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Wiskott-Aldrich Syndrome Protein Is Associated with the Adapter Protein Grb2 and the Epidermal Growth Factor Receptor in Living Cells

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Src homology domains [i.e., Src homology domain 2 (SH2) and Src homology domain 3 (SH3)] play a critical role in linking receptor tyrosine kinases to downstream signaling networks. A well-defined function of the SH3-SH2-SH3 adapter Grb2 is to link receptor tyrosine kinases, such as the epidermal growth factor receptor (EGFR), to the $p21^{ras}$-signaling pathway. Grb2 has also been implicated to play a role in growth factor-regulated actin assembly and receptor endocytosis, although the underlying mechanisms remain unclear. In this study, we show that Grb2 interacts through its SH3 domains with the human Wiskott-Aldrich syndrome protein (WASp), which plays a role in regulation of the actin cytoskeleton. We find that WASp is expressed in a variety of cell types and is exclusively cytoplasmic. Although the N-terminal SH3 domain of Grb2 binds significantly stronger than the C-terminal SH3 domain to WASp, full-length Grb2 shows the strongest binding. Both phosphorylation of WASp and its interaction with Grb2, as well as with another adapter protein Nck, remain constitutive in serum-starved or epidermal growth factor-stimulated cells. WASp coimmunoprecipitates with the activated EGFR after epidermal growth factor stimulation. Purified glutathione *S*-transferase-full-length-Grb2 fusion protein, but not the individual domains of Grb2, enhances the association of WASp with the EGFR, suggesting that Grb2 mediates the association of WASp with EGFR. This study suggests that Grb2 translocates WASp from the cytoplasm to the plasma membrane and the Grb2–WASp complex may play a role in linking receptor tyrosine kinases to the actin cytoskeleton.

INTRODUCTION

Src homology 2 (SH2) and Src homology 3 (SH3) domains are noncatalytic protein modules that play a critical role in linking receptor tyrosine kinases to multiple signaling pathways (Schlessinger, 1994; van der Geer *et al.*, 1994; Pawson, 1995). SH2 domains specifically bind phosphorylated tyrosine and its immediate C-terminal amino acid residues (Pawson, 1995). This interaction is often induced and transient due to the nature of rapid turnover of protein tyrosine phosphorylation. SH3 domains bind proline-rich segments in target molecules (Mayer and Baltimore, 1993; Alexandropoulos *et al.*, 1995). Many signaling molecules contain both SH2 and SH3 domains, such as Src and Src-related tyrosine kinases, phospholipase Cγ

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(PLCγ), Nck, and Grb2. Others have either SH2 domain(s) (such as Syk tyrosine kinase, SHP-1, SHP-2, and SHC) or SH3 domain(s) (such as CDC25/ste6, p80/85 cortactin, and α-spectrin) (Mayer and Baltimore, 1993). On the basis of the presence or absence of any intrinsic catalytic or functional motifs, these SH2/SH3 signaling molecules can be divided into two groups. The first group includes proteins with either an intrinsic enzymatic activity, such as protein tyrosine kinase, phospholipase, and GTPase-activating protein (Pawson, 1995), or a transcription-activating activity, such as STATS (signal transducers and activators of transcription; reviewed by Darnell *et al.*, 1994). The second group includes those that are composed only of SH2 and SH3 domains and do not have any catalytic or DNA-binding activity. Members of this group include CRK (CRK-I, CRK-II, and CRKL), Grb2, and Nck (Mayer and Baltimore, 1993). These proteins act as adapters by linking tyrosine-phosphorylated proteins via SH2 domains to downstream effectors through SH3 domains. A well-characterized example has come from the study of Grb2 in epidermal growth factor receptor (EGFR) signaling. The SH2 domain of Grb2 binds to tyrosine-phosphorylated EGFR, and its SH3 domains interact with proline-rich segments in Sos1, a guanine nucleotide exchange factor for ras. As a result, Grb2 translocates Sos1 from the cytoplasm to the plasma membrane, thereby activating ras (Aronheim *et al.*, 1994; reviewed by Pawson and Schlessinger, 1993). Grb2 has also been shown to bind through its SH2 domain to the platelet-derived growth factor (PDGF) receptor-β (Arvidsson *et al.*, 1994; Li *et al.*, 1994), colony-stimulating factor-1 receptor (van der Geer and Hunter, 1993), hepatocyte growth factor receptor (Ponzetto *et al.*, 1994), insulin receptor substrate-1 (Skolnik, *et al.*, 1993), SHP-2 (Li *et al.*, 1994), receptor tyrosine phosphatase α (den Hertog *et al.*, 1994; den Hertog and Hunter, 1996), SHC (Rozakis-Adcock *et al.*, 1992), bcr-abl tyrosine kinase (Pendergast *et al.*, 1993; Puil *et al.*, 1994), and focal adhesion kinase (Schlaepfer *et al.*, 1994; Clark and Brugge, 1995; Kharbanda *et al.*, 1995). Besides Sos1, SH3 domains of Grb2 were also found to bind vav (a putative guanine nucleotide exchange factor for ras in hematopoietic cells; Ye and Baltimore, 1994), Cbl (the protein product of the c-*cbl* proto-oncogene; Buday *et al.* 1996), C3G (a ubiquitously expressed guanine nucleotide-releasing factor; Tanaka *et al.*, 1994), and dynamin I (a GTPase essential for coated vesicle formation; Kharbanda *et al.*, 1995; Wang and Moran, 1996). Thus, Grb2 may also link the receptor and nonreceptor tyrosine kinases to other small GTPase-related, as well as GTPase-unrelated, signaling pathways. Recent studies have shown that Grb2 plays a part in mediating receptor tyrosine kinase-stimulated actin-cytoskeletal reorganization. Microinjection of anti-Ash/Grb2 antibody abolishes both the S phase entry and organization of actin assembly during ruffle formation in response to EGF and PDGF but not to serum (Matuoka *et al.*, 1993). Wang and Moran (1996) have shown that Grb2 mediates the EGF-stimulated EGFR endocytosis, which apparently involves dynamin but is independent of the ras signaling pathway.

Wiskott-Aldrich syndrome (WAS) is an X-linked recessive immunologic disorder due to functional defects in T and B lymphocytes and platelets (Spitler *et al.*, 1975; Ammann and Hong 1989). Analyses of the hematopoietic cell defects in WAS patients suggest that occurrence of the disease is due to abnormalities in organization of the actin cytoskeleton (Ochs *et al.*, 1980). Using positional cloning, two groups have independently isolated the human *WAS* (*hWAS*) gene and found that mutations in the *WAS* gene of affected patients correlate with occurrence of the disease (Derry *et al.*, 1994; Kwan *et al.*, 1995). A mouse WAS protein (mWASp) homologue shares more than 90% identity with its human counterpart (Derry *et al.*, 1995). Approximately 17% of the amino acids in WASp are proline residues, which constitute at least 10 potential binding sites for SH3 domains. Using radiolabeled Nck SH3 domains as a probe to screen λgt11 expression libraries, Rivero-Lezcano *et al.* (1995) have isolated several cDNA clones, one of which revealed identical sequence to the hWASp. They showed that WASp indeed interacts with the C-terminal SH3 domain of Nck in vitro and coimmunoprecipitates with Nck in intact cells (Rivero-Lezcano *et al.*, 1995). Three groups independently found that WASp interacts with CDC42, a member of the rho and rac family GTPases (Aspenström *et al.*, 1996; Kolluri *et al.*, 1996; Symons *et al.*, 1996). Overexpressed WASp was found in clusters that colocalize with the polymerized actin. Moreover, CDC42-N17, a dominant negative mutant, blocked the WASp clustering (Symons *et al.*, 1996). These results suggest that WASp is involved in CDC42-induced actin assembly. However, an independent study showed that a mutant of CDC42, which no longer binds to WASp, is still able to induce actin cytoskeletal reorganization (Lamarche *et al.* 1996), implying that WASp is not directly involved in the CDC42 function.

Using radiolabeled glutathione *S*-transferase (GST)-Nck to screen a cDNA expression library, we also isolated the full-length mWASp cDNA. Because WASp contains multiple SH3-binding motifs (PXXP), we have tested a panel of SH3-containing signaling molecules for binding to WASp in vitro under high-stringency conditions. We report herein that WASp interacts with Grb2 in vitro and in intact cells at physiological concentrations of these proteins. More important, WASp coimmunoprecipitates with activated EGFR, and this interaction appears to be mediated by Grb2.

MATERIALS AND METHODS

A431 and 293 cells were cultured in DMEM containing 10% fetal bovine serum and 50 IU of penicillin and 50 μg of streptomycin per ml. Human recombinant EGF was purchased from Intergen (Purchase, NY). Rabbit anti-phosphotyrosine (PY) antiserum (no. 72) was used as described previously (Nishimura *et al.*, 1993). Rabbit polyclonal anti-GST antibody and purified rabbit anti-Grb2 antibody (no. 1–68) and rabbit anti-Sos1 antiserum (C-23) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Nck antisera were generated and used as described previously (Li *et al.*, 1992). Bacteria expressing GST-Grb2 fusion proteins, rabbit anti-Grb2 antiserum (no. 327), and anti-EGFR monoclonal antibody (no. 108) were kindly provided by Maria L. Galisteo and Joseph Schlessinger (New York University Medical Center, New York, NY). Rabbit anti-Grb2 peptide antiserum (no. 5647) was a gift from Tony Hunter (The Salk Institute, La Jolla, CA). Rabbit anti-GST-WASp antiserum was generated as described.

Screening cDNA Expression Library with ^{125}I-labeled GST-Nck

Iodination of a purified GST-Nck (full-length) fusion protein was carried out using Iodo-Gen iodination reagent (1,3,4,6-tetrachloro-3α-6α-diphenylglycoluril, Pierce, Rockford, IL). The bottom of a 10-ml Kimax glass tube was coated with Iodo-Gen by adding 100 μl of chloroform containing 1 mg of Iodo-Gen and evaporating the chloroform with nitrogen. The GST-Nck in phosphate-buffered saline (15 μg/100 μl) and Na^{125}I (1 mCi, carrier-free, 3.7GBq/ml, Amersham, Arlington Heights, IL) were added to the bottom of the tube and incubated for 15 min with gentle agitation. The reaction was terminated by the addition of nonradioactive carrier NaI (0.25 mM, final concentration). ^{125}I-labeled GST-Nck (specific activity, 3–5 × 10^4 cpm/ng of protein) in the aqueous part was used as the probe for screening a cDNA expression library. A pEXlox(+) cDNA expression library was obtained from Novagen (Madison, WI), which was constructed from mRNA from 16.5-d embryos of NIH SWISS mice. *Escherichia coli* BL21(DE3)pLysE, which has the T7 polymerase under the lacUV5 control, was cultured in 2× YT containing 0.2% maltose, 10 mM $MgSO_4$, and 25 mg/ml chloramphenicol. In 15-ml tubes, 100 μl of bacteria (OD ~1.0 at 595 nm), 100 μl of 10 mM $MgSO_4$/10 mM $CaCl_2$, and 100 μl of 40 × 10^3 phage diluted in SM (50 mM Tris, pH 7.5, 8 mM $MgSO_4$, 100 mM NaCl, and 0.1% gelatin) were mixed and incubated for 30 min at 37°C. Ten milliliters of LB with 0.7% agarose prewarmed to 50°C were added, mixed, and poured onto 150-mm 2× YT plates. Upon development of the plaques (between 8 and 12 h), plates were overlaid with 137-mm nitrocellulose filters impregnated with isopropyl β-D-thiogalactoside and incubated overnight at 37°C. After three washes in Tris-buffered saline/0.1% Triton X-100, filters were preincubated for 3 h at 4°C in blocking buffer (HEPES, pH 7.5, 5 mM $MgCl_2$, 1 mM KCl, 5 mM dithiothreitol, 5% nonfat dry milk, 10 μg/ml purified GST alone, and 0.02% sodium azide) and then incubated in blocking buffer containing radiolabeled GST-Nck probe (5 × 10^5 cpm/ml ^{125}I-labeled GST-Nck) for 12 h at 4°C. Filters were washed four times in 500 ml per wash of Tris-buffered saline/0.1% Triton X-100, air dried, and visualized by autoradiography. More detailed cloning procedures have been described previously (Skolnik *et al.*, 1991; Margolis *et al.*, 1992). Positive clones were isolated and subjected to secondary and tertiary screening, from which single positive plaques were ultimately picked. Amplified phage DNA was analyzed by *Eco*RI and *Hin*dIII digestion for the directional cloning sites of the cDNA library. DNA sequencing analysis from both the 5′ and the 3′ ends of cDNA inserts was performed by using a Sequenase 2.0 kit (Amersham).

Establishment of Hemagglutinin (HA)-tagged WASp-expressing Cell Line and Generation of Antiserum against WASp

Three copies of an epitope, YPYDVPDYA, derived from the influenza virus HA, was amplified by polymerase chain reaction with addition of a translation initiation signal (ACCAGT) at the 5′ end of the fragment and inserted at the N terminus of the mWASp. Including an additional 11 amino acids between the three copies of the HA tag and a 12-amino acid space before the deduced initiation methionine in the mWASp, there was a total of 50 non-WASp amino acids added to the N terminus of the WASp. The entire cDNA fragment was subcloned into the pRc/CMV eukaryotic expression vector (Invitrogen, San Diego, CA), which contained a cytomegalovirus promoter and neomycin-resistance gene for selection. The construct was transfected into human embryonic kidney cells 293 (30 μg/10-cm tissue culture dish) by the calcium phosphate precipitation technique (Chen and Okayma, 1987). After selection in medium containing Geneticin (G418 sulfate, 1 mg/ml) for 10 d, surviving cells (termed 293-HA-WASp) were amplified and analyzed for HA-WASp expression by immunoblotting (Western) analysis using both anti-HA monoclonal antibody (12CA5) and anti-WASp antiserum (αWASP).

The DNA sequence encoding the N-terminal 279 amino acids was amplified by polymerase chain reaction, linked in-frame to GST in pGEX3X expression plasmid (Li *et al.*, 1992) and transformed into competent bacteria DH5-α. Expression of GST-WASp fusion protein in the bacteria was induced by isopropyl β-D-thiogalactoside (1 μM, 3 h at 37°C) and purified from the soluble cell extract by a glutathione affinity column. The GST-WASp protein was injected into two rabbits (300 μg for the initial injection, followed by two 100-μg booster injections) at Cocalico Biologicals (Reamstown, PA). Antiserum was analyzed by immunoprecipitation and immunoblotting analyses against the HA-WASp expressed in 293-HA-WASp cells. Seven to 10 μl of αWASP antiserum recovered ~85% of the endogenous WASp from a lysate of 7 × 10^6 A431 (human) and BAC1.2F5 (mouse) cells in immunoprecipitation and a 1:1000 dilution provided the clearest results in detection of WASp by Western immunoblot analysis.

Cell Fractionation

293-HA-WASp cells were fractionated into cytosol, membrane, and nuclear fractions by the following procedure. Cells were suspended in 8 volumes of hypotonic buffer (5 mM Tris-HCl, pH 7.4, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 1 mM sodium iodoacetate, 0.5 mM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 0.2 U/ml aprotinin) and broken by homogenization on ice using a Dounce all-glass homogenizer. After achieving more than 90% breakage of the cells, 0.25 volume of compensation buffer (20 mM Tris-HCl, pH 6.7, 0.95 M sucrose, 0.1 M sodium chloride, 30 mM sodium pyrophosphate, 100 mM sodium fluoride, 0.5 mM sodium orthovanadate, 0.025 mM zinc chloride) was added to achieve isotonicity. Nuclei were first separated from the cytosol and membrane by low-speed centrifugation (350 g, 1 min). Membrane and cytosol were further separated by ultracentrifugation (100,000 × *g*, 30 min). The cytosol and the Triton X-100-soluble extract of the nuclear and membrane fractions were immunoprecipitated with αWASP antiserum, resolved on an SDS gel, and transferred to a nitrocellulose membrane. The amount of WASp in these fractions was analyzed by Western blot and α-WASp antiserum, followed by binding to ^{125}I-labeled protein A and autoradiography.

Northern Blot Analysis

A multiple-tissue Northern blot containing 2 μg/lane of mRNA (Clontech, Palo Alto, CA) was prehybridized for 3 h at 42°C according to the manufacturer's instruction. Hybridization was carried out

in the same buffer containing ^{32}P-labeled (random-priming) WASp (nucleotides −37 to +1473) overnight at 42°C, and the blot was washed three times in 0.2× SSC-0.1% SDS at 42°C and three times in 0.1× SSC-0.1% SDS at 65°C prior to exposure to Kodak XAR film at −70°C.

[^{32}P]Phosphate Labeling, Phosphoamino Acid Analysis, and Staphylococcus aureus V8 Protease Digestion

Cells were metabolically labeled with [^{32}P]orthophosphate (1 mCi/ml, for 3 h), as described previously (Li *et al.*, 1991). αWASP immunoprecipitates of the cell lysates were heated for 5 min at 95°C in the presence of 1× sample buffer (1% SDS, 0.14 M 2-mercaptoethanol in Tris base, pH 6.8) and resolved in an SDS gel, which was dried and subjected to autoradiography. Parallel but unlabeled cells were included in the immunoprecipitation as a control for the recovery of WASp by αWASP antiserum. The bands containing ^{32}P-labeled WASp were excised from the dried gel, measured in a β counter, and subjected to phosphoamino acid analysis (Li *et al.*, 1991). V8 protease digestion and peptide mapping analysis were carried out according to the previously published procedures (Cleveland *et al.*, 1977; Beemon and Hunter 1978).

Coimmunoprecipitation and In Vitro Binding Assay

Coimmunoprecipitation experiments to detect protein complexes of Grb2, WASp, and the EGFR were carried out with A431 cells. After solubilization of the cells in lysis buffer (Li *et al.*, 1991), postnuclear extracts were immunoprecipitated with αWASP, anti-Grb2, or anti-EGFR antibodies. After washing with lysis buffer (no bovine serum albumin), the immune complexes were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted by using the indicated antibodies. The results were visualized by incubating with ^{125}I-labeled protein A followed by autoradiography.

For in vitro-binding assays, glutathione-Sepharose beads prebound with either GST alone or GST fused to various signaling molecules were incubated with the postnuclear extract of the 293-HA-WASp cells. After washing the beads, the bound proteins were dissociated by heating at 95°C for 5 min in the presence of 1× sample buffer, resolved by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with 12CA5 monoclonal antibody. After incubation with rabbit anti-mouse IgG, the blot was incubated with ^{125}I-labeled protein A, and results were visualized by autoradiography.

RESULTS

WASp Is Expressed in Both Hematopoietic and Nonhematopoietic Cells

Using radiolabeled GST-Nck (full-length) fusion protein as a probe to screen a cDNA expression library made from a 16.5-d mouse embryo, we isolated the full-length cDNA of the mouse homologue of the *hWAS* gene. The deduced amino acid sequence of our clone is identical to that of the previously reported mWAS cDNA (Derry *et al.*, 1995), except for the three amino acid residues Gly-91, Leu-349, and Pro-357, which were previously reported as Ala-91, Val-349, and Gly-357, respectively.

Rabbit antiserum against GST-mWASp fusion protein (amino acids 1–279) was raised and used to detect the expression of WASp in various types of cells from human, rat, and mouse. The immunological reactivity of this antiserum against WASp was tested with 293 cells, which stably express a HA-tagged mWASp (293-HA-WASp). Lysates of either parental 293 (control or C) or the 293-HA-WASp (transfected or T) cells were immunoprecipitated with the preimmune serum, the αWASP, or anti-HA monoclonal antibody (12CA5). The immunoprecipitates were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted by using either 12CA5 or αWASP antibodies. Figure 1A shows that 12CA5 immunoblotting (lanes 1–3) revealed a 70-kDa protein from both the 12CA5 (lane 1) and the αWASP immunoprecipitates (lane 3) but not from the preimmune serum precipitate (lane 2). This 70-kDa protein was only detected in the lysate of 293-HA-WASp cells (T; lanes 1–3), but not in those of untransfected 293 cells (C, lanes 4–6). The endogenous WASp, which was only recognized by αWASP antiserum and migrated faster than the HA-WASp in SDS-PAGE, was clearly detected in untransfected 293 cells when blotted with the αWASP antiserum (lane 6). These data indicated that our αWASP antiserum was immunologically reactive against both the mouse (HA-WASp) and the endogenous hWASp. Cellular fractionation of the 293-HA-WASp cells, followed by αWASP immunoprecipitation and immunoblotting analysis, showed that both the endogenous WASp and the HA-WASp were predominantly cytoplasmic (Figure 1B, lane 5), consistent with the previous report (Rivero-Lezcano *et al.*, 1995).

Previous studies using Northern blot analysis of total RNA showed that expression of the *WAS* gene was restricted to hematopoietic tissues and cell lines (Derry *et al.*, 1994, 1995). Using the αWASP antiserum, we examined the expression of WASp in a wide range of human and rodent cells, including the human epidermoid carcinoma cells A431, human embryonic kidney cells 293, human breast epithelial cells (MCF7/k1), human keratinocytes (P3), mouse B lymphocytes (A20), mouse T lymphocytes (BW), NIH 3T3 fibroblasts (HER14), mouse macrophage cells (BAC1.2F5), and rat pheochromacytoma cells (PC12). As shown in Figure 1C, αWASP mainly recognized a 68-kDa protein from the human (hWASp, lanes 1–4) and a 65-kDa protein from the rodent (mWASp, lanes 5–10) cells. An additional 66-kDa protein band could sometimes be detected in A431 cells (lane 1). A much smaller amount of the 65-kDa WASp was detected in PC12 cells after a longer exposure to x-ray film (lane 9 versus lane 10). However, no WASp was detectable in NIH 3T3 cells (HER14, lane 7) under these conditions. These data indicated that WASp is expressed in both hematopoietic and nonhematopoietic cells. To confirm the notion that WASp is rather widely expressed, we also carried out Northern blot analysis using mRNA (2 μg/lane, that represents the amount of mRNA in ~200 μg of total RNA) instead of total cellular RNA (20 μg/lane is often used) to increase the sensitivity of

detection. As shown in Figure 1E, almost all of the tissues tested, except for the spleen and liver, exhibit two major mRNA species, one in a range of 2–2.5 kb and the other around 4–7 kb, indicating heterogenities at the transcriptional level of the *WAS* gene in different tissues. However, this heterogenity in mRNA was apparently not reflected by the mWASp products from different types of cells, in which a major 65-kDa mWASp was detected (Figure 1C; a smaller 32-kDa αWASP-reactive species could also be detected in certain cell types; see DISCUSSION).

To test whether or not the 66-kDa species is related to the 68-kDa hWASp in A431 cells, we compared V8 protease peptide maps of these two proteins isolated from [^{35}S]methionine-labeled A431 cells. Figure 1D shows that although these two proteins share some common peptides in a range of 12–19 kDa (lane 2 verses lane 3), there are clearly distinct peptides from the 66-kDa (lower, lane 3) species and from the 68-kDa hWASp (upper, lane 2) between 19 and 40 kDa. The V8 protease peptides of the EGFR from the same cells showed a totally different pattern (lane 1). Since this 66-kDa protein was not always detectable by αWASP and it has significant differences in peptide maps from the 68-kDa hWASp, we believe that it is not related to the 68-kDa hWASp. Another unexpected observation revealed that the mWASp migrated more quickly (65 kDa) than the hWASp (68-kDa) in the SDS-PAGE, even though the amino acid length of the mWASp (520 amino acids) is 18 amino acids longer than that of the hWASp (502 amino acids; Derry *et al.*, 1995). The reason for this discrepancy is unclear. It is possible that the hWASp undergoes some higher degree of posttranslational modifications, such as phosphorylation, than the mWASp.

To examine whether or not WASp gets phosphorylated in cells, A431 cells, which express approximately 2×10^6 surface EGFRs per cell, and A-20 B lymphocytes were metabolically labeled with [^{32}P]orthophosphate and stimulated with EGF. αWASP immunoprecipitates of these cell lysates were resolved in SDS gel, which was dried and subjected to autoradiography. Figure 2A shows that αWASP recognized a ^{32}P-labeled 68-kDa protein from A431 cells that were untreated (lane 1) or treated for 5 or 45 min with EGF (lanes 2 and 3, respectively). The overall intensity of the phosphorylation remains unchanged between the serum-starved (lane 1) and the EGF-stimulated (lanes 2 and 3) cells. When these 68-kDa protein bands were excised from the gel and subjected to phosphoamino acid analysis (Figure 2B), only phosphoserine and phosphothreonine but no PY were detected. The identity of a 200-kDa phosphoprotein that coimmunoprecipitates with WASp is unknown. A similar molecular weight protein could also be detected from [^{35}S]methionine-labeled A431. However, this protein was not directly recognized by αWASP on a nitrocellulose

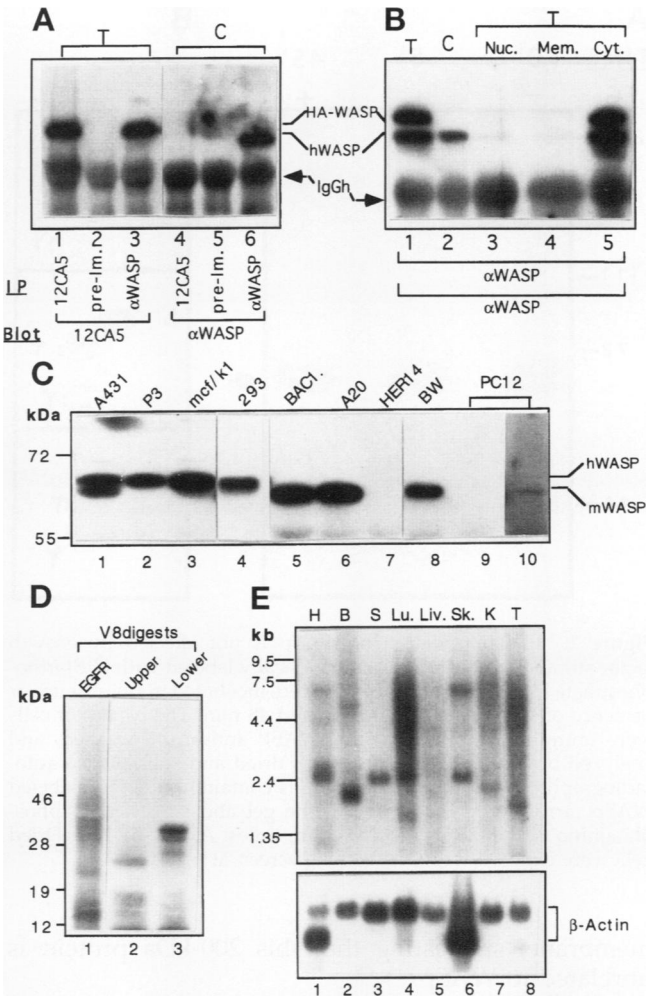

Figure 1. WASp is a widely expressed cytoplasmic protein. (A) Reactivity of anti-WASp antiserum (αWASP) with human and mWASp. GST-WASp (amino acids 1–296) was purified and injected into rabbits (Cocalico Biologicals). The preimmune serum (pre-Im.) and the antiserum (αWASP) were tested for recognizing HA-WASp, which was stably expressed in 293 cells. 12CA5, Monoclonal antibody against HA; C, parental 293 cells; T, transfected 293 cells (293-HA-WASp). (B) 293-HA-WASp cells were fractionated into nuclei, membrane, and cytosol. The Triton X-100 extracts of these fractions were immunoprecipitated with αWASP antibody (lanes 3 and 5). αWASP immunoprecipitates of total lysates of either parental (lane 2) or 293-HA-WASp (lanes 1) cells were included as controls. Samples were analyzed by Western blot analysis using αWASP antiserum. (C) The lysates of ~8×10^6 cells from the various types of cells indicated were immunoprecipitated with αWASP antiserum. The immune complexes were resolved in SDS gels, transferred to nitrocellulose membrane, and immunoblotted with αWASP antiserum. The results were visualized by incubation with ^{125}I-labeled protein A, followed by autoradiography. The αWASP-reactive human (68 kDa) and rodent (65 kDa) WASp are indicated by arrows. (D) The 68-kDa and 66-kDa αWASP-reactive proteins (see C, lane 1) were isolated from [^{35}S]methionine-labeled A431 cells and subjected to V8 protease digestion and SDS-PAGE analysis. The ^{35}S-labeled EGFR from the same cells was included as a control. (E) Multiple tissue blot (2 μg mRNA/lane, Clontech) was blotted with ^{32}P-labeled random primed nucleotides (1×10^6 cpm/ml) using mWASp cDNA (nucleotides −37 to +1473) as the template (top) or β-actin (bottom).

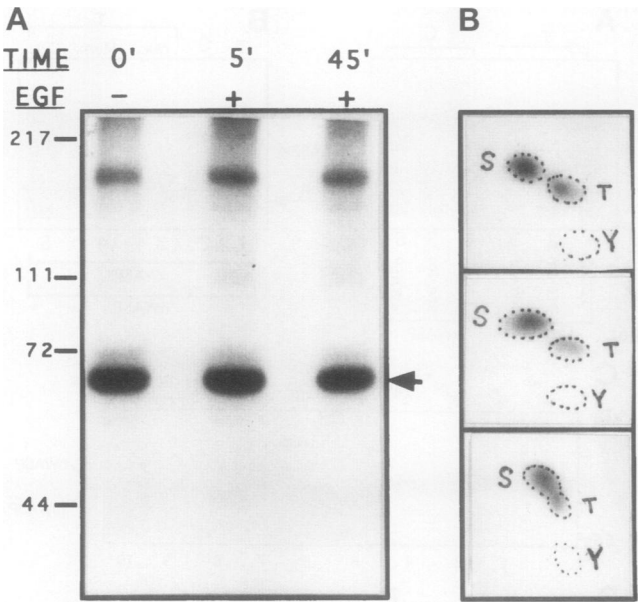

Figure 2. Phosphorylation of WASp is not affected by growth factor stimulation. A431 cells, metabolically labeled with [^{32}P]orthophosphate (1 mCi/ml, 3 h at 37°C), were incubated in the absence or presence of EGF (500 ng/ml) for 5 and 45 min. The lysates of cells were immunoprecipitated with αWASP antiserum, washed, and analyzed by SDS-PAGE. The gel was dried and subjected to autoradiography (A). The individual bands containing the radiolabeled WASp (arrow) was excised from the gel and subjected to phosphoamino acid analysis (B). Exposure times: A, 6 h; B, 40 h. Dried gels were exposed with intensifying screen at −70°C.

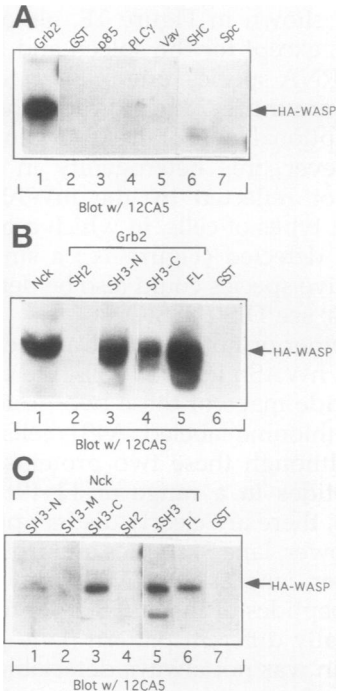

Figure 3. Grb2 binds WASp through SH3 domains. Lysates of the 293-HA-WASp (2.5×10^6 cells) were incubated with 5 μg of GST alone or 5 μg of GST fused to the various signaling molecules indicated (A) or individual domains of Grb2 (B) or Nck (C). After washing, the bound HA-WASp was detected by SDS-PAGE and Western blot analyses using 12CA5 anti-HA mAb. Arrows indicate HA-WASp.

membrane, suggesting that this 200-kDa protein is unrelated to WASp.

Grb2 and Nck Bind, via SH3 Domains, to WASp In Vitro

It has previously been reported that Nck binds through its C-terminal SH3 domain to WASp in vitro and coimmunoprecipitates with WASp in lysates of intact cells (Rivero-Lezcano *et al.*, 1995). Because WASp has multiple PXXP motifs for binding to SH3 domains, we carried out in vitro binding experiments to study whether or not WASp also interacts with other SH3-containing signaling molecules. GST fusion proteins containing the two SH3 domains from the human p85 subunit of phosphatidylinositol 3-kinase (PI 3-kinase), SH2 and SH3 domains of bovine PLCγ, SH2 and SH3 domains of human vav, SH3 domain of human α-spectrin, the C-terminal half (including the SH2 domain) of human SHC, and the full-length Grb2 were tested. Lysates of 293-HA-WASp cells were incubated individually with these GST fusion proteins that were immobilized on glutathione-agarose beads. After a wash, the bead-bound HA-WASp protein was examined by immunoblot analysis using 12CA5 monoclonal antibody. Figure 3A shows that HA-WASp binds strongly to the full-length Grb2 (lane 1) and very weakly to the PLCγ, yet it does not bind at all to the rest of the signaling molecules (lanes 3 and 5–7) or GST alone (lane 2). To identify the domains in Grb2 that are responsible for the interaction, we used GST fusion proteins that contained either the full-length or individual SH3 or SH2 domains of Grb2. The GST-3SH3 domain fusion protein of Nck was included as a positive control, according to the previous report (Rivero-Lezcano *et al.*, 1995; see also Figure 3C). Figure 3B shows that Nck-3SH3 (lane 1), full-length Grb2 (FL, lane 5), the N-terminal SH3 (SH3-N, lane 3), and the C-terminal SH3 (SH3-C, lane 4) of Grb2 bind HA-WASp. Grb2-SH3-N (lane 3) binds WASp significantly stronger than Grb2-SH3-C (lane 4). However, the full-length Grb2 shows the strongest binding to WASp (lane 5), suggesting that both SH3 domains of Grb2 are involved in association with WASp. The SH2 domain of Grb2 (lane 2) and GST alone (lane 6) did not bind WASp. Consistent with the previously published results (Rivero-Lezcano *et al.*, 1995), the C-terminal SH3 of Nck (SH3-C, lane 3) binds to WASp equally as well as the three SH3 domains together (Figure 3C, lane 5) and the full-length (lane 6) Nck. The N-terminal SH3 (SH3-N) and the middle SH3 (SH3-M) of Nck bind

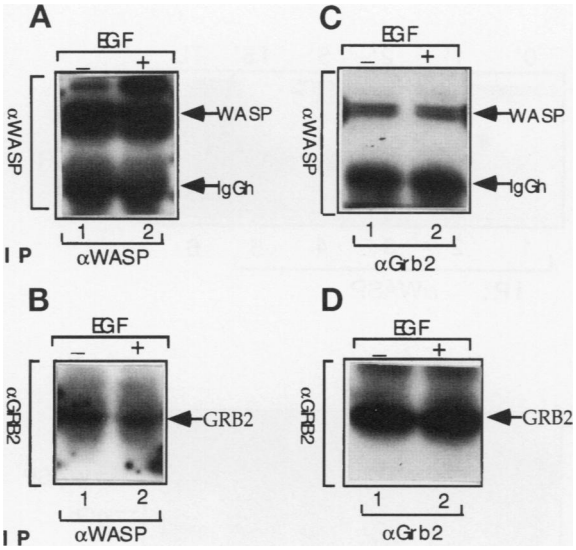

Figure 4. Grb2 associates with WASp in intact cells. The lysates (300 μg pf protein) of A431 cells unstimulated or stimulated with EGF were immunoprecipitated with either αWASP (A and B) or anti-Grb2 (1–68; C and D) antibodies. The immunoprecipitates were resolved in a SDS gel, transferred to a nitrocellulose membrane, and immunoblotted with either αWASP (A and C) or anti-Grb2 (no. 327; B and D) antibodies. The results were visualized by incubating with ^{125}I-labeled protein A, followed by autoradiography. The intensity of each band was measured by densitometry and the radioactivity was measured by gamma counter. The data in A–D were from the same experiment. Exposure was for 30 h at −70°C.

either weakly (lane 1) or did not bind at all (lane 2) to WASp. Thus, these results suggest that WASp is a potential target for SH3 domains of the adapter proteins Nck and Grb2.

WASp and Grb2 Coimmunoprecipitate in Living Cells

We then tested whether the Grb2–WASp complex exists in living cells. The lysates of A431 cells unstimulated and stimulated with EGF were immunoprecipitated with αWASP antiserum. The immunoprecipitates were resolved by an SDS gel, transferred to a nitrocellulose membrane, and immunoblotted with either αWASP or anti-Grb2 (no. 5647) antiserum. Figure 4A shows that αWASP antibody recovered similar amounts of the 68-kDa WASp from both unstimulated and EGF-stimulated cells (lanes 1 and 2). When the same membrane (the lower portion) was blotted with anti-Grb2 antiserum, equal amounts of a 23-kDa protein, which comigrated with the immunoprecipitated Grb2 (Figure 4D; Figure 4, B and D, was from the same amounts of cell lysates and analyzed in the same SDS-PAGE), were detected from both unstimulated and stimulated cells (Figure 4B, lanes 1 and 2). When the same membrane (upper portion) was independently blotted with anti-Sos1 antibody (C-23), no Sos1 protein was detectable, suggesting that Sos1 and WASp do not simultaneously bind to the same Grb2. To estimate the percentage of Grb2 that was associated with WASp, the individual bands on the membrane were excised, and then the radioactivity (dpm) was measured by gamma counting. Comparing the Grb2 band from the Figure 1B and the Grb2 band from Figure 4D revealed that ~7% of the Grb2 was coimmunoprecipitated by αWASP antibodies (if we assume that the α-WASp and anti-Grb2 immunoprecipitations were equal in quantity). To confirm this association, the lysates of A431 cells were immunoprecipitated with anti-Grb2 antibody (1–68) and then the immunoprecipitate was immunoblotted with αWASP antibody (Figure 4C). Equal amounts of a 68-kDa protein, which comigrated with the WASp shown in Figure 4A, were coimmunoprecipitated with Grb2 from both the unstimulated (lane 1) and EGF-stimulated (lane 2) cells. An apparent doublet of the 68-kDa WASp was observed and was most likely due to some difference in posttranslational modifications, such as phosphorylation. Similar amounts of Grb2 were recovered by anti-Grb2 immunoprecipitation (Figure 4D, lanes 1 and 2). Similarly, comparison in radioactivity between the WASp band from Figure 4C and the WASp band from Figure 4A revealed that 5–8% of WASp was coimmunoprecipitated by anti-Grb2 antibodies.

WASp Coimmunoprecipitates with Activated EGFR

It has previously been demonstrated that the SH2 domain of Grb2 binds to activated EGFR upon EGF stimulation (Lowenstein *et al.*, 1992). Therefore, it is possible that EGF induces formation of the EGFR–Grb2–WASp complex in cells. To test this hypothesis, we carried out coimmunoprecipitation experiments using αWASP and anti-EGFR (no. 108) antibodies. αWASP immunoprecipitates of A431 cells, untreated or treated with EGF, were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with either anti-EGFR or anti-PY antibodies. Figure 5A shows that a very small amount of EGFR was detected in the αWASP immunoprecipitate of unstimulated cells (lane 3). EGF stimulation induced a small but significant association of the EGFR with the WASp (lane 4 versus lanes 1 and 2). We estimated that 3–5% of the total EGFR was associated with WASp (see the figure legend for details). When a similar set of the samples was blotted by anti-PY antibody, as shown in Figure 5B, the WASp-associated EGFR was tyrosine phosphorylated (lane 2 versus lane 1).

We analyzed the time course of the association between WASp and EGFR. Figure 5C shows that an increased association of EGFR with WASp was detected as early as 1 min (lane 2) and the maximum association was reached between 2 and 5 min after

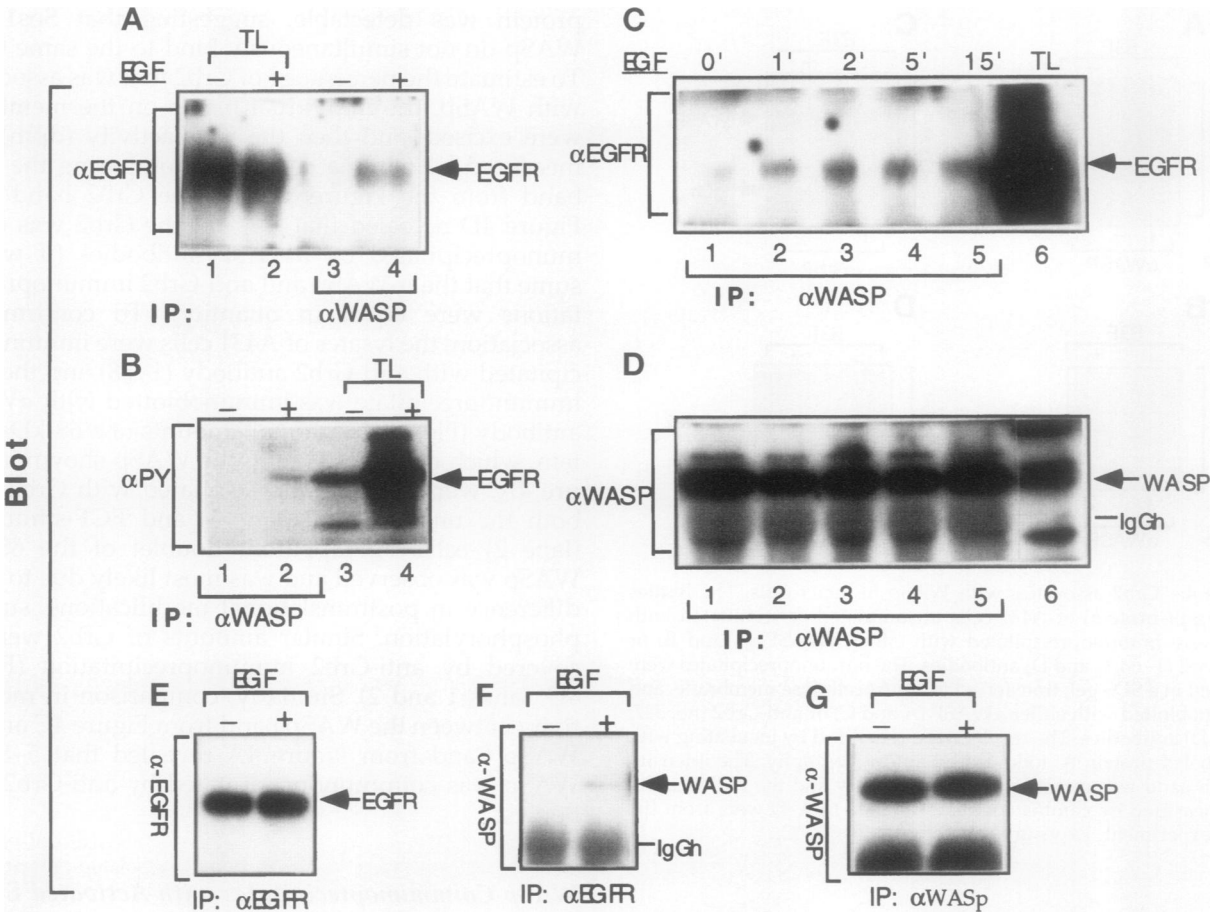

Figure 5. Coimmunoprecipitation of WASp with activated EGFR. The lysates (total cellular proteins, 300 μg) of cells, treated with or without EGF for the indicated times, were immunoprecipitated with αWASP (A–D and G) or anti-EGFR (E and F) antiserum. The immune complexes were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-EGFR (A, C, and E), anti-PY (B), or αWASP (D and G) antibodies. The results were visualized by incubating with ^{125}I-labeled protein A, followed by autoradiography. Total cell lysates (TL, 40 μg in A and B and 120 μg in C) were included in the same SDS-PAGE gel as controls. Individual EGFR bands were subjected to either densitometry or excised and the radioactivity was measured in a gamma counter. Percentage of the EGFR coimmunoprecipitated with WASp was estimated by comparing the dpm of these protein bands. Exposure times: A and B, 36 h; C, 30 h; D, 16 h; E–G, 18 h.

EGF treatment (lanes 3–5). In this experiment, a small amount of EGFR was also coimmunoprecipitated by the αWASP antiserum from the serum-starved cells (lane 1). This "background" was not always reproducible in independent experiments, which was most likely due to variations in quiescence of the A431 cells after the culture in a low serum (0.2%) medium ("serum starvation"). In this case, if the tyrosine phosphorylation of EGFR was measured, a detectable amount of tyrosine-phosphorylated EGFR was often observed even from the unstimulated cells (Figure 5B, lanes 3 and 4). Similar amounts of WASp were recovered from the lysates of these cells (Figure 5D). Total lysates (TL) were included as a control (Figure 5, C and D, lanes 6). It should be pointed out that the intensity of the EGFR bands in the total lysates (Figure 5, A–D) may be underrepresented. Poon *et al.* (1996) have shown that antigen recognition by many antibodies, especially monoclonal antibodies, is reduced when the antigen is in a mixture with other cellular proteins. Thus, any masking effect by other cellular proteins should also be taken into account.

The coimmunoprecipitation between WASp and EGFR was confirmed by a reciprocal experiment, in which anti-EGFR (no. 108) immunoprecipitates were resolved in an SDS gel, transferred to a nitrocellulose membrane, and immunoblotted with αWASP antiserum. Figure 5E shows that the anti-EGFR immunoprecipitation recovered an equal amount of the EGFR from both the unstimulated and the EGF-stimulated cells. When the lower portion of the membrane was immunoblotted with αWASP antiserum, a small but significant amount of WASp was found in the anti-EGFR antibody immunoprecipitates of the EGF-stim-

ulated cells but not of the unstimulated cells (Figure 5F). In comparison to the total αWASP-reactive 68-kDa bands (Figure 5G) in the lysate, ~3% of the WASp was brought down by the anti-EGFR antibodies.

Grb2 Mediates Association of WASp and EGFR In Vitro

Since Grb2 binds directly, via the SH2 domain, to activated EGFR and WASp does not have any PY-binding motifs (i.e., SH2 or PTB domain), it is likely that the association between WASp and EGFR is mediated by Grb2. To test this possibility, we used purified GST-Grb2 to see whether it could enhance a WASp and EGFR interaction in vitro. We first determined the presence of free activated EGFR and WASp in EGF-stimulated cell extracts, based on the fact that GST-Grb2 can only bind the Grb2-free EGFR and Grb2-free WASp molecules and bring them together. Extracts of EGF-stimulated cells were incubated with various amounts of GST-Grb2 on beads, and the bead-bound EGFR and WASp were analyzed by Western blot using either anti-EGFR or αWASp antibodies. Figure 6A shows that GST-Grb2 beads brought down the EGFR in a dose-dependent manner (lanes 1–3). Similarly, increasing amounts of WASp were bound by the GST-Grb2 beads (Figure 6B, lanes 1–3). The higher background in Figure 6B was due to the presence of the anti-GST antibody fraction in the αWASP antiserum, because GST (in this case GST-Grb2) was used in the SDS-PAGE. These results indicate that even in the EGF-stimulated cells, significant amounts of EGFR and WASp were Grb2-free. However, it is unclear whether the same GST-Grb2 bound both EGFR and WASp or if different GST-Grb2 bound EGFR and WASp independently. To address this issue, αWASP immunoprecipitates of the lysates of serum-starved A431 cells were immobilized on protein A-Sepharose beads, and the anti-GST activity of the αWASP was blocked by preincubation with free GST protein (see the figure legend). These beads were extensively washed and incubated with a fresh set of the cell lysates containing purified GST, GST-Grb2 (full length), GST-Grb2-SH2, GST-Grb2-SH3-N, or GST-Grb2-SH3-C fusion proteins. The αWASP-immune-complex–bound EGFR was analyzed by immunoblot analysis using anti-EGFR antibodies. Figure 6C shows that a small amount of activated (lane 2) but not inactive (lane 1) EGFR was coprecipitated by the αWASP antiserum. The addition of GST-Grb2 (full-length) significantly enhanced the amount of the EGFR associated with the αWASP immune complex, which reached the maximum around 20 nM GST-Grb2 and then declined in the presence of 100 nM GST-Grb2 (lanes 4–6). The decreased association at the higher concentration (100 nM) of GST-Grb2 was most likely due to a simultaneous occupancy of the EGFR and

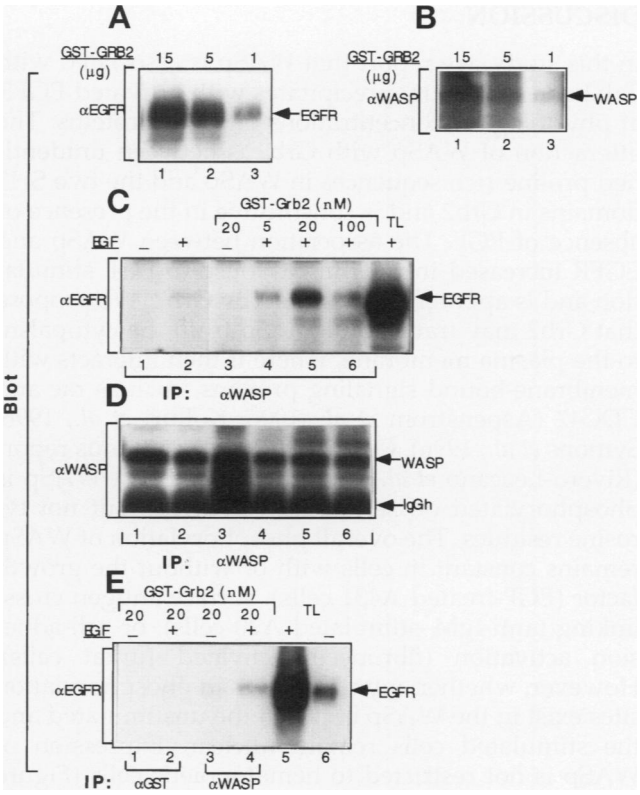

Figure 6. GST-Grb2 enhances interaction between WASp and EGFR in vitro. (A and B) Lysates of EGF-stimulated (500 ng/ml, 5 min) A431 cells were incubated with the indicated amounts of GST-Grb2 on beads. The bead-bound proteins were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-EGFR (A) or αWASP (B) antibodies. (C and E) αWASP immunoprecipitates, immobilized on protein A-Sepharose beads (10 μg/ml GST were added to block anti-GST activity in αWASP antiserum), of lysates of serum-starved A431 cells were washed and incubated with a fresh set of lysates of either unstimulated or EGF-stimulated A431 cells for 2 h at 4°C in the absence or presence of the indicated amounts of purified GST-Grb2 (C) or GST alone (E). The protein A-Sepharose bound αWASP immunoprecipitates were washed again and heated at 95°C for 5 min in 1× sample buffer. The supernatants were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-EGFR (C and E) or αWASP (D) antibodies. Total lysates (TL) of the cells were included in the same SDS-PAGE as a control. Similar but independent experiments using either the SH2 or the SH3 domains of Grb2 were also carried out and the results were negative. Exposure times: A and B, 20 h; C–E, 30 h.

WASp by independent GST-Grb2 molecules, preventing the EGFR and the WASp from forming complexes. GST and the individual SH2 and SH3 domain of Grb2 were unable to increase the amount of the WASp-associated EGFR (Figure 6E, lane 2 versus lane 4). Similar amounts of immunoprecipitated WASp were used for each reaction (Figure 6D). These results strongly suggest that the association between the activated EGFR and WASp in cells is mediated by Grb2.

DISCUSSION

In this study, we report that WASp is associated with Grb2 and coimmunoprecipitates with activated EGFR at physiological concentrations of these proteins. The interaction of WASp with Grb2 is between unidentified proline-rich sequences in WASp and the two SH3 domains in Grb2 and is constitutive in the presence or absence of EGF. The association between WASp and EGFR increased in cells in response to EGF stimulation and is apparently mediated by Grb2. We propose that Grb2 may translocate WASp from the cytopalsm to the plasma membrane, where it then interacts with membrane-bound signaling proteins, such as rac and CDC42 (Aspenström *et al.*, 1996; Kolluri *et al.*, 1996; Symons *et al.*, 1996). Consistent with a previous report (Rivero-Lezcano *et al.*, 1995), we found that WASp is phosphorylated on serine and threonine but not tyrosine residues. The overall phosphorylation of WASp remains constant in cells with or without the growth factor (EGF-treated A431 cells), surface antigen cross-linking (anti-IgM–stimulated A20 cells), or cell-adhesion activation (fibronectin-activated Jurkat cells). However, whether any differences in phosphorylation sites exist in the WASp between the unstimulated and the stimulated cells remain unclear. Expression of WASp is not restricted to hematopoietic cells (Figure 1, C and E). Although WASp was indeed found in T and B lymphocytes and macrophages, comparable amounts of WASp were also detected in epithelial (MCF/k1), epidermoid (A431), keratinocyte (P3), kidney fibroblast (293), and fully differentiated neuronal (H19–7) cells (data not shown). Although in one exception, we did not detect the 65-kDa WASp in NIH 3T3 cells, but instead, a comparable amount of a lower molecular weight (~32 kDa) αWASP-reactive species was reproducibly observed. This 32-kDa protein was also seen with the 65-kDa in the B lymphocyte cell line A-20. Apparently, it is not a degrading product of the 65-kDa WASp, because it still could be detected after immediately lysing the cells in a boiled sample buffer, which instantly denatured all proteins. The nature and function of this 32-kDa αWASP-reactive protein currently remain unknown.

It is clear that the function of SH2- and SH3-containing adapter proteins, such as Grb2 and Nck, is to link receptor tyrosine kinases as well as other PY proteins to their downstream signaling effectors, which in turn get translocated and activated. Translocation of the effectors not only enables them to access their target molecules but also increases the effective local concentrations of these molecules for enzymatic reactions (such as phosphorylation and dephosphorylation; Pawson and Schlessinger, 1993). The role of Grb2 in linking EGFR to the ras pathway, which ultimately leads to DNA synthesis, has been very well documented. Grb2 appears to play a role in tyrosine kinase receptor signaling pathway, which regulates the actin cytoskeleton (Matuoka *et al.*, 1993) and receptor endocytosis (Wang and Moran, 1996), although the mechanisms of these actions remain to be studied.

Similarly, each of the three SH3 domains in Nck binds to distinct signaling molecules. The Abl protein tyrosine kinase was first identified to bind SH3 domains of Nck in vitro (Ren *et al.*, 1994). A novel serine/threonine kinase, NAK (Nck-associated kinase), was identified by coimmunoprecipitation with Nck, followed by in vitro kinase assay (Chou and Hanafusa, 1995). This 65-kDa NAK is most likely related to the $p21^{cdc42/rac}$-activated kinase (PAK1) (Manser *et al.*, 1994), because both NAK and PAK1 have similar molecular weight and bind to the second SH3 domain of Nck in vitro (Bagrodia *et al.*, 1995; Chou and Hanafusa, 1995) and in living cells (Bokoch *et al.*, 1996; Galisteo *et al.*, 1996). PAK1 appears to signal by activating the Jun kinase (JNK) pathway. PRK2, a newly identified protein related to the rho effector PKN, also binds to the middle SH3 domain of Nck (Quilliam *et al.*, 1996). PRK2 binds rho in a GTP-dependent manner and cooperates with rho to induce serum response factor-mediated transcription (Quilliam *et al.*, 1996). Rivero-Lezcano *et al.* (1994, 1995) have shown that the proto-oncogene c-*cbl*, a cellular homologue of the transforming protein of the murine Cas NS-1 retrovirus in B cell and myeloid tumors, and the hWASp coimmunoprecipitates Nck and binds the C-terminal SH3 domain of Nck in vitro. The biological function of Nck has recently been implicated by a study on the Nck homologue in *Drosophila*. Mutations in the *Drosophila* gene *dock*, which is structurally related to Nck, disrupted the photoreceptor cell (R cell) axon guidance and targeting (Garrity *et al.*, 1996), suggesting that Nck links the receptor tyrosine kinase to actin cytoskeleton. Thus, these results have indicated that Nck connects the surface receptors to multiple signaling termini.

Serum and growth factor stimulation causes actin filament organization, which plays a role in many cellular functions such as motility, chemotaxis, cell division, endocytosis, and secretion (Devreotes and Zigmond, 1988; Bretscher, 1991). Previous studies have shown that the small GTP-binding proteins, rho and rac, are required for serum and growth factor-induced focal adhesion and actin stress fiber assembly and membrane ruffling (Ridley and Hall, 1992; Ridley *et al.*, 1992). The mechanism by which serum and growth factors activate rho and rac remains unclear. Nobes *et al.* (1995) have shown that wortmannin, an inhibitor of the p85/p110 PI 3-kinase, blocks PDGF- and insulin-stimulated membrane ruffling, but it does not block actin stress fiber formation in Swiss 3T3 cells, which suggests that PI 3-kinase can act between rac and PDGF and insulin receptors. The downstream

targets for rho/rac GTPases are being intensively studied. It has been reported that the expression of constitutively active rac and CDC42 resulted in the activation of the JNK/SAPK MAP kinase cascade (Coso *et al.*, 1995; Minden *et al.*, 1995). However, a mutant (Y40C) of rac and CDC42 that is no longer able to activate the JNK/SAPK pathway was still able to induce both cytoskeletal changes and G_1 phase progression (Lamarche *et al.*, 1996), suggesting that activation of the JNK mitogen-activated protein kinase pathway may not be required. It has been reported that the hWASp is physically associated with the GTP-bound CDC42 (Aspenström *et al.*, 1996; Kolluri *et al.*, 1996; Symons *et al.*, 1996). Moreover, overexpressed WASp proteins cluster in polymerized actin and this clustering is inhibited by CDC42-Hs-N17 mutant. These results suggest that WASp is a downstream effector of the CDC42 GTPase (Symons *et al.*, 1996). However, the Y40C mutant of rac and CDC42 also failed to bind WASp, implying that binding to WASp is not required for the CDC42-induced filopodia (Lamarche *et al.*, 1996). This apparent discrepancy between these two independent studies may possibly be due to cell type signaling specificities (Lamarche *et al.*, 1996). Nevertheless, our study raises the notion that WASp can be brought to the plasma membrane by adapter molecules such as Grb2 and Nck. But determining whether the Grb2–WASp complex is indeed involved in the EGFR signaling that regulates the actin cytoskeleton awaits future mutagenesis studies.

A novel *WAS*-related gene, termed *N-WASp*, has recently been identified (Miki *et al.*, 1996). Similar to *WASp*, overexpressed *N-WASp* colocalizes with actin filaments, which is apparently mediated through its pleckstrin homology domain. More interestingly, N-WASp coimmunoprecipitates with Grb2/Ash and EGFR (Miki *et al.*, 1996), suggesting that WASp and WASp-related proteins may play a role in transmitting the signal from protein tyrosine kinases to actin cytoskeletal rearrangement. Consistent with this notion, Bunnell *et al.* (1996) recently showed that a T-cell–specific tyrosine kinase, Itk/Tsk, binds WASp via its SH3 domain in vitro.

It would be important to identify the binding sites in WASp for the adapter proteins Grb2 and Nck. The deduced primary amino acid sequence of the WASp revealed at least 10 potential binding sites for the SH3 domains (Derry *et al.*, 1994, Symons *et al.*, 1996). It has been reported that the SH3 domains of Nck share similar binding consensus with Grb2 (Rozakis-Adcock *et al.*, 1993). Consistently, GST-Grb2 and GST-Nck failed to bring down the cellular Nck and Grb2, respectively. However, these data did not provide the proof that Grb2 and Nck share the same binding site(s) in WASp. First, binding by Grb2 or Nck may sterically prevent Nck or Grb2, respectively, from binding to the same WASp molecule. Second, if WASp is in excess in the cells, Nck and Grb2 may simply bind to different pools of WASp. Moreover, at submicromolar concentrations, Grb2 and Nck did not appear to compete for binding to WASp (She and Li, unpublished results). Our preliminary in vitro binding results showed that Nck, but not Grb2, binds to the ^{158}RQLPPPPAPIN^{173} and ^{175}GLPPVPPHPGGD^{186} peptides. It has previously been shown that Grb2 binds to Sos1 at sequence PPXPP**R**, in which the arginine residue determines the specificity (Li *et al.*, 1993; Rozakis-Adcock *et al.*, 1993). Among proline-rich segments in WASp, three segments, ^{314}PLPPPPPPC**R**^{324}, ^{368}PPPPTPP**R**^{376}, and ^{378}PPPPG**R**^{384} are most likely involved in the interaction with Grb2. Identification of the Grb2 and Nck binding sites in WASp is currently being determined in our laboratory.

Note added in proof. While this article was in revision, Quilliam *et al.* (1996) reported that WASp binds Grb2 in vitro.

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