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Autophosphorylation-dependent degradation of Pak1, triggered by the Rho-family GTPase, Chp

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The Paks (p21-activated kinases) Pak1, Pak2 and Pak3 are among the most studied effectors of the Rho-family GTPases, Rac, Cdc42 (cell division cycle 42) and Chp (Cdc42 homologous protein). Pak kinases influence a variety of cellular functions, but the process of Pak down-regulation, following activation, is poorly understood. In the present study, we describe for the first time a negative-inhibitory loop generated by the small Rho-GTPases Cdc42 and Chp, resulting in Pak1 inhibition. Upon overexpression of Chp, we unexpectedly observed a T-cell migration phenotype consistent with Paks inhibition. In line with this observation, overexpression of either Chp or Cdc42 caused a marked reduction in the level of Pak1 protein in a number of different cell lines. Chp-induced degradation was accompanied by ubiquitination of Pak1, and was dependent on the proteasome. The susceptibility of Pak1 to Chp-induced degradation depended on its p21-binding domain, kinase activity and a number of Pak1 autophosphorylation sites, whereas the PIX- (Pak-interacting exchange factor) and Nck-binding sites were not required. Together, these results implicate Chp-induced kinase autophosphorylation in the degradation of Pak1. The N-terminal domain of Chp was found to be required for Chp-induced degradation, although not for Pak1 activation, suggesting that Chp provides a second function, distinct from kinase activation, to trigger Pak degradation. Collectively, our results demonstrate a novel mechanism of signal termination mediated by the Rho-family GTPases Chp and Cdc42, which results in ubiquitin-mediated degradation of one of their direct effectors, Pak1.

Key words: Cdc42 (cell division cycle 42) homologous protein (Chp), GTPase-binding domain, p21-activated kinase (Pak), proteasome, protein stability, Rho-GTPase.

INTRODUCTION

The Paks (p21-activated kinases) are evolutionarily conserved serine/threonine protein kinases that interact specifically with the Rho-family GTPases Cdc42 (cell division cycle 42), Rac and the recently described Cdc42 homologous protein, Chp [1–3]. The Pak proteins consist of two major subgroups. Group I includes three major isoforms, which are activated upon binding to Rho-family GTPases: Pak1 (rat αPak), Pak2 (rat γPak) and Pak3 (rat βPak) [3,4]. Group II consists of Paks 4, 5 and 6, which differ significantly from the group I kinases in their mode of regulation, localization and binding partners [3,4]. In particular, binding to Rho-family GTPases does not enhance their kinase activity, but results in their translocation to the Golgi apparatus [5].

Group I Paks are composed of a C-terminal catalytic domain and an N-terminal regulatory domain, through which Pak interacts with numerous proteins. A number of proline-rich motifs mediate the binding to SH3 (Src homology 3)-containing proteins, including Nck [6], Grb2 (growth-factor-receptor-bound protein 2) [7] and the Pak-interacting exchange factor, PIX [8]. A PBD (p21-binding domain) binds to the activated form of the Rac, Cdc42, TC10 and Chp GTPases [9]. A KI (kinase inhibitory) domain partially overlaps with the PBD. The KI domain binds *in trans* to the C-terminal catalytic domain, thereby inhibiting Pak catalytic activity [10]. Structural data and biochemical studies suggest that binding to Rho-family GTPases causes a conformational change in the KI domain that disrupts its interaction with the catalytic domain [11], followed by Pak autophosphorylation at multiple sites and potentiation of its kinase activity towards specific substrates (summarized in [12]), including LIMK (LIM domain kinase) [13], myosin regulatory light chain [14], MLCK (myosin light-chain kinase) [13] and stathmin [15].

Several studies have addressed the role of autophosphorylation in the activation of Pak1 [16] and Pak2 [17]. Six sites are conserved between Pak1 and Pak2. Full activation of Pak depends on a conserved threonine residue (Pak2-Thr^{402} and Pak1-Thr^{423}) in the activation loop of the catalytic domain as well as phosphorylation of two conserved serine residues (Pak1-Ser^{144}/Ser^{149}) within the KI domain [3]. The precise role of the other autophosphorylation sites is largely unknown.

Pak proteins participate in regulating the actin cytoskeleton, focal adhesion contacts and cell motility, and are implicated in other cellular functions, such as activation of MAPK (mitogen-activated protein kinase) pathways, apoptotic/survival signalling, cellular transformation and HIV pathogenesis [2,18]. Recently, we showed that expression of a Pak inhibitor (Pak^{i}), comprising part of the regulatory domain of Pak1, results in inhibition of SDF-1α (stromal-cell-derived factor-1α)-induced T-cell chemotaxis through restrictive barriers, such that migration through large pores is unaffected, but migration through small pores is inhibited [19]. Pak^{i}-mediated inhibition was partially dependent on the presence of a PIX-binding domain. In contrast, inhibition of the upstream Pak activators, Cdc42 and Rac, resulted in pore-size-independent inhibition of T-cell migration. On the basis of

Abbreviations used: Cdc42, cell division cycle 42; Chp, Cdc42 homologous protein; DTT, dithiothreitol; EGFP, enhanced green fluorescent protein; ERK, extracellular-signal-regulated kinase; HA, haemagglutinin; HEK-293, human embryonic kidney; Hrs, HGF (hepatocyte growth factor)-regulated tyrosine kinase substrate; KI, kinase inhibitory; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; NP-40, Nonidet P40; Pak, p21-activated kinase; Pak^{i}, Pak inhibitor; PBD, p21-binding domain; PI, protease inhibitor; PIX, Pak-interacting exchange factor; POSH, plenty of SH3s; SDF-1α, stromal-cell derived factor-1α; SH3, Src homology 3 domain; tTA, tet-transactivator; WCE, whole-cell extract.

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these results, we suggested that a pathway specifically required for migration through restrictive pores diverges from the general migration pathway at the level of the Rho-family GTPases. Since a direct sole activator of the Pak pathway was not identified, we examined the role of Chp in SDF-1α-induced T-cell migration.

Chp differs from Cdc42 and Rac in having an extended N-terminal domain that is rich in proline residues. Deletion of this domain results in enhanced Pak activation [20]. In addition, Chp lacks a classical CAAX box motif found at the C-terminal end of all other Rho-family GTPases. Instead, Chp contains a unique lipid-modification domain resulting in its localization to the plasma membrane and endomembrane [21]. We now present evidence for a negative-feedback loop initiated by the small Rho-family GTPase, Chp, leading to proteasomal degradation of Pak1.

MATERIALS AND METHODS

Antibodies and reagents

RPE (R-phycoerythrin)-conjugated monoclonal anti-H-2K^k was purchased from Serotec. Anti-HA (haemagglutinin) monoclonal antibody (12CA5) and anti-Myc monoclonal antibody (9E11) were from Babco Inc., anti-α-tubulin and anti-FLAG antibodies were from Sigma–Aldrich and anti-ERK (extracellular-signal-regulated kinase) was from Cell Signaling Technology. Anti-Pak1 antibody has been described previously [19]. SDF-1α was purchased from R&D systems. Protein A–agarose beads and lactacystin were from Sigma–Aldrich. MG132 was purchased from Calbiochem.

Plasmids

Myc-tagged wild-type activated (G40V) and dominant-negative (S45N) Chp were cloned by blunt ligation into the PvuII site of the tetracycline-regulated expression plasmid, pBI-EGFP (Clontech), a bidirectional mammalian expression plasmid co-expressing EGFP (enhanced green fluorescent protein) and the gene of interest. To enable purification of transfected cells, the inserts were also cloned into a derivative of this plasmid, in which EGFP was replaced with a truncated form of the mouse MHC allele, H-2K^k, as described in [19]. For clarity, in the Figure legends, co-expression of a gene 'x' with H-2K^k is designated as x//H-2K^k. Wild-type and activated forms of Chp, Cdc42 (Cdc42 Q61L) and Rac1 (Rac1 Q61L) were cloned into the pcDNA-based (Invitrogen) expression vector, pCAN, in-frame with an N-terminal Myc tag [1]. Chp N-terminal deletion (ΔN), C-terminal deletion (ΔC) and both (ΔNΔC) mutants were as described previously [1]. pEF-BOS-based expression plasmids encoding HA-tagged human Pak1 or the mutant forms, P13A, K299A, H83L/H86L and P192G/R193A, and the regulatory fragment, PakR, have been described previously [22,23]. Mutant forms of HA-tagged rat Pak1, including L107F and the autophosphorylation site mutants S144A/S149A, T422A and S198A/S203A, cloned into the pXJ expression vector, were as described previously [16,24].

Cell culture and transfection

Jurkat- and HEK-293 (human embryonic kidney)-derived cell lines, stably transfected with the tet-transactivator (tTA) were used for expressing the pBI-EGFP- and H-2K^k-based plasmids, described above. Jurkat tTA (previously referred to as Jurkat VP/tetR) were grown and transfected as described in [19], whereas HEK-293 tTA (Clontech) was transfected by calcium phosphate precipitation, and were trypsinized 48 h later and used for purification. Transfected Jurkat tTA or HEK-293 tTA cells were purified using anti-H-2K^k antibodies and magnetic beads, as described in [19]. Jurkat TAg cells and HEK-293T cells are stably transfected with SV40 (simian virus 40) large T-antigen [25]. HEK-293T and NIH-3T3 cells were grown in DMEM (Dulbecco's modified Eagle's medium) with 4.5 g/ml D-glucose, supplemented with 10 % fetal calf serum and penicillin/streptomycin. Cells were plated on 60-mm-diameter plates (3×10^5 cells/dish), and the following day were transfected by conventional calcium phosphate precipitation (HEK-293T) or X-tremeGene reagent (Roche) (NIH-3T3) according to the manufacturer's instructions.

Cell extraction methods

WCE (whole-cell extract) buffer consists of 25 mM Hepes, pH 7.7, containing 0.3 M NaCl, 20 mM 2-glycerophosphate, 0.5 mM DTT (dithiothreitol), 0.1 % Triton X-100, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 0.1 mM Na_3VO_4 and a PI (protease inhibitor) mixture containing 2 μg/ml leupeptin, 100 μg/ml PMSF, 2 μg/ml aprotinin and 5 μg/ml pepstatin A. Cell lysates were rotated for 30 min at 4 °C, followed by a 10 min centrifugation at 16000 ***g*** in a refrigerated microcentrifuge.

Jurkat lysis buffer consisted of 50 mM Tris/HCl, pH 7.5, containing 1 % NP-40 (Nonidet P40), 150 mM NaCl, 50 mM NaF, 2 mM Na_3VO_4, 50 mM 2-glycerophosphate, 20 mM sodium pyrophosphate, 0.5 mM EDTA, 2 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 μg/ml pepstatin A.

RIPA buffer consisted of PBS containing 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % SDS and PI mixture.

Western blot analysis

Protein lysate (30 μg) was prepared with WCE buffer, unless indicated otherwise, and separated by SDS/10 % PAGE followed by immobilization on nitrocellulose membranes (Hybond; Amersham Biosciences). The membranes were incubated in PBS containing 5 % non-fat dried milk powder and probed with primary antibodies, followed by three washes in PBS. Primary antibody was detected using the corresponding horseradish-peroxidase-conjugated secondary antibodies (Sigma–Aldrich).

Pak kinase assay

In vitro kinase assay was performed according to the method described in [26]. Briefly, anti-HA immune complexes prepared from 100 μg of WCE were washed twice with WCE buffer then washed with buffer containing 25 mM Hepes, pH 7.5, 0.2 % Triton X-100 and 1 mM EDTA. Kinase reactions were initiated by resuspending beads in 27 μl of 2× kinase buffer (50 mM Hepes, pH 7.5, 40 mM *p*-nitrophenylphosphate, 40 mM $MgCl_2$, 40 mM 2-glycerophosphate, 1 mM Na_3VO_4 and 4 mM DTT) containing 10 μg of MBP (myelin basic protein), 5 μCi of [γ-^{32}P]ATP and 22 μM ATP. Following incubation for 30 min at 30 °C, the reaction was terminated by the addition of 13 μl of 2× SDS sample buffer. Samples were boiled for 5 min, and proteins were resolved by SDS/10 % PAGE. Gels were stained with Coomassie Blue, dried and exposed to autoradiography, and proteins were quantified using a phosphoimager.

Northern blot analysis

Transfected Jurkat tTA cells were purified as described in [19], and RNA was isolated with TRI Reagent (Sigma–Aldrich) according to the manufacturer's protocol, and size-fractionated by agarose electrophoresis. Specific probes corresponding to Pak1 and β-actin cDNAs were generated with a random primer labelling kit (Beit HaEmek Industries) in the presence of [α-^{32}P]dCTP and purified on Microspin G-25 columns (Amersham Biosciences). mRNA was immobilized on a nitrocellulose membrane using

capillary transfer and pre-hybridized with hybridization buffer (Beit HaEmek Industries) for 60 min at 60 °C, followed by hybridization with either Pak1 or β-actin probes overnight at 60 °C. The next day, the membrane was washed three times in a solution containing 2× SSC (1× SSC is 0.15 M NaCl/0.015 M sodium citrate) and 0.1 % SDS for 5 min each at room temperature (25 °C), followed by a 1 min in 0.2× SSC and 0.1 % SDS at 60 °C. The membrane was subjected to autoradiography and quantified using a phosphoimager.

Pulse–chase analysis

At 40 h following transfection with HA-tagged Pak1, HEK-293T cells were methionine-starved for 1 h and pulse-labelled with [^{35}S]methionine (170 μCi) for 1 h. Subsequently, cells were washed once with PBS and chased with an excess of unlabelled methionine for the indicated times. Cells were lysed, and anti-HA immune complexes were analysed by SDS/10 % PAGE and autoradiography.

RESULTS

Chp inhibits Jurkat T-cell migration in a pore-size-dependent manner

T-cell chemotaxis is known to depend on Pak1 activity [27], but the role of Chp, one of its upstream activators, has not been explored. We tested the effect of wild-type, activated and dominant-negative forms of Chp, on SDF-1α-induced migration of Jurkat T-cells. Chp-expressing cells were marked by co-expression of EGFP, and their migration was compared with that of non-expressing cells from the same transfection, which served as an internal control. Both wild-type and activated Chp reduced SDF-1α-induced migration through 3 μm pores by approx. 60 %, whereas dominant-negative Chp did not substantially affect migration (Figure 1A). Inhibition was less prominent when transwell insets with 5 or 8 μm pores were used (Figure 1B). A similar impairment of chemotaxis through small, but not large, pores is observed upon inhibition of Pak1 [19]. We therefore hypothesized that overexpression of Chp, an activator of Pak1, may paradoxically result in inhibition of Pak1.

Chp down-regulates Pak expression in multiple cell lines

To study the biochemical changes induced by Chp overexpression, we used an expression plasmid that marks Chp-transfected cells with co-expressed H-2K^k on their surface. Transfection was followed by a magnetic-bead-based, rapid and efficient purification of H-2K^k-expressing cells, resulting in a homogenous population of either vector or Chp-expressing cells ([19], and results not shown). Cell lysate, derived from the purified transfected cells, was subjected to SDS/PAGE and Western blotting with anti-Pak1 antibody (Figure 2A). A dramatic reduction in the level of endogenous Pak1 protein was observed in cells expressing activated Chp, as compared with vector-transfected cells. This phenomenon was not cell-type-specific, since it was observed both in Jurkat tTA (Figure 2A, lanes 1 and 2) and in HEK-293 tTA cells (Figure 2A, lanes 3 and 4). In addition, Pak2 expression levels were similarly reduced in Jurkat tTA cells transfected with either wild-type or activated Chp (Figure 2A, lanes 5 and 6). In contrast with Pak1, the level of α-tubulin (Figure 2A, middle panel, lanes 1 and 2, and lower panel, lanes 3–6), ERK (Figure 2A, bottom panel, lanes 1 and 2) and JNK (c-Jun N-terminal kinase) (results not shown) proteins were not affected by Chp expression.

We were also able to observe Chp-induced reduction of Pak1, without using a tetracycline-responsive expression system or

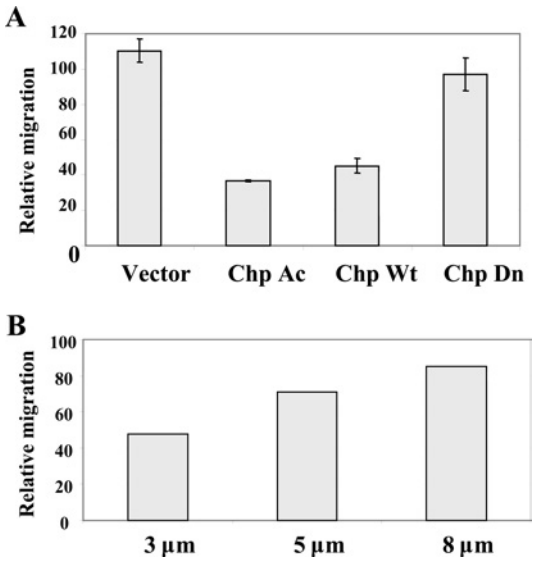

Figure 1 Overexpression of Chp inhibits Jurkat T-cell chemotaxis through restrictive pores

(**A**) Jurkat tTA cells were transiently transfected with wild-type (Chp Wt), activated (Chp Ac) or dominant-negative (Chp Dn) Chp, using a bidirectional promoter to drive expression of both Chp and EGFP. The following day, viable cells were purified with Ficol and the mixture of EGFP-expressing and -non-expressing cells was assayed for SDF-1α-induced migration through 3 μm pores as described in [19]. The migration of EGFP-expressing cells was normalized to the migration of non-expressing cells, from the same transfection and migrating in the same well, and then multiplied by 100 to yield relative migration. Results are the means ± S.E.M. for three independent experiments. (**B**) Migration of Jurkat tTA cells overexpressing wild-type Chp was examined as in (**A**), but using transwell insets with the indicated pore size (3, 5 and 8 μm).

cell-purification procedure. To this end, we repeated the above experiment using HEK-293T cells and a pcDNA-based expression plasmid encoding Myc-tagged activated Chp. HEK-293T cells can be transfected with an efficiency of up to 80–90 %, eliminating the need for purification of transfected cells. Again, Western blot analysis revealed a marked reduction in the level of endogenous Pak1 in cells transfected with activated Chp (Figure 2B).

Remarkably, even ectopically expressed HA-tagged Pak1 was down-regulated by Chp in a similar manner. Upon co-transfection with activated Chp, we observed a decrease in the expression of HA-tagged Pak1, in HEK-293T cells (Figure 2C, lanes 1 and 2) and Jurkat TAg cells (Figure 2C, lanes 3 and 4), as well as in NIH-3T3 cells (see Figure 3A, lanes 1 and 2). In contrast, transfection of dominant-negative Chp mutant S45N, which is locked in the GDP-bound form, did not alter HA–Pak1 expression levels in Jurkat TAg cells (Figure 2C, lane 5). The extent of Pak1 protein reduction in the presence of activated Chp, but not with dominant-negative Chp in three independent experiments is shown (Figure 2D).

One possible explanation for our observations could be a Chp-induced re-localization of Pak1 to a cellular compartment that exhibits lower extraction efficiency, such as lipid rafts or endomembrane. To exclude this possibility, we used multiple methods for the preparation of cell lysates, followed by Western blotting with anti-HA antibody (Figure 2E, top panel). None of the methods recovered equivalent levels of HA–Pak1 in cells transfected with Chp as compared with vector-transfected cells (Figure 2E, top panel, compare lanes 1, 3, 5 and 7 with lanes 2, 4, 6 and 8). In addition, no significant difference was observed regarding the extraction of Chp, except for direct extraction in SDS/PAGE sample buffer that resulted in the highest recovery of

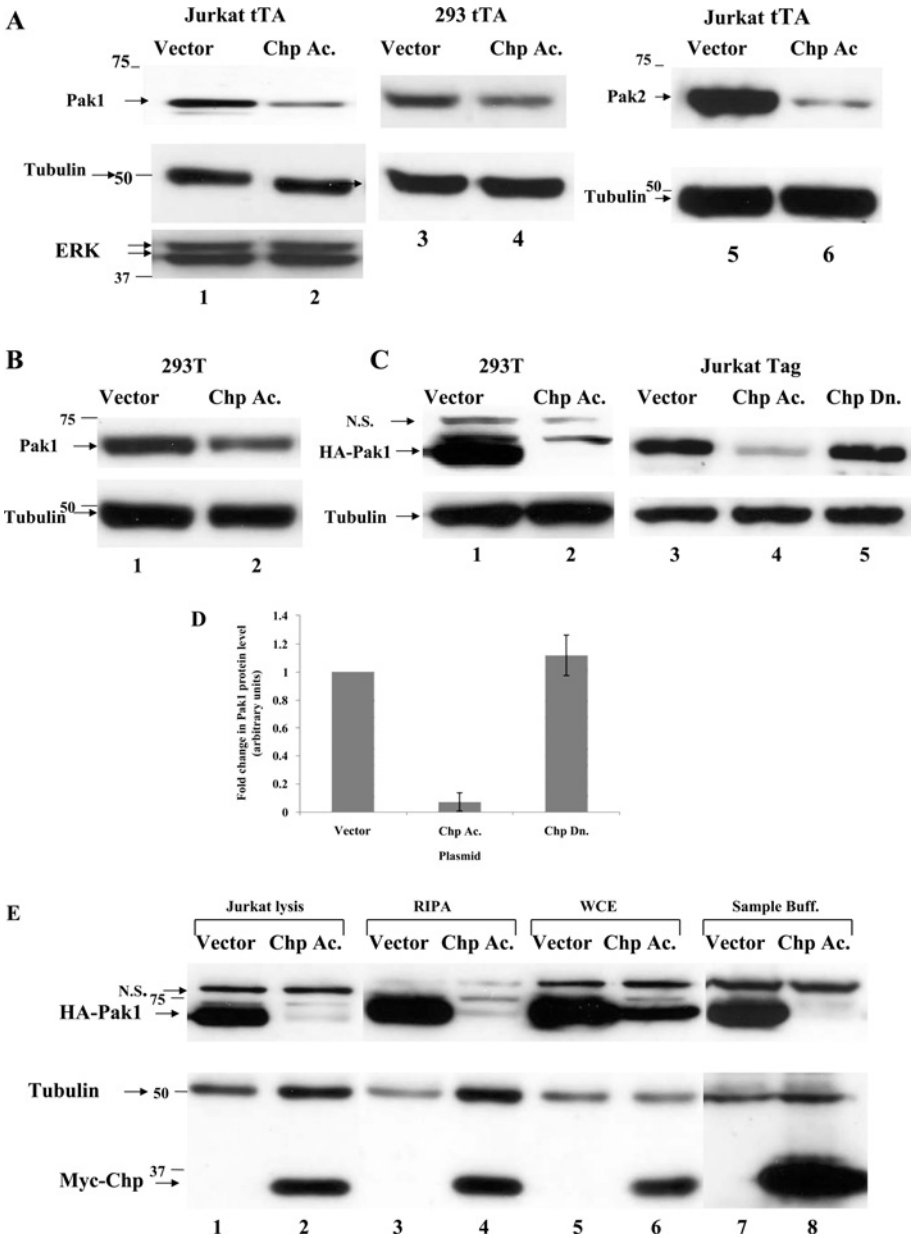

Figure 2 Pak1 protein level is reduced in Chp-transfected cells

(**A**) Cells were transfected with Chp//H-2K^k or empty H-2K^k vector, and H-2K^k-expressing cells were purified using magnetic beads. The following day, cell lysates were subjected to Western blotting with anti-Pak1, anti-Pak2, anti-α-tubulin or anti-ERK1/2, as indicated. Cells were Jurkat tTA (left- and right-hand panels) or HEK-293 tTA (middle panels). (**B**) HEK-293T cells were transfected with a pcDNA-based expression plasmid encoding activated Chp or control vector, and lysates were probed with anti-Pak1 antibody (upper panel) and anti-α-tubulin (lower panel). (**C**) HEK-293T (left-hand panel) or Jurkat TAg (right-hand panel) were co-transfected with HA–Pak1 together with activated Chp, dominant-negative Chp or vector control, and total cell lysates were probed with anti-HA antibody (upper panel) and anti-α-tubulin antibody (lower panel). (**D**) The amount of Pak1 protein found in the lysates of transfected Jurkat TAg cells was quantified by densitometry (arbitrary units). For each transfection, Pak1 was normalized to the amount present in vector-transfected cells in the same experiment, to yield the fold change in Pak1 expression. Results are means $\pm$ S.E.M. for three independent experiments. (**E**) HEK-293T cells were co-transfected with HA–Pak1 together with Myc-tagged activated Chp or vector control. Total cell lysates were prepared using Jurkat lysis buffer (lanes 1 and 2), RIPA buffer (lanes 3 and 4), WCE buffer (lanes 5 and 6) or sample buffer (lanes 7 and 8), and probed with anti-HA (top panel) and anti-α-tubulin (middle panel), followed by anti-Myc (bottom panel). The positions of the molecular-mass markers and the non-specific bands (N.S.) are indicated (values are in kDa). Chp Ac., activated Chp; Chp Dn., dominant-negative Chp.

Chp protein (Figure 2E, bottom panel). Yet, even under these harsh extraction conditions (SDS/PAGE sample buffer), Pak1 expression was below the detection threshold (Figure 2E, lane 8), suggesting that this represents a real reduction in the cellular levels of Pak1.

Together, the above experiments suggest that activated Chp specifically down-regulates the expression of Pak1 in T-cells, epithelial cells and in fibroblast cells.

Chp and Cdc42 Rho GTPases down-regulate Pak1, but not Pak4 family members

We wondered whether the concept of Rho-family GTPase-induced down-regulation of its effector could be generalized to other Rho-family GTPases or other effectors of these GTPases. To this end, we co-transfected NIH-3T3 cells with HA-tagged Pak1 along with Myc-tagged activated Rac1, Cdc42 or Chp, and

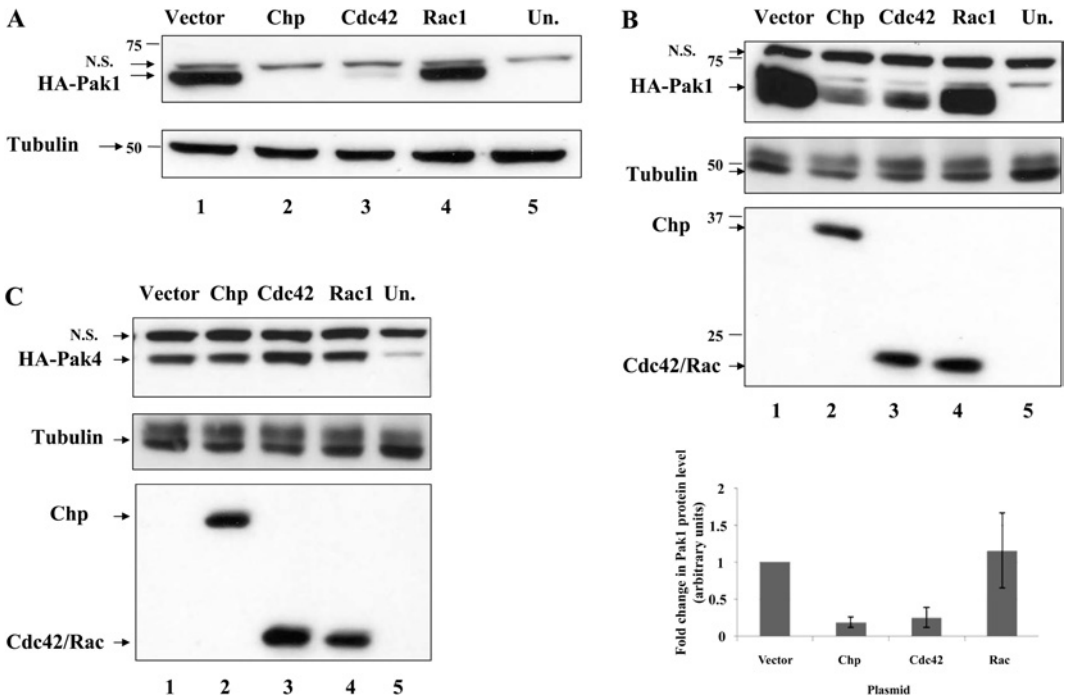

Figure 3 Chp and Cdc42 specifically reduce expression of Pak1, but not Pak4

(**A**) NIH-3T3 cells were co-transfected with HA-tagged Pak1 (lanes 1–4) along with the indicated Myc-tagged activated Rho-family GTPases, or control vector, and lysates were probed with anti-HA (upper panel) or anti-α-tubulin as loading control (lower panel) antibodies. (**B**) HEK-293T cells were co-transfected with HA-tagged Pak1 (lanes 1–4) along with the indicated Myc-tagged activated Rho-family GTPases, or control vector, or were untransfected (Un.) (lanes 5). Lysates were probed with anti-HA (top panel), anti-α-tubulin as loading control (middle panel) or anti-Myc (bottom panel) antibodies. The amount of HA-tagged Pak1 found in the lysates of transfected HEK-293T cells was quantified by densitometry and normalized to the level expressed in vector-transfected cells in the same experiment (as shown in the histogram). Results are means $\pm$ S.E.M. for three independent experiments. (**C**) HEK-293T cells were co-transfected with HA–Pak4 along with the indicated Myc-tagged activated Rho-family GTPase or control vector, or were untransfected (Un.) (lanes 5). Lysates were probed with anti-HA (top panel), anti-α-tubulin (middle panel) or anti-Myc (bottom panel) antibodies. The positions of the molecular-mass markers and the non-specific bands (N.S.) are indicated (values are in kDa).

the effects of each GTPase on Pak1 expression were measured. When compared with control vector, activated Cdc42 reduced HA–Pak1 expression to nearly the same extent as activated Chp, whereas activated Rac1 did not significantly reduce the level of Pak1 (Figure 3A, compare lanes 2 and 3 with lanes 1 and 4). Again, these effects were not cell-type-dependent, as qualitatively similar results were obtained using HEK-293T cells (Figure 3B, upper panels). Western blotting with anti-Myc antibody confirmed that all three transfected Rho-family GTPases were expressed at comparable levels (Figure 3B, lower panel). The average effect of these Rho-GTPases on Pak1 expression in three independent experiments (Figure 3B, lower panel) demonstrates that Chp and Cdc42 share the ability to reduce the expression of their common effector, Pak1, whereas Rac1 did not significantly affect Pak1 expression.

Cdc42 is able to associate with both group I and group II Pak family members. Despite this similarity, these two effectors differ in their mode of regulation. Group I Pak members are activated upon binding to Cdc42, whereas Cdc42 binding to group II Pak members affects their subcellular localization, but not their activity [4,5]. Similarly to Pak1, the expression of both Pak2 and Pak3 was down-regulated upon co-transfection with either Chp or Cdc42 (results not shown). This observation could be part of a negative-feedback loop which results in down-regulation of the activity of group I Pak family member following activation by upstream signals. To test whether Pak4 might conform to a similar mode of regulation by Cdc42 and Chp, HEK-293T cells were co-transfected with HA–Pak4, along with either vector, or each of the Myc-tagged activated GTPases. In this case, none of the activated GTPases substantially affected the expression of HA–Pak4 (Figure 3C, top panel). Thus Chp- and Cdc42-induced down-regulation is unique to the group I Paks (Pak1, Pak2 and Pak3) and is not shared by the group II member (Pak4).

Chp down-regulates Pak expression post-transcriptionally

We observed that endogenous and transfected Pak1 were equally susceptible to Chp-induced down-regulation, despite their expression from different promoters (the endogenous Pak1 promoter and the cytomegalovirus immediate-early promoter respectively). This result suggested that Chp-induced down-regulation of Pak1 probably occurs at a post-transcriptional level. To test this assumption more directly, we used a magnetic-bead-based purification protocol to isolate a homogeneous population of transfected Jurkat tTA cells, following transfection with activated Chp, Cdc42 or vehicle expression plasmids, and assessed the effect of these GTPases on the steady-state level of endogenous *Pak1* mRNA. Northern blot analysis showed no significant change in the level of *Pak1* mRNA (Figure 4A), supporting the notion that Chp- and Cdc42-induced down-regulation of Pak1 occurs post-transcriptionally. To study further the molecular mechanisms by which these Rho-family GTPases reduce Pak1 expression, we decided to focus on Chp as a representative member of the Rho-GTPase family.

Chp targets Pak1 for proteasomal degradation

Pulse–chase experiments, using [^{35}S]methionine to metabolically label HA–Pak1 in HEK-293T cells, revealed that Pak1 is a stable

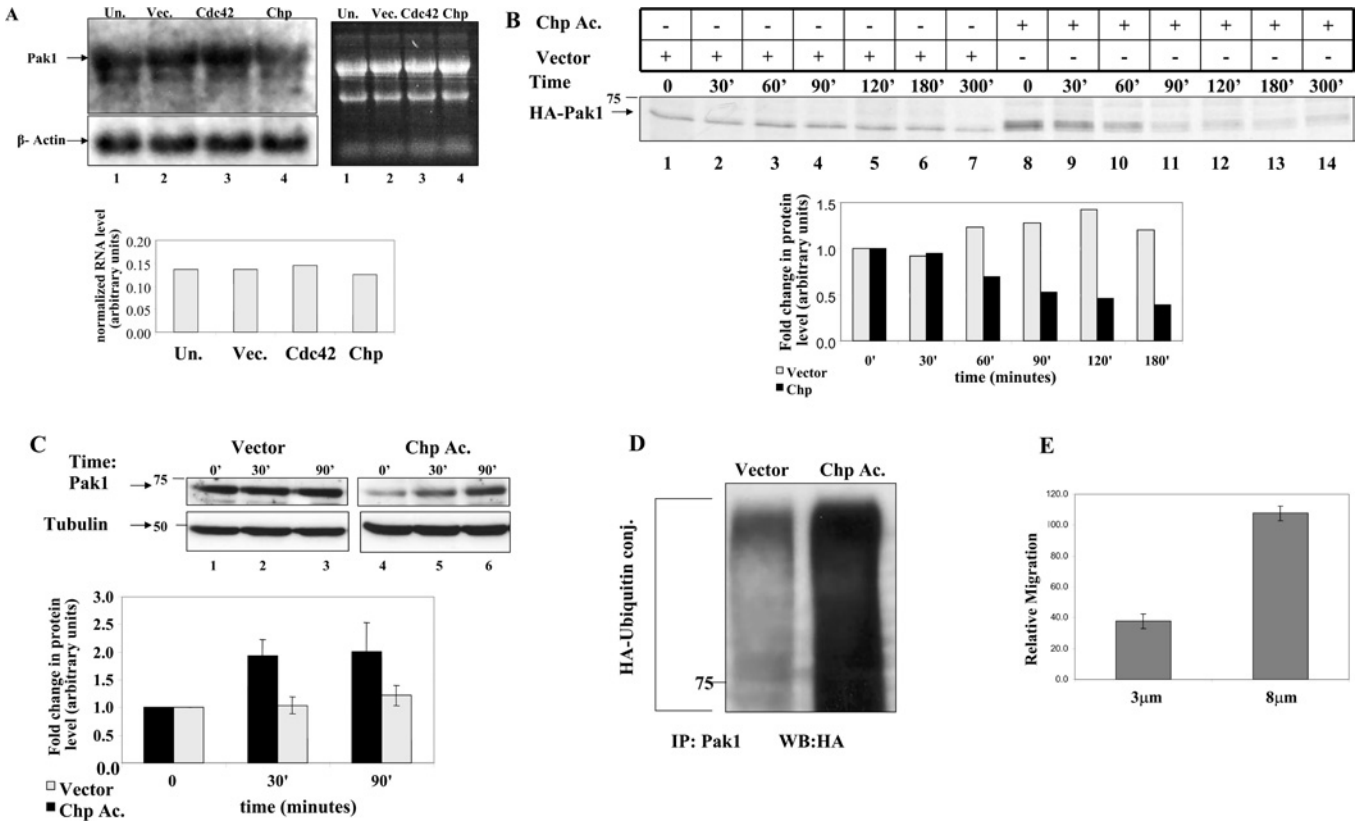

Figure 4 Chp induces ubiquitin-mediated degradation of Pak1

(**A**) Jurkat tTA cells were transiently transfected with activated Cdc42//H-2K^k, activated Chp//H-2K^k or H-2K^k alone (vector; Vec.), or were untransfected (Un.), and were purified as in Figure 2(A), before preparation of mRNA for Northern blot analysis with ^{32}P-labelled DNA probes corresponding to Pak1 (left-hand upper panel) or β-actin (left-hand lower panel). Expression of *Pak1* mRNA was quantified by densitometry and normalized to the level of β-actin, as shown in the histogram. Ethidium bromide staining of the agarose gel is shown (right-hand panel). (**B**) HEK-293T cells were co-transfected with HA–Pak1 together with activated Chp (lanes 8–14) or vehicle expression plasmid (lanes 1–7). Cells were pulse-labelled with [^{35}S]methionine for 1 h and then chased for the indicated time in the presence of unlabelled methionine and lysed. Anti-HA immune complexes, prepared from the cell lysates, were subjected to SDS/PAGE followed by gel drying and autoradiography. The band representing HA–Pak1 was quantified using a phosphoimager, and the average fold change in intensity from two typical experiments is shown in the histogram. (**C**) Jurkat tTA cells were transfected with activated Chp//H-2K^k (lanes 4–6) or empty H-2K^k vector (lanes 1–3), and H-2K^k-expressing cells were purified as in Figure 2(A). The following day, cells were incubated with MG132 (20 μM) for the indicated time before lysis, and lysates were probed with anti-Pak1 (upper panels) or anti-α-tubulin (lower panels) antibodies. The bands were quantified by densitometry, and the ratio of Pak1 to α-tubulin was calculated, and normalized to the value observed in cells before MG132 treatment. Results in the histogram are means $\pm$ S.E.M. for three independent experiments. (**D**) HEK-293T cells were co-transfected with HA-tagged ubiquitin (HA-ubiquitin conj.) along with activated Chp or control vector. Cells were incubated with MG132 (20 μM) for 3.5 h before lysis, and anti-Pak1 immune complexes were probed by Western blotting (WB) with anti-HA antibodies. (**E**) Jurkat T-cells were pre-treated for 3 h with 5 μM lactacystin or with vehicle alone (DMSO), and were allowed to migrate, under the influence of SDF-1α, through 3 or 8 μm pores. Migration of inhibitor-treated cells was normalized to the migration of vehicle-treated cells, migrating in parallel wells of the same pore size, and then multiplied by 100 to yield relative migration. Results are means $\pm$ S.D. for three independent experiments. Chp Ac., activated Chp; IP, immunoprecipitation. Molecular masses are indicated in kDa to the left of the blots.

protein with a half-life of approx. 6 h (Figure 4B, lanes 1–7). Upon expression of activated Chp, the half-life of co-expressed HA–Pak1 was reduced to 1.5 h (Figure 4B, lanes 8–14). Thus Chp appears to destabilize Pak1 protein.

One of the pathways responsible for degradation of cellular proteins is the ubiquitin-dependent proteasomal degradation pathway. A 90 min treatment with the proteasome inhibitor MG132 resulted in roughly 2-fold accumulation of endogenous Pak1 protein in Chp-transfected cells, but did not affect Pak1 expression in vector-transfected cells (Figure 4C). These data suggest that the shorter half-life observed in Chp-transfected cells results from the targeting of Pak1 to a proteasome-dependent degradation mechanism.

Targeting of proteins to proteasomal degradation generally involves their ubiquitination. To test directly whether Chp induces ubiquitination of Pak1, we transfected HEK-293T cells with HA–ubiquitin, alone or together with activated Chp, and MG132 was added 3.5 h before cell harvesting, to inhibit the degradation of ubiquitin-conjugated proteins. A Western blot analysis of anti-Pak1 immune complexes with anti-HA antibodies revealed a high-molecular-mass signal, which was much more prominent in the Chp-transfected cells (Figure 4D). These results provide evidence for the generation of Pak1–ubiquitin conjugates in the presence of activated Chp and the proteasome inhibitor MG132.

To reveal whether proteasome-dependent protein degradation plays a role in T-cell chemotaxis, we examined the effect of proteasome inhibitors on Jurkat T-cell migration through transwell insets bearing 3 or 8 μm pores. Both lactacystin and MG132 produced dose-dependent inhibition of Jurkat T-cell migration through 3 μm pores, but did not affect migration through a larger pore size, nor did they affect SDF-1α-induced ERK activation (Figure 4E, and results not shown). This type of effect on migration is consistent with impaired regulation of Pak1 [19], and suggests that appropriately regulated degradation of either Pak1 or another protein is essential for T-cell migration through restrictive barriers, a process that also depends on Pak1 activity [19].

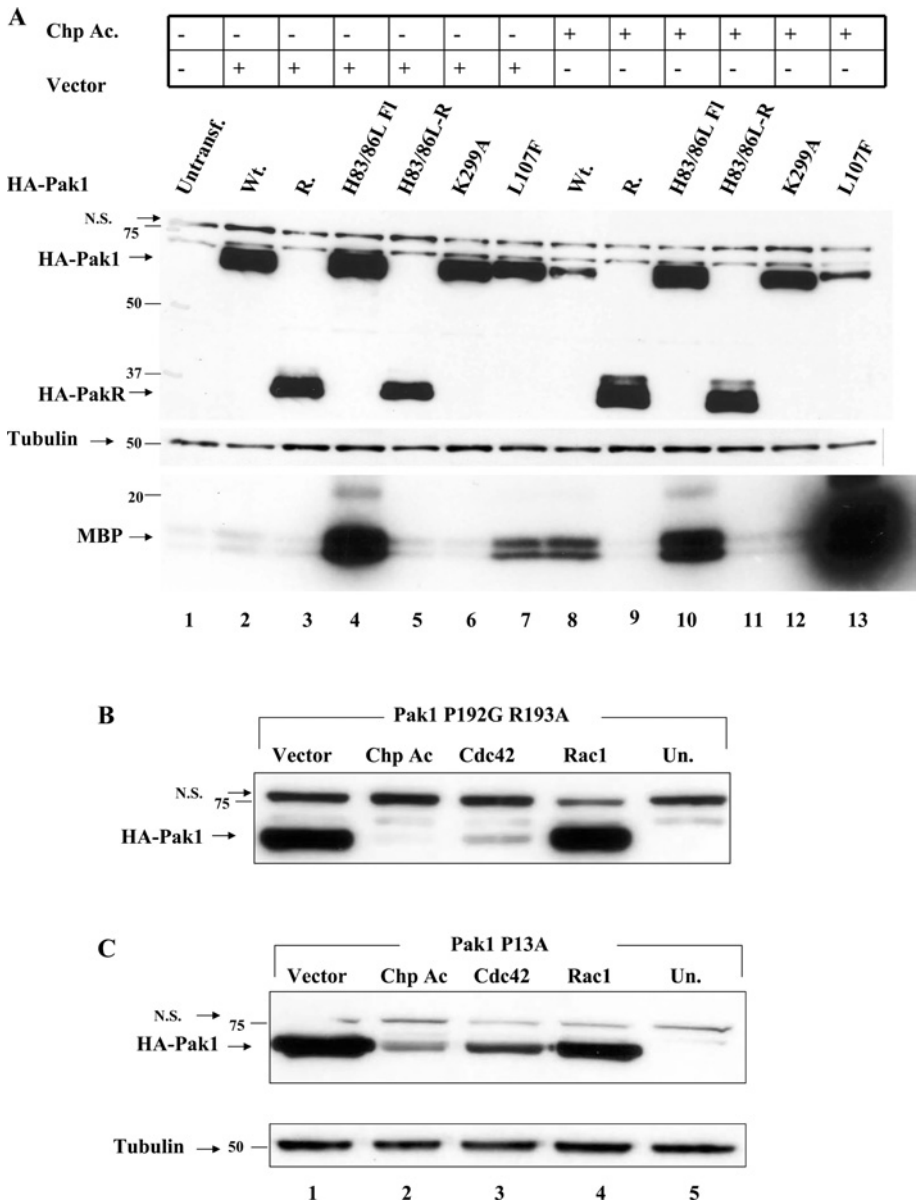

Figure 5 Pak1 domains required for susceptibility to Chp-induced degradation

(**A**) HEK-293T cells were co-transfected with vector (lanes 2–7) or activated Chp (lanes 8–13) along with the indicated HA-tagged Pak1 mutants, or were untransfected (Untransf.; lane 1). Cell lysates were probed with anti-HA (top panel) and anti-α-tubulin (middle panel) antibodies. In parallel, anti HA-immune complexes were subjected to an *in vitro* kinase assay in the presence of [γ-^{32}P]ATP and MBP as a substrate. Reaction products were resolved by SDS/PAGE followed by gel drying and autoradiography (bottom panel). Pak1 mutants are: R., regulatory domain, comprising amino acids 1–265; H83/86L Fl, full-length Pak1 in which the PBD is inactivated (H83L/H86L mutant); H83/86L-R, Pak1 regulatory domain with inactivated PBD (H83L/H86L mutant); K299A, in which the kinase domain is inactivated; L107F, in which the KI domain is inactivated. Wt., wild-type. (**B** and **C**) HEK-293T cells were co-transfected with HA-tagged Pak1 P192G/R193A, in which the PIX-binding site is inactivated (**B**) or Pak1 P13A, in which the Nck-binding site is inactivated (**C**), along with the indicated Myc-tagged activated GTPases or control vector, or were untransfected (Un.). Lysates were probed with anti-HA and anti-α-tubulin antibodies, as indicated. Chp Ac., activated Chp. The non-specific (N.S.) anti-HA cross-reactive bands and molecular-mass markers are indicated (sizes in kDa).

Pak1 kinase activity and Chp binding are required for its Chp-induced degradation

As Chp triggers both activation and degradation of Pak1, we wondered about the mechanistic connection between these two events. We addressed this question using a panel of HA-tagged Pak1 constructs, bearing point mutations with well-characterized effects on Pak1 functionality. Each of the mutants was transfected into HEK-293T cells, along with vector or activated Chp, and cell lysates were assayed in parallel for the expression and activity of each of the transfected Pak1 constructs (Figure 5A, top and bottom panels respectively). Each of the constructs was compared with wild-type Pak1, which exhibited moderate activation and profoundly reduced expression upon co-transfection with Chp (Figure 5A, top panel, lanes 2 and 8).

A comparison of the most and least active Pak1 mutants suggested that Pak1 catalytic activity is important for Chp-induced degradation. Pak1 K299A, bearing an inactivating mutation in the Pak1 catalytic domain, was catalytically inactive and was not susceptible to Chp-induced degradation (Figure 5A, top panel, lanes 6 and 12). In contrast, Pak1 L107F bears an inactivating mutation in the KI domain that renders it constitutively active

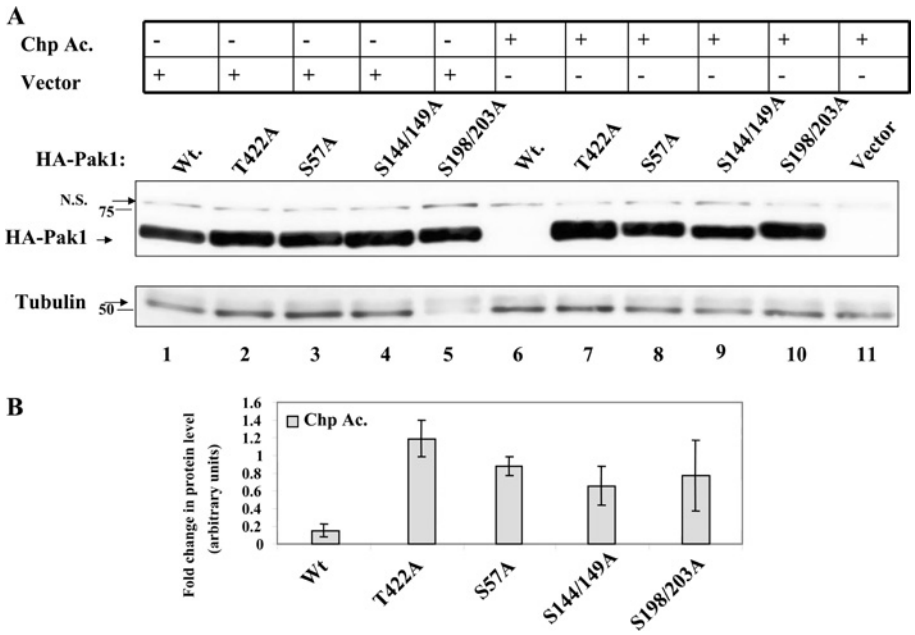

Figure 6 Pak1 autophosphorylation sites are required for Chp-induced degradation

(**A**) HEK-293T cells were co-transfected with vector (lanes 1–5) or activated Chp (lanes 6–11) along with HA-tagged Pak1 mutants bearing point mutations in the indicated autophosphorylation sites (S144/149A, S144A/S149A; S198/203A, S198A/S203A), or control vector. Lysates were probed with anti-HA (upper panel) and anti-α-tubulin (lower panel) antibodies. The non-specific (N.S.) anti-HA cross-reactive bands and molecular-mass markers are indicated (sizes in kDa). (**B**) The expression of the Pak1 mutants shown in (**A**) was quantified by densitometry. The expression of each Pak1 mutant in Chp-transfected cells was normalized to its expression in vector-transfected cells. Results are means $\pm$ S.E.M. for three independent experiments. Chp Ac., activated Chp; Wt., wild-type.

[28]. In the absence of Chp, this mutant was expressed at a lower level than was wild-type Pak1 [10], but exhibited considerable kinase activity (Figure 5A, bottom panel, compare lanes 2 and 7). Upon co-transfection with Chp, expression of Pak1 L107F was reduced further, along with a profound increase in kinase activity (Figure 5A, lanes 7 and 13). Together, these results suggest that activation of Pak may be an important precondition for Chp-induced degradation.

To test the importance of the physical interaction between Pak1 and Chp, we utilized another constitutively active mutant of Pak1, which bears an inactivated PBD. Pak1 H83L/H86L exhibits impaired binding to Cdc42 and Rac [29,30], and impaired binding to Chp, which was confirmed in yeast (results not shown), using the RRS (Ras recruitment system) [31]. As reported previously [29,30], Pak1 H83L/H86L displayed a relatively high basal kinase activity as compared with wild-type Pak1. Consistent with its inability to bind Chp, Pak1 H83L/H86L was not susceptible to Chp-induced down-regulation, nor was its activity augmented in the presence of Chp (Figure 5A, lanes 4 and 10). These results suggest that Pak1 activity is not sufficient to trigger its degradation in the absence of a physical interaction between Chp and Pak1.

In addition to the PBD, the regulatory domain of Pak1 contains binding sites for a number of other signalling proteins; among these, the binding sites for Nck and PIX are the best-characterized. Mutational inactivation of the PIX-binding site (P192G/R193A) [8,23] did not reduce the susceptibility of Pak1 to degradation by activated Chp or Cdc42 (Figure 5B). Likewise, mutational inactivation of the Nck-binding site (P13A) [32] did not substantially affect Pak1 susceptibility to Chp- and Cdc42-induced degradation (Figure 5C). Thus, among the N-terminal protein interaction sites of Pak1, the PBD is uniquely required for Chp-induced Pak1 degradation. Although required, the PBD alone was not sufficient to target Pak1 for degradation. Indeed, a construct encoding the regulatory domain of Pak1 (PakR, comprising amino acids 1–265 of Pak1) was unaffected by Chp (Figure 5A, lanes 3 and 9). Consistently, mutation of the PBD (H83L/H86L) in the context of the Pak1 regulatory domain fragment alone did not alter its expression in the presence or absence of Chp (Figure 5A, lanes 5 and 11). On the basis of the above analysis of seven different Pak1 mutants, we suggest that both the physical interaction with Chp and the catalytic activation of Pak1 are required to target the kinase for degradation.

The importance of Pak1 autophosphorylation for Chp-dependent degradation

Previous studies have identified multiple conserved Pak1 autophosphorylation sites, some of which are involved directly in kinase activation [16]. Since our observations suggested that the kinase activity of Pak1 is crucial for its destabilization by Chp, we examined further the role of specific autophosphorylation sites in targeting the Pak1 protein for degradation. For this purpose, we assessed Chp-induced degradation of a number of Pak constructs bearing alanine mutations at specific autophosphorylation sites, including T422A, S57A, S144A/S149A and S198A/S203A. Strikingly, each of the mutations markedly reduced the susceptibility of Pak1 to Chp-induced degradation (Figure 6A). However, the S144A/S149A mutant consistently remained somewhat susceptible to Chp-mediated Pak1 down-regulation (Figure 6B).

The N- and C-terminal domains of Chp are required for down-regulation of Pak1 expression

The above results suggested that activation and autophosphorylation of Pak1 are required, but not necessarily sufficient, for Chp-induced degradation. We wondered whether a distinct function of Chp may be required to trigger the degradation of Pak1, following its activation. Since Chp differs from other Rho-family GTPases

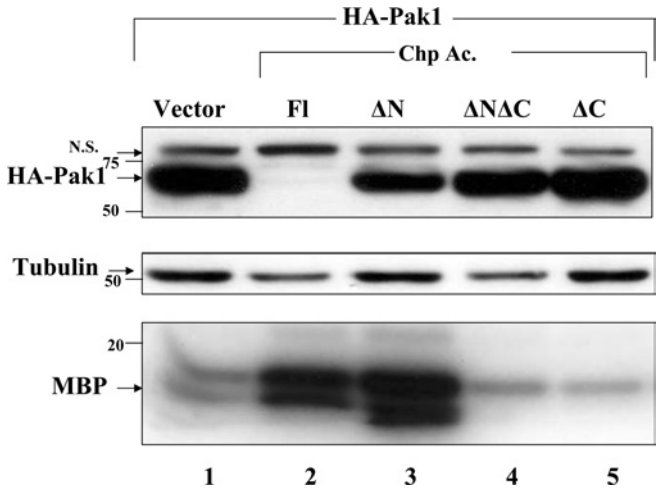

Figure 7 The N- and C-terminal domains of Chp are required to induce Pak1 degradation

HEK-293T cells were co-transfected with HA–Pak1 along with vector, or activated Chp (Chp Ac.) constructs bearing the indicated deletion mutations. Lysates were probed with anti-HA (top panel) or anti-α-tubulin (middle panel) antibodies. The non-specific (N.S.) anti-HA cross-reactive bands and molecular-mass markers are indicated (sizes in kDa). In parallel, anti HA-immune complexes were subjected to an *in vitro* kinase assay in the presence of [γ-^{32}P]ATP and MBP as a substrate. Reaction products were resolved by SDS/PAGE, followed by gel drying and autoradiography (bottom panel). All Chp constructs bore the activating mutation (G40V) in the context of the full-length (Fl) as well as the following deletions: N-terminal truncation (ΔN), C-terminal truncation (ΔC) or both (ΔNΔC).

in its unique N-and C-terminal domains [1], we investigated the role of these domains in mediating Pak1 activation and degradation.

Activated Chp constructs bearing either a C- or N-terminal truncation, or bearing both truncations, were transfected into HEK-293T cells, along with HA-tagged Pak1, and both Pak1 activation and degradation were assessed (Figure 7). As a control, co-transfection of full-length activated Chp resulted in nearly undetectable Pak1 expression, but considerable Pak kinase activity, indicating that the small amount of Pak1 remaining in the cells is highly active (Figure 7, lanes 1 and 2). Deletion of the C-terminal domain of Chp (ChpΔC) abrogates its binding to the regulatory domain of Pak1 [1]; accordingly, this mutant neither activated nor mediated the degradation of Pak1 (Figure 7, lane 5). Similar results were obtained upon deletion of both the N- and C-terminal (ChpΔNΔC) domains (Figure 7, lane 4).

Strikingly, upon deletion of the N-terminal proline-rich domain of Chp (ChpΔN), Pak1 down-regulation was selectively impaired (Figure 7, lane 3). ChpΔN-transfected cells exhibited a high level of Pak1 kinase activity, which surpassed that induced by full-length activated Chp. Unexpectedly, ChpΔN-induced Pak1 kinase activation was not associated with its degradation; indeed, expression of Pak1 in ChpΔN-transfected cells approached the level observed in vector-transfected cells. This result demonstrates the importance of the unique N-terminal domain of Chp in facilitating Pak1 degradation, and suggests further that Chp-induced degradation of Pak1 requires a functional complex, including both activatable Pak1 and full-length Chp.

DISCUSSION

Pak proteins are activated by multiple extracellular signals, resulting in a variety of cellular responses [3,4,33]. The catalytic activation of Pak is generally rapid and transient, peaking at less than 1 min for SDF-1α stimulation [19], at 5 min for lysophosphatidic acid [34] and at 30 min for angiotensin II [35]. Whereas much is known regarding the upstream and downstream signalling pathways involved in Pak kinase activation [3], the regulatory mechanisms enabling its rapid down-regulation are largely unknown. Recent studies place Pak1 as a converging point in transformation induced by various small GTPases, such as Ras, Rac1, Rac3 and Cdc42 [18,36]. Furthermore, an up-regulation of Pak1 kinase activity and expression has been observed in primary tumours, sometimes in the absence of *Pak1* gene amplification [18]. Thus the identification of factors that control the level of Pak protein and activity is of great importance.

The present study describes a novel role for the Rho-family GTPases, Cdc42 and Chp in down-regulating expression of their downstream effector protein, Pak1, via enhanced protein degradation. Upon expression of activated Chp or Cdc42, Pak1 displays a dramatically reduced half-life, which can be attributed to proteasomal degradation. The ability of these Rho-family GTPases to trigger degradation of their effectors is highly selective. Members of the group I Pak family (Pak1, Pak2 and Pak3) are substantially down-regulated by activated Chp or Cdc42 (Figure 2, and results not shown), whereas activated Chp does not trigger degradation of Pak4, an effector that is common to Chp and Cdc42. GTPase-induced degradation of Pak1 is intimately tied to its activation, since mutations in Pak1 that impaired its activation by GTPases also resulted in its stabilization. Thus Chp and Cdc42 appear to exert a dual role in the regulation of group I Pak proteins, both activating them and initiating proteasome-dependent degradation of the active kinase.

Previous publications have provided some evidence for the instability of Pak1 [16,28] and Pak3 [37] and/or low protein expression levels upon transient transfection. Perhaps in an attempt to circumvent problems with reduced Pak expression upon co-expression of activated Cdc42, many studies of Pak activation were performed using GTP[S] (guanosine 5-[γ-thio]triphosphate)-loaded recombinant Rac or Cdc42 to activate Pak1 *in vitro* [38]. To our knowledge, ours is the first study to definitively document the phenomenon of GTPase-induced Pak instability, and to begin to unravel the molecular mechanisms which control the stability of Pak proteins within intact cells.

Rho-family GTPase-induced degradation of Pak1 protein probably represents a negative-feedback loop, leading to signal termination. Under physiological conditions, this mechanism would not be expected to produce a detectable stimulus-induced reduction in the level of Pak1 expression, since extracellular ligands are likely to activate only a fraction of the total cellular Pak, some of which is probably disposed of by dephosphorylation, while the remainder of activated Pak1 would be subject to proteasome-dependent degradation. Thus previous studies have not revealed a reduction in endogenous Pak1 levels upon exposure to stimuli that activate Pak1 in a Rho-GTPase-dependent manner.

Despite the above caveat, some evidence suggests that proteasome-mediated degradation of Pak1 may play an important role in some settings. First, proteasome-dependent degradation of Pak may be part of the cellular processes that enable cell migration through restrictive barriers. The proteasome inhibitors lactacystin (Figure 4E) and MG132 (10 μM, not shown), inhibited Jurkat T-cell migration in a pore-size-dependent manner, a pattern of migration that is characteristic of Pak1-inhibited cells [19]. Secondly, proteasome-dependent degradation of Pak1 may be more easily revealed in the case of GTPase-independent activation of Pak1. When Pak1 is activated in a GTPase-independent manner, by the Ca^{2+}-binding protein CIB1 (calcium and integrin binding 1), its activation can be potentiated in the presence of the non-specific Rho-GTPase inhibitor, toxin B [39]. These unexpected findings may indicate that the Rho-GTPases, inhibited by toxin B, are normally responsible for down-regulating Pak activity,

perhaps via the mechanism that we describe in the present study. We were able to reveal this phenomenon, using transient transfection experiments of activated GTPases, resulting in persistent activation and subsequent degradation of a substantial proportion of total cellular Pak1. This approach has uncovered a unique and novel negative-feedback loop mechanism mediated by the activated form of the Chp and Cdc42 small Rho-GTPases.

Pak activities and domains necessary for Chp-mediated down-regulation

We used a variety of Pak1 mutants to map the domains and activity of Pak1 that determine its susceptibility to Chp-mediated degradation. The PBD, which is responsible for GTPase binding, is necessary, but not sufficient, to target Pak1 for degradation. In contrast, the PIX- and Nck-binding motifs were not required. Mutational inactivation of the KI domain resulted in increased basal activity and lower level of expression; however, the KI domain mutant protein was further activated and destabilized in the presence of Chp. Mutations within Pak autophosphorylation sites resulted in stabilization of Pak1 expression in the presence of Chp, suggesting that only fully autophosphorylated Pak1 protein is subject to degradation. Consistently, kinase-inactive Pak1 mutant was efficiently expressed in the presence of Chp. Although it seems that Pak1 kinase activity is crucial for tagging Pak1 for degradation by its autophosphorylation, one cannot exclude the possibility that the kinase activity is also required for activation of a specific E3 ubiquitin ligase.

Chp N-terminal domain is necessary for Pak degradation

Deletion of the N-terminal domain of Chp impaired its ability to induce Pak1 degradation, but not its ability to activate Pak1 kinase activity towards exogenous substrates, as well as Pak1 autophosphorylation. Thus Pak1 autophosphorylation is not sufficient for Chp-dependent Pak1 degradation. A previous study suggested that the N-terminal domain of Chp negatively regulates its transforming activity [20]. It is possible that the higher transformation rate observed upon deletion of the N-terminus of Chp actually reflects decreased proteasomal degradation of its activated effector, Pak1.

Unique Chp membrane localization

A previous study suggested that Chp is localized to the plasma membrane and endomembrane compartments [21]. The C-terminal domain of Chp is required for its unique lipid modification, responsible for Chp localization, whereas N-terminally truncated Chp is localized to the same cellular compartment as full-length Chp [21]. Although N-terminally truncated Chp binds and activates Pak1, and localizes to the same cellular compartment as wild-type Chp, it fails to down-regulate Pak1. Thus the cellular localization of Chp-bound Pak1 is not sufficient to explain our observations. To support this conclusion further, we examined various extraction methods in the presence of full-length Chp and found that none of them was able to recover higher levels of Pak1 protein. Moreover, the use of the proteasome inhibitor MG132 resulted in increased levels of Pak1 in full-length-Chp-transfected cells, strongly indicating that Pak1 is degraded in a proteasome-dependent manner and not merely re-localized to a different cellular compartment.

Pak2 stability and signalling

Previous studies have documented that Pak2 is subjected to post-transcriptional regulation; however, these observations appear to represent a mechanism which is distinct from the one suggested in the present study. Tyrosine phosphorylation of Pak2 by the non-receptor tyrosine kinase, c-Abl, results in increased stability of Pak2 protein [40]. Subsequently, it was demonstrated that, in response to stress stimuli, a caspase-dependent cleavage of Pak2 releases a Pak2 proteolytic fragment lacking most of the N-terminal regulatory domain. This fragment translocates into the nucleus and is involved in pro-apoptotic cell responses [41]. The expression level of the Pak2 fragment is tightly regulated by an ubiquitin-dependent mechanism. Unlike Pak1 regulation described in the present study, the degradation of the Pak2 fragment is independent of Pak2 kinase activity, autophosphorylation sites and the PBD [41].

Protein phosphorylation and ubiquitination mechanisms

Efficient degradation of Pak1 occurs only in the presence of full-length Chp and activatable Pak1. Continued binding of the activated kinase to Chp may be required for its targeting to degradation, thus the constitutively activated PBD mutant of Pak1 was immune to Chp-induced degradation. Both wild-type and activated Chp were able to target Pak1 for degradation to a similar extent. This is consistent with our previous observation that wild-type Chp efficiently associates with Pak [1]. Truncation of the N-terminal proline-rich region of Chp impaired Chp-induced Pak1 degradation. The E3 ubiquitin ligase responsible for Chp-dependent Pak1 degradation has yet to be identified and is beyond the scope of the present study. Nevertheless, the ligase may require association with the N-terminal proline-rich region of Chp and with Pak1 autophosphorylation sites. An attractive candidate for an E3 ubiquitin ligase which is recruited via a proline-rich domain is the plenty of SH3 domain protein, POSH (plenty of SH3s) [42]. POSH was recently shown to serve as E3 ubiquitin ligase for HGF (hepatocyte growth factor)-regulated tyrosine kinase substrate, Hrs. The POSH SH3 domain binds to Hrs, while the ring finger domain promotes ubiquitination of Hrs [43]. The expression levels of POSH in HEK-293 cells and HeLa cells are below the detection levels and therefore the role of POSH in GTPase-dependent degradation of Pak using POSH siRNA (small interfering RNA) in this process could not be studied. Nevertheless, since Cdc42 lacks a proline-rich domain, it is likely that the E3 ubiquitin ligase responsible for Cdc42-dependent Pak1 degradation is either distinct or alternatively functions via a different mechanisms.

A number of proteins, including cell cycle regulators, transcription factors and other signalling molecules are targeted for proteasomal degradation upon their phosphorylation [44]. Typically, multiple kinases are involved in the generation of a phosphodegron motif recognized by a specific E3 ubiquitin ligase. The autophosphorylation of tyrosine kinase receptors results in their down-regulation by endocytosis. However, this process involves mono-ubiquitination of the receptor by the E3 ubiquitin ligase Cbl [45]. It is becoming apparent that numerous receptors can transmit signals from endocytic compartments which may be different from those initiated at the plasma membrane [46]. Recently, mono- and di-ubiquitination was also observed for H-Ras, resulting in stabilization of Ras association with endosomes and modulation of Ras-mediated activation of the Raf MAPK pathway [47].

In the present paper, we have described a unique mechanism of signalling-dependent self-destruction of Pak1 whereby increased Pak1 kinase activity results in autophosphorylation, which is required for its targeting to proteasomal degradation.

In summary, we have identified a novel mechanism involved in down-regulation of incoming signals mediated by Chp and Cdc42 via their effector Pak. Pak activation by Chp and Cdc42 results in potentiation of Pak kinase activity leading to Pak autophosphorylation on conserved sites, tagging Pak for proteasomal degradation.

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