Activation of the small GTPase Cdc42 by the inflammatory cytokines TNFα and IL-1, and by the Epstein-Barr virus transforming protein LMP1

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SUMMARY

Cdc42, a Rho-family GTPase, has been implicated in several signal transduction pathways, including organization of the actin cytoskeleton, activation of the c-Jun N-terminal MAP kinase (JNK) and stimulation of the nuclear transcription factor kappa B (NFκB). We report here that exposure of fibroblasts to the inflammatory cytokines tumor necrosis factor α (TNFα) and interleukin-1 (IL-1) triggers the activation of Cdc42 leading first to filopodia formation and subsequently to Rac and Rho activation. Inhibition of Cdc42 completely suppresses cytokine-induced actin polymerization, but not activation of JNK or NFκB. The latent membrane protein 1 of Epstein-Barr virus, LMP1, is thought to mimic constitutively activated TNF family receptors. When expressed in fibroblasts, LMP1 stimulates Cdc42-dependent filopodia formation as well as JNK and NFκB activation. Using LMP1 mutants, we show that activation of Cdc42 and JNK/NFκB occur through distinct pathways and that Cdc42 activation is independent of LMP1’s interaction with TRADD and TRAF proteins.

Key words: Cdc42, Epstein-Barr virus, Inflammatory cytokine, LMP1, Rho GTPase

INTRODUCTION

The proinflammatory cytokines TNFα and IL-1 are structurally unrelated and bind to distinct receptors, but share overlapping biological activities. Produced mainly by activated macrophages and monocytes, each can participate in the defence response by causing fever and tissue destruction, activating lymphocytes and promoting trafficking of leukocytes into sites of injury or infection (Dinarello, 1996; Beyaert and Fiers, 1994). A set of crucial intracellular signal transduction pathways is triggered when cells are exposed to TNFα or IL-1, including nuclear translocation and activation of the transcription factor NFκB and activation of the JNK MAP kinase cascade (Thanos and Maniatis, 1995; Whitmarsh and Davis, 1996). TNFα can also kill cells, particularly under conditions where NFκB is not activated, whereas IL-1 generally does not (Beg and Baltimore, 1996; van Antwerp et al., 1996; Wang et al., 1996; Liu et al., 1996).

These pleiotropic biological activities are mediated through TNF and IL-1 receptors, which are distinct and do not share sequence homology. The known TNF-receptors, TNFR1 (p55) and TNFR2 (p75) and IL-1 receptors, IL-1 RI and IL-1 RII, lack intrinsic enzymatic activity and act by recruiting cytoplasmic cellular proteins to initiate signal transduction. Both TNFα and IL-1 receptors engage members of the TNF receptor-associated factor (TRAF) family to transduce signals leading to JNK and NFκB activation, providing a molecular basis for the integration of signals downstream of the two receptors (Baueuerle, 1998; Liu et al., 1996). TNFR1, which plays the major part in the proinflammatory activities of TNFα (Vandenabeele et al., 1995) also interacts through the docking protein TRADD (TNF receptor-associated death domain) with RIP (receptor-interacting protein) and FADD (Fas-associated death domain), proteins which are responsible for the death-inducing activity of this receptor (Hsu et al., 1996; Stanger et al., 1995). Whereas the mechanism by which TNFα and IL-1 activate JNK is not entirely clear, recent studies have revealed that signals from TNF and IL-1 receptors converge at an NFκB-inducing kinase (NIK) to induce NFκB activation via IKK (IκB kinase; Stancovski and Baltimore, 1997).

The Epstein-Barr virus encoded latent membrane protein 1, LMP1, has received much attention since, as well as being an important oncogene involved in human malignancy, it appears to mimic constitutively active TNF receptor family members (Gires et al., 1997; Eliopoulos and Rickinson, 1998). Although it shares little amino acid sequence homology with these receptors, LMP1 interacts with many of the same proteins as the TNFα and IL-1 receptors (e.g. TRAFs 1, 2 and 3 and TRADD) and when expressed in cells, leads to activation of the JNK and NFκB pathways (Hammarskjold and Simurda, 1992; Laherty et al., 1992; Kaye et al., 1995; Mosialos et al.,...
Although they were first characterized as key regulators of the actin cytoskeleton, Rho-like GTPases have also been implicated in NFκB and JNK activation (Hall, 1998). Expression of constitutive active forms of Cdc42 and Rac stimulates JNK-activity in Cos and HeLa cells, while dominant negative versions of these GTPases were reported to have some inhibitory effect on TNFα-induced JNK activation (Wesselborg et al., 1997; Bagrodia et al., 1995; Coso et al., 1995; Minden et al., 1995). The mechanisms by which Cdc42 and Rac lead to JNK activation have been the subject of much investigation; in addition to some of the known components of the JNK cascade (MEKK1, MEKK4, MLK2 and MLK3), Rac and Cdc42 interact directly with at least two other proteins (p65PAK and an SH3-containing structural protein POSH) that have been proposed as mediators of JNK activation (Fanger et al., 1997; Brown et al., 1996; Bagrodia et al., 1995; Tapon et al., 1998; Nagata et al., 1998; Hirai et al., 1997; Teramoto et al., 1996; Rana et al., 1996). Cdc42, Rac and Rho have also been reported to activate NFκB and two reports have suggested that in the case of Rac at least, this is mediated by the production of reactive oxygen species (ROS) (Sulciner et al., 1996; Perona et al., 1997; Tapon et al., 1997; Kheradmand et al., 1998).

In the present study we have analyzed the ability of the TNF and IL-1 receptors, as well as of LMP1, to activate members of the Rho GTPase family in mouse fibroblasts. We show here that all three active Cdc42 leading to actin polymerization and the formation of filopodia. Despite this, activation of JNK and NFκB in fibroblasts by the TNF and IL-1 receptors and by LMP1 occurs independently of Cdc42. LMP1 mutants reveal that signaling to Cdc42 and to JNK/NFκB occurs through distinct pathways and that members of the TRAF and TRADD families are not required for Cdc42 activation.

MATERIALS AND METHODS

Materials

Human recombinant tumor necrosis factor α (TNFα) and human recombinant interleukin-1α (IL-1α) were purchased from R&D Systems and Chemicon, respectively. PDGF-BB was from Amersham. LPA was from Sigma. Clostridium difficile toxin B was a gift from Dr B. Wren (St Bartholomew’s Hospital Medical College, London). The anti-Myc-tag antibody (9E10) was a gift from Dr S. Moss (University College London). Anti-FLAG-tag antibody (M2) and Anti-HA-tag antibody (12CA5) were obtained from Kodak and Boehringer, respectively. Anti-JNK1 (C-17) and anti-NFκB (C-20; recognizing the p65 subunit) antibodies were from Santa Cruz. Anti-LMP1 antibody (CS1.4) has been described earlier (Rowe et al., 1996). OT22 is a mouse monoclonal antibody reactive with the LMP1 antibody (CS1.4) has been described earlier (Rowe et al., 1996; Eliopoulos and Young, 1998). The HA-pCDNA3-1987). OT22 is a mouse monoclonal antibody reactive with the LMP1 antibody (CS1.4) has been described earlier (Rowe et al., 1996; Eliopoulos and Young, 1998). The HA-pCDNA3-HA-JNK1 was a gift from Dr J. Woodgett (The Ontario Cancer Institute, Ontario, Canada). The expression plasmids pSG5-LMP1, pSG5-LMP1AI-43, pSG5-LMP1AI-38 and pSG5-LMP1Δ332-386 were described (Tapon et al., 1997; Jefferies et al., 1985). The pRK5-Myc-expression vector has been previously described (Lamarche et al., 1996) and cDNAs for N17Cdc42 (G25K isotype) or N17Rac (Rac1 isotype) were inserted 3’ of the Myc-tag. pRK5 expression vectors encoding TRADD, RIP, TRAF2, ΔTRAF2 and TRAF6 have been kindly provided by Dr D. V. Goeddel (Tularik, San Francisco, USA). TRAF1 and TRAF3 expression vectors were kindly provided by Dr Eliott Kieff (Harvard Medical School, Boston, USA) and have been described earlier (Devergne et al., 1996).

Expression and purification of recombinant proteins

N17Rac (Rac1 isotype) and C3 transferase were expressed in E. coli as glutathione-S-transferase (GST) fusion proteins and purified as described (Self and Hall, 1995). Proteins were released from the glutathione agarose beads using thrombin as described (Lamarche et al., 1996). c-Jun was expressed as a GST fusion protein and eluted from the beads as a fusion protein using reduced glutathione as described (Nagata et al., 1998).

NIH 3T3 cell transfection, immunoprecipitation, JNK and NFκB assays

NIH 3T3 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% donor calf serum (DCLS), 100 U/ml penicillin and 100 μg/ml of streptomycin. 1.5x10⁶ cells are seeded into 35 mm dishes and transfected with a total of 0.4 μg DNA using Lipofectin (Gibco-BRL) according to the manufacturer’s instructions. After co-transfecting 0.1 μg pRK5-Myc or pRK5-Myc-N17Cdc42 and 0.3 μg pCDNA3-HA-JNK1 for 6 hours, cells were washed twice with PBS, incubated in DMEM (10% DCLS) for 2 hours and in serum free medium for additional 24 hours. Some of the cell samples were then subjected to a 30 minute TNFα (100 ng/ml) or IL-1 (10 ng/ml) treatment or to a 1 minute UV irradiation at 254 nm on a UV transilluminator (UVP, San Gabriel, USA) followed by 30 minutes incubation in serum-free DMEM. After washing with ice-cold PBS, cells were lysed in 100 μl Tris-HCl (pH 8.0), 40 mM Na3P04; 50 mM NaF; 5 mM MgCl2; 100 mM Na3VO4; 10 mM EGTA; 1% Triton X-100, aprotinin (20 μg/ml), leupeptin (20 μg/ml), 3 mM PMSE. HA-tagged JNK1 was immunoprecipitated with 7.5 μg HA-antibody from high-speed supernatants and its kinase activity determined as previously described (Olson et al., 1995). For immunoprecipitation of endogenous JNK, cells were seeded as described above, transiently transfected or serum starved for 24 hours. Some cell samples were then treated with toxin B (50 ng/ml) for 2 hours and with TNFα (100 ng/ml), IL-1α (10 ng/ml), PDGF (5 ng/ml) or LPA (200 ng/ml) for an additional 30 minutes. JNK was immunoprecipitated with an anti-JNK1 antibody and its kinase activity measured as described above. The levels of immunoprecipitated JNK proteins were determined on nitrocellulose filters using anti-HA-tag or anti-JNK1 antibodies and visualized with enhanced chemiluminescence detection (Amersham) according to the manufacturer’s instructions.

For NFκB reporter assays, approximately 3x10⁵ exponentially growing NIH 3T3 cells were seeded into 6 cm dishes and were transfected the following day with 0.2 μg each pBc2A-Luc NFκB responsive luciferase reporter (Arezana-Seisdedos et al., 1993) and β-gal plasmids together with 1 μg pSG5-LMP1 and/or 1 μg pRK5-Myc-N17Cdc42 using Lipofectamin. Twenty four hours later, cells were collected and analyzed for luciferase and β-gal activities as described (Eliopoulos et al., 1997). NFκB activation represents the ratios of luciferase versus β-gal activities.

Swiss 3T3 cell culture, microinjection and cytokine treatment

Swiss 3T3 cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin. Subconfluent, serum-starved cells were prepared as follows: cells were plated at a density of 1x10⁵ cells into 80 cm² flasks and then grown at 37°C in DMEM (10% FCS) for 7-8 days. Before transfection, the cell culture medium was collected and centrifuged.
RESULTS

TNFα and IL-1 stimulate reorganization of the actin cytoskeleton

To determine whether cytokines can induce reorganization of the actin cytoskeleton, serum-starved subconfluent Swiss 3T3 cells were exposed to TNFα and IL-1 for 10 minutes. Filamentous actin was visualized in fixed cells by immunofluorescence using TRITC-labelled phalloidin. Control cells contain little polymerized actin, mainly visible as a fine actin ring displayed at the cell periphery (Fig. 1A), whereas around 90% of TNFα or IL-1 treated cells have developed extending filopodia accompanied by associated lamellipodia within 10 minutes (Fig. 1B,C). 30 minutes after TNFα or IL-1 treatment, filopodia and lamellipodia have largely disappeared and cells retain stress fibres (Fig. 1D,E). Identical results were obtained using serum-starved subconfluent NIH 3T3 cells (data not shown).

The induction of filopodia by TNFα and IL-1 suggests that Cdc42 has been activated (Nobes and Hall, 1995; Kozma et al., 1995). To confirm this, a pRK5 expression plasmid encoding a myc-tagged dominant negative Cdc42 (N17Cdc42) was microinjected into serum-starved Swiss 3T3 cells and after 2 hours, TNFα or IL-1 added for 10 minutes. N17Cdc42 completely blocked filopodia and all other actin changes induced by the two cytokines (Fig. 2B,C) in around 90% of expressing cells. As a control, N17Cdc42 had no effect on PDGF-induced lamellipodia formation (Fig. 2D), a Rac-mediated process (Ridley et al., 1992).

To confirm that the lamellipodia seen in Fig. 1B and C are formed indirectly as a result of Cdc42 mediated Rac activation, a pRK5 expression vector encoding a dominant negative myc-tagged Rac (N17Rac) was microinjected into serum-starved subconfluent Swiss 3T3 cells. Expression of N17Rac itself did not induce any changes in the actin cytoskeleton (Fig. 3A) whereas, as expected, it completely blocked PDGF-induced lamellipodia (compare Fig. 3B with Fig. 2D). Fig. 3C and D show that N17Rac does not interfere with filopodia formation induced by TNFα or IL-1, but that the filopodia are no longer associated with lamellipodia (compare Fig. 3C with Fig. 1B).

To investigate whether the cytokine induced stress fibres are produced as a result of Rac mediated Rho activation, cells were microinjected with the N17 Rac expression vector or with C3...
transferase, a specific inhibitor of Rho. The presence of either protein completely blocked stress fiber production induced by TNFα (Fig. 3E,F) or IL-1 (data not shown), indicating that Rho activity has been induced by endogenous Rac.

To confirm these results, we made use of toxin B from *Clostridium difficile*. This cytotoxin glucosylates and inactivates Rho like GTPases (Prepens et al., 1996; Just et al., 1995). Pretreatment of serum-starved subconfluent Swiss 3T3 cells with toxin B inhibited TNFα triggered filopodia and lamellipodia formation (compare Fig. 4A with B) as well as TNFα-induced stress fiber formation at 30 minutes (data not shown). In the absence of toxin B, 90% of cells responded to TNFα, whereas in the presence of toxin B less than 10% responded. Pretreatment of serum starved cells with toxin B also inhibited PDGF-induced lamellipodia formation (compare Fig. 4C with D).

The results indicate that the effects of TNFα and IL-1 on the actin cytoskeleton are mediated by activation of Cdc42, which in turn, through a GTPase cascade already described, leads to activation of Rac and then Rho (Nobes and Hall, 1995; Kozma et al., 1995).

**Cytokine induced JNK activation is not mediated by Cdc42**

To investigate whether TNFα and IL-1 triggered JNK activation is mediated by Cdc42, NIH 3T3 cells were cotransfected with N17Cdc42 and JNK1 expression plasmids and the cells treated with cytokines for 30 minutes. Using GST-c-Jun as a substrate in an in vitro kinase assay, it can be seen in Fig. 5A that both cytokines induce activation of JNK1 and that high level expression of N17Cdc42 does not inhibit this response. To confirm this observation using non-transfected cells, we again made use of toxin B. Toxin B treated cells were incubated with cytokines and the kinase activity of endogenous JNK1 was assessed after immunoprecipitation. As can be seen in Fig. 5B, TNFα and IL-1 (as well as UV) induced JNK activation was not inhibited by toxin B. Fig. 5B also shows that neither PDGF, which is a strong activator of Rac in these cells nor lysophosphaticid acid (LPA), a strong activator of Rho, lead to activation of JNK. We conclude that Cdc42 and other members of the Rho GTPase family are not required for the activation of JNK by inflammatory cytokines in fibroblasts and that activation of Rac can occur without concomitant activation of JNK.

**Cytokine induced NFκB translocation is not mediated by Rho GTPases**

NFκB is retained in the cytoplasm by inhibitory proteins (IκBs) and upon cell activation, IκBs are phosphorylated and degraded, allowing NFκB to translocate into the nucleus (Verm et al., 1995). To examine whether Rho GTPases are involved in TNFα or IL-1 induced NFκB translocation, Swiss 3T3 cells were first treated with toxin B and then cytokines added. In untreated cells (Fig. 6A), the p65 subunit of NFκB is primarily cytoplasmic, but after addition of TNFα (Fig. 6B) or IL-1 (Fig. 6C) it localizes to the nucleus. Pretreatment of the cells with toxin B does not in itself induce NFκB translocation (not shown), nor does it inhibit TNFα (Fig. 6D) or IL-1 (Fig. 6E) triggered NFκB translocation. We conclude that within the limitations of the immunofluorescence technique, cytokine induced NFκB translocation is not mediated by members of the Rho GTPase family in Swiss 3T3 cells.
Overexpression of TNF and IL-1 receptor associated proteins does not induce reorganization of the actin cytoskeleton

It has been shown that over-expression of TNF/IL-1 receptor interacting proteins such as TRADD, RIP and members of the TRAF family leads to JNK and/or NFκB activation (Hsu et al., 1995, 1996; Song et al., 1997; Cao et al., 1996). To examine whether overexpression of these proteins leads to Cdc42 activation and actin polymerization, serum-starved subconfluent Swiss 3T3 cells were microinjected separately with expression vectors encoding TRADD, RIP, TRAF1, TRAF2, TRAF3 or TRAF6. Two hours later none of the injected cells displayed any changes in filamentous actin (data not shown). As expected, RIP, TRAF2 and TRAF6 strongly activated JNK1 in fibroblasts (Fig. 5C, lanes 3, 5 and 7). In addition, microinjection of ΔTRAF2, a N-terminal TRAF2 deletion mutant known to inhibit TNFα triggered JNK and NFκB activation (Rothe et al., 1995; Reinhard et al., 1997 and Fig. 5C, lanes 9 and 10), did not interfere with TNFα-induced reorganization of the actin cytoskeleton (data not shown). We conclude that the known TNF and IL-1 receptor-interacting proteins are not sufficient to activate Cdc42.

Fig. 5. Rho GTPases are not required for TNFα or IL-1-induced JNK activation. (A) NIH 3T3 cells were non transfected (lane 1) or transfected with HA-pCDNA3-JNK1 along with either pRK5-myc-N17Cdc42 (lanes 4 and 6) or with empty vector (lanes 2, 3 and 5). Cells were serum-starved and left untreated (lanes 1 and 2) or were incubated with 100 ng/ml TNFα (lanes 3 and 4) or 10 ng/ml IL-1 (lanes 5 and 6) for 30 minutes. HA-tagged JNK1 was immunoprecipitated from cell extracts and incubated with GST-c-Jun as a substrate in an in vitro kinase reaction. The amount of JNK1 in the immunoprecipitates was determined by immunoblotting with an anti-JNK1 antibody and is shown in the middle panel. The amount of N17Cdc42 in cell lysates was determined on a separate filter using an anti-myc tag antibody and is shown in the bottom panel. (B) Serum-deprived NIH 3T3 cells were incubated with or without 50 ng/ml toxin B for 2 hours. Cells were then incubated for 30 minutes with 20 ng/ml IL-1, 100 ng/ml TNFα, 10 ng/ml PDGF or 200 ng/ml LPA, or treated for 1 minute with UV light as indicated. The activity of endogenous JNK1 was determined in cell lysates by an immunocomplex kinase assay using GST-c-Jun as substrate (top panel). The level of immunoprecipitated JNK1 was determined by immunoblotting using a JNK1 polyclonal antibody (bottom panel). (C) NIH 3T3 cells were transfected with HA-pCDNA3-JNK1 and with empty vector (lanes 1 and 9) or expression vectors as indicated (lanes 2 to 8, and lane 10). Cells were left untreated (lanes 1 to 8) or were stimulated with TNFα (lanes 9 and 10), and kinase reactions were performed as described in A.
It has been suggested that LMP1, an integral membrane oncoprotein encoded by Epstein-Barr virus, acts as a constitutively activated member of the TNF receptor family (Eliopoulos and Rickinson, 1998; Gires et al., 1997). It self-aggregates in the plasma membrane and associates with TRAF1, TRAF2, TRAF3 and TRADD and it activates the JNK and NFκB pathways (Hammarskjold and Simurada, 1992; Mosialos et al., 1995; Kieser et al., 1997; Eliopoulos and Young, 1998). LMP1 has also been linked to the regulation of the actin cytoskeleton; in lymphocytes, for example, LMP1 expression leads to membrane ruffling and the formation of membrane protrusions (Wang et al., 1985, 1988). To determine whether LMP1, like the TNF and IL-1 receptors, can activate Cdc42 to reorganize the actin cytoskeleton in fibroblasts, an expression vector encoding wildtype LMP1 was microinjected into serum-deprived subconfluent Swiss 3T3 cells. As can be seen in Fig. 7B, LMP1-expressing cells contain many filopodial extensions associated with lamellipodia, as well as numerous stress fibers.

To confirm that LMP1-induced actin polymerization is due to activation of Cdc42, the LMP1 construct was co-injected with an N17Cdc42 expression vector. As shown in Fig. 7C, dominant negative Cdc42 blocked all actin cytoskeletal changes induced by LMP1.

To determine whether Cdc42 mediates LMP1 induced JNK activation, NIH 3T3 cells were co-transfected with LMP1 and an N17Cdc42 expression vector and the activity of endogenous JNK determined. LMP1 activates JNK as expected, but this is not
inhibited by co-expression of dominant negative Cdc42 (Fig. 8A, lanes 3 and 4). To determine whether Cdc42 mediates LMP1 induced NfκB activation, LMP1 and dominant negative Cdc42 were co-transfected with an NfκB reporter plasmid into NIH 3T3 cells. LMP1 activates NfκB-dependent transcription as expected, but this is not blocked by inhibition of Cdc42 (Fig. 8B).

Two regions in the C-terminal cytoplasmic tail of LMP1 (C-terminal Activation Regions) CTAR1 and CTAR2 (see Fig. 7A) have been shown to mediate its effects on the JNK and NfκB pathways by interacting with TRAF1, TRAF2 and TRAF3 (CTAR1) and TRADD (CTAR2) (Eliopoulos and Rickinson, 1998). To identify the LMP1 region responsible for Cdc42 activation, LMP1 mutants were injected into cells. As shown in Fig. 7D and E, both LMP1Δ1-43 (a mutant lacking the short N-terminal cytoplasmic tail) and LMP1Δ332-386AA (a double mutant that can no longer interact with TRAFs or TRADD) activate Cdc42 to produce filopodia (Eliopoulos et al., 1997, 1999). Expression of LMP1Δ194-386 (a mutant that lacks the entire C-terminal cytoplasmic tail) and can no longer induce JNK activation (Fig. 8, lane 2) also leads to activation of Cdc42 (Fig. 7F), though the effect is somewhat reduced compared to cells injected with wild-type LMP1. To confirm that the C-terminal cytoplasmic tail of LMP1 is unable to activate Cdc42, CD2/LMP1 (a chimeric construct in which the extracellular and transmembrane regions of CD2 have been fused to the LMP1 cytoplasmic tail region) was expressed in cells. Antibody-crosslinking of this chimeric protein has been shown to activate JNK1 and NfκB (Eliopoulos et al., 1999). Fig. 7G shows that expression of CD2/LMP1 followed by antibody cross-linking fails to induce filopodia formation, although as previously reported it does activate the translocation of NfκB from the cytoplasm to the nucleus in these cells (data not shown). We conclude that the region responsible for Cdc42 activation, lies within the transmembrane regions or the intervening loops.

DISCUSSION

We show here that the inflammatory cytokines TNFα and IL-1 activate the small GTPase Cdc42 in rodent fibroblasts. Within minutes of TNFα/IL-1 exposure, serum-starved subconfluent Swiss 3T3 cells reorganize their actin cytoskeleton and display filopodia and lamellipodia extensions at the cell periphery. This peripheral membrane activity is transient and by 30 minutes the predominant actin structures visible are stress fibers. Since inhibition of Cdc42 blocks all actin changes, while inhibition of Rac blocks stress fibers but not filopodia, we conclude that these cytokines induce the activation of a GTPase cascade similar to that proposed earlier (Nobes and Hall, 1995; Kozma et al., 1995): initial activation of Cdc42 leads to rapid activation of Rac which in turn leads to a delayed activation of Rho. Although the mechanisms leading to cross-talk between members of this GTPase family are unknown, the almost concomitant activation of Rac with Cdc42 suggests that this is a very direct link (perhaps a single protein), whereas the delayed and weak (relative to LPA) activation of Rho suggests a more indirect effect, perhaps more consistent with the accumulation of a lipid intermediate as previously suggested (Peppelenbosch et al., 1995). Despite the lack of significant filopodia and lamellipodia activity in cells at 30 minutes and later, the maintenance of stress fibers in these cells is dependent on cytokine-induced activation of Cdc42.

The ability of inflammatory cytokines to activate both the JNK MAP kinase cascade and the NfκB pathways is well established and has been proposed to account for many of their biological effects. The time course of JNK activation in rodent fibroblasts by both TNFα and by IL-1 correlates well with the time course of filopodia/lamellipodia formation and since there have been many reports that Cdc42 and Rac can activate JNK when overexpressed in cells, this strongly supports the idea that these GTPases play a role in cytokine-induced JNK activation (Cosó et al., 1995; Minden et al., 1995). Despite this, we have shown that the inhibition of Cdc42 using either a dominant negative expression construct, or toxin B, has no observable effect on the ability of TNFα or IL-1 to induce JNK activation. We conclude that the inflammatory cytokine receptors are capable of activating the JNK MAP kinase pathway independently of Cdc42 or Rac in rodent fibroblasts. Furthermore, activation of Rac by PDGF does not lead to activation of the JNK kinase cascade in these cells.

There have been previous reports that agonist-stimulated JNK activation can be inhibited with dominant negative Cdc42 or Rac. Minden et al. (1995) reported that a dominant negative Rac did not inhibit TNFα-induced JNK activation in HeLa cells, but did inhibit EGF-induced JNK activation by 50%, while Cosó et al. (1995) reported that either dominant negative Rac or Cdc42 inhibited both EGF and TNFα-induced JNK activation in COS cells by around 50%. Bagrodia et al. (1995) found almost complete inhibition of IL-1-induced JNK activation in COS cells after Cdc42 inhibition. There are several possible explanations for the different conclusions reached by us and other groups. First, and unlike ERK, the JNK cascade is complicated by having at least eleven known upstream MAP kinase kinase kinases (ASK1, Tpl-2, DLK, TAK1, MEKK1,2,3,4 and MLK1,2,3) that may regulate the pathway in different cell types and in response to different stimuli (Ip and Davis 1998). Some of these (MEKK1, MEKK4,
and MLK1,2,3) can interact directly with Cdc42 and Rac, though even here, it is not clear that this necessarily leads to stimulation of kinase activity (Teramoto et al., 1996; Nagata et al., 1998). Second, most of the published experiments so far have relied upon transfection of JNK reporter plasmids into cells rather than observing effects on endogenous JNK activity as we have been able to do here using toxin B.

Using an immunofluorescence assay, we have also shown that inactivation of Rho GTPases (by toxin B) does not significantly interfere with either TNFα or IL-1 induced NFκB translocation in NIH 3T3 cells. This is in agreement with a recent publication demonstrating that TNFα induced NFκB activation is not mediated through Rho GTPases using an electrophoretic mobility shift assay for NFκB purified from 293 cells (Wesselborg et al., 1997). However, others have come to different conclusions. Perona et al. (1997) reported that dominant negative versions of Cdc42 and Rho but not Rac could interfere with TNFα induced NFκB activation in COS cells, while Sulciner et al. (1996) found that Rac but not Cdc42 is required for NFκB activation induced by IL-1 in HeLa cells. More recently, Kheradmand et al. (1998) showed that alterations in integrin adhesion could lead to Rac-dependent translocation of NFκB to the nucleus. These contradictory results could again be accounted for either in terms of the assay used (i.e. use of transfected reporters versus observing endogenous proteins) or could reflect different mechanisms of activation of NFκB by different stimuli in different cell types.

In an attempt to identify the cytoplasmic mediators of Cdc42 activation by TNFα/IL-1, we transfected expression constructs encoding known components of signaling pathways activated by these cytokines. Overexpression of TRADD, TRAF1, TRAF2, TRAF3, TRAF6, or RIP produced no detectable changes to the actin cytoskeleton, although RIP and TRADD caused extensive cell death (data not shown). Although it is possible that these proteins might need additional signals for activity, simple overexpression has been reported to lead to JNK and NFκB activation (e.g. Song et al., 1997). In addition, ΔTRAF2, which has been shown to be a potent inhibitor of cytokine induced NFκB and JNK activation (Rothe et al., 1995; Reinhardt et al., 1997), although able to inhibit TNFα-induced JNK-activation could not interfere with activation of Cdc42 by TNFα. Additional TNF receptor binding proteins have recently been cloned, one of these, MADD, stimulates ERK and arachidonic acid release and is more than 94% identical to the Rab3 guanine nucleotide exchange protein (Schievella et al., 1997; Brown and Howe, 1998). This is the first indication of a molecular link between the TNF receptor and a small GTPase but its functional significance has yet to be determined.

Due to the complexity of signals activated by TNFα receptors and since most cells already express receptors making it difficult to do mutational analysis, we have made use of LMP1, an EBV encoded oncprotein that appears to mimic constitutively active TNF family receptors (Eliopoulos and Rickinson, 1998; Gires et al., 1997). LMP1 is known to be a potent activator of JNK and NFκB activity and we have now shown that LMP1 expression triggers Cdc42-dependent actin reorganization. We have shown that the short N-terminal cytoplasmic domain of LMP1 is not required for Cdc42 activation and more interestingly, both Δ332-336AAA LMP1 and the LMP1Δ194-386 mutants, which can no longer interact with TRAFs and TRADD and which do not activate JNK or NFκB (Izumi and Kieff, 1997; Kieser et al., 1997; Devergne et al., 1996; Eliopoulos et al., 1997; Eliopoulos and Young, 1998) still activate Cdc42 and filopodia formation. We conclude that the transmembrane spanning regions or the intervening loops in LMP1 must be responsible for Cdc42 activation and that LMP1-induced actin reorganization and NFκB/JNK activation are mediated by separate signal transduction pathways. We are now attempting to identify molecules capable of interacting with the transmembrane or loop regions of LMP1 as potential candidates for mediating Cdc42 activation.

There are many examples in biology where changes in the actin cytoskeleton are co-ordinately regulated with changes in gene transcription and the Rho GTPase family are in an ideal position to perform this task. One attractive example of this is dorsal closure in Drosophila, which depends both on rearrangement of the actin cytoskeleton and on the JNK MAP kinase pathway (Ip and Davis, 1998). A recent paper suggests that Rac and Cdc42 act upstream of the JNK pathway during Drosophila dorsal closure, though since these experiments were carried out by expressing constitutively activated Rac/Cdc42, they suffer from some of the same potential problems of interpretation as transfected cells in culture (Glise and Noselli, 1997). The relationship between Cdc42 and MAP kinase pathways in S. cerevisiae is somewhat clearer: in response to a signal from the pheromone receptor, Cdc42 and a MAP kinase cascade are co-ordinately activated leading to cytoskeletal and transcriptional changes respectively. Furthermore, activation of these two pathways appears to be spatially co-ordinated, with components from each pathway associating with scaffold proteins to form a multimolecular complex, which localizes to the mating projection (Leberer et al., 1997; Peter et al., 1996). Despite this and the fact that upstream activator of the MAP kinase cascade, Ste20p, has a Cdc42 binding site in its regulatory domain, Cdc42 is not required for MAP kinase activation in this pathway (Leberer et al., 1997; Peter et al., 1996). Superficially at least, this situation looks similar to what we find in fibroblasts: activation of Cdc42 and of JNK by cytokines are not dependent on each other, but filopodia formation and JNK activation appear to be co-ordinated in time. This raises the possibility that while Cdc42 is not required for activation of the JNK pathway per se, the activation of JNK may be co-ordinated with Cdc42-dependent changes to the actin cytoskeleton. We think this possibility deserves consideration and are pursuing the possibility that components of the Cdc42 and JNK signaling pathways may interact.

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