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Tensin Can Induce JNK and p38 Activation

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Cells organize diverse types of specialized adhesion sites upon attachment to extracellular matrix (ECM) components. One of the physiological roles of such cell–ECM interactions is to initiate and regulate adhesion-mediated signal transduction responses. The association of cells with fibronectin fibrils has been shown to regulate the JNK and p38 signaling pathways. We tested whether tensin, a cytoskeletal component localized to both focal contacts and fibronectin-associated fibrillar adhesions, can induce these signaling pathways. We found that tensin overexpression resulted in activation of both the c-Jun amino-terminal kinase (JNK) and p38 pathways. Tensin-mediated JNK activation was independent of the activities of the small GTP binding proteins Rac and Cdc42, but did depend on SEK, a kinase involved in the JNK pathway. We suggest that tensin may directly activate the JNK and p38 pathways, acting downstream or independent of the activities of the small GTP binding proteins Rac and Cdc42. © 2000 Academic Press

Integrins are a family of transmembrane receptors that mediate interactions between cells and the extracellular matrix (ECM) (1). Interactions of integrins with their ligands initiate and regulate cytoplasmic processes that include reorganization of the cytoskeleton and signal transduction responses, e.g., tyrosine phosphorylation of cytoplasmic molecules, and activation of mitogen-activated protein kinase (MAPK) family members that include extracellular signal-related kinase (ERK) and c-Jun amino-terminal kinase (JNK) (for reviews see 2–6). Signals initiated by adhesion are propagated and regulated in the cytoplasm by biochemical mediators that include, among others, the Ras superfamily of small GTP binding proteins, various kinases (e.g., focal adhesion kinase, Src family kinases), adapter proteins (e.g., Shc and Grb2), and phosphatases (e.g., PTEN) (7–12). Several members of the Ras superfamily, e.g., Rho, Rac, and Cdc42, integrate extracellular signals and translate them to cytoplasmic biochemical responses that include reorganization of the cytoskeleton and transcriptional regulation (for reviews see 13–15).

Recent studies have established that molecules residing in focal contacts can directly activate signal transduction pathways. Thus, focal adhesion kinase (FAK) can activate the ERK type of MAPK when overexpressed in fibroblasts, or when *de novo* expressed in primary human macrophages (16, 17). Therefore, focal contacts may serve as specialized structures for activation of MAPK responses.

JNK is another signaling pathway activated upon cell-ECM attachment (18). Activation of Rac or Cdc42, both members of the Rho family of small GTP-binding proteins, can initiate the JNK response (19, 20). The mechanism of initiation and regulation of adhesion-induced JNK responses is not clear. However, a recent study suggested that association of cells with fibronectin fibrils might be required to regulate the JNK response (21). In a previous study, we characterized the sites for the association of cells with fibronectin fibrils termed "fibrillar adhesions," a unique type of cell-ECM attachment site distinct from the well-characterized focal contacts. Unlike the "classical" focal contacts, fibrillar adhesions have low levels of tyrosine phosphorylation and lack the tyrosine-phosphorylated molecules FAK and paxillin. They also contain very low levels of other focal contact-resident molecules, e.g., vinculin and α-actinin (22). In contrast, fibrillar adhesions contain high levels of the cytoskeletal molecule tensin (22).

Tensin is a tyrosine-phosphorylated protein that contains a unique combination of not only actin-binding and actin-capping domains, but also an SH2 domain and a region with homology to the PTEN tumor

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suppressor gene product (23–25). These features suggest a potential role for tensin in signal transduction. The goal of the present study was to examine whether tensin, a molecule particularly prominent in fibrillar adhesions, could induce JNK activation. We found that tensin can activate both JNK and p38 responses upon overexpression. This activation was not suppressed by dominant-negative forms of Rac or Cdc42, indicating that tensin may act downstream, or independently, of events triggered by these small GTP-binding proteins. This is the first report that links directly the cytoskeletal component tensin to the activation of signal transduction pathways.

MATERIALS AND METHODS

Cells. Human embryonic kidney 293T cells were maintained in DMEM supplemented with 10% fetal bovine serum. 293T cells were transfected by the calcium-phosphate precipitation technique as described (19).

DNA constructs. Plasmids containing epitope-tagged forms of MAPK, JNK, and p38 were previously described (19, 26). Expression vectors encoding for GFP or GFP–tensin fusion proteins were previously described (22). Expression plasmids for dominant-negative SEK (pEBG SEK KR) and MKK6 (pCEFL-GST-MKK6 KR) have been described previously (26, 27). Dominant-negative small GTP binding proteins, designated N17Rac and N17Cdc42, have been described previously (19).

Kinase assays. MAPK activity in cells transfected with an epitope-tagged MAPK (HA-ERK2, referred to herein as HA-MAPK) was determined as described previously using myelin basic protein (Sigma, St. Louis, MO) as substrate (28). JNK and p38 assays in cells transfected with epitope-tagged JNK or p38 (HA-JNK, or HA-p38) were carried out as described previously (26) using purified bacterially expressed GST-ATF2 fusion protein as substrate. Samples were analyzed by SDS gel electrophoresis in 12% polyacrylamide gels. Parallel anti-HA immunoprecipitates were processed for Western blot analysis using ERK2, JNK1, or p38 specific antiserum (Santa Cruz Biotechnology, Santa Cruz, CA).

RESULTS AND DISCUSSION

Upon adhesion, cells assemble specialized cell–ECM adhesion sites. These sites provide physical links between the extracellular environment and the cytoplasm, support cell-substrate attachment, and regulate cell spreading and migration. Adhesion sites also promote and regulate signal transduction responses (2–6). Cells can generate simultaneously different types of cell-ECM adhesions, which may deliver specialized cytoplasmic responses (21, 22). While the ERK MAPK response initiated by focal contact-resident molecules such as FAK, Src, and Cas is well-documented, initiation of the JNK response, and especially potential involvement of the unique cytoskeletal component tensin in signal transduction need elucidation. In a recent study, we demonstrated that tensin is the main cytoskeletal component of the fibrillar adhesion, a type of cell-ECM adhesion site distinct from focal contacts (22). We utilized a GFP–tensin fusion protein and demonstrated its localization in both focal contacts and fibrillar adhesions, in an identical manner to the endogenous molecule. We also demonstrated that GFP–tensin fusion protein physically associates with complexes induced by fibronectin-coated beads (B.-Z. Katz, unpublished results).

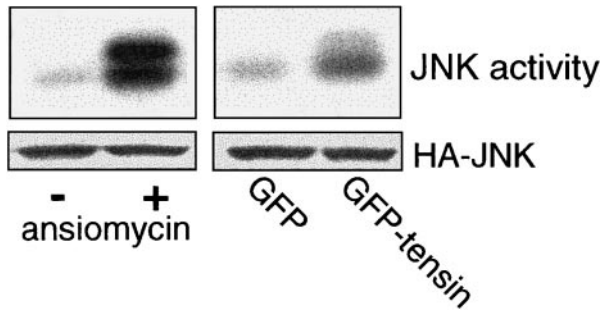

FIG. 1. Activation of the JNK signal transduction pathway by GFP–tensin expression. 293T cells were transfected with plasmids (3 μg plasmid DNA/100-mm dish) encoding either GFP alone or GFP–tensin fusion protein, each with HA-tagged JNK (1 μg DNA/100 mm dish). Twenty-four hours later, the cells were serum restricted with 0.5% FBS. Forty-eight hours after transfection, the adherent cells were lysed, followed by immunoprecipitation with anti-HA monoclonal antibody. JNK activity was then quantified by an *in vitro* kinase assay, using GST–ATF2 fusion protein as substrate. Western blot analysis was performed on anti-HA immunoprecipitate from the corresponding cellular lysate and immunodetected with JNK antibody to confirm similar levels of expression. Anisomycin (10 μg/ml) served as a positive control for the JNK assay (left panels).

In the present study, we first evaluated the activation of the JNK signaling response following expression of exogenous GFP–tensin compared to control nonfused GFP expression. 293T cells were co-transfected with plasmids encoding either GFP alone or GFP–tensin fusion protein, with HA-tagged JNK. Twenty-four hours later, the growth medium was replaced with DMEM supplemented with 0.5% FBS. Forty-eight hours after transfection, the adherent cells were lysed without further activation, followed by immunoprecipitation with anti-HA monoclonal antibody. JNK activity was determined immediately by an *in vitro* kinase assay. As shown in Fig. 1, GFP–tensin expression stimulated JNK activation. Although expressed to a level similar to that of GFP–tensin, GFP alone did not generate the JNK response (Fig. 1). Although FAK overexpression activates the ERK MAPK response (16, 17), we did not observe any FAK-mediated JNK response (data not shown). These results indicate that tensin overexpression stimulates the JNK pathway.

Next, we examined whether tensin-mediated JNK activation depends on Rac, Cdc42, or other mediators of this pathway. 293T cells were transfected with plasmids encoding GFP–tensin fusion protein or GFP alone, accompanied by HA-tagged JNK (HA-JNK). Each of these transfections also included a single dominant-negative form of the following known regulators of the JNK pathway: the small GTP binding

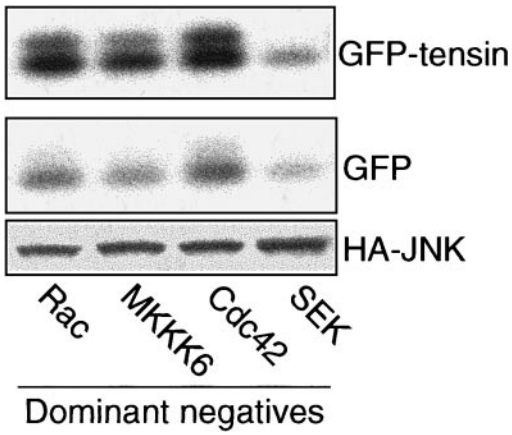

FIG. 2. Activation of JNK signal transduction pathways by GFP–tensin expression depends on SEK activity, but not on the activities of the small GTP binding proteins Rac and Cdc42. 293T cells were transfected with plasmids (3 μg plasmid DNA/100-mm dish) encoding either GFP alone or GFP–tensin fusion protein, each with HA-tagged JNK (1 μg DNA/100-mm dish). Each transfection also included a single plasmid encoding a dominant-negative mutant of Rac, Cdc42, MAP kinase kinase 6 (MKK6), or SEK. Twenty-four hours later, the cells were serum-restricted in 0.5% FBS. After 24 h, the adherent cells were lysed, followed by immunoprecipitation with anti-HA mAb. JNK activity was measured by *in vitro* kinase assay, using GST-ATF2 fusion protein as substrate. Anisomycin (10 μg/ml) served as a positive control for the JNK assay (shown in Fig. 1, which was generated from the same blot). Western blot analysis was performed on anti-HA immunoprecipitate from the corresponding cellular lysate and immunodetected with JNK antibody to confirm similar levels of expression.

proteins Rac (Rac N17) or Cdc42 (Cdc42 N17), the MAP kinase kinase 6 (MKK6, which is involved in activation of the p38 response (29)), and SEK (SEK KR) which is a kinase involved in the JNK pathway (30). As shown in Fig. 2, the dominant-negative forms of the small GTP binding proteins Rac and Cdc42 did not inhibit JNK activation induced by GFP-tensin overexpression. As expected, the dominant negative form of MKK6, which is a kinase involved in the p38 pathway, did not affect JNK activation induced by GFP–tensin overexpression. However, the dominant negative form of SEK reduced JNK activation by GFP–tensin to control levels (Fig. 2).

These results indicate that tensin can specifically activate the JNK response independent of activity of the small GTP binding proteins Rac and Cdc42. However, tensin-mediated activation of JNK does depend on SEK, a kinase involved in the JNK pathway downstream to the activities of the small GTP binding proteins Rac and Cdc42.

We recently found that another cytoskeletal molecule, paxillin, could not stimulate a JNK response unless it was expressed as a transmembrane chimera (31). This result suggests the existence of different mechanisms for triggering adhesion-mediated JNK activation.

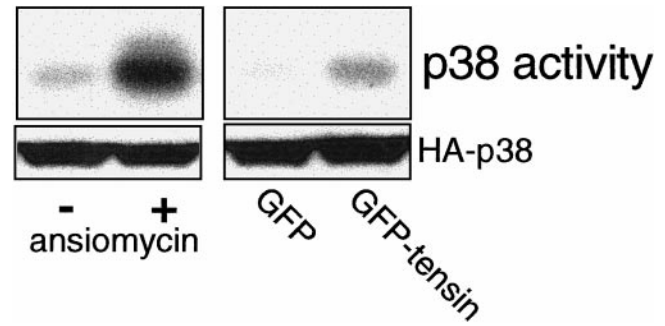

FIG. 3. Activation of the p38 signal transduction pathway by GFP–tensin expression. 293T cells were transfected with plasmids (3 μg plasmid DNA/100 mm dish) encoding either GFP alone or GFP–tensin fusion protein, each with HA-tagged p38 (1 μg DNA/100-mm dish). Twenty-four hours later, the cells were serum restricted with 0.5% FBS. Forty-eight hours after transfection, the adherent cells were lysed, followed by immunoprecipitation with anti-HA mAb. p38 activity was then quantified by an *in vitro* kinase assay, using GST–ATF2 fusion protein as substrate. Western blot analysis was performed on anti-HA immunoprecipitate from the corresponding cellular lysate and immunodetected with p38 antibody to confirm similar levels of expression. Anisomycin (10 μg/ml) served as a positive control for the p38 assay (left panels).

Extracellular signals that stimulate JNK activation may also activate the p38 response (29). Therefore, we evaluated whether tensin can induce additional signaling pathways. 293T cells were co-transfected with plasmids encoding either GFP alone or GFP–tensin fusion protein, with HA-tagged p38. Twenty-four hours later, the growth medium was replaced with DMEM supplemented with 0.5% FBS. Forty-eight hours after transfection, the adherent cells were lysed, without further

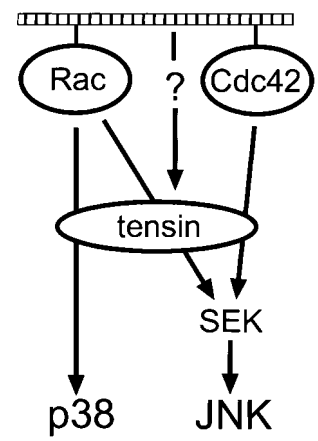

FIG. 4. Tensin can directly activate the JNK and p38 signaling pathways. In the present study, we demonstrated that tensin specifically activates both the JNK and p38 signaling pathways. Tensin-mediated JNK activation is independent of the activities of the small GTP-binding proteins Rac and Cdc42. However, SEK activity is required for tensin to initiate the JNK pathway. Therefore, tensin involvement in the JNK signaling pathway may operate downstream of Rac and Cdc42, or alternatively may respond to other, as yet unidentified signaling mediators.

activation, followed by immunoprecipitation with anti-HA monoclonal antibody. p38 activity was determined immediately by an *in vitro* kinase assay. As shown in Fig. 3, GFP–tensin but not GFP expression stimulated p38 activation. We did not observe any reproducible ERK activation by tensin under similar conditions (not shown).

We suggest that tensin can activate both the JNK and p38 pathways, acting downstream or independently of the activities of the small GTP binding proteins Rac and Cdc42 (Fig. 4). Previous studies indicated that JNK can be activated independently of Cdc42 or Rac (19); however, it may depend on intact fibronectin matrix (21) that generates tensin-containing fibrillar adhesions (22). Tensin mediated signaling activation appears to act upstream to the specific mediators of the p38 or the JNK pathways (e.g., SEK).

Tensin's unique combination of actin-binding and -capping domains, SH2 domain, and PTEN tumor suppressor homology region suggested the possible involvement of tensin in adhesion-mediated signaling events. This report provides the first documentation of tensin activation of signaling pathways.

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