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 NH2-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

*Abbreviations used in this paper: hPAK, human PAK; IBD, integrin-binding domain; IP, immunoprecipitation; KD, kinase domain; LIMK, LIM kinase; MBT, mushroom body tiny gene product; MLCK, myosin light chain kinase; mPAK, mouse PAK; PAK, p21-activated kinase; PII, poly-l-lysine; VN, vitronectin; wt, wild-type.

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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 its 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 upregulated 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 αvβ5 subunit and specifically regulates αvβ5-mediated cell migration.

Table 1. 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) (Faist and Gruss, 1998). Miz-1 is a Myc-interacting zinc finger protein (Seoane et al., 2001). The other two clones have unknown functions.
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 interacted 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 a cell lysate 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 p21CIP1 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-

![Figure 2. Mapping of the PAK4 binding region within the integrin β5 cytoplasmic domain.](image-url)
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-
PAK4 interacts with and modulates integrin αvβ5 function

PAK4 interacts with and modulates integrin αvβ5 function. Zhang et al. 1291

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 underlying 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,
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 reattaching to VN, the majority of cells transfected with PAK4 displayed a relocation to lamellipodia (Fig. 5 C). A similar relocation to lamellipodia upon reattaching 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 reattaching to VN. A quantification of the PAK4 relocation by counting the number of cells with membrane-localized PAK4 is displayed in Fig. 5 D. Taken together, these results suggest that PAK4 relocation 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 reattaching 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 B1–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
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 α6β1 subunit, thereby controlling extracellular ligand-binding affinity of integrin α6β3 (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

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 as measured 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
to mediate integrin oligomerization, inhibition of integrin conformation, and constraining of an integrin in the inactive state (Zage and Marcatonio, 1998; Bodeau et al., 2001; Lu et al., 2001). However, although it is possible that PAK4 binding to integrin B5 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 B5 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 NH2-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 (Kiooses 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 B5 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 B5 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 B5 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-
Family members and found to be specific for PAK4 (unpublished data). 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-SRA3 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 pEGFP-C2 (Clontech Laboratories, Inc.) and confirmed by sequencing. The PAK4 mutants PAK4-M350, PAK4-L19, 22, and PAK4-IDBD (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 NH2-terminal sequence (aa 116–323) was amplified by PCR and cloned into the GST fusion protein expression vector pGEM-1AT (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 β 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 purifed 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 β3 cytoplasmic domain pAb (Chemicon), respectively.

Fluorescent microscopy and time-lapse video microscopy

MCF-7 cells stably expressing EGF–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 Mn2+ (RPMI 1640, 2 mM CaCl2, 1 mM MgCl2, 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 EGF–PAK4 or EGFP cells. Cells 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 (20 µg/ml), or 1% BSA at the bottom surfaces for 2 h at 37°C. MCF-7 cells were transfected with EGFP or EGF–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 × 105) were then added at the top of Transwells. Cells were 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 CaCl2, 1 mM MgCl2, 0.2 mM MnCl2, and 0.5% BSA). After thoroughly cleaning the upper chambers of the Transwells, the migrated cells expressing EGFP or EGF–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 0.5–1.0 µg/ml VN overnight at 4°C. 1% heat-denatured BSA was applied to block nonspecific adhesion. MCF-7 cells stably transfected with EGF–PAK4 or EGFP control were plated into the wells in triplicate at 5 × 104 cells/well in cell adhesion buffer (RPMI 1640, 2 mM

PAK4 interacts with and modulates integrin αvβ5 function | Zhang et al. 1295
CaCl₂, 1 mM MgCl₂, 0.2 mM MnCl₂, 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 phosphorylthrin staining intensity, respectively, by FACScan® flow cytometer using CellQuest software (Becton Dickinson) after staining with anti-integrin ov5 mAb P1F6 and a phycoerythrin-conjugated secondary goat anti-mouse pAb (Jackson ImmunoResearch Laboratories).

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PAK4 interacts with and modulates integrin αvβ5 function | Zhang et al. 1297

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