Inhibition of the Activity of Src and Abl Tyrosine Protein Kinases by the Binding of the Wiskott−Aldrich Syndrome Protein†

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ABSTRACT: The Wiscott−Aldrich syndrome protein, WASP, is an effector through which cdc42, a Rho family GTPase, regulates the actin cytoskeleton in hematopoietic cells. We have found that WASP binds readily to a number of tyrosine protein kinases including the Src kinases and the Abl kinase when the proteins are coexpressed during transient transfection. Binding inhibited the activity of each of these kinases strikingly, both in vitro and in vivo. Surprisingly, the binding was not due to an interaction between the proline-rich domain of WASP and the SH3 domain of these kinases. Rather, residues 83−93 in WASP were found to bind to the catalytic domains of the kinases. Binding did not decrease the affinity of Src kinases for either ATP or a peptide substrate noticeably. Rather, the V_{max} of substrate phosphorylation was reduced by the binding of the peptide. This inhibition represents a novel form of regulation of protein kinase activity and suggests that that the isolation of small molecules that exploit this inhibitory mechanism may be possible.

The human diseases Wiskott−Aldrich syndrome (WAS)[1] and X-linked thrombocytopenia both arise from mutations in the gene encoding the WAS protein (WASP) (*1*, *2*). Both conditions are characterized by reduced platelet size and number. Additionally, eczema, immunodeficiency, and an increased risk of hematopoietic malignancy are observed in WAS (*3*).

WASP is an effector through which cdc42, a rho family GTPase, regulates the actin cytoskeleton. The binding of cdc42 activates WASP by disrupting an autoinhibitory conformation (*4*). Activated WASP is then able to bind to the Arp2/3 complex of actin-regulatory proteins and stimulate Arp2/3-dependent actin polymerization (*5*, *6*). It is likely that the pathology observed in WAS patients results in part from failure of the mutant WASP protein to regulate the actin cytoskeleton properly in the hematopoietic cell lineages to which its expression is restricted (*1*).

In addition to a cdc42 binding domain and an Arp2/3 binding domain termed the verprolin cofilin acidic (VCA) domain, WASP contains an enabled Vasp homology 1 (EVH1) domain at its amino terminus to which the WASP-interacting protein, WIP (*7*), binds. WASP also contains a proline-rich domain that has been shown to bind a number of other proteins involved in the regulation of cell shape and proliferation. These include the adaptor protein Nck (*8*), Tec family tyrosine protein kinases (*9*), and Src family tyrosine protein kinases (*10*−*12*). The Src kinases are reported to bind to WASP through their Src homology 3 (SH3) domains (*10*−*12*).

The Src kinase family is comprised of eight members, including Src, Lck, and Fyn (*13*). At least one member of this family of tyrosine protein kinases is expressed in essentially every vertebrate cell. These kinases are found at the inner face of the plasma membrane where they participate in the response of cells to the binding of a variety of extracellular ligands (*13*). Lck, for example, phosphorylates the T-cell antigen receptor following the recognition of an antigen-presenting cell by a T lymphocyte (*14*). Src kinases contain two protein interaction domains in addition to a kinase catalytic domain. The SH3 domain binds to polyproline II helices, and the SH2 domain binds to sites of tyrosine phosphorylation (*13*). Both domains can undergo intramolecular binding that leads to inhibition of the kinase (*13*).

Src kinases can be activated by the binding of protein ligands to their SH3 domains (*15*−*21*). The binding of these proteins is activating in part because it displaces the SH3 domain of the Src kinase from a binding site in the linker between the SH2 and catalytic domains of the Src kinase (*17*). Because WASP has been found to be a binding partner for Src SH3 domains, we asked whether it also functioned as an activator of Src. Unexpectedly, we found that WASP functioned as an inhibitor of Src kinases.

EXPERIMENTAL PROCEDURES

Cells. Human 293 cells (*22*) were maintained in Dulbecco−Vogt-modified Eagle's medium supplemented with 10% calf serum. The H-SB2 cell line (*23*), derived from a human lymphoblastic leukemia, was grown in RPMI medium supplemented with 10% fetal bovine serum.

Expression Constructs. Human WASP and the Tip protein of the herpesvirus saimiri (HVS) strain 484 were subcloned

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[1] Abbreviations: WAS, Wiskott−Aldrich syndrome; WASP, Wiskott−Aldrich syndrome protein; SH3, Src homology 3; HVS, herpesvirus saimiri; EVH1, enabled Vasp homology domain 1; Arp2/3, actin-related protein 2/3 complex; VCA, verprolin cofilin acidic domain; WIP, WASP interacting protein.

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into pCS3+MT (a gift from Jon Cooper) to append six Myc tags to the amino terminus. Fragments of WASP containing amino acids 1−83, 1−93, 1−104, 1−158, 83−502, 93−502, and 106−502 were generated by PCR and also cloned into pCS3+MT. v-Src was cloned into pRc/CMV (Invitrogen). A carboxy-terminal fragment of v-Src (amino acids 247−526) containing the catalytic subunit was generated by PCR and subcloned into pFLAG (a gift from Han-Kuei Huang) to add three FLAG tags to the amino terminus. Wild-type Lck and the constitutively active Lck F505 were subcloned into pCEP4 (Invitrogen). A carboxy-terminal fragment of Lck F505 (amino acids 229−510) containing the catalytic subunit was generated by PCR and subcloned into pCS3+MT. c-Abl, with a deletion of the SH3 domain (ΔSH3 c-Abl), was cloned into pCMX and provided by Jean Wang. All expression vectors utilized the cytomegalovirus immediate early promoter. The fidelity of all PCR products was verified by sequencing.

Transient Transfections. The 293 cells were transfected with 5 μg of each plasmid using a calcium phosphate-mediated transfection system (Invitrogen) or with Lipofectamine 2000 (Invitrogen). When only one experimental vector was used, 5 μg of an appropriate empty expression plasmid was used to bring the total amount of transfected DNA to 10 μg.

Cell Lysis and Immunoprecipitation. Transfected cells were lysed 48 h after transfection in 1 mL of 1% NP-40 lysis buffer (150 mM NaCl, 1% NP-40, 20 mM Tris, pH 8.2, 2 mM EDTA, and 200 μM Na_3VO_4). Proteins were immunoprecipitated from 200 to 300 μL of lysate with 1 μL of the appropriate antibody [mouse monoclonal anti-Myc antibody 9E10 (*24*), mouse monoclonal anti-Src antibody 2−17 (*25*), rabbit anti-Lck antibodies (*26*), 2 μg of mouse monoclonal anti-Abl K12 (Santa Cruz Biotechnology), or 1.3 μg of mouse monoclonal anti-FLAG antibody M2 (Sigma)] and 30 μL of a 10% Pansorbin suspension (Calbiochem).

Western Blotting. Western blotting with monoclonal anti-Myc antibody, monoclonal anti-Src antibody, rabbit anti-Lck antibodies, or rabbit anti-phosphotyrosine antibodies (*27*) was performed as described previously (*27*). Immunoblotting with anti-FLAG M2 antibody and monoclonal anti-Abl 8E9 (a gift from Jean Wang) was carried out with approximately 2 μg/mL antibody. Immobilon filters were blocked by incubation with 3% BSA prior to incubation with antibody. Bound antibodies were detected with ^{125}I-protein A and a typhoon phosphorimager (Molecular Dynamics) or enhanced chemiluminescence. Quantification was performed using ImageQuant software (Molecular Dynamics).

Production of Soluble Lck Protein. A plasmid encoding wild-type Lck with 62 amino acids deleted from the amino terminus to aid in protein solubilization was subcloned into pFASTBAC HT (Gibco, Life Technologies). The BAC-TO-BAC protocol (Gibco, Life Technologies) was used to generate the recombinant bacmids and baculoviruses. His-tagged Lck was isolated from infected High Five cells as described previously (*28*), and the His tag was removed by incubation with thrombin.

Peptide Synthesis. The WASP peptide amino acids 81−94 (KSYFIRLYGLQAGR) and the scrambled peptide (RLIGNLRFYASGYK) were synthesized on an Applied Biosystems 432A synergy peptide synthesizer using synergy peptide reagents.

In Vitro Kinase Assays. For in vitro protein kinase assays, approximately 30% of an immunoprecipitate was suspended in kinase buffer (40 mM Na-PIPES, pH 7.2, 10 mM $MnCl_2$) with [Val^5]angiotensin II (2 mM) as an exogenous substrate. Reactions were carried out with 15 μCi of [γ-^{32}P]ATP, and the reaction was stopped at the indicated times with 5% trichloroacetic acid. Phosphorylated substrate was collected using phosphocellulose paper, and incorporation was quantified by scintillation counting. Nonspecific kinase background was measured by assaying normal serum immunoprecipitates and/or performing the reactions without angiotensin.

To determine the effect of the WASP peptide on kinase activity, immunoprecipitated Lck or 3.5 μg of purified Lck was preincubated for 1 h at 4 °C with peptide and then assayed in the continued presence of the peptide. Alternatively, the immunoprecipitated kinase was incubated with peptide for 60 min at 4 °C, washed three times with Tris-buffered saline, and then assayed. Substrate affinity and reaction velocity were measured with both immunoprecipitated Lck and purified Lck. The K_m for ATP was measured using 2 mM [Val^5]angiotensin II and 2.5−50 μM ATP. The K_m for the peptide substrate was measured using ATP at a concentration of 15 μM and 0.5−4 mM [Val^5]angiotensin II.

RESULTS

Inhibition of Lck by WASP. To determine whether WASP functioned as an activator of Src kinases, we coexpressed Myc-tagged WASP and Lck by transient transfection of 293 cells. As a positive control, we also cotransfected cells with Lck and the Tip protein of HVS, a known activator of Lck. Immunoprecipitation with anti-Myc antibodies showed considerable complex formation between the WASP and Lck with approximately 20% of the Lck bound to WASP and 11% of WASP bound to Lck (Figure 1A). Strikingly, measurement in vitro of the kinase activity of the Lck protein that co-immunoprecipitated with WASP revealed that WASP functioned as a strong inhibitor of Lck (Figure 1B). The normalized activity of Lck bound to Myc-tagged WASP was reduced by 79% (Figure 1B). This contrasted with the effect of HVS Tip on Lck. The population of Lck bound to Tip exhibited 6-fold increased protein kinase activity when assayed in vitro (data not shown).

To determine whether WASP also inhibited Lck in vivo, we coexpressed WASP with an activated mutant of Lck, Lck F505, and examined the level of tyrosine phosphorylation of cellular proteins in the cotransfected cells. Expression of Lck F505 alone induced a noticeable increase in the level of tyrosine phosphorylation of cellular proteins in 293 cells (Figure 1C, lane 4). Coexpression of WASP reduced the tyrosine phosphorylation of most of these proteins (Figure 1C, lane 6). A similar effect was seen when untagged WASP was coexpressed with Lck F505 (data not shown). WASP itself was a prominent tyrosine-phosphorylated protein in the cotransfected cells (Figure 1C, lane 6). The reduced tyrosine phosphorylation of cellular protein in the cotransfected cells suggested that WASP inhibited Lck kinase activity in vivo as well as in vitro. Again, the inhibition by WASP contrasted with the effect of the HVS Tip protein on Lck. Cells

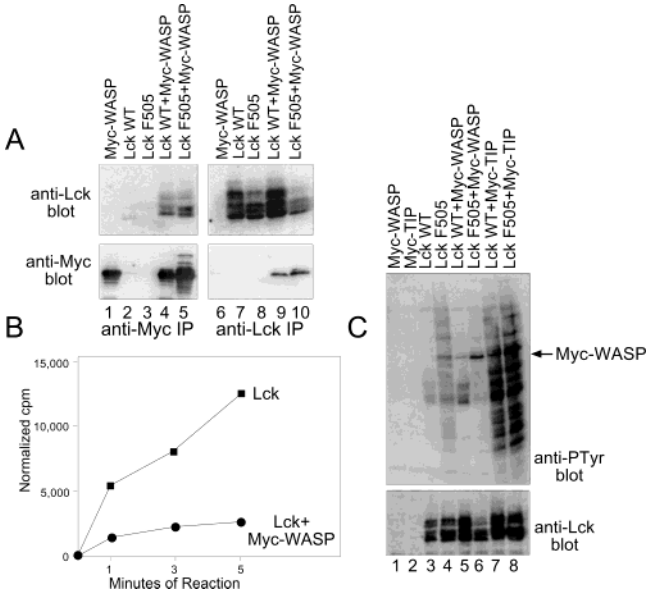

FIGURE 1: WASP binds to and inactivates Lck. (A) Human 293 cells were transfected singly or in combination with plasmids encoding wild-type Lck, Lck F505, and Myc-tagged WASP. Immunoprecipitation was performed with anti-Myc and anti-Lck antibodies, and the precipitates were analyzed by SDS−PAGE and Western blotting with anti-Lck or anti-Myc antibodies and ^{125}I-protein A. (B) Lck was isolated with anti-Lck antibody from 293 cells transfected with only wild-type Lck or with anti-Myc antibody from cells transfected with both wild-type Lck and Myc-tagged WASP. The activity of the two populations was assayed in vitro using [Val^5]angiotensin II as a substrate. A fraction of the immunoprecipitates was also subjected to immunoblotting with anti-Lck antiserum. The rates of substrate phosphorylation were normalized, and the data shown here present rates of angiotensin phosphorylation by equal amounts of Lck protein. (C) Lysates from the transfected cells in panel A or cells transfected with Lck or Lck F505 and Myc-tagged Tip were assayed by SDS−PAGE and Western blotting with anti-phosphotyrosine and Lck antibodies and ^{125}I-protein A. Labeled proteins were detected and quantified with a phosphorimager. The level of Lck F505 in the cells cotransfected with Lck F505 and Myc-WASP was 80% of that in the cells transfected with Lck F505 alone. Key: (A) upper panels, anti-Lck Western blot; lower panels, anti-Myc Western blots; lane 1, Myc-WASP, anti-Myc IP; lane 2, WT-Lck, anti-Myc IP; lane 3, Lck F505, anti-Myc IP; lane 4, WT-Lck + Myc-WASP, anti-Myc IP; lane 5, Lck F505 + Myc-WASP, anti-Myc IP; lane 6, Myc-WASP, anti-Lck IP; lane 7, WT-Lck, anti-Lck IP; lane 8, Lck F505, anti-Lck IP; lane 9, WT-Lck + Myc-WASP, anti-Lck IP; lane 10, Lck F505 + Myc-WASP, anti-Lck IP; (B) filled squares, wild-type Lck, anti-Lck IP; filled circles, wild-type Lck + Myc-WASP, anti-Myc IP; (C) upper panels, anti-PTyr Western blot; lower panels, anti-Lck Western blots; lane 1, Myc-WASP; lane 2, Myc-Tip; lane 3, WT-Lck; lane 4, Lck F505; lane 5, WT-Lck + Myc-WASP; lane 6, Lck F505 + Myc-WASP; lane 7, WT-Lck + Myc-Tip; lane 8, Lck F505 + Myc-Tip.

cotransfected with Tip and wild-type Lck (Figure 1C, lane 7) contained greatly elevated levels of tyrosine-phosphorylated proteins compared to cells expressing only wild-type Lck (Figure 1C, lane 3).

Inhibition of Abl by WASP. To determine whether the ability of WASP to bind to and inhibit protein kinases was unique to Lck, we coexpressed WASP with forms of three other Src kinases: wild-type and activated Fyn, wild-type and activated Src, and activated Hck. In all cases, we observed WASP to bind to and inhibit the kinases to an extent similar to that seen with Lck (data not shown). Cory et al. have also observed the binding of WASP to Lyn and Hck when the proteins are coexpressed under similar

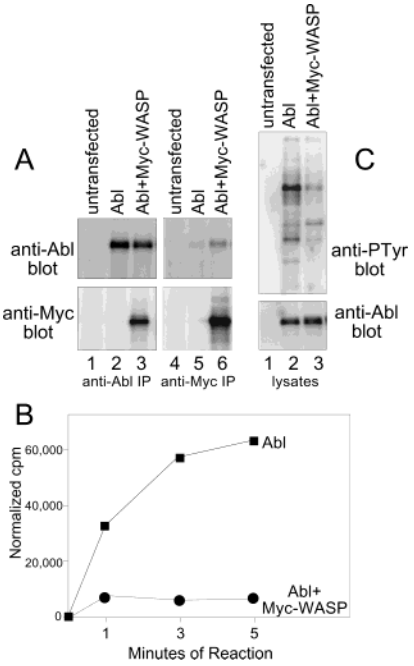

FIGURE 2: WASP binds to and inactivates Abl. Human 293 cells were transfected singly or in combination with plasmids encoding ΔSH3 c-Abl and Myc-tagged WASP. (A) Immunoprecipitation was carried out with anti-Abl and anti-Myc antibodies. The immunoprecipitates were analyzed by SDS−PAGE and Western blotting with anti-Abl or anti-Myc antibodies and ^{125}I-protein A. Labeled proteins were identified and quantified with a phosphorimager. (B) Abl was isolated from 293 cells transfected with Abl using anti-Abl antibody or from cells transfected with both Abl and Myc-WASP using anti-Myc antibody. The activity of the two Abl populations was assayed in vitro using [Val^5]angiotensin II as a substrate. A fraction of each of the immunoprecipitates was assayed by immunoblotting with anti-Abl antiserum for normalization of protein. The data shown here present rates of angiotensin phosphorylation by equal amounts of Abl protein. (C) Lysates were analyzed by SDS−PAGE and Western blotting with anti-phosphotyrosine and anti-Abl antibodies. Key: (A) upper panels, anti-Abl blots; lower panels, anti-Myc blots; lane 1, untransfected, anti-Abl IP; lane 2, ΔSH3 c-Abl, anti-Abl IP; lane 3, ΔSH3 c-Abl + Myc-WASP, anti-Abl IP; lane 4, untransfected, anti-Myc IP; lane 5, ΔSH3 c-Abl, anti-Myc IP; lane 6, ΔSH3 c-Abl + Myc-WASP, anti-Myc IP; (B) filled squares, ΔSH3 c-Abl transfection, anti-Abl IP; filled circles, ΔSH3 c-Abl + Myc-WASP, anti-Myc IP; (C) upper panel, anti-phosphotyrosine blot; lower panel, anti-Abl blot; lane 1, untransfected, lysate; lane 2, ΔSH3 c-Abl, lysate; lane 3, ΔSH3 c-Abl + Myc-WASP, lysate.

conditions (*29*). Consistently, WASP bound more extensively to activated forms of these kinases such as Lck F505 (data not shown). We therefore used activated forms for most of the subsequent experiments. To determine whether the inhibitory binding exhibited by WASP was restricted to Src kinases, we asked whether WASP could bind to the related tyrosine protein kinase Abl when the two were coexpressed in 293 cells during transient transfection. In this experiment, we used a form of c-Abl from which the SH3 domain had been deleted. This form is more active as a protein kinase than is wild-type c-Abl, and this allowed us to estimate the activity of the protein in the transfected cells. Similar to what we had seen with the Src kinases, WASP bound to c-Abl (Figure 2A). Anti-Myc antibody precipitated a third as much Abl as did anti-Abl antibodies (Figure 2A, lane 6), and anti-Abl antibodies precipitated two-thirds as much WASP as did anti-Myc antibodies (Figure 2A, lane 3). The population of Abl bound to WASP was phosphorylated on tyrosine to

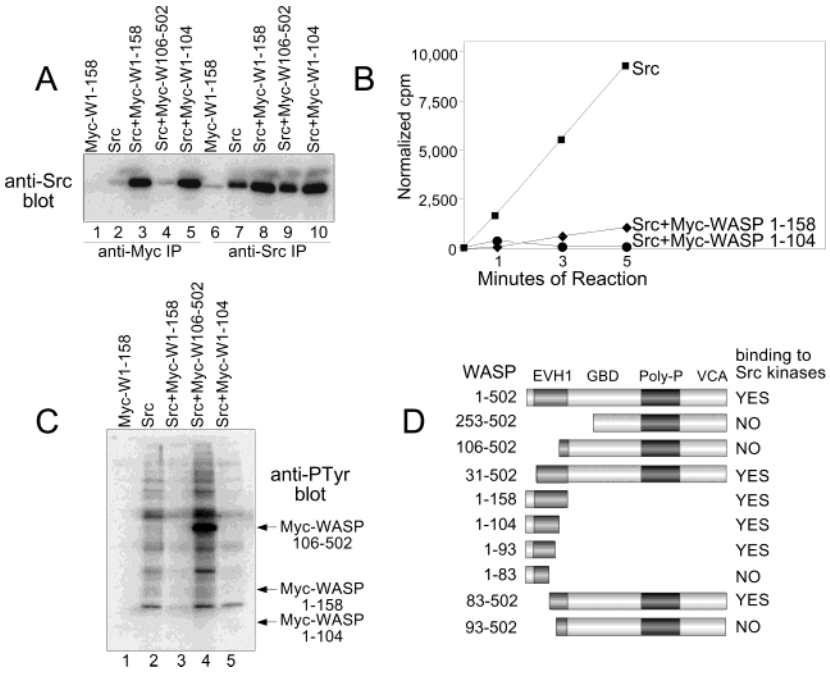

FIGURE 3: The first 104 amino acids of WASP bind to and inactivate Src. 293 cells were transfected with plasmids encoding v-Src alone or together with Myc-tagged truncated WASP containing amino acids 1−158, 106−502, or 1−104. (A) Src was isolated by immunoprecipitation using anti-Myc or anti-Src antibodies and assayed by SDS−PAGE and immunoblotting with anti-Src antibodies and ^{125}I-protein A. Labeled proteins were detected and quantified with a phosphorimager. (B) Src was isolated from 293 cells transfected with Src alone using anti-Src antibody or from cells transfected with both Src and either Myc-tagged WASP 1−158 or WASP 1−104 using anti-Myc antibody. The activity of the three Src populations was assayed in vitro using [Val^5]angiotensin II as a substrate. A portion of each of the immunoprecipitates was assayed by immunoblotting with anti-Src antiserum for normalization of the protein. The data shown here present rates of angiotensin phosphorylation by equal amounts of Src protein. (C) Lysates were analyzed by SDS−PAGE and immunoblotting using anti-phosphotyrosine antibodies and ^{125}I-protein A. (D) Summary of experiments mapping the binding site in WASP responsible for its association to Src kinases. The locations of the EVH1 domain (light gray box), the cdc42 GTPase binding domain (GBD), the proline-rich domain (poly-P, dark gray box), and the ARP2/3 binding domain (VCA) are indicated. Key: (A) anti-Src blot; lane 1, Myc-WASP 1−158, anti-Myc IP; lane 2, v-Src, anti-Myc IP; lane 3, v-Src + Myc-WASP 1−158, anti-Myc IP; lane 4, v-Src + Myc-WASP 106−502, anti-Myc IP; lane 5, v-Src + Myc-WASP 1−104, anti-Myc IP; lane 6, Myc-WASP 1−158, anti-Src IP; lane 7, v-Src, anti-Src IP; lane 8, v-Src + Myc-WASP 1−158, anti-Src IP; lane 9, v-Src + Myc-WASP 106−502, anti-Src IP; lane 10, v-Src + Myc-WASP 1−104, anti-Src IP; (B) filled squares, v-Src, Src IP; filled diamonds, v-Src + Myc-WASP 1−158, anti-Myc IP; filled circles, v-Src + Myc-WASP 1−104, anti-Myc IP; (C) anti-phosphotyrosine blot; lane 1, Myc-WASP 1−158, lysate; lane 2, v-Src, lysate; lane 3, v-Src + Myc-WASP 1−158, lysate; lane 4, v-Src + Myc-WASP 106−502, lysate; lane 5, v-Src + Myc-WASP 1−104, lysate.

a noticeably lower extent than was Abl expressed alone (data not shown). Further, when assayed in vitro, the population of Abl bound to WASP exhibited little or no kinase activity (Figure 2B). Consistent with this, the level of tyrosine phosphorylation of cellular proteins in cells expressing both c-Abl and WASP (Figure 2C, lane 3) was reduced relative to that seen in cells expressing c-Abl alone (Figure 2C, lane 2).

Mapping of the Binding Domain in WASP. WASP has been shown to bind to GST fusion proteins containing the SH3 domain of Src kinases through a proline-rich domain between residues 313 and 416 (*10*). To determine whether this region was involved in the observed inhibitory binding of WASP to tyrosine kinases, we coexpressed a series of deletion mutants of WASP with v-Src in 293 cells. Deletion of 344 or 398 amino acids from the carboxy terminus of WASP, which removed the cdc42 binding domain, the proline-rich domain, and the VCA domain (Figure 3D), had no effect on the binding to (Figure 3A, lanes 3 and 5) or inhibition of v-Src by WASP in vitro (Figure 3B) or in vivo (Figure 3C, lanes 3 and 5). Conversely, deletion of 105 residues from the amino terminus of the protein, which left untouched the cdc42 binding domain, the proline-rich domain, and the VCA domain, abolished the ability of WASP to bind to (Figure 3A, lane 4) and inhibit v-Src (Figure 3C, lane 4). The proline-rich region, as well as the VCA domain, and the cdc42 binding domain were all therefore apparently dispensable for the inhibitory interaction between WASP and tyrosine protein kinases. Rather, binding and inhibition appeared to require only the region of WASP between residues 1 and 104. Because this contains only half of the EVH1 domain, it appeared a functional EVH1 domain was also dispensable for binding. It should be noted that the 1−104 and 1−158 fragments of WASP inhibited the tyrosine phosphorylation of cellular proteins without becoming tyrosine phosphorylated themselves (Figure 3C, lanes 3 and 5). Conversely, the 106−502 fragment of WASP was heavily tyrosine phosphorylated but failed to inhibit the tyrosine phosphorylation of cellular proteins (Figure 3C, lane 4). This indicates that inhibition is not due simply to WASP functioning as an abundant, high-affinity polypeptide substrate. The results of all of the experiments with deletion mutants are summarized in Figure 3D.

Finer mapping of the sequences required for binding showed that whereas residues 1−93 of WASP bound to Lck, residues 1−83 did not (Figure 4A). Additionally, residues 83−502 of WASP bound to v-Src, but residues 93−502 did not (Figure 4B). This suggested that residues 83−93 were necessary for the binding. It is notable that these residues are predicted to be part of a β sheet (β4) within the EVH1

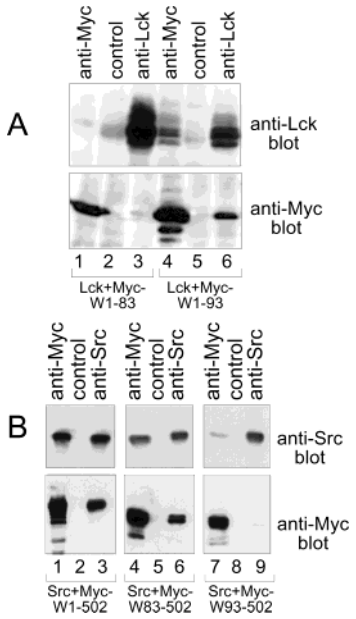

FIGURE 4: Amino acids 83−93 in WASP bind to and inactivate Src kinases. (A) 293 cells were transfected with Lck F505 and either Myc-tagged WASP 1−83 or Myc-tagged WASP 1−93. Protein complexes were isolated by immunoprecipitation with anti-Lck antibodies, anti-Myc antibody, or normal sera as a control. Isolated proteins were subjected to SDS−PAGE and Western blotting with anti-Myc and anti-Lck antibodies and detected by enhanced chemiluminescence. (B) 293 cells were transfected with v-Src and either Myc-tagged wild-type WASP, WASP 83−502, or WASP 93−502. Proteins were isolated by immunoprecipitation with anti-Myc, anti-Src antibodies, or normal sera as a control and analyzed by SDS−PAGE and immunoblotting with anti-Myc and anti-Src antibodies. Proteins were detected by enhanced chemiluminescence. Key: (A) upper panel, anti-Lck blot; lower panel, anti-Myc blot; lane 1, Lck F505 + Myc-WASP 1−83, anti-Myc IP; lane 2, Lck F505 + Myc-WASP 1−83, normal sera IP; lane 3, Lck F505 + Myc-WASP 1−83, anti-Lck IP; lane 4, Lck F505 + Myc-WASP 1−93, anti-Myc IP; lane 5, Lck F505 + Myc-WASP 1−93, normal sera IP; lane 6, Lck F505 + Myc-WASP 1−93, anti-Lck IP; (B) upper panels, anti-Src blots; lower panels, anti-Myc blots; lane 1, v-Src + Myc-WASP 1−502, anti-Myc IP; lane 2, v-Src + Myc-WASP 1−502, normal sera IP; lane 3, v-Src + Myc-WASP 1−502, anti-Src IP; lane 4, v-Src + Myc-WASP 83−502, anti-Myc IP; lane 5, v-Src + Myc-WASP 83−502, normal sera IP; lane 6, v-Src + Myc-WASP 83−502, anti-Src IP; lane 7, v-Src + Myc-WASP 93−502, anti-Myc IP; lane 8, v-Src + Myc-WASP 93−502, normal sera IP; lane 9, v-Src + Myc-WASP 93−502, anti-Src IP.

domain (*30*). To determine whether these amino acids were sufficient for binding and inhibition, we synthesized a peptide identical in sequence to residues 81−94. As a negative control, we synthesized a second peptide using the same 14 amino acids, save for replacing Gln with Asn, in random order (Figure 5A). Incubation of immunoprecipitated Lck from H-SB2 cells with the WASP 81−94 peptide inhibited Lck kinase activity by 65% (Figure 5B). The scrambled peptide had little or no effect on activity. Similar specific inhibition by the WASP peptide was seen with soluble Lck isolated from insect cells and immunoprecipitated v-Src (data not shown). In some experiments, inhibition could be seen with 0.3 mM peptide (data not shown). Half-maximal inhibition generally required 1 mM peptide. Binding of the peptide was apparently quite stable. Excess peptide could be washed away after binding, and the kinase did not regain activity (Figure 5C).

Effect of WASP on the Catalytic Properties of Lck. Finally, we asked whether the inhibition resulting from the binding

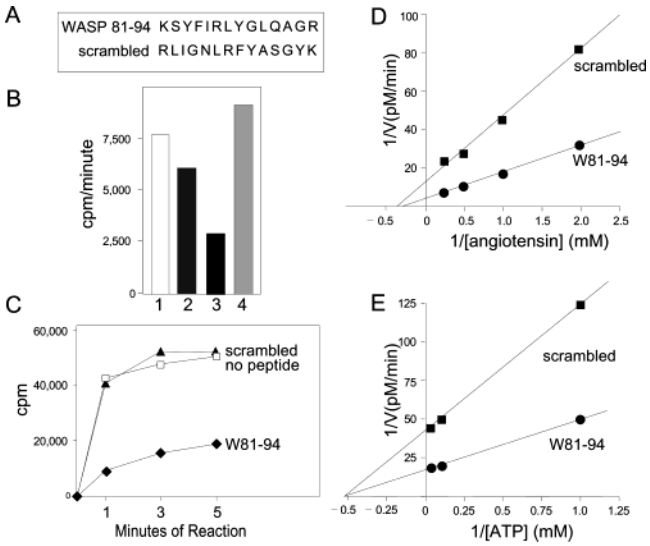

FIGURE 5: A synthetic peptide containing amino acids 81−94 of WASP inhibits the activity of Lck. (A) Sequence of the peptide containing amino acids 81−94 of WASP and the control peptide with similar amino acids in a scrambled order. (B) Lck was isolated from H-SB2 cells, and its kinase activity in the presence of the WASP peptide or scrambled peptide was assayed in vitro using [Val^5]angiotensin II as a substrate. (C) Lck from H-SB2 cells was incubated with WASP peptide, scrambled peptide, or buffer alone, washed extensively, and assayed for its activity in vitro using [Val^5]-angiotensin II as a substrate. (D) The effect of the WASP 81−94 peptide on the affinity of Lck for the peptide substrate [Val^5]-angiotensin II was measured by assaying the kinase using 15 μM ATP and varying amounts of [Val^5]angiotensin II. Each rate of reaction was determined in triplicate. The data are presented in the form of a double-reciprocal plot. (E) The effect of the WASP 81−94 peptide on the affinity of Lck for ATP was measured by assaying the kinase using 2 mM [Val^5]angiotensin II and varying amounts of ATP. Each rate of reaction was determined in triplicate. The data are presented in the form of a double-reciprocal plot. Key: (A) WASP peptide amino acids 81−94, scrambled peptide; (B) column 1, no peptide; column 2, 1 mM WASP peptide; column 3, 3 mM WASP peptide; column 4, 3 mM scrambled peptide; (C) open squares, Lck IP, no peptide; filled triangles, Lck IP, 3 mM scrambled peptide; filled diamonds, Lck IP, 3 mM WASP peptide; (D) closed circles, scrambled peptide; closed squares, WASP 81−94 peptide; (E) closed circles, scrambled peptide; closed squares, WASP 81−94 peptide.

of the WASP 81−94 peptide resulted from competition for substrate binding, a decreased affinity for substrates, or a reduced rate of catalysis. To do this, we measured the K_m of Lck for ATP and for a peptide substrate, angiotensin, in the presence of either the WASP inhibitory peptide or the scrambled peptide. We measured these values using both Lck isolated by immunoprecipitation from the human T leukemia cell line HSB-2 (Figure 5D,E) and a soluble Lck protein purified from insect cells (data not shown). Each assay was performed in triplicate under conditions where substrate phosphorylation exhibited a linear dependence on enzyme concentration and time. The two preparations gave similar results. Binding of the WASP 81−94 peptide had little effect on the K_m for the angiotensin substrate, approximately 4 mM (Figure 5D), or for ATP, approximately 2 μM (Figure 5E). In all experiments, the V_{max} of Lck incubated with the WASP peptide was reduced by 3−4-fold (Figure 5D,E).

Mapping the Binding Domain in Src. To map the region in Src kinases with which the peptide interacts, we examined the binding of WASP to fragments of Src in cotransfected cells. A 1−158 fragment of WASP containing the Src kinase

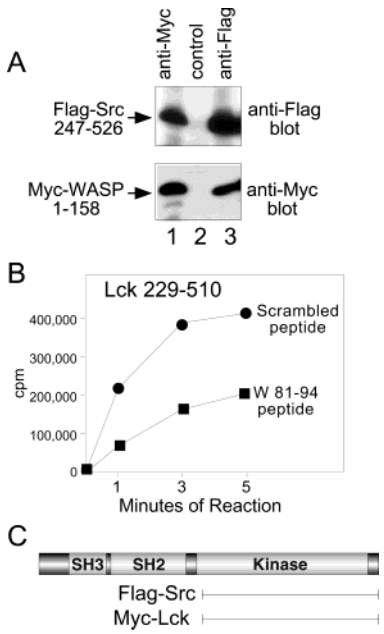

FIGURE 6: WASP binds to the catalytic domains of Src and Lck. (A) 293 cells were transfected with a plasmid encoding Flag-tagged v-Src 247−526 and Myc-tagged WASP 1−158. Protein complexes were isolated by immunoprecipitation with anti-Myc or anti-Flag antibodies and analyzed by SDS−PAGE and immunoblotting with these same antibodies and enhanced chemiluminescence. (B) A Myc-tagged carboxy-terminal fragment of Lck was isolated from 293 cells transfected from a plasmid encoding Lck F505 amino acids 229−510 by immunoprecipitation with anti-Myc antiserum. The activity in the presence of either the scrambled or WASP 81−94 peptide was assayed in vitro using [Val^5]angiotensin II as a substrate. (C) Diagram of Src and Lck indicating the tagged carboxy-terminal fragments used in these experiments. Key: (A) lane 1, Flag-Src 247−526 + Myc-WASP 1−158, anti-Myc IP; lane 2, Flag-Src 247−526 + Myc-WASP 1−158, normal sera IP; lane 3, Flag-Src 247−526 + Myc-WASP 1−158, anti-Flag IP; (B) filled squares, Lck F505 229−510, anti-Lck IP, 3 mM WASP 81−94 peptide; filled circles, Lck F505 229−510, anti-Lck IP, 3 mM scrambled peptide.

binding site bound efficiently to a carboxy-terminal fragment (amino acids 247−526) of Src containing the catalytic domain but lacking the SH2, SH3, and amino-terminal domains (Figure 6A). Similarly, we asked whether the inhibitory WASP 81−94 peptide could inhibit the Lck catalytic domain in vitro. Inhibition of the isolated catalytic domain by the peptide was comparable to that seen with the full-length kinase (Figure 6B). It seems clear therefore that the 81−94 WASP peptide can bind to and inhibit the catalytic domain of Src kinases in the absence of the noncatalytic amino-terminal portion of the kinases.

DISCUSSION

We have found that the WASP protein binds to and inhibits a number of tyrosine protein kinases. The basis of this inhibitory binding is the interaction of amino acids 83−93 in WASP with the catalytic domain of the kinases. We have not yet identified the binding site within the catalytic domain. Inhibition appears to be noncompetitive and to result from reduced catalytic efficiency. The peptide did not alter the K_m of Lck for ATP or peptide substrate significantly.

The ability of a 14 amino acid peptide from WASP to inhibit the catalytic efficiency of Src kinases and Abl suggests that the identification of small, nonpeptidic compounds that bind to the same site and mimic the inhibitory effect of the peptide may be possible. Inhibitors of this sort would be novel and potentially useful because most small molecule inhibitors of kinases do so by interfering with the binding of ATP. The WASP peptide did not compete with ATP. Mapping of the site in the kinase to which the peptide binds is clearly of interest and importance.

We have observed complex formation between WASP and either Src or Abl kinases when the proteins are expressed together by cotransfection of human 293 embryonic kidney cells. The extent to which a similar interaction occurs when the two proteins are expressed at physiological levels is an important question. WASP has been reported to bind to Fyn in human U-937 cells (*10*) and to a Src kinase in human Jurkat cells exposed to SDF-1α (*31*). We, however, have detected little significant binding between WASP and Abl or Src kinases in a number of hematopoietic cell lines. It is possible that we have not looked for binding at a physiologically relevant time or that there are technical problems with detecting the complex through co-immunoprecipitation.

Alternatively, interaction between WASP and Src kinases in hematopoietic cells may be interfered with by competitive binding of the WASP interacting protein, WIP, to the EVH1 domain of WASP. We readily detected a complex between WASP and WIP in most hematopoietic cells (data not shown). A long segment of WIP makes contact with the EVH1 domain of the closely related N-WASP protein (*32*), and residues 480−485 of WIP are likely to occlude the β4 sheet that is homologous to residues 83−93 in WASP. With WIP bound to the EVH1 domain in this manner, it is unlikely that binding to a kinase can also occur. The interaction between WASP and Src and Abl kinases may be easiest to detect when the proteins are expressed at a level greater than that of endogenous WIP.

If WASP is a bona fide inhibitor of Src kinases in vivo, inappropriate or excessive expression of WASP should interfere with Src kinase signaling. The studies of Sato et al. (*33*) are consistent with this prediction. T-cell activation requires the activity of Lck (*34*). Sato and colleagues have developed a strain of transgenic mice expressing the amino-terminal 171 amino acids of WASP, a fragment containing the inhibitory sequences we have described here. These transgenic mice exhibit impaired T-cell activation. Because this phenotype could result from inhibition of the Lck kinase by the WASP fragment in T cells (*33*), this finding suggests that the inhibitory sequence in WASP may exhibit the same activity in mice that we have seen in cell culture.

Src and Abl kinases may also be subject to inhibition by proteins other than WASP that contain sequences similar to residues 81−93 in WASP. A number of proteins, including at least one Rho GAP (*35*), contain a sequence motif that is identical to 7 of 8 consecutive residues between residues 83 and 93 in WASP. One of these proteins might bind to Src kinases or Abl in a manner similar to that that we have seen with WASP. We are investigating this possibility.

Residues 83−93 in WASP are part of an EVH1 domain that binds ligands containing the motif FPPPP (*36*). Similar domains are found in the closely related protein N-WASP (*32*) and in Mena (*37*), among others. We asked whether these two proteins also bound to Lck and Abl but observed no significant binding (data not shown). This may not be surprising in that N-WASP and Mena differ in sequence from

WASP at 5 of 11 and 9 of 11 positions in the β4 strand, respectively.

Despite the fact that WASP binds to GST fusion proteins containing Src kinase SH3 domains (*10*−*12*), we did not observe binding of Src kinases to WASP that involved the Src SH3 domain and the proline-rich domain of WASP in cotransfected cells. Deletion of the SH3 domain of Lck did not abolish binding to WASP (data not shown), and deletion of the proline-rich domain of WASP had no effect on binding to Src (Figure 3A,D). Binding between WASP and the SH3 domains of Src kinases may therefore occur more efficiently in vitro than in cells.

We found WASP to bind to and inhibit all four of the Src kinases we tested. In addition, it bound to and inhibited Abl, a non-Src tyrosine kinase that is closely related to the Src family. We do not know how general is the ability of WASP to bind to protein kinases. Experiments with CDK2/cyclin E and mTOR, both Ser/Thr-specific protein kinases, indicated that the peptide did not inhibit these kinases with the potency observed with Src and Abl (data not shown). It will be interesting to determine how broad is the binding specificity of residues 83−93 in WASP.

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