PAK interacts with NCK and MLK2 to regulate the activation of jun N-terminal kinase

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Abstract The p21-GTPase activated kinase, PAK1, and the mixed lineage kinase, MLK2, have been implicated in the activation of jun N-terminal kinase (JNK). However, the role of PAK1 in JNK activation is still not understood. Here we show that over-expression of the SH3-SH2 adapter Nck 'squelches' JNK activation but this squelching is relieved by over-expression of PAK1. In turn, PAK1 squelches activation of JNK by MLK2 and these kinases interact via their catalytic domains. The data suggest that PAK1 recruits MLK2 to an activated receptor via the adapter Nck, but cannot itself induce activation of the JNK cascade.

Key words: xPAK1; Ste20p; xMLK2; JNK/SAPK1; Stress kinase cascade; Intracellular signalling

1. Introduction

The response of cells to extracellular signals is mediated in large part by the three mitogen activated protein kinase (MAPK) pathways, each terminating in the activation of either the extracellular signal regulated kinases ERK1 and 2 or the stress activated protein kinases SAPK1/JNK (jun N-terminal kinase) and SAPK2/p38/Hog [1,2]. The activated MAPKs regulate gene expression, providing appropriate cellular responses to extracellular signals. The JNK pathway has been shown to be important for Wnt signaling during early development and to mediate responses to cytokines and artificially induced stresses caused by UV irradiation or poisons such as arsenite and anisomycin. While many of the components of the JNK pathway have been identified, the manner in which the signal is transduced from the membrane receptor still remains elusive. The Rac/Cdc42 family of p21-like GTPases have been shown to induce JNK activation [3,4], suggesting that they could do so in a manner resembling the Ras activation of the ERK cascade [5]. However, the link between Rac/Cdc42 and the activation of JNK also remains obscure.

Each of the MAPK pathways consists of a four-level kinase cascade, the MAPK, a MAPK kinase (MAP2K), a MAPK kinase kinase (MAP3K) and often a MAPK kinase kinase kinase (MAP4K). Cascade modules are probably assembled onto a protein scaffold and may be recruited as such to transduce signals from the plasma membrane to the appropriate MAPK [2]. In the case of JNK, the scaffold protein JIP has been shown to assemble the MAP3K mixed lineage kinase 3 (MLK3), the MAP2K, MKK7, and JNK into a module potentially capable of transducing signals from the MAP4K, HPK1 [6,7]. HPK1, a member of the germinal centre kinase (GCK) subfamily of Ste20/p21-GTPase activated kinase (PAK) related kinases, interacts with growth factor receptors such as EGFR via the Src homology domain 2–3 (SH2–SH3) adapter proteins Grb2 and Nck and can directly phosphorylate MLK3 [8,9]. HPK1 is also necessary for TGFβ dependent activation of JNK [10]. However, the GCKs lack a GTPase binding domain (GBD) or CRIB motif and hence cannot transduce signals from Rac/Cdc42. On the other hand MLK3 does contain a Cdc42 specific GBD [11] and this GTPase induces MLK3 activation in vivo [12].

The Ste20 related PAKs were first identified as Rac/Cdc42 activated kinases [13]. PAK1 was later shown to interact constitutively with the adapter Nck and recruitment of this complex to EGF and PDGF receptors led to its activation [14,15]. Over-expression of the mammalian PAKs can cause JNK activation; however, the data supporting an integral role for PAK in the JNK cascade are equivocal. Brown et al. [16] demonstrated that while over-expression of wild-type (wt) hPAK1 did not activate JNK1 in COS7 cells, a 95-fold constitutively over-activated hPAK1 GBD mutant (L107F) did induce an eight-fold activation of JNK. Similarly, Bagrodia et al. [17] demonstrated that in COS1 cells a GBD mutant mPAK3 (F91S,G93A,P95A) activated hJNK1. Over-expression of wt rPAK1 and 2 in human kidney fibroblasts (293 cells) activated JNK1, but in contrast suppressed the activation of JNK by constitutively active Rac and Cdc42, [18,19], and PAK65 was not essential for JNK activation in NIH3T3 cells [20]. MEKK1, a possible MAP3K of the JNK cascade, has also been shown to be activated during coinfection of insect cells with virus vectors expressing MEKK1 and PAK3. However, this effect was clearly indirect as MEKK1 was not a substrate for phosphorylation by PAK3 [21].

Here we show that while *Xenopus* MLK2 activates JNK in an extracellular signal dependent manner, *Xenopus* PAK1 is unable to activate JNK. However, PAK1 relieved Nck medi-

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Abbreviations: PAK, p21-GTPase activated kinase; MLK, mixed lineage kinase; JNK, jun N-terminal kinase; SH2–SH3, Src homology domain 2–3; Squelching, non-stochiometric expression causing disruption of a multi-protein complex; MAPK, mitogen activated protein kinase; SAPK, stress activated protein kinases

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ated suppression of JNK activation but suppressed MLK2 activation of JNK. The data suggest that PAK1 functions as a scaffold linking the JNK cascade to activated RTKs via Nck.

2. Materials and methods

2.1. Expression vectors

The coding region for amino acids 2–527 of *Xenopus* PAK1 [22] (GenBank accession number AF169794) was HA tagged (MYDVPDYASLPGNSNNAL-) and subcloned *Hin*dIIIGenBank*Xba*I into pCDNA3 (Clontech). The coding region for amino acids 2–1005 of *Xenopus* MLK2; [23] (GenBank accession number AF510499) was also HA tagged (MYDVPDYASLPGNDGLP-) and cloned into pCDNA3 *Hin*dIIIGenBank*Xba*I. Expression vectors for human Nckα and JNK2, (SAPK1β) were kindly provided by J. Schlessinger and by J. Landry.

2.2. Cell culture and transfection

COS7 cells [24] were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. The day before transfection, 3×10^5 cells were plated on 100 mm tissue culture dishes. Two hours before the transfection, medium was changed for fresh medium. A standard $CaPO_4$/chloroquine procedure was used for transfection with a total of 10 μg of expression vector. In cases where combinations of vectors were cotransfected, DNA amounts were equalized by the addition of the empty pCDNA3 vector. Twenty-four hours after transfection, cells were lysed in MGE lysis buffer (20 mM MOPS pH 7.0, 10% glycerol, 0.5 mM EDTA, 5 mM EGTA, 1 mM $NaVO_4$, 5 mM sodium pyrophosphate, 50 mM NaF, 80 mM β-glycerophosphate, 1% Triton X-100, 0.1 mM benzamidine, 0.1 mM dithiothreitol (DTT) and 0.1 mM phenylmethylsulfonyl fluoride (PMSF)) and centrifuged at 17 000 rpm for 10 min. Cleared lysates were normalized for equal protein concentration of HA-JNK and, where appropriate, Nckα, PAK1 and MLK2 by Western blotting. For UV irradiation and EGF treatment, cells were transferred to DMEM without serum and immediately treated either with 100 J m^{-2} of UV irradiation using a UVP UVGL-25 (Ultra-Violet Products) or with 50 ng ml^{-1} human EGF (Life Technologies) in DMEM and harvested after a subsequent incubation of 5 min or of 1 h, as indicated.

2.3. JNK kinase assays

Cell cleared lysates were incubated with 2 μg of glutathione–Sepharose immobilized GST-c-jun for 20 min on ice and then were washed five times with MIKI (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1 mM EDTA, 1 mM EGTA 1 mM $MgCl_2$, 1 mM $NaVO_4$, 1% Triton X-100 and 1 mM PMSF). Kinase assays were performed in MPM buffer (150 μM ATP, 15 mM MOPS pH 7, 5% glycerol, 60 mM *p*-nitrophenylphosphate, 15 mM $MgCl_2$, 1 mM DTT and 0.1 mM PMSF) in the presence of 3 μCi of [γ-^{32}P]ATP and incubated for 30 min at 30°C. The reaction was stopped by adding 6×sodium dodecyl sulfate (SDS) gel loading buffer. Phosphoproteins were separated by Tris–glycine–SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes. ^{32}P incorporation into GST-c-jun was visualized and measured by phosphorimaging (Molecular Dynamics).

2.4. PAK kinase assays

Cell cleared lysates were incubated with 5 μl anti-HA-1 ascites (12CA5) for 60 min on ice. Ten μl of a 50% protein A–Sepharose slurry (Pharmacia) in MIKI was then added and the incubation continued for another 30 min. The slurry was then washed three times in 500 μl MIKI and resuspended in one volume (20 μl) of D2X (50 mM HEPES pH 7.4, 50 mM β-glycerolphosphate, 50 mM $MgCl_2$, 0.2 mM $NaVO_4$, 4 mM DTT, 75 μM [γ-^{32}P]ATP (5000 dpm/pmol)) containing 50 ng of myelin basic protein (MBP) (Gibco BRL) and incubated for 30 min at 30°C. Phosphoproteins were separated on Tris–glycine–SDS–PAGE and transferred to nitrocellulose membranes. ^{32}P incorporation into MBP was visualized and measured by phosphorimaging (Molecular Dynamics).

2.5. MLK-PAK binding assay

MLK2 truncation mutants -Sph, -Afl, -Bam and -Eco were expressed by synthetic mRNA (Ambion) injection into *Xenopus laevis* embryos. Embryos were produced by in vitro fertilization [25]. Two cell embryos in 0.2×MMR (20 mM NaCl, 0.5 mM KCl, 0.2 mM $MgSO_4$, 0.5 mM $CaCl_2$, 1 mM HEPES and 0.02 mM EDTA), 5% Ficoll 400, were injected with 2 ng of MLK2 mRNAs and allowed to develop for 18 h. Gastrula embryos were lysed in MGE lysis buffer and were centrifuged at 30 000 rpm for 30 min. The clear protein phase was recovered and analyzed for expression by Western blotting using the monoclonal antibody 12CA5. Twenty μl glutathione–Sepharose (Amersham Biotech) immobilized full length GST-*Xenopus*-PAK1 fusion protein was incubated with the total protein extract from *X. laevis* embryos expressing *Xenopus* MLK2-Sph, -Afl, -Bam and -Eco. After incubation for 20 min on ice, the Sepharose beads were washed five times with 0.1 M TM (50 mM Tris–HCl pH 7.9, 100 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA and 20% glycerol). Sepharose beads were resuspended in SDS–PAGE loading buffer and bound proteins fractionated on Tris–glycine–SDS gels, transferred on nitrocellulose membranes and probed with the monoclonal antibody 12CA5 and developed using a HRP coupled second antibody (Amersham Biotech) in conjunction with the 'Renaissance' ECL reagent kit (Roche). Alternatively, MLK2-SK and -KZG mutants were expressed by in vitro translation (Promega) and 10 μl of the extract incubated with 1 μg of immobilized GST-CDPAK1 or GST alone for 2 h at 4°C in 50 μl of phosphate buffered saline (PBS), 1% Triton X-100. The beads were washed four times with 0.5 ml PBS, 1% Triton X-100, resuspended in SDS–PAGE loading buffer and bound proteins fractionated on Tris–glycine–SDS gels. Gels were fixed and dried and analyzed by phosphoimaging (STORM 860, Molecular Dynamics).

3. Results

3.1. JNK is not activated by PAK1 expression

Consistent with previous studies on mammalian PAKs [19,20,26], expression of wt and both activated and kinase dead mutants of *Xenopus* PAK1, Fig. 1A,B, in COS7 cells did not significantly affect the basal levels of JNK activity, Fig. 1C. Wt PAK1 also did not enhance activation of JNK by UV irradiation (or sodium arsenite, data not shown), while activated PAK1s L98F and T405E actually suppressed UV activation of JNK (up to 30%), and the kinase dead K281A showed a slight enhancement effect.

3.2. Rapid Nck dependent activation of xPAK1 by EGF but not by UV stress

Maximal JNK activity typically occurs 30 to 60 min after UV (or arsenite) treatments, suggesting that JNK activation is not a direct event. However, both UV stress and EGF treatment induce a rapid and direct, albeit weaker, activation of JNK and PAK1 recruitment to the activated EGF receptor via Nck leads to its rapid activation, [14,15,27]. We, therefore, investigated whether the rapid phase of JNK activation coincided with PAK1 activation. Wt and mutant PAK1s were transfected with or without Nck and cells then treated with EGF, UV, or a combination of both and harvested just 5 min later, Fig. 2A. As expected, EGF induced a rapid, five-fold increase in PAK1 activity which was dependent on Nck overexpression. In contrast, UV did not detectably activate PAK1 and EGF and UV treatments did not cooperate in PAK1 activation.

3.3. PAK1 can relieve Nck suppression of the JNK pathway

Fig. 2B shows the relative levels of JNK activation 5 min after a mock treatment, EGF treatment or simultaneous UV and EGF treatments. In the absence of both PAK1 and Nck, EGF induced JNK activation and the combined EGF and UV treatments were found to cooperate in a 'super-activation'

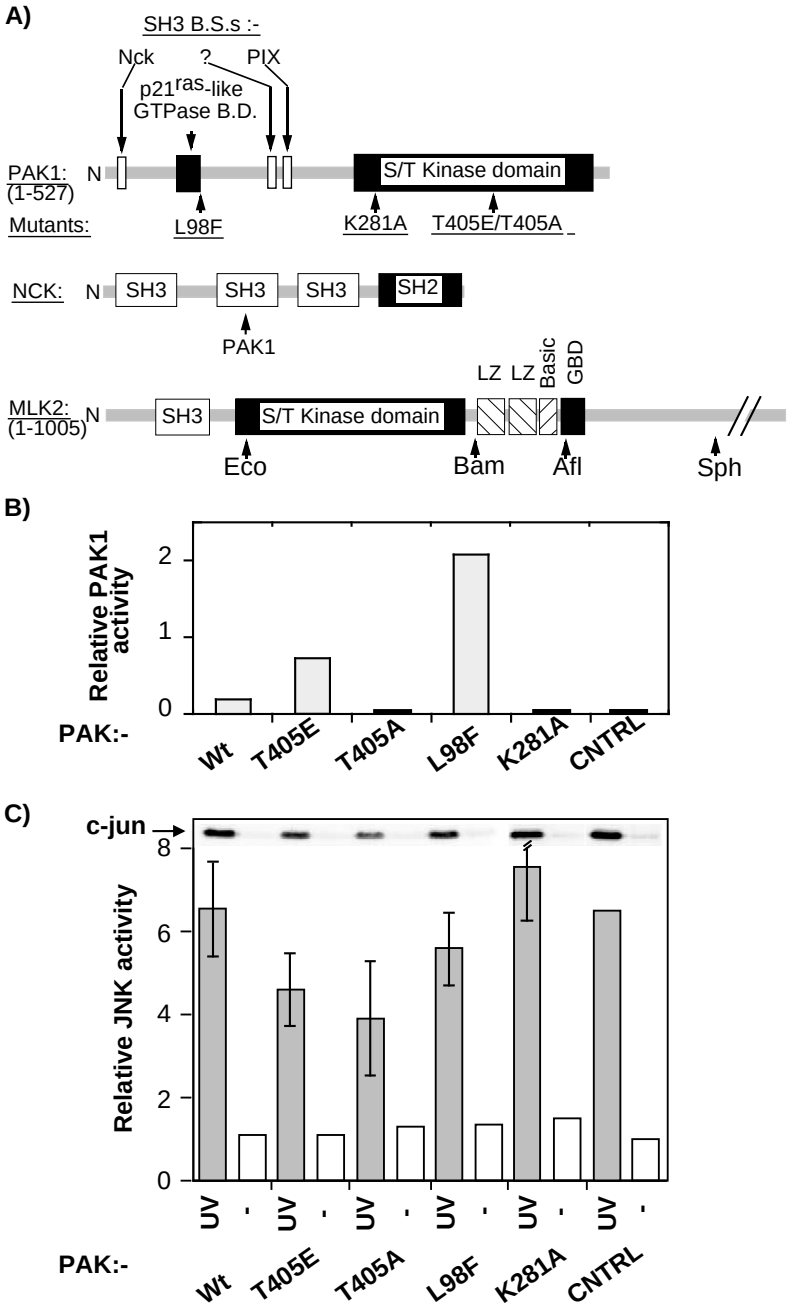

Fig. 1. A: Schematic structure of xPAK1, Nck and xMLK2. xPAK1 schematic shows the positions of known or potential SH3 domain binding sites (SH3 BS) (white boxes) and the p21ras-like GBD (black box). The position of point mutations are indicated (mutants). xPAK1 L98F and T405E are constitutively active mutants, K281A and T405A are kinase inactive mutants. Nck contains three SH3 domains and a SH2 domain. The mixed lineage kinase, xMLK2, contains an N-terminal SH3 domain, a serine/threonine kinase domain, two LZ motifs, a basic region and a GBD. B: Intrinsic activity of the various PAK1 mutants used in this study. C: Activity of xPAK1 does not correlate with JNK activation in COS7 cells. JNK activity was measured in extracts prepared from cells transfected with xPAK1 mutants and irradiated (UV), or not irradiated (−), for 5 s with UV light 1 h before preparation of protein extracts. Expression levels of JNK and PAKs were determined by Western blotting and shown not to vary between transfections. The data represent the results of three independent experiments, each performed in duplicate.

of JNK. When PAK1 was transfected a small enhancement in JNK activity after EGF and EGF/UV treatments was observed ($<20\%$), despite PAK1 not being detectably activated under these conditions, Fig. 2A. Quite unexpectedly, we found that the adapter Nck strongly suppressed JNK activation, Fig. 2B. Nck expression reduced EGF and EGF/UV activation of JNK respectively by 60% and 80%. Nck over-expression was also shown to suppress signaling in other systems [28,29]. The effect is believed to be due to non-stochiometric saturation of upstream or downstream signaling components, a phenomenon which can be likened to 'squelching' (non-stochiometric expression causing disruption of a multi-protein complex) in gene regulation [30].

More surprisingly, PAK1 relieved Nck squelching of JNK activation, Fig. 2B, and re-established the response of JNK to both EGF and EGF plus UV. Though constitutively activated

PAK1s T405E or L98F also relieved Nck squelching, they were no more effective than wt PAK1. Under the conditions in which PAK1 relieved Nck squelching of JNK activation, PAK was also functionally linked to the EGF receptor by Nck. Thus PAK cannot have alleviated Nck squelching by sequestering Nck in an inactive state. Thus despite PAK1's inability to activate JNK, it still plays an integral role in the JNK pathway, probably one that does not require catalytic activity.

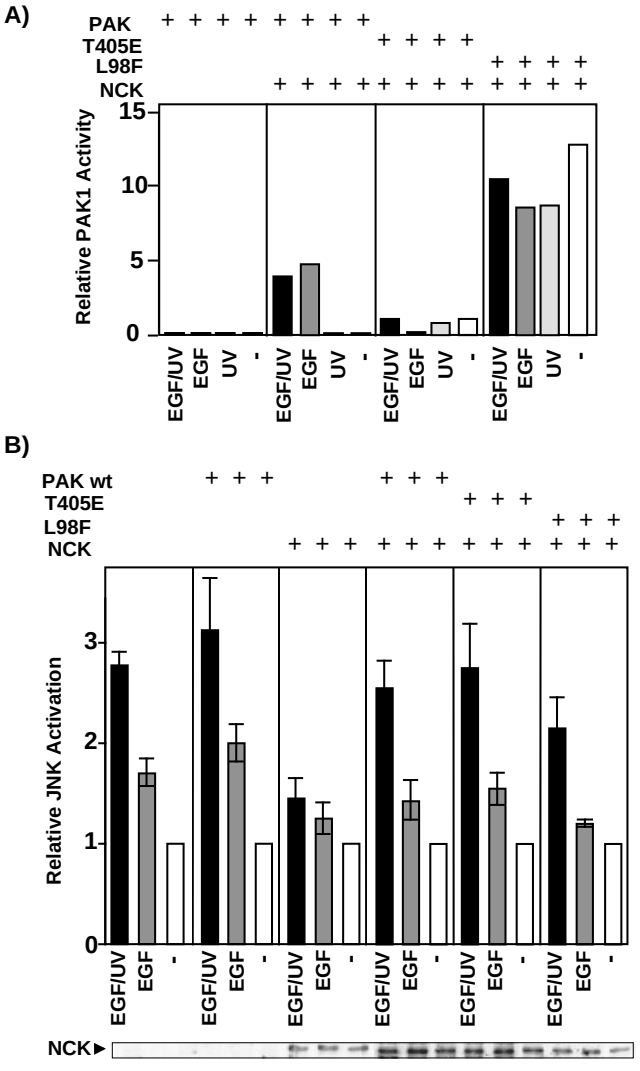

Fig. 2. A: PAK1 rapidly responds to EGF but not to UV stimulation in a Nck dependent manner. Cells transfected with combinations of PAK1, PAK1 mutants T405E or L98F and Nck were treated with UV, EGF or concurrently given both treatments 5 min before preparation of extracts. PAK1 activity was measured using MBP as substrate, see Section 2. B: Nck expression squelches the rapid response of JNK to EGF and EGF plus UV but is counteracted by coexpression of xPAK1. Cells transfected with combinations of xPAK1, xPAK1 mutants T405E, L98F and Nck were treated with UV, EGF or both concurrently 5 min before preparation of extracts. JNK activities were normalized to those for the unstimulated cells. Expression levels of Nck are shown, but those of JNK and PAKs were also determined by Western blotting and shown to be equivalent in the different transfections. The data represent the results of three independent experiments, each performed in duplicate.

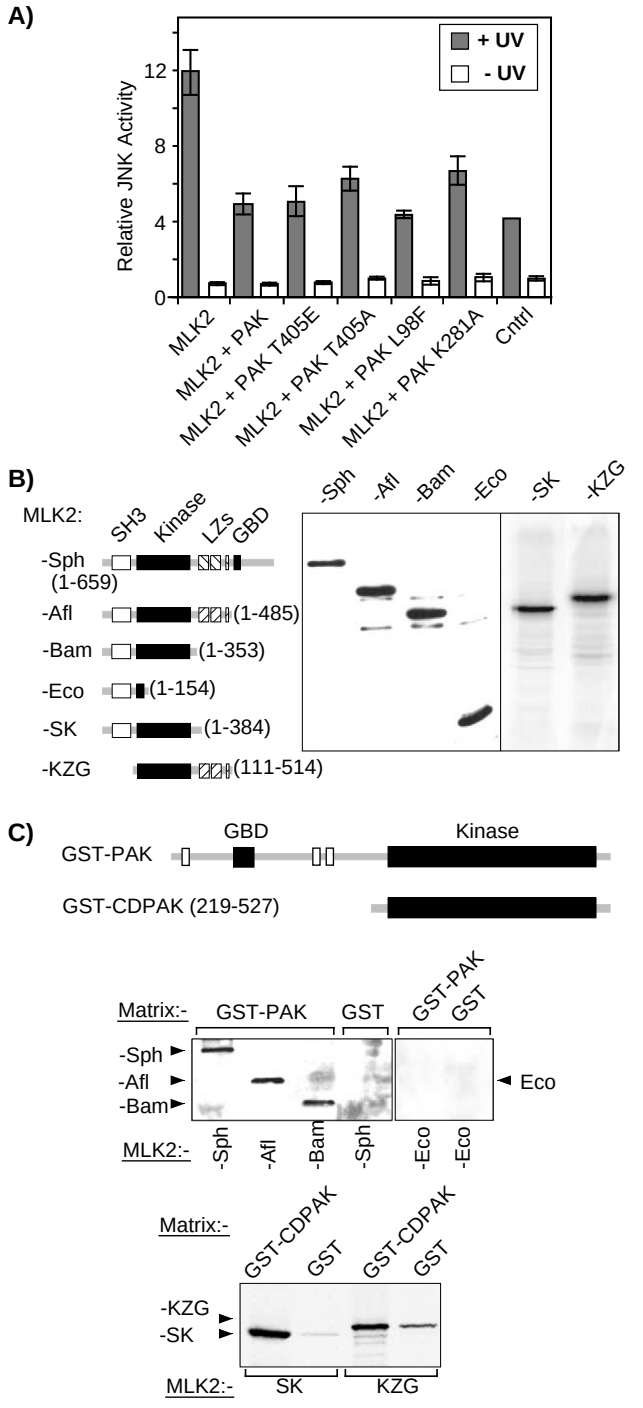

Fig. 3. PAK1 interacts with MLK2 and squelches super-activation of JNK. A: Cells were transfected with combinations of MLK2 and PAK1 or PAK mutants, Fig. 1A, and were treated with UV as in Fig. 1. The data represent the mean of four independent experiments, each performed in duplicate. B: Schematic representation of MLK2 truncation mutants and their expression in whole embryo extracts. MLK2 proteins were visualized by immunoblotting via the common N-terminal HA tag. C: Interaction of MLK2 with PAK1. Whole embryo extracts expressing MLK2 truncation mutants (upper panel) or in vitro translated mutants (lower panel) were incubated with 1 μg of immobilized full length PAK1 (GST-PAK1), the catalytic domain of PAK1 (GST-CDPAK) or GST alone and bound MLK2 was visualized either by immunoblotting using an anti-HA antibody (upper panel) or phosphoimaging (lower panel).

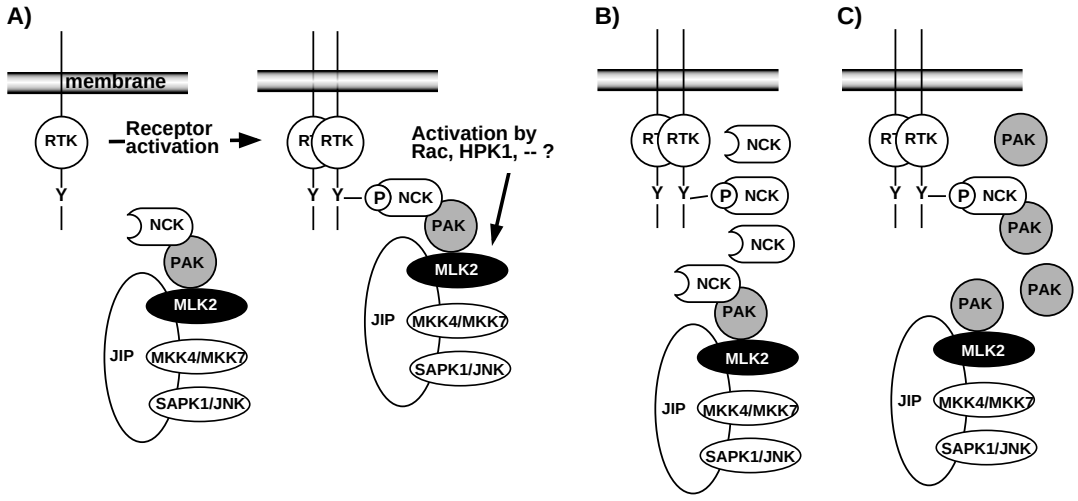

Fig. 4. PAK1 mediates MLK2 recruitment to an activated receptor, see text for discussion. A: Consistent with the data a Nck and PAK complex binds MLK2 and hence the JNK cascade consisting of the JIP scaffold protein, MLK2, MKK4/MKK7 and JNK. Recruitment of the cascade to an activated membrane receptor would localize MLK2 on the plasma membrane where it could be activated by its known upstream effectors HPK-1, or other GCK family kinases, and Rac GTPase. B,C: The model in A predicts that as observed, Nck or PAK over-expression would prevent direct interaction of the JNK cascade with an activated receptor and thus preventing JNK activation.

3.4. PAK1 interacts with MLK2 and regulates its ability to activate JNK

Xenopus MLK2 was recently shown to be a true ortholog of mammalian MLK2 and to function as a MAP3K for JNK, [23]. Low-level over-expression of the *Xenopus* MLK2 did not constitutively activate JNK, rather it enhanced the response of JNK to stress signaling, thus simulating the probable in vivo function of MLK in signal transduction. In contrast, previous work had described the constitutive activation of JNK by high-level over-expression of hMLK2 and hMLK3 [31–34]. However, such high-level over-expression probably led to auto-activation of MLK via its homo-dimerization [35], potentially explaining the apparent contradiction with our recent data [23]. We also observed that during *Xenopus* development PAK1 is often coexpressed with one or other member of the MLK family, (S. Jean and N. Islam, unpublished data). We therefore asked whether PAK1 might play a role in modulating MLK2 activation of JNK.

MLK2 and both wt and mutant PAK1s were cotransfected into COS7 cells and the levels of JNK activation monitored before and after UV treatment. Transfection of MLK2 enhanced UV activation of JNK by three-fold, Fig. 3A. However, all forms of PAK1 inhibited the MLK2 dependent activation of JNK and in several cases reduced it essentially to the control levels, Fig. 3A. Wt PAK1 and the constitutively activated T405E and L98F were somewhat more effective at inhibiting JNK activation than the dominant negative xPAK1-T405A and -K281A, suggesting that in part this effect required PAK1 catalytic activity or perhaps a specific conformation of the catalytic domain. We have also observed that the K281A mutation significantly increases the affinity of xPAK1 for the GTP bound form of Cdc42, suggesting that inhibition of JNK activation was not due to a squelching of upstream GTPases (L. Poitras and N. Bisson, unpublished data).

Whole protein extracts from *Xenopus* embryos expressing MLK2 deletion mutants, Fig. 3C, were incubated with immobilized GST-PAK1 and the retained proteins analyzed. As shown in Fig. 3C, MLK2-Sph, -Afl and -Bam C-terminal deletion mutants all bound specifically to PAK1, while MLK2-Eco, containing only the SH3 domain, did not detectably bind. Thus, the interaction did not require the GBD or leucine zipper (LZ)/basic domains of MLK2, nor was it mediated by its SH3 domain. It did, however, require the MLK2 kinase domain. The PAK1 kinase domain alone was also able to retain the MLK2 truncation mutant -SK, closely similar to -Afl, and the mutant -KZG lacking the N-terminal SH3 domain and all sequences downstream of the basic domain. Thus, in large part the interaction between MLK2 and PAK1 occurred between their kinase domains. Further experiments confirmed that peptides containing the N-terminal SH-3 binding sites of PAK1, see Fig. 1A, did not interact with the SH3 domain of MLK2 (data not shown). MLK2-Sph and -Afl, which both contained a complete kinase domain and LZ, bound somewhat better to PAK1 than did MLK2-Bam, which lacked the LZ motif, Fig. 3C. This suggested that dimerization of xMLK2 might play a role in the interaction between these kinases, possibly by permitting a cooperative interaction of the PAK1 kinase domain with the kinase domains of an MLK2 dimer. Thus PAK1 and MLK2 interact both physically and functionally.

4. Discussion

We have shown that while *Xenopus* PAK1 is unable to function as a MAP4K to activate JNK in COS7 cells, it nonetheless is a component of the JNK signaling cascade. Our evidence is two-fold. Firstly, squelching of the immediate phase of JNK activation by the adapter Nck is alleviated by PAK1 expression. Secondly, PAK1 interacts with the MAP3K MLK2 and suppresses its capacity to activate JNK.

Over-expression of the SH3–SH2 adapter Nck was shown to suppress JNK activation. Similarly, Nck over-expression has been shown to effectively act as a dominant negative mutation in other systems [28,29]. This is probably due to non-stochiometric expression of Nck relative to the other compo-

nents of a signal transduction pathway, e.g. see Fig. 4B, and as such closely resembles 'squelching', an effect in which an excess of a transcriptional activator represses gene transcription by titrating essential coactivators [30]. Strikingly, when PAK1 was coexpressed with Nck it relieved squelching of JNK activation. This was not simply due to a sequestration of Nck into an inactive complex, since at the same time activation of PAK1 by EGF was completely dependent on Nck, though, it could equally be explained by PAK1 being able to displace a factor essential for JNK activation from Nck.

MLK2, [23] mediates a super-activation of JNK in response to UV stress. But in contrast to its palliative effects on Nck squelching, PAK1 squelched MLK2 super-activation of JNK. The effect was in greater part independent of PAK1 kinase activity, though activated forms of PAK1 suppressed the effects of MLK2 somewhat better than did inactivated forms. MLK2 and PAK1 were also shown to interact via their kinase domains, suggesting that PAK1 squelching of JNK super-activation was due to direct interaction with MLK2, e.g. see Fig. 4C. More distantly related members of the Ste20 kinase family such as the GCK subfamily kinase HPK1 also bind MLKs [36,37], though in these cases the interactions do not involve the kinase domains. Thus, the ability to bind MLKs may be a more general property of Ste20 family kinases.

Our data are consistent with a novel non-catalytic function for PAK1 as a scaffolding element in a signal transduction network linking tyrosine kinase receptors to the MLK-JNK cascade, Fig. 4A. In this network, activated receptors could recruit the JNK cascade to the plasma membrane via PAK1 and MLK2. This recruitment would allow the activation of MLK2 by the intervention of the membrane tethered small GTPase Rac or GCK subfamily kinases such as HPK1. In this model, PAK1 would function predominantly as a scaffolding protein. Indeed, the PAKs have been shown to display kinase independent effects [38]. Further, the schematic model in Fig. 4 resembles the bifurcated dorsal closure pathway in *Drosophila* [39]. This pathway, which leads to activation of DMLK (Slipper) and hence DJNK, passes via parallel DRac/DPAK and GCK kinase (misshapen, MSN) dependent branches [40].

Since their discovery, the PAKs have provided a potential but elusive link between the Rho GTPases Rac and Cdc42 and the JNK cascade. However, it is becoming more and more clear that the major phosphorylation substrates of the PAKs do not lie within these cascades [41]. Further, while the PAKs can be activated by Rac and Cdc42, they have also been found to suppress the Rac/Cdc42 dependent activation of JNK [18,19]. This is clearly inconsistent with a role for PAK1 as a MAP4K. Despite this, a range of studies have in fact implicated the PAKs in the modulation of JNK or p38 activation [16–18]. Our data suggest that at least in part these studies, in all of which PAKs have been over-expressed, may be the result of squelching events such as those we describe here.

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