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Identification of Regions of the Wiskott-Aldrich Syndrome Protein Responsible for Association with Selected Src Homology 3 Domains*

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Src homology 3 (SH3) domains have been shown to mediate selected interactions between signaling molecules and are essential for the activation of a number of receptor-driven pathways. The Wiskott-Aldrich syndrome protein was identified as a protein that associated selectively with the SH3 domains derived from c-Src, p85α, phospholipase Cγ1, and c-Fgr. Significantly reduced association was detected to the N-terminal SH3 domain and the tandem SH3 domains of p47^{phox}, and no binding was detected to the SH3 domain of n-Src, the C-terminal SH3 domain of p47^{phox}, or either of the SH3 domains of p67^{phox}. Three peptides corresponding to potential Wiskott-Aldrich syndrome protein SH3 domain binding motifs were found to inhibit its association with c-Src, Fgr, and phospholipase Cγ1 SH3 domains, but not the p85α SH3 domain. These peptides have the sequences MRRQEPLPPPPPPSRG, TGRSGPLPPPPPGA, and KGRSGPLPPVPLGI and show homology with other SH3 domain binding motifs. It is possible that the intracellular association of Wiskott-Aldrich syndrome protein with other signaling proteins is mediated by its SH3 domain-binding regions, and this may play a role in its putative function as a regulatory molecule in immune cells.

It is now well established that intermolecular associations are essential for the generation of intracellular signals resulting from receptor occupancy. In recent years a number of modular domains have been identified which mediate such interactions, and examples include SH2[1] and SH3 domains, originally identified as noncatalytic regions of homology to the v-*src* oncogene (1, 2). SH2 domains bind to specific phosphotyrosine-containing peptide sequences, commonly in transmembrane receptors, and the specificity of this interaction is determined mainly by the residues immediately flanking the phosphorylated tyrosine residue (3–8). SH2 domains all consist of two antiparallel β sheets surrounded by two α-helices, with the phosphotyrosine and +3 residue tightly bound to two pockets on the domain surface (9).

SH3 domains are regions of about 50–75 amino acids, which are commonly found in signaling molecules, in cytoskeletal components, and also in the phagocyte superoxide-forming complex, NADPH oxidase (1, 2). SH3 domains bind to proline-rich sequences with a core motif of P*XX*P where P are conserved proline residues and *X* are commonly aliphatic residues; however, the structural basis for the specificity of SH3-ligand interactions is less well understood compared to SH2 domains (10). Although there is some promiscuity in the binding of some SH3 domains to different proline-rich motifs (11), other SH3 domains are highly selective and will bind to only a single specific sequence (12). All SH3 domain ligands adopt a type II polyproline helix conformation, and structural studies have shown that proline-rich motifs fall into two classes, which can bind to SH3 domains in either forward or reverse orientations (13–24). In phagocytes the activation of the NADPH oxidase is absolutely dependent on the regulated interaction of SH3 domain-containing components, yet the mechanism by which binding is initiated and regulated is not understood (25). In an attempt to identify novel SH3-binding proteins that might be involved in intracellular signaling in the hematopoietic cells, we have identified the product of the gene responsible for Wiskott-Aldrich syndrome (WASP) (26) as a protein that binds to a subset of SH3 domains, and we have characterized the sequences on the WASP protein responsible for SH3 domain binding.

EXPERIMENTAL PROCEDURES

Cells and Reagents—The bacterial expression vector pGEX-4T-1 was purchased from Pharmacia Biotech Inc. Glutathione-agarose beads were obtained from Sigma. Namalwa cells were originally obtained from the American Type Culture Collection and cultured in RPMI 1640 medium (Flow Laboratories) containing 10% heat-inactivated fetal calf serum (Imperial Laboratories) at 37 °C in 5% CO_2. Synthetic peptides with free charged termini were synthesized by Zinsser (Maidenhead, United Kingdom) using Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry and were >80% pure as assessed by HPLC and mass spectrometry. All chemicals were Analar grade or better (BDH), and all other reagents were purchased from Sigma unless stated otherwise.

Glutathione S-Transferase Fusion Proteins—cDNA sequence encoding the SH3 domains of PLCγ1, p85α, c-Src, n-Src, c-Fgr, p47^{phox}, and p67^{phox} as glutathione *S*-transferase (GST) fusion proteins were generated and used as described previously (11, 12). Constructs were transformed into *Escherichia coli* XL1-Blue and expression of GST fusion proteins performed as described previously (12, 27).

GST-SH3 Binding Assays—Affinity matrices were prepared by immobilizing 25 μg of fusion protein on 50 μl of glutathione-agarose beads (Sigma). Cells were pelleted and washed twice in phosphate-buffered saline. Cell pellets were solubilized in lysis buffer (50 mM Tris, pH 7.5, 5 mM EGTA, 2% (v/v) Triton X-100, 75 mM NaCl, 0.5 mM phenylmethanesulfonyl fluoride) and clarified by centrifugation at 14,000 × *g* for 15 min at 4 °C. Cell lysate was mixed with the GST-SH3 affinity matrices for 3 h at 4 °C. The beads were then washed extensively in wash buffer

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[1] The abbreviations used are: SH2, Src homology 2; SH3, Src homology 3; PLCγ1, phospholipase Cγ1; GST, glutathione *S*-transferase; WASP, Wiskott-Aldrich syndrome protein; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.

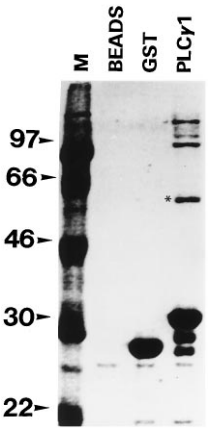

FIG. 1. **Profile of SH3 domain-binding proteins.** GST-SH3 fusion proteins were immobilized to glutathione beads as described under "Experimental Procedures." Lysates from Namalwa cells were adsorbed to the beads, and the bound proteins were separated by SDS-PAGE under reducing conditions followed by Coomassie Blue staining. The 55-kDa protein that was analyzed further is denoted with an *asterisk*. *M*, markers; *BEADS*, glutathione beads alone; *GST*, glutathione beads with immobilized GST; *PLCγ1*, glutathione beads with immobilized with GST-PLCγ1 SH3 domain.

(50 mM Tris, pH 7.5, 0.1% (v/v) Triton X-100, 10% (v/v) glycerol). Binding proteins were eluted by boiling in SDS-PAGE sample buffer, resolved by SDS-PAGE, and prepared for microsequencing or transferred to nitrocellulose filters for immunoblotting. In experiments to investigate the ability of peptides to inhibit WASP-SH3 domain interaction, binding was performed in the presence of the synthetic peptide at the indicated concentration.

Sequencing of 55–60-kDa SH3 Domain-binding Protein—A 55–60-kDa polypeptide that bound to the SH3 domain of PLCγ1 was purified from 10^9 Namalwa cells by SH3 affinity chromatography as described above. The protein sample was separated by SDS-PAGE, identified by Coomassie Blue staining, excised, and digested with Lys-C in the gel slice. Peptides were extracted from the gel and separated by tandem ion exchange and reverse phase HPLC using a Hewlett-Packard 1090M and diode array detection system. Purified peptides were sequenced using fast cycle, automated Edman chemistry on an Applied Biosystems 477A sequencer, modified as described (28).

Western Blotting—Monoclonal antibody 3F3.A5 against a FLAG-WASP fusion protein was generated (29) and used for Western blotting of WASP. SH3 domain-binding proteins were separated by SDS-PAGE, transferred to nitrocellulose filters, and after blocking with 5% nonfat milk powder the filters were overlaid with a 1:1000 dilution of monoclonal anti-WASP in phosphate-buffered saline containing 0.05% Tween 20. The filters were washed three times in phosphate-buffered saline/Tween 20, and the bands were detected using the ECL system (Amersham). Images were scanned using a Microtek Scanmaker II, densitometry was performed, and peaks integrated using ScanAnalysis linked to an Apple Macintosh Quadra.

RESULTS

Identification of a 55-kDa SH3-binding Protein as WASP—Namalwa cells were lysed into Triton X-100-containing buffer, and after centrifugation of insoluble material the lysates were incubated with GST-SH3 domain fusion proteins. Fig. 1 shows the profile of Namalwa cell proteins associating with the SH3 domain of PLCγ1. In addition to bands at 150 kDa and 100 kDa, a prominent band was detected at 55–60 kDa, which specifically interacted with the immobilized SH3 domain fusion protein (*asterisk*). This protein was not present when the cell lysate was incubated with beads alone or GST-immobilized beads. Larger quantities of the 55–60-kDa band were prepared from 10^9 cells and purified by incubation with immobilized PLCγ1 SH3 domain followed by SDS-PAGE. The band was excised and subjected to microsequencing as described under "Experimental Procedures." Two peptides were obtained, and sequences derived from these peptides were found to have 100% identity with residues 237–245 and residues 270–288 of the published sequence of the human Wiskott-Aldrich syndrome protein (WASP) (Fig. 2). Western blotting of whole cell lysates using a monoclonal against WASP (29) confirmed its presence in the Namalwa cells (data not shown).

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MSGGPMGGRP GGRGAPAVQQ NIPSTLLQDH ENQRLFEMLG RKCLTLATAV 50

VQLYLALPPG AEHWTKEHCG AVCFVKDNPQ KSYFIRLYGL QAGRLLWEQE 100

LYSQLVYSTP TPFFHTFAGD DCQAGLNFAD EDEAQAFRAL VQEKIQKRNQ 150

RQSGDRRQLP PPPTPANEER RGGLPPLPLH PGGDQGGPPV GPLSLGLATV 200
     pep 5          pep 6
DIQNPDITSS RYRGLPAPGP SPADKKRSGK KKISKADIGA PSGFKHVSHV 250
          pep 7                      aDIGA PSGFK
GWGPQNGFDV NNLDPDLRSL FSRAGISEAQ LTDAETSKLI YDFIEDQGGL 300
                   sL FSRAGISEAQ LTDAETSK
EAVRQEMRRQ EPLPPPPPPS RGGNQLPRPP IVGGNKGRSG PLPPVPLGIA 350
          pep 1                      pep 3
PPPPTPRGPP PPGRGGPPPP PPPATGRSGP LPPPPPGAGG PPMPPPPPPP 400
                                pep 2
PPPPSSGNGP APPPLPPALV PAGGPGPGGG RGALLDQIRQ GIQLNKTPGA 450
               pep 4
PESSALQPPP QSSEGLVGAL MHVMQKRSRA IHSSDEGEDQ AGDEDEDDEW 500

DD
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FIG. 2. **Alignment of Lys-C peptides derived from the 55–60-kDa protein that bound to the SH3 domain of PLCγ1.** Peptide sequences obtained from Lys-C peptides are indicated in *bold*. Proline-rich sequences, which potentially bind to SH3 domains, and to which homologous peptides were synthesized and used in further studies, are *underlined*.

Selectivity of WASP-SH3 Domain Associations—The interaction of WASP with a panel of immobilized SH3 domains was examined by immunoblotting (Fig. 3). WASP was present in affinity precipitates from the SH3 domains of c-Src, p85α, PLCγ1, and c-Fgr. Significantly less WASP was detected in affinity precipitates using the N-terminal SH3 domain and the tandem SH3 domains of $p47^{phox}$. No WASP was detected when the SH3 domains derived from n-Src, the C-terminal SH3 domain of $p47^{phox}$, or either the N-terminal or C-terminal domains of $p67^{phox}$ were used as affinity matrices. The association of WASP with these SH3 domains is likely to be due to direct binding, since baculovirus-expressed WASP shows a similar SH3 domain interaction profile, and the direct association of the WASP proline-rich domain with these SH3 domains can be demonstrated using a yeast two hybrid assay system.[2] We have shown that Sam68 interacts equally efficiently with the SH3 domains of c-Src, p85α, p47N, and p47N/C but less well with c-Fgr (30), and that $p47^{phox}$ interacts with the p67C SH3 domain (12). Since several of the recombinant SH3 domains that are negative or substantially reduced for WASP association have functional activity in associating with these other proline-rich-motifs, lack of WASP association cannot be due to incorrect folding of the negative SH3 domains.

Characterization of the Motifs in WASP Responsible for the Association with SH3 Domains—The primary sequence of WASP contains multiple potential SH3 binding motifs. In order to identify which of these motifs are responsible for its ability to interact with SH3 domains, peptides corresponding to the WASP proline-rich motifs were prepared. The ability of these peptides to inhibit the interaction of WASP with the SH3 domains of PLCγ1, p85α, c-Fgr, and c-Src was investigated. At 500 μM, peptides 1, 2, and 3 inhibited the association of Namalwa cell-derived WASP with the SH3 domains of c-Fgr, c-Src,

[2] C. Soames and L. Wilson, manuscript in preparation.

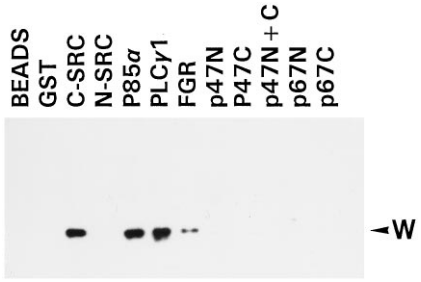

FIG. 3. **Selectivity of WASP binding to SH3 domains.** The binding of WASP from Namalwa cell lysates to a panel of immobilized SH3 domains was performed as described for Fig. 1, and WASP was detected by Western blotting. Glutathione beads were immobilized with GST fusion proteins containing the following SH3 domains. *C-SRC*, c-Src; *N-SRC*, N-Src; *P85α*, the SH3 domain of the 85-kDa subunit of phosphatidylinositol 3′-kinase; *PLCγ1*, the SH3 domain of phospholipase Cγ1; *FGR*, c-Fgr; *p47N*, the N-terminal SH3 domain of $p47^{phox}$; *p47C*, the C-terminal SH3 domain of $p47^{phox}$; *p47N+C*, the tandem N-terminal and C-terminal SH3 domains of $p47^{phox}$; *p67N*, the N-terminal SH3 domain of $p67^{phox}$; *p67C*, the C-terminal SH3 domain of $p67^{phox}$. *W* denotes WASP polypeptide.

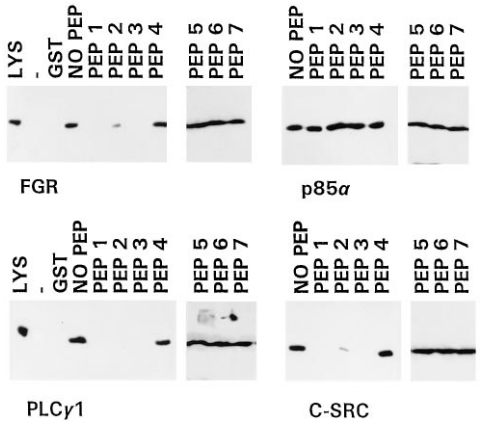

FIG. 4. **Identification of regions of WASP responsible for SH3 domain binding.** Seven proline-rich peptides (*PEP 1–7*) based on potential SH3 domain binding motifs in the WASP amino acid sequence were synthesized and used to determine whether they inhibited binding of WASP from Namalwa cells to the SH3 domains of c-Fgr (*FGR*), the 85-kDa subunit of phosphatidylinositol 3′-kinase (*P85*), phospholipase Cγ1 (*PLCγ1*), or c-Src (*C-SRC*). The peptide sequences are as follows: *PEP 1*, MRRQEPLPPPPPPSRG (residues 307–322); *PEP 2*, TGRSGPLPPPPPGA (residues 375–388); *PEP 3*, KGRSGPLPPVPLGI (residues 336–349); *PEP 4*, GPAPPPLPPALVPA (residues 409–422); *PEP 5*, QSGDRRQLPPPPTPAN (residues 152–167); *PEP 6*, ERRGGLPPLPHPGGDQGG (residues 169–187); *PEP 7*, TSSRYRGLPAPGPSPA (residues 208–223); *LYS* denotes Namalwa cell lysate containing WASP as a marker.

and PLCγ1. In contrast peptides 4, 5, 6, and 7 had no effect on the ability of WASP to associate with any of these SH3 domains (Fig. 4). None of the peptides tested showed any inhibitory activity against WASP-p85α SH3 domain association, suggesting that the interaction of WASP with this SH3 domain was mechanistically different from that of the other SH3 domains. The sequence requirements for inhibitory activity were further investigated using peptides with single amino acid changes. Replacing Arg^{309} or Arg^{321} with lysine had little effect on the ability of peptide 1 to inhibit association with the SH3 domains of Src, Fgr, or PLCγ1. However replacement of Arg^{377} with a lysine in peptide 2 resulted in a significant loss of inhibitory activity for PLCγ1, c-Src, and Fgr SH3 domains (Table I).

Inhibitory Activities of WASP-derived Peptides—The concentration dependence of the abilities of peptides 1, 2, and 3 to inhibit WASP-SH3 domain interactions was investigated. Cell lysates were incubated with immobilized SH3 domains in the presence of 0–250 μM concentrations of peptide and WASP association was detected by Western blotting. Each of the peptides exhibited rather a sharp concentration dependence (Fig. 5). The peptides had no effect on the ability of WASP to associate the SH3 domains of either c-Src or PLCγ1 at or below 100 μM, except for peptide 1, which showed a slight but consistent inhibitory activity on c-Src association at this concentration. All peptides exhibited greater than 80% inhibitory activity at 250 μM.

TABLE I
Inhibitory activities of modified WASP-derived proline-rich sequences

Blots were quantitated by densitometry and categorized as follows: +++, >90% inhibition; ++, 50–90% inhibition; +, 10–50% inhibition; −, <10% inhibition.

Peptide (original)	Sequence	Inhibitory activity (500 μM)			
		PLCγ1	c-Src	c-Fgr	p85α
1	MRRQEPLPPPPPPSRG (307–322)	+++	+++	+++	−
	MR**K**QEPLPPPPPPSRG	+++	+++	+++	−
	MRRQEPLPPPPPPS**K**G	++	+	+++	−
2	TGRSGPLPPPPPGA (375–388)	+++	++	++	−
	TG**K**SGPLPPPPPGA	+	−	−	−

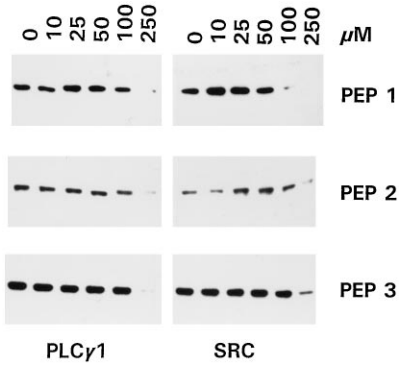

FIG. 5. **Concentration dependence of inhibitory activity of proline-rich peptides 1, 2, and 3.** Binding of WASP from Namalwa lysates to the SH3 domains of PLCγ1 or c-Src was performed in the presence of the indicated concentrations of peptide, and SH3 domain-associated WASP was visualized by Western blotting.

DISCUSSION

When the SH3 domain of PLCγ1 was used as an affinity matrix for Namalwa B cell lysates, an associated 55–60-kDa protein was identified as WASP, the product of the gene defective in Wiskott-Aldrich syndrome. Recently WASP has been shown to be an effector for the small GTP-binding protein Cdc42H, and it appears to regulate actin polymerization (37), consistent with the observations that leucocytes from Wiskott-Aldrich patients have fewer surface microvilli (38). There is a high degree of homology between human and mouse WASP, suggesting evolutionary conservation of function (39). Wiskott-Aldrich syndrome is an X-linked recessive disorder characterized by severe thrombocytopenia, small platelets, excessive bleeding, eczema, recurrent microbial and viral infections, impaired humoral and cellular immunity, and increased susceptibility to lymphoid malignancies (31–36). X-linked congenital thrombocytopenia is also caused by mutations in the Wiskott-Aldrich syndrome gene (40), and the results reported here that WASP interacts with the SH3 domains from a number of signaling molecules found in leucocytes and platelets are consistent with a conserved role in intracellular signaling.

We have shown that WASP can be isolated using immobilized SH3 domains of PLCγ1, c-Src, c-Fgr, and p85α, and to a lesser extent using the tandem SH3 domains of $p47^{phox}$. Recently it has been reported that WASP will also bind to the SH3

TABLE II
Alignment of WASP sequences with sequences known to bind to SH3 domains

These sequences are listed in a manner analagous to that of Sparks *et al.* (24). Conserved residues are printed in boldface.

Sequence		Ref.
Src SH3 class I		
RRQEPLPPPPPP	WASP (Peptide 1)	
TGRSGPLPPPP	WASP (Peptide 2)	
KGRSGPLPPVPL	WASP (Peptide 3)	
RARNLPLPPPP	HnRNPK	44
VTRGVPPPPTV	p62	44
PPRPLPVAPGS	p85α	43, 47
Src SH3 class II		
RQEPLPPPPPSRG	WASP (Peptide 1)	
RQEPLPPPPPSKG	WASP (Peptide 1)	
GGAPPVPSRP	Dynamin (810–819)	11
GPPPQVPSRP	Dynamin (827–836)	11
RAPPGVPSRS	Dynamin (838–847)	11
PLPPPPPPRG	hnRNPK (308–317)	44
APPPPPVPRG	p62 (294–303)	44
GGPPPGPGRR	Shb (50–59)	46
TKSPPQPPRP	Shb (103–112)	46
QPAPALPPKP	p85α (302–311)	43, 45
KPQPAVPPRP	p47^{phox} (360–369)	12

domain of Nck, although the binding motif was not identified (41). Peptide inhibition experiments revealed sequences on WASP that are responsible for its association with c-Src, c-Fgr, and PLCγ1 SH3 domains. These regions correspond to residues 307–322 (peptide 1), 375–388 (peptide 2), and 336–349 (peptide 3), and these appear to be approximately equally effective for these SH3 domains. As expected, peptide 7, which does not have a P*XX*P motif, was inactive, and other WASP-based peptides also showed no activity; therefore, its association with SH3 domains appears to be directed by discrete regions of the protein. None of these peptides inhibited the association of WASP with p85α SH3 domain, and so this association may be mediated by another region of the WASP molecule. It is possible that WASP peptides have a lower affinity for SH3 domains relative to whole WASP protein, as has been observed for the Nef protein interacting with the Hck SH3 domain (42). Peptides also have a lower PPII conformation in solution (43), and these factors might explain the sharp slope and high concentrations of WASP peptides necessary for inhibition. We found no loss of activity if peptides 1 or 2 were preincubated with cell lysate for 3 h, so degradation by cellular enzymes appeared not to be a significant factor. WASP Peptides 2 and 3 have high homology with a consensus of GRSGPLPP*Z*P*Z*G where *Z* is a hydrophobic residue, and whilst some homology is lost when peptide 1 is compared, all three peptides have a consensus of ..*X*R*B*X*P*LPP*Z*P*Z*.. where *X* is any residue and *B* is an uncharged residue. These sequences do not fit perfectly within the consensus sequences of R*X*LPP*Z*P (Class I) or *X*PPLP*X*R (Class II) Src SH3 binding motifs as identified by Feng *et al.* (18); however, all three peptides have an arginine residue N-terminal to the P*XX*P sequence, which is common on Class I ligands (17–22). In addition peptide 1 also has an arginine C terminus to the P*XX*P sequence, consistent with Class I ligands (Table II). Indeed, comparison of the WASP peptides with several peptides known to be Class I or Class II Src SH3 domain-binding proteins show some degree of homology (Table II). WASP peptides 1, 2, and 3 have a conserved arginine and proline spacings with Class I Src-binding peptides derived from heteroribonucleoprotein K, p62, and p85α. Peptide 1 also has proline spacings and an arginine residue conserved in the Class II binding peptides derived from dynamin, heteroribonucleoprotein K, p62, Shb, p85α, and p47^{phox} (11, 12, 43–47). Thus peptides 1, 2, and 3 are all potentially Class I Src binding motifs, but in addition peptide 1 is also potentially a Class II Src binding motif, despite not conforming exactly to the published consensus sequences. The sequence requirements for inhibitory activity were also examined and substitution of Arg^{309} or Arg^{321} in peptide 1 for Lys had little effect, indicating that a positively charged amino acid in these positions is sufficient for activity. In contrast, replacement of Arg^{377} in peptide 2 with Lys virtually abolished activity, indicating a preferential requirement for arginine in this position.

To date there have been no published clinical mutations in the SH3 domain-binding regions of WASP described here in Wiskott-Aldrich syndrome patients. One patient, however, has an insertion-deletion pair in the cDNA sequence coding for the amino acids between peptides 1 and 3, changing the sequence from ^{328}RPPIV^{332} to PAPYC. An Epstein-Barr virus-transformed cell line from this patient produces normal amounts of a normal size protein, suggesting that this region is essential for WASP function (29). It is possible that this mutation might interfere with the SH3 domain binding to the WASP proline-rich regions described here, since regions outside the proline-rich core have been shown to be important for the specificity and affinity of Nef for the Fyn SH3 domain (48).

The specific role of WASP within the cell is at present unknown. The data presented in this paper show that WASP interacts with the SH3 domains of selected signaling molecules, suggesting it might regulate intracellular signaling in addition to associating with the small GTP-binding protein Cdc42 *in vitro* (37, 49, 50). WASP appears to be a member of a larger family of proteins, which includes the profilin-binding vasodilator-stimulated phosphoprotein (VASP) (51, 52), and so WASP might provide a link between SH3 domain-containing signaling molecules such as tyrosine kinases or phospholipases, small GTP-binding proteins, and the cytoskeleton in hematopoietic cells. Further characterization of its intracellular associations will help elucidate WASP function.

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Note Added in Proof—After acceptance of this manuscript, Banin *et al.* (53) reported the association of WASP with the SH3 domains of c-Src family kinases.

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Identification of Regions of the Wiskott-Aldrich Syndrome Protein Responsible for Association with Selected Src Homology 3 Domains

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