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Role of p21-Activated Kinase 4 in Cell Migration

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To the memmory of my mother

Abstract

Cell migration is a cellular process that plays a critical role in various physiological and pathological phenomena, including in cancer metastasis. Understanding at a fundamental level how cancer cells migrate and invade will help to delineate potential targets for the directed development of anti-metastatic therapeutic agents. For example, αv integrins are up-regulated or activated in many migratory cells, and are essential to the processes of wound healing, angiogenesis, and metastasis. Similarly, integrin αvβ5, a vitronectin (VN) receptor, is expressed in most patient carcinoma specimens and is functionally involved in growth factor-induced carcinoma cell migration *in vitro* and metastasis *in vivo*. However, the mechanisms of integrin αvβ5-mediated cell migration are not fully understood. In this project, we aimed to identify proteins that interact with the cytoplasmic tail of integrin β5, and to study their role in cell motility. Firstly, we employed a yeast two-hybrid screening of a mouse embryo cDNA library and thereby identified six proteins specifically interacting with the human integrin β5 cytoplasmic domain. One of the integrin β5-interacting proteins was p21-activated kinase 4 (PAK4), which, in addition to its direct interaction with the integrin β5 cytoplasmic tail, also appeared to functionally regulate αvβ5-mediated migration of the human MCF-7 breast carcinoma cells. Importantly, engagement of integrin αvβ5 by cell attachment to VN led to a redistribution of PAK4 from the cytosol to dynamic lamellipodial structures where PAK4 co-localized with integrin αvβ5. Functionally, PAK4 induced integrin αvβ5-mediated, but not integrin β1-mediated MCF-7 cell migration, without affecting the cell surface levels of integrin αvβ5.

In addition, we found that PAK4 was activated by cell attachment to VN mediated by the PAK4 binding partner integrin αvβ5, and that active PAK4 induced accelerated integrin αvβ5 turn-over within adhesion complexes. Accelerated integrin turn-over was the likely cause of additionally observed PAK4-mediated effects, including inhibited integrin αvβ5 clustering, reduced integrin to F-actin connectivity and perturbed maturation of cell adhesion complexes. These specific outcomes were ultimately associated with reduced cell adhesion capacity and increased cell motility. We thus demonstrate a novel mechanism deployed by cells to tune cell adhesion levels through the auto-inhibitory regulation of integrin-mediated adhesion.

Furthermore, we identified a unique PAK4-binding membrane-proximal β5-SERS-motif in the cytoplasmic tail of β5, and demonstrated a key role for this motif in controlling cell adhesion and migration. We mapped the integrin β5-binding within PAK4 and observed that PAK4 binding to integrin β5 was not sufficient to promote cell migration; instead the PAK4 kinase activity was required for PAK4 promotion of cell motility. In fact, PAK4 specifically phosphorylated the integrin β5 subunit at Ser 759 and Ser 762 within the β5-SERS-motif. Importantly, point mutation of these two serine residues abolished PAK4-mediated promotion of cell migration, indicating a functional role for these phosphorylations in cell migration.

In conclusion, our results demonstrate that PAK4 interacts with and selectively phosphorylates integrin αvβ5 and thereby promotes αvβ5-mediated cell migration, a functional outcome paralleled by a concurrent decrease in total cellular adhesion to VN. Given our finding that PAK4 is activated by αvβ5 ligation to VN, these results delineate an auto-inhibitory negative feedback loop that is initiated by cell adhesion to VN. Binding of integrin αvβ5 to VN drives translocation and activation of PAK4, leading to phosphorylation of αvβ5 and ultimately the limiting of total adhesion between cells and VN and increased cell migration. Thus, our findings provide a new mechanistic characterization of PAK4's role in the functional regulation of integrin αvβ5. This knowledge may ultimately be important for understanding vascular permeability, angiogenesis and cancer dissemination.

List of publications

I. Hongquan Zhang, Zhilun Li, Eva-Karin Viklund, and Staffan Strömblad. (2002). P21-activated kinase 4 interacts with integrin αvβ5 and regulates αvβ5-mediated cell migration. Journal of Cell Biology *158*, 1287-1297.

II. Zhilun Li, John G. Lock, Helene Olofsson, Jacob M. Kowalewski, Steffen Teller, Yajuan Liu, Hongquan Zhang and Staffan Strömblad. (2010). Integrin-mediated cell attachment induces a PAK4-dependent feedback loop regulating cell adhesion through modified integrin αvβ5 clustering and turn-over. Molecular Biology of the Cell *21*, 3317-3329.

III. Zhilun Li, Hongquan Zhang, Lars Lundin, Minna Thullberg, Yajuan Liu, Yunling Wang, Lena Claesson-Welsh and Staffan Strömblad. (2010). P21-activated kinase 4 phosphorylation of integrin β5 Ser 759 and Ser 762 regulates cell migration. Journal of Biological Chemistry *285*, 23699-23710.

List of additional publications (which have relevance to the thesis, but are not included)

I. Hongquan Zhang, Jonathan S. Berg, Zhilun Li, Yunling Wang, Pernilla Lång, Aurea D. Sousa, Aparna Bhaskar, Richard E. Cheney and Staffan Strömblad. (2004). Myosin-X provides a motor-based link between integrins and the cytoskeleton. Nature Cell Biology *6*, 523 – 531.

II. Michelle K. Y. Siu, Hoi Yan Chan, Daniel S. H. Kong, Esther S. Y. Wong, Oscar G. W. Wong, Hextan Y. S. Ngan, Kar Fai Tam, Hongquan Zhang, Zhilun Li, Queeny K. Y. Chan, Sai Wah Tsao, Staffan Strömblad, and Annie N. Y. Cheung. (2010). p21-activated kinase 4 regulates ovarian cancer cell proliferation, migration, and invasion and contributes to poor prognosis in patients. Proceedings of the National Academy of Sciences of the United States of America (PNAS), published online (doi: 10.1073/pnas.0907481107).

Contents

List of abbreviations

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aa	amino acid
BAP	bacterial alkaline phosphatase
BSA	bovine serum albumin
CMAC	cell-matrix adhesion complex
CT	carboxyl-terminal
DMEM	Dulbecco's modified Eagle's medium
ECM	extracellular matrix
EGFP	Enhanced Green Fluorescent Proteins
FA	focal adhesion
FBS	fetal bovine serum
FRAP	fluorescence recovery after photobleaching
GST	Glutathione-S-transferase
HA tag	hemagglutinin tag
His tag	Histidine tag
IB	immunoblotting
IP	immunoprecipitation
mAb	monoclonal antibody
MBP	myelin basic protein
mRFP	monomeric red fluorescent protein
NT	NH2-terminal
pAb	polyclonal antibody
PAKs	p21-activated kinases
PAK4	p21-activated kinase 4
PBS	phosphate-buffered saline
PFA	paraformaldehyde
SD	standard deviation
SEM	standard error of the mean
siRNA	small interfering RNA
shRNA	short hairpin RNA
VN	vitronectin
WT	wild type

1 Introduction

1.1 Cell migration

Cell migration is a cellular event that plays a critical role in various physiological processes and pathological responses, such as embryonic development, angiogenesis, immune function and inflammation, tissue repair, and cancer metastasis (Lauffenburger and Horwitz, 1996; Holly *et al.*, 2000; Dormann and Weijer, 2006; Broussard *et al.*, 2008; Lock *et al.*, 2008). Cancer cells display migratory activity during invasion and metastasis. Metastasis is a late stage of cancer that has a poor clinical outcome with 90% of cancer deaths resulting from metastasized tumors. However, the molecular mechanisms involved in the metastatic process are only partially understood.

Cell migration across a two-dimensional (2D) extracellular matrix (ECM) surface occurs in tissue such as the epithelia during wound healing (Kirfel and Herzog, 2004) and this type of migration is approximated in 2D cell culture conditions (Lauffenburger and Horwitz, 1996). However, in the body, cells can also migrate within three-dimensional (3D) tissue. Cell migration is a dynamic and complex process. In general, cell migration is a cyclic process involving the repetitive extension of a protrusion at the leading edge of the cell, assembly of focal adhesions at the leading edge and disassembly at the cell body and rear, and cytoskeletal contraction to pull the cell body forward (Ananthakrishnan and Ehrlicher, 2007). The process of cellular movement requires highly coordinated interactions and involves a number of different sub-cellular systems (Lock et al., 2008). These sub-cellular systems of cell migration include the extracellular matrix; Cell-matrix adhesion complexes system; the cell polarity system; the microfilament system; plasma membrane (composition and dynamics); vesicular trafficking; microtubules (Figure 1). A number of molecular components in the each sub-cellular system drive the cell movement at different times. However, little is known about how these different sub-cellular systems are integrated within cell migration as a whole.

Cell migration can be classed into two different types: single cell migration (mesenchymal or amoeboid) and collective cell migration (Friedl, 2004; Record Owner, 2010). Cancer cells can migrate either collectively or individually. In most epithelial cancers, the cells can convert to mesenchymal, individual cell migration, known as an epithelial-mesenchymal transition (EMT). EMT is induced by repression of transcriptional regulators such as Snail or Twist that leads to down-regulation of E-cadherin and consequently to loss of the cell-cell adhesion (Kopfstein and Christofori, 2006). Cells with a mesenchymal type of fibroblast-like motility are initiated by the formation of actin-rich filopodia and lamellipodia at the leading edge and exhibit an elongated cell morphology. This process is controlled by the small Rho-GTPases Rac and Cdc42 (Nobes and Hall, 1995). Interestingly, Cancer cells can also undergo a mesenchymal-to amoeboid transition by blocking extracellular proteolysis in a 3D environment (Friedl and Wolf, 2003a; Sahai and Marshall, 2003). Cells usually migrate in a 3D environment *in vivo*; however, the complex process of cell migration in 3D is poorly understood. For this purpose, new and more suitable methods of the

3D cell migration models *in vitro* still need to improve, as well as suitable imaging techniques. A number of differences have been seen in 3D migration compared with 2D migration, For example, in 3D cancer cells can exhibit a rounded shape and an amoeboid mode of migration and have smaller CMACs as compared to 2D, and CMAC components may be specifically involved in either 2D or 3D (Even-Ram and Yamada, 2005; Fraley et al., 2010). By comparing results between 2D with 3D experiments the reasons for these differences may become clearer. It is currently thought that tension has a major influence in regulating CMAC function and since most 3D culture systems exhibit a relatively low tension than a 2D coated glass coverslip this may contribute to some of the differences at the CMAC level (Levental et al., 2009).
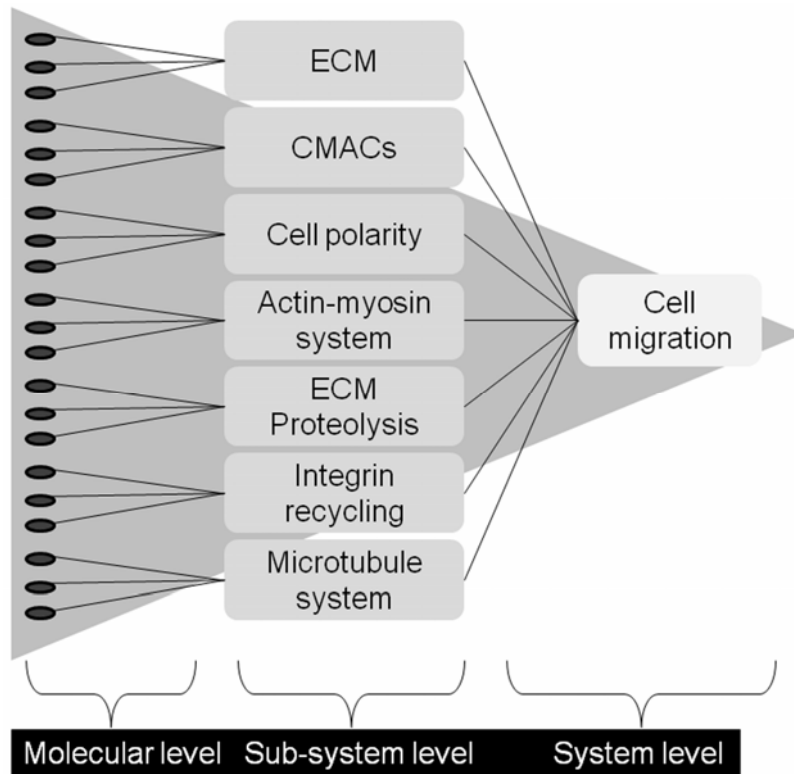
Figure 1. *Regulation of cell migration.* A large number of molecular belong to different sub-cellular systems and dynamically coordinated govern cell migration. ECM: extracellular matrix; CMACs: cell-matrix adhesion complex.

1.1.1 Extracellular matrix

Extracellular matrix (ECM) is a network of proteins. ECM is produced by cells and excreted to the extracellular space within the tissue. The major ECM components include structural proteins (collagen and elastin), specialized proteins (e.g. fibrillin, fibronectin, and laminin) and proteoglycans (Chondroitin sulfate, Heparan sulfate, Keratan sulfate, Hyaluronic acid). Most ECM proteins act as ligands for integrins, with ECM-integrin binding providing both cell adhesion and signaling. Examples of ECM components that bind specific subsets of integrins include fibronectin, laminin and vitronectin (VN) (Buzza et al., 2005). By expressing certain adhesion receptors

2

cells can bind specific ECM proteins which in turn modifies cell behaviour, for example, VN binding by integrin αvβ5, promotes cell adhesion and affects cell morphology, cytoskeletal organization and cell migration (Li et al., 2010b). Overall the ECM provides structural support for tissues, helps cells to bind together and regulates many cellular functions including adhesion and migration (Bornstein and Sage, 2002; Hynes, 2009).

The ECM can also be remodelled in many different ways, such as by proteolytic degradation (Larsen et al., 2006). Studies in experimental conditions show that the ECM density, stiffness, geometrical array and topography affect the properties of migrating cells (Lehnert *et al.*, 2004; Chown and Kumar, 2007; Parsons *et al.*, 2010). For example, increased stromal collagen density increases mammary tumor formation, local invasion, and metastasis in 3D collagen gel and mammary tissues in mice (Provenzano et al., 2008a); parallel arrays of collagen can promote cancer cell invasion, while a non-linear matrix reduces invasion (Provenzano et al., 2008b). Further studies, using a combination of *in vivo* and 3D culture systems with live cell imaging should gain new insights into the relationship between cancer cell invasion, metastasis and ECM remodelling.

1.1.2 Cell-matrix adhesion complexes

When cells attach to ECM, integrins cluster within the plasma membrane and associate with numerous proteins to form organized adhesive contact sites containing large protein networks, called cell-matrix adhesion complexes (CMACs) (Geiger *et al.*, 2001; Campbell, 2008; Lock *et al.*, 2008). To date, more than 150 proteins have been shown to reside in CMACs and related integrin-mediated contacts including many different proteins, such as the actin cytoskeleton associated proteins (tensin, vinculin, paxillin, α-actinin, talin, zyxin, kindlins), tyrosine kinases (Srcs, FAK, PYK2, Csk and Abl), serine/threonine kinases (ILK, PKC and PAK), modulators of small GTPases (ASAP1, Graf and PSGAP), phosphatases (SHP-2 and LAR PTP) and other enzymes (PI 3-kinase and the protease calpain II) (Geiger *et al.*, 2001; Zamir and Geiger, 2001a; Zaidel-Bar *et al.*, 2007; Moser *et al.*, 2009).

CMACs can be classified into different types based on their size, stability, location in the cell. These classifications include nascent adhesions, focal complexes (FCs), focal adhesions (FAs), fibrillar adhesions, and podosomes (Figure 2) (Zamir and Geiger, 2001b; Berrier and Yamada, 2007; Lock *et al.*, 2008). Assembly, maturation and disassembly of CMACs is a sequential process driven by a coordinated interaction of numerous molecules, and the activation of specific signaling pathways (Webb et al., 2002). During cell migrating on ECM-coated surfaces, small GTPase Cdc42 and Rac1 signaling pathways trigger formation of membrane protrusions at the cell leading edge, resulting in formation of nascent adhesions in the lamellipodium behind the leading edge (Raftopoulou and Hall, 2004; Galbraith *et al.*, 2007; Choi *et al.*, 2008). Transient nascent adhesion structures either disappear or develop into larger FCs, which reside at the base of lamellipodium. FCs either disassemble within a short time of their formation, or continue to grow into FAs (Zamir and Geiger, 2001b;

Webb *et al.*, 2002; Berrier and Yamada, 2007; Parsons *et al.*, 2010). FAs connect with actin stress fibers that transmit the contractile forces in the cell that are required for cell movement (Shemesh *et al.*, 2005; Ananthakrishnan and Ehrlicher, 2007).

When fibroblast cells attach on fibronectin-coated surfaces, via α5β1 integrin, they form elongated, rod-shaped adhesions, called fibrillar adhesions. This transition process from FAs to fibrillar adhesions depends on actomyosin-driven contractility. Fibrillar adhesions promote fibrillogenesis (Zamir et al., 2000). During formation of the CMACs, the CMAC protein components present in different types of adhesion structures are not identical. Vinculin for example is present in nascent adhesions and FCs, whereas zyxin only appears in mature FAs, and fibrillar adhesions lack paxillin and vinculin (Zamir *et al.*, 1999; Zaidel-Bar *et al.*, 2003; Zaidel-Bar *et al.*, 2004; Lock *et al.*, 2008).

Other adhesive structures include podosomes and invadopodia. Podosomes are ring structures that have an actin core, which is surrounded by CMAC proteins such as talin and vinculin. Podosomes have been found in osteoclasts, macrophages and endothelial cells (Linder and Kopp, 2005). Invadopodia are podosome-like structures in invasive cancer cells and Src-transformed cells (Weaver, 2006, 2008). RhoGTPases and Src are involved in the assembly and dynamics of invadopodia and podosomes (Burns et al., 2001; Roskoski, 2004). An important function of podosomes and invadopodia is matrix degradation.
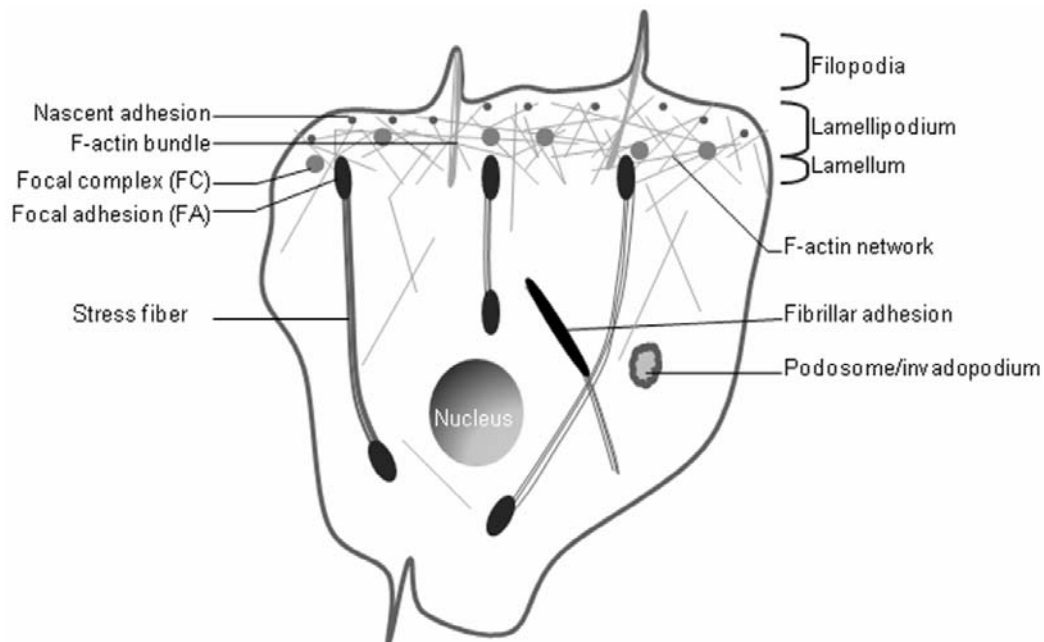
**Figure 2:** The illustration shows different types of CMAC structures. Note particular types of CMACs may only be present in specific cell types.

In migrating cells, the CMACs can sense the extracellular environment transmitting signals into the cell and mediate the traction force to drive cell movement (Geiger *et al.*, 2001; Lock *et al.*, 2008; Askari *et al.*, 2010). The dynamic assembly and

disassembly of adhesion complexes plays a critical role at all stages of cell migration. During cell migration, both the composition and the morphology of the adhesion complexes change. The formation of focal complexes in the lamellipodia of migrating cells is an important step for extending lamellipodia to the ECM (Betapudi, 2010). Adhesion complexes at the leading edge anchor the actinomyosin force generating system to the ECM, resulting in a force on the cell to pull the cell body forward over the ECM. Mechanical tension generated within the cell by the actinomyosin system is clearly important for adhesion maturation, but different adhesion components show differential sensitivity to tension (Pasapera et al., 2010). CMACs also disassemble at the front and rear of the cell to allow cell detachment (Bretscher, 1996b; Carragher and Frame, 2004). The protease calpain also mediates focal adhesion disassembly during cell migration (Franco and Huttenlocher, 2005). However, the mechanism of adhesion dynamic assembly and disassembly in migrating cells is still not well understood, but clearly require tight spatial and temporal regulation.

The attachment strength between the cell and the ECM can be controlled by levels of matrix concentration, integrin abundance (CMACs), and the integrin activation state (Palecek *et al.*, 1997; Holly *et al.*, 2000). In many cell types, the migration rate is optimized at intermediate levels of these three components. Change in any one of these properties will affect the rate of cell migration in a manner that is dependent on the original position of the cell on the bell shaped curve (Figure 3) (Palecek *et al.*, 1997; Holly *et al.*, 2000; Peyton and Putnam, 2005; Gupton and Waterman-Storer, 2006).
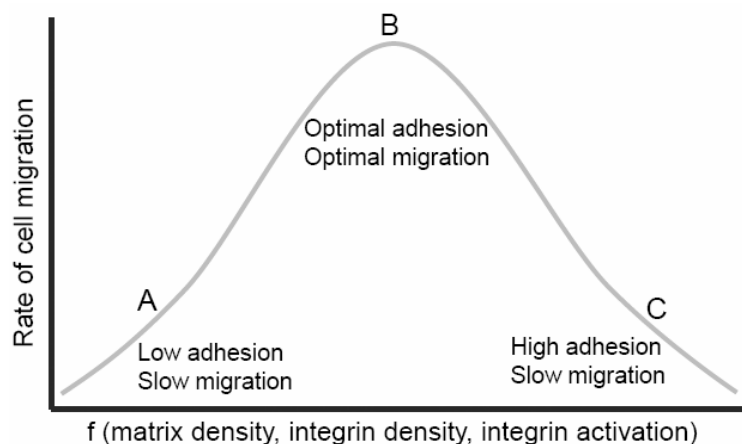
Figure 3. Cell migration in relation to adhesion strength follows an approximate bell-shaped curve (from Holly et al., *Exp. Cell Res. 2000).*

1.1.3   *Polarity in migrating cells*

Polarization is essential for directional cell migration in a variety of cell types. Without polarization, the cells would move in all directions at once, or remain stationary (Ridley et al., 2003). Studies have shown that the Rho family GTPase play a key role in cell polarity (Etienne-Manneville and Hall, 2002; Fukata et al., 2003; Funasaka et al., 2010). When cells respond to a migratory stimulus, such as growth factor (chemotaxis) or ECM (haptotaxis), the cells generate a polarized phenotype and migrate toward regions of higher chemical concentrations. At initial stages of the polarization, actin

5

polymerization at the leading edge generate a protrusive force (Lauffenburger and Horwitz, 1996). Further more, the stabilization of microtubules at the front of the cell, a reorientation of the microtubule organizing center (MTOC) (or centrosome), and the Golgi to a location in front of the nucleus, toward the direction of cell migration, contribute to establishing and maintaining cell polarity in 2D environments (Orlando and Guo, 2009). Although this mechanism of the migrating cell polarization has not been completely clarified, Cdc42, PAR6, PAR3 and a typical protein kinase (αPKC) appears to be involved in the polarization events (Etienne-Manneville and Hall, 2003; Etienne-Manneville, 2004b). Also, the phosphoinositide 3-kinase (PI3K) pathway is involved in the polarization process (Weiner, 2002). However, recent studies found that the Golgi complex and the centrosome are located behind the nucleus during cell migration in 3D environments (Pouthas *et al.*, 2008; Doyle *et al.*, 2009). This suggests that the polarization has distinct mechanisms in 2D and 3D environments. It will also be interesting to investigate whether any of these polarity proteins could be potential drug targets for cancer treatment.

1.1.4   *The actinomyosin system and cell migration*

Tractional force is created at focal adhesion sites by integrins connecting the actinomyosin force-generating system to the ECM. Integrins in focal adhesion sites serve as the both traction force driving the cell movement and as mechanosensors transmitting the physical state of the extracellular environment into the cell and altering F-actin cytoskeleton dynamics (Galbraith et al., 2002). Individual actin filaments can be assembled into two general types of structures: cortical actin networks and stress fibers, which play different roles in cell migration.

The migration cycle begins with the process of protrusions, where actin polymerization promotes extension of two types of protrusions, filopodia and lamellipodia. The thin, spike-like filopodia are formed through the direct polymerization of long parallel actin bundles by members of the formin family (Peng *et al.*, 2003; Pellegrin and Mellor, 2005). The large, broad lamellipodia are formed through actin polymerization of an actin network suggested to be branched (Mullins et al., 1998). However, recent studies show that the actin filament networks in lamellipodia may not be branched, but instead form overlapping arrays where individual actin filaments approach the leading cell edge at angles between 15 and 90 degrees. (Koestler et al., 2008; Urban et al., 2010). In protruding lamellipodia, a high proportion of filaments are oriented at angles orthogonal to the advancing cell edge. In contrast, an increased proportion of actin filaments and microspike bundles are oriented at angles parallel to the cell edge in stable or retracting lamellipodia. This provides a new model for understanding actin-driven protrusion in cell migration. In lamellipodia, the barbed ends of F-actin are oriented towards the leading edge. Continuous assembly at barbed ends and removal of actin monomers from the pointed ends of filaments creates a treadmilling process that generates protrusive force (Neuhaus et al., 1983; Revenu et al., 2004). These F-actin networks may also engage with focal complexes within lamellipodia via adhesion adaptor proteins in a dynamic manner with "clutch-like" properties. This permits the asymmetric orientation of F-actin assembly-derived forces to push out the leading edge

6

membrane; alternatively, when the actin filaments disengage from FCs, F-actin retrograde flow is increased (Theriot and Mitchison, 1991; Atilgan *et al.*, 2005; Mogilner and Rubinstein, 2005; Atilgan *et al.*, 2006). This process may depend on the actin-related protein 2/3 (Arp2/3) complex activation to create free barbed ends (Mullins et al., 1998), and on the actin depolymerizing factor (ADF) cofilin-mediated severing that increases both the number of barbed and pointed ends (Ghosh et al., 2004). Barbed end-capping proteins and depletion of the polymerization-competent pool of actin monomers limit filament growth (Record Owner, 2003). The formation of lamellipodia and filopodia, respectively, are regulated by small GTPases of the Rho family, Rac1 and Cdc42 (Nobes and Hall, 1995). These two forms of protrusion appear to have very distinct functions. The filopodia act as sensors that explore the local environment, whereas the lamellipodia forms broad protrusions in the direction of cell migration to provide a strong foundation over which the cell can move forward (Ridley et al., 2003).

Through Rho-ROCK signaling pathways, myosin II is activated (Watanabe *et al.*, 1999; Wang *et al.*, 2009). The activated myosin II binds to F-actin filaments to form stress fibers (Byers et al., 1984). The stress fibers anchor to mature FAs in lamellae (back of the lamellipodia) via adaptor proteins facilitating a contractile force and pulling the cell body forward. Although myosin II-mediated intracellular mechanical tension is clearly important for adhesion maturation, it also contributes to adhesion disassembly in migrating cells (Broussard et al., 2008). For example, this tension can lead to activation of calpain, which contributes to adhesion disassembly at the cell rear by cleaving a number of focal adhesion proteins, including integrins, talin and vinculin (Franco et al., 2004; Wells et al., 2005). However, these processes are not yet fully understood.

1.1.5 *Proteolysis in cell migration*
Cell migration in 3D requires the overcoming of the physical resistance of three-dimensional tissue networks, one way this can be achieved is via proteolytic degradation of the ECM components in different cell types and cancer. For example, matrix metalloproteinases (MMPs) cleave specific targets in the ECM to facilitate cell migration (Heissig et al., 2005). MMPs have been identified as key secreted enzymes for the breakdown and remodelling of the ECM in both normal and cancer cell migration (Dufour et al., 2008). Cell can migrate as single cells (amoeboid or mesenchymal) or collectively (in cell sheets, strands, tubes, or clusters) (Friedl and Wolf, 2003b). However, when matrix metalloproteinases are inhibited in 3D environments cells can switch towards an amoeboid movement by a mesenchymal-amoeboid transition (Friedl and Wolf, 2003a; Wyckoff et al., 2006).

1.1.6 *Recycling of integrin receptors and plasma membrane in cell migration*
Several studies have shown that cell migration requires recycling of the integrin molecules by internalization from the plasma membrane into endosomal compartments and exocytosis to form new adhesion sites (Lawson and Maxfield, 1995; Pierini *et al.*, 2000; Pellinen and Ivaska, 2006; Howes *et al.*, 2010). Integrins are internalized at the plasma membrane by clathrin-dependent and clathrin-independent endocytic

mechanisms (Caswell et al., 2009). In clathrin-dependent integrin endocytosis, for example, the NXXY motif of the integrin β1 subunit cytoplasmic tail interacts with endocytic adaptor proteins such as AP2, which recruits integrin α5β1 to clathrin-coated structures (Vignoud et al., 1994). In clathrin-independent integrin endocytosis, for example, αLβ2 integrin is internalized and rapidly recycled by a cholesterol-sensitive pathway (Fabbri et al., 2005). However, many integrins can be internalized by more than one mechanism, for instance the αvβ3 integrin be internalized into coated structures or be internalized by other routes (Caswell et al., 2009).

Following endocytosis, integrins travel to early endosomes and can then be targeted to lysosomes for degradation or recycled to plasma membrane. These recycling processes depend on Rab family small GTPases, such as Rab11 and Rab4 (Caswell et al., 2008; Caswell et al., 2009). Although the recycling loop has important roles in cell migration, the regulatory mechanisms remain to be determined.

The plasma membrane recycling system also removes membrane from the cell surface and adds membrane at the front in migrating cell may contribute to extension of the cellular leading edge (Bretscher, 1996a). Plasma membrane trafficking pathways that contribute to cell migration have not been fully elucidated, but recent studies have implicated several SNAREs (SNAP23, VAMP3, VAMP4 and syntaxin13) that are involved in the process (Jahn and Scheller, 2006; Cocucci *et al.*, 2008). In addition, integrins are also involved in the regulation of endocytosis and the recycling of the lipid membrane by Rac-PAK signaling (del Pozo et al., 2004). To clarify the mechanisms of polarized delivery of membranes during cell migration will help to understand the process of cell motility.

1.1.7 *Microtubules in cell migration*
Microtubules are a class of cytoskeletal components that are involved in regulation of cell division, cell migration, vesicle transport and cell polarization (Watanabe *et al.*, 2005; Chien *et al.*, 2009). During cell migration in 2D, microtubules are oriented and organized at the leading edge (Etienne-Manneville, 2004a; Watanabe et al., 2005), while residing at the rear of the nucleus during migration in 3D (Doyle et al., 2009). Microtubules also target to CMACs and regulate cell attachment (Wu et al., 2008). A recent study reports that an AMP-activated protein kinase (AMPK) phosphorylates the microtubule plus end protein CLIP-170 thereby controlling directional cell migration (Nakano et al., 2010).

1.2 Cancer
In the body, normal cells progress through a tightly regulated process of growth, division, and death, which are controlled by the genes and the microenvironment. Sometimes the regulation of this process goes wrong and cancer begins to form. Cancer is a genetic disease caused by sequential accumulation of mutations in genes (Balmain et al., 2003; Michor et al., 2004). Environmental factors increase cancer risk, such as smoking, radiation from the sun, X-rays and chemicals toxins. Cancer also can be caused by viruses, such as the human papilloma virus (HPV) and the epstein barr virus (EBV). Age and hereditary also are important risk factors for cancer.

Alterations in the three different types of genes mainly contribute to cancer progression. These are tumor suppressor genes, oncogenes and DNA repair genes (Balmain et al., 2003; Osborne et al., 2004). These represent the main types of genes involved in cancer; however other mutated genes may also contribute to cancer progression. Data collected from the Network of Cancer Genes (NCG) show 736 genes that are mutated in different cancer types (Syed et al., 2010).

Cancer cells are able to invade nearby tissues and spread to other parts of the body through blood and lymphs system, for example breast cancer can invade to nearby lymph nodes and spread to the liver (Müller et al., 2001). The spread of cancer is called metastasis. Metastasis is the most lethal stage of cancer. Cell attachment to the ECM is a basic requirement to build a multi-cellular organism but is also responsible for a wide range of normal and abnormal cellular activities including cancer cell invasion and metastasis (Hanahan and Weinberg, 2000; Holly *et al.*, 2000; Bergers and Benjamin, 2003). Studies have shown that αv integrins play a role in cancer, for example integrin αvβ3 in melanoma increases lymphatic metastases (Nip et al., 1992), in prostate cancer αvβ3 increases bone metastasis (McCabe et al., 2007); αvβ6 in colon and cervical cancers decrease patient survival (Bates et al., 2005; Hazelbag et al., 2007); and αvβ5 in Glioblastoma increases cancer invasion (Bello et al., 2001). Although integrins are not oncogenic, studies have shown that some oncogenes require integrin signaling to mediate tumorigenesis and metastasis. Also crosstalk between specific integrins and growth factors promote tumour progression (Desgrosellier and Cheresh, 2010).

1.3   The p21-activated kinase family

The p21-activated kinase (PAK) is a family of serine/threonine kinases that was initially identified as binding partners of the Rho GTPases Cdc42 and Rac1 (Manser *et al.*, 1994). The PAK family members play essential roles in cell signaling and control a variety of cellular functions including cell morphology, cytoskeletal dynamics and motility, (Abo *et al.*, 1998; Zhang *et al.*, 2002; Bokoch, 2003; Kumar *et al.*, 2006; Eswaran *et al.*, 2009; Paliouras *et al.*, 2009). However, the role of the PAK family in physiological and pathological processes is not completely understood.

1.3.1   *Structure*

Six PAK isoforms are expressed in the human. Based on their structural and functional similarities, the six members of the human PAK family are classified into two groups: group I consisting of PAK1, PAK2 and PAK3, and group II consisting of PAK4, PAK5 and PAK6 (Manser *et al.*, 1994; Abo *et al.*, 1998; Dan *et al.*, 2002; Lee *et al.*, 2002; Hofmann *et al.*, 2004). All PAKs consist of an N-terminal PBD (p21-GTPase-binding domain) and a highly conserved C-terminal serine/threonine-kinase domain (Figure 4). The group I PAKs contains an N-terminal autoinhibitory domain as a means to inhibit their kinase activity (Lei et al., 2000). This proline-rich region of PAKs is associated with binding to Nck, an adapter protein that is known to be involved in the regulation of actin cytoskeletal dynamics (Zhao *et al.*, 2000). Both Rac and Cdc42 can bind to the p21-GTPase-binding domain in group I PAKs.

The central regions in group II PAKs are less conserved and contain various numbers of proline-rich potential SH3 (Src homology 3) domain-binding sites (Figure 4). A RhoA GEF-binding site has been identified in the central region of PAK4 but this sequence is not present in PAK5 and PAK6 (Callow *et al.*, 2005). We also found an integrin-binding motif within the PAK4 kinase domain that is reasonably conserved in all PAK family members (Zhang *et al.*, 2002).
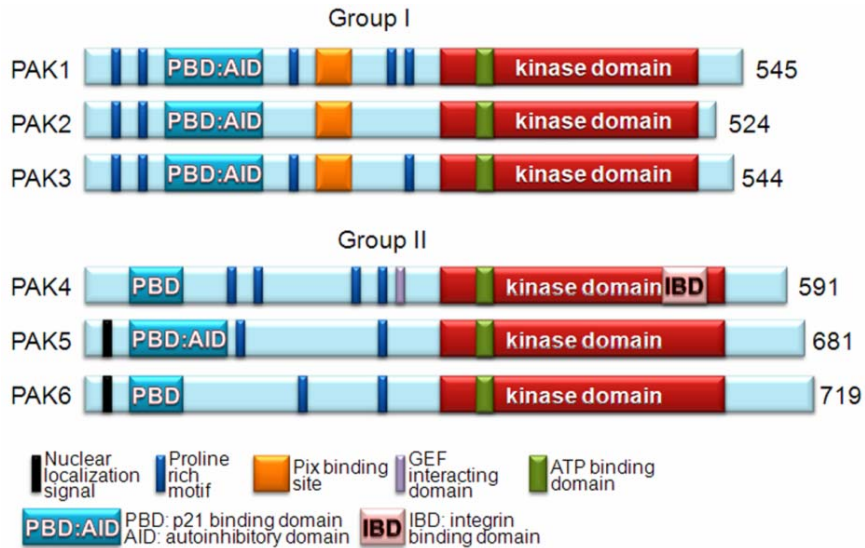
Figure 4. Structure of the two group of p21-activated kinase: All six PAKs have an N-terminal PBD and a C-terminal serine/threonine kinase domain. The central region is more divergent, with various numbers of putative SH3-domain-binding sites.

### 1.3.2   *Activation of PAK4 kinase and its substrates*

PAK1 can be activated by both the small GTPases Cdc42 and Rac (Manser *et al.*, 1994; Bagrodia and Cerione, 1999). Unlike PAK1, PAK4 has a constitutive basal kinase activity (Abo et al., 1998). The PAK4 interaction with Cdc42 only targets the translocation of PAK4 to the Golgi, and it may have no influence on enzymatic activity (Abo et al., 1998; Dan et al., 2001). However, other studies show that PAK4 and PAK5 are activated by small GTPases Cdc42 and Rac1 (Ching et al., 2003; Koh et al., 2008). Although the mechanism of PAK4 activation remains to be elucidated, it is clear that PAK4 can be activated by growth factor stimulation, such as hepatocyte growth factor (HGF) and keratinocyte growth factor (KGF) (Wells *et al.*, 2002; Lu *et al.*, 2003; Ahmed *et al.*, 2008). In addition, the MKK6/p38 MAP kinase pathway also regulates PAK4 activation (Kaur et al., 2005).

A number of PAK4 substrates have been identified. For examples, PAK4 phosphorylates LIMK1 and increases its ability to phosphorylate cofilin resulting in polymerization of actin filaments; PAK4 promotes cell survival by phosphorylating the pro-apoptotic protein BAD; PAK4 phosphorylates GEF-H1 thereby reducing RhoA activity; and PAK4 phosphorylates integrin β5, regulating cell migration (Dan *et al.*, 2001; Gnesutta *et al.*, 2001; Callow *et al.*, 2005; Li *et al.*, 2010c).

10

1.3.3 PAK4 function in cell migration and cancer

The PAK members show different tissue-specific expression patterns (Abo *et al.*, 1998; Callow *et al.*, 2002). PAK1, PAK3, PAK5 and PAK6 have limited tissue expression patterns, whereas PAK2 is ubiquitously expressed (Manser *et al.*, 1994; Teo *et al.*, 1995; Jaffer and Chernoff, 2002; Pandey *et al.*, 2002; Wang *et al.*, 2002). PAK4 is also ubiquitously expressed at low levels in adult tissues but is highly expressed during development (Qu *et al.*, 2003). PAK4 is involved in many cellular functions, such as in organ development, cell growth, cell survival, cell proliferation, neurological disorders, as well as in angiogenesis and luminogenesis (Gnesutta *et al.*, 2001; Qu *et al.*, 2003; Cammarano *et al.*, 2005; Li and Minden, 2005; Danzer *et al.*, 2007; Koh *et al.*, 2008; Tian *et al.*, 2009). PAK4 is also highly over-expressed in most tumor cell lines, and can promote tumorigenesis in vivo (Callow et al., 2002; Kimmelman et al., 2008; Liu et al., 2008; Siu et al., 2010).

Accumulating evidence indicate an important function of PAK4 in regulating cell migration (Figure 5). PAK4 dynamically regulates adhesion complex formation (Zhang *et al.*, 2002; Li *et al.*, 2010b), mediates the induction of linear F-actin polymerization and filopodia in response to Cdc42 (Abo et al., 1998), and induces a increase in stress fibers via LIMK1 and cofilin signaling pathways (Dan et al., 2001). However, other studies show that activated PAK4 decrease the amount of stress fibers (Qu *et al.*, 2001; Wells *et al.*, 2002; Li *et al.*, 2010b), suggesting a more complex role for PAK4 in the regulation of the cell migration. The knowledge of the molecular mechanisms of PAK4´s involvement in cell migration still contains gaps. For example, it still has to be clarified how the multiple functions of PAK4 are regulated in time and space during the different steps of cell migration.
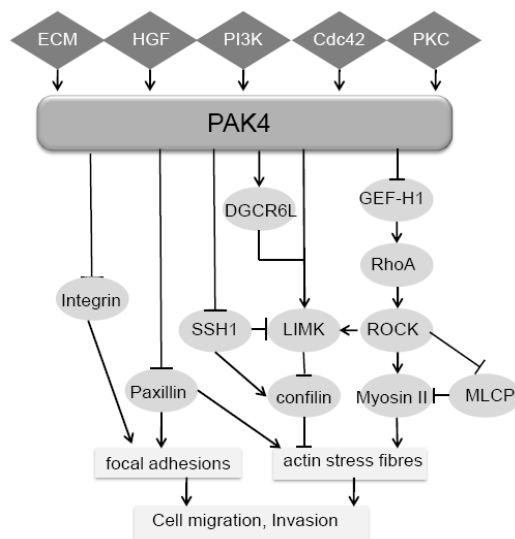

Figure 5. *PAK4 signaling pathways:* PAK4 can be activated by the HGF-Met RTK-PI3K pathway. The activated PAK4 phosphorylates GEF-H1 on serine 885, which inhibits RhoA activity. Actin stress-fibre formation downstream of RhoA activity is mediated by ROCK promotion of myosin II phosphorylation or inactivation of myosin light-chain phosphatase (MLCP). However, PAK4 can also drive the polymerization of actin filaments by the phosphorylation of cofilin through activating LIMK1 and inactivating SSH-1 by phosphorylation. The interaction of PAK4 with a DiGeorge syndrome critical region 6-like protein (DGCR6L) also induces the phosphorylation of LIMK1(Li et al., 2010a). In parallel, activated PAK4 can phosphorylate paxillin on serine 272, driving the dissolution of actin stress fibres and focal adhesions. Cell attachment to the ECM can also activate PAK4 and activated PAK4 can phosphorylate the integrin β subunit to regulate cell-matrix adhesion complexes. Thus, PAK4 dynamically regulates both rearrangement of the actin cytoskeleton and turnover of adhesion complexes that facilitate cell motility.

1.4 Integrins

Integrins are heterodimeric cell surface trans-membrane receptors. They mediate attachment between the cell and the extracellular matrix (ECM) or to other cells (Hynes, 1992). Integrins transmit bi-directional signals across the plasma membrane that are central to many basic cellular functions and pathological processes, such as proliferation, differentiation, apoptosis, wound healing, tumor invasion and metastasis (Hirsch *et al.*, 1996; Clezardin, 1998; Zheng *et al.*, 1999; Holly *et al.*, 2000; Miyata *et al.*, 2000; Zheng *et al.*, 2000; Paulhe *et al.*, 2001; Hollenbeck *et al.*, 2004).
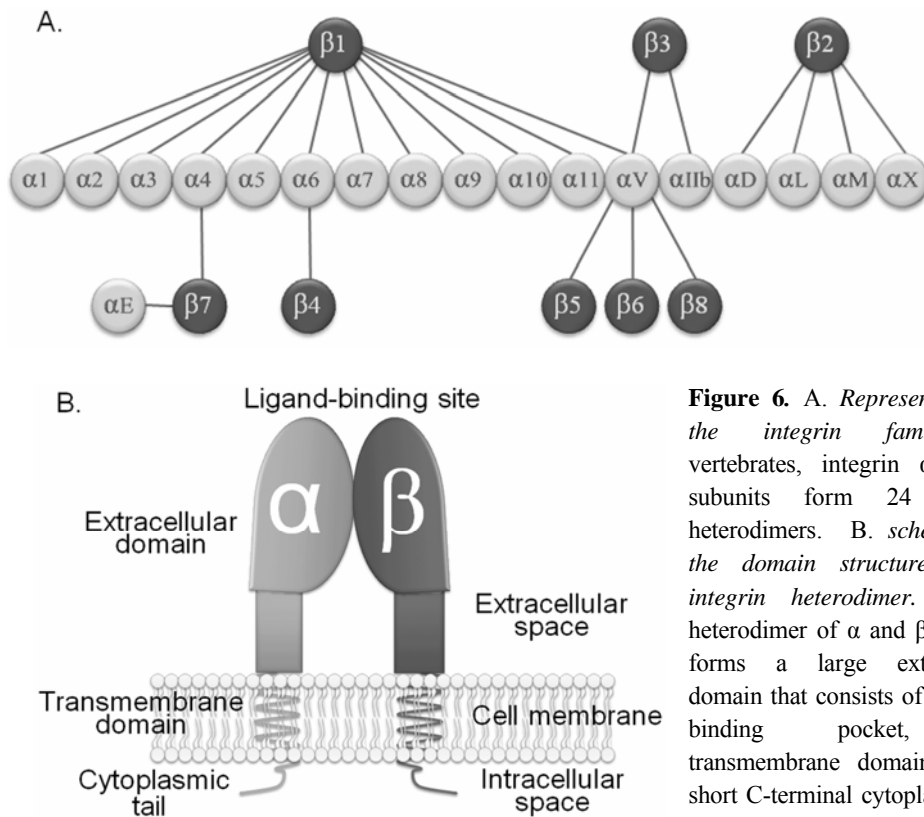
Figure 6. A. *Representation of the integrin family.* In vertebrates, integrin α and β subunits form 24 distinct heterodimers. B. *schematic of the domain structure of an integrin heterodimer.* Integrin heterodimer of α and β subunits forms a large extracellular domain that consists of a ligand-binding pocket, one transmembrane domain, and a short C-terminal cytoplasmic tail for each subunit.

1.4.1 Integrin structure and ligands

Integrins contain two distinct chains, that are termed α and β subunits. In mammals, 18 α and 8 β subunits have been characterized and different combinations of these α and β subunits form 24 distinct integrin hetrodimers (Figure 6A) (Berman and Kozlova, 2000; Humphries, 2000; Takada *et al.*, 2007). However, a cell line normally does not express all 24 integrins. Individual cells selectively express different integrins and modulate their integrin specificity and affinity for ligands (Hynes, 1992). Integrin α and β subunit both contain a large amino-terminal extracellular domain, a single trans-membrane domain, and a short carboxyl-terminal cytoplasmic tail (except the integrin β4) (Figure 6B). Their extracellular domains can bind to either ECM macromolecules or counter-receptors on adjacent cell surfaces (Hynes, 2002). A specific integrin can often bind to several different ligands and different ligands are recognized by more than one integrin, such as the αvβ5 integrin to vitronectin, and the αvβ3 integrin to a variety of ECM proteins containing the peptide sequence arginine-glycine-aspartate (RGD)

12

which can be found in ECM proteins including collagen, vitronectin, fibronectin, fibrinogen.

1.4.2 Integrin activation

Integrins require an activation step to bind to physiological ligands (Hynes, 1992). Structural studies have revealed that integrins may exist in low-, intermediate-, and high-ligand binding affinity states, it is thought that integrins are in a low-affinity state when their extracellular domains are in a bent conformation (inactive) and in a high-affinity state when those are extended conformation (active) (Figure 7) (Xiong *et al.*, 2001; Takagi and Springer, 2002; Xiao *et al.*, 2004). The integrin-ligand binding affinity regulation (activation) can be controlled by the interaction of the integrin cytoplasmic tails with cytoplasmic proteins (inside-out signaling) or the extracellular domain with their ligand (outside-in signaling) (Liddington and Ginsberg, 2002). For example talin, a major cytoskeletal protein at integrin adhesion sites, binds to integrin β subunit cytoplasmic tails and regulates integrin activation, and also kindlin family proteins also contribute to regulate integrin activation (Tadokoro *et al.*, 2003; Wegener *et al.*, 2007; Moser *et al.*, 2009). The activation, and de-activation, of integrins is crucial for cell migration and tumor cell invasion (Hood and Cheresh, 2002).
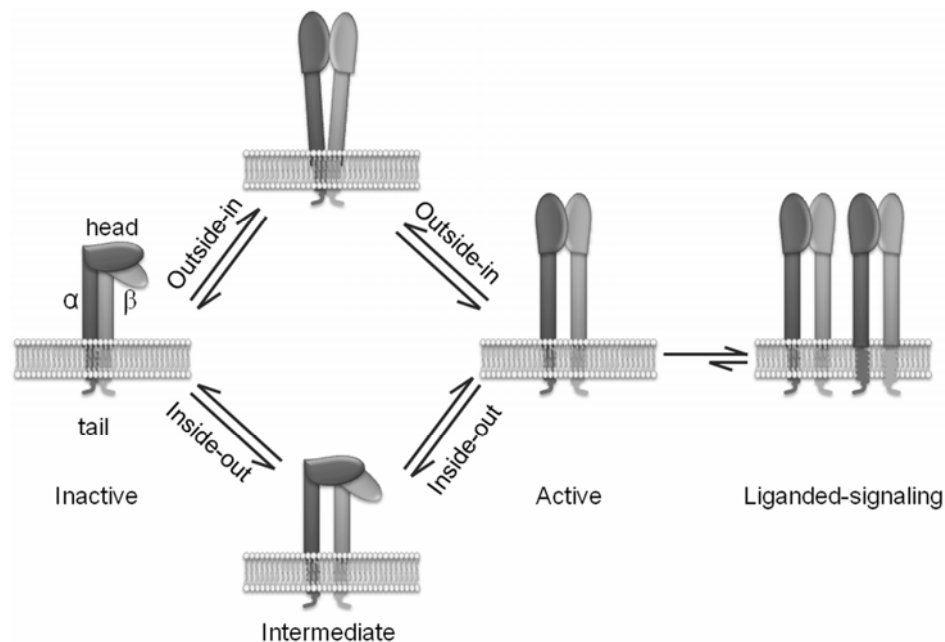
Figure 7. *A schematic diagram of inside-out or outside-in signaling controls integrin activation. Opening of the head or legs/feet opens the other end of the integrin perhaps through an intermediate state(s). The ligand-occupied active integrin causes further conformational changes resulting in clustering and cell signaling. (Based on Xiong 2003* Blood *102)*

1.4.3 Integrin cytoplasmic tails

The integrin α and β cytoplasmic tails are very short and lack enzymatic activities. The membrane-proximal regions of the α and β cytoplasmic domains can interact with each other via a salt bridge (Hughes *et al.*, 1996). Disruption of this salt bridge can change the integrin from low-affinity to high-affinity state, indicating that the short

cytoplasmic domains of integrins are essential for both inside-out and outside-in signaling (Giancotti and Ruoslahti, 1999; Liddington and Ginsberg, 2002).

The integrin cytoplasmic tails (which offer various cytoplasmic protein-binding sites) can transmit signals through interactions with the cytoskeleton, signaling molecules, and other cellular proteins to control integrin activity to regulate cell behavior. So far, a growing number of the integrin cytoplasmic tail-binding proteins have been identified, including actin-binding proteins, such as talin, filamin, zyxin and a-actinin; enzymes, such as ILK and FAK; and adaptor proteins, such as paxillin, viculin (Liu et al., 2000; Lock et al., 2008). The integrin α and β cytoplasmic tails contain many motifs that dynamically interact with specific integrin-binding proteins (Liu *et al.*, 2000). One of the best studied motifs in integrin-signaling events is the NPXY (where X is any amino acid) motif found in many of the integrin β subunit cytoplasmic tails (Reszka *et al.*, 1992). The conserved NPXY motif frequently binds to phosphotyrosine-binding (PTB) domains of other protein, such as talin. Studies indicate that NPXY-talin binding is a key step to integrin activation (Liddington and Ginsberg, 2002; Wegener et al., 2007). In this thesis, six integrin β5 cytoplasmic tail-binding proteins were identified by yeast two-hybrid screening (Zhang *et al.*, 2002). We also determined a membrane-proximal integrin β5-SERS-motif to be involved in controlling cell attachment and migration (Zhang *et al.*, 2002).

1.4.4   Phosphorylation of integrin cytoplasmic tails
Another important mechanism where cellular signaling influences cellular behavior is through the phosphorylation of integrin cytoplasmic tails by intracellular proteins (Liu *et al.*, 2000; Phillips *et al.*, 2001; Fagerholm *et al.*, 2004; Anthis *et al.*, 2009; Legate and Fassler, 2009). Phosphorylation of integrin cytoplasmic tails may also regulate the activation state of integrins, for example, β integrin tyrosine phosphorylation can regulate talin-induced integrin activation (Calderwood *et al.*, 1999; Tadokoro *et al.*, 2003; Wegener *et al.*, 2007; Millon-Fremillon *et al.*, 2008; Anthis *et al.*, 2009). Integrin phosphorylation at tyrosine residues has been found in the cytoplasmic domains of α6A, β1, β3 and β4 (Gimond *et al.*, 1995; Sakai *et al.*, 1998; Cowan *et al.*, 2000; Boettiger *et al.*, 2001; Dans *et al.*, 2001; Datta *et al.*, 2002). Also, serine/threonine phosphorylation of integrin cytoplasmic domains has been found in α4, β1, β2, β3 and β7 subunits (Dahl and Grabel, 1989; Reszka *et al.*, 1992; Barreuther and Grabel, 1996; Valmu *et al.*, 1999a; Valmu *et al.*, 1999b; Kirk *et al.*, 2000; Han *et al.*, 2001; Fagerholm *et al.*, 2002; Hilden *et al.*, 2003). However, so far only a few protein kinases that phosphorylate integrin cytoplasmic domains have been identified. c-Src was found to be responsible for tyrosine phosphorylation, whilst protein kinase C and integrin-linked kinase may mediate serine/threonine phosphorylation of integrins (Novak *et al.*, 1998; Sakai *et al.*, 2001; Fagerholm *et al.*, 2002).

1.4.5   Integrin αvβ5 in cancer
αv integrins are up-regulated or activated in migratory and invasive mechanisms *in vivo*, including in wound healing, angiogenesis, and metastasis (Felding-Habermann and Cheresh, 1993; Brooks *et al.*, 1994; Friedlander *et al.*, 1995; Strömblad and

14

Cheresh, 1996b; Strömblad and Cheresh, 1996a; Brooks *et al.*, 1997). Integrin αvβ5 mediates cell attachment and migration on vitronectin (Wayner *et al.*, 1991; Strömblad and Cheresh, 1996b; Yebra *et al.*, 1996; Hynes, 2002; Lock *et al.*, 2008). Importantly, growth factor activation of integrin αvβ5 mediated cell motility has been functionally linked to angiogenesis as well as carcinoma cell dissemination (Friedlander *et al.*, 1995; Strömblad and Cheresh, 1996b; Brooks *et al.*, 1997). Furthermore, integrin αvβ5 is induced in keratinocytes during wound healing and facilitates vascular endothelial growth factor-mediated vascular permeability (Larjava *et al.*, 1993; Eliceiri *et al.*, 2002; Sheppard, 2004). In addition, most carcinoma specimens from patients express integrin αvβ5 (Lehmann *et al.*, 1994; Jones *et al.*, 1997). However, the role of integrin αvβ5 in cancer is still not clear.

2　Aims of present study

Dissemination by metastasis is the most common cause of death in cancer patients. While the molecular mechanisms involved in the metastatic process are poorly understood; it is clear that integrins are crucial to cell migration and tumor metastasis (Hynes, 2002). To understand how cells migrate may help us to find new ways for development of cancer therapy. The overall aim of this project was to clarify functionality and mechanisms of the integrin β5 cytoplasmic tail in regulating cancer cell adhesion and motility.

Specific aims:

1. To identify integrin β5 subunit cytoplasmic tail binding proteins and address their role in cancer cell lines. **(Paper I)**
2. To elucidate molecular mechanisms of PAK4 regulation of cell-matrix adhesion complex dynamics. **(Paper II)**

3. To determine the influence on cell migration of the PAK4-integrin β5 interaction. **(Paper III)**

3   Methodological considerations

All materials and methods used in this thesis are presented in detail in the corresponding papers. However, some general principles and backgrounds of the methodologies will be described in this section.

3.1   Cell culture

Cell lines derived from tumor or normal mammalian tissues are grown in cell culture under controlled conditions. Cell culture is a core laboratory technique and provides the cellular materials for various biological assays. Cell culture is a relatively simple technique compared to studies using animal organs.

Cell culture *in vitro* is a useful model for studying cell migration. For example, cultured cells can be transfected with specific genes to produce proteins or with RNAi to knock-down specific genes, the cells can be then plated on a variety of ECM substrates, and subcellular structures can be immunostained and observed under a flourescent or confocal microscope. Furthermore, we can select different cell lines for specific purposes. For example, to analyze integrin $\alpha v \beta 5$-mediated cell adhesion on VN, in order to avoid interference from integrin $\alpha v \beta 3$ (both $\alpha v \beta 5$ and $\alpha v \beta 3$ are major vitronectin receptors), we used human MCF-7 breast carcinoma cells (expressing $\alpha v \beta 5$ but not $\alpha v \beta 3$) and CS-1 hamster melanoma cells (expressing endogenous integrin αv but not integrin $\beta 3$ or $\beta 5$) in this study.

Traditional 2D cell culture lacks many features of tissues, such as blood circulation, hormone level, oxygen pressure, loosing original tissue organization and structure; since cells are not entirely in contact with each other.

Because 2D cell culture is not a natural environment for cell growth, 3D cell culture techniques have been developed to more closely mimic *in vivo* tissue environments and has been applied to study cell motility (Even-Ram and Yamada, 2005). In 3D model systems will likely be very helpful in future investigations to understand the mechanisms of cancer cell migration and invasion.

3.2   Flow cytometry analysis

Cell-surface expression of integrins can be regulated by intracellular pools. Flow cytometry or FACS (Fluorescence Activated Cell Sorter) is a powerful technique to detect particular integrins expressed at the cell surface. Cells expressing a particular integrin (or transfected integrin) can also be selected and sterilely sorted for further studies and propagation (Filardo *et al.*, 1996). Through this method, a portion of the cell population can be isolated that have, for example, low or high expression of a particular integrin. Further growth of these selected cells creates a population with slightly higher or lower expression of integrins. In this study, we successfully created stable cells expressing the same level of integrin $\beta 5$-WT and integrin $\beta 5$ mutants in CS-1 cells by FACS sorting after staining with anti-integrin $\alpha v \beta 5$ mAb P1F6 and a FITC-conjugated goat anti-mouse secondary antibody as described (Filardo *et al.*, 1996; Bao and Strömblad, 2004).

3.3 RNA interference

RNA interference (RNAi) was firstly described in plants in the early 1990s, also referred to as post-transcriptional gene silencing (PTGS) (Napoli et al., 1990). It is a powerful tool used for specific gene silencing by siRNA triggering a sequence-specific mRNA degradation in mammalian cultured cells. There are two general methods for producing siRNAs in cultured cells: delivery of synthetic siRNAs, and introduction of a DNA construct that expresses short hairpin RNA sequences (shRNA) (Figure 8). In the cells, a specific cellular enzyme called DICER recognizes the double-stranded RNA and cleaves it to 21-27 nucleotide fragments. One strand of the siRNA (the guide strand) is then assembled into an RNA-induced silencing complex (RISC). The incorporated RNA strand determines the sequence-specificity of the target gene silencing. RISC binds to the mRNA that is targeted by the single RNA strand within the complex and cleaves the mRNA. This cleaved mRNA cannot be translated into protein. Then the RISC dissociates from the mRNA and can cleave other mRNAs. By this way, even a low number of the RNA-induced silencing complex can lead to high-level gene silencing. In this study, we specifically silenced PAK4 gene in MCF-7 cells by delivery of synthetic siRNAs or introduction of a shRNA plasmid.
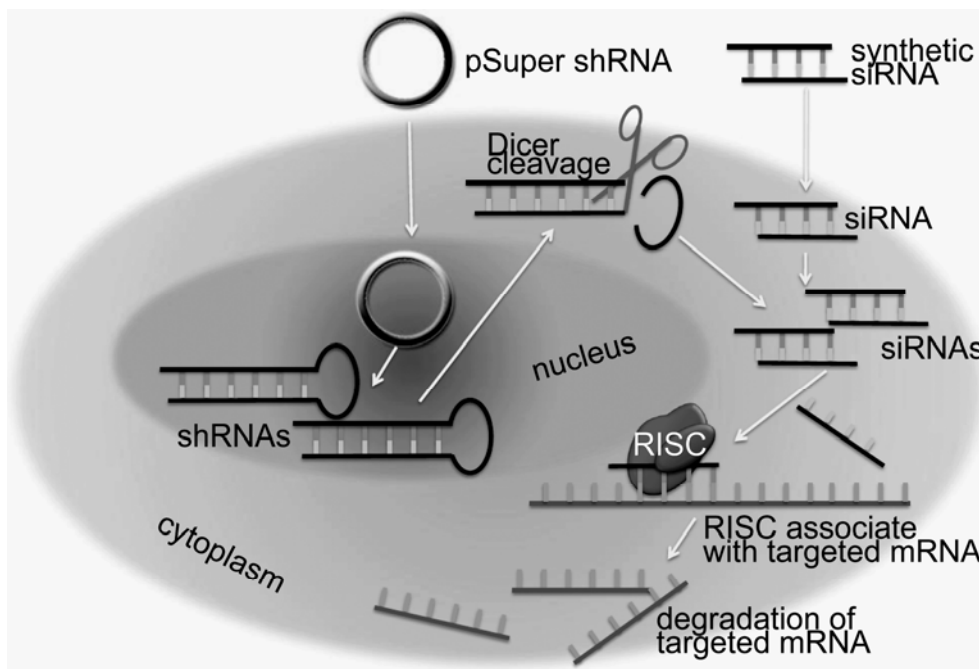
Figure 8. RNA interference (RNAi) triggers mRNA degradation by vector to express short hairpin RNA (shRNA) or by synthetic short interfering RNA (siRNA). In each case, gene silencing results from destruction of mRNA that is complementary to the input siRNA or the siRNA molecules created by Dicer cleavage shRNAs. Dicer: cytoplasmic nuclease; RISC: RNA-induced silencing complex; mRNA: messenger RNA.

However, use of RNAi silencing can cause off-target effects (Jackson et al., 2003; Sledz and Williams, 2004). Off-target effects in RNAi are still poorly understood. Off-target effects by RNAi may occur by sequence overlaps with the target gene, but non-specific effects are also possible (Jackson et al., 2003; Sledz and Williams,

18

2004). High doses of RNAi is cause off-target effects (Jackson et al., 2003). When using siRNA, it is important to use more than one siRNA that have non-overlapping sequences, to use appropriate negative controls, and to confirm depletion of the target protein by Western blot and/or immunofluorescence. An add-back experiment is also a very useful control and can also be used to test the function of a mutated gene, where the wild-type is depleted by RNAi and then the mutant is introduced.

One of the challenges of using this as therapy for medical needs will be the development of methods that will allow for the introduction of interfering RNAs into various cells and tissues of the body. Access to the blood stream and the cells is relatively easy; however it is usually difficult to induce interfering RNAs into most other cells and tissues. Delivery systems that allow this technology to be applied to specific tissues will be a great advantage in the future.

3.4   Yeast two-hybrid screening and yeast mating tests

The yeast two-hybrid assay is a molecular biology technique used to discover protein-protein interactions by testing for interactions between two proteins (Young, 1998). The yeast two-hybrid assay can be used either to screen a cDNA library to find protein-protein interactions or to test for interactions between two previously cloned proteins. Yeast two-hybrid assay used a series of yeast transformations followed by selection of positives in nutrient-deficient media.

The yeast two-hybrid system is an *in vivo* technique testing protein interactions in living cells, and it does not require isolated protein (only the gene) that is fairly easy to perform. The system is also very sensitive and can detect weak protein-protein interactions. On the other hand, the yeast cell environment may not fully mimic mammalian cells (e.g. post-translational modifications may not be replicated in yeast). False positives can also occur in the yeast two-hybrid system. Therefore, after yeast two-hybrid system screening, the results require verification by other methods, such as *in vitro* binding, co-immunoprecipitation and co-localization assays. In this study, we successfully identified an integrin β5 cytoplasmic tail interacting protein, PAK4, by using the DupLEX-A Yeast Two-Hybrid System (OriGen Technologies) (Figure 9).
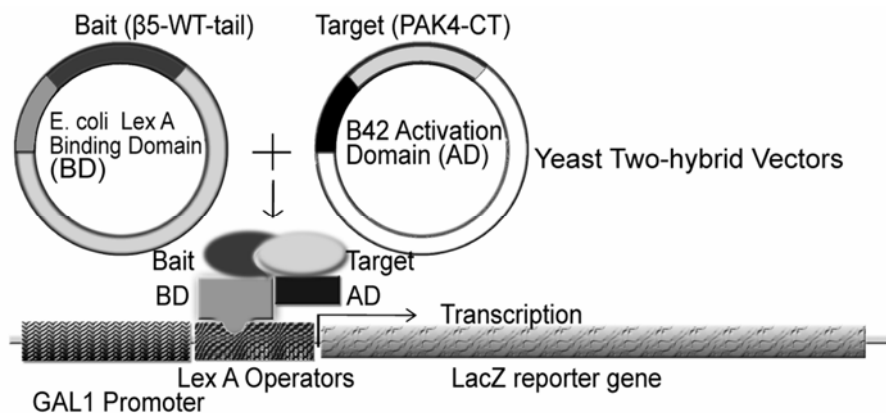
Figure 9. *Schematic diagram of the yeast two-hybrid system to detect interactions between two proteins:* The integrin β5 cytoplasmic domain is fused with *E.Coli* Lex A Binding Domain (BD) in the pEG202 bait vector and the PAK4-CD is fused with B42 Activation Domain (AD) in pJG4-5 prey vector. If the two proteins (Bait and Target) do not interact, there is no expression of the LacZ reporter gene. If they do interact, then the LacZ reporter gene is expressed. (from Li et al., *Journal of Biological Chemistry.* 2010)

3.5 Kinase activity assay

Protein kinases phosphorylate substrate proteins by transferring phosphate groups from ATP to serine, threonine, tyrosine or other residues of the substrate proteins. In particular, protein phosphorylation plays a significant role in a wide range of cellular processes. Thus, assessing the catalytic activity of a specific protein kinase may provide valuable information on signal‐transduction pathways that affect cell behaviors (Brabek and Hanks, 2004). Also identification of specific phosphorylation sites in proteins is important for understanding protein-protein interactions, intracellular signaling pathways, such as in the regulation of cell survival, proliferation, differentiation and death. In this study, we successfully determined the PAK4 kinase activity by *in vitro* and *in vivo* radiometric kinase activity assays as described in our papers (Zhang *et al.*, 2002; Li *et al.*, 2010b; Li *et al.*, 2010c).

In the protein kinase assays, the protein kinase usually is purified by immune-precipitation from a cell extract. However, the purified protein kinase may be contaminated by other kinases, and therefore appropriate negative controls are needed. It should also be noted that some antibodies may inhibit or activate a kinase to affect the kinase assay result. The harmful effects of radioactivity is a concern for human health and environment protection, which makes use of other non-radiometric methods advantageous, for example use of phosphorylation-specific antibodies.

3.6 Cell migration assay

There are different types of cell migration: migration of cells based on chemoattractants (chemotaxis); cell migration towards or within a gradient of substratum (haptotaxis); movement of cells through the vascular endothelium (transmigration); migration of cells into a wound to close the gap (wound healing); and random movement of cells stimulated by chemical reagents (chemokinesis).

The Boyden Transwell chamber was constructed by Stephen Boyden for a leukocyte chemotaxis assay (Boyden, 1962). The Transwell chamber assay has been developed, and widely used in 2D and 3D cell migration and/or invasion assays, including chemotaxis, haptotaxis, as well as chemokinesis. In general, cells are added in the upper chamber and are allowed to migrate through a micro-porous membrane into the lower chamber below the membrane, where addition of growth factors (chemotaxis) and/or coating with ECM (haptotaxis) can be applied. However, for the chemokinesis assay, equal concentrations of an agent are added in both ends. After an appropriate incubation time, the membrane is fixed and stained, and the number of cells that have migrated to the lower side of the membrane is determined (Figure 10).
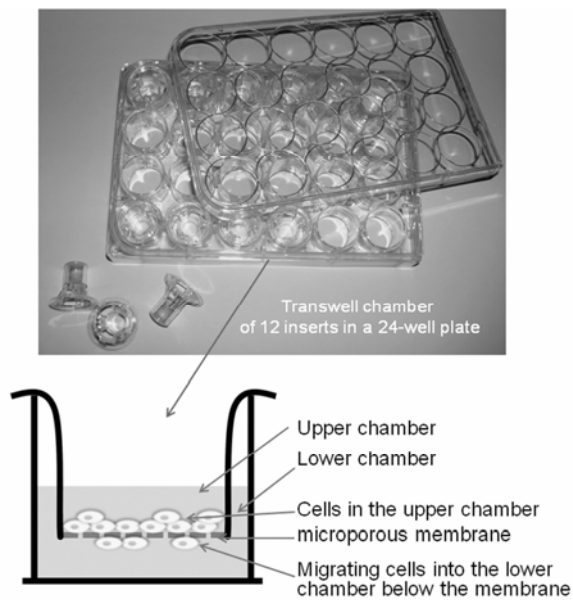
Figure 10. Schematic of Boyden Transwell shows the separation of upper and lower chambers by a microporous membrane. Cells are added in the upper chamber and allow migrating cells through the microporous membrane into the lower side of membrane.

The Boyden Transwell chamber-based cell motility assay is easy to perform and the results are generally very robust. In addition, the results are not affected by cell proliferation. In this study, we use Transwell chambers with 8.0 µm pore size (Costar Inc.) coated with different ECM to analyze haptotactic cell migration in two different cell types (MCF-7 and CS-1 cells). In our experience, the number of cells loaded and the incubation time affect the result. Therefore, this assay needs to be optimized for each individual cell types.

3.7 Immunofluorescent staining and adhesion complex quantifications

Immunofluorescent staining of cultured cells is a useful technique for studying the localization of a specific protein in the cell, using a specific antibody chemically conjugated with a fluorescent dye to bind a specific protein or antigen in cells. Two major types of immunofluorescent staining methods are most commonly used: direct immunofluorescent staining and indirect immunofluorescent staining. The direct immunofluorescent staining uses a primary antibody directly labeled with a fluorescent dye, and indirect immunofluorescent staining uses a secondary antibody linked with a fluorescent dye to recognize a primary antibody. The procedure of direct immunofluorescent staining is more simple and faster than the indirect immunofluorescent staining, and can avoid some non-specific binding that can lead to increased background signal while indirect staining gives an enhanced signal. Immunofluorescent staining can be performed on cells fixed on slides. The stained samples are observed under a fluorescent microscope or a confocal microscope.

In this study, we used indirect immunofluorescent staining method to stain actin and focal adhesion structures in different cell types. The slides were examined by using an IX71 Olympus microscope with a 100×/1.35 oil objective and a Hamamatsu CCD camera or a Zeiss LSM510 confocal microscope with a 63×/1.4 oil objective. The numbers of focal CMACs at the cellular periphery were quantified manually. Because automatic image analysis for the CMACs is more accurate than manual, we also used

21

Patch Morphology Assay 5.2.0 software (Digital Cell Imaging Labs, Edegem, Belgium) to analyze the CMAC number, size and density.

### 3.8   Fluorescence Recovery After Photobleaching (FRAP)

Fluorescence Recovery After Photobleaching (FRAP) is typically used to study the mobility of fluorescently labeled proteins (Axelrod *et al.*, 1976; Reits and Neefjes, 2001). With this technique, the recovery of fluorescence in a defined region of interest (ROI) of a sample is monitored after a bleaching event by taking a time series of images. Bleaching is performed with high intensity light, particularly using lasers, which permit a tightly delimited application of energy. The recovery of fluorescence results from the movement of unbleached fluorophores from the surroundings into the bleached area. To represent the recovery data, the mean intensity in the bleached ROI is normally plotted versus time. In this context, the recovery half-time (time taken to achieve 50% of maximal recovery - $t_{1/2}$) indicates the speed of recovery/mobility, e.g. diffusion speed, and the maximum level of recovered intensity gives information on ratio of mobile/immobile species within the fluorescent molecule population (Figure 11).

It is commonly observed that maximal recovery of a FRAP curve is often lower than the pre-bleach fluorescence intensity. This occurs because some of the bleached molecules within the FRAP ROI are immobile. Hence these immobile proteins do not make available free binding sites within the FRAP ROI, thereby limiting the recruitment of unbleached proteins and reducing maximal fluoresence recovery. The difference (usually expressed as a percentage) between the pre-bleach intensity and post recovery maximal intensity is therefore referred to as the immobile fraction (of the total molecular population within the bleached ROI). Conversely, the fraction of molecules exits the bleached ROI and is replaced by unbleached molecules represent the mobile fraction.

Within biological systems, the combined kinetics of molecular diffusion and molecular interactions affect the mobility of molecules. Therefore, the FRAP recovery curve is determined by both diffusion rates and the chemical interactions of the studied molecules. In general, molecular diffusion is determined by the molecule size, environment viscosity, and the temperature of the surrounding medium, as well as by physical structures. If the diffusion rate is faster than the interaction rate of the molecule, the rate limiting factor for mobility will be the interaction, and in this case the FRAP recovery curve is also dominated by the chemical interaction. If the interaction of the molecule is faster than the diffusion, then the mobility of the molecule is dominated by the diffusion.

We assessed integrin β5 turnover rates within focal adhesion complexes by applying FRAP within MCF-7 cells over-expressing mRFP and β5-EGFP or mRFP-PAK4 and β5-EGFP. Integrin β5-EGFP was bleached using 40 iterations at 40% of total laser power from the 488 nm line of a 4-line argon laser (Coherent). β5-EGFP was imaged pre (×3) and post (×31) bleaching using 0.24% of total 488 nm laser power at an interval of 30 s. mRFP was imaged using 35% of total laser power from a 543 nm laser

22

(Coherent). Entire adhesions were bleached and recovery of adhesions measured using free drawn ROIs and mean intensity quantified in Image J software (version 1.32), followed by analysis using Microsoft Office Excel 2003. We analyzed the random intra-plasma membrane diffusion of individual integrin heterodimers and microclusters (diffusion model), and total recovery including both random intra-plasma membrane diffusion of individual integrin heterodimers and microclusters, and the selective recruitment and concentration of integrins into existing focal adhesion structures (reaction-diffusion model). We also showed the data without diffusion of individual integrin by removing the diffusion recovery from the total recovery (reaction model).
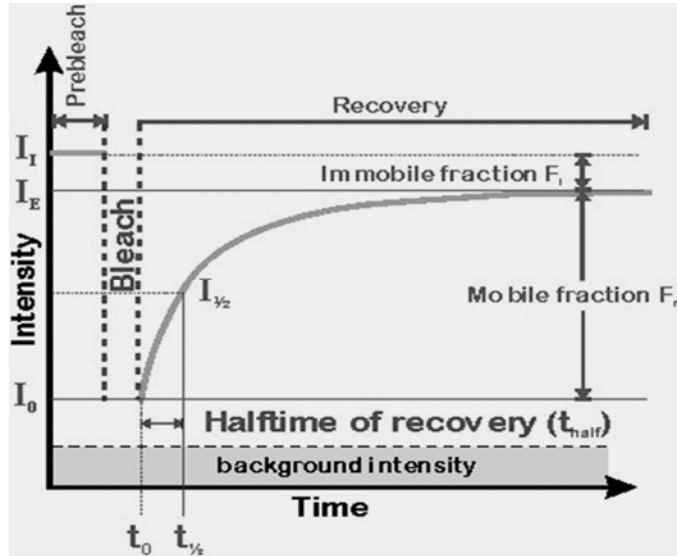
Figure 11. *An idealized plot of a FRAP recovery curve.*
I_I: initial intensity
I_0: intensity at timepoint t_0 (first postbleach intensity)
$I_{1/2}$: half recovered intensity ($I_{1/2} = (I_E - I_0) / 2$)
I_E: end value of the recovered intensity
t_{half}: Halftime of recovery corresponding to $I_{1/2}$ ($t_{1/2} - t_0$)
Mobile fraction $F_m = (I_E - I_0) / (I_I - I_0)$
Immobile fraction $F_i = 1 - Fm$
(from EAMNET FRAP on-line teaching module, EMBL).

4 Summary of results and discussion

4.1 Paper I:
p21-activated kinase 4 interacts with integrin αvβ5 and regulates αvβ5-mediated cell migration.

Rack1 was previously the only protein known to directly interact with the integrin β5 cytoplasmic tail (Liliental and Chang, 1998). In this thesis, we identified six additional proteins specifically interacting with the human integrin β5 cytoplasmic tail by use of yeast two-hybrid screening of a 19-d mouse embryonic cDNA library. One of the proteins was p21-activated kinase 4 (PAK4). The interaction of integrin β5 with PAK4 was then verified by GST pull-down and co-immunoprecipitation assays, concluding that the integrin β5 cytoplasmic tail associates with PAK4 in mammalian cells. Furthermore, we mapped the PAK4 binding to the membrane-proximal region of integrin β5, and identified an integrin-binding domain at aa 505-533 in the C-terminus of PAK4.

Given that PAK4 associated with integrin αvβ5, we analyzed the effect on the cellular distribution of endogenous PAK4 by αvβ5-mediated attachment to VN in MCF-7 cells. We observed a cytosolic distribution of PAK4 in MCF-7 cells during regular culture conditions. However, after replating cells onto VN, we found a remarkable re-distribution of PAK4 to forming lamellipodial structures in the cellular periphery. To examine whether the lamellipodia-localized PAK4 may interact with integrin αvβ5 at the cell membrane, we re-plated MCF-7 cells onto VN and co-stained the cells for endogenous PAK4 and endogenous integrin αvβ5.

PAK4 and integrin αvβ5 in early forming adhesion complexes both localized proximally to the cellular edge but without specific co-localization at the sites of clustered integrins. The presence of PAK4 in the zone where the integrin αvβ5-mediated adhesions were forming would most likely allow interactions between PAK4 and integrin αvβ5, but they occur at a spatial and temporal resolution not able to be detected by light microscopy. Because lamellipodia structures are involved in the process of cell migration, this led us to hypothesize that PAK4 may not only interact with αvβ5, but that PAK4 may also influence integrin-mediated motile events.

The potential effect of PAK4 on αvβ5-mediated cell motility was therefore examined using a Transwell haptotactic cell migration assay. The results indicated that PAK4 specifically induced integrin αvβ5-mediated MCF-7 human breast carcinoma cells motility. Given that cell adhesion strongly affects cell migration, it was interesting to examine whether PAK4 may also impact cell adhesion on VN. Our cell adhesion data showed that MCF-7 cells expressing EGFP-PAK4 showed a markedly decreased cell adhesion on VN compared to EGFP-transfected cells. However, over-expression of PAK4 did not change the abundance of integrin αvβ5 expression on the cell membrane. This suggests that PAK4 inhibition of cell adhesion might be caused by an alteration of integrin αvβ5 binding capacity for its ligand VN.

24

Taken together, this study suggests that PAK4 binds to the integrin β5 cytoplasmic domain in motile cellular structures and that PAK4 modulates integrin αvβ5-mediated cell adhesion and migration. This may be brought about by PAK4 regulation of cytoskeletal components and/or by directly influencing integrin αvβ5 function, thereby modulating cell migration.

In this study, we concluded that:
1. PAK4 can directly interact with the integrin β5 subunit cytoplasmic tail.
2. PAK4 translocates to the lamellipodia upon integrin ligation to VN.
3. PAK4 selectively induces αvβ5-mediated cell motility.

4.2 Paper II:
Integrin-mediated cell attachment induces a PAK4-dependent feedback loop regulating cell adhesion through modified integrin αvβ5 clustering and turn-over.

Cell adhesion to the ECM induces integrin activation and triggers a variety of intracellular signaling cascades, including the activation of PAK1 (Price *et al.*, 1998). By using an *in vitro* kinase assay, we found that PAK4 could also be activated by replating of cells onto the ECM ligand VN using two different cell lines (COS-7 and MCF-7 cells). To examine whether cell attachment-induced PAK4 activation may play a role in integrin αvβ5-mediated cellular functions, we performed cell adhesion and migration assays using the human breast carcinoma MCF-7 cell line (which expresses only integrin αvβ5 as its VN receptor) with either over-expression or RNAi-mediated depletion of PAK4. The results were consistent with our previous results showing that wt PAK4 over-expression reduced cell adhesion capacity and enhanced cell migration (Zhang *et al.*, 2002). Conversely, knock-down of PAK4 increases cell adhesion and reduces cell migration. These results further suggest that PAK4 plays an important role in the regulation of integrin αvβ5-mediated cell adhesion and migration (Zhang *et al.*, 2002; Ahmed *et al.*, 2008).

Cell motility is closely related to cell spreading. Although over-expression of PAK4 markedly promoted cell migration onto VN (Zhang *et al.*, 2002) and a hyper-active PAK4 mutant caused cell rounding (Qu *et al.*, 2001). It has therefore been unclear to what extent PAK4 may affect cell spreading. To this end, we quantitatively compared the area of MCF-7 cells either over-expressing PAK4 or with PAK4 knocked-down by shRNA, after re-plating onto VN. We observed that PAK4 over-expression reduced cell spreading capacity, while knock-down of PAK4 markedly induced cell spreading. Thus, our findings indicate a clear role for PAK4 in the regulation of carcinoma cell spreading.

Integrin-mediated cell-matrix adhesion complex (CMAC) dynamics are critically involved in cell migration (Huttenlocher et al., 1996; Lock et al., 2008). Based on the physical and functional correlations between cell motility and the PAK4-integrin β5 interaction, we hypothesized that PAK4 may play a role in regulating CMAC dynamics. Accordingly, we performed detailed, quantitative, imaging-based analyses of the impact of PAK4 in hundreds of cells and on several thousand cell-matrix adhesion

complexes. We focused on the impact of PAK4 on peripheral CMACs, defined as those found within 5 μm of the cell border, because this region gives rise to the key assemblages of cell motility, including lamellipodia and filopodia (Wehrle-Haller and Imhof, 2003). Our findings indicated that PAK4 over-expression causes a general depletion of CMAC number, size, integrin clustering density and integrin-to-F-actin connectivity, with an especially potent effect on the development of larger adhesion complexes. This is implying a key role for PAK4 in the inhibition of CMAC formation and particularly on the maturation of focal adhesions (FAs). Importantly, PAK4-shRNA expression caused a marked increase in the number and size of CMACs, enhanced integrin clustering, with small CMACs displaying a higher integrin clustering density. PAK4 knock-down also significantly increased the amount of F-actin recruited to CMACs, implying enhanced connectivity of these complexes to the actin cytoskeleton, as compared to control cells. Overall, CMACs in the absence of PAK4 matured more efficiently, as denoted by enhanced integrin clustering with respect to CMAC area, as well as more frequently and with greater actin connectivity than CMACs in control shRNA expressing cells.

Although PAK1 has been suggested to regulate overall CMAC stability (Manser *et al.*, 1997; Kiosses *et al.*, 1999; Stofega *et al.*, 2004), it has been unclear whether PAKs may affect integrin turn-over within existing CMACs. Given that mature, relatively stable FAs have been shown to display higher integrin turn-over rates than smaller/more immature and less stable FCs (Ballestrem *et al.*, 2001; Wehrle-Haller and Imhof, 2003), the inhibitory effect of PAK4 on CMAC maturation was expected to correlate with decreased integrin turn-over. However, surprisingly, PAK4 over-expression accelerated integrin turn-over as determined by fluorescence recovery after photo-bleaching (FRAP) analysis.

Combined with data indicating reduced CMAC number, size, density and F-actin connectivity, these findings clearly identify a role and plausible mechanism for PAK4 in the destabilization of αvβ5-mediated CMACs, ultimately resulting in reduced cell-ECM adhesion strength. Together, our data show that PAK4 is activated by integrin αvβ5 ligation to VN, and that active PAK4 can then act to de-stabilize integrin-mediated adhesion structures to limit cellular adhesion levels. This biological circuit represents a novel auto-inhibitory feedback loop that is intrinsic to the core machinery of cell adhesion, providing cells the capacity to autonomously tune and optimize total cell-extracellular matrix adhesion levels.

In this study, we concluded that:
1. PAK4 can be activated by the cell ligation to integrin αvβ5 ligand vitronectin.
2. Activated PAK4 in turn regulates integrin αvβ5-mediated MCF-7 cell adhesion, spreading and migration.
3. PAK4 enhances integrin αvβ5 molecular turn-over within CMACs, thereby de-stabilizing these CMACs and likely causing the inhibition of integrin clustering, as well as of CMAC maturation and F-actin association.

4. PAK4 is also shown to act upon integrin αvβ5 in environments that are devoid of detectable actin connectivity, such as in nascent CMAC clusters and outside of detectable CMACs, thereby supporting an actin-independent integrin regulatory capacity.

4.3   Paper III:

p21-activated kinase 4 phosphorylation of integrin β5 Ser 759 and Ser 762 regulates cell migration.

Cytoplasmic tails of integrins play key roles in a variety of integrin-mediated events including adhesion and migration (Hynes, 2002). We previously found a role for PAK4 in selective regulation of integrin αvβ5-mediated cell motility (paper I) (Zhang *et al.*, 2002). However, whether PAK4 promotes cell motility through its interaction with integrin αvβ5 and/or its effects on the actin cytoskeleton remained unclear. In this paper, we focus on the molecular mechanisms of this regulation. Firstly, we fine mapped the PAK4 binding site in the integrin β5 cytoplasmic tail and identified a unique PAK4-binding membrane-proximal integrin β5-SERS-motif. We then tested the potential role of the integrin β5 SERS-motif in the regulation of cell adhesion and migration. We found that this integrin β5-SERS-motif was indeed involved in controlling cell attachment and migration.

Secondly, we aimed to clarify whether PAK4 kinase activity and/or its integrin-binding capacity may be responsible for PAK4-induced cell migration. In paper I, we identified an integrin binding domain (IBD) of 29 amino acids in the PAK4 C-terminal region (Zhang *et al.*, 2002). By an *in vitro* kinase assay and GST pull-down experiments combined with mutational and functional analyses, we found that PAK4 binding to integrin β5 was not sufficient to promote cell migration, but that PAK4 kinase activity was required for the PAK4 promotion of cell motility.

Phosphorylation of integrin cytoplasmic tails has been proposed as a means to regulate integrin functions (Fagerholm *et al.*, 2004). Given the role of PAK4 kinase activity in the promotion of cell motility, we examined potential PAK4-mediated phosphorylation of the integrin β5 cytoplasmic tail. We found that the β5 cytoplasmic domain was a specific substrate for PAK4 *in vitro*. Furthermore, we identified two distinct PAK4 phosphorylation sites at serine residues within the membrane-proximal PAK4-binding SERS motif (amino acids Ser 759 and Ser 762). Finally, we found that integrin β5 Ser 759 and Ser 762 are critical for PAK4-induced cell motility and that PAK4-mediated phosphorylation of the β5 cytoplasmic tail appears to regulate cell motility. This may contribute to the understanding of intracellular signaling behind vascular permeability, angiogenesis and carcinoma cell dissemination where activation of integrin αvβ5 has been found to be involved (Lewis *et al.*, 1996; Brooks *et al.*, 1997; Eliceiri *et al.*, 2002).

In this study, we concluded that:
1. A unique PAK4-binding membrane-proximal integrin β5-SERS-motif was identified.

2. The integrin β5-SERS-motif was involved in controlling cell attachment and migration.
3. The β5 cytoplasmic tail SERS-motif can be phosphorylated by PAK4.
4. Phosphorylation of the SERS-motif in the integrin β5 cytoplasmic tail is critical for PAK4-induced integrin αvβ5-mediated carcinoma cell motility.

5 Conclusions

In this thesis, we demonstrated functional mechanisms by which PAK4's interaction with integrin β5 regulates cancer cell adhesion and motility. Our studies suggest a model wherein signaling is initiated by cell adhesion to VN, leading to the translocation of PAK4 from the cytosol to lamellipodia where PAK4 is activated. Activated PAK4 can then phosphorylate the integrin β5 cytoplasmic domain. This reduces CMAC number and size, enhances integrin turnover, inhibits integrin clustering and limits the association of F-actin with CMACs. Exactly how PAK4 may regulate the dynamic linkage between CMACs and F-actin needs to be further investigated. Nonetheless, this array of molecular level effects cumulatively reduces total cell adhesion, while simultaneously facilitating enhanced cell migration (Figure 12). Conceptually, this overall mechanism represents a regulatory negative feedback loop that is initiated by cell adhesion to VN and functions thereafter to limit the level of cell adhesion to VN (Figure 13). This indicates that PAK4 may form part of a tuning mechanism that contributes to the optimization of cell adhesion levels, with strong resulting effects on migration capacity.
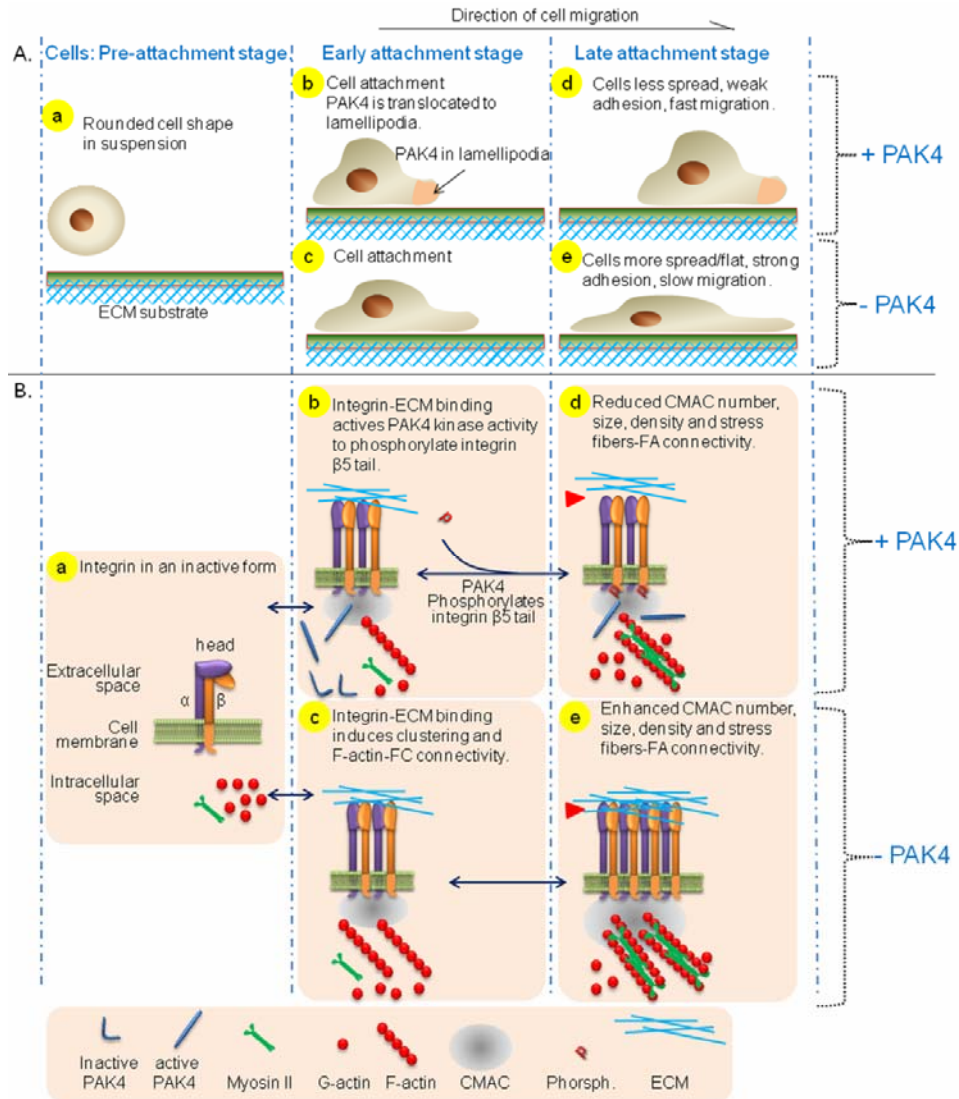
Figure 12: *Regulatory impact of PAK4 at molecular and cellular levels.* A. At the cellular level, when cells in suspension (A-a) are replated and attach onto ECM at an early stage (A-b and c), PAK4 is translocated into lamellipodia in cells over-expressing PAK4 (+PAK4; A-b). At the late attachment stage, PAK4 reduces cell attachment, inhibits cell spreading and induces cell migration in cells over-expressing PAK4 (+PAK4; A-d) compared with control cells (-PAK4; A-e). B. At the molecular level, integrin is initially in the inactive state (B-a). Ligand-binding to the extracellular domain of integrins induce integrin activation, integrin outside-in signaling pathways and integrin clustering (B-b and c). The outside-in signaling may facilitate the translocalisation of PAK4 from the cytosol to lamellipodia, as well as activation of PAK4 (B-b). Activated PAK4 can in turn phosphorylate the integrin β5 cytoplasmic domain and may cause reduction of CMAC number, size and density, as well as inhibition of F-actin-CMAC connectivity (B-d). Thus, PAK4 may induce cell motility through regulating cell-ECM adhesion strength by reducing ECM to CMAC connectivity (as indicated by the red arrows in d and e).
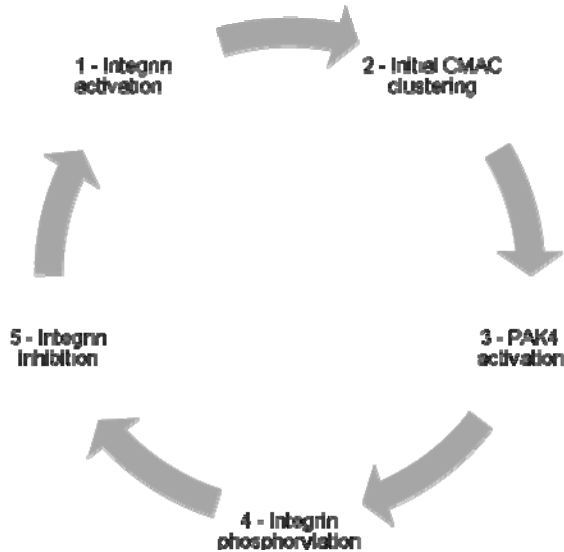
Figure 13: PAK4 is part of a negative feedback loop that limits cell adhesion levels.

30

6   Relevance and perspectives

PAK4-mediated regulation of cell migration can contribute to cancer invasion and metastasis. It will therefore be important to elucidate the role of PAK4 in cancer invasion in 3D environments and metastasis in animal models. Pak4 knock-out mice die in early embryogenesis, indicating that PAK4 is important for early embryonic development (Qu *et al.*, 2003). PAK4 conditional knock-out mice have been constructed, allowing the role for PAK4 to be studied in specific tissues (Liu et al., 2008). A future challenge is to further elucidate PAK4 biological function *in vivo*.

Protein kinases currently constitute a major focus for drug discovery with most major pharmaceutical companies developing inhibitors. In recent years, a number of protein kinase inhibitors have successfully been taken through clinical trials to enter clinical practice. Our studies indicate that PAK4 kinase activity is critical for its function in regulation of cell migration (Zhang *et al.*, 2002; Li *et al.*, 2010c). Although a number of components can activate PAK4, such as VN, HGF and KGF (Wells *et al.*, 2002; Lu *et al.*, 2003; Ahmed *et al.*, 2008; Li *et al.*, 2010b), the mechanism of PAK4 activation is still unclear. It is of importance to elucidate the precise mechanism of regulation of PAK4 kinase activity. Development of PAK4 kinase inhibitors may also facilitate new therapy for cancer. An optimal phosphorylation sequence (RRRRRSWASP) for the group II PAKs has been identified by use of a positional scanning peptide library approach (Rennefahrt *et al.*, 2007). The data provided by peptide library screening may help to identify PAK4-specific phosphorylation sites to identify additional PAK4 substrates.

In addition, a couple of PAK4 down-stream effectors have been identified and they control a variety of cellular functions in different signaling pathways including cell survival, tumorigenesis and cell motility as described in the introduction section of this thesis (Eswaran et al., 2009). Those studies also indicate that PAK4 has a more complex role in regulation of the cell migration (Wells and Jones, 2010). The molecular mechanism of PAK4 in regulation on cell motility, however, still has to be clarified. Therefore, it is important for future studies to focus on the role of PAK4 in dynamic regulation of cell motility in the different steps of cell migration.

A "clutch" model describes temporal-spatial regulation of the connection between F-actin forces and substrate adhesion in the process of cell migration (Giannone et al., 2009). Focal adhesion adaptor proteins provide a dynamic link between F-actin and adhesion complexes, such as vinculin and paxillin (Humphries et al., 2007). We have observed that PAK4 inhibits focal adhesion-actin cytoskeleton connections, suggesting a potential clutch role for PAK4. However, how PAK4 may be involved in the regulation of the clutch between F-actin and adhesion complexes remains to be determined.

Integrin cytoplasmic tails play important roles in integrin-mediated cellular functions (Thiery, 2003; Berrier and Yamada, 2007; Streuli, 2009). In recent years**,** many cellular proteins have been identified that directly or indirectly interact with integrin cytoplasmic tails and this number of CMAC proteins will continue to grow (Zaidel-Bar

et al., 2007). Further studies will be needed to understand how integrins are regulated *in vivo*. It is becoming increasingly clear that CAMC components that can interact with integrin cytoplasmic tails are involved in modulating integrin function. A current challenge is to characterize the effects CMAC components have and to try and understand how CMACs are regulated at the systems level. This may lead to new insights that can be used to provide better clinical therapy for cancer metastasis.

Indeed, some integrin inhibitors, such as cilengitide (an inhibitor of both αvβ3 and αvβ5 integrins) have been used in preclinical and clinical trials (Smith *et al.*, 1990; Alghisi and Ruegg, 2006). In preclinical studies, the cilengitide effectively inhibited angiogenesis and the growth of orthotopic glioblastoma (Yamada *et al.*, 2006). So far, treatment with cilengitide in late-stage glioblastoma patients has shown extending patient survival with minimal side effects (Nabors *et al.*, 2007; Reardon *et al.*, 2008; Desgrosellier and Cheresh, 2010). An oral PAK4 inhibitor, PF-03758309 has also been tested in clinical phase I trials, but these trials have been discontinued for reasons not made public.

PAK4 is over-expressed in an array of different cancers, involves in various oncogenic signaling pathways and links to promote cell survival, growth, tumorigenesis and metastasis (Callow *et al.*, 2002; Li and Minden, 2005; Kimmelman *et al.*, 2008; Eswaran *et al.*, 2009; Siu *et al.*, 2010). Based on these studies, PAK4 appears to be a critical effector in cancer. Our data revealed novel signaling by PAK4 to regulate of cell motility and provides new insights to understand molecular mechanisms involved. In future studies, elucidating the role of PAK4 in caner invasion and metastasis in 3D and animal models will allow the hypotheses generated by this thesis to be tested in more physiological systems. Such refinement of our knowledge of PAK4's function will hopefully be useful for therapeutic development of PAK4 targeting compounds.

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Article

p21-activated kinase 4 interacts with integrin αvβ5 and regulates αvβ5-mediated cell migration

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p21-activated kinase 1 (PAK1) can affect cell migration (Price et al., 1998; del Pozo et al., 2000) and modulate myosin light chain kinase and LIM kinase, which are components of the cellular motility machinery (Edwards, D.C., L.C. Sanders, G.M. Bokoch, and G.N. Gill. 1999. *Nature Cell Biol.* 1:253–259; Sanders, L.C., F. Matsumura, G.M. Bokoch, and P. de Lanerolle. 1999. *Science.* 283: 2083–2085). We here present a novel cell motility pathway by demonstrating that PAK4 directly interacts with an integrin intracellular domain and regulates carcinoma cell motility in an integrin-specific manner. Yeast two-hybrid screening identified PAK4 binding to the cytoplasmic domain of the integrin β5 subunit, an association that was also found in mammalian cells between endogenous PAK4 and integrin

αvβ5. Furthermore, we mapped the PAK4 binding to the membrane-proximal region of integrin β5, and identified an integrin-binding domain at aa 505–530 in the COOH terminus of PAK4. Importantly, engagement of integrin αvβ5 by cell attachment to vitronectin led to a redistribution of PAK4 from the cytosol to dynamic lamellipodial structures where PAK4 colocalized with integrin αvβ5. Functionally, PAK4 induced integrin αvβ5–mediated, but not β1-mediated, human breast carcinoma cell migration, while no changes in integrin cell surface expression levels were observed. In conclusion, our results demonstrate that PAK4 interacts with integrin αvβ5 and selectively promotes integrin αvβ5– mediated cell migration.

Introduction

The p21-activated kinase (PAK)* family contains homologous serine/threonine protein kinases that can act as downstream effectors of the small GTPases Cdc42 and Rac (Lim et al., 1996; Bagrodia and Cerione, 1999; Bar-Sagi and Hall, 2000). So far, six human PAKs (hPAKs) have been identified, and based on homology, they can be classified into two groups: group I including PAK1–3 and group II including PAK4–6 (Dan et al., 2001b). A marked difference between the two PAK groups is the autologous inhibitory sequence in the NH$_2$-terminal regulatory domain found in group I PAKs, with no obvious homologous sequence in group II (Dan et al., 2001b). Due to the presence of an inhibitory sequence that binds to the COOH terminus of group I PAKs, PAK1

displays little or no endogenous kinase activity, but can be activated by the presence of GTP-bound active Cdc42 or Rac, which opens up the folded structure of PAK1 (Lei et al., 2000). PAK1 is known to regulate cell morphology and cytoskeletal reorganization (Sells et al., 1997). Furthermore, membrane-targeted PAK1 has been found to induce neurite outgrowth from PC12 cells (Daniels et al., 1998). In fibroblasts, activated PAK1 has been shown to localize in the leading edge of motile cells (Sells et al., 1999), and in human endothelial cells, PAK1 has been suggested to coordinate the formation of new substrate adhesions at the front of the cell with contraction and detachment at the rear (Kiosses et al., 1999), indicating that PAK1 may be involved in the regulation of cell motility.

PAK4 is the first identified member of the group II PAKs, and is implicated in cytoskeletal reorganization and filopodia formation (Abo et al., 1998). Importantly, PAK4 was recently found to be overexpressed in 78% of a variety of human cancer cell lines, an overexpression that might be mediated by gene amplification and may play a role in Ras-mediated transformation (Callow et al., 2002). In addition, overexpression of a hyperactive PAK4 mutant can protect cells from apoptosis induced by TNFα (Gnesutta et al., 2001). The capability of PAK4 to promote cell survival is

Table I. **Identified interactors with human integrin β5 subunit cytoplasmic domain by yeast two-hybrid screening**

Interacting cDNA clones	Number of clones	GenBank/EMBL/DDBJ accession no.
PAK4	6	AJ011855
Myosin X	5	AJ249706
Bodenin (ICAP)	3	AJ001373
Miz-1	9	AF039567
Mouse cDNA clone	1	AA445617
Mouse myotubes MPLRB5	1	AA816006

Six clones were identical to mPAK4. Five clones represented mouse myosin-X (Yonezawa et al., 2000) and bodenin is the mouse version of human integrin cytoplasmic domain–associated protein (ICAP) (Faisst and Gruss, 1998). Miz-1 is a Myc-interacting zinc finger protein (Seoane et al., 2001). The other two clones have unknown functions.

shared with PAK1, but the anti-apoptotic properties of PAK1 and PAK4 may be mediated by distinct mechanisms (Schurmann et al., 2000; Gnesutta et al., 2001). In addition, hyperactive PAK4 is able to transform fibroblasts to grow in soft agar in an anchorage-independent manner (Qu et al., 2001), perhaps in part due to it's ability to promote cell survival.

Integrins are heterodimeric transmembrane receptors and the major group of receptors for ECM proteins. Integrins are essential during development, in tissue homeostasis, and in the progression of various diseases (Hynes, 1992; Giancotti and Ruoslahti, 1999). By mediating cellular attachment to ECM, integrins are also a central part of the cellular motility machinery, where they are regulated by intracellular signaling molecules, which influence integrin localization, clustering, and binding to the ECM. In addition, integrin engagement to the ECM initiates various signaling events, e.g., activation of the ERK1/2 pathway, which is also important for the regulation of cell motility (Giancotti and Ruoslahti, 1999).

Previous studies have shown that αv integrins are up-regulated or activated in migratory and invasive mechanisms in vivo, including wound healing, angiogenesis, and metastasis (Felding-Habermann and Cheresh, 1993; Brooks et al., 1994; Friedlander et al., 1995; Strömblad et al., 1996; Brooks et al., 1997). Integrin αvβ5 is the predominant vitronectin (VN) receptor for carcinoma cells in vivo, because most carcinoma specimens from patients express αvβ5 but not αvβ3 (Lehmann et al., 1994; Jones et al., 1997). Importantly, integrin αvβ5 has been found to be functionally involved in growth factor–induced carcinoma cell migration in vitro and metastasis in vivo (Klemke et al., 1994; Yebra et al., 1996; Brooks et al., 1997). Furthermore, activation of integrin αvβ5 is implicated in VEGF-induced angiogenesis (Friedlander et al., 1995). In this report, we present a novel role for PAK in cell motility. We found that PAK4 directly interacts with the integrin β5 subunit and specifically regulates αvβ5-mediated cell migration.

Figure 1. **PAK4 interacts with the integrin β5 subunit.** (A) Amino acid sequence comparison of mPAK4 KD, found by the yeast two-hybrid screening with hPAK4, *Drosophila* MBT (MBT), and hPAK1 KDs. Dashes stand for identical amino acids and the variations of amino acids are indicated by letters in hPAK4, MBT, and hPAK1 sequences. The mPAK4 KD is numbered according to that of hPAK4 (Abo et al., 1998). (B) PAK4 association with integrin β5 in GST pull-down assays. GST–PAK4 KD fusion protein pulled down endogenous integrin β5 in cell lysates (top panel). GST–β5 cytoplasmic domain fusion protein pulled down overexpressed HA–PAK4 (bottom). The positive controls represent Western blot of the input lysates for marking the size and showing the presence of integrin β5 and PAK4. (C) PAK4 association with integrins in living cells. (C, top) Anti–integrin β3 mAb AP3 and anti–integrin αvβ5 mAb P1F6 coimmunoprecipitation of PAK4 in COS-7 cells expressing HA–PAK4. (C, middle and bottom) An anti-HA mAb coimmunoprecipitated HA–PAK4 with both αv and β5 integrin subunits. The positive controls represent Western blot of the input lysates for marking the size and showing the presence of integrins and PAK4. (D) Association of endogenous PAK4 with endogenous integrin αvβ5 in MCF-7 cells analyzed by IP. IP using rabbit IgG or α-Rab1 are negative controls.

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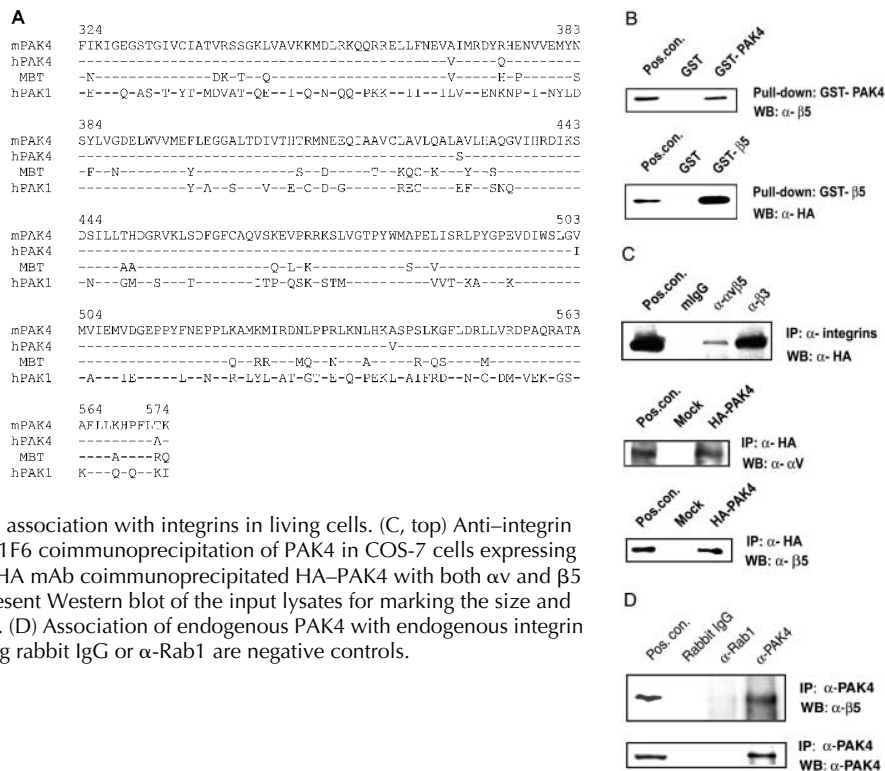
Results

PAK4 directly interacts with the integrin β5 subunit cytoplasmic domain

By means of yeast two-hybrid screening of a 19-d mouse embryo cDNA library and following remating tests, we identified six known or hypothetic proteins specifically interacting with the human integrin β5 cytoplasmic domain (Table I). 25 clones were found to interact with the integrin β5 cytoplasmic domain. Sequence analysis revealed that six of these mouse cDNA clones encoded a sequence highly homologous to hPAK4 kinase domain (KD), and were therefore identified as mouse PAK4 (mPAK4), which strongly and specifically interacted with integrin β5 cytoplasmic domain in the repeated yeast mating tests (unpublished data). An aa sequence comparison of the KD of mPAK4 with hPAK4, hPAK1, and the *Drosophila* PAK homologue mushroom body tiny gene product (MBT) (Melzig et al., 1998) is shown in Fig. 1 A. mPAK4 KD shares 98% homology in aa sequence with hPAK4 KD, 83% with MBT KD, and 56% with hPAK1. The interaction of PAK4 with integrin β5 was then further analyzed by independent biochemical methods both in vitro and in living cells. In GST pull-down assays, we found an association of integrin β5 from cell lysates to a purified GST-fused PAK4 KD and of PAK4 to a purified GST-fused β5 cytoplasmic domain (Fig. 1 B). In addition, HA-tagged PAK4 was coimmunoprecipitated with integrins β3 and αvβ5 in living cells (Fig. 1 C, top). In Fig. 1 C, the β3 immunoprecipitation (IP) appears to bring down more PAK4 than the IP for αvβ5. However, the expression levels of the two integrins are different (unpublished data) and the two antibodies used may be differently efficient for IP. Therefore, differences in PAK4 amounts in this IP cannot be used to indicate relative binding strengths. Furthermore, the reverse IP of HA–PAK4 brought down both integrin αv and β5 subunits from a cell lysate (Fig. 1 C, middle and bottom), whereas IP of an irrelevant HA-tagged p21[CIP1] did not (unpublished data). Importantly, by IP we also found an association of endogenous PAK4 with endogenous integrin αvβ5 in living cells (Fig. 1 D).

PAK4 interacts with the membrane-proximal region of the integrin β5 cytoplasmic domain

To determine which region within the integrin cytoplasmic domain interacts with PAK4, we generated cDNAs encoding various regions of the cytoplasmic domain of integrin β5 by PCR and cloned them into the bait vector pEG202. Yeast mating experiments were performed using a prey vector that contains PAK4 KD (aa 239–591) and the various bait vectors. The PAK4-binding region was mapped to aa 759–767 within the integrin β5 cytoplasmic domain (Fig. 2 A). Furthermore, association of endogenous PAK4 to the membrane-proximal region of integrin β5 subunit was verified by a GST pull-down assay (Fig. 2 B), in which PAK4 associated with GST–β5 cytoplasmic domain, but not with a GST–β5 deletion mutant lacking the PAK4-binding region identified by yeast mating tests. Amino acid sequences of other integrin β subunits corresponding to the PAK4-binding region of integrin β5 cytoplasmic domain were aligned (Fig. 2 C), displaying a moderate sequence homology.

Integrin β5 interacts with a PAK4 COOH-terminal region

To determine the region within PAK4 that interacts with the integrin β5 cytoplasmic domain, we generated cDNAs encoding various regions of the PAK4 KD that were amplified by PCR and cloned into the prey vector pJG4-5. Yeast mating experiments were performed using a bait vector that contains the integrin β5 cytoplasmic domain (aa 753–799) and the various prey vectors. We mapped the integrin-binding region to aa 505–530 within the PAK4 KD by yeast two-hybrid mating tests (Fig. 3 A), and further confirmed the requirement of this region of PAK4 for association with integrin αvβ5 in mammalian cells by IP using a PAK4 deletion mutant (Fig. 3 B). Therefore, we denote this region as the integrin-binding domain (IBD) in PAK4. The aa sequences of other PAK family members, including the *Drosophila* PAK4 homologue MBT, were aligned in comparison with the PAK4 IBD (Fig. 3 C). The IBD region is highly homologous among PAK family members, suggesting that family mem-
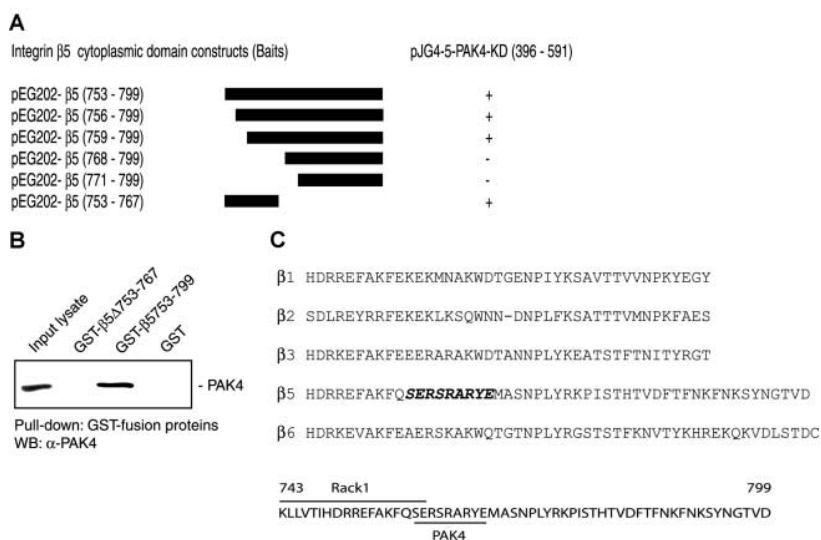
A

Integrin β5 cytoplasmic domain constructs (Baits)   pJG4-5-PAK4-KD (396 - 591)

pEG202- β5 (753 - 799)   +
pEG202- β5 (756 - 799)   +
pEG202- β5 (759 - 799)   +
pEG202- β5 (768 - 799)   -
pEG202- β5 (771 - 799)   -
pEG202- β5 (753 - 767)   +

B

Input lysate / GST-β5₇₅₃₋₇₆₇ / GST-β5₇₅₃₋₇₉₉ / GST

— PAK4

Pull-down: GST-fusion proteins
WB: α-PAK4

C

β1 HDRREFAKFEKEKMNAKWDTGENPIYKSAVTTVVNPKYEGY

β2 SDLREYRRFEKEKLKSQWNN-DNPLFKSATTTVMNPKFAES

β3 HDRKEFAKFEEERARAKWDTANNPLYKEATSTFTNITYRGT

β5 HDRREFAKFQ***SERSRARYE***MASNPLYRKPISTHTVDFTFNKFNKSYNGTVD

β6 HDRKEVAKFEAERSKAKWQTGTNPLYRGSTSTFKNVTYKHREKQKVDLSTDC

743 Rack1   799
KLLVTIHDRREFAKFQSERSRARYEMASNPLYRKPISTHTVDFTFNKFNKSYNGTVD
                  PAK4

Figure 2. **Mapping of the PAK4 binding region within the integrin β5 cytoplasmic domain.** (A) Mapping of the PAK4-binding region in the integrin β5 cytoplasmic domain. Various regions of the integrin β5 cytoplasmic domain were cloned into the bait vector pEG202 and then mated with PAK4 KD in the prey vector in a yeast two-hybrid assay. The PAK4 binding region was mapped to aa 759–767 of the β5 cytoplasmic domain. (B) Association of endogenous PAK4 with the membrane-proximal region within integrin β5 cytoplasmic domain was examined by a GST pull-down assay, including GST fused to a β5 deletion mutant lacking the PAK4-binding region mapped by yeast two-hybrid analysis. (C) Sequence comparison of the PAK4-binding region of integrin β5 with other integrin cytoplasmic domains (top). The Rack1- (Liliental and Chang, 1998) and PAK4-binding regions within integrin β5 are indicated (bottom).
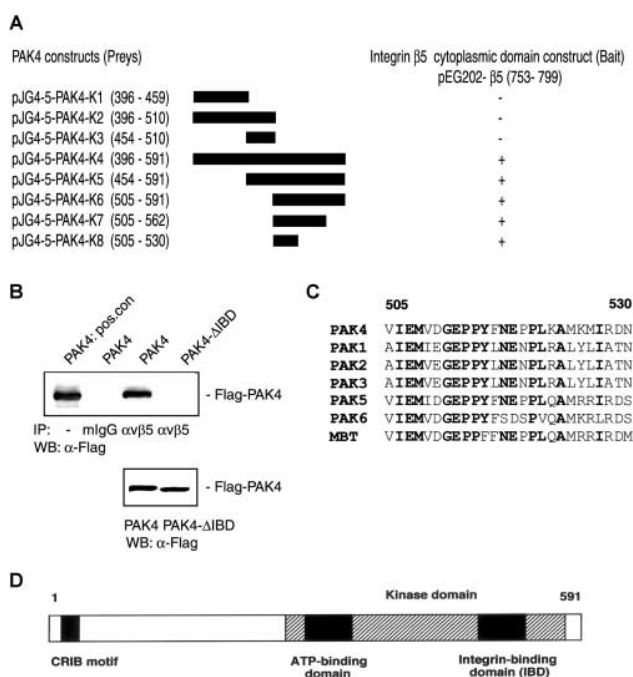
Figure 3. **Mapping of the integrin β5–binding region within the PAK4 KD.** (A) Various regions of the PAK4 KD were cloned into the prey vector pJG4-5 and then mated in a yeast two-hybrid assay with the integrin β5 cytoplasmic domain in the bait vector. Amino acids 505–530 in the PAK4 KD were identified as the responsible region for interaction with the integrin β5 cytoplasmic domain. Therefore, PAK4 aa 505–530 is designated as the IBD. (B) PAK4-ΔIBD with deletion of aa 505–530 was incapable to associate with integrin αvβ5 in mammalian cells, as examined by an IP analysis. Lower panel shows expression levels of PAK4 and PAK4-ΔIBD in lysates used for IP. (C) The corresponding sequences in other PAK family members are compared with PAK4 IBD with identical amino acids in bold. (D) Schematic illustration of the PAK4 structure, including the Cdc42/Rac interactive domain ATP-binding domain, IBD, and KD within PAK4.

bers other than PAK4 might also hold the capacity to bind to integrin cytoplasmic domains. In addition, a schematic illustration of known PAK4 functional motifs indicates the location of IBD within the PAK4 KD (Fig. 3 D).

Translocation of PAK4 to lamellipodia by integrin ligation to VN and colocalization with integrin αvβ5

Given that PAK4 associates with integrin αvβ5, we analyzed the effect on cellular distribution of endogenous PAK4 by αvβ5-mediated attachment to VN in MCF-7 cells, which exclusively use integrin αvβ5 for attachment to VN (unpublished data). Before replating, we observed a cytosolic distribution of PAK4 in MCF-7 cells under normal culture conditions (Fig. 4 A). We then examined the endogenous PAK4 distribution after replating cells onto VN. Interestingly, we found a remarkable redistribution of PAK4 to forming lamellipodial structures in the cellular periphery as early as 10 min after replating on VN. With longer cell attachment, PAK4 was distributed into membrane ruffles and leading edges. However, cells replated onto poly-L-lysine (PLL) that are attached in an integrin-independent manner remained unspread with PAK4 dis-
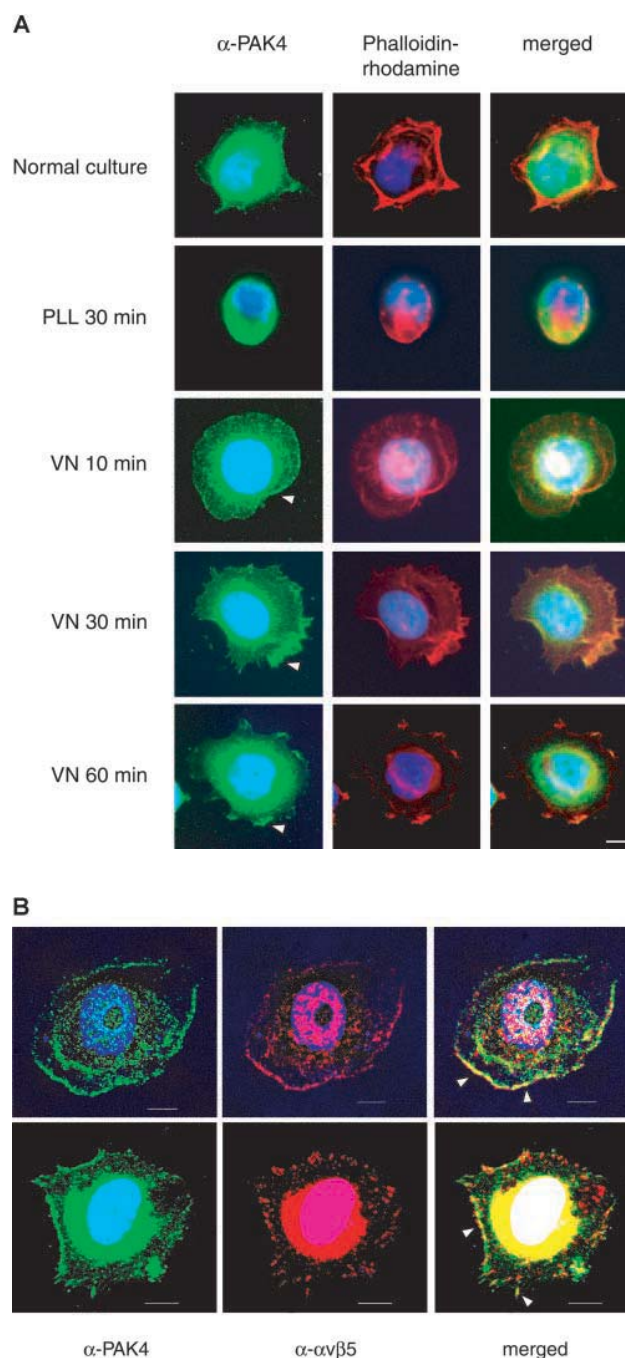
Figure 4. **Relocalization of PAK4 to the cell membrane and colocalization of PAK4 and integrin αvβ5 in MCF-7 cells.** (A) MCF-7 human breast carcinoma cells, under normal culture conditions or replated onto VN or PLL for indicated times, were stained for endogenous PAK4 using an anti-PAK4 pAb (green), for actin using phalloidin–rhodamine (red), and for nuclei by Hoechst (blue). Arrowheads indicate PAK4 localized to lamellipodia or ruffles after replating onto VN. (B) MCF-7 cells were replated onto VN and costained for endogenous PAK4 (green) and endogenous integrin αvβ5 (red). In the top panel (30 min after replating), integrin αvβ5 was found at lamellipodia (arrowheads). In the bottom panel (60 min after replating), integrin αvβ5 has started to form focal complexes at lamellipodia where it colocalized with PAK4 (arrowheads). Bars, 10 μm.
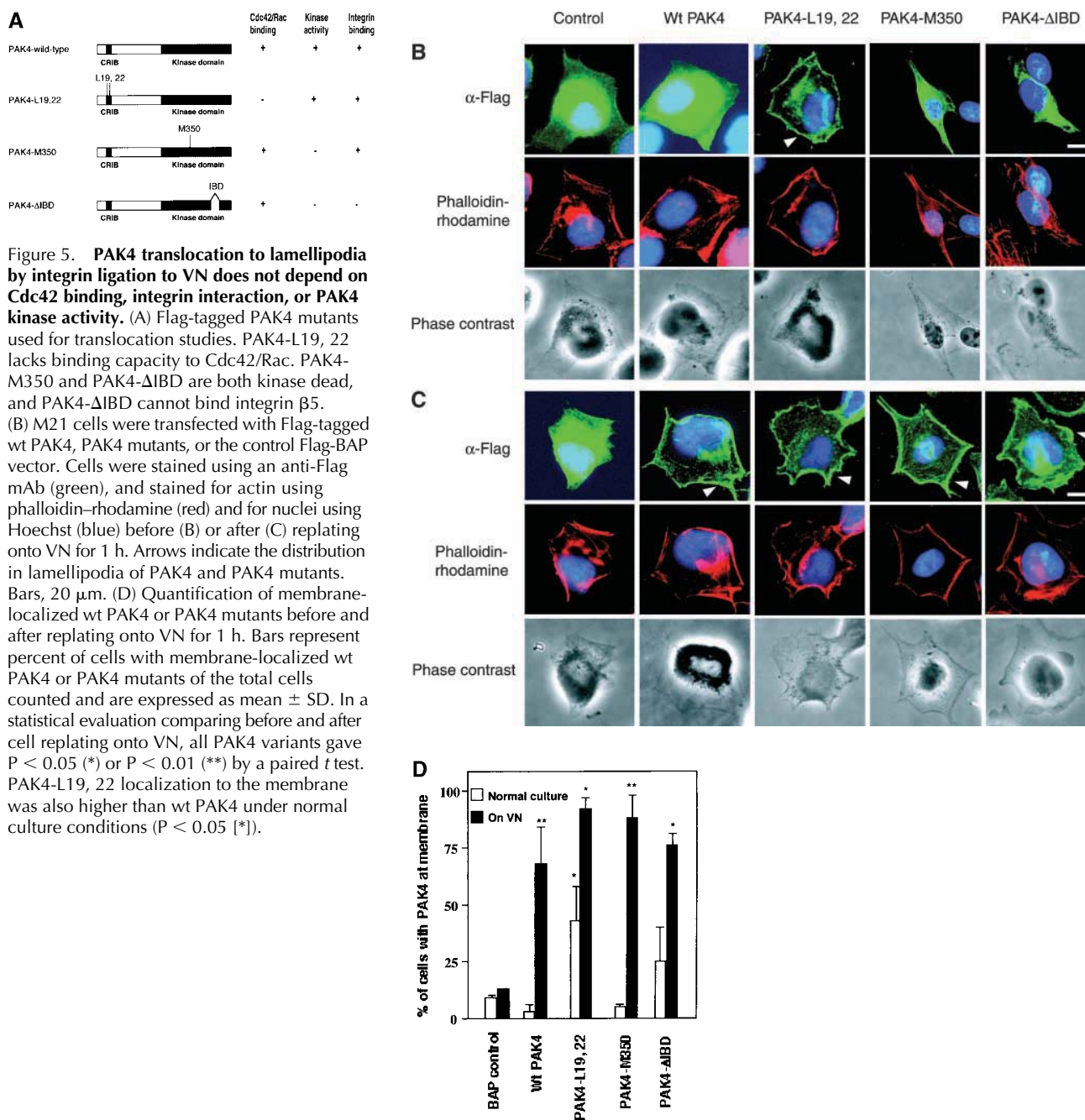
Figure 5. **PAK4 translocation to lamellipodia by integrin ligation to VN does not depend on Cdc42 binding, integrin interaction, or PAK4 kinase activity.** (A) Flag-tagged PAK4 mutants used for translocation studies. PAK4-L19, 22 lacks binding capacity to Cdc42/Rac. PAK4-M350 and PAK4-ΔIBD are both kinase dead, and PAK4-ΔIBD cannot bind integrin β5. (B) M21 cells were transfected with Flag-tagged wt PAK4, PAK4 mutants, or the control Flag-BAP vector. Cells were stained using an anti-Flag mAb (green), and stained for actin using phalloidin–rhodamine (red) and for nuclei using Hoechst (blue) before (B) or after (C) replating onto VN for 1 h. Arrows indicate the distribution in lamellipodia of PAK4 and PAK4 mutants. Bars, 20 μm. (D) Quantification of membrane-localized wt PAK4 or PAK4 mutants before and after replating onto VN for 1 h. Bars represent percent of cells with membrane-localized wt PAK4 or PAK4 mutants of the total cells counted and are expressed as mean ± SD. In a statistical evaluation comparing before and after cell replating onto VN, all PAK4 variants gave P < 0.05 (*) or P < 0.01 (**) by a paired *t* test. PAK4-L19, 22 localization to the membrane was also higher than wt PAK4 under normal culture conditions (P < 0.05 [*]).

tributed in the cytosol (Fig. 4 A). The fact that attachment to VN is mediated by integrin αvβ5, whereas attachment to PLL is integrin independent, indicates that integrin ligation may specifically stimulate relocalization of PAK4 to lamellipodia. Importantly, the redistribution of PAK4 upon cell attachment may allow PAK4 to associate with integrins in lamellipodia, which are sites of integrin attachment to the underlaying ECM. In addition, we observed a similar relocalization of PAK4 in M21 human melanoma cells and ECV 304 human bladder carcinoma cells (unpublished data), suggesting that the relocalization of PAK4 upon replating onto VN occurs in various cell types.

To examine whether the lamellipodia-localized PAK4 could physically meet with integrin αvβ5 at the cell membrane, we replated MCF-7 cells onto VN and costained the cells for endogenous PAK4 and endogenous integrin αvβ5. As shown in Fig. 4 B, PAK4 and integrin αvβ5 colocalized in lamellipodia shortly after replating onto VN. PAK4 may therefore be able to engage in integrin-mediated cellular functions.

Relocalization of PAK4 to lamellipodia does not require its kinase activity, integrin interaction, or Cdc42/Rac binding

Given that PAK4 undergoes membrane relocalization upon attachment onto VN, it was of interest to elucidate whether the relocalization of PAK4 is dependent on its integrin or Cdc42/Rac interaction and/or its kinase activity. Therefore,

A EGFP-PAK4
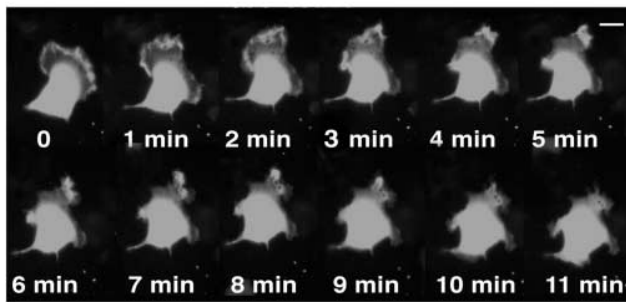
B EGFP
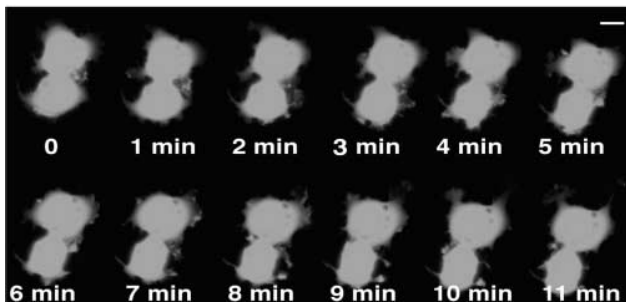
Figure 6. **Dynamic localization of PAK4 in lamellipodia.** MCF-7 human breast carcinoma cells stably transfected with EGFP–PAK4 or EGFP were plated onto VN and visualized by immunofluorescent microscopy after being allowed to attach for 30 min. Images were taken by a CCD camera every minute and the displayed cells are representative for the respective transfection. (A) EGFP–PAK4 is transiently localized in actively reshaping lamellipodia. (B) EGFP control exhibits only nuclear or perinuclear localization. Bars, 20 μm.

we constructed Flag-tagged PAK4 mutants that lack the binding capacity for Cdc42/Rac (PAK4-L19, 22), the IBD (PAK4-ΔIBD), or PAK4 kinase activity (PAK4-M350) as illustrated in Fig. 5 A. Human M21 melanoma cells were transfected with these PAK4 mutants and compared with cells transfected with wild-type (wt) Flag–PAK4 and a vector containing a nonrelated Flag-tagged BAP protein. Under normal culture conditions, wt PAK4 mainly localized in the cytosol (Fig. 5 B). However, upon cell replating onto VN, the majority of cells transfected with PAK4 displayed a relocalization to lamellipodia (Fig. 5 C). A similar relocalization to lamellipodia upon replating was also observed for the kinase-dead and ΔIBD PAK4 mutants, both of them lacking kinase activity (unpublished data) and PAK4-ΔIBD also lacking integrin-binding capacity (Fig. 3 B). However, the PAK4-L19, 22 that lacks GTPase-binding capacity was found in lamellipodia in almost half of the cells in regular culture and was then redistributed to the membrane in the remaining cells upon replating onto VN. A quantification of the PAK4 relocalization by counting the number of cells with membrane-localized PAK4 is displayed in Fig. 5 D. Taken together, these results suggest that PAK4 relocalization to lamellipodia does not require its kinase activity or integrin or Cdc42/Rac binding. However, the Cdc42/Rac binding capacity of PAK4 might be inhibitory for PAK4 localization in lamellipodia.

Dynamic distribution of PAK4 in actively reshaping lamellipodia

To study the temporal and spatial localization of PAK4 in living cells, we established MCF-7 human breast carcinoma cells stably expressing a fluorescent EGFP–PAK4 fusion protein. These cells were plated onto VN-coated glass slides and analyzed by time-lapse fluorescent microscopy. Consistent with our immunofluorescent staining of endogenous PAK4 lamellipodial localization in MCF-7 cells (Fig. 4 A) and of Flag-tagged PAK4 (Fig. 5), EGFP–PAK4 also localized in lamellipodial protrusions after replating onto VN (Fig. 6 A). Interestingly, the PAK4 distribution changed in a highly dynamic fashion, whereas EGFP control cells exhibited only cytoplasmic and nuclear or perinuclear distribution (Fig. 6 B). Furthermore, like endogenous PAK4 (Fig. 4 B), EGFP–PAK4 was also found to partially colocalize with integrin αvβ5 in lamellipodia (unpublished data). The transient localization of PAK4 in lamellipodia coinciding with lamellipodia of actively forming and retracting extensions indicates that PAK4 may modulate these processes. Given that PAK4 associated with integrin αvβ5 and colocalized with integrin αvβ5 in lamellipodia, the dynamic distribution of PAK4 in lamellipodia may reflect a transient and periodic interaction between PAK4 and integrin αvβ5. This led us to hypothesize that PAK4 may not only interact with αvβ5, but that PAK4 may also influence integrin-mediated motile events.

PAK4 stimulates integrin αvβ5–specific cell migration in human breast carcinoma cells

Based on the above hypothesis, we examined the potential effect of PAK4 on αvβ5-mediated cell motility. Integrins αvβ3 and αvβ5 both participate in cell attachment and cell migration toward VN (Wayner et al., 1991). To assess the integrin αvβ5–mediated cell motility, it is ideal to use a cell line expressing αvβ5, but not αvβ3, because αvβ3 usually dominates as VN receptor for cell migration if present, which is the case in most cultured cell lines. Therefore, we chose MCF-7 human breast carcinoma cells, which express αvβ5 but not αvβ3 (unpublished data; Brooks et al., 1997; Wong et al., 1998). In addition, we found that both PAK4 mRNA and protein are highly expressed in MCF-7 cells compared with a number of other tumor cell lines tested (unpublished data), consistent with the recent study by Callow et al. (2002).

In a haptotactic cell migration assay, we found that transient expression of EGFP–PAK4 in MCF-7 cells specifically induced MCF-7 cell migration on VN, but not integrin β1–mediated cell migration on collagen type I (Fig. 7 A). Furthermore, EGFP–PAK4-induced cell migration was blocked by a functional blocking anti-αvβ5 mAb, but not by an anti-αvβ3 mAb (Fig. 7 A, left). Taken together, this demonstrates that PAK4 specifically induces integrin αvβ5–mediated cell motility. Moreover, stable expression of EGFP–PAK4 in MCF-7 cells yielded numerically almost identical results on induction of αvβ5-mediated cell migration as transient PAK4 expression, but did not influence cell motility on collagen (Fig. 7 B). Similarly, stable overexpression of
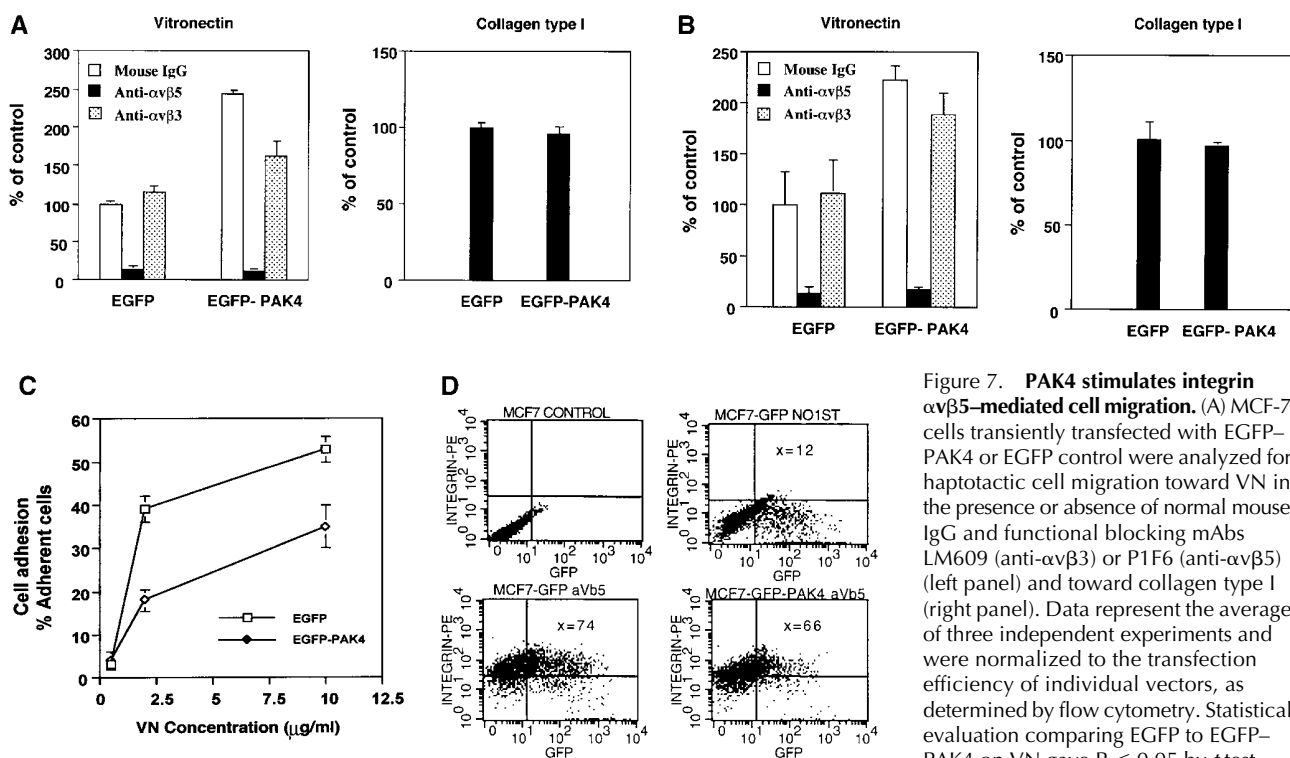
Figure 7. **PAK4 stimulates integrin αvβ5–mediated cell migration.** (A) MCF-7 cells transiently transfected with EGFP–PAK4 or EGFP control were analyzed for haptotactic cell migration toward VN in the presence or absence of normal mouse IgG and functional blocking mAbs LM609 (anti-αvβ3) or P1F6 (anti-αvβ5) (left panel) and toward collagen type I (right panel). Data represent the average of three independent experiments and were normalized to the transfection efficiency of individual vectors, as determined by flow cytometry. Statistical evaluation comparing EGFP to EGFP–PAK4 on VN gave P < 0.05 by t test. (B) Cell migration analyzed as in A of MCF-7 cells stably expressing EGFP–PAK4 or EGFP. All results are expressed as mean values ± SEM of three independent experiments using triplicate analysis in each experiment. Statistical evaluation by t test gave P < 0.05 for EGFP–PAK4 compared with EGFP on VN. (C) Overexpression of PAK4 decreases cell adhesion on VN. Cell attachment of MCF-7 cells stably expressing EGFP–PAK4 or EGFP at different coating concentrations of VN was determined. (D) Transient EGFP–PAK4 expression does not change the membrane expression levels of integrin αvβ5 in MCF-7 cells as measured by flow cytometry. αvβ5 expression was plotted versus EGFP content and the αvβ5 cell surface levels in EGFP-transfected cells (bottom left) was compared with that in EGFP–PAK4 cells (bottom right). Values indicate the mean fluorescence of αvβ5 staining in EGFP-positive cells. Unstained MCF-7 cells (top left) and staining without primary mAb (top right) were used to determine specificity and background.

a Flag-tagged constitutively active PAK4 mutant (S474E; Callow et al., 2002) induced MCF-7 cell migration to VN to the same degree as EGFP–PAK4 (unpublished data). This indicates that overexpression of EGFP–PAK4 may saturate PAK4-inducible motility in this cell type, which might be explained by the observation that a large GST fusion partner at the NH₂ terminus of PAK1 causes constitutive PAK1 activation, suggesting that the EGFP fusion to PAK4 may cause PAK4 activation.

Given that cell adhesion is the basis for cell migration, it was interesting to examine whether PAK4 may also impact cell adhesion on VN. To this end, MCF-7 cells were transfected with EGFP–PAK4 or control EGFP. As shown in Fig. 7 C, overexpression of EGFP–PAK4 markedly inhibited cell adhesion on VN compared with EGFP-transfected cells. One possibility that may explain the inhibition of cell adhesion on VN could be a down-regulation of integrin αvβ5 cell surface expression by PAK4. To examine this, we performed a flow cytometry analysis to determine the cell membrane distribution of integrin αvβ5. However, PAK4 overexpression did not change the abundance of integrin αvβ5 expressed on the cell membrane (Fig. 7 D), or that of integrin β1 (unpublished data). This suggests that PAK4 inhibition of cell adhesion might be caused by an alteration of integrin αvβ5 binding capacity for its ligand VN.

Discussion
PAK interaction with integrins

Previously, only one intracellular molecule, Rack1 (Liliental and Chang, 1998), had been identified to directly interact with the integrin β5 cytoplasmic domain. By yeast two-hybrid screening, we now add six novel intracellular interactors of integrin β5, including PAK4. The PAK4 binding sites within the integrin β5 cytoplasmic domain was mapped to a conserved, membrane-proximal region (aa 759–767). Interestingly, the Rack1-binding site within β5 cytoplasmic domain was also mapped to the membrane-proximal region partially overlapping with the PAK4-binding region, but more extended to the NH₂ terminus (Fig. 2 C). Rack1 is a receptor for PKC and may play a role in linking PKC to integrins. Slightly upstream of the PAK4-binding region, a conserved integrin membrane-proximal region of β3 has been shown to form a hinge with the integrin αIIb subunit, thereby controlling extracellular ligand-binding affinity of integrin αIIbβ3 (Hughes et al., 1995). In addition, a conserved region in the β2 integrin membrane-proximal region (733–742), which almost corresponds to the PAK4-binding region in β5, has been suggested to be critical for endoplasmic reticulum retention, α–β dimerization, and cytoskeletal association of leukocyte integrin αLβ2 (Pardi et al., 1995). The conserved β-integrin membrane-proximal region has also been shown

to mediate integrin oligomerization, inhibition of integrin conformation, and constraining of an integrin in the inactive state (Zage and Marcantonio, 1998; Bodeau et al., 2001; Lu et al., 2001). However, although it is possible that PAK4 binding to integrin β5 membrane-proximal region may affect the association between the integrin αv and β5 subunits or binding between αvβ5 and VN, further studies are required to elucidate if modulation of the PAK4-binding region of β5 can affect integrin hinge formation or the integrin extracellular binding affinity to the ECM.

Intriguingly, interactions between PAK family members and integrins may be conserved, because the IBD within PAK family members is highly conserved (Fig. 3 C) and because we found that in addition to PAK4, both hPAK1 and the *Drosophila* PAK4 homologue MBT are able to interact with various integrin β subunits (unpublished data). The wide interaction spectra between integrins and PAK family members of man and *Drosophila* suggest that the capacity for these interactions might be highly conserved during evolution and may thus fulfill vital functions in various species.

Relocalization of PAK4 to lamellipodia

PAK4 has been found to be localized in the cytosol and in the Golgi apparatus (Abo et al., 1998; Callow et al., 2002). In the current study, we found that PAK4 relocalized to motile structures in the cell membrane upon replating onto VN, including relocalization of PAK4 to lamellipodia and ruffles. In comparison, PAK1 can localize to focal adhesions upon replating (unpublished data; Manser et al., 1997). The NH$_2$-terminal regulatory domain of PAK1:1–329, including its Cdc42/Rac-binding motif, is sufficient to localize PAK1 to focal adhesions, and PAK1 has been indicated to be recruited to focal adhesions dependent on its binding to Cdc42/Rac (Manser et al., 1997; Brown et al., 2002). However, in a substantial part of the cells (>40%), a PAK4 mutant deficient in binding to Cdc42/Rac localized to the membrane before cell replating onto VN, whereas wt PAK4 only localized to the membrane in a few cells before replating. This suggests that binding to Cdc42/Rac might negatively regulate PAK4 membrane relocalization, which is consistent with the finding by Abo et al. (1998) that overexpression of activated Cdc42 results in localization of PAK4 in the Golgi apparatus. In addition, binding to Nck has been shown to mediate PAK1 membrane localization (Lu et al., 1997), whereas Pix, but not Nck, binding is required for PAK1:1–329 localization to focal adhesions (Brown et al., 2002). However, the Nck- and Pix-binding regions of PAK1 are not conserved in PAK4, which might explain why PAK1 can be readily detected in focal adhesions (unpublished data; Manser et al., 1997), whereas PAK4 instead mainly relocalizes to lamellipodia upon replating and is rarely found in focal adhesions (unpublished data). It is unclear how PAK4 relocalization is mediated, but it appears to be independent of its catalytic activity, Cdc42/Rac binding, and integrin binding capacity, because PAK4 mutants deficient in these capacities all still relocalized to lamellipodia upon replating. However, it will be highly interesting to elucidate how relocalization of PAK4 is regulated, because the PAK4 localization at motile cellular structures may be important for its function in motility.

Role of PAK4 in the regulation of cell motility

Cell migration is important in many physiological and pathological processes and the regulation of cell motility is complicated, including both extracellular and intracellular events. Among these, PAK1 has been found to regulate cell motility in mouse fibroblasts (Sells et al., 1999), endothelial cells (Kiosses et al., 1999; Master et al., 2001), and tracheal smooth muscle cells (Dechert et al., 2001). Previous studies have suggested that the regulation of phosphorylation of myosin light chain kinase and LIM kinase (LIMK) by PAK1 and PAK2 might account for the role of these PAK family members in regulation of cell motility on various ECM substrates, but without displaying any apparent integrin specificity (Edwards et al., 1999; Sanders et al., 1999; Goeckeler et al., 2000). However, in this report, we demonstrate that PAK4 specifically induces αvβ5-mediated cell migration on VN, whereas β1 integrin–mediated cell migration on collagen type I is not influenced.

A recent study indicated that unlike PAK1, PAK4 is unable to phosphorylate myosin light chain kinase (Qu et al., 2001), which may rule out one possible route by which PAK4 could stimulate cell migration. Therefore, the mechanisms for induction of cell motility by PAK1 and PAK4 may be distinct, which is also supported by their distinct localization in cell adhesive structures of focal adhesions and lamellipodia, respectively. PAK4 was recently found to interact with LIMK1, to phosphorylate LIMK1 and stimulate the ability of LIMK1 to phosphorylate cofilin (Dan et al., 2001a). As a consequence, PAK4 and LIMK1 may cooperatively regulate cytoskeletal changes that impact cell motility. However, the PAK4 interaction with integrin β5 cytoplasmic domain may also directly modulate the extracellular motility machinery, including cell adhesion to ECM for which PAK4 has been indicated to play a functional role (Qu et al., 2001). PAK4 binding to integrin might directly effect the integrin function, and thereby cell motility, and/or localize PAK4 effects to integrin-proximal sites of migratory regulation. For example, we found that PAK4 can phosphorylate the integrin β5 cytoplasmic domain and this way might affect the integrin αvβ5 extracellular binding capacity (unpublished data).

Taken together, our study suggests a model where PAK4 binds to integrin β5 cytoplasmic domain in motile cellular structures and modulates integrin αvβ5–mediated cell migration. This may be brought about by PAK4 regulation of cytoskeletal components and/or by directly influencing integrin αvβ5 function, thereby facilitating cell migration.

Possible role of PAK4 in tumor progression and metastasis

Intriguingly, PAK4 was recently found to be overexpressed in 78% of an array of human cancer cell lines where its function may be to promote cell transformation (Callow et al., 2002). In addition to this potential function, our study indicates a role for overexpressed PAK4 in breast carcinoma cell migration, suggesting a potential role also in metastasis. The predominant VN receptor in human carcinomas in vivo is integrin αvβ5 (Lehmann et al., 1994; Jones et al., 1997), an integrin that can be activated by growth factors for cell migration (Klemke et al., 1994; Yebra et al., 1996). In fact, growth factor stimulation of breast and pancreatic carci-

noma cells has been shown to cause tumor dissemination and metastasis in vivo, which was functionally linked to activation of integrin αvβ5–mediated cell migration (Brooks et al., 1997). Given that PAK4 stimulated αvβ5-mediated cell migration in breast carcinoma cells, elucidation of its potential role in growth factor signaling pathways governing integrin αvβ5 activation will be very interesting; for example if PAK4 might effect src and/or FAK pathways recently implicated in integrin αvβ5 activation (Eliceiri et al., 2002). In addition, stimulation of angiogenesis by VEGF or TGF-α depends on integrin αvβ5 activation (Friedlander et al., 1995). Therefore, it will also be interesting to assess the potential role of PAK4 in in vivo progression of carcinoma metastasis as well as angiogenesis.

In conclusion, we report a novel cell motility pathway mediated by the serine/threonine kinase PAK4 that directly interacts with integrin αvβ5 and selectively induces αvβ5-mediated cell motility, a mechanism previously demonstrated to mediate carcinoma dissemination.

Materials and methods

Cell culture, cDNA expression vectors, and antibody production

African green monkey kidney COS-7 cells, human breast carcinoma MCF-7 cells, and human melanoma M21 cells were grown in DME supplemented with 10% FCS, 10 μg/ml Gentamycin (Life Technologies). Clones of MCF-7 cells stably expressing EGFP or EGFP–PAK4 were selected in the presence of 0.5 mg/ml G418 (Life Technologies). Pools of G418-resistant EGFP and EGFP–PAK4-expressing clones were used for cell migration studies. hPAK4 expression vector HA–PAK4-SRα3 was provided by Dr. Audrey Minden (Columbia University, New York, NY). Flag-tagged hPAK4 was constructed by cloning the full-length hPAK4 cDNA into the vector p3×FLAG-CMV-10 (Sigma-Aldrich). EGFP–PAK4 expression vector was generated by cloning the full-length hPAK4 cDNA into pEGFP-C2 (CLONTECH Laboratories, Inc.) and confirmed by sequencing. The PAK4 mutants PAK4-M350, PAK4-L19, 22, and PAK4-ΔIBD (deletion of aa 505–530) were created by site-directed mutagenesis using the QuickChange kit (Stratagene), followed by sequence confirmation of the mutated regions. For anti-PAK4 antibody production, a PAK4 NH_2-terminal sequence (aa 116–323) was amplified by PCR and cloned into the GST fusion protein expression vector pGEM-1λT (Amersham Biosciences). GST fusion proteins were purified using glutathione-Sepharose beads (Amersham Biosciences), according to the manufacturer's protocol, followed by separation in PAGE and extraction of the GST–PAK4 fusion protein. Two rabbits were immunized with the GST–PAK4 fusion protein and the sera collected by Dr. Arturo Galvani at Pharmacia Corp. (Nerviano, Italy). The anti-PAK4 antibody was purified by protein G affinity column (Pierce Chemical Co.) after absorption by bacteria powder containing expressed GST protein. The purified anti-PAK4 antibody was tested for reactivity with other PAK family members and found to be specific for PAK4 (unpublished data).

Yeast two-hybrid screening and yeast mating tests

Integrin β5 cytoplasmic domain (aa 753–799; Ramaswamy and Hemler, 1990) was fused to Lex A-DBD in the pEG202 vector (OriGene). The insert sequence and reading frame were confirmed by sequencing. Approximately 2×10^7 yeast transformants of a 19-d mouse whole embryo expression library (OriGene) were pooled and screened by the activation of leucine and β-galactosidase marker genes. 98 clones were His^+ Leu^+ Trp^+ Ura^+ β-gal$^+$ and 25 of them specifically interacted with β5 cytoplasmic domain in yeast mating tests. Sequence analysis and bioinformatics studies indicated that 6 out of 25 β5 cytoplasmic domain–interacting sequences represented mPAK4. For yeast mating tests, different regions of the integrin β5 cytoplasmic domain were cloned into the pEG202 bait vector using yeast strain RFY206 as host, and different regions of the KD of hPAK4 were cloned into pJG4-5 prey vector using yeast strain EGY48 as host.

Protein–protein interaction assays

COS-7 cells were transfected with 4 μg expression vector HA–PAK4-SRα3 by LipofectAmine plus (Life Technologies). Cells were harvested 48 h after transfection and lysed in RIPA buffer (1× PBS, pH 7.4, 0.5% sodium de-

oxycholate, 1% Triton X-100, 0.1% SDS) with protease inhibitors (0.5 μg/ml leupeptin, 1 mM EDTA, 1 μg/ml pepstatin A, 0.2 mM PMSF). Approximately 500 μg of precleared lysates were immunoprecipitated by 4 μg rabbit anti-HA pAb Y11 (Santa Cruz Biotechnology, Inc.), anti-αvβ5 mAb P1F6 (Life Technologies), or anti-β3 mAb AP3 (GTI) and were separated and probed with rabbit anti–human integrin β5 cytoplasmic pAb, anti–integrin αv pAb (Chemicon), or anti–HA tag mAb F-7 (Santa Cruz Biotechnology, Inc.) and then with an HRP-conjugated secondary pAb (Jackson ImmunoResearch Laboratories) and visualized by ECL technique as previously described (Bao et al., 2002). Alternatively, IP of endogenous PAK4 was performed using 2 μg anti-PAK4 pAb. For testing the in vivo association of PAK4-ΔIBD mutant with integrin αvβ5, COS-7 cells were transfected with Flag–PAK4 and Flag–PAK4-ΔIBD. Precleared lysates (100 μg) were immunoprecipitated by 6 μl ascites fluid of anti–integrin αvβ5 mAb P1F6 (Life Technology) or rabbit IgG or α-Rab pAb (C-19; Santa Cruz Biotechnology, Inc.) followed by Western blot analysis using anti-Flag tag mAb M2 (Sigma). As positive controls for the Western blot analyses, 10–15 μg lysates were applied without IP. To make GST fusion proteins, PAK4 KD (aa 324–591) and β5 (aa 753–799) cytoplasmic domains were cloned separately into the GST fusion protein expression vector pGEM-1λT (Amersham Biosciences). GST fusion proteins were purified using glutathione-Sepharose beads (Amersham Biosciences) according to the manufacturer's protocol. To pull down hPAK4, 2 μg of GST β5 tail fusion protein was mixed with 500 μg cell lysate containing HA–hPAK4 in RIPA buffer. Reciprocally, to pull down the endogenous integrin β5 subunit, 200 μg COS-7 cell lysate was mixed with 5 μg purified GST–hPAK4 KD fusion protein in RIPA buffer and incubated overnight at 4°C. Glutathione-Sepharose beads (Amersham Biosciences) were used to capture the GST fusion proteins and the interacting proteins. The bound proteins were visualized by Western blotting with mAb F-7 or rabbit anti–human integrin β5 cytoplasmic domain pAb (Chemicon), respectively.

Fluorescent microscopy and time-lapse video microscopy

MCF-7 cells stably expressing EGFP–PAK4 or EGFP were established under the selection of G418 (0.5 mg/ml). Cells were fixed by 4% paraformaldehyde after attachment on VN. For staining of endogenous PAK4 in MCF-7 cells, rabbit anti-PAK4 pAb was used. For integrin αvβ5 staining, cells were replated in the absence of FCS and Mn^{2+} (RPMI 1640, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.5% BSA), and anti–integrin αvβ5 mAb clone 15F11 (Chemicon) was used for staining. Anti-Flag mAb M2 (Sigma-Aldrich) was used for Flag tag staining. For quantification of cells with lamellipodial PAK4 localization, six microscopic fields were chosen randomly and counted directly through 20× objective. Statistical analysis was performed using Origin version 6.0 (Microcal Software, Inc.). Stained cells were photographed by fluorescent microscopy using a digital camera. Time-lapse studies were performed ~30 min after plating EGFP–PAK4 stably transfected MCF-7 cells onto VN-coated chamber slides in the absence of FCS using a fluorescent microscope (Leica). Pictures were captured every minute using Slidebook software version 2.06 (Intelligent Imaging Innovations, Inc.). The acquired pictures were further processed and assembled using Adobe Photoshop® 5.0 and Adobe Illustrator® 8.0.

Cell migration and cell adhesion assays

Haptotactic cell migration assays were performed using Transwell chambers (Costar Inc.) with 8.0 μm pore size. The Transwell membranes were coated with VN (10 μg/ml), collagen type I (10 μg/ml), or 1% BSA at the bottom surfaces for 2 h at 37°C. MCF-7 cells were transfected with EGFP or EGFP–PAK4 for 48 h, and then cells were trypsinized, washed, and counted in the presence of soybean trypsin inhibitor (0.25 mg/ml). Cells (1×10^5) were then added on the top of Transwell membranes and allowed to migrate toward VN or collagen type I in the presence or absence of anti–integrin αvβ3 mAb LM609 (Chemicon) or anti-αvβ5 mAb P1F6 (25 μg/ml) for 6 h at 37°C in migration buffer (RPMI 1640, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.2 mM $MnCl_2$, and 0.5% BSA). After thoroughly cleaning the upper chambers of the Transwells, the migrated cells expressing EGFP or EGFP–PAK4 were counted using a fluorescent microscope; typically 12 microscopic fields were randomly chosen and counted. For comparison, the number of migrating cells was calibrated to the transfection efficiency within the cell population as determined by flow cytometry. Quantification of stably transfected cells was performed by staining using crystal violet followed by counting of random microscopy fields. For the cell adhesion assay, nontreated 48-well plates (Corning Costar Corp.) were used. Wells were coated with 0.5–10 μg/ml VN overnight at 4°C. 1% heat-denatured BSA was applied to block nonspecific adhesion. MCF-7 cells stably transfected with EGFP–PAK4 or EGFP control were plated into the wells in triplicate at 5×10^4 cells/well in cell adhesion buffer (RPMI 1640, 2 mM

CaCl$_2$, 1 mM MgCl$_2$, 0.2 mM MnCl$_2$, and 0.5% BSA) and allowed to attach for 60 min. After careful washing of nonbound cells using adhesion buffer, MTT was used to quantify the number of stably transfected cells attached.

Flow cytometry analyses

The efficiency for cell transfections and the cell surface expression levels of integrins were analyzed by measurement of EGFP content and phycoerythrin staining intensity, respectively, by FACScan® flow cytometer using CellQuest software (Becton Dickinson) after staining with anti–integrin αvβ5 mAb P1F6 and a phycoerythrin-conjugated secondary goat anti–mouse pAb (Jackson ImmunoResearch Laboratories).

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II

Integrin-mediated Cell Attachment Induces a PAK4-dependent Feedback Loop Regulating Cell Adhesion through Modified Integrin αvβ5 Clustering and Turnover

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Cell-to-extracellular matrix adhesion is regulated by a multitude of pathways initiated distally to the core cell–matrix adhesion machinery, such as via growth factor signaling. In contrast to these extrinsically sourced pathways, we now identify a regulatory pathway that is intrinsic to the core adhesion machinery, providing an internal regulatory feedback loop to fine tune adhesion levels. This autoinhibitory negative feedback loop is initiated by cell adhesion to vitronectin, leading to PAK4 activation, which in turn limits total cell–vitronectin adhesion strength. Specifically, we show that PAK4 is activated by cell attachment to vitronectin as mediated by PAK4 binding partner integrin αvβ5, and that active PAK4 induces accelerated integrin αvβ5 turnover within adhesion complexes. Accelerated integrin turnover is associated with additional PAK4-mediated effects, including inhibited integrin αvβ5 clustering, reduced integrin to F-actin connectivity and perturbed adhesion complex maturation. These specific outcomes are ultimately associated with reduced cell adhesion strength and increased cell motility. We thus demonstrate a novel mechanism deployed by cells to tune cell adhesion levels through the autoinhibitory regulation of integrin adhesion.

INTRODUCTION

Integrins, a cell surface receptor family, mediate cell adhesion to the extracellular matrix (ECM) and trigger intracellular signaling pathways that regulate cell spreading and migration (Hynes, 2002). On cell binding to the ECM, integrins cluster within the plasma membrane and associate with numerous proteins to form organized adhesive contact sites: cell–matrix adhesion complexes (CMACs), containing large protein networks (Lock *et al.,* 2008). Examples of such CMACs include both focal complexes (FCs) and focal adhe-

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Abbreviations used: CMAC, cell–matrix adhesion complex; FC, focal complex; FA, focal adhesion; VN, vitronectin.

sions (FAs; Zamir and Geiger, 2001; Berier and Yamada, 2007; Lock *et al.,* 2008). The abbreviation CMAC is used here to refer to all integrin–ECM adhesions, and the terms FC and FA are reserved for specific CMAC subsets in cases where it is possible to distinguish them. FCs are small, transient adhesions at the cell periphery believed to be important in mediating the attachment of the extending lamellipodium to the ECM (Lauffenburger and Horwitz, 1996). FCs either disassemble within a short time of their formation or mature into more stable FAs (Zamir and Geiger, 2001; Berier and Yamada, 2007). Somewhat counterintuitively, integrins in stable, high-density FAs undergo rapid turnover in comparison to integrins clustered in less stable FCs, indicating a disconnection between the stability of CMACs as a whole and their core integrin components (Ballestrem *et al.,* 2001). Also somewhat surprising is that in FAs, the stabilization of CMACs and stress fibers is associated with an increased adhesion strength that usually counteracts cell motility (Webb *et al.,* 2002). Thus, fast cell migration typically correlates with intermediate rates of CMAC assembly and disassembly as well as with the rapid turnover of structural components such as integrins within CMACs (Gupton and Waterman-Storer, 2006). Although these preconditions for rapid migration are becoming more clearly characterized, the cellular signaling and molecular mechanisms that govern these dynamic properties of the cell adhesion–migration system are still poorly understood.

p21-activated kinases (PAKs) are effectors for the Rho GTPases Cdc42 and Rac. The group 1 PAK family members PAK1 and PAK2 have previously been shown to affect cell migration in distinct manners (Coniglio *et al.,* 2008), and qualitative studies also suggest a possible role for PAK1 in

the regulation of FA morphology (Manser *et al.,* 1997; Kiosses *et al.,* 1999). We now further investigate the mechanisms by which PAK4, a member of the group 2 PAKs, mediates cell adhesion and migration because our previous studies have demonstrated that PAK4 regulates MCF-7 cell migration on vitronectin (VN) and that PAK4 kinase activity is responsible for this reaction (Zhang *et al.,* 2002; Li *et al.,* 2010). PAK4 may also exert an influence on the actin microfilament system, because overexpression of PAK4 can induce localized actin polymerization and filopodia formation (Abo *et al.,* 1998; Dan *et al.,* 2001; Callow *et al.,* 2005), although whether these effects are direct or indirect remains unclear. As a corollary, overexpression of a hyperactive PAK4 mutant (S445N) in fibroblasts or activation of PAK4 by HGF can cause a reduction in stress fiber prominence, decreased adhesion to the ECM, and cell rounding (Qu *et al.,* 2001; Wells *et al.,* 2002). Conversely, mouse embryonic fibroblasts (MEFs) lacking PAK4 display enhanced focal adhesions, indicating a role for PAK4 in CMAC regulation (Qu *et al.,* 2003). However, both the mechanisms and contextual significance of these PAK4 effects remain elusive.

Here, we first determined that PAK4 is activated by ligation of its binding partner, integrin $\alpha v\beta 5$, to the ECM ligand VN. Next, we deployed quantitative imaging-based analyses to characterize the specific effects of PAK4 on adhesion structures and their core adhesive machinery. By these methods we revealed that PAK4 acts at the molecular level of adhesion complexes to accelerate integrin $\alpha v\beta 5$ turnover within CMACs while concurrently reducing integrin clustering density and integrin-to-F-actin connectivity, ultimately destabilizing CMACs and reducing cell attachment strength. Strikingly, these results delineate a novel autoinhibitory negative feedback loop initiated within the core adhesion machinery by integrin $\alpha v\beta 5$ and acting via PAK4 to limit total $\alpha v\beta 5$-mediated cell adhesion to VN. These findings provide a potential mechanism for the intrinsic fine tuning of cellular adhesion levels and in this case are consistent with the facilitation of enhanced cell motility.

MATERIALS AND METHODS

Mammalian Cell Expression Vectors

Hemagglutinin (HA)-PAK4-wild type (WT), Flag-PAK4-WT, Flag-PAK4-K350M, Flag-BAP, and pEGFP-PAK4-WT were previously described (Zhang *et al.,* 2002). An enhanced green fluorescent protein (EGFP)-tagged PAK4–445N, 474E (activated PAK4), was generated using site-directed mutagenesis (Qu *et al.,* 2001). The WT PAK4 was also subcloned into the HindIII/BamHI sites of a monomeric red fluorescent protein vector (pmRFP) kindly provided by Roger Tsien (University of California, San Diego). Full-length integrin $\beta 5$ cDNA kindly provided by Errki Ruoslahti (Burnham Institute) was cloned into the HindIII/EcoRI sites of the pEGFP-N1 vector (Clontech, Palo Alto, CA).

Antibodies and Recombinant Proteins

The anti-HA (Y11) pAb was purchased from Santa Cruz Biotechnology (Santa Cruz, CA); anti-integrin $\alpha v\beta 5$ mAb (clone 15F11) from Chemicon International (Temecula, CA); anti-Flag mAb M2 from Sigma (St. Louis, MO); and tubulin-α Ab-2 mAb from Lab Vision (Fremont, CA). The anti-actin mAb JLA20 was provided by Developmental Studies Hybridoma bank at University of Iowa (Iowa City, IA). The anti-PAK4 (total) pAb was generated in our laboratory.

Cell Culture and Transient Transfections

African green monkey kidney COS-7 cells and human breast carcinoma MCF-7 cells were grown in DMEM, supplemented with 10% fetal bovine serum (FBS) and 10 μg/ml gentamicin (Invitrogen, Carlsbad, CA), and maintained in a humidified incubator at 37°C with 5% CO_2 , 4–8 μg of total DNA was transfected in 100-mm cell culture dishes (80–90% confluence of COS-7 or MCF-7 cells) using LipofectAmine Plus or LipofectAmine 2000 (Invitrogen), according to the manufacturer's protocol.

Generation of Stable PAK4 Clones

MCF-7 cells were stably transfected with Flag-BAP or Flag-PAK4-WT using LipofectAmine Plus (Invitrogen) according to manufacturer's instructions. Twenty-four hours after transfection, selection with 300 μg/ml G418 (Invitrogen) was initiated. After 7–10 d of selection, colonies were isolated and screened for Flag expression by immunoblotting using anti-Flag mouse mAb M2 (Sigma). Clones were maintained in medium supplemented with 150 μg/ml G418 (Invitrogen).

Small Interfering RNA Experiments

For the knockdown of PAK4, two small interfering RNA (siRNA) sequences targeting human PAK4 (no. 273: CAUGUCGGUGACACGCUCCAA and no. 1093: AACGAGGUGGUAAUCAUGAGG) were purchased from Qiagen (Chatsworth, CA); and the control siRNA was previously described (Zhang *et al.,* 2004). MCF-7 cells at 50% confluence were transfected with siRNA using Lipofectamine RNAiMAX reagent according to the manufacturer's protocol (Invitrogen). At 2–4 d after transfection, the endogenous levels of PAK4 were measured by immunoblotting (IB) using anti-PAK4 pAb and tubulin-α Ab-2 mAb (Lab Vision). For PAK4-shRNA constructs and for stable transfection, an shRNA sequencing corresponding to positions 273–291 of the human PAK4 gene was used; the sense oligonucleotide contained the following PAK4 targeting sequence (underlined): 5'-GATCCCCCATGTCGGTGACACGCTCCTTCAAGAGAGGGAGCGTGT-CACCGAGATGTTTTA-3'; and the antisense oligonucleotide: 5'-AGCTTAAA-AACATGTCGGTGACACGCTCCTCTCTTGAAGGAGCGTGTCACCGAG-ATGGGG-3'. To generate a shRNA duplex, the sense and antisense oligonucleotides were annealed, and double-stranded oligonucleotides were cloned into the pSuper vector. Two shRNA-resistant PAK4 cDNAs (EGFP-PAK4-AC285, 288GA and EGFP-PAK4-M350AC285, 288GA) were generated by changing the targeted sequence of shRNA (no. 273) in EGFP-PAK4-WT construct to the sequence 5'-CATGTCGGTGACGCGATCCAA-3' (mutated nucleotides are underlined) by PCR-based site-directed mutagenesis. To establish PAK4 shRNA-stable clones, the pSuper-PAK4 shRNA plasmid or a control shRNA (Numakawa *et al.,* 2004) plasmid with the pCI-neo vector was cotransfected into MCF-7 cells using LipofectAmine Plus. Cells were selected with medium containing 300 μg/ml G418. After selection, all clones were maintained in medium supplemented with 150 μg/ml G418.

PAK4 Activation and In Vitro Kinase Activity Assay

COS-7 cells transiently transfected with HA-PAK4-WT or MCF-7 cells stably expressing Flag-PAK4-WT or Flag BAP were starved in serum-free DMEM overnight to minimize endogenous signaling. The cells were trypsinized and suspended for 30 min in adhesion buffer (RPMI 1640, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.2 mM $MnCl_2$, and 0.5% bovine serum albumin [BSA]) and then plated on vitronectin (VN; 10 μg/ml)-coated suspension dishes for various times. Cells were lysed in a phosphate-buffered saline (PBS)-TDS lysis buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.14 M NaCl, 2.8 mM KCl, 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4). In vitro PAK4 kinase activity analyses were performed as described (Zhang *et al.,* 2002). Briefly, extracts (100–1500 μg of protein) were enriched by immunoprecipitation (IP) using anti-HA (Y11), anti-Flag (M2), or anti-PAK4 (Zhang *et al.,* 2002) antibodies and collected by protein A or protein G-Sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ) and then mixed with 5 μg of MBP substrate (Sigma) in the presence of [γ-^{32}P]ATP (Amersham Biosciences), followed by SDS-PAGE. The radioactivity incorporated into the substrate was then visualized and quantified using a PhosphorImager system (Molecular Imager FX, Bio-Rad, Hercules, CA).

Immunoblotting

Each sample was run on an SDS-polyacrylamide gel, and proteins were transferred to Immobilon-P Membrane (Millipore, Bedford, MA). Membranes were probed with various antibodies as described, and proteins were detected by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech); band intensities were quantified using Kodak 1D image analysis software Eastman Kodak, Rochester, NY).

Cell Adhesion Assay

Cell adhesion assays were performed as described (Yebra *et al.,* 1996). Briefly, nontreated 48-well cluster plates (Corning Costar, Acton, MA) were coated with different concentrations of VN and blocked by 1% heat-denatured BSA. MCF-7 cells (n = 5 × 10^4) per well were seeded in wells and allowed to attach at 37°C in adhesion buffer for 15–45 min. The amount of attached cells was quantified by an MTT assay. For PAK4-siRNA knockdown experiments, MCF-7 cells were transiently transfected with PAK4 siRNA or control siRNA. After 4 d, cell adhesion assays were performed as described above. In RNA interference (RNAi) rescue, a control shRNA MCF-7 clone was transiently transfected with EGFP, and a PAK4 shRNA clone was transiently transfected with EGFP (as a control) or EGFP-PAK4-AC285, 288GA. After 27 h, 5 × 10^4 of these cells were seeded in each well and allowed to attach at 37°C for 15–45 min. The attached EGFP-positive cells were counted using a microscope (10× objective). For comparison, the number of EGFP-containing adhered cells was

calibrated to the transfection efficiency within the cell population as determined by counting of the starting population using fluorescent microscopy.

Immunofluorescence Staining, Microscopy, and Focal Adhesion Quantification

For immunostaining, MCF-7 cells transfected with HA-tagged or Flag-tagged PAK4 or shRNA constructs were detached and plated onto VN-coated cover slips in adhesion buffer for 3–6 h. Cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. The fixed cells were permeabilized with 0.2% Triton X-100, and nonspecific binding was blocked by using 5% BSA in PBS for 1 h at room temperature. The cells were then costained with primary anti-integrin $\alpha v \beta 5$ mAb 15F11 (Chemicon) or anti-vinculin mAb (Sigma), followed by a fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) for adhesion structures and anti-HA pAb Y11 (Santa Cruz Biotechnology) , followed by rhodamine-conjugated secondary antibody (Jackson ImmunoResearch) for PAK4 or rhodamine-conjugated phalloidin (Molecular Probes, Eugene, OR) for actin. For negative controls, the samples were incubated with either of the primary antibodies without the corresponding secondary antibody or the secondary antibodies without any primary antibody. The slides were examined by using an IX71 Olympus microscope with a 100×/1.35 oil objective (Melville, NY) and a Hamamatsu CCD camera (Bridgewater, NJ) or a Zeiss LSM510 confocal microscope with a 63×/1.4 oil objective (Thornwood, NY).

Wasabi software (http://www.wasabisystems.com/) and Zeiss LSM Image Browser Version 4.2.0121 software were used to generate digital images. The numbers of focal CMACs at the cellular periphery were quantified manually or by automation using Patch Morphology Assay 5.2.0 software for systems microscopy (Digital Cell Imaging Labs, Edegem, Belgium; Lock and Strömblad, 2010) or ImageJ software (http://rsb.info.nih.gov/ij/; National Institutes of Health). The peripheral area was defined as a 5-μm-wide region at the cell border. Quantification of the number of adhesion complexes in the cell periphery in control and PAK4-overexpressing cells was performed by counting the number of adhesion complexes in a square of a defined size for each cell among 25–50 cells in three independent experiments. Statistical significance was calculated using an unpaired, two-tailed t test. The 95% confidence intervals were calculated using TEMPLO software (Noraxon, Cologne, Germany).

Determination of Cell Spreading

MCF-7 cells with or without transfections were plated in adhesion buffer for 1–6 h onto VN-coated coverslips. Cells were fixed and stained for F-actin with rhodamine-labeled phalloidin as described above. Images were acquired by an Olympus IX71 microscope with a 20× oil objective and photographed with a Hamamatsu CCD camera. Cell areas were determined for each condition from three separate experiments using ImageJ software. Cells were manually outlined based on phalloidin labeling of actin, and the area was calculated in pixels using an automation tool in ImageJ. This number was then converted to μm^2 by recalculation. Only cells not in contact with neighboring cells were analyzed.

Cell Migration Assay

A haptotactic cell migration assay was performed using Transwell chambers (Corning Costar) with 8.0-μm pore size as described (Yebra et al., 1996). Briefly, MCF-7 cells transiently transfected with EGFP (as a control), EGFP-PAK4-WT, or control shRNA and PAK4 shRNA clones transiently transfected with EGFP (as a control) or EGFP-PAK4-AC285, 288GA, and after 48-h transfection, 1–3 × 10^5 cells were added on top of the Transwell membranes, the bottom surface of which was coated with VN (10 μg/ml), and were allowed to migrate toward VN for 3–6 h at 37°C in adhesion buffer. All of the migrated EGFP-positive cells in each the well were counted using fluorescent microscopy. For comparisons, the migrated cells were calibrated to the transfection efficiency within the cell population as determined by counting using the fluorescent microscope of the starting population. For the knockdown of PAK4, stable PAK4 overexpression clones or transient transfection of PAK4 siRNAs, 1 × 10^5 cells were added on top of the Transwell membranes coated with VN (10 μg/ml) at the bottom and were allowed to migrate toward VN for 3–6 h at 37°C in adhesion buffer. All the migrated cells in each of the wells were stained by crystal violet and were counted using microscopy.

Wound Healing Assay

Forty-eight–well plates were coated with VN (10 μg/ml) at 37°C for 1 h, followed by blocking with 1% BSA for 1 h at 37°C. Cells were trypsinized with trypsin-EDTA, washed twice with PBS, and replated in serum-free DMEM 4 h before wounding at confluence. The cell monolayers were wounded by applying a p200 pipette tip. Cells were then incubated in serum-free DMEM, and images from 3 representative wounded areas per condition were acquired by microscope (Zeiss Axiovert S100) with a digital camera (Axiocam MRC) and software (Axivision AC) at 0, 30,and 55 h. Wound closure rates were determined using the initial and final wound areas during the wounding experiments, with the percentage wound closure calculated as [(initial − final)/initial] × 100.

Fluorescence Recovery After Photobleaching)

MCF-7 cells that were cotransfected 48 h previously with mRFP and $\beta 5$-EGFP or mRFP-PAK4 and $\beta 5$-EGFP were replated onto Matek dishes coated with 10 μg/ml VN in DMEM (Invitrogen). After 16 h, cells were washed three times in F12 medium (Invitrogen) supplemented with 1 mM CaCl$_2$, 1 mM MgCl$_2$, and 0.5% BSA and imaged within this medium at 37°C in 5% CO2 using an LSM510 confocal microscope (Zeiss). Integrin $\beta 5$-EGFP was bleached using 40 iterations at 40% of total laser power from the 488-nm line of a four-line argon laser (Coherent, Palo Alto, CA). $\beta 5$-EGFP was imaged before (×3) and after (×31) bleaching using 0.24% of total 488-nm laser power at an interval of 30 s. Monomeric red fluorescent protein (mRFP) was imaged using 35% of total laser power from a 543-nm laser (Coherent). Entire adhesions were bleached, and recovery of adhesions were measured using free-drawn regions of interest, and mean intensity was quantified in ImageJ software (version 1.32), followed by analysis using Microsoft Office Excel 2003 (Redmond, WA). The mean diffuse plasma membrane intensity of integrin was subtracted from all data points, and corrections were applied to remove the effect of nonspecific bleaching during the recovery process. Maximal diffusive recovery of integrin within the plasma membrane occurs within ~120 s for bleached areas of equivalent size to that of typical focal adhesions (data not shown). The data analysis therefore only includes recovery data starting 2 min into the process. Average values of intensity were calculated at each point in time in the two sets of recovery curves, with and without PAK4, respectively. Using a nonlinear algorithm in the Matlab Curve Fitting toolbox (The Mathworks, Natick, MA), an exponential function on the form:

$$y = a - b \exp(-kt) \qquad (1)$$

was fitted to the curves. This gave the parameter a, the final value of recovery, in the two sets. b is the amount of first-order recovery, and k is the corresponding rate. Curve fitting was also obtained for individual recovery curves. However, nonlinear curve fitting was not possible for the individual recovery curves because the estimate of parameters is sensitive to noise. Instead, in this case, the final value of recovery, a, was kept constant at the number calculated above for each of the two data sets. This made it possible to use linear curve fitting by calculating log [a − I(t)], where I(t) is the intensity at time t, and fitting this expression to log b − kt, where the parameters correspond to those in Equation 1. For statistical analyses, data were analyzed for statistical significance using an unpaired two-tailed t test in Microsoft Office Excel 2003.

RESULTS

Cell Attachment to VN Activates PAK4

Cell adhesion to the ECM triggers a variety of intracellular signaling cascades, including the activation of PAK1 (Price et al., 1998). However, it is unclear whether PAK4 activity is regulated by cell attachment. To this end, COS-7 cells transiently transfected with HA-PAK4-WT were replated onto VN in the absence of serum. PAK4 was markedly activated within 5 min of replating, with a sustained enhancement of activity detected for at least 60 min by an in vitro kinase assay (Figure 1A). Also, stably overexpressed Flag-PAK4-WT and endogenous PAK4 kinase activities in MCF-7 cells were induced by integrin $\alpha v \beta 5$–mediated attachment to VN with a peak activity at about 10 min (Figure 1, B–D). Although WT PAK4 displayed substantial endogenous activity, an inactive PAK4-K350M mutant was analyzed as a control and showed no kinase activity compared with PAK4-WT (Supplemental Figure 1). The level of PAK4 activation in MCF-7 cells upon VN attachment was lower than in COS-7 cells, which may be due to cell type–specific differences. These results demonstrate that integrin-mediated attachment to VN activates PAK4. Considering also that PAK4 is relocalized upon replating of MCF-7 cells from a diffuse cytosolic appearance to be enriched at the cellular periphery where most CMACs are formed (Zhang et al., 2002), the change in the local amount of active PAK4 at these adhesion sites is several times that of the increase in total PAK4 activity.

PAK4 Expression Reduces Cell Attachment to VN

To examine whether cell attachment-induced PAK4 activation may play a role in integrin $\alpha v \beta 5$–mediated cellular functions, we used the human breast carcinoma cell line, MCF-7, which expresses only integrin $\alpha v \beta 5$ as its VN recep-
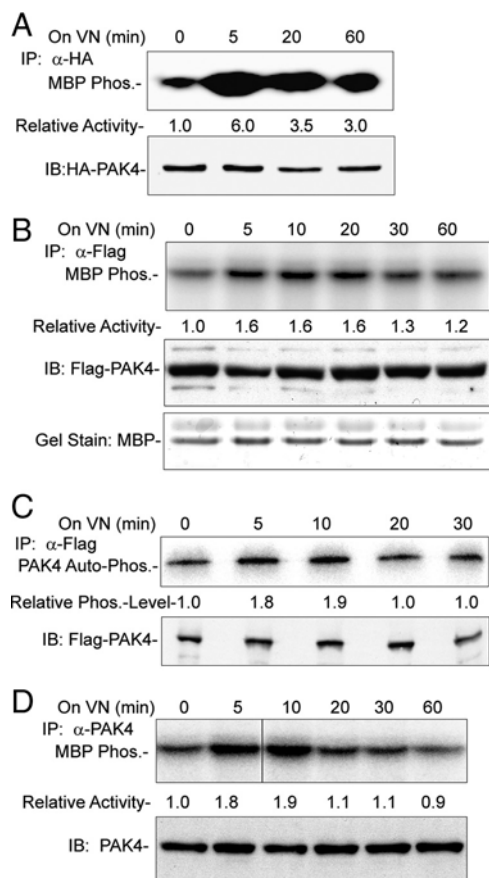
Figure 1. PAK4 activation by cell attachment onto vitronectin (VN). (A) Activation of transiently expressed HA-tagged PAK4 in COS-7 cells. COS-7 cells with transiently transfected HA-PAK4 were plated onto VN-coated dishes for the indicated times. PAK4 was immunoprecipitated (IP) from cell lysates, and kinase activity was determined by an in vitro kinase assay using myelin basic protein (MBP) as substrate (top). Quantified PAK4 kinase activities relative to the zero time point of PAK4 activity are indicated below. Bottom panel, lysate content of HA-PAK4 by immunoblotting (IB). (B) Activation of transiently expressed Flag-PAK4 in MCF-7 cells. Mixed MCF-7 cell clones stably expressing Flag-PAK4 were plated onto VN and analyzed for PAK4 kinase activity as described above (top). Middle, lysate content of proteins detected by anti-FLAG; bottom, Coomassie blue gel staining of MBP loading. (C) Autophosphorylation of stable expressed Flag-PAK4 in MCF-7 cells. Mixed MCF-7 cell clones stably expressing Flag-PAK4 were plated onto VN and analyzed for PAK4 autophosphorylation as described above (top). Bottom, quantified PAK4 autophosphorylation relative to the zero time point of PAK4 autophosphorylation are indicated below. Bottom panel, lysate content of proteins detected by anti-FLAG. (D) Activation of endogenous PAK4 in MCF-7 cells. MCF-7 cells were plated onto VN, and after anti-PAK4 IP, endogenous PAK4 activity was analyzed (top). Bottom, lysate contents of PAK4 protein. The displayed results in Figure 1 are representative among at least three experiments. Note that Figure 1D, top panel, stems from the same gel and was rearranged for display purpose.

tor. In concordance with our previous findings (Zhang *et al.,* 2002), MCF-7 cells stably expressing Flag-PAK4 display reduced cell attachment to VN compared with control cells (Figure 2A). Note that the expression of Flag-PAK4-WT in MCF-7 stable clones produced an approximately twofold increase in PAK4 protein levels compared with control MCF-7 cells, thereby closely mimicking the activity increase induced by replating (Figures 1 and 2A). In addition, it is
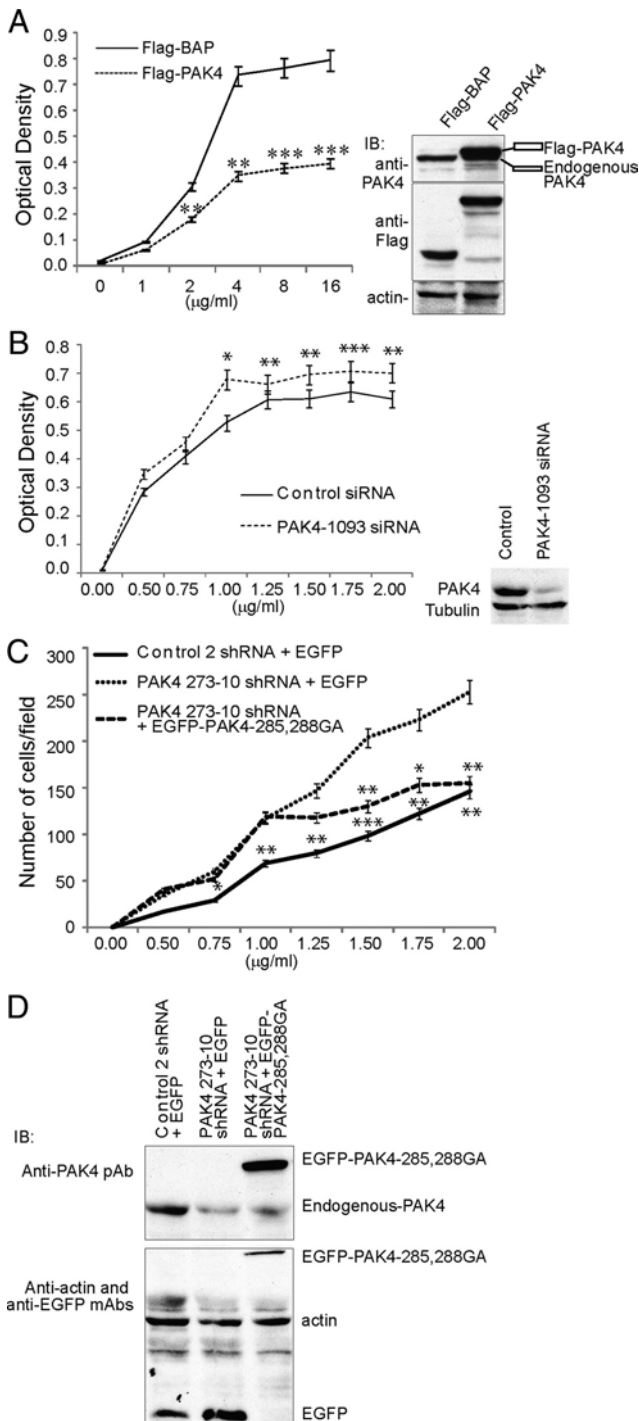
also important to note that unlike group I PAKs, PAK4 has a constitutive basal kinase activity (Supplemental Figure 1; Abo *et al.,* 1998; Li *et al.,* 2010). Together, this indicates that overexpression of PAK4 can mimic the increased activity levels obtained after replating. Conversely, RNAi-mediated knockdown of PAK4 enhanced cell attachment compared with control cells (Figure 2, B and C). On reexpression of an RNAi-resistant mutant of EGFP-PAK4, this phenotype was rescued (Figure 2, C and D). These results suggest that PAK4 plays an important role in the regulation of integrin $\alpha v\beta 5$–mediated cell adhesion.

PAK4 Regulates Integrin $\alpha v\beta 5$–mediated Cell Motility and Spreading

Although overexpression of EGFP-PAK4 markedly promoted cell migration on VN (Zhang *et al.,* 2002), it has remained unclear whether endogenous PAK4 is required for migration of breast carcinoma cells, within which PAK4 has been indicated to be overexpressed in patients (Liu *et al.,* 2008). Consistent with our previous findings, overexpression of WT PAK4 in MCF-7 cells did indeed enhance cell migration on VN in transwell migration assays by ~2–3-fold compared with controls (Supplemental Figure 2, A and B). Two distinct PAK4 siRNAs had the converse effect, suppressing MCF-7 cell migration on VN (Supplemental Figure 2C). Suppression of migration was also seen in stable clones expressing PAK4-shRNA (Supplemental Figure 2D). Critically, this phenotype could be reversed by reexpression of an EGFP-PAK4 siRNA-resistant mutant (Supplemental Figure 2E). PAK4 depletion also caused a marked inhibition of cell migration in wound closure assays (Supplemental Figure 2F). In fact, at 55 h after wounding, PAK4-depleted cells had only migrated to cover 50% of the wounded area, whereas control cells had virtually closed the wound. Together, these results suggest that PAK4 plays a key role in cell migration.

Cell motility is closely related to cell spreading. Although overexpression of EGFP-PAK4 markedly promoted cell migration onto VN (Zhang *et al.,* 2002) and a hyperactive PAK4 mutant caused cell rounding (Qu *et al.,* 2001), it has been unclear to what extent PAK4 may affect cell spreading. To test the influence of PAK4 on cell spreading, MCF-7 cell area was quantitatively compared after replating onto VN in the presence or absence of overexpressed PAK4. HA-PAK4–overexpressing cells displayed significantly less cell spreading than control cells (Supplemental Figure 3A), and transient overexpression of a hyper-active form of PAK4 (EGFP-PAK4–445N, 474E) caused even stronger inhibition of cell spreading than EGFP-PAK4-WT (Supplemental Figure 3, B and C). Stable overexpression of Flag-PAK4-WT also inhibited cell spreading (Supplemental Figure 3D). Furthermore, we created two MCF-7 cell clones stably expressing PAK4-shRNA resulting in more than 80% knockdown of PAK4 protein levels (Supplemental Figure 3E). ShRNA-mediated PAK4 knockdown substantially induced cell spreading compared with control shRNA cells (Supplemental Figure 3F). This phenotype was rescued by reexpression of an EGFP-PAK4 siRNA-resistant mutant, but not by a kinase-dead EGFP-PAK4 siRNA-resistant mutant (Supplemental Figure 3G). Thus, our findings indicate that PAK4 kinase activity is required in the regulation of carcinoma cell spreading and migration. Taken together with the physical and functional links between PAK4 and integrin $\alpha v\beta 5$, these data called for a more detailed elucidation of PAK4 effects and mechanisms within the core migration machinery, namely, integrin-mediated CMACs (Webb *et al.,* 2002; Lock *et al.,* 2008).

PAK4 Inhibits Integrin Clustering, Integrin–F-Actin Connection, and Adhesion Complex Maturation

Detailed, quantitative, imaging-based analyses of the impact of PAK4 in hundreds of cells and on several thousand CMACs were performed. We focused on the impact of PAK4 on peripheral CMACs, defined as those found within 5 μm of the cell border, because this region gives rise to the key assemblages of cell motility, including lamellipodia and filopodia (Wehrle-Haller and Imhof, 2003). Strikingly, HA-PAK4 overexpression resulted in a shift in CMAC populations toward smaller integrin αvβ5–containing structures with the appearance of focal complexes, whereas control cells displayed larger and brighter CMACs (Figure 3A). Semiautomated quantification also revealed a significant decrease in peripheral CMAC numbers in PAK4-overexpressing cells (Figure 3B). It is notable that PAK4 overexpression preferentially inhibited the presence of larger CMACs that in size and structure correspond to FAs (Figure 3, C and D). In fact, the number of FAs (size $>$2 μm^2) per cell was reduced by ~75% in the presence of WT PAK4 overexpression (Figure 3D). Consistent with the results of transient PAK4 overexpression, cells stably overexpressing PAK4 also showed decreased numbers of integrin αvβ5 and vinculin-containing CMACs (Figure 3, E and F). We next used custom-developed software to perform fully automated quantification of CMAC properties, as described in *Materials and Methods* (Figure 4). This revealed that overexpression of EGFP-tagged PAK4 (EGFP-PAK4) reduced CMAC number compared with EGFP control (Figure 5A). Additionally, EGFP-PAK4 expression reduced the density of integrin clustering in CMACs, an effect also observed in the presence of HA- and FLAG-tagged PAK4. In control cells, integrin density (resulting from integrin clustering and indicated by mean αvβ5 mAb labeling intensity per CMAC) increased significantly as CMAC area increased, delineating the progression of CMAC maturation. In contrast, integrin density did not increase substantially as CMAC area increased in cells expressing EGFP-PAK4, resulting in CMACs with reduced densities in all size classes from the smallest, nascent adhesions, to the largest FAs wherein the effect was most pronounced (Figure 5B). Remarkably, EGFP-PAK4 also dramatically inhibited integrin–F-actin connectivity, as indicated by substantially reduced colocalization between αvβ5 mAb and phalloidin labeling within individual CMACs, without significantly altering local F-actin levels (Figure 5, C and D). This effect was also exacerbated in large CMACs. Thus, these findings indicate that PAK4 overexpression causes a general depletion of CMAC number, size, integrin clustering density, and integrin–F-actin connectivity, with an especially potent effect on the development of larger adhesion complexes, implying a key role for PAK4 in the inhibition of CMAC and particularly FA maturation. Importantly, stable PAK4-shRNA expression caused a marked

Figure 2. Role of PAK4 in MCF-7 cell adhesion on VN. (A) Left, cell attachment of MCF-7 cells stably expressing Flag-PAK4 or Flag-BAP at different coating concentrations of VN was determined. Graph shows means of optical density $\pm$ 95% confidence intervals; n = 3 from one representative experiment. Right, the protein levels of endogenous PAK4 and stably expressed Flag-PAK4-WT or Flag-BAP (as a control) in MCF-7 cells were detected by IB using anti-PAK4 pAb (top panel), anti-Flag (M2) mAb (middle panel) with anti-actin mAb as loading control (bottom panel). (B) Left, cell attachment of MCF-7 cells transiently expressing PAK4-siRNA or control siRNA at different coating concentrations of VN was determined. Graph shows means of optical density $\pm$ 95% confidence intervals; n = 3 from one experiment. Right, PAK4 siRNA-mediated knockdown was determined by IB using tubulin as loading control. (C) Cell attachment was determined of MCF-7 stably expressing

control shRNA and stable PAK4 shRNA expressing MCF-7 cells transiently transfected with EGFP (as a control) or EGFP-PAK4-AC285, 288GA (RNAi-resistant PAK4) at different coating concentrations of VN. Graph shows means of number of cells per field $\pm$ 95% confidence intervals; n = 3 from one experiment. (D) PAK4 shRNA-mediated knockdown and expression levels of EGFP and EGFP-PAK4 for cells used in C were determined by IB using actin as loading control. All experiments in Figure 2 were repeated at least three times with similar results. p values are indicated for statistically discernable differences compared with control (A and B) or to PAK4-shRNA cells (C) according to unpaired two-tailed *t* test (*p $\leq$ 0.05; **p $\leq$ 0.01; ***p $\leq$ 0.001).
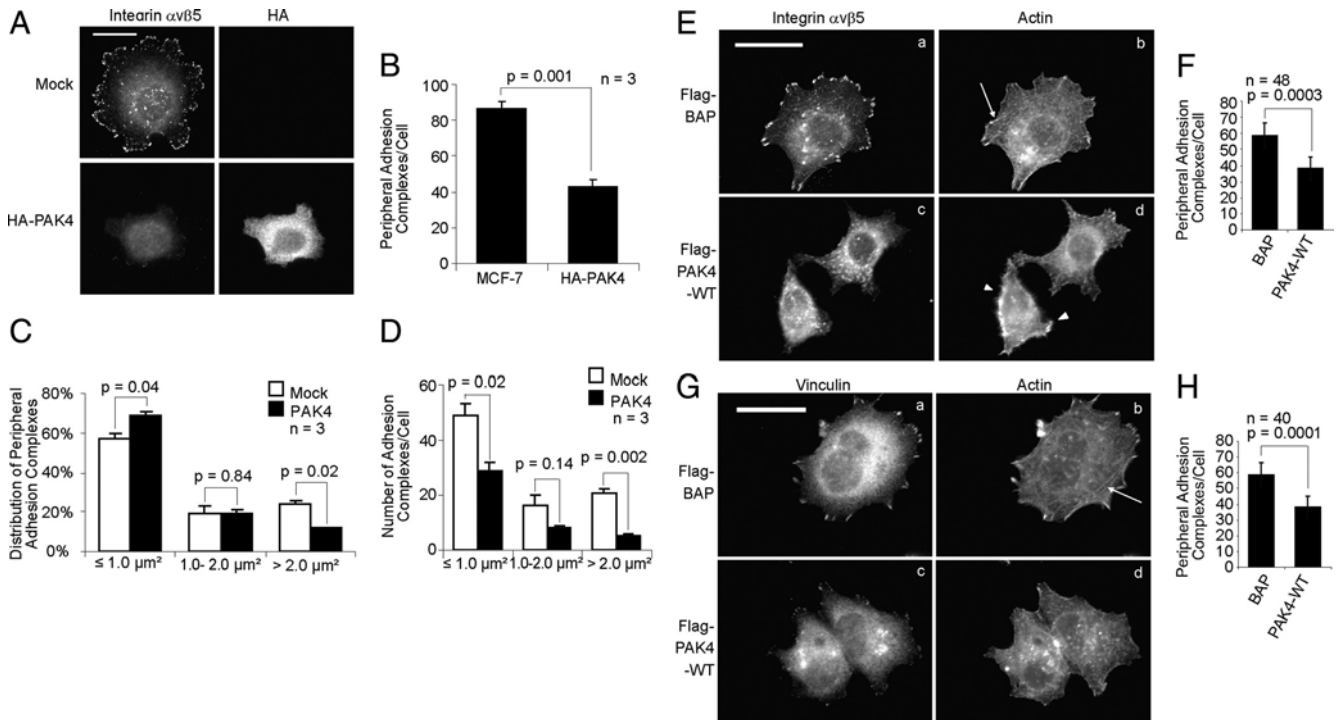
Figure 3. Influence of PAK4 transient or stable overexpression on CMAC size and number. (A) Images from control MCF-7 cells or MCF-7 cells transiently expressing HA-PAK4-WT 3 h after replating onto VN, stained for integrin $\alpha v\beta5$ and HA-tag as indicated. Bar, 20 μm. (B) Quantification of CMAC numbers at the cellular periphery. The results are displayed as means ± SEM of the number of peripheral CMACs within 5 μm of the cell border per cell; between three independent experiments. (C and D) Quantification of CMAC sizes at the cellular periphery. The sizes of the CMACs are categorized into small ($\leq$1 μm^2), medium (from >1 to $\leq$2 μm^2), and large (>2 μm^2) adhesions. Values represent the percentage distribution (C) or number per cell (D) for each group expressed as mean ± SEM between three independent experiments. (E and G) MCF-7 cells stably expressing Flag-BAP or Flag-PAK4-WT were plated onto VN and fixed after 6 h. Cells were stained with anti-$\alpha v\beta5$ (E) or anti-vinculin (G) antibodies and costained with rhodamine-phalloidin. Bar, 25 μm. (F and H) Quantification of the number of CMACs at the cellular periphery as described in *Materials and Methods*. Graphs show means of CMACs per cell ± 95% confidence intervals; n = 48 (F) and n = 40 (H). In addition to effects by PAK4 on CMACs, we observed fewer actin stress fibers and also an induction of filopodia (arrows) in some WT PAK4-overexpressing cells compared with controls. p values indicated according to unpaired two-tailed *t* test.

increase in the number and size of CMACs, in particular among larger adhesions where a 10-fold increase in frequency was observed (Figure 6, A–C). Interestingly, knockdown of PAK4 also promoted enhanced integrin clustering, with small CMACs displaying a higher integrin clustering density than controls (Figure 6D) and an apparently accelerated (with respect to CMAC area) maturation to maximal integrin density. However, PAK4 knockdown did not significantly intensify the maximal clustering density observed in large CMACs, suggesting that both control and PAK4 knockdown conditions permit maximal integrin clustering density, perhaps limited here by other factors such as ECM ligand concentration or allosteric hindrance. PAK4 knockdown also significantly increased the amount of F-actin recruited to CMACs (Figure 6E) without changing colocalization values (Figure 6F), implying an increased total connectivity (indicated by F-actin intensities) of these complexes to the actin cytoskeleton compared with control cells, but with a similar spatial organization (indicated by no detectable change in colocalization values). Overall, CMACs in the absence of PAK4 matured more efficiently, as denoted by enhanced integrin clustering with respect to CMAC area, as well as more frequently and with greater actin connectivity than CMACs in control shRNA-expressing cells.

It is worth noting here that at no time was it possible to detect significant PAK4 association with CMACs, despite the consistent observation that PAK4 is recruited to the cell periphery during the spreading process that follows cell attachment (Zhang *et al.,* 2002). This is true of endogenous and exogenous PAK4 observed via immunofluorescence labeling and of exogenous EGFP- and mRFP-tagged PAK4 observed in live and fixed cells. This suggests that PAK4 acts either indirectly on integrins or that it acts directly but in a location outside of large adhesion complexes, most likely on the nonclustered, diffusing integrin population.

PAK4 Induces Increased Integrin $\alpha v\beta5$ Turnover within CMACs

Although PAK1 has been suggested to regulate overall CMAC stability (Manser *et al.,* 1997; Kiosses *et al.,* 1999; Stofega *et al.,* 2004), it has been unclear whether PAKs may affect integrin molecular turnover within CMACs. Given that mature, relatively stable FAs have been shown to display higher integrin turnover rates than smaller, more immature and less stable FCs (Ballestrem *et al.,* 2001; Wehrle-Haller and Imhof, 2003), the inhibitory effect of PAK4 on CMAC maturation was expected to correlate with decreased integrin turnover. Surprisingly, however, fluorescence recovery after photobleaching (FRAP) analysis of integrin turnover revealed that PAK4 overexpression instead accelerated integrin turnover. MCF-7 cells were cotransfected with integrin $\beta5$-EGFP and mRFP-PAK4 or a control mRFP
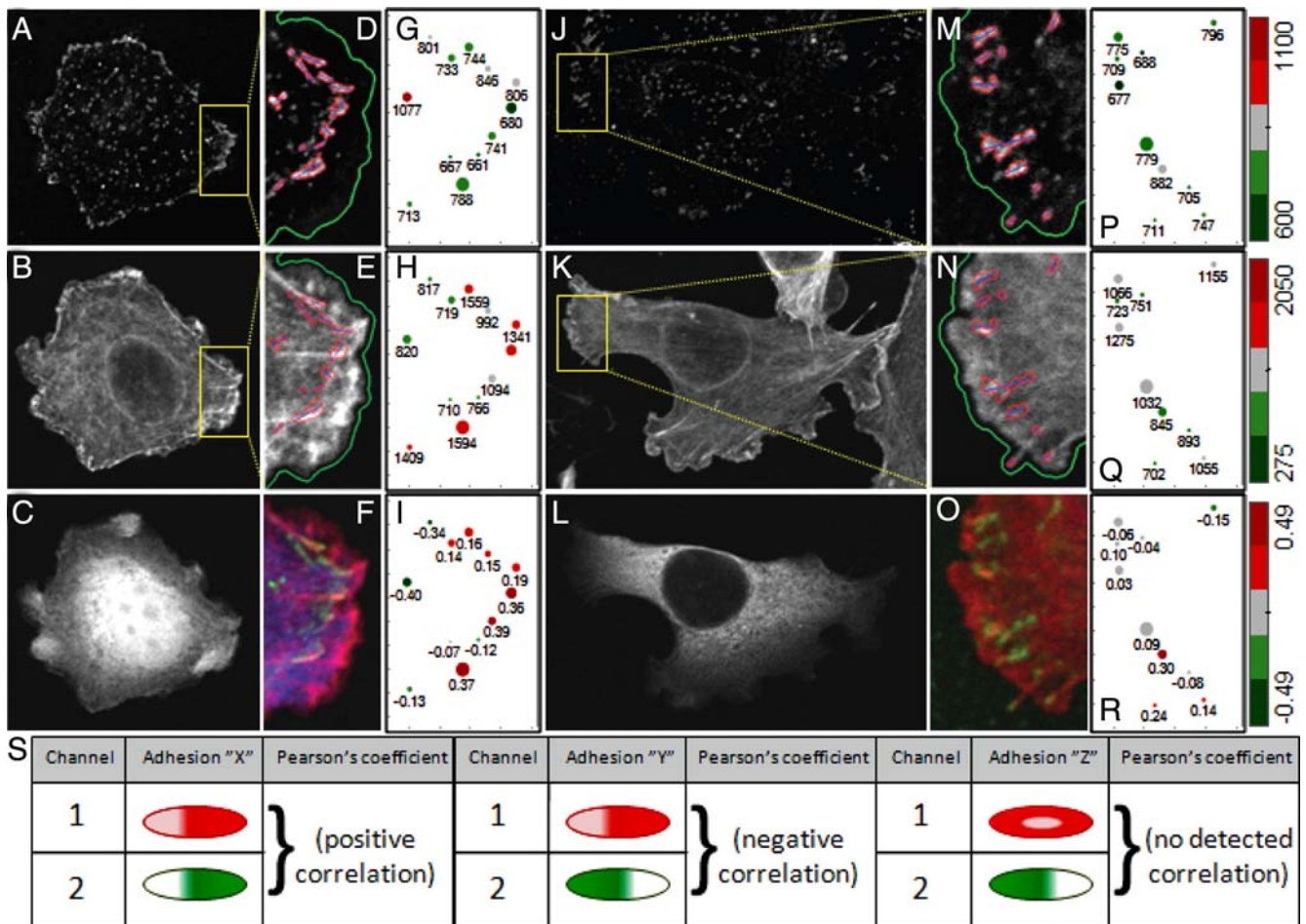
Figure 4. Quantitative image analysis of CMAC component intensity and colocalization. Three-channel confocal images were acquired of a single MCF7 cell expressing either EGFP (A–F) or EGFP-PAK4 (J–O) and labeled for integrin αvβ5 (A, D, J, and M) and F-actin (B, E, K, and N). Cell boundaries were defined using F-actin labeling (green lines in images D, E, M, and N cropped from yellow regions of images A, B, J, and K). CMACs within these boundaries were then detected and defined using the integrin αvβ5 channel (red outlines in D, E, and M, N; blue lines within individual CMACs indicate CMAC major axes). CMAC center of mass coordinates (X and Y), CMAC area, mean CMAC intensity (αvβ5 and F-actin), and intra-CMAC colocalization (as defined by Pearson's r within each CMAC) of αvβ5 and F-actin were measured. Cropped, merged raw images of integrin αvβ5 (green) and F-actin (red) as well as either EGFP (blue, F) or EGFP-PAK4 (blue, O) show F-actin extending in protrusive structures beyond the existing CMACs, as well as strong colocalization (yellow) between integrin αvβ5 and F-actin in EGFP- but not EGFP-PAK4–expressing cells. Quantitative data (G–I and P–R) derived from cropped regions show each detected CMAC distributed according to its original X,Y coordinates, with dot size quantitatively reflecting original CMAC area. Dot color and associated number indicate mean integrin αvβ5 intensity (G, P), mean F-actin intensity (H and Q), or intra-CMAC colocalization of αvβ5 and F-actin (I and R), per CMAC. Color scales for αvβ5 intensity, F-actin intensity, and αvβ5/F-actin colocalization are shown to the right of P, Q, and R, respectively. These data demonstrate the method of quantitative data extraction and show directly the visual and quantitative evidence for reduced colocalization between F-actin and integrin αvβ5 within CMACs in cells overexpressing EGFP-PAK4 (see Figure 5 where equivalent data for 100s of cells and 1000s of CMACs are summarized). (S) The outcomes of Pearson's r analyses of two-channel colocalization. The distributions of red and green intensity information within Adhesion "X" reflect a strong spatial and intensity correlation between these two channels, resulting in a positive correlation score (a perfect positive correlation = 1; however, much lower values are typically detected in biological images due largely to poor signal to noise ratios). Red and green intensity distributions within Adhesion "Y" are inversed, resulting in a negative correlation (a perfect negative correlation = −1). Red and green intensity distributions appear unrelated in Adhesion "Z", resulting in an r-value close to zero.

vector, followed by time-lapse imaging of photobleached regions to allow visualization and quantification of β5-EGFP recovery after bleaching. The recovery kinetics correspond to the replacement of β5-EGFP initially localized in the bleached CMACs and highlight a dramatic increase in total recovery and recovery speed induced by mRFP-PAK4, indicating enhanced β5-EGFP turnover at sites of adhesion (Figure 7, A–C).

It is noteworthy here that fluorescence recovery immediately after photobleaching results from at least two additive processes, one being random intra-plasma membrane diffu-

sion of individual integrin heterodimers and microclusters and the second being the selective recruitment and concentration of integrins into existing CMAC structures. To better analyze the latter process of selective integrin recruitment, we performed FRAP studies of β5-EGFP in plasma membrane regions devoid of CMACs to determine the time frame of diffusional recovery in cells expressing mRFP or mRFP-PAK4 (Figure 7D). This revealed that diffusional recovery is essentially complete under both conditions after 120 s and surprisingly, that PAK4 induces enhanced diffusional recovery of β5-EGFP. This effect implies that PAK4 may either
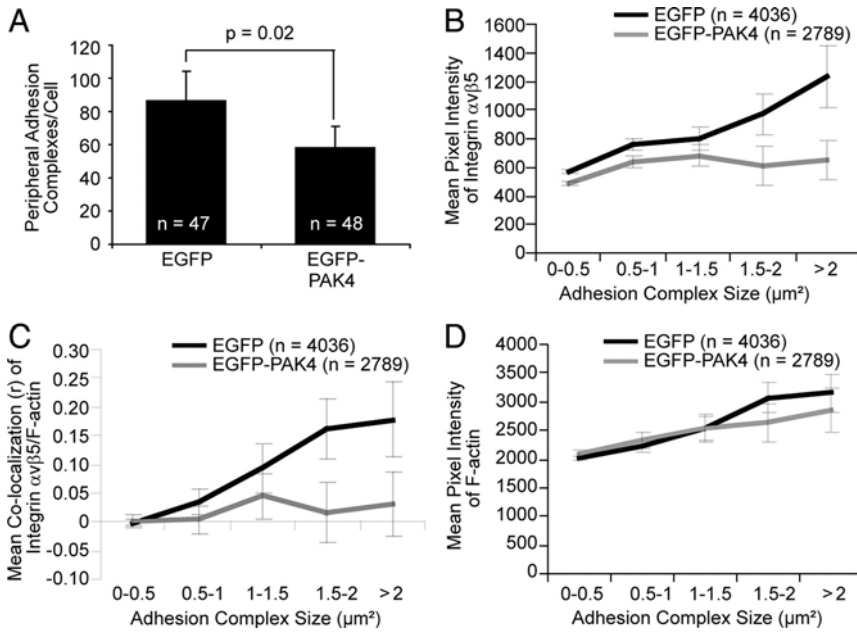
Figure 5. Influence of PAK4 overexpression on CMAC size, number, integrin clustering density, and integrin–actin connectivity. (A) Quantification of CMAC numbers at the cellular periphery from MCF-7 cells transiently expressing EGFP or EGFP-PAK4-WT. Graph shows means of the number of peripheral CMACs per cell ± 95% confidence intervals from 47 (EGFP control cells) and 48 (PAK4-overexpressing cells). (B) Quantification of integrin clustering density in CMACs at the cellular periphery. The mean intensity of endogenous $\alpha v \beta 5$ labeling in CMACs <5 μm from the cell border. The results are displayed as means of all CMACs in the designated size classes (μm^2) ± 95% confidence intervals. (C) Quantification of $\alpha v \beta 5$ versus F-actin colocalization in CMACs <5 μm from the cell border. Calculated and displayed per CMAC using Pearson's r (r) ± 95% confidence intervals. (D) Quantification of F-actin mean intensity (labeled by phalloidin) in CMACs (the area overlaying $\alpha v \beta 5$ labeling) <5 μm from the cell border as displayed in Figure 4G. Panels G, H, and I in Figure 4 are representative images showing the measurement of 4036 CMACs taken from 47 EGFP control cells and 2789 CMACs from 48 EGFP-PAK4-overexpressing cells. All data in Figure 5 are derived from at least three distinct experiments.

alter integrin diffusion by, for example, inhibiting protein–protein interactions or that PAK4 alters membrane lipid ordering to facilitate faster maximal diffusion speeds. To test this, we monitored the recovery of membrane-associated lipid dyes after photobleaching and could distinguish no difference in the presence or absence of overexpressed PAK4 (data not shown). This suggests that PAK4 actively regulates integrin $\alpha v \beta 5$ interactions and dynamics outside of detectable CMACs.

We next utilized these findings to exclude the first 120 s of $\beta 5$-EGFP recovery in CMACs, thereby removing the influence of diffusional $\beta 5$-EGFP recovery within the plasma membrane, allowing the extraction of first order recovery kinetics (Figure 7, E and F). These kinetics revealed an increased maximal recovery (typically called mobile fraction; Wehrle-Haller, 2007) of $\beta 5$-EGFP within the CMACs of mRFP-PAK4–expressing cells (Figure 7E), indicating that more of the clustered integrin population was mobile and available for turnover within the recovery period. Furthermore, the rate of recovery (excluding diffusional recovery) was also greatly enhanced in the presence of mRFP-PAK4 (Figure 7F). Both of these features indicate that the stability of CMAC-associated integrins was reduced under conditions of PAK4 overexpression. Combined with data indicating reduced CMAC number, size, density, and F-actin connectivity, these findings clearly identify a role and plausible mechanism for PAK4 in the destabilization of $\alpha v \beta 5$-mediated CMACs, ultimately resulting in reduced cell-ECM adhesion strength.

DISCUSSION

We here demonstrate that integrin $\alpha v \beta 5$ binding to vitronectin leads to PAK4 activation and that activated PAK4 functions to limit integrin-mediated vitronectin adhesion, thereby reducing total cell–ECM adhesion and facilitating enhanced cell migration. This pathway represents a novel autoinhibitory negative feedback loop that is initiated within the core machinery of cell adhesion and that then acts through an associated kinase, PAK4, to down-regulate adhesion machinery function, at least in part through the mechanism of accelerated or destabilized integrin kinetics. This intrinsic regulatory pathway is distinct from the vast array of pathways that also act on adhesion componentry but are initiated extrinsically to this machinery. Thus our findings crystallize an important new concept in PAK4 function. Integrin activation and ECM binding induce integrin clustering, leading to the formation of CMACs composed of large intracellular protein networks that are fundamental to the adhesive and migratory capacities of cells (Lock *et al.,* 2008). CMACs act as hubs for the input and output of information from the numerous signaling pathways that impinge upon these structures (Lauffenburger and Horwitz, 1996; Hynes, 2002; Kaverina *et al.,* 2002; Lock *et al.,* 2008). CMACs simultaneously transmit and adapt to these signals through structural alteration of their gross morphology and through regulation of their molecular content and kinetics, thereby concurrently reflecting and regulating cellular adhesion status. Thus, CMAC characteristics, such as number, size, density, content, turnover, and distribution are detectable features as well as key factors in the regulation of adhesion strength (Gupton and Waterman-Storer, 2006). For example, several reports demonstrate that a preponderance of small CMACs may facilitate rapid cell migration (Chrzanowska-Wodnicka and Burridge, 1996; Lee and Jacobson, 1997; Beningo *et al.,* 2001; Nayal *et al.,* 2006), whereas larger and temporally more stable focal adhesions tend to inhibit cell migration (Webb *et al.,* 2002; Nayal *et al.,* 2006). Our findings correlate with these reports by showing that WT PAK4 overexpression induced a shift in CMAC populations by reducing the average number, size, and density of CMACs, while concurrently reducing cell spreading capacity and enhancing cell migration. Conversely, knockdown of PAK4 in MCF-7 cells increased the frequency, size, and density of CMACs, while simultaneously inducing greater cell spreading and reduced cell migration. Similar results

Figure 6. Influence of PAK4 knockdown on CMAC size, number, integrin-clustering density, and actin content. (A) MCF-7 cells stably expressing PAK4-shRNA or control shRNA were plated onto VN and fixed 3 h after replating. Cells were stained with an anti-integrin $\alpha v\beta 5$ antibody and costained with rhodamine-phalloidin. Bar, 20 μm. (B) Quantification of the number of CMACs <5 μm from the cellular periphery. The results are presented as mean of number of CMACs per cell ± 95% confidence intervals; n = 36 (shRNA control cells) and 40 (PAK4-shRNA cells); p values according to unpaired two-tailed t test. (C) Distribution of CMAC sizes <5 μm from the cellular periphery; fold change in frequency of different CMAC size classes. (D) Quantification of integrin clustering density in CMACs at the cellular periphery. The mean intensity of endogenous $\alpha v\beta 5$ labeling in CMACs <5 μm from the cell border was analyzed. The results are displayed as means of all CMACs in the designated size classes (μm^2) ± 95% confidence intervals. (E) Quantification of F-actin mean pixel intensity (labeled by phalloidin) in CMACs (the area overlaying $\alpha v\beta 5$ labeling) <5 μm from the cell border, displayed as in D. (F) Quantification of $\alpha v\beta 5$ versus F-actin colocalization in CMACs <5 μm from the cell border. Calculated and displayed per CMAC using Pearson's r (r) ± 95% confidence intervals. Data for D–F are derived from three independent experiments measuring 4666 CMACs (36 shRNA control cells) and 9235 CMACs (40 PAK4 shRNA cells). All of the experiments in Figure 6 were repeated at least three times with similar results.

were obtained by Qu *et al.* (2003) using PAK4-null MEFs, wherein CMAC size was also increased, a molecular effect coinciding with a neuronal migration defect in PAK4 null embryos. Together with our results, this suggests that PAK4 substantially influences cell adhesion strength, and thereby cell spreading and migration, through a general depletion of CMAC populations. Our detailed interrogation of CMAC characteristics and CMAC population distributions, as well as our analyses of integrin turnover kinetics within CMACs, now allow us to more clearly define the mechanisms associated with PAK4 regulation of cellular adhesion and migration. First, by sorting CMACs into subpopulations based on their area, we can clearly observe the enhanced inhibitory effects of PAK4 on larger adhesions, both in terms of their absolute number and their proportion of the total CMAC population. Although total numbers of adhesions are indeed reduced by PAK4 overexpression and increased by PAK4

knockdown, it is clear that the most dramatic effects occur in the larger adhesion classes, with implications both for the function of PAK4 and for the relative functional significance of different CMAC classes. In terms of PAK4, this data implies a clear role for PAK4 in the inhibition of CMAC maturation (to larger, denser adhesions), whereas it is also implicit that larger adhesions have a strong inhibitory impact on the process of cell migration. By depleting these structures selectively, PAK4 has a potent de-inhibitory effect on cell migration.

Mechanistically, our analyses indicate that PAK4 may achieve inhibition of CMAC maturation by inhibiting integrin $\alpha v\beta 5$ clustering capacity, especially in larger adhesions. In PAK4-overexpressing cells, integrin density increased only marginally as CMACs matured to larger sizes, meaning that the limited number of CMACs that achieve a large area display integrin densities equivalent to only the smallest and
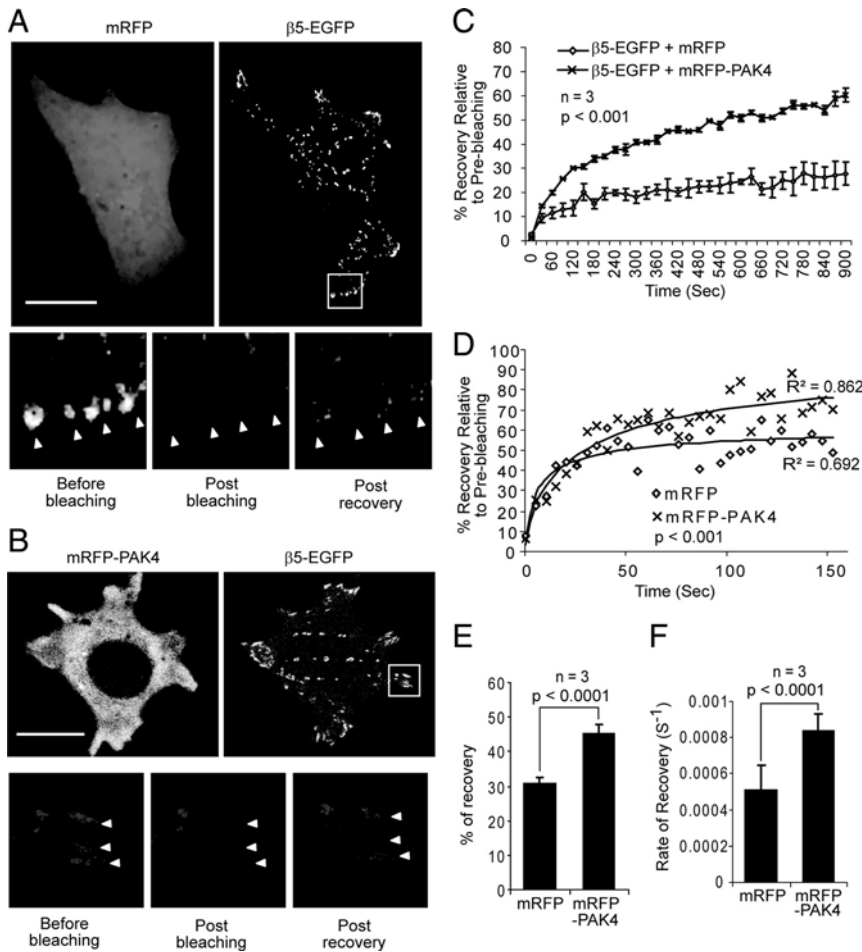
Figure 7. PAK4 promotes integrin $\alpha v \beta 5$ turnover within CMACs. FRAP analysis of integrin $\beta 5$-EGFP turnover in focal adhesions of (A) MCF-7 cells coexpressing mRFP and $\beta 5$-EGFP or (B) mRFP-PAK4 and $\beta 5$-EGFP. Enlarged images are shown (from boxes in A and B) containing individually bleached adhesions before bleaching, immediately after bleaching, and after 900-s recovery (arrowheads in bottom panels of A and B indicates bleached focal adhesions). Bar, 25 μm. (C) Quantified integrin $\beta 5$-EGFP recovery in CMACs after bleaching of cells coexpressing mRFP-PAK4 or mRFP control. The mean fluorescence intensity in the bleached region was quantified and expressed as the percentage recovery relative to the mean of three prebleached values (for that region). Background diffuse integrin intensity within the plasma membrane was subtracted from all values and further corrections were applied for nonspecific bleaching. Values represent means $\pm$ SEM from three experiments, each with a minimum of five cells per condition, with a total of 100 adhesions analyzed after photobleaching. Statistically discernable difference between mRFP and mRFP-PAK4 recovery curves was assessed at each time point; with p = 0.018 at 30 s after bleaching, and p < 0.001 at all times after 30 s according to a two-tailed unpaired *t* test. (D) Equivalent analysis of $\beta 5$-EGFP recovery after bleaching of plasma membrane regions devoid of CMACs in cells coexpressing mRFP-PAK4 or mRFP control. Values represent means from three distinct experiments including eight cells and 13 bleached regions per condition. A two-tailed unpaired *t* test of the means at all time points reveals a statistically discernable difference p = 0.011, and *t* test using all samples and all time points in-dicates p = 1.7×10^{-9}. Fitted lines represent free diffusion recovery functions as previously described (Scott *et al.*, 2006). (E and F) Analysis of the first order recovery (excluding the first 120 s after bleaching that include recovery from free diffusion within the plasma membrane) of $\beta 5$-EGFP in CMACs reveals significantly enhanced percentage of recovery (E) and recovery rate (F) in the presence of mRFP-PAK4. p values were calculated using two-tailed unpaired *t* tests. Representative FRAP Movies for Figure 7 are presented in the supplementary information.

most immature CMACs in control cells. Associated with these effects on integrin density are consistent effects on integrin $\alpha v \beta 5$ to F-actin connectivity. Intriguingly, PAK4 overexpression did not significantly alter the total levels of F-actin detected in individual CMACs, but had a striking inhibitory effect on the degree of colocalization (both spatial and intensity-based) between integrins and F-actin. In contrast, PAK4 knockdown significantly increased the absolute levels of F-actin detectable in individual CMACs. Combined, these data imply that PAK4 perturbs the ability of $\alpha v \beta 5$ integrins to recruit and associate with F-actin, an effect that would naturally inhibit CMAC maturation toward FAs because maturation of these structures is dependent on F-actin–mediated tensile forces and requires strong F-actin association (Albiges-Rizo *et al.*, 2009). This implies initially that PAK4 may alter CMAC structures through a mechanism predominantly dependent on the inhibition of integrin $\alpha v \beta 5$ to F-actin association.

However, our results from integrin $\beta 5$-EGFP FRAP support an alternative hypothesis, when considered in the light of previous findings comparing the kinetics of small (FCs) and large adhesions (FAs), presumably with low and high actin connectivity, respectively. As outlined previously, earlier reports have demonstrated somewhat counterintuitively

that large, temporally stable FA structures contain integrins that undergo relatively rapid turnover when compared with the integrins found in smaller, temporally unstable FCs (Ballestrem *et al.*, 2001). Here, we demonstrated that PAK4 induced reductions in CMAC size, integrin density and F-actin connectivity—morphological changes that ostensibly imply a shift toward a focal complex–oriented CMAC population. However, FRAP analyses indicate a concurrent acceleration of integrin turnover, a phenomenon that does not correlate with typical FC characteristics. We propose that if the effects of PAK4 were mediated predominantly through the inhibition of integrin-to-F-actin connectivity (or through negative regulation of actin filaments or contractility), then the smaller CMACs produced should mimic normal FCs, which combine small area and limited F-actin connectivity with low integrin turnover. In fact, we observe both smaller adhesions and accelerated integrin turnover in the presence of overexpressed PAK4 and surmise instead that accelerated integrin $\alpha v \beta 5$ turnover may be the primary result of PAK4 regulation, with reduced CMAC size, clustering density, and F-actin connectivity being secondary effects. This implies actin-independent regulatory effects of PAK4 on integrin $\alpha v \beta 5$–mediated adhesion. This interpretation is strongly supported by the significant differences in integrin

$\alpha v\beta 5$ clustering density observed with or without EGFP-PAK4 within the smallest CMACs ($0-0.5$ μm^2), which display no detectable F-actin association under either condition (zero colocalization), and hence are likely unresponsive to changes in F-actin connectivity. In addition, our observation that PAK4 enhances $\beta 5$-EGFP diffusional recovery outside of CMACs also supports a regulatory effect of PAK4 independent of actin, because the individual integrin heterodimers and microclusters that comprise the diffusing population are unlikely to be substantially connected to actin filaments. Thus, this PAK4 effect is far more likely to be mediated through inhibitory regulation of transient integrin interactions with either the ECM or cellular binding partners (cytoplasmic or membrane-bound)—interactions that would normally reduce the apparent diffusion rates of plasma membrane–localized integrins and that are prerequisite to the formation of new CMACs. Inhibition by PAK4 of these transient CMAC-precursor interactions may also explain the reduced frequency of small, nascent CMACs observed with PAK4 overexpression. In addition to these results, a direct effect of PAK4 on integrin clustering and function is also supported by our earlier identification of a direct interaction between integrin $\beta 5$ and PAK4 (Zhang *et al.*, 2002) and even more so by our recent findings that PAK4 directly phosphorylates the $\beta 5$ cytoplasmic tail at two serine residues, the mutation of which blocks PAK4-induced cell migration (Li *et al.*, 2010).

The regulation of integrin function within CMACs is the subject of enormous interest because, perhaps unsurprisingly, the majority of direct integrin (and hence CMAC) regulation is thought to occur in this localized context. This view is reinforced by the technical challenges involved in distinguishing clustered and nonclustered integrin populations experimentally, particularly using the traditional biochemical methods deployed to study, for example, protein interactions. Even with modern imaging techniques, it remains difficult to characterize diffuse protein populations and their interactions. However, it is easy to conceptualize possible modes of direct "extra-CMAC" integrin regulation, such as a situation where the binding to or modification (e.g. phosphorylation) of diffusing integrins by a regulatory partner could subtly or potently affect functional outcomes, from signaling to adhesion to motility. The same properties that are extensively regulated within CMACs, such as integrin–ECM affinity and integrin-clustering efficiency, could be modulated by binding of factors that, for example, increase integrin–ECM affinity or cytoplasmic binding partner recruitment. This combinatorial extra-CMAC effect does, in fact, exactly reflect the role of talin. However, because talin binding to integrins is widely regarded as the initiation step in the long cascade of CMAC maturation, the extra-CMAC regulatory role of talin might wrongly be considered an exception, with other direct regulatory interactions thought to occur subsequently within the confines of the CMAC structure (Tadokoro *et al.*, 2003). Intriguingly though, two more putative examples of extra-CMAC integrin regulation already exist. Integrin cytoplasmic domain–associated protein 1α (ICAP-1α) binds to the cytoplasmic domain of integrin β_{1A} and appears to compete with talin binding, resulting in accelerated FA turnover, reduced FA number and maturation, reduced cell adhesion, and enhanced cell motility—effects closely mimicking those of PAK4 (Bouvard *et al.*, 2003; Millon-Fremillon *et al.*, 2008). Remarkably, ICAP-1α is undetectable in CMACs using fluorescence techniques and is instead diffusely distributed within the cell cytoplasm, except during early cell spreading when, like PAK4, it may be recruited to peripheral membrane ruffles. Consequently,

given the strong evidence that the ICAP-1α mechanism is dependent on direct ICAP-1α-integrin β_{1A} interaction, it seems highly probable that ICAP-1α is acting directly on integrin β_{1A} outside of CMACs to perturb integrin recruitment to existing CMACs and possibly also the formation of new CMACs. Remarkably, DOK1, which also competes with talin for binding to $\beta 1$, $\beta 3$, and $\beta 7$ integrin tails (Oxley *et al.*, 2008; Anthis *et al.*, 2009), shows a highly analogous effect profile and mechanism to ICAP-1α. Thus, given that PAK4 shows a similar diffuse distribution and little if any detectable colocalization with $\alpha v\beta 5$-containing CMACs and that PAK4 promotes the diffusion rate of integrins outside of CMACs, it appears likely that PAK4 may also act to regulate integrin $\alpha v\beta 5$ interaction kinetics outside of CMACs, possibly through inhibitory phosphorylation of the $\beta 5$ cytoplasmic tail (Li *et al.*, 2010).

The capacity for integrin clustering may involve regulation of intracellular integrin transport (Caswell and Norman, 2006; Pellinen *et al.*, 2006), and/or altered binding capacity for either intracellular CMAC components or ECM ligands. In each case, the degree of integrin clustering is directly reflected by integrin density within CMACs. Thus, our analysis of integrin density (intensity) is a measure of the integrin-clustering capacity. Further, PAK4-mediated regulation of integrin interactions with VN and/or cellular binding partners inevitably impacts on integrin affinity (of individual heterodimers for VN) and/or valency (the capacity to cluster), which are the two core molecular features underlying overall integrin–ECM binding strength (avidity) (Carman and Springer, 2003). Unfortunately, these mechanisms are tightly intertwined, and we are unable to distinguish the primacy of either mechanism in the current study, because both can result in altered integrin turnover, clustering density, CMAC component recruitment, CMAC maturation, and overall adhesion status.

PAK4 can undoubtedly promote rearrangements of the actin microfilament system, possibly through the regulation of LIM kinase, which inactivates the actin de-polymerizing factor cofilin (Zhdankina *et al.*, 2001; Soosairajah *et al.*, 2005), and/or GEF-H1, affecting Rho A functions (Xu *et al.*, 2003; Callow *et al.*, 2005; Shemesh *et al.*, 2005). For example, an HGF–PAK4–LIMK1–cofilin pathway identified in prostate cancer cells may be responsible for actin filament reorganization (Ahmed *et al.*, 2008). Thus, although we propose a direct molecular effect for PAK4 on integrin clustering and overall integrin–ECM cell adhesion, PAK4 most likely promotes cell motility by exerting a broad-acting motility cue targeting both cytoskeletal remodeling and CMACs, which together represent the two core functional and regulatory machineries of cell motility (Lock *et al.*, 2008). The relative contributions of direct and indirect regulation, perhaps in part through F-actin modulation, need to be further investigated in order to fully understand the role of PAK4. Also the exact mechanism for PAK4 activation upon cell attachment needs further investigation, with upstream regulatory candidates, including the small GTPase Cdc42, which is also activated by ECM attachment (Price *et al.*, 1998; Bao *et al.*, 2002) and can activate PAK4 (Abo *et al.*, 1998; Callow *et al.*, 2002).

In conclusion, we herein demonstrate that PAK4 can be activated by integrin $\alpha v\beta 5$ ligation to VN and that activated PAK4 in turn regulates integrin $\alpha v\beta 5$–mediated adhesion. We show that PAK4 markedly inhibits integrin clustering into dense, actin-connected CMACs, resulting in fewer, smaller, and less mature adhesion complexes. Importantly, PAK4 enhances integrin $\alpha v\beta 5$ molecular turnover within CMACs, thereby de-stabilizing these CMACs and likely caus-

ing the inhibition of integrin clustering, as well as of CMAC maturation and F-actin association. PAK4 is also shown to act upon integrins in environments, such as in nascent CMAC clusters and outside of detectable CMACs, which are devoid of detectable actin connectivity, thereby supporting an actin-independent regulatory capacity. Together, these data show that PAK4 is activated by integrin $\alpha v \beta 5$ ligation to VN and that active PAK4 can then act to de-stabilize integrin-mediated adhesion structures to limit cellular adhesion levels. This biological circuit represents a novel autoinhibitory feedback loop that is intrinsic to the core machinery of cell adhesion, providing cells the capacity to autonomously tune and optimize total cell-ECM adhesion levels.

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Online supplemental material

S-Figure 1: PAK4 activity assay toward MBP. Flag-BAP (as control), Flag-PAK4-WT or Flag-PAK4-K350M were transiently transfected in COS-7 cells. PAK4 was immunoprecipitated (IP) by anti-Flag antibody from cell lysates and kinase activity was determined by an *in vitro* kinase assay using myelin basic protein (MBP) as substrate (top panel). Mid panel shows comassie blue gel staining of MBP loading and bottom panel shows lysate content of proteins detected by anti-FLAG mAb.
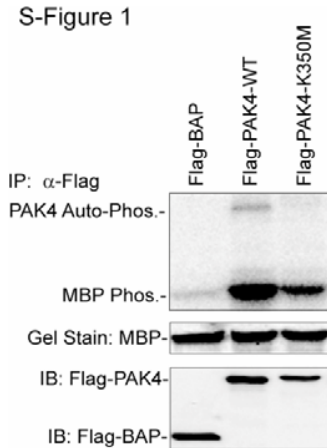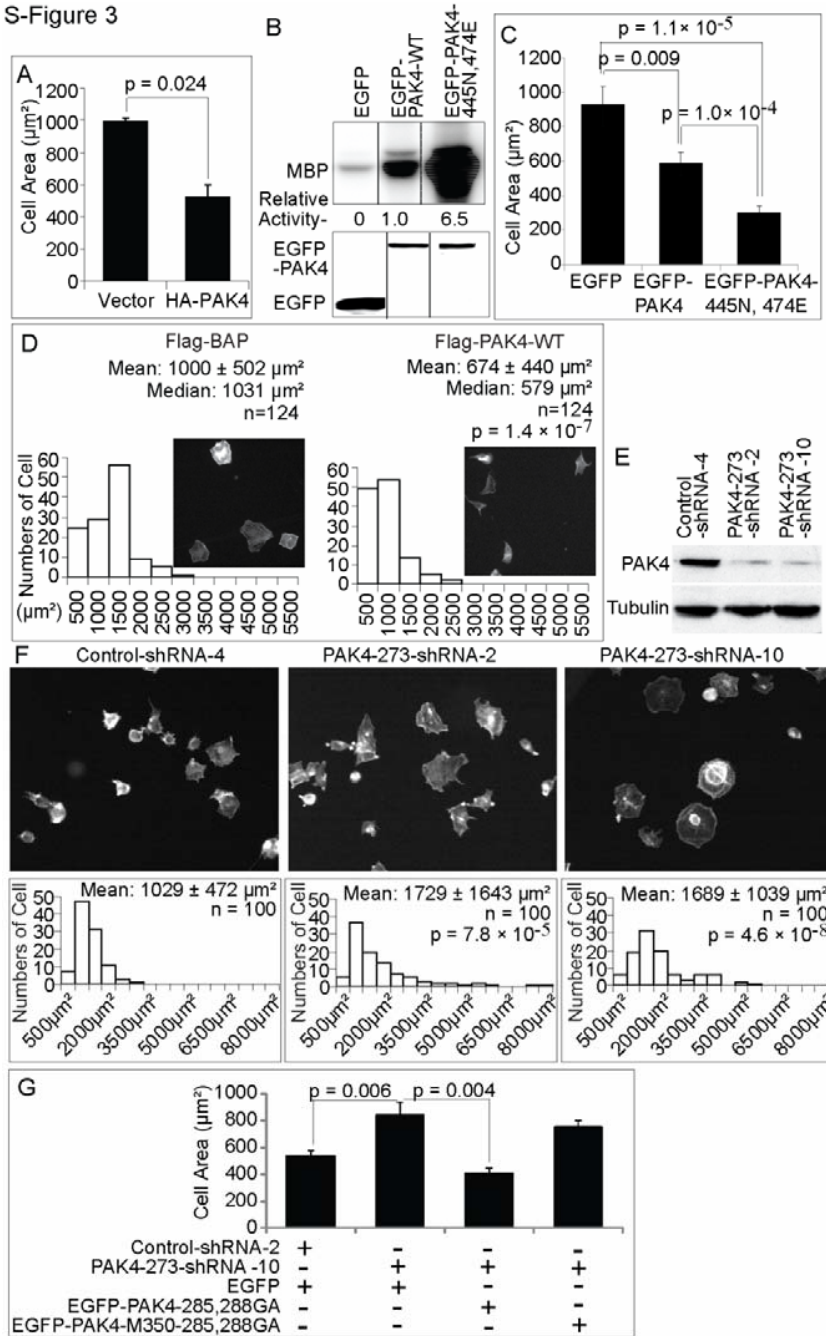

S-Figure 1

S-Figure 2: Role of PAK4 in MCF-7 cell migration onto VN. MCF-7 cells transiently transfected with control EGFP or EGFP-PAK4-WT (A) or stable over-expression with control Flag-BAP or Flag-PAK4-WT (B) as indicated were analyzed for haptotactic cell migration towards VN. Mean values ± SEM are displayed. (C) MCF-7 cells were transfected with a non-targeting negative control siRNA and two different PAK4-siRNAs as described in Materials and methods. After 48 h, haptotactic cell migration towards VN was quantified for each of the siRNA. Left: the graph displays cell migration mean values relative to control ± SEM. Right: PAK4-siRNA-mediated knock-down determined by IB (upper panel), using tubulin as a loading control (lower panel). (D-F) Migration of MCF-7 cells stably expressing PAK4-shRNA. Cells stable expressing control shRNA or PAK4-shRNA was assessed for cell migration. (D) Left graph displays haptotactic cell migration towards VN. Mean ± SEM relative to control of three independent experiments. Right: PAK4 shRNA-mediated knock-down was determined by IB using actin as loading control (lower panel). (E) Rescue of PAK4 shRNA-reduced migration by over-expression of a shRNA-resistant EGFP-PAK4-AC285, 288GA. Left: Bar graph shows haptotactic cell migration onto VN relative to the EGFP control (error bars: mean ± 95% confidence intervals, n = 8). Right: Expression of EGFP and PAK4 was determined by IB. (F) Effects of PAK4 knock-down on MCF-7 cell migration in a wound healing assay. Images were taken at the indicated times after scraping of cell monolayer. The dotted line shows the edge of the wound at the zero time directly after wounding the cell monolayer. Wound closure was measured by comparing the final 'wound' area to the initial area, and the results are expressed as % wound closure (means ± 95% confidence intervals, n = 6); *** : P < 0.001. P-values indicate statistically discernable differences compared to control according to unpaired two-tailed t-test are indicated.

S-Figure 2
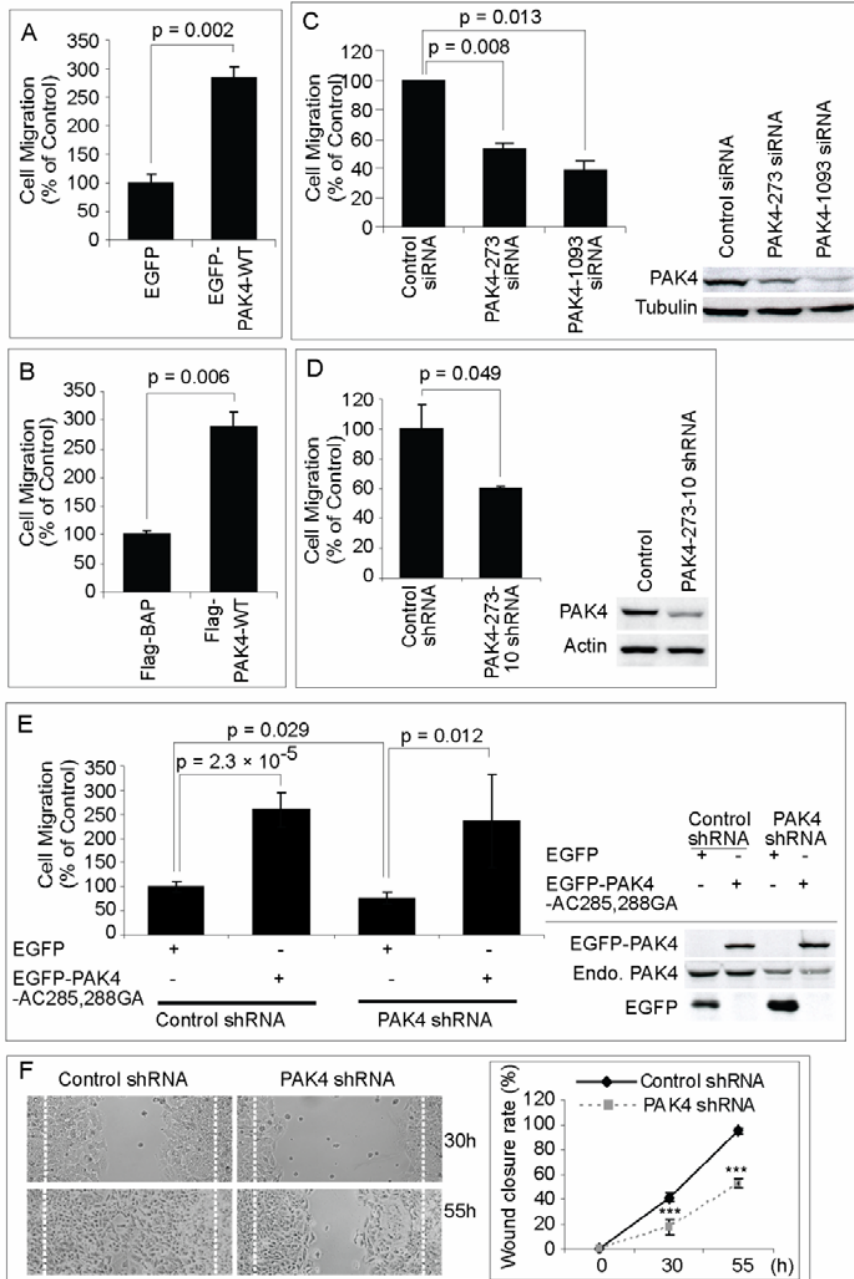
S-Figure 3: PAK4 regulates MCF-7 cell spreading onto VN. (A) Cell areas were quantified in MCF-7 cells with or without transient expression of HA-PAK4 3 h after re-plating onto VN. Error bars represent as means ± SEM between three independent experiments. (B) Kinase activity of the EGFP-tagged PAK4-WT or the double point mutant EGFP-PAK4-445N, 474E was analyzed in an *in vitro* kinase assay using MBP as a substrate (upper panel). Numbers indicate the quantified relative activities. IB shows lysate contents of EGFP-tagged proteins (lower panel). (C) Cell areas were quantified in MCF-7 cells with transient expression of EGFP, EGFP-PAK4-WT or EGFP-PAK4-445N, 474E; 5 h after re-plating onto VN. Error bars represent means ± 95% confidence intervals from 55 (EGFP control), 58 (EGFP-PAK4-WT) and 51 (EGFP-PAK4-445N474E) cells. (D) Images (upper panel) from MCF-7 cells stably expressing Flag-BAP (left column) or Flag-PAK4-WT (right column) re-plated onto VN for 5 h and analyzed for their size as described in Materials and methods. Histograms (lower panel) represent the entire distribution of individual cell areas from three independent experiments with a total of 124 cells per condition. The Mean ± SD, Median and cell numbers (n) are indicated. (E) MCF-7 cells stable expressing PAK4-shRNA were analyzed by IB with anti-PAK4 antibody (upper panel), using tubulin as a loading control (lower panel). (F) Images (upper panel) from MCF-7 cells stably expressing control shRNA clone 4 (left column), PAK4 shRNA clone 2 (middle column) or PAK4 shRNA clone 10 (right column) plated onto VN for 5 h and analyzed for their size as described in Materials and methods. Histograms represent the entire distribution of individual cell areas from three independent experiments with a total of 100 cells per condition (lower panel). (G) Cell areas were quantified in MCF-7 cells stably expressing control shRNA with transient expression of EGFP or PAK4-shRNA MCF-7 clones with transient expression of EGFP, EGFP-PAK4-285, 288GA or EGFP-PAK4-M350-285, 288GA; 1 h after re-plating onto VN. Error bars represent Means ± SEM between three independent experiments. All experiments in Fig. 3 were repeated at least three times with similar results. P-values for statistically discernable differences compared to controls according to unpaired two-tailed t-test are indicated. Note that in S-Fig. 3 B, intermediate lanes have been removed.

S-Figure 3

Supplementary movies for Figure 7

Movie #1. FRAP analysis of β5-EGFP-labelled focal adhesions in MCF-7 cells co-expressing mRFP. Shown here is a representative movie of an MCF-7 cell co-expressing β5-EGFP and mRFP approximately 48 h after transfection and 16 h after replating onto a Matek dish coated with 10 μg/ml of Vitronectin in DMEM growth media plus 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.5% BSA. Cells were imaged at 37°C in 5% CO_2 using a 63× Plan-Apochromat oil immersion objective (NA 1.4) mounted on an LSM510 confocal microscope (Zeiss). 8 bit images were collected at 2048x2048 pixels with no zoom to maximize spatial resolution while minimizing non-specific bleaching effects. Integrin β5-EGFP was bleached within manually selected ROIs delineating individual adhesions using 40 iterations at 40% of total laser power from the 488 nm line of a 4-line argon laser (Coherent). β5-EGFP was imaged pre ($\times$ 3) and post ($\times$ 31) bleaching using 0.24% total 488 nm laser power at an interval of 30 s. mRFP was imaged using 35% total laser power from a 543 nm laser (Coherent). Movie playback is at a rate of 16 frames/s and can be played using Quicktime software.

Movie #2. FRAP analysis of β5-EGFP-labelled focal adhesions in MCF-7 cells co-expressing mRFP-PAK4. Shown here is a representative movie of an MCF-7 cell co-expressing β5-EGFP and mRFP-PAK4. Conditions are the same as for Movie #1.

III

p21-activated Kinase 4 Phosphorylation of Integrin β5 Ser-759 and Ser-762 Regulates Cell Migration[*]

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From the ‡Center for Biosciences, Department of Biosciences and Nutrition, the **Breast Cancer Theme Center, the ¶Department of Laboratory Medicine, and ‖Neurotec, Karolinska Institutet, 141 83 Huddinge and the §Department of Genetics and Pathology, Uppsala University, 751 85 Uppsala, Sweden

Modulation of integrin αvβ5 regulates vascular permeability, angiogenesis, and tumor dissemination. In addition, we previously found a role for p21-activated kinase 4 (PAK4) in selective regulation of integrin αvβ5-mediated cell motility (Zhang, H., Li, Z., Viklund, E. K., and Strömblad, S. (2002) *J. Cell Biol.* 158, 1287–1297). This report focuses on the molecular mechanisms of this regulation. We here identified a unique PAK4-binding membrane-proximal integrin β5-SERS-motif involved in controlling cell attachment and migration. We also mapped the integrin β5-binding site within PAK4. We found that PAK4 binding to integrin β5 was not sufficient to promote cell migration, but that PAK4 kinase activity was required for PAK4 promotion of cell motility. Importantly, PAK4 specifically phosphorylated the integrin β5 subunit at Ser-759 and Ser-762 within the β5-SERS-motif. Point mutation of these two serine residues abolished the PAK4-induced cell migration, indicating a functional role for these phosphorylations in migration. Our results may give important leads to the functional regulation of integrin αvβ5, with implications for vascular permeability, angiogenesis, and cancer dissemination.

Integrins are heterodimers composed of α and β subunits that contain a large extracellular domain with ligand binding capacity, a transmembrane domain, and a short cytoplasmic domain (2). Integrins mediate cell adhesion and thereby play a central role in cell migration. Integrins also participate in bi-directional signaling processes across the plasma membrane where ligand binding to the extracellular matrix generates intracellular signals (outside-in) and where signaling affecting integrin cytoplasmic tails can lead to regulation of the integrin extracellular binding capacity (inside-out) (3–6).

The membrane-proximal regions of the cytoplasmic domains of the integrin heterodimer interact with each other via a salt bridge. Disruption of this salt bridge can change the integrin affinity state, indicating that the short cytoplasmic domains of integrins are essential for both inside-out and outside-in signaling (3, 7). One important mechanism whereby cellular signaling influences cellular behavior is through the interaction and/or phosphorylation of integrin cytoplasmic tails by intracellular proteins (8–12). Interactions and/or phosphorylation of integrin cytoplasmic tails may also regulate the activation state of integrins, for example, β integrin tyrosine phosphorylation can regulate talin-induced integrin activation (11, 13–16).

Integrin phosphorylation at tyrosine residues has been found in the cytoplasmic domains of α6A, β1, β3, and β4 (17–22). Also, serine/threonine phosphorylation of integrin cytoplasmic domains has been found in α4, β1, β2, β3, and β7 subunits (23–31). However, so far only a few protein kinases have been identified that phosphorylate integrin cytoplasmic domains. c-Src was found to be responsible for tyrosine phosphorylation, whereas protein kinase C and integrin-linked kinase may mediate serine/threonine phosphorylation of integrins (29, 32, 33).

Integrin αvβ5 mediates cell attachment and migration on vitronectin (2, 6, 34, 35). Integrin αvβ5 is induced in keratinocytes during wound healing and facilitates vascular endothelial growth factor-induced vascular permeability (36–38). In addition, growth factor activation of integrin αvβ5-mediated cell motility has been functionally linked to angiogenesis as well as carcinoma cell dissemination (39–41). However, how integrin αvβ5 itself may be controlled and the role of its cytoplasmic domains are both unclear.

Overexpression of p21-activated kinase 4 (PAK4)[5] can induce localized actin polymerization and filopodia formation (42) and affect cell adhesion and anchorage-independent growth of rodent fibroblasts (43, 44). We previously found that PAK4 interacts with the integrin β5 cytoplasmic tail and promotes integrin αvβ5-mediated cell migration (1). However,

whether PAK4 promotes cell motility through its interaction with integrin αvβ5 and/or its effects on the actin cytoskeleton remains unknown.

In this study, we used site-directed mutagenesis to map the PAK4-binding site within integrin β5 as well as the integrin β5-binding site within PAK4. Importantly, we also identified PAK4-mediated phosphorylation of two serine residues in the integrin β5 cytoplasmic domain that are involved in the regulation of cell motility. These results provide important information regarding the intracellular regulation of αvβ5 activity.

EXPERIMENTAL PROCEDURES

Reagents—Anti-FLAG mouse mAb M2 was acquired from Sigma, anti-integrin αvβ5 (P1F6) mouse mAb from Invitrogen, rabbit anti-HA (Y11) pAb from Santa Cruz Biotechnology, and rabbit anti-human integrin β5 cytoplasmic tail from Chemicon Int. Cell culture media, Lipofectamine Plus, and Lipofectamine 2000 were purchased from Invitrogen. An ECL detection kit, protein G-Sepharose, and glutathione-Sepharose were from Amersham Biosciences. [γ-^{32}P]ATP was from Amersham Biosciences. Vitronectin was purified from human serum as described previously (45). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, myelin basic protein (MBP), and all other chemicals were obtained from Sigma.

Yeast Two-hybrid Assay—Yeast mating test assay was performed using the DupLEX-A Yeast Two-Hybrid System (OriGen Technologies) as previously described (46). The PAK4 C terminus (CT) (amino acids 396–591) was subcloned into the EcoRI and XhoI sites of the pJG4–5 prey vector and transformed into the EGY48 yeast strain, including the pSH18–34 reporter gene (*lacZ*) plasmid. The integrin β5 cytoplasmic domain (β5-aa-753–799) and various fragments thereof were subcloned into the EcoRI and XhoI sites of the pEG202 bait vector. Point mutations in the integrin β5 tail were generated with the QuikChange site-Directed mutagenesis kit using pEG202-β5-tail as a template and then transformed into the yeast strain RFY206. The correct reading frames and sequences were verified by sequencing. Yeast mating tests were performed by using the RFY206 strain, including different integrin β5-tail constructs with the EGY48 strain, including the PAK4-CT construct.

Mammalian Cell Expression Vectors—FLAG-PAK4-WT, FLAG-PAK4-K350M, FLAG-PAK4-ΔIBD, and FLAG-BAP (bacterial alkaline phosphatase) were previously described (1). The truncated forms of PAK4, N-terminal amino acid 1–322 (NT), kinase domain amino acid 323–591 (KD), and C-terminal amino acid 396–591 (CT), were amplified by PCR using wild-type (WT) human PAK4 cDNA as a template and subcloned into the HindIII/BamHI site of 3 × FLAG-CMV^{TM}-10 expression vector (Sigma-Aldrich). Nine PAK4 point mutations within its integrin-binding domain (IBD) and a PAK4 deletion mutant, PAK4-Δ69–221, were generated using the FLAG-PAK4-WT and QuikChange (Stratagene). To construct Enhanced green fluorescent protein (EGFP)-PAK4-WT and mutants, HindIII/BamHI fragments from FLAG-PAK4 were inserted into the HindIII/BamHI sites of the pEGFP C3 vector (Clontech Laboratories, Inc.). The human integrin β5 full-length cDNA was subcloned into the EcoRI site of the pcDNA3

vector (Invitrogen). Integrin β5 mutants of pCDNA3-β5-ER760,761RE, pcDNA3-β5-ΔSERS, pcDNA3-β5-SS769, 762AA, and pCDNA3-β5-SS759,762EE, were generated by the QuikChange Kit (Stratagene) using pCDNA3-β5-WT as a template. Insertion of CMV-β5 or CMV-β5 mutants into FLAG-PAK4-WT generated FLAG-PAK4-CMV-β5 double cassette constructs.

Cell Culture—African green monkey kidney COS-7 cells and human breast carcinoma MCF-7 cells were grown in Dulbecco's modified Eagle's medium. CS-1 hamster melanoma cells kindly made available by Dr. Caroline Damsky, University of California at San Francisco, were grown in RPMI. All tissue culture media were supplemented with 5% fetal bovine serum and 10 μg/ml Gentamycin (Invitrogen). Cells were maintained in a humidified incubator with 5% CO_2. The CS-1-stable clones were grown under the same conditions as parental CS-1 cells, but in the presence of 500 μg/ml G418 (Invitrogen).

Cell Transfection— 4–8 μg of total DNA was transfected in 10-cm cell culture dishes (80–90% confluence of COS-7 or MCF-7 cells), using Lipofectamine Plus^{TM} (Invitrogen), according to the manufacturer's protocol, and cells were used 30–48 h after transfection. For transfection of CS-1 cells, 4–8 μg of total DNA was transfected in 6-well plates (1×10^6 cells/well) using the Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. CS-1 cells stably transfected with integrin β5 WT or mutant clones were established in medium containing 600 μg/ml G418. Selected cell colonies were transferred to separate culture dishes and were subsequently grown in 500 μg/ml G418 medium. Pools of mixed populations of stable transfectant CS-1 cells expressing comparable levels of each of the wild-type or mutant integrins were established by three times consecutive fluorescence-activated cell sorting with anti-integrin αvβ5 mAb P1F6 performed over a 3-month period.

Pulldown Assay—For *in vitro* GST pulldown assays, the integrin β1-tail, β5-tail (amino acids 753–799), and β5-tail mutants were individually expressed as GST fusion proteins using the bacterial expression vector pGEM-1λT (Amersham Biosciences). GST fusion proteins were produced and purified using glutathione-Sepharose beads (Amersham Biosciences) according to the manufacturer's protocol. GST pulldown assays were performed as described (1). Briefly, 200 μg of lysates from COS-7 cells overexpressing various hPAK4 constructs were incubated with 5 μg of GST fusion proteins. The result was visualized by immunoblotting, and band intensities were measured using Kodak one-dimensional image analysis or ImageJ 1.43 software (National Institutes of Health).

Kinase Activity Assay and Phosphopeptide Mapping—Various PAK4 constructs were expressed in COS-7 cells and lysed in kinase lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl$_2$, 1% Nonidet P-40, 10% glycerol 150 mM NaCl) with addition of fresh protease inhibitors (0.5 μg/ml leupeptin, 1 mM EDTA, 1 μg/ml pepstatin A, 0.2 mM phenylmethylsulfonyl fluoride) and a serine/threonine protein phosphatase inhibitor mixture (Sigma), followed by immunoprecipitation. PAK4 kinase activity was determined in a kinase buffer (50 mM Hepes, pH 7.5, 10 mM MgCl$_2$, 2 mM MnCl$_2$, 0.2 mM dithiothreitol) in the presence of 30 μM cold ATP and 10 μCi of [γ-^{32}P]ATP (3000 Ci/nM,
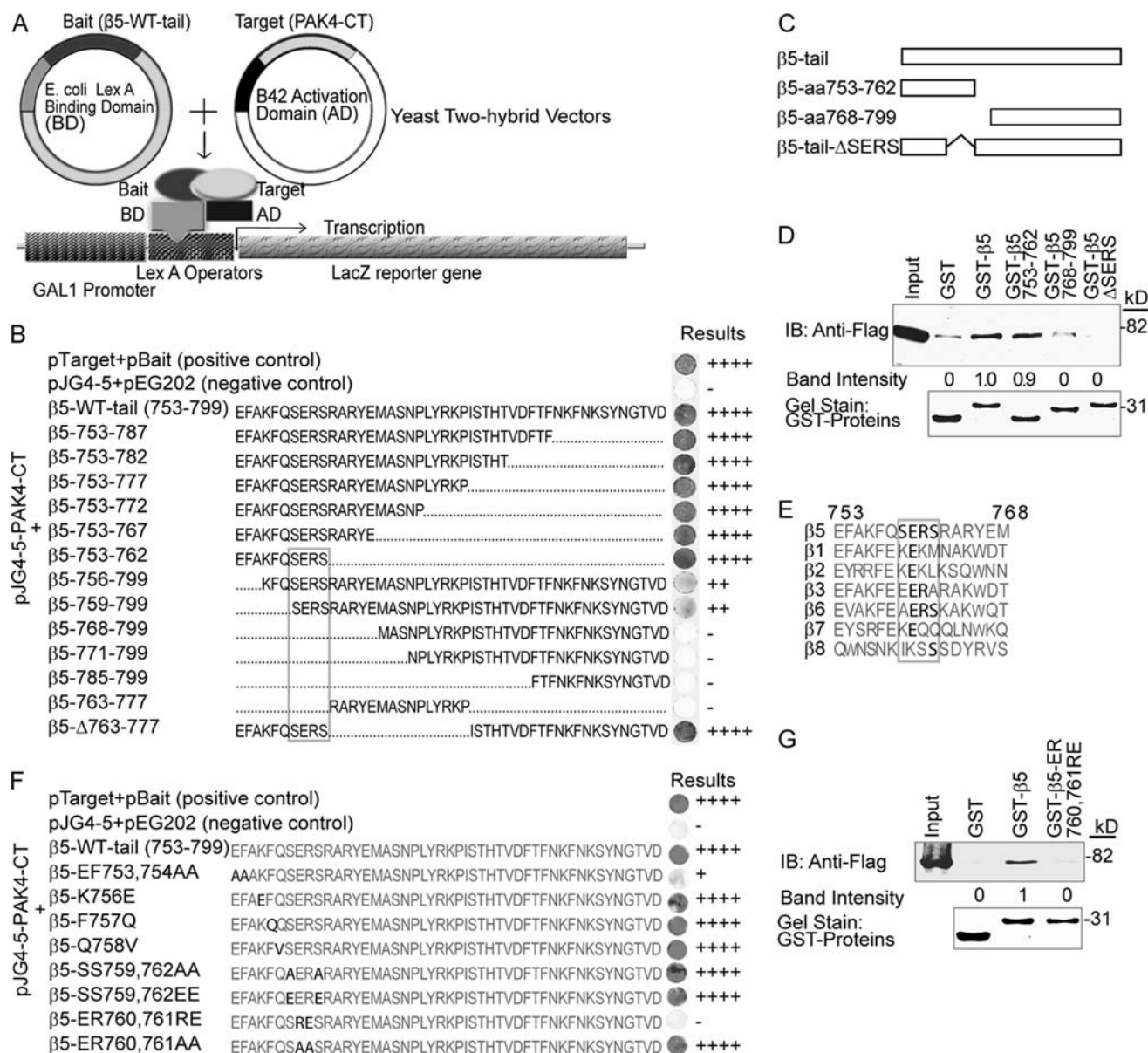
FIGURE 1. **Identification of an integrin β5 SERS-motif in PAK4 binding.** *A*, *schematic diagram* of the yeast two-hybrid system used in Fig. 1. *B*, yeast two-hybrid mating test assay. The integrin β5-tail (aa 753–799) and fragments thereof were mated with PAK4-CT. The strength of the interactions judged by the intensity of blue after 48 h is indicated on the *far right*. *C*, the *schematic diagram* shows the GST-integrin β5 tail and the β5 fragments used in the GST pulldown assay in *panel D*. *D*, GST pulldown assay of PAK4-WT using GST-integrin β5-tail and fragments thereof (*upper panel*). Pulldown of PAK4-WT using GST alone served as a negative control. The *input lane* using a lysate of PAK4 WT-transfected cells marks the size of PAK4-WT. The relative band intensities are indicated below. Coomassie Brilliant Blue gel staining shows the loading of GST proteins (*lower panel*). *E*, an alignment of partial amino acids sequences of integrin β cytoplasmic tails with the corresponding regions of the integrin β5 PAK4-binding motif. The SERS-motif and conserved amino acid within are in *bold*. *F*, the indicated integrin β5-tail point mutations were introduced within the PAK4-binding region, and the resulting products were mated with PAK4-CT as described in *A–B*. *G*, GST pulldown of PAK4-WT using GST, GST-β5-tail, and GST-β5-ER760,761RE mutants. The *input lanes* show the position of PAK4-WT by direct immunoblot of lysate (*upper panel*). The relative band intensities are displayed below. Coomassie Brilliant Blue gel staining shows the loading of GST fusion proteins (*lower panel*). Displayed results are representative of three or more experiments.

Amersham Biosciences) and in the presence of 5 μg of substrate (MBP, GST, GST-β1 tail, or GST-β5 tail) for 30 min at 30 °C. Incubation was stopped in Laemmli buffer, and samples were heated at 95 °C for 4 min. Phosphorylated proteins were separated by 12.5% SDS-PAGE. The gel was dried and visualized by autoradiography. Phosphorylation sites in GST-β5 were mapped as described previously (47). Briefly, phosphopeptides were resolved by 10% SDS-PAGE and transferred to nitrocellulose membrane. GST or GST-β5 corresponding bands were excised and digested with trypsin as described (48). The first

dimension electrophoresis was carried out in a pH 1.9 buffer, and the second dimension separation was performed using TLC in isobutyric acid buffer. The chromatography plates were exposed using Fuji Bas Bio-Imaging Analyzer, and radioactive peptides were scraped off the plate, followed by sequencing and phosphoamino acid analysis. For Edman degradation, phosphopeptides were coupled to Sequelon-AA membranes (Millipore) according to the manufacturer's instructions and sequenced on an Applied Biosystems Gas Phase sequencer. The activity in released phenylthiohydantoin derivatives from each

cycle was quantified using the Bio-Imaging Analyzer. For phosphoamino acid analysis, peptides were lyophilized and thereafter hydrolyzed in 6 M HCl for 1 h at 110 °C, followed by TLC as described (49). To determine PAK4 phosphorylation of the integrin β5 subunit in living cells, COS-7 cells transfected with HA-PAK4 underwent a phosphate starvation for 6 h at 40 h post transfection, followed by metabolic labeling with 300 µCi of [γ-^{32}P]ATP for 2 h at 37 °C. Cells were then washed twice with phosphate-free Dulbecco's modified Eagle's medium and lysed in radioimmune precipitation assay buffer. Integrin αvβ5 was immunoprecipitated by mAb P1F6, and the phosphorylated β5 subunit was visualized by autoradiography.

Cell Adhesion Assay—A cell adhesion assay was performed as described (35). Briefly, non-treated 48-well cluster plates (Corning Costar Corp.) were coated with vitronectin (VN) and blocked by 1% heat-denatured bovine serum albumin. 5×10^4 CS-1 cells/well transfected to express integrin β5, integrin β5 mutants, or co-transfected to express integrin β5 and PAK4, were seeded in wells and allowed to attach at 37 °C for 30 min. The attached cells were counted using a microscope (10× objective) after cell staining by crystal violet or, alternatively, quantified using by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Cell Migration Assay—Haptotactic cell migration assays were performed using Transwell chambers (Costar Inc.) with 8.0-µm pore size as described before (1). Briefly, 1×10^5 MCF-7 cells transiently transfected with EGFP, pEGFP-PAK4, or PAK4 mutants were added on top of the Transwell membranes coated with VN on the bottom and allowed to migrate toward VN for 6 h at 37 °C in adhesion buffer (1, 35). The migrated cells were counted using a fluorescence microscope, and adjustment was made for the transfection rate of each population as determined by flow cytometry or by manual counting using fluorescence microscopy. For CS-1 cell migration assay, 1×10^5 CS-1 cells transiently transfected or stable clones with pcDNA3 empty vector, integrin β5, or β5 mutants were added into the Transwell membranes and allowed to migrate toward VN for 24 h at 37 °C. The migrated cells were stained by 0.5% crystal violet and counted using a microscope (10× objective). For CS-1 cells stably expressing integrin β5 or mutant β5 that were co-transfected to express EGFP or EGFP-PAK4, cell migration was quantified as described above for EGFP/EGFP-PAK4 co-transfected MCF-7 cells.

Flow Cytometry Analysis—Cell surface expression levels of integrins were analyzed by measurement of fluorescein isothiocyanate staining intensity by FACScan® flow cytometer using CellQuest software (BD Biosciences) after staining with anti-integrin αvβ5 mAb P1F6 and a fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories) essentially as described by Bao and Strömblad (50).

*Statistical Analysis*—Data were analyzed for statistical significance using an un-paired two-tailed t test.

RESULTS

Identification of an Integrin β5 Cytoplasmic Tail SERS-motif Involved in PAK4 Binding—We previously found that PAK4 binds to the integrin β5-subunit and promotes integrin αvβ5-
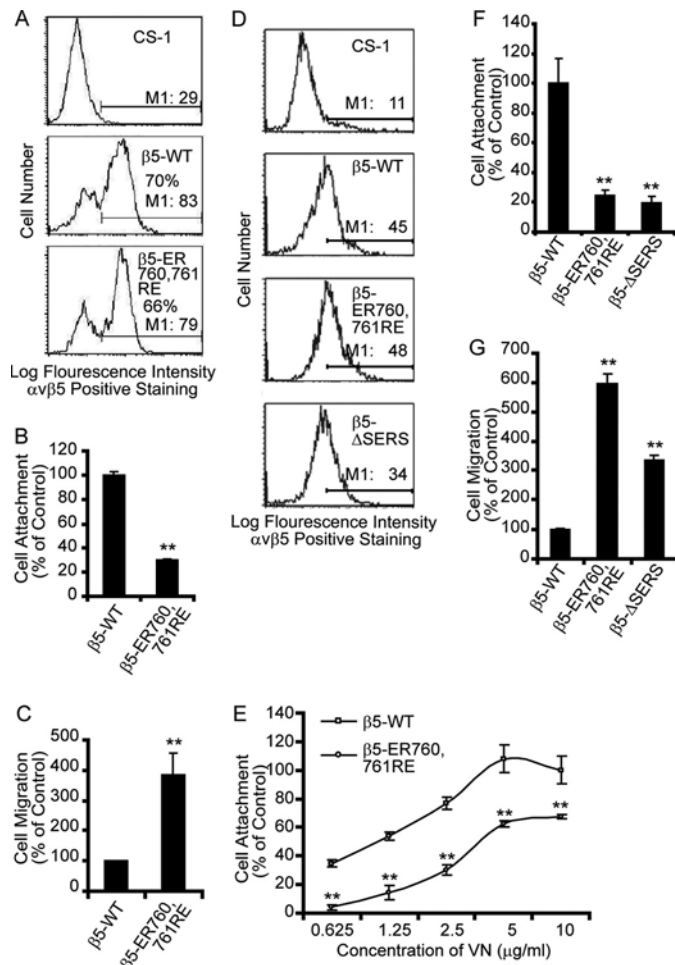
FIGURE 2. **Mutation or deletion of the integrin β5 tail SERS-motif affects cell attachment and migration.** *A*, the integrin αvβ5 cell surface expression levels of CS-1 cells transiently transfected with integrin β5-WT or β5-ER760,761RE were analyzed by flow cytometry. Non-transfected CS-1 cells served as a control for the FACS settings. The given percentages represent the fraction of cells displaying αvβ5-staining above untransfected CS-1 cells, and *M1* shows mean intensity of the cells expressing αvβ5. These cells were then used for cell attachment and migration assays. *B* and *C*, *bar graphs* show quantification of cell attachment (*B*) or cell migration (*C*) on VN of CS-1 cells transiently expressing integrin β5-WT or β5-ER760,761RE, where β5-WT-expressing cells are defined as control. *D*, flow cytometry analysis of integrin αvβ5 cell surface expression in CS-1 cells with or without stable expression of integrin β5 or mutants thereof. *M1* is the mean intensity of the cells expressing αvβ5. *E* and *F*, cell attachment and (*G*) motility on VN of CS-1 cells stably expressing integrin β5 or indicated β5 mutants. *E*, cell attachment onto different VN-coating concentrations and (*F*) to VN-coating using 2.5 µg/ml. *Bars* represent mean values ± S.E. (*B*, *C*, *F*, *G*) or S.D. (*E*) (*n* = 3). Statistically discernable differences as determined by t test are indicated (**, $p \leq 0.01$). Displayed results are representative of three or more experiments.

mediated cell motility (1). We here mapped the PAK4-binding site within the integrin β5-subunit cytoplasmic domain at the amino acid level. Firstly, we analyzed PAK4 interaction in yeast two-hybrid mating tests (a *schematic outline* is shown in Fig. 1*A*) of WT β5 and 13 deletion mutants or fragments thereof as indicated in Fig. 1*B*. The smallest β5 fragment maintaining full binding strength to PAK4 contained a 10-amino acid N-terminal β5-tail sequence (β5–753-762). Any further deletions of this fragment caused loss or partial loss of PAK4 binding. Further, truncation of this 10-amino acid fragment revealed that a β5-SERS motif (β5–759-762) was required for PAK4 binding. Thus, the results suggest that full PAK4 binding requires a
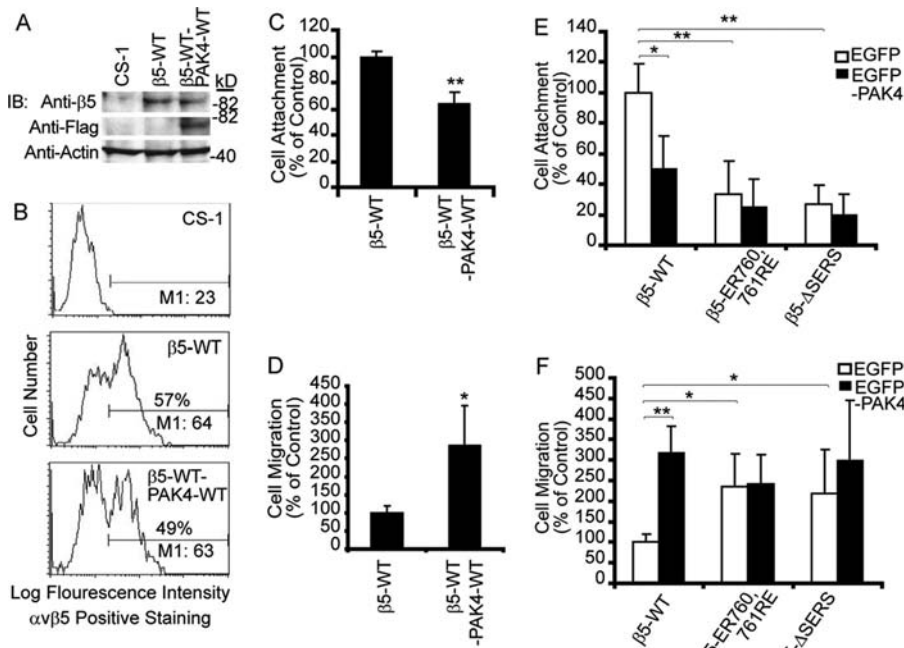
FIGURE 3. **The PAK4-binding integrin β5 tail SERS-motif regulates cell attachment and migration.** *A* and *B*, the expression levels of CS-1 cells transiently transfected with integrin β5-WT or co-transfected with integrin β5-WT and FLAG-PAK4-WT analyzed by immunoblotting (*A*) or flow cytometry (*B*) with untransfected CS-1 cells as negative control and actin as a loading control. Percentages and M1 values are as shown as in Fig. 2. *C*, cell attachment and (*D*) cell motility on VN (10 μg/ml) of CS-1 cells transiently transfected with β5-WT or co-transfected with integrin β5-WT and PAK4-WT. *E*, cell attachment and (*F*) cell motility on VN of stable clones of CS-1-β5-WT, CS-1-β5-ER760,761RE, and CS-1-β5-ΔSERS transiently co-expressing EGFP (*open bars*) or EGFP-PAK4 (*solid bars*). Parental CS-1 cells transfected with EGFP or EGFP-PAK4 served as background control (not shown). Values obtained with CS-1-β5-WT co-transfected with EGFP empty vector were defined as control. *Bars* represent mean values ± S.E. (*n* = 3). Statistically discernable differences as determined by *t* test are indicated (*, *p* ≤ 0.05; **, *p* ≤ 0.01). Displayed results are representative of three or more experiments.

The Integrin β5 Tail SERS-motif Affects Cell Attachment and Motility—To test the potential role of the integrin β5 SERS-motif in regulation of cell adhesion and migration, full-length integrin β5 (β5-WT) and two mutants, β5-ΔSERS and β5-ER760,761RE, were expressed in CS-1 hamster melanoma cells that do not express endogenous integrin β3 or β5 subunits and therefore are unable to attach to VN (51). The cell surface expression levels of WT β5 and β5-ER760,761RE were similar upon transient transfection when analyzed by FACS using anti-integrin αvβ5 mAb P1F6 (Fig. 2*A*). Untransfected CS-1 cells did not attach or migrate on VN (data not shown). Interestingly, CS-1 cells transiently transfected with β5-ER760,761RE displayed a significantly lower attachment to VN as compared with cells transfected with β5-WT (Fig. 2*B*). In parallel, β5-ER760,761RE-transfected CS-1 cells exhibited markedly increased cell motility as compared with WT β5-transfected cells (Fig. 2*C*) displaying an inverse correlation between cell attachment and motility. Likewise, CS-1 cells stably expressing similar levels of WT β5, β5-ER760,761RE or β5-ΔSERS were analyzed (Fig. 2*D*). Cells expressing β5-ER760,761RE or β5-ΔSERS displayed a marked reduction in cell attachment combined with a 3- to 6-fold increase in cell motility on VN as compared with WT β5-transfected cells (Fig. 2, *E–G*). These results indicate that the membrane-proximal SERS motif in the integrin β5 cytoplasmic tail affects the extracellular function of integrin αvβ5.

PAK4 Regulation of Integrin αvβ5-mediated Cell Attachment and Migration May Involve the Integrin β5-tail SERS-motif—Overexpression of WT PAK4 in MCF-7 cells stimulated integrin αvβ5-mediated cell migration on VN (1). To determine whether the SERS-motif in the integrin β5-tail may play a role in PAK4-mediated regulation of cell motility, we co-transfected WT PAK4 with WT integrin β5 or with non-PAK4-binding integrin β5 SERS-motif mutants in CS-1 cells. These transfected cells were analyzed for the effects on integrin αvβ5-mediated cell attachment and migration. When CS-1 cells were transiently transfected with WT FLAG-PAK4 and WT integrin β5 using a double-cassette vector, cell attachment decreased and cell migration increased as compared with cells transfected with WT β5 alone (Fig. 3, *A–D*). Consistently, CS-1 cells stably expressing WT β5 were markedly affected by EGFP-PAK4 overexpression resulting in decreased cell attachment and increased cell migration on VN as compared with EGFP control-transfected cells (Fig. 3, *E* and *F*). Interestingly, CS-1 cells

10-aa region of the integrin β5 tail (aa 753–762) and that the membrane-proximal SERS-motif (aa 759–762) within β5 is critical for PAK4 binding. These results were verified in mammalian cells by GST pulldown analyses, where the β5-753-762 fragment pulled down the same amount of PAK4 as the β5-WT cytoplasmic tail (Fig. 1, *C* and *D*). However, deletion of the β5-SERS-motif (aa 759–762) abolished PAK4 binding (Fig. 1*D*). Among integrins, the SERS amino acid sequence only appears in the integrin β5 cytoplasmic tail, although ERS is found at the same position within integrin β6 and ER within integrin β3 (Fig. 1*E*).

Using yeast two-hybrid mating tests, we then fine-mapped the ten amino acids within the integrin β5 membrane-proximal region involved in PAK4 binding (Fig. 1*F*). Mutations of β5 aa 756, 757, 758, 759, or 762 did not affect its ability to bind PAK4. However, mutation of β5-EF 753 and 754 to alanine residues partially disrupted PAK4 binding, and reversing the charges by swapping ER 760 and 761 to RE completely abolished PAK4 binding. However, mutation of aa ER 760 and 761 to alanine residues did not influence PAK4 binding. The effect of the ER to RE swap of β5 aa 760 and 761 for binding to PAK4 in mammalian cells was verified by a GST pulldown assay (Fig. 1*G*). Together, this identified amino acids 753, 754, 760, and 761 in integrin β5 as involved in PAK4 binding and the SERS-motif as critical for PAK4 binding to the integrin β5 subunit.

stably expressing β5-ER760,761RE or β5-ΔSERS displayed similar levels of cell attachment and migration as when WT β5 expressing cells were co-transfected with PAK4. However, no significant changes in cell attachment or migration were observed when either of the mutant β5 expressing cells were transfected with EGFP-PAK4 as compared with control-transfected cells (Fig. 3, *E* and *F*). This suggests that the PAK4-binding β5-SERS-motif may be involved in PAK4-mediated regulation of cell attachment and migration.

Mapping of the Integrin β5-binding Site within PAK4—To further elucidate the role of the PAK4 to integrin β5 interaction, the β5-binding site in PAK4 was mapped. The PAK4 C-terminal region (PAK4-CT, aa 396–591) directly interacts with integrin β5 in yeast two-hybrid mating tests and the critical IBD was located within aa 505–533 (1). To analyze if this region of PAK4 was also critical for association with integrin αvβ5 in mammalian cells, FLAG-PAK4-WT, a kinase-inactive PAK4 mutant (K350M), FLAG-PAK4-Δ505–533 (ΔIBD), and FLAG-PAK4-Δ69–221 (Fig. 4*A*) were transiently expressed in COS-7 cells, and a GST pulldown assay was performed (Fig. 4*B*). Cell extracts of FLAG-PAK4-WT and Δ69–221 were pulled down by GST-β5 tail fusion protein but not K350M or ΔIBD. This showed that the PAK4-IBD was required for integrin β5-association in mammalian cells. Surprisingly, the kinase-inactive PAK4-K350M that contains the IBD did not associate with integrin β5. This is in contrast with our previous results from yeast two-hybrid mating tests where a C-terminal PAK4 fragment with a K350M mutation could still interact with integrin β5 (1). This may be explained by conformation differences between the K350M-mutated full-length PAK4 and a mutated PAK4 C-terminal fragment. However, the fact that eliminating the PAK4 kinase activity also blocked its integrin binding capacity raised the question whether PAK4 kinase activity may be correlated to integrin binding. Therefore, the kinase activity of the PAK4 constructs was tested. FLAG-BAP, PAK4-WT, K350M, ΔIBD, and Δ69–221 were transiently transfected into COS-7 cells, and the FLAG-PAK4 immunoprecipitates were used in an *in vitro* kinase activity assay. WT PAK4 and PAK4-Δ69–221 displayed autophosphorylation and phosphorylated the substrate MBP, whereas K350M and ΔIBD did not (Fig. 4*C*). Together, this indicates that the PAK4-IBD is required for integrin binding and that the PAK4 kinase activity correlates with integrin β5 binding in mammalian cells.

To further reveal the relationship between the PAK4 integrin binding capacity and its kinase activity, we sought to separate the kinase activity of PAK4 from its integrin binding capacity by introducing nine different point mutations into PAK4-IBD at amino acids conserved among the PAK family (Fig. 5, *A* and *B*), because other PAKs can also bind integrins.[6] To test the effect of these PAK4 mutations on PAK4 association with integrin β5 in mammalian cells, a GST pulldown assay was performed with lysates from COS-7 cells overexpressing FLAG-tagged bacterial alkaline phosphatase (BAP) used as negative control, PAK4-WT, PAK4-K350M, and the nine PAK4 point mutations introduced in full-length WT PAK4. As shown in Fig. 5*C*, the

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[6] H. Zhang and S. Strömblad, unpublished observation.
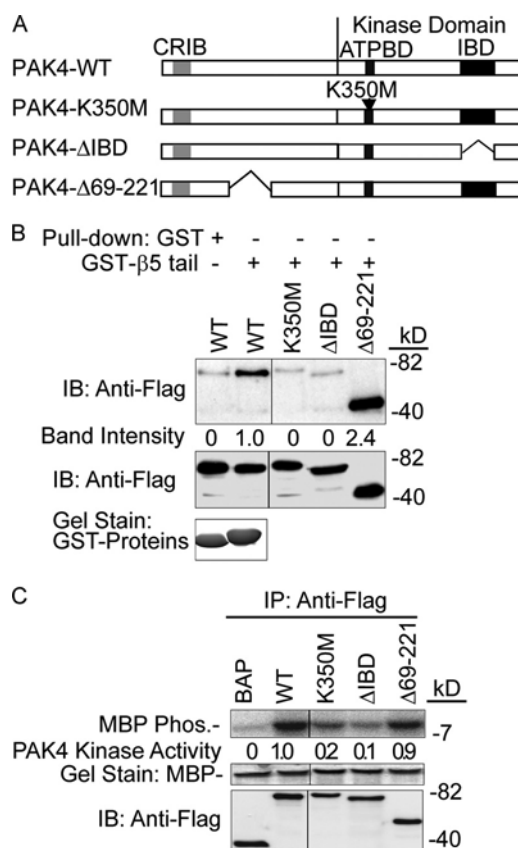
FIGURE 4. **Mapping of the integrin β5 binding region within PAK4 and the role of the IBD for PAK4 kinase activity.** *A*, the *schematic diagram* shows the domain composition of PAK4 and the PAK4 mutants used in this figure. *CRIB* denotes the Cdc42- and Rac-binding domain. The ATP-binding domain (*ATPBD*) and the integrin-binding domain (*IBD*) are situated within the kinase domain (*KD*) of PAK4. *B*, GST pulldown assay of PAK4 mutants. Cell lysates from COS-7 cells transiently expressing FLAG-tagged WT PAK4 (*WT*), PAK4-ΔIBD, PAK4-K350M, or PAK4-Δ69–221 were pulled down by a GST-β5-WT-tail fusion protein (*top panel*). PAK4-WT in combination with GST was used as a negative control (*top panel*). The quantified relative band intensities are shown *below*. The *middle panel* shows the used amounts of overexpressed PAK4 analyzed by immunoblotting (*IB*). Coomassie Brilliant Blue gel staining shows the relative amount of GST fusion proteins (*lower panel*). *C*, PAK4 kinase assay. Immunoprecipitates from COS-7 cells transiently transfected with PAK4 constructs were used in an *in vitro* kinase assay using myelin basic protein (*MBP*) as a substrate (*top panel*). The kinase activities of PAK4 mutants were quantified using a PhosphorImager, and numbers relative to WT PAK4 activity for MBP phosphorylation are indicated *below*. FLAG-BAP was used as a negative control. The *middle panel* shows Coomassie Brilliant Blue gel staining of MBP loading, and the *lower panel* shows loading of overexpressed proteins detected by immunoblot (*IB*).

mutants K350M; PP513,514AA; PP519,520AA; and A523P displayed no or weak association with the integrin β5-tail in mammalian cells, whereas all the other PAK4 point mutants tested displayed a similar positive β5 association as WT PAK4.

The effect of the nine point mutations on PAK4 kinase activity was analyzed using an *in vitro* kinase assay (Fig. 5*D*). We observed that the kinase-defective PAK4-K350M as well as three other PAK4 mutants; PP513,514AA; PP519,520AA; and A523P displayed no significant kinase activity. These were the same four PAK4 full-length mutants that also showed no or weak integrin binding capacity. All other mutants displayed equal or stronger PAK4 kinase activity and autophosphorylation compared with WT PAK4. Given that the same four point mutants that lacked kinase activity were also impaired
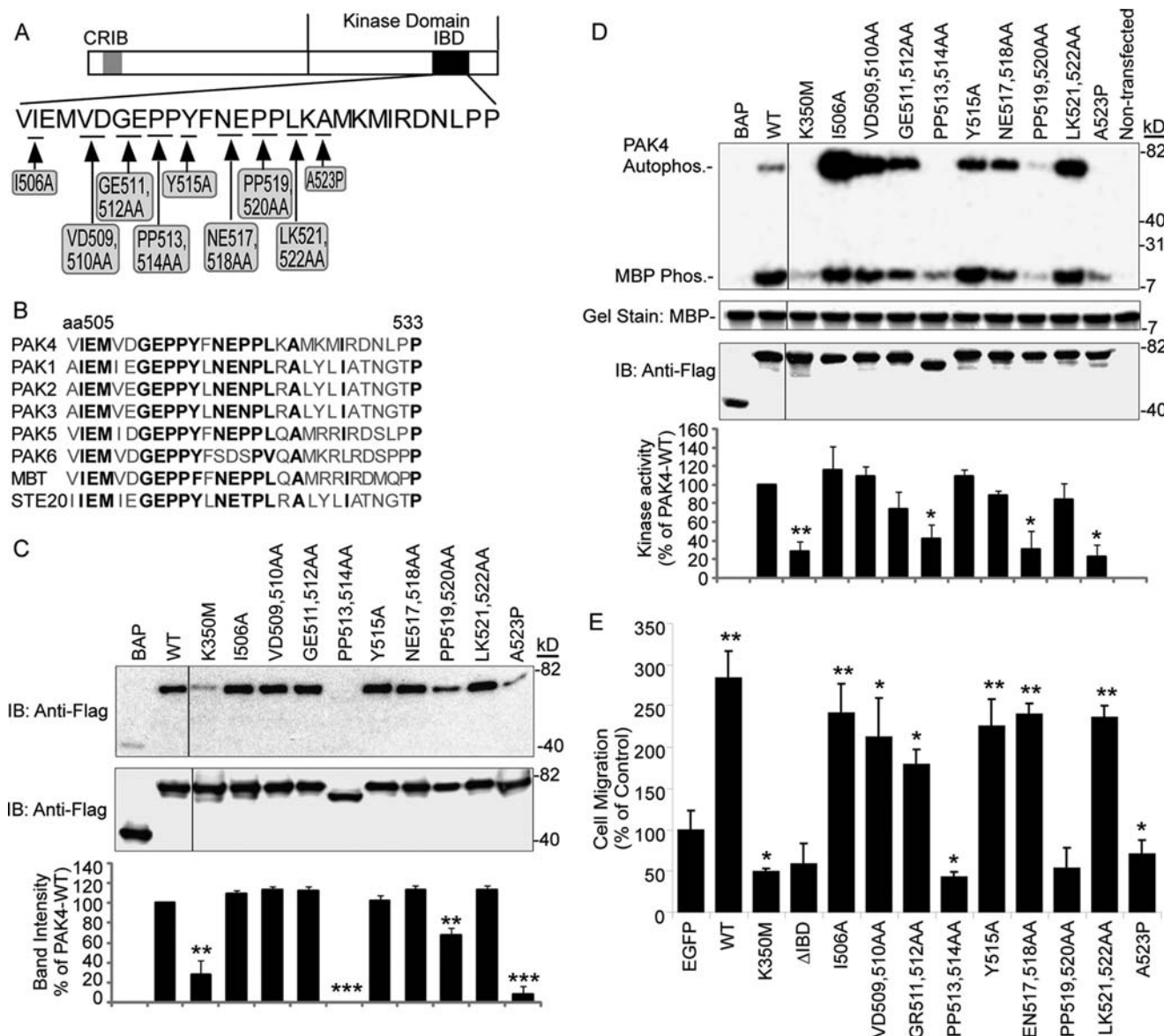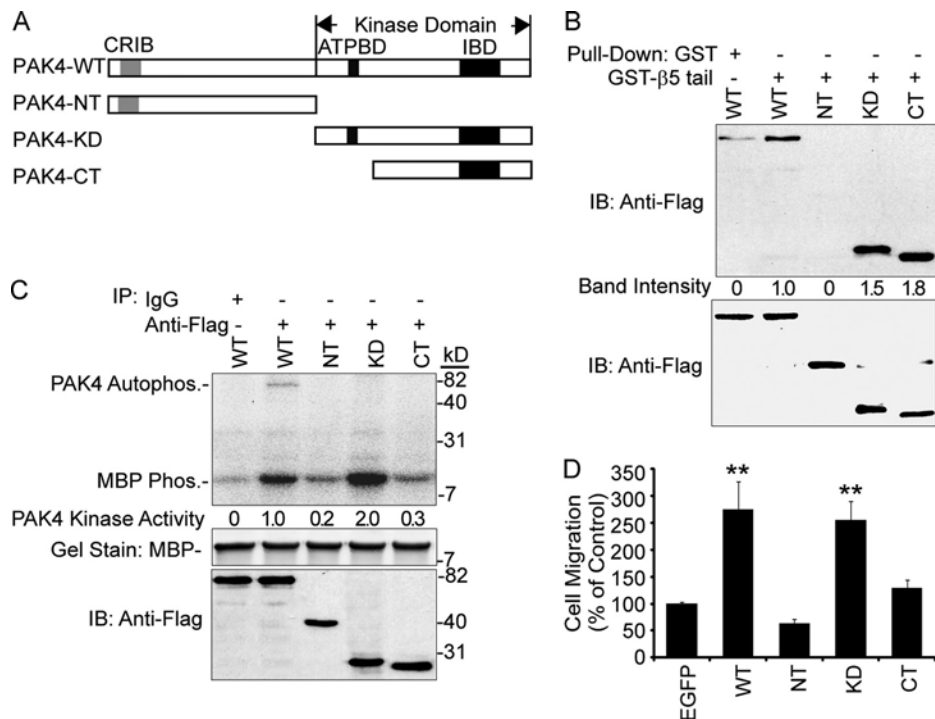
FIGURE 5. **Mapping of the PAK4 integrin-binding site at the amino acid level and function of PAK4-IBD point mutations for PAK4 kinase activity and cell migration.** *A*, the *schematic diagram* shows the PAK4 point mutations created within the PAK4 IBD. *B*, alignment of the integrin-binding motif of PAK4 with the corresponding regions of human PAKs, the *Drosophila* PAK4 homologue mushroom bodies tiny (*MBT*) gene and yeast STE20. Amino acids in *bold* show conservation among the PAKs, which was used as the basis for the PAK4-IBD point mutation design. *C*, GST-β5-tail pulldown of lysates from COS-7 cells transfected with FLAG-tagged full-length PAK4 and point mutants thereof as indicated (*top panel*). FLAG-BAP was used as a negative control. Immunoblotting shows loading of FLAG-tagged proteins (*middle panel*). The migration rate of the mutant PP513,534AA protein consistently appeared higher than other mutants. This may be caused by proteolysis as a result of the mutagenesis. The relative band intensity of pulled down PAK4 is indicated in the *lower bar graph*, and the *band intensity* was set to 0 for the FLAG-BAP control and 100% of control for PAK4-WT. *Bars* represent mean intensity ± S.E. among three experiments. Statistically discernable differences compared with PAK4-WT according to *t* test are indicated (**, $p \leq 0.01$; ***, $p \leq 0.001$). *D*, FLAG-tagged proteins were analyzed in a kinase assay using MBP as a substrate (*top panel*). The FLAG-BAP and lysate of non-transfected COS-7 cells were used as negative controls. Coomassie Brilliant Blue gel staining shows the loading of MBP (*upper middle panel*), and immunoblotting shows the loading of FLAG-tagged proteins (*lower middle panel*). The relative activities quantified by PhosphorImager are shown in the *lower panel* (*bar graph*) for PAK4 MBP substrate phosphorylation activity. The kinase activity was set to 0 for the FLAG-BAP control and 100% of control for PAK4-WT. *Bars* represent mean values ± S.E. for three distinct experiments. Statistically discernable differences compared with PAK4-WT according to *t* test are indicated (*, $p \leq 0.05$; **, $p \leq 0.01$). *E*, MCF-7 cells transiently transfected with control EGFP, EGFP-PAK4 WT, or EGFP-PAK4 mutants as indicated were analyzed for haptotactic cell motility toward VN. *Bar graphs* show quantified cell motility relative to the EGFP control (mean value ± S.E. of at least three experiments). Statistically discernable differences compared with EGFP control according to *t* test are indicated (*, $p \leq 0.05$; **, $p \leq 0.01$).

in associating with integrin β5, we were unable to separate the kinase activity of PAK4 and its integrin binding capacity. Based on this, we hypothesize that the PAK4 integrin-binding site might be located in a substrate-binding pocket of PAK4. Alternatively, PAK4 kinase activity may be required for full-length PAK4 integrin binding.

Effects of PAK4 Mutations on Integrin αvβ5-mediated Cell Motility—We previously demonstrated that overexpression of PAK4 induced integrin αvβ5-mediated carcinoma cell migration (1). We now analyzed if the mutations of PAK4-IBD that disrupted integrin binding also affected the capacity of PAK4 to induce integrin αvβ5-mediated cell migration (Fig. 5*E*). Con-

FIGURE 6. **Elucidation of the role of PAK4 integrin binding capacity for cell motility.** *A*, *schematic diagram* shows common structural features of PAK4 and the PAK4 mutations: PAK4-NT (aa 1–322), PAK4-KD (aa 323–591), and PAK4-CT (aa 396–591). *B*, GST pulldown assay of PAK4 mutants. Cell lysates from COS-7 cells transiently expressing FLAG-tagged WT PAK4 (*WT*), PAK4-NT, PAK4-KD, or PAK4-CT were pulled down by a GST-β5-tail fusion protein (*top panel*). PAK4-WT in combination with GST was used as a negative control (*top panel*). The quantified relative band intensities are shown below. *Lower panel* shows the loading of overexpressed PAK4 analyzed by immunoblotting (*IB*). The GST fusion protein relative amounts used are shown in Fig. 4*B*. *C*, PAK4 kinase assay. Immunoprecipitates from COS-7 cells transiently transfected with FLAG-tagged PAK4-WT, PAK4-NT, PAK4-KD, or PAK4-CT were used in an *in vitro* kinase assay using myelin basic protein (*MBP*) as a substrate (*top panel*). PAK4-WT in combination with a normal mouse IgG was used as a negative control. The kinase activities of PAK4 mutants were quantified using a PhosphorImager, and the numbers relative to WT PAK4 activity for MBP phosphorylation are indicated *below*. The *middle panel* shows Coomassie Brilliant Blue gel staining of MBP loading, and the *lower panel* shows loading of overexpressed proteins detected by immunoblot (*IB*). *D*, cell migration assays of PAK4 mutants. MCF-7 cells were transiently transfected with control EGFP, EGFP-PAK4-WT, EGFP-PAK4-NT, EGFP-PAK4-KD, or EGFP-PAK4-CT mutants. The data represent the mean for three separate experiments ± S.E. Statistically discernable differences compared with EGFP control analyzed by *t* test are indicated (*, $p \leq 0.05$; **, $p \leq 0.01$).

pulled down by the GST-β5 tail fusion protein, PAK4-NT was not. Thus, deletion of the NT domain or the ATP-binding domain did not affect the binding capacity of PAK4 to integrin β5 in mammalian cells. Given that PAK4-CT lacks the PAK4 ATP-binding pocket, it is conceivable that it also lacks kinase activity. To test this, we measured the kinase activity of PAK-CT and compared it to that of PAK4-WT, PAK4-NT, and PAK4-KD (Fig. 6*C*). Although PAK4-WT and PAK4-KD displayed high kinase activities, PAK4-CT and PAK4-NT did not show any significant kinase activity above background. This way, we identified a PAK-CT fragment that lacked kinase activity, but with an intact integrin binding capacity.
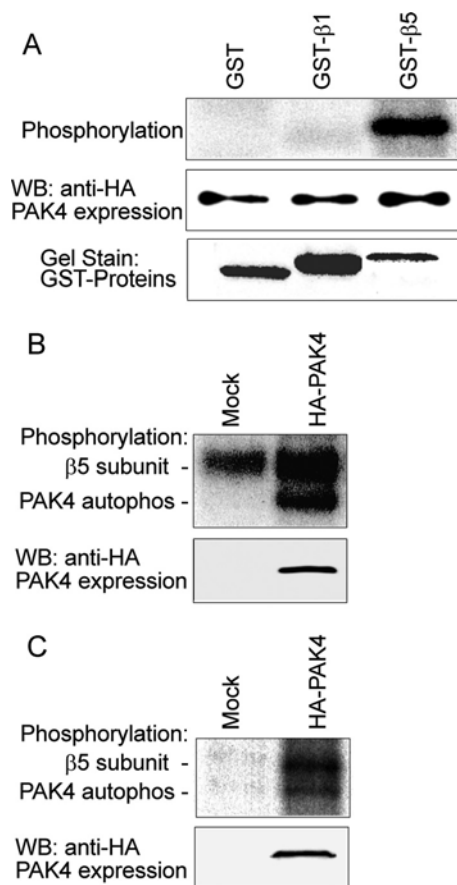
To test if PAK4-CT affected cell motility, we overexpressed PAK-WT, PAK4-NT, PAK-KD, and the PAK4-CT and analyzed integrin αvβ5-mediated cell motility of MCF-7 cells. We found that over-expression of PAK4-WT and PAK4-KD consistently enhanced cell motility (Fig. 6*D*). However, PAK4-CT as well as PAK4-NT failed to induce any substantial cell motility (Fig. 6*D*). This indicates that PAK4 kinase activity is required for promotion of cell motility, and that PAK4 integrin binding is not sufficient. Taken together, our results indicate that PAK4 kinase activity is critical to promote integrin αvβ5-mediated cell motility.

PAK4 Phosphorylates Integrin β5 Cytoplasmic Domain in Vitro and in Living Cells—Given the role of PAK4 kinase activity in promotion of cell motility, we examined PAK4-mediated phosphorylation of the integrin β5 cytoplasmic tail. Using HA-PAK4 immunoprecipitate prepared from transiently transfected COS-7 cells together with a GST-β5 cytoplasmic domain fusion protein, we found that the β5 cytoplasmic domain was a specific substrate for PAK4 *in vitro*, whereas GST-β1 integrin was not (Fig. 7*A*, *top panel*). Likewise, purified αvβ5 from human placenta could also be phosphorylated by PAK4 on its β5 subunit (Fig. 7*B*). Importantly, we also found that integrin β5 was phosphorylated in cells transfected with PAK4, as detected by metabolic ^{32}P labeling, but not in mock-transfected cells (Fig. 7*C*).

PAK4 Phosphorylates Integrin β5 at the Membrane Proximal Ser-759 and Ser-762—To determine the location of PAK4 phosphorylation within integrin β5, we employed phospho-peptide mapping. GST-β5 tail and GST control proteins were treated with PAK4 in the presence of [γ-^{32}P]ATP, trypsinized,

sistent with our previous findings, overexpression of WT PAK4 in MCF-7 cells induced integrin αvβ5-mediated cell migration on VN ~2–3 times above control levels. However, the kinase-inactive PAK4-K350M and the PAK4-ΔIBD mutants failed to stimulate αvβ5-mediated cell migration (Fig. 5*E*). Furthermore, the three point mutations in PAK4-IBD that disrupted the PAK4 integrin binding capacity together with the PAK4 kinase activity did not promote MCF-7 cell migration, whereas WT PAK4 and all other six PAK4-IBD point mutants were able to significantly induce cell motility (Fig. 5*E*). This suggests that the integrin binding capacity, kinase activity, and the capability to induce cell motility of PAK4 are correlated.

PAK4-mediated Integrin β5 Binding Is Not Sufficient to Promote Cell Migration—PAK4-CT, which lacks the PAK4 ATP-binding pocket, strongly interacted with the integrin β5 cytoplasmic domain in yeast mating tests (1). We compared PAK4-NT (N-terminal aa 1–322) and two C-terminal truncated mutants, PAK4-KD (kinase domain, aa 323–591) and PAK4-CT (C-terminal, aa 396–591) (Fig. 6*A*), for association with integrin β5 in mammalian cells by a GST pulldown assay (Fig. 6*B*). Although PAK4-WT, PAK4-KD, and PAK4-CT were
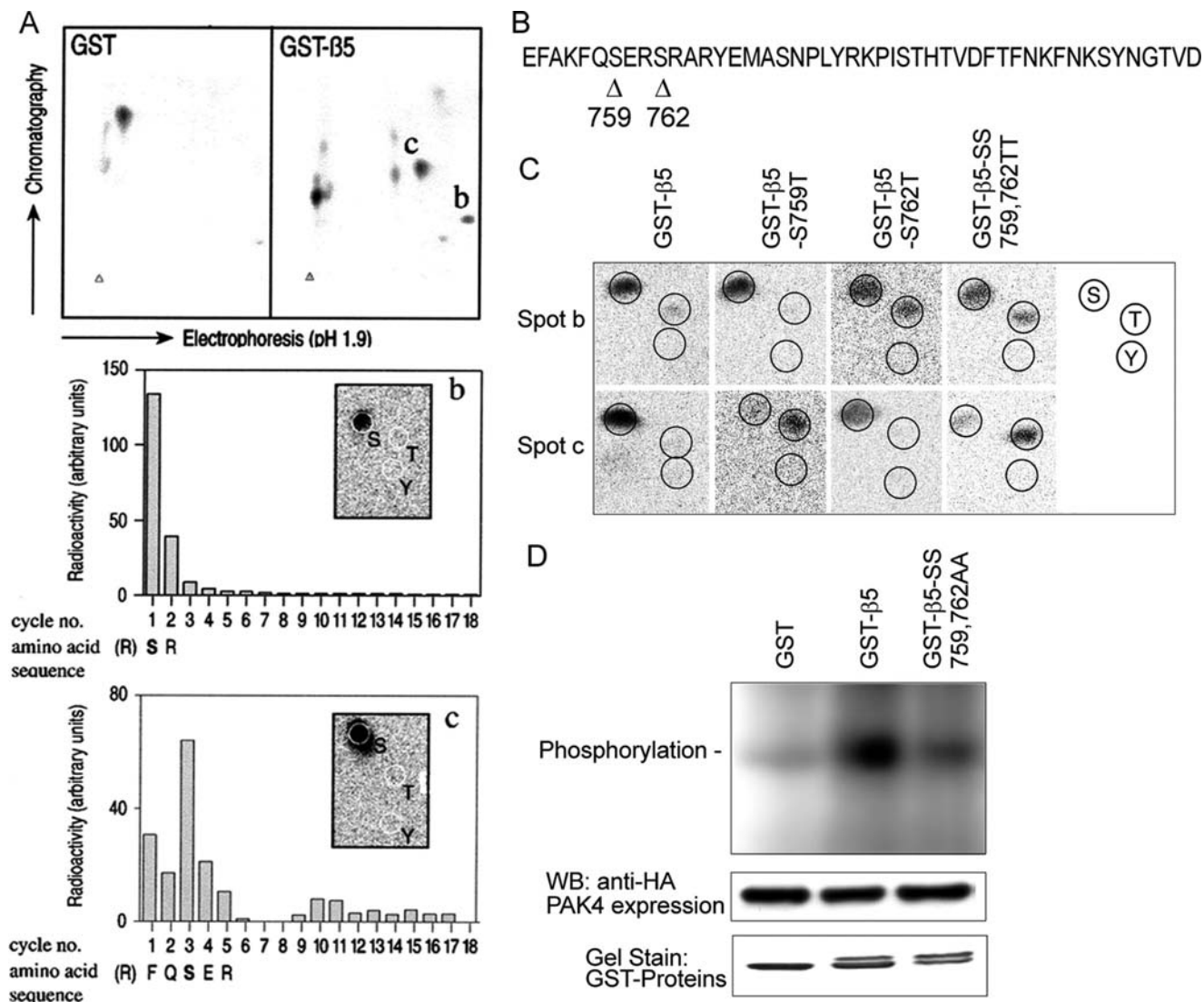
FIGURE 7. **PAK4 phosphorylates the integrin β5 subunit.** PAK4 was immunoprecipitated using an anti-HA mAb from COS-7 cells transfected with an HA-PAK4 vector and incubated with integrins in the presence of $[\gamma\text{-}^{32}P]$ATP. *A*, PAK4 phosphorylation of integrin β5 cytoplasmic domain analyzed by *in vitro* phosphorylation using purified GST, GST-β1, or GST-β5 cytoplasmic domain as substrates. PAK4 levels detected by immunoblot (*middle panel*) and the amounts of GST fusion proteins used are indicated by staining with Coomassie Brilliant Blue (*lower panel*). *B*, in the same manner, 5 μg of purified integrin αvβ5 was analyzed for phosphorylation by immunoprecipitated PAK4, separated by 7.5% SDS-PAGE, and visualized by autoradiography. *C*, PAK4 phosphorylates β5 subunit in living cells. Cells underwent phosphate starvation and then metabolic labeling as described under "Experimental Procedures." Integrin αvβ5 was immunoprecipitated in cells with or without overexpressed HA-PAK4, exposed to SDS-PAGE and autoradiography (*upper panel*). The *lower panel* shows the immunoblot for HA-PAK4 expression.

and subjected to two-dimensional electrophoresis on TLC plates. Two spots (*b* and *c*) appeared in the GST-β5 tail sample that were not found in the GST control (Fig. 8*A*, *upper*). Following phosphoamino acid analysis, both spots *b* and *c* were identified as serines by comparison with the standard marker (Fig. 8*A*, *middle* and *lower* in *boxes*). Edman sequencing of the *spot b* and *c* peptides by 18 cycles of degradation showed that the *spot b* peptide contained a high level of ^{32}P at the first amino acid, whereas the *spot c* peptide at the third amino acid contained the most radioactivity. Thus, we identified two distinct PAK4 phosphorylation sites at amino acids Ser-759 and Ser-762 using the GST-β5 cytoplasmic tail as a substrate (Fig. 8, *A* and *B*). One additional spot immediately to the left of *spot c* was also repeatedly observed and was identified as a serine in the third position, consistent with a phosphorylation of Ser-759 in an incompletely cleaved fragment. However, potential additional phosphorylation site(s) cannot be excluded due to the

built-in limitations of the two-dimensional-gel electrophoretic separation. Given that PAK4 is a serine/threonine kinase, we then mutated Ser-759 and Ser-762 to Thr-759 and Thr-762, and prepared GST-β5-S759T; GST-β5-S762T, and the double mutant GST-β5-SS759,762TT to further examine the specificity of the PAK4-mediated phosphorylation sites in the β5 tail. In the wild type peptide, spot b contained only phosphorylated serine (Fig. 8*C*), whereas in the β5-S762T and β5-SS759,762TT mutants, it contained phosphorylated threonine. Further, *spot c* displayed only serine phosphorylation in WT β5 that was completely reverted to threonine phosphorylation in the β5-S759T and β5-SS759,762TT mutants. This confirms that both the serine residues 759 and 762 of integrin β5 were phosphorylated by PAK4. However, we cannot exclude additional β5 phosphorylation sites, as may be suggested by the observation that *spot b* displayed serine phosphorylation also upon mutation of GST-β5-S762 to threonine. To further test if the two identified phosphorylation sites were responsible for the observed PAK4 phosphorylation, we generated a mutant with mutation of both Ser-759 and Ser-762 to alanine residues. As shown in Fig. 8*D*, PAK4 phosphorylated GST-β5-WT significantly above GST control background, but not GST-β5-SS759,762AA, indicating that Ser-759 and Ser-762 were responsible for the observed PAK4 phosphorylation. Amino acid sequence alignment of the human β integrin cytoplasmic domains sharing high homology with integrin β5 indicates that Ser-759 is unique to β5, whereas Ser-762 is conserved between β5, β6, and β8, but with no corresponding residues in β1, β2, β3, or β7 (Fig. 1*E*). This suggests that phosphorylation of serine residues within the membrane-proximal PAK4-binding SERS-motif is integrin-selective.

Role of Integrin β5 Ser-759 and Ser-762 in PAK4 Regulation of Cell Motility—Mutations of the integrin β5 to β5-SS759,762AA and β5-SS759,762EE did not influence PAK4 binding in yeast two-hybrid mating tests (Fig. 1*F*). To determine whether the two serines in the integrin β5 SERS-motif may play a role in PAK4-mediated regulation of cell motility, we made CS-1 cells stably expressing integrin β5-WT, β5-SS759,762AA, or β5-SS759,762EE. The cell surface expression levels of integrin β5 were similar to β5-WT, as determined by FACS analysis using anti-integrin αvβ5 mAb P1F6 (Fig. 9*A*). We then analyzed the effects on integrin αvβ5-mediated cell migration. When CS-1 cells stably expressing WT integrin β5 were transiently co-transfected with EGFP-PAK4-WT, cell migration increased compared with the same cells co-transfected with EGFP control. CS-1 cells expressing β5-SS759,762AA or β5-SS759,762EE mutants displayed a similar level of cell migration as WT β5-expressing CS-1 cells. However, no significant changes in cell migration were observed when the mutant β5-expressing cells were co-transfected with WT PAK4, in contrast with the WT β5-expressing cells (Fig. 9*B*). This demonstrates that integrin β5 Ser-759 and Ser-762 are critical for PAK4-induced cell motility and thus that PAK4-mediated phosphorylation of β5 cytoplasmic tail appears to regulate cell motility. This also indicates that a negative charge at positions 759 and 762 in the β5 cytoplasmic tail was not sufficient to promote cell migration.

FIGURE 8. **Mapping of PAK4 phosphorylation sites within the integrin β5 cytoplasmic domain.** *A*, PAK4-phosphorylated GST-β5 was separated by two-dimensional-gel electrophoresis after trypsin digestion. *Spots* marked with *b* and *c* appeared consistently in the same location in different experiments and did not occur in the GST control. These two spots were further analyzed by phosphopeptide mapping and identified as serines 759 and 762 (*middle* and *bottom panels*). The *inset* shows results of phosphoamino acid analysis. *B*, *arrowheads* point out the two PAK4-induced phosphorylation sites within the integrin β5 membrane-proximal region at serine residues 759 and 762. *C*, phosphoamino acid content of peptides from *spots b* and *c*, analyzed from GST-β5-WT, GST-β5-S759T, GST-β5-S762T, and GST-β5-SS759,762TT, and GST fusion proteins were phosphorylated *in vitro* by PAK4. Migrating positions of phospho amino acid markers are shown in the *panel* to the *right*. *D*, the two phosphorylation sites at Ser-759 and Ser-762 were mutated to alanine residues to further elucidate the identity of the two phosphorylatable residues. PAK4 phosphorylation of GST-β5 was compared with GST and the GST-β5 alanine mutant.

DISCUSSION

Cytoplasmic tails of integrins play key roles in a variety of integrin-mediated events, including adhesion and migration (2). The phosphorylation of integrin cytoplasmic tails has been proposed as a means of regulating integrin functions (10). Our data show that the integrin β5 subunit cytoplasmic tail is a substrate of PAK4. This is the first example of phosphorylation of integrin αvβ5, and how its extracellular functions may be controlled by intracellular phosphorylation. This also adds to the relatively few known substrates of PAK4 (52, 53), thereby extending the knowledge about the immediate signal transduction capacity of PAK4.

The relationship between cell adhesion and migration is complex. Our results showed that, when compared with WT integrin β5, the β5-SERS mutations resulted in a marked induc-

tion of integrin αvβ5-mediated cell migration accompanied by a decrease in αvβ5-mediated cell attachment. This could potentially be explained by the fact that cell migration compared with attachment strength can follow an approximate bell-shaped curve (54). The rate of cell migration is a function of matrix concentration, integrin abundance, and the integrin activation state. Change in any one of these properties will affect the rate of cell migration in a manner that is dependent on the original position of the cell on the bell-shaped curve (54, 55). Further, overexpression of PAK4 mimicked the effect on cell motility and attachment of the SERS mutations in the β5-tail. Together, αvβ5 in its normal constitution may mediate cell attachment that is too strong for optimal motility, whereas PAK4 phosphorylation or SERS-motif mutation may lower the attachment strength and resultantly increased cell motility.
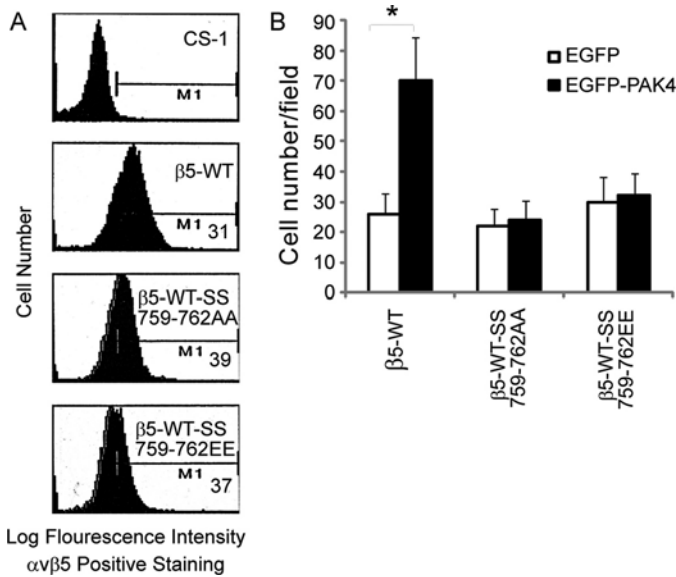
FIGURE 9. **Integrin β5 serines 759 and/or 762 are necessary for PAK4 induced cell migration.** *A,* flow cytometry analysis of integrin αvβ5 cell surface expression in CS-1 cells with or without stable expression of integrin β5-WT, β5-SS759,762AA, or β5-SS759,762EE. *M1* shows mean intensity of the cells expressing αvβ5. *B,* cell migration onto VN of stable mixed clones of β5-WT, β5-SS759,762AA, and β5-SS759,762AA transiently co-expressing EGFP (*open bars*) or EGFP-PAK4 (*solid bars*). *Bars* represent mean values ± S.E. for three experiments. Statistically discernable differences as determined by *t* test are indicated (*, $p \leq 0.05$).

The conserved β-integrin membrane-proximal region where PAK4 binds to and phosphorylates β5 is important for integrin oligomerization, inhibition of integrin conformational changes, and tethering of an integrin in the inactive state (56–58). However, although it is possible that PAK4 binding to and phosphorylation of integrin β5 cytoplasmic tail membrane-proximal region may affect the association between integrin αv and β5 subunits and/or binding between αvβ5 and VN, further studies are required to elucidate if the phosphorylation of β5-subunit by PAK4 can affect integrin hinge formation or change the conformation of integrin extracellular domain.

Two other interesting proteins, theta-associated protein 20 (TAP20) and the scaffolding protein, receptor for activated C kinase (RACK1) can also interact with the integrin β5 cytoplasmic tail. TAP20 binds to the integrin β5 tail and reduces integrin αvβ5-mediated cell migration by a protein kinase C signaling pathway (59, 60). RACK1 interacts with a conserved membrane-proximal region of the integrin β subunit cytoplasmic domain and decreased Chinese hamster ovary cell motility in a manner that may also involve its interaction with protein kinase C (59, 61). The RACK1-binding site in integrin β5 is overlapping with that of PAK4. However, further experiments are required to elucidate whether TAP20 and/or RACK1 may be functionally related to PAK4 in the regulation of integrin αvβ5-mediated cell motility.

Phosphorylation of integrin cytoplasmic tails can have both negative and positive roles in integrin regulation, possibly reflecting the importance of dynamically regulated phosphorylation in the integrin function (10). Integrin β5 tail can also be phosphorylated by protein kinase C, but the one or more specific serines to be phosphorylated were not identified (62). In this study, we demonstrate that PAK4 phosphorylation of serines 759/762 in the integrin β5 cytoplasmic tail promoted CS-1 cell migration. Among integrins, the SERS amino acid sequence only appears in the β5 cytoplasmic tail, although ERS is found at the same position within β6, ER within β3, and Ser-762 within β8. Therefore, PAK4 may also be able to phosphorylate also the integrin β6 and β8 cytoplasmic domains.

We demonstrate that phosphorylation of serine residues 759 and 762 in the integrin β5 cytoplasmic tail is necessary for PAK4-mediated promotion of cell migration. Growth factor stimulation can promote αvβ5-mediated cancer cell migration and dissemination as well as induce αvβ5-mediated angiogenesis and vascular permeability (35, 36, 39, 41, 63). Interestingly, PAK4 kinase can be activated by growth factors, such as Hepatocyte growth factor (64). It will therefore be interesting to elucidate the potential role of PAK4 in growth factor stimulation of angiogenesis, vascular permeability, and cancer cell migration and dissemination and determine if PAK4-mediated phosphorylation of integrin β5 serine residues 759 and 762 may play any role. However, PAK4 may also promote cell motility by phosphorylation of additional substrates involved in the control of cell motility, for example by regulation of the actin microfilament system (43).

In summary, we found that a unique membrane-proximal SERS-motif within the integrin β5 cytoplasmic domain can be phosphorylated by PAK4 and that this phosphorylation regulates integrin αvβ5-mediated carcinoma cell motility. This may also contribute to the understanding of intracellular signaling behind vascular permeability, angiogenesis, and carcinoma cell dissemination (36, 39, 63).

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