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Interaction of Rac Exchange Factors Tiam1 and Ras-GRF1 with a Scaffold for the p38 Mitogen-Activated Protein Kinase Cascade

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Tiam1 and Ras-GRF1 are guanine nucleotide exchange factors (GEFs) that activate the Rac GTPase. The two GEFs have similar N-terminal regions containing pleckstrin homology domains followed by coiled-coils and additional sequences that function together to allow regulated GEF activity. Here we show that this N-terminal region of both proteins binds to the scaffold protein IB2/JIP2. IB2/JIP2 is a scaffold for the p38 mitogen-activated protein (MAP) kinase cascade because it binds to the Rac target MLK3, the MAP kinase kinase MKK3, and the p38 MAP kinase. Expression of IB2/JIP2 in cells potentiates the ability of Tiam1 or Ras-GRF1 to activate the p38 MAP kinase cascade but not the Jnk MAP kinase cascade. In addition, Tiam1 or Ras-GRF1 binding to IB2/JIP2 increases the association of the components of the p38 MAP kinase signaling cassette with IB2/JIP2 in cells and activates scaffold-associated p38. These findings imply that Tiam1 and Ras-GRF1 can contribute to Rac signaling specificity by their ability to form a complex with a scaffold that binds components of one of the many known Rac effector pathways.

The Rac GTPase has the capacity to influence a diverse set of cellular functions, including alterations of the actin cytoskeleton, regulation of cell proliferation, motility and survival, generation of reactive oxygen species, and induction of both Jnk and p38 kinase cascades (for a review, see reference 30). Active GTP-bound Rac carries out these functions by binding to a wide variety of downstream effector proteins, including p65 PAK, phosphatidylinositol 3-kinase, IQ GAP, p67 phox, POR1, S6 kinase, STAT3, POSH, and MLK3 (for a review, see reference 1). Only in some cases has the specific downstream effector protein responsible for a particular cellular phenotype associated with active Rac been elucidated.

The Rac GTPase becomes activated in cells upon interaction with one of multiple Rac-specific guanine nucleotide exchange factors (Rac-GEFs), which promote the release of GDP from Rac, allowing GTP to take its place. Rac-GEFs all have similar Dbl homology (DH) catalytic domains (9). Individual Rac-GEF family members differ in their ability to be activated by distinct upstream signals. One ubiquitously expressed Rac-specific GEF is Tiam1, which was first identified in a screen for genes that promote invasion in murine T lymphoma cells (20). Overexpression of Tiam1 causes oncogenic transformation in fibroblasts and invasiveness in lymphocytes (27). However, in epithelial cells Tiam1 expression suppresses invasion and promotes adhesion through E-cadherin-mediated cell-cell contacts (21). Tiam1 has also been implicated in axon formation in neurons through regulation of growth cone actin organization (24). Finally, Tiam1 expression has been implicated in regulating apoptosis in human leukemia cell lines (23).

Which extracellular signals enhance Tiam1's ability to activate Rac in cells and how this activation is accomplished are not well characterized. Elevated calcium has been reported to increase the protein's intrinsic GEF activity (18), while platelet-derived growth factor has been shown to target Tiam1 to the plasma membrane (5), which could promote its interaction with Rac. The domain structure of Tiam1 implicates specific regions of the protein as mediators of responses to extracellular signals. Near the N terminus are an adjoining pleckstrin homology (PH) domain, a coiled-coil (CC) domain, and an undefined region termed Ex, which cooperatively function to localize Tiam1 to the membrane and which are required for Tiam1-mediated membrane ruffling and Jnk activation (28, 37). Tiam1 has been reported to interact with the hyaluronic acid receptor CD44 through this region, stimulating Tiam1-mediated Rac signaling and tumor cell migration (4). This cluster of motifs is strikingly similar to one found in Ras-GRF1 (36) (also referred to as CDC25Mm [26]), an exchange factor capable of activating both Ras (36) and Rac (22) GTPases. In Ras-GRF1, this region has been shown to play a role in targeting the protein to the membrane fraction of cells and in the activation of the protein by calmodulin binding in the presence of calcium (6).

The fact that Rac proteins can activate multiple downstream target proteins suggests that mechanisms exist to limit Rac target activation to generate signaling specificity. In fact, some evidence has implicated Rac-GEFs in this process since transfections of individual members of this family of GEFs generate different cellular responses even though they activate Rac similarly (46). In addition, one Rac-GEF, PIX, has been shown to bind directly to and help activate the Rac target protein PAK (13).

Another Rac target, MLK3, has been shown to complex with the IB/JIP family of scaffolds for the Jnk mitogen-activated protein (MAP) kinase cascade (45). JIP1/IB1 is required for proper Jnk kinase signaling in mice (43) and could potentially contribute to Rac signaling specificity. Here we show that two related Rac-GEFs, Tiam1 and Ras-GRF1, have unique N termini that allow them to bind to a member of this scaffold

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family, IB2/JIP2. Furthermore, we show that IB2/JIP2 functions as a scaffold for a p38 signaling cascade in mammalian cells. The binding of IB2/JIP2 to these Rac-GEFs connects a specific scaffolding protein to an upstream activator of its kinase components. This interaction influences Rac GTPase signaling by promoting p38 over Jnk activation in cells.

MATERIALS AND METHODS

Molecular cloning of rat IB2/JIP2. Partial rat IB2/JIP2 cDNA clones were obtained using the yeast two-hybrid method in *Saccharomyces cerevisiae* (strain Y190). The bait plasmid was constructed by insertion of sequences encoding amino acids 431 to 670 of Tiam1 cDNA (spanning the N-terminal PH, CC, and Ex domains) in frame with the Gal4 DNA binding domain in the pAS-Cyh vector. Bait DNA was cotransfected into yeast, along with a commercial rat brain cDNA library cloned in frame with the Gal4 DNA activation domain in the pGAD10 vector (Clontech). DNAs from colonies growing under dual selection conditions which also showed histidine auxotrophy and expressed β-galactosidase were retested as described above to confirm the yeast interaction and then sequenced. Overlapping yeast clones spanning nucleotides 302 to 2585 (termed 2/18) were cloned into the pBS vector using standard recombinant DNA techniques. To obtain upstream sequences including the 5′ start site, a commercial rat brain cDNA library ligated to universal adaptors suitable for rapid amplification of cDNA ends (RACE) PCR was used according to the manufacturer's instructions (Clontech). Purified 2/18 sequence and RACE-derived 5′ sequence were then used as templates for overlap PCR, generating a full-length cDNA product, which was subcloned in frame into both pJ3H and pJ3M vectors, containing 5′ hemagglutinin (HA)- and Myc-epitope tags, respectively.

Plasmids. Plasmids encoding cDNAs for HA-Tiam C-1199, ΔPCX-Tiam-1, Dbl, GST-p38, and GST-Jnk were kindly provided by J. Collard, M. Symons, R. Cerione, and T. Roberts. Plasmids encoding Flag-tagged human JIP1 and JIP2 were kindly provided by R. Davis. Plasmids encoding HA-tagged MKK3β, MKK6β, and MKK7 were kindly provided by G. Johnson, and HA-MLK3 was provided by K. Gallo. Ras-GRF1 cDNA has been previously described (36). To obtain plasmids encoding glutathione *S*-transferase (GST) fusions with amino acids 1 to 306 of rat IB2/JIP2 (rIB2), 127 to 202 of JIP1/IB1, and 1 to 229 of human IB2/JIP2, PCR products using appropriate primers on the respective scaffold cDNA templates were ligated into the pGEX5X3 vector. A similar method was used to derive GST-rIB2-511-835. rIB2 cDNAs containing deletions in the Tiam1-binding domain (Δ398-422 and Δ434-522) were derived by overlap PCR and subcloned into the pJ3M vector. rIB2 cDNA with a deletion of the N-terminal 306 amino acids (rIB2-ΔN306) was derived by PCR using appropriate primers on a full-length rIB2 cDNA template. rIB2-1-693, lacking the C-terminal 142 residues spanning the phosphortyrosine binding (PTB) domain, was derived by a similar method.

Antibodies and immunoblotting. Antibodies to Tiam1 (Santa Cruz), Ras-GRF1 (Santa Cruz), Dbl (Santa Cruz), HA-epitope tag (Covance), Myc-epitope (Santa Cruz), Flag-epitope (Sigma), GST (Sigma), p38, phospho-ATF2, phospho-Jun, and phospho-p38 (Cell Signaling) were used according to the manufacturers' instructions. Antibody to IB2 was kindly provided by M. Goldfarb. Secondary antibodies, immunoblotting, and the chemiluminescence protocol for developing blots have been previously described (6).

Cell culture and transfection. COS7 cells were grown in Dulbecco's modified Eagle's medium containing 10% iron-supplemented bovine calf serum (HyClone) in an incubator with humidified air (5% CO_2) at 37°C. HEK 293T cells were grown as described above in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (HyClone). Cells were plated in 60-mm-diameter plastic dishes and transient transfections were performed using DEAE-dextran or Lipofectamine 2000 (Gibco BRL) according to the manufacturer's instructions. Cells were harvested 48 h after transfection. When indicated, cells were deprived of serum for 16 h prior to harvest.

Biochemical assays. (i) GST pull-down assay. Transiently transfected cells were washed with cold phosphate-buffered saline (PBS), pelleted, and lysed in 500 μl of buffer S (50 mM Tris [pH 8.0], 120 mM NaCl, 1 mM EDTA, 0.5% NP-40) containing protease inhibitors (10 μg of aprotinin per ml, 20 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride) and phosphatase inhibitors (50 μM sodium fluoride and 100 μM sodium orthovanadate). Lysates were cleared of unbroken cells and debris by centrifugation at 10,000 × *g* for 10 min and were incubated with equal amounts (usually 5 to 10 μg) of GST fusion proteins immobilized on *S*-hexyl-glutathione–agarose beads (Sigma) for 2 h at 4°C with constant agitation. Aliquots of cleared lysate were retained prior to precipitation for immunoblotting. After washing three times with ice-cold buffer S, bound proteins were eluted in 4× Laemmli buffer, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotted.

(ii) IPs. Transfected cells were harvested, washed with cold PBS, pelleted, and lysed in buffer S containing protease and phosphatase inhibitors as described above. Cleared lysates were incubated with protein A-Sepharose beads (Pharmacia) and appropriate antibody (diluted according to the manufacturer's instructions) for 2 h at 4°C with constant agitation. Aliquots of cleared lysate were again retained prior to immunoprecipitation (IP) for immunoblotting. After washing three times with ice-cold buffer S, bound proteins were eluted in 4× Laemmli buffer, resolved by SDS-PAGE, and immunoblotted. For experiments on endogenous proteins, lysates were precleared on protein A-Sepharose beads loaded with rabbit immunoglobulin G (IgG). For experiments on scaffold-associated proteins, cells were lysed in buffer M (20 mM Tris [pH 7.4], 1% Triton X-100, 1 mM $MgCl_2$, 125 mM NaCl). For quantification of the effects of exchange factor on scaffold binding activity, comparisons were made between signals from serial dilutions of control and experimental samples.

(iii) Immune complex kinase assays. (a) p38 activation. Transfected cells were washed with cold PBS, pelleted, and lysed in buffer M containing protease and phosphatase inhibitors as described above. Cleared lysates were incubated with *S*-hexyl-glutathione–agarose beads (Sigma) for 2 h at 4°C with constant agitation. Immobilized GST-p38 was washed three times with ice-cold PBS containing 1% Triton X-100 and one time with kinase buffer (50 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$). Kinase reactions proceeded for 15 min at room temperature in kinase buffer containing 1 μM dithiothreitol, 50 μM NaF, 100 μM orthovanadate, 50 μM GST-ATF2, and 25 μM ATP. Reactions were stopped with the addition of Laemmli buffer, resolved by SDS-PAGE, and immunoblotted. (b) Jnk activation. Transfected cells were processed as described above and kinase reactions were performed using purified GST-Jun as substrate.

RESULTS

Identification of IB2/JIP2 as a Tiam-binding protein. The PH domain, adjoining CC domain, and Ex region near the N terminus of the Rac exchange factor Tiam1 have been implicated in regulating Tiam1 localization and activity (Fig. 1A) (37). To identify proteins that influence Tiam1 function, these domains of Tiam1 were used as bait in a yeast two-hybrid screen of a rat brain cDNA library. Positive clones included multiple copies of two overlapping sequences that formed a 2,283-nucleotide open reading frame. Additional 5′ cDNA sequence encoding a methionine start site and Kozak sequence was obtained by RACE PCR. The derived amino acid sequence (Fig. 1B) revealed that it is one of the JIP/Islet-Brain family of scaffold proteins and is most closely related to IB2/JIP2. Alignment of human and mouse IB2/JIP2 with the rat cDNA cloned here is shown, with schematic depiction of functional domains previously defined for the more distantly related JIP1/IB1 (Fig. 1C). Overall, the rat cDNA and mouse IB2/JIP2 are 96% identical while human IB2/JIP2 displays 86% sequence identity to the rat clone. Presumably, our clone represents the rat version of IB2/JIP2.

IB2/JIP2 binds to Tiam1 and Ras-GRF1 in cells. To confirm that Tiam1 and IB2/JIP2 interact in cells, coimmunoprecipitation experiments were performed with the pancreatic β-cell line βTC1, in which both proteins are known to be expressed (29). Tiam1 was immunoprecipitated from cell lysates and the precipitates were immunoblotted for the presence of IB2/JIP2 (Fig. 2, left panels). IB2/JIP2 was detected in the lysate (upper left panel, lane 1) and in the Tiam1 immunoprecipitate (lane 2) but not in the control immunoprecipitate (lane 3).

It has been shown previously that the PH-CC-IQ motif found in the N terminus of the Ras and Rac GEF, Ras-GRF1, is functionally similar to the PH-CC-Ex region of Tiam1 in that both regions function to target the GEFs to the membrane fraction of cells (6, 37). This led us to test the hypothesis that

Ras-GRF1 may behave similarly to Tiam1 in its N-terminal-mediated interactions. We therefore performed similar experiments using rat brain lysates, as Ras-GRF1 is most highly expressed in neuronal tissues. As with Tiam1, Ras-GRF1 (Fig. 2, upper right panel, lane 5) but not control IgG immunoprecipitates (Fig. 2, upper right panel, lane 6) from rat brain lysates contained IB2/JIP2.

We then used transfected proteins to map the interaction sites on Tiam1 and rIB2. Myc-tagged rIB2 was transfected with either empty vector, a vector encoding Tiam1 (1,199 C-terminal amino acids lacking the destabilizing N-terminal PEST sequence [27]), or a vector encoding a Tiam1 mutant with an additional deletion of the N-terminal PH, CC, and Ex regions (ΔPCX-Tiam1). Tiam1 was then immunoprecipitated and rIB2 was detected by immunoblotting with anti-Myc antibodies. rIB2 was detected in the immune complexes only when Tiam1 was transfected into cells (Fig. 3A, upper panel, compare lanes 2 and 4). Moreover, when the N-terminal PH, CC, and Ex domains used as bait in the two-hybrid experiments were deleted (ΔPCX-Tiam1), there was little rIB2 observed in immune complexes (Fig. 3A, upper panel, lane 6). Lanes 3 through 6 of the lower panel show that the Tiam1 deletion mutant was expressed at even higher levels than wild-type Tiam1. Myc-rIB2 was also expressed at comparable levels in all experiments (Fig. 3A, upper panel, lanes 1, 3, and 5). These findings confirm that Tiam1 can bind to rIB2 in cells through the PH-CC-Ex motifs of the Rac-GEF.

The two partial cDNA clones isolated from the yeast two-hybrid screen had only a small region of overlap. This finding identified the putative Tiam1-binding site as amino acids 417 to 448, which reside in a previously uncharacterized region of JIP family members. To confirm this hypothesis, we constructed mutants with deletions overlapping the N-terminal (Δ398-422) or C-terminal (Δ434-522) residues of this region (see Fig. 1C). Whereas the wild type and rIB2Δ398-422 bound Tiam1 in cells (Fig. 3B, upper panel, lanes 2 and 4), rIB2Δ434-522 did not (Fig. 3B, upper panel, lane 6). Again, the mutant rIB2s (lanes 3 and 5) were present in cell extracts at levels comparable to wild-type rIB2 (lane 1) and Tiam1 was present at comparable levels in all immunoprecipitates (lower panel, lanes 2, 4, and 6). Taken together, these mutagenic studies argue that residues 434 to 522 of rIB2 interact with sequences in the N-terminal PH, CC, and Ex regions of Tiam1.

Tiam1 binds to other IB/JIP-related proteins. The human proteins JIP1/IB1 and IB2/JIP2 were also tested for their ability to bind to Tiam1, as there is significant amino acid sequence similarity among IB/JIP family proteins in the Tiam-binding region of rIB2. Tiam1 was cotransfected into COS7 cells with either Myc-rIB2, Flag-huJIP1, or Flag-huIB2/JIP2. Tiam1 was immunoprecipitated and the IB/JIP-related proteins were detected by immunoblotting with appropriate epitope antibodies (Fig. 4). Human JIP1 and IB2/JIP2 were also detected in Tiam1 immune complexes as long as Tiam1 was transfected (Fig. 4a, b, and c, compare lanes 2 and 4). Despite being expressed at high levels, human JIP1 was consistently found at lower levels on Tiam1 immune complexes in growing cells, implying possible differences in binding affinity or regulation between Tiam1 interactions with IB2/JIP2 and JIP1.

Ras-GRF1 also binds to IB/JIP-related proteins. Similar results were obtained with the Ras/Rac exchange factor Ras-GRF1. Thus, COS7 cells were cotransfected with Ras-GRF1 or empty vector and scaffold protein (either rIB2, JIP1, or huIB2/JIP2). The exchange factor was immunoprecipitated and immunoblotted for the presence of various scaffold proteins. Figure 4d to f shows that Ras-GRF1 bound all three scaffold proteins in cells. The importance of the unique N-terminal motifs of Tiam1 and Ras-GRF1 in binding IB/JIP proteins was reinforced by the finding that Dbl, a Cdc42/Rac exchange factor that lacks such a motif, did not bind significantly to rIB2 (Fig. 4g, lane 2).

Binding of rIB2 to MAP kinases. Since human IB1/JIP1 and IB2/JIP2 have been reported to act as scaffold proteins for components of the Jnk signaling cascade, rIB2/JIP2 binding properties were assessed. As previously reported (16), a fusion protein of GST and the N-terminal sequences of JIP1 (GST-nJIP1) clearly precipitated Jnk from lysates of transiently transfected COS7 cells (Fig. 5A, lane 4). However, we were unable to demonstrate Jnk binding to a fusion protein of GST with the corresponding region of rIB2 (GSTn-rIB2) (Fig. 5A, lane 3). Moreover, a similar fusion construct of huIB2/JIP2 (GSTn-huIB2) also failed to precipitate Jnk from cell lysates (Fig. 5A, lane 5).

These negative results prompted us to test whether rIB2 could instead be a scaffold protein for p38 MAP kinase. In these experiments, the same N termini of the IB/JIP-related proteins used above were used to precipitate p38α from cell lysates of transiently transfected COS7 cells. As previously reported, GST-JIP1 failed to precipitate p38 (Fig. 5A, lane 9). In contrast, the analogous N terminus of rIB2, which could not precipitate Jnk, did precipitate transiently transfected p38 kinase (Fig. 5A, lane 8). The comparable N terminus of human IB2/JIP2 also precipitated p38 (Fig. 5A, lane 10).

To confirm that rIB2 and p38 can interact in vivo, epitope-tagged scaffold proteins (rIB2, human IB1/JIP1, or human IB2/JIP2) and MAP kinases (GST-p38, GST-Jnk, or empty vector) were transfected into COS7 cells. MAP kinases were precipitated on glutathione beads and then tested for the presence of scaffold protein (Fig. 5B). As predicted from the experiments for Fig. 5A, rIB2 was clearly present on beads when GST-p38 was transfected but not when empty vector was transfected (compare upper panel, lanes 2 and 6). In contrast, only a faint rIB2 signal was observed when GST-Jnk was precipitated from cells (upper panel, lane 4), despite the fact that similar amounts of HA-rIB2 were expressed in cell lysates (compare lanes 1 and 3). Consistent with the results described for Fig. 5A, human IB2/JIP2 behaved like rIB2 and was clearly precipitated with GST-p38 (middle panel, lane 2), only faintly with GST-Jnk (middle panel, lane 4) and not at all when empty vector was transfected (middle panel, lane 6). Finally, as previously reported, human JIP1 was precipitated with GST-Jnk (bottom panel, lane 4) and not with GST-p38 (bottom panel, lane 2) or empty vector (lane 6). These results indicate that both rat and human IB2/JIP2 are p38 binding proteins.

Binding of rIB2 to kinases upstream of MAP kinase. In addition to binding to MAP kinases such as Jnk, IB/JIP family scaffolds also bind to MAP kinase kinases (MKK) and MAP kinase kinase kinases (MKKK). Since p38 is activated by a different set of upstream kinases than Jnk kinases, the binding specificity of rIB2 was investigated. Amino acids 511 to 835 of rIB2, which are analogous to the MKK binding site of JIP1,

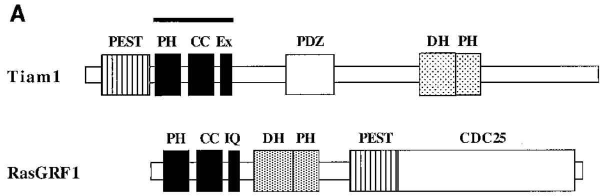

FIG. 1. (A) Schematic depiction of domains of Tiam1 and RasGRF1. The black bar indicates the domains of Tiam1 used as bait in the two-hybrid screen. (B) Sequence alignment of rat, mouse, and human IB2/JIP2 proteins. Differences between rat and mouse IB2/JIP2 are indicated above the sequences (*). Differences between rat and human IB2/JIP2 are indicated below the sequences (♦). (C) Schematic depiction of domains and binding regions on rat IB2/JIP2 based on homology with IB1/JIP1 and schematic depiction of IB2/JIP2 deletion mutants.

B

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                                                                         60
rat IB2   MADRAEMFSLSTFHSLSPPGCRPPQDISLEEFDDEDLSEITDDCGLGLSYDSDHCEKDSL
m IB2     MADRAEMFSLSTFHSLSPPGCRPPQDISLEEFDDEDLSEITDDCGLGLSYDSDHCEKDSL
hu IB2    MADRAEMFSLSTFHSLSPPGCRPPQDISLEEFDDEDLSEITDDCGLGLSYDSDHCEKDSL

                                                                        120
rat IB2   SLGRSEQPHPICSFQDDFQEFEMIDDNEEEEDEEEEEEEEEEEDGDGDGRAGGGSGSQEL
m IB2     SLGRSEQPHPICSFQDDFQEFEMIDDNEEEDDEEEEEEEEEEEDGDRQGKAGGGPGSQAL
hu IB2    SLGRSEQPHPICSFQDDFQEFEMIDDNEEEDDEDEEEEEEEEE-GDGEGQEGGDPGSEAP

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rat IB2   SGESLIPSPSIEESHKLRPTTLHLTTLGAQDSLNNNSNGGFTSAPPSSWQETVLRSPVQE
m IB2     AGDSLIPSPSLEESHKLRPTTLHLTTLGAQDSLNNN-NGGFTSAPPSSWQETVLRSPAQE
hu IB2    APGPLIPSPSVEEPHKHRPTTLRLTTLGAQDSLNNN--GGFDLVRPASWQETALCSPAPE

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rat IB2   PLKELPAPLLPAEEEHHEVQSLARPGCDCEGNQPPEPPAS-GGASPSSDPGIEADLRSHS
m IB2     PLKELPAPLLPAEEERHEVQSLARPGCDCEGNQPPEPPASSGGASPSSDPGIEADLRSHS
hu IB2    ALRELPGPLPATDTGPGGAQSPVRPGCDCEGNRPAEPPAP-GGTSPSSDPGIEADLRSRS

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rat IB2   SGGHEGRRSSQELSSPGSDSEEAGGARLGRMISSISETELELSSDSGSSS-GRSSHLTNS
m IB2     SGGHEGRRSSQELSSPGSDSEDAGGARLGRMISSISETELELSSDGGSSS-GRSSHLTNS
hu IB2    SGGRGGRRSSQELSSPGSDSEDAGGARLGRMISSISETELELSSDGGSSSSGRSSHLTNS

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rat IB2   IEEASSPASEPEPEPEPEPLHEPPRRPAFLPVGQDDTNSEYESGSESEPDLSEDADSPWL
m IB2     IEEASSPASEPEPEPEP-LHEPPRRPAFLPVGQDDTNSEYESGSESEPDLSEDADSPWL
hu IB2    IEEASSPASEPEPP-----REPPRRPAFLPVGPDDTNSEYESGSESEPDLSEDADSPWL

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rat IB2   LSNLVSRMISEGSSPIRCPGQCLSPPAPRPPEEAASQANPVPQDCPDPEAVAGP-HVELV
m IB2     LSNLVSRMISEGSSPIRCPGQCLSP-APRLPEEAASQANSVPQDCQDPE--AGP-HVELV
hu IB2    LSNLVSRMISEGSSPIRCPGQCLSP-APRPPGEPVSPAGGAAQDSQDPEAAAGPGGVELV

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rat IB2   DMDTLCGPPPPAPAAPRLGPAQSGPCLFLSNPTRDTITPLWAPPGRTARPGRSCSAACSE
m IB2     DMDTLCGPPPPAPAAPRLGPAQPGPCLFLSNPTRDTITPLWATPGRTARPGRSCSAACSE
hu IB2    DMETLCAPPPPAPAAPRPGPAQPGPCLFLSNPTRDTITPLWAAPGRAARPGRACSAACSE

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rat IB2   EEEEDEEEDEEDEEDAEDSVIPPGSRTTGSTAPLDASLVYDAVKYTLVVDEHTQLELVSL
m IB2     EEEEDEEEDEEDEEDAEDSVVPPGSRTTGSTAPLDASLVYDAVKYTLVVDEHTQLELVSL
hu IB2    EE---DEEDDEEEEDAEDSAGSPGGRGTGPSAPRDASLVYDAVKYTLVVDEHTQLELVSL

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rat IB2   RRCAGLDNDSEEDSSCEASEEEAGATLLGSDQVPEDASPDSPDLTFSKKFLNVFVNSTSR
m IB2     RRCAGLGNDSEEDSSCEASEEEAGATLLGSDQVPEDASPDSPDLTFSKKFLNVFVNSTSR
hu IB2    RRCAGLGHDSEEDSGGEASEEEAGAALLGGGQVSGDTSPDSPDLTFSKKFLNVFVNSTSR

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rat IB2   SSSTESFGLFSCVVNGEEREQTHRAVFRFIPRHPDELELDVDDPVLVEAEEDDFWFRGFN
m IB2     SSSTESFGLFSCVVNGEEREQTHRAVFRFIPRHPDELELDVDDPVLVEAEEDDFWFRGFN
hu IB2    SSSTESFGLFSCLVNGEEREQTHRAVFRFIPRHPDELELDVDDPVLVEAEEDDFWFRGFN

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rat IB2   MRTGERGVFPAFYAHAVPGPAKDLLGSKRSPCWVDRFDVQFLGSVEVPCHQGNGILCAAM
m IB2     MRTGERGVFPAFYAHAVPGPAKDLLGSKRSPCWVDRFDVQFLGSVEVPCHQGNGILCAAM
hu IB2    MRTGERGVFPAFYAHAVPGPAKDLLGSKRSPCWVERFDVQFLGSVEVPCHQGNGILCAAM

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rat IB2   QKIATARKLTVHLRPPASCDLEISLRGVKLSLSGGGPEFQRCSHFFQMKNISFCGCHPRN
m IB2     QKIATARKLTVHLRPPASCDLEISLRGVKLSLSGGGPEFQRCSHFFQMKNISFCGCHPRN
hu IB2    QKIATARKLTVHLRPPASCDLEISLRGVKLSLSGGGPEFQRCSHFFQMKNISFCGCHPRN

                                                                        835
rat IB2   SCYFGFITKHPLLSRFACHVFVSQESMRPVARSVGRAFLEYYQEHLAFACPTEDIYLE
m IB2     SCYFGFITKHPLLSRFACHVFVSQESMRPVARSVGRAFLEYYQEHLAFACPTEDIYLE
hu IB2    SCYFGFITKHPLLSRFACHVFVSQESMRPVAQSVGRAFLEYYQEHLAYACPTEDIYLE
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MapK binding (residues shown in lines 61–298)

Tiam binding (residues shown in lines 359–477)

MapKK binding (residues shown in lines 478–597)

MapKKK binding (residues shown in lines 598–835)

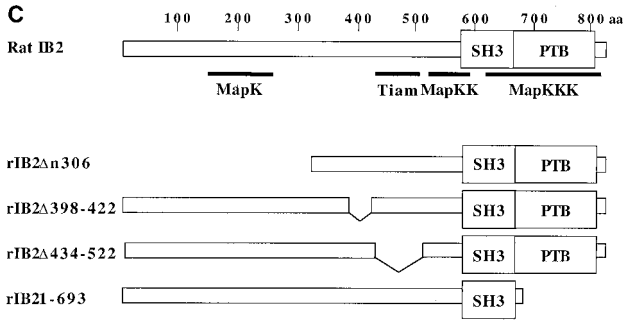

FIG. 1—*Continued.*

were expressed as a fusion protein with GST and tested for the ability to precipitate HA-tagged MKKs, such as MKK3 and MKK6, which activate p38, as well as MKK7, which is known to activate Jnk. As would be expected for a scaffolding protein for p38, this region of rIB2 clearly precipitated the p38-activating MKK3 (Fig. 6A, lane 3) but only weakly precipitated the Jnk-activating MKK7 (Fig. 6A, lane 9). Interestingly, rIB2 does not interact with all p38-activating MKKs because it did not precipitate MKK6 (Fig. 6A, lane 6). In all cases the MKKs were not precipitated by GST itself, confirming the specificity of these binding experiments (Fig. 6A, lanes 2, 5, and 8).

rIB2 was also tested for the ability to bind to the MKKK MLK3. Both human JIP1 and IB2/JIP2 bind to MLK3 through C-terminal sequences, and rIB2 is 96% identical to huIB2/JIP2 in this region. Not surprisingly, rIB2 was present on MLK3 immunoprecipitates (Fig. 6B, upper panel, lane 4) but not on control immunoprecipitates of cells transfected with rIB2 and empty vector (upper panel, lane 2). MLK3 has been shown to activate both p38 and Jnk pathways (33, 40). Taken together, the results in Fig. 5 and 6 support the idea that rIB2 serves as a scaffold for a kinase cascade involving p38 signaling. Our results further indicate that specific Rac GEFs are also components of this signaling complex.

Rat and human IB2/JIP2 facilitate Tiam1 activation of p38 but not Jnk. As a GEF for Rac GTPases, Tiam1 has the potential to activate Rac and thus promote the activation of each of the GTPase's many downstream effector proteins. One such Rac-binding protein is MLK3. Since we showed that Tiam1 can bind to rIB2, a putative scaffold protein with preference for kinases that activate p38 rather than Jnk, we tested whether expression of rIB2 could promote p38 activation by Tiam1. Cells were transfected with plasmids expressing GST-p38 and combinations of Tiam1 and rIB2. The stimulation of GST-p38 in cells was detected by the ability of precipitated p38 to phosphorylate the p38 target ATF2, which was detected with an antibody that specifically recognizes phospho-ATF2 (Fig. 7A). Transfection of Tiam1 alone (Fig. 7A, upper panel, lane 2) or rIB2 alone (Fig. 7A, upper panel, lane 3) did not stimulate p38 activity over control transfection (upper panel, lane 1). However, the presence of both rIB2 and Tiam1 consistently enhanced p38 activation (upper panel, lane 4) approximately threefold. For comparison, in this system an ~10-fold activation was observed for overexpressed MKK3, the direct upstream kinase activator of p38 (upper panel, lane 11). The rIB2-Tiam1 enhancement in p38 activation was not due to changes in expression levels of total p38 (middle panel) or Tiam1 (lower panel, compare lanes 2 and 4).

rIB2 mutants with deletions in specific functional domains were then used in this assay (see Fig. 1C for schematic depiction of rIB2 mutants). A mutant with the first 306 amino acids deleted, which eliminated rIB2 binding to p38 (rIB2-Δn306), failed to fully promote p38 activation when expressed alone or when coexpressed with Tiam1 (Fig. 7A, upper panel, lanes 5 and 8). A rIB2 mutant lacking an MLK3 binding site (rIB2-1-693) also failed to activate p38 fully when transfected alone or when cotransfected with Tiam1 (upper panel, lanes 7 and 10). These findings show that the scaffold function of rIB2 is involved in augmentation of p38 activation. Finally, deletion of residues 434 to 522 (rIB2-Δ434-522), which disrupts rIB2 binding to Tiam1 (Fig. 3B), also failed to activate p38 alone or together with Tiam1 (lanes 6 and 9), indicating that Tiam1 binding to rIB2 has functional significance in p38 regulation.

Similar results were obtained with huIB2/JIP2 (Fig 7B). Thus, expression of either huIB2/JIP2 or Tiam1 alone had no effect on p38 activity (Fig. 7B, upper panel, compare lanes 1, 2, and 3), while their concurrent expression resulted in elevated p38 activity (lane 5). This result gave additional support to the notion that human IB2/JIP2 is a functional homologue of rat IB2 and is a scaffold protein for p38. JIP1 was then tested in the same assay. As expected for a protein that does not bind to p38, JIP1 did not activate p38 on its own (Fig. 7B, upper panel, lane 4). It also did not augment Tiam1 induction of p38 activity in cells (Fig. 7B, upper panel, lane 6) even though it was expressed at levels comparable to those of huIB2/JIP2 (lower panels).

To test the specificity of this phenomenon, the effect of rIB2 on Jnk activity was assessed. Unlike p38, no detectable increase in Jnk activity was observed when Tiam1 was transfected alone or together with rIB2 (Fig. 7C).

Thus, rat and human IB2/JIP2 exhibit identical binding preferences for the p38 MAP kinase over the Jnk MAP kinase as well as identical potential to enhance Tiam1-mediated p38 activation. They likely represent species variants of the same gene product which, despite its assigned name, functions to enhance a specific pathway involving p38.

Increased scaffold-kinase complex formation induced by

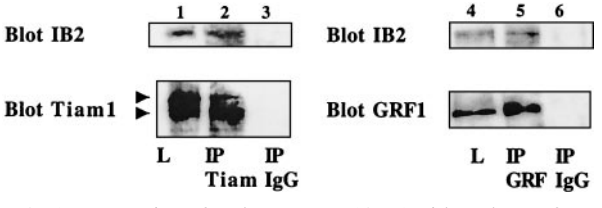

FIG. 2. Interaction of endogenous IB2/JIP2 with exchange factors. Lysates (L) from βTC1 cells (left panels) or rat brain (right panels) were incubated with protein A-Sepharose beads bound to anti-Tiam1 or control IgG antibody (left panels) or anti-GRF or control IgG antibody (right panels) as indicated. Lysates (lanes 1 and 4) and immunoprecipitates (IP) (lanes 2, 3, 5, and 6) were resolved by SDS-PAGE and immunoblotted as indicated. Full-length Tiam1 is very sensitive to proteolysis upon cell lysis, which presumably accounts for the lower-molecular-weight band in cell lysates and immunoprecipitates (lower left panel, arrowheads).

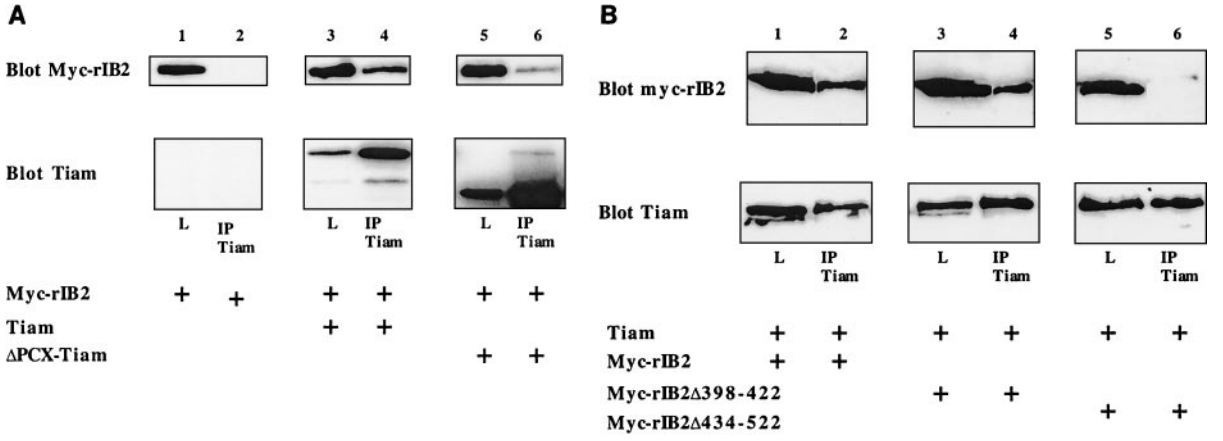

FIG. 3. The N-terminal PH, CC, and Ex domains of Tiam1 and amino acids 434 to 522 of rIB2 are required for binding. (A) COS7 cells were transiently transfected with plasmids expressing Myc-rIB2 and either Tiam1, ΔPCX-Tiam1, or empty vector. After 48 h, Tiam1 was immunoprecipitated and samples were blotted with either anti-Tiam1 antibodies (IP) (bottom panel, lanes 2, 4, and 6) or anti-Myc antibodies (upper panel, lanes 2, 4, and 6). Total cell lysates (L) were also blotted with anti-Myc antibodies (upper panel, lanes 1, 3, and 5) or anti-Tiam1 antibodies (lower panel, lanes 1, 3, and 5). (B) COS7 cells were transiently transfected with plasmids expressing Tiam1 and either Myc-rIB2, Myc-rIB2Δ398-422, or Myc-rIB2Δ434-522. Tiam1 was immunoprecipitated and blotted with either anti-Tiam1 antibodies (bottom panel, lanes 2, 4, and 6) or anti-Myc antibodies (upper panel, lanes 2, 4, and 6). Total cell lysates were immunoblotted with either anti-Myc antibodies (upper panel, lanes 1, 3, and 5) or anti-Tiam1 antibodies (lower panel, lanes 1, 3, and 5). Results are representative of at least three independent experiments.

Tiam1 and Ras-GRF1. Our results indicate that specific Rac-GEFs are also part of the p38 signaling complex recruited by IB2. We have begun to investigate how exchange factors and IB2 cooperate to potentiate p38 activation. Since the hypothesized function of a scaffolding protein is to bring together components of a signaling cascade, we tested whether the binding of Tiam1 to IB2 could enhance this function. In particular, the ability of Tiam1 expression to increase the association of each component of the p38 signaling cascade with IB2 was tested. Flag-IB2 was cotransfected with either MLK3, MKK3, or p38 and then the consequence of adding Tiam1 on the association of each kinase with IB2 was assessed (Fig. 8). The expression of Tiam1 increased the amount of MLK3 bound to IB2 approximately fourfold (Fig. 8A, upper panel, compare lanes 2 and 3), even though similar amounts of MLK3 were present in cell lysates (Fig. 8A, lower panel, compare lanes 2 and 3). Importantly, no increase in MLK3 association with IB2 was observed when the Tiam mutant, ΔPCX-Tiam1, which does not bind to IB2, was used (Fig. 8A, lane 4). A similarity in association was observed when the association of MKK3 (approximately fourfold increase) with IB2 was studied (Fig. 8B) and when the association of p38 (approximately twofold increase) with IB2 was investigated (Fig. 8C, lanes 1 to 4).

We were also interested in determining whether p38 associated with IB2 was activated. To this end, parallel immunoblots of immunoprecipitated scaffold were probed with a phospho-specific p38 antibody that recognizes only the activated form of the protein. Despite the fact that total p38 associated with IB2 increased when Tiam1 was expressed, an increase in the activated form of p38 in the complex was not detected (Fig. 8C, middle IP panel, compare lanes 2 and 3). Thus, Tiam1 binding to IB2 was capable of recruiting p38 MAP kinase to the scaffolding protein even if the kinase was not activated. However, activated p38 protein was not excluded from binding to IB2 since phospho-p38 was detectable in the complex and its amount increased (approximately fourfold) when the Tiam1 signal was amplified by coexpression of MKK3, the direct activator of p38 (Fig. 8C, middle IP panel, lanes 5 to 7). Finally, Ras-GRF1, which also binds to IB2, similarly increased the association of both inactive and active p38 (as well as MKK3 and MLK3) with IB2 in cells (Fig. 8D, top and middle IP panels, lanes 3 and 6; also data not shown).

Interestingly, the ability of Ras-GRF1 to enhance the association of inactive p38 with IB2 was not dependent on Rac activation, as this effect was observed even with Ras-GRF1-DH$^{(-)}$, a mutant protein containing an inactivating point mutation in its Rac-activating DH domain (Fig. 8D, top panel, lanes 4 and 7) (19). However, this mutant did not activate scaffold-associated p38, demonstrating that this effect was dependent on Rac activation (Fig. 8D, middle IP panel, lanes 6 and 7).

DISCUSSION

Studies in yeast were the first to document the existence of a scaffold protein for components of a MAP kinase family of signaling cascades (for a review, see reference 7). In that system, the scaffold, Ste5, was shown to complex with the kinases that lead to the activation of Fus3 and to be required for the formation of a functional Fus3 kinase cascade in cells. More recently, mammalian proteins capable of acting in an analogous manner have been identified. An extensively studied group is the IB/JIP family, members of which complex with components of the Jnk MAP kinase cascade (3, 45). Analogous to Fus3 studies in yeast, recent characterization of a JIP1 knockout mouse has shown that JIP1 is also required for a functional signaling cascade, in this case kainate activation of Jnk (43). The experiments described in the present study have added two new facets to our understanding of scaffold proteins for MAP kinase cascades. First, characterization of IB2/JIP2 has revealed that it can function as a mammalian scaffold for components of a signaling cascade leading to p38 activation.

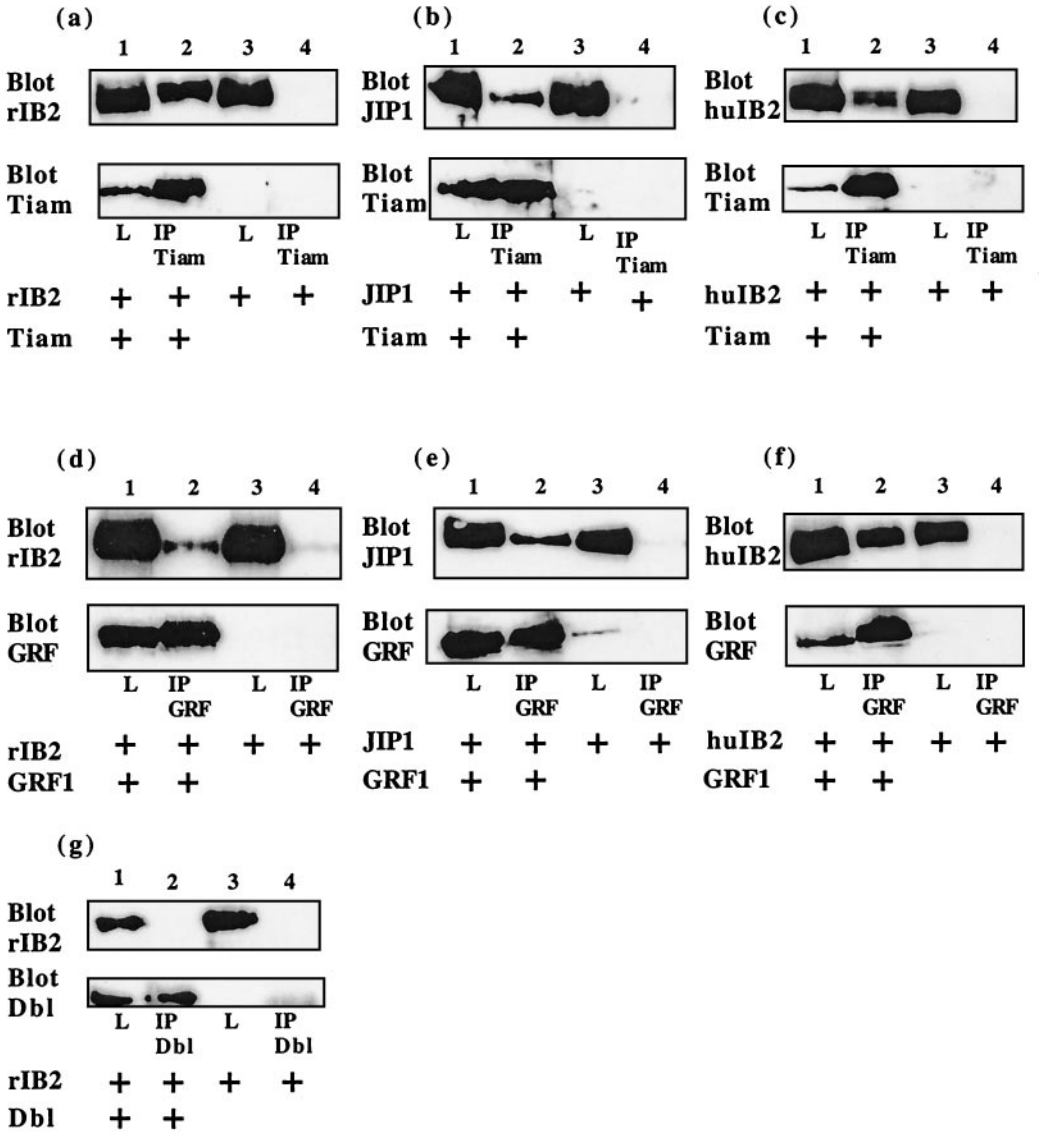

FIG. 4. Both Tiam1 and Ras-GRF1 bind to IB/JIP family members. COS7 cells were cotransfected with plasmids expressing exchange factors Tiam1 (a to c, lanes 1 and 2), Ras-GRF1 (d to f, lanes 1 and 2), or Dbl (g, lanes 1 and 2) or with empty vector (a to g, lanes 3 and 4) and epitope-tagged scaffold proteins rIB2/JIP2 (a, d, and g), JIP1 (b and e), or huIB2/JIP2 (c and f). GEFs were immunoprecipitated and the sample was immunoblotted for either scaffold proteins (top panels, lanes 2 and 4) or GEFs (bottom panels, lanes 2 and 4). Total cell lysates (L) were also immunoblotted with antibodies against either scaffold protein (upper panel, lanes 1 and 3) or GEFs (bottom panel, lanes 1 and 3). Results are representative of at least three independent experiments.

Second, by discovering that IB2/JIP2 can bind to and be modified by the Rac GEFs Tiam1 and Ras-GRF1, a potential mechanism connecting a scaffold for the p38 kinase cascade to upstream regulatory proteins in cells has been revealed.

In all of our experiments the rat cDNA we cloned and human JIP2 displayed indistinguishable properties. Moreover, our cDNA is 86% homologous to human IB2 and 96% homologous to mouse IB2. Finally, no gene in the human database is closer in amino acid sequence to our cDNA than human IB2 and no other gene in the mouse database is closer to our cDNA than mouse IB2. These observations strongly argue that our rat cDNA clone and human IB2 are species variants of the same gene.

The p38 MAP kinases, p38α, -β, -γ, and -δ, were the second mammalian stress- and cytokine-activated MAP kinase family to be identified (for a review, see reference 25). They are homologous to HOG1, the osmo-sensing MAP kinase of *S. cerevisiae*. In most, but not all, cases p38 and Jnk are activated concomitantly; however, the kinase pathways that lead to their activation are distinct. Whereas Jnk is activated via phosphorylation by SEK1 and MKK7 (15, 34), p38 is activated by MKK3 and MKK6 (11, 12, 15, 32). Upstream kinases that activate the MKKs are not always as specific, however. For example, while MEKK1 can activate only SEK1 and MKK7 (44) and TAO can activate only MKK3 (10), MEKK3 can activate both Jnk- and p38-activating kinases (2, 14, 17). Once activated, p38 can activate other kinases such as MAPKAP-K2, PRAK, MSK, and MNK and transcription factors such as CHOP, MEF2, and

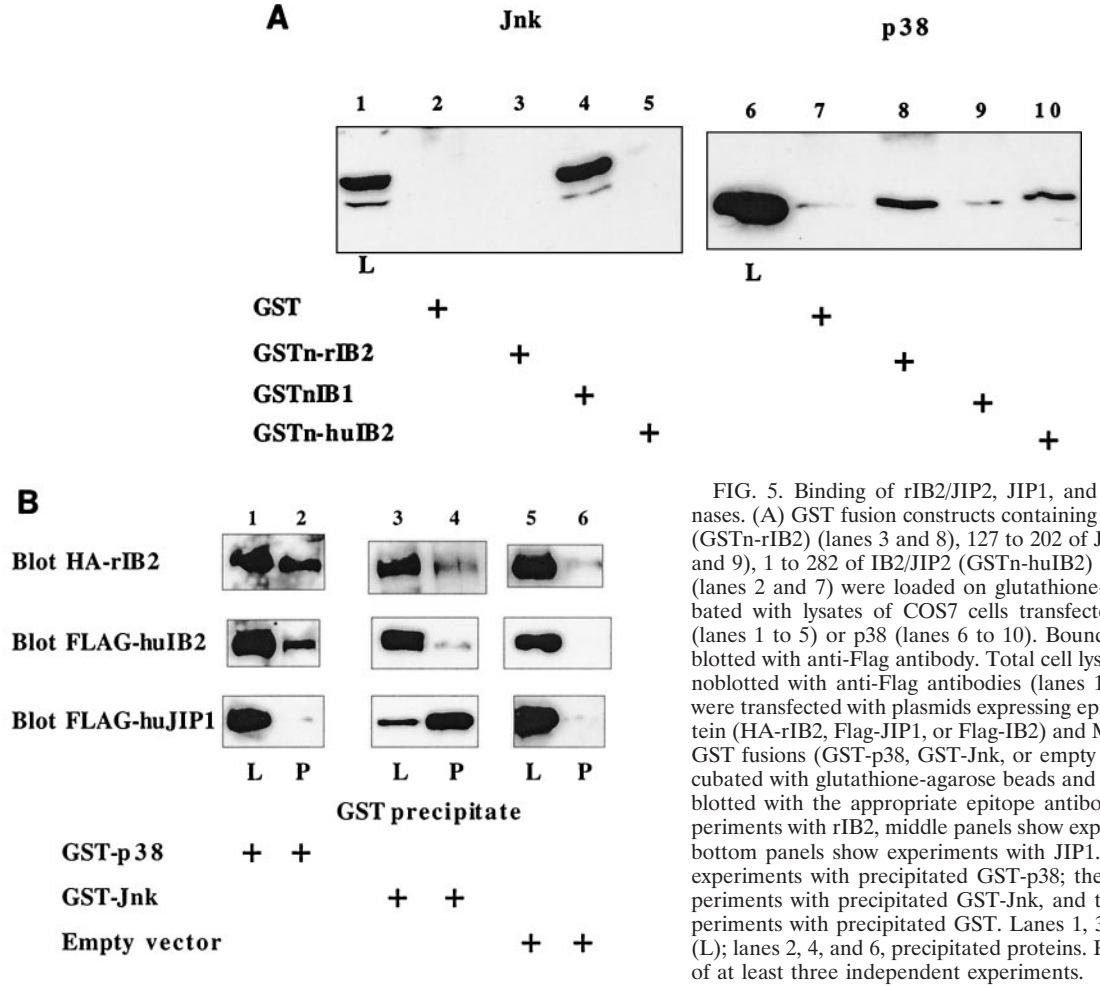

FIG. 5. Binding of rIB2/JIP2, JIP1, and huIB2/JIP2 to MAP kinases. (A) GST fusion constructs containing residues 1 to 306 of rIB2 (GSTn-rIB2) (lanes 3 and 8), 127 to 202 of JIP1 (GSTnJIP1) (lanes 4 and 9), 1 to 282 of IB2/JIP2 (GSTn-huIB2) (lanes 5 and 10), or GST (lanes 2 and 7) were loaded on glutathione-agarose beads and incubated with lysates of COS7 cells transfected with Flag-tagged Jnk (lanes 1 to 5) or p38 (lanes 6 to 10). Bound proteins were immunoblotted with anti-Flag antibody. Total cell lysates (L) were also immunoblotted with anti-Flag antibodies (lanes 1 and 6). (B) COS7 cells were transfected with plasmids expressing epitope-tagged scaffold protein (HA-rIB2, Flag-JIP1, or Flag-IB2) and MAP kinases expressed as GST fusions (GST-p38, GST-Jnk, or empty vector). Lysates were incubated with glutathione-agarose beads and the sample was immunoblotted with the appropriate epitope antibody. Top panels show experiments with rIB2, middle panels show experiments with huIB2, and bottom panels show experiments with JIP1. The left panels indicate experiments with precipitated GST-p38; the middle panels show experiments with precipitated GST-Jnk, and the right panels show experiments with precipitated GST. Lanes 1, 3, and 5, total cell lysates (L); lanes 2, 4, and 6, precipitated proteins. Results are representative of at least three independent experiments.

ATF2. p38 activity can have diverse effects on cells, including regulation of cell cycle arrest, apoptosis, cell migration-invasion, and differentiation.

Our conclusion that IB2/JIP2 is a scaffold for the p38 cascade is based on the observation that both rat and human IB2/JIP2 bound more effectively to p38α and an activator of p38, MKK3, than to Jnk or an activator of Jnk, MKK7, both in vitro and in vivo. In addition, we found that the expression of rat or human IB2/JIP2 in cells potentiated p38 activation but not Jnk activation by the Tiam1 exchange factor. Finally,

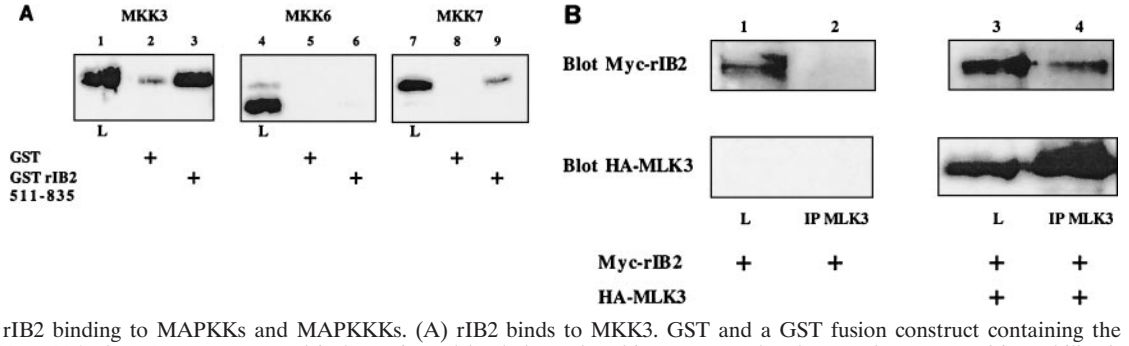

FIG. 6. rIB2 binding to MAPKKs and MAPKKKs. (A) rIB2 binds to MKK3. GST and a GST fusion construct containing the C-terminal residues 511 to 835 of rIB2 were expressed in bacteria and loaded on glutathione-agarose beads. Equal amounts of immobilized GST fusion proteins were incubated with lysates of COS7 cells transfected with HA-tagged MKK3 (left panel), HA-MKK6 (middle panel), and HA-MKK7 (right panel). Bound proteins were eluted, resolved by SDS-PAGE, and immunoblotted with anti-HA antibody. Lanes 1, 4, and 7 contain equal volumes of lysates expressing MKK3, MKK6, and MKK7, respectively. (B) rIB2 binds to MLK3. COS7 cells were transfected with Myc-rIB2 and HA-MLK3 or empty vector. Expressed proteins were immunoprecipitated with HA antibody. Lysates (lanes 1 and 3) and IPs (lanes 2 and 4) were resolved by SDS-PAGE and immunoblotted with the indicated antibodies.

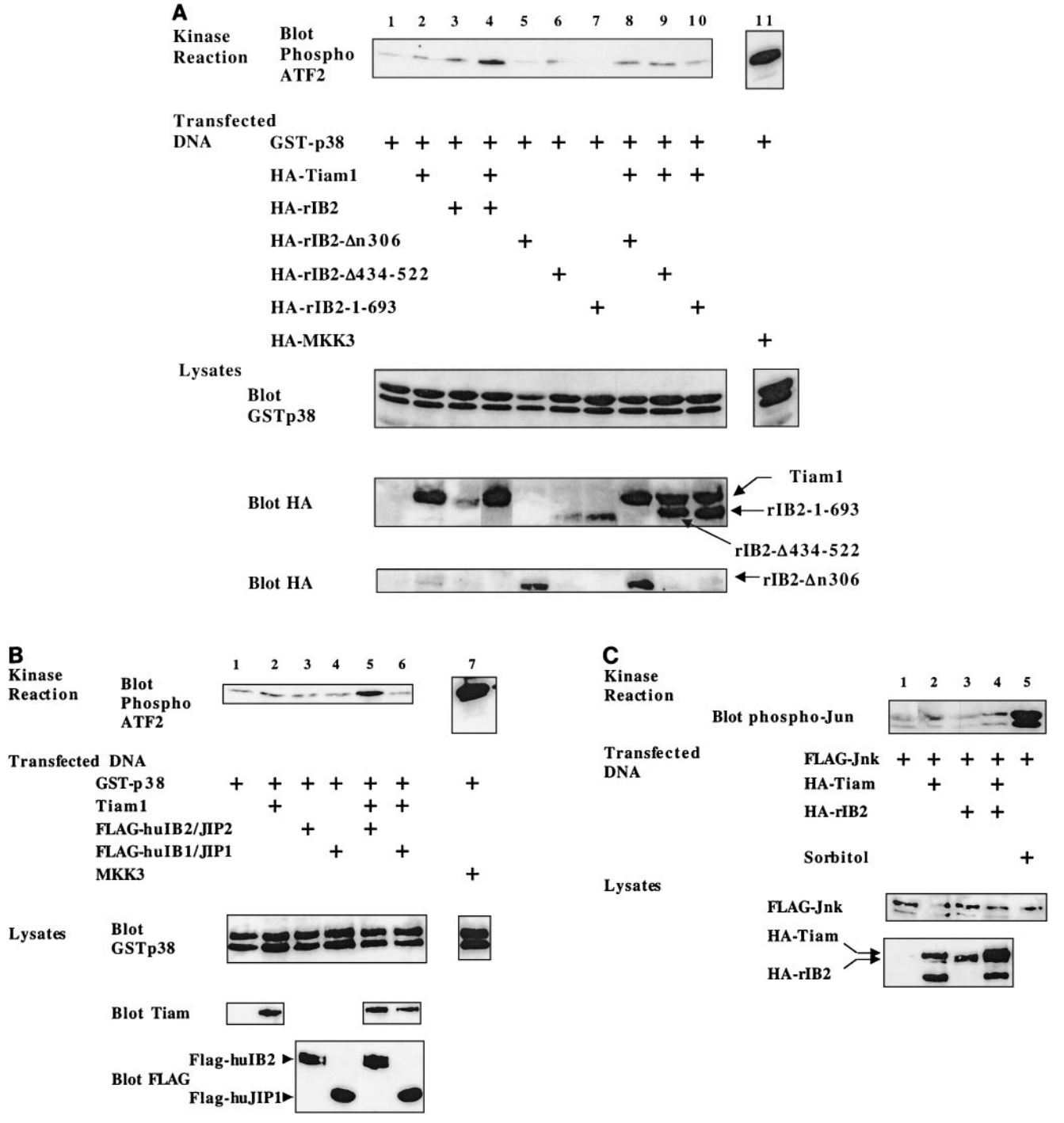

FIG. 7. (A) rIB2 enhances Tiam1 activation of p38. HEK 293T cells were cotransfected with plasmids expressing GST-p38 along with the HA-Tiam1, HA-rIB2, and mutant HA-rIB2 plasmid DNAs as indicated. Total amounts of transfected DNA were equalized with pcDNA3 vector. Cells were serum starved 16 h prior to harvest. GST-p38 purified on glutathione-agarose beads was assayed for its ability to phosphorylate ATF2 as detected with phospho-specific ATF2 antibody (top panel). Equal volumes of lysate were immunoblotted with anti-GST or anti-HA antibody as indicated (middle and lower panels, respectively). Results shown are representative of at least three independent experiments done in duplicate. (B) huIB2/JIP2, but not JIP1, enhances Tiam1 activation of p38. Experiments were performed as for panel A except that huIB2/JIP2 and JIP1 were substituted for rIB2. Results shown are representative of three independent experiments done in duplicate. (C) rIB2 does not enhance Tiam1 activation of Jnk. HEK 293T cells were cotransfected with plasmids expressing Flag-Jnk along with HA-Tiam1 and HA-rIB2 as indicated. Activated Jnk was affinity precipitated from lysates of serum-starved cells by incubation with immobilized GST-Jun. The ability of the precipitated Jnk to phosphorylate Jun was detected using phospho-specific Jnk antibody (top panel). Equal volumes of lysate were immunoblotted with anti-Flag or anti-HA antibody as indicated (middle and lower panels, respectively).

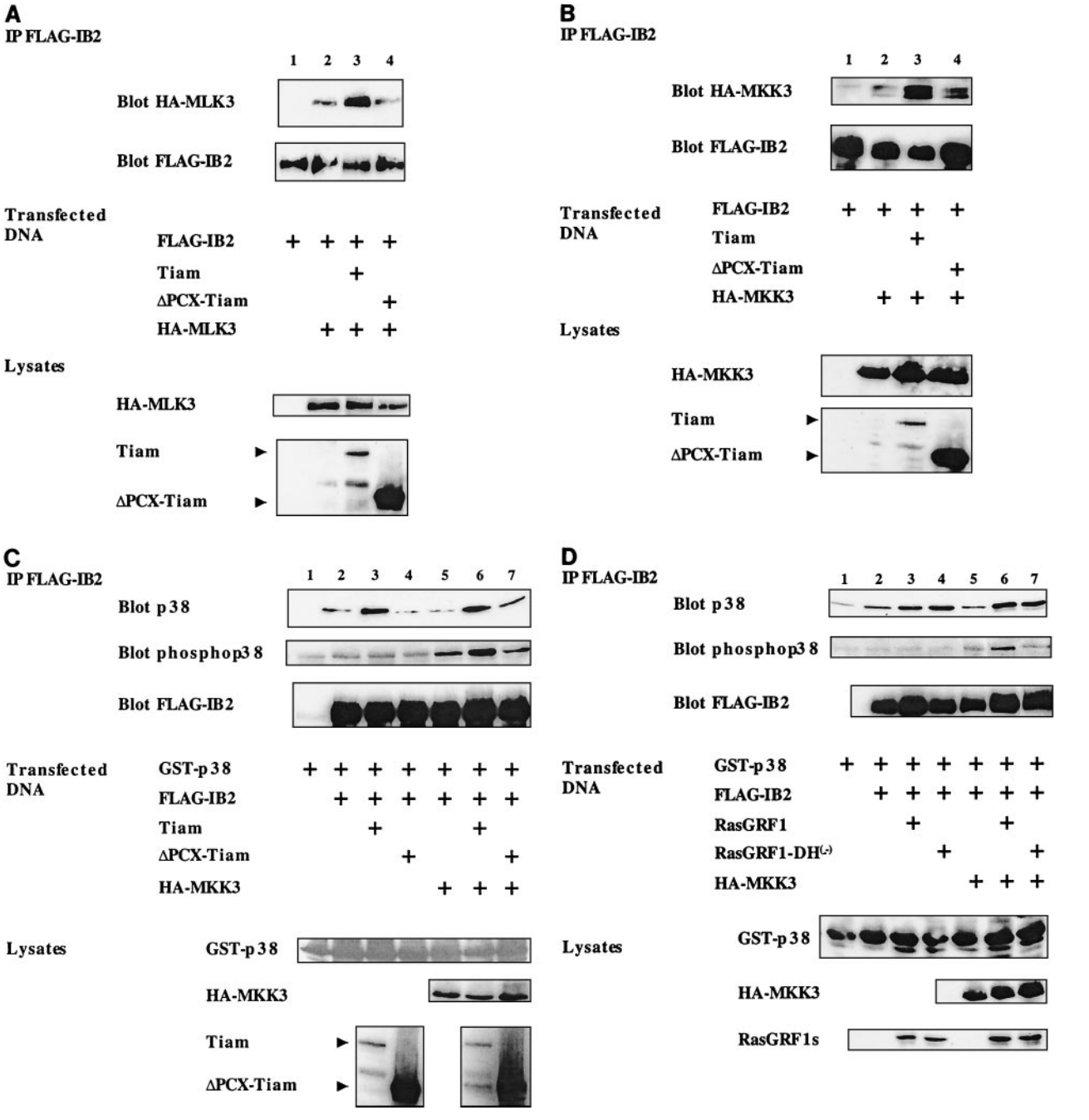

FIG. 8. Exchange factors enhance association of IB2/JIP2 scaffold with MLK3, MKK3, and p38. HEK 293T cells were cotransfected with plasmids expressing Flag-IB2, HA-Tiam1, ΔPCX-Tiam, and/or HA-MLK3 (A), HA-MKK3 (B), or GST-p38 (C) as indicated. Cells were deprived of serum 16 h prior to harvest. IB2 was immunoprecipitated from cell lysates and the presence of individual kinases in the immunoprecipitate was determined by immunoblotting. For p38 (C, middle IP panel), the immunoblots were also probed with phospho-specific p38 antibodies and in some cases MKK3 was transfected along with p38 (C, lanes 5 to 7). The bottom panel shows expression levels of protein in lysates assessed by immunoblotting. (D) Ras-GRF1 or Ras-GRF-DH$^{(-)}$ was substituted for Tiam1 in the experiments described for panel C.

Tiam1 or Ras-GRF1 binding to IB2/JIP2 enhanced the binding of all three components of a p38 MAP kinase signaling pathway to IB2/JIP2. This conclusion was surprising at first because JIP2 and IB2 were originally described as Jnk-binding proteins (29, 45). However, in this earlier work Jnk binding to IB2/JIP2 was much weaker than to IB1/JIP1, suggesting that the scaffold might actually play a different role. In addition, the existence of a p38 scaffold in yeast (31) suggested that there was likely a counterpart in mammalian cells. Finally, recent work has shown that IB2 can bind to p38δ, but not to Jnk, in the presence of fibroblast growth factor homologous factor 1 (35).

Another point of interest regarding the p38 scaffold IB2/JIP2 is its ability to bind to MLK3, since this MKKK has been associated primarily with activation of the Jnk kinase cascade.

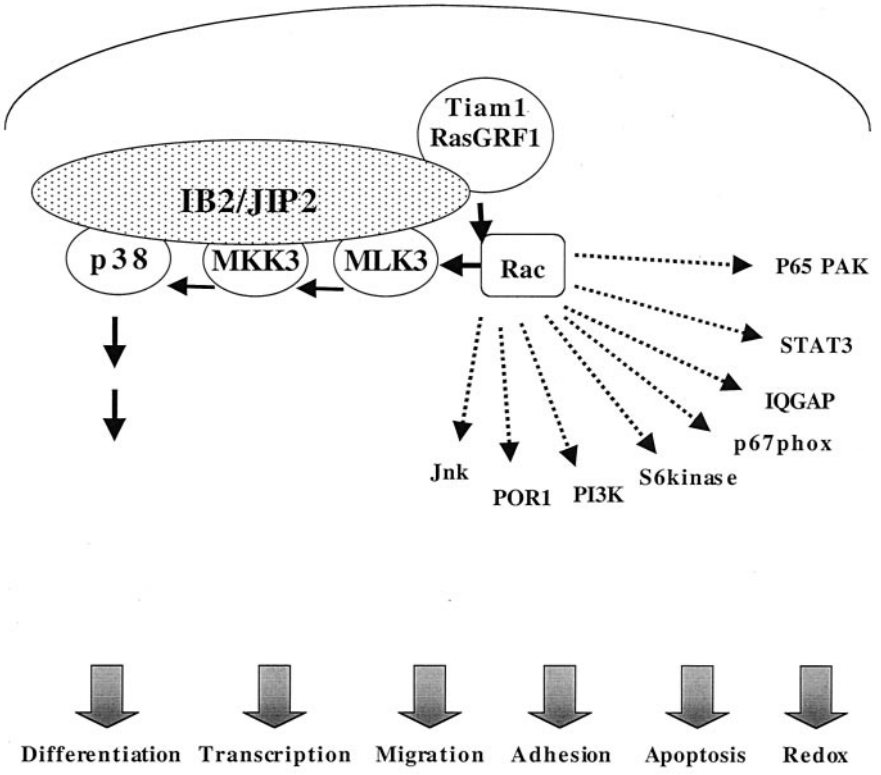

FIG. 9. Model of Tiam/Rac signaling specificity mediated by IB/JIP family members. Rac, which is activated by a variety of GEFs such as Tiam1 or Ras-GRF1, has the capacity to activate a large number of downstream target proteins. By binding to IB2/JIP2, Tiam1 or Ras-GRF1 activates Rac in the spatial context of one of its downstream target proteins, MLK3. Thus, Tiam1 and Ras-GRF1 have the capacity to influence Rac signaling specificity by binding to IB2/JIP2, a scaffold for the p38 signaling cascade.

In fact, overexpression of MLK3 has been reported to activate Jnk much more efficiently than p38 in cells (33). Nevertheless, MLK3 has been shown to stimulate the kinase activity of the p38-activating MKK3 (40). Our finding that IB2 expression enhances p38 activation in cells suggests that MLK3 may require the presence of a scaffolding protein for efficient activation of the p38 signaling cascade. Interestingly, the expression pattern of IB2 is restricted to neurons and pancreatic β-cells, possibly explaining the failure to observe p38 activation by MLK3 in many cell types.

The precise function of scaffolds like the IB/JIP family is still not completely clear. Their overexpression in cells can enhance the magnitude of signaling through a particular MAP kinase cascade, as was shown previously for JIP1 and the Jnk kinase cascade (42), and as we showed here for IB2 and the p38 cascade. However, this may not be their primary function, since changing the ratio of expression of the scaffold to kinases in the cascade can make the scaffolds appear as inhibitors rather than activators of the cascade (16). Alternatively, they may function in directing signaling specificity, rather than as modifiers of signal amplitude. At one level this could occur by promoting the interaction of the correct MKKKs, MKKs, and MAPKs to make a functional cascade. However, in many cases the kinases themselves already have binding sites that recognize their substrates with high affinity (for a review, see reference 25).

Our findings suggest that a function of these scaffolds is to contribute to signaling specificity by coupling Rac activation to one of its many downstream target proteins. This is based on our observation that another component of the IB/JIP scaffold protein complex in cells is a Rac-GEF, either Tiam1 or Ras-GRF1. One of the many target proteins of Rac GTPases is MLK3, the initiating kinase in the cascade that leads to p38 activation (8, 39). In fact, we have found that activation of scaffold-bound p38 by Tiam1 or Ras-GRF1 was dependent upon the Rac-GEF domain of the exchange factors. We also found that the binding of Tiam1 or Ras-GRF1 to IB2/JIP2 contributed to the enhancement of p38 activation through its ability to increase the association of kinases with the scaffold. These findings argue that scaffolds can potentially contribute to signaling specificity by coupling individual upstream activators of Rac to one of the many downstream signaling cascades initiated by Rac. Supporting this notion is our observation that Tiam1 expression did not lead to significant activation of p38 in HEK 293 cells, which do not express IB2/JIP2. Apparently, in the absence of this scaffold activated Rac is targeted toward other effector pathways. However, ectopically expressed scaffold formed a complex with Tiam1 or Ras-GRF1, directing activated Rac toward the components of a p38 kinase cascade rather than a Jnk kinase cascade (Fig. 9).

We also observed that Tiam1 and Ras-GRF1 can bind to IB1/JIP1, the scaffold for kinases involved in Jnk activation. Although we could not demonstrate that expression of IB1/JIP1 enhances Tiam1 or Ras-GRF1-induced Jnk activation (data not shown), it is possible that binding of the GEFs to

JIP1 in cells changes the specificity of downstream signaling toward Jnk. If so, factors that influence whether the GEFs bind to either IB2/JIP2 or IB1/JIP1 could influence whether p38 or Jnk becomes activated in cells upon GEF activation.

Although we have focused the experiments in this paper on the potential role of IB2/JIP2 in mediating signaling specificity downstream of GEFs and Rac, the properties of JIP-related proteins suggest that they may also participate in coupling Tiam1 and Ras-GRF1 to upstream signals. That is because IB2/JIP2 and IB1/JIP1 contain virtually identical PTB domains at their C termini, which have the capacity to bind to various cell surface proteins. One documented IB1/JIP1 and IB2/JIP2 binding partner is ApoER2, a receptor for neuronal Reelin that plays a key role in neuronal development (38). Thus, the PTB domain of IB2/JIP2 or IB1/JIP1 may target Tiam1 or Ras-GRF1 to specific membrane components that can contribute to their activation in cells. For the Rac and Ras GEF, Ras-GRF1, we have already shown that the IB2/JIP2-binding PH-CC-IQ motif is required for its activation by calcium in cells (6). In neurons, where Ras-GRF1 and IB2 are highly enriched, calcium is known to enter cells through a variety of channels which generate distinct intracellular signaling cascades (41). By its ability to bind to cell surface components and Ras-GRF1, it is possible that IB2/JIP2 may couple specific calcium channels to both the Ras/Erk and Rac/p38 signaling cascades.

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REFERENCES

1. **Bishop, A. L., and A. Hall.** 2000. Rho GTPases and their effector proteins. Biochem. J. **348:**241–255.
2. **Blank, J. L., P. Gerwins, E. M. Elliott, S. Sather, and G. L. Johnson.** 1996. Molecular cloning of mitogen-activated protein/ERK kinase kinases (MEKK) 2 and 3. Regulation of sequential phosphorylation pathways involving mitogen-activated protein kinase and c-Jun kinase. J. Biol. Chem. **271:**5361–5368.
3. **Bonny, C., P. Nicod, and G. Waeber.** 1998. IB1, a JIP-1-related nuclear protein present in insulin-secreting cells. J. Biol. Chem. **273:**1843–1846.
4. **Bourguignon, L. Y., H. Zhu, L. Shao, and Y. W. Chen.** 2000. Ankyrin-Tiam1 interaction promotes Rac1 signaling and metastatic breast tumor cell invasion and migration. J. Cell Biol. **150:**177–191.
5. **Buchanan, F. G., C. M. Elliot, M. Gibbs, and J. H. Exton.** 2000. Translocation of the Rac1 guanine nucleotide exchange factor Tiam1 induced by platelet-derived growth factor and lysophosphatidic acid. J. Biol. Chem. **275:**9742–9748.
6. **Buchsbaum, R., J. B. Telliez, S. D. Goonesekera, and L. A. Feig.** 1996. The N-terminal pleckstrin, coiled-coil and IQ domains of the exchange factor, Ras-GRF, act cooperatively to facilitate activation by calcium. Mol. Cell. Biol. **16:**4888–4896.
7. **Burack, W. R., and A. S. Shaw.** 2000. Signal transduction: hanging on a scaffold. Curr. Opin. Cell Biol. **12:**211–216.
8. **Burbelo, P. D., D. Drechsel, and A. Hall.** 1995. A conserved binding motif defines numerous candidate target proteins for both Cdc42 and Rac GTPases. J. Biol. Chem. **270:**29071–29074.
9. **Cerione, R. A., and Y. Zheng.** 1996. The Dbl family of oncogenes. Curr. Opin. Cell Biol. **8:**216–222.
10. **Chen, Z., M. Hutchison, and M. H. Cobb.** 1999. Isolation of the protein kinase TAO2 and identification of its mitogen-activated protein kinase/extracellular signal-regulated kinase kinase binding domain. J. Biol. Chem. **274:**28803–28807.
11. **Cuenda, A., G. Alonso, N. Morrice, M. Jones, R. Meier, P. Cohen, and A. R. Nebreda.** 1996. Purification and cDNA cloning of SAPKK3, the major activator of RK/p38 in stress- and cytokine-stimulated monocytes and epithelial cells. EMBO J. **15:**4156–4164.
12. **Cuenda, A., P. Cohen, V. Buee-Scherrer, and M. Goedert.** 1997. Activation of stress-activated protein kinase-3 (SAPK3) by cytokines and cellular stresses is mediated via SAPKK3 (MKK6); comparison of the specificities of SAPK3 and SAPK2 (RK/p38). EMBO J. **16:**295–305.
13. **Daniels, R. H., F. T. Zenke, and G. M. Bokoch.** 1999. alphaPix stimulates p21-activated kinase activity through exchange factor-dependent and -independent mechanisms. J. Biol. Chem. **274:**6047–6050.
14. **Deacon, K., and J. L. Blank.** 1999. MEK kinase 3 directly activates MKK6 and MKK7, specific activators of the p38 and c-Jun NH2-terminal kinases. J. Biol. Chem. **274:**16604–16610.
15. **Derijard, B., J. Raingeaud, T. Barrett, I. H. Wu, J. Han, R. J. Ulevitch, and R. J. Davis.** 1995. Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms. Science **267:**682–685.
16. **Dickens, M., J. S. Rogers, J. Cavanagh, A. Raitano, Z. Xia, J. R. Halpern, M. E. Greenberg, C. L. Sawyers, and R. J. Davis.** 1997. A cytoplasmic inhibitor of the JNK signal transduction pathway. Science **277:**693–696.
17. **Ellinger-Ziegelbauer, H., K. Brown, K. Kelly, and U. Siebenlist.** 1997. Direct activation of the stress-activated protein kinase (SAPK) and extracellular signal-regulated protein kinase (ERK) pathways by an inducible mitogen-activated protein kinase/ERK kinase kinase 3 (MEKK) derivative. J. Biol. Chem. **272:**2668–2674.
18. **Fleming, I. N., C. M. Elliott, F. G. Buchanan, C. P. Downes, and J. H. Exton.** 1999. Ca^{2+}/calmodulin-dependent protein kinase II regulates Tiam1 by reversible protein phosphorylation. J. Biol. Chem. **274:**12753–12758.
19. **Freshney, N. W., S. D. Goonesekera, and L. A. Feig.** 1997. Activation of the exchange factor Ras-GRF by calcium requires an intact Dbl homology domain. FEBS Lett. **407:**111–115.
20. **Habets, G. G., E. H. Scholtes, D. Zuydgeest, R. A. van der Kammen, J. C. Stam, A. Berns, and J. G. Collard.** 1994. Identification of an invasion-inducing gene, Tiam-1, that encodes a protein with homology to GDP-GTP exchangers for Rho-like proteins. Cell **77:**537–549.
21. **Hordijk, P. L., J. P. ten Klooster, R. A. van der Kammen, F. Michiels, L. C. Oomen, and J. G. Collard.** 1997. Inhibition of invasion of epithelial cells by Tiam1-Rac signaling. Science **278:**1464–1466.
22. **Innocenti, M., R. Zippel, R. Brambilla, and E. Sturani.** 1999. CDC25(Mm)/Ras-GRF1 regulates both Ras and Rac signaling pathways. FEBS Lett. **460:**357–362.
23. **Kawazoe, N., M. Watabe, Y. Masuda, S. Nakajo, and K. Nakaya.** 1999. Tiam1 is involved in the regulation of bufalin-induced apoptosis in human leukemia cells. Oncogene **18:**2413–2421.
24. **Kunda, P., G. Paglini, S. Quiroga, K. Kosik, and A. Caceres.** 2001. Evidence for the involvement of Tiam1 in axon formation. J. Neurosci. **21:**2361–2372.
25. **Kyriakis, J. M., and J. Avruch.** 2001. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol. Rev. **81:**807–869.
26. **Martegani, E., M. Vanoni, R. Zippel, P. Coccetti, R. Brambilla, C. Ferrari, E. Sturani, and L. Alberghina.** 1992. Cloning by functional complementation of a mouse cDNA encoding a homologue of CDC25, a Saccharomyces cerevisiae RAS activator. EMBO J. **11:**2151–2157.
27. **Michiels, F., G. G. M. Habets, J. C. Stam, R. A. van der Kammen, and J. G. Collard.** 1995. A role for Rac in Tiam1-induced membrane ruffling and invasion. Nature **375:**338–340.
28. **Michiels, F., J. C. Stam, P. L. Hordijk, R. A. van der Kammen, L. Ruuls-Van Stalle, C. A. Feltkamp, and J. G. Collard.** 1997. Regulated membrane localization of Tiam1, mediated by the NH2-terminal pleckstrin homology domain, is required for Rac-dependent membrane ruffling and c-Jun NH2-terminal kinase activation. J. Cell Biol. **137:**387–398.
29. **Negri, S., A. Oberson, M. Steinmann, C. Sauser, P. Nicod, G. Waeber, D. F. Schorderet, and C. Bonny.** 2000. cDNA cloning and mapping of a novel islet-brain/JNK-interacting protein. Genomics **64:**324–330.
30. **Nobes, C. D., and A. Hall.** 1995. Rho, Rac and Cdc42 GTPases: regulators of actin structures, cell adhesion and motility. Biochem. Soc. Trans. **23:**456–459.
31. **Posas, F., and H. Saito.** 1997. Osmotic activation of the HOG MAPK pathway via Ste11p MAPKKK: scaffold role of Pbs2p MAPKK. Science **276:**1702–1705.
32. **Raingeaud, J., A. J. Whitmarsh, T. Barrett, B. Derijard, and R. J. Davis.** 1996. MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. Mol. Cell. Biol. **16:**1247–1255.
33. **Rana, A., K. Gallo, P. Godowski, S. Hirai, S. Ohno, L. Zon, J. M. Kyriakis, and J. Avruch.** 1996. The mixed lineage kinase SPRK phosphorylates and activates the stress-activated protein kinase activator, SEK-1. J. Biol. Chem. **271:**19025–19028.
34. **Sanchez, I., R. T. Hughes, B. J. Mayer, K. Yee, J. R. Woodgett, J. Avruch, J. M. Kyriakis, and L. I. Zon.** 1994. Role of SAPK/ERK kinase-1 in the stress-activated pathway regulating transcription factor c-Jun. Nature **372:**794–798.
35. **Schoorlemmer, J., and M. Goldfarb.** 2001. Fibroblast growth factor homologous factors are intracellular signaling proteins. Curr. Biol. **11:**793–797.
36. **Shou, C., C. L. Farnsworth, B. G. Neel, and L. A. Feig.** 1992. Molecular

cloning of cDNAs encoding a guanine-nucleotide releasing factor for Ras p21. Nature **358:**351–354.

37. **Stam, J. C., E. E. Sander, F. Michiels, F. N. van Leeuwen, H. E. Kain, R. A. van der Kammen, and J. G. Collard.** 1997. Targeting of Tiam1 to the plasma membrane requires the cooperative function of the N-terminal pleckstrin homology domain and an adjacent protein interaction domain. J. Biol. Chem. **272:**28447–28454.
38. **Stockinger, W., C. Brandes, D. Fasching, M. Hermann, M. Gotthardt, J. Herz, W. J. Schneider, and J. Nimpf.** 2000. The reelin receptor ApoER2 recruits JNK-interacting proteins-1 and -2. J. Biol. Chem. **275:**25625–25632.
39. **Teramoto, H., O. A. Coso, H. Miyata, T. Igishi, T. Miki, and J. S. Gutkind.** 1996. Signaling from the small GTP-binding proteins Rac1 and Cdc42 to the c-Jun N-terminal kinase/stress-activated protein kinase pathway. A role for mixed lineage kinase 3/protein-tyrosine kinase 1, a novel member of the mixed lineage kinase family. J. Biol. Chem. **271:**27225–27228.
40. **Tibbles, L. A., Y. L. Ing, F. Kiefer, J. Chan, N. Iscove, J. R. Woodgett, and N. J. Lassam.** 1996. MLK-3 activates the SAPK/JNK and p38/RK pathways via SEK1 and MKK3/6. EMBO J. **15:**7026–7035.
41. **West, A. E., W. G. Chen, M. B. Dalva, R. E. Dolmetsch, J. M. Kornhauser, A. J. Shaywitz, M. A. Takasu, X. Tao, and M. E. Greenberg.** 2001. Calcium regulation of neuronal gene expression. Proc. Natl. Acad. Sci. USA **98:** 11024–11031.
42. **Whitmarsh, A. J., J. Cavanagh, C. Tournier, J. Yasuda, and R. J. Davis.** 1998. A mammalian scaffold complex that selectively mediates MAP kinase activation. Science **281:**1671–1674.
43. **Whitmarsh, A. J., C. Y. Kuan, N. J. Kennedy, N. Kelkar, T. F. Haydar, J. P. Mordes, M. Appel, A. A. Rossini, S. N. Jones, R. A. Flavell, P. Rakic, and R. J. Davis.** 2001. Requirement of the JIP1 scaffold protein for stress-induced JNK activation. Genes Dev. **15:**2421–2432.
44. **Yan, M., T. Dai, J. C. Deak, J. M. Kyriakis, L. I. Zon, J. R. Woodgett, and D. J. Templeton.** 1994. Activation of stress-activated protein kinase by MEKK1 phosphorylation of its activator SEK1. Nature **372:**798–800.
45. **Yasuda, J., A. J. Whitmarsh, J. Cavanagh, M. Sharma, and R. J. Davis.** 1999. The JIP group of mitogen-activated protein kinase scaffold proteins. Mol. Cell. Biol. **19:**7245–7254.
46. **Zhou, K., Y. Wang, J. L. Gorski, N. Nomura, J. Collard, and G. M. Bokoch.** 1998. Guanine nucleotide exchange factors regulate specificity of downstream signaling from Rac and Cdc42. J. Biol. Chem. **273:**16782–16786.