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A novel pathway from phosphorylation of tyrosine residues 239/240 of Shc, contributing to suppress apoptosis by IL-3

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Interleukin 3 (IL-3) not only induces DNA synthesis of haematopoietic cells but also maintains their viability by suppressing apoptosis. IL-3 stimulates tyrosine phosphorylation of the Shc adaptor protein and thereby formation of a complex of Shc with Grb2 at phosphorylated tyrosine (Y) residue 317-Shc. This pathway is implicated in Ras/mitogen-activated protein kinase (MAPK) activation towards c-*fos* gene expression. We examined the possible involvement of Shc in the anti-apoptotic activity of IL-3. Conditional overexpression of the Shc SH2 domain, a dominant-negative mutant of Shc, was found to induce apoptosis of IL-3-dependent Ba/F3 cells along with a reduction of c-*myc* gene expression. Apoptosis was rescued by the exogenously introduced c-*myc* gene. Since we identify novel tyrosine phosphorylation sites of Shc: Y239 and Y240, their role on cell survival was tested by mutational analysis. Ba/F3 cells expressing mutant Shc Y317F, which is unable to stimulate efficiently the Ras pathway, still showed resistance to apoptosis. However, cells expressing Shc Y239/240F, which is able to stimulate the Ras pathway, were sensitive to apoptosis. In these cells, induction of the c-*myc* gene was reduced. These findings suggest that a new signalling pathway for cell survival is generated from Y239/240 of Shc to the nuclei involving c-*myc* gene expression.

Keywords: apoptosis/IL-3/Myc/Shc

Introduction

Cytokines, including interleukin-3 (IL-3), regulate several aspects of haematopoiesis and immunity (Metcalf, 1989; Paul and Seder, 1994). They play a central role in the control of survival, differentiation, proliferation and development of haematopoietic cells. Normal haematopoietic cells or even some leukaemic cells rapidly die in the absence of appropriate cytokines, indicating that the maintenance of cell viability is an important function of cytokines (Sachs and Lotem, 1993).

Apoptosis (also called programmed cell death) is normal cell death which occurs at specific sites and a specific time during normal embryonic development and also in many adult tissues (Raff, 1992; Wyllie, 1993). This form of cell death by committing suicide is characterized by DNA degradation into fragments (DNA ladder) and specific morphogenic changes including chromatin condensation and nuclear fragmentation (Kerr *et al.*, 1972; Raff, 1992). Cytokines maintain viability of haematopoietic cells by suppression of apoptosis (Williams *et al.*, 1990). Upon withdrawal of cytokines, haematopoietic cells activate suicide machinery leading to cell death. The signalling pathway for regulating apoptosis is currently being intensively investigated, but still remains unclear.

Although the cytokine receptors lack catalytic domains, they associate with and activate cytoplasmic tyrosine kinases (Ihle and Kerr, 1995; Taniguchi, 1995). The activated tyrosine kinases phosphorylate the receptors which in turn create specific binding sites of various SH2-containing signalling molecules for their phosphorylation and activation. Shc is one such molecule that acts as an adaptor protein, lacking a catalytic domain but containing an SH2 domain (C-terminal) and a PTB domain (N-terminal) for binding to specific phosphotyrosine residues (Pelicci *et al.*, 1992; Cutler *et al.*, 1993; Matsuguchi *et al.*, 1994; Cohen *et al.*, 1995). Grb2 is another SH2-containing adaptor protein, which binds stably to mSOS, a Ras GDP/GTP exchange protein (Cohen *et al.*, 1995; Pawson, 1995). Tyrosine-phosphorylated Shc forms a complex with Grb2-mSOS at phosphorylated 317 tyrosine (Y) residue via the SH2 domain of Grb2 (Rozakis-Adcock *et al.*, 1992; Gotoh *et al.*, 1994; Salcini *et al.*, 1994). This appears to stimulate Ras activation, leading to MAPK activation and c-*fos* mRNA induction (Hill and Treisman, 1995). This is one of the immediate-early response to cytokines, requiring no newly synthesized protein (Rollins and Stiles, 1989). However, the entire role of Shc in cytokine signalling remains unclear.

Another immediate-early response is the induction of c-*myc* mRNA, the mechanism of which remains unknown but is suggested to be rather distinct from the Ras pathway (Roussel *et al.*, 1990; Sato *et al.*, 1993; Barone and Courtneidge, 1995; Taniguchi, 1995). A high level of c-*myc* expression is observed when tyrosine kinase oncogenes such as the v-*abl* abrogate growth-factor requirement of haematopoietic cells, pointing to the possibility that Myc may have an important role in the maintenance of cell viability (Cleveland *et al.*, 1989).

In this report, we investigated the role of Shc in IL-3-signalling, particularly its effect on cell survival. We and others have recently shown that the Shc SH2 domain acts as a dominant-negative mutant by inhibiting phosphorylation of Shc (Baldari *et al.*, 1995; Gotoh *et al.*, 1995). We chose a strategy using conditional expression of Shc SH2, a dominant-negative Shc (DN-Shc) in an IL-3-dependent proB cell line, Ba/F3 cells.

Our results strongly suggest that Shc is one of signalling molecules which induce c-*myc* mRNA expression and contributes to suppress apoptosis in a haematopoietic cell line. Furthermore, we identified novel tyrosine phosphoryl-

ation sites of Shc: Y239/240, and showed that this novel Shc to Myc pathway may arise from Y239/240 in a Ras/MAPK-independent manner.

Results

Induction of apoptosis of Ba/F3 cells by conditional expression of the Shc SH2 domain (DN-Shc)

To examine the role of Shc in cytokine signalling, we transfected IL-3-dependent Ba/F3 cells with an expression vector encoding DN-Shc under the control of metallothioneine (MT) promoter in a Zn^{2+}-inducible manner (Shoji *et al.*, 1994). To compare the effect of the SH2 domain of another molecule, we also transfected a similar construct of Nck SH2. After selection with G418, we obtained mass culture and several clones of stable transformants. All experiments were carried out using both mass culture and stable clones, and similar results were obtained.

After treatment with Zn^{2+} for ~10 h, total cell lysates were immunoblotted with anti-Shc SH2 or anti-Nck SH2 antibody (Figure 1A). Our results showed a high expression of DN-Shc and Nck SH2 in a Zn^{2+}-inducible manner which was several fold that of endogenous proteins. The level of expression continued stably as long as the medium contained Zn^{2+} (data not shown). Interestingly, the Zn^{2+}-induced DN-Shc-expressing cells died in the presence of IL-3 in low serum conditions within 40 h (Figure 1B) due to apoptosis, as shown by chromosomal DNA fragmentation (Figure 1C). Zn^{2+}-induced expression of the Nck SH2 had only moderate effects on cell viability.

IL-3-stimulated total cell lysates and their immunoprecipitates with anti-Shc or anti-IL-3Rβ antibodies were Western blotted with anti-phosphotyrosine antibody (α-PY) (Figure 2). In Zn^{2+}-induced DN-Shc-expressing cells, after stimulation with IL-3, tyrosine phosphorylation of Shc was remarkably reduced (Figure 2A, left panel), but tyrosine phosphorylation of the IL-3Rβ and other proteins was clearly observed at levels similar to that of uninduced cells (Figure 2A, right panel). In mock-transfected cells, IL-3-stimulated tyrosine phosphorylation of Shc and other proteins in total cell lysate was clearly observed either in the presence or absence of Zn^{2+}, confirming that Zn^{2+} treatment alone had no effect on tyrosine phosphorylation of proteins (Figure 2B and data not shown).

We observed the binding of Shc SH2 (DN-Shc) to the phosphorylated IL-3Rβ to which endogenous Shc binds (Lanfrancone *et al.*, 1995), but not to other phosphoproteins in response to IL-3, confirming the specificity of the binding activity of Shc SH2 (data not shown). Thus, it is likely that DN-Shc specifically and competitively inhibited tyrosine phosphorylation of endogenous Shc, and that endogenous Shc contributes to IL-3-dependent suppression of apoptosis.

Inhibition of IL-3-stimulated c-myc mRNA induction by conditional expression of DN-Shc

Shc is involved in Ras activation, leading to MAPK activation and transcription of the c-*fos* mRNA (Hill and Treisman, 1995). We examined the effect on this pathway by DN-Shc by analysing IL-3-stimulated MAPK activity by an 'in-gel' kinase assay. As shown in Figure 3A, the

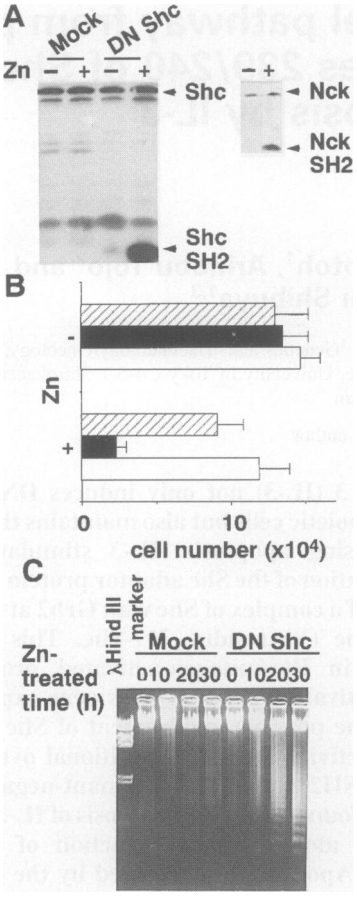

Fig. 1. Induction of apoptosis of Ba/F3 cells by DN-Shc in the presence of IL-3. (**A**) Shc SH2 or Nck SH2 was inserted into the pSVneoHMT vector containing metallothioneine promoter for Zn^{2+}-inducible expression and neomycin-selectable marker. Plasmids were transfected into Ba/F3 cells and G418 resistant-stable transformants were obtained. Cells were incubated with or without $ZnCl_2$ (200 μM) for 10 h. While the medium contains Zn^{2+}, DN-Shc-expression remained constant. Cell lysates of representative clones were Western-blotted with anti-Shc SH2 or anti-Nck SH2, confirming the expression of Shc SH2 (DN Shc) or Nck-SH2 in a Zn^{2+}-inducible manner. (**B** and **C**) Cells were treated or not treated with Zn^{2+} in the presence of IL-3 (0.3 ng/ml). (B) After 40 h, the number of viable cells was counted by a trypan blue dye exclusion technique. Results represent the mean of triplicate cultures of representative clones with standard deviation. Assay of mass cultures and several independent clones showed essentially similar results. Open bar, mock-transfected cells; closed bar, Shc SH2 (DN-Shc)-transfected cells; hatched bar, Nck SH2-transfected cells. (C) Chromosomal DNA fragmentation was analysed by 1% agarose gel electrophoresis at indicated times following induction of DN-Shc. The left lane indicates a λ*Hin*dIII marker.

levels of inhibition of IL-3-stimulated MAPK activity by DN-Shc were only moderate, and IL-3-induced c-*fos* mRNA expression showed moderate inhibition (Figure 3B). These results suggest the possibility of Shc-independent Ras/MAPK/c-*fos* pathways. On the other hand, Northern blot analysis revealed that IL-3-induction of c-*myc* transcript was significantly inhibited in DN-Shc-expressing cells (Figure 3B). Further, the amount of c-*myc* transcript in growing cells, which remains constant as long as IL-3 is present, decreased in DN-Shc-expressing cells (Figure 3C). However, the amount of β-actin did not, confirming that this effect is not due to a general transcriptional repression (Figure 3C). These effects are

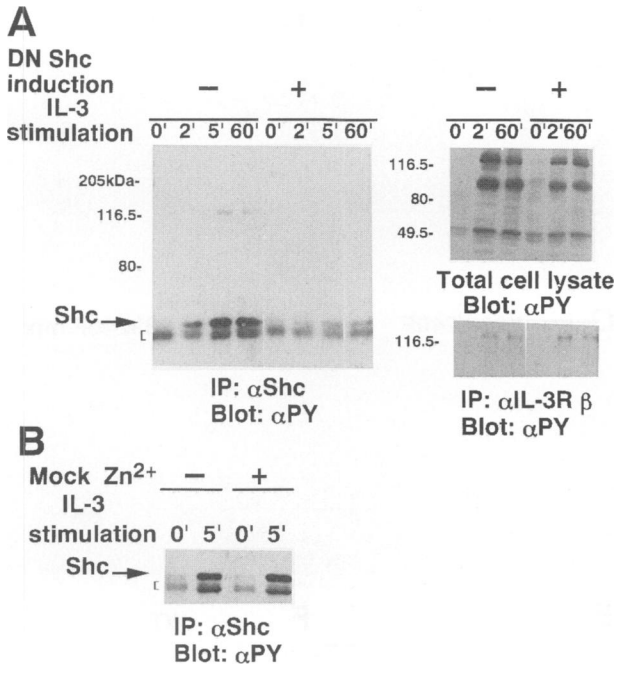

Fig. 2. Inhibition of IL-3-stimulated tyrosine phosphorylation of Shc by DN-Shc. DN-Shc-induced (+) or uninduced (–) cells (**A**) or Zn^{2+}-treated (+) or untreated (–) mock-transfected cells (**B**) were stimulated with or without IL-3 (30 ng/ml) for the times indicated. Immunoprecipitates with anti-Shc (αShc) antibodies (left panel), anti-IL-3Rβ (αIL-3Rβ) antibody (right panel) and total cell lysates (right panel) were resolved by SDS–PAGE, and Western-blotted with anti-phophotyrosine (αPY) antibody. A bracket (left panel) indicates the position of immunoglobulin heavy chain. Reprobing with each antibody confirmed that equal amounts of Shc or IL-3Rβ in each lane were immunoprecipitated (data not shown).

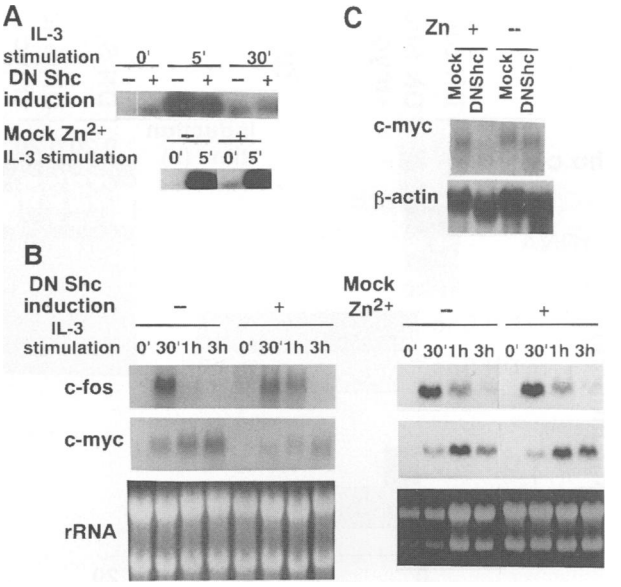

Fig. 3. Moderate inhibition of IL-3-stimulated Ras/MAPK/c-*fos* pathway and inhibition of IL-3-induced c-*myc* expression by DN-Shc. DN-Shc-induced (+) or uninduced (–) cells or Zn^{2+}-treated (+) or untreated (–) mock-transfected cells were stimulated with IL-3 (30 ng/ml) for the times indicated as described in Materials and methods. (**A**) MAPK activity was measured by 'in-gel' kinase assay. (**B**) IL-3-induced immediate-early genes, c-*fos* (top panels) and c-*myc* (middle panels) transcripts were examined by Northern blot analysis using ^{32}P-labelled probes representing portions of c-*fos* and c-*myc*. Loaded total cellular RNA was shown by ethidium bromide staining of ribosomal RNA (bottom panels). (**C**) A constant level of c-*myc* transcripts in Ba/F3 cells in the presence of IL-3 was examined by Northern blot analysis (top panel). Control β-actin transcript was examined using a probe encoding representing portion (bottom panel).

not due to Zn^{2+} treatment, because in mock-transfected cells, Zn^{2+} treatment had no effect on MAPK activity, c-*fos* or c-*myc* mRNA induction in response to IL-3 (Figure 3A and B).

These results suggest that Shc mediates the IL-3 signal toward c-*myc* mRNA induction. There also appear to exist alternative pathways to c-*myc* which are not mediated through Shc, because inhibition of c-*myc* mRNA induction was partial; indeed, ~30% of residual c-*myc* mRNA was present.

Rescue of Ba/F3 cells from DN-Shc induced apoptosis by constitutive expression of c-myc in the presence of IL-3

Because DN-Shc inhibited IL-3-stimulated c-*myc* expression, we hypothesized that greater amounts of c-*myc* transcript may be required to suppress apoptosis in the presence of IL-3. Therefore, we co-transfected the human c-*myc* gene and the hygromycin (hgr) resistance gene into these cells, and obtained several hgr-resistant clones expressing human c-*myc* mRNA (Figure 4A). The human c-*myc*-expressing cells were resistant to apoptosis induced by the conditional expression of DN-Shc, as evidenced by the absence of DNA fragmentation (Figure 4B) and efficient growth (Figure 4C). Thus, constitutive expression of c-*myc* rescued Ba/F3 cells from apoptosis due to the inhibition of phosphorylation of Shc, suggesting that Shc to Myc pathway contributes to suppress apoptosis.

Identification of novel phosphorylation sites of Shc:Y239/240, in response to IL-3

Shc stimulates Ras activation through forming a complex with Grb2 at phosphorylated tyrosine (Y) 317 of Shc (Salcini *et al.*, 1994). We hypothesized that Shc might have other tyrosine phosphorylation sites responsible for c-*myc* induction distinct from that of Ras activation. We searched for possible phosphoacceptor sites of Shc and found Y239/240 as possible candidates, since these residues are located at the intermediate region containing Y317 between the PTB domain and the SH2 domain.

We constructed mutant Shc cDNAs in which Y317 or Y239/240 is substituted to phenylalanine (F) (Y317F and Y239/240F). We introduced the WT, Y317F or Y239/240F Shc cDNA into Ba/F3 cells for constitutive expression and obtained stably expressing cells (Figure 6A). We labelled these cells with [^{32}P]orthophosphate and stimulated with IL-3. The cell lysate was immunoprecipitated with anti-Shc antibodies, and resolved by SDS–PAGE. The labelled Shc proteins were also subjected to tryptic phosphopeptide mapping. The map of the WT Shc exhibited six major spots, 'a, b, b′, c, d and e' and two minor spots 'x and y' (Figure 5). In the map of the Y317F Shc, the intensity of spot 'a' was dramatically decreased as shown by an arrow (Figure 5B). Similarly, in the map of the Y239/240F Shc, the intensity of spots 'b, b′ and c' was strongly decreased (Figure 5C). The mixed map of the Y317F and Y239/240F Shc exhibited essentially an identical pattern to that

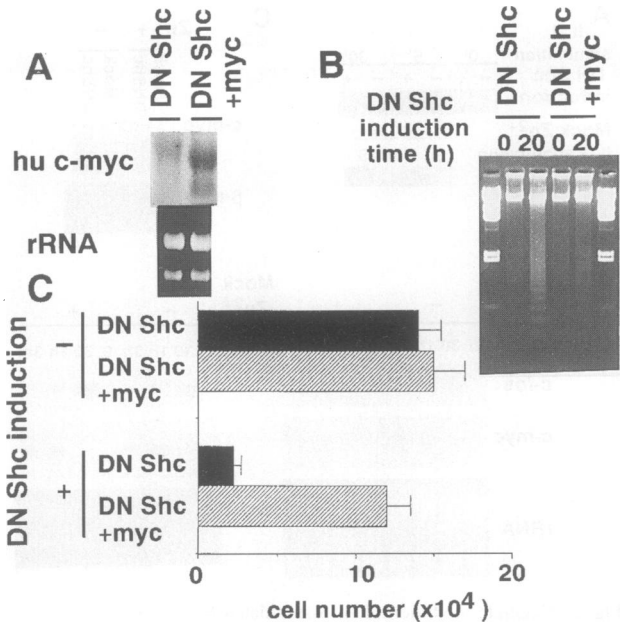

Fig. 4. Rescue of DN-Shc-induced apoptosis by constitutive c-*myc* expression in the presence of IL-3. (**A**) Human c-*myc* cDNA inserted into the vector containing LTR (RSV) promoter for constitutive expression was co-transfected with the vector containing hygromycin-selectable marker into the Zn^{2+}-inducible DN-Shc-expressing Ba/F3 cells. Hygromycin (800 μg/ml)-resistant stable transfectants were obtained. A representative clone and parental DN-Shc-expressing cells were analysed by Northern blot hybridization using a probe containing a representative portion of human c-*myc* cDNA. (**B**) Chromosomal DNA fragmentation was analysed by 1% agarose gel electrophoresis at the times indicated after induction of DN-Shc. (**C**) Constitutive c-*myc*-expressing cells (hatched bar) and parental cells (solid bar) were cultured under the conditions of DN-Shc-induction (+) or no induction (–) in the presence of IL-3 (0.3 ng/ml), as described in the legend to Figure 1B. After 40 h, the number of viable cells was counted by a trypan blue dye exclusion method. Results represent the mean of triplicate cultures of a representative clone with standard deviation. Assay of several clones showed essentially similar results.

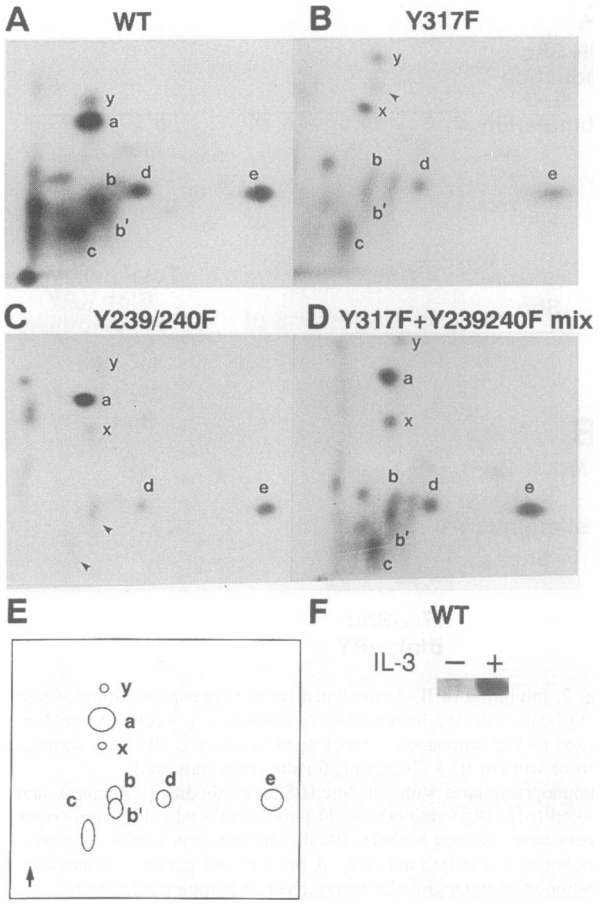

Fig. 5. Two-dimensional tryptic phosphopeptide mapping of the labelled wild-type or mutant Shcs *in vivo*. (**A**) Wild-type Shc. (**B**) Y317F Shc. (**C**) Y239/240F Shc. (**D**) Mixture of Y317F and Y239/240F Shc. (**E**) Schematic representation of each spot. An origin is indicated by an arrowhead. An arrow in (B) indicates spot a. Arrows in (C) indicate spot b, b′ and c. The spots b and b′ are indicated by a single arrow. Counts per min loaded: (A) 1200; (B) 1000; (C) 1000; (D) 800 each of samples in (B) and (C). (**F**) *In vivo*-phosphorylated WT Shc was immunoprecipitated with anti-Shc antibodies from IL-3-stimulated or unstimulated cell lysate, resolved by SDS–PAGE and exposed for autoradiography.

of the WT Shc (Figure 5A and D). In the absence of IL-3, the level of phosphorylation on Shc was very low (Figure 5F), but the pattern of the tryptic map was very similar to that of the WT Shc after IL-3 stimulation (data not shown). The minor spots 'x and y' were occasionally difficult to recognize in the map.

We have also obtained tryptic phosphopeptide maps of phosphorylated Shc protein stimulated with epidermal growth factor (EGF) in fibroblasts (unpublished data). They showed patterns essentially identical to those stimulated with IL-3. The spots 'b, b′ and c' correspond to the singly phosphorylated form and doubly phosphorylated form of Y239 and/or Y240, respectively (data not shown). Thus, it appears that spot 'a' corresponds to phosphorylated Y317 and spots 'b, b′ and c' correspond to the phosphorylated Y239/240-containing peptides. The weak spot 'a' in the map of the Y317F Shc, and faint spots 'b, b′ and c' in the map of the Y239/240F Shc may be derived from co-immunoprecipitated endogenous Shc protein. The spot 'x' might be derived from minor phosphorylation sites or from an alternative phosphoacceptor site when appropriate sites such as Y317 or Y239/240 are lost. We concluded that Y239/240 of Shc became significantly phosphorylated upon stimulation with IL-3.

Resistance to apoptosis and increased c-myc induction of cells expressing WT or Y317F Shc, and enhanced sensitivity to apoptosis and decreased c-myc induction of cells expressing the Y239/240F Shc

We examined the sensitivity of the cells expressing the Shc mutants (Figure 6A) to undergo apoptosis under two conditions in which cells are prone to be apoptotic, namely in the absence of IL-3 but with sufficient serum, and in the presence of IL-3 but with low serum. After withdrawal of IL-3, the WT or Y317F Shc-expressing cells survived longer than mock-transfected or the Y239/240F Shc-expressing cells (Figure 6B). In low serum conditions, in the presence of IL-3, the Y239/240F Shc-expressing cells died faster than the WT or Y317F Shc-expressing or mock-transfected cells (Figure 6C). IL-3-stimulated MAPK activity was enhanced in the WT or Y239/240F Shc-expressing cells but not in the Y317F Shc-expressing cells, confirming that Y317 but not Y239/240 contributes to Ras/MAPK activation (Figure 7A). Furthermore, upon stimulation with low-level IL-3, the initial phase of c-*myc*

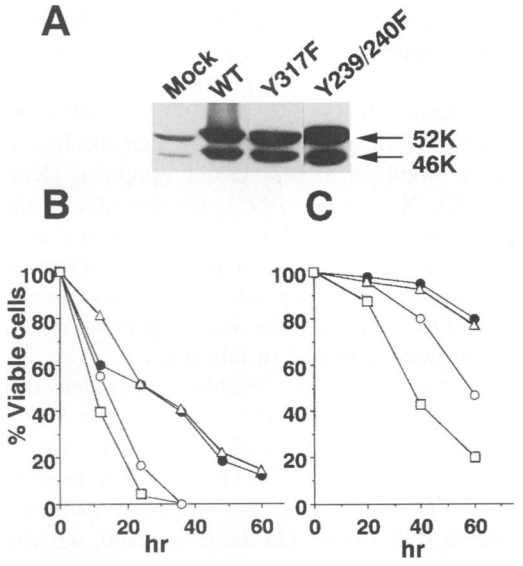

Fig. 6. Resistance to apoptosis of cells expressing the wild-type or Y317F Shc, and enhanced sensitivity to apoptosis of cells expressing the Y239/240F Shc. (**A**) Wild-type, Y317F or Y239/240F Shc cDNA was cloned into the pkUHyg expression vector containing hygromycin selectable marker. Ba/F3 cells were transfected with each construct or vector alone, and then hygromycin-resistant stable transformants were obtained. Cell lysates of Shc transformants and mock-transfected Ba/F3 cells were subjected to Western blot analysis with anti-Shc antibodies, showing overexpression of the wild-type (WT), Y317F or Y239/240F Shc. (**B**) Cells expressing the WT (●), Y317F (△) or Y239/240F (□) Shc or mock-transfected cells (○) were washed free of IL-3 and cultured in the absence of IL-3. (**C**) Cells were cultured in 0.25% serum and IL-3 (0.3 ng/ml). (B, C) The percentage of viable cells were determined by a trypan blue dye exclusion method. Results are the mean of duplicate cultures from one of two to three independent clones for each time point and the independent clones showed essentially similar results within ± 20 standard deviations.

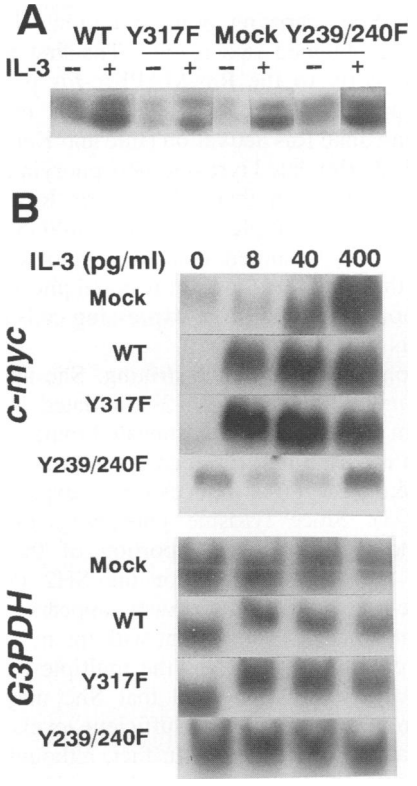

Fig. 7. Increased c-*myc* induction of cells expressing wild-type or Y317F Shc, and decreased c-*myc* induction of cells expressing the Y239/240F Shc. (**A**) Cells were stimulated with IL-3 (0.4 ng/ml), MAPK activity was measured as described in Materials and methods. When analysed in the presence of 4 or 30 ng/ml IL-3, essentially similar results were obtained. (**B**) Following overnight IL-3-deprivation, cells were stimulated with various concentrations of IL-3 for 20 min. Northern blot analysis was performed with probes representing portions of murine c-*myc* or control G3PDH.

induction was reduced in Y239/240F Shc-expressing cells, though it was augmented in the WT or Y317F Shc-expressing cells (Figure 7B). Thus, it appears that the mechanism via which Shc transduces signals to c-*myc* is rather distinct from that of the Ras pathway, possibly through phosphorylation of Y239/240.

Discussion

A number of recent reports showed that Shc becomes tyrosine-phosphorylated in common with a number of activated receptors including cytokine receptors, growth factor receptors and G-protein-coupled receptors, as well as oncogenic tyrosine kinases (Pelicci *et al.*, 1992; Rozakis-Adcock *et al.*, 1992; Cutler *et al.*, 1993; Gotoh *et al.*, 1994; Matsuguchi *et al.*, 1994; Biesen *et al.*, 1995). In most cases, tyrosine-phosphorylated Shc forms a complex with Grb2 at phosphorylated Y317, the high-affinity Grb2-binding site, implicated in contribution for Ras activation (Salcini *et al.*, 1994). In the present study, we identified novel tyrosine phosphorylation sites of Shc: Y239/240, and provide evidence that Shc phosphorylated in these sites appears to have a previously unrecognized role in the induction of c-*myc*, thereby suppressing apoptosis. This novel Shc to Myc pathway appears to be distinct from the Ras/MAPK pathway. This finding seems quite interesting, because it means that tyrosine-phosphorylated Shc in various systems such as those mentioned above may activate this novel pathway in parallel with activation of the Ras pathway.

Shc becomes tyrosine-phosphorylated at Y239, Y240 and Y317 *in vivo* upon stimulation with IL-3, though the phosphorylation level of Y239 and/or Y240 appears to be slightly less than that of Y317 (Figure 5). Also, upon stimulation with EGF, we found that these three tyrosine residues became phosphorylated in EGF receptor-expressing NIH 3T3 cells (N.Gotoh, A.Tojo and M.Shibuya, manuscript in preparation). Hence, Y239, Y240 and Y317 appear to be the major tyrosine phosphorylation sites of Shc. In addition, tryptic phosphopeptide mapping repeatedly revealed spots 'd and e' (Figure 5). Since *in vitro* phosphorylation reaction of Shc with EGF receptor tyrosine kinase showed only spots 'a, b, b′ and c', the spots 'd and e' might represent the peptides phosphorylated on serine or threonine residues within the cells (unpublished data). Furthermore, a recently cloned *Drosophila* Shc homologue conserves Y239/240 but not Y317 (Lai *et al.*, 1995). This suggests that the newly identified phosphorylation sites Y239/240 do have an evolutionarily conserved function of Shc.

To investigate the role of Shc in IL-3-signalling, we employed two strategies to express mutant Shcs defective in signalling for overcoming the endogenous protein. One is inducible expression of the dominant-negative mutant of Shc; the other is stable expression of the wild-type

and mutant Shcs carrying point mutations. From these strategies we obtained two results. The first is that Shc contributes partly to the Ras/MAPK/c-*fos* pathway, but there also appear to exist Shc-independent mechanisms. Vav might mediate Ras activation (Ihle and Kerr, 1995). In addition, IL-3-stimulated tyrosine-phosphorylated PTP1D/Syp may serve as another adaptor for Ras activation through forming a complex with Grb2-mSOS (Li *et al.*, 1994). It should be noted that we cannot exclude the possibility that very low levels of residual phosphorylation of endogenous Shc in DN-Shc-expressing cells might also activate Ras to some extent.

The second result is rather striking, Shc appearing to regulate c-*myc* induction in IL-3-stimulated Ba/F3 cells. So far, from studies using the truncated mutant receptors, it has been reported that the membrane-proximal portion of the cytokine receptors induces c-*myc* expression (Sato *et al.*, 1993). Since tyrosine phosphorylation of Shc requires the membrane-distal portion of the receptors containing the binding sites for the SH2 domains of Shc, this c-*myc* induction pathway appears to be Shc independent. Thus, it is consistent with the notion that the Shc to Myc pathway is one of the multiple pathways to regulate c-*myc* expression, and that Shc may have an important role in induction of sufficient levels of c-*myc*, especially at the initial phase. In fact, although c-*myc* is one of the immediate-early genes, the half-life of Myc is very short and multiple mechanisms appear to operate for regulation of its expression: transcription, translation and degradation (Spencer and Groudine, 1991). At present, we do not know the mechanism by which Shc affects c-*myc* expression, though it is possible that some effector molecules may bind to the phosphorylated Y239 and/or Y240, thereby activating c-*myc* induction.

A possible criticism of this proposal is that the SH2 domain of Shc may bind non-specifically to receptors, preventing the recruitment of the signalling molecules for c-*myc* induction. However, when we expressed the wild-type or point-mutant Shcs in Ba/F3 cells, the former enhanced c-*myc* expression, but the Y239/240F Shc mutant decreased it. Therefore, it is likely that the influence of Shc on Myc is specific to point mutations on Shc proteins.

Recently, it has been reported that receptor tyrosine kinases such as the platelet-derived growth factor (PDGF) receptor requires Src family kinases to induce c-*myc* gene expression through a mechanism distinct from the Ras pathway (Barone and Courtneidge, 1995). Since Shc is a good substrate of Src family kinases, this Src to Myc pathway might utilize Shc as one of the mediators.

We also observed that apoptosis caused by DN-Shc was rescued by the elevated expression of c-*myc* under control of an exogenous promoter (Figure 4). This suggests that sufficient levels of c-*myc* expression contributed by Shc are important for the suppression of apoptosis. Generally, after withdrawal of growth factors or cytokines, the endogenous c-*myc* transcript disappears rapidly. In this growth factor- or cytokine-minus condition, enforced c-*myc* overexpression has been shown to enhance apoptosis (Askew *et al.*, 1991; Evan *et al.*, 1992). In contrast, our results have shown that in the presence of IL-3, c-*myc* expression could suppress apoptosis. Thus, it is possible that Myc may cooperate with some factor whose expression depends on IL-3, such as a Bcl-2-like molecule (Bissonnette *et al.*, 1992; Fanidi *et al.*, 1992). Consistently, it has been reported that the expression of both c-*myc* and *bcl-2* is sufficient to promote proliferation of a haematopoietic cell line (Miyazaki *et al.*, 1995).

Several lines of evidence suggest that the Ras/MAPK pathway is involved in suppressing apoptosis (Kinoshita *et al.*, 1995; Xia *et al.*, 1995). On the other hand, the dominant-negative Ras alone did not induce apoptosis (Terada *et al.*, 1995). One reason for this is that there appear to exist Ras/MAPK-independent pathways to suppress apoptosis. One candidate for such a pathway is the Shc to Myc pathway indicated in this study.

Though the Y317F Shc unable to stimulate the Shc/Ras/MAPK-pathway inhibited apoptosis, the Y239/240F Shc unable to stimulate the Shc to Myc pathway could not suppress apoptosis. In this context, with respect to the anti-apoptotic activity, the Shc to Myc pathway may dominate in Ba/F3 cells. On the other hand, we observed that expression of the Y317F Shc decreased the growth rate of Ba/F3 cells (unpublished data). This is consistent with other reports that granulocyte-macrophage colony stimulating factor (GM-CSF)- or hepatocyte growth factor (HGF)-stimulated cell growth was reduced by the expression of Y317F Shc, suggesting that the Shc/Ras pathway is important for cell growth (Lanfrancone *et al.*, 1995; Pelicci *et al.*, 1995). Therefore, it is possible that these two pathways, Shc to Myc and Shc to Ras, differentially contribute to cell functions.

BCR/ABL, a chimeric fusion protein having enhanced ABL tyrosine kinase activity with Shc phosphorylation, is critical for pathogenesis in Ph1-positive leukaemia (Puil *et al.*, 1994). Conditional expression of DN-Shc in BCR/ABL-transformed Ba/F3 cells also showed apoptosis (unpublished data). From the studies using a temperature-sensitive mutant, it is reported that the primary effect of BCR/ABL in an IL-3-dependent cell line is to prolong survival following growth factor-withdrawal rather than deregulated cell proliferation (Kabarowski *et al.*, 1994). In these cells, Shc is strongly phosphorylated without MAPK activation, as in a case of v-*abl*-mediated suppression of apoptosis (Owen-Lynch *et al.*, 1995). It has been shown that temperature-sensitive mutant v-*abl* induces c-*myc*, but not c-*fos* or c-*jun* (Cleveland *et al.*, 1989). These observations are consistent with the hypothesis that the Shc to Myc pathway may contribute to BCR/ABL or v-*abl*-mediated suppression of apoptosis. Therefore, tyrosine phosphorylation of Shc may be a general mechanism for suppressing apoptosis of haematopoietic cells and targeting it could be a novel approach for the therapy of diseases, including cancer.

In this report, we showed that Shc appears to contribute to sufficient levels of c-*myc*-induction, especially in the initial phase, in response to IL-3, and this may be important for suppression of apoptosis. Moreover, the novel Shc to Myc pathway appears to be generated from the newly identified tyrosine phosphorylation sites of Shc: Y239/240 in a Ras/MAPK-independent manner. Accordingly, the identification of signalling molecules which bind to the phosphorylated Y239/240 of Shc will provide further insights into the mechanisms of this new signalling pathway.

Materials and methods

Construction of Shc mutants and Nck SH2

The human Nck SH2 (methionine followed by amino acids 908 to termination codon) was constructed by PCR using the full-length cDNA as a template and following oligonucleotide primers; 5′-TGCGGTACCA-TGTTTGCTGGCAATCCTTGGTAT-3′/5′-ACCCTATAGTACTATTTA-CGAACTGTTCTA-3′ (*Kpn*I–*Eco*RV fragment) and subcloned into pSP73 vector (Promega). The oligonucleotides contained appropriate restriction sites and termination codon. The *Bam*HI fragment of the Nck SH2 or Shc SH2 (encoding amino acids Met369 to termination codon) (Gotoh *et al.*, 1995) cDNA was cloned into the pSVneoHMT vector (Shoji *et al.*, 1994) containing the metallothioneine (MT) promoter for Zn^{2+}-inducible expression and neomycin-selectable marker. The Y239/240 Shc cDNA was engineered by PCR-mediated mutagenesis. The mutagenic oligonucleotides were 5′-CCATCAGTTCTTTAATGACTT-CCCGGGGAA-3′/5′-AAGTCATTAAAGAACTGATGGTCAGGTGG-CTC-3′, which changes Y239/240 to two phenylalanines. The *Bam*HI–*Pst*I fragment for the Y239/240F Shc was replaced with a PCR-synthesized fragment containing substituted nucleotides and sequenced to ensure that only substituted positions were modified. The *Xho*I–*Xba*I fragment of the WT, Y317F (Gotoh *et al.*, 1995) or Y239/240F Shc cDNA was cloned into the pKU-Hyg expression vector containing hygromycin-selectable marker.

Cell culture and DNA transfection

Ba/F3 cells were maintained in RPMI-1640 (Sigma) containing 10% fetal calf serum (FCS) (Gibco) and 10% WEHI-3-conditioned medium. Transfections were carried out by electroporation method (500 μF, 300 V). Cells were selected for expression of the neomycin resistance gene in 2 mg/ml of G418 (Gibco) or the hygromycin resistance gene in 800 μg/ml of hygromycin (Wako, Tokyo).

Cell survival experiments

In the experiments for Zn^{2+}-induced expression of the Shc SH2 or Nck SH2, cells were placed in 2 ml cultures in 24-well plates (0.7×10^5 cells/ml) and assayed in the medium containing 0.5% FCS supplemented with transferrin (125 μg/ml; Boehringer) and 0.1% bovine serum albumin (Boehringer) for protection of the cells from the toxicity of Zn^{2+}-treatment. Cells were treated with $ZnCl_2$ (200 μM) in the presence of IL-3 (0.3 ng/ml). In the experiments for constitutive expression of the Shc mutants in the absence of IL-3, cells were washed three times and placed in 1 ml of medium containing 10% FCS in 24-well plates (10^5 cells/ml). In the experiments for constitutive expression of the Shc mutants in the presence of IL-3, cells were placed in 1.5 ml of medium containing 0.25% FCS in 24-well plates (0.75×10^5 cells/ml).

Immunoprecipitation and immunoblot analysis

Cells were incubated with or without $ZnCl_2$ (200 μM) in 0.5% FCS overnight and subsequently treated with IL-3 (30 ng/ml) at 37°C for various times. The cells were lysed as described previously (Gotoh *et al.*, 1995). Immunoprecipitation with anti-Shc (UBI) or anti-IL-3Rβ chain antibody prebound to protein-G–Sepharose (Pharmacia) and immunoblot analysis with anti-phosphotyrosine (PY-20; ICN), anti-Shc SH2 (Transduction Laboratories) or anti-Nck SH2 antibody (UBI) were carried out as described previously (Gotoh *et al.*, 1995).

'In-gel' MAPK assay

Cells were starved in 0.5% FCS overnight, treated with various concentrations of IL-3 at 37°C for 5 min, and were lysed as described previously (Gotoh *et al.*, 1995). The cell lysates were separated on SDS–polyacrylamide gel containing 0.5 mg/ml of myelin basic protein (MBP), a specific substrate for MAPK. 'In-gel' MAPK assay was performed as described previously (Gotoh *et al.*, 1994).

Northern blot analysis

Northern blot analysis for total cellular RNA (15 μg for each sample) was carried out as described (Seetharam *et al.*, 1995). The probe DNAs for c-*fos* and c-*myc* were described previously (Seetharam *et al.*, 1995). The probe DNA for β-actin was the rat cDNA (1–160 amino acids). The probe DNA for G3PDH was purchased from Clonetech.

DNA fragmentation

The chromosomal DNA was extracted, analysed by 1% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light.

In vivo labelling of cellular phosphoproteins and stimulation of cells with IL-3

Cells were starved overnight in phosphate-free RPMI1640 containing 0.5% dialysed FCS, labelled with 1.5 mCi/ml $^{32}P_i$ (NEX-053; NEN) for 4 h and were treated with IL-3 (30 ng/ml) at 37°C for 5 min.

Phosphopeptide mapping

Labelled phosphoproteins immunoprecipitated with anti-Shc antibodies were separated on SDS–polyacrylamide gel and the dried gels were visualized by autoradiography. ^{32}P-labelled Shc was eluted from the gel, precipitated by trichloroacetic acid, and subjected to two-dimensional phosphopeptide mapping on thin-layer cellulose plates by the Hunter thin-layer electrophoresis system (CBS Scientific, Del Mar, CA, USA) as described by Boyle *et al.* (1991). Electrophoresis was carried out for 30 min at 1000 V in buffer at pH 1.9.

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