MEK Kinase 1 Interacts with Focal Adhesion Kinase and Regulates Insulin Receptor Substrate-1 Expression*

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MEK kinase 1 (MEKK1) has been shown to contribute to the regulation of cell migration, whereas focal adhesion kinase (FAK) is a major player involved in both cell migration and integrin signaling. Here we show that MEKK1 and FAK are co-immunoprecipitated from mouse fibroblasts. Moreover, the association between MEKK1 and FAK appears to be physiologically relevant, as it is enhanced by treatment with epidermal growth factor (EGF). Targeting FAK to the membrane also enhanced its association with MEKK1, indicating that MEKK1 is localized to a membrane-related subcellular domain, perhaps focal adhesions. Interestingly, the expression of insulin receptor substrate-1 (IRS-1) was diminished in MEKK1-deficient fibroblasts, which is similar to an earlier finding in FAK-deficient fibroblasts. Insulin-like growth factor 1 (IGF-1)-induced ERK activation was diminished in MEKK1-deficient cells, but phosphatidylinositol 3-kinase/Akt activation was not. Although integrin reportedly regulates the transcription of the IRS-1 gene via FAK-mediated JNK activation, no impairment of fibronectin-stimulated activation of FAK, ERK, or JNK was observed in MEKK1-deficient cells. Reconstitution of MEKK1 expression restored IRS-1 expression as well as IGF-1-induced ERK activation. Together, these findings indicate that MEKK1 interacts with FAK in focal adhesions and regulates IRS-1 expression.

MEKK1 is a 196-kDa serine-threonine kinase activated in response to a variety of stimuli, including EGF, lysophosphatidic acid, osmotic stress, and microtubule toxins (1, 2). Upon activation, MEKK1 participates in the regulation of the JNK and ERK pathways and is involved in the activation of NF-κB (3–5). In addition, MEKK1 senses microtubule integrity, protects cells from committing to apoptosis, and contributes to the migration of fibroblasts and epithelial cells. The phenotype of the MEKK1-null mouse includes an eyelid closure defect that is also seen in mice lacking the EGF receptor and TGF-α (6). This suggests the possibility that MEKK1 is required for EGF receptor control of cell migration. Consistent with that idea, overexpression of MEKK1 induces the formation of a large lamellipodia-like structure in epithelial cells (6). Still, the mechanism by which MEKK1 influences cell motility remains unclear.

FAK is protein tyrosine kinase found at sites of adhesion (7). It is activated by integrin-mediated adhesion and serves as a signaling protein within cytoskeleton-associated networks (7). The Src-family protein tyrosine kinases p130Cas, Shc, and Grb2 act in concert with FAK to transduce integrin-generated signals to the ERK/JNK MAP kinase cascades (7). Experiments using FAK-deficient cells have established that FAK is essential for integrin-stimulated cell migration and important for linking activation of the PDGF and EGF receptors to the cellular machinery that promotes directed cell migration (8). The fact that MEKK1 is enriched in membranes and colocalizes with α-actinin along actin stress fibers at focal adhesions (9, 10) prompted us to investigate the possibility that MEKK1 is associated with FAK.

Insulin and IGF-1 exert diverse biological effects by binding to and activating their cognate tyrosine kinase receptors. IRS-1 is a major substrate for the insulin and IGF-1 receptors, which rapidly phosphorylate it on multiple tyrosine residues upon ligand binding. Recently, it was reported that targeted disruption of FAK eliminates IRS-1 expression in MEFs and that interactions between cells and the extracellular matrix regulate the transcription of the IRS-1 gene via FAK-mediated JNK activation (11). Here, we show the following: 1) MEKK1 interacts with FAK in vivo. 2) Like FAK, MEKK1 is required for IRS-1 expression. 3) Targeted disruption of MEKK1 alters IGF-1-induced ERK activation but not PI3K/Akt activation.

EXPERIMENTAL PROCEDURES

Cell Culture—MEFs were harvested from wild-type and MEKK1−/− 14.5 day embryos (6), after which they were immortalized using the 3T3 protocol (12). MEFs and HEK293T cells were cultured in IMDM (Invitrogen) supplemented with 100 units/ml penicillin, 100 mg/ml streptomycin (Invitrogen), 10% fetal calf serum (Gemini Bio-Products, Woodland, CA) and 15 × 10−5 μm monothioglycerol (Sigma).

Materials—Mouse recombinant EGF and IGF-1 were purchased from Sigma. Anti-FAK and anti-IRS-1 Abs were from Santa Cruz Biotechnology; GST, glutathione S-transferase.
MEKK1 Interacts with FAK—After transiently co-transfecting HEK293T cells with expression vectors encoding Xpress-tagged MEKK1 and HA-tagged FAK, we were able to co-immunoprecipitate the expressed MEKK1 and FAK using anti-Xpress and anti-HA Abs (Fig. 1A). We then co-expressed MEKK1 and wild-type or myristoylated FAK in HEK293T cells and found that the latter more readily interacted with MEKK1 than the former (Fig. 1B). Moreover, endogenous FAK and MEKK1 were co-immunoprecipitated from MEFs, and this interaction was enhanced by treatment with EGF (Fig. 1C). EGF induced the activation of both ERK and JNK in MEFs (Fig. 1D). These data suggest that MEKK1 interacts with FAK in vivo and that the interaction is physiologically regulated.

To identify the MEKK1 domain that interacts with FAK, HEK293T cells were transfected with plasmids encoding various Xpress-tagged MEKK1 mutants and HA-tagged, wild-type FAK. The MEKK1 mutants were immunoprecipitated using anti-Xpress Ab and then blotted with anti-HA Ab (Fig. 2).

Co-immunoprecipitation of MEKK1 with FAK was still observed when the MEKK1 kinase domain (amino acids 1171–1493) was deleted; conversely, the MEKK1 kinase domain expressed in HEK293 cells was not co-immunoprecipitated with FAK (data not shown). When we examined the N-terminal regulatory region of MEKK1, we found that the region encompassing amino acids 1–144 (Fig. 2, d1) did not interact with FAK, but regions encompassing amino acids 1–443 (Fig. 2, d2) or more (Fig. 2, d3–d5) were co-immunoprecipitated with FAK. Thus, a MEKK1 domain encompassing amino acids 145–443 is essential for binding to FAK.

MEKK1 Regulates IRS-1 Expression—It was recently reported that IRS-1 is not expressed in FAK-null fibroblasts (11). To further investigate the relationship between MEKK1 and

Technology (Santa Cruz, CA). The anti-IRS-2 polyclonal Ab and the 4G10 anti-phosphotyrosine mAb are described elsewhere (13). Xpress-tagged MEKK1 expression vector (pcDNA3.1/MEKK1) was constructed by subcloning full-length MEKK1 into pcDNA3.1 HisB (Invitrogen, Carlsbad, CA). HA-tagged, wild-type FAK expression vector (pRcCMV/FAK) was a gift from Dr. S. Hanks (Vanderbilt University). A myristoylated FAK expression vector (pCEFL Myr FAK) was a gift from Dr. S. Gutkind (National Institutes of Health). Myristoylated FAK was constructed by fusing the amino-terminal myristoylation signal from c-Src with FAK, resulting in a constitutively active, heavily tyrosine-phosphorylated form of FAK that is targeted to the membrane (15). Transient transfections were performed using FuGENE-6 (Roche Diagnostics). Recombinant retrovirus with pMY vector was prepared as described previously (16). MEKK1+/− MEFs were stably transfected using pMY-HA-tagged, full-length, wild-type MEKK1 or pMY-full-length IRS-1.

Immunoprecipitation and Immunoblotting—Cells were washed twice with PBS and lysed in 20 mM Tris-HCl (pH 7.6), 0.5% Nonidet P-40, 250 mM sodium chloride, 3 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 2 mM sodium orthovanadate, 20 μg/ml aprotinin, 1 mM dithiothreitol, and 5 μg/ml leupeptin. The lysates were centrifuged at 14,000 × g for 10 min at 4 °C, after which the supernatant was incubated first with the appropriate Abs for 16 h at 4 °C and then with protein G-Sepharose (Amersham Biosciences) for an additional 1 h. The Sepharose beads were then washed three times in lysis buffer and resuspended in Laemmli sample buffer. The immunoprecipitates were resolved by electrophoresis. For IFG-1 stimulation, cells were serum starved overnight in medium containing 0.1% fetal calf serum and then exposed to IFG-1 (100 ng/ml) for the indicated times.

Northern Blotting—Total RNA was extracted from cells using ISOGEN (Nippongene, Japan), after which 15-μg samples were denatured with formaldehyde/formamide, resolved by electrophoresis, and transferred to Hybond membranes (Amersham Biosciences). The probes used were an IRS-1 cDNA fragment and the full-length GAPDH cDNA.

Cell Adhesion Assay—Culture dishes were coated with 10 μg/ml bovine plasma fibronectin (Sigma) or 10 μg/ml mouse sarcoma-derived laminin (Sigma) in PBS at 4 °C overnight. The dishes were then rinsed twice with PBS and warmed for 1 h at 37 °C before use. MEFs were serum starved in IMDM with 0.1% fetal calf serum overnight and then resuspended in IMDM containing 0.1% fetal calf serum. The cells were then kept in suspension for 2 h, after which they were then plated on the coated dishes for the indicated times, harvested, and lysed.

Analysis of Kinase Activity—To determine ERK and Akt activities, cell lysates were immunoblotted with either an anti-phospho-MAPK or anti-phospho-Akt Ab (Cell Signaling Technology, Inc.). NIH Image 1.62 was used for quantitative analysis of kinase activity. ERK activity was also measured by in vitro kinase assay with myelin basic protein as substrate (5). PI3K and JNK activities were measured as described previously (1, 13).

RESULTS

MEKK1 Interacts with FAK—After transiently co-transfecting HEK293T cells with expression vectors encoding Xpress-tagged MEKK1 and HA-tagged FAK, we were able to co-immunoprecipitate the expressed MEKK1 and FAK using anti-Xpress and anti-HA Abs (Fig. 1A). We then co-expressed MEKK1 and wild-type or myristoylated FAK in HEK293T cells and found that the latter more readily interacted with MEKK1 than the former (Fig. 1B). Moreover, endogenous FAK and MEKK1 were co-immunoprecipitated from MEFs, and this interaction was enhanced by treatment with EGF (Fig. 1C). EGF induced the activation of both ERK and JNK in MEFs (Fig. 1D). These data suggest that MEKK1 interacts with FAK in vivo and that the interaction is physiologically regulated.

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T. Kitamura, unpublished data.

FIG. 1. Expression of MEKK1 and FAK. A, co-immunoprecipitation of MEKK1 and FAK in 293T cells. 293T cells were transiently transfected with empty vector (pcDNA 3.1 or pRcCMV), Xpress-tagged, full-length recombinant MEKK1 (Xpress-MEKK1), or HA-tagged, full-length recombinant FAK (HA-FAK). Immunoprecipitation (IP) and immunoblotting (IB) were performed with anti-Xpress and anti-HA Abs (12CA5) as indicated. B, enhanced interaction of membrane-targeted FAK with MEKK1. MEKK1 was coexpressed with FAK containing the N-terminal myristoylation signal from c-Src (Myr FAK), wild-type FAK (WT-FAK), or empty vector (Mock) in HEK293T cells. Immunoprecipitation and immunoblotting were performed with the Abs against endogenous MEKK1 or FAK, as indicated. C, co-immunoprecipitation of endogenous MEKK1 and FAK in mouse embryonic fibroblasts. MEFs were treated with 100 ng/ml EGF or without EGF (control) for 10 min. Immunoprecipitation and immunoblotting were performed with Abs against endogenous MEKK1 and FAK as indicated. D, MEFs were treated with 100 ng/ml EGF for the indicated periods. ERK and JNK activations were measured by in vitro kinase assay using myelin basic protein and GST-Jun as substrates, respectively. The autoradiogram shown is representative of three independent experiments yielding similar results.
The N-terminal region of MEKK1 is essential for its interaction with FAK. Xpress-tagged MEKK1 deletion mutants and wild-type FAK were co-transfected into HEK293T cells. Deletion mutants of MEKK1 as follows: d1 (amino acids 1–144), d2 (1–443), d3 (1–785), d4 (1–975) and d5 (1–1230). Cell lysates were prepared, immunoprecipitated (IP) with anti-Xpress mAb, and immunoblotted (IB) with anti-HA mAB. To detect exogenous expression of MEKK1 mutants and FAK, immunoblotting with anti-Xpress and anti-HA Ab was performed.

FAK, we evaluated IRS-1 expression in MEKK1−/− MEFs. Although there was no significant difference in the growth rates of MEKK1−/− and wild-type MEFs (data not shown), expression of the IRS-1 protein was diminished in two independent MEKK1−/− MEF clones (Fig. 3A). By contrast, no changes in the expression of IRS-2, another IRS family protein, or FAK were observed in MEKK1-deficient cells. This indicates that the decreased expression of IRS-1 is not due to altered expression of FAK and that the expression of IRS-1, but not IRS-2, is regulated by MEKK1. As expected, Northern blot analysis showed levels of IRS-1 mRNA to be diminished in the MEKK1−/− MEF clones, whereas levels of GAPDH mRNA were similar in MEKK1−/− and wild-type cells (Fig. 3B).

We found that expression of the IRS-1 protein was increased when MEF cultures reached confluence (Fig. 3A). We therefore tested whether IRS-1 expression was also cell density dependent in HEK293T cells. In one set of experiments, the same numbers of the cells were seeded on day 0 and then harvested on day 1, 2, or 4. To minimize the effect of humoral factors, the culture medium was changed every day. In another set of experiments, different numbers of the cells were seeded on day 0 and then harvested after 48 h. In both experiments, expression of the IRS-1 mRNA and protein was increased as a function of cell density (Fig. 3C). Notably, changes in cell density affected IRS-1 expression similarly in MEKK1−/− and wild-type cells, suggesting that MEKK1 plays a role in regulating basal IRS-1 expression but not cell density-dependent expression. Finally, stably transfecting MEKK1−/− MEFs with full-length MEKK1 cDNA using a retroviral vector showed that expression of the IRS-1 protein was restored by re-expression of MEKK1, confirming that MEKK1 is required for IRS-1 expression in MEFs (Fig. 4).

Cell adhesion to fibronectin and vitronectin reportedly induces the expression of IRS-1 (11). In that regard, the activation of integrin in turn activates FAK, which is required for the fibronectin-stimulated activation of JNK, suggesting that IRS-1 expression is regulated at least in part by a FAK-JNK pathway (11). We therefore investigated whether adhesion to fibronectin would induce activation of FAK, JNK, ERK, and NF-κB in MEKK1−/− cells. We found that fibronectin stimulated FAK, JNK and ERK activation to a similar degree in wild-type and MEKK1−/− cells (Fig. 5) and that NF-κB was not activated in either cell type (electrophoresis mobility shift assay; data not shown). Apparently, MEKK1 is not required for fibronectin-induced FAK, JNK, or ERK activation. We also tested whether laminin, another extracellular matrix protein, would induce FAK, JNK, or ERK activation and found a slight activation of FAK that was not different in MEKK1−/− and
wild-type cells. Neither JNK nor ERK was activated by laminin in either cell type (data not shown). MEKK1 Is Involved in IGF-1–induced ERK Activation but Not PI3K/Akt Activation—IGF-1 exerts a wide variety of effects including cell proliferation, differentiation, survival, and migration, in part via IRS-1, a substrate and downstream signal transduction molecule for the IGF-1 receptor. Because IRS-1 expression was diminished in MEKK1−/− MEFs, we examined IGF-1 signal transduction in MEKK1−/− MEFs. We found that IGF-1 strongly activated ERK and PI3K/Akt and weakly activated JNK in wild-type MEFs. The ERK activation was significantly diminished in the MEKK1−/− MEFs, whereas the PI3K and Akt activation were similar in both cell types (Fig. 6, and data not shown), as was the JNK activation (data not shown). Reconstitution of MEKK1 expression restored IGF-1-induced ERK activation in MEKK1−/− cells (Fig. 7A). MEKK1 thus appears to contribute to IGF-1-induced ERK activation but not to PI3K/Akt activation. It is possible that a decreased IRS-1 protein reduces ERK activation in MEKK1−/− MEFs. It is also possible, however, that there exists both IRS-1-independent and MEKK1-dependent pathways leading to ERK activation. To investigate these possibilities, IRS-1 was stably transfected to MEKK1−/− cells. Although IRS-1 was abundantly expressed in MEKK1−/− cells, IGF-1-induced ERK activation was not restored (Fig. 7B). This indicates that the decreased activation of ERK by IGF-1 in MEKK1−/− cells is not due to the altered expression of IRS-1.

**DISCUSSION**

MEKK1 reportedly binds to a number of signaling proteins including Ras, Raf-1, Rac1, cdc42Hs, Nck-interacting kinase, SEK1, and JNK (1, 17, 18, 19–21); consequently, MEKK1 has been proposed to function, like yeast Pbs2p, as a scaffold protein (22). In the present study, we showed that MEKK1 interacts with FAK in vivo, suggesting that among the subcellular regions to which MEKK1 is localized are focal adhesions. The finding that a membrane-targeted form of FAK (myristoylated FAK) associated with MEKK1 more readily than did wild-type FAK suggests that this idea. In addition, the finding that EGF enhanced the interaction between endogenous MEKK1 and FAK suggests that the interaction is physiologically relevant. In that regard, FAK is known to associate with the activated EGF receptor signaling complex and to be required for EGF-stimulated cell motility (8). MEKK1 has been shown to be activated in the EGF receptor signaling pathway (1), and EGF indeed stimulated activation of ERK and JNK in MEFs. MEKK1 might recruit MEKK1 to focal adhesion complexes containing FAK and EGF receptor. The function of MEKK1 within focal adhesions is unclear, although it is known to regulate JNK activation and survival in cells challenged with mild hypopermissority and microtubule toxins (2, 5). The fact that these stimuli elicit changes in cell shape suggests that MEKK1 localized in focal adhesions might be involved in sensing cytoskeletal dynamics.
MEFs indicates that a MEKK1-independent pathway to IRS-1 expression and that JNK is not activated in FAK-/- MEFs. In the present study, by contrast, adhesion to fibronectin induced a similar activation of JNK, ERK, and FAK in wild-type and MEKK1-/- MEFs, suggesting that integrin induces IRS-1 expression via an integrin-FAK-JNK pathway that is independent of MEKK1. The signaling molecules involved in the regulation of IRS-1 expression thus remain to be determined. It is possible that, like FAK, MEKK1 is involved in organizing the cortical cytoskeleton and that disorganization due to the loss of MEKK1 participates in the down-regulation of IRS-1 expression.

The results of this and our earlier study (6) show that, like FAK, MEKK1 positively regulates IRS-1 expression and cell motility. On the other hand, the ectopic expression of IRS-1 in prostate cancer cells that do not express IRS-1 endogenously increased cell adhesion and decreased cell motility (24). Although this finding is apparently opposite to ours, it is nevertheless consistent with the idea that IRS-1 plays a key role in regulating cell motility. IRS-1 also mediates various metabolic and growth-promoting actions of insulin and IGF-1. For instance, mice lacking IRS-1 display retardation of somatic growth and enhanced β-cell mass (25, 26); effects on cell migration have not been reported, however.

Upon insulin stimulation, IRS-1 interacts with αβ2 integrins (27). FAK binds to both IRS-1 and integrins (7, 28), suggesting that IGF-1/insulin and cell adhesion induce the formation of focal adhesion complexes that include IRS-1, FAK, and MEKK1. We found that IGF-1-induced ERK activation was significantly diminished in MEKK1-/- MEFs. The decreased activation of ERK by IGF-1 in MEKK1-/- MEFs was independent of IRS-1 expression. Although MEKK1 contributes significantly to IGF-1-stimulated ERK activation, its effect is only partial. The activation of other MAPK kinase kinases (e.g. Raf-1 and B-Raf) that also activate ERK might partially compensate for the loss of MEKK1. In contrast to ERK activation, IGF-1-induced PI3K/Akt activation was not diminished in MEKK1-/- MEFs. Although IRS-1 is a key mediator of IGF-1/insulin-induced PI3K signaling, in this case other IRS proteins (e.g. IRS-2 which is abundant in MEFs) might have compensated for the diminished levels of IRS-1. In summary, MEKK1 interacts physiologically with FAK and regulates IRS-1 expression, which in turn contributes to the regulation of IGF-1/insulin-induced signaling and cell migration.

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