Chp, a homologue of the GTPase Cdc42Hs, activates the JNK pathway and is implicated in reorganizing the actin cytoskeleton

Ami Aronheim*, Yehoshua C. Broder*, Aviva Cohen*, Alexandra Fritsch†, Barbara Belisle† and Arie Abo†

The p21-activated protein kinases (PAKs) are activated through direct interaction with the GTPases Rac and Cdc42Hs, which are implicated in the control of the mitogen-activated protein kinase (MAP kinase) c-Jun N-terminal kinase (JNK) and the reorganization of the actin cytoskeleton [1–3]. The exact role of the PAK proteins in these signaling pathways is not entirely clear. To elucidate the biological function of Pak2 and to identify its molecular targets, we used a novel two-hybrid system, the Ras recruitment system (RRS), that aims to detect protein–protein interactions at the inner surface of the plasma membrane (described in the accompanying paper by Broder *et al.* [4]). The Pak2 regulatory domain (PakR) was fused at the carboxyl terminus of a RasL61 mutant protein and screened against a myristoylated rat pituitary cDNA library. Four clones were identified that interact specifically with PakR and three were subsequently shown to encode a previously unknown homologue of the GTPase Cdc42Hs. This ~36 kDa protein, designated Chp, exhibits an overall sequence identity to Cdc42Hs of ~52%. Chp contains two additional sequences at the amino and carboxyl termini that are not found in any known GTPase. The amino terminus contains a polyproline sequence, typically found in Src homology 3 (SH3)-binding domains, and the carboxyl terminus appears to be important for Pak2 binding. Results from the microinjection of Chp into cells implicated Chp in the induction of lamellipodia and showed that Chp activates the JNK MAP kinase cascade.

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Results and discussion

Isolation of a Cdc42Hs homologue that interacts specifically with Pak65

To identify proteins that interact with Pak65, we used a modified form of the Sos recruitment system (SRS) [4,5]. The Pak2 regulatory domain (PakR) was fused to the carboxyl terminus of RasL61 devoid of its membrane attachment signals (Ras(L61)ΔF–PakR) and used to cotransform *cdc25-2* cells with a pituitary cDNA library containing the v-Src myristoylation sequence as previously described [5]. Five galactose-dependent clones were isolated and the specificity of the interaction examined. Clones #1, #2, #5 and #6 were able to grow at 36°C when cotransformed with Ras(L61)ΔF–PakR bait but not with the nonspecific bait Ras(61)ΔF–JDP2, suggesting that the interaction is dependent on the PakR bait (Figure 1a). In contrast, clone #8 grew at 36°C when cotransformed with either bait and was subsequently shown to encode a Sos homologue — Sos is an acknowledged false positive in this assay [4] because when targeted to the membrane it is able to activate the yeast Ras by itself and confer growth at the restrictive temperature. Clones #1, #2 and #5 were identical and exhibited sequence homology to the GTPases Cdc42Hs and Rac1 (Figure 1b). As this cDNA appeared independently three times in our screen, we focused further analysis on this clone; the characterization of clone #6 will be described elsewhere.

DNA from clones #1, #2 and #5 was predicted to encode a ~36 kDa protein, designated Chp (for Cdc42Hs homologue protein), with ~52% amino acid sequence identity to Cdc42Hs. In addition to the homology to Cdc42Hs, Chp contains sequences at the amino and carboxyl termini that are not related to Cdc42Hs. An additional 28 amino acids were found at the amino terminus of Chp that are absent in Cdc42Hs, containing polyproline sequences typical of SH3-binding regions. The carboxyl terminus of Chp does not contain the classical CAAX box that is conserved among all Ras-related GTPases [6]. Instead, Chp contains an additional 32 amino acid sequence that does not share sequence homology to known proteins in the database.

Chp transcription

To determine the tissue distribution of Chp, we used a specific probe corresponding to the GTPase domain of Chp (amino acids 102–151) in northern blot analysis of mRNA from multiple rat tissues. This region exhibits only 62% identity with other members of the GTPase family. A single mRNA species of about 1.5 kb was detected at high

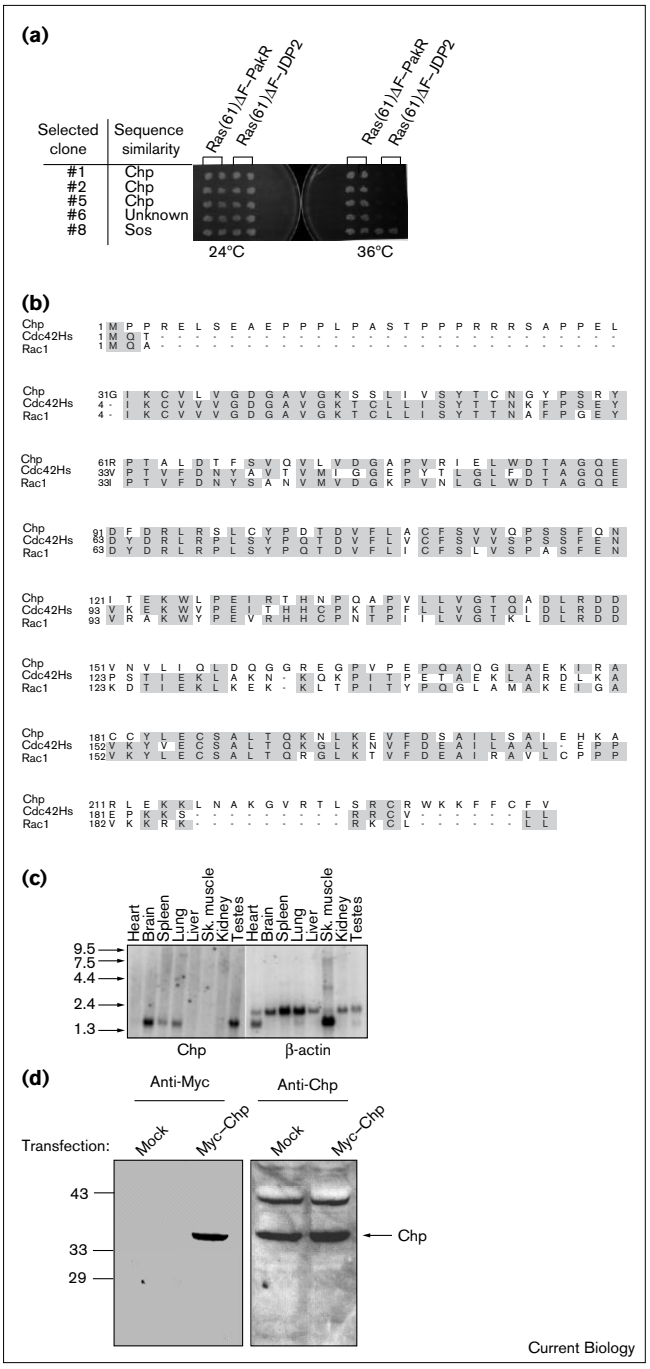


Figure 1

(a) Isolation using the RRS system of a novel protein that interacts with the Pak2 regulatory domain. Ras(61)ΔF–PakR bait was used to screen a rat pituitary cDNA library in a temperature-sensitive *cdc25* yeast strain. Five clones grew efficiently at 36°C only on a medium containing galactose (the conditions under which library expression was induced). Plasmid DNAs were isolated and re-introduced into the same yeast strain with either the specific bait Ras(61)ΔF–PakR or nonspecific bait Ras(61)ΔF–JDP2. Four of the clones – #1, #2, #5 and #6 – grew at 36°C with only the specific bait. **(b)** Amino acid sequence alignment of Chp, Cdc42Hs and Rac1; Cdc42Hs and Rac1 sequences were retrieved from the NCBI *Entrez* database (accession numbers P25763 and 131807 respectively) and the sequences were aligned with Chp using the Laser Gene program. Grey shading indicates residues that are identical in at least two members of the group. **(c)** Tissue distribution of Chp. A northern blot (Clontech Inc.) of RNA from multiple tissues was probed with either the Chp GTPase domain or β-actin DNA fragments. Probing and washing were performed according to the manufacturer's protocols as described in the Supplementary material. The washed filter was exposed to autoradiography for 24 hr. **(d)** Western blot analysis with transfected and nontransfected (mock) cell lines. Chp was transfected into 293 cells with a plasmid encoding Myc-epitope-tagged Chp (Myc–Chp). Whole-cell extracts (50 μg/lane) from transfected and nontransfected cells were separated by SDS–PAGE and blotted onto nitrocellulose. Filters were probed with either anti-Myc or affinity-purified Chp antisera. Size markers in kb (c) or kDa (d) are shown to the left.

levels in brain and testes; lower amounts were also detected in spleen and lung tissues (Figure 1c). Interestingly, small mRNAs (1.0 kb and 1.4 kb) have been reported for other Rac-related proteins [6]. Hybridization with probes corresponding to the amino and carboxyl termini as well as full-length Chp gave similar results (data not shown).

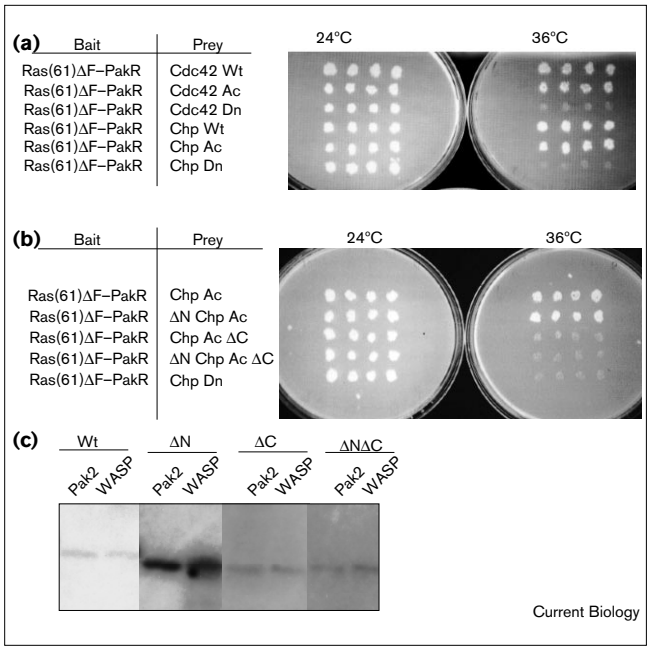
Chp expression

For protein analyses of Chp, polyclonal antibodies were generated against a peptide corresponding to a unique region at the carboxyl terminus of Chp. To compare the size of endogenous Chp with that encoded by the cloned cDNA, we fused the Chp cDNA to a sequence encoding a Myc epitope tag and used this plasmid to overexpress Myc–Chp in 293 cells. Whole-cell extracts derived from either transfected or nontransfected 293 cells were separated on SDS–PAGE and subjected to western blotting with anti-Myc antibodies (Figure 1d, left panel). The nitrocellulose filter was subsequently incubated with affinity-purified anti-Chp antibodies (Figure 1d, right panel) to show that the transfected Chp was identical in size to the protein detected in nontransfected 293 cells using the anti-Chp antibody.

Pak65 interacts with the activated Chp mutant

PakR interacts with the activated forms of Rac and Cdc42 [7–10]. On the basis of its sequence homology to Cdc42 and other Rho GTPases, we generated various Chp mutants that are expected to differ in their GDP/GTP-bound state. To make a constitutively active Chp, we substituted glycine 40 with valine (G40V). The equivalent substitution in Rac and Cdc42Hs (G12V) results in an active allele that is defective in GTPase hydrolysis. In addition, we substituted amino acid 45 with asparagine. Substitution of the corresponding amino acid (at position 17) to aspargine in other Rho GTPases results in a dominant-negative allele that is locked in the GDP-bound inactive form [11]. A *cdc25-2* yeast strain was cotransfected with plasmids encoding different Chp mutants fused to myristoylation sequences together with Ras(61)ΔF–PakR bait. As presented in Figure 2a, constitutively active Chp (Chp Ac) and wild-type Chp, but not the dominant-negative form of Chp (Chp Dn), interacted with PakR.

Figure 2



Interaction of Pak65 with different Cdc42 and Chp mutants. Yeast temperature-sensitive *cdc25-2* cells were transformed with the indicated plasmids and plated on glucose minimal medium supplemented with the appropriate amino acids and bases. Four independent transformants were grown at the permissive temperature, 24°C, replica plated onto appropriately supplemented galactose minimal plates and grown at the restrictive temperature, 36°C. Constructs used are described in the Supplementary material. **(a)** PakR interacts with wild-type (Wt) Cdc42 and Chp and constitutively activated (Ac) Cdc42-L61 and Chp-V40 but not dominant-negative (Dn) Cdc42-N17 and Chp-N45. **(b)** PakR interaction with Chp deletion mutants. Plasmids encoding dominant-negative (Dn) Chp and activated (Ac) Chp or Chp Ac mutants lacking either the amino terminus (ΔN) or carboxyl terminus (ΔC), or both, were cotransfected into *cdc25-2* cells together with Ras(61)ΔF–PakR. **(c)** Recombinant Pak2 and WASP (2–3 μg) were analyzed by the overlay assay and probed with the indicated Chp mutants preloaded with [γ-^{32}P]GTP, as described in the Supplementary material.

Similarly, constitutively active and wild-type Cdc42Hs, but not the dominant-negative form, interact with PakR. The expression levels of the different mutants were similar (data not shown). These data suggest that the specific interaction between Chp and PakR is dependent on the activation state of the GTPase.

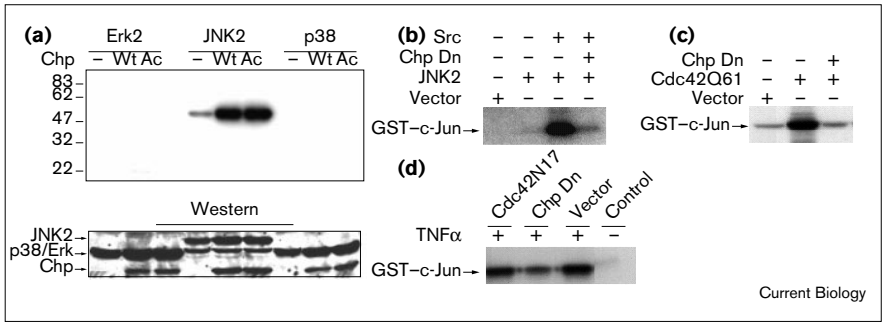
The role of the Chp carboxy-terminal region for Pak65 binding

To investigate the role of the Chp amino-terminal and carboxy-terminal sequences, we further mutated Chp Ac by deletion of the carboxyl or amino terminus, or both. Chp devoid of its carboxy-terminal domain was unable to bind PakR in the two-hybrid system, whereas deletion of the amino terminus had no significant effect on PakR binding (Figure 2b). A similar result was obtained using an overlay assay. Recombinant wild-type Chp was not efficiently loaded with [γ-^{32}P]GTP (data not shown) and as a result the interaction with Pak2 or the Wiskott–Aldrich syndrome protein (WASP) could not be detected. In contrast, a ChpΔN mutant that was efficiently loaded with [γ-^{32}P]GTP exhibited strong interaction with Pak2 and WASP (Figure 2c). Further, deletion of the carboxyl terminus abrograted this interaction with Pak2 and WASP (Figure 2c) despite normal binding to [γ-^{32}P]GTP (data not shown). This result suggests that the extended carboxyl terminus is either involved directly in binding PakR or the loss of this domain results in a conformational change in Chp that prevents PakR binding.

Chp selectively activates the JNK MAP kinase pathway

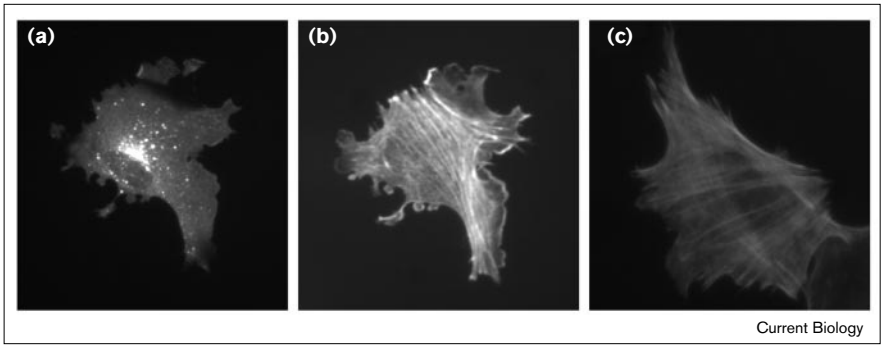
To test whether Chp has a similar effect to Rac and Cdc42 [11,12] on the JNK cascade, we cotransfected 293 cells with various mutants of Chp together with haemagglutinin (HA)-tagged JNK2, extracellular-signal-regulated kinase 2 (Erk2) or p38. Both wild-type and activated forms of Chp stimulated the JNK kinase (Figure 3a). In contrast, the dominant-negative form of Chp did not activate the

Figure 3



(a) Activation of JNK by Chp. 293 cells were cotransfected with plasmids encoding HA-tagged Erk2, JNK2 or p38 together with either an empty expression vector (–) or Myc-tagged wild-type (Wt) or activated (Ac) Chp. Cell extracts were used in an *in vitro* kinase assay (upper panel) using the appropriate substrate (myelin basic protein, GST–Jun and GST–ATF2 respectively). Western blot analysis (lower panel) with anti-HA and anti-Myc antisera was performed to evaluate the expression of the transfected kinases and different Chp proteins. **(b–d)** Dominant-negative (Dn) Chp blocks the activation of JNK by Src, Cdc42Q61 and TNFα. HeLa cells were cotransfected with plasmids encoding Myc-tagged Chp Dn, HA-tagged JNK2 and either Src or Cdc42Q61 as indicated. To measure the effect of TNFα, transfected cells were incubated for 15 min with TNFα, lysed and subjected to the *in vitro* kinase assay (as above).

Figure 4



The activated Chp mutant reorganizes the cortical actin. Porcine endothelial cells were microinjected with **(a,b)** a Myc-tagged Chp expression vector or **(c)** a vector control (50 ng/μl plasmid), fixed 4–6 hr after injection and subsequently visualized by immunofluorescence microscopy. Chp localization in (a) was detected by staining with FITC-tagged Myc antibody. Polymerized actin was visualized in (b,c) by staining with Texas-red-conjugated phalloidin. Reorganization of the actin cytoskeleton can be seen in (b) compared with (c).

JNK pathway in HeLa cells (data not shown) and blocked the activation of JNK by Src and by Cdc42Q61 (Figure 3b,c). A reduction in JNK activation by tumour necrosis factor-α (TNFα) was also detected in cells transfected with dominant-negative Chp (Figure 3d). As described for Cdc42 and Rac GTPases [12,13], Chp did not significantly activate Erk2 and failed to induce the activation of the p38 kinase as well. These data suggest that Chp, in contrast to Rac and Cdc42Hs, is a selective activator of the JNK MAP kinase cascade but not of the Erk or p38 pathways.

Chp is implicated in the induction of lamellipodia

To test whether Chp has a role in the control of the actin cytoskeleton, we microinjected plasmids encoding activated Chp and stained for either Chp expression or polymerized actin. Chp was concentrated on one side of the nucleus at the Golgi apparatus and colocalized with the coatomer protein βCOP at the Golgi in the brefeldin-A-sensitive compartment (data not shown). In addition, injected cells exhibited morphological changes (Figure 4) demonstrated by induction of lamellipodia. Interestingly, the Chp mutant in which the carboxyl terminus had been deleted failed to induce lamellipodia and Chp was not localized to the Golgi (data not shown). These data further support the role of the Chp carboxyl terminus in the localization of Chp to the Golgi and in the induction of lamellipodia, possibly through interaction with Pak2.

The data presented in this paper describe Chp as a new Rho GTPase that interacts with Pak2, that is implicated in the reorganization of the actin cytoskeleton and that activates the JNK pathway. Among the novel features of Chp are the unique amino and carboxyl termini, which are not found on any other known Rho-related GTPases and are implicated in localizing Chp and the interaction of Chp with Pak.

Supplementary material

Additional methodological details are published with this article on the internet.

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References

1. Hall A: **Rho GTPases and the actin cytoskeleton.** *Science* 1998, **279:**509-514.
2. Van Aelst L, D'Souza-Schorey C: **Rho GTPases and signaling networks.** *Genes Dev* 1997, **11:**2295-2322.
3. Nobes CD, Hall A**: Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia.** *Cell* 1995, **81:**53-62.
4. Broder YC, Katz S, Aronheim A: **The Ras recruitment system, a novel approach to the study of protein–protein interactions.** *Curr Biol* 1998, **8:**1121-1124.
5. Aronheim A, Zandi E, Hennemann H, Elledge S, Karin M: **Isolation of an AP-1 repressor by a novel method for detecting protein-protein interactions.** *Mol Cell Biol* 1997, **17:**3094-3102.
6. Didsbury J, Weber RF, Bokoch GM, Evans T, Snyderman R: **rac, a novel ras-related family of proteins that are botulinum toxin substrates.** *J Biol Chem* 1989**, 264:**16378-163826.
7. Martin GA, Bollag G, McCormick F, Abo A: **A novel serine kinase activated by rac1/CDC42Hs-dependent autophosphorylation is related to PAK65 and STE20.** *EMBO J* 1995, **14:**1970-1978.
8. Bagrodia S, Derijard B, Davis RJ, Cerione RA: **Cdc42 and PAK-mediated signaling leads to Jun kinase and p38 mitogen-activated protein kinase activation.** *J Biol Chem* 1995, **270:**27995-27998.
9. Sells MA, Chernof J: **Emerging from the Pak: the p21-activated protein kinase family.** *Trends Cell Biol* 1997, **7:**162-167.
10. Manser E, Huang HY, Loo TH, Chen XQ, Dong JM, Leung T, Lim L: **Expression of constitutively active alpha-PAK reveals effects of the kinase on actin and focal complexes**. *Mol Cell Biol* 1997, **17:**1129-1143.
11. Marshall CJ: **Ras effectors.** *Curr Opin Cell Biol* 1996 **8:**197-204.
12. Minden A, Lin A, Claret FX, Abo A, Karin M: **Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs.** *Cell* 1995, **81:**1147-1157.
13. Coso OA, Chiariello M, Yu JC, Teramoto H, Crespo P, Xu N, *et al.*: **The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway.** *Cell* 1995, **81:**1137-1146.

Supplementary material

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Materials and methods

Plasmids and constructs

For Chp expression vectors, mammalian expression vectors were generated by fusing Chp cDNA in frame with cDNA encoding the Myc epitope tag in the pCan mammalian expression vector. Yeast expression vectors were generated by fusion of Chp cDNA in frame with cDNA encoding the Src myristoylation signal in the pYes2 expression vector (Invitrogen Inc.). Plasmids encoding different Cdc42 mutants were subcloned into the pYes2 expression vector using standard procedures. Chp mutagenesis was performed using the Chameleon mutagenesis kit (Stratagene Inc.) according to the manufacturer's instructions.

Northern blot analysis

Northern blots were purchased from Clontech Inc. and performed as described in the manufacturer's protocols. The specific probes were generated by random primer labelling kit (Stratagene Inc.). Probes were incubated with filters for 2 hr at 68°C. The filters were washed three times with 2 × SSC containing 0.1% SDS at room temperature followed by two washes with 0.1 × SSC containing 0.1% SDS at 55°C.

Western blot analysis

Protein extracts (50 μg/lane) were resolved on an SDS–12.5% polyacrylamide gel, blotted onto nitrocellulose membrane, and probed with anti-Myc antibodies or affinity-purified Chp antibodies. Anti-mouse antibody or protein A conjugated to horseradish peroxidase were used in a secondary incubation. The nitrocellulose blots were developed using the SuperSignal chemiluminescent reaction kit (Promega Inc.).

Chp antibody was generated against the carboxyl terminus peptide 216-KLNAKGVRTLSRCRWKK-232 conjugated to Keyhole-Limpet haemocyanin (KLH). Antibody purification was carried out by coupling 10 mg of the peptide to 10 ml of agarose gel. The gel was equilibrated with PBS and the crude serum was applied to the gel, washed and antibody was eluted by glycine buffer pH 2.0.

Yeast manipulations

Conventional yeast transfection and manipulation protocols were used as described [4,5].

Transfection and in vitro *kinase assays*

293 cells were transfected using the $CaPO_4$ precipitation procedure. Cells were cotransfected with pCan expression plasmids driving the expression of different Chp mutants (5 μg) and Srα expression vectors driving the expression of the different HA-tagged kinases (5 μg). 48 hr following transfection, protein was extracted and *in vitro* kinase assays were performed as described [7]. For HA–Erk2, HA–JNK2 and HA–p38, the following purified substrates (5 μg/assay) were used respectively: myelin basic protein (MBP), GST–c-Jun 1–79, and GST–ATF2Δ.

To study the effect of Chp on the activation of the JNK pathway by Src and Cdc42, we transfected HeLa cells with Chp Dn together with either Src or the activated form of Cdc42Q61 and HA-tagged JNK2. To study the effect of Chp Dn on JNK activation by TNFα, transfected cells were treated for 15 min with 10 ng TNFα/ml of media followed by *in vitro* kinase assay as described above.

Microinjections and immunofluorescence microscopy

Porcine endothelial (PAE) cells grown in DMEM medium containing 10% fetal bovine serum were plated on coverslips. Expression vectors encoding Myc-tagged Chp proteins were diluted to a concentration of 50 ng/μl in injection buffer (5 mM glutamate, 130 mM KCl) and microinjected into the nucleus of approximately 100 subconfluent PAE cells. Injected cells were incubated for 4–6 hr at 37°C. Cells were fixed in 4% formaldehyde in PBS for 10 min and permeabilized by washing in PBS containing 0.1% Triton X-100. For localization studies, cells were cotreated with anti-Myc monoclonal antibody and rabbit anti-βCOP (a gift from Richard Khan) for 45 min. The coverslips were then washed with PBS-TX and cotreated with FITC-conjugated donkey anti-mouse and Texas-red-conjugated donkey anti-rabbit for 30 min. For morphology studies, cells were treated with a primary anti-Myc monoclonal antibody and FITC donkey anti-mouse secondary antibody. Coverslips were then washed in PBS-TX and treated for 10 min with Texas-red-conjugated phalloidin. Fluorescence microscopy was carried out using a Zeiss Axiophot with appropriate filters for fluorescence detection.

Overlay assay

Recombinant proteins (Pak2 and WASP) were separated on SDS–PAGE followed by blotting to a polyvinylidene fluoride (PVDF) membrane, washing, and blocking with PBS containing 1% BSA and 100 mM DTT. Recombinant Chp (2–5 μg) was preloaded with radiolabelled [γ-P^{32}]GTP and incubated for 5–8 min with the PVDF filter. The filter was washed for 5 min and exposed to film for 2 hr. Recombinant proteins (Chp mutants, Pak2 and WASP) were expressed as a Glu–Glu-tagged protein and purified from Sf9 cells as previously described [7].