MOLECULAR AND CELLULAR BIOLOGY, Oct. 1995, p. 5725–5731 Vol. 15, No. 10
0270-7306/95/$04.00+0

Wiskott-Aldrich Syndrome Protein Physically Associates with Nck through Src Homology 3 Domains

OCTAVIO M. RIVERO-LEZCANO, ANTONIO MARCILLA, JEANNE H. SAMESHIMA, AND KEITH C. ROBBINS*

Laboratory of Cellular Development and Oncology, National Institute of Dental Research, Bethesda, Maryland 20892-4330

Received 19 April 1995/Returned for modification 28 May 1995/Accepted 27 July 1995

In the second of a series of experiments designed to identify p47^{nck}-Src homology 3 (SH3)-binding molecules, we report the cloning of SAKAP II (Src A box Nck-associated protein II) from an HL60 cDNA expression library. This molecule has been identified as a cDNA encoding the protein product of *WASP*, which is mutated in Wiskott-Aldrich syndrome patients. Studies in vivo and in vitro demonstrated a highly specific interaction between the SH3 domains of p47^{nck} and Wiskott-Aldrich syndrome protein. Furthermore, anti-Wiskott-Aldrich syndrome protein antibodies recognized a protein of 66 kDa by Western blot (immunoblot) analysis. In vitro translation studies identified the 66-kDa protein as the protein product of *WASP*, and subcellular fractionation experiments showed that p66^{WASP} is mainly present in the cytosol fraction, although significant amounts are also present in membrane and nuclear fractions. The main p47^{nck} region implicated in the association with p66^{WASP} was found to be the carboxy-terminal SH3 domain.

Signal transduction pathways that connect the cell surface with the nucleus utilize protein-protein interactions. Two of the most studied elements that participate in the recognition of specific protein targets are the Src homology 2 (SH2) and Src homology 3 (SH3) domains, first described as noncatalytic components of the Src family of protein tyrosine kinases (36). SH2 domains associate with proteins phosphorylated on tyrosine residues within a specific amino acid sequence motif (4, 44). In contrast, SH3 domains recognize proline-rich segments (8) without any requirements for protein modification, allowing for the constitutive association of this domain with its target proteins.

No common functional features have been found among either the SH3-containing proteins or the SH3-binding proteins. For instance, the SH3 domains of the tyrosine kinases Lck, Fyn, and Abl associate with the p85 subunit of pI3 kinase (23); the adaptor molecule Grb2, which possesses two SH3 domains, links receptor tyrosine kinases to the SH3-binding protein Sos, a regulator of small G proteins (12); and the SH3 domain of the cytoskeletal protein α-spectrin binds to the epithelial sodium channel (41). Such heterogeneity makes it difficult to predict the putative ligands for each of the known SH3 domains.

The *nck* gene was initially isolated from a human melanoma cDNA library, and its product is a 47-kDa cytosolic protein exclusively composed of one SH2 and three SH3 domains (30). This protein is ubiquitously expressed, and its function has not been defined. Although activated tyrosine kinase receptors (6, 31, 34, 35) and insulin receptor substrate-1 (29) have been shown to bind to the SH2 domain of p47^{nck}, the proteins which associate with its SH3 domains are less well characterized. In vitro studies have shown variability in the association of Nck with the Abl protein-tyrosine kinase (17, 38) while no interaction with dynamin, another known SH3-binding protein, has been found (42). Recent studies have shown the association of Nck SH3 domains with two other proteins: Sos, a guanine nucleotide exchange factor for Ras (21), previously described as binding to the SH3-containing protein Grb2 (12); and a serine/threonine kinase, designated NAK (7). Both molecules exhibit preferential binding to the second SH3 domain of Nck.

In order to identify other proteins that specifically bind to p47^{nck}, we expressed its three SH3 domains as a fusion protein to screen Raw 264.7 (a mouse macrophage-like cell line) and HL60 (a human promyelomonocytic cell line) cDNA expression libraries. The first clone characterized, SAKAP I, corresponded to the proto-oncogene c-*cbl* (40), the cellular homolog of v-*cbl*, originally isolated from Cas NS-1, a murine retrovirus that induces pre-B- and pro-B-cell lymphomas (28). The function of the protein product of its cellular counterpart, p120$^{c\text{-}cbl}$, has not yet been determined, but it has been found to associate in vivo with p56^{lyn} and p47^{nck} (32, 40). Recent studies have implicated p120$^{c\text{-}cbl}$ in tyrosine kinase signaling pathways because it is phosphorylated in tumors induced by activated Abl tyrosine kinase (5).

In the present study, we report the analysis of a second cDNA clone, SAKAP II, identified as encoding the protein product of the gene *WASP*. This gene was recently isolated by a positional cloning strategy and was found to be mutated in several Wiskott-Aldrich syndrome (WAS) patients (11, 27). WAS is an X chromosome-linked recessive disorder which is associated with severe thrombocytopenia, eczema, impaired humoral and cellular immunity, and increased susceptibility to lymphoid malignancies (2, 45). Our findings begin to elucidate the molecular nature of WAS protein (WASP).

MATERIALS AND METHODS

Cell culture. Human promyelomonocytic HL60 cells (10) were maintained in RPMI 1640 medium containing 10% fetal bovine serum. To induce differentiation toward the monocytic lineage, exponentially growing cells were subcultured at a density of 10^5 cells per ml for 48 h in the presence of 50 nM 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) (Sigma Chemical Co.). Monkey kidney COS-7 cells (18) were maintained in Dulbecco modified Eagle medium containing 10% fetal bovine serum.

Antibodies. Rabbit polyclonal anti-Fyn (FYN3), anti-Cbl (C-15), and anti-Sp1 (PEP 2) antibodies were purchased from Santa Cruz Biotechnology; rabbit

* Corresponding author. Mailing address: Laboratory of Cellular Development and Oncology, National Institute of Dental Research, National Institutes of Health, Bldg. 30, Rm. 211, 9000 Rockville Pike, Bethesda, MD 20892-4330. Phone: (301) 496-3303. Fax: (301) 402-0823. Electronic mail address: Keith_Robbins@NIH.GOV.

polyclonal anti-Lyn antibody was purchased from Upstate Biotechnology Inc.; goat anti-mouse polyclonal antibody conjugated with horseradish peroxidase was purchased from ICN Biomedical; and goat anti-rabbit polyclonal antibody conjugated with horseradish peroxidase was purchased from Cappel. Rabbit polyclonal anti-Nck and anti-gluthatione *S*-transferase (GST) antibodies have been described elsewhere (40). Antibodies were raised in rabbits against a GST fusion protein containing the first 441 amino acids of WASP.

Cloning and sequencing. Nck SH3-GST fusion protein was used to screen a λZAP (Stratagene) cDNA library obtained from TPA-differentiated HL60 cells as described previously (40). Nucleotide sequencing of the isolated clones utilized dideoxy chain termination technology (Sequenase version 2.0; U.S. Biochemical Corp.). GenBank and EMBL data bases were screened with the BLAST program (3).

Expression vector construction and transfection of COS-7 cells. pcDNA3 vector was purchased from Invitrogen. pcDNA3-*fyn*, a kind gift from Hidemi Teramoto, was constructed by cloning a 2.4-kilobase pair (kb) *Bam*HI fragment derived from Zip-*fyn* (25). pcDNA3-*WASP* was constructed by cloning a 1.8-kb *Asp* 718-*Bam*HI fragment derived from the original pBluescript-*WASP* clone. One microgram of each plasmid DNA was transfected into COS-7 cells by the calcium phosphate precipitation technique, as modified by Wigler et al. (49).

Preparation of GST fusion proteins. Fgr-SH3-GST and Fyn-SH3-GST fusion proteins have been described elsewhere (39). Phospholipase Cγ (PLCγ)-SH3-GST, α-spectrin–SH3–GST, Eps 8-SH3-GST, the three SH3 domains of Nck-GST (3K), and full-length Nck (K) fusion proteins have been previously described (40). The DNA fragment encoding the amino-terminal SH3 domain of Nck (3KI, amino acids 4 to 98) was amplified by PCR from the *nck* cDNA and subcloned into the pGEX-2T vector (Pharmacia) to facilitate expression as a GST fusion protein. The *Hin*dII-*Bst*EII (Klenow end-filled) fragment of *nck*, containing the middle SH3 domain (3KII, amino acids 98 to 170), was ligated into blunt-ended *Eco*RI-cut pGEX-2T. The *Bst*EII-*Stu*I (Klenow end-filled) fragment of *nck*, containing the carboxy-terminal SH3 domain (3KIII, amino acids 170 to 270), was ligated into blunt-ended *Xma*I-cut pGEX-2T. Point mutations within the individual SH3 domains of Nck were generated in a two-step PCR procedure with two internal oligonucleotides encompassing the point mutation and two oligonucleotides upstream and downstream of the SH3 domains, as described by Ho et al. (20). Constructs were subcloned in pGEX-4T-3 (Pharmacia). In this way, fusion proteins were obtained with proline 243 (within the carboxyl-terminal SH3 domain) changed to leucine in the three SH3 domains of Nck-GST (3K); prolines 52, 156, and 243 (one in each SH3 domain), changed to leucines in the three SH3 domains of Nck-GST (3K); and proline 243 changed to leucine in the carboxy-terminal SH3 domain-GST fusion protein (3KIII). The *Eco*RI fragment of WASP (amino acids 1 to 441) was ligated into *Eco*RI-cut pGEX-4T-3. All constructs were verified by sequencing through the entire portion obtained by PCR. Purification of GST fusion proteins has been described previously (39).

Immunoprecipitation and Western blot (immunoblot) analysis. Immunoprecipitation of proteins from HL60 cell lysates and Western blot analysis were performed as previously described (39). When denaturing conditions were necessary, cells were lysed by addition of boiling 1% sodium dodecyl sulfate (SDS)–10 mM Tris-HCl (pH 7.4), transferred to a microcentrifuge tube, and boiled for an additional 5 min. After brief sonication and centrifugation, lysates were diluted 10-fold with regular lysis buffer without SDS (39) and used for immunoprecipitation. For antibody competition experiments, antibodies were incubated overnight with 2 μg of the corresponding GST fusion protein coupled to agarose beads and centrifuged and the supernatant was added to cell lysates for immunoprecipitation. After SDS-polyacrylamide gel electrophoresis (PAGE), proteins were transferred to polyvinylidene difluoride membranes, detected by enhanced chemiluminescence (ECL, Amersham), and exposed to Hyperfilm-ECL (Amersham) as described previously (39). For reprobing, membranes were exhaustively washed with 100 mM glycine (pH 2.5), blocked with 10 mM Tris-HCl (pH 7.5)–150 mM NaCl–4% bovine serum albumin for 1 h, and probed with a different primary antibody.

In vitro transcription and translation. One microgram of DNA was transcribed from the T3 promoter present in pBluescript II (Stratagene), the vector used for the original cloning. Translation in vitro was accomplished in the presence of [^{35}S]methionine with the TNT coupled reticulocyte lysate system from Promega. A total of 5 and 10 μl of the reaction mixture were diluted in 100 μl of lysis buffer and incubated with anti-WASP antibodies and Gammabind G-Sepharose (Pharmacia) or SH3-GST fusion proteins coupled to agarose beads (Sigma), respectively. After extensive washing of the beads, bound material was analyzed by SDS-PAGE.

Subcellular fractionation. HL60 cells were lysed in a hypotonic buffer, and proteins were isolated as nuclear, cytosolic, and membrane fractions as described elsewhere (16). Each fraction was boiled in Laemmli buffer and analyzed for the presence of a variety of proteins by Western blot.

RESULTS

Cloning of SAKAP II, a p47^{nck}-binding protein. In a previous study, we reported the cloning of SAKAP I from a Raw 264.7 cDNA library with the three SH3 domains of Nck-GST fusion protein as a probe. Detection of positive plaques was accomplished by means of anti-GST antibodies and ^{125}I-labeled protein A (40). The same procedure was followed to screen a cDNA library from TPA-differentiated HL60 cells. Four positive plaques were obtained after screening 10^6 recombinant phage. Sequencing and restriction mapping analysis confirmed that all four isolates represented a unique clone of 1.8 kb. A GenBank search revealed homology with *WASP*, a recently isolated gene (11). The 5′ end of our clone corresponded to nucleotide 19 of the published sequence, and no stop codons were present between this nucleotide and the initiation codon at nucleotides 34 to 36. The sequence of our gene differed from the reported sequence (11) but perfectly corresponded with that obtained by Kwan et al. (27).

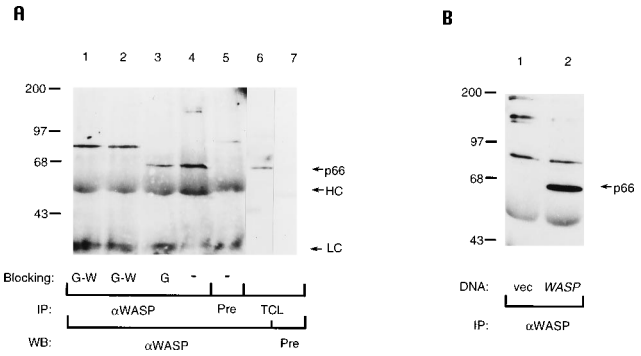

FIG. 1. Western blot analysis of anti-WASP immunoprecipitates. (A) A total of 2 μg of GST-WASP fusion protein (lane 1) and 3 mg of protein from HL60 cell lysates (lanes 2 through 5) were immunoprecipitated (IP) with anti-WASP antibody (αWASP, lanes 1 through 4) or preimmune serum (Pre, lane 5), fractionated by SDS–10% PAGE, and analyzed by Western blotting. A total of 20 μg of protein from total cell lysates (TCL) was identically analyzed (lanes 6 and 7). For competition experiments, anti-WASP antibody was incubated in the presence of GST-WASP fusion protein (G-W, lanes 1 and 2) or GST protein (G, lane 3) as described in Materials and Methods. Membranes were incubated for Western blot (WB) with anti-WASP antibody (αWASP, lanes 1 through 6) or anti-WASP preimmune serum (Pre, lane 7). The positions of p66 and the heavy and light chains of the antibody (HC and LC, respectively) are indicated. (B) COS-7 cells were transfected with either pcDNA3 vector (vec, lane 1) or pcDNA3-*WASP* (*WASP*, lane 2) and lysed after 64 h. Total cell lysates were immunoprecipitated with anti-WASP (αWASP) antibody, fractionated by SDS–10% PAGE, and Western blotted with anti-WASP antibody as a probe. The position of p66 is indicated. Molecular mass standards are shown in kilodaltons.

Anti-WASP antibody recognizes a protein of 66 kDa. In order to characterize the protein encoded by our *WASP* clone, we raised a polyclonal antibody against the first 441 amino acids expressed as a GST fusion protein. After immunoprecipitation and Western blot analysis, this antibody was found to recognize a protein of 66 kDa (Fig. 1A, lane 4), which was not immunoprecipitated by the preimmune serum (lane 5). A protein of the same size can be detected in a total cell lysate probed with anti-WASP antibody (lane 6) but not with preimmune serum (lane 7). To test the specificity of the antibody, we incubated anti-WASP in the presence of GST and GST-WASP fusion protein. In these conditions, GST-WASP (lane 2), but not GST (lane 3), was able to block the capacity of the antibody to recognize p66. The 82-kDa band observed in lane 2 is a bacterial contaminating protein, because it was also detected in an immunoprecipitation in the presence of GST-WASP fusion protein but in the absence of HL60 cell lysate (lane 1). Anti-WASP antibody also detected two bands of approximately 150 kDa in lane 4, but not in lane 3.

To analyze WASP, we expressed the original *WASP* clone in COS-7 cells. Only one extra band was observed in cells transfected with *WASP* (Fig. 1B, lane 2) compared with COS-7 cells

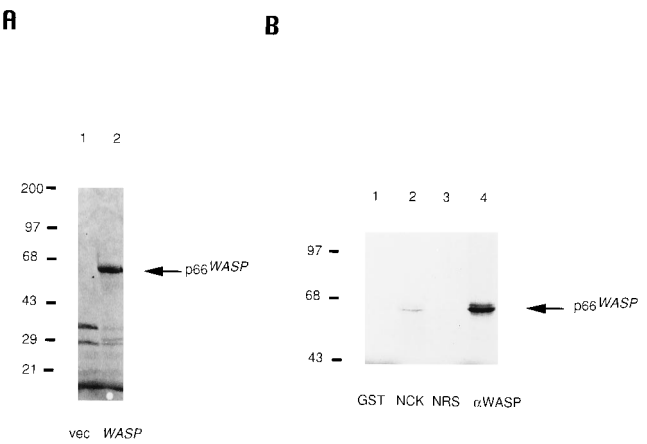

FIG. 2. *WASP* transcription and translation in vitro. A total of 1 μg of DNA was transcribed from the T3 RNA promoter in the pBluescript II vector and translated in a single 50-μl reaction mixture containing [^{35}S]methionine. (A) Transcription and translation of pBluescript II vector (vec, lane 1), and pBluescript II vector containing *WASP* cDNA (*WASP*, lane 2). Reaction mixtures were boiled in Laemmli buffer, fractionated by SDS–5 to 15% gradient PAGE, dried, and exposed to X-Omat AR film (Eastman Kodak Co.). The position of p66^{WASP} is indicated. (B) A total of 10 μl of a coupled transcription/translation reaction mixture containing *WASP* cDNA was incubated in the presence of 5 μg of GST (lane 1) or GST-p47^{nck} fusion protein (lane 2). A total of 5 μl of reaction mixture was immunoprecipitated with nonreactive sera (NRS, lane 3) or anti-WASP antibody (αWASP, lane 4). Reaction mixtures were boiled in Laemmli buffer, fractionated by SDS–7.5% PAGE, dried, and exposed to X-Omat AR film. The position of p66^{WASP} is indicated. Molecular mass markers are indicated in kilodaltons.

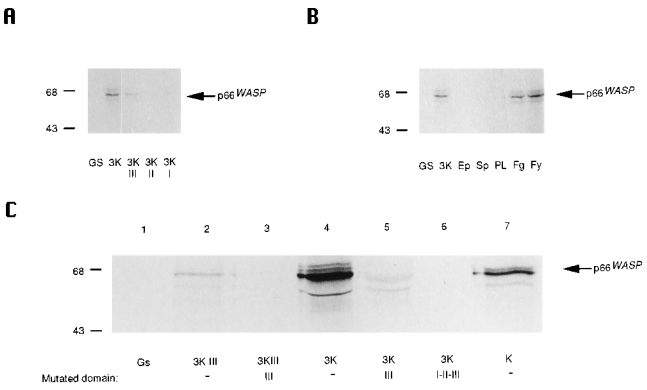

FIG. 3. In vitro binding of p66^{WASP} to GST-SH3 fusion proteins. ^{35}S-labeled p66^{WASP} was synthesized by coupled transcription/translation reactions as described in Materials and Methods, and 10 μl of the reaction mixture was incubated in the presence of 5 μg of various GST-SH3 fusion proteins. Bound proteins were fractionated by SDS–10% PAGE, dried, and exposed to X-Omat AR film. (A) ^{35}S-labeled p66^{WASP} was incubated in the presence of GST (GS), the three Nck SH3 domains-GST (3K), the carboxy-terminal SH3 domain of Nck-GST (3K III), the middle Nck SH3-GST (3K II), and the amino-terminal Nck SH3-GST (3K I) fusion proteins. The position of p66^{WASP} is indicated. (B) ^{35}S-labeled p66^{WASP} was incubated in the presence of GST (GS), the three Nck SH3 domains-GST (3K), Eps 8 SH3-GST (Ep), α-spectrin SH3-GST (Sp), PLCγ 1 SH3-GST (PL), Fgr SH3-GST (Fg), and Fyn SH3-GST (Fy) fusion proteins. The position of p66^{WASP} is indicated. (C) ^{35}S-labeled p66^{WASP} was incubated in the presence of GST (lane 1), the carboxy-terminal Nck SH3-GST (lane 2), the carboxy-terminal Nck SH3-GST with proline 243 changed to leucine (lane 3), the three Nck SH3 domains-GST (lane 4), the three Nck SH3 domains-GST with proline 243 (within the carboxy-terminal SH3 domain) changed to leucine (lane 5), the three Nck SH3 domains-GST with prolines 52, 156, and 243 (one in each SH3 domain) changed to leucine (lane 6), and full-length Nck-GST (lane 7) fusion proteins. The position of p66^{WASP} is indicated. Molecular mass markers are indicated in kilodaltons.

expressing the vector alone (pcDNA3, lane 1). The molecular mass of this band was 66 kDa, accurately matching the size of p66. Proteins of 87 and 150 kDa observed in this experiment were not reproducible (see Fig. 4A, lanes 1 and 2), indicating that these bands are nonspecific. These results provided strong evidence that p66 is encoded by *WASP*. The relationship between the other proteins scored in Western blot analysis and the *WASP* gene remains unclear.

***WASP* is translated in vitro as p66.** The expression of a 66-kDa protein in COS-7 cells showed that p66 was a protein product of *WASP*. This size markedly differed from the predicted molecular mass deduced from the cDNA sequence (53 kDa). This difference might be a consequence of posttranslational modifications in HL60 or COS-7 cells. To address this possibility, we performed in vitro translation experiments with the T3 RNA promoter present in the original cloning vector by using the TNT coupled reticulocyte lysate system. The reaction products were analyzed by SDS-PAGE, in which four ^{35}S-labeled proteins could be observed (Fig. 2A, lane 2). The major band migrated as a 66-kDa protein, the size of the most prominent band detected in anti-WASP immunoprecipitates from HL60 cells and from *WASP*-transfected COS-7 cells. Two other bands of 29 and 34 kDa were also observed when translated RNA from pBluescript vector was analyzed (lane 1), indicating that they are not *WASP* translational products. Finally, a band of 30 kDa detected only as a *WASP* translational product (lane 2) might be a degradation product of WASP or the result of the incomplete translation of a *WASP* mRNA.

In order to confirm that in vitro-translated p66 could associate with Nck, thus reproducing the result obtained in the cloning of SAKAP II, we incubated 10 μl of the translation reaction mixture with 5 μg of a GST fusion protein containing full-length Nck. p66 associated with GST-Nck (Fig. 2B, lane 2) but not with GST alone (lane 1). Moreover, p66 was detected in anti-WASP immunoprecipitates from 5 μl of the translation reaction mixture (lane 4) but not when a nonreactive serum was used for immunoprecipitation (lane 3). This result confirms the reactivity of the anti-GST-WASP antiserum with p66. On the basis of this evidence, we conclude that p66 is the primary translational product of the *WASP* cDNA and that the discrepancy with the theoretical size of the *WASP* gene product might be ascribed to abnormalities in its gel migration or to putative posttranslational modifications that also occur in reticulocyte lysate.

In vitro association of p66^{WASP} with a variety of SH3 domains. To characterize the association of p66^{WASP} with p47^{nck}, we expressed the three SH3 domains together (3K), and each of the SH3 domains of p47^{nck} independently (3KI, 3KII, and 3KIII), as GST fusion proteins. ^{35}S-labeled p66^{WASP} from in vitro translation reaction mixtures was incubated in the presence of beads coupled to each of the described fusion proteins and analyzed by autoradiography after SDS-PAGE. As shown in Fig. 3A, the three SH3 domains of Nck together (3K) and the third SH3 domain (3KIII, closest to the carboxyl terminus) were able to bind p66^{WASP}. In contrast, neither GST nor the other two SH3 domains (3KI and 3KII) appreciably associated with p66^{WASP}. The higher intensity of the signal associated with the three SH3 domains together (3K) compared with that observed in the third SH3 domain alone (3KIII) suggests that although the first (3KI) and second (3KII) SH3 domains do not independently associate with p66^{WASP}, they might cooperate with the third SH3 domain (3KIII) to enhance binding.

We next tested the specificity of SH3 binding to p66^{WASP} by utilizing SH3 domains of a diverse group of proteins including the protein-tyrosine kinases Fyn (25) and Fgr (24), pLCγ 1 (46), the cytoskeletal protein α-spectrin (48), and a substrate of

the epidermal growth factor receptor Eps 8 (15). With the exception of the SH3 element of p59^{fyn} and p55^{fgr}, none of the SH3 domains tested associated with p66^{WASP} (Fig. 3B). The affinity for Fyn-SH3 appeared to be greater than that observed for Fgr-SH3 and Nck-SH3 (3K). These results demonstrate a selective interaction between p66 and the SH3 domains from Nck and members of the Src family of protein-tyrosine kinases Fgr and Fyn.

To analyze the nature of p66^{WASP}-p47^{nck} interaction, we constructed mutated versions of the SH3 domains of Nck. Close to the carboxy terminus of SH3 domains, there is a well-conserved proline which was changed to leucine in a version of *sem-5* (9), a mutation that severely affected the function of the protein. This same mutation impairs the capability of the SH3 domain of both Grb2 and Src to associate with their specific targets (12, 13). As previously shown in Fig. 3A, the carboxy-terminal SH3 domain of Nck (3KIII) associated with p66^{WASP} (Fig. 3C, lane 2), but when proline 243 (equivalent to the one mutated in *sem-5*) was changed to leucine, the association was completely abrogated (lane 3). When this same residue was changed to leucine in the context of the three SH3 domains of Nck (3K, lane 5), the association with p66^{WASP} was greatly affected, but not completely blocked, compared with wild-type domains (lane 4). The inhibition of the interaction was complete when prolines 52, 156, and 243 (one in each of the SH3 domains) were changed to leucines (lane 6). As previously shown in Fig. 2B, full-length Nck (K) associated with p66^{WASP}, but to a lesser extent than the three SH3 domains without the SH2 (3K, Fig. 3C, lane 7), suggesting that the SH2 domain diminishes but does not block the association. These results demonstrate that the p66^{WASP}-p47^{nck} interaction is mediated by the SH3 domains of p47^{nck}.

In vivo association of p66^{WASP} with p47^{nck}. To gain further insight regarding the functional importance of the p47^{nck}-p66^{WASP} association, we asked whether they interacted in vivo. Thus, lysates from COS-7 cells transfected with *WASP* were immunoprecipitated with anti-Nck (p47^{nck} is constitutively expressed in COS-7 cells) and examined by Western blotting with anti-WASP antibodies as a probe. p66^{WASP} could be readily observed in anti-Nck immunoprecipitates (Fig. 4A, lane 3), but not in cells transfected with the pcDNA3 vector alone (lane 1). However, in reciprocal experiments, p47^{nck} could not be detected in anti-WASP immunoprecipitates (lane 5).

As the SH3 domain of p59^{fyn} was also observed to bind p66^{WASP} in vitro (Fig. 3B), we tested whether this binding also occurs in vivo. COS-7 cells were cotransfected with both *WASP* and *fyn*, and cell lysates were immunoprecipitated with anti-Fyn or anti-WASP antibodies (Fig. 4A, lanes 7 through 12). Although p59^{fyn} is naturally expressed at low levels in these cells (Fig. 4A, lane 10), its expression was increased severalfold upon transfection with the *fyn* construct (lane 11). When lysates from cotransfected cells were examined for binding, p59^{fyn} was not detected in anti-WASP immunoprecipitates (lane 12), and reciprocally, p66^{WASP} was not detected in anti-p59^{fyn} immunoprecipitates (lane 9). These results suggest that the direct binding of these molecules observed in vitro might not reflect in vivo association.

To determine whether p66^{WASP} and p47^{nck} might associate in vivo in naturally occurring cells, lysates from HL60 cells, in which both proteins are constitutively expressed, were examined. As expected, both p66^{WASP} and p47^{nck} were detected in HL60 lysates (Fig. 4B, lane 3). When we investigated anti-Nck immunoprecipitates for the presence of p66^{WASP}, the protein was readily detected (lane 1). However, no p47^{nck} was detectable in anti-WASP immunoprecipitates (lane 4), reproducing the result obtained in *WASP*-transfected COS-7 cells. The lack

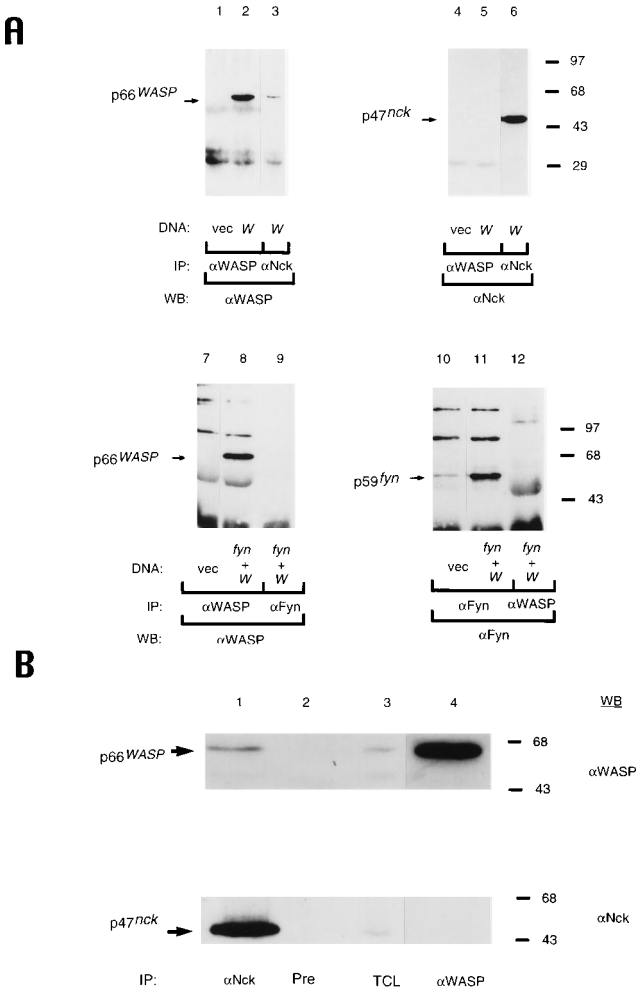

FIG. 4. In vivo association of p66^{WASP} and p47^{nck}. (A) COS-7 cells were transfected with pcDNA3 vector (vec), pcDNA3-WASP (*W*), and pcDNA3-*fyn* (*fyn*) as indicated. Cells were lysed after 64 h, and extracts were immunoprecipitated (IP) with anti-WASP (αWASP), anti-Nck (αNck), or anti-Fyn (αFyn) antibodies. Immunoprecipitates were fractionated by SDS–10% PAGE and Western blotted (WB) with either anti-WASP (lanes 1 through 3 and 7 through 9) or anti-Fyn (lanes 10 through 12) antibodies as probes. Lanes 1 through 3 were stripped and reblotted with anti-Nck antibodies (lanes 4 through 6). The positions of p66^{WASP}, p47^{nck}, and p59^{fyn} are indicated. (B) A total of 3 mg of protein from HL60 cells was immunoprecipitated (IP) with anti-Nck (αNck, lane 1), preimmune serum (Pre, lane 2), or anti-WASP (αWASP, lane 4) antibodies. A total of 40 μg of total cell lysate (TCL, lane 3) as well as immunoprecipitates was fractionated by SDS–10% PAGE and Western blotted (WB) with anti-WASP antibodies (αWASP, upper panel). The membrane was then stripped and reblotted with anti-Nck antibodies (αNck, lower panel). The positions of p66^{WASP} and p47^{nck} are indicated. Molecular mass markers are shown in kilodaltons.

of detectability of p47^{nck} in anti-WASP immunoprecipitates could be explained by several possibilities, including the presence of p47^{nck}, which might affect the recognition of p66^{WASP}, or the stoichiometry and number of complexes recognized by each antibody. Thus, p66^{WASP} was detected in association with p47^{nck} in both HL60 cells and COS-7 cells transfected with *WASP*.

The fact that we did not observe p47^{nck} in anti-WASP immunoprecipitates prompted us to test whether p66^{WASP} was directly detected by anti-Nck antibodies. This would explain the presence of p66^{WASP} in anti-Nck immunoprecipitates. For this purpose, we immunoprecipitated cell lysates from transfected COS-7 cells obtained in either native or denaturing

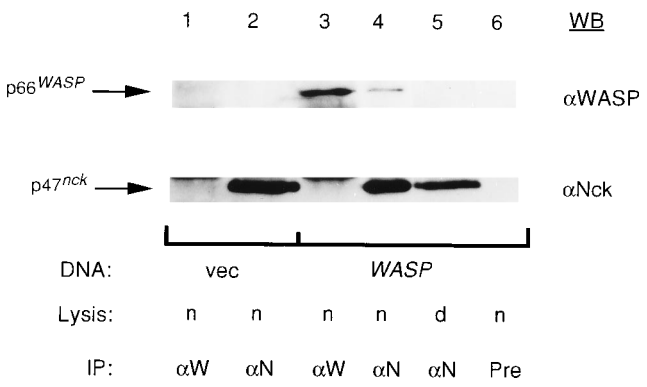

FIG. 5. Western blot analysis of anti-WASP immunoprecipitates from cell lysates obtained in either native or denaturing conditions. COS-7 cells were transfected with either pcDNA3 vector (vec, lanes 1 and 2) or pcDNA3-*WASP* (*WASP*, lanes 3 through 6) and lysed after 64 h in either native (n, lanes 1, 2, 3, 4, and 6) or denaturing (d, lane 5) conditions, as described in Materials and Methods. Total cell lysates were immunoprecipitated (IP) with anti-WASP (αW, lanes 1 and 3), anti-Nck (αN, lanes 2, 4 and 5), or anti-Nck preimmune serum (Pre, lane 6). Immunoprecipitates were fractionated by SDS–10% PAGE and Western blotted (WB) with anti-WASP antibody (αWASP, upper panel). The membrane was then stripped and reblotted with anti-Nck antibody (αNck, lower panel). The positions of $p47^{nck}$ and $p66^{WASP}$ are indicated.

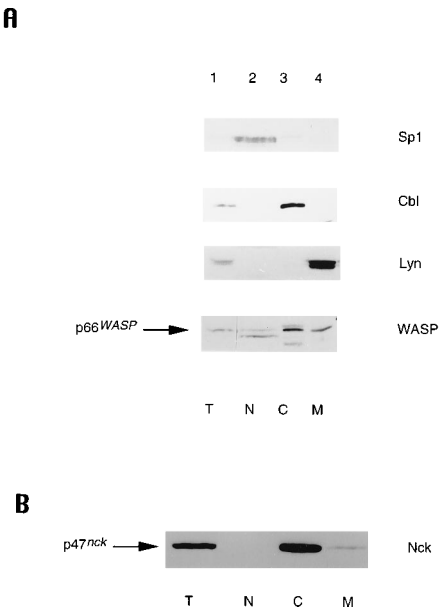

FIG. 6. Subcellular localization of $p66^{WASP}$. Subcellular fractions from HL60 cells were prepared as described in Materials and Methods. N, nuclear fraction; C, cytosolic fraction; M, membrane fraction. Equal quantities of protein (40 μg) for each fraction or the total cellular lysate (T, lane 1) were analyzed by immunoblot with anti-$p95\text{-}106^{sp1}$ (Sp1), anti-$p120^{c\text{-}cbl}$ (Cbl), anti-$p53\text{-}56^{lyn}$ (Lyn), or anti-$p66^{WASP}$ (WASP) antibodies (A) and anti-$p47^{nck}$ antibody (B). The positions of $p66^{WASP}$ and $p47^{nck}$ are indicated.

conditions (Fig. 5). Cells transfected with the vector alone (pcDNA3) did not express $p66^{WASP}$ (lane 1), but $p47^{nck}$ could be readily detected (lane 2). Transfection of cells with pcDNA3-*WASP* allowed the efficient expression of $p66^{WASP}$ (lane 3). Immunoprecipitates obtained with anti-Nck antibodies in native conditions contained significant amounts of $p66^{WASP}$ (lane 4), which could not be observed in denaturing conditions (lane 5). As an additional control, an immunoprecipitate with anti-Nck preimmune sera was included (lane 6). Shadows in lanes 1 and 3 blotted with anti-Nck corresponded to the heavy chain of anti-WASP antibodies. This result demonstrated that the presence of $p66^{WASP}$ in anti-Nck immunoprecipitates was due not to a direct recognition by anti-Nck antibody but to the existence of a complex between $p47^{nck}$ and $p66^{WASP}$ which was disrupted in denaturing conditions.

The specificity of the $p47^{nck}$-$p66^{WASP}$ interaction was further confirmed when $p66^{WASP}$ was not observed in immunoprecipitates from other SH2- and SH3-containing proteins expressed in HL60 cells, including p85 subunit from phosphatidylinositol 3-kinase (14), Crk (33), PLCγ 1 (46), Lyn (50), Syk (47), and Shc (37) (data not shown).

To investigate the possibility that $p47^{nck}$ might bring $p66^{WASP}$ to tyrosine kinases via its SH2 domain, we tested $p66^{WASP}$ for tyrosine phosphorylation. Immunoblots with antiphosphotyrosine antibodies did not detect phosphorylation of $p66^{WASP}$ in HL60 cells activated with a variety of ligands including granulocyte-macrophage colony stimulation factor, interleukin 4, cross-linking of the Fcγ receptor with immunoglobulin G in untreated cells, and H_2O_2 in TPA-differentiated cells (data not shown). Thus, $p66^{WASP}$ does not appear to be a substrate for protein tyrosine kinases.

Localization of WASP in HL60 cells. A nuclear localization signal has been identified in the WASP sequence (11) between amino acids 222 and 235. To determine whether this molecule localizes to the nucleus, HL60 cells were fractionated into nuclear, cytosolic, and membrane fractions, as described in Materials and Methods. The purity of individual fractions was analyzed for specific markers by Western blotting. The transcription factor Sp1 (22) was used as a marker for the nuclear fraction; the Cbl protein (5) was used for the cytosolic fraction; and, for the membrane fraction, Lyn (50), a myristylated protein attached to the cytoplasmic membrane, was used. Each of the proteins was present exclusively in the expected fraction, with the exception of Sp1, which was also present in minor quantities (less than 10% of the total Sp1 protein) in the cytosolic and membrane fractions.

$p66^{WASP}$ was detected in nuclear, cytosolic, and membrane fractions (Fig. 6A, lanes 2, 3, and 4) but unequally distributed among these compartments. An estimation of the amount of $p66^{WASP}$ in each fraction was obtained by densitometry scanning of Western blots, and the signal intensity expressed with respect to the amount of marker protein present in each fraction was compared with that from a total lysate. Thus, the nuclear fraction was calculated to be enriched 10.4-fold, the cytoplasmic fraction was calculated to be enriched 1.8-fold, and the membrane fraction was calculated to be enriched 5.9-fold. In this way, it was estimated that approximately 81% of $p66^{WASP}$ was cytoplasmic, while 16% was present in the membrane fraction and less than 3% was nuclear. Our findings demonstrate that $p66^{WASP}$ is predominantly cytoplasmic, and in light of the purity of our nuclear fraction, we also conclude that a subpopulation of $p66^{WASP}$ is nuclear.

Three other bands of 53, 60, and 68 kDa, which reacted with anti-WASP antiserum, were observed in the enriched fractions. The 53- and 68-kDa proteins were present only in the cytosolic fraction, while the 60-kDa protein was detected exclusively in the nuclear fraction. At this point, the identities of these proteins and their relationships with $p66^{WASP}$, other than at an immunologic level, are unknown.

To further explore the conditions of $p47^{nck}$-$p66^{WASP}$ interaction, we analyzed the localization of $p47^{nck}$. Nck has been initially described as a cytoplasmic protein (30), and this seemed to be the case in HL60 cells (Fig. 6B). However, a small portion of $p47^{nck}$ (approximately 4% of the total

amount) could be readily detected in the membrane fraction. Thus, both p66^{WASP} and p47^{nck} colocalize in the cytoplasmic and membrane fractions, although no trace of p47^{nck} was observed in the nuclear fraction.

DISCUSSION

Very little is known about the signaling pathways in which p47^{nck} participates. As an approach toward understanding its function, we have characterized proteins which associate with its SH3 domains. Previously, we described the association of p120$^{c\text{-}cbl}$ (SAKAP I) with p47^{nck} in vivo (40). In the present study, we have characterized WASP, a new protein found to associate specifically with the SH3 domains of p47^{nck}. The gene which encodes this protein (*WASP*) was also identified in mutant forms in patients with WAS (11, 27).

The predicted amino acid sequence of WASP reveals its proline-rich nature. By virtue of the WASP proline-rich regions, Derry et al. (11) suggested that this protein might associate with SH3-containing proteins. This hypothesis has now been confirmed with the cloning of WASP with a p47^{nck}-SH3-GST fusion protein as a probe. Antibodies raised against WASP detected a major protein of 66 kDa. Evidence that p66 is the protein product of the *WASP* gene derives from transient transfection experiments in which *WASP* cDNA directed the synthesis of a 66-kDa protein, detectable with anti-WASP serum in COS-7 cells. Confirmation that p66 is the primary translational product was obtained when the original clone encoding the full-length *WASP* cDNA was transcribed and translated in vitro, in a cell-free system. The molecular mass of the product was 66 kDa, and it was also recognized by anti-WASP antibodies. This protein, designated p66^{WASP}, has an apparent molecular mass 13 kDa greater than that calculated from the DNA sequence (53 kDa). The anomalous migration in SDS-PAGE may result from its high proline content (18% of the total amino acids). In fact, anomalous gel electrophoretic mobility has been described for other proteins containing an unusually high number of proline residues (1). The origin of the other proteins detected by anti-WASP antibodies is still in question. These proteins do not associate with p47^{nck} and might simply represent cross-reacting species, related or unrelated to p66^{WASP}.

As the probe used for the cloning of SAKAP II (WASP) included the three SH3 domains of Nck, but not the SH2 domain, it was likely that the association with p66^{WASP} was SH3 mediated. We confirmed this hypothesis when mutants of the SH3 domains, impaired in their ability to associate with SH3-binding proteins, were unable to bind to p66^{WASP} in vitro. Furthermore, these analyses demonstrated that the carboxy-terminal SH3 domain seems to play a more important role in the interaction. However, this domain alone is not completely responsible for the association because the p66^{WASP} binding in vitro was much higher when the three SH3 domains were used together than when only the carboxy-terminal SH3 domain was present. Additionally, the three SH3 domains-GST fusion protein, with the carboxy-terminal SH3 mutated, retained a residual, but significant, binding capacity. This result demonstrated that at least one domain, and possibly both of the other SH3 domains, is required for the interaction. The influence of the SH2 domain in the association appeared to be negative, as was also observed for the interaction p47^{nck}-p120$^{c\text{-}cbl}$ (40).

The abundance of proline in WASP makes this protein a likely candidate for association with SH3 domains of other molecules. Yet, specificity for this interaction was demonstrated when the SH3 domains from Fyn, Fgr, and Nck, but not from PLCγ 1, α-spectrin, or Eps 8, associated in vitro with p66^{WASP}. This specificity was even stricter in vivo, because p59^{fyn} and p66^{WASP} did not associate in COS-7 transfected cells. Situations like this, in which in vitro associations have not been observed in vivo, have been reported for other cases, such as the association of C3G with Grb2, dynamin with pI3 kinase, and 3BP1 or 3BP2 with Abl (26). In addition, we did not detect p66^{WASP} in anti-Crk, anti-p85 (pI3 kinase), anti-Lyn, or anti-PLCγ 1 immunoprecipitates from HL60 cells (data not shown). Although all of these proteins are well expressed in HL60 cells, the avidity of the immunoprecipitating antibodies might account for the lack of p66^{WASP} detection. In any case, this result leaves p47^{nck} as the only SH3-containing protein observed thus far to associate with p66^{WASP} in vivo and strongly suggests that the interaction between p47^{nck} and p66^{WASP} is physiologically important.

The finding that p66^{WASP} associates with p47^{nck}, an adaptor molecule, is consistent with previous reports of defective transmembrane signaling in cells from WAS patients (43). B cells from those patients failed to manifest the normal increased proliferation in response to anti-immunoglobulin treatment and displayed a defective mobilization of Ca^{2+}. Other studies have observed that lymphocytes and platelets from WAS patients exhibited an aberrant O-linked oligosaccharide biosynthesis (19), suggesting that this molecule may participate in the regulation of protein glycosylation.

The degree of participation of WASP in signal transduction pathways is unclear. Initial experiments indicate that this molecule is not phosphorylated on tyrosine residues following cellular activation with a variety of ligands (data not shown). Simon et al. (43) previously showed that PLCγ 1 was not phosphorylated after immunoglobulin ligation in B cells from WAS patients in contrast with normal cells. This observation may not be a consequence of impaired association between WASP and the SH3 domain of PLCγ 1, because we were not able to score p66^{WASP} in anti-PLCγ 1 immunoprecipitates.

The finding that a small portion of p66^{WASP} is detectable in the nucleus of the HL60 cells raises interesting possibilities, including the transmission of signals from the membrane, where p47^{nck} can associate with activated tyrosine kinase receptors, to the nucleus. Another p47^{nck}-binding protein, p120$^{c\text{-}cbl}$, also contains a nuclear localization signal in its primary sequence, and its viral form (v-*cbl*) has also been detected in the nucleus (5), supporting the hypothesis of the role of p47^{nck} in transmitting signals from membrane to nucleus. The formation of complexes between p66^{WASP} and p47^{nck} might be also favored because they colocalize in membrane and cytosol fractions.

Evidence that the protein product of *WASP* is responsible for the clinical syndrome is not complete (10, 27). Nevertheless, our study is the first to describe the WASP, and the demonstration that p66^{WASP} physically interacts with p47^{nck} suggests that p66^{WASP} may be normally involved in signal transduction in hematopoietic cells.

ACKNOWLEDGMENTS

O.M.R.-L. was supported by a grant from the Fullbright division of the Spanish Ministry of Health and Consumer Affairs, and A.M. was supported by a NATO Scientific Committee fellowship (Spain).

We thank William T. Wong for expert technical advice.

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