II es una proteína de 36 kDa, codificada por el gen *ANXA2*, sobreexpresado en la mayoría de tumores como: el carcinoma hepatocelular (Frohlich et al., 1990), el adenocarcinoma pancreático (Vishwanatha et al., 1993; Paciucci et al., 1998 a), el glioma de alto grado (Reeves et al., 1992), el carcinoma gástrico (Emoto et al., 2001 a) y la leucemia promielocítica aguda (Menell et al., 1999). La expresión de *ANXA2* es estimulada por insulina, FGF y EGF (Keutzer and Hirschhorn, 1990) e

inhibida por el ácido retinoico (Menell et al., 1999). Una vez sintetizada, AnnII es translocada a la membrana celular de manera dependiente de la expresión de S100A10 y de su fosforilación en la tirosina 23 (Deora et al., 2004). AnnII está unida a la superficie celular por interacciones con fosfolípidos de membrana, como fosfatidilserina, dependientes de Ca^{2+} (Hajjar et al., 1994, 1996). En esta proteína se distinguen el dominio amino terminal (ATD) de treinta residuos (1-30) y el dominio carboxilo terminal del "*core*" (CCD) que va del residuo 31 al 338. El dominio ATD, presenta tres lugares de fosforilación (Ser-11, Tyr-23 y Ser-25), el lugar de unión al dímero S100A10 y una señal de exportación nuclear (NES) esencial para transportar la proteína del núcleo al citosol (Liu et al., 2003 a). El dominio CCD, contiene las regiones de unión a Ca^{2+}, a F-actina (Filipenko and Waisman, 2001), fibrina (Choi et al., 2001), heparina (Kassam et al., 1997) y al RNA (Filipenko et al., 2004).

AnnII puede presentarse como monómero, heterodímero, heterotetrámero con la proteína S100A10 (AIIt) (Waisman, 1995) y según estudios recientes, como un complejo octamérico también con p11 (Schulz et al., 2007). El heterodímero está formado por una subunidad de AnnII unida a una subunidad de 3-fosfoglicerato quinasa (Jindal et al., 1991). El heterotetrámero está formado por dos moléculas de AnnII unidas a dos proteínas S100A10 (**Figura 5**). La proteína S100A10, también llamada p11, es un miembro de la familia de proteínas de unión a Ca^{2+} (*ver apartado 1.2.1.2*). La unión de AnnII a S100A10 que se da en la forma tetramérica es irreversible. La cantidad de la forma AIIt, respecto a la AnnII monomérica depende del tejido examinado, de la síntesis de AnnII respecto a S100A10 y de las modificaciones post-traduccionales que puden impedir su unión (Johnsson et al., 1986). Esto hace que, por ejemplo, la proporción de AIIt/AnnII sea del 100% en células del epitelio intestinal y del 50% en fibroblastos (Gerke and Weber, 1984; Glenney et al., 1985).

En células endoteliales tanto AIIt, como AnnII, favorecen la activación de plasmina de forma Ca^{2+} dependiente, y esta activación se inhibe con εACA (Kassam et al., 1998). El complejo AIIt tiene una capacidad de activación de plasminógeno mayor que AnnII. AIIt estimula la activación de Glu-Plg a Lys-Plg 341 veces cuando el cimógeno está unido, mientras que la forma monomérica solo estimula esta activación 6 veces. Con respecto a tPA en solución, la eficiencia catalítica de tPA/AIIt por Glu-Plg y por Lys-Plg aumenta 90 y 28 veces, respectivamente, comparado con el aumento de tPA/AnnII (21 y 14 veces, respectivamente) (Cesarman et al., 1994; Kassam et al., 1998). AIIt puede unirse a procatepsina B (Mai et al., 2000 a) y a proteínas de la MEC

como colágeno I y tenascina-c, en células tumorales (Chung et al., 1996; Emoto et al., 2001 b). Esta colocalización de proteasas y sus substratos en la superficie celular unidos a AIIt, activa cascadas proteolíticas y la degradación selectiva de proteínas de la MEC incrementándose así la motilidad, la migración y la invasión de células tumorales (Chung et al., 1996; Mai et al., 2000 b). AnnII puede interaccionar también con otra proteína de la familia de las S100, la S100A4, también conocida como metastasina, que se expresa en diferentes tumores como el de mama, el escamoso de esófago, el carcinoma de colon y el adenocarcinoma pancreático, y que al igual que S100A10, unida a AnnII, une tPA y estimula la activación de plasmina en células endoteliales (Semov et al. 2005).

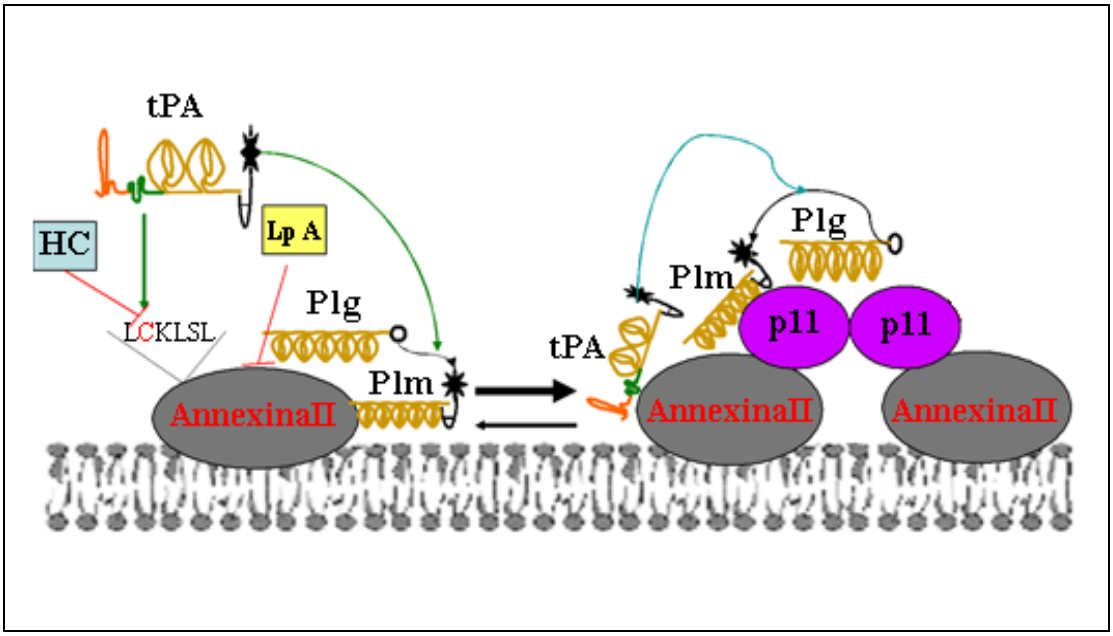

Figura 5. Modelo de la unión de tPA a Anexina II y al heterotetrámero AIIt (2 AnnII y 2 p11). La unión de tPA a Anexina II se da entre el dominio "finger" de la proteasa y la cisteína del hexapéptido LCKLSL de la Anexina II. Esta unión puede ser bloqueada por homocisteína (HC). La unión del Plg se da entre sus dominios "kringle" y la lisina del extremo C-terminal de la Anexina II, pudiendo ser inhibida por Lp A. En el AIIt, tPA se une directamente con la Anexina II, mientras que el Plg se une al extremo C-terminal de p11, dándose una activación de Plg a Plm más efectiva.

AnnII es substrato de proteínas con actividad tirosina quinasa. Algunas de estas proteínas son receptores que fosforilan la AnnII cuando se les une su ligando como, por ejemplo, el receptor de insulina (IR), que se internaliza con la ayuda de AnnII (Biener et al., 1996) o como el receptor del factor de crecimiento plaquetario (PDGFR) (Rothhut, 1997). Cuando AnnII es fosforilada en Tyr23, se rompe el tetrámero AIIt, (Johnsson et al., 1986), mientras que al ser fosforilada en Ser11 y Ser25, AnnII entra en el núcleo (Liu et al., 2003 a).

1.2.1.2 S100A10 (p11).

S100A10 es una proteína dimérica compuesta de dos subunidades de 11 kDa, miembro de la familia de las proteínas S100, que se caracterizan porque presentan dos dominios de unión a Ca^{2+} tipo "*EF-hand*". Esta familia de proteínas se encuentran en el núcleo, en el citoplasma y en la superficie de una gran variedad de células, interviniendo en procesos de progresión y diferenciación celular (Donato, 2001). S100A10 tiene una lisina en el extremo C-terminal a la que se unen tPA y Plg (*ver apartado 1.1.1)*, estimulándose así la producción de Plm, que a su vez se une a esta proteína en otra región provocando su autoproteólisis (Kwon et al., 2005).

Como se ha comentado en el apartado anterior, S100A10 se une a AnnII. Esta unión es Ca^{2+} independiente, y dirige el complejo a la membrana celular, donde se da la interacción de AnnII con fosfolípidos de membrana (Zobiack et al., 2001). Se ha descrito que AnnII, regula la expresión de S100A10 mediante un mecanismo post-traduccional desconocido hasta el momento (Chetcuti et al., 2001; Puisieux et al., 1996).

El grupo de Waisman propone S100A10, y no AnnII, como receptor de tPA y Plg. Según esta hipótesis, AnnII sería necesaria i) para transportar y ancorar S100A10 a la membrana, formando el heterotetrámero AIIt y ii) para controlar su expresión (Kwon et al., 2005).

S100A10 participa además en la regulación de la fosfolipasa A, de la catepsina B, y de los canales de Ca^{2+} (Kwon et al., 2005).

1.2.1.3 Citoqueratinas 8 y 18 (CK18).

Estas proteínas se unen formando heterodímeros y polimerizan, formando parte de los componentes del citoesqueleto (Hatzfeld and Weber, 1990). CK8 puede estar presente en el interior de la célula o en la superficie de células tumorales donde puede unir diferentes proteínas como el plasminógeno (*ver apartado 1.1.1*).

Se ha descrito que ambas, CK8 y CK18, unen tPA, a diferencia de Plg que solo se une a CK8. tPA y Plg se unen al mismo sitio en CK8 y para activar la plasmina, CK8 debe formar un homodímero o un heterodímero con CK18 (Kralovich et al., 1998).

Estas citoqueratinas están sobreexpresadas en diversos tipos de tumores invasivos (Schaafsma et al., 1991 y 1993; Hendrix et al., 1992).

1.2.1.4 Anfoterina.

La anfoterina es una proteína a la que se une heparina. La anfoterina se sobreexpresa durante el desarrollo del cerebro (Rauvala and Pihlaskari, 1987; Merenmies et al., 1991). Esta proteína se localiza específicamente en los filopodios de células neurales e interviene en el crecimiento de neuritas (Merenmies et al., 1991) y en las interacciones entre neuronas y células de la glía (Daston and Ratner, 1991).

En células de neuroblastoma, la anfoterina interacciona con los dominios "*kringle*" de tPA por lo que esta unión se inhibe con εACA, tal y como sucede con el Plg (*ver apartado 1.1.1)* (Parkkinen and Rauvala, 1991). Se ha descrito la intervención de este receptor en procesos tumorales como migración celular, metástasis e invasión celular (Taguchi et al., 2000).

1.2.1.5 Receptores de eliminación fisiológica.

La eliminación fisiológica de tPA ocurre en el hígado (Nilsson et al., 1984). En células hepáticas se ha demostrado la unión de tPA a LPR, en presencia de PAI-1 (Orth et al., 1992) o sin PAI-1 (Bu et al., 1992 a). Una vez unido tPA a LPR, el complejo es endocitado y degradado en los lisosomas, reciclándose posteriormente el receptor (Bu et al., 1992 a). También se ha descrito que la α-fucosa elimina tPA en células de hepatoma (Hajjar and Reynolds, 1994) y el receptor de manosas-6-fosfato hace lo propio en macrófagos (Otter et al., 1991).

1.3 El activador del plasminógeno uroquinasa

La uroquinasa es una glicoproteína de 411 aminoácidos y con una masa molecular de 53 kDa (Lesuk et al., 1965; Wun et al., 1982 a, b). Esta serina proteasa se encuentra en orina, en semen y en plasma, donde su concentración es 2-20 ng/ml (Dano et al., 1985). uPA se secreta en diferentes tipos de cáncer: mama, colon, ovario, gástrico, cérvix, endometrio, vejiga, riñón y cerebro, donde es un marcador de mal pronóstico (Andreasen et al., 1997). Sólo en cáncer de colon se ha demostrado, que son las células tumorales las que secretan la proteasa (Harvey et al., 1999).

El gen *uPA* humano tiene 5.7-6.4 Kb, 11 exones y se encuentra en el cromosoma 10 (Rajput, 1985). El promotor de este gen está regulado por factores de transcripción como SP1 y CTF, factores de crecimiento, hormonas peptídicas, hormonas esteroideas, luz ultravioleta (UV) y el éster de forbol (PMA) entre otros (Dano et al., 1985; Rorth et

al., 1990; Nagamine et al., 1995; Stacey et al., 1995; Besser et al., 1995, 1996). Estos factores actúan sobre la región "*enhancer*" de uPA a través de la vía de transducción de señal Ras/ERKs (Quinasas regulada por factores extracelulares) activando factores de transcripción ETS-1 y ETS-2 que regulan así la transcripción de uPA (Yang et al., 1996; McCarthy et al., 1997; Stacey et al., 1995; D'Orazio et al., 1997; Delannoy-Courdent, 1998; Watanabe T., 1998). Este gen da lugar a un mRNA de 2.5 Kb (Verde et al., 1984) que traduce una proteína monocadena inactiva (sc-uPA, pro-uPA)(Gunzler et al., 1982).

sc-uPA es posteriormente activado por proteólisis entre Lys_{158}-Ile_{159}, catalizada por plasmina (Wun et al., 1982 a; Eaton et al., 1984; Collen et al., 1986; Tapiovaara et al., 1993; Dano et al., 1985). Esta activación también puede darse "*in vitro*" por otras proteasas como la calicreína, la catepsina B, la tripsina o el factor XIIa de coagulación, aunque no se conoce el significado fisiológico de la activación de uPA por estas proteasas (Andreasen et al; 1997). La forma activa de uPA es una doble cadena (tc-uPA) con una actividad catalítica 250 veces mayor a la de la forma monocadena, por lo que pro-uPA se considera un cimógeno, a diferencia de sc-tPA (Eaton et al., 1984; Petersen et al., 1988). La doble cadena del uPA está unida por un puente disulfuro y tiene una estructura terciaria muy similar a las del Plg y tPA. La cadena ligera A está en el extremo N-terminal y la cadena pesada B en el C-terminal. La cadena A, conocida como el fragmento amino terminal (ATF), incluye un dominio del tipo EGF (residuos 1-49), homólogo al de tPA, seguido de un dominio "*kringle*" de unión a varias proteínas (residuos 50-131) (Blasi et al., 1987; Blasi., 1988), similar a los dominios presentes en Plg y tPA. En estos dos dominios de uPA (residuos 1-131) de la cadena A (Stopelli et al., 1985), se encuentra la región de unión a uPAR (Apella et al., 1987). En la cadena B, uPA tiene el dominio serina proteasa (SPD) (residuos 159-411), con la tríada catalítica His_{204}-Asp_{255}-Ser_{356}. A diferencia de tPA las cadenas A y B de uPA están unidas por un péptido conector (residuos 132-158) (Steffens et al., 1982) (**Figura 4).**

Una vez secretado, la mayor parte de pro-uPA, se une a su inhibidor específico (PAI-1), o a su receptor específico (uPAR) (Andreasen et al., 1994), uPA activo convierte el Plg unido a la membrana celular en plasmina (Stopelli et al., 1986) dándose así una retroalimentación positiva entre la activación de uPA y la generación de plasmina (**Figura 7**). uPA puede ser proteolizado de nuevo por plasmina, en el péptido de unión de sus dos cadenas, perdiendo así el fragmento ATF, sin actividad catalítica. La región catalítica y el fragmento restante del péptido conector conservan su actividad, pero pierde la capacidad de unión a la membrana celular, su masa molecular es 33 kDa

y recibe el nombre de uPA de bajo peso molecular (LMW-uPA) (Barlow et al., 1981). La forma no proteolizada de uPA, tiene un peso de 53 kDa, y recibe el nombre de uPA de alto peso molecular (HMW-uPA) (Stump et al., 1986 a).

uPA tiene una especificidad de substrato muy restrictiva. Cataliza la activación de Plm, y de proteínas con secuencias similares pero sin actividad catalítica, como es el caso del factor de crecimiento hepático, también llamado factor de "*scatter*" (HGF/SF), y de la proteína estimuladora de macrófagos (MSP) (Andreasen et al., 2000). uPA también puede proteolizar a su inhibidor específico PAI-1 (Andreasen et al., 1986 a; Nielsen et al., 1986), así como fibronectina (Keski-Oja and Vaheri, 1982). Por último uPA también cataliza el corte proteolítico entre los dominios 1 y 2 de su receptor uPAR (Hoyer-Hansen et al., 1992).

1.3.1 El receptor de uPA

uPAR es una glicoproteína de 270 residuos y un peso molecular de 55-60 kDa (35 kDa la forma no glicosilada) unida a la membrana plasmática por un glicosilfosfatidilinositol (GPI) que proporciona al uPAR movilidad lateral (Lisanti et al., 1994). uPAR tiene tres dominios homólogos (D_1, D_2, D_3), ricos en cisteína. La región de unión entre los dominios D_1 y D_2, es sensible a proteasas, como uPA, "*in vivo*" (Hoyer-Hansen et al., 1992), por lo que puede generar variantes de uPAR con el dominio N-terminal truncado que pueden activar vías de transducción de señal (Solberg et al., 1994). Este receptor, se encuentra en lamelipodios, y zonas de contacto célula-célula y célula-MEC de la membrana celular (Pollanen et al., 1988), de diferentes tipos celulares como monocitos, células epidermoides de carcinoma, fibroblastos, espermatozoides, células del sistema circulatorio y células endoteliales de diferentes tejidos y organismos (Hajjar, 1995).

El gen *uPAR* humano, codifica para un polipéptido de 225 aminoácidos, 21 de los cuáles forman parte del péptido señal. Tras modificaciones post-traduccionales, el receptor maduro, glicosilado, se une a la membrana celular mediante moléculas de GPI (Roldan et al., 1990; Ploug et al., 1991; Andreasen et al., 2000). Existen variantes solubles del uPAR, causados por splicing (Kristensen et al., 1991) o por acción de la fosfolipasa C (PLC) sobre la unión a GPI (Metz et al., 1994).

uPAR puede padecer dos tipos de modificaciones que alteran su función. Una de estas modificaciones es el "*shedding*", llevado a cabo por la PLC, y que separa el receptor del GPI, quedando éste en forma soluble, y manteniendo la afinidad por sus

dos ligandos principales, uPA y vitronectina (Ronne et al., 1994; Wei et al., 1994). La otra es el corte proteolítico entre los dominios D_1 y D_2 del uPAR, causado por uPA y otras proteasas como plasmina, elastasa o MMPs (Behrendt et al 1996; Ploug et al., 1994; koolwijk et al., 2001; Andolfo et al., 2002). Esta hidrólisis entre los dominios D_1 y D_2 del uPAR unido a la membrana celular se acelera por la unión de uPA (Hoyer-Hansen et al., 1997) e impide la unión de este receptor a la Vn (Ploug et al., 1994) y a la integrina (Montuori et al., 2002; Liu et al., 2002).

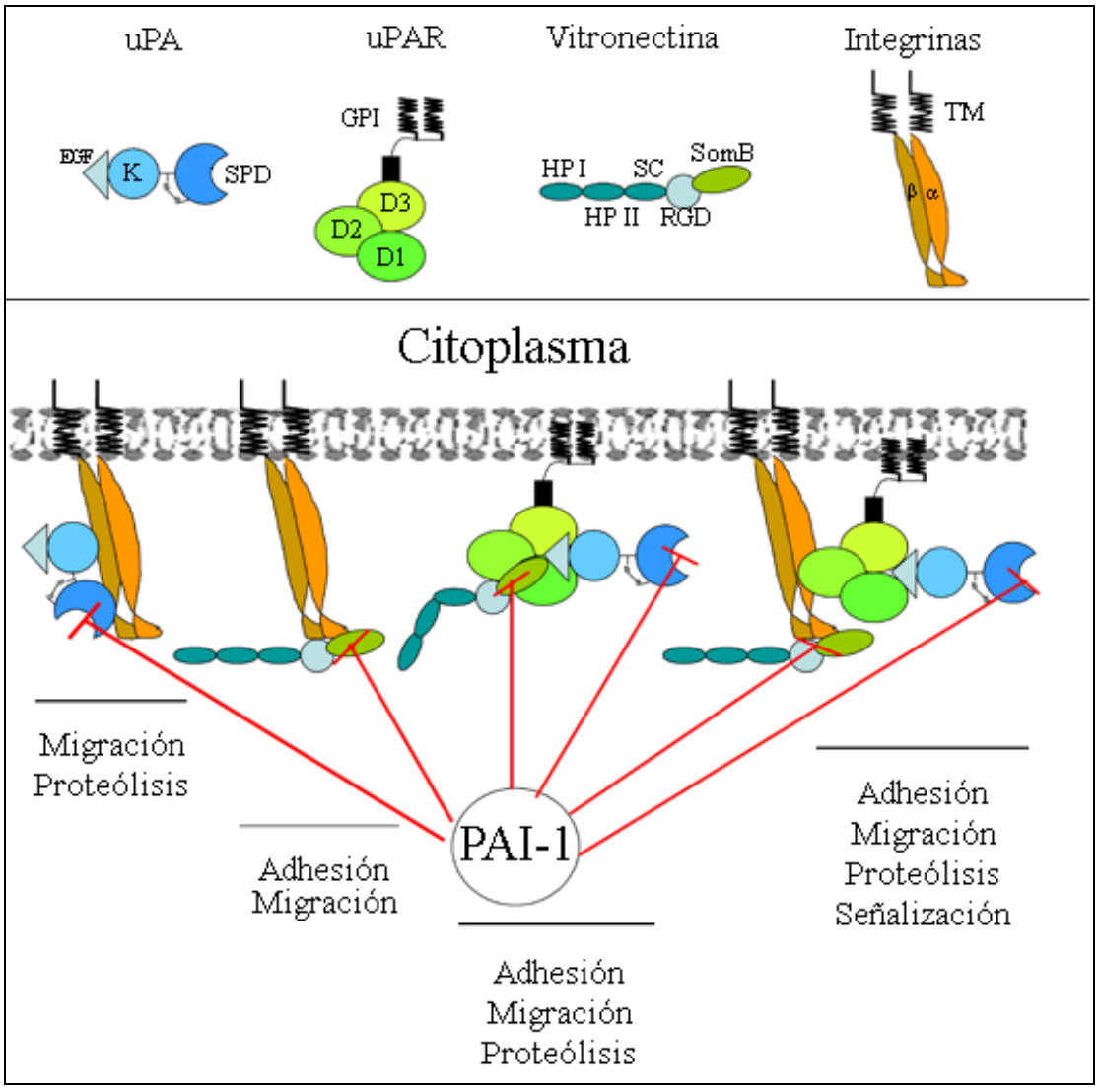

Figura 6. Esquema de las interacciones y los efectos causados por uPA/uPAR, PAI-1, Vitronectina (Vn) e Integrinas.
uPA se une a uPAR mediante su dominio EGF like (EGF), estimulando la unión del receptor al dominio somatomedina B (SomB), de la Vn. Esta unión activa la migración, la proteólisis y la adhesión celular. PAI-1 inhibe este efecto mediante la unión al dominio SomB de la Vn y al dominio serina proteasa (SPD) del uPA. Por otro lado, las integrinas también pueden unirse al dominio SomB de la Vn activando la adhesión y la migración celular, además de la proteólisis y la señalización celular al unirse al complejo uPA-uPAR. Estos efectos también son bloqueados por la mayor afinidad de PAI-1 al dominio SomB de la Vn y al SPD de del uPA. Por último, uPA también puede unirse directamente a las integrinas, activando la migración celular y la proteólisis siempre y cuando su acción no sea inhibida por PAI-1. K (dominio *"kringle"*), $D_{1,2,3}$ (dominios ricos en cisteína), DSP (dominio serina proteasa), EGF (dominio tipo EGF), HPI y HPII (dominios homopexina), SC (Segmento de unión al colágeno), RGD (secuencia de aminoácidos RGD), GPI (glicosilfosfatidilinositol), TM (dominio transmembrana).

El dominio amino terminal (D_1), junto con el dominio D_3 unen uPA, pro-uPA, uPA inactivado con DFP y ATF, con una K_d inferior al rango nanomolar (nM) (Ploug et al., 1993; Ploug and Ellis, 1994; Cubellis et al., 1986). uPA se une a este receptor mediante un loop Ω situado en su dominio tipo EGF (Apella et al., 1987, Ploug et al., 1995). Esta unión es crucial para la activación de uPA en condiciones fisiológicas, ya que sc-uPA unido a uPAR puede activarse a tc-uPA, con una eficiencia mucho más elevada que la activación en solución (Stoppelli et al., 1986; Ellis et al., 1989; Berkenpas et al., 1991; Duval-Jove et al., 1994).

El segundo ligando mejor caracterizado de uPAR es la vitronectina. La interacción de esta glucoproteína con el uPAR es más compleja que la de uPA. Vn interacciona tanto con la forma soluble de uPAR como con la forma unida a membrana por GPI (Wei et al., 1994; Hoyer-Hansen et al., 1997; Waltz et al., 1994; Sidenius and Blasi, 2000). uPAR interacciona, a través de sus tres dominios (Hoyer-Hansen et al., 1997; Sidenius and Blasi, 2000), con el dominio aminoterminal somatomedina B (SomB) de la Vn. Esta interacción viene estimulada por la unión de uPA, ATF y por uPA-PAI, indicando que es independiente de la actividad proteolítica de la proteasa (Del Rosso et al., 1990; Fibbi et al., 1988; Gudewicz and Gillboa, 1987). PAI-1 libre, se une a la Vn con mayor afinidad que uPAR, por lo que la unión de uPAR a Vn depende del balance entre la cantidad de uPA y PAI-1 presentes en el medio (**Figura 6**) (Wei et al., 1994; Hoyer-Hansen et al., 1997; Waltz and Chapman, 1994; Sidenius and Blasi, 2000; Deng et al., 1996; Kanse et al., 1996; Carriero et al., 1997). En esta misma región de la Vn, el dominio SomB, se une la integrina $\alpha_v\beta3$, receptor celular de Vn. Por lo tanto, PAI-1 controla el macrocomplejo formado por uPA/uPAR-Vn-Integrina (Okumura et al., 2002; Deng et al., 1996; Stefansson and Lawrence, 1996; Kjoller et al., 1997). Se ha descrito también que uPAR puede interaccionar directamente con integrinas regulando así su actividad en la membrana celular (Bohuslav et al 1995; Wei et al., 1996; Chapman and Wei, 2001; Ossowski and Aguirre-Ghiso, 2000) y formando complejos uPAR-integrina-caveolina, en los denominados "*lipid rafts*" (Stahl and Mueller, 1995), microdominios de la membrana plasmática ricos en colesterol, importantes en la transducción de señal (Lisanti et al., 1994). Por otro lado, uPA puede unirse a integrinas mediante su dominio "*kringle*" (Pluskota et al., 2003). Todas estas interacciones entre uPA/uPAR-Vn-integrina-caveolina en la superficie celular intervienen en las interacciones célula-célula y célula-MEC, controlando la adhesión, migración e invasión celular (**Figura 6**).

La acción de uPAR puede ser regulada mediante el efecto dual de PAI-1. Por una parte, PAI-1 regula la interacción de uPAR con Vn. Por otro lado, PAI-1 provoca la eliminación de uPA mediante endocitosis, impidiendo así la actividad de uPAR. La unión de uPA con PAI-1 facilita su unión por uPAR (Cubellis et al., 1990). Una vez formado el complejo, PAI-1-uPA-uPAR, es endocitado por medio de LPR (Rodenburg et al., 1998), provocando que tanto uPA como PAI-1, sean degradados en los lisosomas, mientras uPAR es reciclado (Cubellis et al., 1990). Por lo tanto, uPAR es un regulador tan importante del efecto de uPA, que en algunos organismos esta proteasa es patógena únicamente en presencia de su receptor (Zhou et al., 2000).

1.3.2 Mecanismo de actuación.

uPA y uPAR están implicados en la regulación de la proteólisis celular, adhesión, proliferación, invasión tumoral, quimiotaxi y quimiocinesis. Las dos modificaciones proteolíticas del uPAR, pueden tener un efecto negativo en la proteólisis inducida por uPA/uPAR, ya que el corte proteolítico del receptor, impide su unión con la Vn, y el "*shedding*" dificulta la activación del Plg (Sidenius and Blasi, 2003). La internalización de los complejos inactivos uPA/PAI-1 unidos a uPAR puede promover la activación del plasminógeno a plasmina, ya que el uPAR es reciclado manteniendo así la capacidad para unir pro-uPA (*ver apartado anterior*).

En la regulación de la adhesión celular a través de su interacción con la Vn y las integrinas, uPA/uPAR inducen la reestructuración del citoesqueleto de actina y la motilidad celular, donde también interviene Rac (Waltz and Chapman, 1994; Kjoller and Hall, 2001; Wei et al., 1996; Degryse et al., 2001; Kjoller, 2002). PAI-1 también interviene en este proceso controlando la interacción de uPAR, Vn e integrinas (*ver apartado anterior*). Esto indica que el nivel de uPA respecto a PAI-1 determina el grado de migración celular (Kjoller et al., 1997; Czekay et al., 2003). uPAR también puede intervenir en la adhesión celular a través de su interacción con integrinas y caveolina (*ver apartado anterior*), ya que caveolina está asociada a moléculas de señalización celular como Scr y proteínas G (Li et al.,1996; Wary et al., 1998). Tanto la proliferación como la invasión tumoral pueden estimularse por la unión de uPA/uPAR a integrinas activando el EGFR (receptor del factor de crecimiento epidérmico) y la vía de las ERK (Liu et al., 2002) (**Figura 6**). uPA/uPAR inducen quimiotaxis y quimiocinesis en diferentes líneas celulares (Resnati et al., 1996; Fazioli et al., 1997; Busso et al., 1994 a, b), estimulando vías de transmisión de señal como las ERKs, las FAKs y las Scr

(Sidenius and Blasi, 2003). En algunas ocasiones este efecto quimiotáctico depende de la actividad proteolítica de uPA, activando GFs como el pro-HGF (Naldini et al., 1992). La forma soluble del uPAR (suPAR), proteolizada por quimotripsina, también provoca un efecto quimiotáctico al interaccionar con el receptor de quimioquinas, lo que indica que podría actuar como agente autocrino o paracrino (Resnati et al., 1996, 2002; Fazioli et al., 1997).

1.4 Inhibidores del sistema.

La mayoría de los inhibidores que se han descrito como componentes del sistema plasminógeno-plasmina, pertenecen a la familia de las serpinas (Carrell and Travis, 1985) (**Tabla 1**). Estos inhibidores se unen covalentemente al centro activo del enzima, ya que tienen una región muy similar a su substrato. La unión entre estos inhibidores y las serina proteasas forman complejos 1:1 inactivos y muy estables (Laskowski and Kato, 1980).

1.4.1 Inhibidores de la plasmina.

La actividad de la plasmina está regulada por tres tipos de inhibidores: la α_2-antiplasmina, la α_2-macroglobulina y en menor medida por la α_2-antitripsina (α_1-AT).

1.4.1.1 α_2-Antiplasmina

Esta glicoproteína monocadena de 464 aminoácidos se encuentra en plasma a una concentración de μM (Holmes et al., 1987). α_2-antiplasmina es el principal inhibidor de plasmina y es también un miembro de la familia de las serpinas (Collen, 1976; Moroi and Aoki, 1976; Müllertz and Clemmensen, 1976; Wiman and Collen, 1978) (**Tabla 1**). Se une a los sitios de unión a lisina de los dominios "*kringle*" de la serina proteasa, formando así el complejo estequiométrico estable plasmina-α_2-antiplasmina que reduce 100 veces la capacidad de unión de la plasmina a la fibrina (Christensen and Clemmensen, 1978; Wiman and Collen, 1978).

La unión de plasmina-plasminógeno a la superficie celular dificulta la acción de este inhibidor, ya que la proteasa utiliza también dominios "*kringle*" para llevar a cabo la interacción con los receptores de membrana y los demás componentes de la MEC (*ver apartado 1.2.1*).

Inhibidor	*Familia*	*Componentes del sistema Plg-Plm inhibidos*	*Referencia*
PAI-1	Serpinas	PAs	Kruithof et al., 1984; 1986 a, b; 1988 Pannekoek et al., 1986 Andreasen et al., 1986 a, b Lijnen et al., 1991
PAI-2	Serpinas	PAs	Kruithof et al., 1995 Andreasen et al., 1990
PAI-3	Serpinas	PAs	Hebb et al., 1987 Stump et al., 1986 b
α_2*-Antiplasmina*	Serpinas	Plm	Collen, 1976 Moroi and Aoki, 1976; Müllertz and Clemmensen, 1976 Wiman and Collen, 1978
α_2*-Macroglobulina*	No serpina	Plm, PAs	van Leuven et al., 1978; Mosher and Vaheri, 1980; Cummings and Castellino, 1984
α_1*-Antitripsina*	Serpinas	Plm, PAs	Rijken et al., 1983 b
Proteasa nexina	Serpinas	PAs	Scott et al., 1985
Neuroserpina	Serpinas	PAs	Hasting et al., 1997 Osterwalder et al 1998
Proteína inactivadora C	Serpinas	PAs	Irigoyen et al., 1999
Maspina	Serpinas	PAs	Sheng S. et al., 1998

Tabla 1. Inhibidores del sistema de activación del plasminógeno.
Clasificación de los inhibidores de los diferentes componentes del sistema de activación del plasminógeno según la familia a la que pertenecen y los componentes que inhiben.

1.4.1.2 α_2-Macroglobulina

La α_2-Macroglobulina es una glicoproteína de gran tamaño que no pertenece a la familia de las serpinas (van Leuven et al., 1978; Mosher and Vaheri, 1980; Cummings and Castellino, 1984). Este inhibidor se expresa en diferentes líneas celulares, en la circulación sanguínea a elevadas concentraciones (Sottrup-Jensen, 1989) y en otros

fluidos corporales (linfático, pleural, y amniótico) (Starkey and Barret, 1977), lo que muestra su importancia en el control de la proteólisis. Este inhibidor puede unirse a proteinasas libres o unidas a su receptor formando complejos que son internalizados por los LPRs (Borth, 1992), pese a que la unión de plasmina a la superficie de la membrana celular impide su acción. La α_2-macroglobulina puede también inhibir PAs (Stephens, et al., 1991), de manera similar a la **α_1-antitripsina**, que sí pertenece a la familia de las serpinas (Rijken et al., 1983 b) (**Tabla 1**).

1.4.2 Inhibidores de los activadores del plasminógeno

Los inhibidores PAI son glicoproteínas de la superfamilia de las serpinas, perteneciendo al subgrupo de las arg-serpinas, por la presencia de este aminoácido en su centro activo. Existen tres PAIs: el PAI-1, el PAI-2, y el PAI-3 (**Tabla 1**). Otros inhibidores descritos son la proteasa nexina 1, la proteína inactivadora C (Irigoyen et al., 1999) y la maspina (Sheng et al., 1998), todos ellos pertenecientes a la familia de las serpinas.

1.4.2.1 El inhibidor del plasminógeno tipo 1

PAI-1 es una glicoproteína 379 aa, sintetizada preferentemente en el endotelio vascular, pero también se expresa en diferentes tipos celulares como células endoteliales, hepatocitos, fibroblastos, plaquetas y diferentes líneas tumorales (Kruithof et al., 1984; Andreasen et al., 1986 a; Sidenius and Blasi, 2003; Myohanen and Vaheri, 2004). Se secreta en forma inactiva, pero la interacción con vitronectina y heparina, provocan un cambio conformacional de su estructura que estabiliza la forma activa (Salonen et al., 1989). PAI-1 activo se une en complejos 1:1 a los PAs proteolíticamente activos (sc-tPA, tc-tPA y tc-uPA, pero no sc-uPA) (Kruithof et al., 1984, 1986 a, b; Pannekoek et al., 1986; Andreasen et al., 1986 a, b; Kruithof 1988; Lijnen et al., 1991). Este inhibitor puede además unirse a uPA unido a uPAR, inhibiendo así la degradación de la MEC (Cubellis et al., 1989) y a la trombina (Ehrlich et al., 1991). PAI-1 se expresa en diferentes tipos celulares como células endoteliales, hepatocitos, fibroblastos, plaquetas y diferentes líneas tumorales (Sidenius and Blasi, 2003; Myohanen and Vaheri, 2004).

La actividad de PAI-1 puede ser regulada por proteólisis e inactivación mediadas por uPA (Laiho et al., 1987), o por la formación de un macrocomplejo uPAR-uPA-PAI-1- α_2-M/LPR, en el cual uPA-PAI-1 son degradados por los lisosomas y uPAR

reciclado a la superficie celular (*ver apartado 1.3.1*) (Planus et al., 1997; Nykjaer et al., 1997). Interviene la regulación de la fibrinólisis, la adhesión celular, la migración y la angiogénesis tumoral (Irigoyen et al., 1999; Sidenius and Blasi, 2003) (*ver apartados 1.3.1 y 1.3.2)*.

1.4.2.2 El inhibidor del plasminógeno tipo 2

PAI-2 es una proteína de cadena simple perteneciente al subgrupo de serpinas tipo ovoalbúmina (Silverman et al., 2001), que se expresa en placenta (Kawano et al., 1970) y en macrófagos (Kruithof et al., 1986 a). Se han descrito dos formas de PAI-2: una intracelular no glicosilada de 42 kDa y otra de 60 kDa, que se secreta tras ser glicosilada (Antalis et al., 1988; Ye et al., 1988; Belin, 1992, Kruithof et al., 1995). Este inhibidor puede, además, formar polímeros intracelularmente (Mikus and Ny T., 1996). PAI-2 inhibe uPA y tPA, aunque con eficiencias 10 y 100 veces menor que PAI-1, respectivamente (Andreasen et al., 1990; Kruithof et al., 1995).

En el compartimento intracelular, PAI-2 interviene evitando efectos citopáticos para la célula como la apoptosis provocada por el factor tumoral de necrosis α (TNF α) (Kumar and Baglioni., 1991; Dickinson et al., 1995), los efectos del interferón autocrino , IFN- α/β, y la infección por alfa virus (Antalis et al., 1998). Lo que indica, que tanto el PAI-2 secretado como el intracelular intervienen en procesos proteolíticos, como son la remodelación tisular, en el caso de PAI-2 secretado, y la apoptosis en el intracelular.

1.4.2.3 Otros inhibidores de los activadores del plasminógeno.

PAI-3 se ha identificado en orina y en plasma (Hebb et al., 1987). Es un inhibidor inespecífico de serina proteasas y actúa sobre uPA con una actividad 1000 veces menor que PAI-1 (Stump et al., 1986 b) (**Tabla 1**).

La proteasa nexina es un inhibidor inespecífico, que inhibe también otras serina proteasas como plasmina, trombina y tripsina (Scott et al., 1985) (**Tabla 1**).

La neuroserpina, inhibe tPA en el sistema nervioso central (Hasting et al., 1997). Existe controversia respecto a su efecto inhibitorio sobre plasmina (Hasting et al., 1997; Osterwalder et al 1998) (**Tabla 1**).

La maspina, de la familia de las serpinas, se expresa en células epiteliales (Zou et al., 1994). Tiene una región homóloga a PAI-1 y PAI-2, con la que se une a tPA inhibiendo su actividad invasiva (Sheng et al., 1998) (**Tabla 1**).

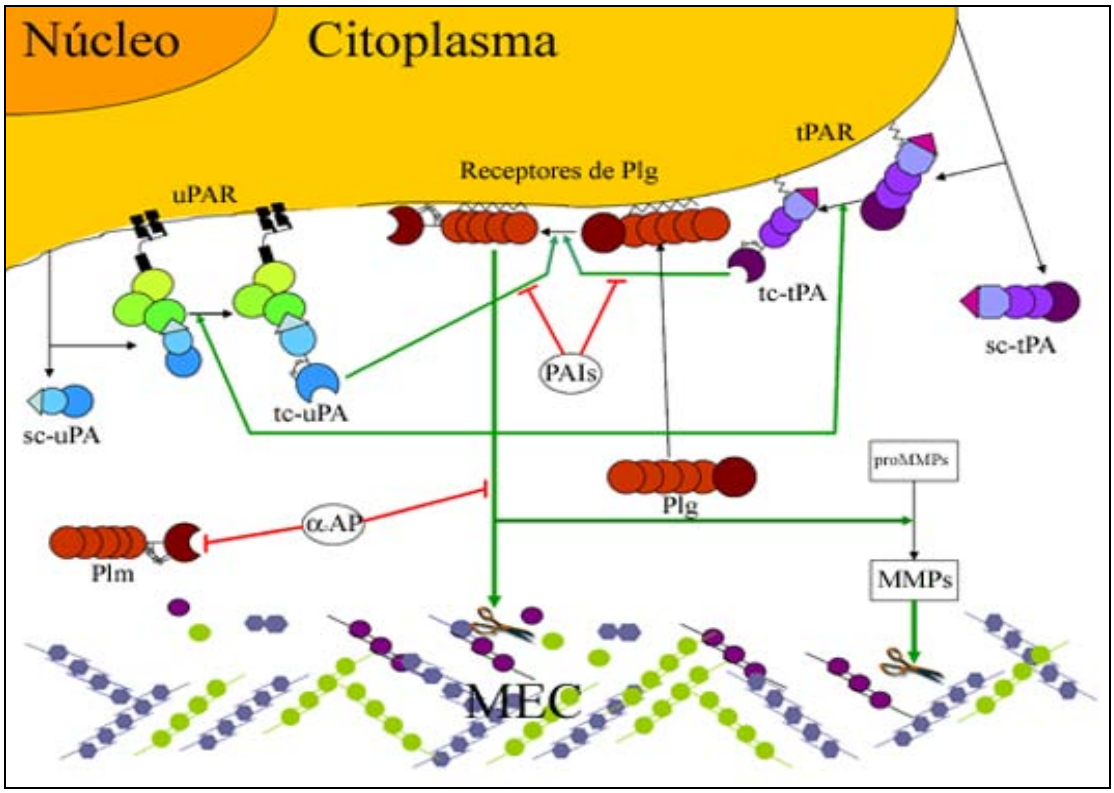

Figura 7. Modelo de regulación del sistema de activación del plasminógeno.
Los activadores del plasminógeno (tPA y uPA) se secretan en forma monocatenaria (sc-PAs). Una vez secretados, una porción de estos activadores se une a su receptor. Estos PAs unidos al receptor son activados por Plm, provocando así la activación de esta ultima al cortar proteolíticamente a su cimógeno, el Plg, que a su vez está unido a su receptor de membrana, dándose una retroalimentación positiva entre la activación de Plm y de los PAs. La regulación del sistema viene dada por la unión de los PAs y el Plg a sus respectivos receptores optimizando la activación de Plm, y por los inhibidores del sistema como PAI-1 y α_2-AP que regulan la actividad de los PAs y la Plm respectivamente. Una vez activada, la Plm cataliza la activación de diversos pro-enzimas como las pro-MMPs junto a las que lleva acabo la degradación de la MEC.

2 Mecanismos de regulación del sistema.

El sistema proteolítico del plasminógeno necesita una regulación muy precisa para evitar una proteólisis descontrolada letal para el organismo. Esta regulación se lleva a cabo a través de la interacción de una gran variedad de proteínas y mecanismos reguladores entre los que se encuentran: i) los inhibidores fisiológicos (*ver apartado 1.4*); ii) los receptores específicos de plasminógeno-plasmina y de los PAs (*ver apartados 1.1.1, 1.2.1 y 1.3.1*); iii) y los reguladores de la expresión génica de los PAs y de sus inhibidores. Además de los ya mencionados componentes del sistema de activación del plasminógeno, existen una serie de proteínas que son indispensables en el funcionamiento y la regulación de éste sistema (**Figura 7)**. Entre estas proteínas se

incluyen proteasas pertenecientes a la familia de las MMPs y algunos factores de crecimiento, como los que llevan a cabo sus efectos a través del EGFR.

2.1 Metaloproteinasas

Las metaloproteinasas, también llamadas matrixinas, son una familia de endopeptidasas dependientes de Zn^{2+} responsables de la proteólisis o degradación de múltiples componentes de la MEC, mayoritariamente colágeno y proteoglicanos (Birkedal-Hansen et al., 1993). La mayoría de estas MMPs se sintetizan como pro-enzimas en forma latente. Se han descrito más de 25 MMPs que pueden ser clasificadas en base a la especificidad de substrato y a la estructura molecular en seis grupos (Visse and Nagase, 2003). Estos incluyen las colagenasas, las gelatinasas, las estromelisinas, las matrilisinas, las MMPs unidas a membrana, y otras menos conocidas y estudiadas (Visse and Nagase, 2003; Chakravorti et al., 2003; Curry and Osteen, 2003; Nelson et al., 2000; Nagase and Woessner, 1999) (**Tabla 2**).

Las MMPs presentan distintos dominios estructurales como un péptido señal, un pro-péptido, un dominio catalítico, una región bisagra y un dominio homopexina (PEX). El péptido señal en el N-terminal dirige la proteína hacia la secrección. Las MMPs se secretan en forma latente, como pro-MMPs que contienen el pro-péptido con la cisteína que interacciona con el dominio de zinc en forma no reactiva (Springman et al., 1990; Van Wart and Birkedal-Hansen, 1990). El dominio catalítico contiene las tres histidinas responsables de la interacción con el zinc esencial para la actividad proteolítica de las MMPs (Woessner, 1994). La región bisagra, rica en prolinas, une el dominio catalítico y el PEX. Este último dominio se encuentra en la región C-terminal y es el responsable de las interacciones con otras proteínas por lo que es indispensable para la unión específica al substrato (Smith et al., 1999). Además de estos dominios, las gelatinasas contienen tres dominios repetidos tipo fibronectina II, junto a su dominio catalítico, que se unen a la gelatina (Collier et al., 1988; Steffensen et al., 1995; Allan et al., 1995). Otra variación, es la presencia de un dominio transmembrana y un lugar de activación por furina en las metaloproteinasas de matriz unidas a la membrana (MT-MMPs) (Polette and Birembaut, 1998). Las adamlisinas o ADAMs (proteína metaloproteinasa y desintegrina), constituyen otra familia de MMPs. Estas proteinasas se caracterizan por tener una función desintegrina además de la metaloproteinasa. Los miembros de esta

familia destacan por tener un papel fundamental en el "*shedding*" de proteínas de membrana (Kheradmand and Werb, 2002; White, 2003; Seals and Courtneidge, 2003).

Componentes	*Activadas por*	*Activadoras de*	*Efectos Biológicos*
COLAGENASAS			
MMP-1, MMP-8, MMP-13, MMP-18 (Colagenasa 1, 2,3 y 4)	MMP-2,-3,-10,-14,-15, Plm, calicreína y quimasa	pro-MMP-1,-2,-8,-9 y -13	Migración y proliferación celular, reepitelización, agregación de plaquetas, procesos inflamatorios, apoptosis, activación de osteoclastos y liberación de FGFb.
GELATINASAS			
MMP-2 y MMP-9 (Geatinasa A y B)	MMP-1,-2,-3,-7,-13,-14,-15, -16,-17,-24, -25, Plm y triptasa	pro-MMP-1,-2,-9 y -13	Crecimiento de las neuritas, procesos inflamatorios, diferenciación, proliferación, migración celular y generación de angiostatina.
ESTROMELISINAS			
MMP-3, MMP-10 y MMP-11 (Estromelisinas 1,2 y 3)	Plm, calicreína, quimasa, triptasa, elastasa, catepsina G y furina	pro-MMP-1,-2,-7,-8,-9, -10 y -13	Migración, diferenciación, proliferación e invasión celular, apoptosis, generación de angiostatina, liberación de FGFb y procesos inflamatorios.
MATRILISINAS			
MMP-7 y MMP-26 (Matrilisinas 1 y 2)	MMP-3,-10 y Plm	pro-MMP-1,-2,-7 y -9	Diferenciación e invasión celular, generación de angiostatina y apoptosis
MMPs unidas a la membrana (MT-MMPs)			
Transmembrana (MT)			
MMP-14, MMP-15, MMP-16, MMP-24 (MT-MMPs 1, 2, 3 y 5)	Plasmina, furina	pro-MMP-2, -8, -13 y MT1-MMP	Tubulogénesis, migración y adhesión celular.
Ancladas por GPI			
MMP-17 y MMP-25 (MT- MMPs 4 y 6)		pro-MMP-2 y -9	
OTRAS MMPs			
MMP-12, MMP-19, MMP-20, MMP-21, MMP-23, MMP-27, MMP-28			

Tabla 2. Miembros de la familia de las Metaloproteinasas (MMPs).
Clasificación de las diferentes MMPs, activadores de MMPs, substratos de MMPs, y efectos biológicos. Resumido de Visse and Nagase, Mandal, Chackraborti, Engelse y Folgueras (Chakraborti et al., 2003; Mandal et al., 2003; Visse and Nagase, 2003; Engelse et al., 2004; Folgueras et al., 2004).

Las MMPs se activan por una proteólisis, entre la cisteína del pro-péptido y el Zn^{2+} del dominio catalítico, producida por otras MMPs, por otras proteinasas (PAs, plasmina, tripsina, calicreína y furina), o por agentes reductores o desnaturalizantes (Springman et al., 1990).

Las MMPs son las responsables de mantener el equilibrio entre la formación y la degradación de la MEC. Su regulación está altamente controlada a nivel transcripcional y a nivel de expresión génica por diferentes moléculas (Reuben and Cheung; 2006; Nagase and Woessner, 1999), ya que un pequeño desajuste puede causar patogénesis y varias enfermedades. Una vez sintetizadas, las matrixinas son reguladas a nivel de la activación y por las interacciones con inhibidores específicos. Los principales inhibidores son proteínas endógenas llamadas TIMPs (inhibidores de las metaloproteinasas). Las MMPs también pueden ser inhibidas por algunos agentes farmacológicos, biofosfonatos, derivados de la tetraciclina y por drogas (Varghese, 2006).

2.2 Vía de activación del receptor del factor de crecimiento epidérmico

El receptor del factor de crecimiento epidérmico es una glucoproteína altamente conservada de 170 kDa, transductora de señales extracelulares. Se han descrito genes homólogos en *C. elegans* (Aroian et al., 1990) y en *D. melanogaster* (Freeman M, 1998). El EGFR forma parte de la familia de receptores tirosina quinasa tipo I, los c-erbB (Mason and Gullick, 1995). EGFR, (HER-1, c-erbB.-1), HER2 (neu, c-erbB.-2), HER3 (c-erbB.-3) y HER4 (c-erbB.-4), son miembros de esta familia de receptores, y presentan estructura y funciones similares (Ullrich et al., 1984; Bargmann et al., 1986; Kraus et al., 1989; Plowman et al., 1990, 1993; Olayioye et al., 2000; Yarden, 2001). Se distribuyen en la región basolateral de células polarizadas y se expresan en tejidos epiteliales, mesenquimales y neuronales, interviniendo en el desarrollo, la proliferación y la diferenciación celular (Singh and Harris, 2005). Esta glucoproteína también se encuentra en vesículas endocíticas (Lenferink et al., 1998).

El EGFR existe con dos afinidades de substrato diferentes, con alta afinidad al EGF (una minoría, 2%-5%) y con baja afinidad (casi todo, 92%-95%), en función de la conformación de este receptor (Ullrich and Schlessinger, 1990). Su estructura es la de los receptores de membrana con actividad tirosina quinasa tipo I. Presenta un dominio

extracelular muy poco conservado; un dominio hidrofóbico transmembrana, implicado en la interacción entre receptores; y un dominio intracelular muy conservado, con actividad tirosina quinasa y con un extremo C-terminal con lugares de autofosforilación y unión de proteínas citosólicas (Mason and Gullick 1995; Yarden, 2001; Yarden and Sliwkowski 2001; Olayioye et al., 2000; Guy et al., 1994; Wells, 1999) (**Figura 8**).

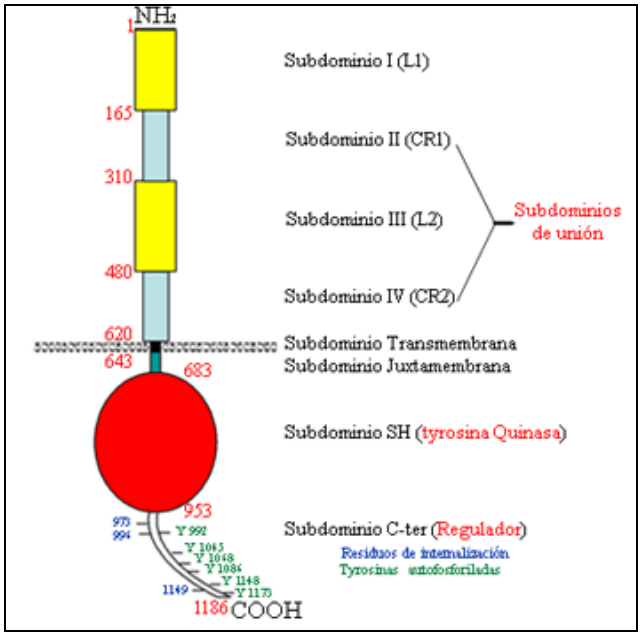

Figura 8. Esquema del receptor del factor de crecimiento epidérmico (EGFR).
El EGFR presenta una estructura en la que se reconocen varios dominios: el extracelular, el transmembrana y el citoplasmático. En el dominio extracelular destacan los subdominios ricos en lisina (L1 y L2) y ricos en cisteína (CR1 y CR2), estos últimos responsables de la unión con otras proteínas de membrana. El dominio transmembrana no presenta subdominios, mientras que el citoplasmático presenta el subdominio SH1 (dominio homólogo de Scr), con actividad citosina quinasa y el subdominio C-terminal que regula la actividad del receptor. En este subdominio se encuentran las tirosinas que se autofosforilan cuando se activa el receptor y los residuos responsables de la internalización del receptor.

El dominio extracelular glicosilado, presenta cuatro subdominios. Dos subdominios ricos en leucina (L1 y L2) que forman la región de unión de ligandos (Ward and Garret, 2001) y dos subdominios ricos en cisteínas (CRI y CRII) que se encargan de la interacción y dimerización con otras proteínas de membrana (Lax et al., 1989; Ward 1995). Estos subdominios, altamente conservados, están intercalados, y unidos por puentes disulfuro (**Figura 8**). El dominio transmembrana tiene estructura de hélice (Rigby et al., 1998), sirve para mitigar el efecto de la proteína quinasa C (PKC) y las quinasas ERK MAPK (proteína quinasa activadora de la mitosis) (Li and Villalobo, 2002; Li et al., 1998; Wells, 1999) y para unir proteínas G (Sun et al., 1997; Daub et al., 1997) (**Figura 8**). En la región citoplasmática se distinguen: i) el subdominio juxtamembrana, similar al dominio transmembrana (Rigby et al., 1998; Li et al., 1998; Wells, 1999); ii) el subdominio SH1 (dominio homólogo 1 de Scr), altamente conservado y con actividad tirosina quinasa, que autofosforila los seis residuos de tirosina del extremo C-terminal; iii) el subdominio C-terminal, poco conservado, y en el que se encuentran las tirosinas autofosforiladas por el subdominio catalítico. El subdominio C-terminal es el responsable de la unión de las diferentes proteínas una vez

el receptor está activado, por lo que es esencial en la regulación del receptor (Thompson and Gill, 1985; Wells, 1999; Hackel et al., 1999). Este subdominio contiene además motivos para la activación proteolítica, la internalización y de degradación del receptor, a través de la endocitosis de vesículas recubiertas de clatrina (Carpenter and Cohen, 1990; Wells, 1999) (**Figura 8**).

El EGFR se activa por la unión de ligandos a los subdominios L1 y L2 extracelulares. Estos ligandos son generalmente sintetizados por la misma célula (secrección autocrina), o por células de alrededor (secrección paracrina), como precursores transmembrana glicosilados, que son posteriormente activados por *"shedding"* mediado por serina proteasas (Pandiella et al., 1992), MMPs (Arribas et al., 1996; Dempsey et al., 1997; Brown et al., 1998) y/o ADAMs (Peschon et al., 1998). Sin embargo, algunos ligandos, son activos sin necesidad de ser procesados, por lo que funcionan también como factores juxtacrinos unidos a la membrana celular (Iwamoto and Mekada, 2000; Anklesaria, 1990; Inui et al., 1997; Tada et al., 1999), lo que indica que este receptor puede ser activado por ligandos de forma autocrina, paracrina o juxtacrina (Iwamoto et al., 1999; Singh and Harris 2005; Pan et al., 2002). Los ligandos se unen mediante su dominio "*EGF like*" al EGFR (Harris et al, 2003). Entre estos ligandos destacan: el EGF (Carpenter and Cohen, 1990), TGF-α (Massague, 1990), la anfiregulina (AR) (Shoyab et al., 1989), la tomoregulina, la decorina (Patel et al., 1998), el epigen (EPI) (Strachan et al., 2001), el hb-EGF ("*Heparin Binding EGF"*) (Higashiyama et al, 1992), la betacelulina (BTC) (Riese et al., 1996) y la epiregulina (EPR) (Shelly et al., 1998) (**Figura 9**).

Una vez unido el ligando, el receptor puede formar un homo- o heterodímero con una estequiometría 2:2, mediante la interacción entre los subdominios CR1 de dos receptores con sendos ligandos unidos (Lemmon et al., 1997; Garret et al., 2002; Ogiso et al., 2002). La formación de estos homo- o heterodímeros está favorecida en las caveolas, donde se encuentran el 50% de los EGFR de la membrana celular (Mineo et al, 1999). Los heterodímeros están formados por un EGFR y otro receptor de membrana como erbB2 (Johannessen et al., 2001) o PDGFR (Saito et al., 2001).

El EGFR puede estar en forma monomérica o dimérica antes de estar unido a su ligando (Sako et al., 2000; Moriki et al., 2001; Yu et al., 2002). Además se han descrito ligandos como AnnII, que están unidos a este receptor antes de ser activado (Blagoev et al., 2003). Pese a esto, la unión de determinados ligandos es necesaria para tener actividad tirosina quinasa (Olayioye, 2000; Yarden, 2001), de lo que se deduce que la

dimerización del EGFR es necesaria, pero no suficiente para activar su dominio tirosina quinasa (Jorissen et al., 2003). Este mecanismo de dimerización permite una amplificación y diversificación de la señal inicial (Normanno et al., 2006).

El EGFR también puede activarse por una transactivación a partir de una gran variedad de estímulos extracelulares, integrándolos así en una sola vía. Estos estímulos van desde el "*stress"* celular (Weiss et al., 1997; Carpenter, 1999), hasta diferentes tipos de hormonas, receptores de citoquinas, integrinas, vitronectina y canales iónicos (Carpenter, 2000; Gschwind et al., 2001; Prenzel et al., 2000). Las proteínas citoplasmáticas que llevan a cabo esta transactivación son tirosinas quinasa como: como JAK2 ("*JAnus Kinase 2"*) (Yamauchi et al., 1997), Src (quinasa del sarcoma virus transformante) (Luttrell et al., 1997; Biscardi et al., 1999), Pyk 2 (proteína tirosina quinasa rica en prolinas 2) (Lev et al., 1995), PKC *(*Tsai et al., 1997; Slack 2000), GPCRs (receptor asociado a proteína G) (Carpenter, 2000; Gschwind et al., 2001; Prenzel et al., 2001) o MMPs (Gschwind et al., 2002; Prenzel et al., 1999), indicando que EGFR funciona como integrador de señales a través de la interacción con diferentes proteínas y receptores de membrana (Normanno et al., 2006) (**Figura 9**).

Una vez activado el receptor, por unión de ligando o por transactivación, se fosforilan los residuos de la cola citoplasmática de EGFR, y éste actúa como una plataforma de reclutamiento de proteínas señalizadoras en respuesta a su activación (Schlessinger 2000). Entre las proteínas que se unen al receptor destacan enzimas directamente activadas por la unión al receptor (fosfolipasas, lipasas y quinasas) y proteínas adaptadoras (Shc, Grb2, Grb7, Crk, Nck, Cbl, Eps 15 y Dok-R) (Jorissen et al., 2003). La mayoría de las interacciones entre estas proteínas adaptadoras y el EGFR se dan a través de sus dominios SH2 o PTB (Pawson and Schlessinger, 1993). Algunas de estas proteínas y/o enzimas están implicadas en la regulación de las vías de transducción de señal que podemos ver en la **figura 9**. La mayor y más conservada vía de señalización activada por el EGFR es la vía MAPK (Alroy and Yarden, 1997), que activa las quinasas ERK1 y ERK2 implicadas en proliferación, supervivencia y transformación celular (Cobb et al., 1994). Esta multiplicidad de vías de señalización activadas por EGFR, puede provocar que más de una sean necesarias para llevar a cabo un determinado efecto, y a la inversa, que una sola vía pueda provocar diversas respuestas biológicas (Singh and Harris, 2005).

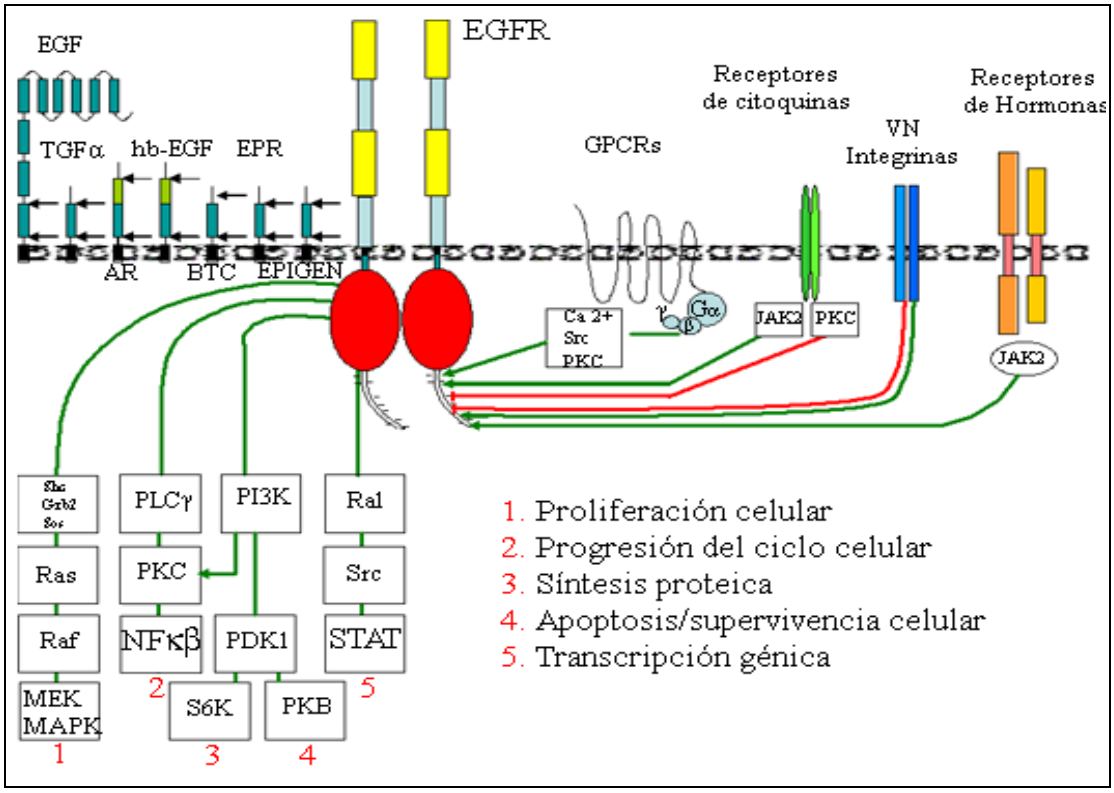

Figura 9. Vías de señalización del receptor del factor de crecimiento epidérmico (EGFR).
En el esquema están representados los posibles ligandos del EGFR. El EGF (Factor de crecimiento epidérmico), el TGFα (Factor de crecimiento transformante α), la AR (anfirregulina), el hb-EGF (*"Heparin Binding" EGF)*, la BTC (betacelulina), la EPR (epirregulina) y el epigen. También se muestra como el EGFR puede ser transactivado a través de la acción de diferentres receptores de membrana como: el receptor asociado a proteína G (GPCR), la vitronectina (Vn), las integrinas y los receptores hormonales y/o de citoquinas. Por último, también se muestran las principales vías que pueden ser activadas por el EGFR así como las funciones en las que intervienen cada una de ellas. Las flechas en verde muestran activación mientras las rojas indican inhibición. Figura resumen a partir de (Patel et al., 1988; Shoyab et al., 1989; Carpenter and Cohen, 1990; Massague, 1990; Higashiyama et al, 1992; David et al., 1996; Pawson and Schlessinger, 1993; Riese et al., 1996 ; Alroy and Yarden, 1997; Soltoff and Candley, 1996; Vivanco and Sawyers, 2002; Belsches et al., 1997; Luttrell et al., 1997; Piiper et al., 1997; Pullen and Thomas, 1997; Robinson and Cobb, 1997; Tsai et al., 1997; Yamauchi et al., 1997; Shelly et al., 1998; Biscardi et al., 1999; Prenzel et al., 1999, 2000; Carpenter, 2000; Slack 2000; Gschwind et al., 2001, 2002; Strachan et al., 2001; Jorissen et al., 2003; Thomas et al., 2003; Normanno et al., 2006).

La actividad del EGFR se regula a diferentes niveles, por: 1) la presencia de ligandos; 2) inhibidores del receptor y proteínas como PKC y proteínas fosfatasa, que impiden la unión de enzimas y proteínas adaptadoras (Casci and Freeman, 1999; Cochet et al., 1984; Davis and Czech, 1985; Milarski et al., 1993); 3) la expresión de mutantes del EGFR sin actividad tirosina quinasa, que forman heterodímeros inactivos (Jaye et al., 1992); 4) y la señal terminal de inactivación, propia del receptor, por la cual se unen al receptor proteínas con actividad ubiquitina ligasa, provocando su internalización en vesículas de clatrina y su ulterior degradación (Joazeiro et al., 1999; Sorkin and Waters,

1993; Prenzel et al., 2001; Normanno et al., 2003; Schlessinger, 2000; Singh and Harris, 2005; Marmor and Yarden 2004).

3 Funciones del sistema plasminógeno-plasmina.

La producción celular del plasminógeno y sus activadores, suele ir asociada con procesos fisiológicos y patológicos que implican migración celular y remodelación tisular (Romer et al., 1996; Bugge et al., 1996).

3.1 Procesos fisiológicos.

El Plg interviene en la degradación de la pared del folículo durante la ovulación (Liu et al., 1987), proceso en el que también participan tPA y uPA (Beers et al., 1975; Strickland and Beers, 1976).

Los activadores de plasminógeno intervienen en procesos de degradación de coágulos en diferentes órganos (Bachmann, 1987; Carmeliet et al., 1994; Bugge et al., 1995; Kitching et al., 1997; Barazzone et al., 1996; Eitzman et al., 1996), y en otros procesos de proteólisis extracelular como: embriogénesis (Strickland et al., 1976; Sappino et al., 1989), reacciones inflamatorias (Vassalli et al., 1979), cicatrización de heridas (Grondahl-Hasnsen et al., 1988; Romer et al., 1996) y angiogénesis (Murata et al.,1991).

tPA tiene un papel más destacado que uPA en la fibrinólisis vascular e interviene en otros procesos como la organización y formación de vasos de células endoteliales (Sato et al., 1994; Schnaper et al., 1995). El papel de esta proteasa es esencial regulando diversos procesos neuronales como la plasticidad sináptica, la permeabilidad neurovascular, el crecimiento del cono axonal, la migración neuronal, la regeneración neural, la memoria, el aprendizaje y el *"stress"* (Krystosek and Seeds., 1981 a, b; Salles et al., 1990; Qian et al., 1993; García-Rocha et al., 1994; Seeds et al., 1995; Strickland, 2001; Tsirka, 2002; Yepes and Lawrence, 2004 a, b; Melchor and Strickland, 2005). uPA en cambio tiene mayor importancia en procesos fisiológicos como la regeneración del músculo esquelético (Lluis et al., 2001), en los que regula la adhesión, la proteólisis celular y las uniones célula-célula y célula-MEC, mediante la localización del efecto de uPA por la unión de uPA/uPAR a integrinas de la membrana celular y a la Vn de la MEC (*ver apartado 1.3.1*).

AnnII como receptor de tPA/Plg y activador de la plasmina, interviene en diferentes procesos como la activación de células de la microglía. También tiene

también funciones independientes al sistema de activación del Plg, como son la regulación de la endo- y la exocitosis, la modulación de la organización de raft lipídicos (Sargiacomo et al., 1993; Schnitzer et al., 1995; Oliferenko et al., 1999; Babiychuck and Draeger, 2000), la contracción de células musculares y la remodelación/organización de la citoarquitectura de la membrana celular (Benaud et al., 2004).

Las MMPs intervienen en la remodelación tisular que ocurre en procesos fisiológicos como el desarrollo tisular, la morfogénesis y la cicatrización de heridas. También participan en la regulación de la comunicación celular, en el *"shedding"* molecular y en el procesamiento de moléculas como otras proteinasas, inhibidores de proteinasas, receptores de membrana, citoquinas, hormonas, moléculas de adhesión y factores de crecimiento, entre otros (Mott and Werb, 2004).

El EGFR forma parte de un complejo sistema de señalización celular fundamental en la fisiología celular. Prueba de ello, es que este receptor interviene en multitud de procesos biológicos como: ciclo celular, mitogénesis, apoptosis, angiogénesis, adhesión celular, funciones homeostáticas, motilidad celular, secrección proteica, procesos de diferenciación/desdiferenciación, invasión y metástasis (Singh and Harris, 2005; Mendelshon and Baselga, 2006). Estos resultados se han obtenido como resultado de estudios en los que se utilizan diferentes estrategias que bloquean la actividad de este receptor. Entre estos mecanismos de inhibición del EGFR, los MAbs (anticuerpos monoclonales) y los TKIs (inhibidores de tirosina quinasas) son los que están en un desarrollo clínico más avanzado (Mendelshon and Baselga, 2006).

3.2 Procesos patológicos.

Varias de las situaciones patológicas están directamente asociadas con la expresión de los componentes del sistema de activación del plasminógeno. Las enfermedades en la que está más estudiado el efecto de los componentes de este sistema son las cardiovasculares (trombólisis y arteriosclerosis) y el cáncer, dónde intervienen en varios procesos de la proliferación tumoral. Además de estas enfermedades, los activadores del plasminógeno también participan en otros procesos patológicos como la reestenosis, la artritis reumatoide, la fibrosis pulmonar y la glomerulonefritis (Werb, 1997; Swaisgood et al., 2000; Busso et al., 1998; Bini and Kudryk 1995; Dano et al., 1985; Mignattii and Rifkin, 1993, 1996; Carmeliet and Collen, 1998; Strickland, 2001; Pepper, 2001 a, b). En el SNC tPA también interviene en diversos procesos patológicos como la degeneración neuronal, la isquemia cerebral o el Alzheimer (Tsirka et al., 1995;

Tsirka, 1997, 2002; Strickland, 2001; Yepes et al., 2002; Yepes and Lawrence, 2004 a, b; Melchor and Strickland, 2005). Estos efectos neurotóxicos de tPA pueden estar asociados a una excesiva producción de plasmina (Chen and Strickland, 1997; Nagai et al., 1999 b; Tsirka 2002), o por su función, independiente de plasmina, como agonista estimulando receptores neuronales o de células de la microglía (Nagai et al., 1999 a; Nicole et al., 2001; Tsirka, 2002; Yepes et al., 2002; Medina et al., 2005)

Las MMPs también interviene en condiciones patológicas como artritis, cáncer, asma, isquemia cerebral, displasias, arteriosclerosis y otras enfermedades cardiovasculares (Galis and Khatri, 2002; Ortega et al., 2003; Luft, 2004; Cunningham et al., 2005).

3.2.1 Progresión tumoral.

No existen diferencias cualitativas en cuanto a los procesos de adhesión/desadhesión celular, migración e invasión de células normales respecto a células tumorales. La única diferencia es que las células tumorales llevan a cabo estos procesos en lugares y momentos totalmente diferentes al comportamiento de las células normales (Werb et al., 1990; Liotta et al., 1991; Van Roy and Mareel, 1992).

La transformación neoplásica es un complejo proceso en el cual múltiples alteraciones genéticas y epigenéticas hacen que células normales pasen a ser tumorales. Una vez establecido, el tumor crece y progresa hacia la invasión y la metástasis. En esta progresión se distinguen diferentes fases como el crecimiento y expansión del tumor primario; seguido de la desadhesión de las células tumorales provocando el escape de las células del tumor primario; la posterior migración e invasión al torrente sanguíneo (intravasación), mediante la degradación de la MEC; el transporte y supervivencia de las células tumorales en el sistema circulatorio; y por último la adhesión, extravasación y crecimiento de estas células tumorales en órganos diana (metástasis) (Chambers and Matrisian, 1997) (**Figura 10**).

Las MMPs intervienen en todos los procesos de la progresión tumoral, en cambio los PAs intervienen en todas las fases exceptuando la extravasación (Chambers and Matrisian, 1997; Rabbani and Mazzar, 2001; Pepper 2001 a, b). La plasmina interviene en la degradación de MEC, la activación de las MMPs y, junto con los PAs, puede activar diversos factores de crecimiento (FGFb, HGF, TGF_{β}, VEGF, PDGF, IGF II), necesarios en diferentes momentos de esta progresión tumoral. A diferencia de las

MMPs, se cree que el sistema de activación del plasminógeno interviene en todos los tipos de cáncer (Romer, 2003; Egeblad and Werb, 2002).

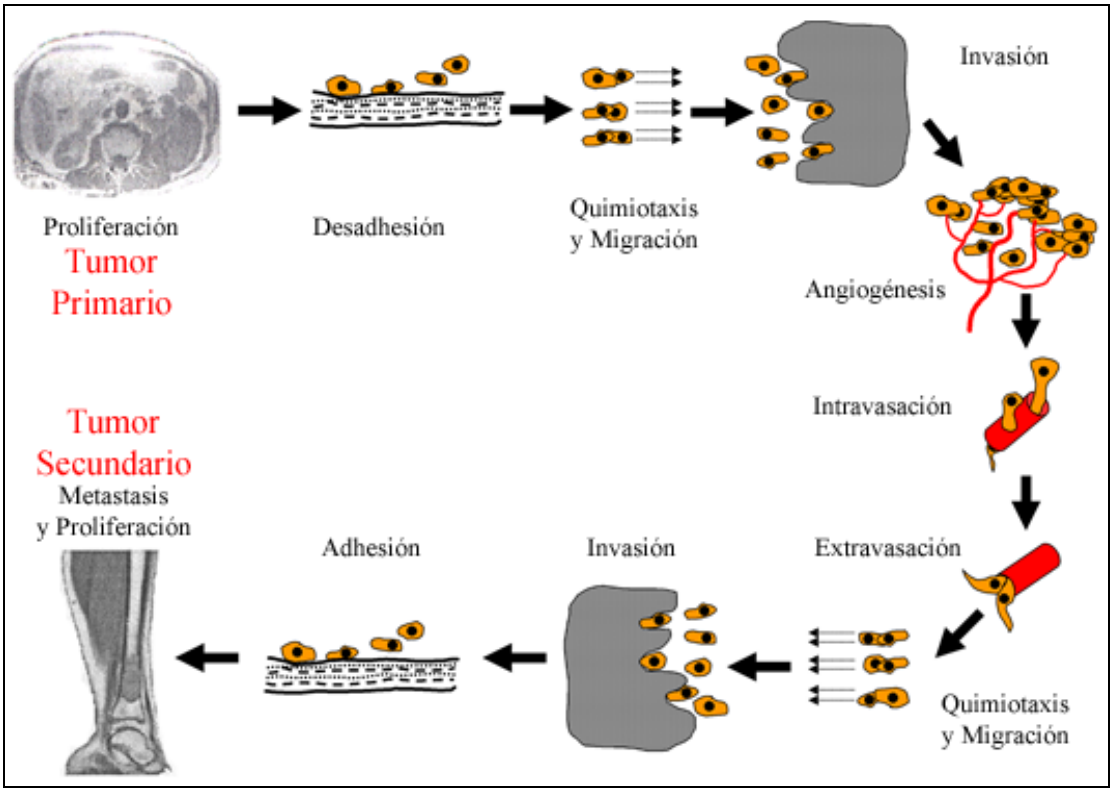

Figura 10. Esquema de la transformación neoplásica.
El proceso de transformación neoplásica, empieza con la proliferación descontrolada de células tumorales, seguida de la desadhesión de éstas y de la migración y posterior invasión mediante la degradación de la matriz extracelular. La inducción de la angiogénesis por parte de estas células, les aporta la posibilidad de recibir nutrientes y de entrar en el sistema circulatorio (intravasación). Una vez en el torrente sanguíneo las células tumorales llegan a los tejidos/órganos diana, por extravasación a través de los capilares, migrando e invadiendo otra vez la MEC y adhiriéndose a los tejidos/órganos diana, en los que finalmente proliferará (metástasis).

En un principio, el activador tisular del plasminógeno estaba únicamente asociado a la fibrinólisis vascular, mientras que la actividad tumoral estaba limitada a uPA. Más adelante se halló que tPA se expresaba en los primeros estadios tumorales de diferentes tipos de tumores como el cáncer de mama (Duffy et al., 1986), en el carcinoma endometrial (Nordengren et al., 1998) y en tumores colorectales (Sier et al., 1991). También se halló relación entre tPA y el fenotipo invasivo y/o metastásico en neuroblastoma (Uchida et al., 2001), melanomas (Kwaan et al., 1988; de Vries et al., 1994, 1995 a), leucemia mieloblástica aguda (Wada et al., 1993) y en cáncer de páncreas (Paciucci et al., 1998 a; Díaz et al., 2002); y un aumento de su concentración en cáncer hepatocelular, carcinomas uterinos y de ovario (De Petro et al., 1998; Saito et al., 1990; Moser et al., 1994).

Numerosos estudios "*in vitro*" e "*in vivo*" indican la implicación de uPA/uPAR en diferentes líneas celulares tumorales y en diferentes tipos de cáncer (Andreasen et al., 1997). Tanto uPA como uPAR se sobreexpresan en diferentes tipos de tumores humanos como leucemias, cáncer de mama, de pulmón, de vejiga, de colon, de hígado, de pleura, de páncreas y tumores cerebrales (Sidenius and Blasi, 2003). Además la síntesis de uPA, se ha asociado a la invasión tumoral (Duffy, 2002, 2004) y altos niveles de uPA, uPAR y PAI-1 son marcadores de mal pronóstico en diversos tipos de cáncer (Schmitt et al. 1997). uPA y uPAR intervienen en un amplio rango de funciones distintas pero interconectadas en el proceso de invasión y metástasis como la proteólisis extracelular, las interacciones célula-célula y célula-MEC y la migración, mediante su actividad quimiotáctica. Estos procesos, comentados anteriormente (*ver apartado 1.3.2*), pueden sufrir una desregulación provocando la proliferación tumoral.

La interacción de las MMPs con los activadores del plasminógeno, en relación a la progresión tumoral, se ha descrito en diversas líneas tumorales como: i) en células de cáncer de mama (Garbett et al., 2000); ii) en células de carcinoma tiroideo (Smit et al., 1999); iii) en células de glioma (Abe et al., 1994); iv) en células de cáncer de colon (Shah et al., 1994); viii) y en células de cáncer de ovario (Moser et al., 1994).

También se ha descrito extensamente la relación del EGFR con los PAs y sus receptores en procesos tumorales. Los receptores uPAR y EGFR están relacionados en la transmisión de la señal proliferativa en diversos artículos. En algunos de estos trabajos EGFR actúa como transductor de la señal de uPAR "*downstream*" (Jo et al., 2002; Liu et al., 2002; Monaghan-Benson, 2006), mientras en otros es uPAR el que actúa "*downstream*", transmitiendo la señal proliferativa que proviene de la activación del EGFR, (Jo et al., 2007). También se ha descrito que la unión del EGF a su receptor induce la expresión y la transducción del uPAR, elevando así la capacidad invasiva de las células (Mamoune et al., 2004; Henic et al., 2006). EGFR también interviene como transductor de la señal de tPA en varios procesos tumorales como la activación de la β-catenina (Maupas-Schwalm et al., 2005) o en la activación de la proliferación (ver capítulo V).

3.2.1.1 Cáncer de Colon.

El tracto intestinal tiene origen endodérmico y está formado por tres capas tisulares: i) una capa externa de musculatura lisa, encargada de la peristalsis; ii) una capa intermedia de tejido estromal; iii) y una mucosa interna, formada por células

epiteliales, que procesa y absorbe los nutrientes. El tracto intestinal está formado por el intestino delgado, dividido en duodeno, yeyuno e ílion, y por el intestino grueso. El intestino delgado, tiene una superficie de absorción de nutrientes muy amplia debido a las protuberancias luminares (villi) y a las invaginaciones en la submucosa (criptas de Lieberkühn). El intestino grueso también presenta criptas, y en lugar de vili, está formado por una superficie epitelial lisa de células epiteliales. El epitelio intestinal padece un recambio celular muy alto, por lo que su homeostasi está perfectamente regulada. Este alto recambio celular se debe a la presencia de "*Stem Cells*", en la base de las criptas en el intestino grueso (Sancho et al., 2004).

El cáncer de colon es el segundo tipo de cáncer más común. Este cáncer tiene un componente social muy elevado, ya que un 65% de los casos se da en paises desarrollados. Otro factor de riesgo elevado es el hereditario ya que el 20% de todos los pacientes con cáncer de colon, tiene un familiar con la misma enfermedad (Lynch and de la Chapelle, 2003). Los síndromes hereditarios de este cáncer están divididos en dos grupos, según la presencia o no de pólipos. La poliposis adenomatosa familiar (FAP), que presenta pólipos, y el cáncer colorectal hereditario sin poliposis (HNPCC), que no presenta estos pólipos. La FAP se caracteriza por un elevado número de adenomas, precursores de los carcinomas en el colon, y por mostrar inestabilidad cromosómica, cariotipo aneuploide y mutaciones en oncogenes (*K*-ras) y genes supresores de tumor (*p53* y *APC*). El HNPCC suele localizarse en el recto proximal, manifestándose entre los 40 y 45 años, y muestra inestabilidad de microsatélites que provocan mutaciones en diferentes genes como *MSH2, MLH1, MSH6, PMS1, PMS2* y *TGFBR2* (Sancho et al., 2004).

El estudio de las diferentes alteraciones genéticas en los diferentes estadios de la progresión tumoral, ha permitido crear un modelo de evolución tumoral, desde el epitelio normal, pasando adenoma, hasta llegar a carcinoma. En un primer estadio se da la activación mutacional de oncogenes y la inactivación de los genes supresores de tumor; seguidamente mutan genes que hacen que el tumor pase a ser maligno; y por último, se producen una serie de alteraciones genéticas que provocan una acumulación de cambios en un orden cronológico que determinan las propiedades biológicas del tumor (**Figura 11**) (Fearon and Vogelstein, 1990). Los últimos estudios muestran que es este cáncer no solo intervienen mecanismos oncogénicos, sino que también participan moléculas y vías fundamentales para la homeostasis fisiológica como WNT, TGF-β, BMP, K-RAS/B-RAF, LKB, Notch/bHLH y Hedgehog (Sancho et al., 2004).

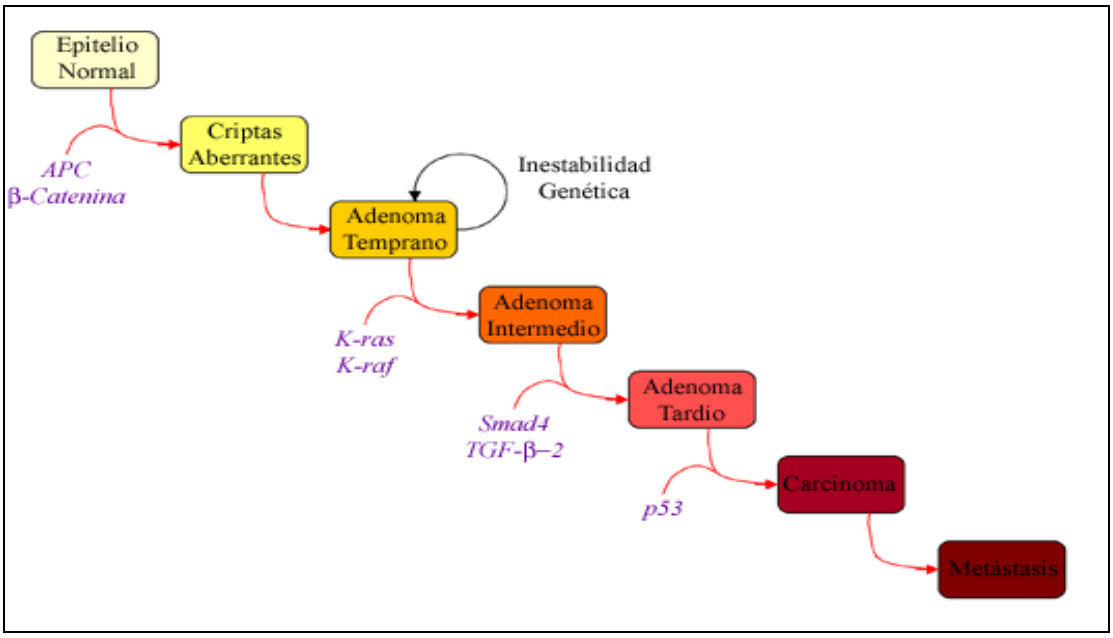

Figura 11. Modelo de evolución tumoral, desde el epitelio normal hasta el carcinoma metastásico Las primeras mutaciones se dan en genes como *APC,* y causan el paso de epitelio normal a criptas displásicas aberrantes. Estas criptas aberrantes evolucionan a adenomas de gran tamaño que llevan consigo mutaciones como la del oncogen *K-ras.* Esta mutación va seguida de alteraciones en los genes *SMAD-4* y TGFβR2, que proporcionan características malignas adicionales a las células del adenoma. Este adenoma sigue progresando, dándose posteriormente la mutación en *p53* que provoca su evolución a carcinoma "*in situ*". Este carcinoma es invasivo, presenta atipia celular y desmoplasia, y acaba provocando metástasis en otros tejidos (Fearon and Vogelstein, 1990).

Las células de cáncer de colon presentan una gran habilidad para invadir y dispersarse debido a la pérdida de sus propiedades adhesivas (Streit et al., 1996; Agrez and Bates, 1994; Citi, 1993). Esta pérdida de adhesión al substrato y aumento de la movilidad celular, que recibe el nombre de "*scatter*", puede ser inducida por el HGF o por PMA, en células de cáncer de colon (Herrera, 1998; Fabre and García de Herreros; 1993). uPA/uPAR, que se sobreexpresan durante la transición de adenoma a carcinoma (De Bruin et al., 1987, 1988; Wang et al., 1994; Cantero et al., 1997; Park et al., 1997; Skelly et al., 1997; Suzuki et al., 1998; Taniguchi et al., 1998), pueden jugar un papel vital en el mecanismo de activación del "*scatter*" , ya que se ha descrito que tanto el HGF como el PMA estimulan su expresión (Pepper et al., 1992; Lund et al., 1995; Jeffers et al., 1996; Paciucci et al., 1998 b), creándose una retroalimentación positiva, dada la capacidad de uPA de activar HGF a partir de pro-HGF (Naldini et al., 1992). A partir de estos estudios previos, en el capítulo III de esta tesis, se estudian: i) las diferentes fases del "*scatter*"; ii) las vías de señalización celular que lo inducen; iii) y la importancia del sistema plasminógeno/plasmina en este efecto.

3.2.1.2 Cáncer de Páncreas.

El páncreas es un órgano endodérmico dividido en una parte exocrina (80% masa tisular) que regula la digestión de proteínas y carbohidratos; y otra parte endocrina que controla la homeostasis de la glucosa. El páncreas exocrino está formado por células acinares, que forman acinos, y células ductales. Estos dos tipos celulares producen enzimas, iones y fluidos que forman el jugo pancreático que se secreta al tracto gastrointestinal. El páncreas endocrino está formado por diferentes tipos de células que secretan insulina, glucagón, somatostatina y el polipéptido pancreático. Estas células endocrinas están agrupadas formando los islotes de Langerhans (Kern, 1993; Bardessy and DePinho; 2002; Hezel et al; 2006)

La gran diversidad fisiológica y celular del páncreas hace que este órgano pueda padecer un amplio espectro de patologías, como el cáncer. El adenocarcinoma ductal pancreático (PDCA) representa más del 85% de neoplasias pancreáticas (Warshaw and Fernández-del Castillo, 1992; Li et al., 2004) y es la cuarta causa de muerte por cáncer en Estados Unidos, con una supervivencia media menor a seis meses y una tasa de supervivencia a los cinco años de entre el 3%-5% (Hezel et al., 2006). El PDCA es tan devastador y agresivo debido a la dificultad de su diagnóstico en estadios tempranos, a la facilidad de diseminación tumoral, al no ser un órgano encapsulado, y a la falta de terapias exitosas.

Diversos estudios muestran que la avanzada edad, el tabaquismo y la pancreatitis crónica, incrementan el riesgo de padecer cáncer de páncreas, mientras que la diabetes y la obesidad, pueden afectar en menor medida (Everhart and Wright, 1995; Fuchs et al., 1996; Gapstur et al., 2000; Michaud et al., 2001; Berrington de González et al., 2003; Stolzenberg-Solomon et al., 2005). También se ha estudiado que la predisposición a padecer este tumor por factores genéticos es inferior al 10% (Schenk et al., 2001; Petersen and Hruban; 2003).

El PDCA aparece en la cabeza del páncreas con infiltración en los tejidos que rodean esta glándula, y con metastasis en hígado y pulmones. Estudios clínicos e histopatológicos han identificado tres lesiones precursoras del PDCA: i) PanIN (neoplasia intraepitelial pancreática), la más estudiada y común; ii) MCN (neoplasia mucinosa enquistada) y iii) IPMN (neoplasia mucinosa papilar intraductal) (Brugge et al., 2004; Maitra et al., 2005). Estas lesiones derivan en el PDCA, que se clasifica histológicamente en tres grados. Los tumores de grado I presentan lesiones tipo PanIN-

1, con células polarizadas y una lámina basal perfectamente diferenciada; los de grado II, que presentan lesiones tipo PanIN-2 mostrando una pérdida de polaridad celular y desorganización de la membrana basal; y los de grado III formados de agregados celulares polimorfonucleados y ya sin lámina basal (PanIN-3) (Kern and Elsässer, 1993; Brat et al., 1998; Hruban et al., 2001; Hezel et al., 2006). La progresión tumoral en el páncreas es muy similar a la ya descrita anteriormente en los tumores de colon (Kinzler and Volgelstein, 1996) (**Figura 11**).

Los análisis moleculares de PDCA muestran un compendio de alteraciones genéticas que se dan en los diferentes estadios de la progresión de este adenocarcinoma. Las alteraciones más estudiadas son las del oncogen *K-ras*, que afecta a las vías de señalización MAPK, PI3K y GTPasas y las de los genes supresores de tumor *INK4A, ARF, p53, SMAD4/DPC4* y *LKB1/STK1*. Además de estas alteraciones genéticas los PDCA también se caracterizan por la sobreexpresión de FCs y de sus receptores como: EGF/TGF α y EGFR, IGF e IGF-IR, c-met y HGF, FGF y FGFR y por último VEGF y VEGFR-1y-2 (Hezel et al., 2006).

El trabajo realizado en los capítulos IV y V de esta tesis, parten del hallazgo de un incremento en la expresión del gen *tPA*, entre otros, en una línea tumoral pancreática respecto a su expresión en tejido y líneas celulares pancreáticas normales (Paciucci et al., 1996). Este resultado se corroboró con la determinación de un incremento de tPA (mRNA y proteína) en células tumorales respecto a las normales, interviniendo en procesos de invasión (Paciucci et al., 1998 a). En estas mismas células tumorales, se halló una elevada expresión de AnnII en la membrana basolateral (Paciucci et al., 1998 a), lo que sugería que, como en las células endoteliales (Hajjar et al., 1994), AnnII podía actuar como receptor de tPA, focalizando la invasión (ver capítulo IV). Estos resultados preeliminares, indicaban que tPA y sus receptores, podían tener un papel determinante en la progresión tumoral del cáncer de páncreas, por lo que nuestro grupo siguió investigando la contribución de tPA sobre la proliferación, apoptosis, angiogénesis, invasión y movilidad. Para ello, se utilizaron diferentes estrategias como secuencias antisentido (AS), un inhibidor químico específico de tPA ("*Pefabloc*"), la proteasa recombinante (rtPA) y por último, el sistema de expresión inducible "*Tet-Off*", que permite generar clones celulares con expresión de tPA inducible y estudiar su comportamiento in vitro e in vivo en ratones inmunodepromidos (Díaz et al., 2002). Los resultados obtenidos mostraban en primer lugar un efecto mitogénico inducido por tPA en las células de cáncer de páncreas. Una vez demostrada la importancia de tPA en la

capacidad mitogénica de estas células, se determinó que este efecto necesitaba la actividad proteolítica de tPA. Estos hallazgos fueron reforzados por los resultados obtenidos en los ensayos "*in vivo*" en ratones inmunodeprimidos, en los que se formaban tumores al inyectarles clones de células que sobreexpresaban tPA (Díaz et al., 2002). En este mismo estudio se confirmó, que tPA estimula la invasión, y que este efecto es mayor en presencia de plasminógeno, indicando que la plasmina, generada vía tPA es un activador de la invasión en estas células. Por último, y tal y como sugerían otros trabajos (Sheng et al., 1998; Stack et al., 1999), se comprobó que tPA estimula la neoangiogénesis asociada a tumor, siendo esta proteasa necesaria para la formación y maduración de los vasos sanguíneos, pero no para el reclutamiento de las células endoteliales (Díaz et al., 2002).

El mecanismo por cual tPA lleva a cabo su efecto proliferativo ha sido el objeto de estudio de la segunda parte de esta tesis (capítulos IV y V)

CAPÍTULO II: Objetivos

En los últimos años, nuestro grupo ha definido la participación de las proteasas uPA y tPA en diferentes momentos de la progresión tumoral en colon y páncreas, respectivamente. A partir de los hallazgos mencionados en la *Introducción*, los objetivos planteados en la presente tesis han sido los siguientes:

1. **Analizar la contribución del sistema de activación del plasminógeno al *"scatter" "in vitro"* estimulado por ester de forbol (PMA) en células de cáncer de colon HT29-M6:**

   Analizar el papel y el mecanismo de acción de las proteasas uPA y plasmina.

2. **Determinar el mecanismo por el cual tPA promueve la progresión del cáncer de páncreas.**

   a. Analizar la existencia de receptor/es específico/s de membrana capaz de trasmitir la señal de tPA en las células tumorales.

   b. Dilucidar el mecanismo por el cual tPA activa la proliferación en células tumorales pancreáticas.

CAPÍTULO III: Requirement of the enzymatic and signaling activities of plasmin for phorbol ester-induced scattering of colon cancer cells

Capítulo publicado como:
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ABSTRACT

Colon cancer progression is associated with the activation of protein kinase C (PKC), the downregulation of functional E-cadherin, and an increased expression of the serine protease urokinase (uPA) and its receptor (uPAR). HT29-M6 intestinal epithelial cells represent an in vitro model to study colon cancer progression. These cells are induced to scatter and to invade by phorbol esters. Using proteolytic and cell signaling inhibitors, we show that HT29-M6 cells require plasminogen for the acquisition of the scattering response to PMA. Our results indicate that, prior to inducing a state of competency for plasminogen-dependent scattering, PMA triggers an ordered succession of events where upregulation of the activity of uPA preceeds proteolysis of uPAR and active degradation of the extracellular matrix (ECM). These events poise HT29-M6 cells to a scatter-competent state that allows the subsequent localized proteolytic activation of plasminogen to plasmin, required for the execution of scattering. Finally, we show that, in addition to its enzymatic activity directed at the degradation of ECM, plasmin generates an intracellular signal resulting in the phosphorylation of ERK 1/2. For a full motogenic activity, plasmin requires this signal, since the use of a MEK inhibitor (PD98059) specifically blocks the plasmin-dependent phase of cell scattering. Our observations suggest that plasmin exerts a dual role in PMA-induced scattering of HT29-M6 cells, one directed extracellularly to promote proteolysis of the ECM, and one directed to generate intracellular signaling.

INTRODUCTION

Cell migration participates in numerous physiological and pathological processes that involve cyclic events of regulated adhesion and detachment of cells to extracellular matrix substrates and the secretion and activation of proteolytic enzymes [1]. The serine proteases urokinase-type plasminogen activator (uPA) and tissue plasminogen activator (tPA) activate plasminogen to plasmin, a protease that plays a prominent role in such processes. Traditionally, the role of tPA has been in fibrinolysis and that of uPA in cell migration, especially in cancer cells [2,3]. uPA is initially released from the cells as pro-uPA, whose proteolytic activity is stimulated upon binding to the receptor uPAR, to enhance the generation of plasmin on the cell surface from nearby bound plasminogen. Plasminogen is ubiquitously distributed and binds, through its kringle domains, to lysine residues of surface proteins. Plasminogen and plasmin bound to the cell surface are protected from inhibition by physiological inhibitors like alpha2-antiplasmin but can be easily displaced by the presence of ε-aminocaproic acid (EACA) that binds to lysine binding sites. uPAR itself can be proteolyzed by uPA, plasmin or metalloproteinases, resulting in the exposure of a chemotactic epitope that activates intracellular signaling [4-6].

Several studies in colon cancer have reported a significant association between high levels of uPA/uPAR, and metastatic disease with a poor prognosis [3]. The goblet cell-like HT29-M6 (M6) human colon carcinoma cell line is a validated model to study molecular mechanisms that control the differentiation process of intestinal epithelial cells [7,8]. Treatment of M6 cells with phorbol 12-myristate-13-acetate (PMA) activates PKC alpha (cPKCα), promoting a rapid change from epithelial to a fibroblastic-like phenotype with the functional inactivation of E-cadherin, overexpression of uPA and a motogenic effect, or scattering [9-12]. These effects have been associated with the known activation of cPKCα by endogenous and dietary factors (such as bile acid and free fatty acids) that may be implicated in colorectal tumorigenesis [13].

In this report we have investigated the contribution of the plasminogen activator system to phorbol ester-induced motility of M6 cells. We show that the motogenic effect of PMA depends on the proteolytic activation of plasminogen to plasmin via uPA, which results in degradation of the extracellular matrix. Furthermore, we show

that plasmin may trigger an intracellular signal that involves the phosphorylation of ERK1/2 kinases. Therefore, our observations indicate that plasmin exerts a dual role in PMA-induced scattering of M6 cells, one directed toward the degradation of the ECM, and one directed intracellularly to activate a signal cascade required for enhanced cell motility.

EXPERIMENTAL PROCEDURES

Cells and reagents. HT29-M6 cells were kindly provided by A. Garcia de Herreros (IMIM, Barcelona, Spain) and were cultured as previously described [8]. Rabbit anti-uPAR antibody and purified D2D3 were obtained as described before [5,6]. PAI-1 was kindly provided by Dr. N. Booth (University of Aberdeen, UK). Anti-β1 integrin monoclonal antibody (LIA1/2) was kindly provided by Dr. F. Sánchez-Madrid (Hospital de la Princesa, Madrid, Spain). Anti E-cadherin antibody (HECD-1) was from Zymed Laboratories Inc. (San Francisco, CA), neutralizing rabbit anti-uPA and goat anti-tPA antibodies from American Diagnostica (Greenwich, CT), and antibodies to total or phosphorylated ERK 1/2 from Upstate Biotechnology (Lake Placid, NY). PMA, pertussis toxin, amiloride, ε-amino caproic acid (EACA), and 1,10-phenantroline were from Sigma Chemical (St. Louis, MO) and were used at the concentration indicated. E64, leupeptin, aprotinin and pepstatin were from Sigma. Galardin (GM 6001) was from Calbiochem (San Diego, CA). Protein A/G sepharose beads, purifed plasmin, Glu-plasminogen and bisindolylmaleimide (GF109203X) were from Roche (Mannheim, Germany). PPI, PD98059 and LY294002 and Tyrphostin AG1478 were from Biomol (Butler Pike, Pennsylvania), gelatin from Merck (Darmstadt, Germany), and recombinant human hepatocyte growth factor (HGF) from R&D Systems (Minneapolis, MN).

Scatter assays. Cells were seeded at $1x10^4$ cells/cm^2 (low density) or $3.5\ x10^4$ cells/cm^2 (high density) in complete medium and cultured for 24 h. Cells were serum starved during 24 h and pre-incubated 2 min at room temperature with 2 mM EACA in PBS to remove serum-derived plasminogen associated with cell membranes [14]. After extensive washes with PBS, cells were treated with PMA (10 nM), or HGF (50 ng/ml), and Glu-plasminogen (5 μg/ml), with or without inhibitors or antibodies, and scattering was evaluated 16 h later under an Olympus inverted phase contrast microscope (Hamburg, Germany). Alternatively, cells were treated with PMA (10 nM) for 6 hours, rinsed with DMEM and treated with plasminogen (2 μg/ml) or plasmin (0.05 U/ml). Photographs were taken at a magnification of x200 or x400 using an Olympus camera.

Quantification of cell scattering. To quantify the cell scattering, we used a modification of our earlier approach [15]. Images were downsampled 50% and then automatically thresholded as described elsewhere [16]. Since DIC imaging emphasizes

edges, the resulting binary mask required processing to fully capture the cellular regions. This was achieved by 5 cycles of closing, 4 cycles of erosion, and 2 cycles of dilation (for details on these standard image processing functions see [17]. Cell clusters were then identified using connected component analysis as previously described [15]. The mean number of pixels in each cluster was computed. As a measure of the degree to which each cell cluster was scattering, the cluster density was computed. The cluster density is the result of the division of the number of pixels occupied by cells within the cluster by the total number of pixels in the bounding rectangle containing the cluster. A higher percentage represents more densely packed cells, while lower percentage represents more dispersed cells. Multiple fields and several replica points for each treatment were used to determine the number of clusters per image (mean cluster density) and their average size in pixels (mean cluster size).

Zymography. The proteolytic activity of conditioned medium was analyzed in gelatin-containing gels with or without plasminogen, as described previously [18,19]. Equal protein loading was achieved by normalization to protein concentration in the cell lysates.

Extracellular matrix degradation assay. Extracellular matrix was obtained from confluent M6 cells seeded on 24 well plates that were labelled overnight with (^{35}S)-methionine/cysteine (80 μCi/ml), ((^{35}S)Trans Label, ICN Costa Mesa, CA) in medium without methionine or cysteine (GIBCO-BRL, Gaithesburg, NY). Cells were rinsed extensively with PBS, EACA treated, and lysed with 1 mM EDTA in distilled H_2O. M6 cells ($3x10^4$) were plated on radiolabelled ECM and treated with different factors and inhibitors in serum free medium. Radioactivity released to medium was monitored using a liquid scintillation counter.

Western blotting. Cells were scraped in lysis buffer (50 mM Tris, 0.1% Triton X100, 5 mM EDTA, 250 mM NaCl) containing phosphatase inhibitors (1 mM sodium othovanadate, 50 mM sodium fluoride) and 1 mM PMSF and protease inhibitor cocktail (Sigma). For electrophoresis, equal amounts of proteins (50 μg) were loaded in each lane. After transfer to PDVF membranes and incubations with antibodies, reactivities were developed with ECL chemiluminiscent substrate (Amersham, Buckinghamshire,

England). For sequential Western blotting, membranes were stripped at 50 ^{o}C according to the manufacturer's instructions.

RESULTS

Active plasmin is required for PMA-induced scattering of HT29-M6 cells.

Cell scattering in M6 cells is induced by PMA at concentrations of 10-200 nM; in the presence of serum, this effect becomes evident at around 6 h [9-11]. Hepatocyte growth factor (HGF) also promotes scattering in the parental HT29 cells [20]. To assess the contribution of the plasminogen activator system to the cell scattering promoted by PMA, experiments were performed in conditions in which plasminogen was completely depleted from cell membranes by pre-elution with the lysine analog EACA. M6 cells were subsequently treated with PMA (10 nM) in the absence of serum during 16-24 h. Under these conditions, cells underwent only a limited scatter, most cell clusters were not disrupted and only cells at the edges of the colonies showed a morphology consistent with a motile phenotype (**Figure 1A, d**). Similar results were obtained using HGF as a motogen (50 ng/ml) (**Figure 1A, g**). The addition of physiological concentrations of Glu-plasminogen (5 μg/ml) [21] but not of fibronectin or laminin (not shown), allowed the full scattering of M6 cells stimulated by PMA or HGF, to a degree comparable to scattering in the presence of complete medium (**Figure 1A, e and h**). In these conditions, cells acquired a fibroblastic aspect with numerous lamellipodial protrusions (**Figure 1 B, a**). The action of plasminogen on cell scattering was reversed by the cell surface plasminogen competitor EACA in both PMA and HGF treated cells (**Figure 1A, f and i**), indicating the contribution of membrane-associated plasmin to the motile phenotype. The addition of plasminogen, in the absence of PMA or HGF, did not affect in other ways the phenotype of the cells (**Figure 1 A, b**). Quantitative analysis of the scattering produced by the addition of plasminogen was performed using a modified Blob algorithm [16] based on numerical quantification of cell clusters (see Materials and Methods). The method identifies non-scattered cells as single clusters, whereas scattered cells present as numerous clusters. For each condition, several replica points from three independent experiments identical to those shown in **Figure 1A** were used to determine the number of clusters per image and their average size in pixels. **Figure 1B** shows the average values for the cluster size obtained in each condition. Scattered cells, as those shown in **Figure 1A, e**, exhibit a significantly smaller cluster size compared to non-scattered cells treated only with PMA, or cells treated with PMA, Plg and EACA (**Figure 1A, d and f**, respectively) that are present as unified, large clusters, containing

many cells. The number of cells per cluster (mean cluster density) was also determined from the same images. The average values for each condition are shown on the right panel in **Figure 1B**. Treatment of cells with PMA or HGF in the presence of plasminogen caused a significantly diminished cluster size and density (**Figure 1B**), corresponding to visibly scattered cells (**Figure 1A, a**), as compared to cells treated with PMA only (**Figure 1A, d**) or treated with PMA, Plg and EACA (**Figure 1A, f**) that have significantly higher cluster size and density (**Figure 1B**). These data confirm that plasmin, generated upon binding of plasminogen to the cell surface, is required for a full scatter phenotype of cells.

We further observed that, on cells plated at higher density, PMA and plasminogen induced their organization as coherent sheets that connected with each other forming a "cord-like" network (**Figure 1C, c**). These results indicate that the motogenic factors PMA and HGF require plasminogen to induce cell dispersion.

We next studied if plasminogen needs to be activated to plasmin in order to participate in the scattering induced by PMA. Neutralizing antibodies to uPA, but not to tPA, treatment with amiloride, an inhibitor of uPA activity that does not affect tPA or plasmin [22], aprotinin (not shown) or the physiological inhibitor PAI-1 [2] blocked cell scattering induced by PMA and plasminogen (**Figure 2A**). In contrast, the addition of the wide-range matrix metalloproteinase inhibitor Galardin (GM6001) [23], aspartyl (E64) and cystein (pepstatin and leupeptin) protease inhibitors showed no effects (**Figure 2A**, and not shown). The corresponding quantitative cluster size and density determinations are shown in Fig 2B. Inhibition of scattering by amiloride is less pronounced compared to PAI-1 or antibody to uPA, suggesting that residual uPA activity is present. As expected, Galardin and anti tPA antibody do not inhibit cell scattering. These data demonstrate that metalloproteinases and tPA activities do not contribute significantly to cell scatter and confirm our previous results that plasmin, produced via uPA, is the protease required for the PMA-triggered scattering of HT29-M6 cells.

uPA is expressed at low levels in unstimulated M6 cells, but can be induced by PMA [12], as well as by other motogenic stimuli in a number of cell types [18,24-26] Therefore, we examined the time-course of induction and secretion of uPA activity by

PMA in M6 cells by gelatin zymography. As shown in **Figure 3A**, PMA stimulated the release of uPA activity, which reached maximum levels at 16 h of treatment, concomitant with the time of appearance of cells with a fully scattered phenotype. To demonstrate that active plasmin is generated only when cells are pretreated with PMA, plasmin generation was analyzed by zymography (**Figure 3B**). The results show that plasminogen added to cells is activated to plasmin only when PMA is present. We conclude that PMA induces uPA secretion, which in turn proteolytically activates membrane-associated plasminogen to plasmin, whose enzymatic activity is required for enhanced cell motility.

Cell scatter associates with ECM proteolytic degradation but not with proteolysis of uPAR or its association to β1 integrins.

For scattering to occur, at least three distinct processes must take place: cell-to-cell adhesions are lost, cells become more motile through cycles of attachment-detachment to substrate, and extracellular matrix proteins are proteolytically degraded. An early effect of the activation of PKC by PMA on M6 cells is the downregulation of functional (cytoskeleton-associated) E-cadherin [9]. However, the decrease of functional E-cadherin promoted by PMA is not prevented by inhibitors of uPA or plasmin (not shown). Therefore, disruption of cell-cell contacts precedes uPA induction by PMA and is independent of proteolytic activites of the plasmin system.

The motogenic response of many cells is mediated by uPAR through different mechanisms. In one mechanism, proteolyzed uPAR acts as a chemotactic receptor that can trigger a migratory response [4-6]. Cell scattering by PMA and activation of the uPA/plasmin system is tightly associated with the appearance of cleaved forms of uPAR (not shown). However, the addition to cells of soluble proteolyzed uPAR, (purified D2D3, active in chemotaxis [5,6]) is not sufficient to promote the scattering of PMA treated cells in the absence of serum or plasminogen (not shown) indicating that additional factors are at play. In a second mechanism, an increased binding of uPA with uPAR favours the association of the latter wih β1 integrins in a complex that modulates the cyclic attachment and detachment of the cell to the substrate [27]. We observed that the interaction between uPAR and integrin β1, as determined by co-immunoprecipitation, was not affected by treatment with PMA (not shown), indicating

that this association is not a target of modulation in the PMA/plasminogen induced motogenesis.

Finally, we determined if the plasminogen-dependent scattering of M6 cells is accompanied by ECM degradation. For this, cells were seeded on plates containing radioactively labeled ECM, previously depleted of plasminogen. After treatment with PMA, or PMA plus plasminogen, proteolysis of ECM was monitored at 4 and 16 hours (**Figure 4**). Four hours after the initiation of the treatments, no significant differences in ECM release were observed under any condition. At this time, loss of functional E-cadherin has already taken place [10] and uPA begins to be detectable in the culture medium (**Figure 3**), but cell scattering was still not observed (not shown). After 16 h of treatment, when full cell scattering was observed in cells treated with PMA and plasminogen, there was an increase in the levels of radioactivity released to the culture supernatant, indicative of ECM degradation (**Figure 4**). This increase was completely inhibited by the addition of EACA, but not by the metalloproteinase inhibitor 1,10-phenantroline. Cells treated with PMA alone did not show degradation of ECM above that of control cells. The addition of the uPA inhibitor amiloride partially inhibited ECM degradation induced by PMA and plasminogen, suggesting the presence of residual uPA not completely inhibited by this treatment (**Figure 2B**). These results indicate that the ECM degradation during the scattering of M6 cells induced by PMA is due to the proteolytic activity of plasmin generated at cell surface sites and lend additional support to the conclusion that enzymatically active uPA and plasmin, but not metalloproteinases, are required for enhanced cell motility.

Plasmin triggers ERK 1/2 activation that is necessary for M6 cell scattering

Several components of the plasminogen system can induce intracellular signaling [28-31]. The scattering of M6 cells exposed to PMA and plasminogen was completely inhibited by the incubation with the MEK inhibitor PD98059 (50 μM) (**Figure 5A, e**) and partially inhibited by the G protein inhibitor pertussis toxin (400 ng/ml), or the specific Src kinase inhibitor PP1 (30 μM) (**Figure 5A, d and f**), but was not affected by the phosphatidylinositol-3 kinase inhibitor LY294002 (30 μM) (**Figure 5A, c**). The corresponding quantitive cell scattering analyses are shown in **Figure 5B**. The inhibition of scatter by ERK inhibitors is significant both for cluster size and cluster

density. By comparison, the inhibition of scattering obtained by PP1 and pertussis toxin, was significant only for cluster size determinations, an indication that these factors are partial inhibitors of cell scattering.

We have investigated if ERK 1/2, Src and cPKCα are involved in the late, plasmin-dependent phase of cell scattering or are required only at initial stages of the process. Cells were treated with PMA for 12 h in the absence of plasminogen to down-regulate cPK-Cα [11] and, after removal of PMA, residual PKC activity was further blocked by treatment with GF109203X for 4 h [9,12]. M6 cells thus treated have disrupted their cell-to-cell homotypic contacts and promoted the secretion of uPA, but they did not undergo scattering (**Figure 5C, b**). Plasminogen added at this stage triggered cell scattering and the formation of cord-like lattices (**Figure 5C, c**), confirming that PKC activation induces early events associated with scattering, but is no longer required at later stages.

We further observed that following the complete downregulation of PKC by the above treatments, the addition of PD98059, but not PP1 or AG1478 (a specific inhibitor of the EGF receptor kinase activity), blocked the scattering induced by plasminogen (**Figure 6A**). These results suggest that the ERK 1/2 pathway is actively involved in the plasmin-dependent phase of scattering, but the EGF receptor pathway, one of the major contributors to the activation of ERKs, is not involved. In addition, they indicate that Src kinase activity is required for the early events induced by activation of PKC but not for the plasmin dependent-phase of scattering. Quantitative scattering analysis confirmed these observations (**Figure 6B**). Thus, cells treated with vehicle, AG1478 or PP1 (**Figure 6A, a, c and d**, respectively) have a significantly lower cluster size (left panel) and cluster density (right panel) as compared with cells treated with PD98059 (**Figure 6A, b**).Therefore, ERK activity is necessary for cell scattering, and Src and EGF receptor activities are not required for the plasmin-dependent phase of PMA-triggered scattering.

To confirm that ERK activation is necessary for cell scattering, we investigated if the addition of plasmin to PKC-depleted cells can induce the phosphorylation of ERK 1/2. **Figure 6C** shows that PMA-treated cells in which PKC was downregulated have low levels of residual ERK 1/2 phosphorylation, comparable to control untreated cells

or to cells pretreated with plasminogen (**Figure 6G**). The addition of plasmin to cells thus treated stimulated ERK 1/2 phosphorylation in a time-dependent manner with a maximum peak at 15 min. Furhermore, incubation with PD98059 significantly inhibited both cell scattering (**Figure 6D and E**) and plasmin-induced ERK 1/2 phosphorylation (**Figure 6F**). In contrast, plasminogen added to PMA pre-treated cells did not induce ERK1/2 phosphorylation (**Figure 6G**), indicating the requirement for active plasmin for this action.

Finally, we tested that ability of plasmin to act directly on cell scattering without pre-treatment with PMA. For this, cells were seeded at low density, such that cell-to-cell contacts were minimal, and plasmin (0.05 U/ml) was added during 16 hours (**Figure 7**). The results show that plasmin promoted cell scattering. At short time treatments, plasmin induced ERK 1/2 phosphorylation that was dependent on MEK activity (**Figure 7**, lower panels). Therefore, plasmin has a motogenic effect on M6 cells that requires intracellular activation of the ERK 1/2 kinases.

In conclusion, our experiments show that the scattering of HT29 M6 cells induced by phorbol esters proceeds as an ordered sequence of events: downregulation of E-cadherin and disruption of cell-cell homotypic contacts; induction and secretion of uPA; proteolytic activation of plasminogen to plasmin by uPA; proteolysis of uPAR; degradation of the extracellular matrix by plasmin. In addition, an intracellular signal generated by plasmin is required to achieve its full cell scattering effect.

DISCUSSION

The constitutive activation of PKC in the colon carcinoma cell line HT29 M6 induces a highly motile phenotype in vitro and a potent capacity for invasion in vivo [12]. In the present work, we demonstrated that full dispersion of M6 cells by the activation of PKC is only observed in the presence of medium containing plasminogen and active plasmin, generated via uPA, and active ERK 1/2 are required for full cell spreading. At least two temporally distinct phases can be distinguished in this scatter event. In the first phase, cells appear flattened but do not spread out from the colonies, the functional E-cadherin is down modulated and there is a requirement for Src activity, in agreement with previous observations [10,12,32]. Plasmin or uPA activities are not required at this stage. In the second phase, starting approximately 4 h after PMA addition, uPA activity is induced and, in the presence of plasminogen, the ECM is proteolyzed and colonies undergo complete dispersion. The latter event is not longer dependent on PKC activity but it is dependent on the activities of plasmin and MEK.

Malignant transformation is often characterized by the disruption of cell-to-cell contacts [33,34]. The down modulation of inter-cellular contacts by motogenic factors has been extensively studied [9-12,18]. In M6 cells, PMA-induced downregulation of functional E-cadherin is a rapid event observed after 2 h of PMA treatment [10]. Secretion of uPA by PMA becomes detectable after 4 hours of treatment, indicating that the requirement of this protease occurs in a later phase of the scattering event. The observation that the inhibition of plasmin, or uPA, in scattering cells does not affect the downregulation of E-cadherin also supports this notion.

The scattering effect by PMA on M6 cells was first described in medium containing serum [9]. Plasminogen, which is present in serum and ubiquitously distributed, can bind to numerous cell surface proteins [14,21,35]. That plasminogen is required for a complete cell scatter effect of PMA was shown in our study by removing it from the cell surface with EACA, and confirmed using blocking antibodies or specific inhibitors. Additional experiments indicate that, in scattering cells, plasminogen is activated to plasmin via uPA. Furthermore, our observations show that the motile phenotype is associated with an increased plasmin-dependent ECM degradation.

The motile phenotype of cells is also associated with increased uPAR levels [18,24,25,36,37]. uPAR is expressed at high levels in invasive colorectal carcinomas [3] and participates in basal and stimulated colonic epithelial cell migration in vitro [38]. The increased association of uPAR with β1 integrins often contributes to colon cancer cell migration [27]. In M6 cells there is a stable association of uPAR with β1 integrins that is not modulated after induction of scattering, suggesting that other events, such as the composition of the extracellular matrix, may contribute to modulate this interaction. The proteolysis of uPAR is directly involved in cell migration [4-6] and the proteolyzed receptor is commonly detected in tumor cells [39,40] and in urine and serum of cancer patiens [41-43]. In scattered M6 cells, there is an increase of proteolyzed uPAR dependent on uPA and plasmin activities that correlates with motility, indicating its possible requirement to focus proteolytic activity at the cell membrane. However, experiments using D2D3 indicate that this proteolyzed form is not sufficient to complete cell scattering.

We have analyzed downstream targets activated by plasmin in scattered cells. Our data indicate that PKC and Src activities are only required at the initial stages of motility, but not in the plasmin-dependent dispersion of cells, in agreement with the rapid down-regulation of PKC after PMA treatment, and with the involvement of Src in the phosphorylation of p120-catenin upon PKC activation [11,32].

We have also shown that for scattering to occur in M6 cells, signaling through ERK 1/2 is required, as indicated by the inhibition of motility by the specific MEK inhibitor PD98059. The addition of active plasmin to PMA treated cells stimulated cell scattering and ERK 1/2 phosphorylation in a time-dependent manner. In addition, plasmin could stimulate ERK 1/2 phosphorylation and cell spreading in the absence of other obvious factors. These observations indicate that plasmin might act as a motogen for colon cancer cells under specific conditions. Thus, in cells with activated PKC, where E-cadherin is downregulated and uPA/uPAR are activated, the motogenic activity of plasmin is clearly manifested. This effect of plasmin is also detected in cells that are sparsely seeded, such that their cell-to-cell contacts are less prominent and do not require to be down modulated by PKC activation. These results are reminiscent of the action of HGF on IMIM-PC-2 pancreas cancer cells where scattering is dependent on uPA and plasmin activities [18]. It has been reported that activation of ERK 1/2 is

associated with the motility induced by uPA/uPAR acting in concert with the EGF receptor [29-31]. To our knowledge, this is the first report demonstrating that plasmin stimulates motility through ERK 1/2 activation in human colon cancer cells, this action being independent of the EGF receptor activity. In fibroblasts, plasmin can trigger ERK 1/2 signaling through activation of the cell surface protease-activated receptor-1 (PAR-1), although this effect has not been studied in the context of cell motility and invasion [44]. In monocytes, plasmin-induced migration requires signaling through PAR-1 and integrin alpha9-beta1 [45]. It remains a possibility that the effect of plasmin on ERK 1/ 2 signaling may be indirect, potentially due to disruption/alteration of matrix interactions, or the generation of a new ligand, which in turn, stimulates ERK 1/ 2 activity. Further work will be needed to determine the putative functional receptors for plasminogen/plasmin in colon cancer cells and the downstream targets activated by plasmin.

Figure 1

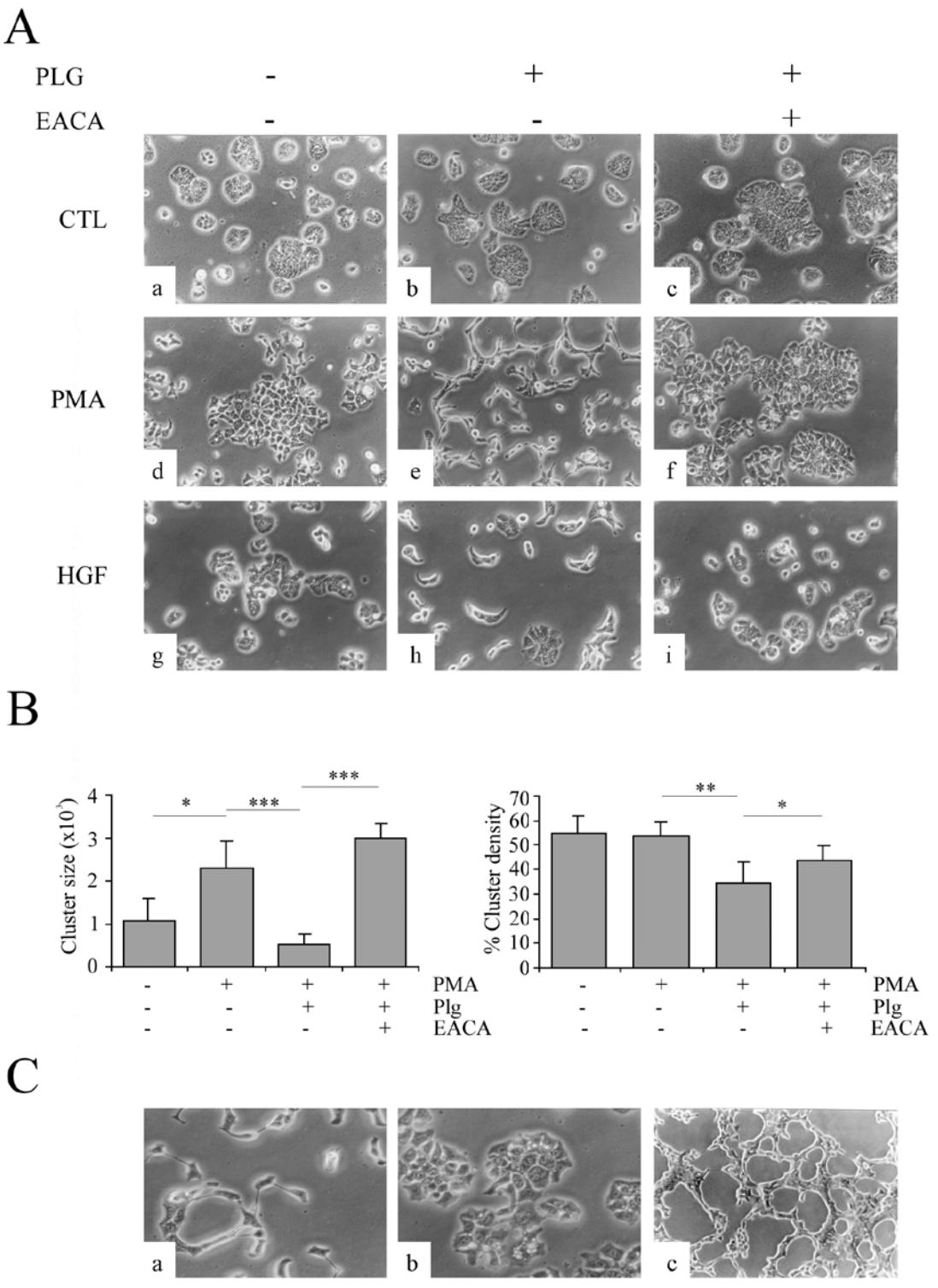
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Figure 1. **A**. PMA and HGF induce HT29 M6 cell motility through activation of plasmin. Cells were serum starved for 24 h, treated with EACA to eliminate membrane-bound plasminogen, washed with DMEM and incubated with PMA (10 nM), with HGF (50 ng/mL), with PMA plus plasminogen (5 μg/mL), or with HGF plus plasminogen for 16 hours. The plasminogen competitor EACA (15 mM) was added to study the role of membrane-bound plasmin on motility. Control cells were incubated in DMEM alone or DMEM supplemented with plasminogen (Plg). A representative experiment of several performed with identical results is shown. Original magnifications: x 200. **B**. Quantification of the cell scattering was performed using a modification of the Blob algorithm, as detailed in Materials and Methods. Left panel: non-scattered cells are observed as large single clusters, and scattered cells as numerous, smaller clusters. The bar graphs correspond to number of clusters per image (mean cluster density) and their average size in pixels (mean cluster size) determined from multiple fields and several replica points for each treatment. The right panel corresponds to mean cluster density determinations for the same images. Statistical significance determined by chi-square tests: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. **C**. Detail of cells treated with PMA plus plasminogen (a), PMA alone (b). Cells, plated at higher density, were treated with PMA plus plasminogen as above and scattering was evaluated 16 h later (c). Original magnification: x 400 (a and b), x 200 (c).

Figure 2

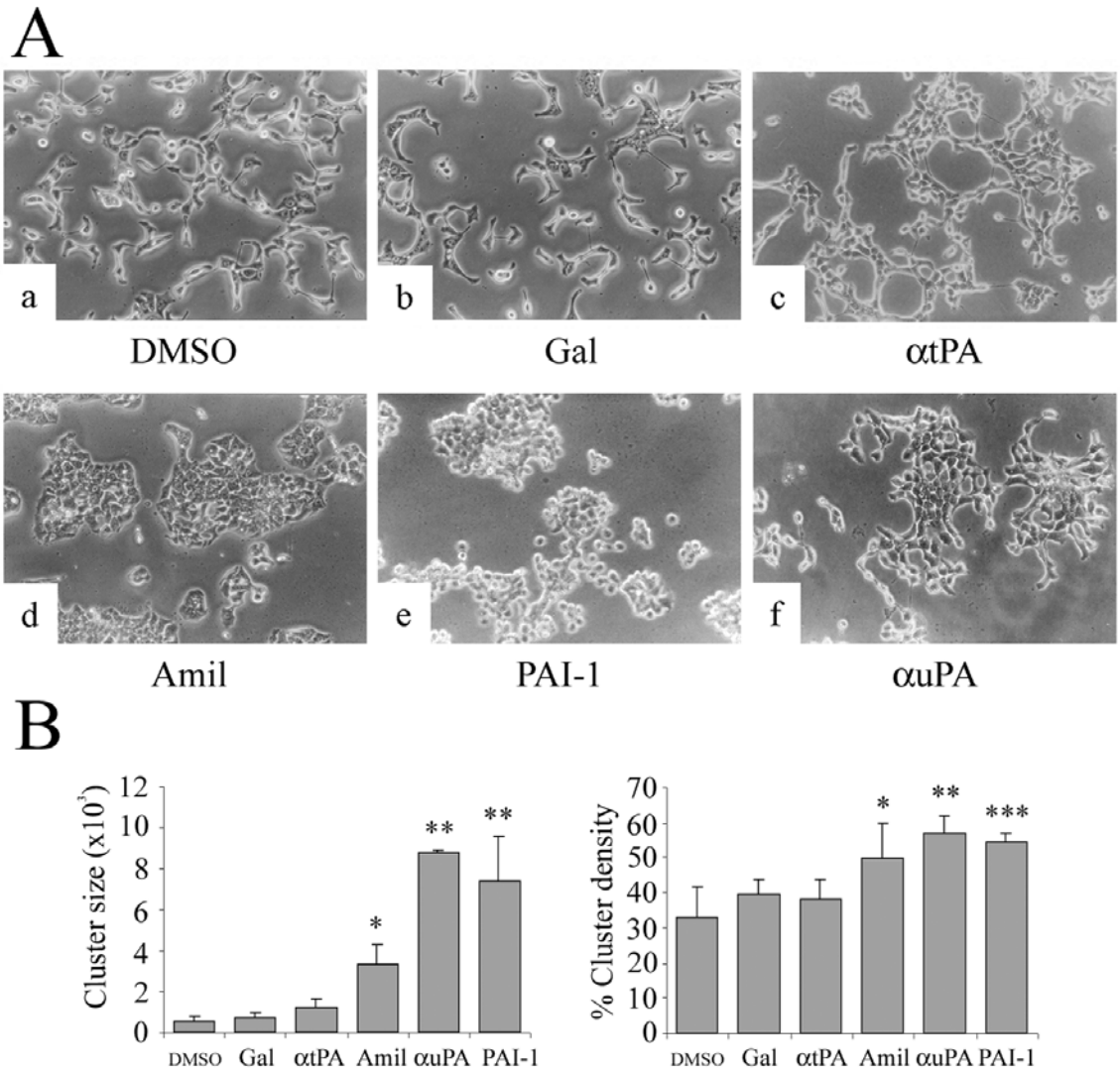

Figure 2. Cell scattering induced by PMA and plasminogen is dependent on uPA activity. **A.** M6 cells were treated with PMA plus plasminogen as above, in the presence of vehicle (DMSO), Galardin (50 μM), neutralizing antibody to tPA (αtPA) (150 μg/ml), amiloride (0.2 mM), PAI-1 (50 μg/mL) and neutralizing antibody to uPA (αuPA) (150 μg/ml). Original magnification: x 200. **B**. Quantification of the cell scattering in the presence of different inhibitors, performed as described in **Figure 1B**. The average cluster size (left panel) and the mean cluster density (right panel) of multiple fields and of several replica points is shown for each treatment. Statistical significance calculated with respect to control cells treated with PMA and plasminogen (DMSO) is: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Figure 3

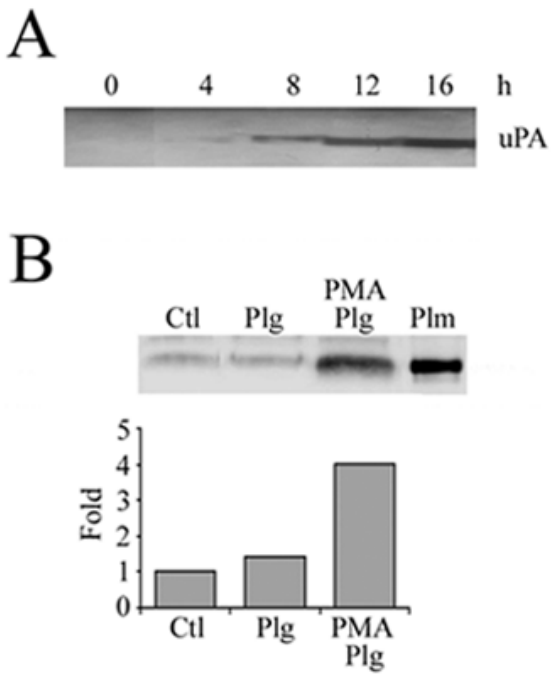

Figure 3. The induction of scatter in M6 cells by PMA is associated with an increased secretion of uPA and conversion of plasminogen to plasmin. **A.** Cells were treated with PMA in medium without serum and culture supernatants were recovered at the indicated times. Equal protein concentrations were analyzed by gelatin zimography in the presence of plasminogen as a substrate. No gelatinase activity was detected in the conditioned medium when zimography was performed in the absence of plasminogen (not shown). **B.** Plasminogen (Plg) was added for 16 h to cells untreated (Ctl, Plg) or treated with PMA in medium without serum. Culture supernatants were recovered and equal protein concentrations were analyzed by gelatin zymography as above. Recombinant plasmin (Plm) (0.01 units) was also loaded in the gel as a positive control. Bottom bars show the densitometric analysis of the gel bands normalized with respect to total protein loaded in the gel.

Figure 4

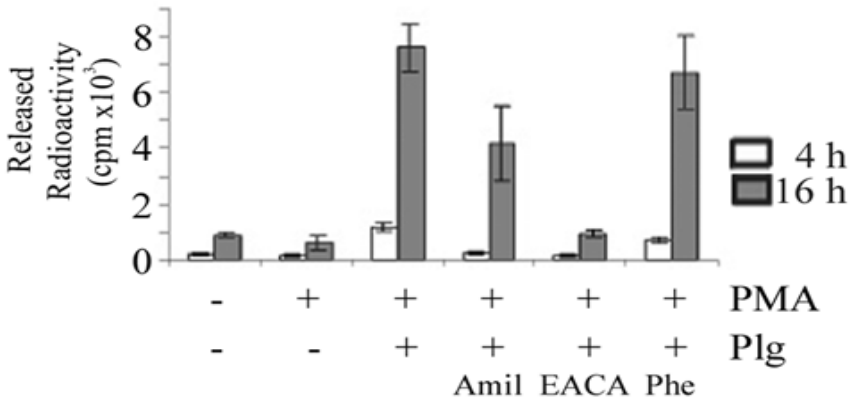

Figure 4. The induction of scatter in HT29 M6 cells by PMA is associated with an increased secretion of uPA and ECM degradation. Cells were plated on radiolabelled and plasminogen-depleted M6 cells from which ECM was prepared as described in Materials and Methods. Radioactivity released to medium was monitored at 4 h and 16 h in cells untreated or treated with PMA, or PMA plus plasminogen (Plg), in the presence of amiloride (Amil), EACA or 1,10-phenantroline (Phe, 50 μM). One representative experiment of two performed in triplicates is shown.

Figure 5

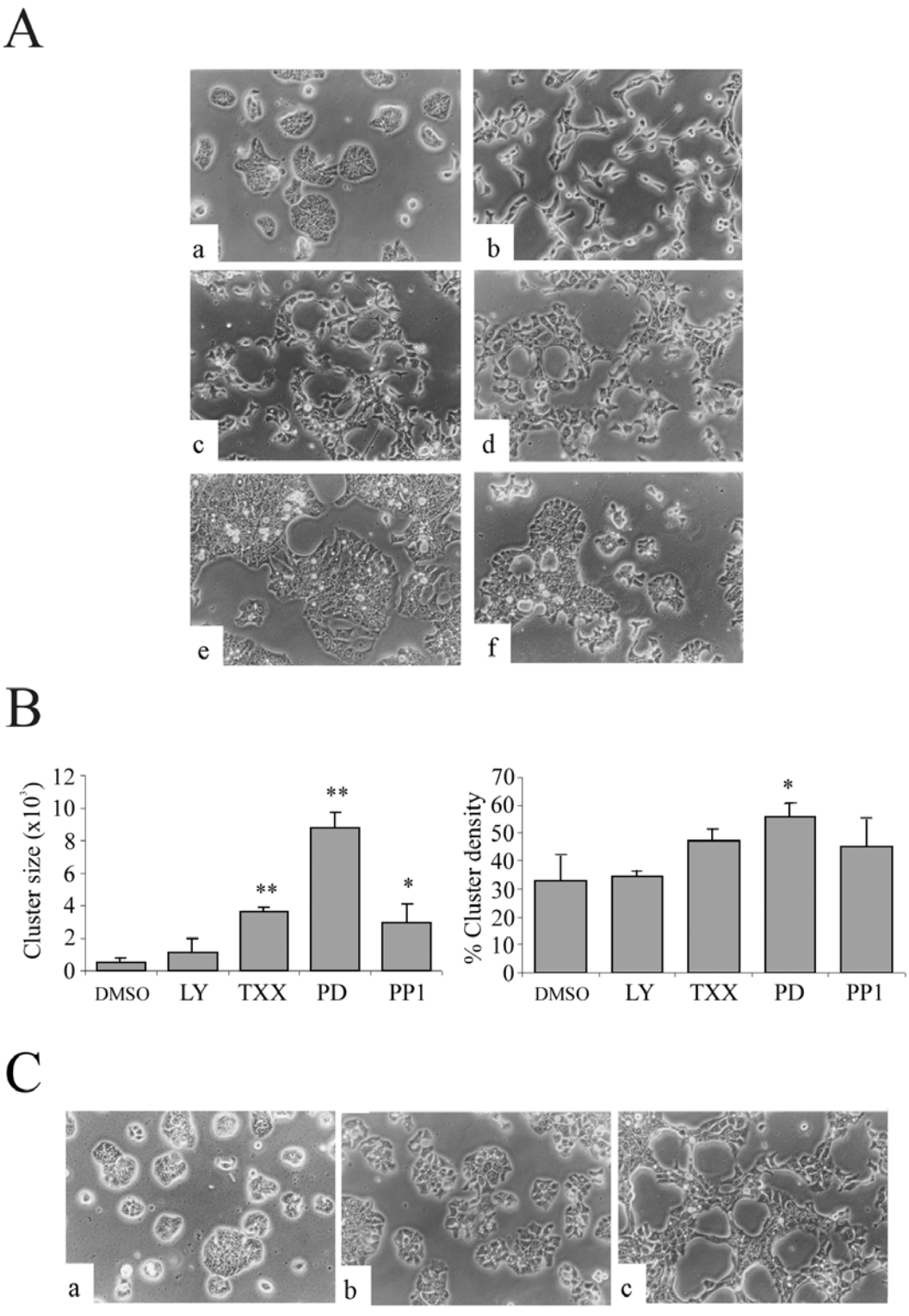
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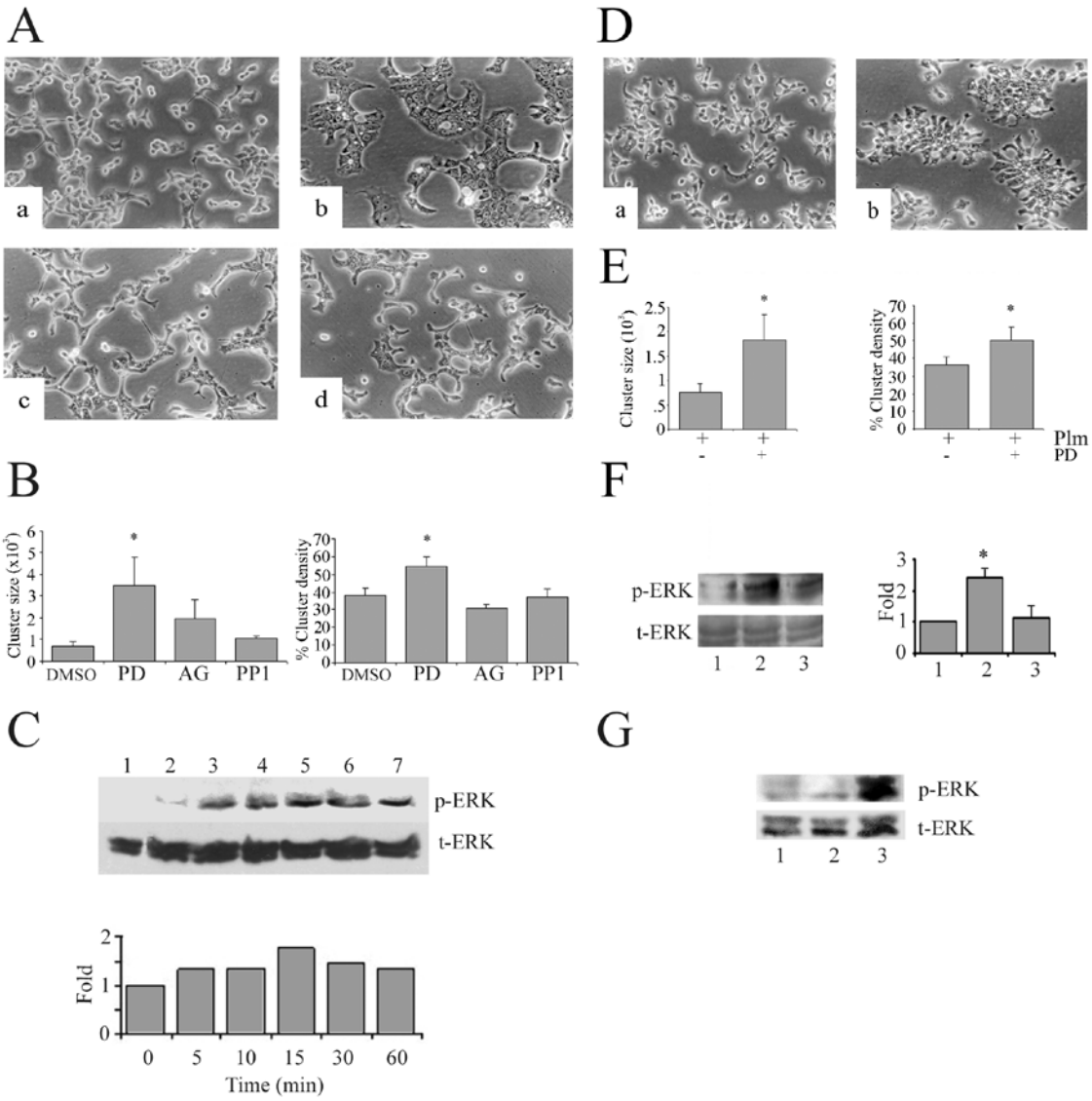
Figure 5. Full cell scattering by PMA and plasminogen is dependent on active Src and ERK 1/2. **A.** M6 cells were treated as described above with PMA and plasminogen in the presence of vehicle DMSO (b) or in the presence of LY-294002 (LY) (30 μM) (c), Pertussis Toxin (TXX) (400 ng/mL) (d), PD98059 (PD) (50 μM) (e), or PP1 (30 μM) (f). Control cells (a) were treated with plasminogen only. Scattering was evaluated 16 h later. **B**. Quantification of the scatter event for the different treatments shown in A was performed as described in **Figure 1B**, measuring the average cluster sizes (left panel) and the average cluster density (right panel) for multiple fields and several replica points for each treatment. Statistical significance, calculated with respect to control cells treated with PMA and plasminogen (DMSO), is: * $P < 0.01$ and ** $P < 0.001$. **C**. Active PKC is not required at later times for the full cell dispersion induced by plasminogen. M6 cells were serum starved and treated with DMEM (a) or PMA (10 nM) (b and c) during 12 h. Subsequently, all cells were treated with 5 μM GF109203X to inhibit PKC activity for 4 h, after which plasminogen was added (a and c) and scattering analyzed 12 h later. Note that as consequence of a higher time of observation with respect to experiments shown in **Figure 1 and 2**, cells start forming "cord-like" structures after plasminogen addition once PKC is inhibited (c). The formation of these "cord-like" structures are indicative of cell scattering as shown in **Figure 1**.

Figure 6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PD
F
p-ERK
t-ERK
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Fold
G
p-ERK
t-ERK
1 2 3

Figure 6. Plasmin induces phosphorylation of ERK 1/2 in PKC-depleted cells. **A**. Cells were treated with PMA for 6 hours, rinsed three times with DMEM to eliminate the drug from the medium, and further treated with plasminogen (2 μg/ml) and vehicle DMSO (a) or plasminogen plus PD98059 (PD) (50 μM) (b), AG1478 (AG) (1 μM) (c), or PP1 (30 μM) (d). Scattering was evaluated 16 hours later. **B**. Quantification of the scatter event shown in A performed as described in **Figure 1B**. Statistical significance, calculated with respect to control cells treated with PMA and plasminogen (DMSO), is *P < 0.05. **C**. Cells were untreated (lane 1) or treated as in A with PMA for 6 hours (lane 2), washed and further treated with plasmin (0.05 U/ml) for 5, 10, 15, 30 and 60 minutes (lanes 3 to 7), lysed and analyzed by Western blotting. ERK 1/2 phosphorylation was determined by Western blotting with a specific anti-phospho ERK 1/2 antibody (p-ERK). Normalization of the transferred proteins was performed using an antibody to total ERK 1/2 (t-ERK). Lower panel indicates the ratio between p-ERK and t-ERK signals after densitometric analysis. **D.** M6 cells pretreated with PMA as in A, were treated with plasmin (Plm) (0.05 U/ml) (a), or PD98059 (PD) (b). **E.** Quantification of the scatter event shown in D, performed as described in **Figure 1B**. Statistical significance is: * P < 0.05. **F**. A representative Western blotting of the induction of ERK1/2 phosphorylation by plasmin on PMA pre-treated cells. Lane 1, M6 cells pre-treated with PMA, lane 2, cells pretreated with PMA and plasmin, and lane 3 indicates the effect of PD98059 added along with plasmin. Bars indicate the average densitometric analyses of three Western blots derived from independent experiments. Statistical significance, with respect to untreated cells, is: * P < 0.01. **G**. Plasmin, but not plasminogen, induces phosphorylation of ERK1/2 in PMA pre-treated cells. Western blot from cells pre-treated with PMA as in A, and further treated with plasminogen (2 μg/ml) lane 2, or plasmin (0.05 U/ml) lane 3, for 15 min. Control cells treated only with PMA are shown in lane 1.

Figure 7

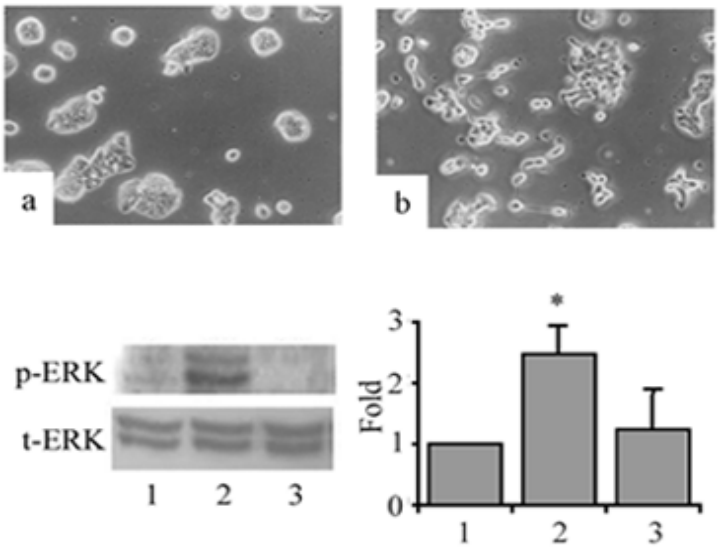

Figure 7. Plasmin-induced phosphorylation of ERK 1/2 is required for full cell scattering. M6 cells were seeded at low density, starved and treated with plasmin at 0.05 U/ml (b) for 16 hours..Scattering was evaluated 16 hours after plasmin addition. Lower panel shows the corresponding Western blot for ERK 1/2 phosphorylation after 15 minutes of plasmin treatment (2) compared to untreated cells (1). Lane 3 shows the effect of PD98059 added along with plasmin in abolishing the phosphorylation of ERK 1/2. Bars indicate the average densitometric analyses of three Western blots derived from independent experiments. Statistical significance, with respect to untreated cells, is: * $P < 0.05$.

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CAPÍTULO IV: Specific interaction of tissue-type plasminogen activator (tPA) with annexin II on the membrane of pancreas cancer cells activates plasminogen and promotes invasion "in vitro"

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ABSTRACT

The overexpression of tissue plasminogen activator (tPA) in pancreas cancer cells promotes invasion and proliferation *in vitro* and tumor growth and angiogenesis *in vivo*. To understand the mechanisms by which tPA favours cancer progression, we analyzed the surface membrane proteins responsible for binding specifically tPA and studied the contribution of this interaction to the tPA promoted invasion of pancreas cancer cells. The property of tPA to activate plasmin and a fluorogenic plasmin substrate was used to analyze the nature of the binding of active tPA to cell surfaces. Specific binding was determined in two pancreas cancer cell lines (SK-PC-1 and PANC-1), and complex formation analyzed by co-immunoprecipitation experiments and co-immunolocalization in tumors. Functional role of the interaction was studied in Matrigel invasion assays. tPA specifically and saturably bound to PANC-1 and SK-PC-1 cells while maintaining its activity. This binding was competitively inhibited by specific peptides interfering with the interaction of tPA with annexin II. The tPA/annexin II interaction on pancreas cancer cells was also supported by co-immunoprecipitation assays using anti tPA antibodies and, reciprocally, with anti annexin II antibodies. In addition, confocal microscopy showed tPA and annexin II colocalization in tumor tissues. Finally, disruption of the tPA/annexin II interaction by a specific hexapeptide, significantly decreased the invasive capacity of SK-PC-1 cells *in vitro*. tPA specifically binds to annexin II on the extracellular membrane of pancreas cancer cells, where it activates local plasmin production and tumor cell invasion. These findings might be clinically relevant for future therapeutic strategies based on specific drugs that counteract the activity of tPA or its receptor annexin II, or their interaction at the surface level.

CAPÍTULO IV: Specific interaction of tissue-type plasminogen activator (tPA) with annexin II on the membrane of pancreas cancer cells activates plasminogen and promotes invasion "in vitro".

INTRODUCTION

Tissue-type and urokinase plasminogen activators (tPA and uPA, respectively) are serine proteases that catalyze the activation of plasminogen *in vivo* [1]. The activity of plasmin is critical in a number of physiological processes, including extracellular proteolysis, cell migration, tissue remodeling and angiogenesis [2-3]. Plasmin activity is also required for tumor progression, and the role of uPA in activating plasminogen is well documented [4-5]. The activity of tPA correlates with a poor prognosis in several cancers, including melanomas [6-8], neuroblastomas [9-10], acute non-lymphocytic leukemia [11] and cancer of the exocrine pancreas, where its overexpression promotes growth and angiogenesis *in vivo* [12-15].

Plasminogen is readily activated by tPA and uPA when both are bound to cell surfaces [16]. The plasmin generated at these sites, protected from the activity of inhibitors such as α_2-antiplasmin and α_2-macroglobulin [17], is responsible for most of the proteolytic activity generated at membrane sites. Several proteins may function as binding sites for plasminogen or as co-binding sites for plasminogen and tPA [18-21]. Annexin II, a cytosolic phospholipid and Ca^{2+} binding protein, is a co-receptor for tPA and plasminogen in endothelial cells [22] that can form heterotetrameric complexes on the surface of HUVEC cells with the annexin II light chain (called S100A10 or p11) and this stimulates the generation of tPA-dependent plasmin [17, 23]. The annexin II/tPA interaction is required for the activation of microglia [24], and for plasmin generation in the NGF activated neuritogenesis [25]. Amphoterin, a heparin-binding protein, binds tPA and promotes neurite outgrowth in neuroblastoma cells [26]. Cytokeratin 18 and 8 bind tPA in hepatocellular and breast carcinoma cells [27]. In addition, interactions of tPA with surface membrane proteins dependent on its kringle domains or the catalytic site have been described [28-31]. Very recently, the transmembrane protein CKAP4 has been identified as a specific tPA binding protein on the surface of vascular smooth muscle cells [32].

We have previously shown that the overexpression of tPA in pancreatic cancer cells potentiates their invasive capacity, and induces proliferation and faster tumor growth *in vivo* [13-15]. The mechanisms by which tPA is able to promote its different

functions in pancreatic cancer cells have not been investigated. In the present work, we focused on the molecules that specifically bind active tPA on the surface of cells and participate in invasion.

EXPERIMENTAL PROCEDURES

Cell culture and reagents.

Cell lines were obtained from American Type Culture Collection (Rockville, MD) and mantained in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO-BRL, Gaithesburg, NY) supplemented with 10% heat inactivated fetal bovine serum (FBS, GIBCO-BRL) at 37^{o}C and 5% CO_2 [15]. Goat antibodies recognizing tPA were from American Diagnostica (Greenwich, CT); antibodies to annexin I and to annexin II, from Transduction Laboratories (Lexington, KY); anti-ERK 1,2, from Upstate Biotechnology (Lake Placid, NY); anti-mouse FITC, biotin-labeled rabbit anti-goat IgG, and mouse IgGs from DAKO (Glostrup, Denmark); anti-goat TRITC from Pierce (Rockford, IL); diisopropylfluorophosphate (DFP), goat IgGs, streptavidin-agarose, and protein G-agarose beads were from Sigma (St. Louis, MO); plasminogen and recombinant tPA (rtPA, Actilyse), were from Boehringer Mannheim (Barcelona, Spain). Peptides LCKLSL and LGKLSL were synthesized at the Serveis Científico Tècnics, Universitat Pompeu Fabra (Barcelona, Spain).

Functional cell membrane binding assay for tPA.

The interaction of tPA with the surface of pancreatic tumor cells was studied by analyzing the plasminogen activating capacity of the bound rtPA [30, 31]. Briefly, cells were seeded ($7x10^4$ cells/well) in 24-well plates and grew to confluence. Endogenous plasminogen activators were removed from cell surfaces with acid glycine buffer [0.05 M glycine (pH 3), 0.1 M NaCl] for 3 min and neutralized with 0.5 M HEPES (pH 7.5), 0.1 M NaCl. Cells were incubated with different concentrations of rtPA for 20 minutes at 37ºC in PBS containing 2% BSA (binding buffer). After washing with binding buffer, bound tPA was determined incubating cells directly in 0.05 M Tris-HCl, 0.1 M NaCl, pH 7.4 with plasminogen (0.2 μM) and H-D-Val-Leu-Lys-7-amido-4-methylcoumarin (AMC 0.2 mM) (Bachem, Budendorf, Switzerland), a plasmin-specific fluorogenic peptide substrate [30].

To determine the linearity of plasminogen activation, fluorescence levels (rfu) were measured at different times after incubation with substrates at excitation and

emission wavelenghts of 380 nm and 480 nm, respectively, using a Fluoroskan Reader (Labsystems, Helsinki, Finland).

Equilibrium, saturation and competition binding assays.

To determine the time needed to reach equilibrium, PANC-1 cells were incubated with rtPA (15 nM) at 37 ºC for different lenghts of time, cells were washed and plasminogen activation was determined. For equilibrium saturation binding assays, PANC-1 cells were incubated with rtPA at different concentrations (0-500 nM) for 20 minutes, and plasmin generation was determined. To convert rfu data to tPA protein concentration, a standard tPA in a fibrin-coupled enzymatic assay was used (DESAFIB-X, Biopool, Sweden) [33]. Competitive binding assays were performed with peptides blocking the interaction of tPA with annexin II (LCKLSL) or control peptides (LGKLSL) used at 0.1-10 mM [34]. To remove annexin II from cell surfaces cells were rinsed with PBS-EDTA (0.5 mM) and incubated with PBS-EDTA plus EGTA (10-50 mM) for 20 minutes [35]. Supernatants from treated cells were concentrated by centrifugation with Ultrafree-4 filters Biomax-10K (Millipore, Bedford, MA). To assay cells in suspension for tPA binding, cells were trypsinized, washed and resuspended in fresh medium for 1 h at 37^0C in polypropylene tubes before proceeding to the binding reaction.

For SK-PC-1 cells, overexpressing tPA, to avoid contamination with the endogenous protein, the bound rtPA was rapidly eluted from cells in acid buffer, neutralized and its activity measured using a fibrin-coupled enzymatic assay.

Analyses of binding data.

Equilibrium and saturation binding data were analyzed by non-linear regression algorithms using Sigma Plot software (SPSS, Chicago, IL). For saturation binding analyses data were fit to a one- or a two-binding site models.

Western blotting and immunoprecipitation.

Western blotting were performed as described [15]. For immunoprecipitations, subconfluent SK-PC-1 cells were lysed in buffer I [20 mM Tris-HCl pH 6.8, 3 mM $MgCl_2$, 50 mM NaCl, 300 mM sucrose**,** 1% Triton X-100 (v/v)] containing 1 mM phenylmethylsulfonyl fluoride (PMSF), and 2 μg/ml each of aprotinin, leupeptin and pepstatin [36]. After centrifugation (13,000 g) the pellet containing annexin II was dissolved in buffer II [50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 10 mM EGTA]. Proteins (400 μg) from the supernatant and pelletted fractions were used for immunoprecipitation with anti annexin II, anti tPA, anti annexin-I antibodies or goat IgGs and recovered by protein G-sepharose beads or anti goat-biotinylated IgG and streptavidine-agarose beads. After washing with buffer II without EGTA, beads were resuspended in Laemmli buffer and immunoprecipitated proteins were identified by Western blotting.

Immunohistochemical staining and confocal microscopy.

Five micron sections from paraffin-embedded tissues were treated with heat in citrate buffer for antigen retrieval. Sections were incubated with anti annexin II and anti tPA antibodies, rinsed and incubated with purified FITC conjugated anti-mouse IgG and TRITC conjugated anti-goat IgG. After rinsing, sections were mounted with Immuno-Fluore Mounting Medium (ICN, Costa Mesa, CA). Fluorescence was visualized on an inverted fluorescence microscope DM IRBE (Leica, Wetzlar, Germany) and captured in a TCS-NT Argon/Kripton confocal laser microscope (Leica). Control sections were incubated with matched mouse or goat IgG and resulted negative. Incubations with rabbit secondary antibodies were also unreactive.

***In vitro* invasion assays.**

The invasive potential of cultured tumor cells was tested using Matrigel-coated Transwell filters (Costar, Cambridge, MA), as previously described [15]. Briefly, $1x10^5$ cells were resuspended in DMEM supplemented with 0.1% BSA and plated on the Matrigel coated filters in the upper chamber, and medium containing 10% FBS was placed in the bottom chamber. Plates were incubated at 37ºC for 24, 48 or 72 h after

which cells that reached the bottom surface of the filters were visualized by staining with crystal violet. Peptides LCKLSL and LGKLSL were tested on cell invasion by mixing them with cells before seeding. Quantitative analyses were performed by counting stained cells on the filters in 10 independent fields/Transwell. Invasion abilities of cells were assigned as follows: highly invasive (+++), $\geq$ 100 cells/field at 24 h; moderately invasive (++), $\geq$ 100 cells/field at 48 h; poorly invasive (+), 50-100 cells/field at 72 h. Quantitative analyses of stained filters were also obtained by elution in 10% acetic acid and measuring absorbance at 595 nm.

Statistics.

Results are expressed as mean $\pm$ S.E.M. and the Student's *t* test was used for statistical analysis. A p value < 0.05 was considered statistically significant in all cases.

RESULTS

tPA binds specifically and saturably to the surface of pancreas cancer cells.

Because pancreatic cancer cells express PAI-1 [15, and data not shown], the physiologic inhibitor of tPA that binds with high affinity and inactivates tPA, and the tPA/PAI-1 complex can bind to cell surface receptors [16], tPA binding sites other than PAI-1 can be analyzed evaluating the fraction of active enzyme bound to cell surfaces [30]. To analyze putative receptors for tPA that maintain the protease proteolytically active in pancreatic cancer cells, we used rtPA and measured the activity of the bound protease by its property to activate plasmin and a fluorogenic plasmin substrate. Firstly, we determined the linearity of plasmin generation by incubating PANC-1 cells with rtPA (15 and 250 nM) during 20 minutes and, after washing out the unbound ligand, measuring the hydrolysis of the AMC substrate at different times. As shown in **Figure 1A**, the generation of proteolyzed AMC substrate is a linear reaction up to 30 min for tPA at 15 nM (r = 0,990). When tPA is used at 250 nM, plasmin generation is linear up to 20 min (r = 0,983). Therefore, all subsequent determinations of bound tPA activity were performed after 15 minutes of incubation with substrates.

Next, we determined the time for tPA binding to reach equilibrium. As shown in **Figure 1B**, the binding of tPA to PANC-1 cells at 37 ºC was time dependent and reached equilibrium after 20 minutes. Using non-linear regression analyses the data depicted in **Figure 1B** fit to a monoexponential curve suggesting a reversible bimolecular interaction.

We next determined the saturability of tPA binding by incubating PANC-1 cells with increasing concentrations of rtPA. As shown in **Figure 2A**, cells demonstrated a dose-related and saturable binding of tPA that reached a plateau at input doses of 100 nM. Non-linear regression analyses of these data showed that the best fit was to a one-site binding model, with a K_D of 42 nM. In parallel experiments, the binding of tPA to SK-PC-1 cells gave very similar results (K_D = 23.5 nM, **Figure 2B**). These values are very similar to the previously reported binding affinity to purified annexin II (K_D = 25 nM, [22]), or to 293 cells transfected with annexin II cDNA (K_D = 48 nM, [**37**]) or to an

unidentified receptor molecule on vascular smooth muscle cells (K_D = 25 nM, [30]). The specificity of this binding is demonstrated by the gradual inhibition of bound tPA in cells treated with increasing concentrations of DFP-inactivated rtPA: at 75 molar excess of competitor tPA, binding is reduced by 73.5 % (**Figure 3A**).

To discard that the observed binding of tPA involved interactions with acellular components such as proteins of the ECM, cells in suspension were also assayed. Results from these experiments indicated a saturable binding of tPA to PANC-1 cells in suspension with a K_D of 34 nM, very similar to the results obtained previously (not shown). Thus, pancreatic cancer cells express a surface protein able to bind tPA specifically and with high affinity while maintaining its activity and thus may be the mediator of tPA activities in these tumors [15].

tPA interacts with annexin II on the surface of pancreas cancer cells.

Because annexin II, the receptor for tPA in endothelial cells, was previously determined to be overexpressed in pancreatic cancer [13, 38-39], we studied its contribution to the binding of tPA to pancreas cancer cells. Earlier work identified the sequence LCKLSL (residues 8-13) from the annexin II protein as the critical region involved in the interaction with tPA [34]. This peptide inhibited 95% of binding of tPA to annexin II [34]. As shown in **Figure 3B**, the binding of rtPA to PANC-1 cells was significatively reduced to 60 $\pm$ 3 % by peptide LCKLSL but no inhibition was observed with the control peptide LGKLSL (binding is 99 $\pm$ 4 %), suggesting that annexin II might be involved in tPA binding to pancreas cells, and confirming that the Cys^9 in the annexin II is required for the interaction [34]. To prove these results, cells were treated with EGTA to reduce the amount of annexin II from the cell surface [35]. In the presence of EGTA, annexin II was recovered in the supernatant (**Figure 3C**, inset) and binding of rtPA to cells was reduced to 65.9 $\pm$ 4.4 % (**Figure 3C**). However, when EGTA was added in combination with peptide LCKLSL, tPA binding was further reduced to 36.8 + 2.4 %. These results support the notion that the interaction with annexin II accounts for most of tPA binding to pancreas cell surfaces.

To confirm the interaction of tPA with annexin II, we performed co-immunoprecipitation experiments using specific antibodies on SK-PC-1 cells that express high levels of both proteins. Isotype matched antibodies to annexin-I were used as a control. Immunoprecipitations with anti annexin II antibody followed by Western blotting analyses with anti tPA antibody, revealed the presence of tPA in the immunoprecipitated proteins (**Figure 4**). In contrast, no detectable tPA was present in the immunocomplexes formed by annexin I antibodies. Conversely, when cell extracts were immunoprecipitated with anti tPA antibody, annexin II was co-immunoprecipitated. However, not all tPA secreted by SK-PC-1 cells coimmunoprecipitated with annexin II, and viceversa, suggesting that they may correspond to molecular forms that do not interact with each other.

tPA and annexin II colocalize at the surface of human pancreatic tumors.

To determine whether the interaction of tPA and annexin II is present *in vivo*, we analyzed the distribution pattern of annexin II and tPA in human tumor tissue sections by double immunofluorescent staining and confocal microscopy. As shown in **Figure 5**, both proteins are expressed at high levels in epithelial cancer cells and co-localization is detected at membrane sites. tPA and annexin II are also found at independent locations, in agreement with our previous results.

Annexin II and tPA overexpression correlate with invasion in pancreas cancer cells.

Annexin II overexpression in pancreatic, colon and gastric cancer cells, correlates with a poor pronostic [13, 38-42]. We studied the expression of annexin II in several pancreas cancer cell lines by Western blotting analyses and the invasion abilities of these cells *in vitro,* on Matrigel coated filters. As shown in **Figure 6**, levels of annexin II were similar in all cell lines, despite differences in their ability to invasion (**Table 1** and [15]). The data summarized in **Table 1** show that the levels of expression of tPA, not those of annexin II, directly relate to a higher capacity for invasion.

The tPA/annexin II interaction is critical for the invasive capacity of cancer cells in vitro.

We analyzed whether the interaction of tPA with annexin II is required for cells to invade through Matrigel *in vitro*. **Figure 7** shows that SK-PC-1 cells efficiently invade Matrigel. However, in the presence of the blocking hexapeptide LCKLSL cell invasion is significatively reduced to 62 ±11 % of control cells at 72 hours. Invasion is also markedly inhibited by the use of this peptide at 24 h and 48 h. Invasion in the presence of a control peptide is no different from untreated cells, suggesting that the interaction of tPA with annexin II is important for the pancreas cell invasion process.

DISCUSSION

We have previously shown that tPA, highly expressed in the majority of pancreatic tumors and cell lines but not in normal pancreatic tissue, is required for the invasion, proliferation and angiogenesis of these tumors [12-13, 15]. We have also shown that annexin II is overexpressed in pancreas adenocarcinomas and localizes at the basolateral compartment of SK-PC-1 cells [13]. Localisation of active tPA at the cell surface permits a local activation of plasmin, imparts proteolytic and invasive advantages to tumor cells and is also essential for the tPA dependent activation of signal transduction [15]. However, so far the identities of the specific cell surface binding molecules for tPA in pancreas cancer cells have not been elucidated.

In the present study, we demonstrate the presence of high affinity binding sites on cell membranes that maintain tPA as an active enzyme and contribute to the invasive properties of SK-PC-1 cancer cells. By measuring only the active tPA bound to extracellular membranes of cells, we have detected a specific and saturable binding for tPA in SK-PC-1, and PANC-1 cells. This binding is significantly inhibited by a specific peptide corresponding to the N-terminus of the annexin II protein (residues 8-13), and also by treatment with EGTA, that removes most of the annexin II from the cell surface, suggesting that annexin II is a binding site for tPA. In addition, the interaction is demonstrated by co-immunoprecipitation in cell extracts *in vitro* and co-immunolocalization in tumors. Finally, the significance of the interaction is functionally demonstrated by the inhibition of invasion detected in the presence of the blocking hexapeptide. Based on these data, we conclude that annexin II is a tPA binding site on the surface of pancreatic cancer cells and that this interaction is important for the invasive capacity of cells.

In HUVEC cells, treatments with EGTA, antisense oligonucleotides to annexin II, or anti annexin II antibodies result in a reduction of specific binding of tPA to 40-50 % of controls, values comparable to our results in pancreatic cells obtained with EGTA or peptide LCKLSL [34, 37]. Interestingly, the inhibitory effect on tPA binding to pancreas cancer cells given by the treatment with EGTA and the peptide LCKLSL together is higher than the inhibition from individual treatments. These results may be

explained by the following: (i) annexin II might be present on surface membranes as monomers or heterotetramers with p11 [17, 23], (ii) in HUVEC cells, a portion of the annexin II, likely the heterotetrameric annexin II/p11, binds to membranes in a Ca^{2+}-independent way and is thus resistant to the EGTA treatment [23, 43], (iii) the activity of tPA is increased when it is bound to the heterotetrameric annexin II with respect to monomeric annexin II [23]. Thus, in our cells the effectiveness of the EGTA treatment on tPA binding to annexin II might be partially occulted by the increased activity of the tPA bound to the tetrameric annexin II. In summary, the inhibition of the interaction obtained by EGTA and the inhibitory peptide is significant, consistent with annexin II being a major binding site for tPA on the surface of pancreas tumor cells.

None the less, in polarized SK-PC-1 cells tPA is also bound to external apical membranes not containing annexin II (V. D. and R.P., unpublished observations), suggesting that additional protein(s) might function as binding sites for tPA in these cells. In vascular smooth muscle cells, a high affinity binding site different from annexin II that maintains the protease active and shows a K_D (25 nM) very similar to the one described here [30], has recently been identified as the transmembrane protein CKAP4 [32]. Amphoterin, that binds tPA in the central nervous system and is implicated in invasion [26, 44], is also expressed by SK-PC-1 cells at high levels (V. D. and R.P., unpublished observations). However, the non-saturable binding kinetics of tPA to this site [26] suggest that it is not the binding site detected in our experiments. Cytokeratin 8 also binds tPA in a specific and saturable manner in breast cancer cells [45] and promotes cell surface plasmin generation, but the reported K_D (250 ± 48 nM) is significantly different from the one determined in our experiments [21]. These observations suggest that pancreatic tumor cells may have more than one membrane molecule, with similar binding affinities for tPA, possibly to focalize plasmin generation at different membrane sites, the relevance of which in the process of tumor progression must be investigated.

Amphoterin, cytokeratin 8, and annexin II are cytosolic proteins lacking a signal sequence and yet extracellular functions for these proteins have been described. In this

regard, annexin II has been found in exosomes, an unconventional secretion pathway for proteins lacking a signal sequence [46].

Our co-immunoprecipitation experiments clearly demonstrate the interaction of tPA with annexin II. However, only a fraction of the two proteins interact with each other, likely corresponding to the fractions localized at the surface compartment of cells. The partial co-immunolocalization of both proteins *in vivo* support this notion, showing both tPA and annexin II overexpression in tumors and colocalization at particular membrane sites.

The LCKLSL peptide inhibits tPA binding to cells and invasion through Matrigel at comparable levels, confirming that the active tPA bound to surface annexin II is needed for invasion. SK-PC-1 and PANC-1 cells express also high levels of uPA and uPAR, that contribute to the invasion process [13, 15, 47]. Thus, the inhibition of invasion obtained by the disruption of annexin II/tPA interaction in the experiments shown here may account for most of the tPA-dependent invasive capacity.

Our results are the first to show the specific interaction of tPA with annexin II on pancreas cancer cells surfaces and its relevance for the invasive process. Understanding the precise mode of interaction of tPA with pancreatic cancer cells and the molecular mechanisms used for invasion will help to understanding the contribution of this protease to cancer progression and in the development future of more effective therapeutic strategies in the future.

Figure 1

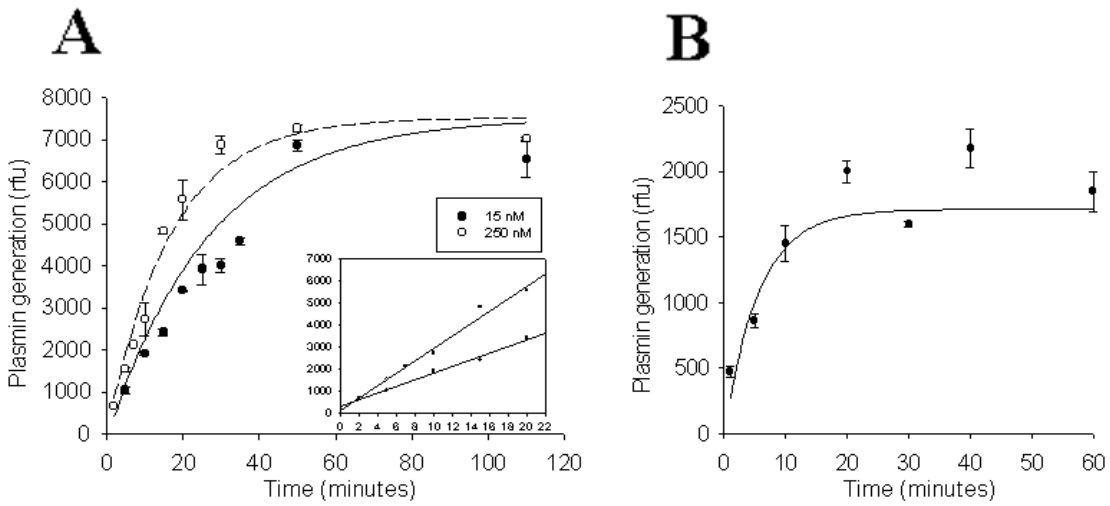


Figure 1. tPA catalyzed plasminogen activation on PANC-1 cells. Cells were grown until confluence and preincubated with 15 nM or 250 nM rtPA during 20 minutes. Cells were then washed to remove unbound tPA, and plasmin generation was determined at different times after the addition of plasminogen (0.2 μM) and H-D-Val-Leu-Lys-7-amido-4-methylcoumarin (AMC, 0.2 mM), as described in Experimental Procedures. Data shown for relative fluorescence units (rfu) represent the generation of active plasmin by active tPA. Inset shows the linearity of plasmin generation during the first 20 minutes of reaction. **B**, Time course of binding of tPA to PANC-1 cells. Cells were preincubated with 15 nM tPA at 37 ºC for the time indicated and plasmin generation was determined after 15 minutes. Equilibrium binding is reached at 20 minutes. Data points are mean (SEM) of values from two experiments performed in triplicate.

Figure 2

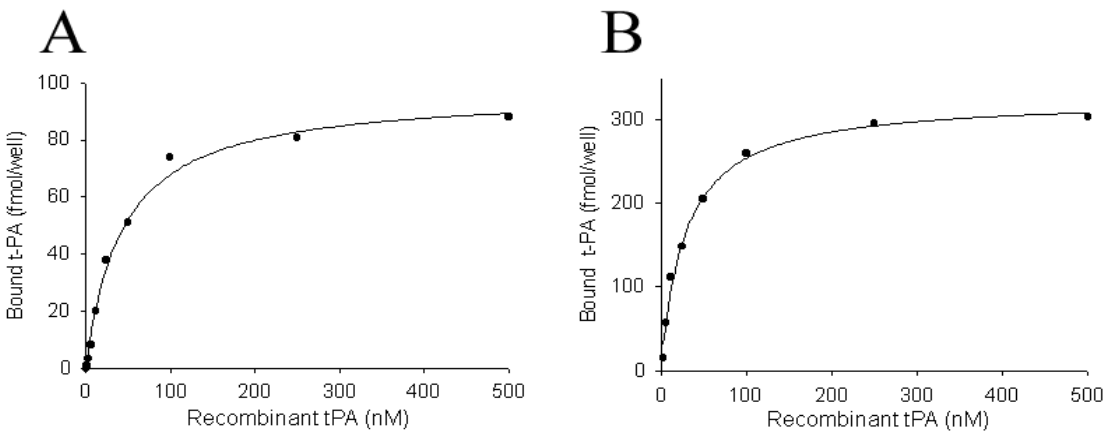
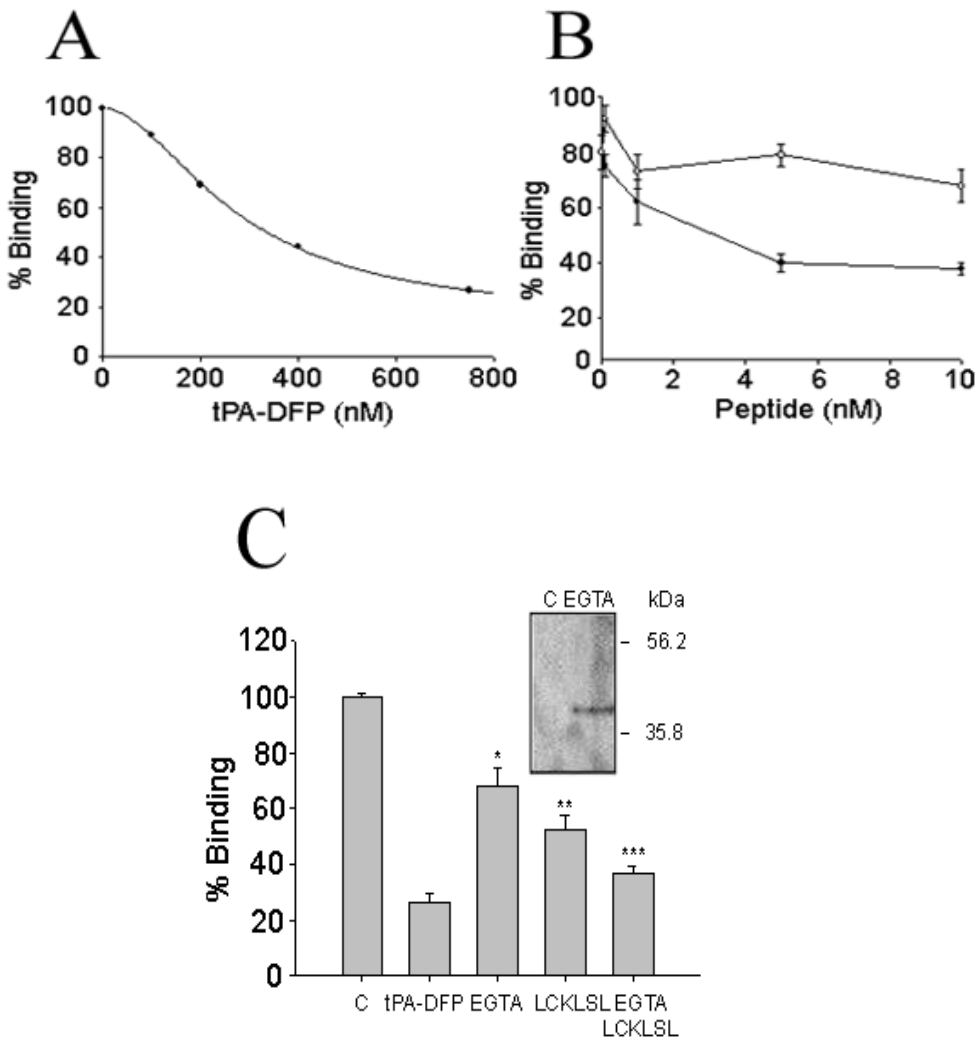


Figure 2. PANC-1 (**A**) and SK-PC-1 (**B**) cells were grown until confluence and incubated with increasing concentrations of rtPA for 20 minutes bound tPA was determined 15 minutes after the addition of substrates. One representative experiment of 4 performed in triplicate samples is indicated. Shown is the best fit of binding models to the data. The binding constants, as revealed by non-linear regression analyses of the data, are indicated in the text.

Figure 3


A
% Binding
100
80
60
40
20
0
0
200
400
600
800
tPA-DFP (nM)
B
% Binding
100
80
60
40
20
0
0
2
4
6
8
10
Peptide (nM)
C
% Binding
120
100
80
60
40
20
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C
tPA-DFP
EGTA
LCKLSL
EGTA
LCKLSL
*
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C EGTA
kDa
56.2
35.8

Figure 3. Experiments were performed with confluent PANC-1 monolayers. (**A**) Specificity of tPA binding was tested using as competitor of rtPA (10 nM), tPA inactivated with DFP at increasing concentrations. (**B**) Specific contribution of annexin II to the binding of tPA to the surface of PANC-1 cells was studied by disrupting the interaction with a blocking peptide (LCKLSL, filled simbol) or a control peptide (LGKLSL, open symbol). Data represent the mean ± S. E. M. (n = 3). Inhibition of tPA binding by LCKLSL is statistically significant (P = 0.007 compared to rtPA only; P = 0.009 compared to control peptide), (**C**) Annexin II was released from the cell surface by treatment with EGTA (50 mM) prior to incubation with rtPA with or without the addition of peptide LCKLSL (5 mM). Data are expressed as the percentage of binding of control samples without competitor and represent the mean ± S. E. M. of triplicate samples from a single experiment. Three experiments were performed with very similar results. Inset shows a Western blot performed with supernatants concentrated from control cells and EGTA treated cells blotted with anti-annexin II antibody. Statistical significance was determined by the Students' t test: *P = 0.009, ** P = 0.0004, *** P = 0.0001.

Figure 4

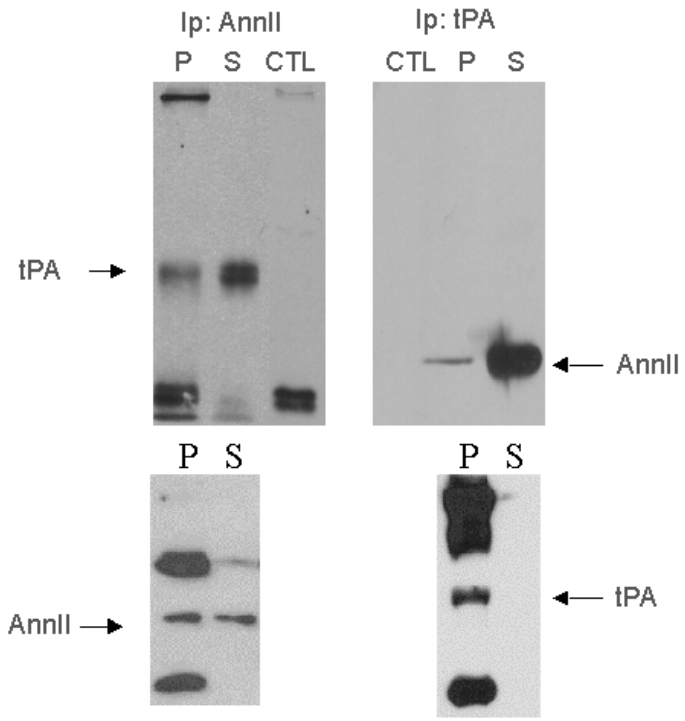

Figure 4. SK-PC-1 cells were maintained in complete medium until 70% confluence and processed as described in Experimental Procedures. **Left panel. Top,** cell lysates were subjected to immunoprecipitation with an antibody to annexin II (lane P represents the pelletted fraction and lane S the supernatant or non-immunoprecipitated fraction), or with isotype-matching anti-annexin I antibody as a control (lane CTL). The electrophoresed proteins were analyzed by immunoblotting with anti-tPA antibody. **Bottom,** the effectivity of the antibody to immunoprecipitate annexin II was checked by blotting the pellet and supernatant fractions with the anti-annexin II antibody. **Right panel. Top,** cell lysates were subjected to immunoprecipitation with a control goat serum (lane Ct) or with anti-tPA antibody (lane P represents the pelletted fraction and lane S, the supernatant or non-immunoprecipitated fraction) and the proteins were analyzed by immunoblotting with a mouse anti-annexin II antibody. **Bottom**, the effectivity of the antibody to immunoprecipitate tPA was checked by blotting the pellet and supernatant fractions with the anti tPA antibody.

Figure 5

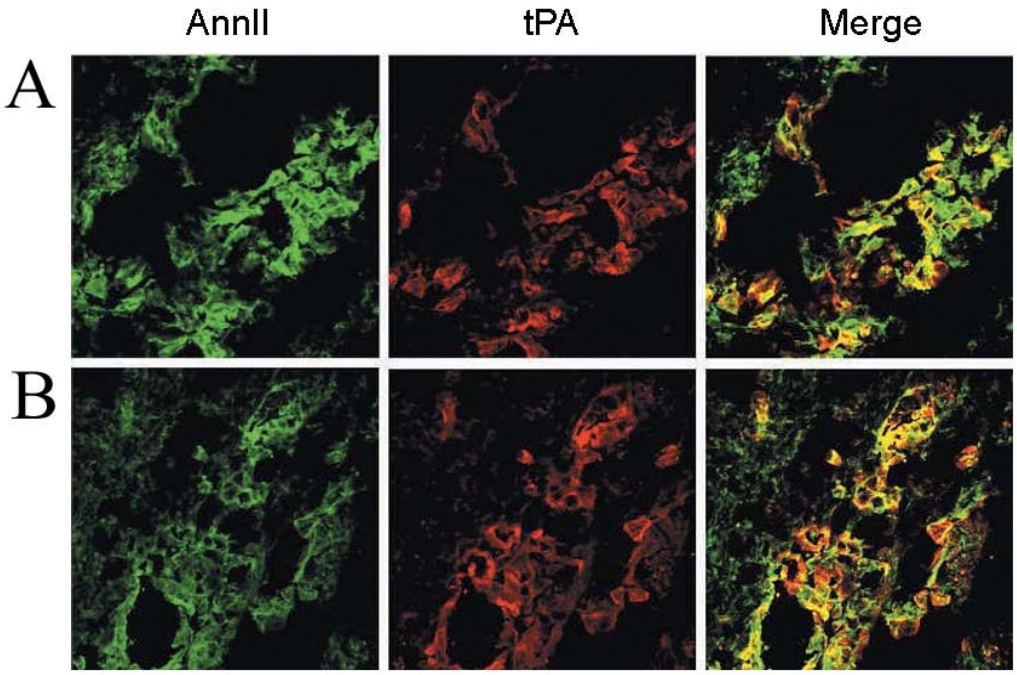

Figure 5. A and **B** show representative images of different tumor samples processed for double-immunofluorescence staining with a mouse anti annexin II and a goat anti tPA antibodies. Immunostaining for annexin II (AnnII) is shown in green, and immunostaining for tPA in red, and colocalization is shown in yellow (Merge). Original magnification, x200.

Figure 6

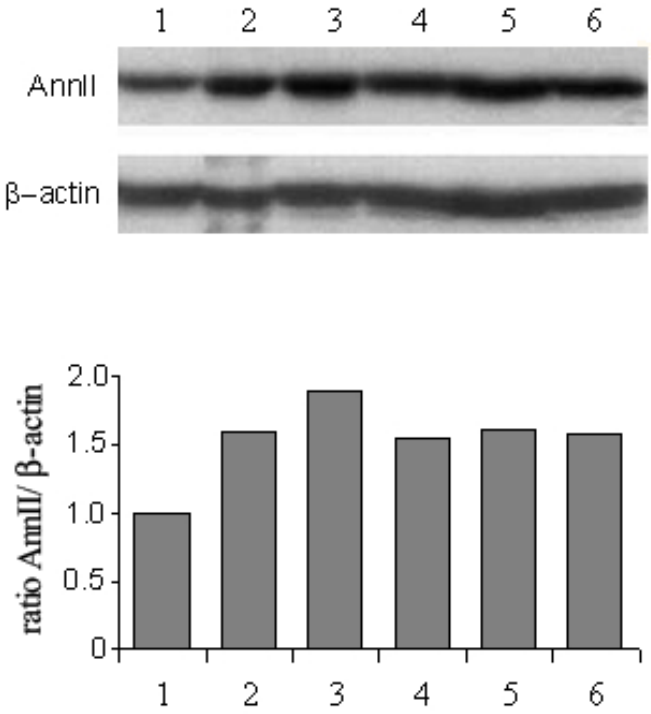

Figure 6. Western blotting with anti annexin II antibody of cellular protein extracts (50 μg) from the cell lines: lane 1, SK-PC-1; lane 2, CAPAN-1; lane 3, RWP-1; lane 4, BxPC-3; lane 5, PANC-1; lane 6, Hs766T. Blots were normalized by subsequent incubations with antibodies to β-actin. The histogram at the bottom shows the ratio of annexin II/ β-actin values obtained by laser scanning densitometry of autoradiograms.

Figure 7

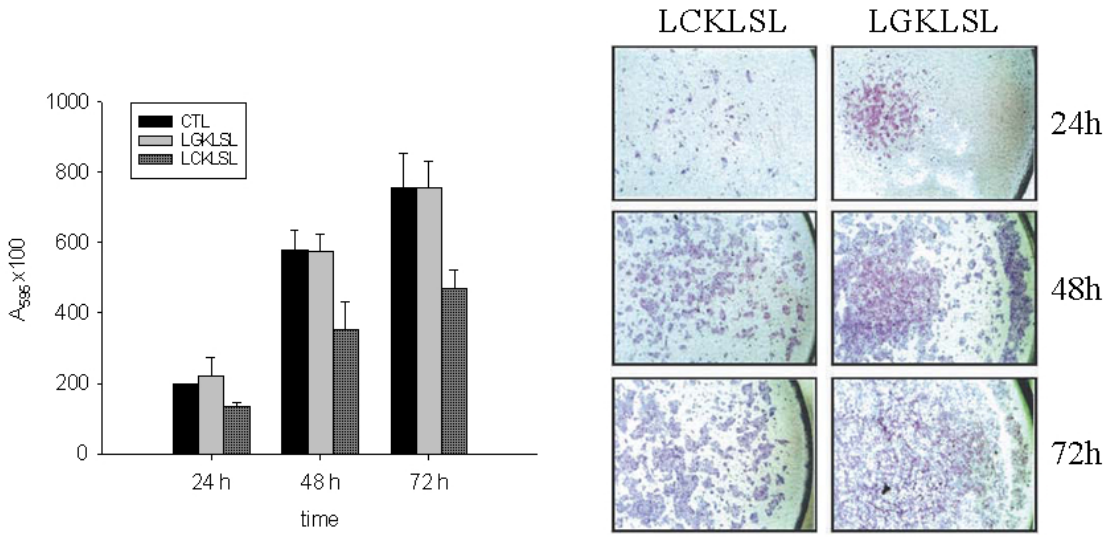

Figure 7. SK-PC-1 cells were cultured on Matrigel-coated Transwell filters for 24, 48, and 72 hours, without or with the peptide LCKLSL (5 mM) or the control peptide LGKLSL (5 mM) added to the upper chamber. Left panel**,** quantitative determinations of invading cells representing the mean ± S. E. M. of two experiments performed in triplicate. Inhibition of invasion given by peptide LCKLSL is significant at 72 h (*P*= 0.084 at 24 h, *P*= 0.056 at 48 h, and *P*= 0.009 at 72 h). Right panel, cells at the bottom surface of Transwell membranes stained with crystal violet in a representative experiment.

Table 1

Cell line	t-PA[a]	Annexina-II[b]	Invasion[c]
SK-PC-1	20	1	+++
CAPAN-1	17.8	1.61	++
RWP-1	5.1	1,9	++
BxPC-3	0	1,54	+
PANC-1	0	1,62	+
Hs766T	0	1.58	+

Expression of tPA and annexin II and invasive capacity of pancreas cancer cells.

[a] Expression of tPA as determined by ELISA assays (13).

[b] Expression of annexin II determined by Western blotting was normalized to the expression of β-actin

[c] The invasive capacity of cells *in vitro* was determined and quantified as described in Experimental Procedures.

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CAPÍTULO V: Activation of the epidermal growth factor signaling pathway by tissue plasminogen activator in pancreas cancer cells

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ABSTRACT

Tissue plasminogen activator (tPA) is the major activator of plasminogen in plasma. This serine protease is overexpressed by exocrine pancreas tumor cells, where it promotes tumor cell proliferation, growth and invasion. Here we have explored the signaling pathways used by tPA to activate the proliferation of pancreatic cancer cells. Transcriptional profiling on cDNA micro arrays was used to analyze the pattern of gene expression in response to tPA compared to the response to epidermal growth factor (EGF) and platelet-derived growth factor (PDGF). Results were confirmed using different biochemical assays in which specific kinase inhibitors or RNA interference were used. The transcriptional profiling showed that tPA modulates the expression of a set of genes commonly regulated by EGF, but distinct from the major set of genes modulated by PDGF. This suggested that tPA and EGF share common signaling pathways, a conclusion supported by further experimental evidence. Firstly, we found that tPA induced a rapid and transient phosphorylation of the EGFR. Secondly, specific EGFR kinase inhibitors, but not PDGFR kinase inhibitors, abolished the tPA-induced phosphorylation of the ERK1/2 kinases and cell proliferation. The mitogenic activity of tPA was also inhibited by siRNA depletion of EGFR, thus confirming the involvement of this receptor and in tPA-triggered signaling. Thirdly, we show that the signaling and mitogenic effects of tPA require its proteolytic activity, the activity of the metalloprotease-9 and active hb-EGF. Our results suggest that tPA induces proliferation by triggering a proteolytic cascade that sequentially activates plasmin, MMP-9 and hb-EGF. These events are required to activate the EGFR signaling pathway and cell proliferation.

INTRODUCTION

pancreatic carcinoma is a devastating disease. About 90% of patients present locally advanced or metastatic disease. Although surgery remains the most successful therapy, less than 10% of patients diagnosed with pancreas cancer actually have a curative resection.[1] Improvements in therapy that can add to surgery and systemic treatments for the advanced disease provide the greatest hope of improving the clinical outcomes in this disease.

Tissue type plasminogen activator (tPA) is the major blood activator of plasminogen required for the degradation of fibrin clots.[2] In addition to their well established fibrinolytic roles, tPA, plasmin, and urokinase type plasminogen activator (uPA), have been implicated in cancer growth and progression *in vivo*.[3] tPA is overexpressed in pancreatic carcinoma, where it stimulates growth and angiogenesis.[4-6] tPA activity correlates with poor prognosis in several other cancers, including melanomas and breast tumors.[7-8]

The mechanisms by which PAs promote cancer cell proliferation are best known for uPA. uPA binds to a specific cell surface receptor (uPAR), that forms part of a multiprotein signaling-receptor complex including integrins, FPR-like receptor-1/lipoxin A4 receptor, and EGFR.[9-11] The binding of uPA to uPAR activates ERK1 and 2, among other kinases.[12-13] This activation is blocked by EGFR tyrosine kinase antagonists.[11,14] In addition, the activation of ERKs in response to uPA requires the activity of a metalloprotease (MMP), suggesting the implication of a released membrane-bound EGFR ligand.[14] For tPA, the signaling mechanisms leading to proliferation are presently unknown. In the brain, it is now becoming clear that tPA exerts both proteolytic and non-proteolytic effects that contribute to various aspects of brain functioning at morphological, biochemical, and functional levels.[15-20]

EGFR, a member of the HER family of receptor tyrosine kinases, is a potent stimulator of cell growth upon binding by its high-affinity ligands, EGF and TGFα. The importance of EGFR in tumor progression has been extensively reviewed. [21]

pancreatic carcinoma is one of many tumors overexpressing EGFR, thereby making it a rational target for antitumor therapy in this disease.[22]

Our previous work has shown that tPA, overexpressed in pancreatic carcinomas, promotes proliferation, angiogenesis and invasion.[4,6] tPA binds to the surface of pancreas cancer cells mostly through annexin II, also abundant in these tumors.[23-24] This interaction maintains tPA active on the cell surface and is required for the cell's invasive capacity.[23]

Because tPA significantly stimulates pancreas cancer cell proliferation *in vitro* and *in vivo* [6], it is important to understand the mechanisms through which this protease induces tumor growth. Here we show that tPA elicits a transcriptional response that significantly overlaps that induced by EGF, which is clearly distinct from the response induced by PDGF. tPA stimulates a rapid phosphorylation and activation of the EGFR and downstream ERKs. By means of siRNA knockdown and the use of chemical inhibitors we show that both the expression and the kinase activity of the EGFR are required for the transmission of the tPA promoted proliferation. Finally, we demonstrate that this process requires proteolytic active tPA, and the activation of plasmin, MMP-9 and hb-EGF, that eventually lead to activation of the EGFR pathway and cell proliferation.

EXPERIMENTAL PROCEDURES

Cell culture and reagents.

Cell lines obtained from the American Type Culture Collection (Rockville, MD) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCOBRL, Gaithesburg, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO-BRL) at 37^0C in an atmosphere of 5% CO2. Galardin (GM 6001), mutant [Glu52]diphtheria toxin CRM197, and active human recombinant MMP-9 were from Calbiochem (Darmstadt, Germany), PD098059, LY294002, and tyrphostin AG1478 from Biomol (Butler Pike, Pennsylvania), recombinant human epidermal growth factor (EGF) from Invitrogen (Carlsbad, CA), p-Aminobenzoyl-gly-pro-D-ala-hydroxamic acid (AHA) from MP Biomedicals (Aurora, OH), plasmin and bisindolylmaleimide (GF109203X) from Roche Diagnostics (Mannheim, Germany), recombinant tPA (Actilyse) from Boehringer Mannheim (Barcelona, Spain), Pefabloc/tPA [2,7-Bis-(4-amidinobenzylidene)-cycloheptanone-(1) dihydrochloride salt] from Pentapharm (Basel, Switzerland). Alpha 2 antiplasmin was from Athens Research and Technology (Athens, GA). Catalytically inactive mutant tPA (S478A) was obtained from Molecular Innovations Inc. (Southfield, MI). Platelet derived growth factor (PDGF), sodium orthovanadate, β-Glycerophosphate, sodium fluoride, protease inhibitor cocktail, diisopropylfluorophosphate (DFP), and tyrphostin AG1296 were from Sigma (St. Luis, MO). Goat anti-tPA antibodies were from American Diagnostica (Greenwich, CT), mouse monoclonal anti-phospho-p44/42 MAPK (Thr 202/Tyr 204) and rabbit anti phospho-Akt (Ser 473) from Cell Signaling (Beverly, MA), rabbit anti-EGF receptor and mouse monoclonal anti-phospho-EGF receptor (Y1173) from Upstate (Lake Placid, NY), goat anti-actin from Santa Cruz Biotechnology (Santa Cruz, CA), rabbit anti-Ki67 from Immunotek (Marseille, France), and peroxidase-coupled anti-goat antibodies from DAKO (Glostrup, Denmark). Neutralizing antibodies to hb-EGF and antibody to PDGFR were from R&D Systems (Minneapolis, MN). Mouse anti-phospho-p38 MAPK was a kind gift from S. Ramon y Cajal (Hospital Vall d'Hebrón, Barcelona, Spain) and anti-ERK5 was kindly provided by N. Gómez (Universitat Autònoma, Barcelona,

Spain). The EGFR-GFP construct was kindly provided by A. Sorkin (University of Colorado).

Microarray hybridization and analysis.

cDNA arrays containing 15,360 cDNAs, of which 13,295 are known genes and 2,257 control genes, were generated and processed as described.[25] PANC-1 cells were grown in low serum (0.1%) medium for 96 h and treated with recombinant tPA (6 nM), EGF (3 nM) or PDGF (1 nM) for 15, 30, 60, 120, or 240 min. The concentration of ligands used are unsaturating with respect to each receptor in these cells.[23, 26-27]. Each treatment and time point was done in duplicate. Dye-swap hybridizations were performed with the same samples labeled with the reciprocal fluorophore. Background was subtracted from the signal, log2(signal) plotted versus log2(ratio), and a lowess normalization applied to adjust most spots to log ratio 0. This value was calculated for all replicates and results tabulated for signal, change (*n*-fold), log ratio, standard deviation of the log ratio, and *z* score. Spots with a SD versus their dye-swap replicate greater than 0.6 were filtered-out. Normalized log2 ratios in gene expression were then used to further analyze and cluster the data.

Clustering of samples.

Q-mode Factor Analysis (FA) was applied on the normalized micro array data.[28-29] Factor Analysis uses the covariance in the transcript levels to group genes and samples, a better indicator than total variance of the occurrence of transcriptional profiles shared by different pathways.[28, 30-31] From the factor model, hierarchical trees were derived by means of UPGMA clustering, starting from the sample coordinates in loadings space. An estimation of the reliability of each branch was obtained by means of a jackknife bootstrap analysis, using 100 replicates and random subsets of 90% of the genes per sample. Genes were considered differentially expressed among groups when their associated two-sided t-test P-value was below 10^{-5}. This cutoff considers a Bonferroni adjustment to take into account multiple testing.

Real-time RT-PCR.

We used RT-PCR with SYBRGreen incorporation to determine the expression levels of selected genes. RNA was isolated from cells, and controlled for quality on a 2100 BioAnalyzer instrument (Agilent, Palo Alto, CA). Total RNA, 2μg, was reverse transcribed by priming with random hexamers at 37 ºC for 60 minutes, followed by RNase treatment at 37 ºC for 20 min. The resulting cDNAs were used as templates in PCR reactions with gene-specific primers. Real-time PCR was performed on ABI PRISM 7700 (Applied Biosystems, Foster City, CA). Thermal cycler conditions were 95 ºC for 15 min, and 45 cycles of 95 ºC for 30 sec, 55 ºC for 30 sec and 72 ºC for 30 sec. All determinations were performed in triplicate and in two independent experiments. Since the relative amplification efficiencies of target and reference samples were found to be approximately equal, the ΔΔCt method was applied to estimate relative transcript levels. Levels of RPS14 amplification were used for endogenous reference to normalize each sample Ct (threshold cycle) value and untreated cells were used as calibrators for growth factor treated cells in each case. The final results, expressed as n-fold differences in target gene expression were calculated as follows:

$$n_{TARGET}=2^{-[(Ct\ target - Ct\ reference)TREATED - (Ct\ target - Ct\ reference)UNTREATED]}$$

Western blotting, immunofluorescence and immunoprecipitation.

Western blotting, immunofluorescence and immunoprecipitation were performed as described previously.[23]

Proliferation assays.

For Ki67 proliferation assays, serum-starved cells were incubated with the specified reagents for 6 h, and fixed and processed for immunocytochemistry as described.[23] Cells positive for nuclear Ki67 staining were scored as proliferating. At least 400 cells were counted for each condition, in at least two independent experiments. To assay the rate of DNA synthesis, cells grown in 0.1 % FBS for 48 h were exposed to

factors for 16 h and labeled with 1 μCi [^{3}H]-thymidine (Amersham Pharmacia Biotech)/well for 4 h. DNA was precipitated with 5% trichloroacetic acid, washed and incorporated radioactivity determined by scintillation counting. The effect of the addition of mitogens was expressed as a fold of incorporation of [^{3}H]-thymidine in cells grown at 0.1 % FBS in quadruplicate samples.

RNA interference.

Small interfering RNA duplexes (siRNA) were synthesized by *in vitro* transcription using the Silencer RNA construction kit from Ambion (Austin, Texas) for the target sequence for EGFR (5'-GAGCTGCCCATGAGAAAT-3'). siRNAs for MMP-9 were purchased from Ambion. Control (scrambled) siRNAs were synthesized corresponding to sequences that did not match any human transcripts or genes by BLAST searches in Genbank. PANC-1 cells were transfected with siRNA duplexes using Lipofectamine Plus Reagent (Invitrogen). Co-transfection with pEGFP vector (0.1 μg/well) (Clontech) monitored transfection efficiency in some experiments.

Statistics.

Results are expressed as mean + SEM, and the Student *t* test was used for statistical analysis. $P < 0.05$ was taken as level of significance.

RESULTS

tPA and EGF elicit a shared transcriptional response in PANC-1 pancreatic carcinoma cells.

Many pancreatic carcinomas express high levels of tPA, and also EGF, PDGF and their receptors, EGFR and PDGFR.[5, 32-33] In order to study the relationship of the proliferative signals induced by tPA on pancreas cancer cells with those triggered by the well-studied growth factors EGF and PDGF, we performed a comparative analysis of transcriptional profiles induced by these factors.[34] In a preliminary experiment, we established the optimal concentration of recombinant tPA (rtPA) in proliferation assays on serum-starved PANC-1 cells, which do not express the endogenous protease [6], showing that the mitogenic effect of rtPA is concentration dependent (**Figure 1A**).

Next, PANC-1 cells were treated with tPA (6 nM), EGF (3 nM), or PDGF (1 nM) for varying times, and transcriptional profiles analyzed. The genes and the samples were clustered by applying an unsupervised approach, based on Factor Analysis.[28-29] This analysis shows that the transcriptional responses to tPA closely group with those to EGF, and are clearly distinct from the responses to PDGF (**Figure 1B-D**). The clustering of the transcriptional responses to tPA and EGF is particularly tight at short times after induction with either factor, followed by a divergence at later times (2 h and 4 h after stimulation). This is reflected by the occurrence of a large number of genes which show shared kinetics and intensity of induction in response to both tPA and EGF, as compared to those genes that respond similarly to tPA and PDGF (**Figure 1C** and **supplementary Table**). The differential induction of several representative genes was further validated by quantitative PCR: NEK2 was predominantly induced by tPA (1.8-fold at 15 min), FOSL at early times by tPA (1.7-fold at 15 min) and at later times by EGF (3.8-fold at 60 min), and FASN by PDGF (4-fold at 30 min) (**Figure 1E**). These results suggest that the signaling pathways induced by tPA in PANC-1 cells significantly overlap those induced by EGF, but much less with those induced by PDGF. We thus hypothesized that tPA directly or indirectly activates one or more components of the EGF signaling pathway.

Signaling by tPA through the EGF receptor.

To test this hypothesis, PANC-1 cells were assayed for the mitogenic activity of tPA, EGF or PDGF in the presence of selective EGFR or PDGFR inhibitors. As shown in **Figure 2A**, all three factors were mitogenic at comparable levels for PANC-1 cells, both by nuclear Ki67 reactivity assays and [^{3}H]-thymidine incorporation assays (not shown). The mitogenic activity of EGF was significantly reduced in the presence of the EGFR-specific inhibitor AG1478, but not when pre-treated with the PDGFR-specific inhibitor AG1296. Correspondingly, the mitogenic activity of PDGF was greatly reduced by the PDGFR specific inhibitor AG1296, and to a lesser extent by the EGFR-specific inhibitor AG1478, in agreement with the known requirement for EGFR for a full mitogenic activity of PDGFR.[35] Relevant to the above hypothesis, the mitogenic activity of tPA on PANC-1 cells was significantly reduced in the presence of the EGFR inhibitor AG1478, while it was unaffected by the PDGFR inhibitor AG1296 (**Figure 2A**). This suggested that the mitogenic activity of tPA requires a catalytically active EGFR, but not PDGFR.

We then determined if tPA could activate EGFR in PANC-1 cells. **Figure 2B** shows that treatment of PANC-1 cells with tPA induced a rapid and transient tyrosine phosphorylation of EGFR reaching maximal levels at 5 min after the induction, and decreased thereafter. The amount of phosphorylated EGFR induced by tPA was comparable to that induced by EGF (**Figure 2B**). In A431 cells, which express high numbers of EGFR molecules on their surface, tPA was also able to induce phosphorylation of EGFR with identical kinetics (**supplementary Figure 1A**), although activation of this pathway by either tPA or EGF in these cells did not result in stimulation of proliferation.

We also expressed a green fluorescent protein-tagged form of EGFR in PANC-1 cells. This chimeric protein behaves as a fully functional receptor, and responds to binding by its ligand by endocytosis and stimulation of the EGFR pathway.[36] In serum-starved cells, EGFR-GFP showed a uniform distribution on the plasma membrane, with little or no intracellular localization (**Figure 2C**). In contrast, after 30

min of exposure to either tPA or EGF, the fluorescent receptor underwent a marked intracellular redistribution, showing a similar pattern of intracellular localization in both treatments. For EGF treated cells, this distribution has been shown to represent early endosomes.[37] As control, the addition of tPA did not induce the internalization of PDGF receptors (**Figure 2C**). Therefore, tPA stimulates the autophosphorylation and internalization of EGFR in PANC-1 cells, with kinetics and effects closely resembling those caused by EGF.

Phosphorylation and activation of the ERK1/2 is part of a cascade that relays signals triggered by activated EGFR.[38] **Figure 3A** shows that MEK activity is required for proliferation by tPA. In contrast, the poor inhibition of the tPA-mediated proliferation by LY294002, pertussis toxin, or GF109203x, indicate that PI3-K, G-proteins and PKC activities, respectively, are not required for this activity of tPA. In addition, tPA can phosphorylate MEK (not shown) and ERK 1/2 in PANC-1 and A431 cells, with maximal induction of the phosphorylated forms 10 min after the addition of tPA (**Figure 3B** and **supplementary Figure 1B**). This induction is reduced by incubation with AG1478, indicating that the phosphorylation of ERK1/2 induced by tPA is mostly dependent on the kinase activity of EGFR.

The state of activation of other signaling kinases in response to tPA was also tested. The phosphoinositide 3'-phosphate-activated kinase AKT (PKB) was activated by EGF but not by tPA (**Figure 3C**). Neither tPA nor EGF were able to promote the phosphorylation of the stress-induced kinase p38 MAPK or ERK5 in PANC-1 cells (**Figure 3C**). These results suggest that the signaling pathways used by tPA, although significantly overlapping, are not identical to the EGF signaling pathway.

Knockdown of EGFR by RNAi prevents tPA-induced mitogenesis.

Next, PANC-1 cells were depleted of EGFR by RNAi, and assayed for tPA-induced proliferation (nuclear Ki67 reactivity). Seventy-two hours after transfection of 80 nM EGFR-specific siRNA duplexes, the protein levels for the receptor decreased by 60% as compared with a control siRNA (**Figure 4A**). Both tPA and EGF produced a marked mitogenic effect (Ki67-positive fraction) on cells expressing the EGFR that either were not transfected, or were transfected with a control siRNAs (**Figure 4B**). After treatment of PANC-1 cells with EGFR-specific siRNAs, but not with control siRNAs, the mitogenic effects of tPA and EGF were significantly reduced (**Figure 4B**). Therefore, the presence of EGFR is required for the mitogenic effect of tPA on PANC-1 cells.

tPA-induced activation of ERK 1/2 requires enzymatically active tPA, plasmin, metalloproteases and the activity of hb-EGF.

It has been reported that EGFR is transactivated by MMP-dependent mechanisms.[11], that involve the proteolytic release of membrane anchored EGF ligands.[39] To test the possibility that tPA transactivates EGFR through the release of ligands for this receptor, we first analyzed the expression of endogenous EGFR ligands in PANC-1 cells by quantitative RT-PCR. **Figure 5A** shows that PANC-1 cells express high levels of hb-EGF, but have low or undetectable levels of TGFα, amphiregulin, and epiregulin, suggesting that hb-EGF might be responsible for the tPA-mediated activation of EGFR in these cells.

To characterize the proteolytic cascade that leads to the release of hb-EGF and the activation of EGFR, specific protease inhibitors were used. Blocking the proteolytic activity of tPA by pre-treatment with the specific inhibitor Pefabloc/tPA, by a neutralizing antibody or irreversibly with DFP (**Figure 5B** and data not shown), markedly reduced the phosphorylation of ERK1/2 by tPA, indicating the requirement for active tPA, as also previously shown.[6] These results were paralleled by those obtained in mitogenic assays. The fraction of proliferating cells (Ki67 positive) induced

by tPA was significatively reduced when its proteolytic activity was inhibited by Pefabloc or DFP treatment (**Figure 5C**). In other cell systems, the effects of tPA on cell signaling appear to require also metalloproteases activities.[40-42] Different metalloprotease inhibitors, including galardin and the gelatinase-specific inhibitor aminobenzoyl-gly-pro-D-ala-hydroxamic acid (AHA), prevented both the activation of ERK1/2 and proliferation by tPA (**Figure 5B and C**). The neutralizing hb-EGF antibody (αhb) and CRM197, a non-toxic diphtheria toxin analog that selectively binds to hb-EGF and inhibits strongly the mitogenic activity of the secreted form of human hb-EGF [43], also inhibited ERK1/2 phosphorylation and proliferation by tPA, indicating that hb-EGF is required for the tPA promoted activation of the EGFR pathway. In addition, the plasmin specific inhibitor α2-antiplasmin also significantly inhibited the tPA promoted proliferation of PANC-1 cells (**Figure 5B**), but not the mitogenic activity of EGF (**supplementary Figure 2**). Finally, a catalytically inactive tPA mutant (tPAS478A) is unable to stimulate activation of ERK1/2 demonstrating the requirement for the proteolytic active tPA in the activation of the pathway (**Figure 5B**). These results suggest that tPA, plasmin, metalloproteases and hb-EGF activities are required for the tPA promoted cell proliferation and the activation of the EGFR pathway.

tPA-induced proliferation requires MMP-9.

Plasmin has been shown to be epistatic to gelatinases and to activate directly MMP-9 in neutrophils.[41-42] Thus, we tested whether MMP-9 is the gelatinase activated by the tPA/plasmin system, by using PANC-1 cells depleted of MMP-9 by RNAi, and assayed for tPA induced proliferation (nuclear Ki67 reactivity). Transfection of the siRNA for MMP-9 caused a specific and significant decrease (48 % reduction for 80 nM siRNA) of secreted protein levels after 48 h (**Figure 6A**). This level of depletion of MMP-9 in PANC-1 cells was sufficient to prevent proliferation by tPA, but not by EGF (**Figure 6B**). Transfection with control siRNA duplexes did not cause any effects on tPA induced proliferation (**Figure 6B**). As shown in **supplementary Figure 2**, the proliferation of PANC-1 cells in response to recombinant plasmin requires the activity

of metalloproteases, and the proliferation induced by MMP-9 requires active hb-EGF. These results confirm that plasmin is epistatic to metalloproteases while MMP-9 is epistatic to hb-EGF in the activation of cell proliferation (**supplementary Figure 2**).

Collectively, our observations suggest that the effects of tPA on cell proliferation and signaling are mediated by the sequential activation of plasmin and MMP-9. In addition, hb-EGF, a EGFR ligand expressed at high levels in PANC-1 cells, is also required to engage the EGFR signaling pathway.

DISCUSSION

Tumors of the exocrine pancreas overexpress tPA and EGFR.[4-5, 22, 32, 44] tPA acts by stimulating cell proliferation in these tumors, promoting their growth and the associated angiogenesis *in vitro* and *in vi*vo.[6] The mechanism by which tPA promotes cell proliferation has remained elusive so far. In this study we provide evidence that tPA induces the activation of the EGFR and the transmission of the proliferative signal to the downstream ERK1/2 kinases through the proteolytically activation of plasmin, MMP-9 and hb-EGF. These are new findings, with potentially important therapeutic implications in pancreas cancer.

The activation of individual biochemical pathways may induce overlapping alterations in the pattern of expressed genes, forming networks of interactions.[45] However, activation of specific pathways can elicit specific transcriptional profiles, such that a given profile corresponds to a phenotypic window that may be used to infer the active pathways and the triggering signals.[46-48] The use of time series is particularly useful in these reverse biochemistry approaches, especially when parallel comparative experiments are performed.[34] Our comparative transcriptional profiling of responses to tPA, EGF and PDGF on PANC-1 pancreas cancer cells shows a significant overlap between responses to tPA and EGF, and also a clear distinction of these two responses from that elicited by PDGF. The similarities in the transcriptional responses to tPA and EGF were more evident at early times of exposure to either factor (from 15 min to 1 h), followed by a relative divergence at later times (2 h and 4 h). This indicates that, despite the use by tPA of pathways common to EGF, additional factors modulate the response of PANC-1 cells to tPA. The physiological relevance of these differences was also supported experimentally. Indeed, activation of ERKs by tPA was less vigorous compared to EGF, and AKT (PKB) was activated by EGF but not by tPA. Nevertheless, the transcriptional profiling analysis was highly suggestive of a significant overlap in the signaling pathways elicited by tPA and EGF, which was confirmed by further experiments. Firstly, chemical inactivation of the catalytic activity of EGFR, but not PDGF, blocked the tPA promoted proliferation of PANC-1 cells. Secondly, tPA induced a rapid phosphorylation of EGFR, of MEK and of ERK1/2, with

kinetics similar to the phosphorylation induced by EGF, and inhibitors of EGFR decreased this induction. Thirdly, tPA promoted specifically the endocytosis of EGFR, also with kinetics comparable to endocytosis induced by EGF. And fourth, knockdown of EGFR with siRNA prevented the mitogenic effect of tPA on PANC-1 cells. Collectively, these observations lead us to conclude that tPA activates the EGF signaling pathway through EGFR.

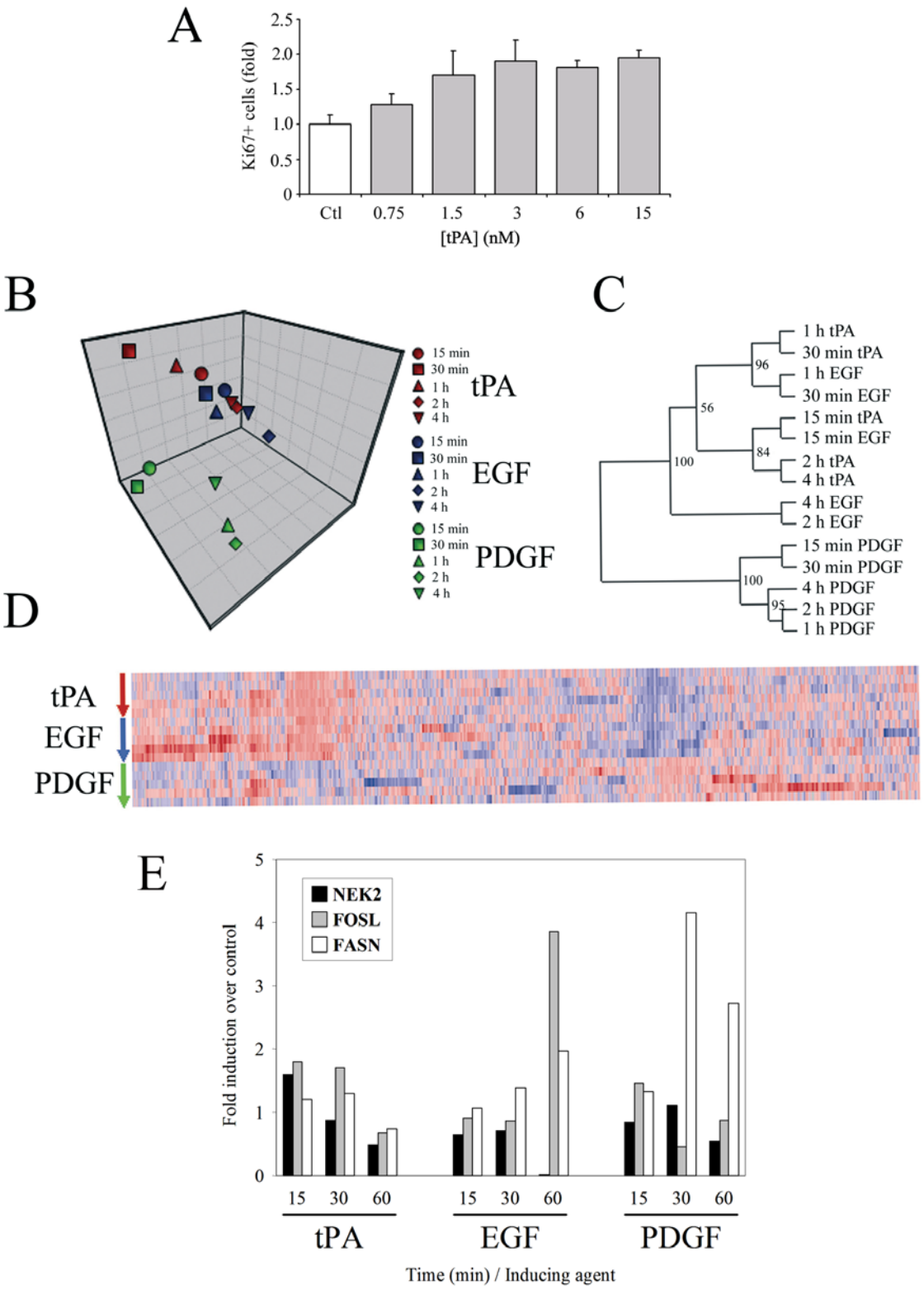
Although tPA contains an EGF-like domain, our results demonstrate that its catalytic activity is required for activation of the EGFR pathway, and therefore a direct activation of EGFR by tPA as a potential ligand for this receptor seems unlikely. Part of the mitogenic stimulus of some receptors can be produced by the transactivation of EGFR by other tyrosine kinase receptors (like PDGFR, IGF1R) [27, 49], protease receptors (uPAR, PAR-1) [11, 14, 50], CAM and integrins [51-52, 13] or hormones acting via heterotrimeric G-proteins.[54-55] The latter is promoted by intracellular signaling components that promote a metalloprotease-dependent proteolytic activation of EGFR ligands.[53-55] Our results show that the proliferative signals generated by tPA, that activate EGFR and ERK1/2, require active tPA, the activity of plasmin and MMP-9. In addition, the use of mutant [Glu52]diphtheria toxin (CRM197) and of a specific neutralizing antibody to hb-EGF, indicate that active hb-EGF is also required. Based on our results, that describe for the first time the signalling pathway for tPA in cancer cell proliferation, we suggest a mechanism by which tPA induces a rapid sequential activation of plasmin and MMP-9. MMP-9 activity is required for the activation of hb-EGF that binds to the EGFR, induces its autophosphorylation [53-54] and initiates a signaling cascade with phosphorylation and activation of MEK and ERK1/2. Recently, a different pathway of transduction of the tPA signal to the nucleus has been reported for the tPA-dependent activation of gene expression and protein secretion in renal interstitial fibroblasts.[56] The mechanism that we propose for tPA in cancer cell proliferation is supported by several findings. Firstly, proteolytic activity of tPA and plasmin are necessary for the tPA promoted EGFR activation and proliferation: both proteases required MMPs for these actions as shown by the use of several inhibitors. Secondly, MMP-9 expression and activity are required for the tPA promoted

EGFR pathway activation and proliferation, as demonstrated by specific inhibitors and RNAi assays. Thirdly, PANC-1 cells express considerably higher levels of hb-EGF compared to other EGFR ligands, and active hb-EGF is required for the tPA promoted EGFR pathway activation and proliferation. Additional evidences by others also lend support to the proposed mechanism. (i) Plasmin can directly activate pro-MMP-9 in infiltrating neutrophils, [41]; (ii) the plasminogen/plasmin system is epistatic to MMP-9 and MMP-2 activation in different cell systems [41-42] and (iii) MMP-2 and MMP-9 may mediate the transactivation of EGFR by G protein-coupled receptors activation of hb-EGF.[51] In addition, MMP-9 is strongly expressed in aggressive pancreatic cancer.[57] Furthermore, serine proteases like plasmin, trypsin or kallikrein are able to release membrane anchored pro-EGF in vivo and in vitro.[58-59]

Recently, tPA has been shown to proteolyze and activate PDGF-CC, generating an efficient PDGFRα ligand that induces proliferation of COS-7 fibroblasts.[60] However, the divergent transcriptional responses induced by tPA and PDGF and the lack of inhibition of the tPA proliferative signal by AG1296, argue against the involvement of this receptor in the tPA mitogenic activity on PANC-1 cells.

Because human pancreatic carcinomas overexpress tPA, MMP-9, hb-EGF, and EGFR, autocrine loops can generate signals that stimulate cell proliferation and anchorage-independent growth.[4-6, 22, 32, 44, 61-62] We have shown that tPA provides an additional autocrine mode of activation of the EGFR pathway that leads to growth stimulation. Our observations provide additional support for targeting the EGFR in antitumor therapy in pancreas cancer.[63] Conceivably, future combination therapies including selective inhibitors or tPA blocking agents could further improve EGFR-targeted therapies.

Figure 1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FOSL
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Fold induction over control
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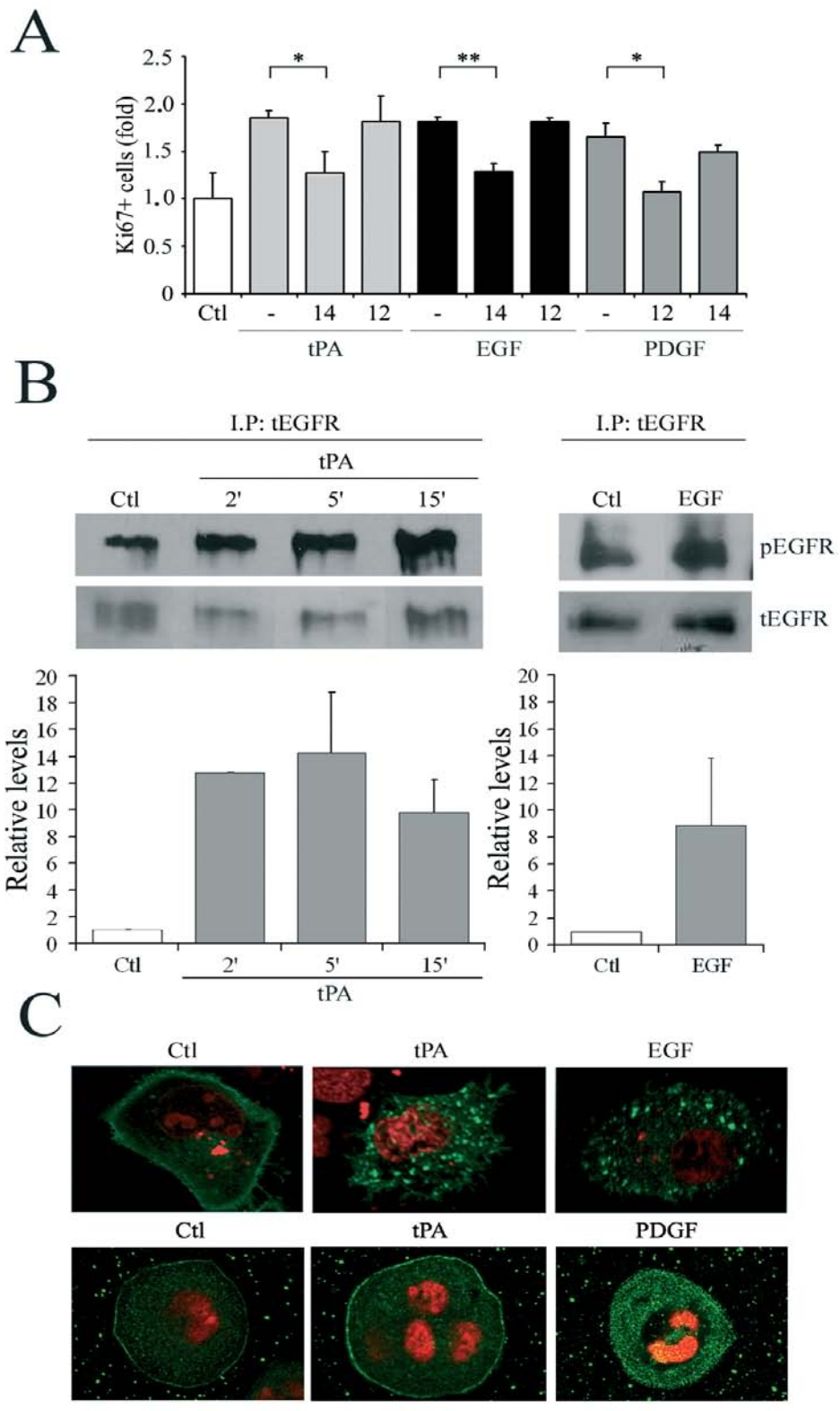
tPA
EGF
PDGF
Time (min) / Inducing agent

Figure 1. tPA and EGF elicit a common transcriptional response in PANC-1 pancreas cancer cells. **A.** PANC-1 cells were serum-starved for 96 h and treated with increasing concentration of the recombinant active tPA for 6 h. Induction of proliferation was analyzed by scoring the number of cells with Ki67 positive nuclei as compared to untreated serum-starved control cells (Ctl). Shown is the fold induction of Ki67 positive cells for each treatment as compared to untreated cells. At least 400 cells were counted for each condition in triplicate samples. Data from two independent experiments were combined. Activation of proliferation is significatively increased starting at 3 nM tPA. The error bars represent the SEM. **B-D.** Serum-starved PANC-1 cells were treated with tPA (6 nM), EGF (3 nM) or PDGF (1 nM) for 15, 30, 60, 120 and 240 min. Total RNA was extracted and used for hybridization on cDNA arrays. After normalization, data were analyzed by the Factor Analysis-based package FADA (Materials and Methods), and represented graphically (**B**) by using as coordinates the first three loading factors obtained for each sample (**C)**, as a phylogenetic tree calculated from distances based on C-index values (Materials and Methods), and (**D**) as a heat map corresponding to normalized expression values of the genes selected by FADA as the most significant for each time-series experiment. **E**. Quantitative RT-PCR to determine levels of NEK2, FOSL and FASN in PANC-1 cells after treatment with tPA, EGF or PDGF at the indicated times, expressed as fold values over control untreated samples. Shown are average values from triplicate determinations and two independent experiments. Standard deviations for DDCt values between replicate determinations were always less than 10%. Primers used were: NEK2, forward AGAACCTGAGAAACAGATGC, reverse TATTGGTCCGGTCAATAATC; FOSL, forward CAAGCATCAACACCATGAG, reverse GCTGTAGTGAGGGTAGGTCA; FASN, forward GACCTGTCTAGGTTTGATGC, reverse GCTTCATAGGTGACTTCCAG.

Figure 2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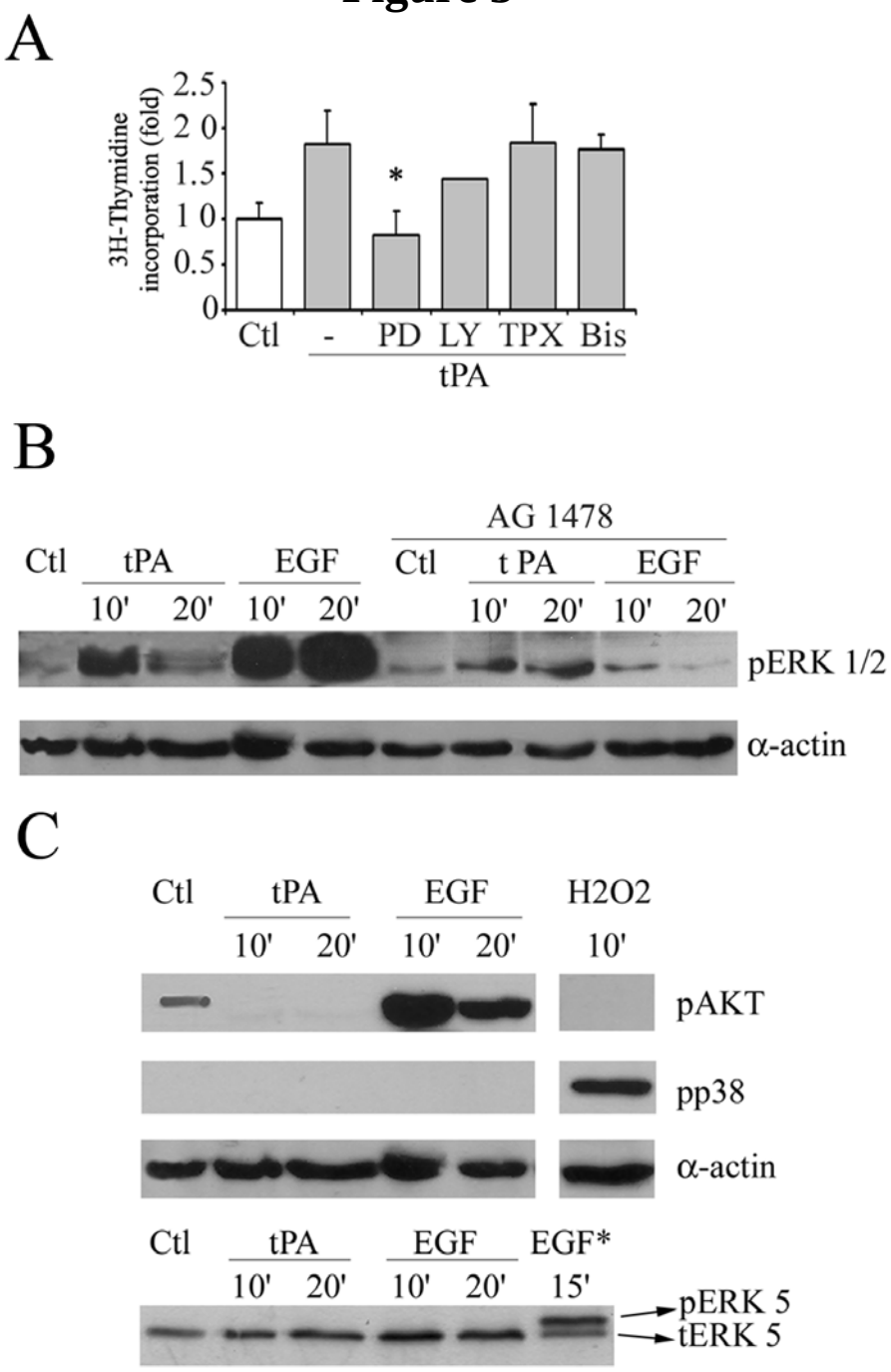
PDGF

Figure 2. Mitogenic signaling by tPA requires active EGF receptor. **A.** Mitogenic activity of tPA, EGF and PDGF on PANC-1 cells. Cells were treated with growth factors as described in **Figure 1**, during 6 h and analyzed by scoring cells with nuclear staining for Ki67. Where indicated, cells were preincubated with the EGFR specific inhibitor AG1478 (14) (2.5 μM) or the PDGFR specific inhibitor AG1296 (12) (2.5 μM) for 30 min before adding the growth factors, and the inhibitors maintained throughout the stimulation time. Shown is the fold of Ki67 positive cells for each treatment as compared to untreated serum-starved control cells (Ctl). At least 400 cells were counted for each condition in triplicate samples. Data from two independent experiments were combined. The error bars represent the SEM. (*P < 0.03 ; **P < 0.009). **B.** TOP: Induction of phosphorylation of EGFR by tPA. Cells were treated with tPA (6 nM) for 2, 5, and 15 min or EGF (3 nM), as a control. Lysates were immunoprecipitated with antibodies to EGFR. The phosphorylated receptor was detected by Western blotting with an antibody specific for pTyr 1173 in EGFR. BOTTOM: The bar graphs show the average from three independent experiments where the densitometric quantitation of the signals corresponding to the phosphorylated receptor were normalized with respect to total EGFR. The error bars represent the SEM. **C.** TOP IMAGES: tPA stimulates EGFR internalization. PANC-1 cells were transiently transfected with GFP-EGFR and serum-starved for 48 hours before the addition of tPA (6 nM) or EGF (3 nM). BOTTOM IMAGES: tPA does not induce PDGFR internalization. As a control, cells were serum-starved for 48 hours and treated with tPA (6 nM) or PDGF (1 nM) for 30 min before staining with anti PDGFR antibody. Nuclei were counterstained with propidium iodide (red). Fluorescent images were obtained after 30 min. of treatment with growth factors.

Figure 3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pp38
α-actin
Ctl
tPA
EGF
EGF*
10'
20'
10'
20'
15'
pERK 5
tERK 5

Figure 3. Active ERK1/2 are required for tPA action on proliferation. **A.** Induction of proliferation was analyzed by the incorporation of [3H]-thymidine and expressed as fold over unstimulated serum-starved controls (Ctl). Serum-starved PANC-1 cells were treated with tPA (6 nM) in the presence of PD098059 (PD) 50 μM, LY294002 (LY) 50 μM, pertussis toxin (TPX) 100 μg/ml, and bisindolylmaleimide (Bis) 5μM. Each treatment is the mean of quadruplicate measurements. At least three independent experiments were performed. The error bars represent the SEM. (*$P < 0.05$). **B.** tPA induces phosphorylation of ERK 1/2. Cells were serum-starved and treated with tPA (6 nM) or EGF (3 nM) for the time indicated, without or with AG1478. Cell lysates (50 μg) were analyzed for ERKs phosphorylation by Western blotting with a phospho-ERK specific antibody. Equal protein loading was verified by re-analyzing the membranes for actin expression levels. A representative blot of three performed is shown. **C**. The ability of tPA to phosphorylate AKT (pAKT), p38 MAP kinase (pp38) or ERK5 (pERK5) was analyzed by Western blotting using specific antibodies to the phosphorylated proteins. The following treatments were performed as controls: H2O2 (1 mM), as a control for p38 MAP kinase phosphorylation; EGF (3 nM), as control for AKT phosphorylation; and HeLa cells treated with 3 nM EGF (EGF*), as a control for ERK5 activation.

Figure 4

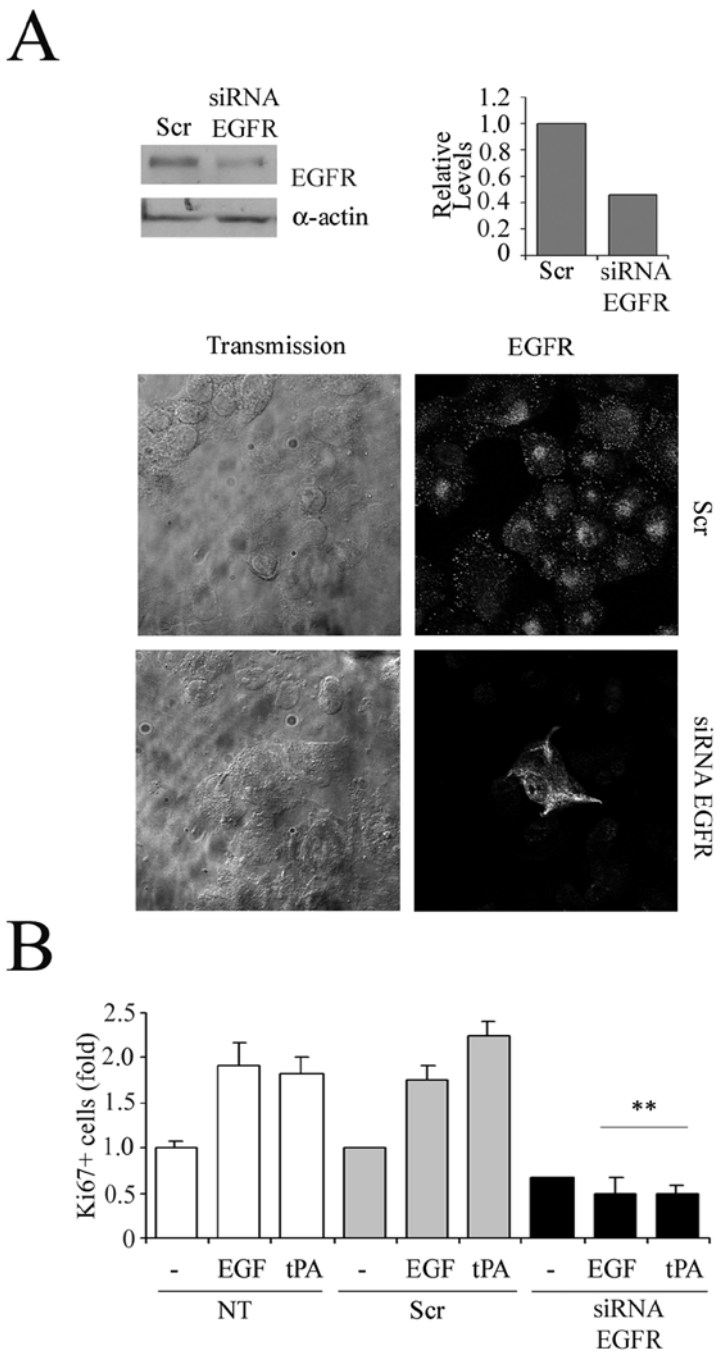

A
siRNA
Scr EGFR
EGFR
α-actin
Relative Levels
1.2
1.0
0.8
0.6
0.4
0.2
0
Scr
siRNA EGFR
Transmission
EGFR
Scr
siRNA EGFR
B
Ki67+ cells (fold)
2.5
2.0
1.5
1.0
0.5
0
**
- EGF tPA - EGF tPA - EGF tPA
NT
Scr
siRNA EGFR

Figure 4. Proliferation induced by tPA requires the expression of the EGFR protein. **A.** Specific depletion of EGFR protein attained by transfecting PANC-1 cells with the indicated double stranded siRNAs. Seventy-two hours post-transfection, EGFR levels were analyzed by Western blotting and quantitated by densitometric scanning of the signal (right graphs). Normalization was done against signals for actin. As controls, non-specific scrambled (Scr) siRNA's were transfected in parallel at the same concentrations. Bottom panels: immunocytochemistry with anti-EGFR antibodies showing the depletion of EGFR protein by the specific siRNA compared with control scrambled siRNA (Scr). **B.** Effect of the depletion of EGFR on the proliferative response to tPA. Untransfected or siRNA-transfected cells were serum-starved for 48 h and then treated with tPA (6 nM) or EGF (3 nM). Cells were double immuno-stained for Ki67 and EGFR and the effect of RNAi was analyzed by scoring the number of Ki67 positive cells in the EGFR negative population. The percentage of positive cells in each treatment with respect to untreated control cells (Ctl), is shown. For proliferation assays, The error bars represent the SEM. At least 400 cells were counted for each condition in two independent experiments. (**$P < 0.001$).

Figure 5

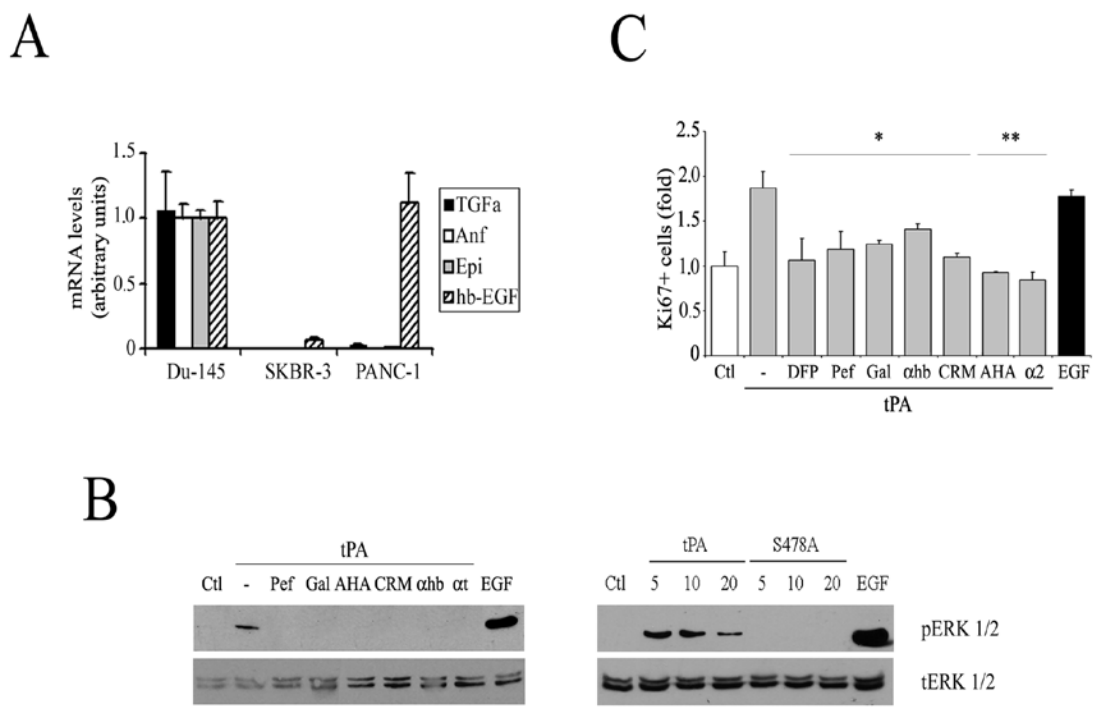


Figure 5. Activation of proliferation and ERK 1/2 phosphorylation by tPA require plasmin, metalloproteases and hb-EGF activities. **A**. Expression levels for EGFR ligands TGFα, amphiregulin (Amp), epiregulin (Epi), and hb-EGF were analyzed in PANC-1 cells by real-time RT-PCR. Data were normalized to actin and are expressed as mean + SEM (*bars*). Du-145 prostate cancer cells, known to secrete these ligands [64], were used as the calibrator. For comparison, SKBR-3 breast cancer cells with very low expression of EGFR ligands are shown. Primers used were: amphiregulin, forward 5'-ATATAGAGCACCTGGAAGCA-3', reverse 5'-TGTCAATCATGCTGTGAGTT-3'; epiregulin, forward 5'-TTCTACAGGCAGTCCTCAGT-3', reverse 5'-ACGTGGATTGTCTTCTGTCT-3'; hb-egf, forward 5'-TTCCACATCATAACCTCCTC-3', reverse 5'-ACCTATGACCACACAACCAT-3'; TGFα, forward 5'-CATTAAAATGGGACACCACT-3', reverse 5'-TCGCTCTGGGTATTGTGT-3'. **B.** tPA proteolytic activity is required for ERKs phosphorylation. LEFT. Cells were treated for 10 min with tPA (6 nM) or EGF (3 nM)

as described in **Figure 1**; specific inhibitors were added 30 min before stimulating with growth factors. Lysates (50 μg) were analyzed by Western blotting with antibodies to phosphorylated ERKs. Ctl, control untreated cells; Pef, pefabloc/tPA (30 μM); Gal, galardin (10 μM); AHA, p-Aminobenzoyl-gly-pro-D-alahydroxamic acid (1 μM); CRM, CRM 197 (5 μg/ml); αhb, neutralizing antibody to hb-EGF (10 μg/ml); αt, neutralizing antibody to tPA (10 μg/ml). RIGHT. Activated ERK1/2 are detected with active tPA (5-20 nM), or EGF (3 nM) but not with the catalytically inactive mutant tPA (tPAS478A) (5-20 nM). Total ERK1/2 signals from the same blots are also shown. **C**. Requirements for the mitogenic effects of tPA. Serum-starved cells were treated with tPA (6 nM) or EGF (3 nM), without or with specific inhibitors, and analyzed after 6 hours by fluorescent staining with anti-Ki67 antibodies. At least 400 cells were scored for each condition. Results are the mean from at least two independent experiments. DFP, diisopropylfluorophosphate-treated tPA (6 nM); Pef, pefabloc/tPA (30 μM); Gal, galardin (10 μM); AHA, p-Aminobenzoyl-gly-pro-D-ala-hydroxamic acid (1 μM); CRM, CRM 197 (5 μg/ml); αhb, neutralizing antibody to hb-EGF (10 μg/ml); □2, alpha 2 antiplasmin, 2 μg/ml. Control isotype-matched antibody used at the same concentration had no effects in these assays (not shown). (**P* < 0.04 ; ***P* < 0.004).

Figure 6

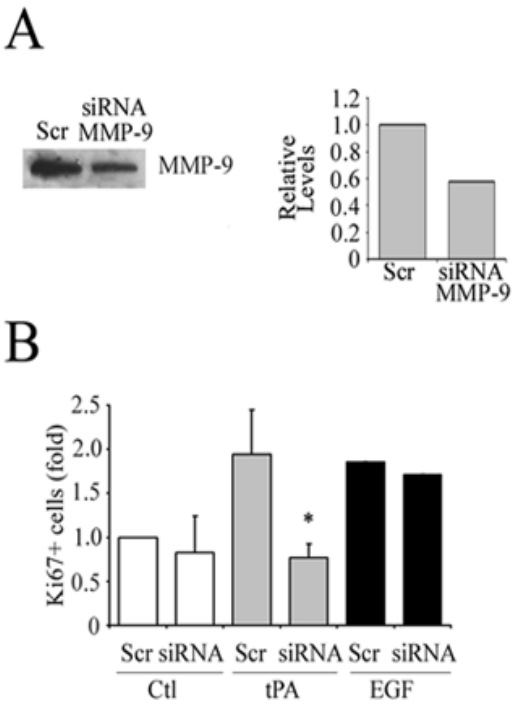
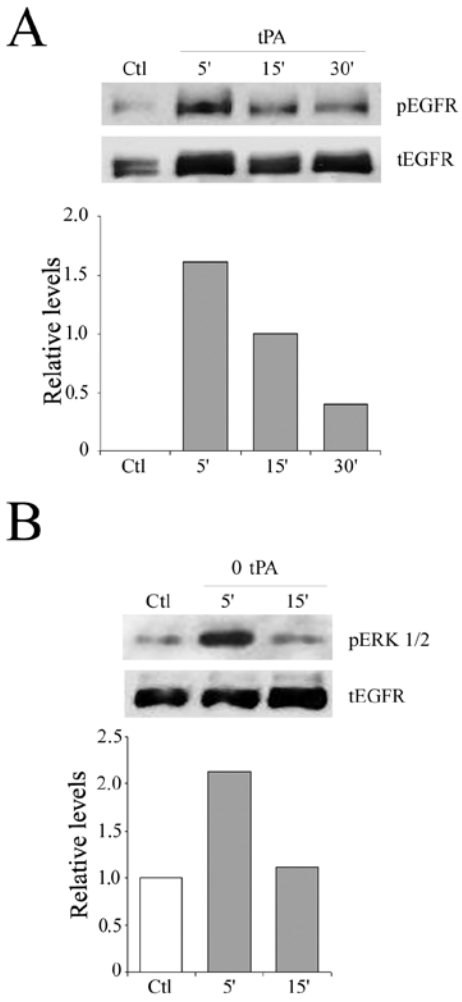


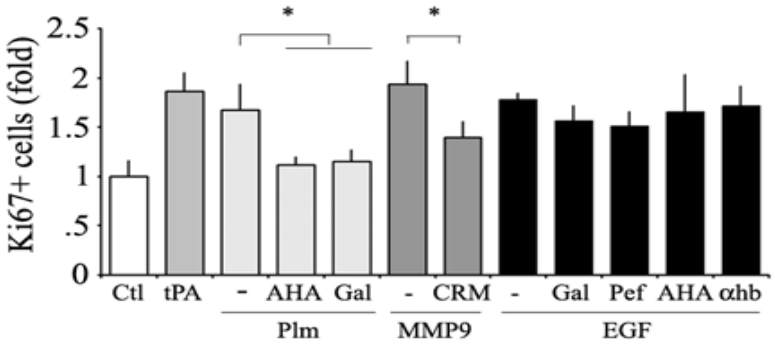
Figure 6. Depletion of MMP-9 inhibits the proliferative response to tPA. **A.** Cells were transfected with pEGFP and siRNA duplexes specifically targeting MMP-9 (siRNA, 80 nM), or control siRNA duplexes with no known targets (Scr, 80 nM). Seventy-two hours post-transfection, culture supernatants were concentrated, and equal amounts of proteins were analyzed by Western blotting. Secreted MMP-9 levels were quantitated by densitometric scanning of the signals (right graph). **B.** Effects of the depletion of MMP-9 on the proliferative status of PANC-1 cells. Cells were transfected with pEGFP and the specific MMP-9 siRNA (siRNA) or a control siRNA (Scr), serum deprived for 48 h and either not treated (Ctl) or treated with tPA (6 nM) or EGF (3 nM). Double fluorescence counting for Ki67 and GFP was used to score the number of proliferating cells in the transfected population. At least 400 cells were counted for each condition in two independent experiments. The error bars represent the SEM. (**P* = 0.04).

Supplementary Figure 1



Supplementary Figure 1. tPA phosphorylates EGFR and ERK 1/2 in A431 cells. **A.** Serum-starved A431 cells were treated with tPA or EGF as described in **Figure 1**. EGFR phosphorylation was analyzed by Western blotting with antibody to phosphorylated Tyr 1173 of EGFR. The signals yielded by this antibody were normalized against the signals yielded by the antibody to total EGFR, and the ratios are shown in the bar graph. **B.** tPA induces the phosphorylation of the ERK 1/2 in A431 cells, as analyzed by Western blotting with antibody to phosphorylated proteins. The bar graph represents the intensity of the signals given by the anti phospho-ERKs normalized for EGFR total protein signals.

Supplementary Figure 2



Supplementary Figure 2. Requirements for the plasmin and MMP-9 induced proliferation. Plasmin (0.05 U/ml) (Plm), MMP-9 (7 nM), tPA (6 nM) or EGF (3 nM) were added to serum-starved PANC-1 cells without or with specific inhibitors, and analyzed after 6 hours by fluorescent staining with anti-Ki67 antibodies. At least 400 cells were scored for each condition. Results are the mean from two independent experiments. Pef, pefabloc/tPA (30 μM); Gal, galardin (10 μM); AHA, p-Aminobenzoyl-gly-pro-D-ala-hydroxamic acid (1 μM); CRM, CRM197 (5 μg/ml); αhb, neutralizing antibody to hb-EGF (10 μg/ml).

Supplementary Table. (Veure Annex) Lists of genes that most significantly discriminate between treatment with one of the growth factors and the other two: PDGF *vs*. tPA and PDGF; EGF *vs*. tPA and PDGF; tPA *vs*. EGF and PDGF. After normalization of the microarray signals, FADA was applied to select for the genes that contribute most significantly to the transcriptional response to each growth factor at each time point. The selected genes were further submitted to a two-sided t-test for the above sample group comparisons. The genes shown have a P-value below 10^{-5}, with Bonferroni adjustment (Materials and Methods).

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ANNEX (SupplementaryTable)

PDGF *vs* tPA+EGF	**tPA *vs* EGF+PDGF**	**EGF *vs* tPA+PDGF**	**tPA *vs* EGF**	**EGF vs PDGF**	**tPA vs PDGF**
$p \leq 0.00001$	$p \leq 0.00001$	$p \leq 0.00001$	$p \leq 0.00001$	$p \leq 0.00001$	$p \leq 0.00001$
PRC1	AA425056	HLF	PLK1	CRADD	RAB26
MAD2L1	TUBB	CRADD	GPR126	YAP1	MAPK1
IFITM1	GPR126	YAP1	PFTK1	RTN1	TUBA1
B7	AA490158	N54960	N54960	NEF3	EBNA1BP2
CES1	NUCKS	DKFZP434I216	COPA	MGC33948	MYBL2
PTTG1	ATXN10	NUP188	TPX2	TM7SF2	EPHB2
STK16	R39616	RTN1	SEMA6C	PIK3CD	CRABP2
SPARC	ZFP36L2	NEF3	MRRF	R40105	LTBP2
ZNF263	SIAT4B	PDE1C	PNOC	HSD17B4	SPARC
MGC23918	W99317	MGC33948	HLF	PLK1	RECQL5
CDC6	PA2G4	R10279	R10279	COL4A6	ITGA3
TUBA3	N50702	TM7SF2	AA088446	DSCR3	IDH2
UBE2C	RFX2	ITM2B	COL4A6	NT5E	MFGE8
LOXL2	AA436565	C12orf8	ITM2B	R53605	RGS11
SPS	NUP88	SEMA6C	C9orf13	N34897	CDC6
LTBP2	MGC4308	AA912416	COL12A1	APOC1	PSMD11
MAPK1	PLA2G12A	AA608907	DNAJC10	RAB26	LOC492304
ACAT2	W95050	TMED8	DNAI1	HOXB6	SPAG5
CRABP2	RAB31	VCX3A	PRKACB	EIF2S1	FOXM1
HMGN2	CASR	TPX2	MTSS1	15E1.2	CAV1
DNAJC4	SIMP	MDK	APOBEC3F	PRKDC	IFITM1
RAB13	PHLDA1	PIK3CD	T83842	MAPK1	ABCA3
PIK3R4	BRD3	R40105	PAIP2	CCNB1	HMGN2
TUBA1	CAPZB	HSD17B4	AA453807	POLR2J2	LOXL2
UBE2H	POU2F2	VAV1	C10orf46	SMARCA3	H2-ALPHA
DHCR24	FDPS	FLJ20811	VCAM1	GCLM	PDCD5
GSTM5	PSMB3	FASTK	DKFZP434I216	DNAJC4	RAB13
MYBL2	MUTYH	CASR	RFX2	TUBA1	UBE2C
EBNA1BP2	Cep63	PLK1	NUP188	EBNA1BP2	CAPN2
DLG7	SERPINB2	PLCXD1	RAD23B	AA479475	PRKCZ
PCNA	MRRF	VAT1	C15orf19	MYBL2	CTSH
PSMD11	RAB31	N50669	PGM1	TRIP	PTK7
RTN1	C9orf13	DFNA5	RIPK1	EPHB2	SPS
MAP3K5	PPP1R7	PAIP2	GAS6	SLAC2-B	TUBA3
H2AFX	GRB2	ANGPTL4	N52549	CRABP2	STK16
EPHB2	DERL1	COL4A6	AA405690	LTBP2	AURKB
IDH2	SLC8A1	PRKACB	VCX3A	SPARC	ZNF263
TIA1	AA029428	DSCR3	FLJ44005	RECQL5	CES1
PBK	NCALD	KRT8	MDK	AA400485	ACAT2
ABCA3	SNRPN	HP1-BP74	TMEM28	GSTM5	PIK3R4
SPAG5	C20orf45	COL12A1	STAC	ITGA3	ADSL
ZNF177	KRT25D	NT5E	ZFP36L2	IDH2	DLG7
MAP4K1	KNTC2	CD68	CASR	MFGE8	PTTG1
AA417911	SLC12A7	OBSCN	OCIL	RGS11	CDC2
AA398757	PSMA2	PROSC	AA425056	CSF3	H80215
PPARG	TNFSF7	N34897	CD109	ZFYVE26	H2AFX
BRRN1	CD109	AA029428	T98056	ARHGEF2	MAD2L1
DLG7	INPPL1	C15orf19	SIAT4B	SFRP4	ZNF286
FOXM1	DNAJB1	STAC	VBP1	FLJ20811	DLG7
PTK7	CTSH	LRPPRC	IMPDH2	RPL21	GARNL3
RGS11	ASGR2	IGFBP7	AA029428	CDC6	EEF2
RECQL5	R40129	CRYAA	SIMP	FLJ10979	TACC3
MFGE8	HLA-DOB	KIAA0317	PDE1C	BMP6	ZNF177

EEF2	KIAA0355	SSB	ANGPTL4	PSMD11	GTSE1
LOC492304	GHRH	RNPC1	CRADD	MAP4K1	GPA33
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CAPÍTULO VI: Discusión

El objeto de estudio de esta tesis ha sido dilucidar los diferentes mecanismos a partir de los cuales las serina proteasas activadoras del plasminógeno, uPA y tPA, llevan a cabo sus efectos en la progresión tumoral del cáncer de colon y de páncreas. Los resultados descritos en los capítulos III, IV y V, muestran de forma detallada las vías de transducción de señal utilizadas por estos PAs. Se muestra por un lado como uPA, proteolíticamente activo, estimula el efecto de *"scatter"* en las células de cáncer de colon HT29-M6, y por otro como tPA mediante la unión a anexina II y la transactivación de EGFR estimula la invasión y la proliferación en células de cáncer de páncreas.

Efectos inducidos por uPA en lás células de cáncer de colon HT29-M6

Las células HT29-M6 de cáncer de colon son un modelo ampliamente utilizado por su capacidad de movilidad "*in vitro*" e invasiva "*in vivo*". El HGF es uno de los factores de crecimiento que provocan cambios morfológicos y de movilidad en estas células. *"In vitro"*, los cambios de movilidad inducidos por HGF o PMA se han definido como "*scatter*" (Herrera, 1998). Se demostró que el fenómeno de "*scatter*" es consecuencia de una serie de cambios que se dan a nivel celular como la activación de la PKC y el descenso del nivel de expresión de la E-cadherina. Estos cambios provocan pérdida de unión entre células y entre células y substrato, con el consecuente aumento de la movilidad celular (Fabre and García de Herreros, 1993; Skoudy and García de Herreros., 1995; Skoudy et al., 1996; Llosas et al., 1996; Batlle et al., 1998). El aumento de la movilidad celular causado por HGF se acompaña de un incremento en la expresión y actividad de uPA (Pepper et al., 1992; Ried et al., 1999) que, a su vez, activa HGF, estableciéndose así una retroalimentación positiva que aumenta el efecto "*scatter*" (Naldini et al., 1992). Los tumores del páncreas exocrino sobreexpresan HGF y su receptor (c-met) pero también uPA y uPAR (Paciucci et al., 1998 a; 1998 b); en paralelo otros autores han observado un descenso en la cantidad de E-caderina en membrana en estos tumores **(**Pignatelli et al., 1994**).** Estos cambios se reproducen *"in vitro"* en el fenómeno de *"scatter"* al tratar líneas celulares derivadas de tumores, IMIM-PC2, con el factor HGF, tal y como sucede en las células de cáncer de colon (Paciucci et al., 1998 b).

En los resultados de esta tesis demostramos que para inducir "*scatter*" en células de cáncer de colon PMA y HGF requieren la actividad de uPA y de plasmina. Además

de la presencia de Plg, la unión de Plm a la MEC es fundamental para el incremento de la movilidad de estas células (Jeffers et al., 1996), por lo que tanto la ausencia de plasminógeno como el bloqueo de los lugares de unión de Plm, inhiben el efecto generado por HGF o PMA (**Figura 1, capítulo III**). Estudiamos cual es la proteasa que, en respuesta a PMA, lleva a cabo la activación del Plg a Plm. Los resultados muestran que uPA es la proteasa implicada en el "*scatter*", coincidiendo plenamente con trabajos previos en los que se describía la importancia de esta proteasa y su receptor en procesos de migración e invasión (**Figura 2, capítulo III**) (Lund et al., 1991; Naldini et al., 1992; Pepper et al., 1992; Wang et al., 1994; Jeffers et al., 1996; Paciucci et al., 1998 b). Comprobamos que la expresión de uPA y Plm, se incrementa después de estimular las células con PMA (**Figura 3, capítulo III**), tal como sucede en las células de cáncer de páncreas donde al ser tratadas con HGF incrementa el nivel de uPA y de uPAR (Jeffers et al., 1996; Paciucci et al., 1998 b).

Como se ha descrito anteriormente, el proceso de "*scatter*" requiere la activación de PKC y un descenso en la concentración de E-cadherina. Fenotipicamente se observan cambios en la movilidad celular y un aumento de la degradación de la MEC. Demostramos así que la degradación de la MEC es necesaria también para permitir la migración de estas células sobre plástico. Nuestro estudio demuestra que en el proceso de "*scatter*" inducido por PMA se pueden identificar dos fases. En la fase temprana, PMA estimula la activación de la PKC, la disminución de expresión de E-cadherina y la ruptura de las uniones entre células vecinas. Estos fenómenos no son alterados por inhibidores específicos de uPA ni de Plm, demostrando que la actividad de estas proteasas se requiere en un segundo momento (**Figura 3, capítulo III**). La fase tardía, se distingue a partir de las 4 horas. En esta fase se observa un aumento progresivo de la expresión de uPA/uPAR y de la movilidad celular acompañado por un aumento de la degradación de la MEC.

Se ha hipotetizado que el aumento de la movilidad celular y los cambios de adhesión de las células a la matriz extracelular podían ser provocados: i) como consecuencia de una proteólisis del uPAR al tratar con uPA, que provocaría un cambio conformacional del receptor, quedando accesible una zona entre los dominios D_1-D_2, responsable de la migración en fibroblastos (Resnati et al., 1996; Fazioli et al., 1997); ii) por una alteración de la unión entre uPAR y β1 integrina, que controla el ciclo de unión-desunión de las células al substrato (Yebra et al., 1996, 1999). En ambos casos está

implicada la unión de uPA a su receptor. Demostramos que en las células HT29-M6 en respuesta a PMA, el dominio D_1-D_2 de uPAR no es el causante de la activación de la migración, puesto que al tratar las células con el fragmento purificado de uPAR, causante de la migración en fibroblastos, (D_2D_3 uPAR purificado) no observamos aumento de la migración celular. Además, tampoco detectamos cambios en la interacción entre uPAR y β1 integrina al tratar las células con PMA (resultados no mostrados, capítulo III) Tanto la actividad de Plm como de uPA, son necesarias para la proteolisis de la MEC y para el fenotipo *"scatter"* (**Figura 4, capítulo III**). Otros grupos habían mostrado anteriormente la capacidad de uPA, sin actividad catalítica, para generar un efecto quimiotáctico en monocitos (Resnati et al., 1996; Fazioli et al., 1997), así como aumentar la expresión de MMPs (Menshikov et al., 2006). En nuestro sistema, el proceso de *"scatter"* requiere la Plm, que proteolíticamente activada por uPA, lleva a cabo la degradación de la MEC (**Figura 4, capítulo III**).

Estudios anteriores a nuestros resultados habían mostrado que en la invasión y en la migración uPA/uPAR interaccionan con integrinas y con vitronectina para activar diferentes quinasas como: FAK, p130, PKC y MAPK (Tang et al., 1998; Wilson and Gibson, 2000; Tarui et al., 2001; Van der Pluijm et al., 2001; Jo et al., 2003 a). Nuestros resultados muestran que la respuesta celular de "*scatter*" inducida por PMA se lleva acabo a través de la activación de ERK 1/2, cPKCα y Src quinasa (**Figura 5, capítulo III**). La activación de cPKCα y Scr quinasa ocurre en la fase temprana del proceso, dependiente de PMA, en la que se rompen los contactos celulares (Llosas et al., 1996; Skoudy et al; 1996) y empieza la secreción y activación de uPA. En la fase tardía del "*scatter*", dependiente del sistema Plg/Plm, interviene la vía de ERK1/2, (**Figura 6, capitulo III**). Esta activación de ERK 1/2 por efecto de uPA-Plm, tiene la peculiaridad de no necesitar la activación previa de EGFR (**Figuras 6 A y B, capitulo III**), en contraste con publicaciones anteriores en las que se había descrito que uPAR, activado por uPA, forma un complejo multiproteico dinámico con diferentes proteínas adaptadoras, llamado matastasoma, en el que el EGFR es indispensable para la activación de ERK 1/2 (Aguirre Ghiso 1999, 2001, 2003; Liu et al., 2002; Jo et al., 2003 b, 2005, Saldanha et al., 2007).

Finalmente, hemos demostrado que plasmina puede estimular "*scatter*" directamente, sin tratamiento previo con PMA (**Figura 7, capítulo III**). El hecho que esto ocurra solamente cuando las células se siembran a baja densidad, indicaría que

PMA es necesario para eliminar las uniones entre células y para activar la expresión de uPA/uPAR. El mecanismo por el cual Plm llevaría acabo el proceso de *"scatter"*, o el receptor de membrana responsable de los efectos observados, no se conocen (**Figuras 6 y 7, capítulo III**). Se ha descrito que la proteasa activa puede inducir migración uniéndose simultáneamente a integrinas y a PAR-1 (receptor activado por proteasa), en diferentes líneas celulares (Tarui et al., 2002; Majumdar et al., 2004), también se ha descrito que PAR-1 y ERK 1/2 son necesarios para que la Plm induzca proliferación y angiogénesis en fibroblastos (Pendurthi et al., 2002; Mandal et al., 2005). Nuestros datos no excluyen que tanto integrinas como PAR-1 puedan estar involucrados en el *"scatter"*.

Vía de señalización y efecto de tPA en células de cáncer de páncreas

tPA se sobreexpresa en líneas celulares de cáncer de páncreas y en tumores de cáncer de páncreas exocrino (Paciucci et al., 1996, 1998 a; Aguilar et al., 2004). Tanto en líneas celulares como en tumores, esta serina proteasa interviene en la proliferación, la invasión y en la angiogénesis (Díaz et al., 2002). El mecanismo mediante el cual tPA lleva a cabo estos efectos se desconocía en el momento en el que se empezó esta tesis. En la segunda parte de los resultados, se estudia el mecanismo de acción de tPA, buscando un receptor específico de tPA en células de cáncer de páncreas (capítulo IV) y, en determinar la vía de transducción de señal utilizada en la activación de la proliferación (capítulo V).

Utilizando ensayos de unión de tPA recombinante sobre células in vivo pudimos observar que tPA se une a las células de adenocarcinoma pancreático (SK-PC-1 y PANC-1) con una K_D 25-48 nM, de manera saturable (**Figura 2, capítulo IV**) y específica (**Figura 3A, capítulo IV**). Esta K_D se aproxima mucho a la descrita para la unión de tPA a anexina II en células endoteliales (Hajjar, 1991). Sabiendo además que la anexina II se sobreexpresa en cáncer de páncreas (Paciucci et al., 1998 a), dirigimos nuestras investigaciones a comprobar si esta proteína podía ser el receptor de tPA en células de adenocarcinoma pancreático. Nuestros resultados muestran que anexina II interacciona con tPA en cáncer de páncreas, las dos proteínas colocalizan en tumores pancreáticos (**Figura 5, capítulo IV**) y coinmunoprecipitan en células SK-PC-1 (**Figura 4, capítulo IV**). Además, la activación de Plm por parte de tPA unido a la superficie celular, desciende si utilizamos un péptido específico que bloquea la unión de

anexina II con tPA (LCKLSL) (**Figuras 3B y C, capítulo IV**) y/o si utilizamos el quelante de Ca^{2+} EGTA (**Figura 3C, capítulo IV**), que impide la unión de anexina II a la membrana celular. Estos resultados confirman que la unión entre tPA y anexina II, se da entre el dominio *"finger"* de la serina proteasa (Cesarman et al., 1994; Hajjar et al., 1994) y la cisteína del hexapéptido LCKLSL de la región N-terminal de la anexina II (Hajjar et al., 1998). Esta unión también puede bloquearse por la formación de puentes disulfuro entre la cisteína de anexina II, y la homocisteína y/u otros péptidos que contengan cisteína (Hajjar et al., 1998; Roda et al., 2003). El conjunto de estas evidencias sugiere que la activación de Plg a Plm ocurre cuando tPA se une a la membrana celular vía anexina II, que a su vez interacciona con los fosfolípidos de la membrana celular, mediante el "*repeat 2*" de su dominio endonexina (Hajjar et al., 1996).

Otros resultados apoyan el papel de anexina II como receptor de tPA en tumores (Kumble et al., 1992; Babbin et al., 2007; Falcone et al., 2001; Emoto et al., 2001 a). Anexina II se sobreexpresa en adenocarcinomas pancreáticos ductales (Paciucci et al., 1998 a; Aguilar et al., 2004) y actúa como receptor específico de tPA en células de cáncer de páncreas (Ortiz-Zapater et al., 2007). La expresión de este receptor está asociada a un mal pronóstico, además de en adenocarcinomas pancreáticos (Davis and Vishwanatha, 1995; Kumble et al., 1992; Vishwanatha et al., 1993; Aguilar et al., 2004), en cáncer de cabeza y cuello (Pena-Alonso et al., 2007), en carcinoma gástrico y colorectal (Emoto et al., 2001 a, b), en cáncer de mama (Correc et al., 1990) y en leucemia promielítica aguda (Menell et al., 1999). En cambio, en cáncer de próstata y en cáncer de esófago la sobreexpresión de esta proteína, está asociada a buen pronóstico (Liu et al., 2003 b; Yee et al., 2007; Qi et al., 2007).

Pese a haber demostrado que anexina II es el receptor mayoritario para tPA en líneas celulares de cáncer de páncreas, nuestros resultados sugieren que una fracción menor de tPA se une a la superficie celular mediante un mecanismo diferente, ya que tanto la colocalización, como la coinmunoprecipitación de anexina II con tPA es parcial; y el bloqueo de la unión de entre ambas proteínas, con LCKLSL y EGTA, no provoca un descenso del 100% en la activación de Plm. Estos resultados podrían explicarse considerando que existe una cierta proporción de anexina II en forma de heterotetrámero II con p11 (AIIt). El AIIt se forma por la unión de p11 a anexina II en la región de unión de tPA y puede unir Plg de forma independiente de Ca^{2+} (Roda et al., 2003; Jost et al., 1997; Kassam et al., 1998). Esto explicaría la inhibición parcial de tPA

a las células de cáncer de páncreas observada en presencia de EGTA, o del péptido LCKLSL (**Figura 3C, capítulo IV**). Se ha descrito en ensayos "in vitro", que la unión de tPA a AIIt se da mediante la unión directa de la serina proteasa a las subunidades p11 (McLeod et al., 2003). Esta unión de tPA a AIIt, podría activar la cascada proteolítica mediante la interacción previa con procathepsina B (Mai et al., 2000 a), y la posterior interacción y activación de uPAR (Kobayashi et al., 1993), caveolina (Schnitzer et al., 1995) e integrinas (Lee et al., 1993), teniendo un comportamiento muy similar al uPAR, en las células de cáncer de colon (capítulo III), focalizando la proteolisis en la membrana celular de forma que se facilita la relación entre la MEC y las funciones intracelulares (Mai et al., 2000 b). Por último, también se ha descrito que tPA puede unirse, independientemente del Ca^{2+}, a la proteína p11 en forma monomérica y a otra proteína de la familia de p11, la S100A4, en células de cáncer de colon (Zhang et al., 2004) y endoteliales (Semov et al., 2005) respectivamente, aumentando así la activación de plasmina. La presencia de estas dos proteínas, de la familia de las proteínas S100 (*ver apartado 1.2.1.2,* capítulo I), en forma monomérica, en las células de cáncer de páncreas, también justificaría la colocalización y coinmunoprecipitación parcial de anexina II con tPA, así como la falta de eficiencia en la inhibición de LCKLSL y EGTA.

Por otro lado, hallazgos previos de nuestro laboratorio muestran que tPA se une parcialmente a la región apical de las células SK-PC-1, región en la que no se expresa anexina II, por lo que tPA está unido a un receptor diferente en la región apical. Esta posibilidad está corroborada en la literatura, donde se ha descrito que tal y como sucede en la unión a fibrina (Van Zonneveld et al., 1986 a; de Vries et al., 1990), tPA también puede unirse a células endoteliales, de melanoma y a monocitos mediante sus dominios kringle (Bizik et al., 1993, 1997; Felez et al 1993) y, con mayor afinidad, aunque en menor número, mediante su dominio catalítico en células musculares (Barnathan et al., 1988; Razzaq et al., 2003). Esto hace suponer que, en células PANC-1, tPA también puede unirse a la galectina-1 (Roda et al., 2009) o a otros receptores de membrana descritos en otras líneas celulares como: la anfoterina, la citoqueratina 8, el receptor de receptor N-metil-D-aspartato (NMDA), la α-enolasa o la proteína-4 asociada al citoesqueleto (CKAP4**).**

Galectina-1 se sobreexpresa en adenocarcinomas pancreáticos ductales humanos (Berberat et al., 2001; Iacobuzio-Donahue et al., 2003; Shen et al., 2004) y en líneas celulares pancreáticas (Roda et al., 2009), por lo que podría ser el receptor expresado en

estas células junto con anexina II. Recientemente, se ha descrito que tPA se une a galactina-1 de manera directa, específica y con alta afinidad, provocando la activación de las ERK 1/2, la proliferación celular y la invasión en líneas celulares pancreáticas (Roda et al., 2009), tal y como sucede en nuestros hallazgos.

La proteína transmembrana CKAP4, también podría intervenir en la unión de tPA, ya que esta proteasa se une a CKAP4 mediante su dominio catalítico, en células musculares, y lo hace, tal y como sucede en nuestros hallazgos, de manera específica y saturable con una K_D =15 nM (Razzaq et al., 2003). El receptor de NMDA (NMDAR) también interacciona con la región catalítica de tPA en neuronas (Nicole et al., 2001), pero no se ha descrito la cinética, ni la K_D, ni el tipo de unión entre ambos. En cambio, la unión de tPA a anfoterina no es saturable (Parkkinen and Rauvala, 1991) y la K_D de unión de tPA tanto a CK8, como a α-enolasa (Hembrough et al., 1996 b; Kralovich et al., 1998; Felez et al., 1991, 1993), es muy diferente a la que se da en células de cáncer de páncreas, indicando que es improbable que estas proteínas funcionen de receptores para tPA en estas células.

A partir de estos resultados concluimos que tPA interacciona de forma mayoritaria, aunque no total, con anexina II en las células de cáncer de páncreas, interviniendo en el proceso de invasión celular (**Figura 7, capítulo IV**).

Una vez demostrado que tPA puede llevar a cabo su efecto invasivo mayoritariamente mediante la unión a anexina II (capítulo IV), estudiamos el modo mediante el cual la proteasa estimula la progresión tumoral, y en particular analizamos el mecanismo por el cual induce la proliferación (capítulo V).

tPA puede activar el EGFR y PDGFR (Maupas-Schwalm et al., 2005; Fredriksson et al., 2004**).** El receptor para EGF se sobreexpresa en diferentes líneas de cáncer de páncreas y en tumores donde tiene un papel clave en la proliferación (Bruns et al., 2000; Sclabas et al., 2003; Murphy et al., 2001; Ho et al., 1999; Korc et al., 1998; Lieberman et al., 1996; Lemoine et al., 1992; Matsuda et al., 2002; Yamanaka et al., 1993; Korc et al 1992). El PDGFR también se sobreexpresa en estos tumores (Ebert et al., 1995), donde interviene en la progresión tumoral (Graves et al., 1996; Herbert et al., 1997; Yu et al., 2000). Nuestros resultados muestran que, el tratamiento de células PANC-1 con tPA o con EGF, induce la expresión de un set de genes similares sobretodo a tiempos cortos (**Figura 1, capítulo V**). En cambio el set de genes activado por PDGF es muy diferente (**Figura 1, capítulo V**). Esto sugiere que la vía de

señalización activada por tPA, se solapa parcialmente con la utilizada por EGF, pero no con la de PDGF.

El tratamiento con tPA resulta en la fosforilación rápida del EGFR (**Figura 2B, capítulo V**). Esta fosforilación es necesaria para la acción de tPA, por lo que el inhibidor específico del receptor (AG 1478) disminuye el efecto mitogénico de tPA un 50% (**Figura 2A, capítulo V**). De manera similar, la inhibición de la expresión del EGFR, mediante siRNA, provoca un descenso muy significativo de los efectos mediados por tPA (**Figura 4, capítulo V**). En cambio, la actividad de PDGFR no se requiere para los efectos inducidos por tPA, ya que tPA no lo fosforila y el uso de su inhibidor específico (AG 1296) no afecta la proliferación mediada por esta proteasa. (**Figuras 2 B y A,** respectivamente**, capítulo IV**). tPA provoca la endocitosis de la proteína de fusión GFP-EGFR (**Figura 2C, capítulo IV**) confirmando que el receptor se activa y sufre un proceso similar al observado después del tratamiento con EGF (Sorkin et al., 2000; Nakashima et al., 1999; Carter et al., 1998; Hayes et al., 2004; Miaczinska et al., 2004). En cambio, el PDGFR no se internaliza tras el tratamiento con tPA (**Figura 2C, capítulo IV**). Estas evidencias indican que tPA promueve la proliferación de las células tumorales PANC-1, mediante la activación del EGFR (**Figura 2, capítulo V**).

El EGFR funciona como integrador de señales (Normanno et al., 2006) y puede activar múltiples vías de señalización celular en función del tipo celular y del ligando, activando así diferentes procesos celulares (Schlessinger 2000; Singh and Harris, 2005; Mendelshon and Baselga, 2006). Nuestros resultados muestran que la activación por tPA tiene un efecto similar a la activación por EGF en estas células, activando principalmente las ERK 1/2 y no la p38 (**Figura 3B, capítulo V**). EGF puede activar ERK 5 y la proliferación en células de cáncer de mama (Kato et al., 1998) y cáncer de páncreas, pero en las células tumorales páncreáticas tPA no activa esta quinasa (**Figura 3B, capítulo V**). Así, pese a tener muchas similitudes, los efectos de tPA y EGF no son exactamente iguales. Otras diferencias se aprecian en la intensidad y el tiempo de activación máxima de ERK 1/2, y en el efecto parcial del inhibidor AG 1478, cuando la fosforilación de ERKs es mediada por tPA (**Figura 3B, capítulo V**). Además, tPA no induce la fosforilación de AKT (**Figura 3C, capítulo V**). Estudios coetáneos de otro grupo corroboran la acción de tPA sobre la inducción ERK 1/2, y no p38, pese a que se observa una ligerísima fosforilación de AKT a tiempos muy cortos (Ortiz-Zapater et al., 2007). Estos estudios también discrepan de nuestros resultados demostrando que la

actividad proteolítica de tPA no se requiere para inducir las quínasas ERK 1/ 2. La concentración de tPA utilizada en estos ensayos (veinte veces mayor a la utilizada en nuestros experimentos) podría explicar las discrepancias observadas. No obstante, estos resultados también ponen en evidencia que tPA y EGF activan vías de transducción de señal que coinciden, aunque no totalmente (Ortiz-Zapater et al., 2007).

Para definir el mecanismo usado por tPA en la activación del EGFR y sabiendo que su acción proteolítica es importante en esta activación, comprobamos si tPA es capaz de transactivar el EGFR por el mecanismo denominado "*the triple membrane-passing signalling"*. En este mecanismo intervienen proteínas unidas al receptor que trasmiten su señal, proteasas (MMPs i/o ADAMs) que catalizan el corte proteolítico de agonistas de EGFR, y por último estos agonistas unidos a la membrana plasmática en forma latente y que una vez proteolizados activan el receptor de EGF (Prenzel et al., 1999, 2000).

El PDGFR ha sido descrito como uno de los receptores más importantes en la transactivación de EGFR tanto en fibroblastos (Li et al., 2000, He et al., 2001), como en células VSMC (Saito et al., 2001). Pese a ello, como ya hemos comentado anteriormente, nuestros resultados muestran que este receptor no interviene en la señalización inducida por tPA. Otros receptores que también han sido descritos como transactivadores de EGFR en diferentes tipos celulares són el IGF-1R (Roudabush et al., 2000; El-Shewy et al., 2004), los receptores de trombina, plasmina y tripsina, PAR-1 y -2, (Kalmes et al., 2000; Gschwind et al., 2001, 2002; Miyata et al., 2000), Fzt (Civenni et al., 2003) y los receptores acoplados a proteínas G (GPCR) de LPA, (Daub et al., 1996, 1997), de ésters de forbol (Umata et al., 2001), de bombesina (Prenzel et al., 1999), de bradiquinina, de carbacol (Gschwind et al., 2002) y de diversos tipos de hormonas (Pai et al., 2002; Filardo et al., 2000). Nuestros estudios no determinan cual de estos receptores interviene en la transactivación de EGFR por acción de tPA, pero solo el PAR-1 ha sido descrito como catalizado directamente por tPA (Nagai et al., 2006) y no se descarta su implicación en la acción de tPA sobre la activación de EGFR, tal como sucede con la trombina en las células HT29-M6 (Darmoul et al., 2004).

Una vez activado el receptor, una proteína G acoplada a éste (mayoritariamente la G_i, sensible a la toxina pertussis, lleva a cabo la posterior activación de metaloproteinasas latentes (Daub et al., 1996, 1997; Prenzel et al., 1999, 2000; Kanda et al., 2001; Filardo et al., 2000; Pai et al., 2002). La toxina pertussis no tiene efecto alguno sobre la activación de la proliferación mediada por tPA en células PANC-1,

indicando que tPA no activa el EGFR vía proteínas G_i (**Figura 3A del capítulo V**). Tampoco participan proteínas G_q, que intervienen en la activación del EGFR a través de la activación de PKC (Eguchi et al., 1998), (**Figura 3A, capítulo V**), ya que el inhibidor de PKC (GF 109203X) no afecta la activación de la proliferación mediada por tPA. La proteína $G_{\alpha 13,}$ podría llevar a cabo la activación de las MMPs en nuestra línea celular, tal como sucede en fibroblastos, donde LPA transactiva EGFR, mediante la activación de esta proteína $G_{\alpha 13}$ (Gohla et al., 1998).

Prenzel y colaboradores (1999; 2000) demostraron que las proteínas G, llevan acabo su efecto a través de la activación de MMPs, al observar que el efecto de los diferentes ligandos de GPCRs sobre EGFR desaparece al tratar con Batimastat, inhibidor de MMPs. Estos resultados también coinciden con nuestros ensayos, en los que la Galardina, otro inhibidor de amplio espectro para MMPs, impide la fosforilación y la proliferación inducidas por tPA (**Figuras 5B y C,** respectivamente**, capítulo V**). Por otro lado, se había descrito que la metaloproteinasa MMP-9 se sobreexpresa en células de cáncer de páncreas (Gress et al., 1995; Haq et al., 2000; Takada et al., 2004); y que tPA incrementa su expresión (Wang et al., 2003; Hu et al., 2006), y su activación en diferentes tipos celulares (Zhao et al., 2004; Ueda and Matsushima, 2001). Estas observaciones sugerieron que en células PANC-1, tPA podría activar en primer lugar pre-MMP-9 para transactivar EGFR, tal como hace la gonadotropina en células de carcinoma prostático (Roelle et al., 2003). En las **figuras 5B y C** del capítulo V vemos que el inhibidor específico de MMP-9, AHA, provoca la inhibición de la fosforilación de ERK 1/2 y de la proliferación inducida por tPA. Por último, en la **figura 6** de este mismo capítulo, corroboramos la importancia de esta metaloproteinasa al mostrar que su expresión es imprescindible para la proliferación provocada por tPA, pero no es necesaria para la acción de EGF, reafirmando las diferencias anteriormente comentadas entre la vía de señalización activada por la serina proteasa y el factor de crecimiento (Roelle et al., 2003).

El hbEGF, importante en la progresión tumoral (Miyamoto et al., 2006; 2008), es probablemente el agonista del EGFR activado por MMP-9. El resultado de la *"real time PCR"* (**Figura 5A, capítulo V**) muestra que hbEGF se expresa a niveles más altos que anfiregulina, epiregulina y TGF alfa en las células PANC-1, tal y como se había demostrado anteriormente en diferentes líneas páncreáticas (Kobrin et al., 1994). Comprobamos que el efecto de tPA se da mediante la acción de este agonista porque el tratamiento tanto con el inhibidor de su actividad, CRM 197, como con un anticuerpo

bloqueante específico, anulan el efecto de tPA en la señalización y en la proliferación celular (**Figura 5B y C,** respectivamente**, capítulo V**).

Todos los hallazgos anteriores confirmarían la hipótesis de que en las células PANC-1, tPA activa la fosforilación de ERK 1/2 y la proliferación celular siguiendo un mecanismo similar al postulado por el grupo de Ullrich (Prenzel et al., 2000). tPA unido a Anexina II activa un GPCR (probablemente PAR-1) unido a una proteína G insensible a toxina pertussis (posiblemente $G_{\alpha 13}$) que a su vez provoca el paso de pro-MMP-9 a MMP-9. Alternativamente, la MMP-9 puede ser activada por plasmina más directamente (Zhao et al., 2004). MMP-9 induce "*shedding*" de hbEGF que una vez solubilizado se une y activa el EGFR con posterior fosforilación de ERK 1/2. Esta cascada de activaciones resulta en un incremento de la proliferación. Por último, se ha descrito que anexina II, que une de forma específica y saturable tPA **(Figuras 2 y 3A, capítulo IV**), también interviene en la fosforilación de ERK 1/2 en PANC-1, estimulada por esta proteasa, aunque no se conoce el mecanismo que utiliza para llevar a cabo este efecto (Ortiz-Zapater et al., 2007).

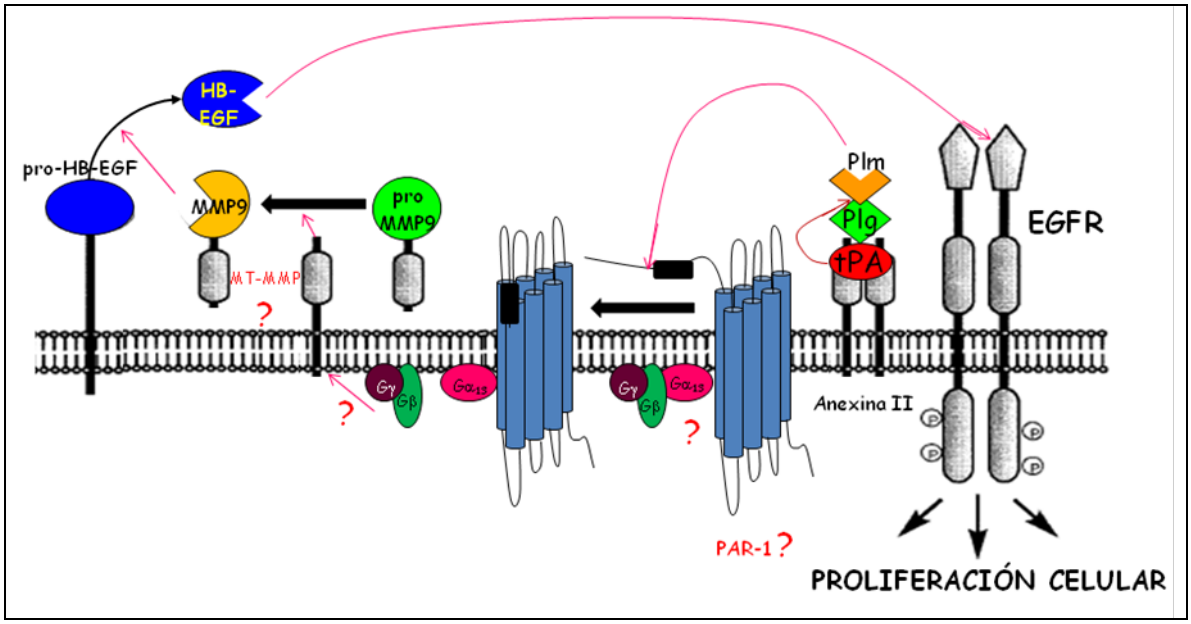

Figura 11. Modelo de transactivación de EGFR inducida por tPA en células de cáncer de páncreas. El modelo que propomemos, postula que tPA se une a su receptor específico de membrana Anexina II que a su vez une plasminógeno, este complejo promueve la generación de plasmina activa en la superficie celular. La plasmina activaría el receptor de PAR-1 (Receptor activado por una proteasa 1), el cual a su vez provocaría el paso de una small GDP (proteína guanosina-5'-bifosfato pequeña) a small GTP (proteína guanosina-5'-bifosfato pequeña). Las subunidades β y γ de la small GTP podrían activar la pro-MMP-9 a través de una MMP de membrana (MT-MMP). La plasmina podría también activar pro-MMP-9 directamente. Una vez activa la MMP-9, ésta procesaría el pro-hb-EGF a hb-EGFque una vez liberado se uniría y activaría al EGFR.

Finalmente analizamos si tPA necesita ser catalíticamente activa para llevar a cabo su efecto. Al tratar las células con tPA inactivado, unido a Pefabloc o DFP, la fosforilación de ERK 1/2 desaparece (**Figura 5B, capítulo V**). Lo mismo sucede si en lugar de tPA catalíticamente activo, utilizamos un mutante de tPA con el dominio catalítico mutado, tPA S478A (**Figura 5B, capítulo V**). En ensayos de proliferación, estos inhibidores también reducen más del 80% la proliferación inducida por tPA proteoliticamente activo (**Figura 5C, capítulo V**). También comprobamos que la actividad catalítica de plasmina es necesaria para este efecto, puesto que su inhibidor, α_2-antiplasmina, inhibe la proliferación inducida por tPA totalmente (**Figura 5C, capítulo V**). Estos resultados indican que tPA activa la proliferación vía activación del plasminógeno a plasmina, coincidiendo con otras publicaciones que indican que Plm activa el receptor PAR-1 en neuronas del hipocampo (Mannaioni et al., 2008). Esto apoya la hipótesis de que PAR-1 podría intervenir en el mecanismo mediante el cual tPA transactiva el EGFR. El requerimiento de plasmina, explicaría también la activación de MMP-9, tal como sucede en neutrófilos (Zhao et al., 2004). Efectivamente, pudimos comprobar que la MMP-9 se requiere para la activación de la proliferación de las células PANC-1 mediada por tPA, pero no en la proliferación mediada por EGF (**Figura 6, capítulo V**). Los resultados mencionados se han integrado en un modelo de acción para la proteasa en cáncer de páncreas como se muestra en la **Figura 11**.

La necesidad de actividad catalítica de tPA, que muestran nuestros ensayos, se contrapone a los resultados de otros grupos (Ortiz-Zapater et al., 2007). En células neuronales humanas tPA fosforila ERK 1/2 vía PKC y sin que sea necesaria su actividad catalítica (Medina et al., 2005). También se ha visto en células de carcinoma humano que tPA sin actividad catalítica induce la transactivación de EGFR, pero a diferencia de nuestro caso, este mecanismo es dependiente de la proteina G_i (Maupas-Schwalm et al., 2005). Estos resultados indican que tPA podría actuar por mecanismos diferentes dependiendo de su concentración y del tipo celular, cáncer de páncreas o neuronas.

A partir de los resultados obtenidos en los capítulos III, IV y V de esta tesis, podemos concluir que la actividad catalítica de uPA en cáncer de colon y de tPA en cáncer de páncreas, es necesaria para la activación de vías de transducción de señal que son comunes a las dos proteasas. La plasmina, activada por uPA o tPA, interviene en ambos casos activando un receptor específico (posiblemente PAR-1) y posteriormente

las quinasas ERK 1/2. También se infiere a partir de nuestros resultados que, pese a la mutua necesidad de la actividad catalítica, ambas proteasas llevan acabo sus respectivos efectos en la progresión tumoral a partir de vías de señalización diferentes. De esta manera vemos que mientras, en células de cáncer de colon, uPA activa PKC y lleva a cabo su efecto independientemente de EGFR, tPA, en células de cáncer de páncreas, activa una vía de señalización independiente de PKC, en la que la activación del EGFR juega un papel primordial. Mostrando así la variedad de mecanismos en los cuales pueden actuar los activadores del plasminógeno en función del tipo celular y del proceso de proliferación celular.

CAPÍTULO VII: Conclusiones

1. Uroquínasa y plasminógeno son necesarios para el *"scatter"* inducido por ester de forbol y HGF en células de cáncer de colon.

2. En el proceso de *"scatter"* inducido por PMA pueden distinguirse dos fases: una fase temprana, independiente de uPA y Plm, en la que se reducen las uniones células-células mediadas por E-Caderina; y una fase tardía, en la que aumenta la expresión de uPA y de su receptor (uPAR), no se requiere la actividad de PKC, y, en presencia de plasminógeno, se activa la Plm. En esta segunda fase se produce un aumento de la movilidad celular.

3. En la fase temprana del "scatter" es necesaria la actividad de PKC y de Src quínasa; en la fase tardia, se requiere la actividad de ERK 1/2 inducidas vía uPA-Plm y no se requiere la actividad del EGFR.

4. En células sembradas a baja densidad, que dan lugar a formación de colonias pequeñas, la Plm activa las ERK 1/2 y produce *"scatter"* sin tratamiento previo con PMA.

5. La actividad catalítica de uPA, a través de la activación de ERKs, induce un aumento de la movilidad celular en cáncer de colon .

6. tPA interacciona con Anexina II de forma mayoritaria aunque no total, en células de cáncer de páncreas. Esta interacción es importante para la invasión celular.

7. tPA promueve la proliferación de células PANC-1 mediante la activación y la internalización del EGFR.

8. Las vía de señalización celular utilizada por tPA para provocar la estimulación de la proliferación en células PANC-1, coincide parcial aunque no totalmente con la utilizada por EGF para activar este mismo efecto en estas células.

9. La actividad proteolítica de tPA es necesaria para la activación de las quínasas ERK 1/2 y para la proliferación de células PANC-1. La actividad proteolítica de tPA también se requiere para la transactivación del EGFR, en la que intervienen la activación proteolítica secuencial de plasmina, MMP-9 y la liberación al medio de cultivo de hb-EGF.

10. La actividad catalítica de tPA, a través de la activación de ERKs, induce la invasión y la proliferación en cáncer de páncreas.

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