Synergistic activation of JNK/SAPK by interleukin-1 and platelet-derived growth factor is independent of Rac and Cdc42

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The c-Jun N-terminal kinases (JNKs) are activated strongly by inflammatory cytokines and environmental stresses, but only weakly by growth factors. Here we show that platelet-derived growth factor (PDGF) strongly potentiates activation of JNK by interleukin 1 (IL-1) in human fibroblasts and a pig aortic endothelial (PAE) cell line. This synergistic activation of JNK by IL-1 and PDGF was unaffected by bacterial toxins that inactivate Rho proteins and Ras. Since Rho proteins have been implicated in JNK activation, their possible involvement was investigated further using stably expressed, inducible N17 or V12 mutants in PAE cell lines. N17 Rac non-selectively reduced JNK activity by 30% in resting or stimulated cells (IL-1 alone, or with PDGF). N17 Cdc42 had no effect. V12 Rac weakly activated JNK and synergized with IL-1, but not with PDGF. V12 Cdc42 weakly activated JNK, but synergized with PDGF and not IL-1. Our results imply that Rho GTPases are not directly involved in mediating IL-1-induced JNK activation, or in the potentiation of this activation by PDGF.

Key words: lethal toxin, mitogen-activated protein kinase, Ras, Rho GTPases, toxin B.

INTRODUCTION

Inflammatory stimuli such as the cytokines interleukin 1 (IL-1) and tumour necrosis factor (TNF) and cellular stresses activate similar sets of protein kinases. Prominent among these are the c-Jun N-terminal kinases (JNKs), also known as stress-activated protein kinases (SAPKs), which are members of the mitogen-activated protein kinase (MAPK) family. JNKs are one of three distinct sub-groups of the family, in which the motif phosphorylated by activating MAPK kinases (MKKs) comprises Thr-Pro-Tyr [1].

Mammalian JNKs are the products of three distinct genes (human JNK 1, 2 and 3, equivalent to rat SAPK γ, α and β respectively) from which a total of ten different cDNAs have so far been shown to arise [2]. Each JNK may be expressed as either a full-length (54 kDa) protein or as a C-terminally truncated (46 kDa) form which arises from differential mRNA splicing [1]. Additional splice variants of JNK1 and JNK2 (but not JNK3) are generated by use of an alternative exon within the kinase domain [2]. The individual JNK proteins are highly homologous [1] and the physiological significance of the existence of so many isoforms is not understood. JNKs phosphorylate and activate transcription factors, including activating transcription factor-2 (ATF-2) and c-Jun [2,3], and are involved in regulating expression of genes controlled by these factors [4].

Generally JNKs are activated strongly by the cytokines IL-1 and TNF, but only weakly by growth factors such as epidermal growth factor (EGF), or by PMA [5,6]. EGF and PMA strongly activate the p42/p44 [or extracellular signal-regulated kinase (ERK)] MAPK pathway [7,8]. IL-1 activates the p42/p44 MAPKs, JNKs and the third sub-type, p38 MAPK, in fibroblasts [9]. However, only JNK is activated in tissues of animals after IL-1 injection [9,10], suggesting that the JNK pathway may be of particular importance for IL-1 regulation of inflammatory genes. JNKs are activated by specific MKKs. MKK4 and MKK7 have both been identified as potential JNK kinases [4]. *In vivo*, IL-1 utilizes MKK7, which has recently been identified as the IL-1-activated JNK kinase in rabbit liver [9].

The upstream mechanisms involved in activation of the JNK pathway are not clearly understood. A role for Rho subfamily GTPases has been suggested by experiments involving over-expression of dominant negative (N17) mutants of Rac and Cdc42. N17 Rac diminished levels of active JNK in cells treated with TNF or EGF [11,12], and N17 Cdc42 had similar effects on cells treated with IL-1 [13]. These investigators also noted that over-expression of constitutively active mutants of Rac and Cdc42 caused activation of the JNK pathway. Their conclusions, that IL-1 and TNF signalling to JNK may involve Rac and Cdc42, are consistent with early studies in which GTP binding to cell membrane proteins increased after treatment with IL-1 or TNF [14,15].

In experiments aimed at identifying agonists that modulate IL-1-induced JNK activation, we have found that platelet-derived growth factor (PDGF), although itself only a weak activator of the JNK pathway, strongly synergized with IL-1. PDGF is a known physiological activator of Rac [16], and this prompted us to examine the potential role of Rho proteins in potentiation of IL-1-induced JNK activation by the growth factor. Results of these studies have also led to a re-appraisal of the role of Rho proteins in JNK activation by IL-1 itself.

EXPERIMENTAL

Materials

Human IL-1α was made as described [17]. [γ-^{32}P]ATP (Redivue), X-ray film (Hyperfilm) and PDGF BB were from Amersham International (Slough, U.K.). Puromycin hydrochloride and

Abbreviations used: ATF-2, activating transcription factor-2; CMV, cytomegalovirus; EGF, epidermal growth factor; ERK extracellular signal-regulated kinase; FCS, fetal calf serum; GST, glutathione S-transferase; IL-1, interleukin 1; IPTG, isopropyl β-D-thiogalactoside; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MKK, MAPK kinase; PAE cell, pig aortic endothelial cell; PDGF, platelet-derived growth factor; SAPK, stress-activated protein kinase; TNF, tumour necrosis factor.

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hygromycin B were from Calbiochem UK (Nottingham, U.K.). Fetal calf serum (FCS), fatty acid-free BSA, Protein A–agarose, myelin basic protein (MBP) and DMSO were from Sigma (Poole, Dorset, U.K.). Culture media were from BioWhitaker (Wokingham, U.K.). Isopropyl β-D-thiogalactoside (IPTG) was from the Alexis Corporation (Nottingham, U.K.). Lethal toxin and toxin B were prepared by M. Popoff (Institut Pasteur, Paris, France) and were kindly supplied by L. O'Neill (Trinity College Dublin, Republic of Ireland). cDNA for human glutathione S-transferase–ATF-2 (GST–ATF-2) (18–96) was a gift from N. Jones (ICRF, London, U.K.) and was expressed in *Escherichia coli*. Rabbit antiserum to JNK was to the synthetic peptide GVVKGQPSPSAQVQQ (amino acids 370–384 of SAPK β). This antibody immunoprecipitated the main peak of JNK activity identified by anion-exchange chromatography of pig aortic endothelial (PAE) cell lysates. It is not specific to any JNK isoform (results not shown). Two rabbit antibodies to p42 MAPK were used: serum raised against the synthetic peptide EETARFQPGYRS was a gift from C. Marshall (ICR, Chester Beatty Laboratories, London, U.K.); and affinity-purified antibody to an amino acid sequence mapping within the C-terminus of rat ERK-2 was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.)

Cell culture

MRC-5 fibroblasts were maintained in Dulbecco's modified Eagle's medium with 10% FCS. PAE cells expressing wild-type PDGF β receptor [18] were maintained in Ham's F12 with 10% FCS. PAE cells express no endogenous receptors for PDGF [19]. PAE cells expressing N17 or V12 Rac (clones 32 and 84 respectively) under the control of an IPTG-inducible cytomegalovirus 3 (CMV3) promoter were made as described [20] from a clone (P23) expressing wild-type PDGF β receptor and lac repressor protein. PAE cells expressing N17 or V12 Cdc42 were made using a similar strategy. The N17 or V12 Cdc42 cDNAs were subcloned into the pCMV3 hygro expression vector with an in frame N-terminal EE tag [20] using standard PCR and subcloning strategies. The plasmids were sequenced to verify in-frame cloning of the desired constructs and were transfected into PAE clone P23. Stable cell lines were isolated after a round of selection (hygromycin B, 100 μM). Screening for inducible expression of Cdc42 mutants was done using anti-EE immunoprecipitates from [^{35}S]methionine-labelled cells (0.14 mCi of ICN 'Trans Label' reagent per 25 cm^2 flask in methionine- and cysteine-free F12 medium) treated with or without 15 mM IPTG for 16 h [20]. A number of clones showing strong induction from a low basal expression were expanded and stored. Two clones, 26 expressing N17 Cdc42 and 14 expressing V12 Cdc42 were used in subsequent experiments. The levels of mutant Cdc42 protein observed in these clones after 16 h induction with 15 mM IPTG (shown in Figure 1) were comparable with those reported for the Rac expressing clones [20]. Clones expressing mutant GTPases (32, 84, 26 and 14) were maintained in Ham's F12 with 10% FCS containing 500 nM puromycin/HCl and 100 μM hygromycin B. All cells were grown to confluence (75 cm^2 flask per point). Cells were serum-starved in medium containing 0.1% fatty acid-free BSA for 16 h before experimentation; where appropriate, expression of mutant GTPases was induced in PAE clones by including IPTG (15 mM). Where indicated (see Results section), cells were then pre-incubated for 60–120 min with lethal toxin (400 ng/ml) or toxin B (400 ng/ml) in medium containing 0.1% fatty acid-free BSA and 10 mM Hepes, pH 7.4. Cells were then treated (in the same medium) with IL-1 (20 ng/ml) or PDGF (75 ng/ml), alone or in combination, for 10 min at 37 °C.

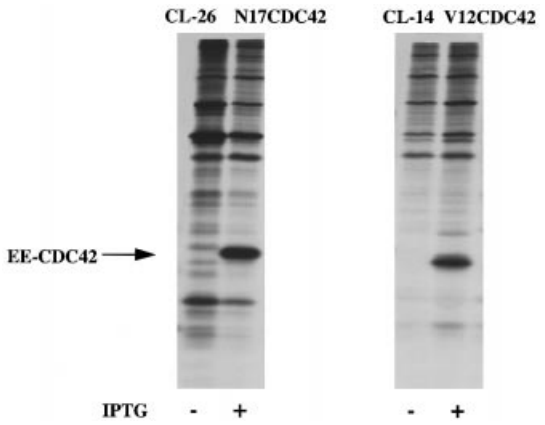

Figure 1 Inducible expression of Cdc42 mutants in PAE cell lines

Clone 26 (N17 Cdc42) and clone 14 (V12 Cdc42) were made as described in the Experimental section. An autoradiogram of anti-EE immunoprecipitates from [^{35}S]methionine-labelled cells, either treated with 15 mM IPTG for 16 h, or not treated, and separated by SDS/PAGE is shown. The positions of EE-Cdc42 proteins are indicated by arrows (apparent molecular mass approx. 25 kDa).

Immunoprecipitation

Cells were lysed in RIPA buffer {20 mM Hepes, pH 7.4/20 mM β-glycerophosphate/0.5 M EDTA/0.5 EGTA/0.1 mM Na_3VO_4/0.2 M NaCl/2 mM PMSF/10 μM E-64 [*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane]/2 μg/ml aprotinin/1% Nonidet P40/1% sodium deoxycholate/0.1% SDS}. Lysates were microfuged at 16000 ***g*** for 15 min at 4 °C. Antiserum (10 μl) and 30 μl of Protein A–agarose were added to each cleared lysate (corrected to 0.1 mg of total protein in a volume of 1 ml). Samples were agitated for 2 h at 4 °C and the beads were washed three times in RIPA buffer and twice in kinase buffer (25 mM Hepes, pH 7.4/25 mM β-glycerophosphate/25 mM $MgCl_2$/0.1 mM Na_3VO_4/0.1% 2-mercaptoethanol).

Kinase assay

Pellets were resuspended in 40 μl of kinase buffer containing 50 μg/ml GST-ATF-2 (JNK immunoprecipitates) or MBP (ERK immunoprecipitates). The kinase reaction was started by addition of a further 10 μl of kinase buffer containing 100 μM ATP and 0.5 μCi/μl [γ-^{32}P]ATP. Samples were shaken for 20 min at 20 °C, the assay was stopped and phosphorylated substrate was visualized on a PhosphorImager (Fuji FLA2000), and by autoradiography, following SDS/PAGE. All experiments were performed at least three times; a representative autoradiograph from each series is presented in Figures 2–6.

RESULTS

PDGF synergizes with IL-1 in activation of JNK

As observed in many other cell types, JNK was strongly activated by IL-1 (20 ng/ml) in both a primary human fibroblast line (MRC-5, Figure 2a, lane 2) and in a transformed PAE cell line expressing a transfected PDGF β receptor (Figure 2b, lane 2). In contrast, activation of JNK by PDGF (75 ng/ml) alone in either

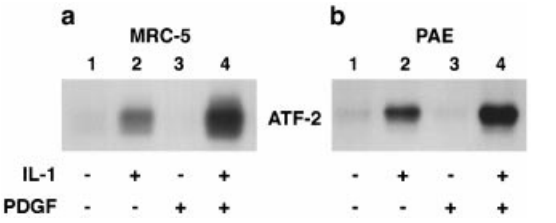

Figure 2 IL-1 and PDGF synergistically activate JNK

MRC-5 (**a**) or PAE cells expressing wild-type PDGF β receptor (**b**) were treated for 10 min with IL-1 (20 ng/ml), PDGF (75 ng/ml) or both as indicated. JNK activity was measured by immune complex assay using [γ-^{32}P]ATP and GST–ATF-2 (18–96) as substrate. Products were visualized by autoradiography and quantified on a PhosphorImager after SDS/PAGE.

cell type was weak (Figures 2a and 2b, lanes 3). PDGF, however, was found to potentiate JNK activation by IL-1 in both cell lines (Figures 2a and 2b, lanes 4). The additional presence of PDGF both prolonged and enhanced maximal IL-1-stimulated JNK activation (results not shown). A 2-fold enhancement of the signal (compared with that with IL-1 alone) was reproducibly observed at 10 min in PAE cells over many experiments. In comparison, human fibroblasts showed a 3-fold enhancement at 10 min. Although maximal JNK activation was not observed until between 15 and 20 min (both with IL-1 alone, and with IL-1 and PDGF), the potentiating effect of PDGF was less pronounced and more variable at these time points (results not shown). The 10 min time point was therefore used subsequently to investigate the synergy between IL-1 and PDGF. PAE cells were used in preference to fibroblasts to investigate the signalling pathways involved, because their rapid growth conveniently provided sufficient cells for the experiments, and because PAE lines expressing dominant negative and constitutively active Rac [20] were available.

JNK activation by IL-1 and PDGF is unaffected by *Clostridium* toxins

Toxins from species of *Clostridium* bacteria that specifically inactivate Rho subfamily GTPases (in addition to *C. botulinum* C3 toxin, which has been used as an inhibitor of Rho itself for some years) have recently been isolated and characterized [21]. These include toxin B from *C. difficile*, which specifically glucosylates and inactivates all Rho subfamily GTPases [22], and lethal toxin from *C. sordellii*, which has a broader specificity and will glucosylate Ras and some Rap and Ral proteins as well as Rho [23]. We have used these two toxins to investigate the possible dependence of JNK activation by IL-1 and PDGF on small GTPases. After pre-incubation of PAE cells with toxin B for 120 min, morphological changes consistent with inactivation of Rho (rounding up of cells, and some loss of attachment) were clearly visible, indicating that the cells were sensitive to the action of this toxin (results not shown). Some loss of attachment was also observed in cells incubated with lethal toxin for 120 min. However, cells still responded normally to IL-1 administration by strong activation of both JNK (Figure 3a, lanes 6 and 10) and ERK (Figure 3b, lanes 6 and 10). ERK activation by PDGF (alone, or in combination with IL-1) was strongly (although not completely) inhibited by lethal toxin (Figure 3b, lanes 7 and 8), but not by toxin B (Figure 3b, lanes 11 and 12), consistent with Ras inactivation by lethal toxin but not toxin B. Levels of active JNK in IL-1-stimulated cells were enhanced by toxin B treatment (Figure 3a, lanes 10 and 12); quantification by PhosphorImager revealed that levels in unstimulated and PDGF-stimulated cells were also increased (see legend to Figure 3). Neither toxin inhibited potentiation of IL-1-induced JNK activation by PDGF (Figure 3a, lanes 8 and 12). These results therefore suggest that JNK activation by IL-1 and PDGF (alone or in combination) is independent of Rho family GTPases, and also independent of the other GTPases inactivated by lethal toxin, including Ras.

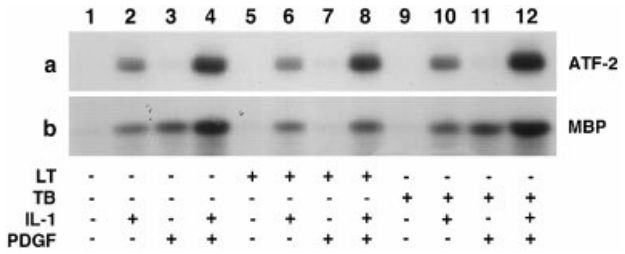

Figure 3 JNK activation by IL-1 and PDGF is unaffected by *Clostridium* toxins

PAE cells expressing wild-type PDGF β receptor were treated for 10 min with IL-1 (20 ng/ml), PDGF (75 ng/ml) or both, after a 2 h pre-incubation with lethal toxin (400 ng/ml), toxin B (400 ng/ml) or vehicle, as indicated. JNK activity (**a**) was measured as in Figure 2. ERK activity (**b**) was measured by immune complex assay using [γ-^{32}P]ATP and MBP as substrate. The average JNK activity increase in the presence of toxin B for each treatment (over four experiments) was: 95% for control, 28% for IL-1, 79% for PDGF, 41% for IL-1 and PDGF. The average fold ERK activation by PDGF (over five experiments) was 11.8 ± 3.8 in the absence of lethal toxin, and 3.1 ± 1.0 after treatment with lethal toxin.

JNK activation by IL-1 and PDGF is not specifically inhibited by N17 Rac or by N17 Cdc42

The conclusion that IL-1 activates JNK independently of Rho family GTPases appears to contrast with some previous reports which, on the basis of transient transfection of dominant negative mutants of Rac and Cdc42, have suggested that Rho family GTPase activation may be required for JNK activation by pro-inflammatory cytokines [11,12], and specifically that IL-1 may activate JNK via Cdc42 [13]. We have investigated further the dependence of JNK activation by IL-1 on Rho GTPases using the same mutants stably transfected into PAE cells under the control of an IPTG-inducible CMV3 promoter [20]. Induction of N17 Rac expression in PAE clone 32 resulted in an approx. 30% reduction in JNK activity in cells treated with IL-1 (Figure 4a, lanes 2 and 6), or in those treated with a combination of IL-1 and PDGF (Figure 4a, lanes 4 and 8). A similar reduction in JNK activity in resting cells (Figure 4a, lanes 1 and 5) and those treated with PDGF alone (Figure 4a, lanes 3 and 7) was also caused by induction of N17 Rac. Thus, whereas induction of N17 Rac expression reduces levels of activated JNK in these cells, it does not specifically inhibit the activation of JNK by IL-1. It should be noted that PDGF-induced membrane ruffling in clone 32 is essentially completely inhibited after overnight incubation with 15 mM IPTG [20], showing that sufficient N17 Rac is induced to act as a dominant negative in blocking signalling pathways downstream of Rac. Induction of N17 Cdc42 expression in PAE clone 26 had no effect on levels of activated JNK in either unstimulated cells (Figure 4b, lanes 1 and 2) or those treated with IL-1 (Figure 4b, lanes 3 and 4), PDGF (Figure 4b, lanes 5 and 6) or with the two agonists in combination (Figure 4b, lanes 7 and 8). This suggests that both IL-1 and PDGF signal to the JNK pathway independently of Cdc42.

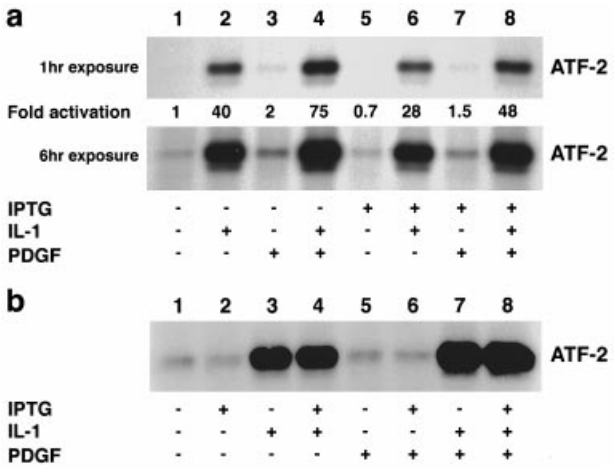

Figure 4 JNK activation by IL-1 and PDGF is not specifically by N17 Rac or N17 Cdc42

PAE cells clone 32 (**a**) or clone 26 (**b**) (expressing N17 Rac or N17 cdc42 respectively, under the control of an IPTG-inducible CMV3 promoter) were treated for 10 min with IL-1 (20 ng/ml), PDGF (75 ng/ml) or both, after a 16 h pre-incubation with IPTG (15 mM) where indicated. JNK activity was measured as in Figure 2. In (**a**) two autoradiographs from the same gel, a 1 h exposure and a 6 h exposure, are shown, and fold-activation is indicated for each treatment.

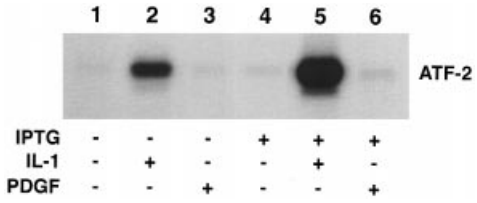

Figure 5 V12 Rac synergizes with IL-1 in activation of JNK

PAE cells (clone 84, expressing V12 Rac under the control of an IPTG-inducible CMV3 promoter) were treated for 10 min with IL-1 (20 ng/ml) or PDGF (75 ng/ml), or both, after a 16 h pre-incubation with IPTG (15 mM) where indicated. JNK activity was measured as in Figure 2.

V12 Rac synergizes with IL-1 in activation of JNK

Induction of V12 Rac in PAE clone 84 (overnight incubation with 15 mM IPTG) resulted in only weak JNK activation (Figure 5, lane 4). However, induction of V12 Rac strongly potentiated the JNK response to IL-1 (Figure 5, lanes 2 and 5), suggesting that IL-1 and Rac act independently in activation of the JNK pathway. These results imply that IL-1 signals to JNK independently of Rac.

V12 Cdc42 synergizes with PDGF in activation of JNK

Surprisingly, while not affecting the strength of the IL-1 signal, V12 Cdc42 strongly potentiated the (weak) PDGF-induced JNK activation (Figure 6, lane 6). This finding clearly demonstrates that the JNK pathway is modulated differently by the different Rho subfamily GTPases Rac and Cdc42. Thus, whereas constitutively active Rac appears to act on the IL-1 branch of the signal, upstream of where the IL-1 and PDGF signals are integrated, V12 Cdc42 acts on the PDGF signal, again upstream of the integration point.

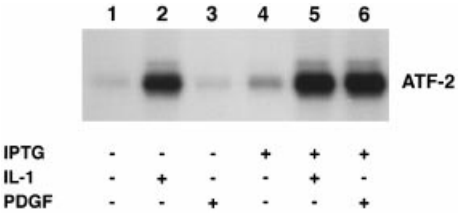

Figure 6 V12 Cdc42 synergizes with PDGF in activation of JNK

PAE cells (clone 14, expressing V12 Cdc42 under the control of an IPTG-inducible CMV3 promoter) were treated for 10 min with IL-1 (20 ng/ml) or PDGF (75 ng/ml), where indicated, after a 16 h pre-incubation with IPTG (15 mM) where indicated. JNK activity was measured as in Figure 2.

DISCUSSION

The synergistic effect of PDGF on the activation of JNK by IL-1 may be physiologically significant: PDGF released from platelets at sites of injury or inflammation could augment the actions of IL-1 secreted by monocytes or macrophages. It remains to be determined whether the additional presence of PDGF, and consequent synergistic JNK activation, affects expression of IL-1 responsive genes in target cells.

In view of the known activation of Rac by PDGF [16], and the suggestions that both IL-1 [13] and PDGF [24] signal to JNK via Rho family proteins, we sought to determine whether these GTPases were crucial for the input of either IL-1 or PDGF (or both) into synergistic JNK activation. We have used two separate approaches to investigate this possibility: treatment of cells with specific bacterial toxins that inactivate Rho proteins, and expression of dominant negative and constitutively active mutants of Rac and Cdc42.

The recent identification of a novel set of toxins from species of *Clostridium* bacteria, which inactivate specific sets of small GTPases [21], has provided a useful additional tool for the investigation of pathways dependent on these signalling molecules. The specificities of these toxins are well defined, and they are efficiently internalized by mammalian cells [21]. As with any inhibitor, however, care is required in interpreting results where the targets may not be completely inactivated. This appears to be the case with Ras inactivation by lethal toxin, since a small amount of activity of the ERK pathway (stimulated via Ras by PDGF) remains in these cells after treatment with the toxin (Figure 3). The inhibition by this toxin, however, does appear to be completely specific to PDGF-stimulated ERK activity. ERK stimulation by IL-1, and JNK stimulation by either agonist, is unaffected. The lack of any observable inhibition of JNK activity shows that stimulation of this pathway by IL-1 and PDGF (alone or in combination) does not require maximal levels of Ras, and suggests that it is independent of the GTPase. Although no direct measure of Rho GTPase inactivation by toxin B is available, the appearance of the cells after a 2 h incubation with the toxin clearly indicated substantial inactivation of Rho itself, as judged by extensive rounding up and some loss of attachment by the cells. The observation that active JNK levels are slightly raised in these cells suggests that the toxin may also be acting as a cellular stress (this effect is far less pronounced in cells treated

with lethal toxin), further complicating interpretation of the data. It is clear, however, that stimulation of the JNK pathway by IL-1 and/or PDGF is not specifically inhibited by treatment of cells with toxin B. As with lethal toxin therefore we can conclude that GTPases which are targets of toxin B (i.e. all Rho family members) are not required, at least at maximal levels, for activation of JNK by IL-1 or for potentiation of this response by PDGF. Our results therefore suggest that IL-1 and PDGF signal to JNK independently of these GTPases, at least in PAE cells.

The evidence for involvement of Rac and Cdc42 in JNK activation arises from work with dominant negative and constitutively active mutants of the GTPases. We have therefore, as an additional approach, expressed these mutants in PAE cells and investigated their effects on JNK activation. However, in place of transient co-transfections of mutant GTPases with epitope-tagged JNK, we have employed stably integrated, inducible mutant GTPase constructs. This system offers two advantages. First, levels of expression of mutant GTPases will be less variable among the population of cells studied and will be considerably lower than those present in transiently transfected cells [20]. This may overcome, at least in part, problems caused by lack of specificity of highly overexpressed mutant proteins, which may potentially interact with activators or effectors of closely related proteins, as well as those of the target protein itself. Secondly, the stable inducible system allows the activity of endogenous JNK (as opposed to epitope-tagged, transfected JNK) to be measured.

Induction of V12 Rac caused an approx. 2-fold activation of JNK (Figure 5). This relatively weak activation, compared with that observed in transient transfections of L61 Rac [11] or V12 Rac [12], may reflect lower expression of the mutant GTPase. However, the levels of V12 Rac induced in this clone have been shown to be in excess of the endogenous protein [20] and sufficient to cause extensive membrane ruffling (considerably more extensive than the ruffling induced by PDGF treatment in these cells) [20]. Crucially, activation of JNK by V12 Rac is at least an order of magnitude lower than that observed after stimulation with IL-1, showing that activation of Rac is not a sufficient signal for maximal JNK activation. The synergy between V12 Rac and IL-1 also strongly suggests that the GTPase and the cytokine activate JNK via independent signalling pathways.

Induction of N17 Rac in PAE cells resulted in an approx. 30% reduction in the observed JNK activity after IL-1 stimulation (Figure 4). This reduction is of a similar magnitude to that reported with transfected N17 Rac and TNF-induced JNK in HeLa cells [12] or COS cells [11]. In HeLa cells, as here, basal JNK activity was also reduced by N17 Rac [12]; the result is not reported for COS cells. None of these results directly supports the conclusion that IL-1 or TNF signals to JNK via Rac. Indeed, our results show that a similar reduction in JNK activity by N17 Rac is also observed in cells treated with PDGF alone, or with PDGF and IL-1. This suggests that N17 Rac does not specifically inhibit activation of JNK by IL-1, but has the effect of reducing levels of activated JNK in both resting and stimulated cells. It is possible that this reflects a reduction in expression of either JNK or an upstream component of the JNK signalling pathway caused by overexpression of the dominant negative GTPase; certainly the 16 h incubation with IPTG would give sufficient time for induced N17 Rac to effect cellular gene expression. This could perhaps explain why N17 Rac has a partial inhibitory effect on JNK activity, whereas toxin B, which should equally act as a Rac inhibitor, has no effect. Similarly, it is possible that the observed synergy between IL-1 and V12 Rac is mediated by up-regulation of expression of the same component of the signalling pathway, caused by overexpression of the constitutively active Rac mutant over 16 h. Whatever the explanation, our results show that IL-1-induced JNK activation is not specifically inhibited by N17 Rac, and therefore support the conclusion drawn from the use of the V12 mutant, that IL-1 acts independently of Rac. The similarity of our results to those obtained with transiently transfected N17 Rac and TNF-induced JNK [12] suggest that these conclusions may extend to TNF.

As with V12 Rac, we found that V12 Cdc42 caused a modest (2–3-fold) JNK activation in PAE cells (Figure 6), again considerably lower than that observed in IL-1-stimulated cells or that reported in cells transiently transfected with V12 Cdc42 [12] or L61 Cdc42 [11,13]. However, there was no synergy between IL-1 and V12 Cdc42. We also observed no reduction in JNK activity, in either resting or stimulated cells, after induction of N17 Cdc42 (Figure 4). In contrast, a strong inhibition of IL-1-induced JNK activity has been reported in COS cells transiently transfected with N17 Cdc42 [13]. The explanation for this discrepancy is not clear, but one possibility is that, at the high levels present in transiently transfected COS cells, N17 Cdc42 may be acting non-specifically and inhibiting the action of Rac or other small GTPases. It is also possible that the activity of transiently transfected JNK may be modulated by additional signals (in this case dominant negative Cdcd42) to those which regulate activity of the endogenous kinase. A third possibility is that N17 Cdc42 is only able to modulate the JNK pathway in certain cell types (including COS cells, but not PAE cells). Whatever the explanation, our failure to find any inhibitory effect with the dominant negative GTPase suggests that IL-1 does not utilize Cdc42 in signalling to JNK in PAE cells. As with Rac, therefore, our data with Cdc42 mutants are compatible with the lack of inhibition we observe with toxin B, and strongly argue against a role for the GTPase in IL-1 signalling in these cells.

The physiological significance of the modulation of JNK by these two GTPases remains undetermined. The surprising finding that V12 Cdc42 synergizes with PDGF, but not with IL-1, in activation of JNK clearly indicates that they modulate the JNK pathway independently of one another, as well as independently of IL-1. We have, however, been unable to identify any agonists which activate JNK via either small GTPase.

In summary, we have shown that PDGF, despite being a weak activator of the JNK pathway, strongly potentiates activation of JNK by IL-1. We have further shown, using both bacterial toxins and dominant negative mutants, that neither of the Rho family GTPases Rac and Cdc42 are required for synergistic activation of JNK by IL-1 and PDGF in PAE cells. However, active mutants of both GTPases appear to be capable of modulating the activity of the JNK pathway independently, with V12 Rac able to synergize with IL-1, and V12 Cdc42 with PDGF. Whether this reflects a physiological role for Rac and Cdc42 in JNK activation by agonists other than IL-1 and PDGF remains to be determined.

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REFEERENCES

1 Kyriakis, J. M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E. A., Ahmad, M. F., Avruch, J. and Woodgett, J. R. (1994) Nature (London) **369**, 156–160
2 Gupta, S., Barrett, T., Whitmarsh, A. J., Cavanagh, J., Sluss, H. K., Derijard, B. and Davis, R. J. (1996) EMBO J. **15**, 2760–2770
3 Derijard, B., Hibi, M., Wu, I. H., Barrett, T., Su, B., Deng, T., Karin, M. and Davis, R. J. (1994) Cell **76**, 1025–1037
4 Ip, Y. T. and Davis, R. J. (1998) Curr. Opin. Cell Biol. **10**, 205–219
5 Karin, M. (1995) J. Biol. Chem. **270**, 16483–16486
6 Raingeaud, J., Gupta, S., Rogers, J. S., Dickens, M., Han, J., Ulevitch, R. J. and Davis, R. J. (1995) J. Biol. Chem. **270**, 7420–7426

7 Rossomando, A. J., Payne, D. M., Weber, M. J. and Sturgill, T. W. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 6940–6943
8 Boulton, T. G., Nye, S. H., Robbins, D. J., Ip, N. Y., Radziejewska, E., Morgenbesser, S. D., DePinho, R. A., Panayotatos, N., Cobb, M. H. and Yancopoulos, G. D. (1991) Cell **65**, 663–675
9 Finch, A., Holland, P., Cooper, J., Saklatvala, J. and Kracht, M. (1997) FEBS Lett. **418**, 144–148
10 Kracht, M., Truong, O., Totty, N. F., Shiroo, M. and Saklatvala, J. (1994) J. Exp. Med. **180**, 2017–2025
11 Coso, O. A., Chiariello, M., Yu, J. C., Teramoto, H., Crespo, P., Xu, N., Miki, T. and Gutkind, J. S. (1995) Cell **81**, 1137–1146
12 Minden, A., Lin, A., Claret, F. X., Abo, A. and Karin, M. (1995) Cell **81**, 1147–1157
13 Bagrodia, S., Derijard, B., Davis, R. J. and Cerione, R. A. (1995) J. Biol. Chem. **270**, 27995–27998
14 Imamura, K., Sherman, M. L., Spriggs, D. and Kufe, D. (1988) J. Biol. Chem. **263**, 10247–10253
15 O'Neill, L. A. J., Ikebe, T., Sarsfield, S. and Saklatvala, J. (1992) J. Immunol. **148**, 474–479
16 Hawkins, P. T., Eguinoa, A., Qiu, R. G., Stokoe, D., Cooke, F. T., Walters, R., Wennstrom, S., Claesson-Welsh, L., Evans, T., Symons, M. et al. (1995) Curr. Biol. **5**, 393–403
17 Kracht, M., Shiroo, M., Marshall, C. J., Hsuan, J. J. and Saklatvala, J. (1994) Biochem. J. **302**, 897–905
18 Wennstrom, S., Hawkins, P., Cooke, F., Hara, K., Yonezawa, K., Kasuga, M., Jackson, T., Claesson-Welsh, L. and Stephens, L. (1994) Curr. Biol. **4**, 385–393
19 Westermark, B., Siegbahn, A., Heldin, C. H. and Claesson-Welsh, L. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 128–132
20 Welch, H., Eguinoa, A., Stephens, L. R. and Hawkins, P. T. (1998) J. Biol. Chem. **273**, 11248–11256
21 Aktories, K. (1997) J. Clin. Invest. **99**, 827–829
22 Just, I., Selzer, J., Wilm, M., von Eichel-Streiber, C., Mann, M. and Aktories, K. (1995) Nature (London) **375**, 500–503
23 von Eichel-Streiber, C., Boquet, P., Sauerborn, M. and Thelestam, M. (1996) Trends Microbiol. **4**, 375–382
24 Lopez-Ilasaca, M., Li, W., Uren, A., Yu, J. C., Kazlauskas, A., Gutkind, J. S. and Heidaran, M. A. (1997) Biochem. Biophys. Res. Commun. **232**, 273–277

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