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CrkL is an adapter for Wiskott-Aldrich syndrome protein and Syk

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Wiskott-Aldrich syndrome (WAS) and X-linked thrombocytopenia are caused by mutations of the WAS protein (WASP) gene. WASP may be involved in the regulation of podosome, an actin-rich dynamic cell adhesion structure formed by various types of cells. The molecular links between WASP and podosomes or other cell adhesion structures are unknown. Platelets express an SH2-SH3 adapter molecule, CrkL, that can directly associate with paxillin, which is localized in podosomes. The hypothesis that CrkL binds to WASP was, therefore, tested. Results from coprecipitation experiments using anti-CrkL and GST-fusion proteins suggest that CrkL binds to WASP through its SH3 domain and that the binding was not affected by WASP tyrosine phosphorylation. The binding of GST-fusion SH3 domain of PSTPIP1 in vitro was also not affected by WASP tyrosine phosphorylation, suggesting that the binding of the SH3 domains to WASP is not inhibited by tyrosine phosphorylation of WASP. Anti-CrkL also coprecipitates a 72-kd protein, which was identified as syk tyrosine kinase, critical for collagen induced-platelet activation. CrkL immunoprecipitates contain kinase-active syk, as evidenced by an in vitro kinase assay. Coprecipitation experiments using GST-fusion CrkL proteins suggest that both SH2 and SH3 domains of CrkL are involved in the binding of CrkL to syk. WASP, CrkL, syk, and paxillin-like Hic-5 incorporated to platelet cytoskeleton after platelet aggregation. Thus, CrkL is a novel molecular adapter for WASP and syk and may potentially transfer these molecules to the cytoskeleton through association with cytoskeletal proteins such as Hic-5. (Blood. 2001; 97:2633-2639)

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Introduction

Mutations in the Wiskott-Aldrich syndrome protein (WASP) gene may result in the classic Wiskott-Aldrich syndrome (WAS) or in a milder phenotype, X-linked thrombocytopenia.[1-3] Recently, significant progress in the understanding of its physiologic functions has been made.[4-6] The carboxy terminal region of WASP binds to the Arp2/3 complex, which is considered critical for its ability to reorganize actin strands.[7,8] WASP also specifically binds to guanosine triphosphate (GTP)-bound CDC42Hs, a Rho family GTPase.[9,10] Recently, Linder et al[11] reported that WASP and CDC42Hs are concentrated in the core of podosomes in normal human macrophages and that podosomes are absent in macrophages from patients with WAS. Intracellular introduction of either constitutively active CDC42Hs or the carboxyl terminal region of WASP resulted in the disassembly of podosomes.[11] Taken together, it appears that WASP is essential for the formation of podosomes and, upon binding to CDC42Hs, also for their disappearance. The potentially critical role of WASP in the regulation of podosomes may be directly related to the trafficking defect of macrophages and possibly other hematopoietic cells from WAS patients.[4-6] Because the binding of CDC42Hs to WASP results in the disassembly of podosomes, Linder et al[11] concluded that the CDC42Hs that colocalizes with WASP may be in a guanosine diphosphate-bound form and not in direct contact with WASP. If this conclusion is correct, there must be a "non-CDC42Hs" molecular link that concentrates WASP in the podosomes. WASP has a well-characterized proline-rich domain that interacts with the SH3 domains of a number of proteins in vitro, though only a few of them were actually demonstrated to be associated with WASP in vivo.[4-6] Podosomes express paxillin,[12,13] which in its tyrosine-phosphorylated form is a ligand for the SH2 domain of CrkL in hematopoietic cells.[12] It appears that the amino terminal SH3 domain of CrkL can bind to BCR/ABL or c-Abl, linking the kinases to paxillin.[14]

We have previously reported that platelets express CrkL, that thrombopoietin induces tyrosine phosphorylation of CrkL, and that CrkL associates with tyrosine-phosphorylated Stat-5.[15,16] On the other hand, we and others have reported that collagen or collagen-related protein induced tyrosine phosphorylation of WASP in platelets.[17-19] Other stimuli, such as thrombin or thrombopoietin, also induced tyrosine phosphorylation of WASP, but the degree of the phosphorylation was relatively modest.[17,18] Although reports have shown that WASP becomes inducibly tyrosine phosphorylated, presumably by Btk, in cell lines,[20,21] to date, platelets are the only primary cells in which inducible tyrosine phosphorylation of WASP has consistently been demonstrated. It is possible that prolonged activation of Btk/Tec kinases in collagen-treated platelets, in contrast to that in antigen-activated B lymphocytes, is the reason for the consistent observation of WASP phosphorylation.[19] Thus, platelets provide a unique system to investigate the physiologic functions of WASP and CrkL and to test the possibility that CrkL binds to WASP. We show here that CrkL is an adapter for

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WASP in vivo and in vitro. The binding was apparently mediated through the SH3 domain of CrkL. During these investigations, we found that CrkL is also a ligand for platelet-associated syk tyrosine kinase, which is essential for platelet function.[22]

Materials and methods

Prostaglandin E_1 (PGE-$_1$), ATP, Arg-Gly-Asp-Ser (RGDS) peptide, dimethyl sulfoxide, aspirin, apyrase (type VIII), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes), sodium dodecyl sulfate (SDS), 2-mercaptoethanol, sodium orthovanadate, bovine serum albumin (BSA), protein A-Sepharose, Triton X-100, and Tris (hydroxymethyl) aminomethane (Tris) were purchased from Sigma (St Louis, MO). Nitrocellulose membranes were from Bio-Rad Laboratories (Hercules, CA). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) molecular standards and enhanced chemiluminescence (ECL) reagents, including secondary antibodies, were from Amersham (Arlington Heights, IL). The polyclonal anti-WASP antibody 503 was previously characterized.[3,17] Anti-CrkL serum, its immunizing peptide, and an anti-GST monoclonal antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-syk monoclonal antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Anti-c-Cbl and antipaxillin monoclonal antibodies were purchased from Transduction Laboratories (Lexington, KY). The antipaxillin monoclonal antibody cross-reacts with Hic-5. An antiphosphotyrosine antibody, 4G10, was a gift from Dr Brian J. Druker (Oregon Health Sciences University, Portland, OR). An anti-CD32 monoclonal antibody, IV.3, was purchased from MEDAREX (Annandale, NJ). F(ab')2 fragment of goat antimouse IgG was purchased from ICN Pharmaceutical (Aurora, OH). Horse tendon collagen was obtained from Hormon Chemie (Munich, Germany).

Platelet preparation

Blood from healthy volunteers was drawn by venipuncture, after informed consent was obtained, and placed into 1:10 volume of 3.8% (wt/vol) trisodium citrate and gently mixed. Platelet-rich plasma (PRP) was prepared by centrifugation of whole blood at 200*g* for 20 minutes. PRP was aspirated and incubated with 2 μM aspirin for 30 minutes at room temperature. After the addition of PGE-$_1$ at a final concentration of 1 μM (from a stock solution of 1 mM in absolute ethanol), the PRP was spun at 800*g* to form a soft platelet pellet. The pellet was suspended in 1 mL modified Hepes-Tyrode buffer (129 mM NaCl, 8.9 mM $NaHCO_3$, 0.8 mM KH_2PO_4, 0.8 mM $MgCl_2$, 5.6 mM dextrose, and 10 mM Hepes, pH 7.4), supplemented with apyrase 0.6 U/mL, and washed twice. Platelets (3×10^8 cells/mL) were suspended for experiments in modified Hepes-Tyrode buffer supplemented with apyrase (0.6 U/mL) and RGDS peptide (200 μg/mL).

Platelet stimulation and protein analysis

Aliquots of platelets (0.5 mL, 3×10^8 cells/mL) were incubated in modified Hepes-Tyrode buffer supplemented with apyrase (0.6 U/mL) and RGDS peptide (200 μg/mL). Platelet stimulation by agonists was terminated by the addition of an equal volume of lysis buffer (15 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM PMSF, 10 mM EGTA, 1 mM sodium orthovanadate, 0.8 μg/mL leupeptin, 2% Triton X-100 [vol/wt]). After 20 minutes on ice, the lysates were centrifuged at 10 000*g* at 4°C for 20 minutes. The lysate-soluble supernatant was recovered, incubated with protein A-Sepharose (40 μL of 50% slurry) for 1 hour, and centrifuged to obtain the cleared supernatant. Immunoprecipitating antibody (1-5 μg/sample) was added to the cleared supernatants for 2 to 3 hours on ice, and the immune complexes were bound by the addition of protein A-Sepharose (40 μL of 50% slurry/mL supernatant). Immunoprecipitates were washed 3 times with 1 mL cold buffer (the same as the lysis buffer except that the concentration of Triton X-100 [vol/wt] was 1%). Proteins were denatured by heating at 95°C for 5 minutes in modified Laemmeli sample buffer (10% glycerol, 1% SDS, 5% 2-mercaptoethanol, 50 mM Tris-HCl, pH 6.8, 0.002% bromophenol blue, 10 mM EGTA, and 1 mM sodium orthovanadate) and then separated by one-dimensional SDS-PAGE with 10% or 7.5% to 15% polyacrylamide gels. Proteins were transferred from the gel onto nitrocellulose membranes in transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol). To block residual protein binding sites, membranes were incubated in TBST (10 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Tween 20) with 10% BSA. Membranes were washed with TBST and incubated overnight with primary antibodies diluted in TBST as indicated—1.0 μg/mL 4G10, 1.0 μg/mL anti-CrkL antibody, 1.0 μg/mL anti-syk monoclonal antibody, and 1:1000 dilution rabbit anti-WASP antibody. Membranes were washed with TBST and incubated with horseradish peroxidase–conjugated secondary antibody, diluted 1:3000 in TBST. Antibody binding to proteins was visualized with ECL chemiluminescence according to the manufacturer's instructions.

In vitro kinase assays

CrkL immunoprecipitate was washed twice with kinase buffer (150 mM NaCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM Na_3VO_4, and 10 mM Hepes, pH 7.4) and incubated for 30 minutes at room temperature in 30 μL kinase buffer containing 1 mM ATP. Reactions were terminated with an equal volume of 2 × concentrated modified Laemmeli sample buffer, proteins were separated by SDS-PAGE, and tyrosine-phosphorylated proteins were examined by immunoblotting with 4G10.

GST binding assays

The constructs for GST-CrkL fusion proteins, a gift from Dr Brian J. Druker, and for PSTPIP1 were described previously.[23,24] Production of GST-fusion proteins and binding experiments using cell lysates were performed as described.[15] The GST-fusion proteins were isolated from sonicated bacterial lysates using glutathione Sepharose beads. Coomassie-stained gels were used to normalize for the expression of the various GST fusion proteins.

Far Western blotting

GST fusion proteins were purified on glutathione Sepharose beads and eluted with 20 mM reduced glutathione. Nitrocellulose membranes, which were blocked and washed, were then incubated overnight with GST-fusion proteins at a final concentration of 1.0 mg/mL in Tris-buffered saline (10 mM Tris, 150 mM NaCl, 0.5% Tween 20, pH 7.4). After extensive washing, the membrane-bound GST-fusion proteins were probed with anti-GST.

Isolation of platelet cytoskeleton

Triton X-100 insoluble cytoskeleton was isolated as described.[15] An equal amount of lysis buffer was added to platelet suspensions to solubilize platelets. After 5 minutes on ice, the lysates were centrifuged at 10 000*g*, and the resultant pellet was washed twice in washing buffer. For one-dimensional SDS-PAGE, the Triton X-100 insoluble residue was solubilized in SDS sample buffer. The supernatant was diluted with an equal volume of 2 × concentrated SDS sample buffer.

Results

Anti-CrkL precipitates multiple tyrosine-phosphorylated proteins from the platelet lysates

We have reported that collagen induces a robust increase in tyrosine phosphorylation of WASP after treatment of platelets with collagen. We first examined the presence of tyrosine-phosphorylated proteins in the CrkL immunoprecipitates from platelet lysates prepared before and after collagen treatment. The phosphotyrosine immunoblots of the CrkL immunoprecipitates demonstrate numerous proteins—with apparent masses (all approximate) of 150 kd, 120 kd, 100 to 90 kd, 72 kd, 64 kd, and 39 kd—when CrkL from extracts of collagen-stimulated platelets was precipitated (Figure

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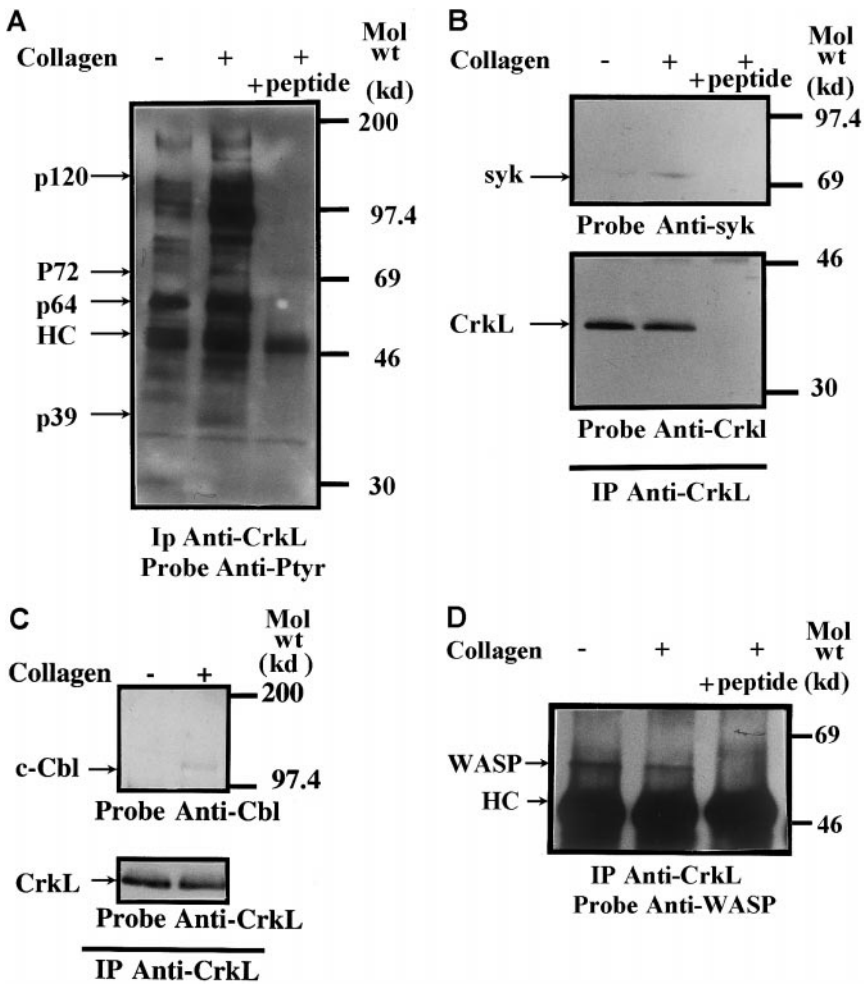

Figure 1. CrkL is associated with WASP and syk. (A-D) Aliquots of platelets were treated with collagen 50 μg/mL or buffer for 5 minutes and then were lysed in detergent buffer, as described in "Materials and methods." CrkL was purified from the soluble extracts by immunoprecipitation (IP), and the denatured samples were divided into 4 equal aliquots. The immunizing peptide (30 μg/mL) was added during precipitation, as indicated. Replicate samples (with or without collagen treatment and with or without the immunizing peptide) were separated by 7.5% to 15% SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with either 4G10 total phosphotyrosine antibody (A), anti-CrkL (B, lower panel), anti-syk (B, upper panel) anti-c-Cbl (C), or anti-WASP (D).

1A, lane 2). The immunizing peptide for the CrkL antiserum strongly inhibited the precipitation of virtually all these bands (Figure 1A-C, lane 3) and CrkL (Figure 1B, lane 3, lower panel), suggesting that these proteins specifically represent either CrkL or CrkL-associated proteins. The 39-kd protein may be CrkL itself. The 120-kd band may contain c-Cbl, which becomes tyrosine phosphorylated in stimulated platelets.[25-27] Indeed, CrkL immunoprecipitate from the lysate prepared from platelets stimulated with collagen, but not resting platelets, did contain a 120-kd protein reactive with an anti-c-Cbl monoclonal antibody (Figure 1C). Thus, as described in other systems, collagen induced the association of CrkL with c-Cbl. The broad 100- to 90-kd band contained little Stat-5, in contrast to the experiments using thrombopoietin (Oda et al[15] and data not shown), and the identity of the band remains to be determined. Anti-WASP recognized a 64-kd band (Figure 1D), suggesting that the 64-kd tyrosine-phosphorylated band contains WASP. A similar amount of WASP was present in the lysates prepared before and after collagen-induced platelet stimulation. We were also interested in the identification of the minor 72-kd tyrosine-phosphorylated band because the syk tyrosine kinase is known to have a similar molecular weight and is critically involved in platelet activation by collagen.[22] Anti-syk monoclonal antibody recognized a 72-kd band in the CrkL immunoprecipitates, and the degree of the coprecipitation was enhanced when the extracts were prepared after platelet stimulation with collagen (Figure 1B, upper panel).

SH3 domains of CrkL and PSTPIP1 bind to WASP in vitro

WASP has a well-documented, proline-rich domain that is a docking site for several SH3-containing proteins. CrkL has one amino terminal SH2 domain followed by 2 SH3 domains.[28-30] To date, no specific ligand for the carboxy terminal SH3 domain has been identified. To determine the site involved in the association of CrkL with WASP, we performed in vitro pull-down assays using bacterially expressed GST-CrkL-SH2 and GST-CrkL-amino terminal SH3 proteins (Figure 2A).WASP was precipitated by the SH3 domain of CrkL but not the SH2 domain or GST alone. The data suggest that the amino terminal SH3 domain of CrkL by itself may account for CrkL-WASP interactions, though a role of the carboxy terminal SH3 domain of CrkL in in vivo interactions cannot be ruled out. The binding of the SH3 domain to WASP was not affected by the activation of platelets before preparation of the extracts. Because the binding of the SH3 domain of PSTPIP1 to WASP is uniquely inhibited by tyrosine phosphorylation of PISPIP1,[24] we investigated the effect of WASP phosphorylation on the interactions of the 2 molecules. The in vitro binding of the SH3 domain of PSTPIP1 to WASP or 64-kd tyrosine-phosphorylated protein was not inhibited by the treatment of platelets with collagen (Figure 2B-C).

Coprecipitation of CrkL with kinase active syk

When syk is activated, its activity in autophosphorylation or transphosphorylation in vitro is also enhanced. Syk in resting

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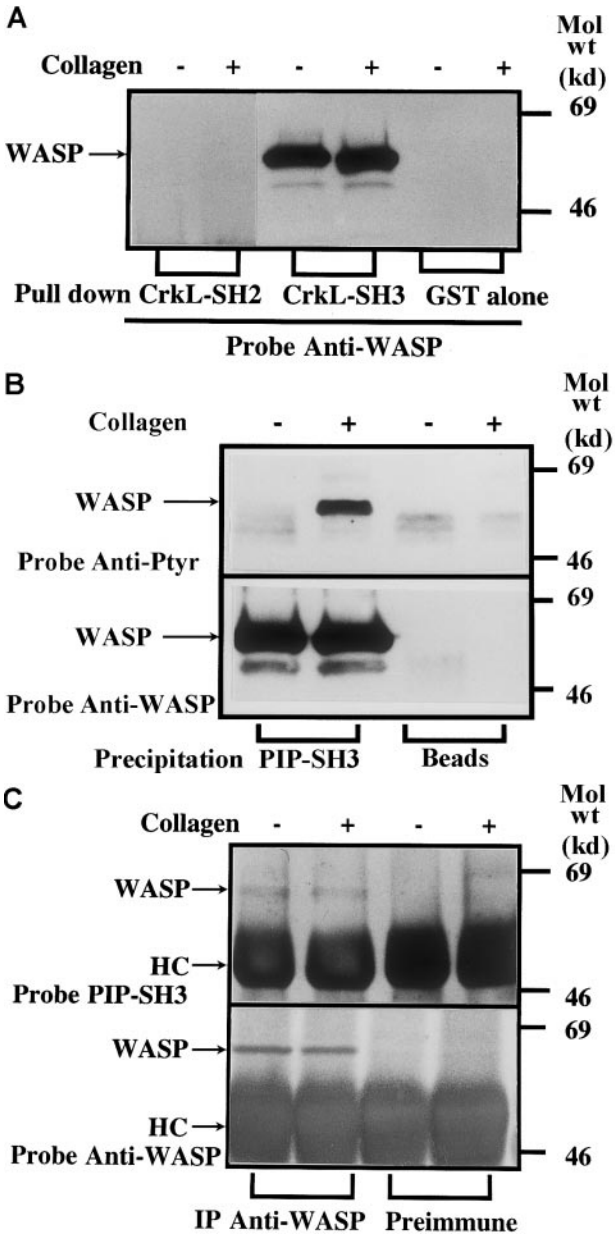

Figure 2. SH3 domains of CrkL and PSTPIP1 bind to WASP in vitro. (A) Platelet lysates prepared with or without collagen stimulation were subjected to GST-CrkL pull-down assay. Precipitates eluted from glutathione Sepharose, which had immobilized GST, GST-CrkL-SH2, or GST-SH3 (the amino terminal SH3), were examined for the presence of CrkL. (B) The same as in panel A, except that GST-SH3 domain of PSTPIP1 or beads alone were used. (C) Direct binding of PSTPIP1 to immobilized WASP. Platelet lysates prepared before and after stimulation with collagen were divided in half. Anti-WASP or preimmune serum was added. Precipitates were then divided in half, separated by 7.5% to 15% SDS-PAGE, and transferred to nitrocellulose membrane. Membranes were probed with the GST-SH3 domain of PSTPIP1 or with anti-WASP. To detect the membrane-bound GST fusion proteins, membranes were further probed with anti-GST. HC indicates heavy chains of antibodies.

platelets is weakly but measurably activated, and its activity is further enhanced by platelet activation.[22,31,32] To test the possibility that syk in the CrkL immunoprecipitates is kinase active, we performed immune complex kinase assays. CrkL was immunopurified from resting or stimulated platelets, then incubated in the presence or absence of ATP. Tyrosine phosphorylation of the immunoprecipitates was detected by immunoblotting. The exposure of ECL film was intentionally short to minimize the contribution of endogenous tyrosine phosphorylation that can be detected in the absence of ATP incubation. Tyrosine phosphorylation of a 72-kd protein was detected in the presence of ATP (Figure 3A). The intensity of the band is enhanced after the treatment of platelets with collagen, most likely reflecting the increased amount of syk in the CrkL immunoprecipitates (Figure B, lane 2, upper panel). We then performed pull-down assays to determine the mode of CrkL binding to syk. In contrast to the interaction between CrkL and WASP, both the SH2 and the amino terminal SH3 domains of CrkL were able to pull down syk, and the degree of the binding was enhanced by activation in both cases (Figure 3B). A small amount of syk was precipitated by GST-coated beads, reflecting unspecific interaction.

Translocation of Hic-5, WASP, syk, and CrkL after platelet aggregation

It was reported that platelets express paxillin-like Hic-5.[33] Given the smallness of platelets, it was difficult to morphologically examine the colocalization of WASP, syk, CrkL, and paxillin-like Hic-5 in platelets under our experimental conditions. However, as shown in Figure 4, we confirmed that CrkL, syk, and WASP translocated to the thrombin-activated actin cytoskeleton, depending on platelet aggregation, and that the same was true for Hic-5, suggesting the possibility that these proteins may indeed colocalize under certain situations in platelets.

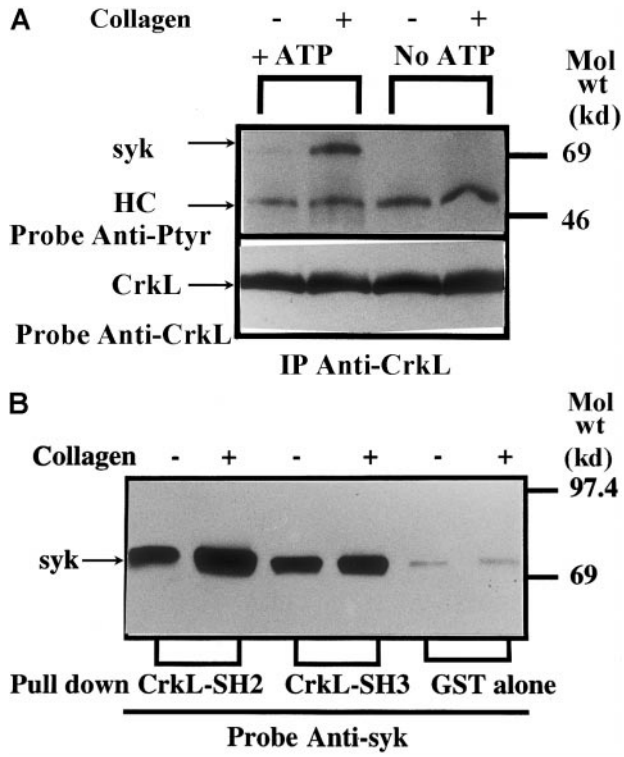

Figure 3. CrkL is associated with kinase active syk. (A) Platelets were treated with collagen or buffer for 5 minutes. CrkL was purified from the soluble extracts by immunoprecipitation as described above. Immune complexes were incubated in kinase buffer with or without 1 mM ATP for 30 minutes. Tyrosine phosphorylation was then examined by immunoblotting with 4G10. (B) Both SH2 and amino terminal SH3 domains of CrkL are involved in the association of CrkL with syk in vitro. The procedures are the same as in Figure 2A, except that anti-syk monoclonal antibody is used to detect syk in the precipitates.

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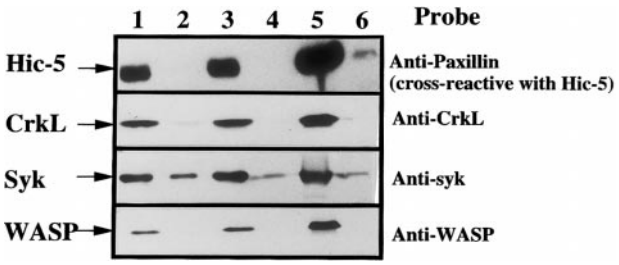

Figure 4. Association of WASP, CrkL, Hic-5, and syk with the Triton X-100 insoluble residue. Platelets were lysed with Triton X-100–EGTA buffer before or after stimulation with thrombin (1 U/mL), with or without stirring. Lysates were separated by high-speed centrifugation into soluble and insoluble residues. Proteins from each fraction were separated by 10% SDS-PAGE and immunoblotted with anti-WASP, anti-CrkL, anti-paxillin (cross-reactive with Hic-5), or anti-syk antibodies as indicated. Lane 1, Triton X-100 soluble residue of resting cells (7.5×10^6 cells). Lane 2, Triton X-100 soluble residue of cells (7.5×10^6 cells) prepared 10 minutes after exposure to thrombin (1 U/mL) with stirring. Lane 3, Triton X-100 soluble residue of cells (7.5×10^6 cells), prepared 10 minutes after exposure to thrombin (1 U/mL) without stirring to prevent aggregation. Lane 4, Triton X-100 insoluble residue of resting cells (3.0×10^7 cells). Lane 5, Triton X-100 insoluble residue from 3.0×10^7 cells, prepared 10 minutes after exposure to thrombin (1 U/mL) with stirring. Lane 6, Triton X-100 insoluble residue from 3.0×10^7 cells, prepared 10 minutes after exposure to thrombin (1 U/mL) without stirring.

Phosphorylation of WASP and CrkL following cross-linking of CD32

Recent observations indicate that, in macrophages, WASP and tyrosine-phosphorylated proteins are involved in Fcγ receptor-mediated phagocytosis.[34,35] Macrophages from WAS patients were found to exhibit reduced phagosome-specific actin polymerization and to virtually lack the recruitment of tyrosine-phosphorylated proteins, suggesting that WASP is responsible for an accumulation of tyrosine-phosphorylated proteins.[34] Not addressed in these experiments was the possibility that WASP itself becomes tyrosine phosphorylated after the cross-linking of Fcγ receptor. Human platelets uniquely express Fcγ receptor IIA (CD32). CD32 protein was cross-linked with monoclonal antibody (IV.3), and WASP and CrkL were then immunopurified from platelet extracts and analyzed by antiphosphotyrosine immunoblot (Figure 5A-B). Cross-linking of CD32 resulted in increased tyrosine phosphorylation of both proteins. Cross-linking of CD32 also resulted in coprecipitation of CrkL with several tyrosine-phosphorylated proteins (apparent masses [all approximate]: 150 kd, 120 kd, 78 kd, 64 kd, and 39 kd), most of which, except the 64-kd band, increased in the extent of their phosphorylation. The CrkL immunizing peptide abolished the precipitation of these proteins, suggesting that the 39-kd proteins may be CrkL and that other proteins are specific CrkL-associated proteins.

Discussion

Our ongoing efforts to understand the physiologic functions of WASP led us to investigate its potential interaction with CrkL, an SH2-SH3–containing protein, involved in various signaling processes.[28-30] CrkL lacks catalytic domains and is a typical adapter protein. To better understand the functions of the molecule, it is essential to identify the ligands that may be of physiologic importance. In the current study, we have demonstrated that CrkL associates with WASP and with syk, respectively, both in vivo and in vitro. Recent reports suggest that CrkL plays a role in the adhesion and migration of hematopoietic cells.[36,37] In support of the potentially important role of CrkL and WASP in cell adhesion processes, we showed that both proteins are incorporated into the cytoskeleton of aggregated platelets (Figure 4).[15,17] We have also reported that CrkL is constitutively tyrosine phosphorylated in platelets from patients with chronic myeloid leukemia who occasionally have platelet dysfunction and bleeding problems.[15] The role of CrkL in cell adhesion is partly mediated by the SH2 domain, which can interact with paxillin, Cas, or Cas-L/Hef-1. These are enriched in cell adhesion structures such as podosomes or focal contacts,[14,28-30] in which CrkL may recruit other molecules through its amino-terminal SH3 domain. Because our data indicate that WASP-CrkL interaction can be mediated solely through the SH3 domain of CrkL, leaving the SH2 domain free, CrkL-WASP interaction should provide ample molecular basis for targeting WASP to podosomes or focal adhesions through the interaction of CrkL with paxillin or other proteins. Btk, a recently identified WASP kinase,[18,20,21] may play an important role in this process—a constitutively active mutant of Btk was found to be a potent inducer of lamellipodia and membrane ruffle formation when transfected into cultured cells.[38] Furthermore, the introduction of constitutively active forms of Rac1 and Cdc42 induced colocalization of Btk with actin in these regions.[38] Thus, it is possible that WASP, a Btk

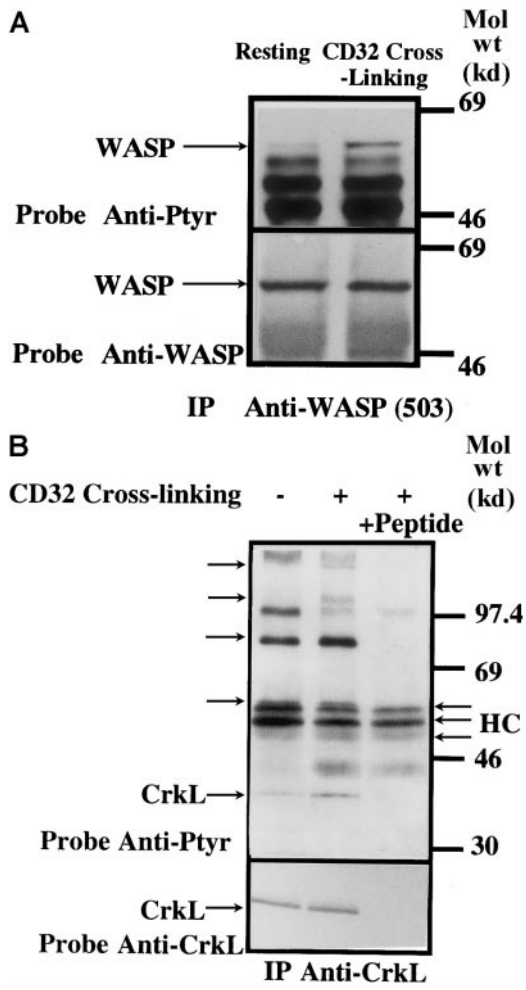

Figure 5. Influence of CD32 cross-linking on WASP and CrkL phosphorylation modulated by PI 3-kinase. Anti-CD32 (3 μg/mL) was added to platelet suspensions for 10 minutes, followed by the addition of (Fab')2 goat antimouse IgG (30 μg/mL) for 5 minutes, as indicated. WASP (A) or CrkL (B) were purified from the soluble extracts by immunoprecipitation as described above, then immunoblotted with 4G10 (top panels) and anti-WASP or anti-CrkL (bottom panels). In panel B, the CrkL immunizing peptide (30 μg/mL) was added during precipitation as indicated. Arrows indicate bands of interest; HC, heavy chains of antibodies.

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substrate, mediates the organization of actin cytoskeleton under these experimental conditions.[38] Although it is of great interest to examine whether the CrkL-WASP complex actually binds to phosphorylated paxillin, the recent report that platelets do not express paxillin affects our model.[33] Thus, more studies using different systems are necessary to determine whether the CrkL-WASP complex is actually recruited to the podosomes in vivo. However, because both CrkL and WASP are incorporated into the actin-rich platelet cytoskeleton after platelet aggregation, it is possible that the CrkL-WASP complex plays a role in cell adhesion structures.[15,17] Although platelets lack paxillin, they do express cas and paxillin-like Hic-5, which may provide the crucial scaffold for CrkL in the cytoskeleton of platelets.[33,39] The possibility that Hic-5, WASP, CrkL, and syk may colocalize in platelets was supported by the observations that all these proteins redistribute to the platelet cytoskeleton after platelet aggregation (Figure 4). CrkL-WASP interaction may not be limited to the cytoplasm. A small portion of WASP and CrkL has been located in the nuclear fraction, where CrkL can interact with the Stat-5–DNA complex.[16,40-42] The significance of the presence of WASP in the nuclei has not been addressed. Interaction between CrkL and Stat-5 is solely mediated by the SH2 domain of CrkL,[16,41,42] leaving the amino terminal SH3 domain free. Thus, it is possible to speculate that WASP interacts with the CrkL-Stat-5 complex in the nuclei.

Interaction of the amino terminal SH3 domain of CrkL with WASP appeared not to be inhibited by the phosphorylation of WASP. Similarly, the binding of the SH3 domain of PSTPIP1 to WASP in vitro was not affected by tyrosine phosphorylation of WASP. Recently, it was reported that, in platelets, the binding of the SH3 domains of Btk and phospholipase Cγ1 to WASP in vitro was also not affected by WASP phosphorylation.[18,21] Thus, it appears that the interactions through SH3 domains are generally not affected by tyrosine phosphorylation of WASP, whereas tyrosine phosphorylation of PSTPIP1 decreases the degree of the WASP-PSTPIP1 interaction.[24]

It was recently reported that WASP may play a critical role in Fcγ receptor–mediated phagocytosis and recruitment of tyrosine-phosphorylated proteins to the phagosome.[34] We found that WASP and CrkL become tyrosine phosphorylated and that CrkL is also associated with other tyrosine-phosphorylated proteins after cross-linking of CD32 in platelets. Because WASP and CrkL appear constitutively associated, it is possible that WASP is essential to recruit tyrosine-phosphorylated proteins to the phagosome.

Finally, we found an association of CrkL with the kinase active syk in vivo and in vitro. The pull-down assays revealed that the SH2 domain and the amino terminal SH3 domain of CrkL are involved in their association. In contrast, Gelkop and Isakov[43] recently reported that, in T lymphocytes, the SH2 domains of Crk family proteins directly bound to phosphorylated Zap-70, a syk-like kinase. In preliminary Far Western blot analysis, we observed that GST-CrkL interaction with immobilized syk was not affected by tyrosine phosphorylation of syk. Because CrkL inducibly associates with c-Cbl (Figure 1C) and c-Cbl is known to directly bind to syk,[44,45] it is possible that CrkL-syk interaction is partially mediated by c-Cbl. Thus, the relation between Zap-70 and CrkL in T lymphocytes appears different from that between syk and CrkL in platelets. Platelets do not express Zap-70.[46] Thus, it remains to be seen whether these differences result from the use of different cells in the experiments or from differences in the structures of these kinases. Nevertheless, the association of CrkL with syk suggests the potentially important role of CrkL in platelet activation by collagen because syk has been shown to be critical in this process.[22] If it is true in vivo, as it is in vitro, that either the SH2 or the amino terminal SH3 domain of CrkL is sufficient for interaction with syk, CrkL may be able to recruit numerous proteins (Figure 1A) to syk using either of the domains without disrupting its association with kinase active syk. Indeed, syk has been demonstrated to translocate to the platelet cytoskeleton, and the translocation was significantly dependent on platelet aggregation.[32] The mechanisms of this translocation are unknown. Our data suggesting that CrkL associates with syk may provide an explanation for the translocation of syk to the cytoskeleton during platelet aggregation. Because syk has pleiotropic functions in different hematopoietic cells (reviewed in Turner et al[47]) and CrkL is also widely expressed by these cells, our current observations may be extended to other hematopoietic cells.

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