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PAK4, a novel effector for Cdc42Hs, is implicated in the reorganization of the actin cytoskeleton and in the formation of filopodia

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The GTPases Rac and Cdc42Hs control diverse cellular functions. In addition to being mediators of intracellular signaling cascades, they have important roles in cell morphogenesis and mitogenesis. We have identified a novel PAK-related kinase, PAK4, as a new effector molecule for Cdc42Hs. PAK4 interacts only with the activated form of Cdc42Hs through its GTPase-binding domain (GBD). Co-expression of PAK4 and the constitutively active Cdc42HsV12 causes the redistribution of PAK4 to the brefeldin A-sensitive compartment of the Golgi membrane and the subsequent induction of filopodia and actin polymerization. Importantly, the reorganization of the actin cytoskeleton is dependent on PAK4 kinase activity and on its interaction with Cdc42Hs. Thus, unlike other members of the PAK family, PAK4 provides a novel link between Cdc42Hs and the actin cytoskeleton. The cellular locations of PAK4 and Cdc42Hs suggest a role for the Golgi in cell morphogenesis.
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Introduction

Members of the Rho family of small GTPases Rac and Cdc42Hs have been implicated in diverse biological processes. These include roles in cell proliferation, progression through the cell cycle and oncogenic transformation (Van Aelst and D'Souza-Schorey, 1997). Cdc42Hs and Rac also play important roles in signal transduction cascades such as those that lead to activation of both the JNK and the p38 families of MAP kinases, and thus lead to long-term changes in gene expression (Bagrodia *et al*., 1995; Coso *et al*., 1995; Minden *et al*., 1995; Zhang *et al*., 1995; Brown *et al*., 1996). One of the most important functions of Rac and Cdc42Hs is the regulation of the organization of the actin cytoskeleton. Microinjection of Cdc42Hs into fibroblasts and a variety of other cell types causes the transient induction of filopodia protrusions followed by the formation of lamellipodia. While the induction of filopodia is caused by Cdc42Hs activation, the induction of lamellipodia is probably due to the ability of Cdc42Hs to activate Rac. Thus, co-expression of Cdc42Hs with a dominant-negative Rac mutant results in the sustained induction of filopodia without the subsequent induction of lamellipodia. Furthermore, microinjection of activated Rac leads to the induction of lamellipodia, but not filopodia. In addition to the formation of polymerized actin structures, both Cdc42Hs and Rac induce the formation of focal complexes that are associated with the filopodia and lamellipodia (Nobes and Hall, 1995). Finally, in some cells, both Cdc42Hs and Rac have been shown to have a role in the dissolution of stress fibers (Kozma *et al*., 1995; Dutartre *et al*., 1996; Manser *et al*., 1997), which may be due to antagonism between these GTPases and a third GTPase, RhoA (Manser *et al*., 1997).

A great deal of effort has been made to identify the downstream molecular targets for Rac and Cdc42Hs. Several proteins were shown to interact with the activated forms of Rac and Cdc42Hs, including PAK65, p67-phox, WASP, IQGAP and MLK3 (Manser *et al*., 1994; Bagrodia *et al*., 1995; Burbelo *et al*., 1995; Martin *et al*., 1995; Aspenstrom *et al*., 1996; Hart *et al*., 1996; Kuroda *et al*., 1996; Rana *et al*., 1996; Symons *et al*., 1996; Teramoto *et al*., 1996; Van Aelst *et al*., 1996). PAK was the first protein kinase that was shown to be a target for Rac and Cdc42Hs, and consequently drew much attention. Activated Rac and Cdc42Hs stimulate PAK autophosphorylation and stimulate its kinase activity. Several PAK family members have been identified, and all were shown to interact with GTP-bound forms of Rac and Cdc42Hs. These include human PAK1 and 2, mouse PAK3 and the rat homologs PAK α, β and γ (Manser *et al*., 1994; Bagrodia *et al*., 1995; Martin *et al*., 1995; Brown *et al*., 1996). The PAKs are all similar in structure, containing an N-terminal regulatory domain and a C-terminal kinase domain. They are also all quite similar in sequence, exhibiting 73% overall sequence identity and ~92% sequence identity within the kinase domain (Sells and Chernof, 1997). The regulatory domains of the PAKs contain a GTPase-binding domain [GBD (Symons *et al*., 1996), also known as Cdc42Hs/Rac-interactive binding (CRIB) domain (Burbelo *et al*., 1995)] that is necessary and essential for their direct interaction with both Cdc42Hs and Rac.

The functions of the PAKs are not yet entirely known. The sequence similarities between the PAKs and yeast STE20, however, suggest a role in transcription activation or cell morphogenesis. In *Saccharomyces cerevisiae*, STE20 is activated by Cdc42p, and is an important component of the KSS/FUS3 MAP kinase pathway. STE20 and the related CLA4 may also mediate cytoskeletal changes induced by Cdc42p, such as those that occur during cytokinesis (Cvrckova *et al*., 1995). Because of

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the evolutionary conservation between many yeast and mammalian signaling enzymes, it seems likely that the PAKs may have functions similar to the yeast STE20 and CLA4 proteins. In fact, the PAKs have been shown to activate the JNK MAP kinase pathway weakly in some cells (Bagrodia *et al*., 1995; Zhang *et al*., 1995; Brown *et al*., 1996). This suggests that, like STE20, the PAKs may be involved in MAP kinase pathways. Some groups have shown, however, that the PAKs are not necessary for JNK activation, and thus their roles in MAP kinase pathways are as yet unclear (Joneson *et al*., 1996; Lamarche *et al*., 1996; Westwick *et al*., 1997). The PAKs may also be involved in cytoskeletal organization. PAK1 was reported to induce filopodia and membrane ruffles similar to those induced by Cdc42Hs and Rac (Sells *et al*., 1997), and to localize to polymerized actin (Dharmawardhane *et al*., 1997; Sells *et al*., 1997). Interestingly, however, these cytoskeletal changes are partly independent of PAK1′s kinase activity, and they also occur independently of PAK1′s ability to bind the Rho GTPases (Sells *et al*., 1997). Thus, while overexpressed PAK1 can promote cytoskeletal changes, it may not specifically mediate the cytoskeletal changes induced by Rac and Cdc42Hs. Others have found that PAK1 does not induce filopodia or lamellipodia but instead has a role in the dissolution of stress fibers and down-regulation of focal adhesions (Manser *et al*., 1997). Finally, effector mutants of Rac and Cdc42Hs [such as RacL61(Y40C) and Cdc42HsL61(Y40C)] that do not bind the PAKs, maintain the ability to induce lamellipodia and filopodia (Joneson *et al*., 1996; Lamarche *et al*., 1996). Taken together, these results suggest that the induction of lamellipodia and filopodia by Rac and Cdc42Hs can occur independently of the known PAKs.

Here we report the cloning and characterization of a novel serine/threonine kinase, PAK4. Like other members of the PAK family, PAK4 contains an N-terminal regulatory domain and a C-terminal kinase domain. The kinase domain of PAK4 shares 53% sequence identity with those of the other PAKs. Outside of this region, however, PAK4 is entirely different in sequence from the other PAKs, except for a short stretch containing a modified GBD motif. PAK4 is the first member of the PAK family to be identified that differs significantly in sequence from the other PAKs, and thus represents an entirely new member of the PAK family. PAK4 interacts specifically with the GTP-bound form of Cdc42Hs via its GBD motif and weakly activates the JNK family of MAP kinases. Co-expression of PAK4 with Cdc42Hs causes PAK4 to translocate from a diffuse perinuclear area to the Golgi membrane and subsequently induce actin polymerization and the sustained formation of filopodia. Our results indicate that PAK4 is an important mediator of filopodia formation by Cdc42Hs and that translocation of PAK4 to the Golgi by Cdc42Hs may be important for its ability to induce filopodia. Furthermore, PAK4 interacts with the Cdc42Hs effector mutant, Cdc42HsL61(Y40C), which was previously shown to induce filopodia independently of other known PAKs. Our results indicate, therefore, that PAK4, rather than the previously identified PAKs, provides a link between Cdc42Hs and the actin cytoskeleton.

Results

Identification of PAK4, a novel member of the PAK family

To identify new PAK-related proteins, we designed degenerate primers corresponding to regions of homology between the kinase domains of yeast STE20 and mammalian hPAK2. These primers were used to generate PCR products from Jurkat cell cDNA. The PCR products subsequently were subcloned and sequenced, and sequence homologies were obtained by Blast searches of the GenBank. Using this technique, we identified partial sequences for several novel putative protein kinases. We used one of the partial cDNAs as a probe to screen human Jurkat cell and fetal brain cDNA libraries as described in Materials and methods. We isolated two identical clones that hybridize with this cDNA. One of the clones contained a complete open reading frame of ~1.7 kb as shown in Figure 1A. Like the PAK family of kinases, the C-terminal portion of the predicted protein sequence contains the 11 subdomains that are characteristic of serine/threonine protein kinases. Blast searches of the GenBank confirmed that this region is most similar to the kinase domains of the PAK family members. This putative kinase domain has 53% identity and 73% similarity with human PAK2, and 49% identity and 71% similarity with yeast STE20 (Figure 1B). The N-terminal putative regulatory domain of PAK4 does not share homology with any other known proteins except for a short sequence resembling a modified GBD/CRIB domain. This loosely conserved sequence of ~16 amino acids is found in many proteins that bind Rac and Cdc42Hs (Burbelo *et al*., 1995). This sequence has been shown to be essential and necessary for interactions of these proteins with the GTPases (Burbelo *et al*., 1995). The GBD/CRIB domain found on PAK4 in comparison with those found on several other Cdc42Hs/Rac-binding proteins is shown in Figure 1C. To determine the expression profile of PAK4, we probed a Northern blot with a cDNA probe that corresponds to the kinase domain of PAK4. A band of ~3 kb was seen in all of the human tissues that we analyzed. PAK4 appears to be most highly expressed in prostate, testis and colon (Figure 1D). A band of the same size was detected when the Northern blot was probed with a cDNA corresponding to the PAK4 regulatory domain (not shown).

PAK4 interacts with the GTP-bound form of Cdc42Hs

The finding that PAK4 has a putative GBD/CRIB motif suggests that it is a new target for Cdc42Hs and/or Rac. We used an overlay assay to determine whether PAK4 interacts with either of these GTP-binding proteins. We found that PAK4 interacts tightly with the GTP-bound form of Cdc42Hs and not with the inactive GDP-bound form of Cdc42Hs. A much weaker interaction was detected with the GTP-bound form of Rac, and no binding was observed with Rho. In contrast, PAK2 interacts with similar affinity to the GTP-bound forms of either Rac or Cdc42Hs (Figure 2A). PAK4 lacking the GBD/CRIB domain (PAK4ΔGBD) does not bind to Cdc42Hs, indicating that the GBD/CRIB domain is required for binding (Figure 2B). Interestingly, although PAK4 binds Cdc42Hs,

unlike other PAKs its autophosphorylation is not stimulated by GTP-loaded Cdc42Hs (see Figure 2C).

PAK4 autophosphorylates and phosphorylates an exogenous substrate

The analysis of PAK4′s kinase activity has revealed that it behaves similarly to other serine/threonine kinases. In order to determine whether the transfected PAK4 can autophosphorylate, NIH 3T3 cells were transfected with hemagglutinin (HA)-tagged PAK4 expression vector. PAK4 was immunopurified from cell lysates and incubated in kinase buffer with [γ-^{32}P]ATP. Autophosphorylation was analyzed after SDS–PAGE and autoradiography (Figure 3A). The presence of a band of the exact same size as PAK4 strongly suggests that, like the other PAKs, PAK4 can autophosphorylate. PAK4 also co-immunopurified with a 42 kDa protein that became phosphorylated in the kinase assay. The significance of this band is not known. Since PAK2 can be cleaved by caspases, one possibility is that this band represents a cleavage product of PAK4. We believe this to be unlikely, however, because it is not recognized by antibodies that we have generated against the PAK4 kinase domain or by antibodies directed against the epitope tag at the N-terminus. Furthermore, we still see this band even when we use a truncated PAK4 (PAK4Δ), which has only the kinase domain (not shown). Another possibility that we have yet to investigate is that this band represents a PAK4-interacting protein that becomes phosphorylated either by PAK4, or by another protein in the immune complex.

Although the physiological substrates for PAK4 are unknown, other PAK family members have been shown to phosphorylate substrates such as histone H4 or myelin basic protein (MBP) *in vitro*. To see whether this is the case for PAK4, NIH 3T3 cells were transfected with either empty vector or HA-tagged expression vectors for wild-type PAK4 or various PAK4 mutants. After transient expression, PAK4 was immunopurified from cell lysates and incubated with kinase buffer and [γ-^{32}P]ATP together with histone H4. As illustrated in Figure 3B, wild-type PAK4 efficiently phosphorylates histone H4. We also tested the activity of a PAK4(M350) mutant. This mutant contains a mutation in which the conserved lysine in subdomain II is converted to a non-phosphorylatable residue, methionine. Mutation of this conserved lysine disrupts the ATP-binding site of nearly all serine/threonine kinases that have been analyzed (Hanks *et al*., 1988). Immunopurified PAK4(M350) was completely unable to autophosphorylate or, as shown in Figure 3B, to phosphorylate histone H4. Most serine/threonine kinases contain a conserved serine or threonine residue within the linker region between kinase subdomains VII and VIII. This residue usually becomes phosphorylated either by autophosphorylation or by an upstream kinase. Phosphorylation of this residue is essential for the activities of most serine/threonine kinases (Marshall, 1994; Johnson *et al*., 1996; Pelech, 1996). Consistent with this, mutation of the corresponding serine in PAK4 to a methionine results in a completely inactive PAK4(M474) (Figure 3B). Many protein kinases become further activated when the regulatory domain is removed. We have generated PAK4Δ which contains only the kinase domain. PAK4Δ exhibits at least 5-fold more kinase activity than wild-type PAK4, suggesting that the N-terminal portion of PAK4 contains a regulatory domain (Figure 3B). Like the wild-type protein, however, mutation of the lysine in subdomain II to generate PAK4Δ(M350) results in a completely inactive kinase (Figure 3B). We obtained similar results when we used MBP as a substrate (not shown). Finally, we have found that unlike other PAKs, the kinase activity of transfected PAK4 is not stimulated by Cdc42HsV12. Cdc42HsV12 was not able to stimulate PAK4′s ability to phosphorylate either histone H4 or MBP, although the basal level of histone H4 and MBP phosphorylation by PAK4 is already somewhat high (Figure 3C). These results are consistent with our finding that Cdc42Hs does not stimulate PAK4 autophosphorylation (see Figure 2C). For all of the experiments described above, similar results were obtained in Cos cells.

PAK4 activates the JNK pathway

One of the functions of Cdc42Hs and Rac is to activate the JNK and p38 MAP kinase pathways (Bagrodia *et al*., 1995; Coso *et al*., 1995; Minden *et al*., 1995; Zhang *et al*., 1995; Brown *et al*., 1996). Since PAK4 is a target for Cdc42Hs, we tested its ability to activate mammalian MAP kinase pathways. NIH 3T3 cells were transfected with increasing doses of an expression vector containing the PAK4 cDNA. These were co-transfected with expression vectors containing epitope-tagged JNK, ERK or p38 cDNAs. After transient expression, JNK, ERK or p38 were immunopurified from cell lysates using antibodies against the epitope tag, followed by an *in vitro* kinase assay. The results indicate that overexpression of the wild-type PAK4 leads to activation of the JNK pathway, although this activation is somewhat weak compared with other JNK activators such as Rac or Cdc42Hs (Figure 3D). PAK4 has little activity towards ERK and p38 (Figure 3E and F).

PAK4 induces localized actin polymerization and induces the formation of filopodia

Since PAK4 is a novel target for Cdc42Hs, we studied its role in the induction of cytoskeletal changes. Microinjection of fibroblasts with purified constitutively active Cdc42HsV12 protein has been shown to lead to a transient induction of filopodia (Kozma *et al*., 1995; Nobes and Hall, 1995). Within a short time (by 15–30 min) after microinjection of the protein, the filopodia are replaced by lamellipodia. The formation of lamellipodia is due to the ability of Cdc42Hs to activate Rac. Thus, only in the presence of dominant-negative RacN17 does Cdc42Hs induce the sustained formation of filopodia (Kozma *et al*., 1995; Nobes and Hall, 1995). Another consequence of Cdc42HsV12 microinjection is the dissolution of stress fibers, possibly caused by antagonism with the RhoA GTPase (Dutartre *et al*., 1996; Manser *et al*., 1997). To see whether PAK4 can potentiate the cytoskeletal effects triggered by the GTPases, porcine aortic endothelial (PAE) cells were microinjected with expression vectors for HA-tagged PAK4 either alone or together with Myc-tagged Cdc42HsV12 or Rac1V12 expression vectors (Symons *et al*., 1996). The experiments described below were also carried out with NIH 3T3 fibroblasts, with similar results. As shown in Figure 4A, when expressed alone, PAK4 was localized in a perinuclear area and did not affect the actin cytoskeleton. Cells microinjected with expression vectors for constitutively active Rac1V12 or Cdc42HsV12 alone induced extensive lamellipodia (as shown in Figure 4B). Filopodia were not observed in the Cdc42Hs-injected cells because cells were analyzed

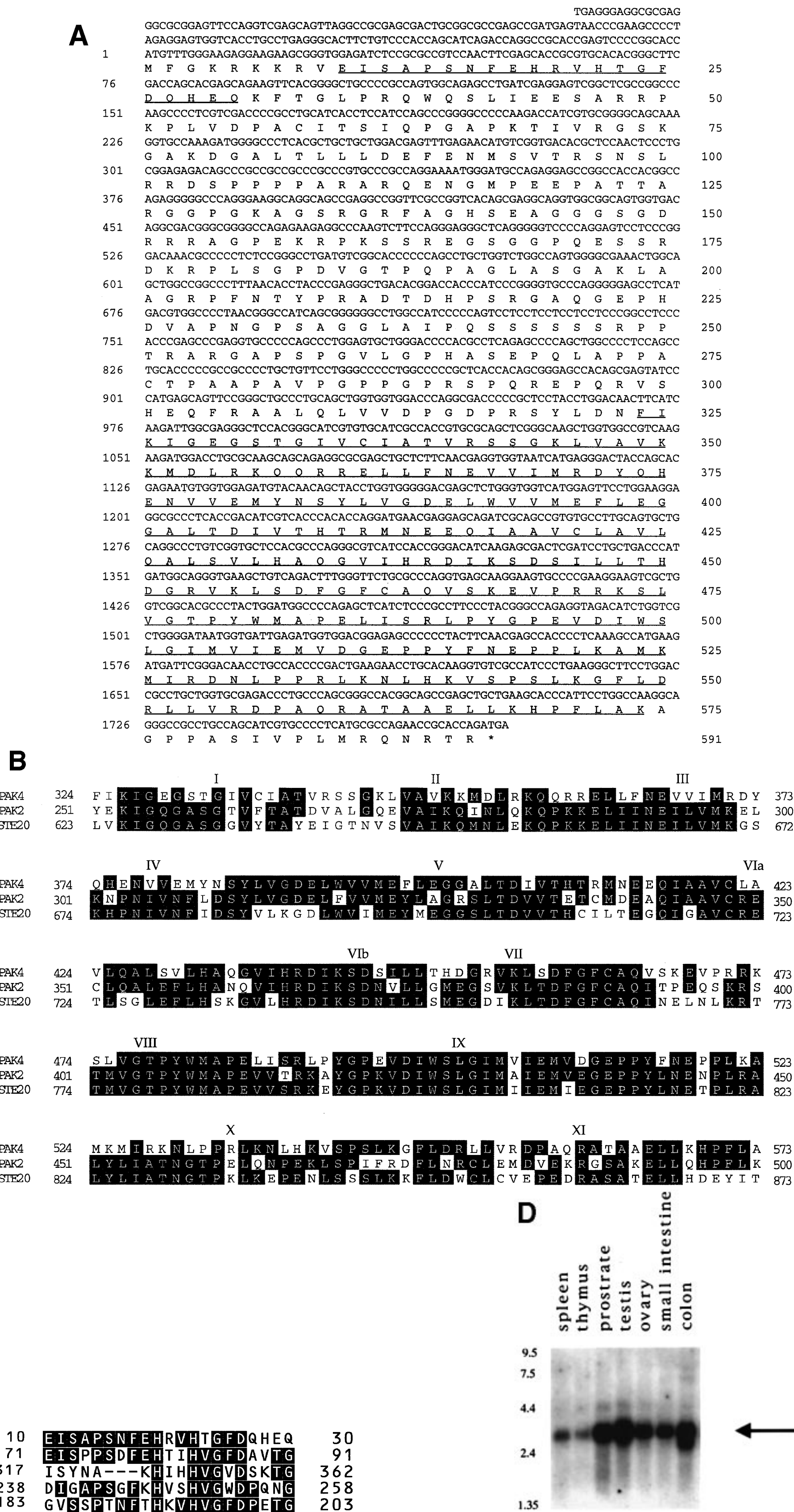

Fig. 1. Sequence and expression pattern of PAK4. (**A**) Nucleotide sequence of PAK4, a novel serine/threonine kinase. The CRIB domain (amino acids 10–30) and the kinase domain (amino acids 323–574) are underlined. (**B**) Alignment of the kinase domain of PAK4 with those of PAK2 and STE20. (**C**) Alignment of the GBD/CRIB motif of PAK4 with the corresponding regions of several other mammalian (PAK65 and WASP) and yeast (STE20 and CLA4) Rac- and Cdc42Hs-interacting proteins. (**D**) Northern blot analysis of PAK4. A Northern blot containing mRNA from various human tissues was probed with a cDNA containing the kinase domain of PAK4. A band of ~3 kb is indicated.

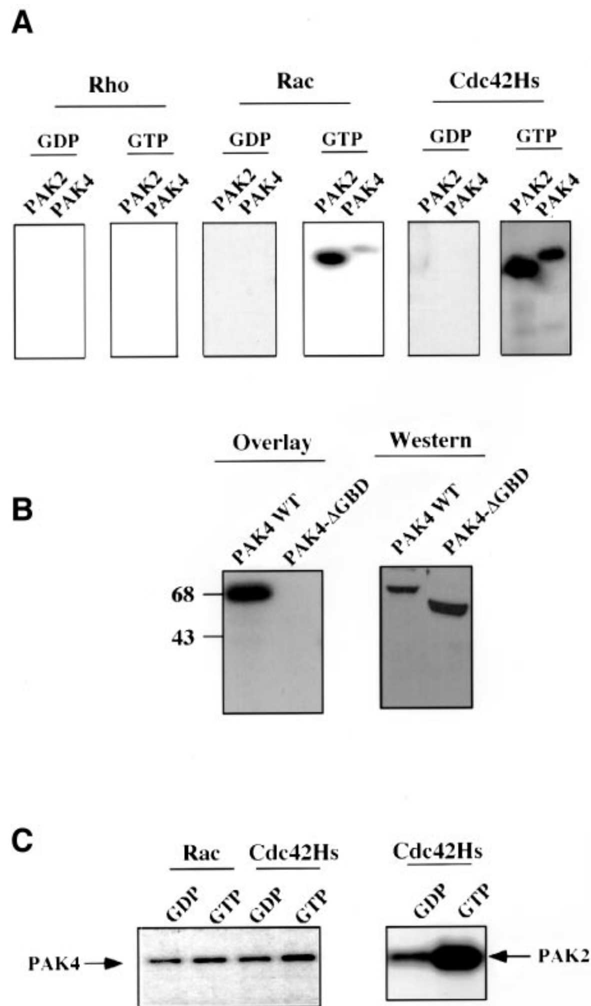

Fig. 2. PAK4 interacts with the activated Cdc42Hs through its GBD/CRIB domain. (**A**) Recombinant PAK4 and hPAK2 (2–3 μg) were analyzed by the overlay assay and probed with the indicated GTPase pre-loaded with either [γ-^{32}P]GTP or [β-^{32}P]GDP as described in Materials and methods. (**B**) Cos-1 cells were transiently transfected with expression vectors containing HA-tagged PAK4 or PAK4ΔGBD (a deletion mutant lacking the GBD/CRIB domain). After transient expression, Cos-1 cells were harvested, immunopurified with the anti-HA antibodies, separated by SDS–PAGE and transferred to PVDF membranes. Binding of PAK4 or PAK4ΔGBD to [γ-^{32}P]GTP-loaded Cdc42Hs was assessed as in (A). To ensure that both wild-type PAK4 and PAK4ΔGBD were expressed at approximately equivalent levels, cell extract (25 μg) was analyzed by Western blots probed with anti-HA antibody. (**C**) A 2 μg aliquot of purified PAK4 or PAK2 immobilized on beads in 40 μl of kinase buffer was incubated with 1–2 μg of the indicated GTPases which were pre-loaded with either GTP or GDP. The reaction was incubated for 20 min at 30°C with 50 μM ATP and 5 μCi of [γ-^{32}P]ATP. Phosphorylated proteins were analyzed by SDS–PAGE followed by autoradiography.

between 16 and 20 h after microinjection, after filopodia should have been replaced by Rac-induced lamellipodia. Interestingly, co-expression of PAK4 with Cdc42HsV12 caused dramatic changes in the actin cytoskeleton and redistribution of PAK4. When co-injected with Cdc42HsV12, PAK4 became concentrated on one side of the nucleus in an area that resembles the *trans*-Golgi compartment. Dual staining with phalloidin and HA antibody revealed a striking co-localization of PAK4 with polymerized actin clusters (Figure 4A) and, in cells expressing lower levels of PAK4, polymerized actin was detected around vesicles (data not shown). Strikingly, co-expression of PAK4 and Cdc42HsV12 also induced the sustained formation of actin-enriched filopodia protrusions and enriched cortical actin in >80% of the injected cells. These results are illustrated in Figure 4A. The results from three separate experiments are also summarized in Table I. Co-expression of PAK4 with a dominant-negative Cdc42HsN17 had no effect on PAK4 localization or the reorganization of the actin cytoskeleton (data not shown). Co-expression of PAK4 and the constitutively active Rac1V12 resulted in a very similar phenotype to that observed with Rac1V12 alone. Rac1V12 induced the formation of lamellipodia and relocalization of a small percentage of PAK4 from the perinuclear area to the lamellipodia (Figure 4A). To test whether these phenotypes are specific for PAK4, we co-injected PAK2 expression vector with either Rac1V12 or Cdc42HsV12 expression vector. PAK2 was localized in the cytosol and the nucleus and was not redistributed by Rac or Cdc42Hs (data not shown). In addition, cells co-expressing Rac1V12 or Cdc42HsV12 together with PAK2 had similar cytoskeletal phenotypes as cells expressing Rac1V12 or Cdc42Hs alone, both of which contained lamellipodia but no filopodia (Figure 4B). These data strongly suggest that in contrast to PAK2, PAK4 is an effector for Cdc42Hs that leads to the induction of filopodia and actin polymerization. Our results suggest that PAK4 drives the Cdc42Hs→filopodia reaction rather than the Cdc42Hs→Rac→lamellipodia reaction (see Figure 8).

PAK4 is recruited to the Golgi apparatus by activated Cdc42Hs

PAK4 appears to be regulated by specific recruitment by Cdc42Hs to an area resembling the *trans*-Golgi. It is interesting to note that the endogenous Cdc42Hs recently was shown to be localized primarily at Golgi membranes, and to localize with the Golgi membrane coatomer protein β-COP (Erickson *et al.*, 1996). In addition, we recently identified a Cdc42Hs-related protein that was also localized to the Golgi structure (A.Abo, unpublished observation). To determine whether PAK4 also localizes to the Golgi, we co-stained injected cells with anti-HA and anti-β-COP antibody. As presented in Figure 5, in cells expressing PAK4 alone, PAK4 was localized to perinuclear areas and was not co-localized with β-COP. In cells co-expressing PAK4 and Cdc42HsV12, however, PAK4 was co-localized with β-COP in the Golgi. Since the levels of endogenous PAK4 are quite low in the cells we have analyzed, we have not been able to analyze the endogenous protein by immunofluorescence. This low endogenous level of PAK4 may in fact partly explain the transient nature of filopodia in most cells. However, our finding that PAK4 localizes to the Golgi only in the presence of Cdc42HsV12, and only when it contains an intact GBD/CRIB motif (discussed below), strongly supports the idea that cellular PAK4 is recruited to the Golgi by Cdc42Hs.

Next we tested the effects of the drug brefeldin A (BFA) on PAK4 location. Golgi coatomer proteins normally are redistributed when cells are exposed to BFA (Orci *et al.*, 1991). Likewise, Cdc42Hs is redistributed from its Golgi location when cells are treated with BFA (Erickson *et al.*, 1996). Interestingly, we found that treatment of injected cells with BFA also caused a rapid redistribution of PAK4 from the Golgi to the cytoplasm and the nucleus. This is nearly identical to the effects of BFA on β-COP in these

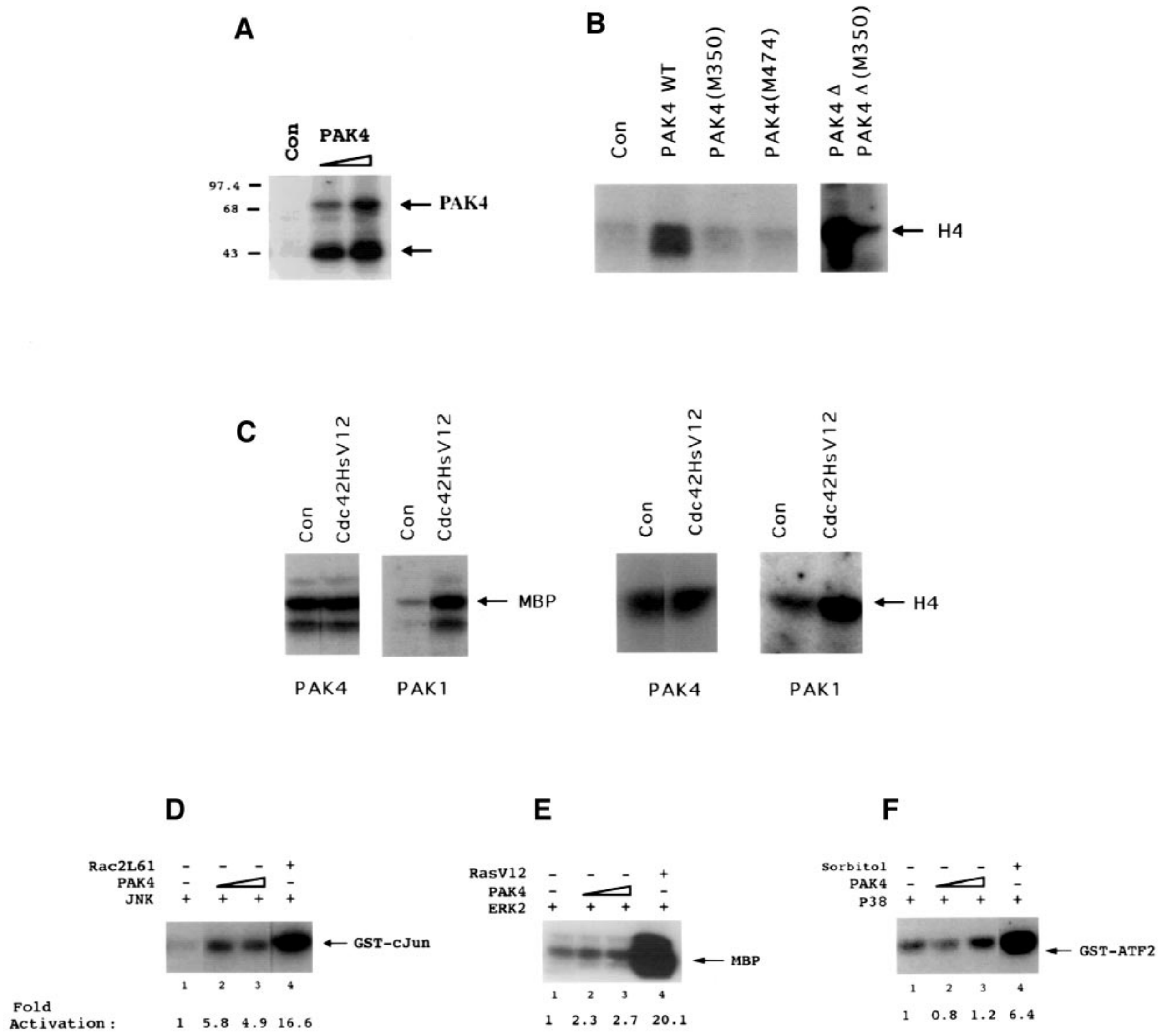

Fig. 3. Analysis of PAK4 kinase activity. (**A**) NIH 3T3 cells were transfected with either empty SRα vector (control) or with increasing doses of SRα expression vectors containing the PAK cDNA fused to an HA epitope tag (0.5 and 1 μg). After transient expression, PAK4 was immunopurified from cell lysates using anti-HA antibody. Immunopurified PAK4 was incubated with histone H4, or without any substrate in the presence of [γ-^{32}P]ATP and kinase buffer (Minden *et al*., 1994). Substrate phosphorylation was analyzed after SDS–PAGE and autoradiography. Autophosphorylation of the 68 kDa PAK4 and a band of ~42 kDa that co-purifies with PAK4 is indicated. (**B**) Cos-1 cells were transfected with 1 μg of expression vectors containing either wild-type PAK4 or the indicated PAK4 mutants, all fused to HA tags. After transient expression, immunopurified PAK4 was used to phosphorylate histone H4 in the presence of kinase buffer and [γ-^{32}P]ATP. Substrate phosphorylation is indicated. Similar results were obtained with NIH 3T3 cells. (**C**) NIH 3T3 cells were transfected with 1 μg of Myc-tagged PAK4 or PAK1 expression vectors together with 1 μg of Cdc42HsV12 expression vector. After transient expression, PAKs were immunopurified from cell lysates using anti-Myc antibody. Immunopurified PAKs were incubated with histone H4 or MBP in the presence of [γ-^{32}P]ATP and kinase buffer (Minden *et al*., 1994). Substrate phosphorylation was analyzed after SDS–PAGE and autoradiography. PAK4 activation was not seen even when other doses of Cdc42Hs were used, ranging from 0.2 to 2 μg. (**D**–**F**) NIH 3T3 cells were transfected with empty SRα vector (control) or increasing doses of SRα Myc-tagged PAK4 expression vector (0.2, 0.6 or 1 μg) together with 1 μg of HA-tagged JNK, ERK or p38 expression vectors. As positive controls, cells were either transfected with expression vectors for Rac2L61 or RasV12 or treated with 400 mM sorbitol, as indicated. After transient expression, JNK, ERK or p38 were immunopurified from cell lysates using anti-HA antibody. Immunopurified proteins were incubated in kinase buffer and [γ-^{32}P]ATP together with either GST–c-Jun, MBP or GST–ATF2 as substrates, respectively (Minden *et al*., 1995). Substrate phosphorylation was visualized after SDS–PAGE and autoradiography. Substrate phosphorylation was quantitated by phosphorimager analysis. The numbers indicated are the averages of three independent experiments.

cells (Figure 5). These data strongly suggest that in the presence of Cdc42Hs, PAK4 is localized at a BFA-sensitive component of Golgi membranes. In addition, BFA-treated cells contained less filopodia and polymerized actin. These data suggest that the localization of PAK4 by Cdc42Hs to the Golgi may play an important role in the reorganization of actin.

PAK4 kinase activity is required for its ability to induce localized actin polymerization and to potentiate filopodia formation by Cdc42Hs

Next we tested the effect of PAK4 mutants on the formation of filopodia and actin polymerization. The kinase-inactive PAK4(M350) was microinjected into cells either alone or together with Cdc42HsV12. Like PAK4 wt, PAK4(M350)

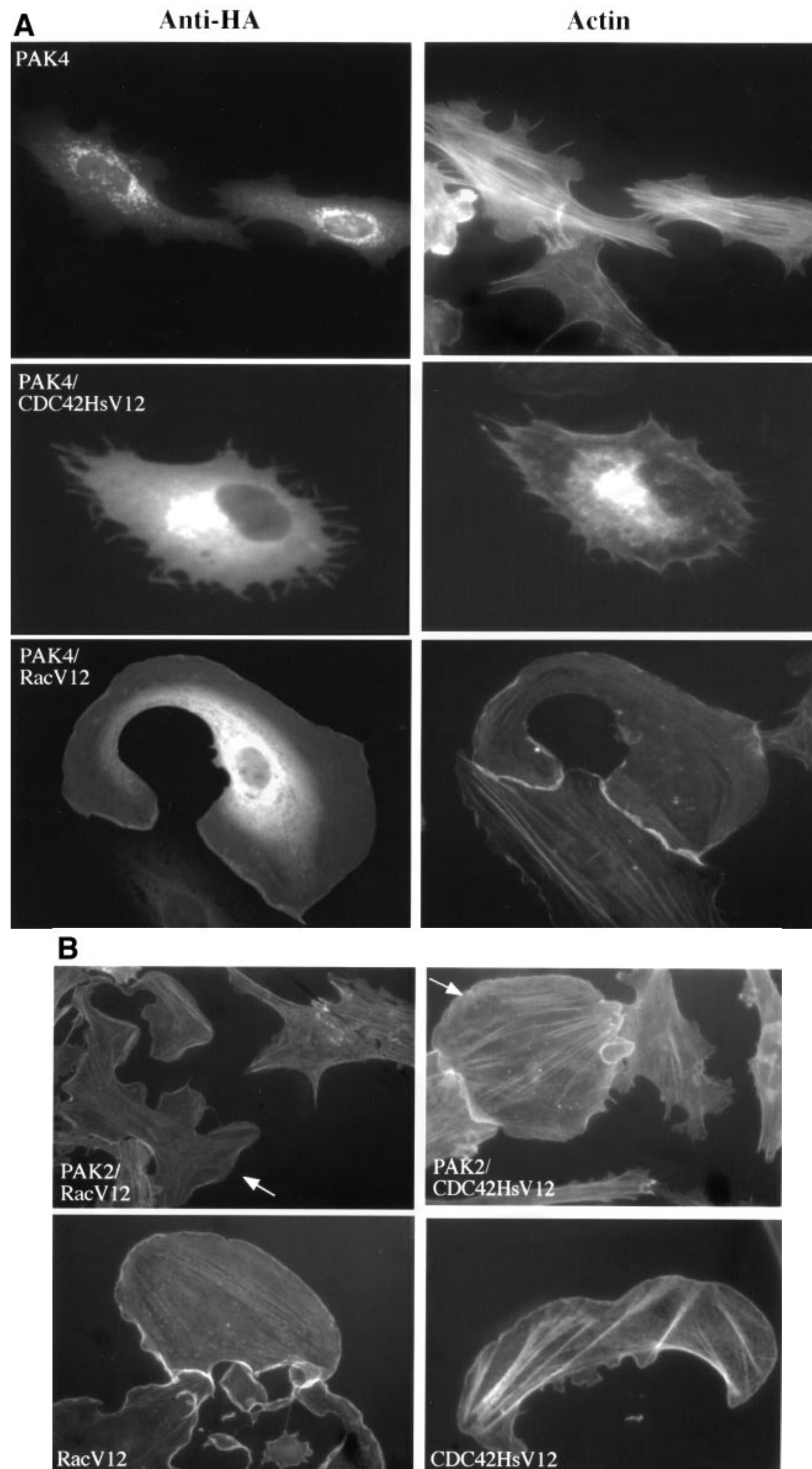

Fig. 4. Localization of PAK4 and its effects on the actin cytoskeleton. (**A**) Porcine endothelial cells (PAE) were microinjected with HA-tagged PAK4 expression vector alone or in combination with Myc-tagged Cdc42HsV12 or RacV12 expression vectors (50 ng/μl plasmid). Cells were fixed 16–20 h after injection and PAK4 was visualized by immunofluorescence microscopy after staining with rhodamine-tagged HA antibody. Polymerized actin was visualized after staining with FITC-conjugated phalloidin. (**B**) PAE cells were microinjected with Myc-tagged PAK2 expression vector alone and in combination with Cdc42HsV12 or RacV12 expression vectors. Polymerized actin was visualized as described in (A). Arrows indicate the injected cells.

was localized in the perinuclear area and was recruited by Cdc42Hs to the BFA-sensitive compartment of the Golgi (Figure 6A). However, in contrast to PAK4 wt, PAK4(M350) was unable to induce localized actin polymerization or the formation of filopodia, and did not lead to a reduction in stress fibers. In fact, PAK4(M350) inhibited some of the effects normally triggered by Cdc42Hs such as the reduction of stress fibers and induction of lamellipodia (Figure 6A). These data suggest that PAK4(M350) cannot mediate the effects of Cdc42Hs, but

Table I. Summary of cell injections with CDC42HsV12 and PAK4

Plasmids injected	Cdc42HsV12	PAK4	Cdc42HsV12 + PAK4
Total no. of cells injected in three experiments	91	119	123
Percentage of injected cells containing filopodia	20.7 ± 5.5	3.9 ± 3.1	84.4 ± 8.9
Percentage of injected cells containing lamellipodia	85.4 ± 7.7	1.9 ± 2.4	5.1 ± 5
Percentage of injected cells in which PAK4 is localized at the Golgi	N/A	0	82 ± 13.5

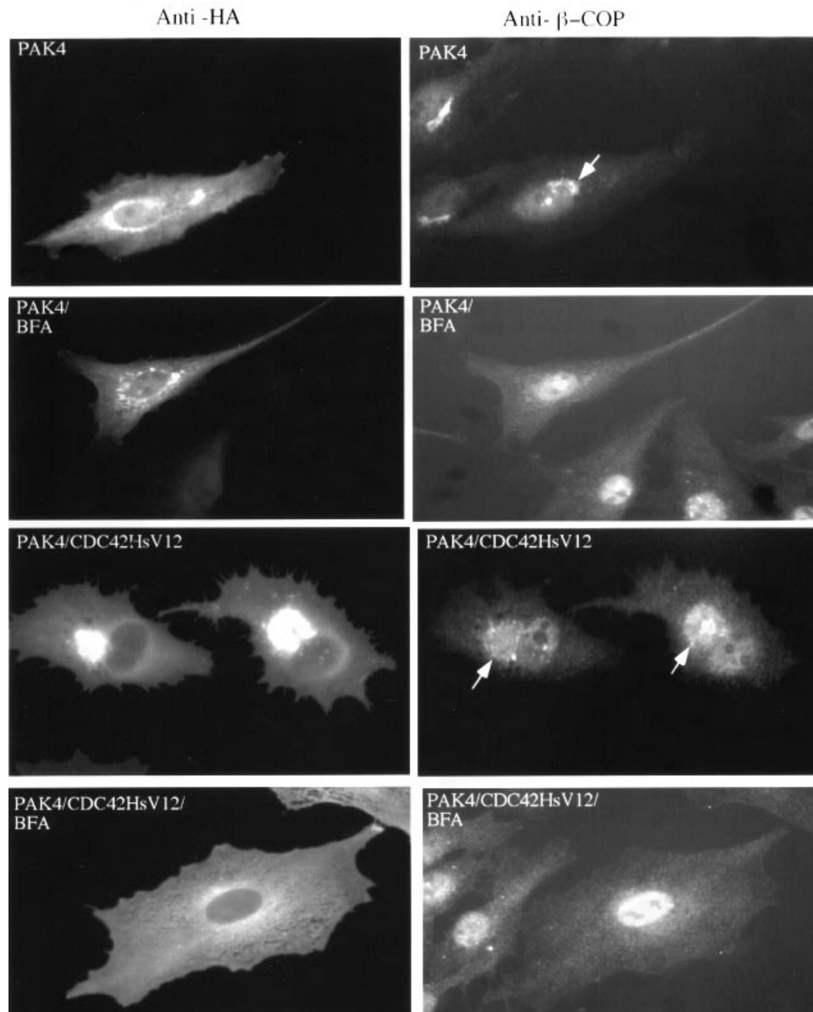

Fig. 5. PAK4 is recruited by Cdc42Hs to the BFA-sensitive compartment of the Golgi. PAE cells were microinjected with HA-tagged PAK4 expression vector alone or in combination with Cdc42HsV12 expression vector. After 16–20 h, cells were either left untreated or treated with 5 μg/ml BFA for 3–5 min at 37°C followed immediately by fixation. β-COP was visualized by a specific antibody against β-COP protein (gift of Richard Kahn). To compare the localization of PAK4 with that of β-COP, compare the fluorescence micrograph of anti-HA staining with that of anti β-COP staining.

can instead function as a dominant-negative mutant which blocks some of the downstream events normally triggered by Cdc42Hs. We also generated a putative constitutively active PAK4 mutant. This mutant was generated by mutation of Ser474 (in the linker region between subdomains VII and VIII) to a glutamic acid. The resulting PAK4(E474) mutant had a greatly enhanced autophosphorylation activity (data not shown). Mutation of this conserved site to a negatively charged amino acid has been found to generate constitutively active mutants of many serine/threonine kinases including the PAKs (Benner *et al.*, 1995; Manser *et al.*, 1997; Szczepanowska *et al.*, 1997). When expressed alone, PAK4(E474) localized to similar areas as PAK4 wt and PAK4(M350), and did not have any effect on the actin cytoskeleton. Like PAK4 wt, PAK4(E474) was recruited specifically by Cdc42HsV12 to the Golgi and induced the formation of filopodia and polymerization of actin to the same extent as wild-type

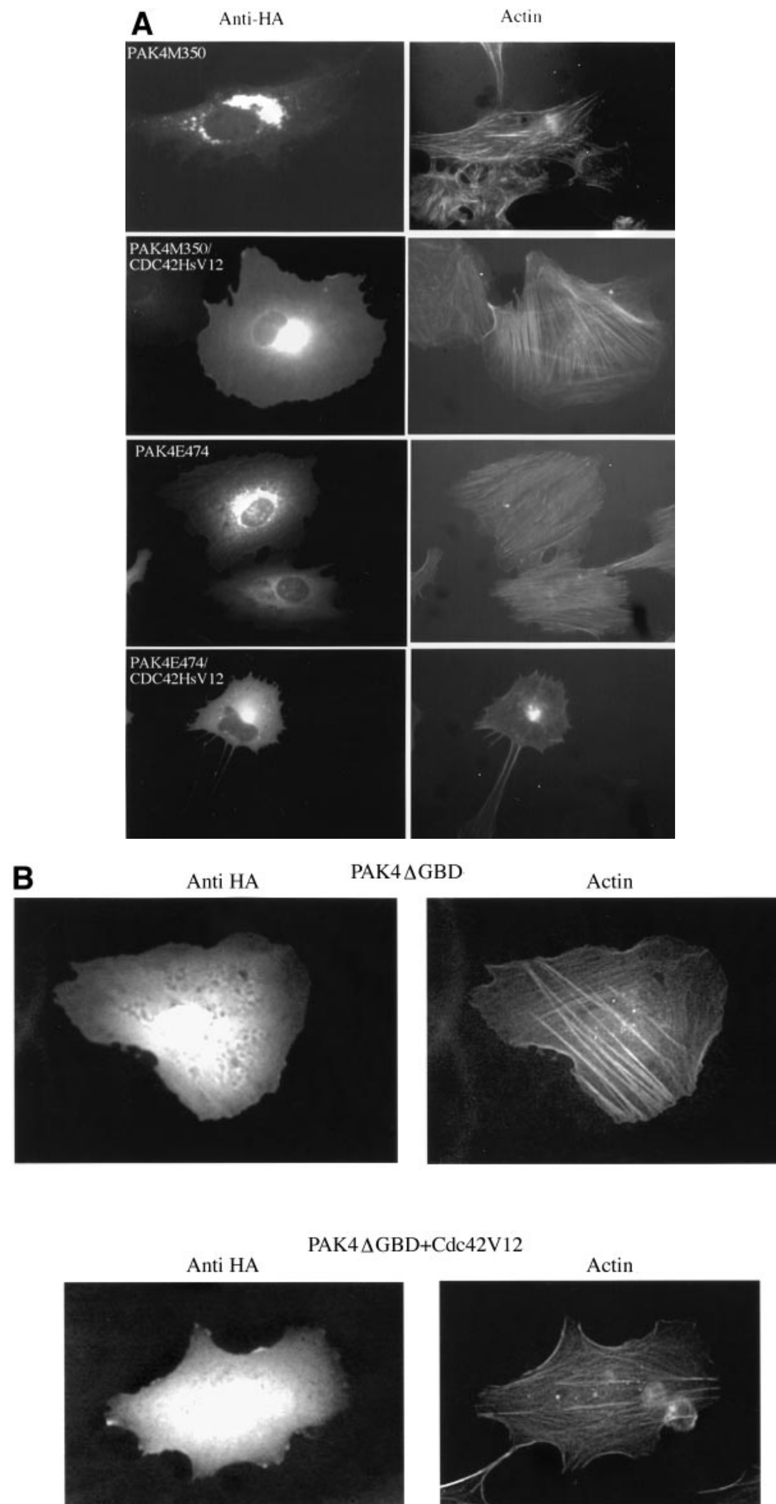

Fig. 6. PAK4 localization to the Golgi and kinase activity are essential for actin polymerization. Fluorescence micrographs of PAE cells microinjected with expression vectors containing HA-tagged kinase inactive PAK4(M350) (**A**), PAK4(E474) (A) or PAK4ΔGBD (**B**), alone or in combination with Cdc42HsV12 expression vector. PAK4 was detected by HA antibody and polymerized actin was visualized by phalloidin staining.

PAK4 (Figure 6A). Finally, we tested PAK4ΔGBD. This mutant is lacking the GBD domain and can no longer bind Cdc42Hs. Its kinase activity is intact, however; it autophosphorylates and phosphorylates histone H4 even more efficiently than the wild-type PAK4 (not shown). By using immunofluorescence, we found that this deletion mutant was localized throughout the entire cell including the nucleus. When cells were co-injected with Cdc42HsV12, the location of PAK4ΔGBD did not change, and it was not concentrated in the Golgi area as we saw with the full-length protein. Importantly, PAK4ΔGBD did not potentiate the formation of filopodia in response to Cdc42HsV12 (see Figure 6B). This result was seen in nearly 100% of the injected cells. Taken together, these

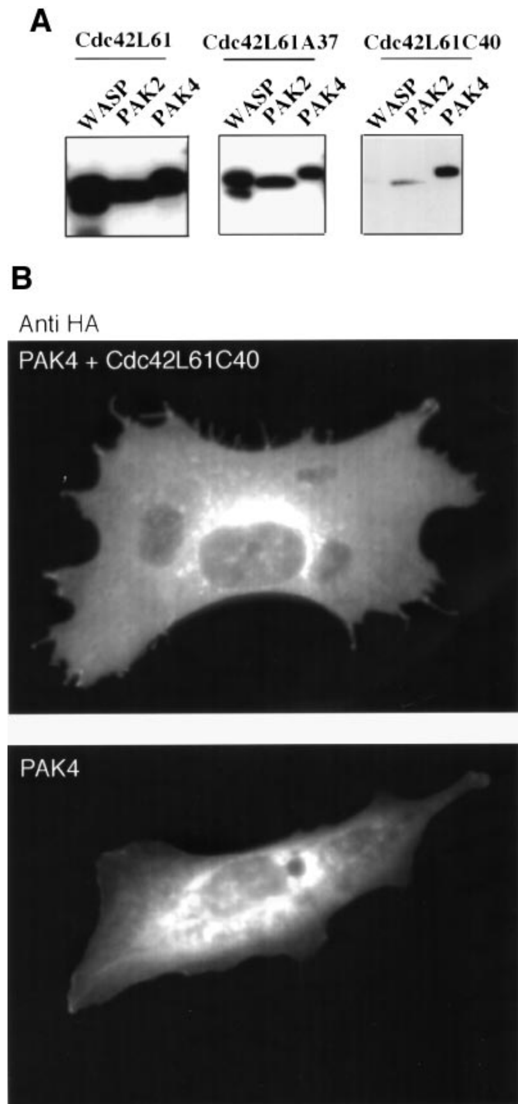

Fig. 7. Analysis of Cdc42Hs effector mutants. (**A**) Recombinant PAK4, hPAK2 and WASP (2–3 µg) were analyzed by the overlay assay and probed with the indicated Cdc42Hs mutant pre-loaded with [γ-^{32}P]GTP (Martin *et al.*, 1995). (**B**) Porcine aortic endothelial (PAE) cells were injected with HA-tagged PAK4 expression vector alone or in combination with Myc-tagged Cdc42HsL61(C40) mutant (100 ng/µl plasmid). Cells were fixed 16–20 h after injection and PAK4 was visualized by immunofluorescence microscopy after staining with rhodamine-tagged HA antibody.

data indicate that both PAK4′s kinase activity and its interaction with Cdc42Hs are required for its ability to induce filopodia. However, neither the kinase activity nor cellular localization alone are sufficient. In fact, even a constitutively active PAK4(E474) mutant had no enhanced effect on actin polymerization and filopodia formation and did not bypass the need for recruitment to the Golgi by Cdc42Hs.

PAK4 binds Cdc42Hs effector mutants to induce filopodia and actin polymerization

Two effector mutants of constitutively active Cdc42HsL61 were examined previously to assess the role of PAK and other effectors in filopodia formation (Lamarche *et al.*, 1996). Both mutants had single amino acid substitutions in the effector loop. One mutant, Cdc42HsL61(Y40C), could not bind PAK or several other GBD/CRIB domain-containing proteins. The other mutant, Cdc42HsL61 (F37A), maintained the ability to bind PAK and other GBD/CRIB domain-containing proteins. Interestingly, both effector mutants were equally efficient in the ability to induce filopodia when microinjected into fibroblasts together with dominant-negative Rac (Lamarche *et al.*, 1996). These results would tend to suggest that PAK binding to Cdc42Hs is not necessary for filopodia formation. To see whether Cdc42HsL61(Y40C) could be mediating its effects through PAK4, we tested PAK4 binding to Cdc42HsL61 and to the two effector mutants. For comparison, we also analyzed PAK2 and another GBD/CRIB-containing protein, WASP (Symons *et al.*, 1996). As shown in Figure 7A, all three proteins bound to Cdc42HsL61. Likewise, as expected, they also all bound to Cdc42Hs(F37A), though with a slightly lower affinity. Surprisingly, however, PAK4 bound Cdc42HsL61(Y40C) with an affinity similar to that detected with Cdc42HsL61(F37A), although WASP and PAK2 did not bind Cdc42HsL61(Y40C) efficiently. Moreover, when Cdc42HsL61(C40) was co-injected into PAE cells with PAK4, PAK4 was recruited to the Golgi area, induced actin polymerization and promoted the formation of filopodia (see Figure 7B). Our results suggest a mechanism whereby the effector mutant Cdc42HsL61(Y40C) can induce filopodia formation. Although it cannot bind PAK2 efficiently, this mutant maintains the ability to interact with PAK4. Importantly, we have found that PAK4, rather than the other known PAKs, is the important mediator of filopodia formation by Cdc42Hs.

Discussion

We have identified PAK4 as a novel member of the PAK family. Like the other PAKs, PAK4 contains a GBD/CRIB domain at the N-terminus and a kinase domain at the C-terminus. The overall sequence identity to other PAKs, however, is significantly different. Although the kinase domain of PAK4 is more similar in sequence to the kinase domains of PAK 1, 2 and 3 than to other known proteins, it shares only 53% sequence identity. PAK4 exhibits no sequence homology in the regulatory domain outside the GBD/CRIB sequences. Even the GBD/CRIB motif is similar, but not identical, to the GBD/CRIB motif in the other PAKs. This suggests that PAK4 may have a different function from that of the known PAKs. We have shown that PAK4 interacts preferentially with the GTP-bound form of Cdc42Hs and activates the JNK family of MAP kinases. Moreover, microinjection of PAK4 and Cdc42Hs plasmids into cells demonstrates that PAK4 has a profound effect on the actin cytoskeleton. When co-injected with Cdc42Hs, PAK4 is recruited by Cdc42Hs to the BFA-sensitive compartment of the Golgi, and subsequently induces actin polymerization at the Golgi. Strikingly, while Cdc42Hs induction of filopodia is quite transient and cannot be seen 16–20 h after microinjection of PAE cells, co-injection of PAK4 greatly stimulates the sustained induction of filopodia by Cdc42Hs. In contrast to other PAKs, PAK4 kinase activity and its interaction with activated Cdc42Hs are essential for the induction of this

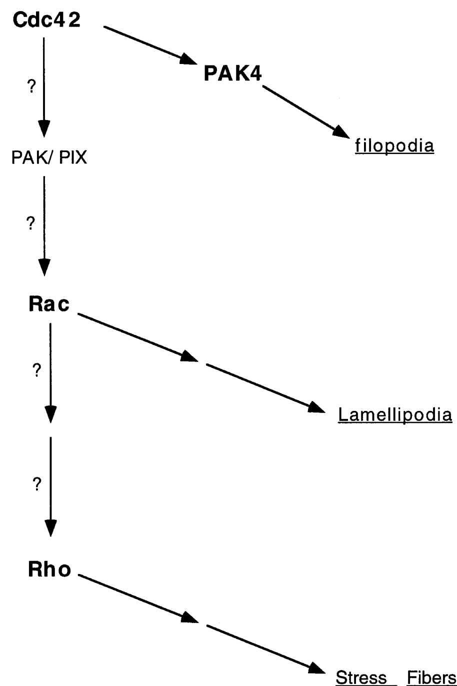

Fig. 8. Model for the role of PAK4 and Rho GTPases in cytosletal organization. See text for details.

phenotype. In addition, PAK4 interacts with the Cdc42Hs effector mutant [Cdc42L61(C40)] which previously was shown to induce filopodia but which failed to bind other PAKs (Lamarche *et al*., 1996). Our data provide a novel link between Cdc42Hs, PAK4, actin polymerization and filopodia formation. Our results also support the idea that the Golgi apparatus plays a role in cytoskeletal reorganization.

The Rho family of GTPases play key roles in the control of cell morphology. By using microinjection techniques, it was demonstrated that Cdc42Hs triggers the formation of microspikes and filopodia followed by activation of Rac, which leads to the formation of lamellipodia. A third GTPase, RhoA, is implicated in the formation of stress fibers. In addition, all three GTPases regulate the assembly of focal complexes. The cytoskeletal changes triggered by these GTPases play important roles in cell motility and division and in the maintenance of cell shape. The identification of molecular targets which mediate these cytoskeletal effects is therefore of great importance. Several proteins were shown to interact with the activated form of Cdc42Hs; however, the molecular effector that links Cdc42Hs to the formation of filopodia has not yet been identified. Recent experiments with various effector mutants of Cdc42Hs demonstrated that the cytoskeletal changes induced by Cdc42Hs are independent of PAK1, 2 or 3 (Lamarche *et al*. 1996).

Here we have shown that a novel PAK-related protein, PAK4, interacts only with the GTP-bound form of Cdc42Hs and not with other Rho members. The interaction between Cdc42Hs and PAK4 is essential for targeting PAK4 to the Golgi compartment and subsequently for the reorganization of the actin cytoskeleton and filopodia formation. In contrast to PAK1, 2 and 3, the kinase activity of PAK4 is not regulated by Cdc42Hs. Rather, PAK4 appears to have constitutive kinase activity in the absence or presence of Cdc42HsV12. Thus, it appears to be recruitment of PAK4 by Cdc42Hs, rather than stimulation of its kinase activity, which is important for actin polymerization and cytoskeletal changes. PAK4 does not induce filopodia merely by binding to Cdc42Hs, however, because a kinase-inactive PAK4 mutant was relocalized to the Golgi by Cdc42Hs, but failed to reorganize the actin cytoskeleton. PAK4′s kinase activity is thus essential for its ability to potentiate filopodia formation by Cdc42Hs. Kinase activity alone, however, is not sufficient to induce the cytoskeletal changes. Even a constitutively active PAK4 could not bypass the need for recruitment by Cdc42Hs to the Golgi. This mutant localized to the same area in the cell as wild-type PAK4, and only when it was recruited to the Golgi compartment by Cdc42Hs did it induce the formation of filopodia and actin polymerization. We have also found that two different dominant-negative mutants of PAK4 inhibited filopodia formation induced by co-injection of Cdc42Hs and dominant-negative Rac (not shown). A mutant containing only the regulatory domain of PAK4 blocked filopodia formation in 100% of the injected cells while a mutant in which the kinase domain is mutated and the GBD/CRIB domain is deleted dramatically inhibited filopodia in at least 50% of the injected cells. This lower level of inhibition by this construct may be due to its inability to localize near its putative substrate, presumed to be near the Golgi membrane. Taken together, our results strongly support the idea that PAK4 is an important mediator of filopodia induction by Cdc42Hs, and that its activity depends on its translocation to a specific site, presumably in close proximity to a putative substrate.

This mechanism of regulation of PAK4 is substantially different from the one proposed for other PAK members. PAK1 was shown to be recruited to the focal complexes induced by Cdc42Hs, but the recruitment to these sites did not require a direct interaction with Cdc42Hs (Manser *et al*., 1997). Recently, it was demonstrated that PAK1 is recruited to the focal complexes by interacting with a novel GTP/GDP exchange factor PIX (Manser *et al*., 1998). A specific proline-rich sequence on the PAK regulatory domain interacts with the SH3 domain of PIX. Furthermore, it was proposed that the PAK–PIX complex activates the Rac signaling pathways. Interestingly, PAK kinase activity was not required for activation of the Rac pathway, but was shown to be important in the disassembly of the focal complexes (Manser *et al*., 1998). PAK1 has also been reported to induce filopodia and lamellipodia similar to those induced by Cdc42Hs and Rac (Sells *et al*., 1997). However, these cytoskeletal changes occur independently of PAK1′s ability to bind Cdc42Hs and Rac, and are partly independent of its kinase activity. These results, coupled with the results from experiments using effector mutants of Rac and Cdc42Hs, suggest that, while PAK1 may be able to induce cytoskeletal changes when it is overexpressed, it might not be the link between GTPases and the cytoskeleton. In contrast, PAK4 is recruited specifically to the Golgi by Cdc42Hs and appears to be an important link between Cdc42Hs and filopodia formation. We have not yet determined whether PAK4 has a direct role in the dissolution of stress fibers although it does appear to potentiate the dissolution of stress fibers by Cdc42Hs. Unlike other PAKs, however, it cannot

induce the dissolution of stress fibers on its own. PAK4 is quite different from the other PAKs in sequence, even within the kinase domain. Consistent with this, PAK4 appears to be regulated differently from the other PAKs, and may mediate its effects via different substrates.

Recent studies indicated that Cdc42Hs is localized to the BFA-sensitive compartment of the Golgi apparatus. The role of Cdc42Hs in the Golgi is poorly understood. The Golgi apparatus is known mostly for its roles in the formation of transport vesicles which carry cargo to a receiving compartment. Non-clathrin coat proteins such as β-COP and the GTPase ARF were shown to be implicated in Golgi-mediated transport (Donaldson *et al*., 1992a; Schekman and Orci, 1996). Disruption of the coatomer complexes by treating cells with BFA or expressing dominant-negative forms of ARF blocked the transport of vesicles to the plasma membrane (Orci *et al*., 1991; Donaldson *et al*., 1992b; Helms and Rothman, 1992; Dascher and Balch, 1994; C.J.Zhang *et al*., 1994). Our data indicated that PAK4 is also co-localized with β-COP at the Golgi and is redistributed by treating the cells with BFA. Furthermore, BFA treatment affected the filopodia formation and actin polymerization induced by PAK4 and Cdc42HsV12. Interestingly, it was reported previously that fibroblasts treated with BFA failed to make filopodia and lamellipodia, and subsequently cells were defective in motility (Bershadsky and Futerman, 1994). It has been proposed that the disruption of the Golgi apparatus by BFA may affect the supply of vesicles containing the components necessary for the formation of filopodia (Bershadsky and Futerman, 1994; Erickson *et al*., 1996). Because it is localized by Cdc42Hs to the Golgi, PAK4 is a good candidate for an effector molecule that transduces a Cdc42Hs-dependent signal from the Golgi. It is conceivable that PAK4 and Cdc42Hs regulate the reorganization of actin and the formation of filopodia by controlling the transport of vesicles containing the proteins and/or lipids necessary for the induction of morphological changes. Interestingly, in addition to the induction of filopodia, PAK4 also leads to localized actin polymerization at the Golgi area. In future studies, it will be interesting to determine whether actin polymerization in this area is important for vesicle fusion or other aspects of Golgi function.

We propose the following model for PAK4 and Rho GTPases in cytoskeletal organization (see Figure 8). Activated Cdc42Hs interacts with PAK4, bringing it into the proximity of substrates necessary for filopodia formation. Cdc42Hs next activates Rac which leads to the induction of lamellipodia. Rac activation by Cdc42 may involve PAK–PIX complexes (Manser *et al*., 1998). Rac then leads to the formation of lamellipodia, although the mechanism by which it does so is still not known. Rac and Cdc42Hs may also have a role in activating a third GTPase, Rho, which is involved in the formation of actin stress fibers. Filopodia formation by Cdc42Hs is normally quite transient. This is probably due partly to the fact that Cdc42Hs leads to rapid activation of Rac and subsequent lamellipodia formation. Another possible reason is the relatively low abundance of PAK4 in the cell. Hence, microinjection of excess PAK4 into the cell can drive the Cdc42→PAK4→filopodia reaction over the Cdc42Hs→Rac→lamellipodia reaction. In summary, PAK4 provides a molecular link between Cdc42Hs and actin rearrangement, and suggests involvement of the Golgi apparatus in cell morphogenesis. Understanding how PAK4 together with Cdc42Hs and putative targets at the Golgi control the reorganization of the actin cytoskeleton will contribute to our understanding of the molecular mechanism of morphogenesis

Materials and methods

Isolation of PAK4

To isolate PAK4, a pair of degenerate oligonucleotide primers were synthesized based on the amino acid sequences conserved among the kinase domains of yeast STE20 and human PAK65 (the two oligos corresponded to the amino sequences 'KKELIINE' and 'VGTPYWMA', respectively). The two primers were used to generate a PCR product using Jurkat cell cDNA as a template. Low stringency conditions were used so that diverse products could be obtained. PCR products were gel purified and subcloned. Inserts were sequenced using the dideoxy chain termination method. A 400 bp PCR product containing significant homology to the catalytic domain of STE20 was used as a probe to screen both a human Jurkat cell cDNA library (in the ZAP Express™ *Eco*RI vector) and a human fetal brain cDNA library (in λTriplEX™ vector). Nylon transfer filters containing 1×10^6 recombinant plaques were hybridized with the randomly primed [α-^{32}P]dCTP-labeled probe (Prime-It II kit, Stratagene) overnight at 42°C in 6× SSC, 50% formamide, 0.1% SDS, and washed at 65°C in 2× SSC, 0.1% SDS according to a standard protocol. Positive plaques (~40) were taken through further purification and excised *in vivo* as plasmids. The positive inserts were sequenced on both strands. 5′ RACE was carried out to determine that the sequence upstream of the start codon contains an in-frame stop codon.

Cell culture and transfection

All cells lines were grown at 37°C in 5% CO_2 and cultured in Dulbecco's modified Eagle's medium (DMEM) containing appropriate serum [HeLa, 10% fetal bovine serum (FBS); NIH 3T3, 10% bovine calf serum (BCS); Cos-1, 10% newborn calf serum (NCS)]. All media were supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 1 mM glutamine. Transient transfection assays were carried out using the lipofectamine (Gibco-BRL) method according to the manufacturer's protocol.

Protein kinase assays

To assay the kinase activity of PAK4, NIH 3T3 cells were transfected with either empty SRα expression vector or expression vectors containing HA-tagged PAK4. Cells were harvested in M2 buffer (Minden *et al*., 1994) 48 h after transfection. Approximately 100 μg of cell extracts were mixed with anti-HA antibody and protein A–Sepharose and incubated 2 h to overnight at 4°C with rotating. The immune complexes were washed twice with M2 buffer and twice in 20 mM HEPES, pH 7.5, and incubated in kinase buffer (described in Minden *et al*., 1994) containing 20 μM ATP and 5 μCi of [γ-^{32}P]ATP together with either 5 μg of histone H4 or MBP (Boehringer Mannheim) or no substrate, at 30°C for 20 min. The reaction was stopped by boiling in 4× SDS loading buffer. Proteins were resolved by SDS–PAGE, and substrate phosphorylation and autophosphorylation were visualized by autoradiography. JNK, ERK and p38 activity were measured as described (Minden *et al*., 1995). To measure autophosphorylation of purified PAKs, recombinant PAK2 or PAK4 (2 μg bound to protein G–Sepharose conjugated with monoclonal glu-glu antibody) was washed once and incubated in 40 μl of kinase buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM $MnCl_2$) with 2 μg of either Rac1 or Cdc42Hs, all previously loaded with GTP or GDP. The reaction was initiated by adding 10 μl of kinase buffer containing 50 μM ATP and 5 μCi of [γ-^{32}P]ATP and incubated for 20 min at 30°C. The reaction was stopped by adding 10 μl of 5× SDS–PAGE sample buffer and boiling for 5 min. Samples were applied to a 14% SDS–PAGE gel and exposed to film.

Northern blots

The Northern analysis was performed using a human tissue blot (Clontech). Hybridization and washes were carried out as recommended by the manufacturer. The probe was prepared by labeling a 400 bp cDNA fragment corresponding to the kinase domain of PAK4 with

[α-^{32}P]dCTP (Amersham International PLC) by random priming (Stratagene).

Overlay assay

The overlay assay is described in Martin *et al*. (1995). Recombinant and immunopurified proteins were separated on SDS–PAGE followed by blotting to a PVDF membrane, washing and blocking with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 100 mM dithiothreitol (DTT). Recombinant GTPases (2–5 μg) were pre-loaded with the indicated radiolabeled nucleotide and were incubated for 5–8 min with the PVDF filter. The filter was washed for 5 min and was exposed to a film for 2 h. Recombinant proteins were expressed and purified from Sf9 cells as previously described (Martin *et al*., 1995). Various Cdc42Hs effector mutants were prepared in *Escherichia coli* as GST fusion proteins and were purified on glutathione–Sepharose beads and eluted from the beads by 5 mM reduced glutathione.

Western blot

Cos-1 cell lysates (25 μg) were resolved by SDS–PAGE and transferred to PVDF membrane. The membrane was blocked in PBS containing 0.2% Tween-20 (PBST) and 4% non-fat milk for 1 h and then incubated with anti-HA antibody diluted in PBST containing 4% non-fat milk for 1 h. After washing three times with PBS, the membrane was probed with secondary antibody, peroxidase-conjugated goat IGG fraction to mouse IGG for 1 h. After washing three times with PBS, the immunocomplexes were visualized by enhanced chemiluminescence (ECL) reagent (Amersham Corp.).

Microinjections and immunofluorescence microscopy

Microinjections and immunofluorescence microscopy were carried out essentially as described (Symons *et al*., 1996). Briefly, PAE cells were grown in DMEM containing 10% FBS and were plated on a coverslip. Expression vectors encoding various PAK4, PAK2, Cdc42Hs and Rac mutants diluted to a concentration of 50 ng/μl in injection buffer (5 mM potassium glutamate, 130 mM KCl) were microinjected into the nucleus of ~100 subconfluent PAE cells. Injected cells were incubated for 16–20 h at 37°C and fixed in 4% formaldehyde. Cells were permeabilized with PBS containing 0.1% Triton X-100 and incubated in the presence of the primary monoclonal antibodies anti-HA or anti-Myc for 60 min. The coverslips were washed with PBS containing 0.1% Triton X-100 and incubated for 30 min with the second antibody, rhodamine-conjugated anti-mouse antibody. To visualize F-actin, cells were washed again and were incubated with fluorescein isothiocyanate (FITC)-conjugated phalloidin. Fluorescence photomicroscopy was carried out on a Zeiss Axiophot with appropriate filters for fluorescence detection.

Preparation of recombinant proteins

To prepare recombinant PAK4, a *Bam*HI–*Not*I fragment containing the full-length PAK4 was subcloned into the pAcO-3 baculovirus expression vector containing a 5′ Glu-Glu tag. Recombinant protein was then prepared and purified as described in Martin *et al*. (1995). Recombinant PAK2 was generated as described in Martin *et al*. (1995).

Plasmids

To construct HA-tagged PAK4, an *Eco*RI fragment of the cDNA was ligated in-frame into the *Eco*RI site of Bluescript II KS(+) vector containing a 5′ HA tag. HA-PAK4 was then removed from Bluescript II KS(+) as a *Hin*dIII–*Stu*I fragment and subcloned into the *Hin*dIII–*Sma*I site of expression vector SRα3 (Takebe *et al*., 1988). To construct Myc-tagged PAK4, an *Eco*RI fragment containing the entire coding sequence of PAK4 was inserted into the *Eco*RI site of the pCAN Myc2 vector. A *Hin*dIII–*Eco*RV fragment (including the Myc tag and the entire PAK4-coding sequence) was then inserted into SRα at the *Hin*dIII–*Sma*I site.

PAK4(M350), PAK4(M474) and PAK4(E474) were generated by site-directed mutagenesis (Stratagene QuickChange kit) of K(350) or S(474) to methionine or glutamate. Rac2L61, JNK, p38, ERK and ERK are described in Minden *et al*. (1995).

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