Interaction of Nck-associated protein 1 with activated GTP-binding protein Rac

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Bacterially expressed glutathione S-transferase fusion proteins containing Rac1 were used to identify binding proteins of this Rho family GTPase present in a bovine brain extract. Five proteins of 85, 110, 125, 140 and 170 kDa were detected, all of which were associated exclusively with guanosine 5′-[γ-thio]-triphosphate-bound Rac1, not with GDP-bound Rac1. The 85 and 110 kDa proteins were identified as the regulatory and catalytic subunits respectively of phosphatidylinositol 3-kinase. Several lines of evidence suggested that the 125 kDa protein is identical with Nck-associated protein 1 (Nap1). The mobilities of the 125 kDa protein and Nap1 on SDS/PAGE were indistinguishable, and the 125 kDa protein was depleted from brain extract by preincubation with the Src homology 3 domain of Nck to which Nap1 binds. Furthermore, antibodies to Nap1 reacted with the 125 kDa protein. Nap1 was co-immunoprecipitated with a constitutively active form of Rac expressed in Chinese hamster ovary cells. The observation that complex formation between activated Rac and PAK, but not that between Rac and Nap1, could be reproduced *in vitro* with recombinant proteins indicates that the interaction of Nap1 with Rac is indirect. The 140 kDa Rac-binding protein is a potential candidate for a link that connects Nap1 to Rac. The multimolecular complex comprising Rac, Nap1 and probably the 140 kDa protein might mediate some of the biological effects transmitted by the multipotent GTPase.

INTRODUCTION

Members of the Rho family of GTPases have been implicated in the regulation of cell morphology and motile activity [1]. Rac regulates the accumulation of actin filaments at the plasma membrane and induces the formation of lamellipodia and membrane ruffles [2], and Rho is required for the formation of focal adhesions and the regulation of actin stress fibres [3]. Furthermore, CDC42Hs, when microinjected into fibroblasts, induces both the formation of filopodia as well as the activation of Rac and, subsequently, Rho [4], probably through arachidonic acid metabolism [5], suggesting that the three GTPases are activated sequentially.

Recently, Rac was shown to be involved in cell transformation [6,7]. Furthermore, Rac stimulates cell cycle progression and DNA synthesis [8], activates stress-activated protein kinases (also known as c-Jun N-terminal kinases) [8–11], mediates growth-factor-stimulated arachidonic acid release [5], activates membrane NADPH oxidase [12,13] and regulates axonal growth in the cerebellum [14], properties indicative of the multipotency of this small GTPase.

Little is known of the molecular mechanisms by which Rac achieves these various biological effects. The GTP-bound forms of Rac and CDC42Hs bind the 85 kDa regulatory subunit of the PtdIns 3-kinase *in vitro* [15] and PtdIns3*P* 5-kinase and PtdIns4*P* 5-kinase activities in intact cells [16], and might regulate the activities of these enzymes. The protein kinase PAK, a mammalian homologue of *Saccharomyces cerevisiae* STE 20, was also identified as a target of Rac and CDC42Hs [17–21]; the GTPases bind directly to and activate PAK in a GTP-dependent manner, activation resulting from the stimulation of autophosphorylation of the kinase. Another direct target of Rac is $p67^{phox}$, which is required for activation of NADPH oxidase [21–23]; Rac associates directly with $p67^{phox}$ and the two proteins then form a multimolecular complex with $p47^{phox}$, $p21^{phox}$ and $gp91^{phox}$ that regulates the production of superoxide radicals in phagocytic cells [12,13,24,25].

To identify additional targets of activated Rac and further elucidate the mechanism of Rac signalling, we now describe the characterization of proteins in bovine brain extract that bind in a GTP-dependent manner to an immobilized glutathione S-transferase (GST) fusion protein containing Rac1.

MATERIALS AND METHODS

Preparation of GST fusion proteins

cDNAs encoding GST fusion proteins containing Rac1, V12Rac1 (with a valine residue substituted for Gly^{12}), N17Rac1 (with an asparagine residue substituted for Ser^{17}), CDC42Hs or RhoA were kindly provided by A. Hall (University College London, London, U. K.), and a cDNA encoding GST fusion protein containing H-Ras was kindly provided by Y. Takai (Osaka University, Osaka, Japan). GST fusion proteins containing the first, second or third Src homology 3 (SH3) domain of human Nck or full-length Nck [NckSH3(1), NckSH3(2), NckSH3(3) and Nckfull respectively] have been described previously [26]. Mutant GST–NckSH3 fusion proteins with a leucine residue substituted for Pro^{52} in NckSH3(1), Pro^{156} in NckSH3(2) and Pro^{243} in NckSH3(3) [NckSH3(1)P52L, NckSH3(2)P156L and

Abbrevations used: GST, glutathione S-transferase; GTP[S], guanosine 5′-[γ-thio]triphosphate; HA, haemagglutinin; Nap1, Nck-associated protein 1; SH3, Src homology 3.

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NckSH3(3)P243L respectively] were constructed with the use of In Vitro Mutagenesis System, version 2.1 (Amersham).

Affinity purification of Rac-binding proteins

Bacterially expressed GST fusion proteins were immobilized on glutathione beads, incubated with either 0.1 mM GDP or guanosine 5′-[γ-thio]triphosphate (GTP[S]) in buffer A [50 mM Tris/HCl (pH 7.5)/50 mM NaCl/10 mM EDTA/5 mM $MgCl_2$/1 mM dithiothreitol] for 30 min at room temperature and then washed twice with buffer A. The immobilized GDP- or GTP[S]-bound GST fusion proteins were then incubated for 2 h at 4 °C with Triton X-100 extracts of bovine brain prepared as described previously [26]. After extensive washing, proteins bound to the beads were released by boiling in SDS sample buffer, separated by SDS/PAGE and revealed by silver staining.

Production of recombinant proteins in Sf9 cells

A full-length rat PAK cDNA, the corresponding amino acid sequence for which was identical with rat brain PAK [17], with the exception that His^{73} and Cys^{92} were replaced by proline and valine residues respectively, was isolated from 3Y1 rat fibroblast cDNA libraries. Full-length rat PAK cDNA and a full-length rat Nck-associated protein 1 (Nap1) cDNA [26] were subcloned into the pAcG2T vector [27] and transfected into Sf9 cells with a BaculoGold transfection kit (Pharmingen). The expressed GST–PAK and GST–Nap1 fusion proteins were isolated from Sf9 cell lysates by incubation with glutathione beads, and were then either eluted from the beads with reduced glutathione or cleaved to remove GST by incubation with thrombin.

Preparation of antibodies

Rabbit polyclonal antibodies to PAK and to the 110 kDa catalytic subunit of PtdIns 3-kinase were generated against baculovirus-expressed full-length GST–PAK fusion protein and a peptide corresponding to the C-terminus of the 110 kDa subunit (KMDWIFHTIKQHALN) respectively. Polyclonal antibodies to Nap1 [26] and a monoclonal antibody to the 85 kDa subunit of PtdIns 3-kinase [28] have been described previously. A monoclonal antibody (12CA5) to the haemagglutinin (HA) epitope tag and a monoclonal antibody to the T7 epitope tag were obtained from Boehringer Mannheim and Novagen respectively.

Transfection and immunoblot analysis

COS-7 cells were transiently transfected with plasmid pSRHis-T7 containing PAK cDNA with the use of Lipofectamine (Gibco-BRL); 48 h after transfection, cells were lysed and insoluble material was removed by centrifugation. The resultant supernatant was subjected to binding assays with immobilized GST fusion proteins or to immunoprecipitation. CHO-IR cells [29] were co-transfected with pSV40-hgh, a plasmid that confers resistance to hygromycin, and the pSRα Nap1 plasmid. Transfected cells were selected by the addition of hygromycin B to the culture medium, and drug-resistant cells stably expressing Nap1 were screened by immunoblot analysis with antibodies to Nap1 and cloned by limiting dilution.

Adenovirus vectors encoding HA epitope-tagged V12Rac1 or N17Rac1 [the HA epitope sequence (YPYDVPDYASL) was introduced by PCR at the N-terminus of the GTPases] were generated by cloning the cDNA species into pAxCAwt [30], which contains the CAG promoter [31], and by co-transfection into 293 cells with DNA-TPC as described previously [32]. Protein-encoding viruses were screened by immunoblot analysis and cloned by limiting dilution. Once 48 h had elapsed after infection with adenovirus vectors, cells were lysed and subjected to immunoprecipitation.

Rac binding assay

Dot-blot assays were performed essentially as described previously [33,34]. In brief, 0.5 μg of either recombinant Nap1 or PAK was spotted on nitrocellulose filters and incubated for 30 min at room temperature in a blocking buffer containing 5% (v/v) fetal bovine serum, 1 M glycine and 5% (w/v) dry skimmed milk. The filters were washed twice with a solution containing 50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$ and 0.1 mM dithiothreitol, and then incubated for 10 min at 4 °C with [γ-^{32}P]GTP-bound recombinant V12Rac1 prepared as described [22]. After being washed twice with a solution containing 100 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.1% Triton X-100 and 5 mM $MgCl_2$, the filters were dried and the bound GTPases were detected with a Fuji BAS-2000 image analyser. For binding assays in solution, 0.5 μg of recombinant V12Rac1 was loaded with 10 μCi of [^{3}H]GTP or [^{3}H]GDP (8 Ci/mmol and 10 Ci/mmol respectively; Amersham) as described [33], after which the labelled GTPases were incubated for 5 min at 4 °C with immobilized GST–PAK or GST–Nap1 on glutathione beads. The beads were washed three times with 50 mM Tris/HCl, pH 7.5, containing 100 mM NaCl, 5 mM $MgCl_2$ and 0.1 mM dithiothreitol, and the remaining radioactivity associated with the beads was determined with a scintillation counter.

Overlay assay

GST–NckSH3(2) fusion protein was biotinylated with the use of the Enhanced Chemiluminescence (ECL) Protein Biotinylation System (Amersham); 5 μg of either recombinant Nap1 or PAK was separated by SDS/PAGE and transferred to Immobilon-P membranes (Millipore). After blocking, the membrane was probed with biotinylated GST–NckSH3(2) resolved at 1 μg/ml in a buffer containing 50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Tween-20. The bound protein was revealed by horseradish peroxidase-conjugated streptavidin and the ECL detection kit (Amersham).

RESULTS

To identify potential targets of activated Rac, we prepared an affinity matrix by immobilizing a GST fusion protein containing Rac1 on glutathione beads and then incubating the beads with either GDP or the non-hydrolysable GTP analogue GTP[S]. These matrices were then used to isolate proteins from Triton X-100 extracts of bovine brain. The bound proteins were analysed by SDS/PAGE and silver staining. GTP[S]–Rac1, but not GDP–Rac1, associated with proteins of 85, 110, 125, 140 and 170 kDa (designated p85, p110, p125, p140 and p170 respectively) (Figure 1, upper panel). Although a 70 kDa protein was sometimes detected in the fraction bound by GTP[S]–Rac1, the specific association of this protein was not reproducible; it was often present in the fraction isolated with the GDP-bound GTPases.

Given that the 85 kDa subunit of PtdIns 3-kinase binds to Rac and CDC42Hs [15], we analysed the brain protein fractions that interacted with GTP[S]–Rac1 or GDP–Rac1 by immunoblot analysis with antibodies to the 85 kDa or to the 110 kDa subunits of PtdIns 3-kinase. Immunoreactive proteins of 85 and 110 kDa were detected by the respective antibodies in the fraction isolated with GTP[S]–Rac1 but not in that isolated with GDP–Rac1

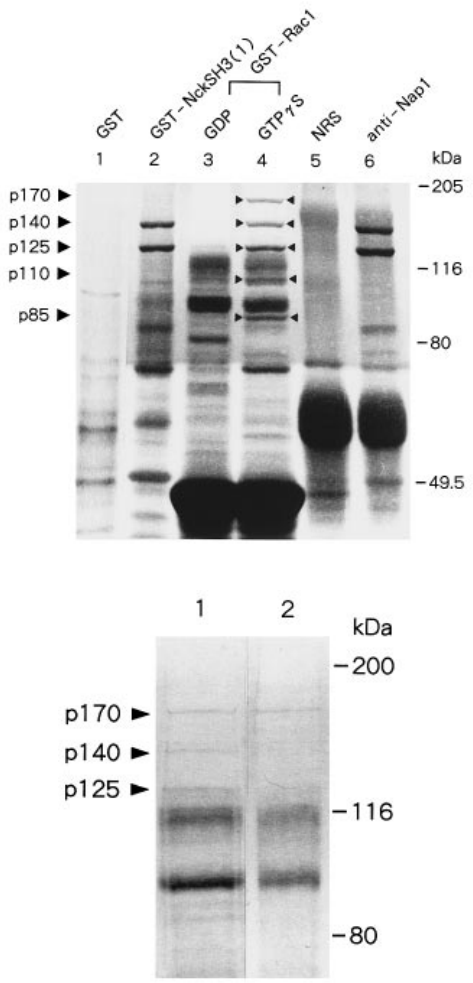

Figure 1 Interaction of brain proteins with affinity matrices prepared from GST fusion proteins containing Rac1, and depletion of p125 and p140, but not p170, from brain extract by incubation with NckSH3(1)

Upper panel: 5 μg of immobilized GST (lane 1), GST–NckSH3(1) (lane 2), GST-Rac1 bound to GDP (lane 3) or GTP[S] (GTPγS) (lane 4), or 2 μl of normal rabbit serum (NRS) (lane 5) or 5 μg of anti-Nap1 polyclonal antibodies to Nap1 (anti-Nap1) (lane 6) preadsorbed on Protein G beads, were incubated with brain extract and the bound proteins were analysed by SDS/PAGE and silver staining. The p85, p110, p125, p140 and p170 proteins are indicated by arrowheads; the positions of molecular-mass standards are shown at the right. Lower panel: brain extract was incubated with either unfused GST (lane 1) or GST–NckSH3(1) (lane 2) immobilized on glutathione beads. After a brief centrifugation, the resulting supernatants were incubated with affinity matrices containing GTP[S]-bound GST–Rac1. The bound proteins were analysed by SDS/PAGE and silver staining. Arrowheads indicate the positions of p125, p140 and p170.

(results not shown), suggesting that the p85 and p110 proteins that bound to GTP[S]–Rac1 are probably the 85 and 110 kDa subunits respectively of PtdIns 3-kinase.

We recently showed that 125 and 140 kDa proteins form a specific complex with Nck [26], an adaptor protein that contains SH2 and SH3 domains [35]. These two proteins exclusively associated with the first SH3 domain of Nck [NckSH3(1)] [26]. We have purified the 125 kDa protein, designated Nap1, and isolated its cDNA [26]. Because the molecular sizes of p125 and p140 were similar to those of Nap1 and the 140 kDa Nck-associated protein, the brain fractions isolated with Rac1 and NckSH3(1), as well as an immunoprecipitate prepared from brain extracts with antibodies to Nap1, were analysed by

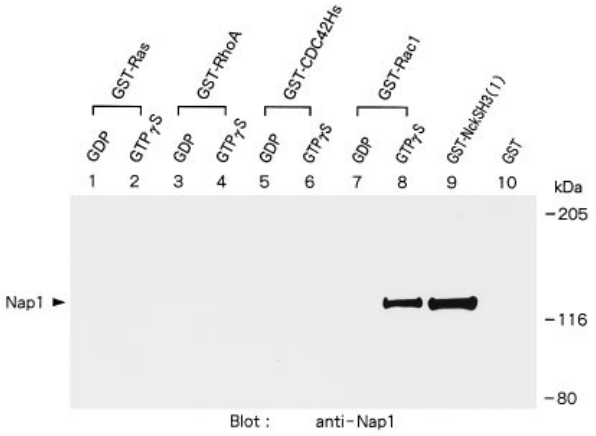

Figure 2 Recognition of p125 by antibodies to Nap1

Affinity matrices prepared from GST fusion proteins containing various GTPases loaded with GDP or GTP[S] (GTPγS) (lanes 1–8) or NckSH3 (lane 9), or GST alone (lane 10), were incubated with brain extract. The bound proteins were resolved by SDS/PAGE and subjected to immunoblot analysis with polyclonal antibodies to Nap1. The position of Nap1 is indicated by an arrowhead; the positions of molecular-mass standards are shown at the right.

SDS/PAGE and silver staining. The mobilities of p125 and p140 that associated with GTP[S]–Rac1 were indistinguishable from those of Nap1 and the 140 kDa Nck-associated protein that bound to NckSH3(1) (Figure 1, upper panel). Pronounced 125 and 140 kDa bands, as well as several minor bands, were apparent in the anti-Nap1 immunoprecipitate (Figure 1, upper panel), whereas the antibodies reacted with a single 125 kDa protein in brain extracts by immunoblot analysis [26], suggesting that Nap1 and the 140 kDa protein form a stable complex. Again, the mobilities of these proteins seemed identical with those of p125 and p140 (Figure 1, upper panel), prompting us to investigate whether the two sets of proteins are related.

Bovine brain extract was incubated with immobilized GST–NckSH3(1) or unfused GST, after which the supernatants were incubated with the affinity matrix containing GTP[S]–Rac1. Proteins that bound to GTP[S]–Rac1 were analysed by SDS/PAGE and silver staining. Both p125 and p140, but not p170, were almost completely depleted from the brain extract by preincubation with GST–NckSH3(1) (Figure 1, lower panel). In contrast, prior incubation of the brain extract with unfused GST had no effect on the amounts of p125 and p140 that bound to GTP[S]–Rac1. Moreover, preincubation of brain extract with the GTP[S]–Rac1 affinity matrix resulted in the depletion of Nap1 and the 140 kDa Nck-associated protein (results not shown).

We next investigated whether antibodies to Nap1 react with p125. Brain extract was incubated with various affinity matrices containing the GTP[S]-bound or GDP-bound GTPases, and the bound proteins were subjected to immunoblot analysis with antibodies to Nap1. An immunoreactive 125 kDa protein was apparent only in the fractions that bound to GTP[S]–Rac1 (Figure 2); the mobility of this protein was indistinguishable from that of a 125 kDa immunoreactive protein that bound to NckSH3(1). These results indicated that p125, a protein that binds to activated Rac1, is identical with or closely related to Nap1.

To determine whether the interaction between Rac and Nap1 occurs in intact cells, we infected CHO-IR cells stably expressing Nap1 with adenovirus vectors encoding an HA epitope-tagged

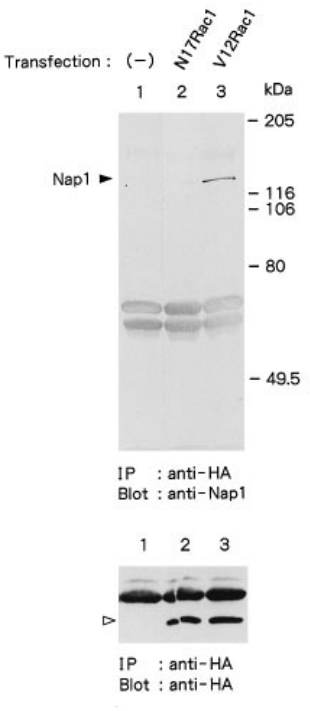

Figure 3 Interaction of Nap1 with active Rac1 in intact cells

CHO-IR cells stably expressing Nap1 were transfected with or without (lane 1) adenovirus vectors encoding HA-tagged N17Rac1 (lane 2) or HA-tagged V12Rac1 (lane 3). The cells were lysed and subjected to immunoprecipitation (IP) with antibodies to HA and immunoblot analysis with antibodies to Nap1 (upper panel) or to HA (lower panel). The position of Nap1 is indicated by an arrowhead; the positions of molecular-mass standards are shown at the right.

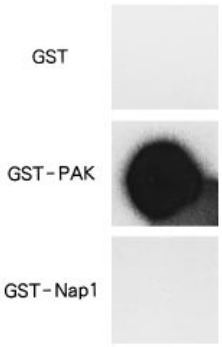

Figure 4 Interaction of Nap1 and PAK with GTP-bound V12Rac1 in the dot-blot assay

GST, GST–PAK or GST–Nap1 (0.5 μg of each) was spotted on nitrocellulose filters and incubated with 0.5 μg of V12Rac1 preloaded with [γ-^{32}P]GTP. The filters were washed and dried, and the amount of bound radioactivity was determined with a Fuji BAS-2000 image analyser.

constitutively active Rac mutant (V12Rac1) or an HA epitope-tagged dominant negative Rac mutant (N17Rac1). The Rac1 proteins were immunoprecipitated with antibodies to HA and the immunoprecipitates were subjected to immunoblot analysis with antibodies to Nap1 or to HA. Immunoprecipitate prepared from the infected cells expressing V12Rac1, but not those from the cells expressing N17Rac1, contained Nap1 (Figure 3), demonstrating that Nap1 forms a complex with activated Rac in intact cells.

Table 1 Interaction of Nap1 and PAK with GTP-bound V12Rac1 in the glutathione beads assay

Thrombin-cleaved V12Rac1 was loaded with [^{3}H]GDP or [^{3}H]GTP and incubated with GST, GST–PAK or GST–Nap1 immobilized on glutathione beads. The radioactivity associated with the beads was determined as described in the Materials and methods section and expressed as a percentage of input counts. The results shown represent the means ± S.E.M. for three experiments.

Immobilized protein	Loaded nucleotide	Percentage of input counts
GST	[^{3}H]GDP	2.1 ± 1.1
	[^{3}H]GTP	1.6 ± 0.9
GST–PAK	[^{3}H]GDP	2.6 ± 1.0
	[^{3}H]GTP	18.8 ± 4.2
GST–Nap1	[^{3}H]GDP	4.8 ± 2.0
	[^{3}H]GTP	3.9 ± 1.7

We next attempted to reconstitute the association of Nap1 or PAK with activated Rac by two different approaches. First, recombinant Nap1 or PAK produced as GST fusion proteins by baculovirus expression in insect cells were spotted on a nitrocellulose membrane and probed with [γ-^{32}P]GTP-bound V12Rac1 (Figure 4). Alternatively, recombinant Nap1 or PAK fusion proteins were immobilized on glutathione beads, which were then incubated in the presence of V12Rac1 preloaded with either [^{3}H]GTP or [^{3}H]GDP (Table 1). Consistent with the results of previous studies [33,36], recombinant PAK interacted with GTP-bound V12Rac1 in both approaches. However, neither approach revealed an association between recombinant Nap1 and V12Rac1, suggesting that Nap1 does not interact directly with Rac and that the association is mediated by unidentified molecules.

Recently PAK was also shown to be associated with Nck [20]. Because Nap1 probably binds indirectly to Rac (Figure 4 and Table 1) and Nck [26], we examined whether PAK mediates these interactions. We first investigated the binding specificity of PAK with each of the three SH3 domains of Nck GST fusion proteins containing NckSH3(1), NckSH3(2), NckSH3(3) or NckSH3 GST fusion proteins in which proline residues in P(S/K)NY motifs in the three SH3 domains were converted into leucine [NckSH3(1)P52L, NckSH3(2)P156L and NckSH3(3)P243L] were immobilized on glutathione beads. Similar mutations in the SH3 domains of Sem5 or Grb2 inhibit the function of these proteins or their association with ligands [37,38]. The immobilized fusion proteins were incubated with lysates of COS-7 cells that had been transfected with a plasmid encoding T7 epitope-tagged PAK (Figure 5, upper panel) or with brain extract (Figure 5, lower panel), and bound proteins were subjected to immunoblot analysis with antibodies to T7 or to Nap1 respectively. A 65 kDa immunoreactive protein was recognized by antibodies to T7 in the bound protein fraction isolated with Nckfull and NckSH3(2), but not in those isolated with NckSH3(1), NckSH3(3) or the mutant SH3 domains (Figure 5, upper panel). When recombinant PAK produced by baculovirus expression was separated by SDS/PAGE and subjected to an overlay assay with the use of biotinylated NckSH3(2), PAK reacted to the labelled NckSH3(2) (results not shown), demonstrating that PAK predominantly binds directly to the second SH3 domain of Nck. In contrast, only the brain fraction that bound to Nckfull and NckSH3(1) reacted with antibodies to Nap1 (Figure 5, lower panel). These results indicate that the specificities of PAK and Nap1 binding to

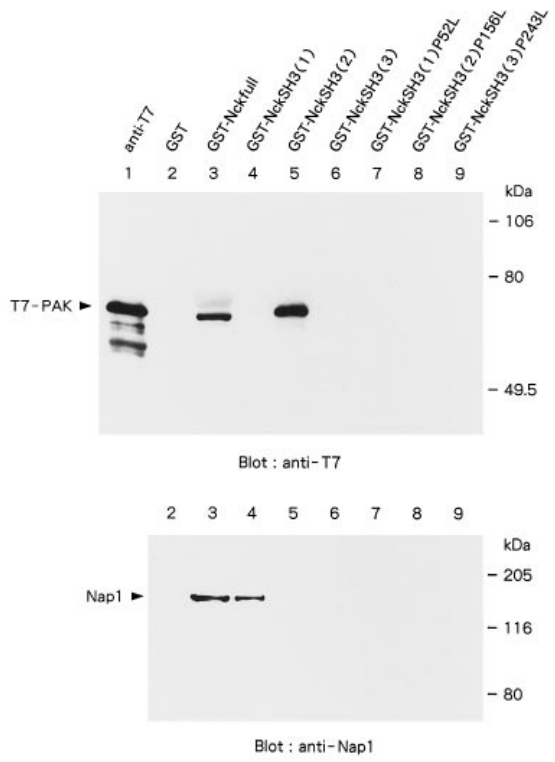

Figure 5 Specificities of Nap1 and PAK binding to the SH3 domains of Nck

GST or GST fusion proteins (5 μg) containing Nckfull, NckSH3(1), NckSH3(2), NckSH3(3), NckSH3(1)P52L, NckSH3(2)P156L or NckSH3(3)P243L coupled to glutathione beads, or 5 μg of anti-T7 antibody coupled to Protein G beads, were incubated either with lysates of COS-7 cells transiently expressing T7-tagged PAK (upper panel) or with bovine brain extract (lower panel). The bound proteins were resolved by SDS/PAGE and subjected to immunoblot analysis with antibodies to T7 (upper panel) or to Nap1 (lower panel). Arrowheads indicate the positions of T7-tagged PAK and Nap1; the positions of molecular-mass standards are shown at the right.

the SH3 domains of Nck are distinct. We further investigated whether PAK can bind directly to Nap1 by incubating immobilized GST–PAK with recombinant Nap1 or brain extract, and then subjecting the bound proteins to immunoblot analysis with antibodies to Nap1. Neither experiment detected Nap1 association with PAK (results not shown). These results support the hypothesis that PAK does not mediate the association of Nap1 with Rac or Nck.

DISCUSSION

We have shown that activated Rac specifically associated with various proteins, including p85, p110, p125, p140 and p170, in brain extract. The first two of these proteins are most probably the 85 and 110 kDa subunits of PtdIns 3-kinase, given that they were recognized by specific antibodies to these subunits. The association of PtdIns 3-kinase with Rac and CDC42Hs has been demonstrated previously [15,16]. The 85 kDa subunit of this enzyme contains the Rho GAP (GTPase-activating protein) homology domain, through which the interaction with the GTPases is mediated [15]. Indeed, our preliminary experiments revealed that mutant 85 kDa subunits that lack the binding site for the 110 kDa subunit or the SH3 domain were still able to interact with Rac, whereas a mutant 85 kDa subunit that lacks the Rho GAP homology domain did not bind activated Rac (results not shown).

Several lines of evidences indicated that p125 is identical with Nap1: (1) the mobilities of the two proteins on SDS/PAGE were indistinguishable; (2) preincubation of brain extract with the first SH3 domain of Nck, which binds Nap1, resulted in depletion of p125; and (3) antibodies to Nap1 reacted with a 125 kDa protein in the brain fraction that bound specifically to GTP[S]-bound Rac1. Moreover, circumstantial evidence suggests that p140 is identical with the 140 kDa Nck-associated protein.

We showed that Nap1 was co-precipitated with a constitutively active Rac protein from cells overexpressing these molecules, demonstrating that the putative target of Rac forms a complex with this protein in mammalian cells. However, our attempts to reconstitute complex formation with recombinant Nap1 and Rac or Nck were unsuccessful, suggesting that unidentified molecules mediate these interactions. However, these results do not exclude the possibility that some essential post-translational modification of Nap1 and/or Rac is required for a direct interaction of these two proteins. Although PAK has been shown to bind to Rac and to Nck [17–21], our observation that the binding specificity of PAK to the three SH3 domains of Nck differs from that of Nap1, together with our failure to detect direct binding of PAK to Nap1, indicates that PAK does not mediate the interaction between Nap1 and Rac or Nck.

Our results demonstrated that PAK binds directly to the second SH3 domain of Nck. Chou and Hanafusa [39] showed that a protein-serine/threonine kinase of approx. 65 kDa specifically associates with the second SH3 domain of Nck, and they named this enzyme NAK (Nck-associating kinase). Because its molecular size and the binding specificities for the SH3 domains of Nck are similar, NAK may be identical with or related to PAK. Chou and Hanafusa [39] were not able to stimulate the kinase activity of NAK with a variety of mitogenic stimuli; similarly, with the exception of weak activation by fMet-Leu-Phe in neutrophils [19], activation of PAK by extracellular stimulation has not been demonstrated.

The 140 kDa protein that associates with both activated Rac and Nck is a potential link for connecting Nap1 to Rac and to Nck. p140 is the only protein that binds in similar amounts, as revealed by protein staining, to an affinity matrix prepared with Nck or to one prepared with activated Rac. Moreover, immunoprecipitates prepared from brain extract with antibodies to Nap1 also contain comparable amounts of p140, suggesting that Nap1 and p140 form a stable complex. Because several minor bands (60–80 kDa) were also observed in these fractions, we cannot exclude the possibility that one or more of these proteins might contribute to the interactions. Recently we purified sufficient p140 for partial amino acid sequence analysis, the results of which suggest that p140 is a previously uncharacterized protein (T. Kitamura, Y. Kitamura, K. Yonezawa and M. Kasuga, unpublished work). The molecular cloning of p140 might clarify the role in the interactions of Nap1 with Rac and Nck.

Although the function of Nap1 is unknown, WASP, another target of a Rho family GTPase, was recently shown to bind to the third SH3 domain of Nck [40]. WASP, a protein implicated in Wiskott–Aldrich syndrome [41], binds directly to activated CDC42Hs [34] and is important in the regulation of cytoskeletal organization by this GTPase [42]. Given that Nck and Rho family GTPases are thought to contribute to growth-factor signalling [1,5,43–47], this newly identified class of molecules that interact with both the adaptor protein and the GTPases might mediate cross-talk between their respective signalling pathways.

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