Src Homologous and Collagen (Shc) Protein Binds to F-actin and Translocates to the Cytoskeleton upon Nerve Growth Factor Stimulation in PC12 Cells*

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Immunoprecipitates of metabolically labeled PC12 cells consistently contained a 43-kDa protein that was associated with Shc, a signal-transducing protein with a single SH2 domain. Following affinity chromatography with immobilized recombinant glutathione S-transferase (GST)-Shc fusion protein, the 43-kDa protein was identified as actin by mass spectrometry and immunoblotting. Cosedimentation experiments using purified actin and GST-Shc showed that Shc binds directly to F-actin, confirming Shc-actin interaction in vivo. Various GST-truncated Shc fusion proteins were prepared and used in actin cosedimentation assays. Constructs containing the SH2 and collagen homology domains were not precipitated, and those containing the amino-terminal domain were. Thus, Shc-actin interactions do not occur in the region of tyrosine phosphorylation and leave the SH2 domain free to bind to other tyrosine-phosphorylated molecules. Although the major pool of Shc in unstimulated PC12 cells is soluble, two other pools are associated with the cytoskeleton and the submembranous cytoskeleton. Upon nerve growth factor stimulation, approximately 50% of the soluble Shc translocates to both cytoskeleton environments within 2 min, decreasing thereafter. When cells were pretreated with cytochalasin D, a drug that disrupts actin filaments, Shc translocation to the cytoskeleton was abolished. However, in the submembranous fraction, the Shc level was elevated in resting cells following cytochalasin D treatment. The kinetics of translocation, compared to mitogen-activated protein kinase activation, and the nature of the Shc-actin interaction suggest that the cytoskeletal association of Shc, induced by growth factors, may be related to membrane ruffling and actin fiber reorganization.

Shc protein is part of a family of adapters involved in the mitogenic and differentiation signaling of a variety of receptor tyrosine kinases including those for EGF (1), NGF (2, 3), PDGF (4), and insulin (5, 6). Shc also transduces the signal of other receptor classes such as those for interleukin 2 (7) and the T-cell (8). In addition, FGF, growth hormone, thrombopoietin, interleukin 3, granulocyte-macrophage colony-stimulating factors, erythropoietin, and hepatocyte growth factor induce Shc phosphorylation, but the direct interaction of Shc with their respective receptors has not yet been observed (9–15).

The Shc adapter protein is composed of one SH2 domain at the carboxy-terminal end, one collagen homology (CH) domain and one amino-terminal domain. It exists in two main forms of 46 and 52 kDa, resulting from alternative translation start sites, and a 66-kDa isoform whose origin is less clear (1). When a receptor such as that for NGF is activated, Shc binds rapidly to a specific phosphotyrosine of the receptor and becomes phosphorylated on a tyrosine residue (1, 16, 17). The adapter protein Grb-2 then associates with Shc which allows p21ras activation via the guanine nucleotide release protein, Sos, leading to the activation of the MAPK pathway (16–26). Initially, the Shc SH2 domain was presumed to be responsible for the binding of Shc to the activated growth factor receptor, although a discrepancy existed between Shc recognition site and Shc SH2 domain specificity (2, 27–29). Several recent studies have shown that Shc binds the activated receptor in the NPY sequence by its amino-terminal or phosphotyrosine binding domain (30–35). This mechanism suggests that Shc SH2 domain remains free to interact with other yet unidentified phosphotyrosine-containing proteins. In addition, it has been shown that the Shc phosphotyrosine binding domain is able to recognize several other non-receptor phosphoproteins (36). Recent studies indicate that when a growth factor receptor contains a binding site for Shc and Grb-2, the Shc pathway is dominant (5, 20). This could be explained by the higher affinity of the phosphotyrosine binding domain for the NXPY motif compared with the affinity that the SH2 domains display for their proper binding sites (32). Besides its binding to activated receptors, Shc has been shown to associate with intracellular molecules such as phosphatidylinositol 3-kinase (37), Sos (38), c-Abl (39–41), and the tyrosine phosphatase PEST (42) as well as to several unidentified phosphotyrosine-containing proteins (10, 39, 43). However, the physiological relevance of these associations is still unclear.

Shc has oncogenic properties since its overexpression induces transformation in fibroblasts and differentiation in PC12 cells (1, 16). Differentiation of PC12 cells induced by NGF requires Shc activity. When Tyr 490 of TrkA, the binding site for Shc, is mutated to phenylalanine, NGF can no longer promote PC12 cell differentiation (3).

In the present study, Shc synthesis in PC12 cells stimulated by NGF and other growth factors was examined. It was observed that a 43-kDa protein was always found in Shc immu-
Fig. 1. Schematic representation of Shc domains expressed as GST-fusion proteins. Full-length human Shc cDNA and cDNAs fragments were cloned by reverse transcription-PCR of HepG2 cell total RNA and subdivided in the pGEX-3X expression vector. The arrow indicates the two translation initiation starts. N, Shc amino-terminal domain (hatched area); CH, collagen homology Shc domain (filled area); SH2, Shc Src-homology type 2 domain (open area).

1. EXPERIMENTAL PROCEDURES

Reagents—Mouse EGF and jngF were prepared by the method of Savage and Cohen (44) and Mabey et al. (45). A bgf analog in all which half-cystine residues were replaced by serines (46) was a gift from G. Michael Fox (Amgen Inc., CA). Polyclonal anti-Shc and monoclonal anti-phosphotyrosine (4G10) antibodies were purchased from Upstate Biotechnology (Lake Placid, NY), monoclonal anti-Shc and monoclonal anti-Grb-2 antibodies were purchased from Transductional Laboratories (Lexington, KY), monoclonal anti-actin antibody, rabbit muscle actin, poly-L-lysine, and cytochalasin D were from Sigma. Immobilon-P and Immobilon-CD membranes were from Millipore. pGEX-3X bacterial expression vector and glutathione-agarose were from Pharmacia Biotech Inc. All other chemicals were of the highest quality available.

Cell Culture—PC12 cells were obtained from E. Shooter (Stanford University) and grown in Dulbecco’s modified Eagle’s medium containing 10% horse serum and 5% fetal calf serum (Life Technologies, Inc) and 1% Pen-Strep solution (Life Technologies, Inc) (complete medium) in a 5% CO2 humidified atmosphere.

Metabolic Labeling of Cells—Cells were plated in collagen-coated 6-well plates in complete medium, grown to 70% confluence, and serum-starved in Dulbecco’s modified Eagle’s medium containing 1% horse serum for 48 h. Growth factors were added to the medium (EGF, 20 ng/ml; NGF, 100 ng/ml; bgf, 10 ng/ml) for the indicated period of time. For the last 2 h of growth factor stimulation, the medium was replaced by methionine-free medium containing 20 μCi/ml of [35S]methionine (ICN). Cells were then washed in ice-cold phosphate-buffered saline and lysed in RIPA buffer (50 mM Tris-HCl, pH 7.8, 1% Nonidet P-40, 140 mM NaCl, 0.1% SDS, 0.1% deoxycholic acid, 1 mM Na2VO4, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin). After centrifugation for 10 min at 30,000 x g at 4°C, the expressed GST fusion proteins were isolated from bacterial lysates by affinity chromatography using glutathione-agarose beads, washed 4 times in lysis buffer, and stored at 4°C on agarose beads in TBS containing 1% Triton X-100, 1 mM diethylthiourea, 1 mM phenylmethylsulfonyl fluoride, and 10% glycerol. CDNA fragment encoding Shc-1-232(N) was also subdivided in pGEX expression vector. However, this fragment could not be expressed as a GST-fusion protein.

Purification and identification of p43—A RIPA buffer PC12 cell extract corresponding to 50 x 150 μm dishes was incubated with GST-p52Shc-agarose for 4 h at 4°C. The agarose beads were washed several times in the same buffer, and GST-p52Shc eluted with reduced glutathione. The eluted material was mixed with a [35S]methionine radiolabeled Shc immunoprecipitate, separated on a 10% SDS-polyacrylamide gel, and transferred onto an Immobilon-CD membrane. The membrane was negatively stained according to the manufacturer’s recommendations and exposed with a x-ray film. After 48 h, the film was developed, and the band corresponding to the expressed GST-shc-immunoprecipitated 43-kDa protein was excised. The band was digested with endoprotease Lys-C (Boehringer Mannheim) using a modification of the method of Zhang et al. (47) as described (48). Following digestion, the supernatant was removed and stored, the membrane was sequentially extracted with acetonitrile (30% followed by 60%trifluoroacetic acid (25%), and the extracts were stored separately. Approximately 10% (0.3 μl) of the supernatant and of the acetonitrile extracts were applied to the mass spectrometer slide and mixed with an equal volume of matrix (α-cyan-4-hydroxycinnamic acid, 33 mM, Hewlett-Packard). The MALDI-MS spectra were internally calibrated with oxidized insulin B chain (Sigma) as described (49). The spectra were obtained on a Kratos Kompact MALDI-II (Kratos Analytical, Manchester, UK) in positive ion mode using both the linear and reflector settings as described previously (49). Sequence-specific fragmentation was obtained by post-source decay (PSD-)MALDI-MS (50) using a Voyager RP Biospectrometry Workstation (Perspective/Vestec Products) with parent-ion selection, which allows fragmentation of a specific ion from a mixture. The sample was prepared as above for MALDI-MS, and reflected spectra were recorded at various mirror ratios (reflector voltage/linear voltage) decreasing from 1 to 0.11 while maintaining a constant linear accelerating voltage of 25 kV and a grid voltage of 90%. The spectra were concatenated and calibrated using the manufacturer’s software.

Actin Sedimentation Assay—GST-fusion proteins (Fig. 1) were eluted from glutathione-agarose beads with reduced glutathione as described (51). The eluted fractions were quantitated on Coomassie Blue-stained gel using a dilution series of bovine serum albumin. Actin sedimentation assays were performed essentially as described by Hartig et al. (52). Briefly, 2 μg of GST-fusion proteins, GST, or a mixture of proteins were incubated with 10 μg of rabbit muscle G-actin for 10 min in 200 μl of buffer containing 2 mM Tris-HCl (pH 7.4), 0.2 mM CaCl2, 0.2 mM dithiothreitol, and 0.5 mM ATP. Actin polymerization was induced by adding 75 mM KCl and 2 mM MgCl2 and, after 1 h at room temperature, the samples were centrifuged at 100,000 x g for 1 h at 25°C. The supernatant was carefully removed, concentrated with Microcon ultrafiltration units (Amicon Inc., Beverly, MA), and mixed with SDS-PAGE loading buffer. Pellets and supernatants were analyzed on a 10% SDS-polyacrylamide gel and stained with Coomassie blue. The sedimentation assay was performed with the CH domain of Shc, proteins contained in the supernatant and the pellet were blotted onto polyvinylidene difluoride membrane, and the presence of GST-CH was detected with an anti-GST monoclonal antibody (Santa Cruz Biotechnology) and the enhanced chemiluminescence system. To determine whether Shc could bind to phosphorylated G-actin, the prepared Blue-stained GST-Shc fusion protein was mixed with 10 μg of G-actin in the same condition as for the sedimentation assay in the absence of KCl and MgCl2. After a 1-h incubation at room temperature with a continuous gentle agitation, agarose-bound GST-Shc fusion proteins were washed 2 times in incubation buffer with or without 1% Triton X-100. Agarose beads were then boiled in loading buffer, and proteins were separated on a 10% SDS-polyacrylamide gel.

Isolation and Analysis of Triton X-100-soluble and -insoluble Fractions—PC12 cells were plated on poly-L-lysine-coated 150-mm dishes in the published sequences in codon 399 with no effect on the coding potential of the CDNA. The CDNA were then dropped in the pGEX-3X bacterial expression plasmid, which was used to transform the protease-deficient BL21 Escherichia coli strain. The resulting glutathione S-transferase (GST) fusion proteins were induced by 1 mM isopropyl-1-thio-β-D-galactopyranoside for 4 h. Bacteria were lysed by sonication in Tris-buffered saline (TBS) containing 1% Triton X-100, 1 mM diethylthiourea, 1 mM benzamidine, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. After centrifugation for 10 min at 30,000 x g at 4°C, the expressed GST fusion proteins were isolated from bacterial lysates by affinity chromatography using glutathione-agarose beads, washed 4 times in lysis buffer, and stored at 4°C on agarase beads in TBS containing 1% Triton X-100, 1 mM diethylthiourea, 1 mM benzamidine, and 10% glycerol. CDNA fragment encoding Shc-1-232(N) was also subdivided in pGEX expression vector. However, this fragment could not be expressed as a GST-fusion protein.

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complete medium, grown to 70% confluence, and serum-starved for 18 h in Dulbecco’s modified Eagle’s medium containing 1% horse serum. Cells were then stimulated for the indicated period of time with 100 ng/ml of NGF. When cells were treated with cytochalasin D, the drug was added to the culture medium at a final concentration of 2 μM 2 h before adding NGF. After stimulation, dishes were immediately placed on ice, and cells were washed once with ice-cold phosphate-buffered saline containing 100 μM Na3VO4 and 50 mM NaF, harvested, and pelleted by centrifugation at 800 × g for 5 min at 4 °C. Cell pellets were then immediately lysed in 200 μl of extraction buffer (EB) (53) containing 100 mM Tris-HCl (pH 7.4), 2% Triton-X-100, 10 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 20 μg/ml apro- tinin, and 2 mM Na3VO4. After incubation on ice for 10 min, the Triton-insoluble residues were pelleted at 10,000 × g for 10 min and washed twice with 1 ml of 2 × diluted EB. The two first supernatants were mixed, and the submembranous cytoskeleton was sedimented from this fraction by further centrifugation at 100,000 × g for 30 min (54). These pellets were washed once with 2 × diluted EB. Triton-insoluble and submembranous cytoskeletal extracts were incubated for 30 min on ice in 0.5 ml of 2 × diluted EB containing 0.5 M NaCl and 0.3% deoxycholic acid and centrifuged at 100,000 × g for 30 min. The supernatants were added 1 ml of EB in order to lower the concentration of NaCl and deoxycholic acid. Shc was immunoprecipitated by incubating with 3 μg of anti-Shc polyclonal antibodies for 2 h at 4 °C followed by protein A-agarose precipitation. Immunoprecipitated materials were boiled in SDS-PAGE loading buffer, separated on a 10% SDS-polyacrylamide gel, and transferred onto Immobilon-P membranes. Membranes were blocked for at least 2 h at room temperature in TBS containing 0.05% Tween 20 and 4% BSA before being probed with 4G10 anti-phosphotyrosine monoclonal antibody and analyzed with the enhanced chemiluminescence detection system (Amersham). After detection of phosphotyrosine, membranes were stripped in glycine-HCl (pH 2.5). 0.05% Tween 20 for 2 h at 80 °C. Membranes were then reblocked and reprobed with anti-Shc monoclonal antibody. Exposed films were digitalized with a scanner (Hewlett Packard) and analyzed with the Image Quant software ( Molecular Dynamics).

RESULTS

A 43-kDa Protein Is Found in Shc Immunoprecipitates—PC12 cells, metabolically labeled with [35S]methionine and stimulated for the indicated period of time either with EGF, NGF, or bFGF, were lysed, sonicated, and immunoprecipitated with an anti-Shc polyclonal antibody and run on a 10% SDS-polyacrylamide gel. As shown in Fig. 2, 46- and 52-kDa forms of Shc were easily detected in unstimulated PC12 cells, indicating that Shc synthesis is active in resting cells. A doublet at 68 kDa was also detected, and, although the molecular size of this doublet suggests it could correspond to the previously described 66-kDa Shc-related protein (1), this band was not detected on Shc immunoblots (data not shown). Therefore, it cannot be ruled out that this doublet corresponds to another Shc-unrelated protein that is co-immunoprecipitated with Shc. Following growth factor stimulation, the level of Shc synthesis was not significantly altered with time. However, a band at 43 kDa (p43), which was already detectable in resting cells, clearly appeared in Shc immunoprecipitates. Interestingly, p43 synthesis seemed to be regulated upon NGF stimulation. As shown in Fig. 2, p43 reached a maximal level after 4 h of NGF stimulation and decreased thereafter but remained high up to 24 h following NGF stimulation. The same result was obtained for bFGF, but not for EGF for which the level of p43 synthesis remained stable (data not shown). These data indicated that a 43-kDa protein associated in vivo with Shc and that its synthesis is stimulated and regulated by neurotrophic growth factors, suggesting it is related to the neurotrophic response of the cells.

p43 Coimmunoprecipitated with Shc Is Actin—Human Shc cDNA obtained from HepG2 cells by reverse transcription-PCR, was subcloned in the pGEX-3X bacterial expression vector to produce a GST-p52Shc fusion protein. The agaro-immobilized GST-p52Shc was used to affinity purify p43 from a RIPA buffer extract of NGF-stimulated PC12 cells. Bands corre-
binds actin, the behavior of various GST-Shc and -truncated Shc fusion proteins (see Fig. 1) in cosedimentation assays with actin were tested. Aliquots of GST-Shc fusion proteins and purified rabbit muscle actin were incubated at room temperature in the presence of KCl and MgCl₂ for 60 min as indicated under "Experimental Procedures." Subsequently, the actin filaments were sedimented and the supernatant containing unpolymerized actin was removed and concentrated by ultrafiltration. Both pellet and supernatant were separated on SDS-PAGE and analyzed after Coomassie Brilliant Blue staining or, respectively, as described under "Experimental Procedures." Shc immunoprecipitations were performed with GST-Shc and F-actin dependent manner—To determine whether Shc associates with the cytoskeleton in vivo, an equal number of PC12 cells were extracted as described under "Experimental Procedures." Three fractions, the Triton-soluble fraction, the 10,000 × g cytoskeleton-rich fraction, and the membrane skeleton fraction were obtained. Shc immunoprecipitations were performed with an anti-Shc polyclonal antibody directly on the Triton-soluble fraction and after extraction in 0.5 M NaCl plus 0.3% deoxycholic acid for both cytoskeleton fractions. The amounts of Shc protein and tyrosine-phosphorylated Shc were analyzed by immunoblotting with anti-Shc and 4G10 anti-phosphotyrosine monoclonal antibodies. Fig. 8 (left panel) shows that, in resting PC12 cells, Shc is mainly (66% of total Shc amount) present in the soluble fraction (TS); however, low but detectable amounts are also found in cytoskeleton (TI) and submembranous (SM) fractions (13% and 21%, respectively). Following NGF stimulation, the level of Shc in the soluble fraction quickly decreased and reached a minimum level 2 min after NGF was added to the culture medium (31% of total Shc amount). At 5 min, Shc concentration increased but did not reach its initial level after 10 min of NGF stimulation (42% of total Shc amount). Shc tyrosine phosphorylation was detectable as soon as 30 s post-NGF stimulation, and the amount of tyrosine-phosphorylated Shc protein increased progressively up to 10 min. In contrast with the Triton-soluble fraction, NGF stimulation induced an increase in Shc concentration in both cytoskeleton and submembranous fractions which peaked at 2 min (27% and 41%, respectively) and decreased thereafter (24% and 34% after 10 min). The amount of tyrosine-phosphorylated Shc protein increased in a similar fashion, reaching a maximal level 2 min post-NGF stimulation and decreasing thereafter. These results indicate that NGF stimulation induces Shc to transiently translocate from the soluble compartment of the cell to the cytoskeleton and the submembranous compartments. The fact that, initially, the actin levels in Shc immunoprecipitates were found stable following NGF stimulation is probably due to the difference in the way cellular extracts were prepared in this
Fig. 4. Sequence information on one of the Lys-C fragments. Sequence of specific fragment ions were obtained from PSD-MALDI-MS analysis of the selected ion 1961.7 (↑) from the sample shown in Fig. 3B. The fragment ions consistent with b series ions are labeled, and the b series ions expected from the given sequence are shown as well. The strong ion at m/z 124 represents the methylated histidine ammonium ion.

Fig. 5. Confirmation by immunoblotting of the presence of actin in Shc immunoprecipitate. To further assess the result obtained by mass spectrometry, Shc was immunoprecipitated from PC12 cells stimulated with 100 ng/ml NGF for the indicated periods of time. Blot of Shc-immunoprecipitated materials was probed with an anti-actin monoclonal antibody. 50 μg of protein from a PC12 cell total extract was run in parallel.

Because Shc acts as an actin-binding protein and is transiently translocated to the cytoskeleton upon growth factor stimulation, its redistribution in cells with a disrupted actin cytoskeleton was examined. For this purpose, resting PC12 cells were treated with 2 μM cytochalasin D for 2 h prior to growth factor stimulation and Shc distribution as well as tyrosine phosphorylation were analyzed as above. Fig. 8 (right panel) shows that, in resting cells, Shc was found in the soluble (TS) and the submembranous (SM) fractions but was not detectable in the cytoskeletal fraction (TI) even after a longer exposure (data not shown). Upon NGF stimulation, Shc concentration in the soluble fraction remained unchanged. However, the kinetics and amount of Shc tyrosine phosphorylation were not significantly affected by cytochalasin D treatment. In the cytoskeletal fraction, Shc was not detectable even after 2 min of NGF stimulation, indicating that Shc could not translocate in cytochalasin D-treated cells. In the submembranous fraction of cytochalasin D-treated cells, Shc was found present at a level comparable to the soluble fraction. The concentration of Shc in this fraction did not change following NGF stimulation. Although Shc tyrosine phosphorylation occurred, it was weak compared with the amount of Shc present. This result indicates that growth factor-induced Shc translocation depends on the integrity of the actin cytoskeleton.

DISCUSSION

Shc is a member of a diverse group of adaptor molecules that link many tyrosine kinase-containing receptors to downstream signaling entities. In PC12 cells, it is rapidly bound to Tyr-490 of the TrkA receptor following NGF stimulation and, following phosphorylation, provides the assembly site for the formation of the "activated" Ras complex that is necessary for the activation of MAPK cascades and their translocation to the nucleus (2, 16–26). Recent evidence has established that the interaction with TrkA is through the amino-terminal region rather than the SH2 domain located in the carboxyl-terminal domain of the molecule (30, 35).

The observation that Shc can also bind to filamentous actin and that this interaction is rapidly stimulated by growth factors suggest a new role for this adaptor. As with the receptor interaction, binding occurs through the amino-terminal domain. However, it does not interact with phosphorytrosine residues on actin and does not require self-phosphorylation. This suggests that the binding to receptor and actin would be eventually exclusive and that the cytoskeletal-bound Shc is also able to bind additional phosphorytrosine-containing proteins through its SH2 domain as is presumably the case for the receptor-bound form (30, 31, 35). Although we have demonstrated that in vitro Shc can bind directly to F-actin, the possibility that in vivo Shc could also associate indirectly with the actin cytoskeleton cannot be ruled out.

The large increase in actin associated with Shc detected after 4 h of NGF and FGF stimulation in PC12 cells appears to be a function of the long term response of these cells to produce neurites. EGF-treated cells do not show this response and do not go on to produce neurites. However, this peak in apparent actin association appears to be due primarily to an increase in synthesis (determined by an increase in [35S]methionine labeling) as judged by the concentration of actin detected by Western blot (Fig. 5). The rapid mobilization (within 2 min) of soluble Shc to cytoskeleton appears to represent the early growth factor response. Although Shc translocation was observed when PC12 cells were stimulated with NGF, we do not think that this phenomenon reflects any NGF-specific effect. The reason we did not perform our experiments with EGF is that the EGF receptor is an actin-binding protein and that an important percentage of the total EGF receptor population is linked to the actin cytoskeleton (52). Therefore, we think that this phenomenon reflects any NGF-specific effect. The result we did not perform our experiments with EGF is that the EGF receptor is an actin-binding protein and that an important percentage of the total EGF receptor population is linked to the actin cytoskeleton (52). Therefore, we think that this phenomenon reflects any NGF-specific effect.
present in the different cytoskeletal preparations.

Shc redistribution upon growth factor stimulation has previously been described in cells stimulated by interleukin 3 (11) or PDGF (57) where phosphorylated Shc was found in membrane preparations supposedly in association with activated receptors. Our findings suggest that a part of Shc found in membrane preparations could be associated with the actin cytoskeleton.

What induces Shc translocation? It is interesting to note that the level of tyrosine-phosphorylated Shc in the cytoskeletal fractions parallels the amount of Shc that translocates to these compartments. This could suggest that tyrosine phosphorylation of Shc increases its affinity for F-actin and consequently induces its translocation to the actin cytoskeleton. However, if true, a decrease in the amount of tyrosine-phosphorylated molecules should also have been detected in the soluble fraction or at least there should have been an accumulation of tyrosine-phosphorylated Shc in the soluble fraction. Instead, the amount of tyrosine-phosphorylated Shc in the soluble fraction increases continuously up to 10 min of NGF stimulation while the translocation phenomena is transitory. Therefore, it seems more likely that Shc translocation is independent of Shc phosphorylation as the in vitro actin cosedimentation assay suggested.

One hypothesis is that there are two different Shc subpopulations that are independently phosphorylated and that another early intracellular event which follows TrkA activation and is independent of Shc binding to the receptor and of its tyrosine phosphorylation, is responsible for Shc translocation. It is tempting to suggest that the rapid and transitory intracellular calcium influx that follows the activation of a growth factor receptor (58–62) could be responsible for Shc translocation. Consistent with this view, phospholipase Cγ activation, leading to the formation of the second messengers diacylglycerol and inositol triphosphate, which in turn activate protein kinase C and mobilize intracellular calcium, respectively (59, 60), could modify Shc affinity for F-actin, resulting in the translocation of a Shc subpopulation (phosphorylated or not) to the actin cytoskeleton. Once Shc has been translocated, it could then be phosphorylated in situ by a cytoskeletal associated non-receptor tyrosine kinase. Such a non-receptor tyrosine kinase could be represented by the Src family of tyrosine kinases, c-Abl or Syk, which are known to associate and/or to translocate to the cytoskeleton under some circumstances, and Shc is known to be a substrate for them (17, 41, 63–68).

What is the function of Shc translocation? It is now well established that Shc phosphorylation by the activated receptor tyrosine kinase is the first step of a cascade of events that leads eventually to MAPK activation (16–26). As shown here, Shc translocates to two different cytoskeletal fractions and may become phosphorylated on tyrosine by other non-receptor tyro-
sine kinases present on the cytoskeleton. Thus, in addition to soluble Shc, it is possible that the bound Shc also functions in initiating the activation of the Ras/MAPK pathways. In support of this idea, Grb-2 is found in Shc immunoprecipitates at a level that correlates with the amount of phosphorylated Shc (data not shown). However, the kinetics of MAPK activation, which coincides in PC12 cells with the level of tyrosine-phosphorylated Shc found in the soluble fraction (data not shown), argues against this interpretation. MAPK activation reaches a maximum 10 min after NGF stimulation and remains at a high level up to 1 h. In contrast, in the cytoskeletal fraction, phosphorylated Shc concentration is maximal after 2 min following NGF stimulation and decreases quickly thereafter. This difference may in fact reveal the involvement of cytoskeleton-associated Shc in other functions.

Shc is an adapter protein which has been found recently to associate with several identified (37–42) and unidentified phosphotyrosine-containing proteins (10, 36, 43). In addition, Shc has several potentially phosphorylatable tyrosine residues that could be targets for Grb-like molecules and is, in some circumstances, phosphorylated on serine residues (17, 69). Thus, Shc is clearly complex, and it is likely that it can initiate pathways other than the Ras/MAPK pathway, occurring at different times and in different locations inside the cell. One such role is the reorganization of actin stress fibers and membrane ruffling that occurs in response to growth factor stimulation. Both phenomena are under the control of the small GTP-binding proteins, Rho (70) and Rac (71), respectively. Recently, Grb-2 has been linked to the formation of membrane ruffles probably by modulating Ras and Rac signaling pathways (72). In addition, it has been reported that a GST-Grb-2 fusion protein, microinjected into cells, localizes to membrane ruffles (73). Although this localization seems to be mediated by the Grb-2 SH3 domain, the presence of tyrosine-phosphorylated Shc in the membrane ruffles may account in part for the Grb-2 co-localization. Therefore, Shc may also participate in membrane ruffling and the early actin reorganization following growth factor stimulation by coupling Grb-2 and small GTP-binding proteins to the actin cytoskeleton.

A direct role for Shc in the regulation of actin polymerization should also be considered. Shc does not have any known catalytic activity; and, therefore, it is unlikely to be directly involved in the regulation of actin polymerization or the reorganization of the cytoskeleton that follows growth factor stimulation (74–77). However, the possibility that Shc binding to the actin-cytoskeleton could regulate actin polymerization by competing with agents like gelsolin, which are inhibitors of actin polymerization, is a possibility since this function has been envisaged, for example, for the non-receptor tyrosine kinase, c-Abl, another actin-binding molecule (66).

Many other proteins involved in the signal transduction of growth factors can also be found associated with the cytoskeleton. Grb-2 has been localized specifically with the membrane ruffles (73), and phospholipase Cγ, phosphatidylinositol 3-kinase, c-Src, and c-Abl whose enzymatic activities or presence have been detected in the cytoskeletal fraction in various situations (63–66, 73, 78–80). Interestingly, all these proteins contain an SH3 domain, which plays an important role for subcellular compartmentalization and cytoskeletal localization as has been shown for Grb-2 and phospholipase Cγ (73, 81). However, this is not always true. The catalytic and the SH2 domains of v-Src are required for its binding to the cytoskeleton (65), and c-Abl contains a specific F-actin binding sequence (66). In this study, Shc has been shown to bind F-actin directly.
through its amino-terminal domain in the absence of any other protein. Experiments are now in progress to determine precisely the F-actin binding of Shc amino terminus.

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