

Formation of Shc-Grb2 complexes is necessary to induce neoplastic transformation by overexpression of Shc proteins

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The mammalian SHC gene encodes three overlapping proteins which all contain a carboxy-terminal SH2 domain. Shc proteins are phosphorylated on tyrosine by a variety of receptor and cytoplasmic tyrosine kinases. Phosphorylated Shc proteins form a complex with the SH2-SH3 containing Grb2 protein which is implicated in the regulation of Ras, suggesting that Shc is involved in the intracellular transmission of growth signals from activated tyrosine kinases to Ras. Overexpression of Shc proteins in cultured fibroblasts induces a transformed phenotype. We now report that, *in vitro*, the high affinity binding of Grb2 to Shc proteins requires phosphorylation of Shc at Tyr317, which lies within the high affinity binding motif for the Grb2 SH2 domain, pYVNV, where Asn at the +2 position is crucial for complex formation. *In vivo*, Tyr317 is the major, but not the only, site for Shc phosphorylation, and is the sole Shc high affinity binding site for Grb2. Mutant Shc proteins with substitution of the Tyr317 by Phe lose the capacity to be highly phosphorylated on tyrosine upon growth factor receptor activation, to bind Grb2 and to induce neoplastic transformation. In contrast, Shc proteins that have an extensive aminoterminal deletion, but retain the Tyr317 site and the SH2 domain conserve the capacity to be phosphorylated, to bind to Grb2 and to induce cell transformation. These data indicate that the formation of the Shc-Grb2 complex is a crucial event in the transformation induced by overexpression of Shc and support the notion that Shc proteins can deliver activation signals to RAS.

Introduction

The mammalian gene SHC encodes at least three widely expressed proteins of 46, 52 and 66 kDa (p46^{shc}, p52^{shc} and p66^{shc}). These Shc polypeptides all contain a carboxy terminal Src homology 2 (SH2) domain and an adjacent glycine/proline rich region with homology to the collagen α 1-chain (CH domain). Because of the differential use of translational initiation sites and alternative splicing, the three Shc isoforms differ in the extent of their amino-terminal sequences (Pelicci *et al.*, 1992 and unpublished results).

Many of the substrates of protein-tyrosine kinases possess an SH2 domain that directs their interactions with phosphotyrosine-containing sites on activated growth factor receptors (Pawson & Schlessinger, 1993). The SH2 containing Shc proteins are substrates for a

considerable number of receptor tyrosine kinases, and become phosphorylated on tyrosine in cells stimulated with growth factors such as the epidermal growth factor (EGF), platelet-derived growth factor, hepatocyte growth factor, Steel factor and insulin, as well as in cells with activated Neu/ErbB2 and Sea tyrosine kinases (Pelicci *et al.*, 1992; Segatto *et al.*, 1993; Pronk *et al.*, 1993 and unpublished results). Shc proteins become physically associated with activated receptors via their SH2 domains both *in vitro* and in living cells (Pelicci *et al.*, 1992; Segatto *et al.*, 1993). They are, in fact, among the most highly tyrosine phosphorylated proteins in the tissues of mice injected with EGF, which suggests that phosphorylation of Shc proteins is an important aspect of the response to EGF in this animal (Wada *et al.*, 1993). Shc proteins also appear to be involved in signalling from cytoplasmic tyrosine kinases, since they are constitutively phosphorylated on tyrosine in cells that are transformed by transforming tyrosine kinase variants such as v-Src and v-Fps (McGlade *et al.*, 1992). In addition, Shc proteins are rapidly phosphorylated on tyrosine after ligand stimulation of surface receptors that have no intrinsic tyrosine kinase activity, but are thought to signal by recruiting and activating cytoplasmic tyrosine kinases (e.g. IL-2, T-cell, CD4, GM-CSF and erythropoietin receptors) (Burns *et al.*, 1993; Damen *et al.*, 1993; Ravichandran *et al.*, 1993; Sato *et al.*, 1993).

Because tyrosine phosphorylation of Shc proteins induces the formation of a stable complex between Shc and the adaptor protein Grb2 in growth factor stimulated and v-Src transformed cells, Shc proteins are thought to be implicated in the regulation of the Ras pathway (Rozakis-Adcock *et al.*, 1992). Grb2 is the mammalian member of a conserved family of proteins (Sem-5 in *Caenorhabditis elegans*, drk in *Drosophila melanogaster*) that contain a single SH2 domain flanked by two SH3 domains (Clark *et al.*, 1992; Lowenstein *et al.*, 1992; Matuoka *et al.*, 1992; Olivier *et al.*, 1993; Suen *et al.*, 1993). Genetic studies carried out in *C. elegans* and *D. melanogaster* have demonstrated that activation of the Ras pathway by receptor tyrosine kinases requires the Sem-5/drk/Grb2 protein (Clark *et al.*, 1992; Olivier *et al.*, 1993). In mammalian cells, Grb2 is constitutively associated with mSos1 by binding of its SH3 domains to proline-rich motifs in the mSos1 tail (Buday & Downward, 1993; Chardin *et al.*, 1993; Egan *et al.*, 1993; Gale *et al.*, 1993; Li *et al.*, 1993; Olivier *et al.*, 1993; Rozakis-Adcock *et al.*, 1993; Simon *et al.*, 1993). mSos1 is a mammalian homologue of a *D. melanogaster* guanine nucleotide releasing protein, Sos, which is essential for Ras activation by the *D. melanogaster* receptor tyrosine kinases DER and sevenless (Simon *et al.*, 1991; Bonfini

et al., 1992; Bowtell *et al.*, 1992; Chardin *et al.*, 1993). The Grb2 SH2 domain binds inducibly to both auto-phosphorylated receptors and tyrosine phosphorylated Shc, leading to the formation of heterotetrameric complexes containing the activated receptor, phosphorylated Shc protein, Grb2 and mSos1 (Rozakis-Adcock *et al.*, 1993; Skolnick *et al.*, 1993a,b). One biochemical function of Shc is, therefore, the formation of complexes with Grb2/mSos1 following phosphorylation by activated tyrosine kinases.

Consistent with a role in signalling through the Ras pathway, experimental evidence suggests that Shc stimulates the intracellular transmission of growth and differentiation signals. The overexpression of Shc proteins in cultured fibroblasts induces a transformed phenotype. Unlike parental cells, fibroblasts overexpressing a human SHC cDNA acquire partial independence from exogenous growth factors, display anchorage-independent growth and form tumours in nude mice (Pelicci *et al.*, 1992). When overexpressed in PC12 pheochromocytoma cells, SHC induces terminal differentiation. The ability of SHC to induce neurite outgrowth in PC12 cells is dependent on Ras activation since this phenotype is blocked by co-expression of a dominant inhibitory Ras mutant (Rozadis-Adcock *et al.*, 1992). It would, therefore, seem that Shc does, indeed, exert a biological effect by stimulating the Ras pathway.

The present research addressed the question of whether the interaction between Shc and Grb2 proteins is necessary for induction of mitogenesis by overexpression of Shc proteins. The Grb2 binding site on Shc proteins was mapped and the transforming potential of Shc mutants defective for Grb2-binding investigated.

Results

Phosphorylation of Shc Tyr317 creates a Grb2-binding site

Analysis of the primary Shc protein sequence revealed a putative phosphorylation site at Tyr317, located amino-terminal to the SH2 domain within the CH region (Pelicci *et al.*, 1992; Figure 1). The specificity of SH2-binding appears to be primarily dictated by the three residues immediately C-terminal to the phosphotyrosine (in the +1, +2 and +3 positions) (Pawson & Schlessinger, 1993). When employed to screen a degenerate phosphopeptide library, the Grb2-SH2 domain showed a strong preference for Asn at the +2 position (Songyang *et al.*, 1994). Indeed the EGFR and IRS-1 Grb2-SH2 binding sites both have Asn at position +2 (Buday & Downward, 1993; Skolnick *et al.*, 1993b). Similarly, the *C. elegans* Sem-5 SH2 domain preferentially selected pY-L/V-N-V/P (Songyang *et al.*, 1993). The Shc Y317 site contains the sequence YVNV, which corresponds well with the consensus Grb2 SH2-binding motif predicted from the phosphopeptide library screen. Hence, phosphorylation of Tyr317 might be responsible for creating the high affinity binding site for the Grb2 SH2 domain.

This hypothesis was tested by assaying the binding capacity of a Shc GST-fusion protein containing amino acids 280 to 473 (GST-SHC+) (Figure 1). The GST-

SHC+ protein was inducibly phosphorylated on tyrosine in bacteria by co-expression with a β -galactosidase fusion polypeptide containing the catalytic domain of the Elk protein-tyrosine kinase (Reedjik *et al.*, 1992). The GST-SHC+ protein contained no detectable phosphotyrosine prior to induction of the Elk tyrosine kinase, but became tyrosine phosphorylated following its expression (Figure 2). As a mutant version of the GST-SHC+ protein, GST-SHC+Y317F, in which Tyr317 was substituted by phenylalanine, was not phosphorylated under similar conditions (Figure 2), Tyr317 appears to be the only site in this protein which is phosphorylated by Elk. Like results were obtained when GST-SHC+ and GST-SHC+Y317F were purified and used as the substrates for the purified EGF-receptor (EGFR) kinase domain, or the v-Src tyrosine kinase (data not shown). Furthermore, when different Shc polypeptide fragments were expressed as GST fusion proteins, and incubated with purified v-Src tyrosine kinase, only those containing the region surrounding Tyr317 were substrates for *in vitro* tyrosine phosphorylation (data not shown).

To test Grb2-binding, tyrosine phosphorylated GST-SHC+ was immobilized, incubated with a lysate of Rat-2 fibroblasts, and the resulting complexes immunoblotted with anti-Grb2 antibodies. This approach showed that Grb2 bound specifically to the phosphotyrosine-containing form of GST-SHC+ (Figure 2). In contrast, the GST-SHC+Y317F variant, that lacks the Tyr317 phosphorylation site, did not bind detectably to Grb2 under the same conditions (Figure 2). The involvement of the Tyr317 site in the binding of Grb2 to wild type Shc was investigated in greater detail by assessing the capacity of a 15 amino acid phosphopeptide corresponding to the Shc sequence encompassing Tyr317 to compete for binding of tyrosine phosphorylated GST-SHC+ to Grb2. Addition of the Tyr317 phosphopeptide to a concentration of 0.2 μ M inhibited the binding of phosphorylated GST-SHC+ to Grb2 in a Rat-2 cell lysate whereas an unrelated phosphotyrosine-containing peptide (pY771) corresponding to the binding site for Ras GTPase activating protein on the β PDGF-receptor did not (Figure 2).

These results are strong evidence that the Shc phosphorylation at Tyr317 creates a binding site for the Grb2 SH2 domain. Since high affinity SH2-binding apparently requires specific amino acids C-terminal to the phosphorylated tyrosine (Pawson & Schlessinger, 1993), substitution of residues at the +1 to +3 positions relative to Tyr317 might be expected to modulate Grb2-binding. As Asn at the +2 site (Asn319) is characteristic of Grb2-binding sites (Buday & Downward, 1993; Skolnick *et al.*, 1993b; Songyang *et al.*, 1993, 1994), this speculation was tested by mutating the codon for Asn319 of p52^{shc} to encode alanine, and introducing it into the GST-SHC+ vector. The mutant was efficiently phosphorylated by both the Elk tyrosine kinase in bacteria, and the EGFR kinase domain *in vitro*. Despite its efficient phosphorylation on tyrosine, the Ala319 Shc mutant did not bind to Grb2 in a Rat-2 cell lysate as efficiently as wild-type GST-SHC+ (data not shown). This result suggests that the high affinity binding of Grb2 to Shc proteins requires phosphorylation of Shc at Tyr317, which lies within the high affinity binding motif for the Grb2 SH2 domain,

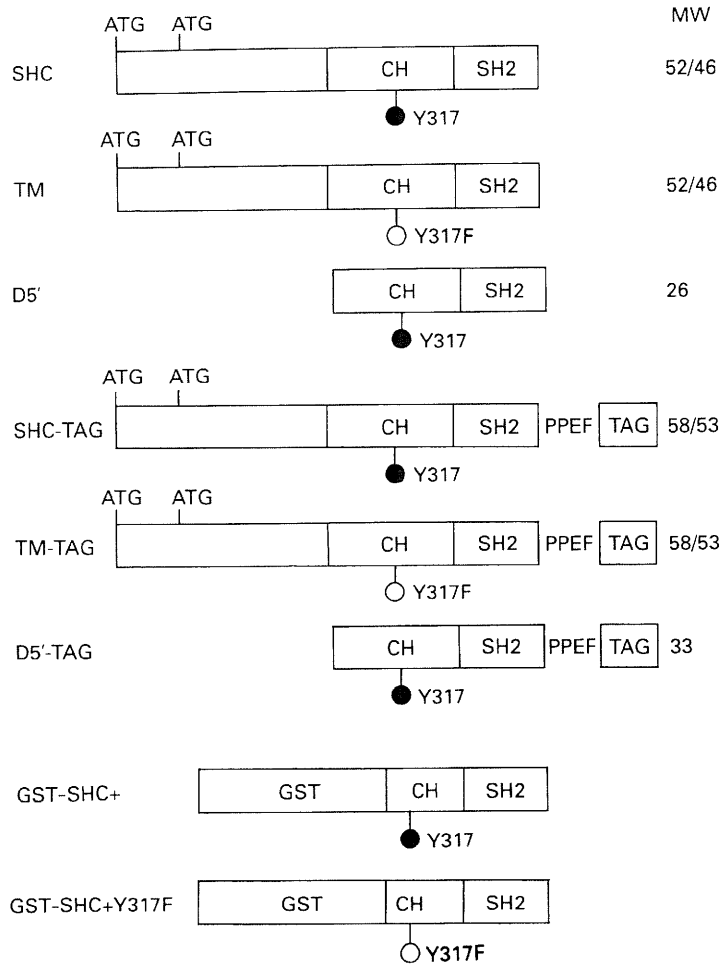


Figure 1 Schematic representation of wild type and mutant Shc proteins. The two in-frame ATG from which p46^{shc} and p52^{shc} originate, the $\alpha 1$ homologous collagen region (CH), the SH2 domain (SH2), the tyrosine at position 317 (Y317), the tyrosine-phenylalanine substitution at position 317 (Y317F) and the tagging polypeptide (PML) are indicated. The predicted molecular weight (MW) for each protein is given on the right. SHC indicates the wild-type protein; TM the protein with the Y317F mutation and D5' the one with the deleted amino terminal amino acids. The suffix TAG denotes the same proteins tagged with a PML epitope. P (proline), E (glutamic acid) and F (phenylalanine) are the amino acids that were inserted into the Shc-PML junction. The two lower diagrams show the carboxy-terminal portion (amino acids 280–473) of the wild-type (GST-SHC+) and the Y317F mutant (GST-SHC-Y317F) Shc expressed in bacteria as GST fusion proteins

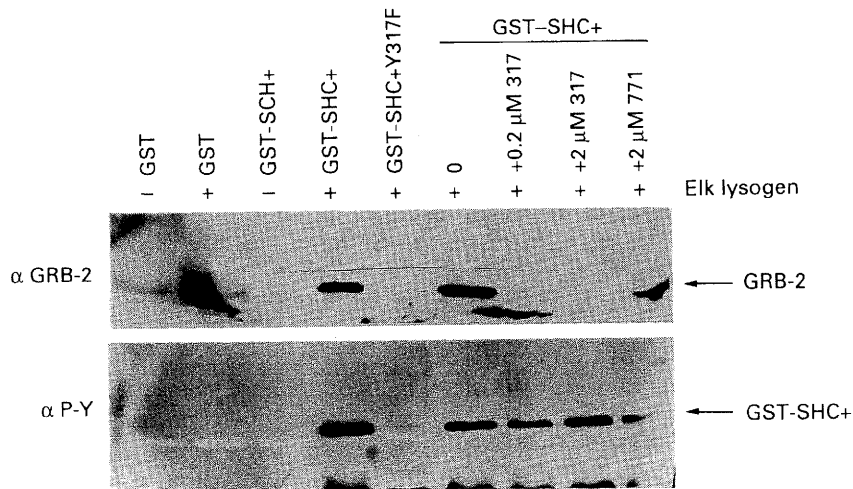


Figure 2 Expression and tyrosine phosphorylation of Shc proteins in *E. coli* and binding to Grb2. GST, GST-SHC+ or the mutant GST-SHC-Y317F were expressed in bacteria in the presence (+) or absence (-) of expression of the Elk tyrosine kinase domain (Elk lysogen). The purified proteins were immobilized and incubated with cell lysates from R2 fibroblasts. The resulting protein complexes were analysed by SDS-PAGE and immunoblotted with either anti-GRB2 antiserum (α GRB2) or antiphosphotyrosine (P-Y) antiserum, as indicated. In some cases, 0.2 or 2 μ M phosphopeptides corresponding to the SHC Y317 (317) phosphorylation site or to the PDGF-R phosphorylation site at Y771 (771) were added to the incubation

pYVNV, where Asn at the +2 position is crucial for complex formation. Shc contains another motif that is a putative candidate Grb2-binding site, YYND (positions 239–242). Although we can offer no evidence to support this proposition, the presence of a negative Asp at the +3 position would likely destabilize binding to the Grb2 SH2 domain.

Shc tyrosine 317 is required for GRB-2 association in vivo

Having established that phosphorylation of Shc Tyr317 is sufficient to promote Grb2-binding *in vitro*, we investigated the involvement of this site on the formation of a Shc-Grb2 complex in cells by constructing mammalian expression vectors with a wild type human SHC cDNA that encodes the p52^{shc} and p46^{shc} proteins or a mutant SHC cDNA in which the codon for Tyr317 was replaced with Phe (TM). In addition, both the SHC and TM cDNAs were tagged with a foreign epitope to allow the ectopically expressed Shc proteins to be immunologically distinguished from endogenous Shc polypeptides, which are ubiquitously expressed. To obtain SHC-TAG and TM-TAG cDNAs, SHC and TM sequences were fused in-frame with a 162 bp fragment of the human PML cDNA (Pandolfi *et al.*, 1991), which encodes a 54 amino acid PML peptide of 6.2 kDa (the anti-PML antibody does not cross-react against mouse PML proteins [data not shown]). Note that two proline residues were introduced as a flexible linker between the Shc and PML sequences (Figure 1). The two tagged cDNAs, therefore, had the potential to encode two PML-tagged Shc proteins of 53 and 58 kDa (p53^{shc-TAG} and p58^{shc-TAG}) containing wild type Shc sequences, and p53^{TM-TAG} and p58^{TM-TAG} with a Phe317 substitution (Figure 1).

To test the tyrosine phosphorylation and Grb2-binding properties of these Shc proteins, the SHC-TAG and TM-TAG cDNAs were introduced into rodent fibroblasts that overexpress the EGFR (SAA cells) (Di Fiore *et al.*, 1987). As anticipated, western blot analysis of the transduced SAA cells (SAA-TM-TAG and SAA-SHC-TAG) with anti-Shc antibodies revealed the endogenous Shc proteins (p46^{shc}, p52^{shc}, p66^{shc}) and the two PML-tagged exogenous Shc proteins of approximately 53 and 58 kDa (p53^{shc-TAG} and p58^{shc-TAG}, or p53^{TM-TAG} and p58^{TM-TAG}, respectively) (Figure 3a). To ensure that the tagging procedure did not interfere with antibody recognition of either the Shc or PML epitopes, SAA-TM-TAG and SAA-SHC-TAG cell lysates were immunoprecipitated with anti-PML antibodies and western blotted with anti-Shc antibodies. The anti-Shc antibody recognized two proteins (53 and 58 kDa approximately) in the anti-PML immunoprecipitates, that comigrated with the p53^{shc-TAG} and p58^{shc-TAG} detected by western blotting of the SAA-SHC-TAG whole cell lysate with anti-SHC antibody (Figure 3b).

The tyrosine phosphorylation of the wild type and TM Shc proteins, and their potential to bind Grb2, were then examined in EGF-stimulated SAA-TM-TAG and SAA-SHC-TAG cells. Lysates prepared from serum-starved cells, or from cells stimulated for 5 min with EGF, were immunoprecipitated with anti-PML antibodies and the immunoprecipitates immunoblotted with anti-SHC, anti-phosphotyrosine or anti-GRB2 antibodies. EGF stimulation induced a marked retardation of the electrophoretic mobility of the p53^{shc-TAG} and p58^{shc-TAG} proteins, which contain wild type Shc sequences (Figure 4a); such a reduction in the mobility of Shc proteins has previously been attributed to their tyrosine phosphorylation following growth factor

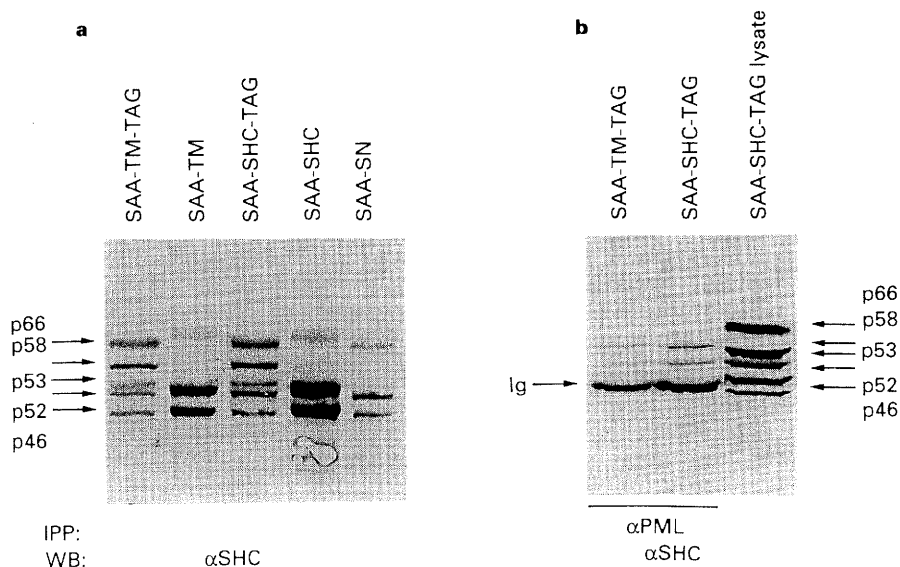


Figure 3 (a) Expression of wild type tagged (SHC-TAG) and Tyr317 mutated (TM-TAG) Shc proteins in SAA cells. Western blot analysis of Shc expression of SAA cells transfected with the LXS expression vector (SAA-SN) or the LXS vector containing wild-type (SAA-SHC), PML-tagged (SAA-SHC-TAG), mutated TM (SAA-TM) and PML-tagged TM (SAA-TM-TAG) Shc cDNAs. Arrows indicate the Shc tagged polypeptides that migrate with molecular weights of approximately 53 and 58 and the endogenous Shc proteins (p46^{shc}, p52^{shc} and p66^{shc}). The exogenous wild-type and TM mutated Shc p46 and p52 polypeptides comigrate with the endogenous p46^{shc} and p52^{shc}. (b) Immunoprecipitation of tagged Shc polypeptides. Anti-PML immunoprecipitates from lysates of the indicated cell lines were immunoblotted with anti-Shc antibodies. A cell lysate from SAA-SHC-TAG cell was run in parallel

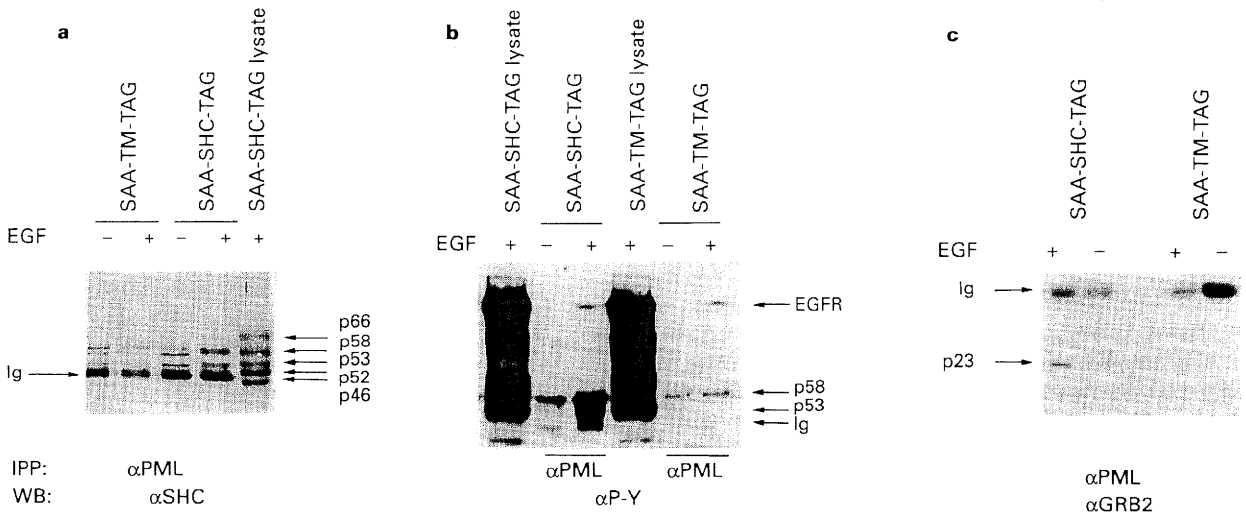


Figure 4 *In vivo* phosphorylation and binding to Grb2 of wild-type and TM Shc proteins. (a) Anti-PML immunoprecipitates from the indicated cell lines were blotted with anti-SHC antibodies. +/– indicated whether cells were stimulated with 16 nm EGF prior to lysis or not. Anti-SHC immunoreactive polypeptides are arrowed. The band that migrates between the p46^{shc} and p52^{shc} in immunoprecipitates is due to rabbit IgG heavy chain cross-reacting with the anti-mouse anti-serum used for detection (Ig). A lysate from SAA-SHC-TAG cells was run in parallel. (b) Anti-PML immunoprecipitates from the indicated cell lines were blotted with anti-phosphotyrosine antibodies (α P-Y). Lysates from SAA-SHC-TAG and SAA-TM-TAG cells were run in parallel. (c) Anti-PML immunoprecipitates from the indicated cell lines were blotted with anti-Grb2 antibodies. The α GRB2 23 kDa immunoreactive polypeptide is arrowed

stimulation (Pelicci *et al.*, 1992). Indeed, the phosphotyrosine content of p53^{shc-TAG} and p58^{shc-TAG} increased considerably upon EGF stimulation (Figure 4b). In addition, phosphorylated p53^{shc-TAG} and p58^{shc-TAG} formed stable complexes with Grb2 in EGF-stimulated cells (Figure 4c).

In contrast, both the reduction in electrophoretic mobility and the increase in tyrosine phosphorylation were far more limited for the p53^{TM-TAG} and p58^{TM-TAG} proteins after EGF stimulation (Figure 4a and b), and no binding of these mutant proteins to Grb2 was detected (Figure 4c). However, it should be noted that the degree of basal phosphorylation of p53^{TM-TAG} and p58^{TM-TAG} was never truly zero and it slightly increased upon EGF stimulation (Figure 4b). Also note that a novel phosphotyrosine containing polypeptide of approximately 175 kDa stably complexed with both wild-type and TM Shc tagged proteins upon EGF stimulation (Figure 4). Probing of the same anti-PML immunoprecipitates with anti-EGFR antibodies revealed that the 175 kDa polypeptide is indeed the EGFR (data not shown). It, therefore, appears that the stability of the Shc/activated EGFR complex does not depend on the extent of Shc phosphorylation and Shc/Grb2 complex formation.

Overall these results provide strong evidence that, *in vivo*, Tyr317 is the major, but presumably not the only, site for Shc phosphorylation by the EGFR, and that it is the sole Shc high affinity binding site for Grb2.

Mutation of the Shc Grb2-binding site abrogates Shc transforming potential

To ascertain whether phosphorylation of Tyr317 and consequent binding of Grb2 are essential for the transforming activity of Shc proteins, the effects of the wild type Shc and TM proteins on the growth properties of cultured fibroblasts were investigated. For this pur-

poses SHC and TM cDNAs were expressed in NIH3T3 cells and clones overexpressing the Shc or TM proteins (NIH-SHC, NIH-TM clones) analysed for their ability to escape to physiological G₁ arrest produced by serum and capacity to form colonies in soft agar. Two clones that overexpressed the wild type Shc proteins, and four that overexpressed the TM proteins, were selected because they expressed similar levels of Shc proteins. Four clones transfected with the expression vector only (NIH-SN clones) were used as controls (Figure 5).

The effects of wild type or TM Shc proteins on cell cycle indices were investigated by analysing the DNA content of propidium iodide (PI)-stained nuclei isolated from wild type NIH3T3, two NIH-SHC, four NIH-TM and four NIH-SN cell lines in flow cytometry studies (Fried *et al.*, 1978). The data obtained from three experiments of each cell clone were separately summed and statistically compared. Data are expressed as the mean of percent cycling cells (S-G₂-M \pm SE) (Figure 6). The cells were cultured in 10% serum until they were almost confluent and were then serum-deprived for 24 h. The cell-cycle phase distribution was similar in the exponentially growing NIH-SN, NIH-SHC and NIH-TM cell lines (data not shown). In contrast, there was significantly ($P < 0.002$) larger proportion of cycling cells in the serum-free NIH-SHC cells than in the serum-free NIH-SN or NIH-TM cell lines (Figure 6).

NIH-SHC, NIH-TM and NIH-SN cell lines were plated in triplicate at varying cell concentrations in soft agar medium supplemented with 20% serum and colonies scored after 14 days. Whereas the NIH-SHC clones formed colonies at a frequency comparable with that previously reported (Pelicci *et al.*, 1992), the NIH-TM and NIH-SN clones did not form colonies (Table 1).

These data indicate that integrity of the Tyr317 site is required for Shc transformation of cultured fibro-

blasts. They also suggest that formation of the Shc-Grb2 complex is an obligatory step in the chain of events through which overexpression of Shc proteins leads to neoplastic transformation. The correlation between Grb2-binding and Shc-induced transformation supports the notion that transformation by Shc involves activation of the Ras signalling pathway.

Deletion of the Shc amino-terminus does not affect the Shc transforming potential

Whether interaction with Grb2 is, by itself, sufficient for the transformation of NIH3T3 cells by Shc, or whether other regions of Shc might be involved in this activity, was investigated by expressing a Shc mutant (D5') that lacked the amino-terminal 232 amino acids, but retained the CH domain and Tyr317 site as well as the SH2 domain (D5' cDNA, Figure 1). The D5' cDNA, which encodes a 26 kDa protein, was expressed in NIH3T3 cells (NIH-D5', Figure 7a), tagged with the

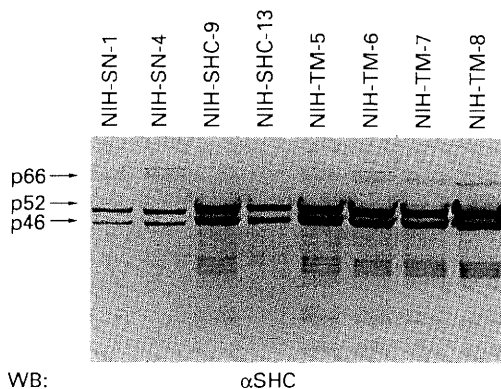


Figure 5 Western blot analysis of Shc expression in NIH3T3 cells transduced with the LXSXN empty expression vector (NIH-SN clones), wild-type (NIH-SHC clones) or TM (NIH-TM clones) cDNAs

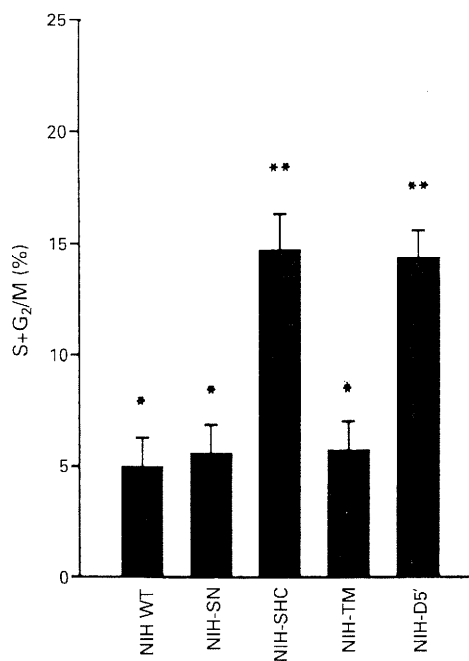


Figure 6 Effects of overexpression of wild-type and mutant Shc proteins on the growth properties of NIH3T3 fibroblasts. The fraction of cycling (S + G₂/M) cells was evaluated in the following cell lines: wild-type NIH3T3 (NIH WT); NIH-SN-1, NIH-SN-4, NIH-SN-5, NIH-SN-7 clones (NIH-SN) transfected with the empty LXSXN expression vector; NIH-SHC-9; NIH-SHC-13 (NIH-SHC) clones overexpressing the wild-type Shc protein; NIH-TM-5; NIH-TM-6, NIH-TM-7, NIH-TM-8 (NIH-TM) clones expressing the Y317F Shc mutant; NIH-D5'-d; NIH-D5'-1, NIH-D5'-2, NIH-D5'-3 (NIH-D5') clones expressing the D5' aminoterminally truncated Shc mutant. Cells were cultured in 10% serum and then serum deprived for 24 h. Columns (+/- SE) show the percent of S + G₂/M cells as derived from DNA fluorescence histograms of PI stained-nuclei from the indicated groups of cell lines after 24 h of serum-deprivation. Results are the means of three separate experiments for each cell line. The values of the NIH-SHC and NIH-D5' cell lines (**) and those of the NIH WT, NIH-SN and NIH-TM (*) were homogenous and differed statistically ($P < 0.002$) according to the Kruskal-Wallis' analysis of variance

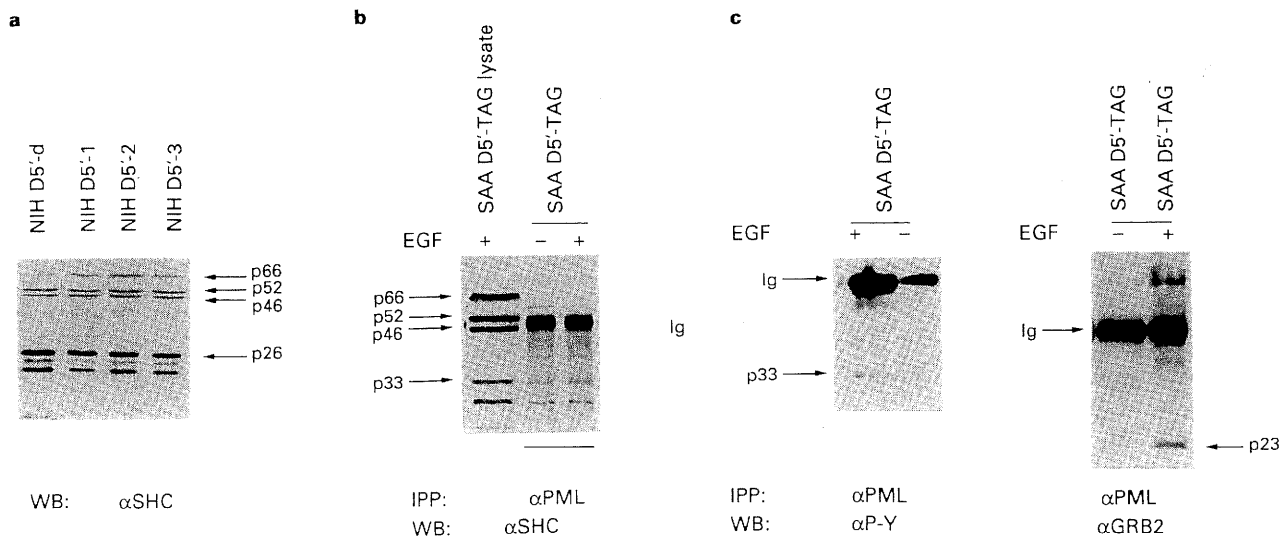


Figure 7 Expression of amino-terminally truncated Shc protein into NIH3T3 (a) and SAA (b) cells and evaluation of its potential to become tyrosine phosphorylate (c) and bind Grb2 (d) in response to EGF. In (a) lysates from the indicated cell lines were analysed by western blotting using anti-SHC antibody. In the remaining panels, lysates from the indicated cell lines were immunoprecipitated with anti-PML and blotted with anti-SHC (b) and anti-Grb2 (d) antibodies. An anti-Shc polypeptide migrating faster than p26 and p33 was detected (a and b) that is likely to represent a Shc partial degradation product.

Table 1 Cloning efficiency of control NIH3T3 clones (NIH-SN) and NIH3T3 clones expressing wild type (NIH-SHC), Y317F (NIH-TM) and 5' deleted (NIH-D5') Shc cDNAs

Cells	Cloning efficiency (%)
NIH-SN-1	<0.001
NIH-SN-4	<0.001
NIH-SN-5	<0.001
NIH-SN-7	<0.001
NIH-SHC-9	3.1
NIH-SHC-13	0.5
NIH-TM-5	<0.001
NIH-TM-6	<0.001
NIH-TM-7	0.002
NIH-TM-8	<0.001
NIH-D5'-d	3.0
NIH-D5'-1	0.3
NIH-D5'-2	0.3
NIH-D5'-3	0.25

Colonies were examined 14 days after cells were seeded in triplicate at 10^3 , 10^4 , 10^5 cells per culture dish. Each result is the average of five separate experiments

PML epitope (to give D5'-TAG, Figure 1), and the resulting 33 kDa product expressed in SAA cells (SAA-D5'-TAG; Figure 7b). Following stimulation of the SAA-D5'-TAG cells with EGF, the electrophoretic mobility of the p33^{D5'-TAG} was retarded (Figure 7b) and its tyrosine phosphorylation increased (Figure 7c). Immunoprecipitation of the p33^{D5'-TAG} polypeptide by anti-PML antibodies and blotting with α GRB-2 revealed that the potential of this mutant to bind Grb2 was unchanged (Figure 7d).

Flow cytometry analysis of the DNA content of PI-stained nuclei from serum-starved NIH-D5' cells revealed a high proportion of cycling cells, comparable to that seen with NIH-SHC cells (Figure 6). Agar plating of NIH-D5' cells demonstrated that all clones examined were capable of forming colonies, although the size of these colonies was slightly inferior to those of the NIH-SHC cells (Table 1).

It, therefore, appears that the 232 amino terminal amino acids of the Shc protein are not essential to the transforming potential of SHC and that interaction with Grb2 and, by inference, activation of Ras, may be sufficient to induce cellular transformation.

Discussion

Identification of a Grb2-binding site on Shc

Because overexpressed human Shc proteins have the capacity to induce the neoplastic transformation of rodent fibroblasts, Shc polypeptides are thought to interact with some component of the intracellular mitogenic signalling pathway (Pelicci *et al.*, 1992). Moreover, the ubiquitous expression of Shc proteins in normal cells, and their frequent tyrosine phosphorylation in response to growth factors and other extracellular signals known to activate tyrosine kinases, leads one to suspect that endogenous Shc proteins are implicated in signal transduction downstream from the tyrosine kinases. These postulated functions of Shc proteins prompted us to investigate whether cytoplasmic signalling proteins can be found to associate with Shc in cells stimulated with growth factors or trans-

formed by oncogenic tyrosine kinases. One such protein is Grb2 which, through its SH2 domain, binds inducibly to tyrosine phosphorylated Shc (McGlade *et al.*, 1992; Rozakis-Adcock *et al.*, 1992). As Grb2 is implicated in Ras activation by virtue of its interaction with mSOS1 and mSOS2, these results suggest that Shc proteins might also couple to the Ras pathway, and the fact that the ability of Shc to induce neurite outgrowth in PC12 cells is dependent on Ras activation (Rozakis-Adcock *et al.*, 1992) supports this concept. It was for this reason that we mapped the Grb2-binding site on Shc and investigated its contribution to Shc transforming activity.

The binding of Grb2 to Shc is potentially explained by the finding that Shc Tyr317 is located within a consensus binding motif for the Grb2 SH2 domain, typified by the presence of an Asn two residues C-terminal to a phosphotyrosine (Songyang *et al.*, 1993, 1994). In fact, when we substituted Tyr317 with Phe, tyrosine phosphorylation of Shc proteins was abolished both *in vitro* and *in vivo*, and binding to Grb2 concomitantly annulled. The finding that substitution of Asn319 with Ala abolished binding to Grb2 without affecting phosphorylation of Tyr317 is consistent with the view that specificity for the Grb2 SH2 domain is conferred by residues C-terminal to the phosphorylation site (Pawson & Schlessinger, 1993). Moreover, as a phosphopeptide containing the Tyr317 phosphorylation site competed efficiently for binding to Grb2 *in vitro*, binding of Shc to Grb2 appears to be primarily, if not entirely, dependent on phosphorylation of Shc Tyr317 and the resulting creation of a high affinity binding site for the Grb2 SH2 domain. The Grb2-binding motif identified in Shc, pTyr-Val-Asn-Val, is also present at an autophosphorylation site located at Tyr177 within the Bcr region of the oncogenic Bcr-Abl tyrosine kinase. We and others have shown that phosphorylation of Bcr-Abl at the same site also induces Grb2-binding; formation of this complex is implicated in Ras activation and lymphoid transformation by Bcr-Abl (Pendergast *et al.*, 1993; Puil *et al.*, 1994; Tauchi *et al.*, 1994). This specific SH2-binding motif is, therefore, common to at least two Grb2-binding sites.

Grb2-binding correlates with Shc transforming activity

Overexpression of the 46 and 52 kDa human Shc proteins in rodent fibroblasts induces morphological transformation, continued transit through the cell cycle in the absence of growth factors and malignant transformation (Pelicci *et al.*, 1992). Identification of the Grb2-binding site on Shc, and the ability to express mutant Shc proteins lacking this site due to the substitution of Tyr317 by Phe, allowed us to assess the potential importance of Grb2-binding for Shc transforming activity. Mutant Shc proteins with Phe at residue 317 could be abundantly expressed in fibroblasts, but lost the capacity to be highly phosphorylated on tyrosine in response to EGF, bind Grb2, and induce neoplastic transformation. Furthermore, the amino-terminal region of Shc could be removed without markedly affecting these properties. These data are fully consistent with the proposition that Shc overexpression exerts its transforming activity by forming a constitutive complex with Grb2, and thereby delivering a continuous mitogenic stimulus through the Ras path-

way. The mechanism by which the interaction of Shc with Grb2 might activate the Ras pathway remains unclear.

Shc function in normal and transformed cells

This scheme does not rule out the possibility that Shc interacts with other proteins through its CH domain, Tyr317 phosphorylation site or SH2 domain, which contribute to its transforming properties. Nor does it exclude a role for the Shc amino-terminal region in signal transduction in normal cells. The finding that Shc binds to the EGFR through its SH2 domain, and, in its tyrosine phosphorylated form to Grb2, is something of a conundrum, since the EGFR has an independent binding site for the Grb2 SH2 domain (Buday & Downward, 1993). One can postulate that tyrosine kinases which cannot bind directly to Grb2, such as v-Src, might employ Shc as an intermediary through which to activate the Grb2-mSOS1 complex. However, this argument does not hold for the EGF-receptor or Bcr-Abl, which are able to both bind Grb2 directly and induce the formation of a Shc-Grb2 complex (Pendergast *et al.*, 1993; Puil *et al.*, 1994; Tauchi *et al.*, 1994). If Grb2 and the associated mSOS1 protein are activated purely as a consequence of the interaction of the Grb2 SH2 domain with a phosphotyrosine-containing site, Shc may act to amplify the signal from the EGFR to Ras by increasing the number of complexes formed with the Grb2 SH2 domain. However, as it is highly probable that Shc has one or more functions other than its ability to bind Grb2, it could be involved in regulating a distinct signaling pathway, that is integrated with the Ras pathway through the interactions of phosphorylated Shc with Grb2.

Materials and methods

Plasmid constructions

The TM cDNA was generated by changing the TAT codon (tyrosine 317) of wild-type Shc cDNA (Pelicci *et al.*, 1992) to a TTT codon (phenylalanine) by polymerase chain (PCR) reactions based protocols for oligonucleotide-directed mutagenesis (Bowman *et al.*, 1990). The D5' cDNA was constructed by substituting the 5' portion of the SHC cDNA (corresponding to AA 1–232) with a 15 bp sequence containing an eukaryotic initiation site for translation (TAAAG-CACTATGGGC). SHC, TM and D5' cDNAs were cloned into the EcoRI site of the LXSN retroviral vector (Miller & Rosman, 1989) to obtain the L-SHC-SN, L-TM-SN and L-D5'-SN expression vectors.

The SHC, TM and D5' cDNA's were tagged with a PML epitope. The PML sequence from nucleotide 550 to 712 was excised from the PML cDNA (Pandolfi *et al.*, 1991). A EcoRI site was added at the 5' and a termination codon and a BamHI site at the 3' end of this sequence. The modified EcoRI–BamHI PML sequence was cloned into the 5'-EcoRI–BamHI-3' sites of the LXSN eukaryotic expression vector (LXSN-PML). The SHC, TM, D5' cDNAs were modified so that their in-frame termination codon was substituted by a 12 bp sequence (GAATTCTGGCGG) containing an EcoRI site and two codons for proline and then cloned into the EcoRI site of the LXSN-PML plasmid to obtain the L-SHC-TAG-SN, L-TM-TAG-SN, L-D5'-TAG-SN expression vectors.

In vitro phosphorylation and Grb2 binding of Shc proteins

pGEX GST-SHC+ was constructed by PCR subcloning of the region encoding amino acid 280 to 473 of the SHC cDNA into pGEX2T. The mutant pGEX GST-SHC + Y317F was created by PCR subcloning amino acids 280–473 from the TM mutant cDNA into the pGEX2T plasmid. pGEX2T, pGEX-SHC + or pGEX-SHC + Y317F were transfected into C600HFL bacteria, and infected with *gt11* expressing a β gal-Elk fusion protein, to obtain stable lysogens as previously described (Reedjick *et al.*, 1992). To obtain tyrosine phosphorylated GST fusion proteins, bacteria were grown and the expression of GST-fusion proteins induced with 1 mM IPTG at 30°C. They were then heat shocked at 42°C for 15 min and incubated for a further 30 min at 37°C to allow expression of the Elk Kinase. The bacteria were lysed by sonication in PLC-LB and fusion proteins were purified by glutathione agarose as described previously (Pelicci *et al.*, 1992). The immobilized proteins were analysed by SDS-PAGE and quantitated by Coomassie Blue staining.

In vitro mixing experiments were carried out by incubating 5 μ g of fusion protein with R2 fibroblast lysates prepared from approximately 5×10^6 cells using PLC-LB, for 90 min at 4°C. When phosphopeptides were added, they were preincubated with the R2 lysates for 30 min before the addition of the GST fusion proteins. The resultant protein complexes were washed 3–5 times with PLC-LB and analysed by SDS-PAGE and immunoblotting which has been described in detail elsewhere (Pelicci *et al.*, 1992). Anti-GRB-2 serum was used at 1:500 dilution and the affinity purified anti-P-Y antibodies were used at 1 μ g ml⁻¹. Immunoblots were developed using anti-rabbit HRP conjugate and ECL (Amersham). The phosphopeptides used were a generous gift of Steve Shoelson (Harvard Medical School). The peptide corresponding to SHC tyrosine 317 contained the sequence: E-L-F-D-D-P-S-pY-V-N-V-Q-N-L-D-K.

Antibodies and cell lines

Two anti-Shc polyclonal antisera (anti-SHC-SH2 and anti-SHC-C) were obtained by immunizing rabbits with a bacterially expressed Shc SH2 peptide (anti-SHC-SH2) or a Shc synthetic peptide (APRDLFDMKPFEDALRVC) (anti-SHC-C). These antisera were used at 1:5000 (anti-SHC-SH2) or 1:1000 (anti-SHC-C) dilution for immunoblot analysis and 0.5 μ g mg⁻¹ lysates in immunoprecipitation procedures. Monoclonal antibodies to phosphotyrosine were purchased from Upstate Biotechnology and used at 1 μ g ml⁻¹ for western blot analysis. A polyclonal anti-Grb2 was obtained by injecting rabbits with a bacterially expressed Grb2 SH2 peptide (anti-Grb2).

The LXSN, L-SHC-SN, L-TM-SN, L-D5'-SN, L-SHC-TAG-SN, L-TM-TAG-SN, L-D5'-TAG-SN retrovirus expression vectors were transfected into the PA317 retrovirus packaging cell line by the calcium phosphate precipitation procedure. After 48 h, the PA317 supernatants were used to infect NIH3T3 fibroblasts and SAA cells. The cells were then selected with G418 containing medium. SAA cells are NIH3T3 fibroblasts engineered to over-express the EGFR (Di Fiore *et al.*, 1987).

Immunoprecipitation and western blotting procedures

Lysates were prepared from serum starved cultures of the appropriate cell lines. Cells were lysed on ice in PY buffer (20 mM Tris HCl pH 7.8, 50 mM NaF, 50 mM NaCl, 30 mM Na₂P₂O₇, 5 mM sodium orthovanadate, 1% vol/vol Triton X-100) containing freshly added protease inhibitors (1 mM phenylmethyl sulfonyl fluoride, 10 μ g ml⁻¹ leupeptin and 5 mg ml⁻¹ aprotinin). Lysates were clarified by centrifugation at 4°C and protein concentration determined by BCA reagent (Pierce). For immunoprecipitation experiments, appropriate

antibodies were adsorbed on Protein A Sepharose (Pharmacia) and then incubated with cell lysates for 2 h at 4°C. Immune complexes were washed 3–5 times with ice cold PY buffer, eluted and denatured by heating for 3 min at 95°C in reducing Laemmli buffer; proteins were then resolved on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). For immunoblot analysis, either specific immunoprecipitates or 50 µg total cell lysates were transferred onto nitrocellulose filters after SDS-PAGE. After blocking nonspecific reactivity with 2% nonfat dry milk dissolved in TBST (20 mM Tris-HCl pH 7.8, 150 mM NaCl, 0.02% Tween 20) (1 h incubation at 22°C), filters were probed for 1 h at 22°C with specific antibodies diluted in TBST. After extensive washing, immune complexes were detected with horseradish-peroxidase conjugated species-specific secondary antiserum (Bio Rad) followed by enhanced chemiluminescence reaction (Amersham).

Cell cycle distribution and soft agar colonization

A quantitative measure of cell cycle distribution was obtained by flow cytometric analysis of DNA histograms (Fried *et al.*, 1978). Briefly, the various cell lines were cultured in 100 mm plastic dishes in DMEM medium supplemented with 10% FCS. At the pre-established time points, the cells were washed twice with cold PBS and 2 ml fluorochrome solution (propidium iodide 0.05 mg ml⁻¹ dissolved in 0.1% sodium citrate with 0.1% Triton-X100) added. The plates were placed at 4°C in the dark for 60–90 min, the cells dislodged by repeated pipetting and the stained cells transfer-

red to test tubes for DNA analysis. Cell fluorescence was measured in a FASCAN flow cytometer (Becton Dickinson, Mountain View, USA) by an argon ion laser at 488 nm for excitation. Orange-red DNA fluorescence due to PI staining of isolated nuclei was detected in the band above 600 nm and measured as pulse area of the particles. A doublet discrimination module was used to distinguish doublets from single cells. Data were recorded in a Hewlett-Packard (HP9000 model 310) computer. The percentage of nuclei in the different phases of the cell cycle (G₀-G₁, S and G₂/M) was calculated from the histogram with DNA cell cycle analysis software (CellFIT, Becton Dickinson). A minimum of 10⁵ cells per sample were analysed.

For the soft-agar assay, cells were trypsinized and washed in Ca⁺⁺, Mg⁺⁺-free phosphate-buffered saline (PBS), and three scalar concentrations of cells (1 × 10³, 1 × 10⁴, 1 × 10⁵) were plated in 1 ml of DMEM medium containing 20% FCS, 100 mg ml⁻¹ gentamicin and 0.3% (w/v) Noble agar, over 2 ml layer of DMEM medium with 0.6% agar in six well plates.

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