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CrkL Directs ASAP1 to Peripheral Focal Adhesions*

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Searching for proteins in platelets that can interact with the N-terminal SH3 domain of CrkL (using a combination of a pull-down assay followed by mass spectrometry), we have found that human platelets express an ADP-ribosylation factor (Arf)-specific GTPase-activating protein (GAP), ASAP1, as a CrkL-binding protein. In spreading platelets, most endogenous ASAP1 is localized at peripheral focal adhesions. To determine the physiologic significance of the CrkL-ASAP1 association, we overexpressed CrkL, ASAP1, or both in combination in COS7 cells. Unlike endogenous ASAP1 in platelets, overexpressed ASAP1 showed diffuse cytoplasmic distribution. However, when co-expressed with wild-type CrkL, both endogenous and expressed ASAP1 accumulated at CrkL-induced focal adhesions. An SH2-mutated CrkL, which cannot localize at focal adhesions, failed to recruit ASAP1 into focal adhesions. Thus, CrkL appears to be a lynchpin between ASAP1 and peripheral focal adhesions.

CrkL is a Src homology (SH)2[1] and SH3 adapter (1–3). Through its SH2 domain, CrkL binds to focal adhesions proteins like paxillin and Cas (1–3). CrkL also binds to a Rap-specific guanine nucleotide exchange factor, C3G, through its N-terminal SH3 domain and, thus, conveys C3G to focal adhesions. C3G activates a small GTPase Rap1 and regulates cell adhesion and spreading, indicating that the CrkL-C3G complex is a critical component of focal adhesions (4). We previously reported that CrkL is present in human platelets, and that it is an adapter for WASP, syk, or phosphorylated STAT5 (5–7).

On the other hand, ADP-ribosylation factors (Arfs) are also members of the Ras-related small GTPases and function in the regulation of membrane trafficking and actin cytoskeleton (8, 9). Similar to other GTPases, the activity of Arfs is regulated positively by GEFs and negatively by GTPase-activating proteins (GAPs). Recently, several Arf GAPs have been cloned and characterized and found to have phosphoinositide-dependent GAP activity toward Arfs (10). ASAP1 (also called DEF-1, for differentiation enhancing factor-1), the prototype of the phosphoinositide-dependent Arf GAP family, is a multidomain protein with pleckstrin homology, Arf GAP, ankyrin repeat, proline-rich region, and SH3 domains. ASAP1 binds to phosphatidylinositol (4, 5)P_2 through its PH domain and shows GAP activity toward Arf (11–13). In NIH3T3 cells, endogenous ASAP1 localizes in focal adhesions and, when overexpressed, ASAP1 affects cell spreading of NIH3T3 cells on fibronectin (14). Although the function of Arf in focal adhesions is not clear yet, the localization of ASAP1 and its effect on cell spreading suggests the importance of Arf signaling on the dynamics of focal adhesions.

During our continual efforts to clarify the role of CrkL in the regulation of signal transduction, we found that the SH3 domain of CrkL binds to ASAP1. The data obtained from studies using platelets and COS7 cells overexpressing ASAP1 revealed that CrkL is a critical lynchpin between ASAP1 and focal adhesions.

EXPERIMENTAL PROCEDURES

Blood from healthy volunteers, after obtaining written informed consent, was drawn by venipuncture into a one-tenth volume of 3.8% (w/v) trisodium citrate and gently mixed. Alternatively, buffy coat, provided by the Hokkaido Red Cross Blood Center (Sapporo, Japan), was used instead of whole blood. Washed human platelets were prepared as described previously (7) and suspended in a modified Hepes-

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[1] The abbreviations used are: SH, Src homology domain; Arfs; ADP-ribosylation factors; FAK, focal adhesions kinase; GAP, GTPase-activating protein; ASAP1, ARF GAP-containing SH3, ANK repeat, and pleckstrin homology domains; DEF-1, differentiation enhancing factor-1; ECL, enhanced chemiluminesence; GST, glutathione *S*-tranferase; STAT, signal transducer and activator of transcription; MALDI-TOF/MS, matrix-assisted laser desorption/ionization time-of-flight/mass spectrometry.

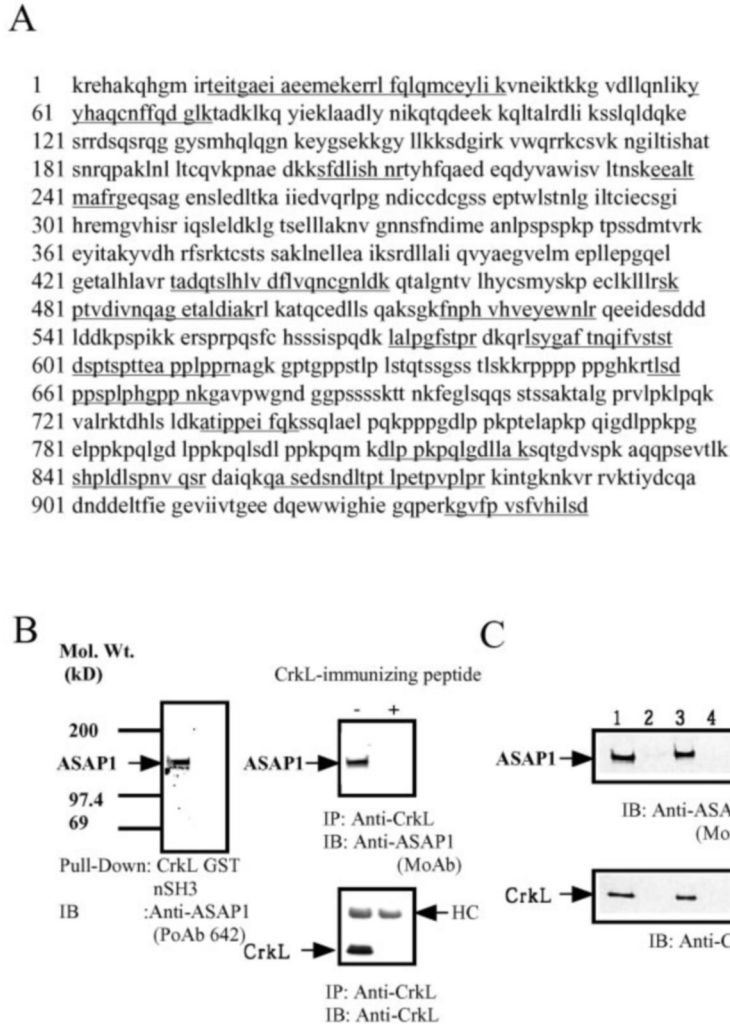

FIG. 1. **Identification of ASAP1 as a CrkL ligand in human platelets.** *A*, the partial amino acid sequence of human ASAP1. The sequence was predicted from an incomplete cDNA sequence of the KIAA1249 clone. The *underlined* peptides were identified by a mass spectrometry analysis of the trypsin-digested 140-kDa protein co-precipitated with GST-CrkL. *B*, *in vitro* and *in vivo* interaction of CrkL with ASAP1. *Left panel*, platelet proteins co-precipitated with GST-CrkL were separated by SDS-PAGE (7.5–15% polyacrylamide). Western blot analysis was conducted with an anti-ASAP1 polyclonal antibody (642). *Right panels*, platelet lysates were prepared using the detergent buffer as described under "Experimental Procedures," immunoprecipitated with anti-CrkL monoclonal antibody and separated by SDS-PAGE (7.5–15% polyacrylamide). The CrkL-immunizing peptide (30 μg/ml) was added during immunoprecipitation as indicated. Western blot analysis was conducted with an anti-ASAP1 monoclonal antibody (*upper panel*) or an anti-CrkL (*lower panel*) polyclonal antibody. *HC*, heavy chains of the anti-CrkL antibody, used for immunoprecipitation. *C*, translocation of ASAP1 to the cytoskeletal fraction upon activation of platelets by thrombin (1 unit/ml). Platelets were lysed with Triton X-100-EGTA buffer before (*lanes 1* and *4*) or after stimulation by thrombin with (*lanes 2* and *5*) or without (*lanes 3* and *6*) stirring. Lysates were separated by high-speed centrifugation into soluble fractions (*lanes 1–3*) and insoluble (*lanes 4–6*) pellets. Proteins from each fraction were separated by SDS-PAGE and immunoblotted with anti-ASAP1 monoclonal or anti-CrkL polyclonal antibodies as indicated.

Tyrode buffer (129 mM NaCl, 8.9 mM $NaHCO_3$, 0.8 mM KH_2PO_4, 2 mM KCl, 0.8 mM $MgCl_2$, 5.6 mM dextrose, and 10 mM Hepes, pH 7.4) at a concentration of 3×10^8 cells/ml with apyrase (2 units/ml) at 37 °C. Specific affinity-purified anti-ASAP1 antibody (642) was prepared as previously described (12). Anti-ASAP1, anti-phospho-FAK, and anti-paxillin (cross-reactive with Hic-5) monoclonal antibodies were from Transduction Laboratories (Jackson, KY). Anti-CrkL and anti-C3G polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). An anti-CrkL monoclonal antibody was from Upstate Technologies (Lake Placid, NY). An anti-FLAG monoclonal antibody, thrombin, and other reagents were from Sigma.

Immunoprecipitation and Immunoblotting—Platelets in suspension (0.5 ml) were lysed by the addition of an equal amount of lysis buffer (15 mM Hepes, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 mM EGTA, 1 mM sodium orthovanadate, 0.8 μg/ml leupeptin, 2% Triton X-100 (v/w), pH 7.4). Immunoprecipitation and immunoblotting using the enhanced chemiluminesence (ECL) methods were performed as previously described (7).

GST Binding Assays—GST-CrkL N-terminal SH3 fusion protein was a gift from Dr. Brian J. Druker (Oregon Health Sciences University). Production of GST fusion proteins and binding experiments using cell lysates were performed as previously described (7). GST fusion proteins were isolated from sonicated bacterial lysates using glutathione-Sepharose beads. Coomassie Brilliant Blue-stained gels were used to normalize the expression of the various GST fusion proteins.

MALDI-TOF/MS—From platelet lysates, we precipitated proteins that bind to the N-terminal SH3 domain of CrkL. Following digestion by trypsin, the bound proteins were analyzed by MALDI-TOF/MS using a Voyager-DE/STR (Applied Biosystems, Foster City, CA). The proteins were identified by comparison between the molecular weights determined by MALDI-TOF/MS and the theoretical peptide masses from the proteins registered in NCBInr.

Isolation of Platelet Cytoskeleton—The Triton X-100-insoluble cytoskeleton from thrombin-stimulated platelets was isolated as previously described (15). An equal amount of lysis buffer was added to the platelet suspensions to solubilize the platelets. After 5 min on ice, the lysate was centrifuged at 10,000 $\times$ *g*. The resulting pellet was washed twice in washing buffer. For one-dimensional SDS electrophoresis, the Triton X-100-insoluble pellets were solubilized in SDS sample buffer. The supernatant was diluted with an equal volume of 2$\times$ concentrated SDS sample buffer.

Ectopic Expression in COS7—The full-length cDNA of a murine ASAP1β expression plasmid was constructed in the pCI (FLAG tag) vector. Human CrkL cDNA, a gift from John Groffen (Children's Hospital, Los Angeles, CA), was subcloned into pCXN2 with FLAG tag and designated as pCXN2-Flag-CrkL. SH2 mutant of CrkL, which was mutated on codon 38, CGC (Arg) to GTC (Val), was generated by PCR, subcloned into pCXN2 vector with FLAG tag, and designated as pCXN2-Flag-CrkLR38V. The expression vector of FLAG tag C3G (a gift from Dr. Michiyuki Matsuda) was described previously (4). To obtain cell lysates, COS7 cells were transfected with 2 μg of recombinant plasmids using FuGENE 6 (Roche Diagnostics, Indianapolis, IN). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. After 24 h, the cells were harvested with SDS sample buffer and sonicated for SDS-PAGE analysis. Alternatively, the cells were harvested with the lysis buffer as described above for immunoprecipitation.

Localization of ASAP1 in Spread Platelets and COS7 Cells—Platelets from diluted platelet-rich plasma were allowed to spread on glass coverslips for 1 h at 37 ° or COS7 cells grown on glass coverslips were fixed in 3.7% paraformaldehyde. Following treatment with 100 mM Tris-HCl (pH 7.4) for 15 min, the cells were permeabilized in 0.15% Triton X-100 for 1 min and blocked with Block Ace (Snow Brand, Tokyo) for 30 min. The samples were incubated with primary and secondary antibodies, and subjected to extensive washing. Alexa-labeled secondary antibodies and Alexa-conjugated phalloidin were purchased from

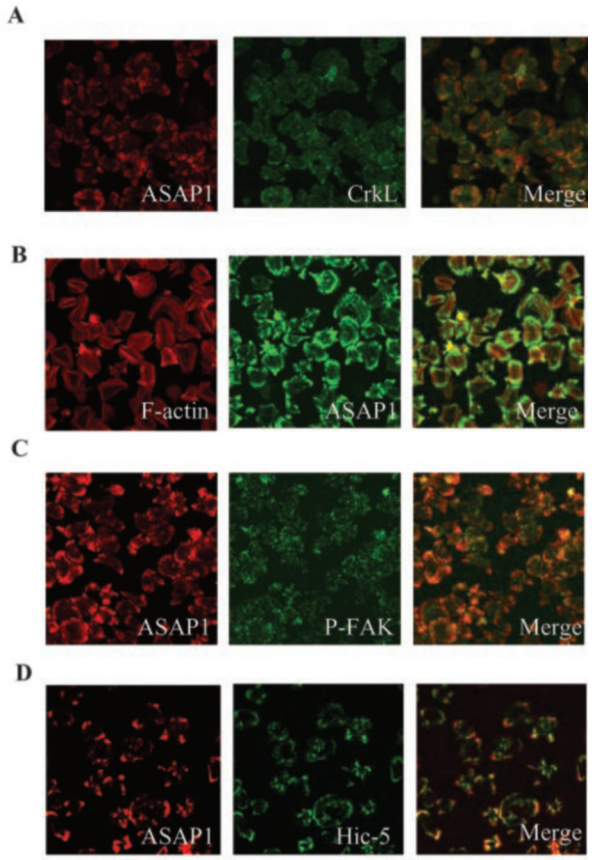

FIG. 2. **Colocalization of ASAP1 with CrkL, phosphorylated-FAK, and Hic-5 at focal adhesions.** Platelets in diluted platelet-rich plasma were allowed to attach and spread on glass coverslips, and were fixed and stained for ASAP1, CrkL, phosphorylated FAK, Hic-5, and F-actin as indicated.

Molecular Probes (Eugene, OR). Images were taken with an inverted confocal laser-scanning microscope (Zeiss LSM 510) with a ×100 oil objective lens and processed by Adobe Photoshop version 7.0.

RESULTS

Mass Spectrometry Following a Pull-down Assay—Platelet proteins were precipitated by the N-terminal SH3 domain of CrkL, expressed as a GST fusion protein, and subjected to SDS-PAGE analysis followed by Coomassie Brilliant Blue staining. A 140-kDa band was observed, which was consistently precipitated by GST-CrkL but not GST alone. A mass spectrometry analysis following trypsin digestion revealed that the 140-kDa band contained several peptides derived from the human homologue of mouse ASAP1 (KIAA1249 clone) (Fig. 1*A*). The anti-ASAP antibody consistently recognized the 140-kDa protein precipitated by GST-CrkL (Fig. 1*B*, *left panel*). Furthermore, ASAP1 was also specifically immunoprecipitated from platelet lysates by anti-CrkL antibody, indicating the *in vivo* interaction of CrkL with ASAP1 (Fig. 1*B*, *right panels*).

Translocation of ASAP1 Following Platelet Activation—We next examined whether ASAP1 translocates to the Triton X-100-insoluble pellets in activated platelets, which represent the operationally defined actin cytoskeleton (15, 16), as reported for CrkL (5). We found that ASAP1 translocated to the thrombin-activated actin cytoskeleton depending upon platelet aggregation (Fig. 1*C*). We also confirmed and extended our previous observations that CrkL also translocates to aggregation-dependent Triton X-100-insoluble pellets (Fig. 1*C*). These data suggest that the ASAP1-CrkL complex may be involved in the organization of the actin cytoskeleton following platelet aggregation.

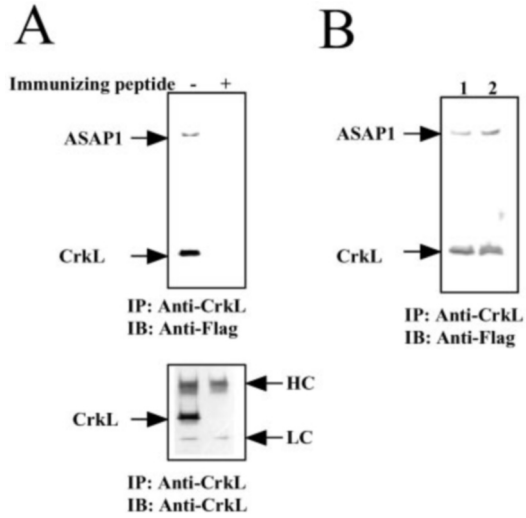

FIG. 3. **Specific association of CrkL with ASAP1 in COS7 cells.** *A*, ASAP1 and wild-type CrkL were overexpressed in COS7 cells. CrkL was immunoprecipitated from the lysates with an anti-CrkL polyclonal antibody. The CrkL-immunizing peptide (30 μg/ml) was added during precipitation as indicated. Replicate samples (± the immunizing peptide) were separated by 7.5–15% SDS-PAGE, transferred to nitrocellulose membranes, and then immunoblotted with an anti-FLAG monoclonal antibody (*upper panel*) or with an anti-CrkL polyclonal antibody (*lower panel*). *B*, wild-type CrkL (*lane 1*) or SH2-mutated CrkL (*lane 2*) was overexpressed with ASAP1 in COS7 cells. CrkL was purified from the soluble extracts by immunoprecipitation, and the denatured samples were separated by 7.5–15% SDS-PAGE, transferred to nitrocellulose membrane, and then immunoblotted with an anti-FLAG monoclonal antibody.

The Localization of ASAP1 in Spreading Platelets—In glass-activated spreading human platelets, most of ASAP1 was localized in the areas where stress fibers terminate (Fig. 2*B*). The ASAP1-rich structures were also enriched with CrkL, Hic-5, and phosphorylated FAK, suggesting that, in platelets, ASAP1 is accumulated at focal adhesions (Fig. 2, *A*, *C*, and *D*). This result is consistent with the previous report of ASAP1 localization in NIH3T3 cells (12). It should be noted that platelets express Hic-5, a focal adhesions protein, instead of paxillin (17). As the spreading of platelets on glass coverslips is also an $\alpha_{IIb}\beta_3$ integrin-dependent process (18–20), the data further suggest that the ASAP1-CrkL complex is involved in reorganization of the cytoskeleton following ligation of $\alpha_{IIb}\beta_3$ integrin. As we cannot express proteins in platelets, we further investigated the physiologic significance of the CrkL-ASAP1 interaction in ectopic expression systems.

Ectopic Expression of CrkL and ASAP1 in COS7 Cells—Wild-type CrkL and ASAP1 were overexpressed in COS7 cells. CrkL was purified from cell lysates by immunoprecipitation. The immunoprecipitates were subjected to Western blotting following SDS-PAGE analysis. FLAG-tagged CrkL and ASAP1 were present in the CrkL immunoprecipitate, and the immunizing peptide for the CrkL antiserum strongly inhibited the precipitation of both bands (Fig. 3*A*), suggesting that CrkL and ASAP specifically associate in COS7 cells. Both wild-type and SH2-mutated CrkL were coprecipitated with ASAP1, suggesting that the intact SH2 domain is not necessary for CrkL-ASAP1 interaction (Fig. 3*B*). Next, we examined the effects of CrkL overexpression on the localization of both endogenous ASAP1 and CrkL. In the cells where CrkL was not overexpressed, both CrkL and ASAP1, expressed at low levels, diffusely distributed as small dots, which were relatively enriched at the peripheral portion of the cells (Fig. 4*A*). However, on uncoated glass coverslips, CrkL overexpression led to the formation of peripheral focal adhesions, presumably through the

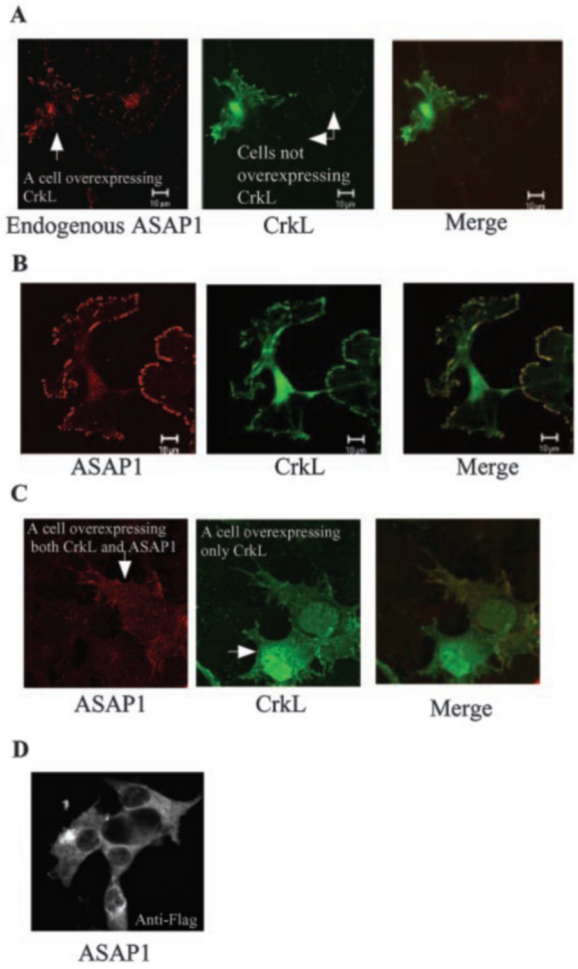

FIG. 4. **Fluorescence micrographs showing the localization of ASAP1 and CrkL in COS7 cells.** COS7 cells were allowed to attach and grow on glass coverslips overnight and were transfected with expression vectors. After a further overnight incubation, the cells were fixed and stained for CrkL and ASAP1 as indicated. *A*, wild-type CrkL was overexpressed. *B*, both wild-type CrkL and ASAP1 were overexpressed. *C*, both SH2-mutated CrkL and ASAP1 were overexpressed. *D*, only ASAP1 was overexpressed and identified by a monoclonal anti-FLAG antibody.

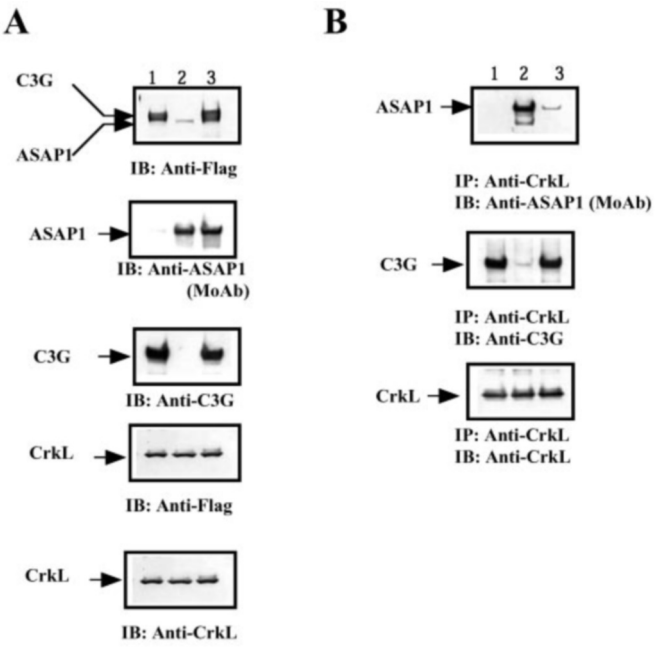

FIG. 5. **Overexpression of C3G inhibits the association of CrkL with ASAP1.** Wild-type CrkL was co-expressed with C3G (*lane 1*), ASAP1 (*lane 2*), or both (*lane 3*). The Triton X-100-soluble extracts (20 μg/ml/lane) were denatured and subjected to SDS-PAGE followed by immunoblotting. Five separate membranes were prepared and treated with anti-FLAG, anti-C3G, anti-CrkL, or anti-ASAP1 antibodies as indicated. *B*, CrkL was purified from cell lysates separated by 7.5–15% SDS-PAGE, transferred to nitrocellulose membranes, and the membranes were then immunoblotted with anti-ASAP1, anti-C3G, or with anti-CrkL antibodies as indicated.

CrkL-mediated increased avidity of integrin (21, 22) to adhesive proteins in bovine serum adsorbed on glass coverslips. Both CrkL and endogenous ASAP1 exactly colocalized at these focal adhesions (Fig. 4*B*). It was reported that the overexpression of ASAP1 led to destruction of focal adhesions in NIH3T3 cells cultured on fibronectin-coated glass coverslips (14). Interestingly, the overexpression of ASAP1 and CrkL did not inhibit the formation of focal adhesions or colocalization of these proteins (Fig. 4*B*). CrkL is known to accumulate at focal adhesions through its interaction with Cas or paxillin, via its SH2 domain (1–3). In agreement with the critical role of the SH2 domain, the SH2-mutated CrkL did not induce the formation of peripheral focal adhesions or accumulation of ASAP1 at the structures (Fig. 4*C*). The overexpression of ASAP1 alone did not induce peripheral focal adhesions and it was diffusedly distributed (Fig. 4*D*) as was reported previously (14). It appeared that ASAP1 does not disrupt focal adhesions if enough CrkL is present to accommodate ASAP1. In other words, overexpressed ASAP1 may remove other CrkL ligands from focal adhesions. The prime candidate of such molecules is C3G (4), which is a ligand for CrkL and accumulates at focal adhesions, through its interaction with CrkL.

Overexpression of C3G Inhibits the ASAP1-CrkL Association—C3G binds to CrkL through specific interaction of the proline-rich domain of C3G with the N-terminal SH3 domain of CrkL (4). If ASAP1 binds to the N-terminal SH3 domain of CrkL *in vivo*, then the overexpression of C3G should compete with ASAP1 for binding to CrkL. The results shown in the Fig. 5 supported this hypothesis. The overepression of C3G inhibited co-precipitation of ASAP1 and CrkL (Fig. 5), suggesting that the binding of both proteins to CrkL may be mutually exclusive.

DISCUSSION

Despite the recognized importance of Arf in cell biology (8, 9), the role of Arf in platelet function has received surprisingly little attention. Our finding that ASAP1 is a novel CrkL ligand, which may be involved in $\alpha_{IIb}\beta_3$ signaling, should set the molecular basis for further studies on Arf and its GAPs in platelets. Mass spectrometry analyses following two-dimensional electrophoresis of platelet protein were performed, and the presence of a number of hitherto undescribed platelet proteins was reported (23, 24). Our approach confirms that mass spectrometry is indeed a powerful approach to identify platelet proteins. Furthermore, our approach also employs a pull-down assay before mass spectrometry, thus enabling us to identify a novel protein-to-protein interaction.

The mechanisms of accumulation of ASAP1 at focal adhesions were unclear. Our data suggest that CrkL is one of the carriers of ASAP1 to focal adhesions, not ruling out the possibility that adapters like Crk (1–3) have similar roles in other situations. Recently, Liu *et al.* (25) reported that ASAP1 directly binds to FAK and suggest that ASAP1 may be a component of multimolecular complexes at focal adhesions. The overexpression of wild-type ASAP1 reportedly retarded the spreading of REF52 cells plated on fibronectin (25). The over-

expression of a truncated variant of ASAP1 unable to bind to FAK resulted in a less pronounced inhibition of cell spreading (25). These data suggest that while FAK is a major scaffolding protein for ASAP1, there must be focal adhesions proteins other than FAK responsible for localization of ASAP1. As CrkL binds to paxillin or Cas, and locates at focal adhesions (1–3), the adapter may contribute to "FAK-independent" functions of ASAP1 at focal adhesions. Interestingly, it was recently reported that the overexpression of ASAP1 inhibited spreading of NIH3T3 cells, and the effect was independent of its Arf GAP activity (26). It is possible that physical replacement of C3G by ASAP1 is involved in such an effect. More recently, a RalBP1-binding protein was shown to interact with mouse ASAP1 (27). In the same report, the association of PAG2 (a human homologue of ASAP1) and POB1 was demonstrated. It was suggested that POB1 interacts with PAG2 through its proline-rich motif, similar to FAK, thereby regulating cell migration. Taken together, CrkL is a critical regulator of localization of ASAP1, likely through its interaction with focal adhesions proteins like paxillin or Cas, and, through cooperation with FAK and POB1, the adapter may be critically involved in the regulation of the cytoskeleton at focal adhesions.

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