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Shc Proteins Are Localized on Endoplasmic Reticulum Membranes and Are Redistributed after Tyrosine Kinase Receptor Activation

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The intracellular localization of Shc proteins was analyzed by immunofluorescence and immunoelectron microscopy in normal cells and cells expressing the epidermal growth factor receptor or the EGFR/erbB2 chimera. In unstimulated cells, the immunolabeling was localized in the central perinuclear area of the cell and mostly associated with the cytosolic side of rough endoplasmic reticulum membranes. Upon epidermal growth factor treatment and receptor tyrosine kinase activation, the immunolabeling became peripheral and was found to be associated with the cytosolic surface of the plasma membrane and endocytic structures, such as coated pits and endosomes, and with the peripheral cytosol. Receptor activation in cells expressing phosphorylation-defective mutants of Shc and erbB-2 kinase showed that receptor autophosphorylation, but not Shc phosphorylation, is required for redistribution of Shc proteins. The rough endoplasmic reticulum localization of Shc proteins in unstimulated cells and their massive recruitment to the plasma membrane, endocytic structures, and peripheral cytosol following receptor tyrosine kinase activation could account for multiple putative functions of the adaptor protein.

The Shc locus is highly conserved throughout evolution and codes for three overlapping proteins of 66, 52, and 46 kDa ($p66^{shc}$, $p52^{shc}$, and $p46^{shc}$) (38). They are encoded by two distinct transcripts that derive from the same locus but differ in the presence of 5′ alternative coding exons or parts of exons (34). $p66^{shc}$, $p52^{shc}$, and $p46^{shc}$ have a common carboxy-terminal SH2 domain, flanked by a glycine- and proline-rich region of approximately 145 amino acids (collagen homologous region 1 [CH1]) (38). At the N terminus, adjacent to the CH1 region, they possess a region with functional properties of a phosphotyrosine-binding domain (PTB) (4, 6, 24, 25, 47, 54). The binding specificities of the Shc PTB and SH2 domains differ (3, 24, 44, 54). The unique amino-terminal portion of $p66^{shc}$ contains an additional collagen homologous region (CH2 region). The CH1 and CH2 regions are putative SH3 binding sites (34).

Shc proteins are phosphorylated by all activated receptor tyrosine kinases (RTKs) tested to date: epidermal growth factor receptor (EGFR) (38, 39), platelet-derived growth factor receptor (55), hepatocyte growth factor receptor (37, 39), erbB-2 receptor (47), insulin receptor (23, 26, 40, 41, 48), fibroblast growth factor receptor (53), nerve growth factor receptor (7, 50), and sea receptor (13). Phosphorylated Shc proteins bind to activated receptors through their PTB and/or SH2 domains (25, 26, 54). Shc proteins are also involved in signalling from cytoplasmic TKs, since they are constitutively phosphorylated in cells that express activated Lck, Src, or Fps (2, 17, 32, 42, 51). In addition, Shc proteins are rapidly phosphorylated on tyrosine after ligand stimulation of surface receptors that have no intrinsic TK activity but are thought to signal by recruiting and activating cytoplasmic TKs (e.g., interleukin 2 erythropoietin, granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, B- and T-cell receptors, CD4, and CD8) (2, 9, 14, 27, 31, 43).

The major site of Shc phosphorylation is Tyr-317 (46), which mediates interactions with the SH2 domain of the Grb2 adaptor protein (12, 30). Grb2 is constitutively complexed with Sos (4, 8, 10, 18, 22, 36, 45), a ubiquitously expressed Ras guanine nucleotide exchange factor for Ras. Recruitment of the Grb2/Sos complex by phosphorylated Shc proteins results in the membrane relocalization of Sos, an event considered sufficient to induce Ras activation (1), suggesting that Shc proteins are implicated in the regulation of Ras. Tyrosine phosphorylation of Shc proteins and association with the Grb2-Sos complex are growth factor-dependent events that occur mainly in endosomes (16).

Here we show that Shc proteins are localized intracellularly either scattered throughout the central cytoplasm or associated with the cytosolic side of rough endoplasmic reticulum (RER) cisternae. Treatment with EGF induces redistribution of Shc proteins to the cell periphery and their association with the plasma membrane and endocytic structures. Evidence that receptor autophosphorylation, but not Shc phosphorylation, is required for redistribution of Shc proteins was obtained with both erbB2 and Shc tyrosine phosphorylation-defective mutants.

MATERIALS AND METHODS

Cell lines. ZIP-SHC13 and ZIP-SHC9 are NIH 3T3 clones obtained by transfecting $p52^{shc}/p46^{shc}$-encoding cDNA (38). ZIP-1 is a control NIH 3T3 clone transfected with the empty expression vector (a Moloney murine leukemia virus-based retroviral vector) (38). NIH-p66-8 is an NIH 3T3 clone transfected with $p66^{shc}$-encoding cDNA (34). NR6-EGFR/erbB2 and NR6-EGFR/erbB2-5P are NR6 polyclonal cell populations overexpressing the EGFR/erbB2 or the EGFR/erbB2-5P chimera and $p52^{shc}/p46^{shc}$ (47). SAA-SHC, SAA-TM, and SAA-SN are

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polyclonal cell populations obtained by transfecting the EGFR-overexpressing SAA cells with the $p52^{shc}/p46^{shc}$ cDNA, the Tyr-317→Phe TM $p52^{shc}/p46^{shc}$ cDNA, and the LXSN empty expression vector, respectively (46). Cells were cultured in Dulbecco's modified EagleS medium supplemented with 10% fetal calf serum and antibiotics. For EGF treatment, SAA-SHC, SAA-TM, NR6-EGFR/erbB2, and NR6-EGFR/erbB2-5P cells were serum starved for 48 h and then incubated with 10 nM EGF (Gibco, Grand Island, N.Y.) (10 ng/ml) at 37°C for 10 min before fixation. Treatment with nocodazole (20 μg/ml) to depolymerize microtubules and with cytochalasin B (5 μg/ml) to depolymerize microfilaments was performed for 1 h at 37°C before EGF addition and prolonged during incubation with EGF for 10 min at 37°C.

Anti-Shc antibodies. The anti-SHC-SH2 polyclonal antibody was raised against the Shc SH2 region (38). The anti-SHC-CH1 polyclonal antiserum was raised against the Shc APRDLFDMKPFEDALRVC synthetic peptide derived from the CH1 region (46). The anti-SHC-SH2 and the anti-SHC-CH1 antibodies react against the three Shc isoforms. The anti-SHC-CH2 polyclonal antibody was raised against the Shc CH2 region (34) and specifically interacts with $p66^{shc}$ (34). The anti-SHC-SH2 monoclonal antibody was raised against the Shc SH2 domain (19).

Immunoprecipitations and Western blotting procedures. For whole-cell lysates, cells were directly lysed in sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris [pH 6.8], 2% [vol/vol] SDS, 10% glycerol, and 5% [vol/vol] β-mercaptoethanol) and boiled for 5 min. For immunoprecipitation studies, cells were lysed in ice in 50 mM Tris HCl (pH 8.0)–150 mM NaCl–1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] (pH 8.0)–100 mM NaF (pH 8.0)–10% glycerol–1.5 mM $MgCl_2$–1% (vol/vol) Triton X-100–1 mM sodium orthovanadate containing freshly added protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin per ml, and 5 mg of aprotinin per ml). Unsolubilized materials were removed by centrifugation for 10 min at 12,000 × *g* at 4°C, and protein concentration was determined with the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, Calif.). For immunoprecipitation experiments, appropriate antibodies were adsorbed on protein A–Sepharose CL-4B (Pharmacia LKB, Uppsala, Sweden) and then incubated with cell lysates for 90 min at 4°C. Immune complexes were washed three times with cold NET gel buffer (50 mM Tris HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA [pH 8.0], 0.1% [vol/vol] Nonidet P-40, 0.25% gelatin, 1 mM sodium orthovanadate, and proteinase inhibitors), eluted, and denatured by heating for 3 min at 95°C in reducing Laemmli buffer. Samples were then resolved by SDS-polyacrylamide gel electrophoresis. For immunoblot analysis, either specific immunoprecipitates or 20 μg of total cell lysate was electrotransferred onto nitrocellulose filters (Bio-Rad Laboratories). After blocking of nonspecific reactivity, filters were probed overnight at 4°C with specific antibodies diluted in TBS-T (20 mM Tris HCl [pH 7.8], 150 mM NaCl, 0.02% Tween 20) containing 1% bovine serum albumin (BSA) (fraction V; BDH Chemicals Ltd., Poole, England). After extensive washing, immunocomplexes were detected either with horseradish peroxidase-conjugated species-specific secondary antiserum (Bio-Rad Laboratories) followed by enhanced chemiluminescence reaction (Amersham International plc) or with goat alkaline phosphatase-conjugated anti-rabbit immunoglobulin G (Fc) antibody (Promega, Madison, Wis.) followed by color reaction performed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (Promega). The anti-SHC-SH2 monoclonal antibody was used at a 1:100 dilution for immunoblot analysis and 0.5 mg/ml in immunoprecipitation procedures. The anti-erbB2 antibody was used at a 1:1,000 dilution for Western blotting (immunoblotting) analysis, as previously described (47). Monoclonal antibodies to phosphotyrosine were purchased from Upstate Biotechnology Inc. (Lake Placid, N.Y.) and used at 1 mg ml^{-1} for immunoblot analysis.

MAP kinase activation assay. T31-1 (NR6-EGFR/erbB2) and T31-3 (NR6-EGFR/erbB2 5P) cells were serum starved for 24 h and then treated with 10 ng of EGF per ml for 1, 5, and 15 min. Cells were lysed, and 50-μg samples of total lysates were electrophoresed on a 11% polyacrylamide gel, blotted onto nitrocellulose, and incubated with anti-mitogen-activated protein kinase (anti-MAP kinase) antiserum. Polyclonal antibodies to MAP kinase were purchased from Upstate Biotechnology Inc. and used at 1 mg ml^{-1} for Western blot analysis. Results shown are representative of data obtained in three experiments.

Immunofluorescence. All cells, grown on coverslips, were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) (30 to 60 min, 25°C) and permeabilized with 0.1% Triton X-100 in PBS for 10 min. Cells were incubated for 1 h at 25°C with the following primary antibodies: anti-SHC-SH2 and anti-SHC-CH1 polyclonal antisera (1:100 in PBS), anti-SHC-CH2 polyclonal antibody (1:1,000 in PBS), anti-SHC-SH2 monoclonal antibody (1:10 in PBS), antiphosphotyrosine monoclonal antibody (1:100 in PBS) (Py69; ICN Pharmaceuticals Inc., Costa Mesa, Calif.), anti-EGFR 108.1 monoclonal antibody (1:100 in PBS), anti-ER polyclonal antibody (29) (1:200 in PBS), and anti-cathepsin D polyclonal antibodies (1:100 in PBS) (kindly provided by Ciro Isidoro, University of Turin). For early endosome localization, cells were incubated with human transferrin (hTf) (5 μg/ml) (Sigma Chemical Co., St. Louis, Mo.) for 1 h at 37°C before fixation and permeabilization; the internalized hTf was then detected with anti-hTf (1:500 in PBS) (Sigma). After washing with PBS, all bound antibodies were visualized with anti-mouse immunoglobulin G (fluorescein isothiocyanate [FITC]) (1:10 in PBS for 30 min at 25°C) or with anti-rabbit immunoglobulin G FITC (1:100 in PBS for 30 min at 25°C). Anti-hTf antibodies were visualized with anti-goat immunoglobulin G FITC (Cappel Research Products, Durham, N.C.). For Golgi apparatus localization, cells were incubated with the lectins HPA-FITC or with WGA-FITC (1:10 in PBS for 1 h at 25°C) (Sigma).

Immunoelectron microscopy. ZIP-SHC9 and ZIP-1 cells, as well as SAA-SHC and SAA-SN cells, untreated or treated with EGF as above, were processed for postembedding immunocytochemistry as described elsewhere (28). Briefly, cells were fixed with 1% glutaraldehyde in PBS for 1 h at room temperature, partially dehydrated in ethanol, and embedded in LR White resin. Thin sections were collected on nickel grids, immunolabeled with the antibodies followed by protein A-gold (18 nm) prepared by the citrate method (49). The sections were stained with uranyl acetate and lead citrate.

Conventional electron microscopy. ZIP-SHC9 cells, incubated with BSA-gold particles (18-nm) for 15 min at 37°C to allow their internalization in early endocytic structures, and ZIP-1 cells were postfixed in 1% osmium tetroxide in veronal acetate buffer, pH 7.4, for 2 h at 4°C, stained with uranyl acetate (5 mg/ml), dehydrated in acetone, and finally embedded in Epon 812. Thin sections were examined unstained or poststained with uranyl acetate and lead hydroxide.

RESULTS

$p52^{shc}/p46^{shc}$ and $p66^{shc}$ are either scattered throughout the central cytoplasm or associated with the cytosolic side of RER cisternae. The intracellular localization of Shc proteins was first investigated by indirect immunofluorescence microscopy using two different anti-Shc polyclonal antibodies (anti-SHC-SH2 and anti-SHC-CH1; see Materials and Methods) and NIH 3T3 fibroblasts transfected with a $p52^{shc}/p46^{shc}$-encoding Shc cDNA (ZIP-SHC9 and ZIP-SHC13 clones; Fig. 1A). The two antibodies gave identical results. The staining pattern was reticular and localized in the perinuclear portion of the cytoplasm. A weaker cytoplasmic diffuse signal was also seen in some cells (Fig. 2A and B). The intensity of the staining was greater in the ZIP-SHC9 cells (Fig. 2A) than in the ZIP-SHC13 cells (Fig. 2B), and this correlated with the higher levels of Shc expression in the ZIP-SHC9 clone (Fig. 1A). Identical patterns of the immunofluorescence signal were obtained by staining v-src-transformed fibroblasts (32) with the anti-SHC-SH2 antibody and $p66^{shc}$-overexpressing NIH 3T3 cells (NIH p66-8 clone; Fig. 1A) with an anti-$p66^{shc}$-specific antibody (anti-SHC-CH2; see Materials and Methods) (Fig. 2C). None of the three anti-Shc polyclonal antibodies recognized endogenous Shc proteins in wild-type NIH 3T3 cells (data not shown). However, a monoclonal anti-Shc antibody stained the parental NIH 3T3 cells (ZIP-1 clone; Fig. 1A) faintly with a cellular distribution identical to that described above (Fig. 2D).

The subcellular localization of Shc proteins was first investigated by comparing the Shc staining pattern with that of antigens commonly used as markers of specific intracellular structures in ZIP-SHC9 cells. Specific polyclonal antibodies were used to stain the ER, WGA and HPA lectins were used to stain the Golgi complex, anti-hTf antibodies were used to identify early endosomes, and polyclonal antibodies against cathepsin D were used to reveal late endosomes and lysosomes. Except for the lack of the additional cytosolic signals frequently seen with the various anti-Shc antibodies, the reticular pattern of ER staining was very similar to that of Shc protein (Fig. 2E). The other markers gave distinctly different staining patterns. WGA (Fig. 2F) and HPA (not shown) were typically concentrated and localized in the perinuclear area of the Golgi complex. The anti-hTF staining, performed after hTF internalization for 1 h at 37°C, was punctate and dispersed throughout the cytoplasm (Fig. 2G). Staining of late endosomes and lysosomes was localized in discrete punctate structures distributed in both central and peripheral portions of the cytoplasm (Fig. 2H).

To analyze the endocellular localization of Shc proteins in greater detail, we performed gold immunolabeling electron microscopy using ZIP-SHC9, control NIH 3T3 fibroblasts, and

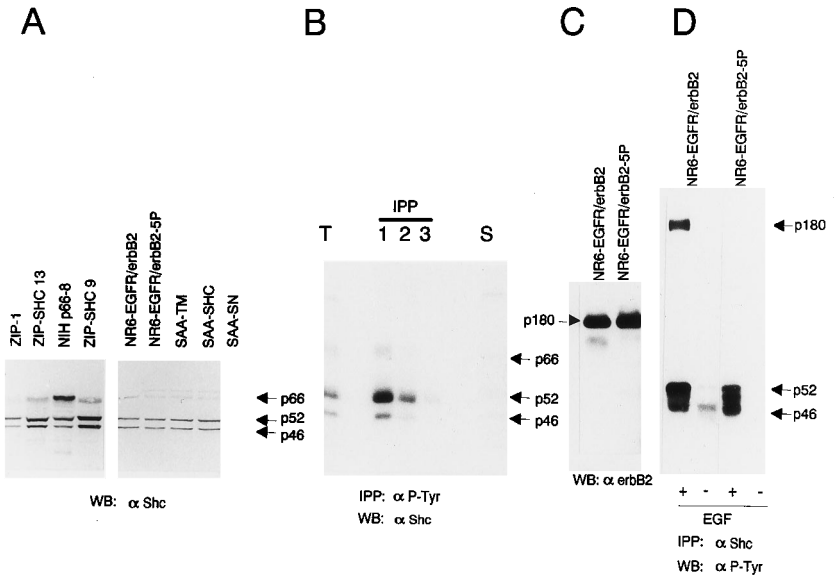

FIG. 1. (A) Western blot (WB) analysis of Shc expression in NIH 3T3 and SAA cells transfected with various Shc cDNAs. ZIP-SHC13 and ZIP-SHC9, $p52^{shc}/p46^{shc}$-overexpressing NIH 3T3 clones; ZIP-1, NIH 3T3 control clone; NIH p66-8, $p66^{shc}$-overexpressing NIH 3T3 clone; NR6-EGFR/erbB2, EGFR/erbB2- and $p52^{shc}/p46^{shc}$-overexpressing NR6 polyclonal cells; NR6-EGFR/erbB2-5P, EGFR/erbB2-5P- and $p52^{shc}/p46^{shc}$-overexpressing NR6 polyclonal cells; SAA-SHC, $p52^{shc}/p46^{shc}$-overexpressing SAA polyclonal cells; SAA-TM, Tyr-317→Phe TM mutant-overexpressing SAA cells; SAA-SN, SAA control polyclonal cell population. αShc, anti-SHC-SH2 monoclonal antibody. The TM mutated polypeptides comigrated with the endogenous $p46^{shc}$ and $p52^{shc}$. (B) Stoichiometry of in vivo phosphorylation of Shc proteins. SAA-SHC cells were treated with EGF (10 ng/ml) for 5 min at 37°C. Total cellular proteins (1 mg) were then immunoprecipitated three times with the antiphosphotyrosine antibody (IPP 1 to 3), and the immunocomplexes were recovered after each immunoprecipitation. Immunoprecipitates were analyzed in immunoblot with anti-SHC-SH2 monoclonal antibodies. The supernatant of the immunoprecipitation reaction (lane S; 1/5 of the total supernatant, corresponding to 200 μg of initial material) and an aliquot of the total cellular proteins (lane T; 200 μg of proteins) were also analyzed. Autoradiographic signals were quantified in a phosphorimager scanner. Stoichiometry of in vivo phosphorylation was calculated in two different ways according to either the formula (ipt × 100)/(ipt + supernatant × 5) or the formula (ipt × 100)/(total × 5). The two formulas yielded similar results. The positions of specific substrate bands are indicated. (C) WB analysis of erbB2 expression in NR6-EGFR/erbB2 and NR6-EGFR/erbB2-5P cells. The p180 EGFR/erB2 chimeric protein is indicated. (D) Anti-Shc immunoprecipitates (IPP) from NR6-EGFR/erbB2 and NR6-EGFR/erbB2-5P cells were immunoblotted with antiphosphotyrosine antibodies.

the anti-SHC-CH1 polyclonal antibody. Gold particles were located in the cell cytoplasm, either scattered throughout the central cytoplasm or associated with the cytosolic side of dilated RER cisternae (Fig. 3A). The lumen in the RER cisternae was unlabeled. As the swollen morphology of the RER cisternae was also observed in conventional electron microscopy of both ZIP-SHC9 (Fig. 3B) and parental NIH 3T3 cells (ZIP-1 clone) (not shown), the RER dilation does not seem to depend on overexpression of Shc proteins. Cytoplasmic or RER-associated gold immunolabeling was also present, although less dense, in more peripheral areas of the cell (Fig. 3C). There was no clear association of the labeling with the plasma membrane or the cytosolic side of other intracellular membranes, such as Golgi cisternae, endosomal structures, or mitochondrion membranes (Fig. 3A and C). Surprisingly, the outer nuclear membranes, which are contiguous with the RER, were also unlabeled (Fig. 3A). Late endosomes were unequivocally identified in parallel gold immunolabeling experiments using anti-cathepsin D antibodies (Fig. 3D). Early endocytic structures, labeled with BSA-gold particles internalized for 15 min at 37°C (Fig. 3B), were also easily identified. None of the endocytic compartment structures appeared to be labeled by anti-Shc antibodies or protein A-colloidal gold.

EGF treatment induced redistribution of Shc proteins. The effects of RTK activation on Shc localization were determined by analyzing the cellular distribution of Shc proteins upon EGF treatment in cells overexpressing both EGFRs and Shc proteins. For this purpose, EGFR-overexpressing mouse fibroblasts (SAA cells) (15) were transfected with $p52^{shc}/p46^{shc}$-encoding Shc cDNA (38). Shc protein expression levels in the resulting polyclonal cell population (SAA-SHC cells) and the parental SAA cells transfected with the empty expression vector (SAA-SN cells) are shown in Fig. 1B.

The optimal conditions for Shc phosphorylation and the stoichiometry of Shc phosphorylation were determined in EGF-treated SAA-SHC cells. EGF induced maximal Shc phosphorylation at a concentration of 10 nM (not shown). At this concentration, Shc phosphorylation was half maximal after 10 s of stimulation, reached a plateau at 1 min, and remained stable until 15 min of stimulation (not shown). We therefore measured the stoichiometry of Shc phosphorylation after 10 min of 10 nM EGF treatment in the SAA-SHC cells. Cell lysates from EGF-treated SAA-SHC cells were subjected to three sequential cycles of immunoprecipitation with antiphosphotyrosine antibody followed by immunoblotting with the anti-SHC-SH2 monoclonal antibody (Fig. 1B). Aliquots of the supernatant from the immunoprecipitation reaction and the total initial material were also analyzed in immunoblot (Fig. 1B). After autoradiography, the signals were quantified by phosphorimaging, and the percentage of in vivo phosphorylation was calculated after correction for the amount of material loaded. We estimated that 40 to 50% of the total Shc pool was phosphorylated, under our analysis conditions.

To analyze the effects of TK activation on Shc localization, SAA-SHC cells were serum starved for 48 h and then stimulated with 10 ng of recombinant EGF for 10 min at 37°C. Activation of EGFRs was monitored by labeling cells with an antiphosphotyrosine antibody (data not shown). The anti-Shc staining of serum-starved SAA-SHC cells was similar to that of ZIP-SHC9 cells (Fig. 4A), while it became punctate, peripheral, and frequently associated with the cell surface in EGF-treated cells (Fig. 4B). Like results were obtained upon EGF

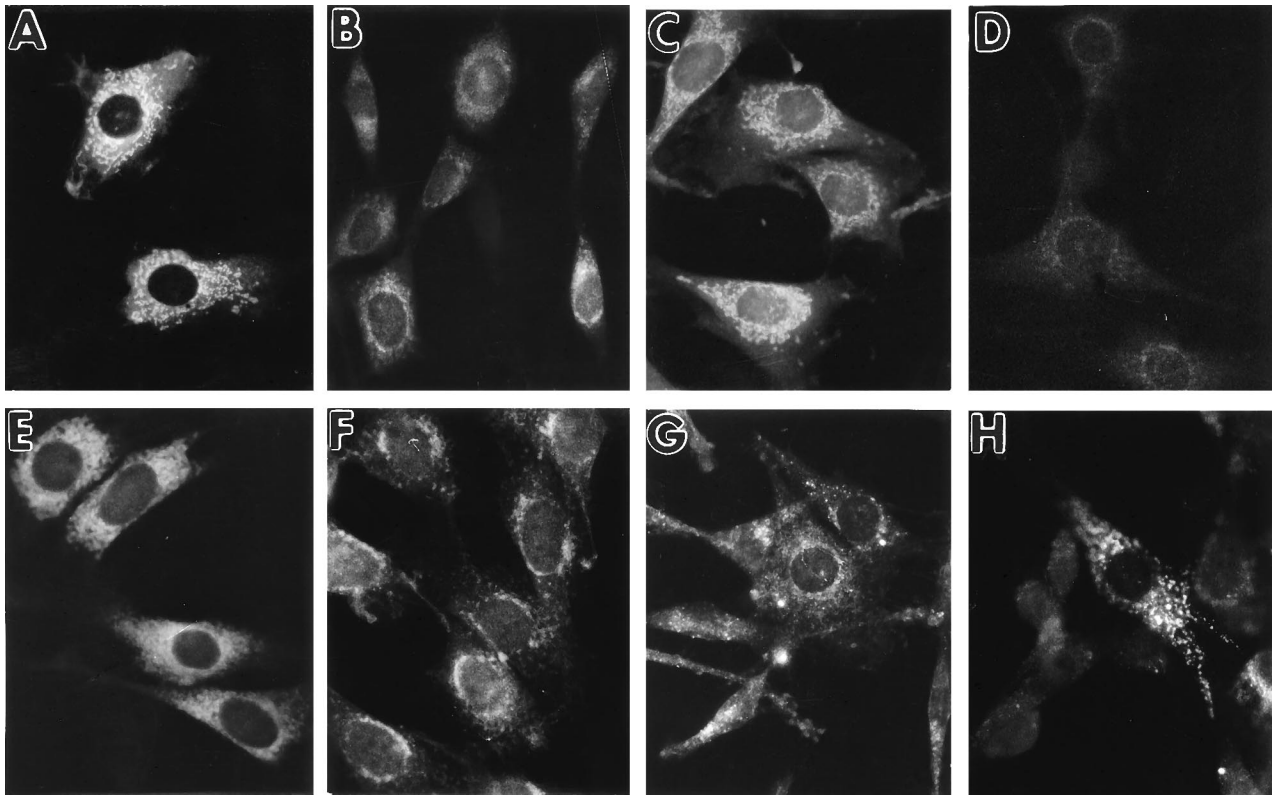

FIG. 2. Immunofluorescence analysis of the localization of Shc proteins. Staining of NIH 3T3 cells transfected with the $p52^{shc}/p46^{shc}$-encoding cDNA (ZIP-SHC9 cells [A] or ZIP-SHC13 cells [B]) and control NIH 3T3 cells (ZIP-1 cells) (D) with anti-SHC-CH1 antibodies and of NIH 3T3 cells transfected with the $p66^{shc}$-encoding cDNA (NIH p66-8 cells) (C) with the anti-SHC-CH2 antibody. In all cell types, the pattern of staining is mostly reticular and only partially cytosolic and is localized in the central portion of the cell. Parallel staining with anti-ER antibodies (E), WGA lectin (F), anti-Tf antibodies (G), and anti-cathepsin D antibodies (H), used as markers of ER, Golgi complex, early endocytic structures, and late endocytic structures, respectively, shows that only the signal corresponding to ER is comparable to the reticular signal of Shc proteins.

stimulation of SAA-SHC cells for 1 h at 4°C (data not shown). The basal localization and EGF-dependent redistribution of Shc proteins did not depend on the integrity of microtubules and microfilaments, since they were not affected by either nocodazole or cytocalasin B treatment (data not shown).

The EGF-induced Shc protein redistribution was further probed by immunolabeling thin sections of untreated and EGF-treated SAA-SHC cells with the anti-SHC-CH1 polyclonal antibody, followed by protein A-colloidal gold conjugates, and studied by electron microscopy. In untreated cells, the gold labeling was localized in the central perinuclear region of the cell and associated with RER cisternae (Fig. 5A), as described above for ZIP-SHC9 cells. Plasma membranes and endocytic structures were consistently unlabeled (Fig. 5A). Upon EGF treatment, the gold particles became more peripheral and were frequently associated with the inner side of the plasma membranes (Fig. 5B, arrows). Specific labeling was also detected on the cytosolic side of coated pits (Fig. 5C and D) and around endosomal structures (Fig. 5E). There was no labeling of the Golgi complex in either untreated or EGF-treated cells (Fig. 5F).

Receptor autophosphorylation, but not Shc phosphorylation, is required for redistribution of Shc proteins. Receptor TK activation induces Shc phosphorylation and binding of Shc proteins to specific autophosphorylation sites in the cytoplasmic tail of the activated receptors. To test the roles of Shc phosphorylation and receptor-Shc complex formation on the ligand-dependent redistribution of Shc proteins, the cellular localization of Shc proteins was investigated in NIH 3T3 fibroblasts transfected with tyrosine phosphorylation-defective Shc or receptor mutants.

Tyr-317 is the major site of in vivo phosphorylation of Shc proteins (46). To obtain cells overexpressing both EGFR and a tyrosine phosphorylation-defective Shc mutant, SAA cells were transfected with the Tyr-317→Phe Shc mutant (TM) (46) (the levels of Shc expression of the resulting SAA-TM polyclonal cell population are shown in Fig. 1A). The immunofluorescence staining of untreated SAA-TM cells was not unlike that in ZIP-SHC9, ZIP-SHC13, and SAA-SHC cells overexpressing wild-type Shc proteins (Fig. 4C). Treatment of SAA-TM cells with EGF induced a redistribution of the signal analogous to that in the SAA-SHC cells (Fig. 4D). Note that the anti-SHC-CH1 antibody does not react with the mouse Shc proteins (not shown) and, therefore, the anti-Shc labeling of the SHC-TM cells corresponds to the TM Shc proteins.

Shc localization was next investigated in NR6 cells overexpressing wild-type Shc and either the EGFR/erbB2 or the EGFR/erbB2-5P receptor but devoid of endogenous EGFR. In the EGFR/erbB2 receptor, the transmembrane and intracellular domains of the erbB2 kinase are fused to the extracellular domain of EGFR and the erbB2 kinase is, therefore, regulated by EGF binding. In the EGFR/erbB2-5P receptor the major erbB2 tyrosine-phosphorylation sites (tyrosine residues 1139, 1197, 1221, 1222, and 1248) were mutagenized to phenylalanine (5P mutation) to generate EGFR/erbB2 molecules still endowed with TK activity but virtually devoid of

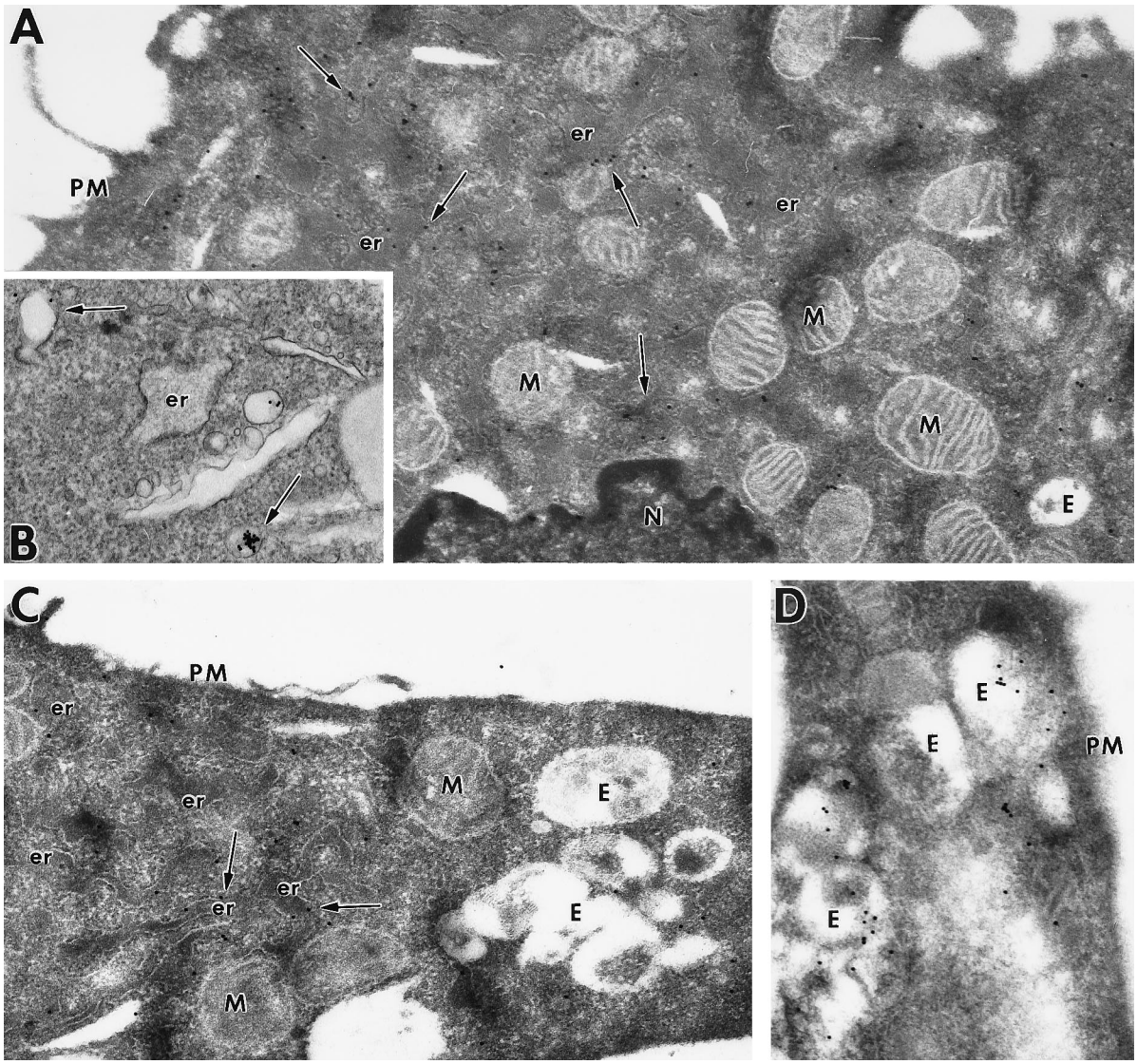

FIG. 3. Immunoelectron-microscopic localization of Shc proteins in the ZIP-SHC9 NIH 3T3 cells using the anti-SHC-CH1 antibody. The immunolabeling is more dense in the central portion of the cell (A) than in more peripheral areas (C). Most gold particles are associated with the cytosolic side of RER cisternae (A and C, arrows), while only a few are scattered throughout the central portion of the cytoplasm. The outer nuclear membrane (A), mitochondria (A and C), and the plasma membrane (A and C) are unlabeled. Early endosomal structures, identified in parallel conventional thin sections by internalization of BSA-gold particles for 15 min at 37°C (B), and late endosomal structures, identified in parallel immunolabeling with anti-cathepsin D antibody (D), were not labeled with anti-Shc antibodies. PM, plasma membrane; N, nucleus; M, mitochondria; E, endosomes. Magnification, ×30,600.

phosphotyrosine (47). The EGFR/erbB2-5P mutation completely abrogates binding of Shc proteins to the receptor (47) (Fig. 1C). NR6 cells overexpressing the EGFR/erbB2 (T31-1) or the EGFR/erbB2-5P (T31-3) receptor were engineered to overexpress wild-type Shc proteins. Comparable levels of EGFR/erbB2 chimeras (Fig. 1C) and Shc proteins (Fig. 1A) were present in the NR6-EGFR/erbB2 and NR6-EGFR/erbB2-5P cell populations.

Analysis of Shc localization in untreated NR6-EGFR/erbB2 (Fig. 4E) and NR6-EGFR/erbB2-5P (Fig. 4G) cells revealed a prevalently reticular signal in the central cell portions. EGF treatment induced a massive relocation of the signal toward the cell periphery in the NR6-EGFR/erbB2 cells (Fig. 4F), while it did not induce any modifications in Shc localization in the NR6-EGFR/erbB2-5P cells (Fig. 4H).

To determine whether the 5P mutation interferes with the potential of the EGFR/erbB2 chimera to stimulate Ras, we measured levels of EGF-induced MAP kinase activation into the NR6-EGFR/erbB2 and NR6-EGFR/erbB2-5P cells. As shown in Fig. 6, T31-1 and T31-3 cells were stimulated with 10 ng of human recombinant EGF per ml for 1, 5, and 15 min. Western blotting with anti-MAP kinase antibody showed that EGF induced a mobility shift of the 42- and 44-kDa forms of MAP kinase in T31-1 cells only. Taken together, these results indicate that the mutagenesis at the major erbB2 tyrosine phosphorylation sites abolishes activation of the RAS-dependent signalling pathway and recruitment of Shc to the plasma membrane. Therefore, receptor autophosphorylation and formation of receptor-Shc complexes, but not Shc tyrosine phosphorylation, are required for redistribution of Shc proteins induced by RTK activation.

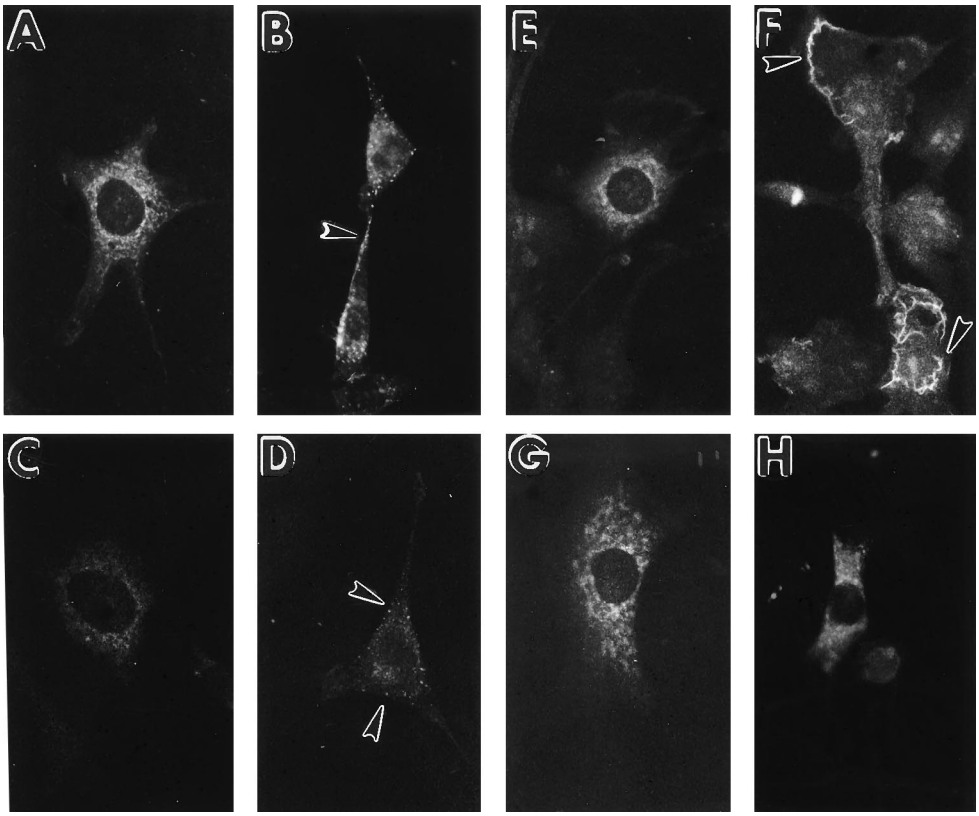

FIG. 4. Immunofluorescence analysis of the redistribution of Shc proteins after receptor activation. The pattern of staining of SAA-SHC cells with the anti-SHC-CH1 antibodies is perinuclear and mostly reticular in untreated cells (A) and becomes punctate, peripheral, and associated with the cell surface in EGF-treated cells (B, arrowhead). Redistribution to the cell periphery of Shc signals after EGF treatment also occurs in cells expressing the Shc TM mutant (SAA-TM) (untreated SAA-TM cells [C] and EGF-treated SAA-TM cells [D]). Shc signals are redistributed by EGF into the NR6-EGFR/erbB2 cells (untreated NR6-EGFR/erbB2 cells [E] and EGF-treated NR6-EGFR/erbB2 cells [F]) but not into the NR6-EGFR/erbB2 5P cells (untreated NR6-EGFR/erbB2-5P cells [G] or EGF-treated NR6-EGFR/erbB2 5P cells [H]. Note the association of Shc labeling with membrane ruffles in EGF-treated NR6-EGFR/erbB2 cells (F, arrowheads).

DISCUSSION

Our study on the intracellular localization of Shc proteins demonstrated that p52^{shc}/p46^{shc} and p66^{shc} were localized mainly intracellularly on the RER and occasionally in the cytosol. As four distinct anti-Shc antibodies gave this unique staining pattern both in cells engineered to overexpress Shc proteins and in normal cells, it almost certainly reflects the physiological distribution of Shc proteins.

Shc protein localization was extremely specific, since not all the RER cisternae were labeled by the anti-Shc antibodies. Labeling was concentrated within the most central perinuclear cisternae, while the outer nuclear envelope, which is continuous with and functionally equivalent to the ER cisternae, appeared mostly devoid of associated Shc labeling. Shc proteins, therefore, appear to be associated with distinct RER subdomains.

The RER is the intracellular site of protein neosynthesis, glycosylation, folding, and oligomerization. Accumulation of unfolded proteins in the ER triggers a signalling pathway from the ER to the nucleus which involves activation of the Ern1p kinase (35). The localization of several protein kinases, such as the eta and delta isoforms of protein kinase C, to the RER membranes (11, 33) suggests that their ER association reflects substrate specificity and local activity. ER localization and local regulation by redox changes of the receptor-like Ltk TK have also recently been reported (5). Therefore, signal transduction events controlled by phosphorylation at either serine-threonine or tyrosine residues may occur at the RER similarly to the events that occur at the plasma membrane. This hypothesis is supported by the ER subcellular localization of the PTP-1B tyrosine phosphatase (21), which would allow it to participate in the regulation of signal transduction pathways through dephosphorylation of specific substrates. Shc proteins, and presumably other SH2-containing proteins, may also function at the ER level as part of these signalling mechanisms. Alternatively, the perinuclear ER location of Shc proteins might be involved in the regulation of the adaptor function at the plasma membrane by controlling the availability of Shc proteins for interactions with activated RTKs.

Both immunofluorescence and electron microscope studies showed that few Shc proteins were present in the cytosol. Like the ER Shc proteins, the cytosolic Shc proteins were not scattered throughout the cytoplasm but were limited to the central portion of the cell. The cytosolic fraction may coexist in equilibrium with the RER-associated fraction, the latter being predominant.

Activation of RTKs, the EGFR, and the EGFR/erbB2 chimera induced Shc proteins to undergo redistribution from the perinuclear location toward the cell periphery. Shc labeling was found to be associated with the cytosolic surface of the plasma membrane, clathrin-coated pits and endosomes, and the peripheral cytosol. While the association of Shc proteins with the plasma membrane and the endocytic structures likely reflects the physical interaction of Shc with the activated re-

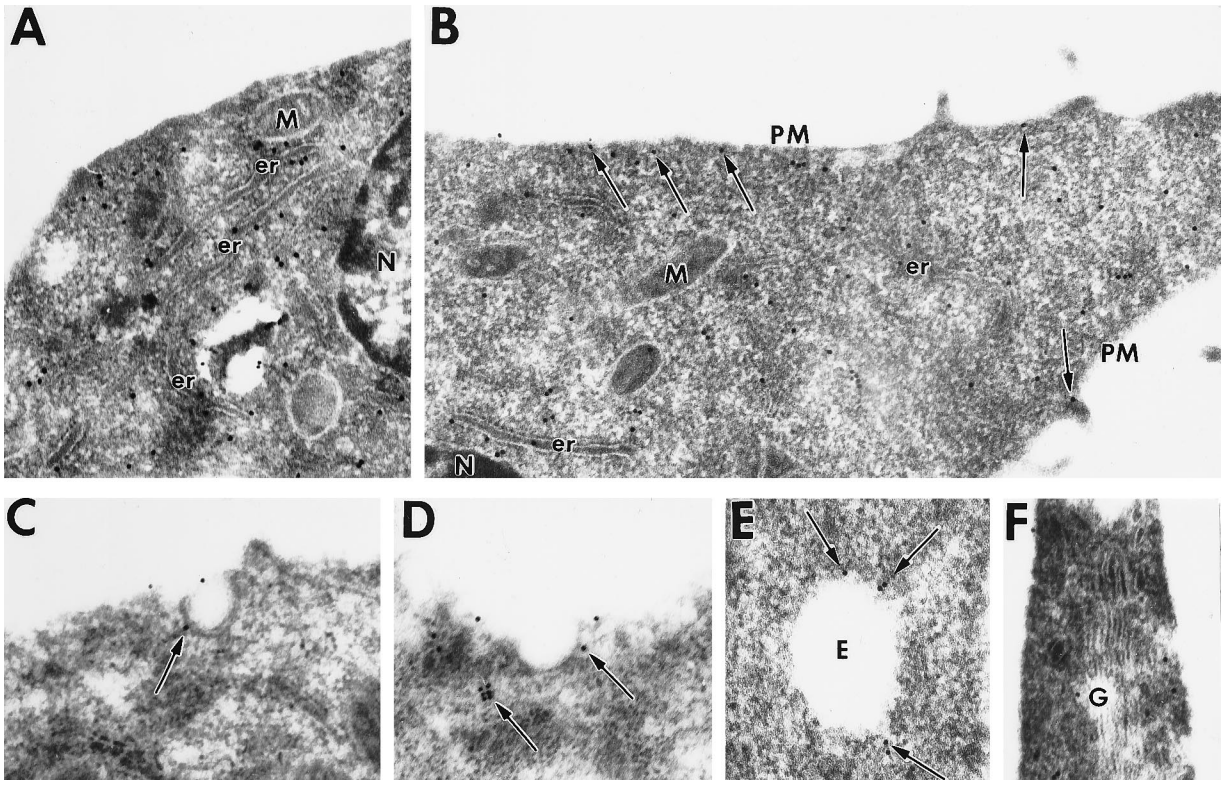

FIG. 5. Immunoelectron-microscopic analysis of Shc protein redistribution after EGF treatment of SAA-SHC cells. Gold immunolabeling with anti-SHC-CH1 antibodies appears to be associated with the cytosolic side of RER cisternae in the perinuclear region of the cell (A). After EGF treatment, gold particles are located mostly at the cell periphery, in proximity of and associated with the plasma membrane (PM) (B, arrows) and clathrin-coated pits (C and D, arrows) and around endosomes (E) (E, arrows). N, nucleus; M, mitochondria. The Golgi complexes (G) appear unlabeled (F). Magnification: ×37,800 (A), ×35,100 (B), or 30,600 (C to F).

ceptors, their localization in the peripheral cytosol suggests that Shc proteins are involved in additional growth factor-dependent interactions. erbB2 activation induces the formation of Shc-activated receptor and receptor-free Shc/Grb2 complexes (44, 47). Although Shc proteins are tyrosine phosphorylated by activated erbB2 at high stoichiometry (40 to 50%), only 2 to 4% of Shc proteins are complexed with the activated erbB2 (44). It is, therefore, conceivable that the Shc proteins redistributed to the peripheral cytosol upon growth factor stimulation correspond to the receptor-free Shc/Grb2 complexes. The recruitment of Shc toward the peripheral cytosol and around endosomes may account for additional putative functions of the Shc adaptor protein, such as the control of endosomal sorting of internalized RTKs or the regulation of cytoskeletal actin organization.

Tyrosine phosphorylation of Shc proteins, and, presumably, formation of the Shc/Grb2 complexes, does not appear to play any role during the process of Shc protein redistribution. Relocalization occurred in cells expressing a tyrosine phosphorylation-defective Shc mutant, while it did not occur in cells expressing wild-type Shc proteins and the EGFR/erbB2-5P chimera. In these cells, despite the mutational inactivation of the erbB2 major autophosphorylation sites, 10-min EGF stimulation induced Shc phosphorylation to an extent comparable to that in the cells expressing the parental EGFR/erbB2 (Fig. 1C). Phosphorylation of Shc proteins by the erbB2-5P receptor likely reflects the high affinity of the erbB2 kinase for Shc polypeptides (47).

The redistribution of Shc proteins upon growth factor stimulation depends on receptor autophosphorylation, as demonstrated by the finding that Shc redistribution was lost in cells that expressed the EGFR/erbB2-5P mutant. This implies that the interaction of Shc proteins with specific phosphotyrosine residues in the cytoplasmic tail of the activated receptor is crucial to target Shc proteins to the cell periphery. The interaction of Shc proteins with activated erbB2 or EGFR involves the binding of both the SH2 domain and the PTB domain of Shc to specific tyrosine-phosphorylated residues (24, 44, 54). Notably, the binding activity of the PTB domain is dispensable for the growth factor-mediated redistribution of Shc proteins, as shown by the fact that EGF treatment induces relocalization of Shc mutant proteins with deletion of the PTB (52).

The mechanism(s) involved in the relocation of Shc proteins

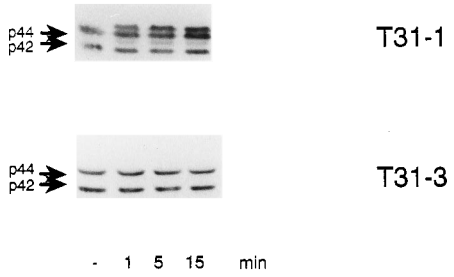

FIG. 6. Effect of Shc relocalization on activation of MAP kinases. Western blot analysis of MAP kinase expression in NR6-EGFR/erbB2 (T31-1) and NR6-EGFR/erbB2 5P (T31-3) cells at various times (1, 5, and 15 min) after stimulation with EGF (10 ng/ml).

remains to be identified. Transport of Shc proteins mediated by vesicular intracellular traffic is unlikely because our data show that (i) the Golgi complex, the intracellular compartment classically located between the ER and the plasma membrane along the exocytic pathway, was never labeled by anti-Shc antibodies and (ii) Shc relocation does not require integrity of microfilaments and/or microtubules, as would be expected for vesicular traffic along microtubule or actin tracks (20). Our results support the hypothesis that relocation of Shc is a consequence of a reequilibrium between a cytosolic pool and a pool which may associate with either the RER membranes or the endosomal and plasma membranes. The availability of phosphotyrosine residues on membrane proteins in different cellular locations, as after RTK activation or during their internalization, may govern the shift in equilibrium among the different compartments of Shc proteins.

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