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Signal-Transducing Adaptor Protein-2 Regulates Integrin-Mediated T Cell Adhesion through Protein Degradation of Focal Adhesion Kinase[1]

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Signal-transducing adaptor protein-2 (STAP-2) is a recently identified adaptor protein that contains pleckstrin homology- and Src homology 2-like domains as well as a YXXQ motif in its C-terminal region. Our previous studies demonstrated that STAP-2 binds to STAT3 and STAT5, and regulates their signaling pathways. In the present study, we find that STAP-2-deficient splenocytes or T cells exhibit enhanced cell adhesion to fibronectin after PMA treatment, and that STAP-2-deficient T cells contain the increased protein contents of focal adhesion kinase (FAK). Furthermore, overexpression of STAP-2 induces a dramatic decrease in the protein contents of FAK and integrin-mediated T cell adhesion to fibronectin in Jurkat T cells via the degradation of FAK. Regarding the mechanism for this effect, we found that STAP-2 associates with FAK and enhances its degradation, proteasome inhibitors block FAK degradation, and STAP-2 recruits an endogenous E3 ubiquitin ligase, Cbl, to FAK. These results reveal a novel regulation mechanism for integrin-mediated signaling in T cells via STAP-2, which directly interacts with and degrades FAK. ***The Journal of Immunology,*** **2007, 179: 2397–2407.**

Recently, we cloned signal-transducing adaptor protein-2 (STAP-2)[4] as a c-*fms*-interacting protein (1). Human STAP-2 is identical with an adaptor molecule, BKS. BKS is a substrate of breast tumor kinase protein tyrosine kinase, which is closely related to members of the Src family of nonreceptor protein tyrosine kinases (2). STAP-2 shows high sequence and structural similarities to STAP-1, which we cloned as a c-*kit*-interacting protein (3). Both STAP-1 and STAP-2 contain an N-terminal pleckstrin homology (PH) domain and a region weakly related to the Src homology 2 (SH2) domain (overall amino acid identity, 33%). The N-terminal PH domains of STAP-2 and STAP-1 share 36% identity and 58% similarity. The central region of STAP-2 is distantly related to the SH2 domain. This region of STAP-2 shares 40% sequence identity with that of STAP-1 and 29% sequence identity with the SH2 domain of human phospholipase C-γ2. However, STAP-2 has a C-terminal proline-rich region and a YXXQ motif, both of which are absent from STAP-1. We previously reported that STAP-2 interacts with STAT3 through its YXXQ motif and enhances STAT3 transcriptional activity (1). STAP-2 also interacts with STAT5 through its PH and SH2 domains (4). In addition, thymocytes from STAP-2-deficient mice show enhanced IL-2-dependent as well as TCR-mediated cell growth (4).

Focal adhesion kinase (FAK) is a ubiquitously expressed nonreceptor protein tyrosine kinase, which has emerged as a crucial molecule for integrating signals from integrins and receptor tyrosine kinases in processes such as cell survival, proliferation, and motility (5, 6). Genetic ablation of FAK results in early embryonic lethality in mice, and cells derived from these knockout embryos demonstrate severe migration and survival defects (7–10). Conversely, enhanced FAK signaling up-regulates cell motility, and promotes cell survival in an anchorage-independent manner (11, 12). Moreover, dysregulated activation of FAK is often observed in invasive cancer cells (13). In contrast, ligand binding of integrins results in catalytic activation of FAK and its autophosphorylation at Y-397, which serves as a binding site for Src family kinases through their SH2 domains. These SH2 domains also link FAK to the Grb2 adaptor protein, and then to the Ras pathway (14). Thus, ligand ligation of integrins transduces external stimuli from the extracellular matrix to the nucleus, thereby controlling cell survival, motility, and proliferation (5, 6).

In this study, we have now found that STAP-2-deficient splenocytes or T cells demonstrate the enhanced cell adhesion to fibronectin (FN) after PMA treatment and that the increased protein contents of FAK are observed in STAP-2-deficient T cells. In addition to the novel potential of STAP-2 to regulate T cell adhesion to FN after integrin stimulation, we describe a physiological role for the interaction between STAP-2 and FAK. Overexpression of STAP-2 induces a dramatic decrease in the protein contents of FAK and integrin-mediated T cell adhesion to FN in Jurkat T cells via the degradation of FAK. Regarding the mechanism for this effect, we found that STAP-2 associates with FAK and enhances its degradation, proteasome inhibitors block degradation of FAK, and STAP-2 recruits an endogenous E3 ubiquitin ligase, Cbl, to FAK. All the results of the present study

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[4] Abbreviations used in this paper: STAP-2, signal-transducing adaptor protein-2; FAK, focal adhesion kinase; FN, fibronectin; HA, hemagglutinin; PH, pleckstrin homology; SH2, Src homology 2; siRNA, small interfering RNA; WT, wild type.

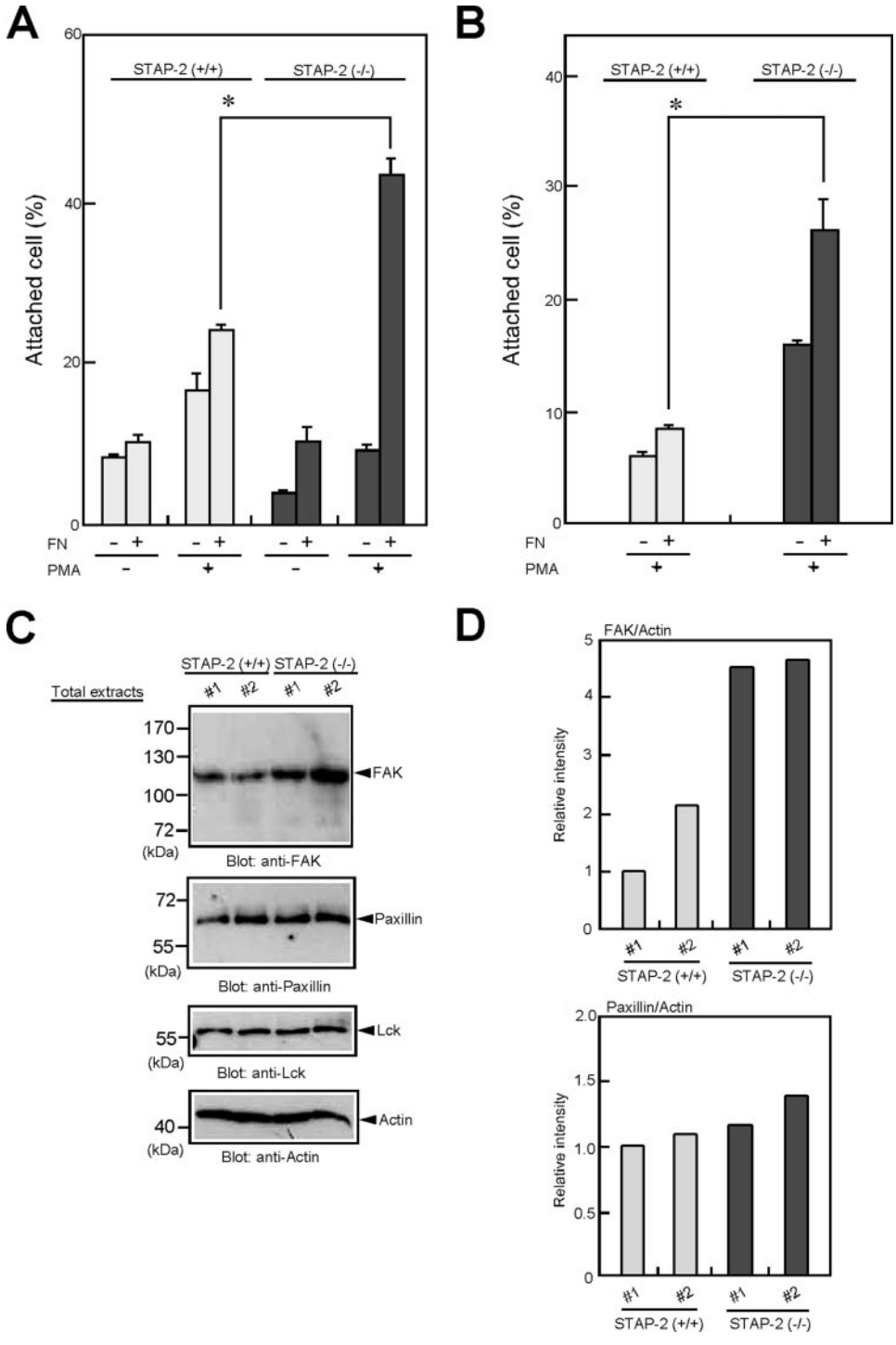

FIGURE 1. STAP-2 down-regulates cell adhesion of lymphocytes to FN and protein contents of FAK in T cells. *A*, Isolated splenocytes (1×10^6) from WT- or STAP-2-deficient mice were analyzed for adhesion to FN after stimulation with (+) or without (−) PMA (10 ng/ml) for 20 min. Results are representative of three independent experiments, with SDs. *, $p < 0.05$. *B*, Isolated T cells (2×10^6) from WT- or STAP-2-deficient mice were also analyzed for adhesion to FN after stimulation with (+) PMA (10 ng/ml) for 20 min. Adhesion assays were conducted for 30 min at 37°C, and the attached cells were stained with WST and quantitated by reading the absorbance at 450 nm. Results are representative of three independent experiments, with SDs. *, $p < 0.05$. *C*, Isolated T cells (2×10^6) from WT- or STAP-2-deficient mice were lysed and analyzed for protein levels of FAK, paxillin, Lck, and actin. Results are representative of three independent experiments. *D*, Densitometric quantification of the above results was also shown. Relative intensity of FAK or paxillin was normalized to total actin of the same sample.

clearly indicate that STAP-2 protein is a potential novel regulator of integrin/FAK-mediated signaling in T cells.

Materials and Methods

Reagents, Abs, and mice

FN and PMA were purchased from Sigma-Aldrich. The proteasome inhibitors MG115 and MG132, the pan-caspase inhibitor Z-VAD, and the caspase 8 inhibitor IETD were purchased from Peptide Institute. Expression vectors for hemagglutinin (HA)-tagged FAK wild type (WT), FAK Y397F, and FAK K454R were provided by H. Sabe (Osaka Bioscience Institute, Osaka, Japan) and S. Hanks (Vanderbilt University School of Medicine, Nashville, TN) (15). Epitope-tagged STAP-2, GST-fusion STAP-2 mutants (GST-STAP-2 (1–147 aa), GST-STAP-2 SH2 (148–243 aa), GST-STAP-2 C (692–1052 aa)), Myc-tagged Cbl, and Cbl70Z constructs were described previously (4, 16). Myc-tagged FAK mutant cDNAs (FAK-N (1–415 aa), FAK-ΔN (415–1052 aa), FAK-C (1–690 aa), and FAK-related nonkinase (692–1052 aa)) (17) were generated by PCR methods and sequenced (primer sequences are available upon request). Anti-Myc, anti-GST, anti-Cbl, and anti-Lck Abs were obtained from Santa Cruz Biotechnology. Anti-FLAG mAb (M2), anti-HA, anti-Pyk2, and anti-paxillin Ab were from Sigma-Aldrich. Anti-actin mAb was purchased from Chemicon International. Anti-phosphotyrosine mAb (PY20), anti-mouse integrin β_1 mAb (HMβ1-1), and anti-phospho FAK Y397 Ab were purchased from BD Pharmingen. Anti-FAK Ab was purchased from Upstate Biotechnology. Anti-human integrin β_1 mAb (TS2/16)-producing murine hybridoma TS2/16.2.1 (18) was a gift from Cell Resource Center for Biomedical Research (Tohoku University, Sendai, Japan) and used as culture supernatant. Anti-polyubiquitin mAb, FK2, was a gift from H. Yokosawa (Hokkaido University, Sapporo, Japan) (19). Anti-human STAP-2 Ab was

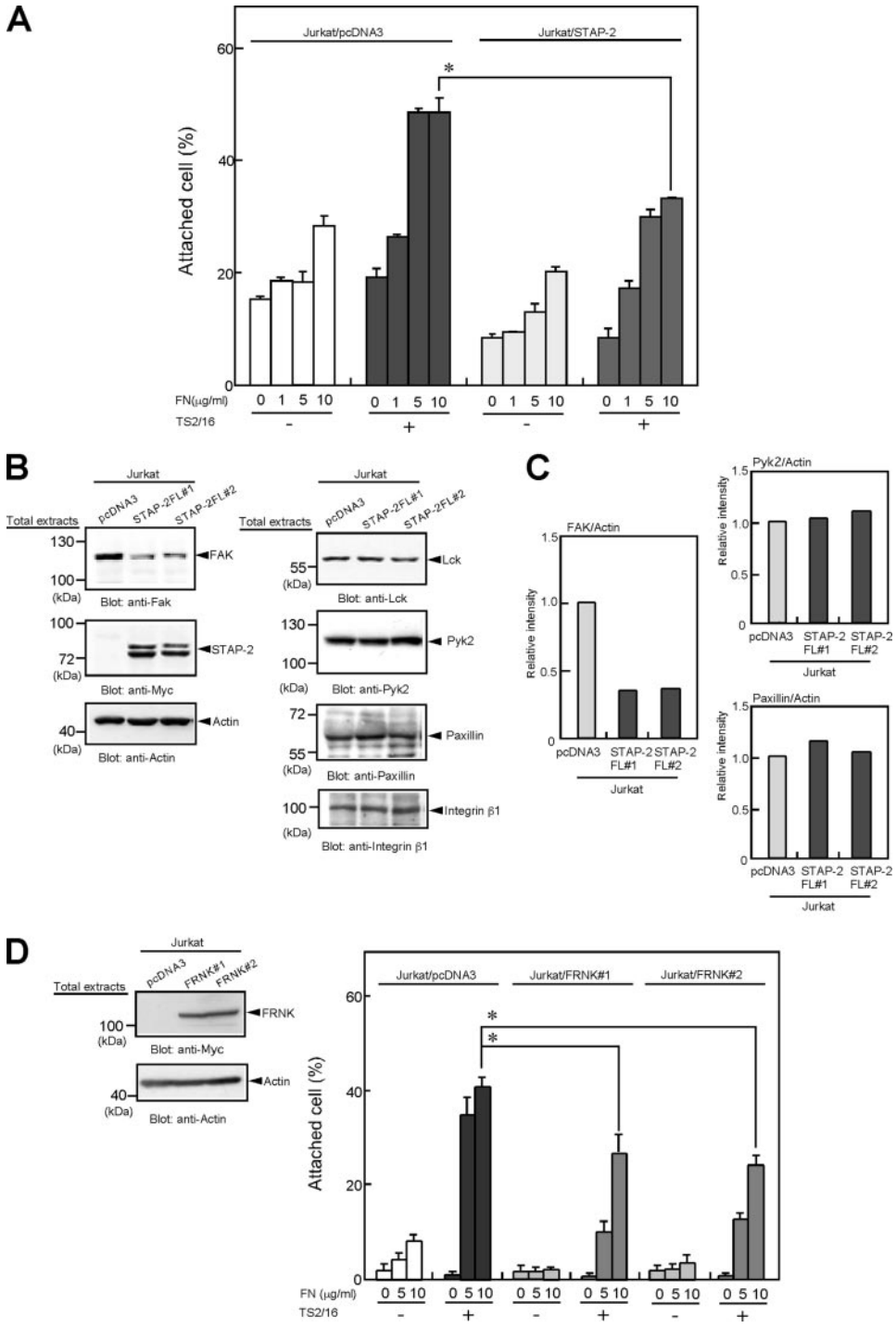

FIGURE 2. Forced expression of STAP-2 down-regulates cell adhesion and protein contents of FAK in Jurkat T cells. *A*, Jurkat/pcDNA3 or Jurkat/STAP-2 cells (2×10^5) were analyzed for adhesion to the indicated concentrations of FN after stimulation with (+) or without (−) TS2/16 for 10 min. Adhesion assays were conducted for 3 h at 37°C, and the attached cells were stained with WST and quantitated by reading the absorbance at 450 nm. Results are representative of three independent experiments, with SDs. ∗, $p < 0.05$. *B*, Jurkat/pcDNA3 or Jaurkat/STAP-2 (FL#1 and #2) cells (1×10^7) were lysed, and total cell lysates were blotted with anti-FAK, anti-Myc, anti-actin, anti-Lck, anti-Pyk2, anti-paxillin, and anti-integrin β_1 (TS2/16) Abs. *C*, Densitometric quantification of the above results was also shown. Relative intensity of FAK, Pyk2, or paxillin was normalized to total actin of the same sample. *D*, Jurkat/pcDNA3, Jurkat/FRNK#1, or Jurkat/FRNK#2 cells were lysed, and then an aliquot of total cell lysates was blotted with anti-Myc (*upper panel*) or anti-actin Ab (*lower panel*). Jurkat/pcDNA3, Jurkat/FRNK#1, or Jurkat/FRNK#2 cells (2×10^5) were analyzed for adhesion to the indicated concentrations of FN after stimulation with (+) or without (−) TS2/16 for 10 min. Adhesion assays were conducted for 3 h at 37°C, and the attached cells were stained with WST and quantitated by reading the absorbance at 450 nm. Results are representative of three independent experiments, with SDs. ∗, $p < 0.05$.

described previously (20). The generation of STAP-2-deficient mice was described previously (1). Mice were housed and bred in the Pharmaceutical Sciences Animal Center of Hokkaido University. All animals were maintained under pathogen-free conditions and in compliance with national and institutional guidelines. All protocols were approved by the Hokkaido University animal ethics committee.

Cell culture, cell purification, and treatment

Human T cell leukemia cell line, Jurkat, was maintained in RPMI 1640 medium supplemented with 10% FCS. Stable Jurkat transformants expressing STAP-2 WT, STAP-2 ΔSH2, STAP-2 WT/FAK WT, FRNK, Cbl, or Cbl70Z were established, as described previously (4), and maintained in the above medium in the presence of G418 (0.5 mg/ml). Human T cell lymphoma, HUT78, was maintained in RPMI 1640 medium supplemented with 10% (FCS). Human embryonic kidney carcinoma cell line, 293T, was maintained in DMEM containing 10% FCS, and the indicated plasmids were transfected with the standard calcium precipitation protocol (4). For isolation of splenocytes and T cells, single-cell suspensions of splenocytes from 6- to 12-wk-old mice were prepared, as described previously (21). Splenic T cells were purified (>95% $CD3^+$ cells) by negative selection by EasySep Mouse T cell Enrichment kit (StemCell Technologies). For ubiquitination or protease inhibition experiments, cells were pretreated with the protease inhibitors MG115, MG132, IETD, and Z-VAD at 10 μM for 1–3 h before harvest and maintained throughout the experiment.

Immunoprecipitation and immunoblotting

The immunoprecipitation and Western blotting assays were performed, as described previously (4). Briefly, cells were harvested and lysed in a lysis buffer (50 mM Tris-HCl (pH 7.4), 0.15 M NaCl, containing 1% Nonidet P-40, 1 mM sodium orthovanadate, 1 mM PMSF, and 10 μg/ml each of

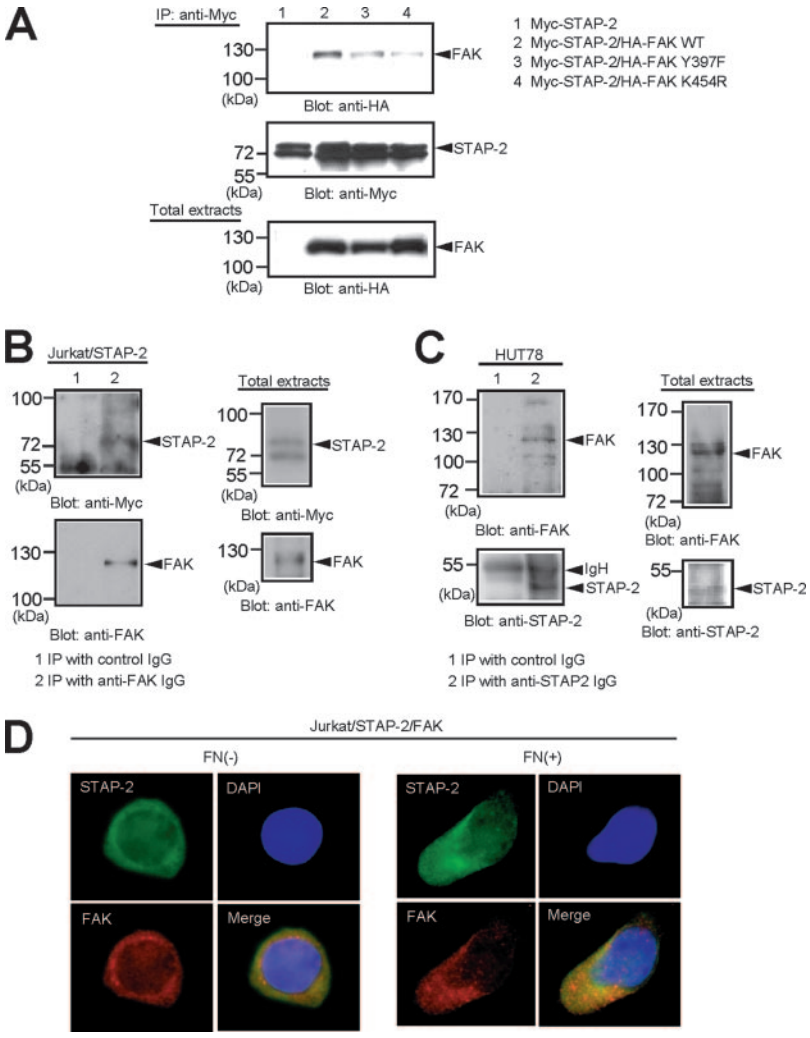

FIGURE 3. STAP-2 associates with FAK. *A*, 293T cells (1×10^7) were transiently transfected with Myc-tagged STAP-2 together with or without HA-tagged FAK WT, FAK Y397F, and FAK K454R. Forty-eight hours after transfection, the cells were lysed, immunoprecipitated with anti-Myc Ab, and immunoblotted with anti-HA (*upper panel*) or anti-Myc Ab (*middle panel*). Total cell lysates were blotted with anti-HA (*lower panel*). *B*, Jurkat/STAP-2 cells (2×10^7) were pretreated with MG132 (10 μM) for 1 h. The pretreated cells were lysed, immunoprecipitated with control IgG or anti-FAK Ab, and immunoblotted with anti-Myc or anti-FAK Ab (*left panels*). Total cell extracts were also blotted with anti-Myc or anti-FAK Ab (*right panels*). *C*, Human T cell lymphoma, HUT78 cells (2×10^7) were lysed, immunoprecipitated with control IgG or anti-STAP-2 Ab, and immunoblotted with anti-FAK or anti-STAP-2 Ab (*left panels*). Total cell extracts were also blotted with anti-FAK or anti-STAP-2 Ab (*right panels*). IgH, H chain of Ig. *D*, Jurkat/STAP-2/FAK cells (6×10^5) were placed in suspension for 10 min with TS2/16, and then respread onto coverslips coated with or without FN for 3 h at 37°C. The cells were fixed and reacted with anti-HA and rabbit anti-Myc Abs, and visualized with FITC- or rhodamine-conjugated secondary Ab. The same slide was also stained with 4′,6′-diamidino-2-phenylindole for the nuclei staining.

aprotinin, pepstatin, and leupeptin). The immunoprecipitates from cell lysates were resolved on SDS-PAGE and transferred to polyvinylidene difluoride transfer membrane (PerkinElmer). The filters were then immunoblotted with each Ab. Immunoreactive proteins were visualized using an ECL detection system (Millipore).

Indirect immunofluorescence microscopy

To analyze the subcellular localization of STAP-2 and FAK proteins, we used a stable Jurkat transformant, Jurkat/STAP-2/FAK, expressing both Myc-STAP-2 and HA-FAK. Jurkat/STAP-2/FAK cells were treated with TS2/16 for 10 min, and plated on coverslips coated or uncoated with FN (10 μg/ml) for 3 h at 37°C, and fixed. Immunofluorescence stainings were performed, as described (4). The following primary Abs were used: mouse anti-HA and rabbit anti-Myc Abs. Two secondary Abs were used: FITC-conjugated anti-rabbit IgG or rhodamine-conjugated anti-mouse IgG (Chemicon International). DNA was visualized by 4′,6′-diamidino-2-phenylindole (Wako Pure Chemical) staining. Confocal laser-scanning microscopy was performed with a LSM510 microscope (Zeiss) with an Apochromat ×63/1.4 oil immersion objective, using excitation wavelengths of 543 nm (rhodamine red) and 488 nm (FITC).

Cell adhesion assays

Cell adhesion assays were performed, as described elsewhere (22, 23, 24), using flat-bottom high-binding 96-well microtiter plates (Nunc) for FN. Assays were conducted at 30-min incubation for murine splenocyes and T cells or at 3-h incubation for Jurkat cells at 37°C, and after washing, the attached cells left in each well were estimated by using a WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt) assay (Cell Counting Kit-8; Wako Pure Chemical). Ten microliters of WST-8 solution was added into each well, and the cells were incubated for another 1 h. The absorbance was measured at a test wavelength of 450 nm (OD_{450}) and a reference wavelength of 650 nm using a microplate reader (Bio-Rad). Percent binding was calculated as: OD_{450}, attached cells/OD_{450}, total number of cells to well. Integrin activation with TS2/16 mAb was performed by incubating the cells with 1/10 dilution of hybridoma supernatant for 10 min at 37°C before the attachment assay. In these conditions, no significant cell attachment to BSA was observed. We also tested several time courses (30 min, 1 h, 2 h, and 3 h) for Jurkat T cell attachment to FN. The maximum of Jurkat T cell attachment was observed at 1 h and sustained until 3 h. The cell viability of Jurkat or its transformants was not changed during adhesion assays.

Small interfering RNA (siRNA)-mediated silencing of Cbl

Silencing of Cbl expression in Jurkat/STAP-2 cells was achieved by a Cbl-specific siRNA. Jurkat/STAP-2 cells (4×10^6) were nucleofected with control or Cbl siRNA (Santa Cruz Biotechnology; SC-29242) using the Cell Line Nucleofector Kit V (Amaxa Biosystems). Thirty-six hours after transfection, cells were lysed and immunoblotted with anti-Cbl Ab.

Statistical methods

The significance of differences between group means was determined by Student's *t* test.

Results

STAP-2 down-regulates cell adhesion of lymphocytes to FN

In our previous study, we demonstrated that thymocytes from STAP-2-deficient mice showed enhanced IL-2- or TCR-dependent cell proliferation, indicating that STAP-2 may play roles in T cell-mediated systems (4). These findings led us to investigate the effects of STAP-2 on integrin-mediated T cell adhesion to FN, which regulates lymphocyte trafficking as well as immune responses. As

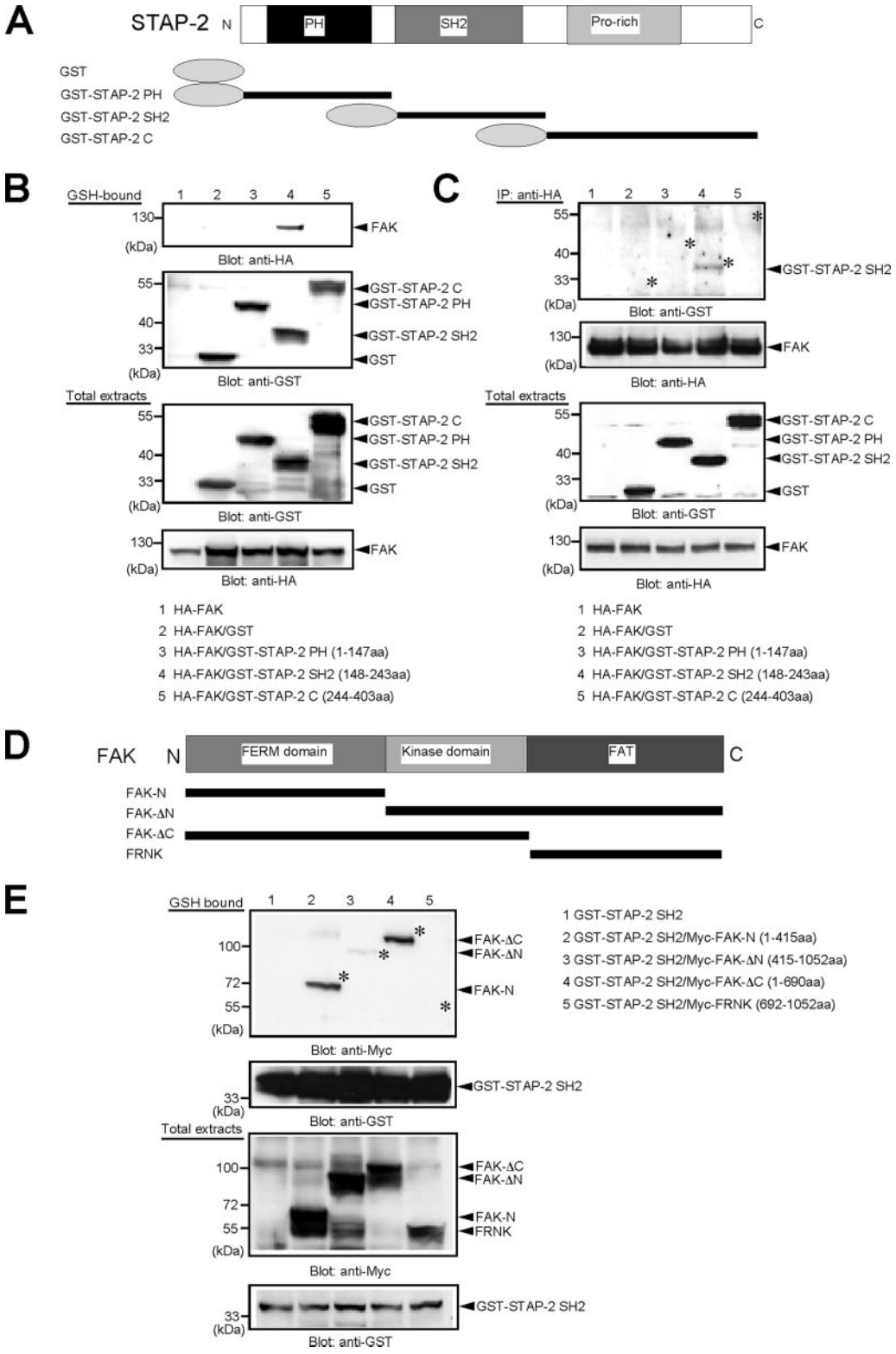

FIGURE 4. Mapping of the interacting domain between STAP-2 and FAK. *A*, Domain structure of STAP-2 and GST-fused mutant fragments is schematically shown. *B*, 293T cells (1×10^7) were transfected with HA-FAK together without or with GST or GST-fused STAP-2 deletion mutants. Forty-eight hours after transfection, the cells were lysed, pulled down with glutathione (GSH)-Sepharose, and blotted with anti-HA (*upper panel*) or anti-GST Ab (*middle panel*). Total cell lysates were blotted with anti-GST or anti-HA Ab (*lower panels*). *C*, 293T cells (1×10^7) were transfected with HA-FAK together with or without GST or GST-fused STAP-2 deletion mutants. Forty-eight hours after transfection, the cells were lysed, immunoprecipitated with anti-HA Ab, and blotted with anti-GST (*upper panel*) or anti-HA Ab (*middle panel*). Total cell lysates were blotted with anti-GST or anti-HA Ab (*lower panels*). The asterisks indicate the migration positions of the respective mutants. *D*, Domain structure of FAK and its mutant fragments is schematically shown. *E*, 293T cells (1×10^7) were transfected with GST-fused STAP-2 SH2 together with or without Myc-FAK-N, Myc-FAKΔN, Myc-FAKΔC, or Myc-FRNK, respectively. Forty-eight hours after transfection, the cells were lysed, pulled down with GSH-Sepharose, and blotted with anti-Myc (*upper panel*) or anti-GST Ab (*middle panel*). Total cell lysates were blotted with anti-Myc or anti-GST Ab (*lower panels*). The asterisks indicate the migration positions of the respective mutants.

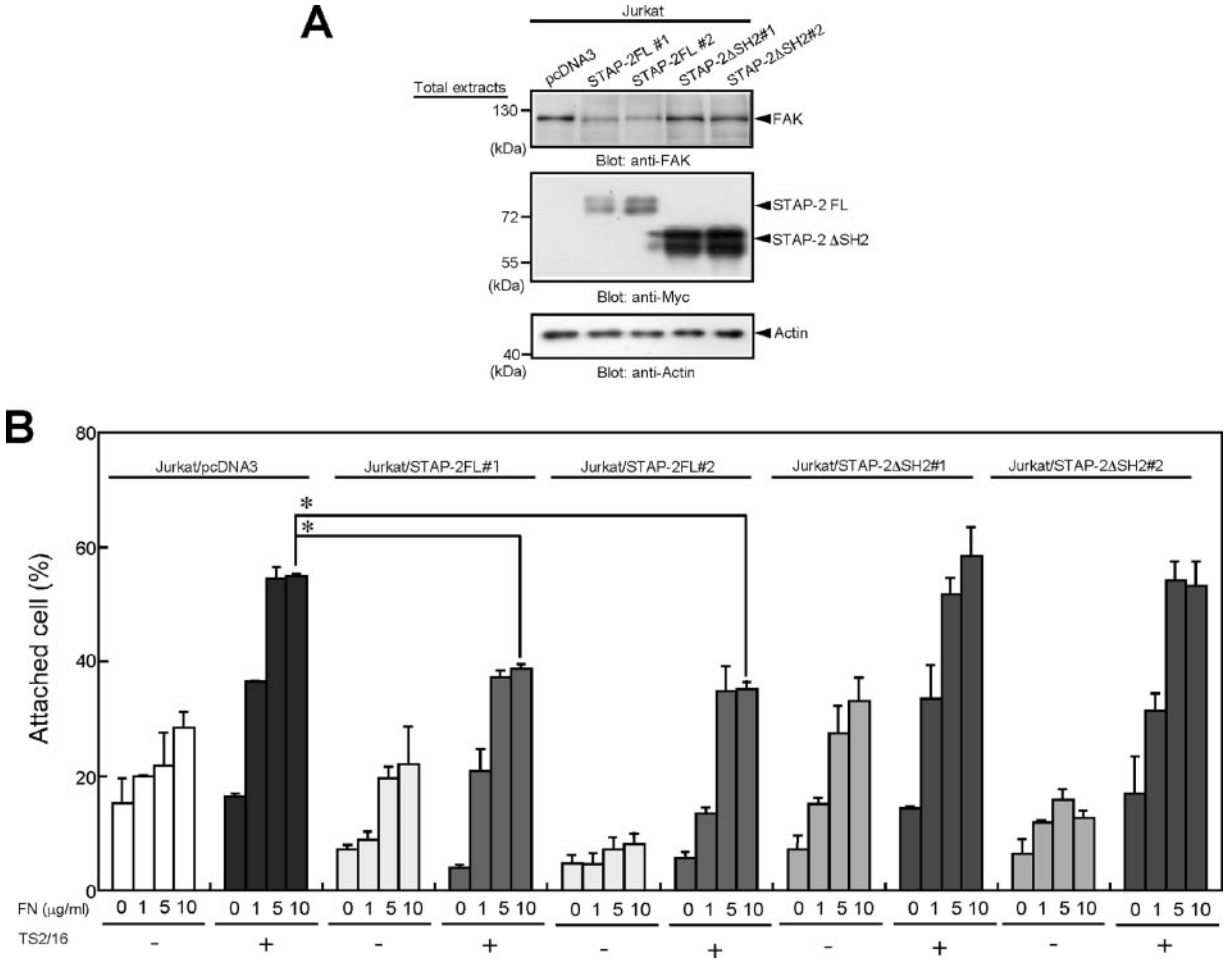

FIGURE 5. The SH2-like domain of STAP-2 is involved in the down-regulation of cell adhesion and FAK content. *A*, Jurkat/pcDNA3, Jaurkat/STAP-2 (FL#1 and #2), and Jaurkat/STAP-2 ΔSH2 (#1 and #2) cells were lysed, and total cell lysates were blotted with anti-FAK, anti-Myc, and anti-actin Abs. *B*, Jurkat/pcDNA3, Jaurkat/STAP-2 (FL#1 and #2), or Jaurkat/STAP-2 ΔSH2 (#1 and #2) cells (2×10^5) were analyzed for adhesion to the indicated concentrations of FN after stimulation with (+) or without (−) TS2/16 for 10 min. Adhesion assays were conducted for 3 h at 37°C, and attached cells were stained with WST and quantitated by reading the absorbance at 450 nm. Results are representative of three independent experiments, with SDs. ∗, $p < 0.05$.

shown in Fig. 1*A*, only small numbers of freshly isolated splenocytes adhered to FN. When integrins on the splenocytes were activated by PMA treatment, they acquired the ability to adhere to FN. Importantly, much higher cell attachment to FN was observed for splenocytes derived from STAP-2-deficient mice compared with those from WT mice (43.3 ± 3.3% in STAP-2-deficient splenocytes vs 17.0 ± 2.3% in WT splenocytes). Similar results were obtained for T cells derived from STAP-2-deficient mice compared with those from WT mice (26.1 ± 2.7% in STAP-2-deficient T cells vs 5.7 ± 0.4% in WT T cells) (Fig. 1*B*). Therefore, STAP-2 negatively controls the adherence of lymphocytes to FN. Ligand ligation of integrins leads to the formation of focal adhesion complexes, which are characterized by signaling proteins such as FAK and paxillin. Thus, we examined the protein contents of FAK and paxillin in STAP-2-deficient splenocytes. As shown in Fig. 1*C*, significantly increased protein contents of FAK were observed in STAP-2-deficient splenocytes. Densitometric analysis revealed that the FAK contents were ~2.0- to 4.6-fold higher in STAP-2-deficient T cells than in the control T cells (Fig. 1*D*). In contrast, no alterations were observed in the protein contents of either paxillin or a T cell-specific *src* family kinase, Lck, between WT- and STAP-2-deficient mice. No alteration of integrin β_1 expression was observed between WT- and STAP-2-deficient splenocytes by FACS analysis using anti-mouse integrin β_1 mAb (HMβ1-1) (data not shown). Therefore, STAP-2 selectively affects the protein contents of FAK in vivo, which is critical for T cell adhesion to FN.

To clarify the relationship between STAP-2 and FAK in T cells, we established Jurkat T cells overexpressing STAP-2 (Jurkat/STAP-2), because Jurkat cells are known to adhere strongly to FN in combination with stimulation of integrins such as $\alpha_4\beta_1$ and $\alpha_5\beta_1$. After preincubation with or without the β1-specific Ab TS-2/16, Jurkat transfectants were seeded onto FN-coated plates. We observed significant cell attachment to the FN-coated plates in a FN dose-dependent manner after a 30-min incubation (Fig. 2*A*), whereas no significant adherence to the control plates coated with BSA was detected (data not shown). The cell adhesion of Jurkat/STAP-2 cells to FN-coated plates was much lower than that of Jurkat/pcDNA3 cells. Next, we examined the FAK protein contents in the Jurkat/STAP-2 (FL#1 and #2) cells. A significant decrease in the endogenous FAK protein level was observed in the Jurkat/STAP-2 cells compared with that in the Jurkat/pcDNA3 cells (Fig. 2, *B* and *C*), although no significant differences in the FAK mRNA expression levels were observed among the three types of Jurkat cells (data not shown). Regarding the protein levels of Lck, another FAK family kinase Pyk2, paxillin, and integrin β_1,

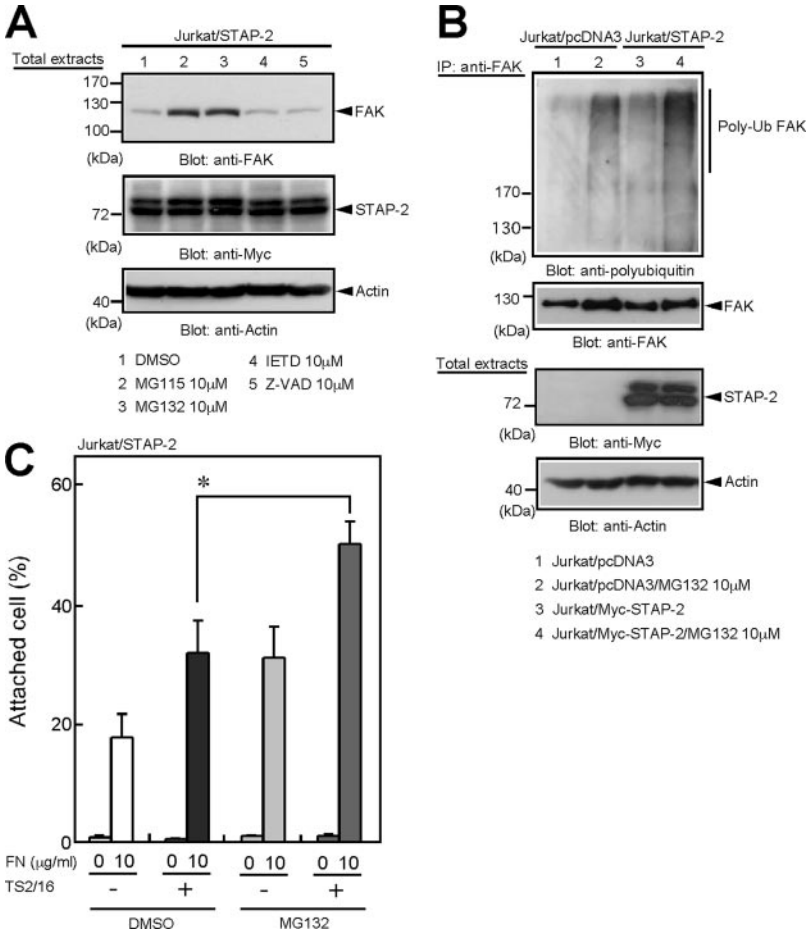

FIGURE 6. STAP-2 enhances ubiquitin-dependent degradation of FAK. *A*, Jaurkat/STAP-2 cells (1×10^7) were pretreated with DMSO, MG115, MG132, IETD, or Z-VAD (10 μM) for 1.5 h, as indicated. The pretreated cells were lysed, and total cell lysates were blotted with anti-FAK, anti-Myc, and anti-actin Abs. *B*, Jurkat/pcDNA3 or Jaurkat/STAP-2 cells (1×10^7) were pretreated with DMSO or MG132 for 3 h, as indicated. The pretreated cells were lysed, immunoprecipitated with anti-FAK Ab, and immunoblotted with anti-polyubiquitin or anti-FAK Abs (*upper panels*). Total extracts were also blotted with anti-Myc or anti-actin Abs (*bottom panels*). *C*, Jaurkat/STAP-2 cells (2×10^5) were pretreated with DMSO or MG132 (10 μM) for 3 h. The pretreated cells were analyzed for adhesion to the indicated concentrations of FN after stimulation with (+) or without (−) TS2/16 for 10 min. Adhesion assays were conducted for 3 h at 37°C, and the attached cells were stained with WST and quantitated by reading the absorbance at 450 nm. Results are representative of three independent experiments, with SDs. *, $p < 0.05$.

no differences were observed in Jurkat cells with or without STAP-2 overexpression. Furthermore, the FAK protein level did not show a significant change during adhesion assays (data not shown). However, there is the possibility that the decrease of FAK protein contents may reflect movement of FAK protein from a soluble to insoluble compartment in cells. We then examined the FAK protein content in the 1% Nonidet P-40-insoluble fraction of Jurkat cells. However, we could not detect any FAK protein in the 1% Nonidet P-40-insoluble fraction of Jurkat cells (data not shown). These results suggest that STAP-2 negatively modulates the adherence of T cells to FN via down-regulation of the FAK protein contents. To further confirm the involvement of FAK on adhesion of Jurkat T cells to FN, we established Jurkat T cells overexpressing FRNK (Jurkat/FRNK#1 and #2), which is the C-terminal domain of FAK and acts as an endogenous FAK inhibitor (5, 6). As shown in Fig. 2*D*, the cell adhesion of Jurkat/FRNK cells to FN-coated plates was lower than that of Jurkat/pcDNA3 cells. These results indicate that FAK plays a critical role on integrin-mediated T cell adhesion to FN.

Association of STAP-2 with FAK

STAP-2 interacts with STAT3 through its YXXQ motif and STAT5 through its PH- and SH2-like domains (1, 4). Because STAP-2 was able to regulate the protein contents of FAK, as described above, we next examined whether there was an association between STAP-2 and FAK. Expression vectors for Myc-tagged STAP-2 were transfected into 293T cells with or without HA-tagged FAK WT, FAK Y397F, which carries a mutation at a major phosphorylation site, or FAK K454R, which carries a mutation at a conserved lysine residue in the kinase domain. Each transfectant was lysed and immunoprecipitated with an anti-Myc Ab and blotted with an anti-HA Ab. As shown in Fig. 3*A*, the immunoprecipitates contained FAK WT protein. FAK Y397F and FAK K454R proteins were also present in the immunoprecipitates, although at lower levels than FAK WT protein. These results suggest that FAK activation may be required for their interactions. A similar interaction between STAP-2 and FAK was also observed in Jurkat/STAP-2 cells expressing Myc-tagged STAP-2. The Jurkat/STAP-2 cells were lysed, immunoprecipitated with control IgG or an anti-FAK Ab, and immunoblotted with an anti-Myc Ab. As shown in Fig. 3*B*, the immunoprecipitates contained Myc-STAP-2 protein, whereas those with a control Ab did not. To confirm endogenous association of both proteins, we used a T cell lymphoma cell line, HUT78, which abundantly expressed both endogenous STAP-2 and FAK. Because we could not detect endogenous STAP-2 protein in Jurkat cells, it seemed to be due to the low detection sensitivity of anti-STAP-2 Ab immunoblotting. The HUT78 cells were lysed, immunoprecipitated with control IgG or an anti-STAP-2 Ab, and immunoblotted with an anti-FAK Ab. The immunoprecipitates with an anti-STAP-2 Ab contained endogenous FAK protein, whereas those with a control Ab did not (Fig. 3*C*). Therefore, STAP-2 associates with FAK, and kinase activation of FAK may be required for the full association between STAP-2 and FAK.

To characterize the nature of the interaction between STAP-2 and FAK, we attempted to determine where this interaction occurred in Jurkat cells. To this end, we used a Jurkat transfectant expressing both Myc-tagged STAP-2 and HA-tagged FAK (Jurkat/STAP-2/FAK). In TS-2/16-untreated Jurkat/STAP-2/FAK cells, both Myc-STAP-2 and HA-FAK proteins were diffusely distributed in the cells, although a small amount of FAK showed punctate structures (Fig. 3*D*, *left panels*). However, treatment of Jurkat/STAP-2/FAK cells with

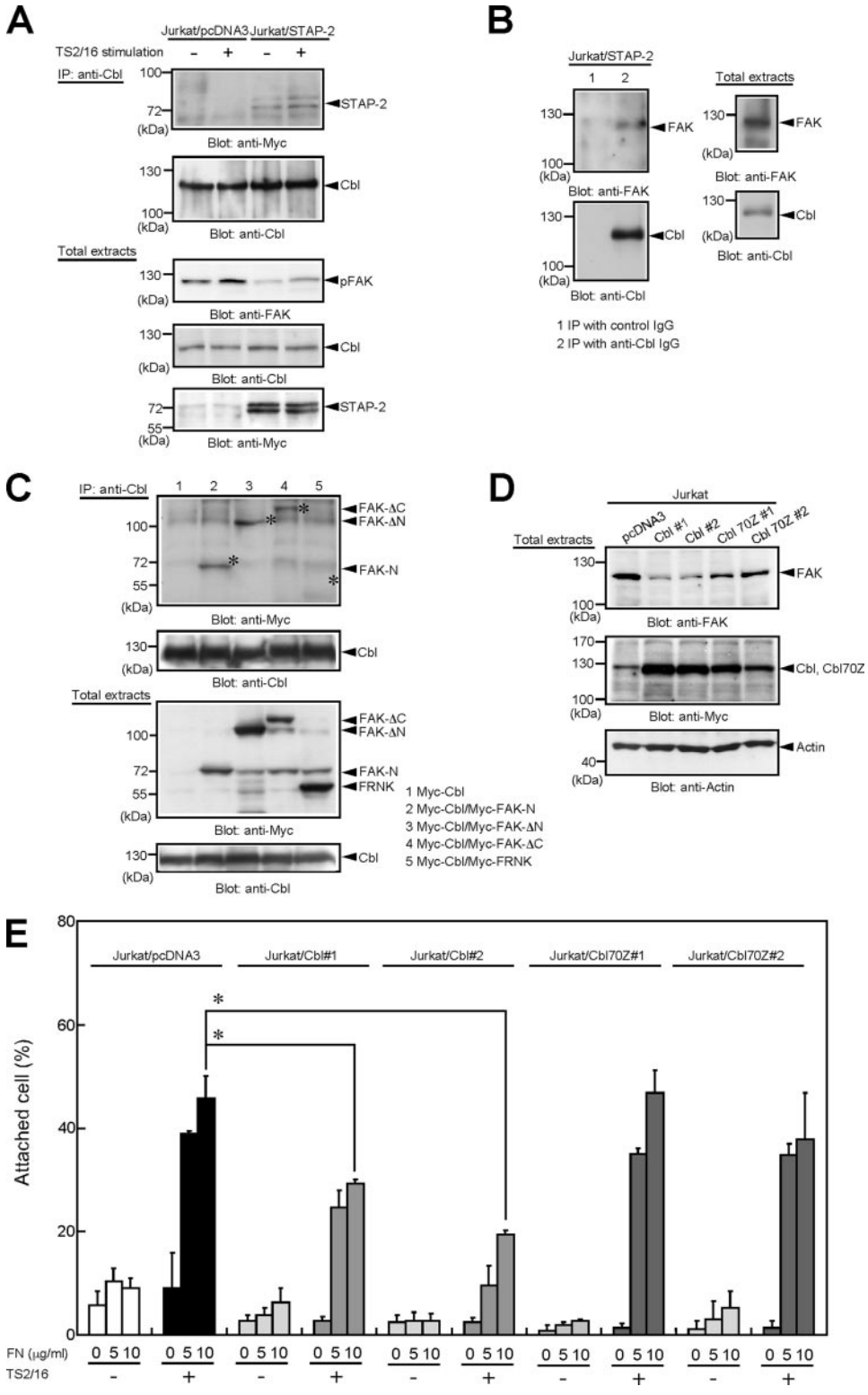

FIGURE 7. STAP-2 recruits an E3 ubiquitin ligase Cbl to FAK. *A*, Jurkat/pcDNA3 or Jaurkat/STAP-2 cells (1×10^7) were pretreated with MG132 (10 μM) for 1 h. The pretreated cells were lysed after stimulation with (+) or without (−) TS2/16 for 1 h. The cell lysates were immunoprecipitated with anti-Cbl Ab and immunoblotted with anti-Myc or anti-Cbl Ab (*upper panels*). Total extracts were also blotted with anti-pFAK, anti-Cbl, or anti-Myc Ab (*bottom panels*). *B*, Jurkat/STAP-2 cells (2×10^7) were pretreated with MG132 (10 μM) for 1 h. The pretreated cells were lysed, immunoprecipitated with control IgG or anti-Cbl Ab, and immunoblotted with anti-FAK or anti-Cbl Ab (*left panels*). Total cell extracts were also blotted with anti-FAK or anti-Cbl Ab (*right panels*). *C*, Mapping of the interacting domain between Cbl and FAK. The 293T cells (1×10^7) were transfected with Myc-Cbl together without or with Myc-FAK-N, Myc-FAKΔN, Myc-FAKΔC, or Myc-FRNK. Forty-eight hours after transfection, the cells were lysed, immunoprecipitated with anti-Cbl Ab, and blotted with anti-Myc or anti-Cbl Ab (*upper panels*). Total cell lysates were blotted with anti-Myc or anti-Cbl Ab (*lower panels*). The asterisks indicate the migration positions of the respective mutants. *D*, Jurkat/pcDNA3, Jaurkat/Cbl#1 Jaurkat/Cbl#2 Jaurkat/Cbl 70Z#1, or Jaurkat/Cbl 70Z#2 cells (1×10^7) were lysed, and total cell extracts were blotted with anti-FAK, anti-Myc, and anti-actin Abs. *E*, Jurkat/pcDNA3, Jurkat/Cbl#1 Jurkat/Cbl#2 Jurkat/Cbl 70Z#1, or Jurkat/Cbl 70Z#2 cells (2×10^5) were also analyzed for adhesion to the indicated concentrations of FN without stimulation (−), or after stimulation with TS2/16 for 10 min (+). Adhesion assays were conducted for 3 h at 37°C, and attached cells were stained with WST and quantitated by reading the absorbance at 450 nm. Results are representative of three independent experiments, with SDs. *, $p < 0.05$.

FN resulted in concentration of both tagged proteins to focal adhesion foci as punctate structures (Fig. 3*D*, *right panels*). Consistent with the in vivo interaction data presented above, these results suggest that activated FAK interacts with STAP-2 in focal adhesions. Therefore, STAP-2 colocalizes with FAK after integrin activation.

Domains for the interaction between STAP-2 and FAK

To determine the domains of STAP-2 involved in its association with FAK, a series of STAP-2 deletion mutants fused with GST (GST-STAP-2 PH, GST-STAP-2 SH2, and GST-STAP-2 C) were used (Fig. 4*A*). The respective mutants, together with HA-tagged FAK WT, were transiently expressed in 293T cells. The binding potentials of these proteins with HA-tagged FAK WT were examined by pull-down assays with glutathione-Sepharose or coimmunoprecipitation assays with an anti-HA Ab, followed by Western blot analysis with an anti-HA or anti-GST Ab. The precipitates for the GST-STAP-2 SH2 protein contained FAK protein (Fig. 4*B*), and so did the immunoprecipitates with an anti-HA Ab (Fig. 4*C*). However, no FAK

protein was detected in the precipitates for GST-STAP-2 PH protein or GST-STAP-2 C protein. To determine the domains of FAK involved in its association with the SH2-like domain of STAP-2, 293T cells were transfected with GST-STAP-2 SH2 and/or a series of Myc-tagged FAK deletion mutants (FAK-N, FAK-ΔC, FAK-ΔN, and FRNK) (Fig. 4*D*). Lysates of the transfectants were pulled down with glutathione-Sepharose, and subjected to Western blot analysis with an anti-Myc Ab. As shown in Fig. 4*E*, the FAK-N and FAK-ΔC mutants interacted with the SH2-like domain of STAP-2, whereas the FRNK mutants did not. Therefore, the SH2-like domain of STAP-2 interacts with the N-terminal domain of FAK.

STAP-2 enhances ubiquitin-dependent degradation of FAK

To assess the physiological roles of the association between STAP-2 and FAK, we established stable transfectants of Jurkat cells expressing an empty vector (pcDNA3), STAP-2 or STAP-2 ΔSH2, which has a deletion of the SH2-like domain (Fig. 5*A*). A significant decrease in the FAK protein level was observed in Jurkat/STAP-2 cells compared with Jurkat/pcDNA3 cells. However, the protein contents of FAK in Jurkat/STAP-2 ΔSH2 cells were similar to those in Jurkat/pcDNA3 cells. In parallel, Jurkat/STAP-2 cells showed impaired the ability to enhance the adherence to FN, whereas Jurkat/STAP-2 ΔSH2 cells did not (Fig. 5*B*). To clarify the molecular mechanisms for the STAP-2-mediated decrease in the FAK protein contents, we examined the effects of the proteasome inhibitors MG115 and MG132, or the caspase inhibitors IETD and V-ZVAD on FAK protein in Jurkat/STAP-2 cells. As shown in Fig. 6*A*, the endogenous FAK protein contents in Jurkat/STAP-2 cells were increased after treatment with MG115 or MG132. These results indicate that the decrease in the FAK protein contents in Jurkat/STAP-2 cells is due to proteasome-dependent degradation. Indeed, in the presence of MG132, larger amounts of FAK protein with polyubiquitination were detected in Jurkat/STAP-2 cells than in Jurkat/pcDNA3 cells (Fig. 6*B*). We next examined the effect of MG132 on the T cell adhesion to FN. As shown in Fig. 6*C*, pretreatment of Jurkat/STAP-2 cells with MG132 showed a significant increase in the cell adhesion to FN. Therefore, the association of STAP-2 with FAK enhances the ubiquitination of FAK, thereby leading to its protein degradation. Furthermore, proteasome-dependent protein degradation may also regulate T cell adhesion to FN.

STAP-2 recruits the E3 ubiquitin ligase Cbl to FAK

Recently, an E3 ubiquitin ligase, Cbl, has been shown to regulate T cell functions via its E3 ubiquitin ligase activity, which results in the ubiquitination of its binding partners. This observation prompted us to investigate the involvement of Cbl in STAP-2-mediated degradation of FAK. To this end, we tested whether STAP-2 interacts with Cbl in Jurkat cells expressing Myc-tagged STAP-2 (Jurkat/STAP-2). Lysates of the cells were immunoprecipitated with an anti-Cbl Ab and subjected to Western blot analysis with an anti-Myc Ab. As shown in Fig. 7*A*, the immunoprecipitates contained Myc-tagged STAP-2 protein. We also tested association of Cbl with FAK in Jurkat/STAP-2 cells. The Jurkat/STAP-2 cells were lysed, immunoprecipitated with control IgG or an anti-Cbl Ab, and immunoblotted with an anti-FAK Ab. The immunoprecipitates with an anti-Cbl Ab contained FAK protein, whereas those with a control Ab did not (Fig. 7*B*). Furthermore, we also demonstrated that STAP-2 interacts with FAK in Jurkat/STAP-2 cells, as shown in Fig. 3*B*. These results show the possibility that STAP-2, FAK, and Cbl interact with each other in Jurkat/STAP-2 cells. To further determine the domains of FAK involved in its association with Cbl, 293T cells were transfected with Cbl and/or a series of Myc-tagged FAK deletion mutants. As

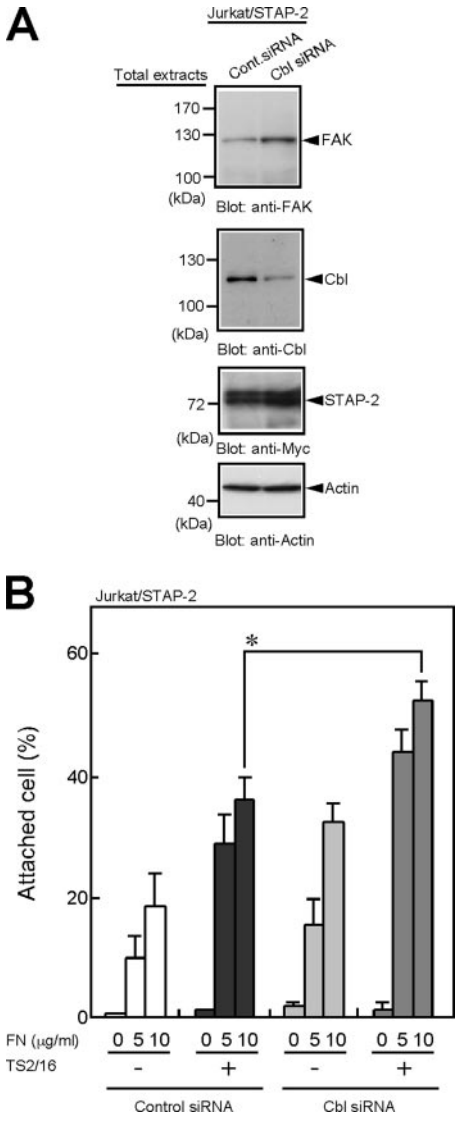

FIGURE 8. Reduction of endogenous Cbl enhances the FAK protein content and cell adhesion in Jurkat/STAP-2 cells. *A*, Jurkat/STAP-2 cells (4×10^6) were nucleofected with 200 pmol control or Cbl siRNA by nucleofection with cell line Nucleofector Kit V, according to their optimized protocol. The cells were harvested after 36 h and lysed. Total cell extracts were blotted with anti-FAK, anti-Cbl, anti-Myc, and anti-actin Abs. Densitometric analysis reveals a decrease of 60% in Cbl content in Jurkat/STAP-2 cells treated with Cbl siRNA. *B*, Jurkat/STAP-2 cells (4×10^6) were pretreated with control or Cbl siRNA by nucleofection with cell line Nucleofector Kit V, as described above. The pretreated cells were analyzed for adhesion to the indicated concentrations of FN after stimulation with (+) or without (−) TS2/16 for 10 min. Adhesion assays were conducted for 3 h at 37°C, and the attached cells were stained with WST and quantitated by reading the absorbance at 450 nm. Results are representative of three independent experiments, with SDs. *, $p < 0.05$.

shown in Fig. 7*C*, the immunoprecipitates with the anti-Cbl Ab contained the FAK-N, FAK-ΔN, and FAK-ΔC mutants, but not the FRNK mutant, indicating that FAK interacts with Cbl through its large N-terminal portion. We then examined the effects of overexpression of Cbl or its oncogenic mutant, 70Z/3 Cbl, in Jurkat cells (Jurkat/Cbl or Jurkat/70Z3 Cbl, respectively). The oncogenic mutant 70Z/3 Cbl lacks a functional really interesting new gene finger domain due to deletion of amino acid residues 366–382 and is unable to mediate ubiquitination. As shown in Fig. 7*D*, Jurkat/Cbl cells showed a significant decrease in FAK protein, whereas

Jurkat/70Z3 Cbl cells did not. These findings suggest that Cbl participates in FAK degradation via its ubiquitin ligase activity. In addition, transfectants overexpressing Cbl displayed a significant decrease in the cell adhesion to FN, whereas those overexpressing 70Z/3 Cbl did not (Fig. 7*E*). Therefore, these results suggest that STAP-2, FAK, and Cbl interact with each other to modulate integrin-mediated T cell adhesion.

Reduction of endogenous Cbl enhances the FAK protein content and cell adhesion in Jurkat/STAP-2 cells

To directly verify the roles of Cbl in STAP-2-mediated degradation of FAK, we knocked down Cbl protein in Jurkat/STAP-2 cells (Fig. 8*A*). Approximately 60% reduction of the Cbl protein content was observed following Cbl siRNA expression. In this situation, Jurkat/STAP-2 cells expressing the Cbl siRNA showed a 1.6-fold higher FAK protein content than those expressing a control siRNA. We also examined the effect of Cbl knockdown on the cell adhesion to FN. As shown in Fig. 8*B*, reduction of endogenous Cbl displayed a significant increase in the cell adhesion to FN. Therefore, STAP-2 negatively modulates integrin-mediated T cell adhesion through FAK degradation via the Cbl-dependent proteasomal pathway.

Discussion

The domain structure of STAP-2 contains several potential tyrosine phosphorylation sites as well as PH- and SH2-like domains, and STAP-2 shows the characteristics of an adaptor protein that can interact with several other signaling molecules. Indeed, STAP-2 binds to not only STAT3 and STAT5, but also MyD88 and IκB kinase (25). These interactions lead to modification of IL-2-dependent cell growth of T cells, IL-6-induced expression of acute-phase genes in hepatocytes, and LPS/TLR4-mediated enhancement of NF-κB activity in macrophages (1, 4, 25). In the present study, we have identified new functions of STAP-2, specifically that it enhances Cbl-dependent degradation of FAK and down-regulates integrin/FAK-mediated cell adhesion to FN in T cells. Because FAK is a crucial molecule for integrin-mediated signaling for cell survival, proliferation, and motility, STAP-2 is likely to play roles in a wide range of biological processes in vivo via negative control of the FAK protein contents.

During focal adhesion turnover, integrin-mediated phosphorylation of FAK recruits a number of signaling and structural proteins, and promotes the initial assembly of focal adhesions (5, 6). The activated FAK recruits JNK to focal adhesion sites, where JNK mediates the phosphorylation of paxillin, which promotes focal adhesion remodeling and cell motility (26). FAK also recruits ERK2 and calpain-2 to focal adhesion sites, which facilitates ERK2-induced activation of calpain-2, leading to cleavage of focal adhesion components and promotion of focal adhesion disassembly (27, 28). During actin remodeling, small GTPases of the Rho family control the actin polymerization/depolymerization and actin-stress-fiber assembly/disassembly cycle. FAK can regulate these Rho-family GTPases by modulating various upstream regulators, such as Rho guanine nucleotide exchange factor of 190 kDa, ARF-GTPase-activating protein 1, GTPase regulator associated with FAK, and neuronal Wiscott-Aldrich syndrome protein (29–31). Thus, FAK occupies an important position within the focal adhesion sites. We have demonstrated that the FAK protein contents are increased in STAP-2-deficient T cells, and that overexpression of STAP-2 reduces the protein level of FAK in Jurkat T cells. Moreover, our confocal microscope analysis revealed that STAP-2 colocalizes with FAK at focal adhesion sites after integrin activation. Thus, STAP-2 is likely to regulate the FAK protein contents within focal adhesion sites, resulting in a modification of the cell adhesion to FN.

FAK consists of a central kinase domain flanked by large N- and C-terminal domains, which contain binding sites for many signaling molecules (5, 6). The N-terminal domain of FAK contains a band 4.1, ezrin, radixin, and moesin homology domain, whereas the C-terminal domain is composed of two proline-rich regions and a focal adhesion targeting domain. Our immunoprecipitation experiments have revealed that the N-terminal domain of FAK interacts with both STAP-2 and Cbl. Interactions between STAP-2 and Cbl were also observed. These interactions may allow STAP-2 to recruit Cbl to FAK, thereby leading to an enhancement of the ubiquitin-dependent degradation of FAK. This possibility was supported by our data that the protein level of FAK in Jurkat/STAP-2 cells is increased after treatment with proteasome inhibitors, overexpression of Cbl reduces the FAK protein contents, and down-regulation of Cbl increases the FAK protein contents. Although Cbl has been shown to regulate T cell functions via its E3 ubiquitin ligase activity (29, 32, 33), this is the first report that FAK is degraded through the Cbl-dependent proteasomal pathway. Cbl has a TKB domain and a RING finger domain at its N terminus, which represent the basic functional unit of a ubiquitin ligase for activated tyrosine kinases. The RING finger domain can interact with ubiquitin-conjugating enzymes (E2). The TKB domain binds to a consensus phosphotyrosine motif, N/DXpYXXXϕ, in which ϕ is a hydrophobic residue (34). In the case of FAK, its activation starts with autophosphorylation at Y-397 after integrin stimulation. The phosphorylated Y-397 acts as a binding site for the SH2 domain of Src-family kinases, which subsequently phosphorylate FAK at six tyrosine residues (Y-397, Y-407, Y-576, Y-577, Y-861, and Y-925). Among these tyrosine residues, the sequence surrounding Y-397, namely DDYAEII, fits a consensus phosphotyrosine motif for recognition by Cbl. Thus, Cbl may recognize the sequence around Y-397 of FAK.

FAK-deficient cells spread more slowly on extracellular matrix proteins and migrate poorly in response to chemotactic and haptotactic signals (7). Although FAK-positive cells respond to exerted forces by reorienting their movement and forming prominent focal adhesions, FAK-negative cells fail to exhibit such responses. Conversely, overexpression of FAK in Chinese hamster ovary cells enhances their cell migration (11). In addition to the regulation of cell adhesion and migration, FAK is also linked to the protection of cells from anoikis (35). Overexpression of FAK also protects kidney epithelial cells against UV light-induced cell death (36). Thus, disruption of the FAK protein contents should cause a variety of diseases, such as immunological disorders and malignancies. Indeed, a direct requirement of FAK during tumor progression has been shown using a mouse model of skin carcinogenesis (37). Overexpression and/or phosphorylation at specific tyrosine residues of FAK have been reported to be associated with human cancers, and the levels of FAK expression are correlated with the invasive potential of tumors. A recent report indicated that binding of p53 to the FAK promoter suppresses the gene expression of FAK, suggesting that loss or mutation of p53 may contribute to the alerted FAK expression (38). Alternatively, posttranscriptional modification of FAK may be involved in the regulation of its activation. In this situation, STAP-2 and/or Cbl may be suitable candidates for FAK regulators.

The present data that the protein level of FAK is significantly higher in splenocytes from STAP-2-deficient mice than in those from control mice may indicate the physiological importance of the interactions among STAP-2, FAK, and Cbl. Although activated calpain-2 is known to cleave FAK, the ubiquitin ligase Cbl is another functional regulator of FAK (26). After integrin stimulation,

the phosphorylated FAK is recognized by Cbl and then degraded via the Cbl-dependent proteasomal pathway. During this process, STAP-2 enhances the recruitment of Cbl to FAK. Thus, Cbl is likely to negatively regulate integrin/FAK-mediated signaling by promoting focal adhesion disassembly. The protein level of STAP-2 is likely to determine focal adhesion turnover as well as actin remodeling by controlling the FAK protein contents. If this is the case, STAP-2 may regulate a variety of FAK-mediated cellular events besides integrin-induced T cell adhesion to FN, because it is abundantly expressed in the body. In contrast, the 5′ region of the STAP-2 genomic sequence contains several potential binding sites for c-Rel, AP-1, p65/NF-κB, and STATs. In the murine myeloid leukemia cell line M1, STAP-2 mRNA expression is strongly induced by LIF in parallel with its differentiation into macrophages. STAP-2 can be also induced in hepatocytes by LPS or IL-6 (1). These cell type-restricted expressions of STAP-2 may have a benefit toward controlling the FAK protein contents in specific cellular circumstances. Further investigations will clarify when and how STAP-2 acts as a regulator of the interaction between Cbl and FAK in vivo.

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Disclosures

The authors have no financial conflict of interest.

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