Opposite effects of the p52^{shc}/p46^{shc} and p66^{shc} splicing isoforms on the EGF receptor–MAP kinase–fos signalling pathway

Enrica Migliaccio¹, Simonetta Mele², Anna E. Salcini¹, Giuliana Pelicci¹, Ka-Man Venus Lai³, Giulio Superti-Furga⁴, Tony Pawson³, Pier Paolo Di Fiore^{1,5}, Luisa Lanfrancone¹ and Pier Giuseppe Pelicci^{1,2,6}

¹European Institute of Oncology, Department of Experimental Oncology, Via Ripamonti, 435-20141 Milan, Italy, ²Istituto di Medicina Interna e Scienze Oncologiche, University of Perugia, 06100 Perugia, Italy, ³Division of Molecular and Developmental Biology, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario M5G 1X5, Canada, ⁴EMBL, Heidelberg, Germany and ⁵Istituto di Microbiologia, University of Bari, Italy

⁶Corresponding author

Shc proteins are targets of activated tyrosine kinases and are implicated in the transmission of activation signals to Ras. The p46^{shc} and p52^{shc} isoforms share a C-terminal SH2 domain, a proline- and glycine-rich region (collagen homologous region 1; CH1) and a N-terminal PTB domain. We have isolated cDNAs encoding for a third Shc isoform, p66^{shc}. The predicted amino acid sequence of p66^{shc} overlaps that of p52^{shc} and contains a unique N-terminal region which is also rich in glycines and prolines (CH2). p52^{shc}/p46^{shc} is found in every cell type with invariant reciprocal relationship, whereas p66^{shc} expression varies from cell type to cell type. p66^{shc} differs from p52^{shc}/p46^{shc} in its inability to transform mouse fibroblasts *in vitro*. Like p52^{shc}/p46^{shc}, p66^{shc} is tyrosine-phosphorylated upon epidermal growth factor (EGF) stimulation, binds to activated EGF receptors (EGFRs) and forms stable complexes with Grb2. However, unlike p52^{shc}/p46^{shc} it does not increase EGF activation of MAP kinases, but inhibits fos promoter activation. The isolated CH2 domain retains the inhibitory effect of p66^{shc} on the fos promoter. p52^{shc}/p46^{shc} and p66^{shc}, therefore, appear to exert different effects on the EGFR–MAP kinase and other signalling pathways that control fos promoter activity. Regulation of p66^{shc} expression might, therefore, influence the cellular response to growth factors.

Keywords: fos/p52^{shc}/p46^{shc}/p66^{shc}/phosphorylation/SH2 proteins

Introduction

The SH2-containing Shc proteins (Pelicci *et al.*, 1992) are cytoplasmic substrates of activated tyrosine kinases (TK) (Lotti *et al.*, 1996) and have been implicated in the transmission of activation signals from TKs to Ras proteins (Bonfini *et al.*, 1996). Shc proteins are phosphorylated by all receptor TKs (RTKs) tested to date, including the EGF

receptor (EGFR) (Pelicci *et al.*, 1992), the platelet-derived growth factor receptor (Yokote *et al.*, 1994), the hepatocyte growth factor receptor (Pelicci *et al.*, 1995a), the erbB-2 receptor (Segatto *et al.*, 1993; Ricci *et al.*, 1995), the insulin receptor (Pronk *et al.*, 1993; Skolnik *et al.*, 1993), the fibroblast growth factor receptor (Vainikka *et al.*, 1994), and the nerve growth factor receptor (Borrello *et al.*, 1994; Stephens *et al.*, 1994). Shc proteins are also involved in signalling from cytoplasmic TKs, since they are constitutively phosphorylated in cells that express activated Lck, Src, Fps or Sea (McGlade *et al.*, 1992; Crowe *et al.*, 1994; Dilworth *et al.*, 1994; Baldari *et al.*, 1995; Pelicci *et al.*, 1995b). 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. IL-2, erythropoietin, G-CSF, GM-CSF, B- and T-cell receptors, CD4, CD8) (Burns *et al.*, 1993; Damen *et al.*, 1993; Ravichandran *et al.*, 1993; Baldari *et al.*, 1995; Lanfrancone *et al.*, 1995; Matsuguchi *et al.*, 1994).

Upon phosphorylation, Shc proteins form stable complexes with cellular tyrosine-phosphorylated polypeptides, including RTKs and receptors devoid of intrinsic TK activity. These interactions are mediated by the Shc SH2 and/or PTB domains (Pelicci *et al.*, 1992, 1995b; Segatto *et al.*, 1993; Blaikie *et al.*, 1994; Borrello *et al.*, 1994; Kavanaugh and Williams, 1994; Stephens *et al.*, 1994; Yokote *et al.*, 1994; Baldari *et al.*, 1995; Lanfrancone *et al.*, 1995; van der Geer *et al.*, 1995). Phosphorylated Shc proteins also associate with the Grb2 adaptor protein (Clark *et al.*, 1992; Lowenstein *et al.*, 1992) through direct binding of the Grb2 SH2 domain to the major Shc tyrosine-phosphorylation site (Tyr317) (Rozakis-Adcock *et al.*, 1992; Salcini *et al.*, 1994). Grb2 is constitutively complexed with SOS (Batzner *et al.*, 1993; Buday and Downward, 1993; Chardin *et al.*, 1993; Egan *et al.*, 1993; Gale *et al.*, 1993; Olivier *et al.*, 1993; Rozakis-Adcock *et al.*, 1993), a ubiquitously expressed Ras guanine nucleotide exchange factor for Ras (Simon *et al.*, 1991; Bonfini *et al.*, 1992; Bowtell *et al.*, 1992; Chardin *et al.*, 1993). Recruitment of the Grb2–SOS complex results in the membrane relocalization of SOS, an event considered sufficient to induce Ras activation (Aronheim *et al.*, 1994), thus suggesting that Shc proteins are involved in the regulation of Ras. This hypothesis is supported by the findings that Shc overexpression induces increased proliferative response, mitogen-activated (MAP) kinase and fos activation by EGF (A.E. Salcini and P.G. Pelicci, unpublished results), GM-CSF (Lanfrancone *et al.*, 1995) or thrombin (Chen *et al.*, 1996). Furthermore, transformation of NIH-3T3 fibroblasts by overexpression of Shc proteins depends on the presence of Tyr317 and Grb2-binding (Salcini *et al.*, 1994), and neuronal terminal differentiation

Fig. 1. Partial DNA and primary amino acid sequence of the λ K9 cDNA. **(A)** Schematic representation and limited restriction enzyme map of the λ K9 and λ GF11 cDNA sequences. The box represents the longest ORF starting from the ATG positions 195 (ATG1; λ K9) and 83 (ATG2; λ GF11) with the PTB, SH2 and the collagen-homologous (CH) 1 and 2 regions (CH1 and CH2) indicated. The ATGs at amino acid positions 525 and 660 of the λ K9 sequence and 83 and 218 of the λ GF11 (ATG2 and ATG3, respectively) (see text) are also indicated. P, *Pvu*II; A, *Ava*I; B, *Bam*HI. **(B)** Nucleotide and deduced amino acid sequences of the λ K9 5' extremity. The nucleotide sequence corresponds to the λ K9 clone from nucleotides 1–566. The translation of the λ K9 sequence from the first in-frame ATG (ATG1 at position 195; bold type) is shown below the nucleotide sequence. The 5' in-frame termination codon at position 75 is underlined. Nucleotide and amino acid positions are indicated on the right of each lane. The region of the λ K9 sequence common to that of the λ GF11 is underlined (the bold ATG at nucleotide position 525 corresponds to ATG2 of λ K9 and λ GF11). **(C)** Comparison of the λ K9 collagen-related sequence (CH2) with that of the α 1-collagen chain (Coll). |, identical residues; ., conserved residues.

of PC12 cells by Shc overexpression is prevented by the co-expression of a Ras dominant negative mutant (Rozakis-Adcock *et al.*, 1992).

Two Shc isoforms of 52 kDa (p52^{shc}) and 46 kDa (p46^{shc}) have been characterized (Pelicci *et al.*, 1992). They share a carboxy-terminal SH2 domain, an adjacent glycine/proline-rich region (CH) and, in their N-terminal region, a recently identified phosphotyrosine binding domain (PTB) (Blaikie *et al.*, 1994; Kavanaugh and Williams, 1994; van der Geer *et al.*, 1995). The modular organization of the p52^{shc}/p46^{shc} proteins is shown in Figure 1A. Consistent with a general function of Shc proteins in the transmission of signals from activated TKs, p52^{shc} and p46^{shc} are ubiquitously expressed. However, antibodies raised against the Shc SH2 domain, also detect an ~66 kDa polypeptide, expressed mainly in epithelial

cells (Pelicci *et al.*, 1992). We report here the structural characterization and preliminary functional properties of p66^{shc}.

Results

Isolation and sequence analysis of the λ K9 cDNA clone

To isolate cDNAs representative of p66, a human cDNA library prepared from p66-expressing cells was screened with a DNA probe representative of the Shc SH2 domain. Twenty-two clones were isolated from the 500 000 phage plaques of the Calu1 cDNA library. The insert of one clone (λ K9) was longer than the λ GF11 clone which encodes p52^{shc}/p46^{shc}, and differed from λ GF11 (Pelicci *et al.*, 1992) in its 5' extremity at restriction mapping analysis (Figure 1A). The λ K9 insert was subcloned into the pGEM-3 vector (pK9 plasmid) and sequenced. Analysis of the λ K9 DNA sequence and comparison with that of λ GF11 revealed that the two clones were identical from nucleotide position 525 of λ K9 and nucleotide position 76 of λ GF11 to their ends. The sequences of the 5' extremity of the λ K9 clone, including its unique 524 bp region, is shown in Figure 1B.

The longest open reading frame (ORF) predicted from clone λ K9 is 1749 bp with the first in-frame ATG at position 195 (ATG1 in Figure 1B) and an in-frame TGA at position 1944 (not shown). ATG¹⁹⁵ is probably the translation initiation codon of λ K9, since it is flanked by sequences (TCAACTATGG) that match the Kozak consensus for translation initiation in eukaryotes (Kozak, 1989) and is preceded by an in-frame TGA codon at nucleotide position 75 (Figure 1B). The protein predicted from the λ K9 clone sequence is 583 amino acids in length, and has a molecular mass of 62 898 Da. Comparison between the λ K9 and the λ GF11 (p52^{shc}/p46^{shc}) proteins disclosed an amino-terminal region of 110 amino acids unique to λ K9, which continued into a common region starting at residue 1 of λ GF11 and residue 111 of λ K9, and terminated at the ends of both proteins. The first amino acid common to both proteins is the Met translation initiation codon of p52^{shc} (see the partial amino acid sequence of λ K9 shown in Figure 1B and ATG2 in Figure 1A).

A search in the genebank database for sequences homologous to the amino-terminal-unique region of K9 identified human $\alpha 1$ collagen (20% amino acid identity and 33% similarity; Figure 1C). This collagen-homologous λ K9 region was rich in glycine (24%) and proline (37%) residues, both of which are also abundant in the collagen $\alpha 1$ chain (57% and 46% in the λ K9-homologous collagen $\alpha 1$ chain region, respectively). Some 31% of glycine and 50% of proline $\alpha 1$ collagen residues are conserved in the λ K9 collagen-homologous region. Because the p52^{shc}/p46^{shc} protein contains a glycine/proline-rich region that is also partially homologous with the collagen $\alpha 1$ chain (CH region), we renamed it CH1 and called the λ K9 region CH2 (Figure 1A). Not only do these two regions fail to display a high degree of homology, but they share homology with two adjacent regions of the collagen $\alpha 1$ chain which overlap for 34 amino acids (bold sequence in Figure 1C).

Fig. 2. RNase protection analysis of the expression of the $p66^{shc}$ encoding transcript. (A) Schematic representation of the used RNase probes (R1 and R2) and their positions with respect to the λ K9 cDNA. (B) RNAs derived from the indicated cell samples were hybridized to the R1 (left panel) or R2 (right panel) RNA antisense probes. The protected fragments are shown by arrows; the origin (p66; p52/p46) of the fragment is also indicated. Riboprobe contained variable lengths of vector plasmids (10–40 bp) and therefore migrated slightly slower than fully protected fragments. Molecular weight markers are given on the right. tRNA, transfer RNA used as control of RNase digestion.

Identification of the λ K9 cDNA clone as the $p66^{shc}$ cDNA

To ascertain whether the λ K9 clone is, indeed, representative of the transcript encoding p66, the expression of λ K9 corresponding mRNA was determined by RNase protection experiments and compared with $p66^{shc}$ expression. A 498 bp RNA probe containing a 454 bp region unique to λ K9 (probe R1, Figure 2A) gave a strong 454 bp protection fragment in murine NIH-3T3 cells stably transfected with a λ K9 expression vector (NIH-p66) and in a series of human p66-expressing cell samples (the A-172, NCTC, SK-N-MC and Calu1 cell lines and in mesothelial primary cells) (compare cell samples of Figure 2B, left panel and Figure 3). A fainter protection fragment was observed in cell lines that express lower levels of p66 (K562, GTL16 and PEER; Figures 2B and 3). tRNA and mRNAs from mouse NIH-3T3 fibroblasts or human cells that do not express p66 (U937, HL-60, KG1) were not protected by the R1 probe (Figures 2B and 3). Similar results were obtained with a second λ K9 RNA probe that spans the junction between the unique λ K9 sequences and the sequences common to λ K9 and λ GF11 (probe R2, Figure 2A). The 281 bp R2 probe

Fig. 3. Western blotting analysis of $p66^{shc}$, $p52^{shc}$ and $p46^{shc}$ expression. Whole-cell lysates were prepared from the indicated cell lines and 50 μ g of total protein separated by 10% SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-SH2 (upper panel) or anti-CH2 (lower panel) Shc antibodies (α SH2 and α CH2). The SHC proteins (p66, p52 and p46) are indicated on the left.

yielded a 240 bp fully protected fragment in cells expressing $p66^{shc}$ (NIH-p66 fibroblasts and A-172 cells), a 40 bp protection fragment in $p52^{shc}/p46^{shc}$ expressing cells [NIH-3T3 transfected with the λ GF11 cDNA (NIH-p52/p46) A-172 and HL-60 cells] and no protection of tRNA and RNA from mouse cells (NIH-3T3 cells). The 40 bp protection fragment corresponds to the partial protection of the R2 probe by the $p52^{shc}/p46^{shc}$ transcript (Figure 2B, right panel).

We next generated a polyclonal antibody against the CH2 portion of λ K9 (α CH2, see Materials and methods). A bacterial glutathione *S*-transferase (GST)-CH2 fusion protein was produced by inserting the sequence of the λ K9 cDNA encoding amino acids 1–110, corresponding to its CH2 region, into the bacterial expression plasmid pGEX-2T to generate polyclonal antibodies in rabbit. The resulting serum reacted specifically with the λ K9 cDNA products that had been translated *in vitro* and transiently expressed in COS-1 cells, as well as with a polypeptide of similar size in the Calu1 cell line (not shown). The anti-CH2 antibody also recognized a protein of ~66 kDa in all cell samples that expressed p66 (Figure 3, lower panel). Moreover, the translation products of the λ K9 cDNA were recognized by the anti-SH2 Shc antibody both *in vitro* and *in vivo* (not shown). Taken together, these data indicate that the λ K9 clone encodes for the $p66^{shc}$ isoform.

Origin of the $p66$ cDNA

The origin of the human $p66^{shc}$ transcript was investigated by isolating the human Shc locus and mapping the various Shc exons. A genomic library from normal human embryo lung fibroblasts (WI38) was screened with the Shc SH2 DNA probe and two overlapping λ -clones (λ -JO and λ -PA1) were isolated (not shown). The inserts from these phages spanned ~15 kb of human DNA (Figure 4A). To map Shc exons, the two λ -genomic clones were digested with the *Eco*RI, *Xba*I and *Bam*HI restriction enzymes and hybridized to different portions of the Shc cDNA. The hybridizing restriction fragments were subcloned into the

Fig. 4. (A) Organization of the murine and human Shc locus and exon assembly of Shc transcripts. A limited restriction enzyme map of the mouse and human loci is shown at the top. Boxes indicate Shc exons and the exon numbers are given above [numbering is temporary since the Shc cap site(s) has not been mapped]. A schematic representation of the exon assembly in the p66^{shc} and p52^{shc}/p46^{shc} encoding transcripts is given below. Exons are indicated by boxes and the splicing events are shown by the broken zig-zag line. The position of the three Shc ATGs (see text) is given. E, *EcoRI*; B, *BamHI*; X, *XbaI*. (B) Nucleotide sequences of exon 1–2a and exon 1–2b donor/acceptor splice junctions in both the human (Hu.) and mouse (Mu.) loci. The donor and acceptor sites are underline; exons 1 and 2 and intervening sequences (intron 1 and the retained intron) are indicated.

plasmid vector pGEM3 and the exon/intron boundaries sequenced. The restriction enzyme map and exon/intron organization of the Shc locus are reported in Figure 4A. The Shc locus contains 13 exons. Exon 1 is non-coding and contains only sequences that correspond to the p52^{shc}/p46^{shc} transcripts (λ GF11 cDNA from nucleotides 1–75). The 5' 521 bp of exon 2 contain sequences corresponding to the CH2 p66^{shc} region and includes both the 5' untranslated region and the p66^{shc} ATG1. The 3' 168 bp of exon 2 contain sequences corresponding to the 56 amino acids common to the p66^{shc} and p52^{shc}/p46^{shc} isoforms (including ATG2 and ATG3) (exon 2a). Exons 3 to 13 encode the remaining p66^{shc} and p52^{shc}/p46^{shc} common sequences. It appears, therefore, that only the 168 bp 3' portion of exon 2 (exon 2a) is incorporated within the mature transcript encoding p52^{shc}/p46^{shc}, as found in the λ GF11 cDNA clone. An acceptor consensus site for splicing was found 5' to the Shc exon 2a (Figure 4B), suggesting that the λ GF11 transcript is formed by the juxtaposition of exons 1 and 2a. In contrast, transcripts encoding p66^{shc} would incorporate the entire exon 2.

A similar Shc genomic organization and Shc transcript assembly was found in mice. Alignment of the predicted mouse p66^{shc} sequence, as derived from analysis of mouse p66 cDNAs (not shown), with human p66^{shc} showed a high degree of amino acid identity and identical overall organization of the two proteins (not shown). In the mouse Shc locus, exon 1 encodes p52^{shc}/p46^{shc} unique sequences, the 5' end of exon 2 encodes p66^{shc} unique sequences, exon 2a–13 sequences common to all Shc isoforms (Figure 4A). An acceptor consensus site for splicing is also found 5' to the mouse Shc exon 2a at the same position as in the human Shc exon 2a (Figure 4B).

Overexpression of the p66^{shc} cDNA does not induce transformation of NIH-3T3 fibroblasts

Overexpression of the p52^{shc}/p46^{shc} isoforms induces transformation of NIH-3T3 fibroblasts, as determined by their acquired capacity to grow in semi-solid media and to form tumours in nude mice (Pelicci *et al.*, 1992). The biological activity of the p66^{shc} and the p52^{shc}/p46^{shc} isoforms was compared by expressing the p66^{shc} and p52^{shc}/p46^{shc} cDNAs into NIH-3T3 fibroblasts and evaluating the capacity of the resulting transformants to form colonies in soft agar.

NIH-3T3 fibroblasts express all three Shc isoforms. As expression of the p66^{shc} cDNA into NIH-3T3 fibroblasts yielded a marked increase in the level of p66^{shc} and p52^{shc} and, to a lesser extent, p46^{shc} (NIH-p66 clone; Figure 5A); all three isoforms are presumably encoded by the p66^{shc} cDNA. Indeed, the protein predicted by the translation of the p66^{shc} cDNA contains three in-frame ATGs, the second and third corresponding to the first and second of the p52^{shc}/p46^{shc} predicted protein (Figure 1A). To determine the potential of isoform translation of the λ GF11 and λ K9 cDNAs, ATGs were variously mutagenized. *In vitro* translation of the λ GF11(p52^{shc}/p46^{shc} cDNA) yielded two polypeptides of 52 and 46 kDa that reacted specifically against the anti-SH2 Shc antibody (not shown). Mutation of the ATG3 of λ GF11 to TTG (GF11TTG) abrogates translation of p46, while mutagenesis of ATG2 (GF13) abrogates translation of p52 (Pelicci *et al.*, 1992). Expression of the GF11TTG cDNA in NIH-3T3 cells resulted in expression of the p52^{shc} isoform (NIH-p52 clone), while expression of the GF13 cDNA resulted in the production of the p46^{shc} protein (NIH-p46 clone) (Figure 5A). These results suggest that p52^{shc} and p46^{shc} isoforms are trans-

Fig. 5. Potential of isoform translation of the λ GF11 and λ K9 cDNAs. The following cDNAs were expressed into NIH-3T3 cells (A and B): GF11, encoding p52^{shc}/p46^{shc} (Pelicci *et al.*, 1992); K9, encoding p66^{shc}; GF11TGG, derived from the GF11 cDNA by phenylalanine/alanine substitution of ATG3 (Pelicci *et al.*, 1992); GF13, starting between ATG2 and ATG3; K9TTGs, derived from the K9 cDNA by phenylalanine/alanine substitution of ATG2 and ATG3. (A) Western blotting analysis of Shc expression of NIH-3T3 cells stably transfected with the LXSN expression vector (NIH-SN) or with the same vector expressing the GF11 (NIH-p52/p46), GF13 (NIH-p46), GF11TGG (NIH-p52), K9 (NIH-p66), K9TTGs (NIH-p66-TTGs) cDNAs. (B) Western blotting analysis of Shc-overexpressing clones. Lysates from one control NIH-3T3 clones (NIH-SN) and clones overexpressing p52^{shc}/p46^{shc} (NIH-SHC-13 and NIH-SHC-9), p66^{shc}/p52^{shc} (NIH-p66-8), p66^{shc} (NIH-p66-TTGs5 and NIH-p66-TTGs7) were blotted against the anti-SH2 Shc antibody (α SH2).

lated from the same transcript by alternative usage of two in-frame ATGs. *In vitro* translation of the λ K9 (pK9 plasmid) yielded p66 and p52 proteins and, to a lesser extent, a p46 polypeptide, all of which reacted with the anti-SH2 antibody (not shown). Simultaneous mutagenesis of ATG2 and ATG3 of λ K9 (K9TTGs) did not affect translation of the p66 polypeptide, but abrogated p52 and p46 expression, as assayed by both *in vitro* translation (not shown) and stable expression in NIH-3T3 fibroblasts (NIH-p66-TTGs clone; Figure 5A). Taken together, these data provide evidence that the three in-frame ATGs are all used as translation initiation sites both *in vitro* and *in vivo* and that the λ K9 cDNA encode all three Shc isoforms.

To test for transformation capability, NIH-3T3 clones overexpressing the wild-type p52^{shc}/p46^{shc} (NIH-SHC-9 and NIH-SHC-13), the wild-type p66^{shc} (NIH-p66-2 and NIH-p66-8), or a mutant version of p66 with phenylalanine substitutions at the second and third ATGs (NIH-p66-TTGs5 and NIH-p66-TTGs7), or control NIH-3T3 clones (NIH-SN and NIH-SN-5) were plated in triplicate at various concentrations in 0.3% agar medium supplemented with 20% serum, and colonies scored after 14 days. Western blot analysis of the various Shc-overexpressing clones and one of the two control clones (NIH-SN) is reported in Figure 5B. The two NIH-SHC clones formed colonies in soft agar at the expected frequencies (Pelicci *et al.*, 1992), whereas the control NIH-SN clones, the NIH-p66 and NIH-p66-TTGs clones did not (Table I). It, therefore, seems that the p66^{shc} isoform does not retain the transforming activity of the p52^{shc}/p46^{shc} isoforms.

p66^{shc} is phosphorylated upon EGF stimulation and binds to activated EGFR and Grb2

In order to investigate the molecular basis of the lack of transforming potential of p66^{shc}, its ability to mimic the effects of p52^{shc}/p46^{shc} on known signal transduction mechanisms was investigated by comparing the capacity of p66^{shc} and p52^{shc}/p46^{shc} to bind activated EGFR, to be phosphorylated upon EGF stimulation, to bind Grb2 and activate MAP kinases.

The p66^{shc} (K9TTGs cDNA) and p52^{shc}/p46^{shc} (GF11

Table I. Cloning efficiency ^a	
Cells	Cloning efficiency (%)
NIH-SN	<0.001
NIH-SN-5	<0.001
NIH-SHC-13	>0.5
NIH-SHC-9	>3
NIH-p66-2	<0.001
NIH-p66-8	<0.001
NIH-p66-TTGs5	<0.001
NIH-p66-TTGs7	<0.001

^aCloning efficiency was monitored in control NIH-3T3 clones (NIH-SN-1 and NIH-SN-5) and NIH-3T3 clones expressing p52^{shc}/p46^{shc} (NIH-SHC-9 and NIH-SHC-13), wild-type p66^{shc} (NIH-p66-2 and NIH-p66-8) and mutant p66TTGs (NIH-p66-TTGs5 and NIH-p66-TTGs7) cDNAs. Colonies were examined 14 days after cells were seeded in triplicate at 10³, 10⁴ and 10⁵ cells per culture dish. Each result is the average of five separate experiments.

cDNA) coding sequences were cloned under the control of the adenovirus promoter in a plasmid containing the SV40 origin of replication (pMT2) and transiently transfected into COS-1 cells. At 16 h after transfection, the cells were washed and kept in serum-free media for an additional 24 h and either treated for 5 min or not with 30 ng/ml of EGF. Lysates prepared from serum-starved or EGF-stimulated cells were analysed for Shc protein expression (Figure 6B) and immunoprecipitated with anti-Shc antibodies. The immunoprecipitates were immunoblotted with anti-phosphotyrosine or anti-Grb2 antibodies (Figure 6A), EGF stimulation induced a comparable increase in the phosphotyrosine content of p66^{shc} and p52^{shc}/p46^{shc} proteins (Figure 6A, upper panel). A novel phosphotyrosine-containing polypeptide of ~175 kDa stably complexed with both p66^{shc} and p52^{shc}/p46^{shc} upon EGF stimulation (Figure 6A, upper panel). Probing of the same anti-Shc immunoprecipitates with anti-EGFR antibodies revealed that the 175 kDa polypeptide is indeed the EGFR (data not shown). In addition, phosphorylated p66^{shc} and p52^{shc}/p46^{shc} formed stable complexes with Grb2 in EGF-stimulated cells (Figure 6A, lower panel). It can, therefore, be concluded that p66^{shc} is phosphorylated by

Fig. 6. Tyrosine phosphorylation and Grb2 binding of $p52^{shc}/p46^{shc}$ and $p66^{shc}$. (A) Anti-phosphotyrosine Western blotting of anti-Shc (α SH2) immunoprecipitates from lysates of Cos-1 cells transfected with the pMT2-p66-TTGs and pMT2-p52/p46 expression vectors and subsequently treated (+) or not (-) with EGF (upper panel). The same blot was reprobed with anti-Grb2 antibodies (α Grb2) (lower panel). The EGFR, $p66^{shc}$, $p52^{shc}$ and $p46^{shc}$ polypeptides and the α Grb2 25 kDa immunoreactive polypeptide are indicated by arrows. Immunoglobulin cross-reactive polypeptides are also indicated (Ig). (B) Western blotting analysis of Shc proteins expression in Cos-1 cells transfected with the pMT2-p66-TTGs and pMT2-p52/p46 expression vectors and treated (+) or not (-) with EGF.

activated EGFR and binds to activated EGFR and Grb2 similarly to $p52^{shc}/p46^{shc}$.

The effects of $p66^{shc}$ and $p52^{shc}/p46^{shc}$ proteins on MAP kinase activation were investigated by co-transfecting the various Shc expression vectors and a reporter plasmid ($p44^{mapk}$) encoding an HA-epitope tagged p44 MAP kinase (Meloche *et al.*, 1992), in HeLa cells. As the epitope tag on MAP kinases allows the exogenous proteins to be distinguished immunologically from the endogenous MAP kinase, the transfected cells can be selectively monitored. The tagged MAP kinase was then immunoprecipitated with the anti-HA monoclonal antibody and assayed for activity using the myelin basic protein (MBP) or a synthetic peptide containing a MAP kinase phosphorylation site (PLS/TP) as substrates. The assay for MBP and the synthetic peptide were performed in the gel and in solution, respectively. Cells were transfected with $p44^{mapk}$ and the expression vector alone or expression vectors for $p52^{shc}/p46^{shc}$, wild-type $p66^{shc}$ or the $p66$ -TTGs mutant. Post-transient transfection expression, levels of $p52^{shc}/p46^{shc}$, wild-type $p66^{shc}$ and $p66$ -TTGs were similar and those of $p44^{mapk}$ equal in the various samples (see Figure 7B and D for a typical experiment). Cells were serum-starved for 24 h after transient transfection and then treated or not with 30 ng/ml EGF for 5 min. EGF treatment of cells transfected with the vector induced only modest MBP phosphorylation (Figure 7A) and an ~6-fold rise in phosphorylation of the synthetic peptide (Figure 7C). The expression of $p52^{shc}/p46^{shc}$ caused a marked increase in

MAP kinase phosphorylation and a 14- to 16-fold rise in the synthetic peptide, whereas stimulation with EGF in the presence of wild-type $p66^{shc}$ or the $p66$ -TTGs mutant had either no, or very little, effect on EGF-induced $p44^{mapk}$ activation.

Taken together, these findings suggest that $p66^{shc}$ binds activated receptors and Grb2 similarly to $p52^{shc}/p46^{shc}$, but is not able to transmit MAP kinase activation signals.

p66^{shc} negatively regulates c-fos promoter activity

The inability of $p66^{shc}$ to signal through the MAP kinase signalling pathway, leaves open the question of its physiological function. Since expression of $p52^{shc}/p46^{shc}$ is ubiquitous while $p66^{shc}$ levels differ in the various cell lines, we postulated that $p66^{shc}$ served a different regulatory role in signalling. This hypothesis was tested by determining the ability of $p52^{shc}/p46^{shc}$ and $p66^{shc}$ to induce transcription from the fos promoter. The reason for selecting this assay was that because fos activation occurs through different pathways (Treisman, 1995), it may detect interference of a given molecule in several signalling mechanisms. Cos-1 and HeLa cells were transiently transfected with various Shc expression vectors and a reporter plasmid where the luciferase gene is transcriptionally controlled by c-fos promoter sequences. Cells were then serum-starved and analysed before and after EGF stimulation. EGF stimulated luciferase activity to ~2-fold above basal values in both the Cos-1 (Figure 8A, left panel) and HeLa (Figure 8B, left panel) cells transfected with the vector alone or a vector with the $p52^{shc}/p46^{shc}$ cDNA cloned in the opposite transcriptional orientation. Transfection with the $p52^{shc}/p46^{shc}$ expression vector stimulated luciferase activity 3- to 4-fold that of control values, whereas with $p66^{shc}$ or the $p66$ -TTGs mutant luciferase values were considerably lower than those obtained with the vector alone, both before and after EGF stimulation (Figure 8A and B, left panels). Expression levels of the various constructs used in these experiments were similar (the results of a representative experiment are shown in Figure 8A and B, right panels). It, therefore, seems that $p66^{shc}$ exerts an inhibitory effect on c-fos promoter regulation.

The inhibitory effect of $p66^{shc}$ on c-fos promoter activation is mediated by its CH2 region

As the presence of the CH2 region is the only feature that distinguishes $p66^{shc}$ from $p52^{shc}/p46^{shc}$, we wondered whether the isolated CH2 domain would manifest the same properties as $p66^{shc}$ in the functional assays just described (MAP kinase and fos promoter activation by EGF). The CH2 coding region was isolated from the $p66^{shc}$ cDNA and cloned into the pMT2 expression vector. Figure 9D shows the transient expression of CH2 and compares it with that of $p52^{shc}/p46^{shc}$. Upon EGF stimulation, CH2 overexpression had no effect on the phosphorylation of the MBP or the synthetic peptide by MAP kinases (Figure 9A and B), while it displayed the same inhibitory effect on fos promoter expression as the $p66^{shc}$, both on unstimulated and EGF-stimulated promoter activity. Overexpression of the homologous CH1 region had no inhibitory effect on fos promoter activity (not shown). It appears, therefore, that the inhibitory effect of $p66^{shc}$ on fos activation is mediated by its CH2 domain.

Fig. 7. Effects of overexpressed Shc proteins on EGF-induced MAP kinase activation. HeLa cells were transfected with 0.5 μ g of HA-tagged p44^{mapk} expression vector and 5 μ g of pMT2-GF11(p52/p46), pMT2-K9 (p66-wt), pMT2-K9-TTGs (p66-TTGs) or the empty pMT2 expression vector. 48 h later, cells were treated (+) or not (-) with 30 ng/ml EGF for 5 min. Anti-HA (α HA1) immunoprecipitates were evaluated for their ability to phosphorylate either the MBP substrate directly on agarose gels (A) or a synthetic peptide containing a MAP kinase phosphorylation site in an immune complex kinase assay (C), as described in Materials and methods. The same anti-HA immunoprecipitates were also resolved by SDS-PAGE and blotted with an anti-Erk1 (α Erk1) polyclonal antibody (B). The in-gel kinase assay shown in (A) is representative of three experiments that gave similar results. Data in (C) represent the mean of three independent experiments performed in triplicate and are expressed as fold increase of MAP kinase activation with respect to the vector-transfected cells (solid bars, EGF-stimulated samples; open bars, unstimulated samples). The blots in (A) and (B) derive from two aliquots of the same anti-HA immunoprecipitates. Cell lysates from the same experiment shown in (A) and (B) were blotted with anti-Shc (α SH2) antibodies (D). p44 MAP kinase polypeptides, p66^{shc}, p52^{shc} and p46^{shc} are indicated by arrows.

Discussion

This paper reports the sequence and the structural organization of the human and mouse p66^{shc} and presents an initial description of its biochemical and functional properties. Since p66^{shc}, except for its unique N-terminal region, completely overlaps with p52^{shc}/p46^{shc}, it contains three functional regions in common with p52^{shc}/p46^{shc}: an SH2 carboxy-terminal region; an adjacent region rich in glycine and proline (CH1 region) which includes the Grb2 interaction site; and the SH2-like amino-terminal region (PTB). The unique amino-terminal portion of p66^{shc} also contains a collagen-homologous region rich in glycine and proline (CH2 region) (see Figure 1A).

p66^{shc} and p52^{shc}/p46^{shc} are encoded by two distinct transcripts that derive from the same locus but differ in the use of alternative 5' coding exons, or parts of exons. p66^{shc} is formed following the assembly of exons 2–13 while p52^{shc}/p46^{shc} originates from the assembly of exon 1 with the 3' portion of exon 2 (exon 2a), and then with exons 3–13. The exon 1–2a assemblage is made possible by the presence of a retained-intron corresponding to the 5' region of exon 2. There is a consensus splicing acceptor in front of exon 2a in both the human and murine Shc loci. Another mechanism that regulates expression of the three Shc isoforms is the alternative usage of in-frame ATGs. The transcript encoding p66^{shc} has three in-frame ATGs that are responsible for the translation of p66^{shc},

p52^{shc} and, to a lesser extent, p46^{shc}. The p52^{shc}/p46^{shc} transcript has two in-frame ATGs that are responsible for the translation of the p52^{shc} and p46^{shc}.

Nothing is known about the mechanisms that regulate the expression of the three Shc isoforms *in vivo*. Different regulatory mechanisms could control the expression of the two main Shc transcripts in different cell types, since p52^{shc}/p46^{shc} are found in every cell type with invariant reciprocal relationship, whereas p66^{shc} expression varies from cell type to cell type and is lacking in some cells. It is not, for example, expressed in haemopoietic cells (Pelicci *et al.*, 1992).

An important distinguishing feature is that, unlike p52^{shc}/p46^{shc}, p66^{shc} is unable to transform NIH-3T3 fibroblasts. The capacity of p52^{shc}/p46^{shc} to transform fibroblasts depends on the integrity of its Grb2 site and, by inference, the capacity to stimulate the Ras/MAP kinase signalling pathway (Salcini *et al.*, 1994). The present data demonstrate that overexpression of p52^{shc}/p46^{shc} enhances EGF-induced MAP kinase activation, whereas p66^{shc} overexpression has no effect. The inability of p66^{shc} to increase MAP kinase activation does not appear to be the consequence of a defect in its capacity to become phosphorylated following EGF stimulation, or to bind to activated EGFR or form stable complexes with Grb2. Activation of Ras in mammalian cells is thought to depend on the relocation of the Grb2–SOS complex to the plasma membrane (Buday and Downward, 1993; Aronheim *et al.*,

Fig. 8. Effects of overexpressed Shc proteins on c-fos promoter activity. Cos-1 (A, left panel) or HeLa (B, left panel) cells were co-transfected with 0.5 μ g of the pADneo-fosluc reporter plasmid and 5 μ g of the indicated Shc expression vector and treated (solid bars) or not (open bars) with 100 ng/ml EGF. Each point is the average of three independent experiments performed in triplicate, and standard deviations of results are given. Right panels show Western blot analysis of Shc protein expression in transfected Cos-1 and HeLa cells. p52/p46-as represents the p52^{shc}/p46^{shc} encoding cDNA cloned into the pMT2 expression vector in the opposite transcriptional orientation.

1994; Quilliam *et al.*, 1994). However, other mechanisms could be involved in the Grb2–SOS regulation of Ras. It has, for example, been shown that phosphorylation of SOS upon growth factor stimulation decreases binding of the Grb2–SOS complex to Shc and EGFR in mammalian cells (Cherniak *et al.*, 1994; Rozakis-Adcock *et al.*, 1995) and there is evidence for Grb2-independent SOS activation in *Drosophila* (Karlovich *et al.*, 1995; McCollam *et al.*, 1995). Despite the capacity of p66^{shc} to bind Grb2 efficiently, it could interfere with the function of the recruited p66^{shc}–Grb2–SOS complex by, for example, influencing the affinity of Grb2–SOS binding or the state of SOS phosphorylation. All these possibilities are the subject of ongoing experiments.

Regardless of the mechanism implicated, the fact that p66^{shc} does not stimulate the MAP kinase signalling pathway could explain why it fails to transform rodent fibroblasts, as do p52^{shc}/p46^{shc}, but provides no information on the physiological function of p66^{shc}. Overexpression of p66^{shc} inhibits fos promoter activity and this activity

contrasts with that of p52^{shc}/p46^{shc}, which induces an increase in EGF-stimulated fos promoter activation. Instead, p66^{shc} slightly inhibits the basal fos promoter activity and completely abolishes its activation by EGF. Therefore, p66^{shc} could, unlike p52^{shc}/p46^{shc}, exert an antagonistic effect on fos activation, and be part of a complex inhibitory–stimulatory network that converges on growth factor-regulated genes, like fos. This positive–negative signal balance of Shc proteins might be regulated by the growth factor-induced tyrosine phosphorylation of p52^{shc}/p46^{shc} and by the intracellular levels of p66^{shc} expression.

No information is available on the mechanism through which p66^{shc} negatively influences fos promoter activity. The involvement of Shc in the Ras–MAP kinase–fos signalling pathway depends on the integrity of the Grb2 interaction site, which is situated within the CH1 region (Salcini *et al.*, 1994; Chen *et al.*, 1996). The fact that overexpression of p66^{shc} as well as the CH2 region itself have the same regulatory effect on the fos promoter seems to indicate that p66^{shc} acts on fos through Ras–MAP kinase-independent pathways. MAP kinase activates fos by phosphorylation of the TCF–SRF complex, which binds the serum response element (SRE) in the fos promoter. An additional signalling pathway that is triggered by external signals (interferon, cytokines) and converges to the fos promoter is the STAT signalling pathway, that binds the sis-inducible element (SIE). Other less-characterized fos promoter regulatory regions are a binding site for an uncharacterized AP1/ATF family member and sequences in the vicinity of the TATA box which bind poorly characterized cellular factors, some of which are also responsive to extracellular signalling pathways (Cano and Mahadevan, 1995; Treisman, 1995; Hill and Treisman, 1995).

In vitro binding experiments using the GST–CH2 fusion protein demonstrates that the CH2 region behaves as a protein–protein interaction interface (E.Migliaccio and P.G.Pellicci, unpublished results). This functional activity could underlie the mechanism through which the CH2 domain may interfere with the fos promoter activity. The CH2 binding activity appears to be specific, in that it binds a subset of cellular proteins distinct from those bound by the homologous CH1 region (E.Migliaccio and P.G.Pellicci, unpublished results). As there are three putative SH3 binding regions (PXXP motif; Yu *et al.*, 1994) in the human and mouse CH2 regions, some of the cellular proteins that bind CH2 could be SH3-containing proteins. We found that, *in vitro*, the p66^{shc} CH2 region binds SH3 domains (from PLC γ and p120^{GAP}) and that, *in vivo*, p66^{shc} forms stable complexes with PLC γ and p120^{GAP} (E.Migliaccio and P.G.Pellicci, unpublished results).

To summarize, the functional characteristics of the p66^{shc} isoform are apparently diverse from those of p52^{shc}/p46^{shc}, in that their expression and both biochemical and biological properties differ. Remarkably, p66^{shc} and p52^{shc}/p46^{shc} appear to exert opposite effects on the regulation of the fos promoter. The physiological relevance of this alternative fos promoter regulation, and of other genes of the immediate-early class, must now be defined.

Fig. 9. Effect of expression of the isolated p66^{shc} CH2 region on MAP kinase activation and c-fos promoter activity. HeLa cells were transfected with 0.5 µg of HA-tagged p44^{mapk} expression vector and 5 µg of pMT2-GF11(p52/p46), pMT2-CH2 (pCH2) or the empty pMT2 expression vector and subsequently treated (+) or not (-) with EGF. MAP kinase activity was evaluated as described in the legend to Figure 7 by using the MBP substrate in an in-gel kinase assay (A) or a synthetic peptide in an immune complex kinase assay (B). The in-gel kinase assay shown in (A) is representative of three experiments that gave similar results. Data in (B) are the mean of three independent experiments performed in triplicate and are expressed as fold increase of MAP kinase activation with respect to the vector-transfected cells (solid bars: EGF-stimulated samples; open bars: unstimulated samples). (C) HeLa cells were co-transfected with 0.5 µg of the pADneo-fosluc reporter plasmid and 5 µg of the indicated Shc expression vector and subsequently treated (solid bars) or not (open bars) with 100 ng/ml EGF. Each point is the average of three independent experiments performed in triplicate, and standard deviation of results is given. (D) Anti-Shc Western blotting of cell lysates from HeLa cells transiently transfected with the p52^{shc}/p46^{shc} (αSH2 antibody) or p66^{shc} CH2 region (αCH2 antibody) expression vectors.

Materials and methods

Isolation of human p66^{shc} cDNAs

Human p66^{shc} cDNAs were isolated from a cDNA library prepared from Calu1 cells. Double-stranded (ds) cDNA was synthesized from poly(A)⁺ RNA isolated from the Calu1 cells and cloned into the λgt11 vector using a commercial kit (Invitrogen Librarian TMXI cDNA Library Construction System; Invitrogen, San Diego, CA). The cDNA library was screened with a DNA probe representative of the human Shc SH2 domain (the region of the SHC GF11 cDNA encoding amino acids 366–473, isolated using PCR and cloned into the PCRII plasmid). Mouse p66 cDNA clones were isolated from an NIH-3T3 cDNA library using the human Shc SH2 DNA probe. Hybridization was performed at 37°C in 50% formamide, 3× SSC, 5× Denhardt's, 10% dextran sulfate containing 100 µg/ml sonicated and denatured salmon sperm DNA. The final wash was 0.2× SSC, 0.1% SDS at 60°C. Isolated plaques were grown and phage DNA analysed by restriction enzyme mapping according to established procedures (Sambrook *et al.*, 1989). Inserts were subcloned in plasmid vector pGEM3 for further analysis. DNA sequence analysis was performed on subclones using the dideoxy technique with different polymerases (Klenow, Sequenase, *Taq* polymerase).

Isolation of human and mouse Shc genomic clones

A commercially available human genomic library from the WI-38 lung cell line (Stratagene, La Jolla, CA, USA) and a mouse placenta genomic library (gift from R.Lowell-Badge), both cloned in the Lambda FIXII vector (Stratagene, La Jolla, CA, USA), were screened with the Shc SH2 DNA probe described above. Several overlapping human and mouse clones were analysed by restriction enzyme mapping. Exons were located by subsequent hybridization to different DNA probes derived from the λGF11 and λK9 cDNAs. *Xba*I fragments from the phage inserts were

cloned in pGEM-3 vector and used for subsequent subcloning. DNA sequencing analysis was performed along the exons, at the exon/intron boundaries and at the 5' and 3' flanking regions of the human and mouse loci.

RNAse protection experiments

Total RNA from different cell lines was prepared according to established procedures (Sambrook *et al.*, 1989). ³²P-labelled sense and anti-sense transcripts from the resulting subclones were obtained using an *in vitro* transcription commercial kit (Promega Corporation, Madison, WI) according to manufacturer's instructions. The labelled RNA probes were hybridized to 20 µg of total RNA at 56°C. RNase digestion and electrophoresis on 8% 7M urea-polyacrylamide gels were carried out as previously described (Melton *et al.*, 1984).

Plasmids

The K9 plasmid was obtained by cloning the insert of the λK9 phage into the *Eco*RI site of the pGEM-3 vector (Promega, Madison, USA). The R2 and R1 probes were obtained by subcloning the *Apa*I–*Ava*I 240 bp and the *Eco*RI–*Pvu*II 454 bp fragments from K9 (nucleotide positions 321–560 and 1–454, respectively) into the pGEM-3 vector. The plasmids encoding p52^{shc}/p46^{shc} (GF11), p52^{shc} (GF11TTG) and p46^{shc} (GF13) have been described previously (Pellicci *et al.*, 1992). The K9TTGs clone was generated by changing the two in-frame ATG codons (Met111 and Met156) of the wild-type p66^{shc} cDNA (K9) into a TTG codon by PCR-based oligonucleotide-directed mutagenesis. The K9, K9TTGs, GF11, GF13 and GF11TTG were also cloned into the LXSN expression vector. The K9 cDNA, K9 TTGs cDNA, GF11 cDNA, GF11-antisense and CH2 region of p66^{shc} cDNA encoding amino acids 1–110 were subcloned into the pMT2 vector for transient expression into COS-1 and HeLa cells. The plasmid p44^{mapk} encodes an epitope-tagged p44 MAP kinase (Meloche *et al.*, 1992) (the epitope tag corresponds to

a sequence from the influenza haemagglutinin HA1 protein). The pADneo-fosluciferase containing the entire c-fos promoter region clones upstream of the *Photinus pyralis* luciferase gene was kindly provided by Dr E. Van Obberghen-Schilling.

Expression of Shc cDNAs

The K9 cDNA, K9 TTGs cDNA, GF11 cDNA, GF11-antisense and CH2 region of p66^{shc} cDNA encoding amino acids 1–110 were subcloned into the pMT2 vector and transiently expressed in COS-1 cells lipofectin (BRL) and in HeLa cells by using the calcium phosphate co-precipitation protocol. The empty LXSN expression vector and LXSN subclones of the K9, K9TTGs, GF11, GF13, GF11TTGs cDNAs were transfected into the PA317 retrovirus packaging cell line and after 48 h the PA317 supernatants were used to infect NIH-3T3 fibroblasts (Miller and Rosman, 1989). The cells were then selected with G418-containing medium.

Antibodies

A region of the p66^{shc} cDNA encoding amino acids 1–110 (CH2 region) was isolated using PCR and cloned into the *Bam*HI-*Eco*RI sites of the bacterial expression plasmid pGEX-2T. GST-CH2 fusion protein was purified as previously described (Pellicci *et al.*, 1992). A polyclonal anti-p66^{shc} antibody was prepared by immunizing New Zealand White rabbits with purified GST-CH2 polypeptide. The Shc anti CH2 and anti-SH2 antiserum were used at 1:5000 dilution for immunoblot analysis and 1:500 in immunoprecipitations. Monoclonal antibodies to phosphotyrosine were purchased from Upstate Biotechnology Inc. and used at 1 µl/ml for Western blot analysis. Polyclonal anti-human-Grb2 was purchased from Santa Cruz Biotechnology and used for Western blot at 1:1000. 12CA5 monoclonal antibody directed against a sequence of influenza haemagglutinin HA1 protein was provided by Dr E. Van Obberghen-Schilling. Polyclonal antibody anti-ERK 1 (p44^{mapk}) protein was purchased from Santa Cruz Biotechnology and used for Western blot at 0.5 µg/ml.

Immunoprecipitations and Western blotting procedures

For whole-cell lysates, cells were directly lysed in SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS v/v, 10% glycerol and 5% v/v β-mercaptoethanol) and boiled for 5 min. 50 µg of total protein was analysed by SDS-PAGE. For immunoprecipitation, cells were lysed on ice in PY buffer (20 mM Tris-HCl, pH 7.8, 50 mM NaF, 50 mM NaCl, 30 mM Na₄P₂O₇, 5 mM sodium orthovanadate, 1% v/v Triton X-100) containing freshly added protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin and 5 mg/ml aprotinin). Lysates were clarified by centrifugation at 4°C and protein concentration determined by BCA reagent (Pierce). Appropriate antibodies were adsorbed on protein A-Sepharose (Pharmacia) and then incubated with cell lysates for 2 h at 4°C. Immunoprecipitates were washed three times with ice-cold NET buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% NP-40, 1 mM EDTA, 0.25% gelatine), eluted and denatured by heating for 3 min at 95°C in reducing Laemmli buffer. Proteins were resolved by 10% SDS-PAGE and transferred to nitrocellulose filters. Blots were blocked and probed with specific antibodies. After extensive washing, immune complexes were detected with horseradish peroxidase conjugated with specific secondary antiserum (Bio-Rad) followed by enhanced chemiluminescence reaction or developed with alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (Promega).

Transient transfection assays

Luciferase assay. Cos-1 and HeLa cells were grown in six-well culture plates and co-transfected with 5 µg of the various Shc expression vectors, 0.5 µg of the pADneo-fosluciferase reporter plasmid and 0.5 µg of the pCH110 β-galactosidase reporter plasmid (Pharmacia) by lipofectamine (Gibco-BRL) for Cos-1 cells and the calcium phosphate co-precipitation for HeLa cells. After 16 h, transfected cells were rinsed twice with PBS and placed in serum-free DMEM. 24 h later, cells were treated with 100 ng/ml EGF or not for an additional 8 h. Luciferase activity was determined in cell lysates of transfected cells according to the manufacturer's recommendations (Promega Biotech).

MAP kinase activation assays. HeLa cells were grown in six-well culture plates, co-transfected with 5 µg of the various Shc expression vectors, 0.5 µg of the p44^{mapk} and 0.5 µg of the pCH110 plasmid, serum deprived, as described above, and stimulated or not with 30 ng/ml EGF for 5 min. MAP kinase activity was determined by immune complex kinase assays, according to established procedures. Briefly, anti-HA immunoprecipitates from lysates of transfected cells were tested for their capacity to phosphorylate a synthetic peptide containing a MAP kinase phosphorylation site (Biotrak kit; Amersham Life Science) or the MBP in an in-

gel kinase assay. Transfection efficiency was monitored by measuring β-galactosidase activity.

Soft agar colonization

Cells were trypsinized and washed in Ca²⁺-, Mg²⁺-free, phosphate-buffered saline (PBS), and three scalar concentration of cells (1×10³, 1×10⁴ and 1×10⁵) were plated in 1 ml of DMEM medium containing 20% FCS, 100 mg/ml gentamicin and 0.3% (w/v) Noble agar, over 2 ml of DMEM medium with 0.6% agar in six-well plates.

Accession number

Nucleotide sequence for the K9 cDNA (p66shc) has been submitted to DDBJ/EMBL/GenBank, and can be retrieved under accession number U73377.

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