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In Vivo Interaction of AF-6 with Activated Ras and ZO-1

Takaharu Yamamoto, Naozumi Harada, Yoji Kawano, Shinichiro Taya, and Kozo Kaibuchi[1]
Division of Signal Transduction, Nara Institute of Science and Technology, Ikoma, Nara 630-0101, Japan

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AF-6 contains two putative Ras-associating domains (RA domains) which are seen in several Ras effectors such as RalGDS and RIN1. We previously showed that an AF-6 fragment containing the amino-terminal (N-terminal) RA domain directly binds to activated Ras and ZO-1 *in vitro.* In this study, we showed that a single amino acid mutation in the N-terminal RA domain of AF-6 abolished the interaction of AF-6 with activated Ras and that the sites of this critical amino acid residue were similar to those for Raf-1 and RalGDS. The overexpression of the N-terminal RA domain of AF-6 inhibited the Ras-dependent c-*fos* promoter/enhancer stimulation in NIH3T3 cells. Endogenous AF-6 was coimmunoprecipitated with activated Ras from Rat1 cells expressing activated Ras. Moreover, we showed that AF-6 was coimmunoprecipitated with ZO-1 from Rat1 cells. Taken together, these results indicate that the Ras-interacting region on AF-6 is structurally similar to that on Raf-1 and on RalGDS and that AF-6 interacts with activated Ras and ZO-1 *in vivo.* © 1999 Academic Press

Ras (H-Ras, K-Ras, N-Ras) is a signal-transducing small guanosine triphosphatase (GTPase) that plays critical roles in the control of cell growth and differentiation (1-3). Mutations in H-Ras, K-Ras and N-Ras have all been found in human tumors, and the frequency of Ras mutations is among the highest for any gene in human cancers (4). Ras is inactive in its GDP-bound form and active in its GTP-bound form. Activated Ras transmits its signal by interacting with diverse downstream effectors including Raf-1 (5), phosphatidylinositol-3-OH kinase (6), RalGDS (7-9) and RIN1 (10).

We previously identified AF-6 as a novel Ras effector (11). AF-6 was identified as the fusion partner of acute lymphoblastic leukemia-1 (ALL-1) protein (12). The ALL-1/AF-6 chimeric protein is a critical product of the t(6;11) abnormality associated with some human leukemias. Recently, various splicing variants of AF-6 have been reported (13) and the complete genomic structure of the human *AF-6* gene has been determined (14).

Our previous study showed that the amino-terminal (N-terminal) region of AF-6 directly binds to activated Ras (11). This Ras-binding domain of AF-6 is included in a large family of homologous Ras-associating domains (RA domains), which are seen in RalGDS and RIN1, and AF-6 contains two RA domains, N-terminal and carboxyl-terminal (C-terminal) RA domains (15). Recently, it was reported that the three-dimensional overall structure of the Ras-binding domain in Raf (Raf-RBD) (16) and the Ras-interacting domain in RalGDS (RalGDS-RID) are very similar and that the locations of critical amino acid residues for their interactions with activated Ras are identical, suggesting that Ras/Raf-RBD and Ras/RalGDS-RID complexes are structurally very similar (17). The amino acid residues on RalGDS-RID critical for its interaction with activated Ras are also conserved in AF-6 RA domains, but it is unknown whether the Ras/AF-6 RA domain complex is structurally similar to the Ras/Raf-RBD and Ras/RalGDS-RID complexes.

AF-6 has a PDZ (PSD-95/Dlg/ZO-1) domain, which is thought to localize AF-6 at specialized sites of the plasma membrane such as cell-cell contact sites. In fact, AF-6 accumulates at various cell-cell contact sites, such as the point of cell-cell adhesion (13, 18) and synaptic junctions (19). We previously showed that ZO-1, one of the peripheral components of cell-cell adhesion, directly binds to the RA domain of AF-6 *in vitro* (18), but it remains to be clarified whether AF-6 interacts with Ras and/or ZO-1 *in vivo.*

[1] To whom correspondence should be addressed at Division of Signal Transduction, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma, Nara 630-0101, Japan. Fax: +81-743-72-5449. E-mail: kaibuchi@bs.aist-nara.ac.jp.

Abbreviations used: RA domain, Ras-associating domain; N-terminal, amino-terminal; C-terminal, carboxyl-terminal; Raf-RBD, Ras-binding domain in Raf; RalGDS-RID, Ras-interacting domain in RalGDS; GST, glutathione-*S*-transferase; MBP, maltose-binding protein; DTT, dithiothreitol; IPTG, isopropyl-*β*-D-thiogalactoside; GTP*γ*S, guanosine 5′-(3-*O*-thio)-triphosphate; DSP, dithiobis(succinimidylpropionate); RGL-RID, RGL (RalGDS like) Ras-interacting domain.

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In light of these observations, we here examined *in vivo* interaction of AF-6 with activated Ras and ZO-1 in intact cells.

MATERIALS AND METHODS

Materials and chemicals. The expression plasmid of glutathione-*S*-transferase (GST)-mouse ZO-1 (1-862 aa) was kindly provided by Dr. S. Tsukita (Kyoto University, Kyoto, Japan). pMNC301-1 harboring the cDNA of Raf-1 kinase defective mutant was kindly provided by Dr. U. R. Rapp (Frederick Cancer Research and Development Center, Frederick, MA) (20). Human AF-6 cDNA was kindly provided by E. Canaani (Weizmann Institute of Science, Rehovot, Israel). The preparation of GST and maltose-binding protein (MBP) fusion proteins and the loading of GST-H-Ras with guanine nucleotides were performed as described previously (11).

Site-directed mutagenesis. The cDNA fragment encoding human AF-6 (1-506 aa) was amplified by polymerase chain reaction and subcloned into pMal-c2 (New England Biolabs, Beverly, MA). The substitutions of Leu for Lys at 58 or 265 on AF-6 (1-506 aa) were achieved using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) and DNA sequences were verified with an ABI PRISM 377 DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA).

In vitro binding assay. To examine the interaction of GST-H-Ras and MBP-AF-6 (1-506 aa) mutants, GST, GDP · GST-H-Ras or GTPγS · GST-H-Ras (750 pmol each) was mixed with MBP-AF-6 (1-506 aa) wild type or MBP-AF-6 (1-506 aa) mutants (150 pmol each) in 750 μl of buffer A (20 mM Tris/HCl at pH 7.5, 1 mM EDTA, 1 mM dithiothreitol (DTT) and 5 mM $MgCl_2$). To examine the interaction of GST-ZO-1 (1-862 aa) and MBP-AF-6 (1-506 aa) mutants, GST or GST-ZO-1 (1-862 aa) (480 pmol each) was mixed with MBP-AF-6 (1-506 aa) wild type or MBP-AF-6 (1-506 aa) mutants (150 pmol each) in 750 μl of buffer A. After incubation at 4°C for 1 h, the protein complexes were collected with glutathione Sepharose 4B (Amersham Pharmacia Biotech. AB, Uppsala, Sweden). After being washed with buffer A, the bound MBP-AF-6 (1-506 aa) mutants were coeluted with GST-fusion proteins by the addition of buffer A containing 10 mM reduced glutathione. Aliquots of the eluates were subjected to SDS-PAGE and the eluted MBP-AF-6 (1-506 aa) mutants were detected by silver staining or immunoblotting with anti-MBP antibody (New England Biolabs).

Luciferase assay. Transfection of plasmids into NIH3T3 cells was carried out by the standard calcium-phosphate method (21). Luciferase assay was performed as described previously (22).

Immunoprecipitation assay. Rat1 RasVal A1 and Rat1 RasAsn A3 cells, kindly provided by Drs. Y. Kaziro and H. Itoh (Tokyo Institute of Technology, Yokohama, Japan), were maintained as described previously (18). To examine the interaction of AF-6 with Ras, subconfluent Rat1 derivatives were incubated either with or without 5 mM isopropyl-β-D-thiogalactoside (IPTG) for 24 h. These cells were washed with PBS twice, suspended in TX buffer (50 mM Tris/HCl at pH 7.5, 1 mM EDTA, 5 mM $MgCl_2$, 50 mM NaCl, 0.5% TritonX-100, 10 μM (*p*-amidino-phenyl) methanesulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin). The immunoprecipitation using Rat anti-Ras monoclonal antibody Y13-238 or Rat control IgG (2 μg each) was performed as described previously (23). The immunoprecipitates were subjected to immunoblot analysis using anti-GST-AF-6 antibody (18), anti-Raf-1 antibody (E-10; Santa Cruz Biotech., Inc. Santa Cruz, CA) or anti-Ras antibody (RASK4).

Chemical cross-linking. Chemical cross-linking with 0.5 mM dithiobis(succinimidylpropionate) (DSP) was performed as described previously (24). Rat1 cells were solubilized with extraction buffer (50 mM Tris/HCl at pH 7.5, 1 mM EDTA, 50 mM NaCl, 0.5% Triton X-100, 10 μM (*p*-amidino-phenyl) methanesulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin) and separated to Triton-soluble and -insoluble fractions by centrifugation. The Triton-insoluble pellet was suspended in 100 μl of SDS IP buffer (50 mM Tris/HCl at pH 7.5, 5 mM EDTA, 2.5 mM EGTA, 1% SDS), incubated at 100°C for 10 min and then diluted to 1 ml with extraction buffer. The immunoprecipitation using anti-ZO-1 antibody (Zymed Lab. Inc. South San Francisco, CA) or rabbit IgG (2 μg each) was performed as described above. The immunoprecipitates were subjected to immunoblot analysis using anti-GST-AF-6 antibody or anti-ZO-1 antibody.

RESULTS

The substitution of Leu for Arg at 89 in Raf-1 prevents the Ras-dependent biological activity of Raf-1 (25) and the mutation of the analogous residue in D-Raf results in a partial loss of D-Raf function in *Drosophila* (26). This Arg residue corresponds to Lys 32 in RalGDS-RID and the substitution of Ala for Lys at 32 abrogated the interaction of RalGDS-RID with activated Ras. Since Lys 32 in RalGDS-RID is conserved in both AF-6 RA domains (Lys 58 and Lys 265), we examined whether mutations at same sites of AF-6 RA domains affect the interaction of AF-6 with activated Ras. For this purpose, we generated three MBP-AF-6 (1-506 aa) mutants by site-directed mutagenesis: (i) 58L, where Leu was substituted for Lys at 58 in the N-terminal RA domain; (ii) 265L, where Leu was substituted for Lys at 265 in the C-terminal RA domain; (iii) 58L265L, where Leu was substituted for Lys at 58 and 265 in the RA domains. We examined *in vitro* binding of GST-H-Ras to these MBP-AF-6 (1-506 aa) mutants. MBP-AF-6 (1-506 aa) bound to GTPγS · GST-H-Ras, but not to GST alone and little to GDP · GST-H-Ras, as described previously (11). The 58L and 58L265L mutations in AF-6 RA domains abolished the interaction with activated Ras, but the 265L mutation did not affect it (Fig. 1A). These results indicate that the N-terminal RA domain is necessary for the interaction of AF-6 with activated Ras and that the critical residue for the interaction with activated Ras on this domain is located at sites similar to those on Raf-RBD and RalGDS-RID. Since we previously showed that MBP-AF-6 (36-206 aa) directly binds to the N-terminal half of ZO-1, we examined whether the mutations of AF-6 RA domains affect the interaction of AF-6 with ZO-1. MBP-AF-6 (1-506 aa) bound to GST-ZO-1 (1-862 aa), but not to GST alone, as described previously (18). All mutants bound to GST-ZO-1 (1-862 aa) as wild type of MBP-AF-6 (1-506 aa) did (Fig. 1B). These results indicate that the substitution of the residue critical for the interaction of AF-6 with activated Ras does not affect the interaction of AF-6 with ZO-1 and suggest that the site of AF-6 for the recognition by activated Ras is different from that for ZO-1.

Activated Ras stimulates the c-*fos* promoter/enhancer in NIH3T3 cells as described previously (27-30). This Ras-dependent stimulation has been reported to be inhibited by the overexpression of the Ras-

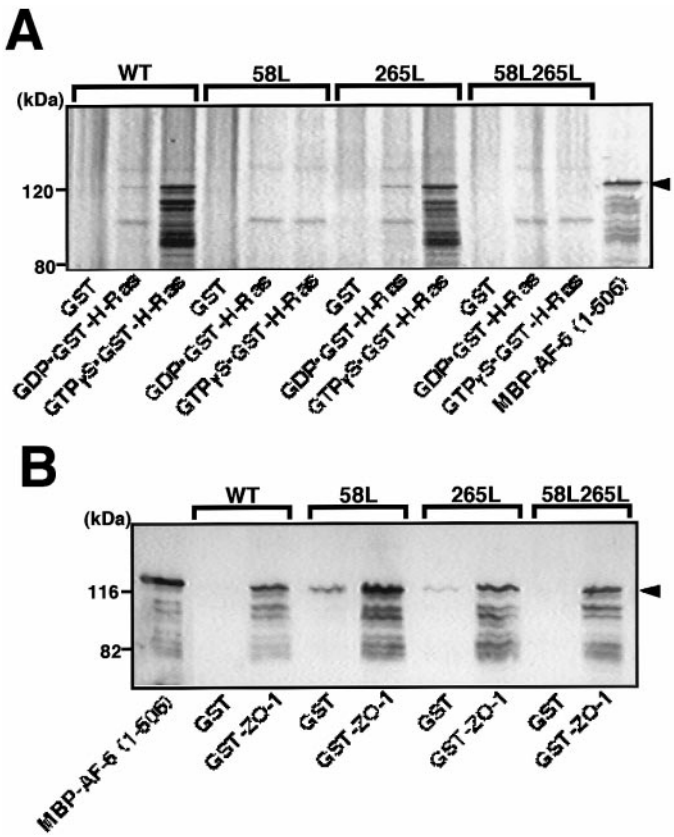

FIG. 1. Interactions of various mutants of AF-6 RA domains with activated Ras and ZO-1. The interactions of various MBP-AF-6 (1-506 aa) mutants with GST-Ras (A) or GST-ZO-1 (B) were examined by *in vitro* binding assay as described in Materials and Methods. The bound MBP-AF-6 (1-506 aa) mutants were eluted by the addition of reduced glutathione and detected by silver staining (A) or by immunoblotting with anti-MBP antibody (B). WT: MBP-AF-6 (1-506 aa) wild type; 58L: MBP-AF-6 (1-506 aa) (58L); 265L: MBP-AF-6 (1-506 aa) (265L); 58L265L: MBP-AF-6 (1-506 aa) (58L265L). The arrowheads denote the position of MBP-AF-6 (1-506 aa) or MBP-AF-6 (1-506 aa) mutants. The results shown are representative of three independent experiments.

interacting domain in RGL (RalGDS like) (RGL-RID), indicating that activated Ras interacts with RGL-RID in intact cells (31). Since we already demonstrated that the N-terminal RA domain of AF-6 directly binds to activated Ras *in vitro* (11), it is possible that the N-terminal RA domain of AF-6 has an inhibitory activity against Ras-dependent c-*fos* promoter/enhancer stimulation as does RGL-RID. To examine this possibility, a fusion gene (c-*fos*-luciferase), which contains 445 bp of the c-*fos* gene 5′-flanking sequence linked to the luciferase gene, was used as a reporter of transcriptional activity of c-*fos* promoter/enhancer as described previously (22). NIH3T3 cells were cotransfected with c-*fos*-luciferase, H-Ras^{12V} (constitutively activated H-Ras) cDNA and either pSRαneo-HA-AF-6 (36-206 aa) or pMNC301-1, which harbors the cDNA encoding kinase defective Raf-1, and pME18S-lacZ as an internal control. The expression of HA-AF-6 (36-206 aa) or kinase defective Raf-1 alone did not affect the c-*fos*-luciferase expression. In the cells transfected with H-Ras^{12V}, the c-*fos*-luciferase expression increased significantly. HA-AF-6 (36-206 aa) inhibited the H-Ras^{12V}-stimulated c-*fos*-luciferase expression as kinase defective Raf-1 did (Fig. 2). These results indicate that the N-terminal RA domain of AF-6 functions as the activated Ras-interacting domain in intact cells as RGL-RID does.

To examine whether activated Ras binds to the intact molecule of AF-6, we performed immunoprecipitation assays using activated Ras-overexpressing Rat1 fibroblast cells. We used Rat1 cell lines which contain the constitutively activated ras (ras^{12V}) or dominant negative ras (ras^{17N}) gene under Lac repressor control, designated as Rat1 RasVal A1 and Rat1 RasAsn A3 cells, respectively. The addition of IPTG efficiently induced the expressions of Ras^{12V} and Ras^{17N} and their expression levels were nearly equal (data not shown). To assess the interaction of AF-6 with activated Ras, we immunoprecipitated the expressed Ras^{12V} or Ras^{17N} with Y13-238, antibody against Ras. As shown in Fig. 3, AF-6 was coimmunoprecipitated with Ras^{12V} by Y13-238 in the presence of IPTG, but not with Ras^{17N}. When the same immunoprecipitates were subjected to immunoblot analysis using antibody against Raf-1, we found that Raf-1 was also coimmunprecipitated with Ras^{12V} by Y13-238 in the presence of IPTG, but not with Ras^{17N}. These results indicate that the intact molecule of AF-6 interacts with activated Ras *in vivo*.

Since Ras acts at the plasma membrane and AF-6 binds to ZO-1 *in vitro* and is colocalized with ZO-1 at cell-cell adhesion sites as we described previously (18), it is possible that the Ras-AF-6 pathway modifies the function of ZO-1 in cell-cell adhesion. To examine whether AF-6 interacts with ZO-1 *in vivo* and, if so,

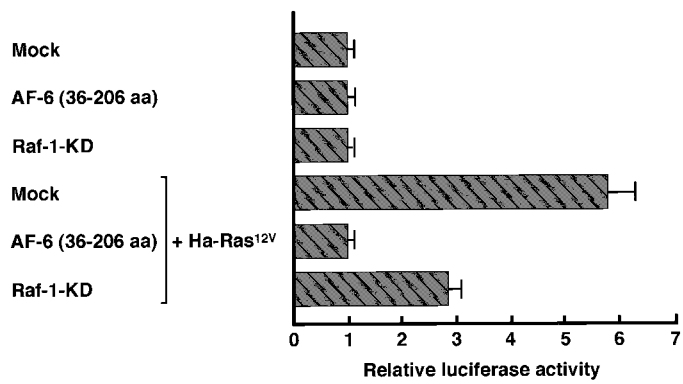

FIG. 2. Inhibition of c-*fos* activation by the N-terminal RA domain of AF-6. NIH3T3 cells were cotransfected with pCEV4-H-Ras^{12V} (17 ng), c-*fos*-luciferase (1 μg), pME18S-lacZ (2.33 μg), and pSRαneo-HA-AF-6 (36-206 aa) (650 ng) or pMNC301-1 (kinase defective Raf-1; Raf-1-KD) (650 ng). After 24 h, the luciferase activity in the cells was measured and presented as relative activity to the cells transfected with the control vectors alone. The values are means ± standard errors of three independent experiments.

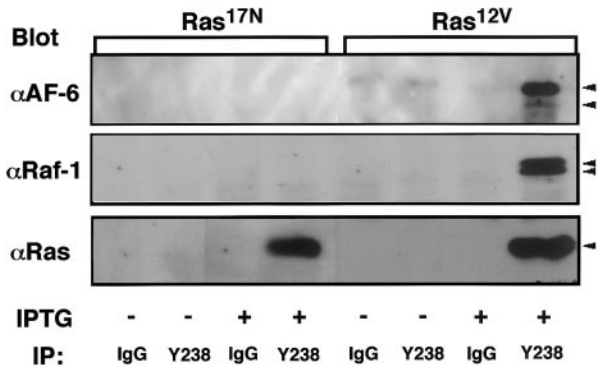

FIG. 3. Interaction of endogenous AF-6 with constitutively activated Ras in activated Ras-expressing Rat1 cells. Subconfluent Rat1 RasVal A1 or Rat1 RasAsn A3 cells were incubated for 24 h in the presence (+) or absence (−) of IPTG. The cells were solubilized with TX buffer and incubated with control IgG (IgG) or Y13-238 (Y238). The immunocomplexes were precipitated with protein G Sepharose and subjected to immunoblot analysis using anti-GST-AF-6 antibody (top), anti-Raf-1 antibody (middle) or anti-Ras antibody (RASK4) (bottom). The arrowheads denote the positions of AF-6 (top), Raf-1 (middle) or mutated Ras (bottom), respectively. The results shown are representative of three independent experiments.

whether this AF-6/ZO-1 complex is involved in cell-cell adhesion, we performed immunoprecipitation assays. Since ZO-1 is highly resistant to mild extraction using a nonionic detergent such as 0.5% Triton X-100 and strong extraction for solubilizing the Triton-insoluble cell fraction (1% SDS at 100°C) may destroy the AF-6/ZO-1 complex, we performed immunoprecipitation after chemical cross-linking with DSP, a homobifunctional thio-cleavable cross-linker. The immunoprecipitation after cross-linking with DSP previously revealed that the cadherin/catenin complex is present in the Triton-insoluble fraction as well as in the Triton-soluble fraction (24, 32). In Rat1 cells, 80% of ZO-1 existed in the Triton-insoluble fraction (data not shown). In the absence of DSP, AF-6 was coimmunoprecipitated with ZO-1 from the Triton-soluble fraction, but not from the Triton-insoluble fraction. In the presence of DSP, AF-6 was coimmunoprecipitated with ZO-1 from both fractions (Fig. 4). These results indicate that AF-6 forms a complex with ZO-1 in the Triton-insoluble fraction as well as Triton-soluble fraction.

DISCUSSION

AF-6 has two RA domains (15). The present study showed that a single amino acid substitution in the N-terminal RA domain of AF-6 abolished the interaction of AF-6 with activated Ras and this critical residue was located at a similar site in Raf-RBD and RalGDS-RID. This result suggests that the N-terminal RA domain is indispensable for the interaction of AF-6 with activated Ras and that the site for the recognition by activated Ras in the N-terminal RA domain on AF-6 is structurally similar to that in Raf-RBD and in RalGDS-RID. The mutation of a similar residue in the C-terminal RA domain did not affect the interaction of AF-6 with activated Ras. Therefore, it is possible that activated Ras binds to the N-terminal RA domain but not to the C-terminal RA domain of AF-6. Since Canoe (33) and Ce-AF-6 (34), which are homologues of *Drosophila* and *C. elegans* respectively, have two RA domains structurally similar to that of AF-6, these proteins have properties similar to AF-6 in the interaction with activated Ras.

In the present study, we showed *in vivo* interaction of AF-6 with activated Ras using two strategies, by showing the inhibition of the Ras-dependent c-*fos* promoter/enhancer in NIH3T3 cells and by showing coimmunoprecipitation with activated Ras in Rat1 cells expressing activated Ras. These results indicate that AF-6 interacts with activated Ras *in vivo* as Raf-1 and RalGDS do, but we should note that these experiments were performed under the conditions where activated Ras or AF-6 was overexpressed. We were unable to detect the interaction of AF-6 with activated Ras induced by natural stimulation with growth factors such as EGF and NGF under these conditions. It remains to be elucidated what stimuli cause the interaction of AF-6 with activated Ras.

AF-6 is colocalized with ZO-1 at cell-cell contact sites and directly binds to ZO-1 *in vitro*. In this study, we showed that AF-6 is coimmunoprecipitated with ZO-1 from the Triton-insoluble fraction as well as the Triton-

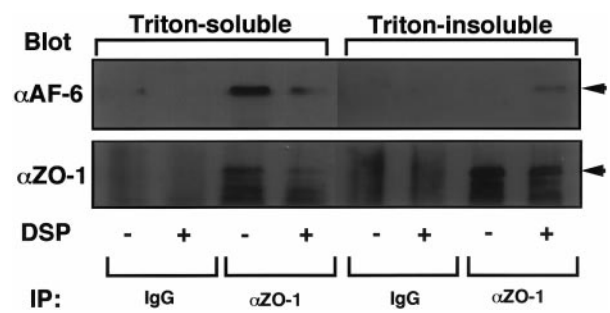

FIG. 4. Interaction of endogenous AF-6 and ZO-1 in Rat1 cells. Confluent Rat1 cells were washed with PBS and incubated with chemical cross-linker DSP (+) or vehicle (−). The cells were solubilized with extraction buffer and a Triton-soluble fraction and Triton-insoluble fraction were prepared as described in Materials and Methods. Equivalent aliquots of the Triton-soluble and -insoluble fractions were subjected to immunoprecipitation assay using control Rabbit IgG (IgG) or anti-ZO-1 antibody (αZO-1). The immunocomplexes were precipitated with protein A Sepharose and subjected to immunoblot analysis using anti-GST-AF-6 antibody (top) or anti-ZO-1 antibody (bottom). Ten volumes of the immunoprecipitate from the Triton-insoluble fraction was subjected to immunoblot analysis against one volume of that from the Triton-soluble fraction. The arrowheads denote the positions of AF-6 (top) or ZO-1 (bottom), respectively. The results shown are representative of three independent experiments.

soluble fraction using a chemical cross-linker, suggesting that various pools of AF-6/ZO-1 complex are present. ZO-1 directly interacts with occludin and serves as an essential peripheral component at tight junctions (35). In cells lacking tight junctions, ZO-1 is localized at cell-cell contact sites with the cadherin/catenin complex, where ZO-1 interacts with α-catenin (36). Moreover, ZO-1 is suggested to form a complex with catenins during early stages in the assembly of tight junctions of epithelial cells (37). These observations suggest that ZO-1 is involved in various cell-cell adhesions and the rearrangement of cell-cell adhesions. It remains to be clarified which cell-cell adhesion machinery, the AF-6/ZO-1 complex is a part of. We previously showed that activated Ras inhibits the interaction of AF-6 with ZO-1 *in vitro*. Therefore, it is possible that activated Ras prevents the interaction of AF-6 with ZO-1 when it regulates cell-cell adhesion. However, further study is necessary to clarify whether activated Ras modulates the AF-6/ZO-1 complex during the rearrangement of cell-cell adhesion.

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