Delineation of the Cdc42/Rac-Binding Domain of p21-Activated Kinase

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ABSTRACT: p21-activated kinases (PAKs) serve as effector proteins for the GTP-binding proteins Cdc42 and Rac. They are serine/threonine kinases containing the Cdc42/Rac interactive binding (CRIB) motif. The main aim of this study was to define the minimal domain of αPAK required for Cdc42/Rac binding. Eight stable PAK fragments of varying lengths, each containing the CRIB motif (residues 75−88), were expressed in *Escherichia coli*, and their ability to interact with Cdc42 and Rac was assessed using scintillation proximity assays, isothermal titration calorimetry, and fluorescence techniques. The shortest fragments examined (residues 70−94 and 75−94) bound only weakly to either Cdc42 or Rac. A longer fragment starting at residue 75 and ending at residue 105 showed binding to Q61L Rac.GTP with K_d = 1.9 μM. Highest affinity binding ($K_d \sim 0.05$ μM) was seen with longer fragments ending at residue 118 or 132. A small increase in affinity was seen with those fragments starting at residue 70 rather than residue 75. PAK fragments bound with ∼3−10-fold higher affinity to Cdc42 than to Rac and bound Q61L variants with 5−10-fold higher affinity than wild type. The dissociation rates of Q61L Rac.mant-GTP and of Q61L Cdc42.mant-GTP from PAK fragment residues 70−132 were measured to be 0.66 and 0.25 min^{-1}, respectively, which are 100-fold lower than dissociation rates for Ras:Ras-effector domains, although their affinities are similar. Calorimetric measurements revealed that binding was associated with a relatively slow heat change. It is suggested that these PAK fragments (in the absence of Cdc42 or Rac) might exist predominantly in an inactive conformation that slowly interconverts with an active conformation and/or a slow conformational change may occur upon binding to Cdc42/Rac. In conclusion, the PAK CRIB motif itself is insufficient for high-affinity binding to Cdc42/Rac, but a 30 amino acid region of PAK (residues 75−105), containing this motif, is sufficient.

Cdc42 and Rac are members of the Rho family of small GTP-binding proteins that control many cellular processes such as morphogenesis and cytoskeletal organization, neutrophil activation, mitogenesis and transformation, and protein kinase cascades (*1*, *2*). These proteins act as molecular switches linking extracellular stimuli to the activation of intracellular signaling pathways. Hence, activation of Cdc42 by bradykinin stimulation induces the formation of filopodia (*3*), whereas activation of Rac by platelet-derived growth factor induces membrane ruffling (*4*). Both Cdc42 and Rac also participate in signaling pathways leading to the activation of the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38 kinase (*5*−*8*).

Cellular changes induced by Cdc42 and Rac are mediated by the binding of their GTP forms to effector proteins that are subsequently activated and thereby participate in downstream signaling events. The p21-activated kinases (PAKs)[1] are a family of serine/threonine kinases that bind to Cdc42 and Rac (*5*, *9*−*11*). Originally isolated from rat brain, PAK was recognized as a homologue of the yeast kinase STE20, which is involved in the mating/pheromone MAP kinase cascade in *Saccharomyces cerevisiae* (*10*). Three PAK isoforms ranging in size from 62 to 68 kDa have been identified in mammalian tissues. These are αPAK (PAK1), which is found mainly in brain, muscle, and spleen tissues; βPAK (mPAK3), which is present in brain tissue; and γPAK (hPAK, PAK2), which has a ubiquitous distribution. There are several lines of evidence to support the role of PAK as an effector molecule for Cdc42 and Rac (*5*, *9*): For example, PAK has a higher affinity for the GTP forms of these proteins rather than for the GDP forms, and binding to Cdc42/Rac.GTP elicits its kinase activity (*10*, *11*). Also, the overexpression of PAK or the introduction of constitutively active PAK mutants into mammalian cells results in cellular changes normally associated with Cdc42/Rac activation (*8*, *12*−*14*).

PAK proteins have at their C terminus a serine/threonine kinase domain that exhibits 70% homology to the kinase domain in STE20 and is also related to kinases that do not interact with Cdc42/Rac (*5*, *9*−*11*). The N-terminal region of PAK contains two to four proline-rich regions that recognize and allow binding to Src-homology 3 (SH3) domains

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[1] Abbreviations: PAK, p21-activated kinase; GST, glutathione *S*-transferase from *Schistosoma japonicum*; SPA, scintillation proximity assay; ITC, isothermal titration calorimetry; CRIB, Cdc42/Rac interactive binding; mant-GTP, 2′(3′)-*O*-*N*-methylanthraniloyl-GTP; DTT, dithiothreitol.

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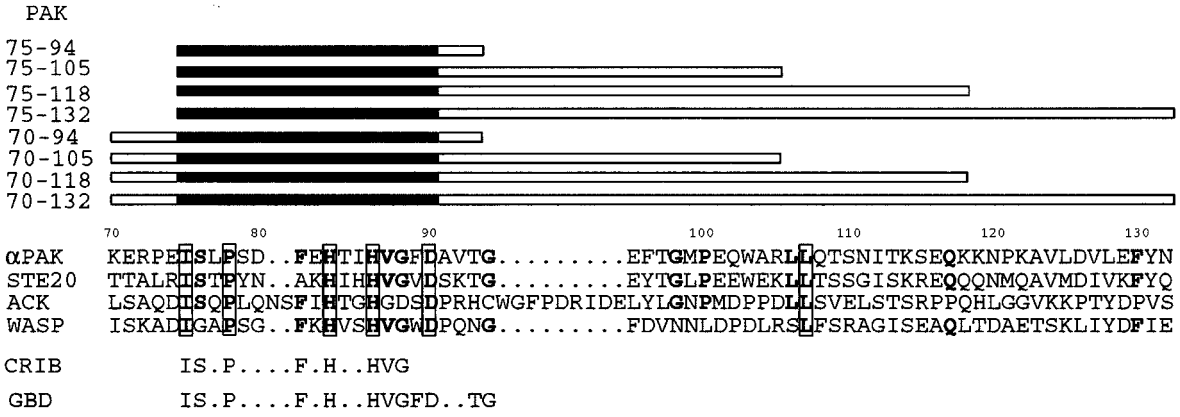

FIGURE 1: Sequence alignment of αPAK with other Cdc42/Rac-interacting proteins containing the CRIB motif. The numbers above the alignment represent the numbering of the PAK residues. The residues and accession numbers for each of the regions shown are as follows: αPAK 70−132, U24152 as modified in ref *18*; STE20 332−392, L04655; ACK 505−578, L13738; WASP 233−295, P42768. Those residues conserved in three of the sequences are shown in bold, whereas those present in all of the sequences are in bold and boxed. The CRIB motif as defined in ref *15* and the GBD consensus as defined in ref *16* are shown below the alignment. The regions of PAK expressed as GST fusions are shown above the sequence alignment. The black box represents the most highly conserved region around the CRIB motif.

and a highly conserved region (*15*, *16*) that has been termed the Cdc42/Rac interactive binding (CRIB) motif (*15*). This motif consists of ≈16 amino acids and has the sequence I-S-X-P-$(X)_{2-4}$-F-X-H-X-X-H-V-G, which corresponds to residues 75−88 in αPAK (Figure 1). Similar motifs are also present in other proteins that are known to bind to Cdc42 or Rac. These proteins include serine/threonine kinases, for example, STE20 and mixed-lineage kinases, tyrosine kinases, for example, ACK, and non-kinase proteins, for example, WASP. Although it is likely that the CRIB motif is intimately involved in the binding to Cdc42 and Rac, we are not aware of studies to show that this minimal domain alone is sufficient for binding.

Manser et al. (*10*) showed that a fragment of αPAK (residues 67−150) retained the Cdc42/Rac-binding properties of full-length PAK and concluded on the basis of sequence comparisons with STE20 and ACK that residues 75−132 constitute a Cdc42/Rac-binding domain. Leonard et al. (*17*) expressed residues 65−137 of mPAK3, homologous to residues 70−142 of αPAK, and demonstrated its ability to bind Cdc42. However, shorter fragments have not been characterized.

A major aim of this study was to delineate the minimum region of PAK required for interacting with Cdc42 and Rac and, in particular, to establish whether the CRIB motif of PAK is sufficient for binding. We describe here the use of a scintillation proximity assay, coupled with other biophysical methods, to measure protein−protein interactions and thus to quantify the binding affinities of Cdc42/Rac to PAK fragments.

MATERIALS AND METHODS

Cloning of PAK Fragments. The plasmid pGEX-PAK_{61-150} encoding αPAK residues 61−150 from rat brain was a gift from Thomas Leung and Ed Manser. The encoded protein sequence of PAK is as described in Figure 1 of ref *18*. This plasmid was used as a template to generate several different sized PAK regions, each encompassing the CRIB motif, by PCR using *Taq* DNA polymerase (Promega). Oligonucleotide primers obtained from Life Technologies were designed to amplify the coding sequence of the various PAK regions, flanked directly by a 5′ *Bam*HI site and a 3′ stop codon followed by an *Eco*RI site to facilitate subcloning into pGEX-4T and allow the expression of GST-PAK fusions. The resulting PCR products were digested with *Bam*HI and *Eco*RI, ligated into pGEX-4T linearized with the same enzymes, and then transformed into *Escherichia coli* (DH5α). The sequences of the recombinants were verified by using an automated DNA sequencer (Applied Biosystems Inc.).

Protein Expression and Purification. Protein expression was carried out using *E. coli* BL21 as the host strain. GST-fusion proteins were expressed, purified, and, where required, cleaved with thrombin essentially as previously described (*19*, *20*). Protein concentrations of GST-fusions were determined with the Bio-Rad protein assay kit using bovine serum albumin as a standard. Protein concentrations of the thrombin-cleaved PAK proteins were determined from A_{280} using their amino acid compositions and the extinction coefficients of Tyr, Phe, and Trp (*21*). The quality of the purified proteins was assessed by SDS−PAGE, and their molecular weights were verified by electrospray mass spectrometry.

Wild-type Cdc42 (residues 1−188) was obtained by thrombin cleavage of a GST-Cdc42 fusion protein (*22*). Q61L Cdc42 (residues 1−188) was obtained by factor Xa cleavage of a His-tagged fusion protein produced by a pET16b-Cdc42 expression clone. Wild-type Rac (*23*) and Q61L Rac (*24*) were obtained by thrombin cleavage from GST fusion proteins expressed using vectors that were gifts from Professor Alan Hall and Sohail Ahmed, respectively.

Nucleotide Complexes. [^{3}H]GTP complexes of Q61L Rac and Q61L Cdc42 were made as described for Q61L Ras (*19*), except that 5−7 h incubation periods were used because the dissociation rate of GTP from the Q61L proteins was very slow. GTP complexes were made according to a similar procedure, except that [^{3}H]GTP was replaced with GTP (10-fold molar excess over protein). Mant-GTP complexes were made by incubating a 5-fold excess of mant-GTP (gift of Dr. John Eccleston) with either Q61L Rac or Q61L Cdc42 in the presence of 17 mM EDTA and 190 mM ammonium sulfate for 5 h at 37 °C. GDP complexes of Q61L Rac or Q61L Cdc42 were made by incubating 10 mM GDP (Sigma)

with the protein (0.2−0.5 mM) in the presence of 17 mM EDTA and 190 mM ammonium sulfate for 7 h at 37 °C. Wild-type Rac and Cdc42 were already GDP complexes when purified. GMPPNP complexes were made as follows: Cdc42 or Rac (0.7 mM) was incubated, with gentle agitation, at 37 °C in the presence of 4.4 mM GMPPNP (Boehringer), 0.3 M ammonium sulfate, and alkaline phosphatase−agarose (Sigma Product P0762, 100 units). After 30 min (wild type) and 5 h (Q61L variants), $MgCl_2$ was added to 20 mM. For all complexes, unbound nucleotide was removed on a Sephadex G-25 centrifuge gel filtration column, equilibrated in 10 mM Tris-HCl, pH 7.5, 1 mM DTT, and 1 mM $MgCl_2$. The purity of nucleotide complexes was checked by HPLC using a Partisphere SAX column (125 × 4.6 mm, 5 μm, Whatman) eluted with 0.6 M ammonium phosphate, pH 4.0. All complexes were >99% pure with the exception of the Q61L Rac and Q61L Cdc42.GDP complexes, which showed >98% GDP and ∼1% GTP. The GDP used for nucleotide exchange contained ∼0.3% GTP, and this is likely to be the source of the GTP contamination in the Q61L.GDP complexes, as GTP binds with higher affinity than GDP.

Scintillation Proximity Assay (SPA). This assay was performed and data were analyzed essentially as described for Ras/Raf binding (*25*), except Ras was replaced by 0.026 μM Q61L Rac.[^{3}H]GTP or Q61L Cdc42.[^{3}H]GTP and GST-Raf by 0.03 μM GST-PAK. Where the affinites were measured by competition for binding of Rac.[^{3}H]GTP or Cdc42.[^{3}H]GTP to GST-PAK_{75-132}, SPA data were fitted by nonlinear regression using GraFit to equations defining competitive binding for two ligands, taking the K_d values for GST-PAK binding to Rac or Cdc42 to be 60 and 20 nM, respectively. The equation used was

$$\text{SPA signal} = [S_{max}[(K_iP + K_dI_0) - \sqrt{(K_iP + K_dI_0)^2 - 4K_i^2A_0B_0}]]/[K_i(P - \sqrt{P^2 - 4A_0B_0})]$$

where $P = (A_0 + B_0 + K_d)$, S_{max} is the maximum SPA signal, that is, the signal when GST-PAK is fully complexed with radiolabeled Rac (Cdc42), A_0 is the total concentration of GST-PAK, B_0 is the total concentration of Rac (Cdc42), K_d is the equilibrium dissociation constant for Rac (Cdc42) binding to GST-PAK, and K_i is the equilibrium dissociation constant for binding of inhibitory protein to Rac (or Cdc42 or PAK). This equation does not take into account inhibitor depletion by binding to the protein. Hence, for high-affinity interactions, the K_i value obtained will represent an upper estimate of the true value. For the interactions reported here with K_d > 20 nM, this error is likely to be small.

Fluorescence Measurements. Fluorescence of mant-GTP complexes was followed in a Perkin-Elmer LS-5B fluorometer at 25 °C with λ_{ex} = 365 nm and λ_{em} = 435 nm. Experiments were performed in 50 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, and 1 mM DTT. To measure fluorescence changes associated with binding of GST-PAK to Rac or Cdc42, GST-PAK_{70-132} was added in small aliquots to a solution of either Q61L Rac.mant-GTP (2 μM) or Q61L Cdc42.mant-GTP (2 μM) such that the volume was not significantly altered. Fluorescence intensity was measured after each addition. Titration data were fitted to a simple binding isotherm for a bimolecular association. To measure the kinetics of dissociation, either Q61L Rac.mant-GTP (0.2 μM) or Q61L Cdc42.mant-GTP (0.2 μM) was mixed with 0.1 μM GST-PAK_{70-132}. A large excess of Q61L Rac.GTP (8 μM) was added to displace mant-GTP labeled protein from PAK, and the increase in fluorescence intensity followed with time. Data were fitted to an equation describing a first-order rate process.

Isothermal Titration Calorimetry (ITC). ITC was performed in a Microcal MCS calorimeter (*26*) at 37 °C. Q61L Cdc42.GMPPNP (0.18 mM) was placed in the injection syringe and injected into thrombin-cleaved PAK protein (10 μM) in the cell. Both proteins were dissolved in 50 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, and 0.1 mM DTT. A titration consisted of a preliminary 2 μL injection followed by 14 10 μL injections so as to achieve a final molar ratio of Cdc42 to PAK of 2.1:1. Blank runs were also performed in which PAK was omitted. The heat changes for each injection in the absence of PAK protein was subtracted from those in its presence, and the data were fitted to an equation describing binding at a single site, using the Origin software supplied so as to yield estimates of K_a, ΔH, and apparent stoichiometry of interaction.

RESULTS

Expression of Cdc42/Rac-Binding Regions of PAK. Residues 61−150 of PAK are sufficient for binding to Rac or Cdc42 (*10*). However, when expressed as a GST-fusion, it is badly degraded (*27*). Therefore, on the basis of sequence homologies between PAK and other CRIB-containing proteins (Figure 1), we constructed expression systems for GST-fusions with eight different length PAK regions, all of which contained the CRIB motif (residues 75−88). These regions started with either residue 70 or 75 and ended at residue 94, 105, 118, or 132. All eight GST-PAK fusions were much more homogeneous than GST-PAK_{61-150} (*27*). The six smallest GST fusions with PAK residues 70−94, 75−94, 70−105, 75−105, 70−118, and 75−118 were all purified as single intact proteins of the anticipated M_r. The two largest regions, GST-PAK_{70-132} and GST-PAK_{75-132}, showed some degradation, suggestive of cleavage between residues 118 and 105.

Determination of Affinity of PAK Fragments for Rac or Cdc42 Using SPA Technology. Scintillation proximity assays (SPAs), which can measure protein−protein binding under equilibrium conditions without the requirement of a separation step, have been previously used to quantify the affinity of interaction between Ras and NF1 (*19*) or Raf (*25*). Here we have used similar assays to measure interaction between Cdc42 or Rac with the various regions of PAK. In these assays a GST-fusion with a PAK region is bound to protein A SPA beads via anti-GST. An SPA signal is obtained when binding of GST-PAK to Cdc42 (or Rac).[^{3}H]GTP occurs. Experiments were performed using the low GTPase Q61L mutants of Cdc42 or Rac so that stable [^{3}H]GTP complexes could be used.

Using Q61L Rac.[^{3}H]GTP, a SPA signal was readily obtained with GST-PAK_{70-132}, GST-PAK_{75-132}, GST-PAK_{70-118}, and GST-PAK_{75-118}, suggesting high-affinity binding of these four proteins (Figure 2). A weak signal was obtained with GST-PAK_{70-105}. No signal above background was obtained with GST-PAK_{75-105}, GST-PAK_{75-94}, or GST-PAK_{70-94}. The

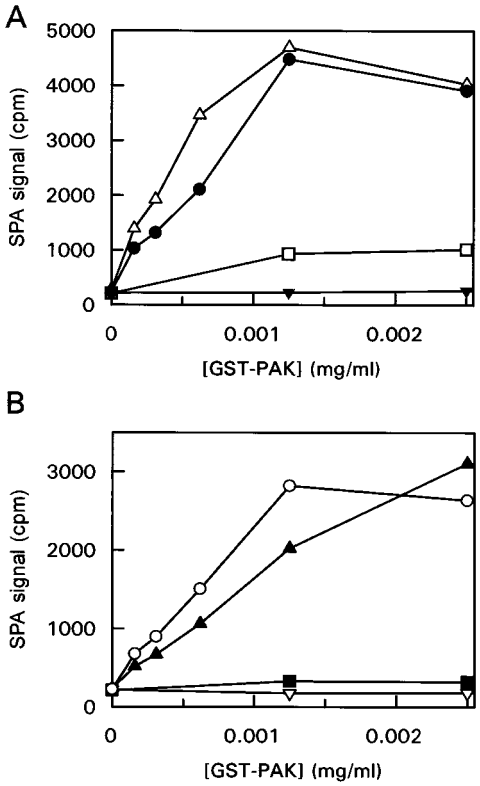

FIGURE 2: SPA showing binding of Q61L Rac.GTP to GST-PAK fragments. The indicated concentrations of GST-PAK were incubated with 0.026 μM Q61L Rac.[^{3}H]GTP, anti-GST, and protein A SPA beads as described under Materials and Methods. The GST-PAK proteins used were as follows: (A) PAK_{70-94} (▼), PAK_{70-105} (□), PAK_{70-118} (△), and PAK_{70-132} (●); (B) PAK_{75-94} (▽), PAK_{75-105} (■), PAK_{75-118} (▲), and PAK_{75-132} (○).

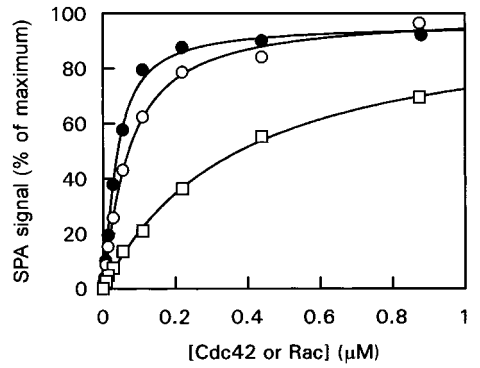

FIGURE 3: Measurement of affinity of Q61L Rac.GTP and Q61L Cdc42.GTP for GST-PAK fragments. The indicated concentrations of Q61L Rac.[^{3}H]GTP or of Q61L Cdc42.[^{3}H]GTP were incubated with 0.03 μM GST-PAK in SPAs. The SPA signal was corrected by subtraction of a blank in which GST-PAK was omitted. The effect of [Rac] or [Cdc42] on this corrected SPA cpm signal was fitted to a binding isotherm to give the apparent K_d and the signal at saturating concentration of Rac (or Cdc42). The data are expressed as a percentage of this maximum signal. The lines shown are the best fit, and the K_d values obtained from these and similar experiments are summarized in Table 1. The proteins used were Cdc42 + GST-PAK_{75-132} (●), Rac + GST-PAK_{75-132} (○), and Rac + GST-PAK_{70-105} (□).

affinities of the five GST-fusion proteins, which gave a measurable signal for Q61L Rac.GTP binding, were determined in SPAs in which the concentration of Rac was varied at a constant concentration of GST-fusion (Figure 3 and data not shown). The apparent K_d values obtained are summarized in Table 1. GST-PAK constructs, starting at residue 70 or 75 and ending at residue 132 or 118, all bound to Q61L Rac.GTP with similar high affinities ($K_d \sim 0.02-0.06$ μM). GST-PAK_{70-105} bound ~10-fold more weakly with a K_d of 0.37 μM.

Table 1: Affinities of PAK Fragments for Q61L Rac[a]

	K_d (μM)			K_d (μM)	
PAK	GST-fusion[b]	cleaved[c]	PAK	GST-fusion[b]	cleaved[c]
70−94	no signal[d]	≈100	75−94	no signal[d]	
70−105	0.37	0.7	75−105	no signal[d]	1.9
70−118	0.03	0.05	75−118	0.04	0.12
70−132	0.02		75−132	0.06	

[a] The apparent K_d values for binding of either GST-PAK or thrombin-cleaved PAK to Q61L Rac.GTP were measured using the SPA. [b] K_d estimated by varying the concentration of Q61L Rac.[^{3}H]GTP at a fixed concentration of the indicated GST-PAK fusion. [c] K_d estimated by using thrombin-cleaved PAK fragments as a competitive inhibitor of Q61L Rac.[^{3}H]GTP binding to GST-PAK_{75-132}. [d] No signal detected, suggesting $K_d \geq 2$ μM.

In similar experiments, SPA was used to measure the interaction between Q61L Cdc42.[^{3}H]GTP and GST-PAK_{75-132} (Figure 3). An apparent K_d of 0.02 μM was determined, showing that the affinities of Q61L Rac and Cdc42 for GST-PAK_{75-132} were similar.

To check whether the GST-tag had any effect on the interaction between Cdc42/Rac and PAK, the affinity of PAK regions purified after thrombin cleavage from the GST-fusions was measured. This was performed using SPAs in which the cleaved protein was used to compete with GST-PAK_{75-132} for binding to Q61L Rac.[^{3}H]GTP or Q61L Cdc42.[^{3}H]GTP (data not shown). The calculated apparent K_d values for interaction with Rac are summarized in Table 1. The apparent K_d values for interaction of Cdc42 with PAK_{70-118}, PAK_{70-105}, PAK_{70-94}, and PAK_{75-94} were 0.01, 0.07, ≈40, and ≈40 μM, respectively. Thus, the affinities for Cdc42/Rac are not greatly different from those estimated above using the GST-fusion proteins. Using this competition assay, the affinity of PAK fragments ending at residue 105 for either Cdc42 or Rac was lower than that for equivalent fragments ending at residue 118. The interaction between PAK_{75-105} and Q61L Rac.GTP was particularly weak, with K_d of 1.9 μM. This observation is consistent with the lack of signal with these proteins seen in Figure 2 and our previous experience that it is difficult to obtain a direct SPA signal with interactions of $K_d > 1$ μM. PAK_{70-94} and PAK_{75-94} showed only very weak binding to either Cdc42 or Rac ($K_d = 40-100$ μM), confirming that the CRIB motif alone is insufficient for high-affinity binding.

A similar competitive inhibition procedure was used to compare the affinities of wild-type and Q61L Rac and Cdc42 proteins and to examine how the nature of the bound nucleotide affected the interaction. The data obtained with GDP, GMPPNP, and GTP complexes of these proteins are summarized in Table 2. GTP and GMPPNP complexes with Q61L Rac bound equally well to GST-PAK_{75-132} with an apparent K_d of 0.09 μM, suggesting that GMPPNP serves as a good analogue of GTP. The Q61L Rac.GMPPNP bound 5-fold more strongly than the equivalent form of wild-type Rac, and the GMPNP form of wild-type Rac bound ~10-fold more strongly than the GDP form (Table 2). Similar experiments with Cdc42 as a competitive inhibitor of Rac binding to GST-PAK_{75-132} showed that GTP and GMPPNP

Table 2: Affinity of Different Nucleotide Complexes of Rac and Cdc42 for GST-PAK_{75-132}[a]

Rac/Cdc42 complex	K_d (μM)		Rac/Cdc42 complex	K_d (μM)	
	Rac	Cdc42		Rac	Cdc42
Q61L.GTP	0.09	0.03	WT.GMPPNP	0.5	0.2
Q61L.GMPPNP	0.09	0.02	WT.GDP	5.4	2.0
Q61L.GDP[b]	0.3	0.3			

[a] The apparent K_d values for binding of GST-PAK_{75-132} to Rac or Cdc42 were measured using the SPA. The indicated nucleotide forms of either wild-type or Q61L forms of Rac or Cdc42 were titrated as inhibitors into an SPA containing fixed concentrations of Q61L Rac.[^{3}H]GTP and GST-PAK_{75-132}. [b] The GDP complexes of Q61L Cdc42 or Rac were contaminated with ~1% of the GTP complex.

complexes of Q61L Cdc42 bound with equal affinities to GST-PAK_{75-132} and ~3-fold higher affinity than the equivalent Q61L Rac complexes. Similarly, the GDP and GMPPNP complexes with wild-type Cdc42 bound to GST-PAK_{75-132} with 2−3-fold higher affinity than did the Rac complexes. PAK_{70-105} and PAK_{70-118} competitively inhibited the binding of Q61L Cdc42.[^{3}H]GTP to GST-PAK_{75-132} with apparent K_d values of 0.07 and 0.01 μM, respectively. The equivalent K_d values with Q61L Rac were 0.7 and 0.05 μM, respectively (Table 1). Therefore, Q61L Cdc42.GTP binds with 5−10-fold higher affinity than does Q61L Rac.GTP to these shorter PAK fragments.

Kinetics of Dissociation of PAK from Rac and Cdc42. While establishing the experimental procedure for the PAK SPAs, we tested whether there was any time dependence in the assay. In contrast to Ras-based SPAs, upon addition of a competitive inhibitory protein to a Rac/PAK SPA the signal took several minutes to decrease to its final equilibrium level. As the time resolution of the SPA was not sufficient to quantitate the dissociation rate, we decided to use fluorescent mant-nucleotides to measure binding and dissociation of PAK.

Addition of GST-PAK_{70-132} to mant-GTP complexes with Q61L Cdc42 (or Rac) led to a saturable decrease in fluorescence (data not shown). At the concentrations of Cdc42 and Rac used, we could only estimate an upper limit for K_d of 0.1 μM with either protein. The kinetics of dissociation of GST-PAK from the PAK.Cdc42 (or Rac).mant-GTP complex were then followed by continuously monitoring the increase in fluorescence after addition of a large molar excess of Q61L Cdc42 (or Rac).GTP (Figure 4). The fluorescence changes fitted single exponentials with rate constants of 0.25 min^{-1} for Cdc42 and 0.66 min^{-1} for Rac.

Isothermal Titration Calorimetry. ITC is a technique that not only measures thermodynamic parameters for a binding reaction but also measures the apparent stoichiometry of reaction (*26*). If the stoichiometry is known, or can be assumed, as in the case of binding of PAK to Cdc42, one can estimate the concentration of active protein in a sample. Thus, we used ITC to assess whether the short PAK fragments comprised fully active protein and also to verify that the affinities of interaction estimated using the heterogeneous SPA bead system were similar to those in true solution.

We first used ITC to examine the interaction between Q61L Cdc42.GMPPNP and thrombin-cleaved PAK_{70-118}. The interaction was exothermic and could be fitted to a K_d of 0.15 μM (Figure 5A) and an apparent stoichiometry of

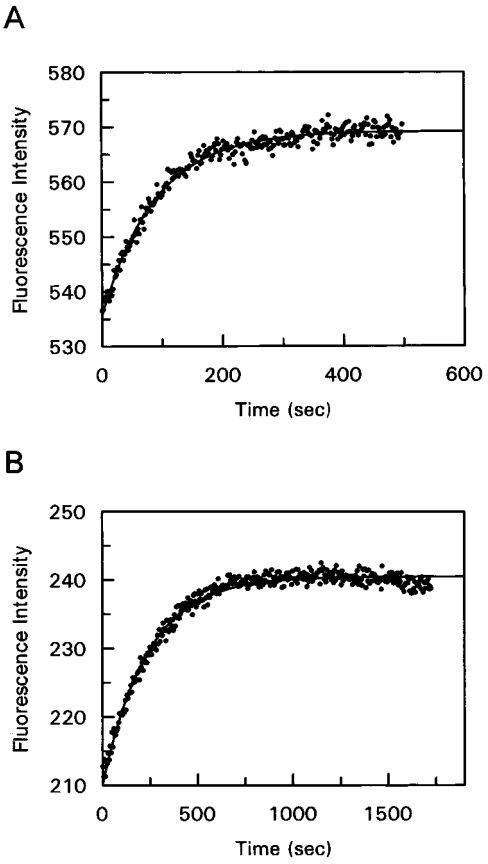

FIGURE 4: Dissociation kinetics of PAK from Rac and Cdc42. GST-PAK_{70-132} (0.1 μM) was added to a solution of either 0.2 μM Q61L Rac.mant-GTP (A) or 0.2 μM Q61L Cdc42.mant-GTP (B). A large excess of Q61L Rac.GTP (8 μM) was added to displace mant-GTP labeled protein from PAK and the increase in fluorescence intensity followed with time. The continuous lines are the best fits to an equation describing a first-order rate process.

interaction 0.97 mol of Cdc42/mol of GST-PAK. This suggested that the PAK preparation was fully active in binding Cdc42. For a rapid binding process, the width of the peaks obtained during a titration reflect the mixing and reaction time of the ITC instrument. With PAK_{70-118} the observed peaks (Figure 5B, trace 1) were a little broader than this (width at half-height = 0.4 min). However, with shorter PAK fragments the peak width became larger, so that with the shortest fragment examined PAK_{75-105} the heat change lasted over 10 min (width at half-height = 4min) (Figure 5B, trace 2). By allowing a long time between injections, calorimetric titrations were performed with PAK_{70-105} and PAK_{75-105}, although the broad peaks reduced the accuracy of the curve fitting. K_d values of about 2 and 2.6 μM were obtained, respectively.

DISCUSSION

PAK belongs to a larger family of proteins that bind to the Cdc42/Rac group of Ras-related proteins and also have the CRIB motif (*15*, *16*). There has been some assumption that the CRIB motif region might be sufficient for binding to Cdc42/Rac, and hence a primary aim of this study was to find the minimal stable region of PAK capable of high-affinity interaction with Cdc42/Rac.

Whereas GST-PAK_{61-150} was subject to heavy degradation during expression in *E. coli* (*27*), we found that PAK

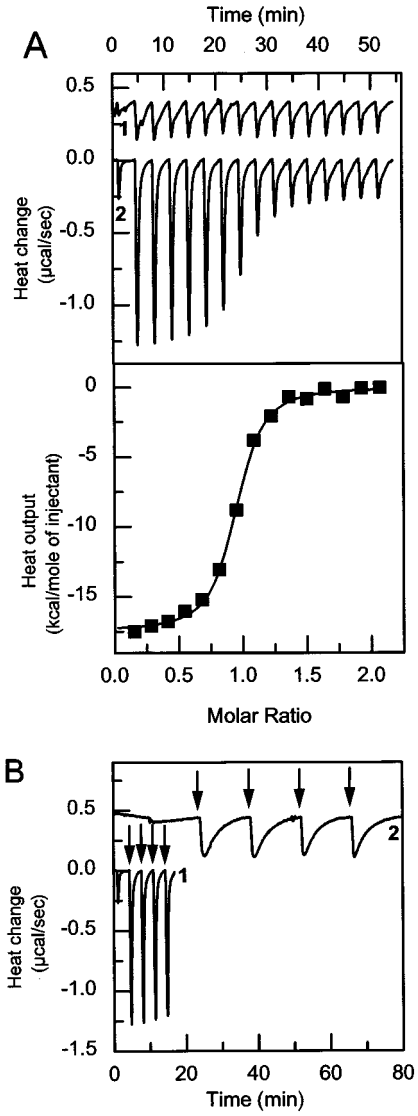

FIGURE 5: Isothermal titration calorimetry measurements of binding of Cdc42 to PAK. Q61L Cdc42.GMPPNP (0.18 mM) was titrated either into a solution of thrombin-cleaved PAK (10 μM) or into buffer, and heat changes were recorded continuously with time on a Microcal isothermal titration calorimeter as described under Materials and Methods. In panel A, upper graph, the raw data from injections into buffer (trace 1) or PAK_{70-118} (trace 2) are shown. The peaks were integrated to give the heat change associated with each injection and the buffer control subtracted from the experiment with PAK. The resultant data are plotted in panel A, lower graph, as molar ratio of Cdc42 injected to PAK against the heat output per mole of injected Cdc42. The line through the points is a least-squares fit to the data assuming a single-site binding model. In panel B, the raw data from injections into PAK_{70-118} (trace 1) or PAK_{75-105} (trace 2) are shown.

fragments starting at either residue 70 or 75 and ending at residue 94, 105, 118, or 132 can be expressed in *E. coli* as stable GST-fusion proteins. This is consistent with the shorter fragments containing a defined domain structure.

The data presented here show that on its own the CRIB region of PAK is insufficient to give high-affinity binding to Cdc42 and Rac, whereas fragments which are extended at the C terminus to residue 105, 118, or 132 bind both Rac and Cdc42. The inclusion of residues 70−74 gives no or, at most, a modest increase in affinity. Fragments terminating at residues 118 and 132 have similar affinities for Rac or Cdc42, whereas those terminating at residue 105 bind about an order of magnitude weaker. There was some evidence that PAK was susceptible to proteolytic cleavage in the region between residues 105 and 118, suggesting that the boundaries of the minimal stable Rac-binding domain are close to residues 75 and 105. Cdc42 bound with 3−10-fold higher affinity than Rac to any particular PAK fragment, with slight differences in the relative affinity depending upon the length of the PAK fragment. Manser et al. (*10*, *28*) have also reported that Cdc42 binds more tightly to full-length PAK than does Rac, although this was not quantified. Using an overlay assay, Martin et al. (*11*) reported a 3−4-fold higher affinity of full-length recombinant hPAK65 for Cdc42 as compared with Rac. Thus, the recombinant PAK fragments used in our study retain the binding specificity of full-length PAK.

The affinity we report here for Cdc42.GMPPNP ($K_d = 2$ μM) is similar to that reported by Leonard et al. (*17*) for Cdc42.mant-GMPPNP binding to a fragment of βPAK ($K_d \sim 1$ μM). We measured the affinity of the GDP forms of wild-type Cdc42 and Rac to be about an order of magnitude weaker than those of the GMPPNP forms. This difference is similar to that found by Leonard et al. (*17*) for the difference in affinity between PAK binding to Cdc42.mant-GDP and Cdc42.mant-GMPPNP. We also observed that the Q61L mutation in either Rac or Cdc42 confers an increase in affinity of ∼5−10-fold.

It has previously been reported that a 53 residue fragment of ACK (residues 499−551 of full-length ACK) (*29*) and a 34 residue fragment of WASP (residues 235−268) (*16*) are sufficient for Cdc42 binding. It can be seen from the sequence alignments in Figure 1 that the minimal region of PAK required for binding to Cdc42/Rac closely corresponds with the region of similarity between PAK, STE20, ACK, and WASP. These alignments also show that the most conserved region is longer than the CRIB motif defined by Burbelo et al. (*15*), and Symons et al. (*16*) have suggested that there is a longer consensus, which they termed GBD (Figure 1). Within the consensus motifs of the four proteins shown in Figure 1, there are only five invariant residues. Examination of the proteins cited by Burbelo et al. as binding Cdc42 and/or Rac shows that of these only two (corresponding to Ile-75 and His-83 of α-PAK) are invariant.

We have measured for the first time the kinetics of dissociation of PAK from Cdc42 and Rac. The rate constants obtained using PAK_{70-132} and mant-GTP complexes with the Q61L variants were 0.25 min^{-1} for Cdc42 and 0.66 min^{-1} for Rac. Although the affinity of interaction between PAK and Q61L Rac/Cdc42 is similar to that between Q61L Ras and effectors, the rates with PAK are several orders of magnitude slower than for Q61L Ras dissociating from Raf (180 min^{-1}; C. Gorman, personal communication) or NF1 [∼50 min^{-1} with Q61L Ras (*31*)]. This suggests the possibility that the association phase with PAK is also slow, which is consistent with the time-dependent heat changes seen in calorimetry (see below).

The ITC experiments confirm that the recombinant proteins are nearly fully active in terms of their protein−protein binding properties and also confirm the affinities measured using the SPA. Similarly, we have shown that the affinities for Ras binding to its effector proteins as measured using SPA are very similar to those values obtained using ITC (*30*). More interestingly, the calorimetry has revealed a slow

interaction process between Cdc42 and PAK, which was not seen with Ras interactions. Thus, with PAK_{75-105} the exotherm occurs over several minutes. It is interesting to note that NMR data suggest that these isolated PAK fragments have little or no secondary structure (Helen Mott, personal communication). We can envisage several hypotheses consistent with these observations: PAK might exist predominantly in an inactive conformation, or in many inactive conformations, that are kinetically slow to interconvert to the conformation competent to bind. Alternatively, PAK might have a binding determinant (say, the CRIB region) that allows a rapid weak interaction, followed by a slow conformational rearrangement to give high-affinity binding concomitant with an exotherm. We are currently studying the kinetics and structure of the PAK/Cdc42 complex to help to distinguish between these alternatives.

In summary, we have identified a 30 amino acid region of PAK, containing the CRIB motif, capable of binding Cdc42/Rac. However, the CRIB region itself is insufficient for Cdc42/Rac binding. In contrast to other small G protein effectors such as Raf, RalGDS, RasGAP, and RhoGAP, the Cdc42/Rac-interacting domain of PAK may not be fully structured in the absence of the small G protein.

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