Opposite effects of the p52shc/p46shc and p66shc 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 Pawson2, Pier Paolo Di Fiore1,5, Luisa Lanfrancone1 and Pier Giuseppe Pelicci1,2,6

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 p46shc and p52shc 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, p66shc. The predicted amino acid sequence of p66shc overlaps that of p52shc and contains a unique N-terminal region which is also rich in glycines and prolines (CH2). p52shc/p46shc is found in every cell type with invariant reciprocal relationship, whereas p66shc expression varies from cell type to cell type. p66shc differs from p52shc/p46shc in its inability to transform mouse fibroblasts in vitro. Like p52shc/p46shc, p66shc is tyrosine-phosphorylated upon epidermal growth factor (EGF) stimulation, binds to activated EGF receptors (EGFRs) and forms stable complexes with Grb2. However, unlike p52shc/p46shc it does not increase EGF activation of MAP kinases, but inhibits fos promoter activation. The isolated CH2 domain retains the inhibitory effect of p66shc on the fos promoter. p52shc/p46shc and p66shc, therefore, appear to exert different effects on the EGFR–MAP kinase and other signalling pathways that control fos promoter activity. Regulation of p66shc expression might, therefore, influence the cellular response to growth factors.

Keywords: fos/p52shc/p46shc/p66shc/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; Baldrari et al., 1995; Lanfrancone et al., 1995; Matsuguichi et al., 1994). Upon phosphorylation, Shc proteins form stable complexes with cellular tyrosine-phosphorylated polypeptides, including RTKs and receptors devoted 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; Blakie 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., 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., 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., 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., 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., 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).
of PC12 cells by Shc overexpression is prevented by the co-expression of a Ras dominant negative mutant (Rozakis-Adock et al., 1992). We report here the structural characterization and preliminary functional properties of p66shc.

Results

Isolation and sequence analysis of the λK9 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 (λK9) was longer than the λGF11 clone which encodes p52shc/p46shc, and differed from λGF11 (Pelicci et al., 1992) in its 5' extremity at restriction mapping analysis (Figure 1A). The λK9 insert was subcloned into the pGEM-3 vector (pK9 plasmid) and sequenced. Analysis of the λK9 DNA sequence and comparison with that of λGF11 revealed that the two clones were identical from nucleotide position 525 of λK9 and nucleotide position 76 of λGF11 to their ends. The sequences of the 5’ extremity of the λK9 clone, including its unique 524 bp region, is shown in Figure 1B.

The longest open reading frame (ORF) predicted from clone λK9 is 1749 bp with the first in-frame ATG at position 195 (ATG1 in Figure 1B) and an in-frame TGA codon at position 1944 (not shown). ATG1 is probably the translation initiation codon of λK9, since it is flanked by sequences 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 λK9 clone sequence is 583 amino acids in length, and has a molecular mass of 62 898 Da. Comparison between the λK9 and the λGF11 (p52shc/p46shc) proteins disclosed an amino-terminal region of 110 amino acids unique to λK9, which continued into a common region starting at residue 1 of λGF11 and residue 111 of λK9, and terminated at the ends of both proteins. The first amino acid common to both proteins is the Met translation initiation codon of p52shc (see the partial amino acid sequence of λK9 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 λK9 identified human α1 collagen (20% amino acid identity and 33% similarity; Figure 1C). This collagen-homologous λK9 region was rich in glycine (24%) and proline (37%) residues, both of which are also abundant in the collagen α1 chain (57% and 46% in the λK9-homologous collagen α1 chain, respectively). Some 31% of glycine and 50% of proline α1 collagen residues are conserved in the λK9 collagen-homologous region. Because the p52shc/p46shc protein contains a glycine/proline-rich region that is also partially homologous with the collagen α1 chain (Figure 1A), we renamed it CH1 and called the λK9 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 α1 chain which overlap for 34 amino acids (bold sequence in Figure 1C).
E. Migliaccio et al.

Fig. 3. Western blotting analysis of p66\textsuperscript{shc}, p52\textsuperscript{shc} and p46\textsuperscript{shc} expression. Whole-cell lysates were prepared from the indicated cell lines and 50 μg 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 (αSH2 and αCH2). The SHC proteins (p66, p52 and p46) are indicated on the left.

**Identification of the λK9 cDNA clone as the p66\textsuperscript{shc} cDNA**

To ascertain whether the λK9 clone is, indeed, representative of the transcript encoding p66, the expression of λK9 corresponding mRNA was determined by RNase protection experiments and compared with p66\textsuperscript{shc} expression. A 498 bp RNA probe containing a 454 bp region unique to λK9 (probe R1, Figure 2A) gave a strong 454 bp protection fragment in murine NIH-3T3 cells stably transfected with a λK9 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 (K562, GTL16, KG1) were not protected by the R1 probe (Figures 2B and 3). Similar results were obtained with a second λK9 RNA probe that spans the junction between the unique λK9 sequences and the sequences common to λK9 and λGF11 (probe R2, Figure 2A). The 281 bp R2 probe yielded a 240 bp fully protected fragment in cells expressing p66\textsuperscript{shc} (NIH-p66 fibroblasts and A-172 cells), a 40 bp protection fragment in p52\textsuperscript{shc}/p46\textsuperscript{shc} expressing cells [NIH-3T3 transfected with the λGF11 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\textsuperscript{shc}/p46\textsuperscript{shc} transcript (Figure 2B, right panel).

We next generated a polyclonal antibody against the CH2 portion of λK9 (αCH2, see Materials and methods). A bacterial glutathione S-transferase (GST)-CH2 fusion protein was produced by inserting the sequence of the λK9 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 λK9 cDNA products that had been translated \textit{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 λK9 cDNA were recognized by the anti-SH2 Shc antibody both \textit{in vitro} and \textit{in vivo} (not shown). Taken together, these data indicate that the λK9 clone encodes for the p66\textsuperscript{shc} isoform.

**Origin of the p66 cDNA**

The origin of the human p66\textsuperscript{shc} transcript was investigated by isolating the human Shc locus and mapping the various Shc exons. A genomic library from normal human embryonic 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 EcoRI, XbaI and BamHI restriction enzymes and hybridized to different portions of the Shc cDNA. The hybridizing restriction fragments were subcloned into the...
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 p52shc/p46shc transcripts (λGF11 cDNA from nucleotides 1–75). The 5' 521 bp of exon 2 contain sequences corresponding to the CH2 p66shc region and includes both the 5' untranslated region and the p66shc ATG1. The 3' 168 bp of exon 2 contain sequences corresponding to the 56 amino acids common to the p66shc and p52shc/p46shc isoforms (including ATG2 and ATG3) (exon 2a). Exons 3 to 13 encode the remaining p66shc and p52shc/p46shc common sequences. It appears, therefore, that only the 168 bp 3' portion of exon 2 (exon 2a) is incorporated within the mature transcript encoding p52shc/p46shc, as found in the λGF11 cDNA clone. An acceptor consensus site for splicing was found 5' to the Shc exon 2a (Figure 4B), suggesting that the λGF11 transcript is formed by the juxtaposition of exons 1 and 2a. In contrast, transcripts encoding p66shc would incorporate the entire exon 2.

A similar Shc genomic organization and Shc transcript assembly was found in mice. Alignment of the predicted mouse p66shc sequence, as derived from analysis of mouse p66 cDNAs (not shown), with human p66shc 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 p52shc/p46shc unique sequences, the 5' end of exon 2 encodes p66shc 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 p66shc cDNA does not induce transformation of NIH-3T3 fibroblasts**

Overexpression of the p52shc/p46shc 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 p66shc and the p52shc/p46shc isoforms was compared by expressing the p66shc and p52shc/p46shc 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 p66shc cDNA into NIH-3T3 fibroblasts yielded a marked increase in the level of p66shc and p52shc and, to a lesser extent, p46shc (NIH-p66 clone; Figure 5A); all three isoforms are presumably encoded by the p66shc cDNA. Indeed, the protein predicted by the translation of the p66shc cDNA contains three in-frame ATGs, the second site for splicing was found 5' to the Shc exon 2a (Figure 4B), suggesting that the λGF11 transcript is formed by the juxtaposition of exons 1 and 2a. In contrast, transcripts encoding p66shc would incorporate the entire exon 2.

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 p66shc and p52shc/p46shc 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.
lathed from the same transcript by alternative usage of two in-frame ATGs. In vitro translation of the λK9 (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 λK9 (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 λK9 cDNA encode all three Shc isoforms.

To test for transformation capability, NIH-3T3 clones overexpressing the wild-type p52hc/p46hc (NIH-SHC-9 and NIH-SHC-13), the wild-type p66hc (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 p66hc isoform does not retain the transforming activity of the p52hc/p46hc isoforms.

p66hc 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 p66hc, its ability to mimic the effects of p52hc/p46hc on known signal transduction mechanisms was investigated by comparing the capacity of p66hc and p52hc/p46hc to bind activated EGFR, to be phosphorylated upon EGF stimulation, to bind Grb2 and activate MAP kinases.

The p66hc (K9TGGs cDNA) and p52hc/p46hc (GF11 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 in Western blot analysis of Shc-overexpressing clones. Lysates from one control NIH-3T3 clones (NIH-SN) and clones overexpressing p52hc/p46hc (NIH-SHC-13 and NIH-SHC-9), p66hc/p52hc (NIH-p66-8), p66hc (NIH-p66-TTGs5 and NIH-p66-TTGs7) were blotted against the anti-SH2 Shc antibody (αSH2).

![Fig. 5. Potential of isoform translation of the λGF11 and λK9 cDNAs. The following cDNAs were expressed into NIH-3T3 cells (A and B): GF11, encoding p52hc/p46hc (Pelicci et al., 1992); K9, encoding p66hc; 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 LXS 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 p52hc/p46hc (NIH-SHC-13 and NIH-SHC-9), p66hc/p52hc (NIH-p66-8), p66hc (NIH-p66-TTGs5 and NIH-p66-TTGs7) were blotted against the anti-SH2 Shc antibody (αSH2).

<table>
<thead>
<tr>
<th>Cells</th>
<th>Cloning efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIH-SN</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>NIH-SN-5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>NIH-SHC-13</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>NIH-SHC-9</td>
<td>&gt;3</td>
</tr>
<tr>
<td>NIH-p66-2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>NIH-p66-8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>NIH-p66-TTGs5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>NIH-p66TGGs7</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*Cloning efficiency was monitored in control NIH-3T3 clones (NIH-SN-1 and NIH-SN-5) and NIH-3T3 clones expressing p52hc/p46hc (NIH-SHC-9 and NIH-SHC-13), wild-type p66hc (NIH-p66-2 and NIH-p66-8) and mutant p66TGGs (NIH-p66-TTGs5 and NIH-p66-TTGs7) cDNAs. Colonies were examined 14 days after cells were seeded in triplicate at 10^3, 10^4 and 10^5 cells per culture dish. Each result is the average of five separate experiments.
The EGFR, p66shc, p52shc and p46shc polypeptides and the presence of the CH2 region is the only feature that distinguishes p66shc from p52shc/p46shc, whereas with p66shc or the p66-TTGs mutant luciferase values were considerably lower than those obtained with activated EGFR and binds to activated EGFR and Grb2 similarly to p52shc/p46shc.

The effects of p66shc and p52shc/p46shc proteins on MAP kinase activation were investigated by co-transfecting the various Shc expression vectors and a reporter plasmid (p44mapk) encoding an HA-epitope tagged p44 MAP kinase (Meloch 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 p44mapk and the expression vector alone or expression vectors for p52shc/p46shc, wild-type p66shc or the p66-TTGs mutant. Post-transfection expression, levels of p52shc/p46shc, wild-type p66shc and p66-TTGs were similar and those of p44mapk 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 increase in phosphorylation of the synthetic peptide (Figure 7C). The expression of p52shc/p46shc 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 p66shc or the p66-TTGs mutant had either no, or very little, effect on EGF-induced p44mapk activation.

Taken together, these findings suggest that p66shc binds activated receptors and Grb2 similarly to p52shc/p46shc, but is not able to transmit MAP kinase activation signals.

**p66shc negatively regulates c-fos promoter activity**

The inability of p66shc to signal through the MAP kinase signalling pathway, leaves open the question of its physiological function. Since expression of p52shc/p46shc is ubiquitous while p66shc levels differ in the various cell lines, we postulated that p66shc served a different regulatory role in signalling. This hypothesis was tested by determining the ability of p52shc/p46shc and p66shc to induce transcription of 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 p52shc/p46shc cDNA cloned in the opposite transcriptional orientation. Transfection with the p52shc/p46shc expression vector stimulated luciferase activity 3- to 4-fold that of control values, whereas with p66shc 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 p66shc exerts an inhibitory effect on c-fos promoter regulation.

**The inhibitory effect of p66shc on c-fos promoter activation is mediated by its CH2 region**

As the presence of the CH2 region is the only feature that distinguishes p66shc from p52shc/p46shc, we wondered whether the isolated CH2 domain would manifest the same properties as p66shc in the functional assays just described (MAP kinase and fos promoter activation by EGF). The CH2 coding region was isolated from the p66shc cDNA and cloned into the pMT2 expression vector. Figure 9D shows the transient expression of CH2 and compares it with that of p52shc/p46shc. 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 p66shc, 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 p66shc on fos activation is mediated by its CH2 domain.
Discusstion

This paper reports the sequence and the structural organization of the human and mouse p66shc and presents an initial description of its biochemical and functional properties. Since p66shc, except for its unique N-terminal region, completely overlaps with p52shc/p46shc, it contains three functional regions in common with p52shc/p46shc: 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 p66shc also contains a collagen-homologous region rich in glycine and proline (CH2 region) (see Figure 1A).

p66shc and p52shc/p46shc 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. p66shc is formed following the assembly of exons 2–13 while p52shc/p46shc 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 p66shc has three in-frame ATGs that are responsible for the translation of p66shc, p52shc and, to a lesser extent, p46shc. The p52shc/p46shc transcript has two in-frame ATGs that are responsible for the translation of the p52shc and p46shc.

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 p52shc/p46shc are found in every cell type with invariant reciprocal relationship, whereas p66shc expression varies from cell type to cell type and is lacking in some cells. It is not, for example, expressed in haemopoietic cells (Pellicci et al., 1992).

An important distinguishing feature is that, unlike p52shc/p46shc, p66shc is unable to transform NIH-3T3 fibroblasts. The capacity of p52shc/p46shc 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 p52shc/p46shc enhances EGF-induced MAP kinase activation, whereas p66shc overexpression has no effect. The inability of p66shc 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., 1992).
shc and Fos activation

contrasts with that of p52shc/p46shc, which induces an increase in EGF-stimulated fos promoter activation. Instead, p66shc slightly inhibits the basal fos promoter activity and completely abolishes its activation by EGF. Therefore, p66shc could, unlike p52shc/p46shc, 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 p52shc/p46shc and by the intracellular levels of p66shc expression.

No information is available on the mechanism through which p66shc 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 p66shc as well as the CH2 region itself have the same regulatory effect on the fos promoter seems to indicate that p66shc 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.Pelicci, 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.Pelicci, 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 p66shc CH2 region binds SH3 domains (from PLCγ and p120GAP) and that, in vivo, p66shc forms stable complexes with PLCγ and p120GAP (E.Migliaccio and P.G.Pelicci, unpublished results).

To summarize, the functional characteristics of the p66shc isoform are apparently diverse from those of p52shc/p46shc, in that their expression and both biochemical and biological properties differ. Remarkably, p66shc and p52shc/p46shc 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 p66shc CH2 region on MAP kinase activation and c-fos promoter activity. HeLa cells were transfected with 0.5 μg of HA-tagged p44MAPK 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 p52shc/p46shc (αSH2 antibody) or p66shc CH2 region (αCH2 antibody) expression vectors.

Materials and methods

Isolation of human p66shc cDNAs

Human p66shc 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 plage 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. XbaI 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 antisense 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% TMA 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 EcoRI site of the pGEM-3 vector (Promega, Madison, USA). The R2 and R1 probes were obtained by subcloning the Apad–Aval 240 bp and the EcoRI–PvuII 454 bp fragments from K9 (nucleotide positions 321–560 and 1–454, respectively) into the pGEM-3 vector. The plasmids encoding p52shc/p46shc (GF11), p52shc (GF11TTG) and p46shc (GF13) have been described previously (Pelicci et al., 1992). The K9TTGs clone was generated by changing the two in-frame ATG codons (Met11 and Met156) of the wild-type p66shc cDNA (K9) into a TTG codon by PCR-based oligonucleotide-directed mutagenesis. The K9, K9TTGs, GF11, GF13 and GF11TTG were also cloned into the LXSφ expression vector. The p9 cDNA, p9 TTGs cDNA, GF11 cDNA, GF11-antisense and CH2 region of p66shc cDNA encoding amino acids 1–110 were subcloned into the pMT2 vector for transient expression into COS-1 and HeLa cells. The plasmid p44MAPK encodes an epitope-tagged p44 MAP kinase (Melobe et al., 1992) (the epitope tag corresponds to
a sequence from the influenza haemagglutinin HAI1 protein. The pADneo-fosloci containing the entire c-fos promoter region clones upstream of the Photinus pyralis luciferase gene was kindly provided by Dr E Van Obbergen-Schilling.

Expression of Shc cDNAs
The K9 cDNA, K9TTGs cDNA, GF11 cDNA, GF11-antisense and CH2 region of p66shc 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 Rossman, 1989). The cells were then selected with G418-containing medium.

Antibodies
A region of the p66shc cDNA encoding amino acids 1–110 (CH2 region) was isolated using PCR and cloned into the BamHI-EcoRI sites of the bacterial expression plasmid pGEX-2T. GST-CH2 fusion protein was purified as previously described (Pelici et al., 1992). A polyclonal anti-p66shc antibody was prepared by immunizing New Zealand White rabbits with purified p66shc CH2 polypeptide. The Shc anti CH2 and anti-SH2 antisera 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 HAI1 protein was provided by Dr E Van Obbergen-Schilling. Polyclonal antibody anti-ERK 1 (p44mapK) 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. 30 µg of total protein was analysed by SDS-PAGE. For immunoprecipitation, cells were lysed on ice in PV buffer (20 mM Tris–HCl, pH 7.8, 50 mM NaF, 50 mM NaCl, 30 mM Na3PO4, 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 absorbed 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 antisera (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 pDneo-fosloci reporter plasmid and 0.5 µg of the pCH110 β-galactosidase reporter plasmid (Pharmacia) by lipofection (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 p44mapK 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.

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

Acknowledgements
We thank Dr A Romano, G Scita and R Chiarini for their many helpful discussions and advice. This work was supported by grants from HSPO and AIRC.

References
Crowe, A., McGlade, J., Pawson, T. and Hayman, M.J. (1994) Phosphorylation of the SHC proteins on tyrosine correlates with the
E.Migliaccio et al.

transformation of fibroblasts and erythroblasts by the v-sea tyrosine kinase. Oncogene, 9, 537–544.


Received on August 14, 1996; revised on October 18, 1996.