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Urokinase Regulates Migration of Human Vascular Smooth Muscle Cells via Tyk2-PI-3K Interaction

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Summary

Janus kinases Jakl and Tyk2 play an important role in urokinase-type plasminogen activator (uPA)-dependent intracellular signaling. Recent findings demonstrate that both kinases are associated with the uPA receptor (uPAR) and mediate uPA-induced activation of signal transducers and activators of transcription (Statl, Stat2, and Stat4) in human vascular smooth muscle cells (VSMC). Janus kinases are not only required for Stat activation but may also interfere with other intracellular signaling pathways. In the present study, one novel molecular mechanism for a cross-talk of the Janus kinase Tyk2-directed signaling cascades was elucidated. It was demonstrated that in VSMC, Tyk2 interacts with a downstream signaling pathway involving phosphatidylinositol 3-kinase (PI3-K) and that this interaction is required for the uPA-related cell migration.

With the help of the developed adenoviral gene transfer it was demonstrated that the uPA-induced PI3-K activation was abolished in VSMC expressing the dominant negative form of Tyk2. The interactions of PI3-K and Tyk2 were studied using immunoprecipitation and pull-down assays. The regulatory subunit p85 of PI3-K co-immunoprecipitated with Tyk2 but not with Jakl, Jak2, or Jak3, and uPA stimulation increased the PI3-K activity in Tyk2 immunoprecipitates. Tyk2 directly bound to either of the two Src homology 2 (SH2) p85 domains in a uPA-dependent fashion.

The participation of Tyk2 and PI3-K in uPA-related cell migration was studied using chemotaxis assay in modified Boyden chamber and by means of time-lapse videomicroscopy. These experiments demonstrate that the Tyk2-mediated PI3-K activation in response to uPA was required for VSMC migration. Thus, two unrelated structurally distinct specific inhibitors of PI3-K, wortmannin and LY294002, prevented VSMC migration induced by uPA. No migratory effect of uPA was observed in VSMC expressing the dominant negative form of Tyk2.

With the help of immunocytochemistry combined with time lapse vidoemicroscopy in wounding model, cytoskeletal reorganization in migrating VSMC was revealed that required association of Tyk2 and PI3-K. Formation of actin-rich lamellipodial extentions at the leading edge of migrating cells and abundant stress fibers in response to uPA was abrogated in VSMC expressing a Tyk2 mutant form. Similar effects were induced by cell treatment with the PI3-K inhibitors, wortmannin and LY294002.

These results underscore the versatile function of Tyk2 in uPA-related intracellular signaling and indicate that PI3-K plays a selective role in the regulation of VSMC migration.

The functional role of endogenous uPA for VSMC migration was studied in coculture model, as far as uPA expressed by monocytes is a potent chemotactic factor for VSMC and might serve for the acceleration of vascular remodeling. This study demonstrates that coculture of human VSMC with freshly isolated peripheral blood-derived human mono-

cytes results in significant VSMC migration that increases during the coculture period. Accordingly, VSMC adhesion was inhibited with similar kinetics, whereas VSMC proliferation was arrested. The increase of VSMC migration in coculture was equivalent to the uPA-induced migration of monocultured VSMC and was blocked by addition into coculture of soluble uPAR (suPAR) that served as a competitor for the uPA binding.

These results lead to proposal that the upregulated uPA production at the place of vascular injury acts most likely as an endogenous activator of VSMC migration contributing to the remodeling of vessel wall. The underlying molecular mechanism involves activation and association of Tyk2 and PI3-K leading in turn to cytoskeletal rearrangement and increase in cell motility.

Zusammenfassung

Die Janus Kinasen Jak1 und Tyk2 spielen eine wichtige Rolle in der urokinase-type plasminogen activator (uPA)-abhängigen intrazellulären Kommunikation. Neueste Befunde demonstrieren, das beide Kinasen mit dem uPA-Rezeptor assoziert sind und die uPA-induzierte Aktivierung von Proteinen der „signal transducers and activators of transcription"-Familie (Stat1, Stat2 und Stat4) in humanen glatten Muskelzellen (VSCM) vermitteln. Janus Kinasen sind nicht nur für die Stat-Aktivierung notwendig, sondern können auch mit anderen intrazellulären Signalwegen interagieren. In der vorliegenden Arbeit wurde ein neuartiger molekularer Mechanismus für eine Querverbindung der Janus-Kinase Tyk2 beleuchtet. Es konnte gezeigt werden, das Tyk2 mit einem Phostphatidylinositol-3-Kinase (PI3-K) beinhaltenden Signalweg interagiert und das diese Interaktion in VSCM für mit uPA zusammenhängende Zellmigration notwendig ist.

Mit Hilfe des entwickelten adenoviralen Gentransfers konnte gezeigt werden, dass die uPA-induzierte PI3-K Aktivierung durch Expression der dominant negativen Form von Tyk2 in VSCM aufgehoben werden konnte. Die Interaktion von PI3-K und TYk2 wurde sowohl durch Immunpräzipitation als auch durch Pull-Down Assays überprüft. Die regulatorische Untereinheit p85 der PI3-K konnte mit Tyk2 co-immunopräzipitiert werden, jedoch nicht mit Jak1, Jak2 oder Jak3. Weiterhin wurde die Aktivität der co-immunopräzipitierten PI3-K durch uPA erhöht. Tyk2 wurde uPA-abängig an beiden Src homology2 (SH2) Domänen der p85 Untereinheit gebunden.

Die Beteiligung von Tyk2 und PI3-K an der mit uPA zusammenhängenden Zellmigration wurde in einem Chemotaxis Assay in einer modifizierten Boyden-Kammer und durch Zeitraffer Videomikroskopie untersucht. Diese Versuche zeigten, dass Tyk2-vermittelte PI3-K Aktivierung nach uPA Stimulation für die Migration von VSMC notwendig ist. Die uPA induzierte Migration von VSCM wurde von zwei nicht verwandten, strukturell unterschiedlichen spezifischen Inhibitoren von PI3-K, Wortmannin und LY294002, verhindert. Bei Expression der dominant negativen Form von Tyk2 wurde kein migratorischer Effekt der uPA Behandlung gesehen.

Durch Immunocytochemischen Methoden verbunden mit Zeitraffer Videomikroskopie wurde in einem Verwundungsmodell belegt, das die zytoskelletale Reorganisation in migrierenden VSCM eine Assoziation von Zyk2 und PI3-K bedarf. Die Bildung von Actin-reichen, lamellipodalen Ausstülpungen am ventralen Ende der Zelle und zahlreichen Stressfasern als Antwort auf uPA-Stimulation wurde durch die Expression einer Tyk2 Mutante aufgehoben. Ähnliche Effekte wurden durch die Behandlung von VSCM mit den PI3-K Inhibitoren Wortmannin und LY294002 erzielt.

Diese Resultate unterstreichen die vielseitige Funktion von Tyk2 in der uPA-verwandten, intrazellulären Signaltransduktion und zeigen, das PI3-K eine Rolle in der

Regulation der Migration von VSCM spielt.

Die funktionelle Rolle von endogenem uPA für VSCM Migration wurde in einem Co-Kultur Modell untersucht. Hier wurde überprüft, in wie weit von Monozyten exprimiertes uPA, in der Co-Kultur ein potenter chemotaxischer Faktor für VSMC sein und eine Beschleunigung des Gefäß-Remodeling herbeiführen könnte. Diese Untersuchung zeigte, dass Co-Kultur von humanen VSMC mit frisch isolierten humanen Monozyten aus peripherem Blut zu einer signifikanten Erhöhung der Migration von VSMC während der Co-Kultur Periode führte. Folglich wurde die Adhäsion von VSMC mit einer ähnlichen Kinetik inhibiert, während die Proliferation von VSCM gestoppt wurde. Die Zunahme der Migration von VSMC in Co-Kultur war äquivalent zur Induktion durch uPA von VSMC Migration in Monokultur und wurde durch Zugabe von löslichem uPAR in die Kultur geblockt, das um die uPA-Bindung kompetierte?

Diese Resultate führen zu der Annahme, dass die bei einer Gefäßverletzung hochregulierte uPA Produktion sehr wahrscheinlich als ein endogener Aktivator der Migration von VSMC beiträgt, die zum Remodeling des Gefäßwand beiträgt. Die unterliegenden molekularen Mechanismen schließen die Aktivierung und Assoziation von Tyk2 und PI3-K ein, die wiederum zu zytoskelletaler Reorganisation und zunehmender Zellbeweglichkeit führen.

Abbreviations

% (v/v)	percent by volume
% (w/v)	percent by weight
A:	Ampere (Unit)
A_{260}:	Absorbance at 260 nm
A_{280}:	Absorbance at 280 nm
Ab:	Antibody
Amp:	Ampicillin
APS:	Ammonium persulphate
ATCC:	American-Type Culture Collection (Rockville, US)
ATP:	Adenosine Triphosphate; Adenosine-5 -Triphosphate
bp:	base pair
BSA:	Bovine Serum Albumin
C:	Degree Centigrade
CASMC:	Coronary Artery Smooth Muscle Cell
cDNA:	Complementary Deoxyribonucleic Acid
Ci:	Curie (Unit)
cpm:	Counts per Minute
DMEM:	Dulbecco's Modified Eagle Medium
DMPC:	Dimethylpyrocarbonate
DMSO:	Dimethyl sulfoxide
DNA:	Deoxyribonucleic Acid
DNase:	Deoxyribonuclease
dNTP:	2'-deoxynucleotide-5'- triphosphate
DTT:	Dithiothreitol
ECL:	Enhanced chemiluminescence
EDTA:	Ethylenediaminetetraacetic Acid
ELISA:	Enzyme-Linked Immunosorbent Assay
FACS:	Fluorescence-Activated Cell Sorter
g:	gram(me)
GPI:	Glycosylphosphatidylinositol anchor
GTP:	Guanosine Triphosphate; Guanosine 5 -Triphosphate
h:	hour
HEPES :	N-(2-Hydroxylethyl)piperazine-N -(2-Ethanesulfonic Acid)
IgG:	Immunoglobulin G
k:	kilo=10^3
kb:	kilobase
l:	liter

LB:	Luria Bertani
m:	milli=10^{-3}
M:	Molar
min:	minute
moi:	multiplicity of infection
MOPS:	3-(N-Morpholino)propanesulfonic Acid
n:	nano=10^{-9}
p:	pico=10^{-12}
PAGE:	Polyacrylamide Gel Electrophoresis
PBS:	Phosphate-Buffered Saline
PCR:	Polymerase Chain Reaction
pfu	plaque forming units
PMSF:	Phenylmethanesulfonyl Fluoride
PVDF:	Polyvinylidene Fluoride
RNA:	Ribonucleic Acid
RNase:	Ribonuclease
rpm:	revolutions per minute
RT:	Room Temperature
RT:	Reverse Transcription
scuPA:	single chain uPA
SDS:	Sodium Dodecyl Sulfate
sec:	second
TAE:	Tris Acetate EDTA
tcuPA:	two chain uPA
TEMED:	N,N,N',N'-Tetramethylethylenediamine
TRIS:	Tris(hydroxymethyl)aminomethane
Tris:	Tris (hydroxymethyl)-aminomethane
uPA:	Urokinase
uPAR:	Urokinase Receptor
V:	Volt (Unit)
x g:	*g* force
µ	micro=10^{-6}

Abbreviations for Amino Acids

Amino Acid	Three-Letter Abbreviation	One-Letter Symbol	Amino Acid	Three-Letter Abbreviation	One-Letter Symbol
Alanine	Ala	A	Isoleucine	Ile	I
Arginine	Arg	R	Leucine	Leu	L
Asparagine	Asn	N	Lysine	Lys	K
Aspartic acid	Asp	D	Methionine	Met	M
Asparagine or aspartic acid	Asx	B	Phenylalanine	Phe	F
Cysteine	Cys	C	Proline	Pro	P
Glutamine	Gln	Q	Serine	Ser	S
Glutamic Acid	Glu	E	Threonine	Thr	T
Glutamine or glutamic acid	Glx	Z	Tryptophan	Trp	W
Glycine	Gly	G	Tyrosine	Tyr	Y
Histidine	His	H	Valine	Val	V

Contents

1 Introduction

1.1 Atherosclerosis and it's Progression

Atherosclerosis, a disease of the large arteries, is the primary cause of cardiovascular diseases and stroke. The key event of atherosclerosis is vascular remodeling resulting in the development of neointimal fibrocellular lesions, which can severely restrict blood flow and cause stenosis. Vascular reconstruction, such as coronary angioplasty, endatherectomy, bypass surgery and vascular stents is often successful initially. However, periodic stenosis with an extracellular matrix (EM) ranging from loose collagen fibrils and proteoglycans to a dense collagen scar with few inflammatory cells remains one of the major limitations of these procedures. Restenosis occurs in 30-50% of patients within 6 months after the operation (Forrester *et al.*, 1991; Ellis *et al.*, 1998).

Pathological studies have revealed a series of changes in the blood vessels during atherogenesis. The hypothesis that an injury to the endothelium may have a key role in the atherosclerotic process was proposed in 1973 (Ross and Glomset, 1973). It was shown that a primary initiating event in this pathophysiological process is the accumulation of the low-density lipoprotein (LDL) in the subendothelial matrix. Accumulation is increased when the level of circulating LDL is raised. LDL diffuses through endothelial cell junctions and its retention in the vessel wall involves interaction between the LDL component apolipoprotein B (apoB) and matrix proteoglycans (Boren *et al.*, 1998). In addition to LDL, other apoB-containing lipoproteins can accumulate in the intima and promote atherosclerosis. It has been shown that trapped LDL undergoes aggregation and oxidation, and that these modifications contribute to inflammation and foam-cell formation. Oxidized LDL (oxLDL) may not only directly injure the endothelium but also stimulate endothelial cells to produce adhesion molecules, chemotactic proteins such as monocyte chemotactic protein-1 (MCP-1) and growth factors such as macrophage colony-stimulating factor (M-CSF), resulting in the recruitment of monocytes and T lymphocytes to the vessel wall (Fig. 1). It has been reported that adhesion molecules such as ICAM-1, P-selectin, E-selectin (Dong *et al.*, 1998; Collins *et al.*, 2000), VCAM-1 and VLA-4 (Shih *et al.*, 1998) are important for the adhesion of leukocytes and monocytes to endothelium. These cells then migrate through the endothelium and localize subendothelially. The cytokine M-CSF (Smith *et al.*, 1995) stimulates the proliferation and differentiation of macrophages, and in cooperation with tumor necrosis factor-α (TNFα) and interferon-γ (IFN-γ) (Tontonoz *et al.*, 1998) it increases the expression of scavenger receptors. Macrophages internalize oxLDL through both scavenger receptors (Goldstein *et al.*, 1979) and a postulated oxLDL receptor. Intracellular accumulation of lipids leads to the formation of foam cells. Such fatty streaks are the early lesions of atherosclerosis. In such lesions, activated macrophages,

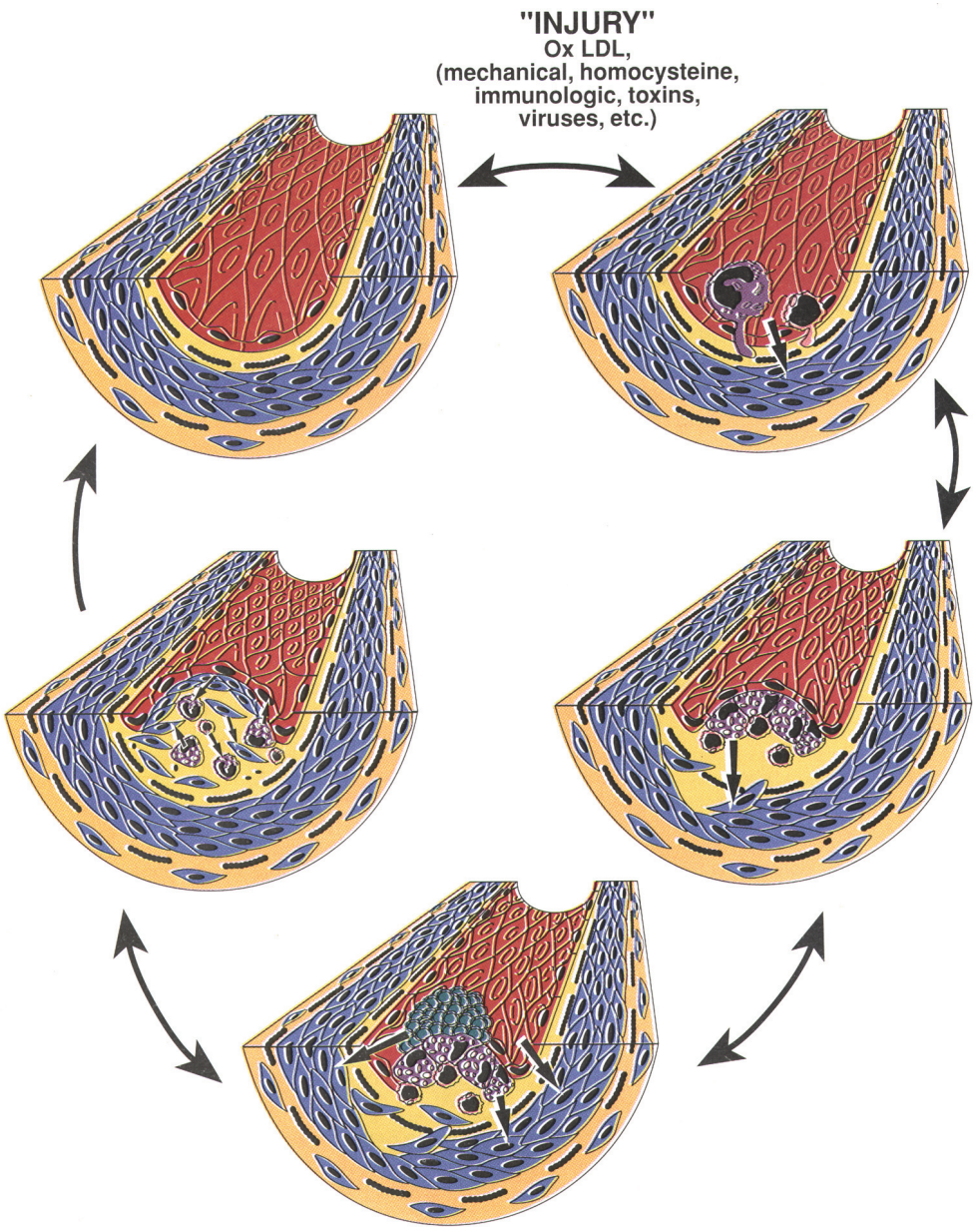

Fig. 1 The development of atherosclerosis process (Ross, 1993).

The dysfunction of endothelial cells is an important step in the beginning of atherosclerotic process. The adherence of monocytes, macrophages, T-lymphocytes to the injured endothelium is increased. Adhered cells migrate into the subendothelial space. The macrophages accumulate lipids and become foam cells. T cells and foam cells form a fatty streak lesion. The numerous growth-regulatory molecules and cytokines produced by the cells in fatty streaks, lead to proliferation and migration of smooth muscle cells from the media into the subendothelial space. The lesion is progressing to a fibrofatty lesion and then to a fibrous plaque. Secreted fibrous components result in increased lesion size. Activated macrophages, smooth muscle cells, T-cells and endothelium proceed with releasing regulatory molecules. This leads to advanced lesions, platelet adherence and to a thrombus formation. The advanced lesion is characterized by lipid-rich necrotic debris and smooth muscle cells, which are also, form a fibrous cap. The platelets release different regulatory molecules, which enhance the pathological process. In this figure: VSMC, blue; endothelium, red; macrophage, violet; platelet, green, T cells, pink.

T-cells, and endothelium continue to release numerous growth-regulatory molecules and cytokines that contribute to the progression of atherosclerosis. MCP-1 (Yla-Herttuala *et al.*, 1991) oxLDL (Quinn *et al.*, 1988) and TGFβ (Sporn *et al.*, 1987) can induce macrophage chemotaxis, whereas growth factors such as PDGF (Hart *et al.*, 1988) bFGF (Folkman *et al.*, 1988) IGF-1 (Banskota *et al.*, 1989), IL-1 (Raines *et al.*, 1989) TNFα (Old, 1985) and also TGFβ (Moses *et al.*, 1990) can induce VSMC proliferation. None of these factors work alone in the process of atherogenesis. Through a network of cellular interactions, the release of one factor can stimulate the expression of another one.

As a result, the formed fatty streak can progress to fibrofatty lesions and then to a fibrous plaque. A fibrous plaque is caracterized by a subendothelial mass of extracellular lipids and by the accumulation of VSMC and VSMC-derived extracellular matrix. It is important to note that accumulation of intimal VSMC is fundamental to the entire process. The endothelial injury increases the concentration of plasma proteins in the subendothelial space. VSMC migrate into the intima. VSMC migrate from the media layer into the subendothelial space and change their function from contractile to secretory. Connective tissue matrix components are the secretory products of VSMC. With the secretion of fibrous components by VSMC fibrous plaques develop and increase in size, resulting in the progressive narrowing of the vessel lumen. Thus, migrating VSMC and their products are some of the central players in the development of vasculopathy. VSMC migrated into the intima are able to further proliferate and to accumulate modified lipoproteins, thus contributing to foam cells formation (Hayashi *et al.*, 1992). With time, foam cells undergo necrosis that leads to the formation of a necrotic core and accumulation of extracellular lipids. Production of matrix metalloproteinases by macrophages contributes to the weakening of the fibrous plaque (Galis *et al.*, 1994). Plaque rupture exposes lipids and tissue factors to blood components, initiating coagulation, platelet adherence, and the formation of a thrombus. Advanced atherosclerotic lesions can lead to ischemic symptoms, myocardial infarction and stroke (Lee and Libby, 1997).

It has been shown that the severity of vascular damage is dependent on the fibrinolytic system (Carmeliet and Collen, 1997; Carmeliet *et al.*, 1997a; Carmeliet *et al.*, 1997b), which plays a prominent role in the progression of atherosclerotic lesions, most likely by affecting VSMC proliferation, migration and adhesion (Waltz *et al.*, 1997; Blasi, 1999).

1.2 Functions of uPA and uPAR in the Plasminogen Activation System

Extracellular proteolysis plays a key role in blood coagulation, fibrinolysis, vascular remodeling, angiogenesis, wound healing, and growth and metastasizing of malignant tumors. These proteases are located on the cell surface due to specific interaction with membrane-bound proteins and are responsible for degradation of the pericellular matrix

components and for destruction of cell-cell contacts. Thus, these proteases remove fibrin deposits and also provide the cell invasion/migration within tissues.

The urokinase-type and tissue-type plasminogen activators (uPA, or urokinase, and tPA, respectively) are important components of the extracellular protease system because they specifically convert plasminogen into plasmin. Plasmin is a serine protease with wide substrate specificity. Upon the generation of intravascular thrombi mainly constituted of polymeric fibrin, the system of plasminogen activators is triggered to recover the blood flow, and the plasmin directly degrades fibrin that leads to the thrombus dissolving. The plasminogen activators are believed to be key components of the fibrinolysis system. On the cell surface plasmin activates a number of metalloproteinases that degrade the extracellular matrix proteins and the components of basal membrane, such as collagen, fibronectin, and laminin. The combined effects of the plasminogen activators, plasmin, and metalloproteinases on the plasma membrane promote a vector cell movement due to destruction of the cell—cell contacts and the matrix and also due to the activation or releasing of latent or matrix-bound growth factors possessing chemotactic properties.

The tissue-type plasminogen activator is mainly involved in fibrinolysis, whereas the urokinase-type plasminogen activator, in addition to the fibrinolytic function, plays the most important role in cell migration and tissue remodeling.

On the cell surface urokinase binds to the high affinity receptor (uPAR), which is located on the leading edge of the migrating cells. The binding of urokinase to the receptor provides a strictly local proteolysis of the extracellular matrix proteins in the direction of the cell movement. Moreover, the urokinase—receptor complex activates the intracellular signaling and thus regulates the cell adhesion, migration, and proliferation. Furthermore, the urokinase receptor and its specific inhibitor (PAI-1) can interact with adhesion receptors, extracellular matrix proteins, and also with proteins which mediate the activation of intracellular signaling. These findings allowed considering the system of plasminogen activators as a separate group of regulators of cell movement and communication, which provide proteolytic and “signaling” functions.

1.3 The Structure and Processing of Urokinase

Urokinase is synthesized by vascular endothelial and smooth muscle cells (SMC), by epithelial cells, fibroblasts, monocytes/macrophages, and also by cells of malignant tumors of different origin (Gelehrter *et al.*, 1983; Golder and Stephens, 1983; Angles-Cano *et al.*, 1985; Clowes *et al.*, 1990). Cells secrete this protein as a single-chain polypeptide with molecular weight of 54 kD that consists of 411 amino acid residues. The urokinase structure is subdivided into three domains: the N-terminal domain homologous to the epidermal growth factor (amino acids 9-45), the kringle domain (amino acids 46-143),

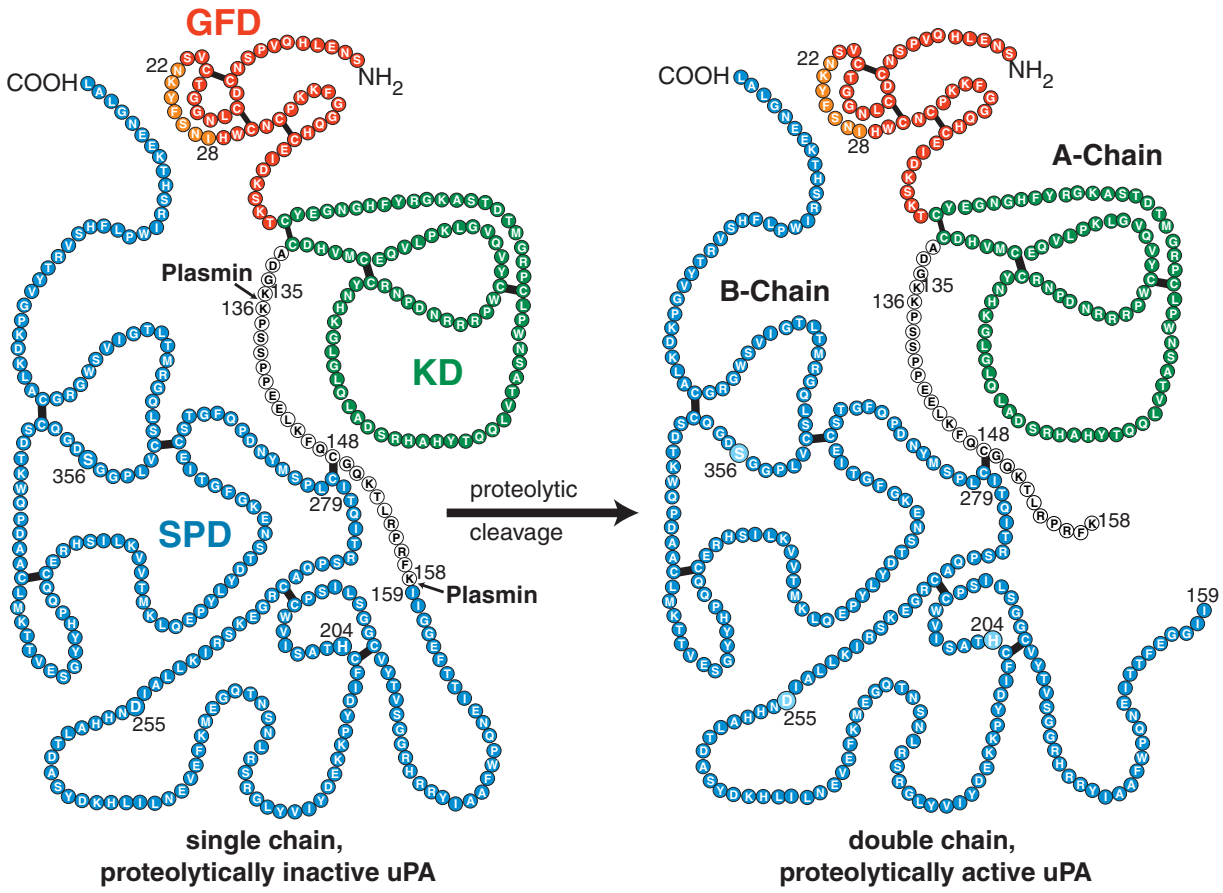

Fig. 2 Aminoacid structure of uPA.

The arrows denote the sites of pro-uPA plasminolysis. The cleavage of the Lys158-Ile159 peptide bond of pro-uPA converts a proteolytically inactive single-chain uPA into the double chain proteolytically active uPA. Active uPA consists of two polypeptide chains, which are connected to each other through a disulfide brige (Cys148-Cys279). The A-chain includes the epidermal growth factor like domain (EOF, aminoacids 1-49, red) and the kringle domain (KD, aminoacids 50-131, green) whereas the B-chain includes the serine protease domain (SPD, amonoacids 159-411, blue). The aminoacid triad Ser356-His204-Asp255 is responsible for the proteolytic activity of uPA. Splitting of the peptide bond not only in Lys158-Ile159 but also in Lys135-Lys136 results in the ATF fragment of uPA.

and the C-terminal catalytic domain (amino acids 144-411) (Fig. 2). The “growth factor-like domain” (GFD) is responsible for the interaction of urokinase with the uPAR receptor (Appella *et al.*, 1987). The protease domain of urokinase includes the active site of the enzyme represented by a specific for serine proteases amino acid triad His204, Asp255, and Ser356. The kringle domain of urokinase contains a sequence that interacts with the specific inhibitor PAI-1 (Mimuro *et al.*, 1992). It has also been found that urokinase binds to heparin through the kringle domain, but the functional significance of this interaction is yet unclear (Stephens *et al.*, 1992). The interaction of urokinase with this receptor results in the migration of SMC, (Pollanen *et al.*, 1988), fibroblasts and of a number of other cells

(Clowes *et al.*, 1990).

The single-chain urokinase has no peptidase activity to synthetic substrates. Therefore, this urokinase form was for a long time considered as the enzyme precursor, or prourokinase (Wun *et al.*, 1982). However, prourokinase can convert plasminogen into plasmin. Plasmin, in its turn, activates urokinase because it cleaves the Lys158—Ile159 peptide bond and converts the single-chain urokinase into the two-chain form (Fig. 2). The two-chain urokinase reveals protease activity with respect to both synthetic substrates and plasminogen, and the rate of plasminogen cleavage by the two-chain urokinase is more than 200-fold higher than the rate of cleavage by the single-chain form (Lijnen *et al.*, 1990). Other extracellular proteases, such as kallikrein, blood coagulation factor XIIa, and cathepsin B, also cleave the Lys158—Ile159 bond of urokinase. The polypeptide chains A and B (light and heavy chains, respectively) are connected by the Cys148-Cys279 disulfide bond in case of the two-chain urokinase The A-chain includes the "growth" and the kringle domains, whereas the proteolytic domain is a part of the B-chain. Upon cleavage of the Lys158—Ile159 bond a newly generated amino terminal tail of the B-chain with Ile 159 on the end is relocated into the region of the substrate-binding pocket of the protease. This is associated with the formation of anion pair between the NH2-group of the Ile 159 and the side chain of Asp255. These conformational changes result in the opening of the substrate-binding pocket and, possibly, of the active site of the enzyme (Goldberg *et al.*, 1990).

A 32-kD urokinase was isolated from adenoma cell culture (Stump *et al.*, 1986a). This low-molecular-weight form of the protein is a single-chain fragment of urokinase containing amino acids 144-411. The enzymatic properties of this fragment are virtually the same as the properties of the full-length urokinase. The low-molecular-weight two-chain urokinase is generated after the cleavage of the Lys135—Lys136 bond by plasmin in the region between the kringle and protease domains, and its proteolytic activity is comparable to the activity of the full-length two-chain urokinase (Stump *et al.*, 1986b). Because the urokinase cleavage in this region can separate the A- and B-chains, it is obvious that in addition to the low-molecular-weight urokinase an amino terminal fragment of the protein is generated (Kobayashi *et al.*, 1985). Nevertheless, up to now the amino terminal fragment is prepared by the cleavage of the full-length urokinase by protease V8. Thrombin hydrolyzes the Arg156—Phe157 peptide bond that results in formation of a two-chain urokinase variant that is proteolytically inactive and fails to be activated by other proteases (Fig. 2). Such processing is a mechanism for inactivation of the enzyme (Ichinose *et al.*, 1986; Braat *et al.*, 1999). However, the resulting inactive form probably has other functions on the cell surface that are not associated with proteolysis.

Thus, a number of bonds in urokinase undergo proteolytic cleavage with formation of several variants of the protein on the cell surface (Fig. 2). The full-length, single-, and two-chain forms of urokinase and its amino terminal fragment can bind to the uPAR receptor on the cell surface.

1.4 The Urokinase Receptor

The discovery of the urokinase receptor in 1985 (Bajpai and Baker, 1985; Stoppelli *et al.*, 1985) opened a new dimension in the study of extracellular proteolysis and matrix degradation. The proteolytic cascade system of plasminogen activation had long been recognized as performing a central role in these processes (Andreasen *et al.*, 1997). It was soon demonstrated that the novel receptor, uPAR, could localize uPA on a wide range of cell types (Nielsen *et al.*, 1988) and further to specific compartments on the surface of the cells, typically at sites involved in focal cell-substratum and cell-cell contacts (Pollanen *et al.*, 1988). It was realized that at these sites the bound ligand remained enzymatically active (Appella *et al.*, 1987), and kinetic studies showed that the plasminogen activation cascade system became accelerated when the reactants were cell-bound (Ellis *et al.*, 1989; Stephens *et al.*, 1989). These observations were highly relevant for the hypothesis that the uPA system does have an involvement in processes of pericellular tissue degradation such as those occurring in cancer invasion. Subsequent work led to the elucidation of several aspects, of the processes in which uPAR is involved and even the identification of novel roles of the receptor system, in addition to those directly related to proteolysis.

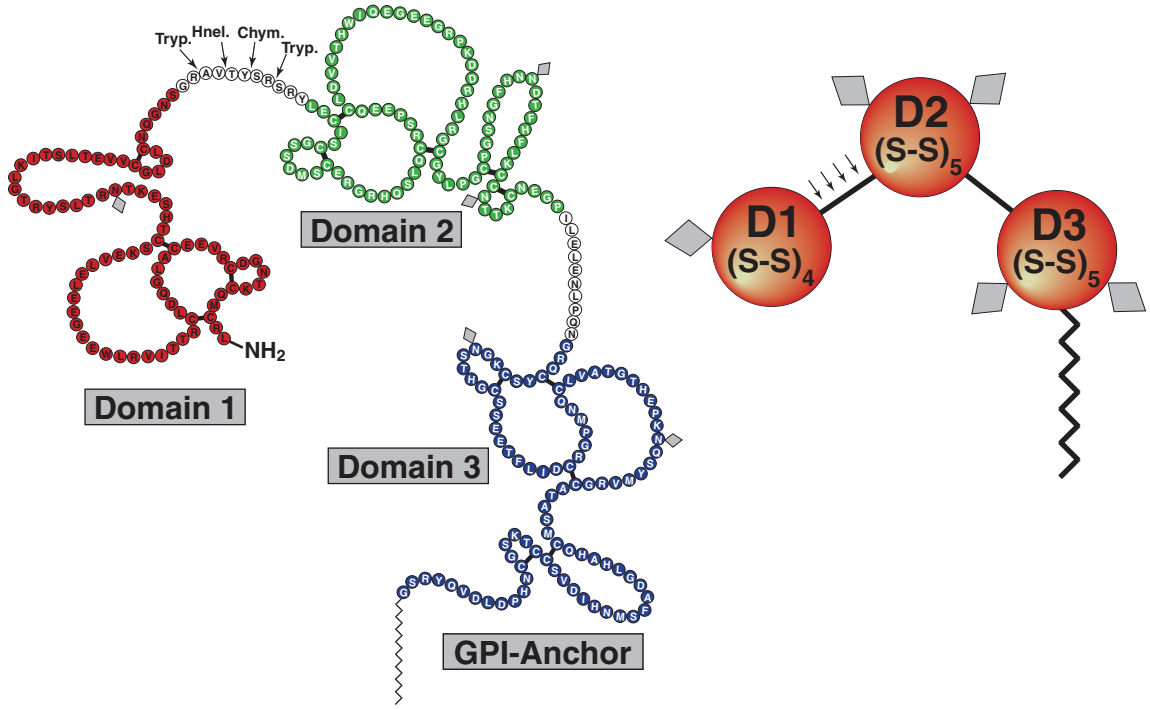

Fig. 3 Aminoacid structure of uPAR.

The polypeptide chain is composed of three domains (domain 1 (D1), blue; domain 2 (D2), green; domain 3 (D3) red) connected by two short linker regions. Four aminoacid bonds represent the protease sensitive part of linker region between domains 1 and 2. uPAR is linked to the membrane by a GPI anchor. The solid short black lines depict disulfide bonds, which are holding uPAR in a proper conformation. The symbol (S-S) indicates the number of disulfide bonds in each domain. Rhombi correspond to the potential glycosylation sites.

The number of uPAR molecules per cell varies markedly, with values from a few thousands to several hundred thousands being reported (Picone *et al.*, 1989). Ligand binding is reversible with a K_d in the range of 0.1-1 nM and occurs with active uPA as well as the zymogen pro-uPA (Stoppelli *et al.*, 1985; Vassalli *et al.*, 1985). The interaction allows the proteolytic activation of the bound zymogen (Cubellis *et al.*, 1986) at the very site where plasminogen activation also occurs. More recently, it has become clear that uPAR also binds to vitronectin in a manner that allows this ligand and uPA to be bound simultaneously (Wei *et al.*, 1994)

The human (Behrendt *et al.*, 1990) and murine (Solberg *et al.*, 1992) uPARs have been purified and characterized and uPAR-encoding cDNAs from human (Roldan *et al.*, 1990), murine (Kristensen *et al.*, 1991) , rat (Rabbani *et al.*, 1994) and bovine (Reuning and Bang, 1992; Kratzschmar *et al.*, 1993) cells have been cloned and sequenced. The human cDNA encodes a 335 amino acid residue polypeptide, including an NH_2-terminal signal peptide of 22 residues and a COOH-terminal segment that is removed during processing for glycolipid membrane anchorage. The mature receptor protein probably comprises 283 residues (Ploug *et al.*, 1991; Moller *et al.*, 1993).

The uPAR protein (Fig. 3) is composed of three extracellular domains (Behrendt *et al.*, 1991; Behrendt *et al.*, 1996) with a low but distinct mutual sequence homology (Palfree, 1991). The consensus sequence pattern characterizing the domains of uPAR is refound in a group of proteins currently referred to as the uPAR/Ly-6 protein family. In addition to uPAR and the murine Ly-6 differentiation antigens, this family comprises the complement component CD59 (membrane inhibitor of reactive lysis; MIRL), the murine thymocyte / B-cell antigen ThB (with the homologous Ag E48 on human keratinocytes) and gene product of herpesvirus saimiri HVS-15 (Behrendt *et al.*, 1991; Palfree, 1991; Ploug *et al.*, 1995). uPAR, however, is the only family member identified so far which has more than one domain. The disulfide pairing pattern of the first domain of uPAR (Ploug *et al.*, 1993) and that of CD59 (Sugita *et al.*, 1993) have been solved, and the patterns identified clearly confirm the relatedness of these proteins .

uPAR is anchored in the plasma membrane through a glycosyl-phosphatidylinosityl (GPI) moiety (Ploug *et al.*, 1991). The protein thus has no intracellular domain and no membrane-spanning peptide segment, a property central to any understanding of the membrane distribution and to the discussion of functions such as cellular signal transduction. The GPI anchor is coupled covalently during a post-translational processing event in which the COOH-terminal part of the nascent polypeptide is removed and the GPI structure becomes attached to a novel COOH-terminus which, in the case of human uPAR, is most likely Gly233 as concluded from mutagenesis studies (Moller *et al.*, 1993). The primary structure of the human uPAR contains five potential N-glycosylation sites (Roldan *et al.*, 1990), of which four have been found to be utilized. Indeed uPAR is heavily

glycosylated, giving rise to a pronounced molecular heterogeneity, and leading to a substantial shift in electrophoretic mobility upon enzymatic deglycosylation (Behrendt *et al.*, 1990). A number of molecular studies have been done using a genetically-engineered, water-soluble uPAR variant (amino acid residues 1-277) which is devoid of the GPI moiety (Ronne *et al.*, 1994). This protein has the same ligand-binding properties as wild-type uPAR (Ploug and Ellis, 1994), and in the following it is considered functionally equivalent at the molecular level to the GPI-containing uPAR.

1.5 uPAR-mediated Signaling in Regulation of the Actin Cytoskeleton and the Effects on Cell Motility

Several of the signaling pathways affected by uPAR-mediated signaling are known to be involved in the regulation of the actin cytoskeleton and of integrin activation in connection with cell migration. In some cases these pathways have now been functionally connected to uPAR-mediated promotion of cell motility.

Most evidence stems from studies on uPA-mediated ERK activation in cell motility. A transient uPA-induced activation of ERK via a pathway requiring FAK, Src, She and subsequent engagement of the classical, Grb2/Sos/Ras/Raf/MEK pathway has been linked to an increase in migration of MCF-7 breast cancer cells in assays where serum was used as a chemoattractant and VN as the haptotactic stimulus (Nguyen *et al.*, 1998; Nguyen *et al.*, 1999; Nguyen *et al.*, 2000). FAK, Src, and She are all needed for uPA-induced ERK activation in these cells, though FAK and She have been reported to activate ERK through parallel independent pathways in other settings (Schlaepfer *et al.*, 1998; Giancotti and Ruoslahti, 1999) ERK activation is also necessary for the chemotaxis of rat smooth muscle cells towards pro-uPA (Degryse *et al.*, 2001).

In MCF-7 cells, the ERK activation leads to phosphorylation and activation of myosin light chain kinase (MLCK) and an MLCK-dependent increase in chemo-and haptokinetic cell motility (Nguyen *et al.*, 1999). MLCK phosphorylates myosin II regulatory light chain, leading to the assembly of actomyosin filaments and contraction in smooth muscle cells (Kamm and Stull, 2001).The induction of cell contractility induced by MLCK mediated activation of the actin-myosin motor is thus one downstream event by which uPA ligation of uPAR could be connected to the cellular machinery mediating cell motility.

Additional pathways regulate both MEK/ERK-dependent and obviously MEK/ERK-independent cell migration. Current evidence indicates that uPAR might also be involved in motility regulation through some of these. There is evidence that one such pathway involves activation of Rac, a member of the Rho-family of small GTPases (Hall, 1998). Like other Rho GTPase family members, Rac plays an important role in the regulation of the actin cytoskeleton. Constitutively active Rac induces lamellipodia and protrusion for-

mation in various cell types through activation of downstream effectors (Bishop and Hall, 2000) and Rac activity is essential for cell migration (Nobes and Hall, 1999). Rac can be activated through a pathway where phosphorylation of the substrate domain of the scaffolding protein p130Cas creates a binding site for the SH2 domain of the adaptor protein Crk (O'Neill *et al.*, 2000). The further coupling of another adaptor protein DOCK180 to this complex leads to activation of Rac through an unknown mechanism (Kiyokawa *et al.*, 1998). The functional consequences of this pathway has been demonstrated in COS-7 cells where lamellipodia formation and increased cell motility are induced by p130Cas/Crk co-expression or insulin treatment in a manner dependent on p130Cas/Crk association and Rac activity (Klemke *et al.*, 1998; Cheresh *et al.*, 1999). Interestingly, some uPAR-mediated effects seem to be mediated via a similar pathway dependent on p130Cas and Rac. Increased expression of human uPAR in murine Swiss 3T3 fibroblasts induces prominent protrusions/lamellipodia in a p130Cas and Rac-dependent manner (Kjoller and Hall, 2001). In addition uPAR expression also induces a 4-fold increase in the levels of active Rac and increases random cell motility in a Rac-dependent manner. These events seem to be initiated by uPAR binding to vitronectin (VN). As mentioned above it has also been reported that in rat SMC, soluble VN induces chemotaxis and increases the number of cells showing a motile morphology in a uPAR-dependent manner (Degryse *et al.*, 2001). In contrast to the uPA/uPAR induced effects on cell motility descibed above, the VN-induced and uPAR-mediated or uPAR-dependent responses are insensitive to the inhibition of MEK/ERK. It is thus an interesting possibility that uPA and VN ligation of uPAR could regulate cell motility through at least partially different signaling pathways. The available data suggest that uPA would then be primarily responsible for inducing signaling to MEK/ERK while VN would induce signaling through p130Cas/ Crk/DOCK180/Rac. This is in agreement with the observation that increased uPAR expression per se in MCF-7 cells induces a 6-fold increase in cell migration onto VN independently of MEK/ERK activity, which can be further increased (2-fold) by addition of uPA in a MEK/ERK sensitive manner (Nguyen *et al.*, 1999).

The fact that uPA binding to uPAR leads to an increase in uPAR binding to VN (Waltz and Chapman, 1994; Kanse *et al.*, 1996) adds an additional level of complexity to the possible cooperation between uPA- and VN-induced pathways (Hoyer-Hansen *et al.*, 1997). uPA might induce signaling through one pathway by causing a conformational change of uPAR that leads to interaction with one transmembrane protein, while it simultaneously promotes uPAR association to VN, an interaction which could induce an additional pathway (a pathway leading to p130Cas dependent Rac activation) through a different transmembrane mediator.

The notion that uPA and VN would induce two separate pathways is, however, undoubtedly an oversimplification as there is extensive cross-talk between the suggested

pathways. For example FAK and Src has been implicated not only in ERK activation but also in p130Cas activation (Vuori *et al.*, 1996), and Rac has been reported to mediate cytoskeletal changes downstream of Ras (Ridley and Hall, 1992) results that are not immediately consistent with the model. The regulation of cell migration through separate sigaling pathways involving MEK/ERK and p130Cas, respectively, have however previously been suggested from a number of studies (Anand-Apte and Zetter, 1997; Cheresh *et al.*, 1999; Gu *et al.*, 1999) using different migration stimuli.

Signaling via a heterotrimeric G_i protein has also been implicated in cell motility and concomitant morphology changes induced by ATF or su-PAR-D2D3 (Resnati *et al.*, 1996; Fazioli *et al.*, 1997; Blasi, 1999; Degryse *et al.*, 1999; Degryse *et al.*, 2001). The signaling pathways downstream of G_i have in this case been proposed to involve one or more kinases of the Src-family due to the need for p56/59 Hck activation in monocytic cells (Fazioli *et al.*, 1997; Chiaradonna *et al.*, 1999) and the inhibition of chemotaxis and/or cytoskeletal changes by herbimycin A in RSMC (Degryse *et al.*, 1999). Also Src-deficient fibroblasts do not respond chemotactically to uPA (Fazioli *et al.*, 1997; Degryse *et al.*, 1999). Both G_i and Src-type kinases have previously been found to have roles in chemotaxis and/or cell motility control. G_i thus has a well-established role in chemotaxis (Mellado *et al.*, 2001). This appears to be independent of $G_{\alpha i}$ but instead involves the released $G\beta\gamma$ subunit complex after activation of the heterotrimeric G_i (Neptune *et al.*, 1999). A reconstruction of the events after chemoattractant-induced G_i activation by transfection of COS-7 cells suggested that the $G\beta\gamma$ complex can induce cytoskeletal reorganization via a pathway leading to Rac activation through the γ isoform of PI3-K and the guanine nucleotide exchange factor Vav (Ma *et al.*, 1998). This is thus one plausible way in which G_i could be involved in mediating uPA-induced changes in cell morphology and motility. PI3-K inhibitors have at least a partially inhibitory effect on ATF/uPA induced cell migration and chemotaxis (Degryse *et al.*, 2001).

The function for Src in cell motility is believed to involve a role in focal adhesion turnover (Fincham and Frame, 1998), and Src-deficient fibroblasts spread and migrate less well than wild-type cells (Hall *et al.*, 1996), so it is perhaps not surprising that uPA is not able to induce chemotaxis in Src-deficient cells (Fazioli *et al.*, 1997). The evidence for activation of Src-like kinases in response to uPA/uPAR induced signaling is, however, quite sparse. Only p56/p59Hck, which is primarily expressed in hematopoietic cells (Martin, 2001) has been reported to be activated upon uPA binding. In hck-/-, fgr-/- macrophages, cell motility is impaired consistent with a role of Hck in uPA-induced motility in monocytes (Suen *et al.*, 1999). Src is also implicated in the activation/regulation of other key players in motility regulation such as FAK, ERK and p130Cas (O'Neill *et al.*, 2000; Martin, 2001), but it is not yet clear whether Src-type kinases are generally involved in uPA-induced signaling to control cell motility in non-hematopoietic cells.

Finally, several lines of research suggest that also one or more isoforms of PKC may have a role in uPAR-mediated regulation of cell motility. In a number of different cell types the induction of chemotaxis upon uPA or ATF treatment coincides with de novo formation of the PKC activator diacylglycerol (Del Rosso *et al.*, 1993; Anichini *et al.*, 1994) and sometimes translocation of PKC activity to the membrane (Fibbi *et al.*, 1998). The involvement of PKCs has also been suggested from at least a partial inhibition of uPA-induced phosphorylation of cytokeratin (Busso *et al.*, 1994), changes in the actin cytoskeleton (Carriero *et al.*, 1999) and chemotaxis (Anichini *et al.*, 1994; Degryse *et al.*, 2001) after inhibiton of PKC. Several different PKC isoforms have been implicated in actin cytoskeleton regulation (Keenan and Kelleher, 1998) and PKC activation may thus be yet another pathway by which uPAR might affect cell motility.

With our current knowledge, it appears that uPAR at least has the potential to mediate signaling through several pathways that involve control of cell motility in general and the connected events such as actin cytoskeleton reorganization and integrin function in particular. This could be due to both activation of several different trans-membrane adaptors or result from activation of one major signaling pathway resulting in the relay of changes through cross-talk within the intracellular signaling network.

1.6 Structure-Function Analysis of the Janus Kinases

Discovery of the Janus kinases (JAKs) occurred at a time when a variety of approaches were being tested in attempts to identify novel protein tyrosine kinases. The first JAK kinase, which was named TYK2 (tyrosine kinase 2), was obtained upon screening a T-cell library using low stringency hybridization techniques (Krolewski *et al.*, 1990; Firmbach-Kraft *et al.*, 1990). The unique structure and function of TYK2 became obvious only after other members of the JAK family were identified and characterized. Polymerase chain reaction (PCR) using degenerate oligonucleotides spanning the heavily conserved kinase domains of members of the Src family of protein tyrosine kinases resulted in identification of partial cDNA clones of JAK1 and JAK2 (Wilks, 1989). Full-length cDNA clones of JAK1 and JAK2 were subsequently cloned using the partial cDNA fragments as probes (Wilks, 1991; Harpur *et al.*, 1992). Their discovery, along with several other members of the tyrosine kinase family resulted in the usage of the acronym JAK (Just Another Kinase). Subsequent sequencing studies revealed that the JAK family of protein tyrosine kinases (PTKs) differs markedly from other classes of PTKs by the presence of an additional kinase domain. To denote this unique structural feature, these kinases were renamed as ‘Janus kinases’ in reference to an ancient two-faced Roman God of gates and doorways (Darnell, 1998; Darnell *et al.*, 1994; Ihle *et al.*, 1995, 1997; Pellegrini and Dusanter-Fourt, 1997; Schindler and Darnell, 1995; Heim, 1999; Leonard and O’Shea, 1998; Schindler,

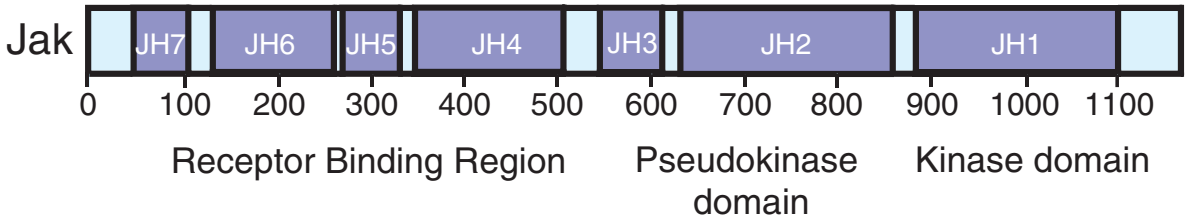

Fig. 4 JAK structure.

The JAKs share seven regions of high homology,JH1-JH7. JH1 encodes the kinase, JH2 represents a pseudokinase domain, which appears to regulate JH1 catalytic activity. JH3-JH7 is implicated in receptor association.

1999; Leonard and Lin, 2000; Ward *et al.*, 2000).

Expression studies indicated that JAK1, JAK2 and TYK2 are ubiquitously expressed and are encoded by transcripts of 5.4 kb (JAK1), two transcripts of 5.3 and 5.0 kb (JAK2), and a 4.4 kb transcript (TYK2), respectively. Several independent groups identified and cloned a fourth member of the JAK family (Johnston *et al.*, 1994; Rane *et al.*, 1994; Kawamura *et al.*, 1994; Takahashi and Shirasawa, 1994; Witthuhn *et al.*, 1994). JAK3 is encoded by a 4.2 kb transcript that codes for a 120 kD protein that is expressed predominantly in cells of hematopoietic origin. Recently, reports describing JAK3 expression in normal and transformed human cell lines of various origins have been published (Lai *et al.*, 1995; Verbsky et al, 1996).

The unique structure of the JAK kinases clearly distinguishes them from other members of the protein tyrosine kinase family (Fig. 3). The most intriguing feature of these proteins is the presence of two domains (JH1 and JH2), with extensive homology to the tyrosine kinase domains. A second interesting feature is the absence of any SH2 or SH3 domains. Instead, these proteins encode a group of well-conserved domains termed as JAK homology (JH1-JH7) domains that follow a non-conserved amino terminus of about 30-50 amino acids. Of the dual kinase domains identified only the JH1 domain appears to be functional. The JH2 domain, which harbors considerable homology to the tyrosine kinase domains lacks certain critical amino acids required for a functional kinase and does not appear to be associated with a kinase activity. Both the tyrosine kinase domain (JH1) and the pseudo-kinase domain (JH2) are housed at the carboxy terminus of the protein (Fig. 3). The other conserved blocks of sequences (JH3-JH7) that are characteristic to members of the JAK family, comprise approximately 600 N-terminal amino-acids residues. The precise functions of the JH3-JH7 domains as well as the pseudokinase domain (JH2) are currently under investigation. The sequences of the JH3-JH7 domains bear no resemblance to any characterized protein motif. Considering the variety of interactions and functions performed by the JAK kinase family members it seems plausible that these domains facilitate some key functions like protein-protein interactions, recruitment of substrates, etc.

1.7 Activation of Jak Kinases by Cytokines/Interferons and Growth Hormones

Janus kinases are generally believed to be present in unstimulated cells in an inactive form. Ligand-induced receptor oligomerization, such as cytokine interaction with its specific receptor, serves as a trigger to signal the recruitment of JAKs to close proximity of the receptors. Local aggregation of the JAK kinases and their subsequent activation by either auto or trans phosphorylation is an event which either involves other members of the JAK kinase family, Src family of kinases and/or receptor tyrosine kinases. The JAK kinases (either alone as monomers or as homo or heterodimers) have been implicated in signal transduction processes initiated by a variety of growth factors and cytokines. A number of studies show that JAK kinases are involved in signaling by interleukins such as IL-2, IL-4, IL-7, IL-9 and IL-15 (Witthuhn *et al.*, 1994; Zeng *et al.*, 1994; Johnston *et al.*, 1994; Kirken *et al.*, 1994; Yin *et al.*, 1994). JAK2 is activated following erythropoietin stimulation of its receptor (Witthuhn *et al.*, 1993) while JAK1 and JAK2 have been implicated in signaling by IL-3, GM-CSF and IL-5 (Silvennoinen *et al.*, 1993a; Quelle *et al.*, 1994; Lutticken *et al.*, 1994). Stimulation by the cytokines IL-6, CNTF, and LIF results in the activation of JAK1, JAK2 and TYK2 kinases (Stahl *et al.*, 1994; Guschin *et al.*, 1995). Also, JAK2 and TYK2 are activated upon IL-12 stimulation and are proposed to play a critical role in IL-12 mediated T-cell differentiation (Bacon *et al.*, 1995). Similarly, JAK kinases have been shown to play a key role in the signal transduction pathways initiated in response to stimulation by growth hormone (Argetsinger *et al.*, 1996), prolactin (Rui *et al.*, 1994; Dusanter-Fourt *et al.*, 1994) and G-CSF (Nicholson *et al.*, 1995; Shimoda *et al.*, 1997). The interferons also transduce their signals via the JAK kinases, JAK1, JAK2 and TYK2 (Velazquez *et al.*, 1995; Watling *et al.*, 1993). JAK3 conveys signals transduced from IL-2, IL-4, IL-7, IL-15 and IL-19. In summary it appears that specific JAK kinases, either alone or in combination with other JAK kinases, may be preferentially activated depending on the type of the receptor that is being activated.

1.8 STATs

STATs are among the best characterized JAK substrates and activation of virtually all cytokine/interferon/growth hormone receptors lead to the activation of one or more STATs. For example, IL-3 activation of hematopoietic cells appears to lead to the activation of multiple STATs, which include STAT-1, STAT-3, STAT-5 and STAT-6 (Reddy *et al.*, 2000). The nature of STATs that are activated appear to be more dependent of the cell line that is used in the study, rather than the cytokine or the nature of JAK activated by cytokine/receptor interactions. This observation suggests that neither the cytokine recep-

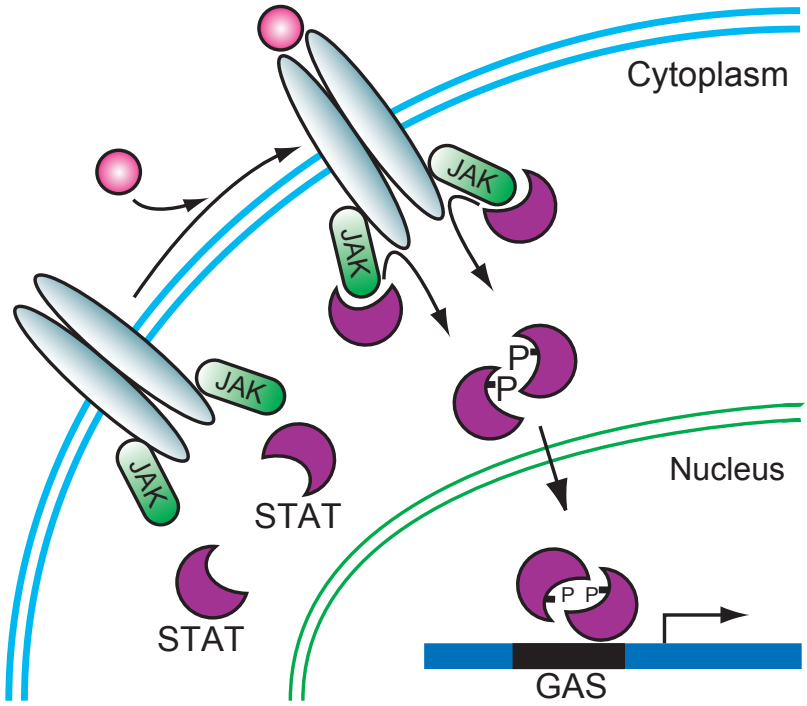

Fig. 5 The JAK-STAT signaling pathway.

Upon binding ligand, receptor-associated JAKs become activated and mediate phosphorylation of specific receptor tyrosine residues. This leads to the recruitment of specific STATs, which are then also tyrosine-phosphorylated. Activated STATs are released from the receptor, dimerize, translocate to the nucleus, and bind to members of the γ-activated site (GAS) family of enhancers.

tors nor the JAKs by themselves dictate the nature of STATs that are activated by a given cytokine. It is also interesting to note that a C-terminal deletion mutant of IL-3 βc chain, termed βc541 was found to be deficient in its ability to activate STAT-5, while still being able to activate JAK2 (Burfoot *et al.*, 1997). These observations also imply that while JAK2 activation is a critical step in IL3/IL5/ GMCSF mediated activation of their cognate receptors; it is by itself not adequate for STAT activation.

1.9 Cross-Talk among Jaks and Components of other Signaling Pathways

Signal transduction in response to a variety of ligands involves the activation of the Ras signaling pathway (Egan and Weinberg, 1993). In the Ras pathway, typified by signal transduction in response to Erythropoietin as well as IL-3, cytokine stimulation results in the recruitment and tyrosine phosphorylation of She. Following phosphorylation, Grb2 associates with She and with Sos. This results in an increase in GTP-bound Ras, activation of Raf-1, followed by activation of the mitogen-activated (MAP) kinases and induction of primary response genes such as c-myc, c-fos, etc. Recently, several studies have explored the interdependence between the JAK kinase signaling pathway and the Ras-linked to MAP kinases pathway as well as other key signaling molecules such as Vav, PI-3 kinase and the protein tyrosine phosphatases such as SH-PTP1. Evidence from these studies

suggests that there may be interactions amongst the diverse components of the cells' signal transduction repertoire. Also, data indicates that cells have equipped themselves with 'safety routes' in the event of malfunction of a particular 'primary' signaling pathway. The availability of an optional signaling route, available via interactions with multiple signaling molecules, enable cells to survive the loss of the 'primary' signaling route. Thus, although the Ras and JAK kinase pathways mediate distinct signals it is becoming evident that there may be interdependence between the two pathways. Thus, phosphorylation of the receptors by JAK kinases creates potential docking sites for adaptor molecules such as She, the p85 subunit of PI-3K, STAT proteins and other kinases such as Src-kinases which can associate with the phosphorylated receptors via their SH2 domains. Once recruited to the receptor complex, the JAK kinases can phosphorylate multiple downstream signaling molecules to further propagate the Ras and JAK kinase pathways.

The abilities of various receptors to integrate activation of the JAK kinase pathway to elements of the Ras pathway have been extensively documented (Alam *et al.*, 1995; Bates *et al.*, 1996; Kumar *et al.*, 1994). Carboxy terminal truncations in the IL-3 receptor β-chain resulted in the loss of activation of the Ras pathway, although the ability to activate JAK2 and induce mitogenesis was retained providing evidence for the existence of alternate signaling pathways in cytokine signaling (Quelle *et al.*, 1994). In a similar study, it was demonstrated that distinct regions of the G-CSF receptor are required for tyrosine phosphorylation of JAK2, STAT3 as well as p42, p44 MAP kinases. MAP kinase tyrosine phosphorylation correlated with both the proliferative response and JAK2 activation (Nicholson *et al.*, 1995). Recently, (Mizuguchi *et al.*, 2000) described the JAK kinase mediated activation of both the Ras and STAT pathways in cell proliferation. Conditional activation of JAK kinases confers IL-3 independence to BA/F3 cells that requires functional Ras and STATS proteins. This observation indicates an obligatory role for the Ras and STAT proteins as targets of JAK activity. In agreement with the above studies, overexpression of Ras or STATS alone does not confer IL-3 independence whereas concomitant activation of Ras and STATS is sufficient to confer IL-3 independence. The same group also, elucidated the role of JAK kinase activity in Ras signaling using a TYK2 protein that was modified by the addition of a membrane localization sequence and a chemical dimerizer (coumermycin)-dependent dimerization sequence (Mizuguchi and Hatakeyama, 1998). The modified TYK2, upon activation by dimerization, conferred IL-3 independence to pro-B lymphoid cells that was abolished by expression of dominant negative Ras indicating the mandatory role for Ras proteins as downstream targets of JAK kinase signals. Interferon stimulation led to activation of the Ras pathways that also depend on JAK kinase activities (Sakatsume *et al.*, 1998; Stancato et al, 1997). Sakatsume *et al.* (1998) showed that activation of the Raf-1 protein by interferon gamma was Ras-independent but required the kinase activity of JAK1. Similarly, Stancato *et al.* (1997) reported that

activation of Raf-1 and the MAP kinase pathways, upon stimulation of cells with interferon beta and oncostatin M, required JAK1 kinase activity. The above studies, taken together indicate that JAK kinases target both the Ras and STAT pathways to exert their biological effects.

Activation of Ras and PBKinase as well as STAT proteins leads to increased induction of transcription factors such as c-jun. Also, c-jun levels are elevated in B-lymphoid cells exposed to ionizing radiation (IR). Goodman *et al.* recently demonstrated that the induction of c-jun in IR treated cells required the activation of JAK3 but was impervious to the activation status of the Btk, Syk and Lyn tyrosine kinases (Goodman *et al.*, 1998).

Interaction of JAK2 with the SH2 domain of p95 Vav, a protein expressed in hematopoietic cells, was reported in cells stimulated with GM-CSF (Matsuguchi *et al.*, 1995). Also, in IL-7 stimulated Tcells, members of the JAK family were shown to regulate the activity of PI-3 kinase. Coupling of the JAK signaling pathway and She phosphorylation was demonstrated in signal transduction events from c-Mpl, a member of the cytokine receptor family and the receptor for thrombopoietin (Gurney *et al.*, 1995). Integration of the JAK and Ras pathways was evident in IL-6 induced signals in a B-cell line AF10 in which JAK1, p52Shc, Raf-1 and MEK-1 were activated (Kumar *et al.*, 1994). Studies involving signal transduction by growth hormone (GH) indicated that JAK2, Ras and Raf are required for activation of the MAP kinases (Winston and Hunter, 1995). Based on this study, it was suggested that JAK kinases might represent a common component during activation of the ERK2/ MAPK and STAT signaling pathways, which appeared to bifurcate upstream of Ras activation but converged with the phosphorylation of STATs by the ERK/ MAPK proteins. The role of JAK kinases in insulin signaling demonstrated that JAK1 and JAK2 constitutively associate with Grb2 via interactions with the SH3 domains of Grb2 (Giorgetti-Peraldi *et al.*, 1995). Several studies have reported the involvement of JAK kinase activity in the modulation of PI-3 Kinase function. GM-CSF stimulation of neutrophils triggers the activation of Jak2, STAT5, STAT5B, and PI-3K. Treatment of these cells with a JAK2 inhibitor AG-490 resulted in abrogation of phosphorylation of the p85 subunit of PI-3K in response to GM-CSF stimulation (Al-Shami and Naccache, 1999). Further studies indicated that the p85 subunit associated with JAK2 and this interaction was not dependent on the two SH2 domain of PI-3K. Also, Yamauchi *et al.* (1998) using cells either deficient in JAK2 or harboring dominant negative JAK2, showed that the insulin-receptor substrate (IRS) proteins, IRS-1, IRS-2 and IRS-3, are phosphorylated by JAK2. Furthermore, these authors elegantly demonstrated that upon phosphorylation by JAK2, the phosphorylated IRS proteins serve as scaffolding intermediates to allow docking and subsequent activation of PI3-Kinase. Stimulation of cardiac myocytes by LIF promotes interaction between JAK1 and PI-3K that leads to increased PI-3K activity in JAK1 immunoprecipitates suggesting that JAK1 mediates the activation of the PI3 pathway through

the gp130 subunit (Oh *et al.*, 1998). Taken together, these studies indicate that the cellular signal transduction machinery is programmed to respond to changes in stimulus by integrating diverse signaling pathways to generate an orchestrated response.

1.10 Phosphatidylinositol-3 Kinase

Phosphatidylinositol-3 kinase (PI3-K) is one of the most important regulatory proteins that are involved in different signaling pathways and controlling of key functions of the cell. The double enzymatic activity of PI3-K (lipid kinase and protein kinase) as well as the ability of this enzyme to activate a number of signal proteins including some oncoproteins determines its fundamental significance in regulation of cell functions such as growth and survival, aging, and malignant transformation. Among the main effectors of PI3-K are the mitogen-transducing signal proteins (protein kinase C, phosphoinositide-dependent kinases, small G-proteins, MAP (mitogen activated protein) kinases), which are activated either via their interaction with lipid products of PI3-K or through PI3-K-dependent phosphorylation of proteins. The anti-apoptotic effect of PI3-K is realized by activation of proteins from another signaling pathway—protein kinase B(PKB) and/or PKB-dependent enzymes (GSK-3, ILK). PI3-K plays a critical role in malignant transformation. PI3-K itself possesses oncogenic activity and also forms complexes with some viral or cellular oncoproteins (src, ras, rac, alb, T-antigen), whose transforming activities are realized only in presence of PI3-K. The transforming effect of PI3-K is supposed to occur on the basis of complex alterations in cellular signaling pathways: appearance of constitutively generated PI3-K-dependent mitogen signal and activation of some protooncogenes (src, ras, rac, etc.), PI3-K/PKB-pathway stimulation resulting in delay of apoptosis and increase of cell survival, and actin cytoskeleton reorganization.

Initially PI3-K was the subject of interest because of its known ability to form complexes with some viral oncoproteins such as v-src and v-ros (Macara *et al.*, 1984; Sugimoto *et al.*, 1984) and also because of involvement of intracellular PI3-K in the viral transformation process (Cantley *et al.*, 1991). Later, in 1997, the possibility of malignant transformation of cells as the result of transfection with DNA containing a fragment of a viral or cellular PI3-K gene was shown (Chang *et al.*, 1997). Parallel investigations of biochemical properties of PI3-K led to rather unexpected results. The two-subunit (regulatory p85 and catalytic p110) molecule of PI3-K appears to possess both lipid kinase and protein kinase activity (Carpenter *et al.*, 1993b). Activation of the dimeric p85/p110 PI3-K molecule occurs through phosphorylation of a tyrosine residue by either receptor (platelet, insulin-like, or epidermal growth factor receptors) or non-receptor (p60-src) tyrosine kinases (Fukui and Hanafusa, 1989; Ruderman *et al.*, 1990; Kapeller and Cantley, 1994). Experiments with the use of some specific inhibitors of PI3-K and/or cell transfection with different PI3-K

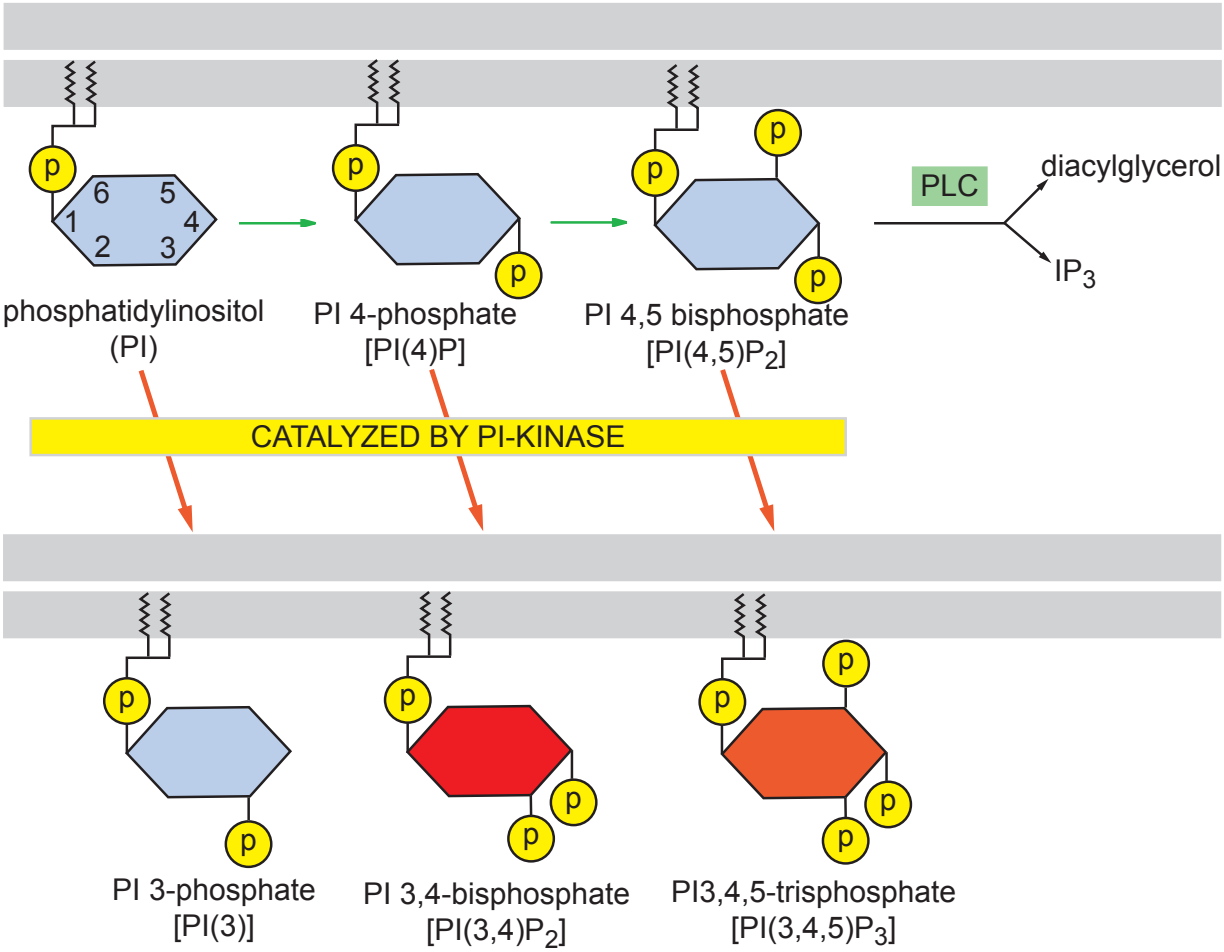

Fig. 6 The generation of inositol phospholipid docking sites by PI3–K.

PI3-K phosphorylates the inositol ring on carbon atom 3 to generate the inositol phospholipids shown at the bottom of the figure; the two lipids shown in red can serve as docking sites for signaling proteins with PH domains. The phosphorylations indicated by the green arrows are catalyzed by other inositol phospholipids kinases. Phospholipase C (PLC) can cleave $PI(4,5)P_2$ to produce two small signaling molecules diacylglycerol and inositol 1,4,5-trisphosphate (IP_3).

gene variants reveal PI3-K being a mediator in the control of at least two very important cell functions, namely, cell division (as a necessary component of the signaling pathway initiated by growth factors) and apoptosis (the progress of which is inhibited by PI3-K) (Cantley *et al.*, 1991; Scheid *et al.*, 1995).

Recently, clear progress in the investigation of the mechanism of PI3-K action has revealed the main mediators of its action. For instance, the event cascade leading to the delay of apoptosis is initiated by complex formation between Ptdlns-phosphate products of the PBK-catalyzed reaction and protein kinase B (PKB), also named Akt or Akt/PKB) (Franke *et al.*, 1997; Klippel *et al.*, 1997). The latter enzyme plays an important role in the regulation of the activity of many genes controlling, directly or indirectly, the apoptotic

process (Kauffmann-Zeh *et al.*, 1997; Khwaja *et al.*, 1997; Kulik *et al.*, 1997). PI3-K-mediated transduction of mitogen signal is realized in another way. In spite of the traditional view of PtdIns and its derivatives as the main components of mitogen signal, the role of PI3-K in the regulation of cell division appears not to be restricted to synthesis of these compounds. Not so long ago it was shown that PI3-K might directly control the activities of individual components of the RAS/RAF/ERK-mitogenic pathway by complex formation with some signal proteins; the enzyme acts in this case as a serine-threonine protein kinase (Carpenter *et al.*, 1993b; Dhand *et al.*, 1994; Bondeva *et al.*, 1998).

Among the other important PI3-K functions involved directly or indirectly in mitogen signal transduction, the involvement of PI3-K in receptor down-regulation (endocytosis and degradation of activated growth factor receptors), in control of lysosomal enzyme synthesis (Joly *et al.*, 1994; Brown *et al.*, 1995), and in reorganization of actin cytoskeleton during the course of malignant transformation process and/or mitogen stimulation of cells should also be emphasized (Rodriguez-Viciana *et al.*, 1997).

In general, PI3-K is now considered as one of the most important regulatory proteins, being involved in a number of diverse signaling pathways and controlling the main functions of the cell. PI3-K activation in malignant cells after exposure to radiation or other stress (Carpenter *et al.*, 1990; Kapeller and Cantley, 1994) and also the above-mentioned anti-apoptotic effect of PI3-K indicate the important role of this enzyme in the control of both malignant cell resistance to damaging agents and the sensitivity of malignant tumors to chemo or radiotherapy.

1.11 PI3-K: Structure and General Properties

Phosphatidylinositol-3 kinase is a heterodimer of two subunits, catalytic and regulatory, with molecular weights of 110 kD (p110) and 85 kD (p85), respectively (Carpenter *et al.*, 1990; Kapeller and Cantley, 1994). Cloning experiments with the use of PI3-K cDNA have revealed at least five isoforms of each subunit (Escobedo *et al.*, 1991; Skolnik *et al.*, 1991; Pons *et al.*, 1995; Inukai *et al.*, 1996). The regulatory p85 subunit consists of several domains including the SH3 domain, two proline rich fragments, and two SH2 domains separated by the iSH2 (inter SH2) sequence (Fig. 8). The iSH2 domain provides the interaction between the p85 and p110 subunits, and the two SH2 domains are responsible for binding of the p85/p110 heterodimer with receptor tyrosine kinases (Kapeller and Cantley, 1994; Yu *et al.*, 1998). It is supposed that due to the ability of the regulatory p85 subunit to interact with both the catalytic p110 subunit and receptor tyrosine kinases directed membrane targeting of p110 occurs, initiating complex formation between the enzyme and its phospholipid substrate (Skolnik *et al.*, 1991; Kapeller and Cantley, 1994).

The catalytic p110 subunit of PI3-K is homologous to protein kinases and pos-

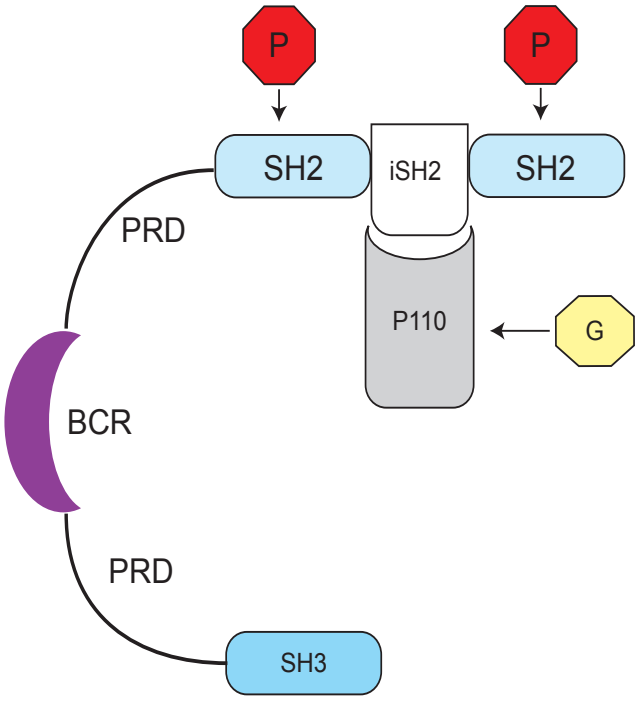

Fig. 7 Schematic structure of the PI3-K p85/p110-heterodimer.

The regulatory p85 subunit consists of (from the N-terminus) SH3-domain, two PRDs (proline rich domain), separated by BCR-homologous domain (BCR, breakpoint cluster region), and two SH2-domains, separated by the iSH2 (inter SH2)-sequence, which is responsible for binding with the p110 subunit. Arrows show the binding sites of heterodimer to the main PI3K activators: phosphotyrosine proteins (P) and small G-proteins (G).

sesses both serine-threonine protein kinase and phosphoinositide kinase activities (Whitman *et al.*, 1988; Auger *et al.*, 1989; Carpenter *et al.*, 1990; Carpenter *et al.*, 1993b). Phosphorylation of PtdIns and phospho-inositides PtdIns(4)P and PtdIns(4,5)P_2 occurs in the D3-position of the inositol ring leading to formation of PtdIns(3)P, PtdIns(3,4)P_2, and PtdIns(3,4,5)P_3, respectively. Three classes of the PI3-K protein superfamily are now known. All of these possess the protein kinase activity; the difference is preferentially in the substrate specificity of the phosphoinositide kinase site. Thus, the first class includes p85/p110 heterodimers reacting with all phosphoinositides, PtdIns, PtdIns(4)P, and PtdIns(4,5)P_2. These are now often referred to as phosphoinositide-3 kinases; the term reflects their substrate specificity more correctly than the traditional name, phosphatidylinositol-3 kinases. The second class involves enzymes phosphorylating preferably PtdIns and PtdIns(4)P. Finally, the third class includes PI3-K that possesses additionally a specific protein transfer function and has a structural and functional resemblance with the yeast analog of PI3-K, vps34p (vacuolar protein sorting). Unlike the enzymes from the first and the second classes, this one uses only PtdIns as a substrate (MacDougall *et al.*, 1995; Stoyanov *et al.*, 1995).

Two main processes lead to PI3-K activation: p85/p110 heterodimer assembly and interaction of the heterodimer with activator proteins. As mentioned above, the binding of the catalytic and regulatory subunits occurs via the iSH2 domain of the latter. The p85/p110 heterodimer assembly does not result itself in marked enzyme activation. Moreover, some investigators reported the activity of the catalytic subunit being decreased when the p85/p110 complex is formed in vitro (Yu *et al.*, 1998). An additional interaction with specific activator proteins is required for the subsequent activation of the heterodimer

(Backer *et al.*, 1992; Carpenter *et al.*, 1993a; Rordorf-Nikolic *et al.*, 1995). The main activator proteins have tyrosine-phosphorylated amino acid sequences including both some receptor (receptors of platelet, epidermal, or insulin-like growth factors), and non-receptor (p60-src) tyrosine kinases (Fukui and Hanafusa, 1989; Ruderman *et al.*, 1990; Kapeller and Cantley, 1994). The binding of phosphotyrosine sites of activator proteins with SH2 domains of the PI3-K regulatory subunit causes a conformational change of the heterodimer leading to enzyme activation (Yu *et al.*, 1998). There are, however, other mechanisms of PI3-K activation. An extra activation of the heterodimer may occur while direct interaction between the PI3-K catalytic subunit and one of several cellular proteins takes place. A good example is the complex formation between p21-ras and p110 resulting in activation of PI3-K (Kodaki *et al.*, 1994; Rodriguez-Viciana *et al.*, 1994; Rodriguez-Viciana *et al.*, 1996). The diversity of ways for PI3-K activation to occur and also the multi-substrate specificity and double-enzymatic activity (lipid kinase and protein kinase) of the enzyme likely determine its key role in the control of cell growth and survival.

1.12 Position of PI3-K among General Signaling Pathways

Investigations of recent years have shown the involvement of PI3-K in the control of cell division being realized through at least two possible mechanisms: the first includes phosphoinositide production (PI3-K lipid kinase activity), the second includes direct interaction of PI3-K with some cellular signal proteins, when the protein kinase activity of PI3-K may play a critical role (Whitman *et al.*, 1988; Auger *et al.*, 1989; Carpenter *et al.*, 1990; Carpenter *et al.*, 1993b; Dhand *et al.*, 1994).

Traditional opinions concerning the role of phosphoinositides in cell growth control were based on their role as substrates of phospholipase C, the enzyme that is activated by receptor tyrosine kinases during cell division. When accumulated in the cells due to phospholipase C action, the hydrolysis products of phosphoinositides (diacylglycerol and inositol phosphates) activate protein kinase C, thus stimulating one of the most important signaling pathways of the cell (Hug and Sarre, 1993; Cazaubon *et al.*, 1994; Orr and Newton, 1994).

However, as was shown later, the phosphoinositides may have an independent significance in mitogen signal transduction, because of their ability for direct interaction with some signal proteins. The role of phosphoinositides in activation of protein kinase B (PKB), which is involved preferentially in the control of cell apoptosis, will be considered below. As for the protein mediators of cell division, protein kinase C (PKC) should be distinguished first of all. The PKC activation appears to occur not only via binding with diacylglycerol formed by hydrolysis of phospholipids, but also via the interaction with PI3-K lipid products (Carpenter and Cantley, 1996). Great progress in the study of the control mech-

anisms of PI3-K was achieved following the discovery of a new family of serine-threonine protein kinases, phosphoinositide-dependent kinases (PDK) (Alessi *et al.*, 1997; Alessi *et al.*, 1998). These are activated by the lipid products of PDK, 3OH-phosphoinositides (hence the name of the family), and responsible for the phosphorylation and activation of a number of signaling protein kinases, including both PKB and PKC (Alessi *et al.*, 1997; Alessi *et al.*, 1998; Le Good *et al.*, 1998). Thus, two steps of PI3-K-dependent activation of protein kinase C may be distinguished: PKC interaction with diacylglycerol, the phos-

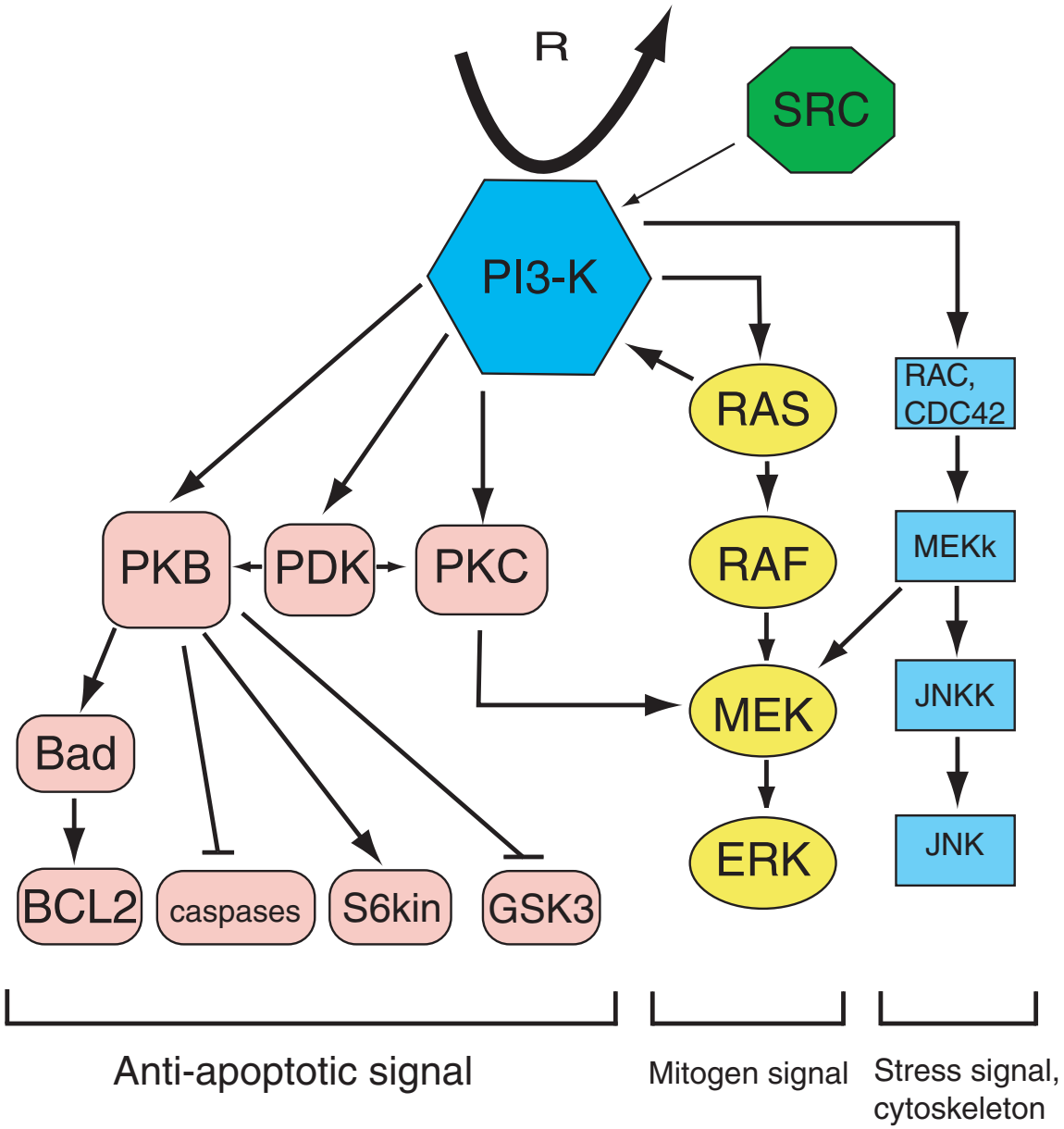

Fig. 8. The involvement of PI3K in the control of intracellular signaling pathways.

PI3K is activated via interaction with receptor (growth factor receptors) or non-receptor (p60-src) tyrosine kinases. Among the main "down-stream" effectors of PI3K are: PKB, responsible for anti-apoptotic signal transduction; RAS/ERK, the main mitogen-conducting pathway; RAC/JNKK/JNK, pathway partially controlling the mitogen signal transduction but involved mainly in control of other cell functions, such as stress reaction or actin cytoskeleton reorganization.

phoinositide hydrolysis product, and phosphorylation of PKC by PDK family enzymes. Also, the data obtained from the studies on binding of phosphoinositides with SH2-containing proteins should be taken into account. These indicate one of the PDK products, PtdIns(3,4,5)P_3, interacts with SH2-domains of proteins competing with phosphotyrosine peptides (Rameh *et al.*, 1995). The same effect may exist for an additional pathway of activation of SH2-containing signaling proteins, which is independent from receptor tyrosine kinases.

The subject of particular interest is the involving of PDK in receptor "down-stream" processes, including endocytosis and degradation of activated growth factor receptors. As known, binding of ligand with growth factor receptor and activation of its phosphotyrosine kinase domain is followed by internalization of the receptor into intracellular vesicles and its consequent degradation in lysosomes (Chang *et al.*, 1993; Lamaze and Schmid, 1995; Opresko *et al.*, 1995). The whole process and, in particular, the activated receptor transfer into lysosomes appear to be under PDK control (Joly *et al.*, 1994; Joly *et al.*, 1995). For instance, the studies on the down-stream handling of platelet growth factor receptor have revealed that some mutations in the phosphotyrosine site of the receptor molecule responsible for PDK binding may cause an almost total blockage of the receptor transfer into lysosomes (Joly *et al.*, 1994). The same effect is caused by wortmannin, a specific PDK inhibitor (Joly *et al.*, 1995). However, PDK possesses an ability to direct control of lysosomal enzyme activity by stimulation of the transfer of de novo synthesized hydrolases into lysosomes (Brown *et al.*, 1995).

The ability of PDK to direct binding with some cellular proteins and also the fact that the enzyme possesses not only lipid kinase, but also protein kinase activity opened new opportunities for studying its role in intracellular signaling pathways. The p85/p110 heterodimer in vivo forms complexes with a broad spectrum of cellular molecules including tyrosine kinases, Grb2, p21-ras, rac, Cdc42, tubulin, etc. (Wennstrom *et al.*, 1994c; Zheng *et al.*, 1994; Hawkins *et al.*, 1995b; Nobes *et al.*, 1995; Tolias *et al.*, 1995). The subject of principal significance for understanding the role of PI3-K in the control of RAS/RAF/ERK signaling pathway is complex formation between the catalytic subunit of PI3-K and p21-ras. PI3-K binds only with the GTP-form of ras resulting in PI3-K activation observed both in vitro and in vivo. The same PI3-K activation effect occurs when the heterodimer p85/p110 binds some other G-proteins, for instance, rac or Cdc42 (Wennstrom *et al.*, 1994c; Zheng *et al.*, 1994; Hawkins *et al.*, 1995b; Nobes *et al.*, 1995; Tolias *et al.*, 1995). And, on the other hand, complex formation between PI3-K and p21-ras is accompanied by an increased amount of activated (GTP-bound) form of ras (Hu *et al.*, 1992). Moreover, the presence of PI3-K appears to be necessary both for stimulation of the RAS/RAF/ERK pathway and for induced transformation of cells, and, in addition, in some cases PI3-K activity inhibition may cause total blockage of transformation (Rodriguez-Viciana *et al.*,

1997). It should be mentioned that the mutual control between PI3-K and p21-ras is rather complex and does not corresponded to a linear model of mitogen signal transfer. PI3-K is supposed to activate p21-ras (possibly via membrane targeting of SOS-proteins) and is activated simultaneously via its binding with the GTP-form of ras or other G-proteins (Carpenter and Cantley, 1996).

However, the significance of PI3-K for the RAS/RAF/ERK signaling pathway is not limited by its influence on p21 -ras. Recently, the important role of serine-threonine protein kinase activity of PI3-K in the control of cellular MAP-kinases was demonstrated. Experiments using different classes of PI3-K whose lipid and protein kinase activity components differ revealed that only the protein kinase activity of PI3-K causes the activation of cellular MAP-kinases (Bondeva *et al.*, 1998). The level of the synthesis of phosphoinositides (lipid products of PI3-K) did not influence markedly the MAP-kinase activities (Bondeva *et al.*, 1998). Thus, the general scheme of PI3-K-dependent control of cellular mitogen-transduction signaling pathways consists of several stages, the main being: PI3-K activation via the binding of p85/p110 heterodimer with tyrosine-phosphorylated proteins and/or small G-proteins (p21-ras, rac, Cdc42); the synthesis of 3-OH phosphoinositides which are the sources of both diacylglycerol and inositol phosphates and activators of some protein kinases (PKB, PKC, PDK) they can directly interact with; serine-threonine phosphorylation of secondary PDK messengers and the activation of MAP-kinases (Fig. 9). Also, the scheme of PDK involvement in cellular metabolism should be supplemented with PDK-dependent control of stress-activated signaling pathways.

1.13 Evidence for a Role of PI3-K in Cell Migration

Elegant molecular and pharmacological evidence first suggested that PI3-K and its lipid products might play an important role in platelet-derived growth factor (PDGF)-dependent actin polymerization and cell migration.(Kundra *et al.*, 1994; Wennstrom *et al.*, 1994a; Wennstrom *et al.*, 1994b; Hawkins *et al.*, 1995a). Moreover, selective activation of PI3-K using constitutively active PI3-K mutants or the addition of exogenous Ptdlns(3,4,5)P_3 can initiate cell motililty and membrane ruffling (Reif *et al.*, 1996; Derman *et al.*, 1997). However, the very first evidence for the involvement of PI3-K in chemokine-stimulated cell migration was the demonstration that chemotaxis and polarization of T cells induced by Regulated on Activation, Normal, T-cell Expressed, and Secreted (RANTES)/CCL5 could be inhibited by PI3-K inhibitors such as wortmannin and LY294002. Subsequent studies by several groups have shown that other CC chemokines (e.g. macrophage inflammatory protein [MIP]-3α/CCL20 and monocyte chemoattractant protein [MCP]-1/CCL2) as well as CXC chemokines (e.g. interleukin [IL]-8/CXCL1 and stromal-cell-derived factor 1 [SDF]-1/CXCL12) stimulate wortmannin-sensitive chemotaxis of eosinophils, THP-1

cells, as well as neutrophils and T lymphocytes, respectively (Knall *et al.*, 1997; Turner *et al.*, 1998; Sotsios *et al.*, 1999; Sullivan *et al.*, 1999). It seems probable that the production and degradation of 3'-phosphoinositide lipids is crucial in maintaining chemotactic signalling gradients. This interpretation was reinforced by subsequent evidence from mice deficient in SHIP (Src homology 2 [SH2]-containing inositol 5-phosphatase), an enzyme that hydrolyses PtdIns(3,4,5)P_3. These SHIP$^{-/-}$ mice suffer from lethal infiltration of the lungs by macrophages and neutrophils and therefore persistently high levels of PtdIns(3,4,5)P_3 and subsequent activation of its downstream effectors might lead to excessive inflammation (Helgason *et al.*, 1998). Finally, elegant studies using green fluorescent protein-tagged PH domains that bind selectively with PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 revealed that PtdIns(3,4,5)P_3 accumulated at the leading edge of chemoattractant-stimulated HL-60 cells (Servant *et al.*, 2000). Similarly, PtdIns(3,4,5)P_3 localization at the leading edge of polarized cells was observed using a PtdIns(3,4,5)P_3-specific antibody (Ab) (Rickert *et al.*, 2000) This accumulation of PtdIns(3,4,5)P_3 at the leading edge correlates with the polarization of chemokine receptors that are involved in detecting a chemoattractant gradient.

1.14 PI3-K Activation Regulate Cell Migration

Reorganization of the actin cytoskeleton is an important step in cell migration, and different chemokines are able to induce the polarization of lymphocytes with generation of specialized cell compartments. Given that Rho GTPases Rho, rac and Cdc42 are regulators of actin cytoskeleton and cellular polarity, there has been much interest in ascertaining the role of the Rho GTPases in chemokine signalling and their relationship with the PI3-K-dependent signalling cascade (Hall, 1998). Indeed, there is considerable evidence to indicate that Rho family kinases are regulated by PI3-K in several systems. For instance, Rac and Cdc42 have been reported to associate with p85/p110 (Tolias *et al.*, 1995), whilst expression of active mutants of p110α in fibroblasts can induce actin reorganization in the form of Rac-mediated lamellipodia and focal complexes and Rho-mediated stress fibres and focal adhesions (Reif *et al.*, 1996). Similarly, reorganization of the actin cytoskeleton and membrane ruffling induced by overexpression of wild-type PI3-Kγ or expression of an active mutant of PI3-Kγ required Rac but not Cdc42 (Reif *et al.*, 1996), whilst the PI3-K homologue TOR2 controls Rho activation in Saccharomyces cerevisiae (Ma *et al.*, 1998) PI3-K has been shown to be involved in the regulation of actin cytoskeleton by growth factors such as PDGF and insulin (Kotani *et al.*, 1994).

Cdc42 appears to have a major role in the control of directional migration of leucocytes, as a dominant-negative mutant of Cdc42 displays a much more potent inhibitory effect on leukaemic T-cell line chemotaxis towards SDF-1 gradients than dominant-nega-

tive mutants of RhoA and Rac (del Pozo *et al.*, 1999). In addition, expression of Cdc42 mutants in monocytic cells demonstrated that rearrangement of the actin cytoskeleton in response to CC chemokines (MCP-1 and MIP-1α) is regulated via Cdc42 (del Pozo *et al.*, 1999). Interestingly, MCP-1 and MIP-1α but not Cdc42-stimulated cytoskeletal reorganization, can be inhibited by wortmannin, indicating the involvement of PI3-K upstream of Cdc42 in chemokine-stimulated cell migration (Weber *et al.*, 1998). There are also remarkable similarities between the phenotype of mice lacking the small GTPases Rac2 (which in mammals is usually restricted to expression in haematopoietic cells) and that of the PI3-Kγ-deficient phenotype. Hence, Rac2-deficient animals have a higher leucocyte blood count, their leucocytes are less able to infiltrate the peritoneum in experimental inflammatory models and less able to migrate in vitro in response to chemoattractants such as fMLP and IL-8 (Roberts *et al.*, 1999). The overlap of phenotypes suggests that Rac2 may be in the same leucocyte signalling pathway as PI3-Kγ. However, whilst the effect of chemokines on Rac activation was not assessed, it should be noted that Rac activation in response to the non-chemokine chemoattractant fMLP still occurs in POKγ-deficient cells. One probable explanation for this, however, is that fMLP stimulation of the p85/p110 heterodimer is able to sustain coupling to Rac in PHKγ-deficient mice (Stephens *et al.*, 1993).

2 Materials and Methods

2.1 Materials

Chemicals of high quality commercial grade were purchased from Sigma-Aldrich (St.Louis, MO, USA), Amersham Pharmacia Biotech (Piscataway, NJ, USA), Merck & Company (West Point, PA, USA), Serva Biochemicals (Hauppauge, NY, USA). Radiochemicals and chemiluminescent signal enhancers were obtained from NEN Reseach Products, Dupont (Hertfordshire, UK). Vectashield mounting medium was purchased from Vector Laboratories (Burlingame, CA, USA). GST-(PI3-K)-p85-N-SH2 domain (amino acids 333-428) and GST-(PI3-K)-p85-C-SH2 domain (amino acids 624-718) were obtained as an agarose conjugate from Upstate Biotechnology (UBI) (Walham, MA, USA). Wortmannin and LY294002 were from Sigma-Aldrich; uPA and ATF were from American Diagnostica (Greenwich, CT, USA). Restriction endonucleases HindIII, XbaI, PacI, EheI, and T4 DNA ligase were from New England BioLabs (Beverly, MA, USA); the Expand High Fidelity PCR system was from Roche Diagnostics (Indianapolis, IN, USA); plasmid pCR2.1TOPO was purchased from Invitrogen (Carlsbad, CA, USA); and plasmids pTGBKCMV and pAD1 were from HepaVec (Berlin, Germany), Plasmid pBluescript II SK- was bought from Stratagene (La Jolla, CA, USA). Reagents for cultivation E. Coli cells were purchased from Difco Laboratoris, division Becton Dickinson (Franclin Lakes, NJ, USA).

2.2 Antibodies

Mono- and polyclonal anti-STAT1 phosphotyrosine and phosphoserine antibodies were from Affinity Research Products Ltd. (Exeter, UK) and Pierce Chemical (Rockford, IL, USA); mono- and polyclonal anti-p85 PI3-K antibodies were from BD Transduction Laboratories (Lexington, KY, USA) and Upstate Biotechnology (UBI) (Waltham, MA, USA) Mono- and polyclonal anti- Janus kinases antibodies were purchased from BD Transduction Laboratories and Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-uPAR (CD87) monoclonal antibody was purchased from American Diagnostica, Inc. (Stamford, CT, USA). Peroxidase-conjugated affinipure goat anti-mouse and goat anti-rabbit IgG and Cy3-conjugated anti-rabbit and anti-mouse IgG were purchased Jakson Immunoresearch Laboratoris (West Grrove, PA, USA). Alexa 488-conjugated phalloidin was from Molecular Probes, Inc. (Eugene, OR, USA).

2.3 Cell Culture and Cell Treatment

2.3.1 Cell Culture

Human VSMC from coronary artery were obtained from Clonetics (Walkersville, MD, USA). The cells were grown in SmGM2 medium (Clonetics) supplemented with 5% fetal calf serum (FCS) and were used between passages 5 and 7. For uPA stimulation experiments, the cells were cultured for 24 h in serum-free medium and were then treated with uPA as described below. Human transformed kidney epithelial cells HEK 293 were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). Monocytes were isolated from human peripheral blood from healthy volunteers using Biocoll Separation Solution (Biochrom, Berlin, Germany) according to the standard protocol. FACS analysis using FITC-conjugated mouse anti-human CD14 monoclonal antibody resulted in about 70-80% CD14 positive cells that were resuspended directly after separation in SmGM medium (Clonetics, San Diego, USA) and used for coculture. For the transfilter coculture experiments, VSMC were grown up to 30% confluency in 6-well plates. Filter inserts (25 mm diameter, 0.4 μm polycarbonate membrane; Nunc, Roskilde, Denmark) containing $1.8x10^6$ monocytes were transferred into wells containing VSMC and both cell types were cocultivated up to 4 days.

2.3.2 Cell Stimulation

Subconfluent and serum-starved VSMC were treated with 1 nM uPA for 5-180 min at 37° C, lysed, and precleared as described in immunoprecipitation protocol. In some experiments, cells were pretreated with 100 nM wortmannin for 20 min at 37° C.

2.4 Molecular Biology Methods

2.4.1 Bacterial Strains Used for Adevirus Construction

In order to produce and amplify recombinant adenovirus with the adenoviral system, two different prokaryotic host strains are required. The first, BJ5183, is recA proficient and supplies the machinery necessary to execute the recombination event between the shuttle vector and the pHV1 vector. The second strain, DH5α (Clontech Laboratories, Palo Alto, CA) competent cells, is used to amplify the recombined adenovirus plasmid.

2.4.2 The Hanahan Method of Preparation Competent E. Coli

The desired E. coli cell strain were streak directly from a frozen stock on the surface of an SOB agar plate (2% bactotryptone; 0.55% yeast extract; 10 mM NaCl; 10 mM KCl;

10 mM $MgCl_2$; 10 mM $MgSO_4$; 1.5 % bactoagar, pH 7,6). The plate was incubated for 16 h at 37° C. Four or five well-isolated colonies were transferred into 1 ml of SOB (2% bactotryptone; 0.55% yeast extract; 10 mM NaCl; 10 mM KCl; 10 mM $MgCl_2$, pH 7,6) containing 20 mM $MgSO_4$. The bacteria were dispersed by vortexing at a moderate speed, and then the culture was diluted in 100 ml of SOB containing 20 mM $MgSO_4$ in a 1-liter flask. The cells were grown for 2.5-3.0 h at 37° C, monitoring the growth of the culture E. coli: in early- to mid-log phase (OD_{600} = 0.4). The cells were transferred to sterile, disposable, ice-cold 50-ml polypropylene tubes. The culture was cooled < to 0° C by storing the tubes on ice for 10 min. The cells were recovered by centrifugation at 2,700 x g (4,100 rpm in a Sorvall GSA rotor) for 10 min at 4°C. The medium was decanted from the cell pellets. The pellets were resuspended by swirling or gentle vortexing in ~20 ml (per 50-ml tube) of ice-cold FSB (potassium acetate, 10 mM; $MnCl_2$-$4H_2O$, 45 mM; $CaCl_2$-$2H_2O$, 10 mM; KCl, 10 mM; hexamminecobalt chloride, 100 mM; Glycerol 10% (v/v); pH 6.4) transformation buffer. The resuspended cells were stored on ice for 10 min. The cells were recovered by centrifugation at 2,700 x g (4,100 rpm in a Sorvall GSA rotor) for 10 min at 4°C. The buffer was decanted from the cell pellets. The pellets were resuspended by swirling or gentle vortexing in 4 ml (per 50-ml tube) of ice-cold FSB. 140 μl of DMSO was added per 4 ml of resuspended cells. It was mixed gently by swirling, and the suspension was stored on ice for 15 min. An additional 140 μl of DMSO was added to each suspension. It was mixed gently by swirling and then the suspensions were returned to an ice bath. Aliquots of the suspensions were dispensed into chilled, sterile microfuge tubes or tissue culture vials. The competent cells were immediately snap-freezed by immersing the tightly closed tubes in a bath of liquid nitrogen. The tubes were stored at -70° C until needed.

2.4.3 Transformation of Competent Cells

The transforming DNA (up to 25 ng per 50 μl of competent cells) was added in a volume exceeding 5% of that of the competent cells. The tubes were swirled gently several times to mix contents. The tubes were stored on ice for 30 min and then were transferred to a rack placed in a preheated 42°C circulating water bath. The tubes were stored in the rack for exactly 90 sec. The tubes were rapidly transfered to an ice bath for 1-2 minutes. 800 μl of SOC (SOB plus 20 mM glucose) medium were added to each tube. The tubes were transfered to a shaking incubator set at 37° C. The cultures were incubated for 45 min allowing the bacteria to recover and to express the antibiotic resistance marker encoded in plasmid with gently shaking (<225 cycles/min). The appropriate volume (up to 200 μl per 90-mm plate) of transformed competent cells was transferred onto agar SOB medium containing 20 mM $MgSO_4$ and the appropriate antibiotic.

2.4.4 Small–Scale Preparation of Plasmid DNA

Mini preparations of plasmid DNA were obtained by alkaline lysis method. This protocol is a modification of the method of Birboim and Doly (1979) and Ish-Horowitz and Burke (1981). Single bacterial colony was transferred into 3 ml of LB (bacto yeast extract, 0.5%; bacto tryptone, 2%; $MgSO_4$ 0.5%; pH 7.6) medium containing the appropriate antibiotic in a loosely capped 15 ml tube. The culture was incubated overnight at 37° C with vigorous shaking. The culture was poured into 1.5 ml microfuge tube and centrifuged at 5,000 rpm for 5 min in a microfuge. The remainder of the culture was stored at 4° C. The medium was removed by aspiration, living the bacterial pellet as dry as possible. The bacterial pellet was resuspended in 100 μl of ice-cold Solution I (50 mM glucose; 25 mM Tris-HCl, pH 8.0; 10 mM EDTA; pH 8.0) by vigorous vortexing. The freshly prepared 200 μl Solution II (0,2 N NaOH; 1% SDS (w/v)) was added to cell suspension, mixed contents by inverting the tube rapidly five times. The tubes were stored on ice. The ice-cold solution III (5 M potassium acetate, 2 M acetic acid) was added to the tubes, which were then vortexed gently in an inverted position for 10 sec to disperse Solution III through the viscous bacterial lysate. The tubes were stored on ice for 3-5 min. After the centrifugation for 5 min at 13000 rpm, 4° C the supernatant was transferred to a fresh tube. An equal volume phenol: chloroform was added and mixed by vortexing. After centrifugation for 2 min at 13,000 rpm, 4° C in a microfuge, the supernatant was transferred to a fresh tube. Double stranded DNA was precipitated with 2 volumes ethanol at room temperature. Tubes were spinned for 5 min at 13,000 rpm, 4° C in a microfuge and the precipitate was rinsed with 1 ml of 70 % ethanol at 4° C. The pellet of nucleic acid was dried in the air for 10 min and redissolved in TE (10 mM Tris-HCl; 1 mM EDTA; pH 8,0) containing DNAase-free pancreatic RNAase (20 μl/ml). DNA was stored at –20° C.

2.4.5 RNA Isolation

Total RNA extracted from cultured smooth muscle cells was used as a template in reverce transcription reactions. During the work with RNA a series of precautions were taken to avoid RNase contamination. Glassware was baked at 200° C for at least 4 h and water was treated with DMPC (Dimethylpyrocarbonate). A 10% DMPC stock was prepared in ethanol and was then diluted 1:100 in double distilled water, left at room temperature overnight and finally autoclaved.

Total RNA extractions were done with a TRISOL reagent (GibcoBRL). Cells were lysed directly in a culture dish by adding 1 ml of TRIZOL reagent to a 3.5 cm diameter dish, and passing the cell lysate several times through a pipette. The amount of TRIZOL reagent added is based on the area of the culture dish (1 ml per 10 cm^2) and not on the number

of cells present. The homogenized samples were incubated for 5 min at RT to permit the complete dissociation of nucleoprotein complexes. 0.2 ml of chloroform was added per 1 ml of TRIZOL Reagent. Tubes were shaked vigorously by hand for 15 sec and incubated then at RT for 2 to 3 min. The samples were centrifuged at 12,000 x g for 15 min at 2 to 8° C. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. The volume of the aqueous phase is about 60 % of the volume of TRIZOL Reagent used for homogenization. The aqueous phase was transferred to a fresh tube. The RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol. 0.5 ml of isopropyl was used alcohol per 1 ml of TRIZOL reagent used for the initial homogenization. Samples were incubated at RT for 10 min and centrifuged at 12,000 x g for 10 min at 2 to 8° C. The supernatant was removed. The RNA pellet was washed once with 75 % ethanol, adding at least 1 ml of 75 % ethanol per 1 ml of TRIZOL reagent used for the initial homogenization. The sample was mixed by vortexing and centrifuged at 7,500 x g for 5 min at 2 to 8° C. At the end of the procedure, the RNA pellet was briefly dried by air or vacuum for 5-10 min. RNA was redissolved in 100 % formamide (deionized) and stored at -70° C.

The purity of total RNA was checked by running ~ 1μg of total RNA on a 1 % agarose gel made in TAE buffer (40mM Tris-acetate;1mM EDTA, pH 8) with 0,5 μg/ml ethidium bromide. The two ribosomal RNA bands (23S and 18S) were clearly detected when the gel was viewed under ultraviolet light (UV) and smears (as indicator of RNA degradation) were not seen between the two bands. To quantify the total RNA, an aliquot was measured by UV absorbance at 260 nm (A_{260}) and 280 nm (A_{280}) where the absorbance of 1 in a 1 cm path length corresponds to a RNA concentration of 40 μg/ml. The absorbance ratio of 260 nm and 280 nm gave an estimate of the purity of the solution. Pure RNA solutions had A_{260}/A_{280} values between 1.7-2.

2.4.6 Reverse Transcription

To assay for Tyk2 gene expression, total RNA from human VSMC was reverse transcribed into cDNA. For this reaction hexamer primers of random sequence as well as oligonucleotides of defined sequence were used. The following components were mixed in a total volume of 20 μl to perform a reverse transcription reaction: 1 μg of total RNA was mixed with 1 x reaction buffer (100 mM Tris/ 500 mM KCl pH 8.3), 5 mM $MgCl_2$, 2-10 pmol of primer, 1 mM each dNTP and 20 units of avian myeloblastosis virus (AMV) reverse transcriptase enzyme (Roche Molecular Biochemicals). The mixture was then briefly vortexed and incubated at 25° C for 10 min to allow the primer to anneal to the RNA, followed by incubation at 42° C for 1 h during which the RNA was reverse transcribed resulting in cDNA synthesis. The reaction mixture was incubated at 99° C for 5 min to denature the AMV enzyme and then the mixture was cooled down to 4°C for 5 min.

2.4.7 RT-PCR for uPA and uPAR

RT-PCR analysis for uPA and uPAR mRNAs in monocytes and VSMC in coculture was performed using the TaqMan method. RNA was isolated using the RNeasy mini prep kit (Qiagen, Santa Clarita, USA) and on-column DNase digestion according to the manufacturer's protocol. RNA was eluted from columns in 50 µl RNAse-free water. Gene-specific TaqMan primers and probes for human uPA, uPAR and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed using Primer Express software (PE Biosystems, Foster City, USA), according to the manufactures guidelines. All primers and probes were purchased from BioTeZ GmbH (Berlin, Germany), with each GAPDH, uPA or uPAR probe being syntesised with a fluorescent 5'-reporter dye (FAM; 6-carboxyfluorescein) and 3'-quencher dye (TAMRA; 6-carboxy-tetramethyl-rhodamine). The following primers were used: (a) for uPA, ACTGCAGGAACCCAGACAACC; TGGACAAGCGGCTTTAGGC; TaqMan probe AGGCGACCCTGGTGCTATGTGCAG; (b) for uPAR, ACCACCAAATGCAACGAGG; GTAACACTGGCGGCCATTCT; TaqMan probe CAATCCTGGAGCTTGAAAATCTGCCG; (c) for GAPDH, GAAGGTGAAGGTCGGAGTC; GAAGATGGTGATGGGATTC; TaqMan probe CAAGCTTCCCGTTCTCAGCC. Reverse transcription reaction and TaqMan PCR reaction were performed according to the single-tube protocol (PE Biosystems). TaqMan PCR assays for each gene target were peformed on total RNA in optical tubes with an ABI Prism 7700 Sequence Detection system (PE Biosystems). For each 25 µl TaqMan reaction, 3 µl total RNA was used. Parallel assays were run for the housekeeping gene GAPDH in addition to target genes of interest. All TaqMan RT-PCR data were captured using Sequence Detector Software (SDS version 1.6; PE Biosystems). For each mRNA template, reactions were prepared in triplicates and relative quantitation of gene expression was calculated using the comparative C_T method for separated tubes, according to the standard protocol. Data from each sample were normalized by dividing the quantity of target RNA by the quantity of housekeeping mRNA (GAPDH), to correct for differences in RNA quantity and quality.

2.4.8 In Vitro Mutagenesis Using Double-Stranded DNA

Two oligonucleotide primers were used for single point mutation. For the design of oligonucleotide primers were used recomindation of Sratagene Company for QuickChange mutagenesis kit.

The template DNA used for mutagenesis was a circular plasmid containing the cDNA of interest. In sterile 0.5-ml microfuge tubes, a series of reaction mixtures containing dif-

ferent amounts (e.g., 5, 10, 25, and 50 ng) of plasmid DNA were set up and a constant amount of each of the two oligonucleotide primers.

10^x Pfu polymerase buffer (Stratagene)	5µl
template plasmid DNA	5-50 ng
oligonucleotide primer 1 (20 mM)	2.5 µl
oligonucleotide primer 2 (20 .mM)	2.5 µl
dNTP mix (dNTPs, each at 5 mM)	2.5 µl
H_20	to 50 µl

2.5 units of Pfu DNA polymerase (Stratagene) were added. The tubes were placed in the thermal cycler. The nucleic acids were amplified using the denaturation, annealing, and polymerization times and temperatures listed in the table.

Cycle Number	Denaturation	Annealing	Polymerization
1 cycle	1 min at 95°C		
2-18 cycles	30 sec at 95°C	1 min at 55°C	2 min/kb of plasmid DNA at 68°C
Last cycle	1 min at 94°C	1 min at 55°C	10 min at 72°C

For single-base substitutions, were used 12 cycles of linear amplification; for substitution of one amino acid with another (usually two or three contiguous base substitutions), were used 16 cycles; for insertions and deletions of any size, were used 18 cycles. After amplification of the DNA, the reactions were placed on ice. Adding 10 units of Dpnl directly to the remainder of the amplification reactions digested the parent's plasmid DNAs. The reagents were mixed by pipetting the solution up and down several times, the tubes were centrifuged for 5 sec in a microfuge, and then were incubated for 1 h at 37°C. Competent cells (DH5α strain prepared by Rubidium Chloride method) were transformed with 1, 2, and 5 μl of digested DNA according to the procedure described earlier.

2.4.9 Sequencing

Plasmids were sequenced to verify that they contained the desired inserts and to control for unwanted mutations. Samples were processed by the dye terminator method (Applied Biosystems, division PE Biosystems) and purified from unintercalated labeled dNTPs by Centri-Sep spin columns (Applied Biosystems, Foster City, CA). The samples were then mixed with 4 ml buffer (deionized formamide and 25 mM EDTA pH 8.0 containing 50 mg/ml Blue dextran in a ratio 5:1 formamide: EDTA-Blue dextran), denatured

by heating at 94° C for 4 min, and loaded on sequencing gel on an automated DNA sequencer (Applied Biosystems, Foster City, CA). The samples were then separated on a polyacrylamide gel and the fluorescent nucleotides excited with a laser beam. The emitted fluorescence was collected by detectors (Applied Biosystems, Foster City, CA) and sent to a computer, where the appropriate software converted the data into nucleotide sequence. For sequence assembling, editing and alignments, the Lasergene software programs EditSeq, SeqMan and MegAlign (DNASTAR, Madison, USA) were used. Some samples were sequenced by the InViTek Company (Berlin, Germany).

2.4.10 Probe Labeling

cDNA fragments subcloned into pCRI2.1TOPO vectors were prepared by restriction digestion of plasmids and isolated by gel purification. For radioactive labeling of DNA fragments, the random primer extension kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) was used and the enzyme required for this process was Escherichia coli DNA polymerase I (Klenow fragment). The reaction mixture contained: 25 ng of DNA (which has been previously gel purified) and 5 μl of random primers (Amersham Pharmacia Biotech), which were brought to 50 μl final reaction volume with double distilled water. The initial mixture was denatured by boiling for 5 min. Keeping the tube at RT, the unlabeled dNTPs (0.1 mM each, omitting those to be used as label) and reaction buffer (from Amersham Pharmacia Biotech, containing Tris-HCl pH 7.5, 2-mercaptoethanol and $MgCl_2$) followed by the radiolabeled dNTPs and DNA polymerase I (Klenow fragment) enzyme (0.04 units/μl) were added. The reaction mixture was then incubated at 37° C for 1 h. Phosphorus-labeled dNTPs (3000 Ci/mmol [α-^{32}P] dCTP) were used. Non-incorporated labeled nucleotides were removed from the probe by precipitation with 0.1 volume of 4 M LiCl and 2.5 volumes of chilled absolute ethanol and incubation at –70° C for 30 min and centrifugation at 13,000 x g for 15 min at 4° C. The pellet was then washed with ~ 100 μl of cold 70 % ethanol, air dried and dissolved in 20 μl of double distilled water. The efficiency of the labeling reaction was monitored by a scintillation counter in multi-sample β-spectrometr (Beckman Instruments Inc., Palo Alto, CA, USA).

2.4.11 RNA Preparation, and Northern Blotting

VSMC were grown to 50% confluence and infected for 1h with recombinant AdTyk2 or AdTyk2KE adenovirus stock at a multiplicity of infection of 500 plaque-forming units/cell (PFU). Immunological staining with anti-Tyk2 antibody assessed the efficiency of infection. Cells were serum-starved overnight 1 day after infection and used for total RNA preparation on the second day after infection. Total RNA was prepared from VMSC using

standard protocol with TRIZOL reagent from Life Technologies (Rockville, MD, USA). The RNA was subjected to electrophoresis through an agarose-formaldehyde gel, transferred to a gene-screen plus membrane (DuPont NEN Products, Boston, MA, USA), and hybridized with nick-translated BamHI fragment from Tyk2 cDNA as described (Maniatis et al, 2000). An actin probe was used to confirm equal RNA loading on the gel.

2.5 Biochemical Methods

2.5.1 Protein Determination by Bradford Method

96 microwell plate (Macrowell Plate, Nunc,Naperville, IL, USA) was used. The diluted samples of BSA at concentrations of 4 - 40 μg/ml for the standard curve and of unknown protein were added to the wells in a volume of 50 μL. 200 μl of Bradford reagent Protein Assay Dye ((Bio-Rad Laboratories, Hercules, CA, USA) at a dilution with water of 1:5 were added per well for 5 min. Each sample was measured in triplicates. The absorption was measured at 595 nm using a microplate reader ThermoMax from Molecular Devices (Sunnyvale, CA, USA). The software SOFTmax PRO was used.

2.5.2 Tyrosine Phosphorylation, Western Blotting

Subconfluent and serum-starved human VSMC were washed twice with HEPES-NaCl buffer (10 mM HEPES, pH 7.5; 150 mM NaCl) and then treated with 1 nM uPA (Sigma) or 1 nM ATF (kindly provided by Dr J. Henkin, Abbott Laboratories, Abbott Park, USA) at 37° C for 5 to 30 min. Cells were put on ice; washed with ice-cold, HEPES-buffered saline containing 0.3 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.1 mM quercetin, and 0.1 mM N-carbobenzyloxy-L-phenylalanine chloromethyl ketone; and harvested by scraping. After centrifugation (1,500 x g, 3-5 min, 4° C), the pellets were lysed in lysis buffer (20 mM Tris-HCl, pH 8.0; 138 mM NaCl; 10% glycerol; 2 mM EDTA; 1% Triton X-100; and protease inhibitors as indicated above) and left on ice for 10 min. Lysates were sonicated at maximum intensity for two 10 sec periods and centrifuged (8 min at 15,000 x g, 4° C). Supernatants were used for polyacrylamide gel electrophoresis (PAGE) and Western blotting. The blots were developed with the appropriate antibody; the immune complexes were visualized by an enhanced chemiluminescence detection system Pierce Chemical (Rockford, IL, USA).

2.5.3 Immunoprecipitation

For immunoprecipitation, cell lysates containing 600 to 1000 µg protein were pre-cleared for 2 h at RT with Gamma-Bind Sepharose (Amersham Pharmacia Biotech) and then immunoprecipitated overnight at 4° C by using 5 or 10 µg polyclonal antibody coupled to protein A–agarose (Santa Cruz Biotechnology, Santa Cruz, CA, USA). For monoclonal antibody protein A-agarose were replased by protein G-agarose (Santa Cruz Biotechnology). Precipitates were washed three times in PBS buffer (137 mM NaCl; 2,7 mM KCl; 10 mM Na_2HPO_4; 1,8 mM KH_2PO_4; pH 7,4) with 0,05% (v/v) Tween20 (PBS-$T_{0.05\%}$). Precipitated proteins were eluted with Laemmli sample buffer (1% SDS, w/v; 11% glycerol, w/v; 125 mM DTT; 0.01% bromophenol blue, w/v; 50 mM Tris-HCl, pH 6.8; 95° C, 5 min) and were used for PAGE and Western blotting.

2.5.4 SDS-Polyacrylamide-Gel-Elektrophoresis (SDS-PAGE)

The standard Laemmli (Laemmli, 1970) method was used for discontinuous gel electrophoresis under denaturing conditions, that is, in presence of sodium dodecyl sulfate (SDS). The standard method for full-size (e.g., 14x14 cm) was adapted for the minigel format (7x10 cm). Assemble the glass plate sandwich of the electrophoresis apparatus was done according to manufacturer's instruction used two clean glass plates and two 1-mm spacers. The desired percentage of acrilamide in separating gel depends on the molecular size of the protein being separated. The 3% stacking (125 mM Tris-HCl, pH 6,8; 3% (v/v) acrilamide/bisacrilamide (37,5:1); 0,1 %(w/v) SDS; 0,1% (w/v) APS and 0,05 % (v/v) TEMED) and 10 % separating gel (375 mM Tris-HCl, pH 8,8; 10% (v/v) acrilamide/bisacrilamide (37,5:1); 0,1 %(w/v) SDS; 0,1% (w/v) APS and 0,05 % (v/v) TEMED) were used for usual procedure. Gel run was carried out in electrophoresis camera Mini-Protean II with Power Supplier model 1000/500 Bio-Rad Laboratories (Hercules, CA, USA) at constant current 20 mA per gel.

2.5.5 Semi-dry Blotting

The electrotransfer of proteins from polyacrylamid gel to PVDF membrane (Roche Diagnostics, Indianapolis, IN, USA) was performed in semi-dry modification in Trans-Blot SD apparatus with Power Supplier model 1000/500 (Bio-Rad Laboratories, Hercules, CA, USA) at 110 mA per blot-sandwich (50 min) with transfer buffer (Tris 48 mM; Glycin 39 mM; SDS 0,037 % (w/v); Methanol 20% (v/v)).

2.5.6 Immunologic Detection of Transferring Proteins

Immobilized proteins were probed with specific antibodies to identify and quantitate any antigenic presents. The membrane was immersed in blocking buffer (PBS-$T_{0.05\%}$, 1% BSA (w/v), RT, 1h) to fill all protein-binding sites with a nonreactive protein or detergent. Next, it was placed in solution containing the antibody (in PBS-$T_{0.05\%}$, 1% BSA (w/v), RT, 2h or 4° C overnight) directed against the antigen.

The blot was washed 10 min, three times with PBS-$T_{0.05\%}$, and exposed for 1h the peroxidase-antibody conjugate directed against the primary antibody (secondary antibody in PBS-$T_{0.05\%}$, 1% BSA (w/v), RT, 1h). Membrane was washed as describe above. Antigens were identified by luminescent visualizations. After the final wash, the blot was immersed in substrate solution (Renaissance©, DuPont NEN, Boston, MA, USA) containing luminol for horseradish peroxidase (HRPO) systems, sealed in thin plastic wrap, and placed against film Kodak X-Omat Blue XB-1 (Eastman Kodak Company, Rochester, NY, USA).

2.5.7 Reprobing of PVDF- Blotmembrane

"Stripping" of the membranes was performed with 200 mM β-mercaptoethanol, 62.5 mM Tris-HCl (pH 6.8), and 2% SDS (w/v) for 45 min at 50°C. Membrane were washed three times in PBS-$T_{0.05\%}$, blocked in 3% (w/v) BSA (1h, RT), and used for immunoblotting as indicated above.

2.5.8 In Vitro Phosphorylation Assay and Reimmunoprecipitation

For the in vitro phosphorylation assay, 400-800 µg of protein of precleared cell lysates were incubated with 4-5 µg of the indicated antibody coupled to the protein A-agarose for 2 h. Precipitates were washed four times with lysis buffer and twice with kinase buffer (20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM dithiothreitol, 300 µM sodium orthovanadate, 10 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride). The kinase assay was performed in 40 µl of kinase buffer containing 3-5 µCi of [α-^{32}P]ATP (3000 Ci/mmol) for 10 min at 30° C. Precipitates were washed once with lysis buffer and twice with PBS-Tween buffer. The phosphorylated products were eluted in 150 µl of lysis buffer containing 0.5 % SDS and 5 mM sodium orthovanadate and then subjected to a second round of immunoprecipitation with 1 µg of the indicated antibody coupled to protein A-agarose overnight at 4° C. Phosphorylated proteins were analyzed by SDS-PAGE and autoradiography. In some experiments, gels containing phosphorylated proteins were soaked in 1 N KOH at 55° C for 2 h to hydrolyze phosphate on serine and threonine.

2.5.9 PI3-K Assay

Precleared cell lysates containing 600-800 µg of protein were incubated for 2 h at 4° C with 4 µg of anti-PI3-K antibody against the p85 regulatory subunit and then precipitated on protein A-agarose. Precipitates were washed once with lysis buffer containing 300 µM sodium orthovanadate and 1 mM phenylmethylsulfonyl fluoride and then four times with 10 mM HEPES, 100 mM NaCl, pH 7.4, and then subjected to the in vitro kinase reaction in a final volume of 50 µl of reaction mixture containing 1 mg/ml L-phosphatidylinositol, 3 mM $MgCl_2$, 15 mM ATP, and 3-5 µCi of [α-^{32}P] ATP (3000 Ci/mmol) for 10 min at 30° C. Phase separation of lipids was performed in two steps by n-hexane/isopropyl alcohol (26:14) and 2N KCl/HCl (8:0.25). Phosphorylated lipids were separated by thin layer chromatography on aluminum sheets silica gel 60W (Merck, Germany) in chloroform/methanol/H_2O/25% ammonium (45:35:7.5:2.8; v/v/v/v) and visualized by autoradiography.

2.5.10 Fusion Protein Precipitation Assay

GST fusion proteins containing the single C- or N-terminal p85 SH2 domain (GST-p85-C-SH2 and GST-p85-N-SH2) bound to glutathione-agarose beads were used for affinity precipitation. Precleared cell lysates containing 400-800 µg of protein were incubated with 3-4 µg of SH2 domain conjugates for 2 h at 4° C. Precipitates were washed three times with PBS-Tween buffer. Precipitated proteins were eluted with 2-fold Laemmli sample buffer containing 20 mM dithiothreitol and 10 mM glutathione and were used for PAGE and Western blotting.

2.5.11 Enzyme immunoassay (ELISA) and flow cytometry

Enzyme immunoassay for quantitative measurement of human uPA and uPAR in mono- and coculture cell-free fractions was performed using commercial kits from Monozyme (Hoersholm, Denmark) according to standard protocols from supplier.

Single cell-associated fluorescence for uPAR (CD 87) was quantified by flow cytometry with a FACScan flow cytofluorometer (Becton-Dickinson). Binding of R-phycoerythrin (R-PE)-conjugated mouse anti-human CD87 monoclonal antibody (PharMingen, San Diego,USA) to surface receptors on monocytes and VSMC was determined on freshly detached, unfixed cells according to the standard protocol from antibody supplier.

2.6 Microscopy

2.6.1 Time-Lapse Video Microscopy

Before wounding, cells were cultured in serum-free SmGM2 medium for 6 h. In migration experiments with PI3-K inhibitors, cells were pretreated with wortmannin or LY294002 in serum-free medium for 30 min before wounding, and then inhibitors were added again to the serum-free medium after wounding with or without uPA. Cell migration was monitored by time-lapse imaging using an endoscope (telecam PAL 20210036, Storz, Germany) attached to an Axioplan microscope (Zeiss) and acquisition and analysis software (Avid Videoshop, Avid Desktop Software Inc., Microsoft Excel). The wounds were viewed inside an environmental chamber under constant temperature (37° C) and humidified in 5% CO_2 and air (CTI Controller 3700; Zeiss). Microscopic recordings were started immediately after wounding, and then further images were taken every 30 min for 9 h following wounding. Results are the mean number of migrated cells ± S.D. at indicated time points or -fold stimulation of at least five separate experiments.

2.6.2 Immunofluorescence Microscopy

Cells were seeded and cultured on glass coverslips, and wounding was performed as indicated above. Cells were allowed to migrate for 8 h at 37°C and then treated for the different periods of time with appropriate inhibitors or stimulators. After incubation, cells were fixed with 4% paraformaldehyde in PBS for 20 min, RT, washed three times with PBS, and permeabilized with 0.1% Triton X-100 in PBS for 3 min, RT. Then cells were stained with Alexa 488-conjugated phalloidin for 20 min, RT. For staining of Tyk2, Jak1, STAT1, and uPAR, appropriate primary antibody against individual proteins in the concentration 5 μg/ml was used. Cells were washed three times with PBS and incubated 1 h RT with appropriate Alexa 488 (or Cy3) secondary antibody in the concentration 5 μg/ml. Cells were washed as described above and coverslips were mounted with Vectashield mounting medium. Color images were captured using a SensiCam 12-bit CCD camera (PCO Computer Optics, Kelheim, Germany) and acquisition and analysis software AxioVision and KS 300 (Carl Zeiss, Thornwood, NY, USA) running on a PC 586/200MMX-64MB (Inteq, Berlin, Germany). Images for Alexa 488 and Cy3 staining were captured digitally and were imported as TIF files into Adobe Illustrator for analysis and printing.

2.7 Functional Studies

2.7.1 Wound Assay

The wound assay was performed as described elsewhere (Okada *et al.*, 1995). Briefly, VSMC were plated confluently on 14-mm diameter glass coverslips (Menzel-Glaser, Braunschweig, Germany) and were placed in 35-mm tissue culture dishes. A wound width of approximately 3 mm was made using a razor blade. VSMC were allowed to recover for 4—24 h and processed as noted below.

2.7.2 Chemotaxis Assay

Chemotaxis assay was performed using modified Boyden chambers with polyvinylpyrrolidone-free polycarbonate filter membranes, 8-µm pore size. 30,000 cells in serum-free SmGM2 medium were added to the upper well of the Boyden chamber. uPA was diluted in serum-free SmGM2 and added to the lower well of the Boyden chamber, and migration was allowed for 4h. When chemotaxis was performed in the presence of the PI3-K inhibitors wortmannin or LY294002, these substances were added to the upper well. All experiments were performed in triplicates. Cell migration was quantified by densitometry, and cell migration in the absence of chemoattractant was taken as 100%.

2.7.3 Adhesion Assay

The assay was performed in 96-well plates coated with bovine vitronectin at the concentration of 0.35 pg/ml at 37 °C for 1 h and then blocked with 3% BSA in PBS for 30 min. Wells treated with 3% BSA only served as background samples. VSMC were detached with 5 mM EDTA inPBS, pH 7.4 and then washed once with PBS. Cells were resuspended in serum-free medium, plated at $1.5x10^4$ cells/well and allowed to incubate at 37 °C for 20 min. Then the wells were rinsed twice with PBS containing Ca^{2+} and Mg^{2+} to remove nonadherent cells and then PBS without Ca^{2+} and Mg^{2+} was added together with hexoseamidasereagent (15 mM p-nitrophenyl N-acetyl-β-D-glucosaminide, 0.5% Triton-X 100, 100 mM sodium citrate buffer, pH 5.0) for 40 min at 37 °C. The reaction was stopped by adding 0.2 M NaOH containing 5 mM EDTA and absorbance was measured at 405 nm.

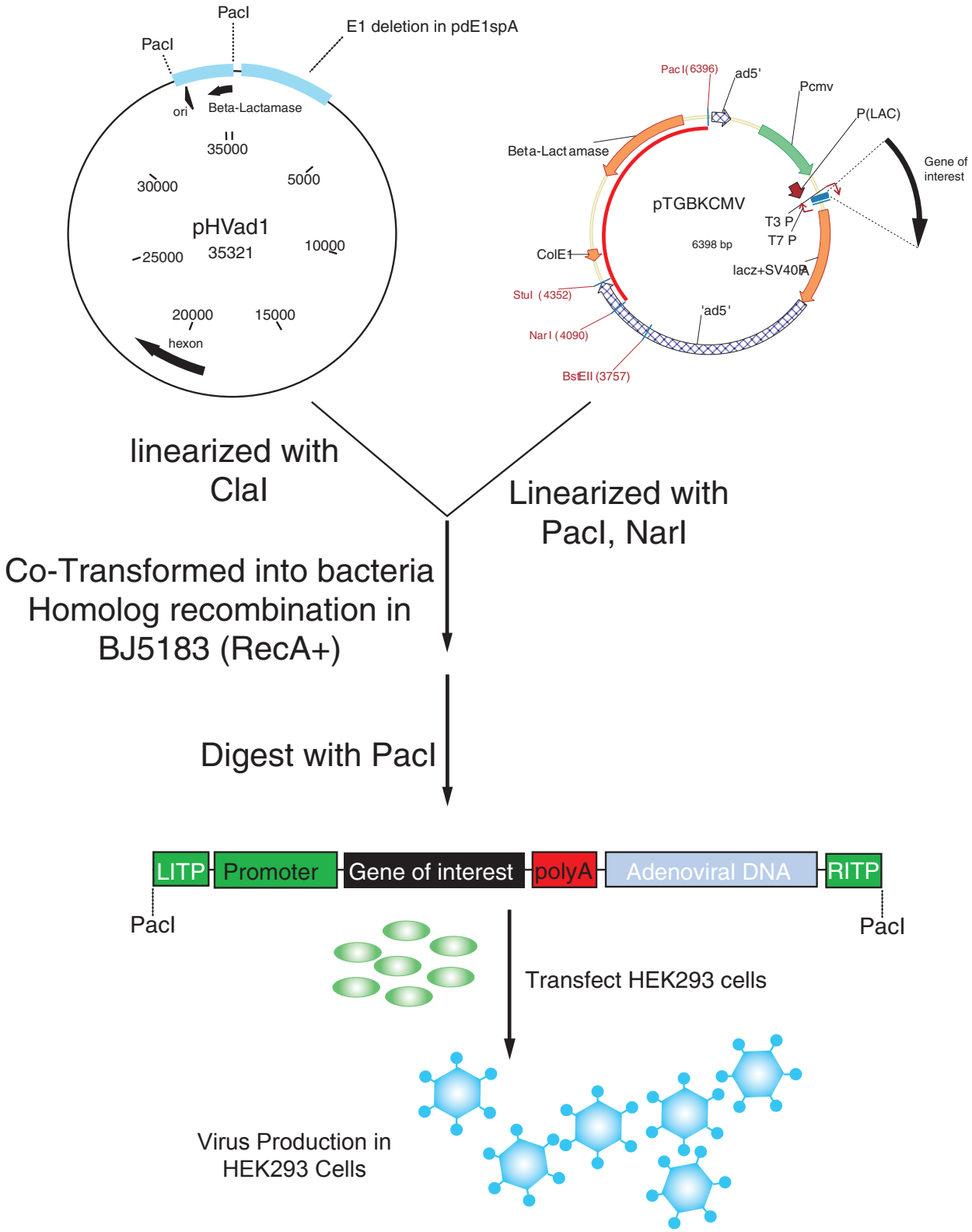

Fig. 9 Schematic overview of the adenovirus technology.

The gene of interest is first cloned into a shuttle vector, e.g., pTGBKCMV. The resultant plasmid is linearized by digesting with restriction endonuclease PacI, and subsequently cotransformed into E. coli. BJ5183 cells with an adenoviral backbone plasmid, e.g., pHVad1. Recombinants are selected for ampicillin resistance, and recombination confirmed by restriction endonuclease analyses. Finally, the linearized PacI recombinant plasmid is transfected into adenovirus packaging cell lines, e.g., 293 cells.

2.7.4 Proliferation Assay

DNA synthesis was measured using the 5-bromo-2-deoxy-uridine (BrdU) labeling and detection kit III from Roche Diagnostics (Indianapolis, IN, USA) as described by supplier.

2.8 Recombinant Adenovirus

2.8.1 Recombinant Adenovirus Construction

A schematic overview of the production of recombinant adenovirus is shown (Fig. 10). In the adenovirus generation system (Chartier *et al.*, 1996), the cDNA of interest is cloned into either of two shuttle vectors pHV1 or pTGBKCMV. Once constructed, the shuttle vector is linearized with Pme I and Ehe I (can be replaced by Stu I, Not I, XcaI or BstEII). After restriction digestion two fragments were separated by electrophoresis in agarose gel. Plasmid fragment with origin replication and ampicillin resistanse gene was discarded. Fragments from transfer vector was cotransformed into BJ5183 together with pHV1, the linearized by Cla I viral DNA plasmid. Transformants are selected for ampicilin resistance, and recombinants are subsequently identified by restriction digestion. Once a recombinant is identified, it is produced in bulk using the recombination-deficient DH5α (Clontech), strain. Purified recombinant Ad plasmid DNA is digested with Pac I to expose its inverted terminal repeats (ITR), and then used to transfect HEK293 cells where deleted viral assembly genes are complemented in vivo.

The concentration of the recombinant adenovirus was assessed based on the absorbance at 260 nm and plaque assay (Stratford-Perricaudet, Makeh *et al.* 1992).

2.8.2 Calcium-Phosphate-Mediated Transfection of HEK 293 Cells

Twenty-four hours before transfection, exponentially growing cells were harvested by trypsinization and replated at density of 8 x 10^5 cells in 60-mm tissue culture dishes (near 50 % confluence) complete DMEM medium with 10% FCS. The cultures were incubated for 20-24 h at 37° C in a humidified incubator with an atmosphere of 5 % CO_2. The medium was changed 1 h before transfection.

The calcium phosphate-DNA coprecipitate was prepared as follows: Combine 25 μl of 2.5 M $CaCl_2$ with of plasmid DNA (4 μg pAd digested PacI and 10 μg pUC18 as carrier) in a sterile 15-ml plastic tube and, if necessary, the final volume was brought to 250 μl with sterile H_2O. This 2x calcium-DNA solution was mixed with an equal volume of 2x Hepes Transfection Buffer “HTB” (275 mM NaCl; 28 mM Na_2HPO_4 x2 H_2O; 10 mM KCl; 42 mM HEPES, pH 6.75) at RT. The ingredients were quickly mixed and allowed to stand

for 1 min. The calcium phosphate-DNA suspension was immediately transferred into the medium above the cell monolayer. The plates were rocked gently to mix the medium, which will become yellow-orange and turbid. After 6 h incubation, the medium and DNA precipitate was removed by aspiration. 3 ml of warmed (37°C) complete growth medium with 0.8% SeaPlaque agarose (FMC Bioproducts, Rockland, ME, USA) was overlayed, and the cells were put into the incubator for 3 days. 3 ml complete DMEM medium was added to cells after 3 days. Plaques were appeared within 10-21 days. To maintain cell viability throughout plaque formation, additional agarose/DMEM mix was overlayed onto each dish every 4 to 5 days or when medium started turning yellow.

2.8.3 Purification of Recombinant Adenovirus by Double Cesium Chloride Gradient

Purification of an Ad was a three-step process:

1. ultracentrifugation on a discontinuous CsCl gradient which removes the majority of cellular contaminants and defective viral particles

2. ultracentrifugation on a continuous CsCl gradient to completely separate infectious from defective viral particles

3. removal of CsCl by desalting.

2.8.4 Discontinuous Gradient

HEK 293 confluent cells were transferred from the 5 x 175 cm^2 flasks to the 40 x 15 cm dishes (d0). After 3 days (d3) cells were 70-80% confluent ($5x10^7$ cells/dish). Total amount of cells was about $2x10^9$. Virus (50 ml virus cell lisat) was added to 240 ml DMEM and redistributed to each dish in 7 ml virus solution. Cells were incubated 4-5 h 37° C, in 5% CO_2, then 20 ml fresh DMEM with 10% FCS was added to each dish and incubated 36-48 h 37° C, 5% CO_2. After several days cells showed strong cyttopathic effect (CPE). Cells were suspended by pipetting and collected by centrifugation (20 min, 1,500 rpm, 4° C). Ten dishes were used for the preparation of single cesium chloride gradient centrifugation. Cell pellet from ten dishes was resuspended in 5 ml DMEM medium with 10% FCS and was extracted viral particles by performing three freeze/thaw cycles at -20°C/37°C. Cell debris were removed by centrifugation (15 min 5,500 rpm, 4°C). 2 ml of CsCl ρ1.5 (10 mM Tris-HCl, pH 7.9) were slowly poured into polyallomer tubes with a 2 ml pipette. Very gently, with 2 ml of CsCl ρ1.35 (10 mM Tris-HCl pH 7.9) and 1 ml CsCl ρ1,25 (10 mM Tris-HCl pH 7.9) were overlayed. On the top of each discontinuous gradient up to 7 ml of viral particle stock in DMEM 10% was loaded. The tubes were centrifuged at

100,000 x g (35,000 rpm in SW 40 rotor, Beckman Instruments, Palo Alto, CA) for 2 h at 10° C, deceleration rate = 0. In a laminar flow hood, the tubes from the rotor were carefully removed. Using a 10 ml pipette, most of the impurities from the top of the gradient were aspirated. Using a 1 ml syringe with an 18G needle the viral band (about 1 ml) was aspirated, avoiding collecting other bands and impurities. The solution containing the viruses was transferred the to a sterile 15 ml polypropylene tube and mixed with 11 ml CsCl ρ1,35 (10 mM Tris-HCl pH 7.9).

2.8.5 Continuous Gradient

The 11-12 ml diluted viral suspension was loaded in 12 ml polyalomer tubes and centrifuged at 100,000 x g (35,000 rpm with SW 40 rotor) at 4° C for 20 h (deceleration rate=0). Using a 10 ml pipette, most of the gradient and impurities were removed from the top of the tube while avoiding the lowest (bottom) bluish white band containing the viruses. The lowest (bottom) bluish white band containing the viruses was collected using syringe with 20G needle.

2.8.6 Virus Desalting and Concentration by Pharmacia NAP-25 Column

Cesium chloride was removed by gel filtration. The Pharmacia NAP-25 column (Amersham Pharmacia Biotech, Piscataway, NJ) was equilibrated the approximately 15 ml of the “Ad” buffer (10 mM Tris-HCl, pH 8.0; 135 mM NaCl; 3 mM KCl; 1 mM $MgCl_2$; 10 % (v/v) glycerol). The sample was mixed with “Ad” buffer for final volume 2,5 ml and put onto the column in a maximum volume of 2,5 ml. The buffer was allowed to enter the gel completely. The bad column volume (near 1,5 ml) was discard. Fractions containing adenovirus were collected. Additional 2 ml “Ad” buffer was added in column to completely elute adenovirus. The desalted virus was kept at -80°C for long-term storage. A small aliquot was kept separately to determine the stock titer with the remainder aliquoted in convenient volumes for use in upcoming experiments.

3 Results

3.1 Urokinase Stimulates Human Vascular Smooth Muscle Cell Migration via Phosphatidylinositol 3-Kinase —Tyk2 Interaction

uPA/uPAR system is known to be involved in tissue remodeling and wound healing by affecting cell migration, adhesion and proliferation (Ossowski and Aguirre-Ghiso, 2000), most likely via induction of intracellular signaling. One of the major uPA/uPAR-related signaling cascades in human VSMC is the Jak/STAT pathway, however, the molecular mechanisms underlying the interplay between Jak/STAT signaling and uPA-directed VSMC migration remains unexplored.

3.1.1 Jak1 and Tyk2 are Polarized with uPAR to the Leading Edge of Migrating VSMC

To explore the proposed interplay between the uPA/uPAR system and the Jak/STAT pathway upon cell migration, we used a migration wound assay. VSMC migration in one general direction following wounding was observed by 4 h after injury and was pronounced at 24 h (Fig. 10). In confluent VSMC, uPAR was distributed over the body of the cell in a punctate pattern, with some increased staining at the periphery. However, the staining was not restricted to a specific site on the cell membrane (Fig. 10). In response to injury, about 25% of migrating cells demonstrated a highly asymmetric distribution of uPAR to the leading edge (Fig. 10).

To obtain evidence that Jaks might be associated with uPAR within the same signaling complex upon cell migration, double immunolabeling experiments were performed using anti-uPAR mAb and pAb to individual kinases. Antibodies to Jak2 and Jak3 demonstrated a homogeneous punctate staining pattern (data not shown), with increased staining at the sites of cell-cell contacts. However, the staining was not restricted to the leading edge of the cells either in wounded and non-wounded culture. In contrast, strongly pronounced polarization of Jak1 and Tyk2 to the leading edge of migrating cells and colocalization with polarized uPAR could be seen already 6 h after wounding. Control antibodies showed no staining above background autofluorescence (data not shown).

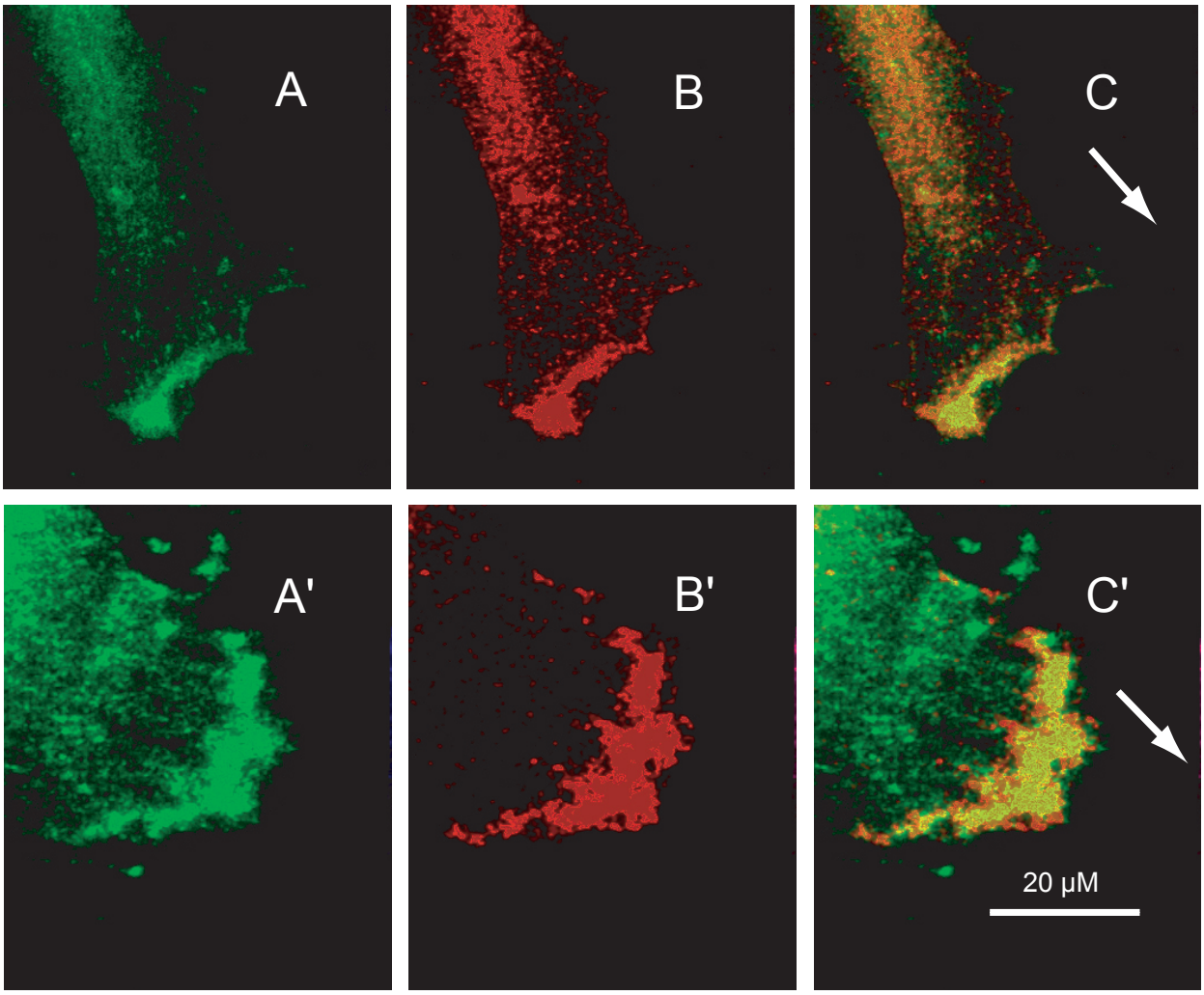

Fig. 10 Polarized distribution of uPAR, Jak1 and Tyk2 in the wounded VSMC cultures.

Wounded cultures with directionally migrating cells were stained 18 h after wounding with antibodies to the following proteins: uPAR (A,A') in green, Jak1 (B) in red, and Tyk2 (B') in red. In the third column (C,C') are superimposed composite figures of either Jak1 and uPAR or Tyk2 and uPAR from the first and second columns. Yellow color denotes the colocalization of two proteins. Bar 20 μm. In each panel, arrows indicate the direction of the wound.

3.1.2 Tyk2 and PI3-K are Associated

To examine the potential link between uPA/uPAR-directed Jaks and PI3-K, we first performed immunoprecipitation with anti-Jak antibodies and looked for the p85 subunit of PI3-K in the immunoprecipitates. Proteins that were coprecipitated with Jaks were subjected to in vitro kinase assay, and the phosphorylated proteins were reimmunoprecipitated with anti-p85 antibody. As a control for p85 recovery, both rounds of immunoprecipitation were performed with anti-p85 antibody. As shown in (Fig. 11), upper panel, one band corresponding to p85 was detected in Tyk2, but not in Jak1, Jak2, or Jak3

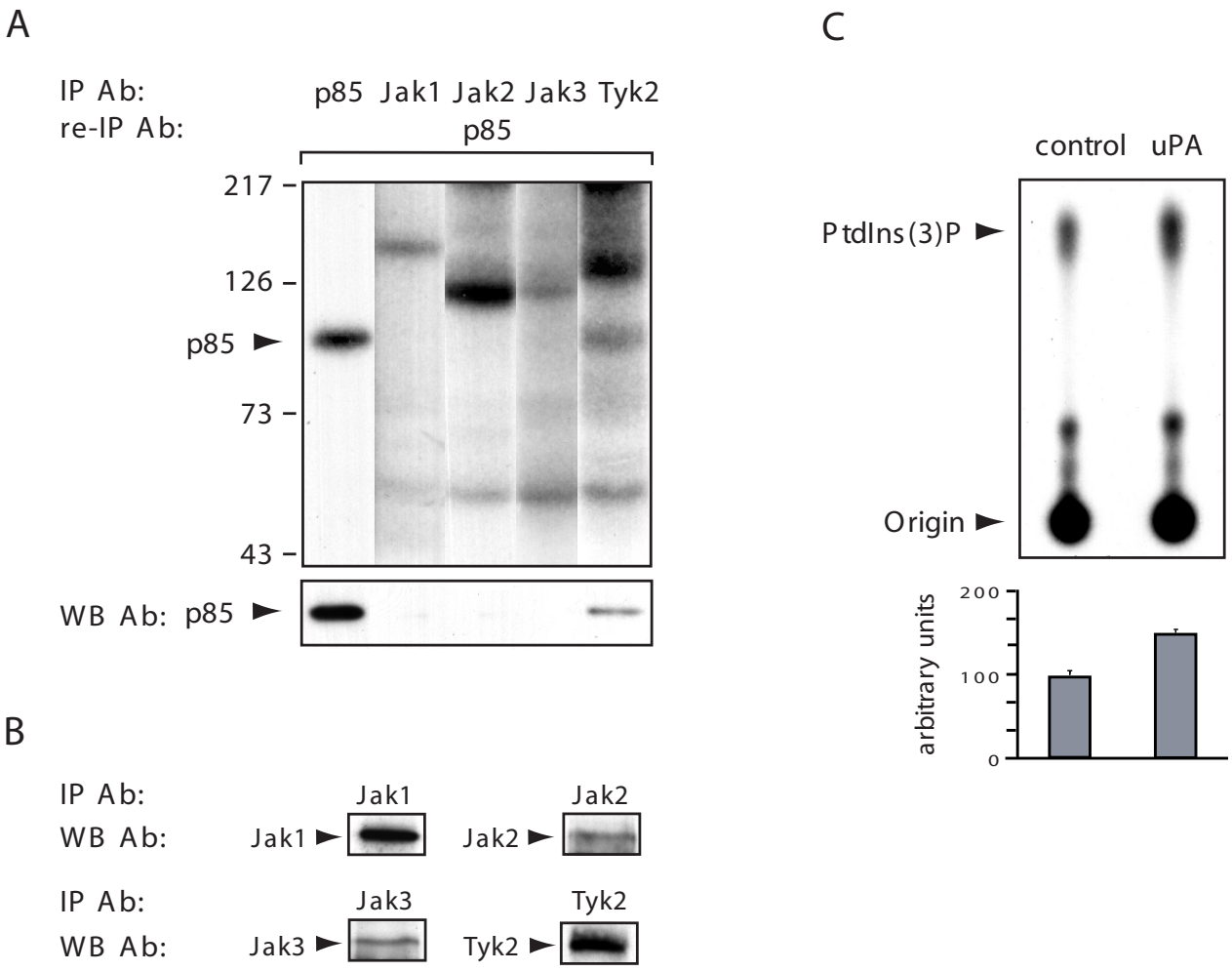

Fig.11 Tyk2 and PI3-K are associated in VSMC.

A, proteins co-precipitated (IP) with antibodies (Ab) to Jak1, Jak2, Jak3, or Tyk2 were subjected to in vitro kinase assay and reimmunoprecipitated (re-IP) with anti-p85 antibody (upper panel). The left lane shows the results of the in vitro kinase assay combined with two rounds of immunoprecipitation with anti-p85 antibody as control for p85 recovery. Phosphorylated proteins were subjected to SDS-PAGE and autoradiography. The identity of the immunoprecipitated p85 band was confirmed by immunoblotting with anti-p85 antibody (lower panel). B, Janus kinases were immunoprecipitated from VSMC lysates under the same conditions as used in A and were identified by immunoblotting as indicated. C, PI3-K activity was measured in Tyk2 immunoprecipitates of unstimulated (control) or uPA-stimulated (uPA) VSMC (upper panel). Products of PI3-K were separated by thin layer chromatography and visualized by autoradiography. The lower panel shows the results of densitometric quantification. WB, Western blot.

immunoprecipitates. The identity of the immunoprecipitated p85 band was confirmed by immunoblotting with anti-p85 antibody (Fig. 11, lower panel). To verify that antibodies to Jak1, Jak2, and Jak3 were behaving appropriately under our experimental conditions, the additional positive controls were performed, confirming that all three kinases might be precipitated from the VSMC lysates (Fig. 11 B). These data demonstrate that p85 can directly and constitutively associate with Tyk2 in VSMC.

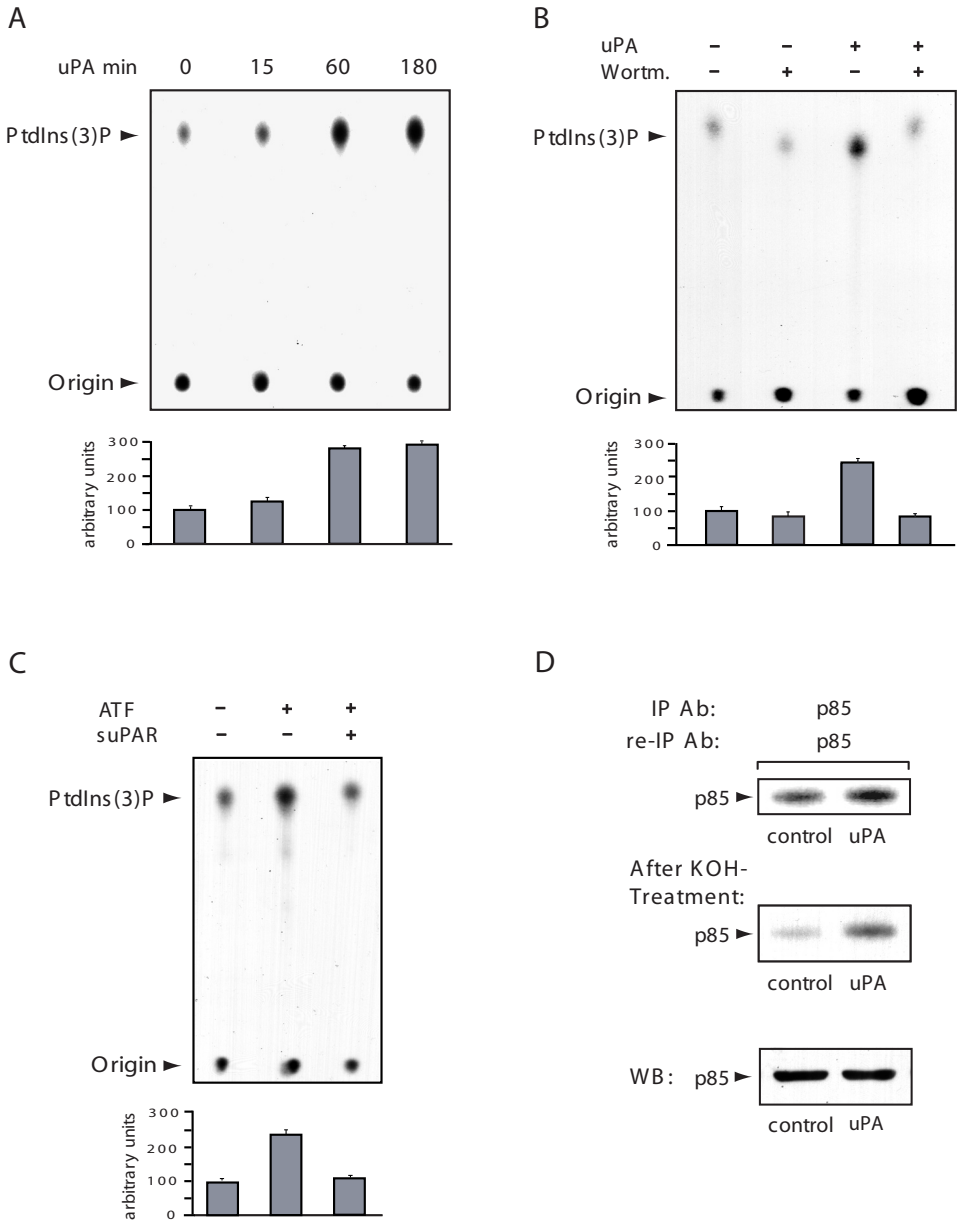

Fig. 12 uPA induces PI3-K activation and tyrosine phosphorylation of p85 in VSMC.

A, VSMC were treated with 1 nM uPA for the indicated times at 37 °C, and the PI3-K assay was performed with subsequent thin layer chromatography and autoradiography visualizing phosphatidylinositol 1,4,5-trisphosphate (PtdIns(3)P) products. B, cells were untreated or treated with uPA for 30 min and/or with wortmannin, as indicated. C, VSMC were stimulated with 1 nM ATF for 45 min in the presence or absence of 20 nM soluble recombinant uPAR. Quantification of the results by densitometry is shown below each panel. D, phosphorylation of p85 was assayed in control VSMC and after cell activation with 1 nM uPA by in vitro kinase assay combined with two rounds of immunoprecipitation (IP and re-IP) with anti-p85 polyclonal antibody (Ab). Phosphorylated proteins were subjected to SDS-PAGE and autoradiography before (upper panel) and after (middle panel) KOH treatment. The equal loading of precipitated proteins on the gel was confirmed by immunoblotting (lower panel).

3.1.3 uPA Induces PI3-K Activation and Tyrosine Phosphorylation of p85

To address the potential role of PI3-K in the uPA-mediated signaling, we assayed PI3-K activity directly by measuring the levels of the product, phosphatidylinositol phosphate, in serum-starved VSMC stimulated with uPA at different time points. As shown in (Fig. 12) uPA induced a significant increase (up to 3-fold) in PI3-K activity in a time-dependent manner with a sustained PI3-K activation observed after 3 h. The PI3-K-specific inhibitor wortmannin, which binds covalently to the catalytic p110 subunit of PI3-K and inhibits PI3-K irreversibly, at nanomolar concentrations completely blocked the uPA-induced stimulation of enzyme activity (Fig. 12). To assess a potential contribution of uPA's proteolytic activity to the mechanisms of PI3-K activation, the amino-terminal fragment of uPA, ATF, was used for cell stimulation. ATF provided the same effect (Fig. 12), confirming the involvement of proteolytically inactive uPA in signaling events. We also examined the requirement for uPAR upon PI3-K activation. For this purpose, we used soluble recombinant uPAR, which is a known competitor for the uPA- and ATF-uPAR binding. The data presented in Fig. 12 clearly demonstrate that the pretreatment of VSMC with soluble recombinant uPAR significantly decreased the uPA/ATF-induced PI3-K stimulation (shown for ATF).

We next examined PI3-K p85 subunit phosphorylation after uPA stimulation using an in vitro kinase assay combined with two rounds of immunoprecipitation with anti-p85 antibody (Fig.12, upper panel). The results demonstrate that uPA markedly increased phosphorylation of the p85 protein. The band was resistant to alkaline hydrolysis, indicating tyrosine phosphorylation (Fig.12, middle panel). The equal loading of precipitated material on the gel was confirmed by immunoblotting (Fig. 12, lower panel).

3.2.1 Overexpression of Wild Type Tyk2 and Mutant Forms Tyk2 in VSMC

To further analyse the revealed interaction between Tyk2 and PI3-K, we overexpressed wild type Tyk2 in VSMC. The overexpression enabled us to obtain cell lysates enriched in Tyk2, which is otherwise expressed at a low level in native VSMC. Primary cultured smooth muscle cells from different origins display a strongly toxic reaction in response to transfection by commercially availably transfection reagents (such as FuGene, Lipofectamine) (Nabel *et al.*, 1991; Keogh *et al.*, 1997). To overcome these difficulties, we generate adenovirus constructs that can be effectively used for infection of VSMC and overexpression in these cells of wild and mutant forms of Tyk2.

To obtain full-length cDNA copy for Tyk2, a long PCR protocol was used. For am-

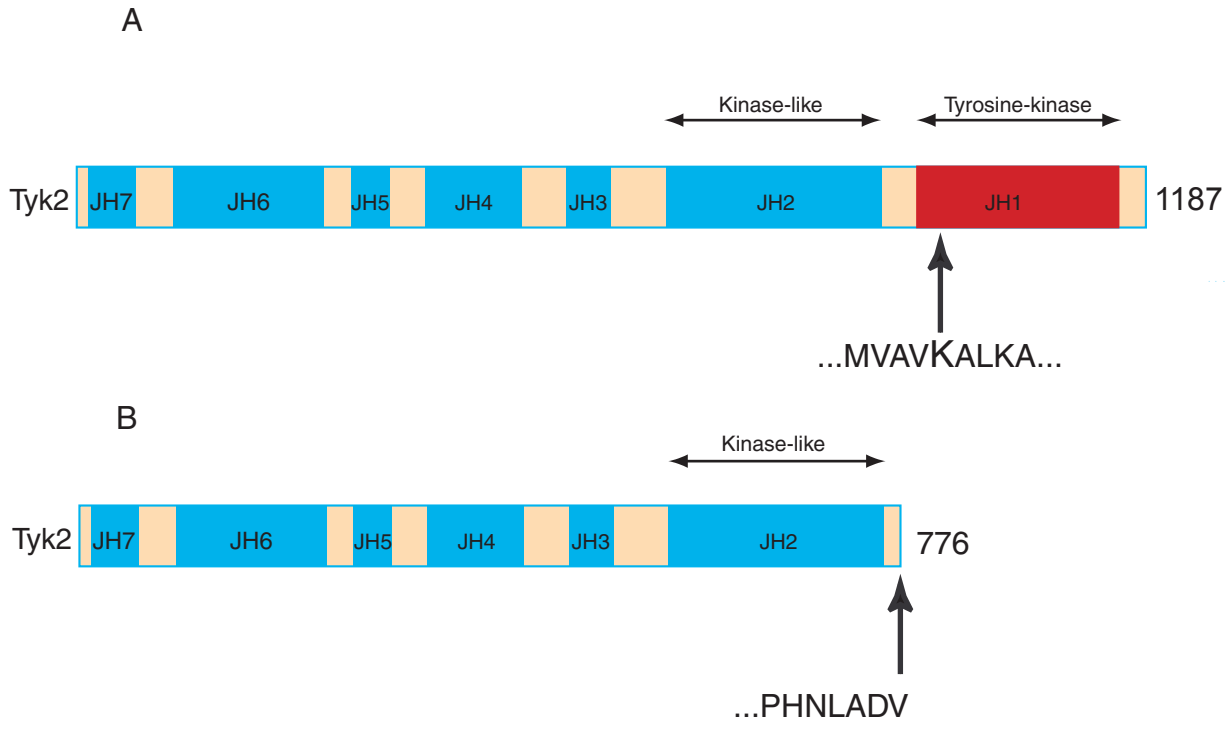

Fig. 13 Structures of mutant forms of the Janus kinase Tyk2.

A, Point mutation in kinase domain of the Tyk2-kinase deficient form. B, Deletion in carboxyl terminus completely abrogates kinase activity of Tyk2.

plification, cDNA samples were prepared by reverse transcription reaction on mRNA isolated from primary culture of human VSMC. After amplification, cDNA was subjected to electrophoresis in 1% agarose gel, recovered from gel, cloned in pBluescriptII SK+ and sequenced. The results confirmed structure for full length Tyk2. Mutant form of Tyk2 (Ad5Tyk2KE) was obtained by site-directed mutagenesis in the catalytic active center of the kinase domain Lysine 930 was replaced by glutamine (AAG->GAG) (Fig. 13) .

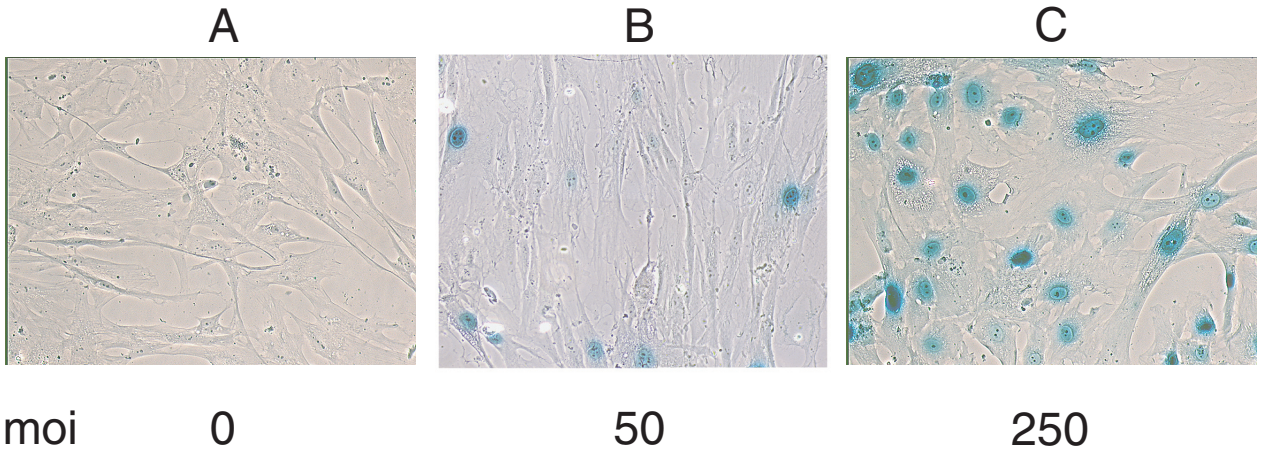

Fig. 14 Adenoviral-mediated overexpression of β-lactosidase enzyme in VSMC.

The cells were infected with replication-defective adenovirus expressing β-galactosidase at a multiplicity of infection of 0 (panel A), 50 (panel B) and 250 (panel C) viral particles/cell (moi). More than 95% of the cells expressed the transgene after 48 h. Cells were photographed under phase contrast microscopy in a light field.

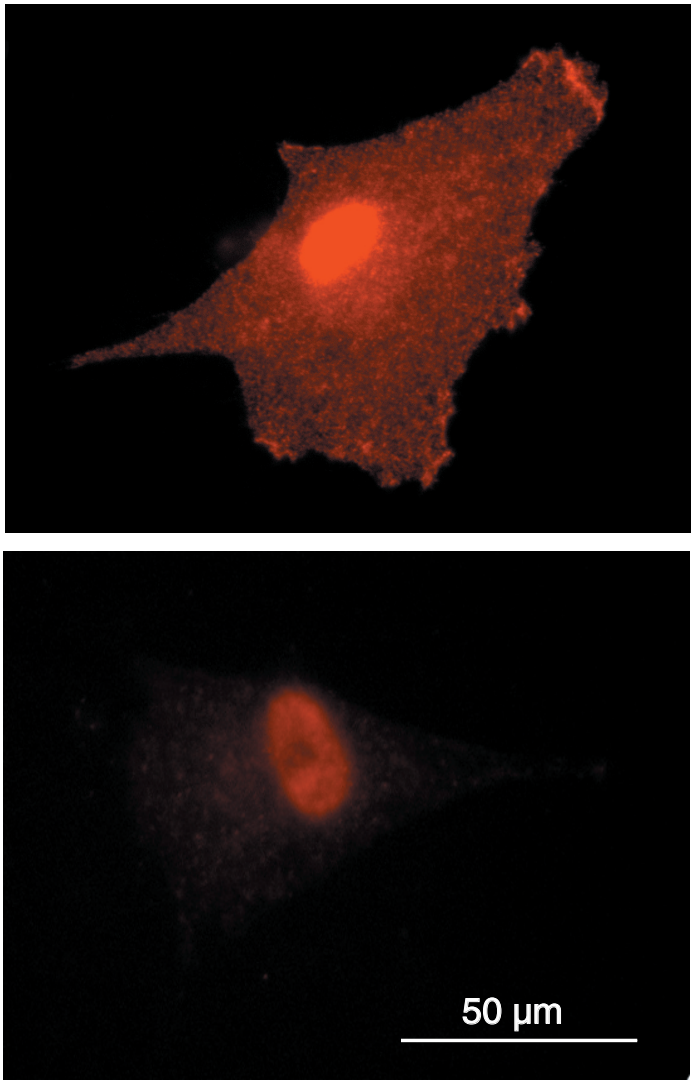

Fig. 15 Overexpression of Tyk2 in VSMC.
VSMC were infected with Ad5Tyk2, and immunofluorescent staining for Tyk2 was performed in noninfected cells (lower image) and Ad5Tyk2-VSMC (upper image) using anti-Tyk2 monoclonal antibody (Ab) and corresponding Cy3-conjugated secondary antibody.

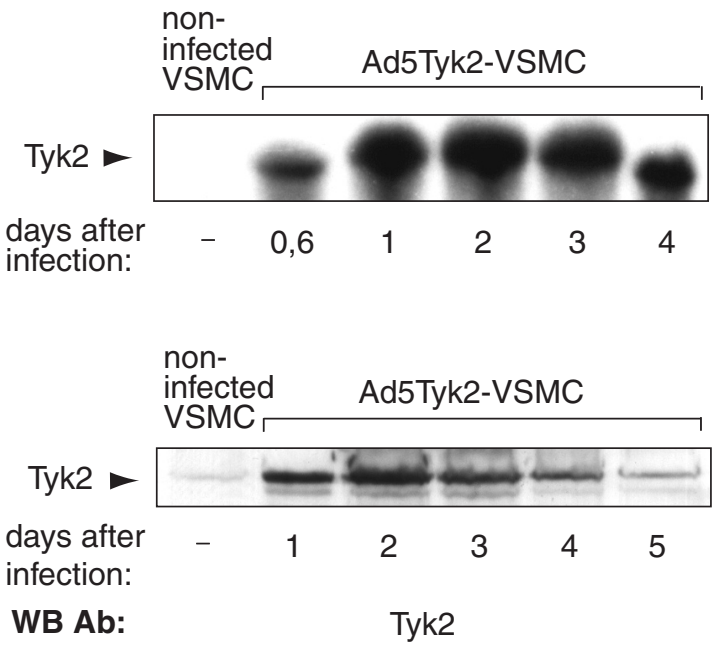

Fig. 16 Time-dependent expression of Tyk2 in adenovirus-infected VSMC.
Northern (upper panel) and Western (lower panel) blot (WE) time course analysis of Tyk2 mRNA and protein expression in adenovirus-infected VSMC.

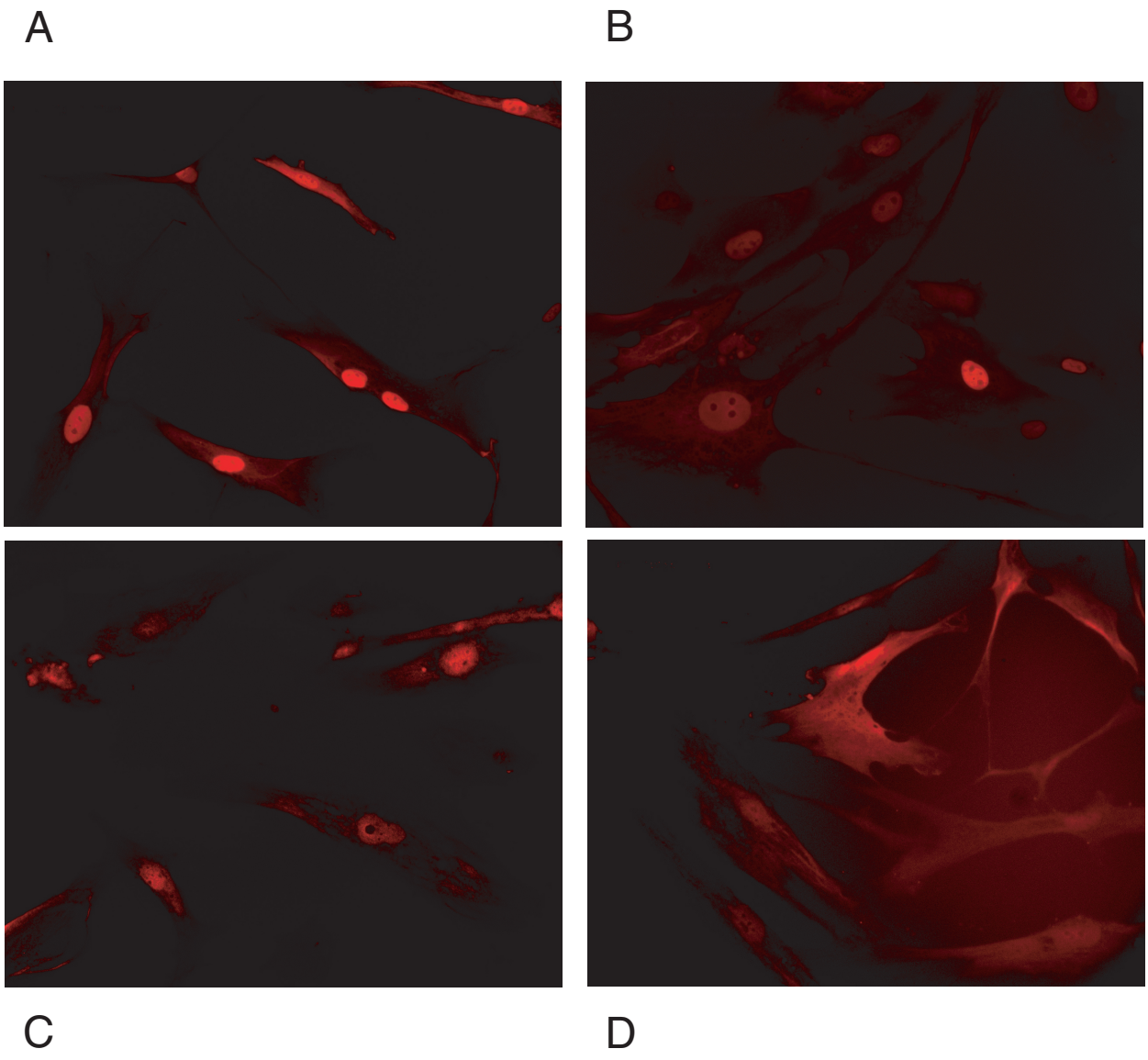

Fig. 17 Functional validation of Tyk2 constructs.

A-C: VSMC were stimulated with interferon-α (IFNα) for 30 min, fixed and stained with anti-STAT1 monoclonal antibody and corresponding Cy3-conjugated secondary antibody.

A, non-infected cells; B, AdTyk2KE-infected cells; C, AdTyk2ΔC-infected cells; D, non-stimulated cells.

Mutation of this site has been shown to abrogate the kinase activity of Tyk2 (Colamonici *et al.*, 1994). One more kinase-deficient construct (Tyk2ΔC) was generated by removing kinase domain after 776 aa Fig. 13. As a control virus, adenovirus-expressed β-galactosidase (AdβGal) was used. This virus was used to establish and optimize infection protocol (Fig. 14). The optimal infection was found to correspond 250 pfu/cell and was correspondingly used in our experiments.

Adenoviruses for expression of wild and mutant forms Tyk2 were generated as described in method section. After purification of recombinant adenoviruses, their activity was determined 1-3*10^{11} pfu/ml by titration assay.

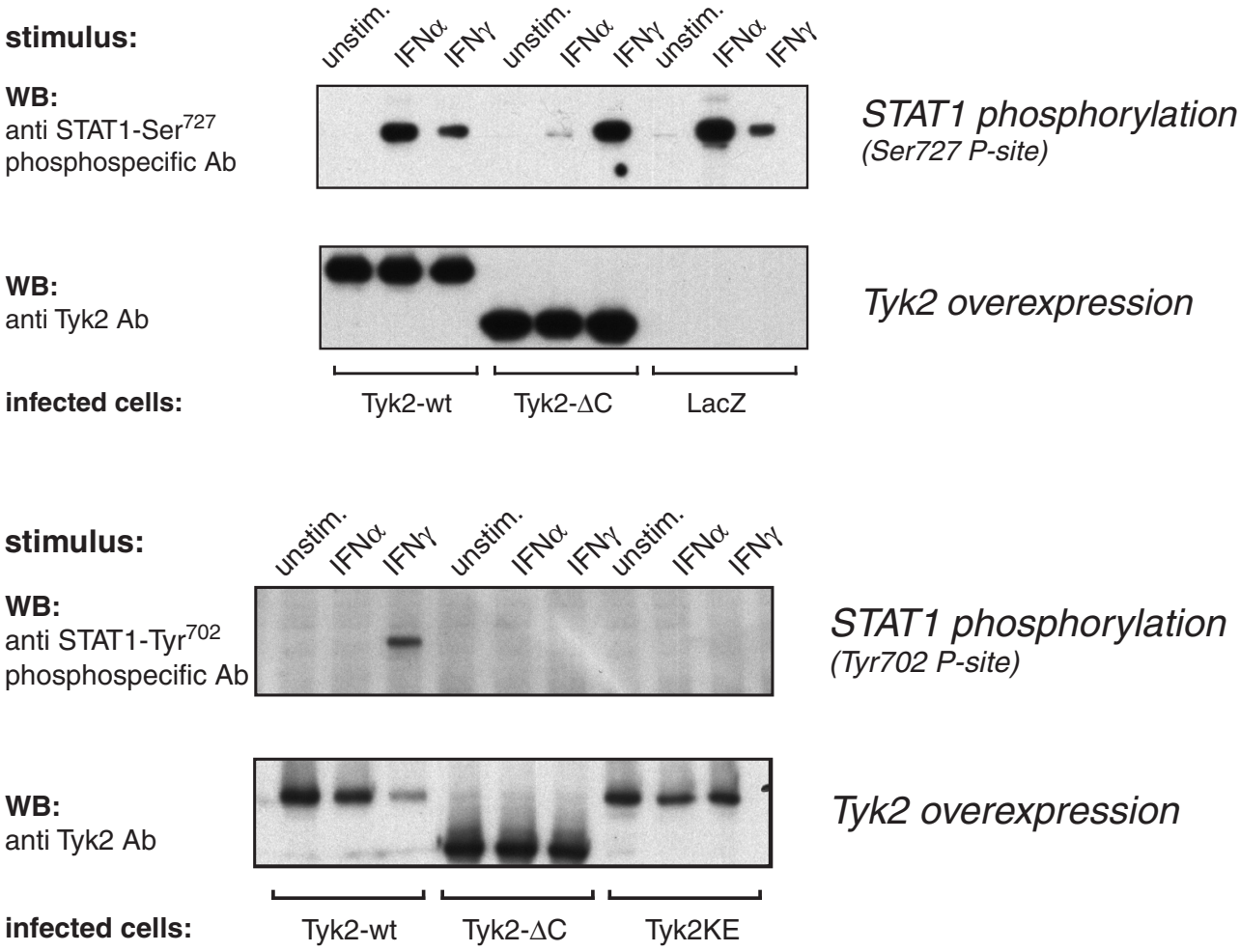

Fig. 18 Overexpression of Tyk2 dominant-negative forms inhibits STAT1 phosphorylation.

Protein extracts from VSMC were analyzed by Western blot to detect expression and phosphorylation of STAT1 using anti-STAT1, anti- phosphoserine STAT1 (Ser 727 P site) and anti- phosphotyrosine (Tyr 702 P site) STAT1 monoclonal antibodies, respectively. Cells were also infected with a control virus β-LacZ. STAT1 phosphorylation levels were evaluated by Western blot analysis. The cells infected with a control virus, wild type Tyk2 expressed adenovirus (AdTyk2wt), defected in kinase domain Tyk2 adenovirus (AdTyk2KE) and kinase domain truncated form Tyk2 (AdTyk2ΔC) were stimulated with or without 1 ng/ml interferon-α (INFα) or interferon-γ (INFγ) for 30 min.

Immunocytochemical study of control uninfected cells and Ad5Tyk2-VSMC using anti-Tyk2 antibody confirmed the efficiency of cell infection with the recombinant adenovirus under these conditions Fig. 15 . Northern and Western blot analysis were further used to confirm the expression of Tyk2 mRNA and protein in VSMC infected with adenovirus and

cultured for different time periods. As shown in Fig.16, VSMC infection with Ad5Tyk2 resulted in a dramatic increase in Tyk2 mRNA and protein levels relative to uninfected cells. The Tyk2 protein expression reached a maximum 2 days after infection and decreased within the next 3 days. In further experiments, we therefore used VSMC cultured for 2 days after infection with Ad5Tyk2.

3.2.2 Overexpression of Mutant Forms Tyk2 Leads to Inhibition of Endogenous Tyk2 Kinase Activity

Up to date there is no convenient direct method to measure the kinase activities of Jaks. Therefore an indirect method was used, namely phosphorylation of STAT proteins that serve as substrates for Jaks. VSMC were infected with control virus or with virus constructs of Tyk2 mutant forms (Ad5Tyk2KE; Ad5Tyk2ΔC). IFN-γ was used as a strong positive control for the Jak/STAT activation (Shuai *et al.*, 1993) Cells were treated with 1000 U/ml IFN-α or IFN-γ at 30 min or left untreated as indicated and used for immunoblotting with anti-STAT1 phosphotyrosine 701 or anti-STAT1 phosphoserine 727 antibodies. To control Tyk2 expression, reprobing with anti-Tyk antibody was used. As shown in Fig. 18, overexpression of truncated forms of Tyk2 reduced IFN-γ - induced STAT1 phosphorylation on Ser727 and Tyr701.

In order to confirm dominant negative properties of mutant form, VSMC expressing transgenes were activated by IFN-γ. In response to IFN-γ, activated and phosphorylated STAT1 normally undergoes translocation to cell nucleus (Fig.17, control VSMC). In contrast, STAT1 nuclear translocation was blocked in VSMC overexpressing mutant forms of Tyk2 (Fig. 17) These data confirm the dominant negative expression of Ad5Tyk2KE and Ad5Tyk2ΔC leading to inhibition of endogenous Tyk2 kinase activity (Fig. 18).

3.2.3 Tyk2 and PI3-K Directly Interact via SH2 Domains of PI3-K

To investigate the interaction mechanisms between Tyk2 and p85, in vitro binding experiments were performed. We used two GST fusion proteins containing the single C- and N-terminal Src homology 2 (SH2) domains of the human p85 subunit of PI3-K (GST-p85-C-SH2 and GST-p85-N-SH2) immobilized on the glutathione-agarose beads. The beads were incubated with the lysates of unstimulated and uPA-stimulated Ad5Tyk2-VSMC, and the precipitated proteins were analyzed by immunoblotting with anti-Tyk2 antibody. As shown in Fig. 19, one major band corresponding to Tyk2 was detected in both GST-p85-C-SH2 and GST-p85-N-SH2 precipitates from uPA-stimu-lated cells. Tyk2 bands identified in unstimulated cells were significantly weaker. These results strongly suggest that Tyk2 directly associates with PI3-K through the binding to both C-and N-terminal SH2 domains of p85 and that this association is uPA-inducible.

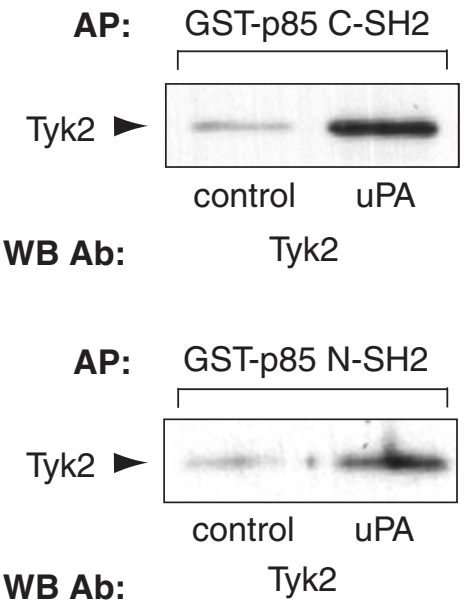

Fig.19 Tyk2 and PI3-K regulatory subunit are directly associated.

Cell lysates from uPA-stimulated and control Ad5Tyk2-VSMC were subjected to affinity precipitation (AP) with GST-p85-C-SH2 (upper panel) or GST-p85-N-SH2 (lower panel). Precipitated proteins were separated by 7.5% SDS-PAGE and analyzed by immunoblotting with anti-Tyk2 monoclonal antibody.

3.2.4 PI3-K and Tyk2 Are Required for the uPA-induced VSMC Migration

Recent reports imply that PI3-K is involved in cell migration control (Adam *et al.*, 1998; Reiske *et al.*, 1999; Vanhaesebroeck and Waterfield, 1999). Although uPA is known to regulate cell migration, the underlying molecular mechanisms are unclear. Therefore, we next examined the possible impact of PI3-K on VSMC migration in response to uPA. For this purpose, two main approaches were used, namely directional VSMC migration in Boyden chambers and cell movement in a wounded VSMC monolayer monitored by time-lapse imaging. Fig. 20 A displays the data on directional migration of VSMC along the uPA gradient tested in a microchemotaxis Boyden chamber. After 4 h, cell migration in response to uPA was significantly enhanced about 3-fold, compared with migration in the presence of medium alone. To examine the requirement of PI3-K for uPA-promoted cell migration, VSMC were subjected to migration assays in the presence of two unrelated structurally distinct specific PI3-K inhibitors, wortmannin and LY294002. Cell treatment with both inhibitors decreased uPA-related cell migration with no statistically significant effect on the basal migration (Fig. 20).

To further characterize VSMC migration, an in vitro injury assay was performed. This cell migration model was used to assess the potential effects of constant uPA doses and to analyze the role of PI3-K in this process. The cell migration rate was calculated from the cell number migrating into the wounded area within 9 h after wounding. In these ex-

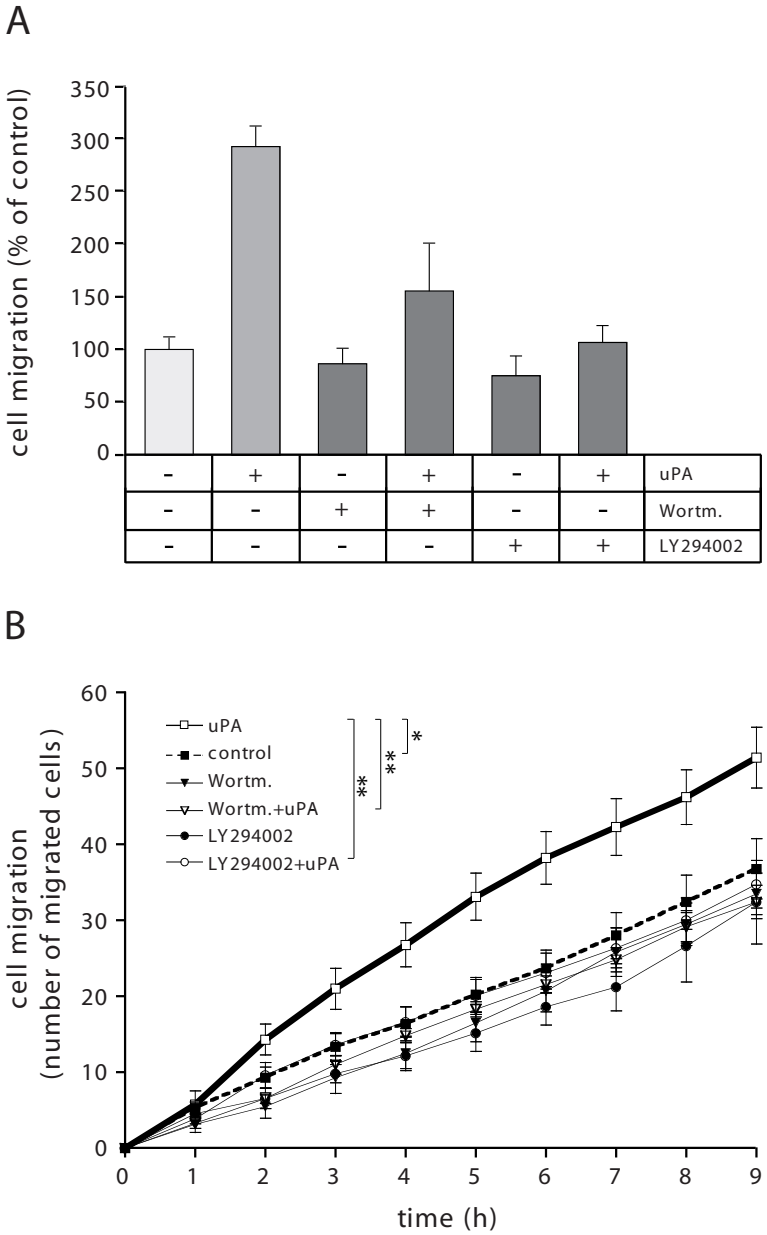

Fig. 20 PI3-K is required for the VSMC migration in response to uPA.

A, VSMC were serum-starved for 24 h and then subjected to chemotaxis assay using a modified Boyden chamber. Cells were resuspended in serum-free medium alone or in serum-free medium containing 200 nM wortmannin or 50 mM LY294002, as indicated, and were then left to migrate toward serum-free medium alone or 100 nM uPA. Random cell migration toward medium alone without any chemoattractant was considered as 100% migration.

B, VSMC were wounded, and cell migration was evaluated using time lapse videomicroscopy for non-treated cells, after pretreatment of cells with 200 nM wortmannin or 50 mM LY294002 and in the presence of 50 nM uPA as indicated. Results of cell migration are shown as numbers of migrated cells at the indicated time points ± S.D. of n= 10 (control), n = 9 (uPA), n=6 (wortmannin (wortm.)), n= 7 (wortmannin + uPA), n= 5 (LY294002), and n= 8 (LY294002 + uPA). *,$p < 0.02$ uPA versus the control; **,$p < 0.006$ uPA versus wortmannin + uPA, uPA versus LY294002 + uPA (unpaired t test).

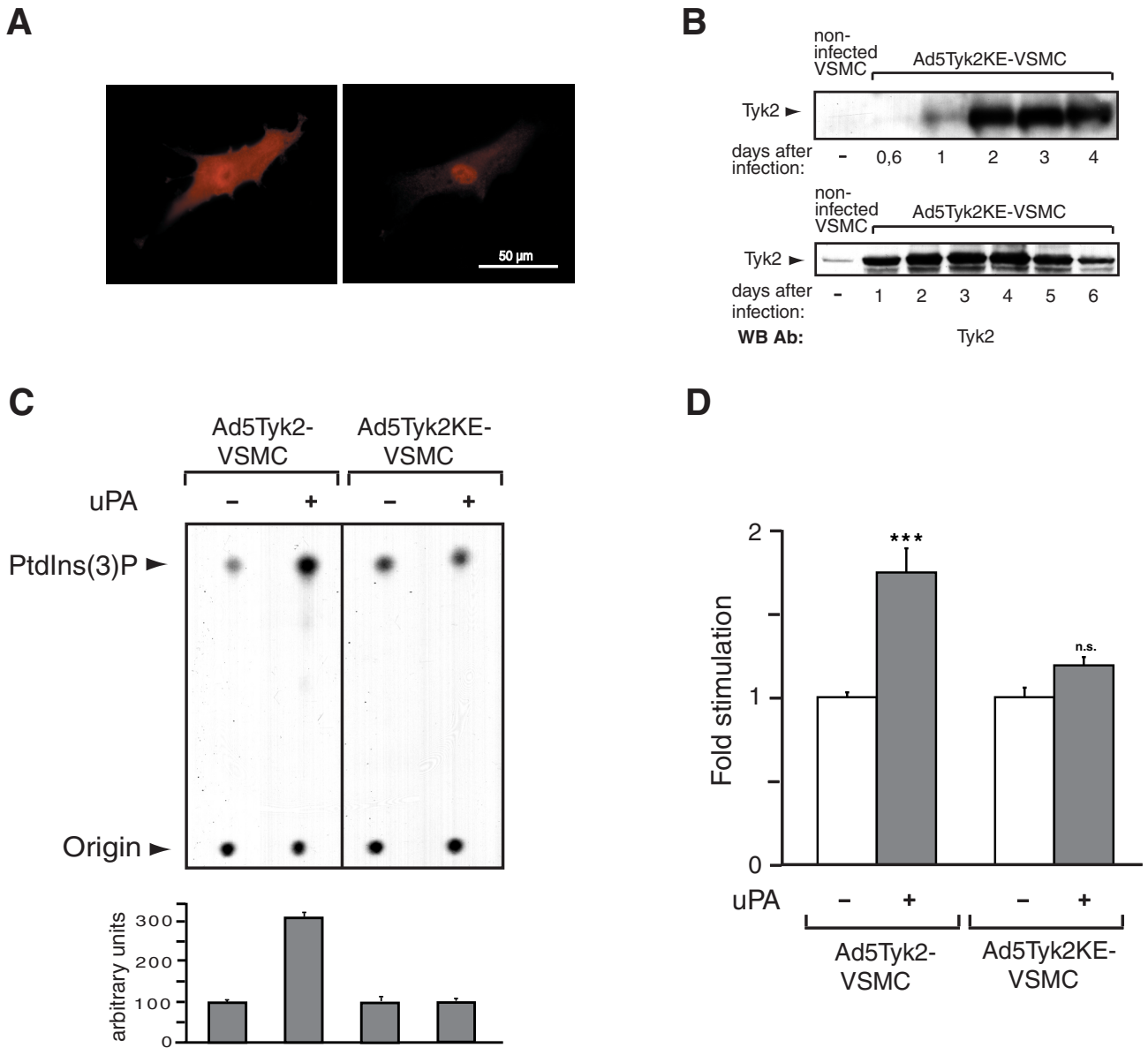

Fig. 21 Tyk2 is required for the uPA-induced PI3-K activation and VSMC migration.

A, VSMC were infected with Ad5Tyk2KE, and immunofluorescent staining for Tyk2 was performed in noninfected cells (right image) and Ad5Tyk2KE-VSMC (left image). B, Northern (upper panel) and Western (lower panel) blot (WE) time course analysis of Tyk2KE mRNA and protein expression in adenovirus-infected VSMC. C, VSMC expressing Tyk2 wild type or mutant form were stimulated with 1 nM uPA for 45 min, and the PI3-K assay was performed with subsequent thin layer chromatography and autoradiography of phosphatidylinositol 1,4,5-trisphosphate (PtdIns(3)P) products (upper panel) and their quantification by densitometry (lower panel). D, VSMC expressing Tyk2 wild type or mutant form were wounded, and cell migration was evaluated using time lapse videomicroscopy as indicated above. Results of cell migration are shown as -fold stimulation ± S.D. of n= 5 (Ad5Tyk2-VSMC, control), n = 6 (Ad5Tyk2-VSMC, uPA), n= 7 (Ad5Tyk2KE-VSMC, control), or n = 5 (Ad5Tyk2KE-VSMC, uPA). n.s., uPA nonsignificant versus the control; ***,p < 0,0003 uPA versus control.

periments, VSMC migration significantly increased in the presence of uPA compared with migration with medium alone as early as 2 h after wounding (Fig. 20 B). Furthermore, VSMC treatment with either wortmannin or LY294002 completely abolished the effects of uPA. Thus, regulation of cell migration by uPA is PI3-K-dependent.

The next series of experiments were performed to determine whether Tyk2 is required for PI3-K-mediated VSMC migration in response to uPA. VSMC were adenovirally infected to express the dominant negative form of Tyk2 devoid of kinase activity, as described above. The efficiency of cell infection was confirmed by immunostaining, Northern and Western blotting (Fig. 21, A and B). As shown in Fig. C, uPA-induced PI3-K activation was blocked in Ad5Tyk2KE-expressing cells, whereas in wild type Ad5Tyk2-expressing VSMC, PI3-K activity increased after uPA stimulation. These results demonstrate that Tyk2 is required for the uPA-related PI3-K activation and that Tyk2 functions presumably upstream of PI3-K in VSMC.

To elucidate whether Ad5Tyk2KE mutant also inhibits PI3-K-mediated VSMC migration in response to uPA, cell migration assays were performed using the wounding model, as described above. As shown in Fig. 21 D , VSMC expressing wild type Tyk2 demonstrated an approximately 2-fold increase in cellular migration in the presence of uPA. By contrast, uPA did not affect cell migration in Ad5Tyk2KE-expressing VSMC.

3.2.5 Association of Tyk2 and PI3-K Is Essential for uPA-related Cytoskeletal Reorganization in Migrating VSMC

Cell motility is generally associated with polarization of initially unpolarized cells. An active leading edge facing the cell-free area is formed. In addition, focal contacts, stress fibers, and actin filaments are rearranged (Lauffenburger and Horwitz, 1996). To examine the cytoskeletal reorganization resulting from uPA-induced activation of Tyk2 and PI3-K in migrating VSMC in the wounding model, we again performed time-lapse video microscopy and immunofluorescent staining with Alexa 488-conjugated phalloidin. As reported by others (Degryse *et al.*, 1999) and as shown in Fig. 22, VSMC migrating in the presence of uPA developed a polarized morphology, with extended lamellipodia extrusions at the leading edge facing the open space (Fig. 22). Formation of actin-rich lamellipodial extensions at the leading edge and abundant stress fibers in response to uPA was observed also in VSMC expressing wild type Tyk2 (Fig. 22). The dynamic cytoskeletal activity of both cell types migrating without uPA was significantly weaker (Fig. 22 control). By contrast, lamellipodial activity was completely different in VSMC expressing a Tyk2 mutant form. Ad5Tyk2KE-VSMC did not display a strong morphological polarity with clear leading edge and revealed in the presence of uPA mainly small lateral protrusions localized at the sides and not at the leading edge of migrating cells (Fig. 22).

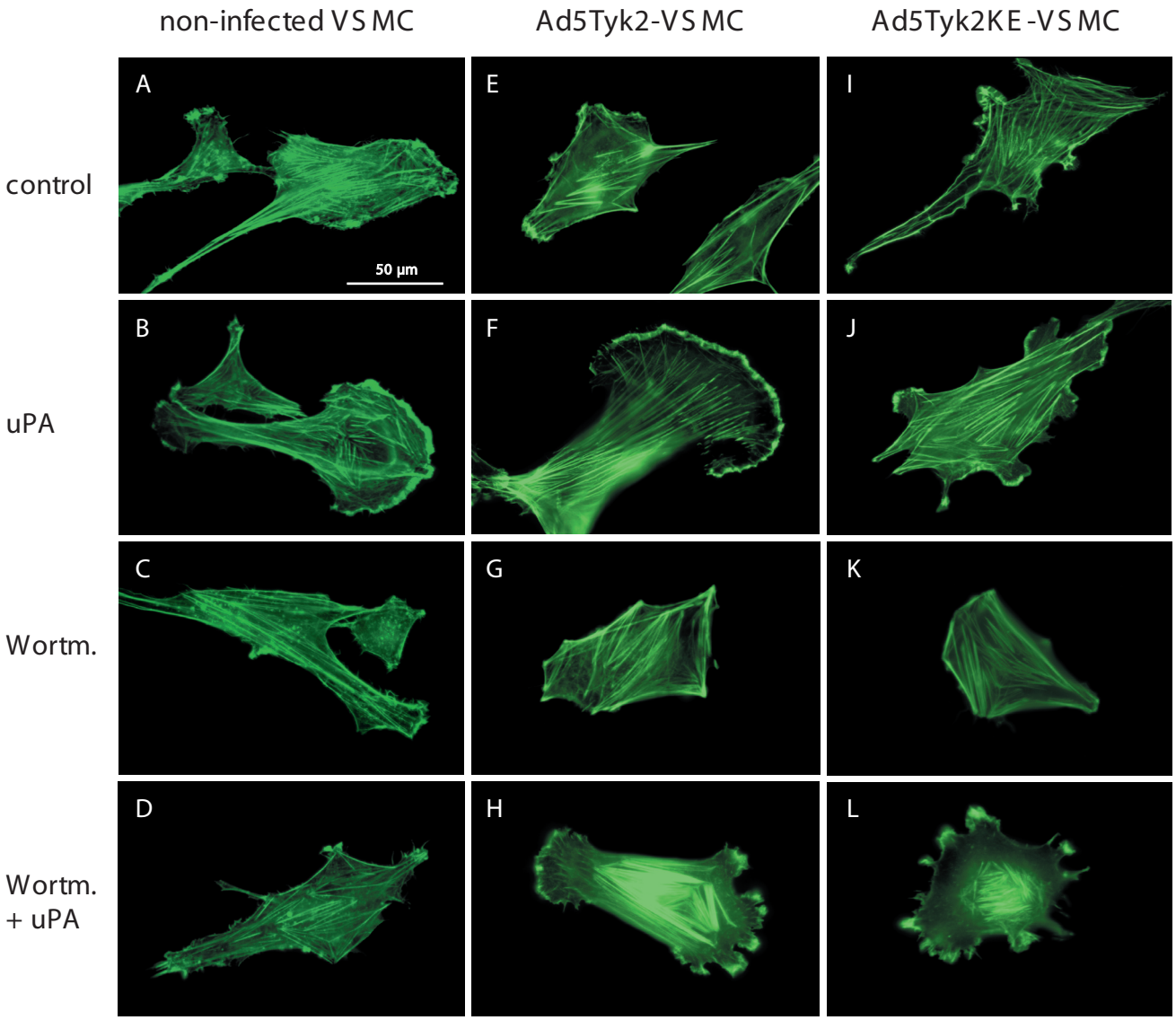

Fig.22 Tyk2 and PI3-K are required for the uPA-induced cytoskeletal reorganization in migrating VSMC. Noninfected VSMC (left column) or VSMC expressing Tyk2 wild type (middle column) or negative form (right column) migrated within 20 h after wounding were left unstimulated or were stimulated with 50 nM uPA without or with pretreatment with 100 nM wortmannin (wortm.) as indicated. The cells were then fixed and immunofluorescently stained with Alexa 488-conjugated phalloidin. Results are representative of 3—5 separate experiments.

To examine the contribution of PI3-K to the observed cytoskeletal rearrangements, cells were treated with PI3-K inhibitors. VSMC treatment with wortmannin caused a complete loss of uPA-induced polarity Fig. 22. In Ad5Tyk2-VSMC treated with wortmannin, cell polarity was also disturbed; the protrusive leading edge, clearly developed in the presence of uPA and absence of wortmannin, was damaged Fig. 22. These cells came to be similar to Ad5Tyk2KE-VSMC, whose uPA-induced lamellipodial activity can be seen all around the cells' periphery (Fig. 22). Moreover, wortmannin treatment of VSMC expressing wild type and the mutant form of Tyk2 resulted in the reorganization of stress fibers.

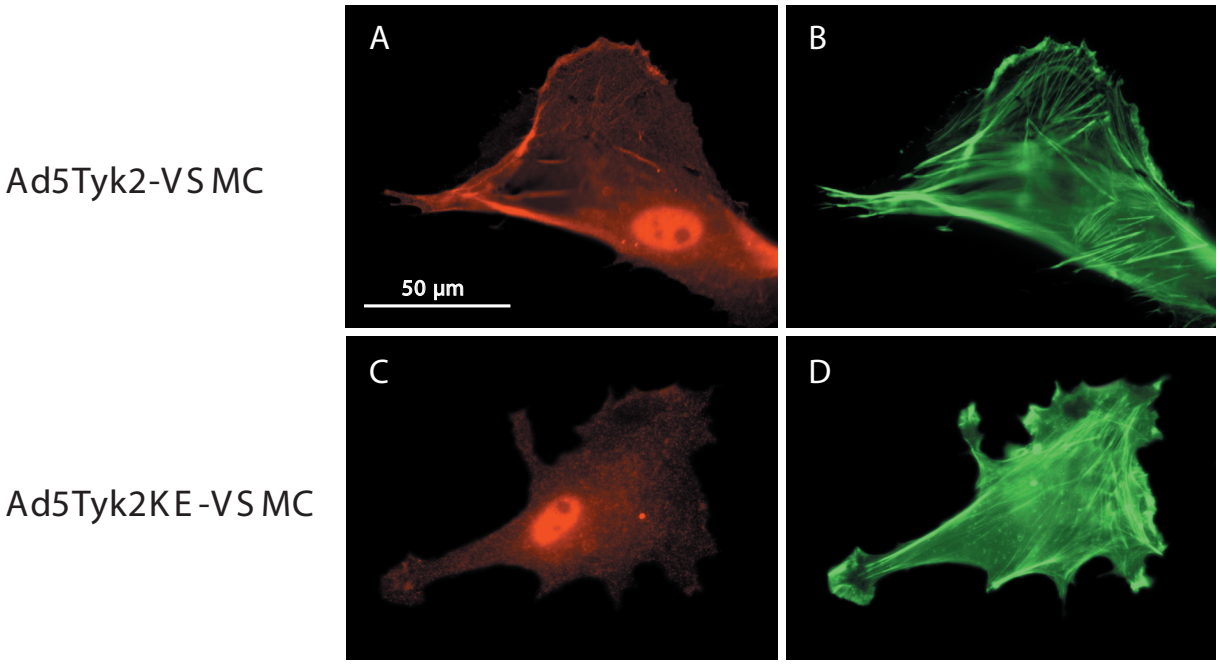

Fig. 23 Redistribution of Tyk2 in migrating VSMC.

Redistribution of Tyk2 is essential for the leading edge formation in migrating VSMC. VSMC expressing Tyk2 wild type (A, B) or negative form (C, D) migrated within 20 h after wounding and were left unstimulated or were stimulated with 50 nM uPA. The cells were then fixed and stained with anti-Tyk2 monoclonal antibody and corresponding Cy3-conjugated secondary antibody (A, C) or with Alexa 488-conjugated phalloidin (B, D).

Interestingly, these effects were especially pronounced in the presence of uPA. As can be seen in Fig. 22, uPA stimulation of wortmannin-pretreated VSMC resulted in disassembly of stress fibers, which lost organization into typical long bundles of parallel or radially directed stress fibers.

Our findings demonstrate that in migrating VSMC Tyk2 is polarized to the leading edge (Fig. 23). The strong polarity in Tyk2 intracellular redistribution was also observed in migrating VSMC expressing wild type Tyk2 (Fig. 23 A,B). By contrast, in VSMC expressing the Tyk2 mutant form (C,D), Tyk2 was randomly distributed within the cell that might explain the loss of cytoskeletal polarity of these cells (Fig. 23).

3.3 VSMC Migration is Stimulated by Endogenous, Monocyte-Expressed uPA in Coculture Model

Although uPA is well-proved migratory signal for different cell types, its most biological activities have been studied in the above documented experiments and in the studies of others using exogenous recombinant or purified protein or peptides (Fazioli *et al.*, 1997; Mukhina *et al.*, 2000; Ossowski and Aguirre-Ghiso, 2000). The aim of this part of the study was to get evidence that VSMC migration might be regulated by endogenous uPA. We

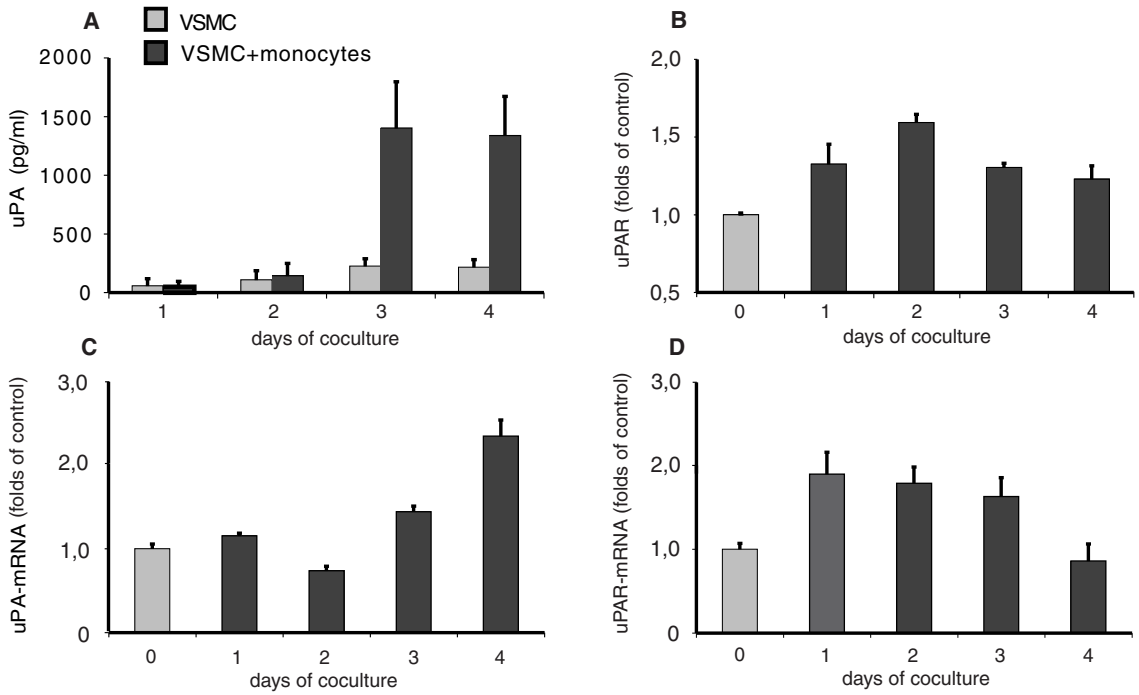

Fig. 24 uPA and uPAR expression in cocultured monocytes and VSMC.

A, Enzyme immunoassay (ELISA) for quantitative measurement of human uPA in mono- and coculture cell-free fractions.

B, Single cell-associated fluorescence for uPAR (CD 87) was quantified by flow cytometry with a FACScan flow cytometers to surface receptors on VSMC was determined on freshly detached, unfixed cells. Results are shown as folds of mean fluorescence of respectively monocultured cells (control).

C, D, RT-PCR analysis for uPA (C) and uPAR (D) mRNAs in monocytes and VSMC in coculture was performed using the TaqMan® method.

addressed a coculture model of VSMC with peripheral blood-derived monocytes, as far as uPA expressed by monocytes is a potent chemotactic factor for VSMC and might serve for the acceleration of vascular remodeling.

3.3.1 uPA/uPAR Expression in Coculture Model

VSMC were grown in a filter separated coculture with freshly isolated peripheral blood-derived monocytes for 1, 2, 3 or 4 days, and then the amount of uPA secreted upon cocultivation in cell-free fractions was measured by means of the quantitative ELISA. The results shown in Fig. 24 demonstrate the fivefold increase in the uPA production during cocultivation. The expression of uPAR on monocytes was not changed upon cocultivation (Fig. 24), whereas expression of the cell surface uPAR in VSMC was increased on day two, as measured by flow cytometric analysis (Fig. 24). To check definitely for the

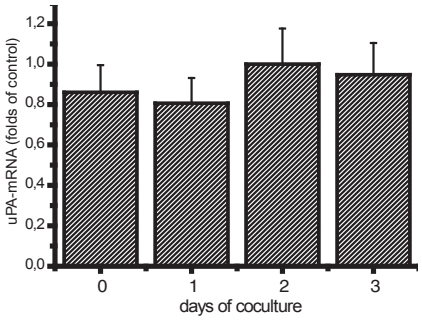

Fig. 25 RT-PCR analysis for uPA in VSMC.

RT-PCR analysis for uPA mRNAs of VSMC in coculture was performed using the TaqMan method.

probable source of the increased uPA expression in coculture, RT-PCR was performed separately in cocultivated VSMC and in monocytes. As shown in Fig. 24, monocytes cocultivated with VSMC display strong upregulation of the uPA expression. At the same time, VSMC revealed no quantitative difference in the uPA expression profile upon cocultivation with monocytes (Fig. 25). The results of RT-PCR analysis of uPAR expression in both cell types (Fig. 24) are consistent with the above shown data obtained by flow cytometric analysis (Fig. 24). Thus, uPAR was highly expressed in VSMC cocultivated with monocytes whereas its expression in monocytes was not changed.

3.3.2 Monocyte-Expressed uPA Stimulates VSMC Migration

To study the functional properties of VSMC upon cocultivation with monocytes, VSMC grown in a filter separated coculture with monocytes for 1, 2, 3 or 4 days were used for mi-

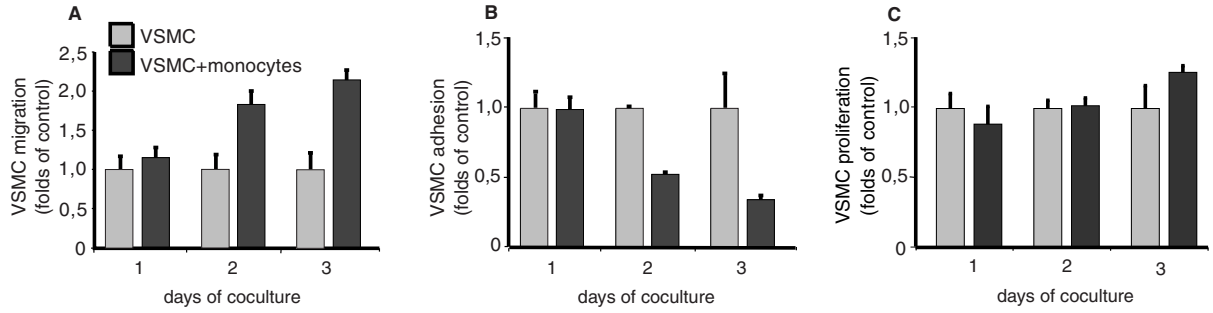

Fig. 26 Monocytes affect VSMC functional behavior.

A, VSMC were monocultured or cocultured with monocytes for indicated times and then subjected to chemotaxis assay. The assay was performed using modified Boyden chambers and migration was allowed for indicated times. Results are mean values±SD of one representative experiment performed in triplicates.

B, VSMC were mono- or cocultured with monocytes for indicated times and then subjected to adhesion assay. All experiments were performed in triplicates. Results are given as mean values±SD of one representative experiment performed in triplicate.

C, VSMC were cocultured with monocytes as indicated above, and DNA synthesis was measured using the 5-bromo-2-deoxy-uridine (BrdU) labeling and detection kit III. Experiments were performed in triplicate.

gration, adhesion and proliferation assays. As shown in Fig. 26, cocultivation of both cell types resulted in the two- to threefold increase in VSMC migration in a time-dependent fashion. In contrast, VSMC adhesion was decreased in a similar time-dependent manner (Fig. 26). These observations imply endogenous signal that decreased VSMC adhesive capacity and served for promotion of cell motility. The observed inhibition of VSMC adhesion reflects their reduced cell-cell or cell-matrix adhesive properties in coculture contributing to facilitation of migration process. To investigate whether proliferation of VSMC might be also affected by cocultivation with monocytes, DNA synthesis in VSMC was measured (Fig. 26). No changes in cell proliferation were found suggesting that coculture does not provide mitogenic signals utilized by VSMC. These data are also in agreement with the report of others where no stimulatory effect of monocytes on smooth muscle cell growth was found (Proudfoot *et al.*, 1999).

In the next experimental settings we observed, that changes in VSMC migration monitored by time-lapse video microscopy in a direct coculture was equivalent to, but not additive with, the maximal effect achieved in VSMC monoculture stimulated with uPA (Fig. 27). Moreover, the increase in cell motility was completely abolished in both cases after addition to mono- and coculture of the soluble uPAR (suPAR) served as a competitor for

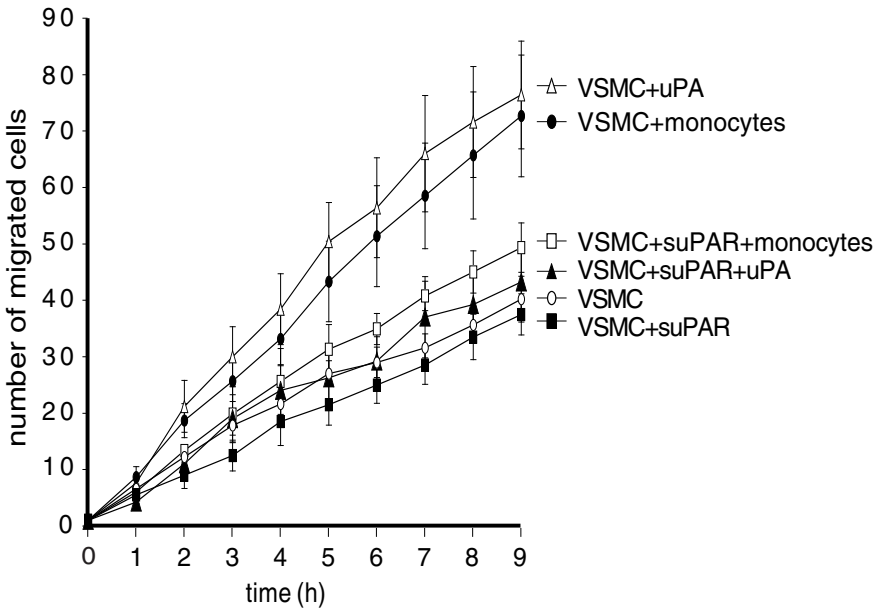

Fig. 27 Increased VSMC migration in coculture is related to the uPA/uPAR system.

Wounded monocultured or cocultured VSMC were stimulated with 50 nM uPA and pretreated with 40 nM suPAR, as indicated. Cell migration was monitored by time-lapse imaging. Results are the mean number of migrated cells at indicated time-points±SD of five (VSMC monoculture) or six (VSMC-monocyte coculture) experiments. **, $p<0.0095$ coculture versus monoculture; ***, $p<0.0009$ uPA versus control; **, $p<0.001$ coculture + suPAR versus monoculture + suPAR; *, $p<0.04$ coculture versus coculture + suPAR (unpaired f-test).

the uPA binding (Fig. 27). These data provide evidence that the observed effects of increased VSMC migration in coculture might be induced by the upregulated expression of uPA by monocytes in coculture.

4 Discussion

4.1 uPA-directed Signaling in Cell Migration

In this study, evidence is provided that implies novel roles for the Janus kinase Tyk2 and PI3-K in human coronary VSMC. As the main finding of this work, a direct association of Tyk2 with PI3-K through the Scr homology 2 domains of p85 subunit is demonstrated. These data show that Tyk2, in addition to known regulation of cell transcription via STAT proteins, is required for PI3-K activation thereby providing a crucial link between two signaling pathways. Importantly, the revealed Tyk2 and PI3-K association within the cell and their activation were urokinase-responsive and were required for the uPA-related VSMC migration. This observation is the first demonstration that PI3-K is essential for at least some of the uPA/uPAR functions in VSMC and that Tyk2 is the main uPA-dependent pathway of PI3-K activation. The functional role of endogenous uPA for VSMC migration was studied in coculture model, as far as uPA expressed by monocytes is a potent chemotactic factor for VSMC and might serve for the acceleration of vascular remodeling. This study demonstrates that coculture of human VSMC with freshly isolated peripheral blood-derived human monocytes results in significant VSMC migration and cell proliferation arrest. Evidence is provided that these changes in VSMC functional behavior were induced by the monocyte-expressed uPA.

VSMC proliferation and migration into the intima after vascular injury, as well as their formation of neointima, contribute to vessel narrowing and are pivotal to the atherosclerotic process (Ross 1993). uPA and uPAR are active participants in these processes by regulating wound healing, tissue remodeling, and immune responses (Blasi 1999). In addition to the effects mediated by proteolysis, uPA and uPAR also display biological functions that are not directly attributable to the formation of plasmin but rather to the induction of cell migration and proliferation control (Dear and Medcalf 1998). The link between uPA/uPAR and cell motility was established over a decade ago (Gudewicz and Gilboa 1987; Fibbi et al., 1988; Del Rosso et al., 1993). Current data confirm the uPA-dependent cell migration in a wide variety of cell types (Odekon et al., 1992; Busso et al., 1994; Fazioli et al., 1997; Nguyen et al., 1998; Chiaradonna et al., 1999; Nguyen et al., 1999; Webb et al., 2000). Nevertheless, the proposed molecular mechanisms have been conflicting. The uPA/uPAR-related migratory responses seem to be highly cell specific, implying some structural specificity and diversity of underlying signaling events. Thus, in human breast cancer cells MCF-7 and HT 1080 fibrosacroma cells, uPA-initiated cellular motility required activation of signaling cascade including Ras, mitogen-activated protein kinase kinase, extracellular signal-regulated kinase, and myosin light chain kinase as downstream effectors (Nguyen et al., 1998; Nguyen et al., 1999). On the contrary, mi-

gration of human epithelial cells seemed to involve the uPA-activated protein kinase C (Busso et al., 1994), whereas in cells of monocytic lineage these effects were attributed to the activation of protein tyrosine kinases of the Src family (Resnati et al., 1996; Fazioli et al., 1997). In addition, uPA/uPAR-related cell migration is integrin-dependent (Carriero et al., 1999; Yebra et al., 1999).

The molecular machinery of uPA/uPAR-related VSMC migration remains sparsely explored, although the recent generation of transgenic mice deficient in uPA and uPAR demonstrated that VSMC migration is dependent on the fibrinolytic system and is decisive for the severity of vascular damage (Carmeliet et al., 1997; Carmeliet and Collen 1998). Separate recent findings confirm migration of rat VSMC initiated by uPA/uPAR binding and suggest involvement of Src tyrosine kinase and G protein in this process (Degryse et al., 1999). We suggested a role for the Janus kinases in VSMC migration and showed that Jakl and Tyk2 were co-localized with the uPAR to the leading edge of migrating VSMC (Dumler et al., 1998). However, to generate migratory responses, Jaks were expected to signal independently of STAT activation most likely through a link to an additional unknown pathway.

4.2 Tyk2 Associates with PI3-K in VSMC

PI3-K is central to cell migration processes regulated by cytokines and growth factors in diverse cell types including VSMC (Imai and Clemmons 1999). Moreover, PI3-K possesses a high capacity to cooperate with other signaling pathways to mediate a required functional response (Vanhaesebroeck and Waterfield 1999). Interestingly, several recent reports provide evidence for the interference of PI3-K and Jak/STAT signaling cascade. Thus, the PI3-K p85 regulatory subunit was shown to bind directly to STAT5 protein, which served as an adapter to couple the PI3-K signaling pathway to the interferon receptor in Daudi cells (Pfeffer et al., 1997). A similar interaction was demonstrated for the p85 subunit and STAT5 protein in a bone marrow-derived Ba/F3 cell line, where both pathways cooperated to mediate interleukin-3-dependent suppression of apoptosis (Rosa Santos et al., 2000). Several other reports provided further evidence for coordinated activation of PI3-K and STAT proteins leading to the functional cooperation of both signaling cascades (Bao et al., 1999; Chen et al., 1999). Moreover, recent studies demonstrated the ability of Jaks to associate with and to regulate the p85 subunit of PI3-K, as was shown for Jak3 in human T cells (Sharfe et al., 1995), Jak1 in cardiac myocytes (Oh et al., 1998), and Jak2 in human neutrophils (Al-Shami and Naccache 1999).

Consistent with these reports, we find that Janus kinase Tyk2, but not Jakl, Jak2, or Jak3, is specifically associated with PI3-K in human VSMC. Moreover, this association is uPA-dependent. The fact that Jak1 was not co-immunoprecipitated with PI3-K is of in-

terest because in our previous studies we did not observe any difference between Tyk2 and Jak1 in terms of uPA stimulation, uPAR association, and polarization to the leading edge of migrating cells (Dumler et al., 1998; Dumler et al., 1999). The inability to reveal Jak1-PI3-K complexes suggests that this kinase is most likely involved exclusively in the transcriptional regulation via STAT proteins or might perform an additional function in the uPA/uPAR-related signaling. Although we found no association between Jak1 and PI3-K, their cooperation cannot be completely excluded. Nevertheless, two kinases performing the same task would be redundant.

To address the molecular basis of Tyk2-PI3-K interaction, we performed an adenovirus-mediated Tyk2 overexpression in VSMC. We performed Tyk2 pull-down assays using GST-p85 fusion proteins composed of either the C- or N-terminal SH2 domains of p85. Tyk2 bound to both N- and C-terminal SH2 domains of the p85 regulatory PI3-K subunit. This result is consistent with the findings of others demonstrating that these domains mediate specific protein-protein interactions (Vanhaesebroeck, Leevers et al. 1997). Tyk2 has a candidate motif, YXXM, which is a potential site for p85 binding. However, determination of whether or not SH2 p85 domains are responsible for the interaction with Tyk2 in the context of above motif requires further verification. Our data are the first to demonstrate that following uPA stimulation, Tyk2 and PI3-K form a specific protein complex in VSMC. Although Tyk2 constitutively associates with p85, uPA induces a strong activation of this interaction. Moreover, uPA induces PI3-K activation within the complex, which we believe is mediated by tyrosine phosphorylation of the PI3-K p85 subunit. This hypothesis, which we are currently pursuing, is supported by the fact that growth factor-promoted p85 phosphorylation increases PI3-K activity (Thakker et al., 1999). However, the increase in PI3-K activity we measured in Tyk2 precipitates in response to uPA was significantly less than the uPA-induced PI3-K activation in whole cell lysates. An intriguing question is raised by these observations, which imply additional still unknown uPA/uPAR-dependent PI3-K functions.

4.3 PI3-K in the Regulation of Cell Migration in Response to uPA

Our results highlight the importance of PI3-K in the regulation of cell migration in response to uPA. We demonstrate the involvement of PI3-K in uPA-induced signaling leading to cell migration in two in vitro models, the Boyden chamber and the wounding assay. Both techniques allowed us to analyze different parameters of cell migration. Specific PI3-K antagonists, LY294002 and wortmannin, completely abrogated the uPA-responsive increase in cell motility. The observed ability of uPA to initiate sustained PI3-K activation might facilitate the long-term migration process.

One of the issues raised by these observations is whether PI3-K lies upstream or

downstream of Tyk2 in the reavealed pathway. Our demonstration that in VSMC expressing the Tyk2 dominant negative form PI3-K activation in response to uPA is completely blocked implicates that PI3-K activation acts broadly downstream of Tyk2 in the signal transduction cascade. Moreover, these data support the concept that in VSMC Tyk2 is central to the PI3-K regulation. This positioning is further supported by our finding that a dominant negative Tyk2 blocks uPA-related cell migration. Upon stimulation, Tyk2, as certain other players in signaling machineries, relocates to the leading edge of the cell membrane that might enhance reaction-limited signal transduction. The results of our study on VSMC morphology reported here imply that polarization of Tyk2 might contribute to the lamellipodial activity required for a spatial asymmetry of migrating cells. However, it appears that the loss of PI3-K activity has similar consequences on lamellipodial activity and migration process as blockage of Tyk2 by its mutant. Overall, these data favor the view that a functional relationship between two kinases is decisive for VSMC cytoskeletal reorganization and migration. Our data are consistent with other reports demonstrating the central role of PI3-K in initiating actin cytoskeletal rearrangements, cell polarization, and cell migration in several cell systems (Hooshmand-Rad et al., 1997; Vicente-Manzanares et al., 1999). During these processes, PI3-K is generally translocated from the cytosol to the cytoskeleton-associated fraction presumably via a link to additional proteins (Meng and Lowell 1998). It is likely that Tyk2-PI3-K association via the SH2 domains of the p85 subunit is responsible for bringing PI3-K to the cytoskeleton-associated subcellular fraction, since loss of Tyk2 polarization in Ad5Tyk2KE-VSMC correlates with the loss of PI3-K activation and of leading edge formation. However, it is also possible that other adaptor molecules may facilitate translocation of PI3-K. In rat VSMC, Src kinase was presumed to cooperate functionally with uPAR and integrins at the leading edge upon the migration process, since uPA-caused a c-Src redistribution from the cytoplasm to plasma membrane (Degryse et al., 1999). These data, coupled with the recent demonstration that the integrin-dependent cell migration requires both Src family kinases and PI3-K (Meng and Lowell 1998), as well as with our findings, suggest the existence of a complex functional unit formed at the leading cell membrane in response to uPA. The localized regulation of signaling molecules, such as Src, Tyk2, and PI3-K, by uPA/uPAR and integrins may provide an efficient mechanism for targeting downstream functional effects of these kinases as well as for the balance between these pathways.

4.4 Importance of uPA/uPAR System for VSMC Migration in a Coculture Model

In the last decade a great variety of animal studies were performed to establish a therapeutic strategy for the treatment of atherosclerosis and for prevention of restenosis after balloon angioplasty (Muller et al., 1992). Despite several promising animal studies

showing a reduction of neointima formation after the administration of antiproliferative compounds, the transfer of these protocols to the human situation did not lead to a breakthrough in restenosis therapy. One of the main reasons responsible for this failure is the fact that the test models used are inadequate and do not allow a transfer to the human situation. To overcome these obvious limitations, the alternative in vitro models might be very helpful to study communication between two or more cell types, which are neighbored in the arterial vessel wall. After vascular injury, the endothelial integrity is disturbed and monocytes infiltrate into the injured vessels. The monocytes promote an inflammatory response with generation of migratory and proliferative signals that converge on VSMC. The VSMC first migrate into the normally thin intimal layer and thereafter proliferate, causing neointimal formation and restenosis. The underlying molecular mechanisms remain, however, poorly defined. In this study, we have therefore established and used the coculture model with human VSMC and peripheral blood-derived monocytes that allowed us to analyze the early cellular events in vascular remodeling. We show here that the uPA/uPAR system is upregulated upon VSMC-monocyte interaction and serves as a promigratory signal thus contributing to increased VSMC motility.

The uPA/uPAR system is known to be involved in tissue remodeling and wound healing by affecting cell adhesion, migration, and proliferation (Ossowski and Aguirre-Ghiso, 2000). It has been also demonstrated in different in vitro models that uPA induces accelerated migration of VSMC (Noda-Heiny and Sobel, 1995; Degryse et al., 1999; Kusch et al., 2000; Mukhina et al., 2000). By time-lapse video microscopy performed directly on the cocultures, we observed, surprisingly, that changes in VSMC migration due to coculture were equivalent to the maximal effect achieved in VSMC monoculture stimulated with uPA (Fig. 27). Moreover, the increase in cell motility determined by either uPA or monocytes was completely abolished after addition to mono- and coculture of the soluble uPAR (suPAR) as a competitor for the uPA binding (Fig. 27). These data suggest that the increased VSMC migration in coculture might be induced by an upregulated expression of uPA. To verify this hypothesis, we measured the amount of uPA secreted upon coculture in cell-free fractions. The results of the quantitative ELISA shown in Fig. 24A demonstrate a five-fold increase of uPA production during coculture. Interestingly, the maximal uPA secretion was reached on day three of coculture, whereas the pronounced changes in cell adhesion and migration were observed already on day two (Fig. 24). One possible explanation for this discrepancy might be the increased expression of the cell surface uPAR, as detected by flow cytometry in VSMC on day two (Fig. 24). As uPAR provides high affinity binding sites for uPA, this increase would amplify uPA-dependent migratory signals. The expression of uPAR on monocytes was not changed upon coculture (data not shown). To determine the source of the increased uPA expression in coculture, RT-PCR was performed separately in cocul-tured VSMC and in monocytes. As shown in Fig. 24 cocultured monocytes display strong upregulation of uPA expression, whereas cocultured VSMC revealed no quantitative differences of uPA expression (data not shown). The results of RT-

PCR analysis of uPAR expression in VSMC (Fig. 24) are consistent with the data discussed above obtained by flow cytometry (Fig. 24). Thus, uPAR is highly expressed in VSMC cocultured with monocytes, whereas its expression in monocytes does not change.

Taken together, our findings indicate that coculture of human VSMC and peripheral blood-derived monocytes leads to the increase of VSMC motility through a mechanism(s) involving increased expression of uPA by monocytes and of cell-surface uPAR by VSMC. These findings might have important implications for several vascular diseases, such as restenosis and arteriosclerosis involving infiltration of inflammatory cells followed by VSMC migration at sites of vascular injury. This study supports and extends the usefulness of the in vitro coculture model, which overcomes the obvious limitations of animal models and allows to study the communication between two interacting cell types (Axel et al., 1997; Bhatia et al., 1999; Streblow et al., 1999). Using this experimental system, we found that monocytes were able to affect VSMC function by either direct contact or transfilter coculture. The fact that a direct interaction was not mandatory suggests that the monocyte-interacting cells might specify the regulatory effects of monocytes. It has been recently demonstrated that both peripheral blood cells and endothelial cells can release su-PAR, and that this release was enhanced when either mononuclear cells or thrombocytes were cultured together with endothelial cells (Mustjoki et al., 2000). In this case, however, coculture without cell contact failed to enhance suPAR release. Although uPA is a well-documented migratory signal for VSMC, most biological activities of uPA have been studied using exogenous recombinant or purified protein or peptides (Fazioli et al., 1997; Degryse et al., 1999; Kusch et al., 2000; Mukhina et al., 2000). Our study is the first to demonstrate the net effect of the monocyte-secreted endogenous uPA on VSMC migration. The effects observed in our coculture model might have a counterpart in vivo during the early phase of monocyte entry into the arterial wall under pathological conditions.

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Specialisation:

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Methods:

- Protein Chemistry Techniques: Gel electrophoresis, Western immunoblot analysis, Imunostaining.
- Molecular biology techniques: Small and large scale preparation of plasmid DNA, Restriction Digestion, Transformation of E.coli, Regular cloning, DNA sequencing, Site directed mutagenesis, PCR Technique, Hybridization, Procariotic expression, Real-Time PCR, RNA extraction, siRNA.
- Cell biology: Transient cell transfection- FuGene, Superfect, Ca/PO_4 precipitation,

Maxifectin, Cell culture works- CASMC, HUVEC, HEK293, primary fibroblasts.

- Gene therapy: Generation of recombinant adenoviruses, Production adenovirus high titer stock, Adenovirus titration, Generation recombinant retroviruses, Infection cells by recombinant retroviruses.
- Videomicroscopy: Axioplan II microscope, Image processing- Axiovision program.
- Computer analysis: DNA Star, VectorNTI.
- Computer techniques: Windows 9*, NT, OS/2, Linux, MacOS, Sun Sparc Station-Solaris.

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3. Second advanced course of Signal Transduction. Species, Greece, 2001.

4. FEBS Advanced practical course of Yeast Two-Hybrid system, Moscow, Russia, 2002.

5. 1st Hannover Seminar on Transplantation and Vascular Biology. 2000.

LIST OF MAIN PUBLICATIONS

Kunigal, S., Kusch, A., Tkachuk, N., Tkachuk, S., Jerke, U., Haller, H., and Dumler, I. (2003). Monocyte-expressed urokinase inhibits vascular smooth muscle cell growth by activating Stat1. Blood.

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Selected abstracts:

1. Small GTP-binding proteins of vertebrate and invertibrate visual systems. Interaction of exoenzyme C substrates with photoactivated rodopsin. V.Petrov, A.Dergachev, S.Tkachuk, V.M. Lipkin. 9th international conference on second messengers & phospoproteins. 1995, Nashville, USA

2. Urokinase regulates gene transcription by formation of homo- and heterodimers of Stat proteins in human vascular smooth muscle cell.,Dumler, A. Kopmann, K. Wagner, S. Tkachuk, H. Haller, and D.-C. Gulba Franz Volhard Clinic and Max-Delbruck Center for Molecular Medicine, Humboldt University at Berlin, Germany

3. Immunocytochemical visualization of transfected DNA in cultured cells. S. Zaitsev, I. Buchwalow, A. Haberland, S. Tkachuk, M. Bottger, Acta histochem. 102 ,49-55, 2000

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