AF-6 Controls Integrin-mediated Cell Adhesion by Regulating Rap1 Activation through the Specific Recruitment of Rap1GTP and SPA-1*

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In the present study, we showed that SPA-1, a Rap1 GTPase-activating protein (GAP), was bound to a cytoskeleton-anchoring protein AF-6. SPA-1 and AF-6 were co-immunoprecipitated in the 293T cells transfected with both cDNAs as well as in normal thymocytes. In vitro binding studies using truncated fragments and their mutants suggested that SPA-1 was bound to the PDZ domain of AF-6 via probable internal PDZ ligand motif within the GAP-related domain. The motif was conserved among Rap1 GAPs, and it was shown that rapGAP I was bound to AF-6 comparably with SPA-1. RapV12 was also bound to AF-6 via the N-terminal domain, and SPA-1 and RapV12 were co-immunoprecipitated only in the presence of AF-6, indicating that they could be brought into close proximity via AF-6 in cells. Immunostaining analysis revealed that SPA-1 and RapV12 were co-localized with AF-6 at the cell attachment sites. In HeLa cells expressing SPA-1 in a tetracycline-regulatory manner, expression of AF-6 inhibited endogenous Rap1GTP and β1 integrin-mediated cell adhesion to fibronectin in SPA-1-induced conditions, whereas it affected neither of them in SPA-1-repressed conditions. These results suggested that AF-6 could control integrin-mediated cell adhesion by regulating Rap1 activation through the recruitment of both SPA-1 and Rap1GTP via distinct domains.

Rap1 is a member of Ras family GTPases and is suggested to play roles in the regulation of cell proliferation, differentiation, and cell adhesion (reviewed in Ref. 1). Rap1 is activated by a wide variety of extracellular stimuli through different kinds of specific guanine nucleotide exchange factors, which are coupled with various receptors or the second messengers via distinct interaction motifs (1). The amounts and duration of intracellular Rap1GTP, on the other hand, are controlled by specific Rap1GAPs (16–18). Although these features imply that AF-6 may function as a molecular scaffold integrating the signals related to cell adhesion and cytoskeletal reorganization, its exact functions remain to be seen. We provide evidence that AF-6 binds both SPA-1 and its specific substrate Rap1GTP via distinct domains and can control integrin-mediated cell adhesion by regulating Rap1 activation.

**EXPERIMENTAL PROCEDURES**

Cells and Antibodies—Thymocytes, splenic T and B cells were obtained from normal BALB/c mice. 293T, HeLa, and Caki-2 (human kidney cancer) cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. HeLa cells stably transfected with SPA-1 cDNA (HeLa/Tet-SPA-1) in a pTRE vector were used.

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(Clontech) were reported before (9) and maintained in complete Dulbecco’s modified Eagle’s medium containing 10 ng/ml doxycycline (Dox) (Sigma), unless indicated specifically. Antibodies used in the present study included anti-SPA-1 (9), anti-AF-6 (19), anti-Rap1, anti-Rap1 GAP (Santa Cruz Biotechnology), and anti-T7 antibodies (Novagen). Biotinylated anti-SPA-1 antibody was prepared using EZ-link sulfo-NHS-LC biotin (Pierce). Anti-VLA-4 and anti-VLA-5 antibodies were provided by Dr. T. Kinashi, Kyoto University, Kyoto, Japan.

**Plasmid Construction and cDNA Transfection—**cDNA of AF-6 lacking Ras/Rap1-binding domain (ARBD AF-6) was obtained by deleting an N-terminal region between Nofl and AfII sites (1–128 residues) from a full-length AF-6 cDNA. cDNA of AF-6 lacking PDZ domain (ΔPDZ AF-6) was also prepared by deleting a fragment between BamHI and XhoI sites (910–1612 residues) from a full-length AF-6 cDNA, to which PCR-amplified fragments (910–990 and 1078–1612 residues) were ligated back consecutively. SPA-1 cDNA lacking GRD (ΔGRD SPA-1) and C3G cDNA bearing a CAAX box sequence at the C terminus (C3G-F) were reported before (9). These plasmids were transfected into 293T or HeLa cells using a CaPO4 precipitation method or Effectene Transfection Reagent (Qiagen).

**In Vitro Binding of SPA-1 and AF-6—**cDNAs of truncated SPA-1 fragments, fragment 1 (residues 1–211), 2 (residues 212–532), 3 (residues 533–680), 4 (residues 681–1038), and 5 (residues 748–1038), were amplified by PCR and subcloned into BamHI/XhoI sites of a pSP73 vector. PCR-amplified cDNAs of subfragments of SPA-1 (ΔGRD fragment 2), G1 (residues 338–532), G2 (398–459), and G3 (residues 435–489) as well as the mutants of G2 fragment, M1 (V432A) and M2 (F433A) generated by a site-directed mutagenesis kit (Stratagene Quick), were subcloned into BglII/EcoRI sites of a pSP73 vector. A cDNA fragment of rapGAP 1 (residues 263–322) corresponding to the G2 fragment of SPA-1 was amplified by PCR and also subcloned into a pSp73 vector. cDNAs of a series of truncated AF-6 fragments, fragment 1 (residues 36–494), 2 (residues 495–909), 3 (residues 914–1129), and 4 (residues 1130–1612), as well as AF-6 and ΔPDZ AF-6 cDNAs were subcloned into KpnI and SalI sites of a pSP73 vector. In *in vitro* transcription and translation (IVTT) of each cDNA was performed using T7/SP6-coupled wheat germ extract system (omega) in the presence of [35S]methionine. In *in vitro* binding assay was performed as follows. Cell lysate of the 293T cells transfected with pEF-BOs-AF-6 or pSR6 were immunoprecipitated with anti-AF-6, anti-AF-1, or preimmune IgG as a control followed by the precipitation with protein A-Sepharose beads (Amersham Biosciences). The labeled IVTT products above were incubated with such conjugated beads for 1 h at 4°C with gentle rotation. The beads were extensively washed, eluted with SDS sample buffer, and electrophoresed in regular SDS-PAGE or Tricine-buffered SDS-PAGE for smaller molecular mass IVTT products followed by autoradiography.

**Immunoprecipitation and Immunoblotting—**Cells were lysed with lysis buffer (0.5% Triton X-100, 10 mM Tris-HCl, pH 7.6, 150 mM NaCl, protease inhibitor mixture), incubated with specific antibodies overnight at 4°C with gentle rotation, and then precipitated with protein A-Sepharose beads for 30 min at 4°C. After extensive washing, the beads were eluted with SDS sample buffer, boiled, and electrophoresed in SDS-PAGE followed by immunoblotting and ECL detection (Amersham Biosciences). To detect intracellular Rap1GTP, cell lysates (0.8–1 mg of proteins) were incubated with a GST fusion protein of RapGDS-RBD coupled with glutathione-Sepharose 4B (Amersham Biosciences) on ice for 1 h, washed, and eluted with SDS sample buffer followed by immunoblotting with anti-Rap1 antibody.

**Immunostaining—**HeLa/Tet-SPA-1 cells were transfected with pEF-BOs and selected with puromycin (Sigma) to establish a stable cell line (HeLa/Tet-SPA-1/ARF-6). The latter cells were transfected transiently with T7-tagged RapV12 cDNA in a pSR vector by using Effectene Transfection Reagent (Qiagen). These cells were cultured on coverslips in the presence of Dox at an either inductive (0.1 ng/ml) or non-inductive (1 ng/ml) dose for 24 h. The cells were rinsed with Tris-buffered saline solution with 3% paraformaldehyde, permeabilized by 0.5% Triton X-100/Tris-buffered saline, blocked with 2% bovine serum albumin/Tris-buffered saline, and incubated with anti-AF-6 or anti-Rap1 antibody followed by AlexaFluor 546 anti-rabbit IgG (Molecular Probes). After washing, the cells were incubated further with biotinylated anti-SPA-1 or anti-T7 antibody followed by AlexaFluor 488-streptavidin or mouse IgG. Normal rabbit or mouse IgG was used as a control for the corresponding primary antibody. The stained cells were analyzed using a confocal microscopy (Olympus).

**Cell Adhesion Assay—**96-Well flat-bottom plates were coated with 5 μg/ml fibronectin (FN) (Sigma) overnight at 4°C followed by blocking with 3% bovine serum albumin/phosphate-buffered saline for 1 h at 37°C. 293T or HeLa/Tet-SPA-1 cells were treated with trypsin/EDTA, washed, resuspended in serum-free Dulbecco’s modified Eagle’s medium containing 0.02% bovine serum albumin and 10 mM Hepes, rotated in suspension for 2 h, and plated in triplicate onto FN-coated wells at 0.5 × 10⁴ cells per well. After incubation for 30 min at 37°C, nonadherent cells were removed gently by aspiration, and the remaining adherent cells were fixed with 3.7% paraformaldehyde followed by staining with 0.5% crystal violet in 20% methanol. After extensive washing with distilled water, dye was extracted with extract solution (50% ethanol in 50 mM sodium citrate, pH 4.5) and measured using an enzyme-linked immunosorbent assay reader (Molecular Devices). Absorbance in uncoated wells was subtracted from that in the FN-coated wells to show specific adhesion.

**Statistics Analysis—**Statistics analysis was done by Student’s *t* test.

**RESULTS**

**Association of SPA-1 and AF-6 in Normal Cells—**By yeast two-hybrid screening of a mouse spleen cell cDNA library using a full-length SPA-1 as bait, AF-6 was identified as a potential SPA-1-binding protein (data not shown). We therefore examined the association of SPA-1 and AF-6 by transient gene expression in 293T cells, which expressed only marginal SPA-1 and AF-6 if any. In the 293T cells co-transfected with SPA-1 and AF-6 cDNAs, SPA-1 (130 kDa) was co-immunoprecipitated by anti-AF-6, and reciprocally AF-6 (~200 kDa) was co-immunoprecipitated by anti-SPA-1 antibody, although AF-6 tended to be degraded in overexpression system (Fig. 1A). Neither protein was immunoprecipitated by control preimmune IgG.

We then investigated whether the association of SPA-1 and AF-6 occurred physiologically in normal cells. As shown in Fig. 1B, thymocytes abundantly expressed both SPA-1 and AF-6 among normal lymphoid cells, and the endogenous SPA-1 and AF-6 were co-immunoprecipitated with anti-AF-6 and anti-SPA-1 antibodies, respectively, indicating that SPA-1 and AF-6 were associated physiologically in normal cells. It was estimated that around 40% of SPA-1 and 15% of AF-6 were associated with AF-6 and SPA-1, respectively, in normal thymocytes.

**Involvement of GRD of SPA-1 and PDZ Domain of AF-6 for Binding—**To identify the domains involved in the association of SPA-1 to AF-6, binding of truncated SPA-1 fragments to AF-6 was examined in *vitro*. Among the fragments, a GRD fragment of SPA-1 (fragment 2, residues 212–532) was bound to the AF-6-coated beads, whereas none of the other fragments were bound (Fig. 2A). Although not shown, fragment 2 was not bound to the control beads. Similar analysis was performed using truncated fragments of AF-6. As also indicated in Fig. 2A, only a fragment containing the PDZ domain of AF-6 (fragment 3, residues 914–1129) was bound specifically to the SPA-1-coated beads. To confirm these results, we generated a GRD-deletion mutant of SPA-1 (ΔGRD SPA-1) and a PDZ-deletion mutant of AF-6 (ΔPDZ AF-6) by IVTT. As shown in Fig. 2B, ΔGRD SPA-1 and ΔPDZ AF-6 failed to be bound to the beads coated with AF-6 and SPA-1 respectively, whereas full-length proteins were bound to the partner proteins. These results strongly suggested that SPA-1 and AF-6 were associated via interaction between GRD of SPA-1 and PDZ domain of AF-6.

**Binding of SPA-1 to PDZ Domain of AF-6 via Probable Internal PDZ Ligand Motif, a Common Property of Rap1 GAPs**—We next intended to investigate whether the binding of SPA-1 and AF-6 occurred by PDZ-mediated protein interaction. By further truncation of a SPA-1 GRD fragment (fragment 2), it was shown that smaller fragments, G1 (residues 338–532) and G2 (residues 398–459), were bound to AF-6-coated beads, whereas another overlapping fragment G3 (residues 435–489) was not (Fig. 3B), suggesting the binding site was in the region between the residues 398 and 434. By using peptide libraries, it was
reported that the AF-6 PDZ preferred a class 2 ligand motif (ψ-X-ψ, where ψ is a hydrophobic residue), and a hydrophobic residue was also preferred at −1 position (20). In the G2 fragment, it was noticed that a stretch of residues at 432–434, IVF, fitted the predicted PDZ ligand motif, which was in a probable β-sheet immediately followed by a turn and a β-sheet as predicted by Chou-Fasman secondary structure prediction (Fig. 3A). To investigate possible involvement of this motif, we generated mutant proteins of G2, M1 (V433A), and M2 (F434A) by IVTT, and examined their binding to AF-6-coated beads. As shown in Fig. 3B, M1 was bound barely to AF-6-coated beads and M2 with markedly reduced efficiency as compared with G1, suggesting that both Val-433 and Phe-434 were required for the binding to AF-6 PDZ domain. Among known Rap1 GAPs, the motif was conserved (IVF for SPA-1, anti-AF-6, or preimmune IgG) and immunoblotted with the indicated antibodies. Relative intensities of the immunoprecipitated bands of heterologous antibody combinations to those of homologous antibody combinations are indicated.

**Fig. 1. Intracellular association of SPA-1 and AF-6.** A, 293T cells were transfected with SPA-1, AF-6, or both cDNAs (1 μg each), and 2 days later aliquots of cell lysate of each group were immunoprecipitated (IP) with anti-SPA-1, anti-AF-6, or preimmune IgG followed by immunoblotting. SPA-1 was detected as a 130-kDa band and AF-6 as an ~200-kDa band with a ladder of smaller bands likely representing protein degradation. The experiments were repeated three times with similar results. B, left, freshly isolated mouse thymocytes and splenic T and B cells were lysed and immunoblotted with indicated antibodies. Right, thymocytes lysate was immunoprecipitated with anti-SPA-1, anti-AF-6, or preimmune IgG and immunoblotted with the indicated antibodies. Relative intensities of the immunoprecipitated bands of heterologous antibody combinations to those of homologous antibody combinations are indicated.

Co-localization of SPA-1 and Rap1 with AF-6 at Cell Attachment Sites—We next examined the intracellular localization of SPA-1 in relation to AF-6 by using HeLa/Tet-SPA-1 cells and those stably transfected with AF-6 (HeLa/Tet-SPA-1/AF-6), in which SPA-1 expression was repressed at the undetectable level in the presence of 1.0 ng/ml Dox while induced strongly in the presence of 0.1 ng/ml Dox within 24 h. In HeLa/Tet-SPA-1 cells cultured with 0.1 ng/ml Dox, SPA-1 was expressed diffusely at the cortical area as well as in the cytosol with little expression of AF-6 (Fig. 4A). On the other hand, AF-6 was localized predominantly at the cell attachment sites with undetectable SPA-1 expression in the HeLa/Tet-SPA-1/AF-6 cells cultured with 1.0 ng/ml Dox (Fig. 4B). It was noted that those cells expressing both SPA-1 and AF-6 tended to become slender, which was followed by the cell detachment from a dish in 2 days, much earlier than HeLa/Tet-SPA-1 cells.

**Fig. 2. Requirement of GRD of SPA-1 and PDZ domain of AF-6 for the binding.** A, a series of truncated fragments of SPA-1 (left) or AF-6 (right) were generated by IVTT in the presence of [35S]methionine and analyzed with SDS-PAGE followed by autoradiography (left panel of each). Lysate of 293T cells transfected with AF-6 or SPA-1 cDNA was immunoprecipitated with specific antibody and protein A-Sepharose beads. Each IVTT fragment was incubated with the AF-6- or SPA-1-coated Sepharose beads, and the specific binding was determined by a pull-down assay followed by autoradiography (right panel of each). Although not shown, none of the fragments were precipitated by the control beads incubated with the cell lysates and preimmune IgG. The experiments were repeated twice with identical results. B, left, wild type (wt) and AGRD SPA-1 proteins generated by IVTT (left panel) were incubated with AF-6-coated beads, and the washed beads were eluted with SDS sample buffer followed by autoradiography (right panel). Right, wild type and ΔPDZ AF-6 proteins generated by IVTT (left panel) were incubated with SPA-1-coated beads, and the washed beads were eluted with SDS sample buffer followed by autoradiography (right panel).
The Tricine-buffered SDS-PAGE. Input of each IVTT product is shown in H9262 AF-6 with or without rapGAP I cDNA (1A control (IgG) beads, and the binding were analyzed as in Fig. 2 of rapGAP corresponding to G2 were incubated with AF-6-coated or of G1, G2, G3, M1 (V432A), and M2 (F433A) of SPA-1 and a fragment man secondary structure prediction is also indicated.

B for M1 fragment and V mutated residues (sequence of G3 fragment. Aligned sequences of rapGAP and E6TP1 are tion of G1, G2, and G3 fragments of SPA-1 GRD, and amino acid residues (V for M1 fragment and P for M2 fragment) are indicated in boldface. A β-finger-like structure predicted by Chou-Fasman secondary structure prediction is also indicated. B, IVT products of G1, G2, G3, M1 (V432A), and M2 (F433A) of SPA-1 and a fragment of rapGAP corresponding to G2 were incubated with AF-6-coated or control (IgG) beads, and the binding were analyzed as in Fig. 2A using Tricine-buffered SDS-PAGE. Input of each IVTT product is shown in the left panel of each group. C, left, 293T cells were transfected with AF-6 with or without rapGAP I cDNA (1 µg), and the lysates were immunoprecipitated with anti-AF-6 followed by immunoblotting with anti-rapGAP. Expression of transfected cDNAs was confirmed by straight immunoblotting. Right, Caki-2 parental cells were lysed, immunoprecipitated with anti-AF-6, anti-rapGAP, or control IgG, and immunoblotted with anti-AF-6 antibody. These experiments were repeated twice with reproducible results.

(see below). As shown in Fig. 4E, SPA-1 was co-localized also with endogenous Rap1 under this condition. These results strongly suggested that both SPA-1 and Rap1GTP were recruited to the cell attachment sites by AF-6.

AF-6 Induces the Association of SPA-1 and Rap1GTP and Enhances Rap1 Inactivation—A possibility that AF-6 recruited both SPA-1 and its substrate Rap1GTP was investigated more directly. 293T cells were transfected with SPA-1 and T7-tagged RapV12 with or without AF-6 cDNA, and the lysate was immunoprecipitated with anti-T7 antibody. In the absence of AF-6, SPA-1 was co-immunoprecipitated barely with RapV12 (Fig. 5A), conforming to a general consensus that the interaction of GAPs with the substrate was only transient (21). In the presence of AF-6, however, a significant proportion of SPA-1 was co-immunoprecipitated with RapV12 along with AF-6 (Fig. 4A). On the other hand, expression of ΔRBD AF-6, which failed to be co-immunoprecipitated with RapV12, hardly induced the co-immunoprecipitation of SPA-1 with RapV12 either (Fig. 4A). The results indicated that AF-6 could bind both SPA-1 and Rap1GTP simultaneously via distinct domains, leading to the efficient association of SPA-1 and Rap1GTP. We then examined the effect of AF-6 expression on the efficiency of Rap1 inactivation by SPA-1 in the cells. Expression of SPA-1 in 293T cells suppressed the amount of endogenous Rap1GTP generated by the transfection of C3G-F cDNA in a dose-dependent manner as reported previously (9). Additional expression of AF-6 significantly enhanced the Rap1 inactivation by SPA-1 (Fig. 4B). Thus, transfection of 0.3 µg of SPA-1 cDNA resulted in almost complete inhibition of Rap1GTP generation in the presence of AF-6, whereas 40% of Rap1GTP still remained in the absence of AF-6 (Fig. 4B). As also shown in Fig. 4B, the enhancing effect of AF-6 on the Rap1 inactivation by SPA-1 was reduced markedly by the deletion of the PDZ domain.

AF-6 Enhances the Inhibitory Effect of SPA-1 on β1-Integrin-mediated Cell Adhesion—We finally investigated functional effects of the association of SPA-1 and AF-6 on cell adhesion. 293T cells in suspension adhered to fibronectin (FN)-coated plates in a manner dependent on VLA4 and VLA5 (Fig. 6A). Although expression of SPA-1 in 293T cells significantly inhibited the cell adhesion as reported before (9), that of AF-6, ΔRBD AF-6, or ΔPDZ AF-6 alone hardly affected the cell adhesion (Fig. 6A). However, co-transfection of AF-6 with SPA-1 cDNA resulted in the greater inhibition of cell adhesion than that of SPA-1 alone (Fig. 6A). Neither ΔRBD AF-6 nor ΔPDZ AF-6...
affected the SPA-1-induced inhibition of cell adhesion, suggesting that the effect of AF-6 was dependent on the association with SPA-1 and Rap1. Similar experiments were performed using HeLa/Tet-SPA-1 cells. HeLa/Tet-SPA-1 cells were transfected with a control vector, AF-6, or ΔPDZ AF-6 cDNA and cultured for 1 day in the presence of Dox at 1.0 or 0.1 ng/ml. At 1.0 ng/ml Dox, SPA-1 expression was almost completely repressed (Fig. 6B, left). Under this condition, expression of AF-6 or ΔPDZ AF-6 induced a slight increase in Rap1GTP, and the cell adhesion tended to be enhanced marginally although with statistical insignificance (Fig. 6B, right). When the cells were cultured in the presence of 0.1 ng/ml Dox, on the other hand, SPA-1 expression was induced, and concomitantly Rap1GTP was reduced, leading to the significant decrease in the cell adhesion (Fig. 6B). Expression of AF-6 under this condition resulted in the much greater decrease in both cell adhesion and Rap1GTP level, whereas that of ΔPDZ AF-6 was without effect at all (Fig. 6B). These results suggested that AF-6 could contribute to the inhibition of β1 integrin-mediated cell adhesion by enhancing the efficiency of SPA-1-mediated Rap1GTP inactivation.

**DISCUSSION**

In the present study, we demonstrated that a Rap1 GTPase-activating protein SPA-1 was bound to AF-6. SPA-1 was co-immunoprecipitated specifically with AF-6 and vice versa not only in the 293T cells co-transfected with SPA-1 and AF-6 cDNAs but also in normal thymocytes, indicating that the association was physiological. In vitro binding studies revealed that the binding was mediated by the interaction between GRD of SPA-1 and the PDZ domain of AF-6. Most known PDZ-mediated protein interactions occur through recognition of short C-terminal PDZ ligand motifs of partner proteins (22, 23). However, exceptional interactions of PDZ domain with internal motifs of partner proteins have been also reported (24–27). It
was reported that neuronal nitric-oxide synthase was bound to a PDZ domain of syntrophin via the internal region, in which a PDZ ligand motif in a sharp β-finger structure of neuronal nitric-oxide synthase mimicked a C-terminal ligand motif as a pseudo-peptide (27). It was reported that a class 2 ligand motif (Q-X-X-β) with hydrophobic residues also at the −1 position was preferred by AF-6 PDZ domain by using peptide libraries (20). SPA-1 GRD contained a stretch of residues IVP (residues 432–434), which fitted the predicted ligand motif for AF-6 PDZ, and the mutations of Val-433 to Ala or Phe-434 to Ala markedly reduced the binding of a minimal fragment of SPA-1 (G2) to AF-6 in vitro. Marginally retained binding activity of a F434A mutant fragment may be due to the residual activity of alanine as an anchoring residue. Chou-Fasman secondary structure prediction suggested that the motif was located in a β-finger-like structure. These results suggested, but did not prove, that the interaction of internal PDZ ligand motif in SPA-1 GRD with the PDZ domain of AF-6 mediated the specific binding of SPA-1 to AF-6. The motif was conserved in rapGAP and E6TP1, and present results indicated that rapGAP was bound to AF-6 comparably with SPA-1, suggesting that the binding to AF-6 was a shared feature of Rap1 GAPs. On the other hand, mutations of RKKR at positions 421–431 reduced the binding of a minimal fragment of SPA-1 (G2) to AF-6 irrespective of the presence of SPA-1, conforming to the previous report (16) that RapV12 was bound to AF-6. The present results further indicated that SPA-1 and Rap1V12 could be co-immunoprecipitated significantly in the presence of AF-6 but not of Rap1. The present results using HeLa/Tet-SPA-1 indicated that expression of AF-6, but not Rap1, inactivating Rap1. The present results using HeLa/Tet-SPA-1 indicated that expression of AF-6, but not Rap1, inactivating Rap1. The present results using HeLa/Tet-SPA-1 where SPA-1 could negatively regulate the cell adhesion by functional inhibition of p120 RasGAP was inhibited in vivo by an RBD fragment of AF-6 due to competitive binding of the two fragments to overlapping sites of RasGTP (15). Independent binding of SPA-1 and Rap1GTP to the distinct domains of AF-6 may prevent such a possible interference with the GAP catalytic activity of SPA-1 by AF-6. Rather, it was shown that the GAP activity of SPA-1 in vivo was enhanced significantly in the presence of AF-6, indicating that AF-6 recruited SPA-1 and its substrate Rap1GTP into close proximity in the cells and facilitated the efficient catalytic interaction between them. We reported previously (9–11) that Rap1GTP activated β1 and β2 integrins and induced the integrin-mediated cell-cell and cell-matrix adhesion, whereas SPA-1 could negatively regulate the cell adhesion by inactivating Rap1. The present results using HeLa/Tet-SPA-1 cells indicated that expression of AF-6, but not ΔPDZ AF-6, induced the decrease in Rap1GTP levels and significant inhibition of β1 integrin-mediated cell adhesion to FN in an SPA-1-induced condition, whereas AF-6 did not affect either of them in an SPA-1-repressed condition. These results have suggested strongly that AF-6 plays a role in the control of integrin-mediated cell adhesion by enhancing the efficiency of Rap1 inactivation by SPA-1 at the cell adhesion sites. Integrin-mediated cell adhesions are regulated dynamically during various cellular functions. In the immune system, for instance, leukocyte movement and migration involve a spatio-temporally organized regulation of cell adhesion (28, 29). Also, T cell activation in immune responses depends on the integrin-mediated intimate cell-cell interactions between T cells and antigen-presenting cells called immunological synapse, the extent and duration of which profoundly affect the modes and intensity of immune responses (30). We reported previously (12) that Rap1 activation and its regulation by SPA-1 played crucial roles in the immunological synapse formation through LFA-1/ICAM-1 interaction. The present results indicated that AF-6 was expressed abundantly in the normal thymocytes partly in association with SPA-1, and our results (31) using SPA-1 transgenic mice showed that Rap1 activation was essential for the differentiation and expansion of thymocytes in vivo. It thus seems possible that AF-6 plays a significant role in the fine control of integrin-mediated cellular functions via regulation of Rap1 activation in the immune as well as other systems.

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