Roles of Cell-Cell Adhesion-dependent Tyrosine Phosphorylation of Gab-1*

Masahiko Shinohara‡, Atsuko Kodama‡§, Takashi Matozaki§, Atsunori Fukuhara, Kouichi Tachibana, Hiroyuki Nakanishi, and Yoshimi Takai¶

From the Department of Molecular Biology and Biochemistry, Osaka University Graduate School of Medicine/Faculty of Medicine, 2-2 Yamada-oka, Suita 565-0871, Japan

Gab-1 is a multiple docking protein that is tyrosine phosphorylated by receptor tyrosine kinases such as c-Met, hepatocyte growth factor/scatter factor receptor, and epidermal growth factor receptor. We have now demonstrated that cell-cell adhesion also induces marked tyrosine phosphorylation of Gab-1 and that disruption of cell-cell adhesion results in its dephosphorylation. An anti-E-cadherin antibody decreased cell-cell adhesion-dependent tyrosine phosphorylation of Gab-1, whereas the expression of E-cadherin specifically induced tyrosine phosphorylation of Gab-1. A relatively selective inhibitor of Src family kinases reduced cell-cell adhesion-dependent tyrosine phosphorylation of Gab-1, whereas expression of a dominant-negative mutant of Csk increased it. Disruption of cell-cell adhesion, which reduced tyrosine phosphorylation of Gab-1, also reduced the activation of mitogen-activated protein kinase and Akt in response to cell-cell adhesion. These results indicate that E-cadherin-mediated cell-cell adhesion induces tyrosine phosphorylation by a Src family kinase of Gab-1, thereby regulating the activation of Ras/MAP kinase and phosphatidylinositol 3-kinase/Akt cascades.

The cell junction plays essential roles in various cell functions, including cell adhesion and migration (1–5). In polarized epithelial cells, the cell-cell junction shows a specialized membrane structure comprising tight junctions, AJs, and desmosomes. AJs play a particularly important role because the formation of AJs subsequently leads to the formation of other cell junctions. At AJs, cadherin, a Ca2⁺-dependent homophilic cell adhesion molecule, plays a fundamental role in cell-cell adhesion (2, 5, 6). Cadherin forms Ca2⁺-dependent homophilic

cis dimerization and trans interaction, which cause cell-cell adhesion (7–11). The cytoplasmic tail of cadherin interacts with β-catenin, which in turn interacts with α-catenin. α-Catenin interacts directly with F-actin (12). α-Catenin furthermore interacts with other F-actin-binding proteins, α-actinin and vinculin, through the NH2-terminal half (13–16), and ZO-1 through the COOH-terminal half (17). The linkage of cadherin to the actin cytoskeleton strengthens cadherin-based cell-cell adhesion (2, 5, 6, 18).

In addition to its role in cell-cell adhesion, the cadherin-catenin system may play an important role in the signal transduction system. β-Catenin has been shown to play a crucial role in the Wnt-mediated signaling pathway (19). In addition, the cadherin-catenin system may be closely linked to tyrosine phosphorylation of proteins, which exist at cell-cell adhesion sites. In fact, α-, β-, and γ-catenins and p120ctn, which all associate directly or indirectly with the cytoplasmic region of E-cadherin, are known to be tyrosine phosphorylated in v-src-transformed cells (20–22) or in response to epidermal growth factor and HGF/SF (23). The cadherin-catenin system has been shown to form a complex with the epidermal growth factor receptor or the c-Erb-B-2 gene product (24, 25). Moreover, protein tyrosine phosphatases, such as LAR-PTP, PTPμ, PTP1B-like phosphatase, and SHP-2, have been shown to associate with the cadherin-catenin system (26–28). Increased tyrosine phosphorylation of catenins and p120ctn is correlated with the decrease of cell adhesion activity which occurs upon cell transformation or mitogenic growth factor stimulation (20–23). In contrast, it has been shown recently that cell-cell adhesion induces tyrosine phosphorylation of these junctional proteins in nontransformed cells (29, 30). However, it remains to be determined whether tyrosine phosphorylation of cadherin-associated proteins is involved in the regulation of cell-cell adhesion.

Gab-1 is a docking protein that undergoes tyrosine phosphorylation and subsequently binds Grb2, SHP-2, and the p85 subunit of PI3-kinase in response to various growth factors such as HGF/SF, epidermal growth factor, insulin, and insulin-like growth factor (31, 32). Gab-1 also contains a pleckstrin homology domain in its NH2-terminal region; this domain mediates protein interaction with cellular membranes, possibly by binding to polyphosphoinositides (33, 34). Gab-1 furthermore contains a c-Met-binding domain, which may mediate protein-protein interactions by binding to phosphotyrosine-containing motifs of the cytoplasmic portion of c-Met (32). Gab-1 has recently been demonstrated to be localized at cell-cell adhesion sites of MDCK cells (35). The pleckstrin homology domain of Gab-1 or PI3-kinase activity is required for the proper membrane localization of Gab-1 (35). We and others have demonstrated recently that both E-cadherin and c-Met are colocalized.

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‡ Present address: Howard Hughes Medical Institute, Dept. of Molecular Genetics and Cell Biology, The University of Chicago, Chicago, IL 60637.

§ Present address: Biosignal Research Center, Institute for Molecular and Cellular Regulation, Gunma University, 3-39-15 Showa-Machi, Maebashi, Gunma 371 8512, Japan.

¶ Present address: Molecio Research Institute, by guest on July 27, 2018http://www.jbc.org/ Downloaded from

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at cell-cell adhesion sites of MDCK cells (36, 37). In addition, HGF/SF or a phorbol ester, TPA, induces disruption of cell-cell adhesion, which is accompanied by endocytosis of both E-cadherin and e-Met. Thus, Gab-1 and the cadherin-catenin system could interact with each other.

In this study, we show that cell-cell adhesion stimulates tyrosine phosphorylation of Gab-1 and that this effect may be mediated through E-cadherin and a Src family kinase. Thus, cell-cell adhesion as well as growth factors regulates the activation of the downstream signaling pathway of Gab-1.

EXPERIMENTAL PROCEDURES

Antibodies—The FLAG-tagged human cDNA of Gab-1 was provided by Dr. T. Hirano (Osaka University, Suita, Japan) (38). MDCK cells were supplied by Dr. W. Birchmeier (Max-Delbruck Center for Molecular Medicine, Berlin, Germany). Human recombinant HGF/SF was provided by Dr. T. Nakamura (Osaka University, Suita, Japan). The anti-Gab-1 rabbit polyclonal Ab was obtained from Upstate Biotechnology (Lake Placid, NY) and also provided by Dr. T. Hirano (Osaka University). An anti-FLAG mouse mAb (M2) was from Eastman Kodak. The horseradish peroxidase-conjugated anti-anti-phosphotyrosine mAb PY20 (PY20) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-tyrosine-phosphorylated form of MAP kinase rabbit polyclonal Ab, the anti-MAP kinase rabbit polyclonal Ab, the anti-serine-phosphorylated form of Akt rabbit polyclonal Ab, and the anti-Akt rabbit polyclonal Ab were obtained from New England BioLabs (Beverly, MA). Other materials and chemicals were obtained from commercial sources.

Cell Culture—MDCK cells, mouse mammary tumor MTD-1A cells, and mouse keratinocyte 308R cells were maintained at 37 °C in a humidified atmosphere of 10% CO2 and 90% air in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum (Life Technologies, Inc.), 100 units/ml penicillin, and 100 μg/ml streptomycin. L, CL, and EL cells were kindly supplied by Drs. S. Tsukita and A. Nagafuchi (Kyoto University, Kyoto, Japan). These cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. For transient transfection experiments, L, CL, and EL cells were seeded at a density of 1 × 10^5 cells/dish onto 10-cm dishes and transfected with 1 μg of pCMV vector containing the FLAG-tagged Gab-1 cDNA by the use of LipofectAMINE and PLUS Reagent (Life Technologies, Inc.) at 24 h after seeding. The recombinant adenosine, Ax1CATcsk-ΔK, was obtained from Dr. M. Okada (Osaka University). MDCK cells were infected with the adenovirus vector at the indicated multiplicity of infection as described previously (39).

Immunoprecipitation and Immunoblotting—All cultured cells (in a 10-cm plate) were frozen in liquid nitrogen and then lysed on ice in 1 ml of an ice-cold lysis buffer (20 mM Tris-HCl at pH 7.6, 140 mM NaCl, 2.6 mM CaCl2, 1 mM MgCl2, 1% (v/v) Nonidet P-40, and 10% (v/v) glycerol) containing 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 1 mM sodium vanadate. Whole cell lysates were centrifuged at 10,000 × g at 4 °C for 15 min, and the resulting supernatants were mixed with the SDS sample buffer, boiled, and subjected to SDS-PAGE. The extent of tyrosine phosphorylation of GST proteins was analyzed by immunoblotting with the horseradish peroxidase-conjugated anti-anti-phosphotyrosine mAb PY20.

RESULTS

Cell-Cell Adhesion-dependent Phosphorylation of Gab-1—MDCK cells were serum starved in the culture medium containing 2 mM Ca^{2+} for up to 24 h, after which the whole cell lysates were prepared and subjected to immunoprecipitation with the anti-Gab-1 polyclonal Ab, and the resulting immunoprecipitates were subjected to immunoblotting with the horseradish peroxidase-conjugated anti-anti-phosphotyrosine mAb PY20 (upper panel) or the anti-Gab-1 polyclonal Ab (lower panel). The results shown are representative of three independent experiments.

Fig. 1. Cell-cell adhesion-dependent tyrosine phosphorylation of Gab-1 in MDCK cells. A, MDCK cells were treated as indicated in the figure. The whole cell lysates were then subjected to immunoprecipitation (IP) with the anti-Gab-1 polyclonal Ab (αGab-1), and the resulting immunoprecipitates were subjected to immunoblotting with the horseradish peroxidase-conjugated anti-anti-phosphotyrosine mAb PY20 (αPY) (upper panel). The same blot was reprobed with the anti-Gab-1 polyclonal Ab (lower panel) to ensure that similar amounts of endogenous Gab-1 were present in each lane. High, MDCK cells were deprived of serum for 12 h in medium containing 2 mM Ca^{2+}. Low, MDCK cells were deprived of serum in the medium containing 2 mM Ca^{2+} for 12 h and subsequently cultured in medium containing 2 μM Ca^{2+} for the indicated time. Low → High, cells pretreated with 2 μM Ca^{2+} for 4 h were cultured further in the medium containing 2 mM Ca^{2+} for the indicated time. B, serum-deprived MDCK cells were incubated in the presence or absence of 10 ng/ml HGF/SF for 0–18 h. The whole cell lysates were then subjected to immunoprecipitation with the anti-Gab-1 polyclonal Ab, and the resulting immunoprecipitates were subjected to immunoblotting with either the horseradish peroxidase-conjugated anti-anti-phosphotyrosine mAb PY20 (upper panel) or the anti-Gab-1 polyclonal Ab (lower panel). The results shown are representative of three independent experiments.
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Effects of Cell-Cell Adhesion on the Downstream Signaling of Gab-1—We next examined the effects of cell-cell adhesion on the downstream signaling of Gab-1. Decreasing the Ca\(^{2+}\) concentration in the medium induced a marked reduction of the tyrosine phosphorylation of Gab-1 (Fig. 5A). We then determined the activation of MAP kinase by immunoblotting the whole cell lysates with the anti-serine-phosphorylated MAP kinase polyclonal Ab. The significant activation of MAP kinase in MDCK cells without any stimulation was observed when cells were cultured in the culture medium containing 2 mM Ca\(^{2+}\) (Fig. 5B). In contrast, reduction of the Ca\(^{2+}\) concentration in the medium inhibited MAP kinase activation markedly compared with that observed in the 2 mM Ca\(^{2+}\) medium (Fig. 5B). Tyrosine-phosphorylated Gab-1 binds P13-kinase (48, 49), which subsequently activates Akt/PKB kinase, a downstream target of P13-kinase (50). Thus, we next examined the effect of reduction of medium Ca\(^{2+}\) on Akt activation, as determined by immunoblotting the whole cell lysates with the anti-serine-phosphorylated Akt polyclonal Ab. The significant activation of Akt was observed in MDCK cells without any stimulation when cells were cultured in the culture medium containing 2 mM Ca\(^{2+}\) (Fig. 5B). In contrast, reduction of the Ca\(^{2+}\) concentration in the medium inhibited MAP kinase activation markedly compared with that observed in the 2 mM Ca\(^{2+}\) medium (Fig. 5B).

In the present study, we have demonstrated that cell-cell adhesion reversibly induces the tyrosine phosphorylation of Gab-1 in cultured epithelial cells. The extent of the tyrosine phosphorylation of Gab-1 is increased markedly in EL cells compared with that observed in L cells, whereas the anti-E-
polyclonal Ab (lower panel).

B 

2mM Ca\textsuperscript{2+} 

IP: α Gab-1 

Blot: kD 120- - Gab-1 - Gab-1 

α E-Cadherin 

C 

2mM Ca\textsuperscript{2+} 

IP: α Gab-1 

Blot: kD 120- - Gab-1 - Gab-1 

Low → High → TPA Ca\textsuperscript{2+} in Medium

FIG. 3. E-Cadherin-dependent tyrosine phosphorylation of Gab-1. A, serum-deprived MDCK cells were cultured in the medium containing 2 μM Ca\textsuperscript{2+} for 4 h and then cultured further in the medium containing 2 mM Ca\textsuperscript{2+} for 2 h in the presence or absence of the anti-E-cadherin mAb Arc-1 (aE-Cadherin). The whole cell lysates were then subjected to immunoprecipitation (IP) with the anti-Gab-1 polyclonal Ab (αGab-1), and the resulting immunoprecipitates were subjected to immunoblotting with either the horseradish peroxidase-conjugated anti-phosphotyrosine mAb PY20 (αPY, upper panel) or the anti-Gab-1 polyclonal Ab (lower panel). B, L, CL, and EL cells were transfected with pCMVFLAG-Gab-1. At 48 h after transfection, these cells were serum deprived in the medium containing 2 mM Ca\textsuperscript{2+}, and the whole cell lysates prepared from the cells were subjected to immunoprecipitation with anti-FLAG mAb (αFLAG). The resulting immunoprecipitates were subjected to immunoblotting with either the horseradish peroxidase-conjugated anti-phosphotyrosine mAb PY20 (αPY, upper panel) or the anti-Gab-1 polyclonal Ab (lower panel). C, MDCK cells were treated as indicated in the figure. The whole cell lysates were then subjected to immunoprecipitation with the anti-Gab-1 polyclonal Ab, and the resulting immunoprecipitates were subjected to immunoblotting with either the horseradish peroxidase-conjugated anti-phosphotyrosine mAb PY20 (upper panel) or the anti-Gab-1 polyclonal Ab (lower panel). Low → High, MDCK cells were deprived of serum in the medium containing 2 mM Ca\textsuperscript{2+} for 12 h and subsequently cultured in the medium containing 2 μM Ca\textsuperscript{2+} for 4 h. The cells were cultured further in the medium containing 2 mM Ca\textsuperscript{2+} for 2 h. Low → TPA, cells pretreated with 2 μM Ca\textsuperscript{2+} for 4 h were cultured further in the medium containing 2 μM Ca\textsuperscript{2+} and 100 nM TPA for 2 h. The results shown are representative of three independent experiments.

We have also explored the mechanism by which cell-cell adhesion stimulates the tyrosine phosphorylation of Gab-1. 4-Amino-5-(4-methylphenyl)-7-(t-buty1)pyrazolo[3,4-d]pyrimidine, a relatively selective inhibitor of Src family kinases, reduces the tyrosine phosphorylation of Gab-1, whereas expression of a dominant-negative mutant of Csk increases its response. These data suggest that a Src family kinase, at least in part, contributes to the tyrosine phosphorylation of Gab-1 in response to cell-cell adhesion. In fact, Src family kinases have been found at AJs (51). In addition, tyrosine phosphorylation of AJ proteins, such as β-catenin, γ-catenin, and α-catenin, has been shown to be increased markedly in response to cell-cell adhesion in mouse keratinocytes (30). In contrast, in fyn-deficient keratinocytes, the tyrosine phosphorylation of these AJ proteins has been shown to be decreased markedly, and structural and functional abnormalities at the cell-cell adhesions similar to those caused by tyrosine kinase inhibitors have been observed (30). Thus, Src family kinases may generally contribute to regulate the tyrosine phosphorylation of AJ proteins in response to cell-cell adhesion.

We have also demonstrated that the tyrosine phosphorylation of Gab-1 by cell-cell adhesion correlates well with the activation of MAP kinase and Akt activation in response to cell-cell adhesion. Activation of Akt by the formation of E-cadherin-mediated cell-cell junctions has also been demonstrated in MDCK cells (52). It has been reported that cell-cell adhesion, which is mediated by E-cadherin, can promote cell survival in a variety of cell types (53, 54). Compelling evidence suggests that both Ras/MAP kinase and PI3-kinase/Akt cascades are involved in cell survival (55). Thus, our present results provide an interesting mechanism whereby E-cadherin-mediated cell-cell adhesion stimulates the tyrosine phosphorylation of Gab-1, which then induces the activation of both Ras/MAP kinase and PI3-kinase/Akt cascades. This mechanism could be involved in cell-cell adhesion-dependent cell survival.

It remains unknown how the E-cadherin-mediated cell-cell adhesion induces the Src family kinase-mediated tyrosine phosphorylation of Gab-1. It is possible that the E-cadherin-mediated cell-cell adhesion induces the activation of a Src family kinase. In contrast, the E-cadherin-mediated cell-cell adhesion stimulates the tyrosine phosphorylation of AJ proteins in response to cell-cell adhesion.

Thus, Gab-1 might be a new member of the group of proteins that are localized at AJs and undergo tyrosine phosphorylation in response to cell-cell adhesion.
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adhesion promotes the recruitment of this kinase to the place where Gab-1 is localized. In fact, it has been shown that the binding of extracellular matrix to integrin also induces tyrosine phosphorylation of several proteins by Src, which is recruited to integrin-based focal adhesions through its binding to FAK, another focal adhesion-associated tyrosine kinase (56). Further efforts are clearly necessary to clarify the molecular mechanism by which the E-cadherin-mediated cell-cell adhesion leads to the tyrosine phosphorylation of Gab-1.

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REFERENCES
1. Takeichi, M. (1988) Development 102, 639–655
2. Takeichi, M. (1991) Science 251, 1451–1455
3. Luna, E. J., and Hitt, A. L. (1992) Science 258, 955–964
4. Jockusch, B. M., Bubeck, P., Giehl, K., Kroemker, M., Moschner, J., Rothkegel, M., Rudiger, M., Schluter, K., Stanke, G., and Winkler, J. (1995) Annu. Rev. Cell Dev. Biol. 11, 379–416
5. Gumbiner, B. M. (1996) Cell 84, 345–357
6. Takeichi, M. (1995) Curr. Opin. Cell Biol. 7, 619–627

Fig. 4. Effect of an inhibitor for Src family kinases or expression of a dominant-negative mutant of Csk on the tyrosine phosphorylation of Gab-1. A, serum-deprived MDCK cells were cultured at 2 μM Ca²⁺ for 4 h with or without 1 μM 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (Src Inhibitor), a compound reported to act as a relatively selective inhibitor of Src family kinases, for 30 min, and then cultured further at 2 mM Ca²⁺ for 2 h. The whole cell lysates were then subjected to immunoprecipitation (IP) with the anti-Gab-1 polyclonal Ab (α-Gab-1), and the resulting immunoprecipitates were subjected to immunoblotting with either the horseradish peroxidase-conjugated anti-phosphotyrosine mAb PY20 (αPY, upper panel) or the anti-Gab-1 polyclonal Ab (lower panel). B, MDCK cells were infected with either Ax1CAT-lacZ (Control) or Ax1CAT-csk-ΔK (CSK-ΔK) at 100 multiplicity of infection. At 48 h after the transfection, the infected MDCK cells in the medium containing 2 mM Ca²⁺ were subjected to immunoprecipitation with the anti-Gab-1 polyclonal Ab, and the resulting immunoprecipitates were subjected to immunoblotting with either the horseradish peroxidase-conjugated anti-phosphotyrosine mAb PY20 (upper panel) or the anti-Gab-1 polyclonal Ab (lower panel). The results shown are representative of three independent experiments.

Fig. 5. Effects of cell-cell adhesion on the downstream signaling of Gab-1. A, serum-deprived MDCK cells were treated as indicated in the figure. The whole cell lysates were then subjected to immunoprecipitation (IP) with the anti-Gab-1 polyclonal Ab (α-Gab-1), and the resulting immunoprecipitates were subjected to immunoblotting with either the horseradish peroxidase-conjugated anti-phosphotyrosine mAb PY20 (αPY, upper panel) or the anti-Gab-1 polyclonal Ab (lower panel). High, MDCK cells were deprived of serum for 12 h in the medium containing 2 mM Ca²⁺. Low, MDCK cells were deprived of serum in the medium containing 2 mM Ca²⁺ for 12 h and subsequently cultured in the medium containing 2 mM Ca²⁺ for 4 h. B, the whole cell lysates were subjected to immunoblotting with either the anti-tyrosine-phosphorylated-MAP kinase polyclonal Ab (p-MAPK, upper panel) or the anti-MAP kinase polyclonal Ab (p-MAPK, lower panel). C, the whole cell lysates were also subjected to immunoblotting with either the anti-serine-phosphorylated-Akt polyclonal Ab (p-Akt, upper panel) or the anti-Akt polyclonal Ab (p-Akt, lower panel). The results shown are representative of three independent experiments.
