Recovery of anoikis in Src-transformed cells and human breast carcinoma cells by restoration of the SIRPα1/ SHP-2 signaling system

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Abstract
Src kinase dysregulation contributes to cancer progression but mechanistic understanding for this contribution remains incomplete. Signal regulatory protein alpha1 (SIRPα1) is a tumor suppressor that is constitutively suppressed in v-Src-transformed cells, where restoration of SIRPα1 expression inhibits anchorage-independent growth. In this study, we investigated the role of the protein tyrosine phosphatase SHP-2 in SIRPα1 activity. SHP-2 suppression resulted in a blockade of SIRPα1-mediated inhibition of anchorage-independent growth. Notably, we found that SIRPα1 did not act in v-Src-transformed cells by triggering cell growth arrest but by eliciting a suspension-selective apoptosis (anoikis), and that SHP-2 was required for this effect. Furthermore, we found that SHP-2 was crucial for recovery of stress fiber and focal contact formation by SIRPα1 in v-Src-transformed cells. Finally, we found that SIRPα1/SHP-2 signaling regulates anoikis in human breast carcinoma cells with activated c-Src. Taken together, our findings define SHP-2 as an essential component of tumor suppression and anoikis mediated by SIRPα1 in human breast carcinoma cells as well as v-Src-transformed cells.
Introduction

v-Src, the oncogene product of Rous sarcoma virus, is a protein tyrosine kinase that mediates cell transformation (1). Acquisition of anchorage-independent growth, the ability to grow without attachment to substrate, is one of the critical phenotypic properties associated with cell transformation (1). We have previously reported that expression of signal regulatory protein alpha1 (SIRPα1) was constitutively suppressed in v-src-transformed cells in a transformation-specific manner. Furthermore, forced expression of SIRPα1 inhibited anchorage-independent growth of transformed cells, suggesting its role as a tumor suppressor (2). In addition, we found that expression of SIRPα was frequently suppressed in human breast carcinoma and forced expression of either SIRPα1 or SIRPα2 substantially suppressed the anchorage-independent growth of breast carcinoma cells (3).

SIRPα1, a member of SIRP family proteins (4) is a receptor-type transmembrane glycoprotein with three immunoglobulin (Ig)-like domains in its extracellular region and tyrosine phosphorylation sites in its cytoplasmic region (5). SIRPα1 was originally identified as a docking protein for Src homology 2 (SH2) domain-containing protein tyrosine phosphatase-2 (SHP-2) (6). SHP-2 is a phosphatase composed of two SH2 domains and catalytic domain in the C-terminus (7). While intramolecular association of the catalytic domain with the SH2 domain inhibits its catalytic activity (8), association of SHP-2 with phosphorylated tyrosine receptors or docking proteins activates phosphatase, which, consequently, activates the Ras-MAPK pathway (9-11). In v-src-transformed cells, SHP-2 is required for the activation of anti-apoptotic protein kinase Akt (12). Recent report (13) showed that mutated form of SHP-2 worked as an oncogenic phosphatase in Hodgkin's lymphoma associated with Noonan syndrome. It is, therefore, of interest whether SHP-2 is simply a cancer-promoting phosphatase or also required for the SIRPα1-dependent inhibition of anchorage-independent growth of v-src-transformed cells.

Here, we show the evidence that SHP-2 is required for the SIRPα1-dependent suppression of anchorage-independent growth and the SIRPα1/SHP-2 signaling system restores anoikis, a form of cell apoptosis induced by detachment from extracellular matrix (14, 15) in human breast
carcinoma cells as well as v-src-transformed cells. Moreover, we show that SHP-2 is required for the recovery of stress fiber formation and focal adhesion induced by SIRPα1-expression in v-src-transformed cells.

**Materials and Methods**

*Immunoblotting, Immunofluorescent analysis and antibodies*

Immunoblotting and immunofluorescent analysis were performed as described (16, 17). Anti-SHP-2 and Erk2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Src, anti-phospho Src and anti-vinculin antibodies were obtained from Cell signaling (Tokyo, Japan). Anti- SIRPα antibody was generated as described previously (2). Stress fiber formation was performed by rhodamine-conjugated phalloidin (Sigma-Aldrich, Tokyo, Japan).

*Plasmid and establishment of cell lines*

SR3Y1 was transfected with Rat SIRPα1 cDNA ligated with pBabepuro expression vector and drug-resistant colonies were selected as previously (2). To establish SHP-2 knockdown cells, oligonucleotides encoding shRNA specific for SHP-2 (5’-CGCCACCCTGTGCGGAAAT-3’) and Luciferase (5’-CTTACGCTGAGTACTTCGA-3’) were cloned into pSilencer2.1™neo (Ambion, Austin, TX). Cells were transfected with plasmid and drug-resistant colonies were obtained. To establish V3Tet that express SIRPα1 by the removal of tetracycline, SIRPα1 cDNA was cloned into pTRE2pur vector (Clontech, Mountain View, CA) and transfected to V3, (Balb3T3 transformed with v-src) together with pTet-Off vector (Clontech) (16). Drug-resistant colonies that expressed SIRPα by the removal of tetracycline were selected. Human breast carcinoma cells, Hs578T, and Hs578T expressing human SIRPα1, Hs578T/ SIRPα, cells were cultured as described (3).

*siRNA transfection*

Transfection of siRNA was described previously (18). siRNAs were obtained from Sigma. The sequence of mouse SHP-2 siRNA is 5’-GUGACAUCGACGUUCCUAAAA-3’, human SHP-2 is 5’-CGCUCAUGACUAUACGCUATT-3’ and Luciferase siRNA is 5’-CUUACGCUGAGUACUUCGATT-3’.
Soft agar colony formation and Suspension culture

Soft agar colony formation was described previously (2, 3). For suspension culture, cells were mixed with 1.68 % methyl cellulose in medium and incubated for 48 h. After the incubation, cells were collected by centrifugation.

TUNEL assay and treatment with caspase inhibitors

Cells cultured either on dishes or in suspension for 48 h were subjected to a TUNEL assay according to the manufacturer’s protocol (Promega, Madison, WI). To examine the effect of caspase inhibitors, cells were incubated overnight with indicated inhibitor at a concentration of 2 μM, and further incubated with same inhibitor in suspended condition for 48 h. A pancaspase inhibitor, Z-VAD-FMK, caspase-3 inhibitor, Z-DEVD-FMK, caspase-8 inhibitor, Z-IETD-FMK, caspase-9 inhibitor, Z-LEHD-FMK, from BioVision Inc. (California) were used.

Rho activation assay

Rho activity was measured as described previously (4). Cells were lysed and incubated with GST-Rhotekin-RBD (residues 7-89) fusion protein bound to glutathione-agarose beads. Beads were washed and subjected to immunoblotting with anti-Rho antibody.

Result and Discussion

SR3Y1, a rat fibroblastic cell line 3Y1 transformed with v-src, was used. SR3Y1 stably expressing SIRPα1 was first established by transfection of SIRPα1. To investigate the role of SHP-2, two cell lines, SR3Y1 expressing SIRPα1 transfected with plasmids that encoded shRNAs targeting either SHP-2 (SR/SIRP/shSHP-2) or Luciferase (SR/SIRP/shLuc), were established. We also established SR3Y1 that expressed shRNA targeting either SHP-2 or Luciferase (SR/shLuc, SR/shSHP-2). As shown in figure 1A, expression of SHP-2 was reduced in SR/SIRP/shSHP-2 and SR/shSHP-2, but not in cells, SR/SIRP/shLuc and SR/shLuc, that expressed Lucifearse shRNA. Since three independent clones of each transfection showed similar levels of SHP-2 expression, we used clone 1 of each cell line for further experiments.

We first examined requirement of SHP-2 in the SIRPα1-mediated
suppression of anchorage-independent growth (figure 1B). Cells were cultured in soft agar for 1 week and formation of colonies was examined. SR/SIRP/shLuc cells, which overexpress SIRPα1 in SR3Y1, showed clear suppression of colony formation in soft agar. In contrast, silencing of SHP-2 in SIRPα1-expressing SR3Y1 (SR/SIRP/shSHP-2) restored the ability of cells to grow in soft agar to the level similar to SR/shLuc. These results indicate that SHP-2 is required for the SIRPα1-mediated suppression of anchorage independent growth.

Anoikis, which is specifically observed in normal cells but lost in transformed cells, is a form of cell apoptosis induced by detachment from extracellular matrix (14, 15). To examine whether anoikis was induced by SIRPα1 expression in SR3Y1, cells were cultured in attached or suspended condition, and cell death was examined by trypan blue exclusion test. SR/SIRP/shLuc showed increased rate of cell death in a suspension-specific manner to the level similar to that of 3Y1 (figure 2A), whereas clear increase of cell death by suspension was not observed in SR/shLuc. In contrast to SR/SIRP/shLuc, SR/SIRP/shSHP-2 did not show substantial increase of cell death in suspended culture. To confirm these observations, cells were examined by TUNEL assay to detect DNA fragmentation. As shown in figure 2B, SR/SIRP/Luc showed increased rate of condensation and fragmentation of DNA, i.e. apoptosis, in a suspension-specific manner. In contrast, SR/SIRP/shSHP-2 did not show the suspension-specific apoptosis. Moreover, treatment of SR/SIRP/Luc with a pancaspase inhibitor, Z-VAD-FMK, clearly inhibited suspension-induced apoptosis. These results suggest that forced expression of SIRPα1 restores suspension-specific apoptosis, anoikis, in v-src-transformed cells and SHP-2 is required for the apoptosis induced by SIRPα1. We examined the effect of caspase inhibitors on the suspension-specific apoptosis of SR3Y1 mediated by SIRPα. SR/SIRP/Luc was cultured in suspension in the presence or absence of the indicated inhibitors and apoptosis was examined. We found that, at least in part, caspase-3 and caspase-9 (19) are involved in apoptosis mediated by SIRPα1/SHP-2 signaling (figure 2C).

Since the morphology of SR3Y1 recovered to be flat by forced expression of SIRPα, we next examined formation of actin stress fibers and focal adhesions. Cells were cultured on the fibronectin-coated glass coverslides and stained for actin and vinculin. As shown in figure 3A, actin stress fibers and focal
adhesions was restored to some extent in SR/SIRP/Luc, although cells were overlapped each other. In contrast, SR/SIRP/shSHP-2 had disorganized actin stress fibers and loss of focal adhesions.

Since Rho, a small GTPase, is required for the organization of stress fibers, we next examined the Rho activity by pull-down assay (figure 3B, C). Consistently, activity of Rho was increased in SR/SIRP/Luc compared to that of SR/shLuc, and SR/SIRP/shSHP-2 showed reduced Rho activity similar to SR/shLuc. These results suggest that the SIRPα/SHP-2 signaling regulates Rho to modulate actin stress fiber formation in v-Src-transformed cells. On the other hand, in 3Y1 with reduced SHP-2 expression (3Y1/shSHP-2), silencing of SHP-2 did not affect activity of Rho, indicating that Rho activity is independent of SIRPα/SHP-2 signaling in normal fibroblasts.

To confirm our observations, we established v-Src transformed cells in which SIRPα1 expression was reversibly controlled by tetracycline. V3Tet, v-Src-transformed Balb3T3 in which SIRPα1 was expressed only in the absence of tetracycline, was established. As shown in figure 4A, expression of SIRPα1 was clearly induced in the absence of tetracycline but suppressed by the presence of tetracycline. While V3Tet showed clear increase of soft agar colony formation in the presence of tetracycline, colony formation was significantly suppressed by the removal of tetracycline. In these cells, SHP-2 expression was transiently suppressed by siRNA. As shown in figure 4A, cells transfected with SHP-2 siRNA showed reduction in SHP-2 expression. Furthermore, knockdown of SHP-2 expression restored anchorage-independent growth in the presence of SIRPα1 expression.

We next examined organization of stress fibers. V3Tet cells, transfected with siRNAs and cultured with or without tetracycline for 2 days, were subjected to immunofluorescent analysis. As shown in figure 4C, organization of actin stress fibers was restored by SIRPα1 expression, whereas silencing of SHP-2 canceled the SIRPα1-dependent recovery of stress fiber formation.

To extend our observations, we examined whether SIRPα/SHP-2 signaling regulates anoikis in human carcinoma cell line with activated c-Src. Hs578T and Hs578T with overexpressed SIRPα1 (Hs578T/ SIRPα) (3) was used for this study. Hs578T and Hs578T/ SIRPα were transfected with Luciferase or SHP-2 siRNA and 24 h later, cells were cultured in suspension for 72 h, and the ratio of dead cells was assayed by trpae blue exclusion test. As shown in
In addition, silencing of SHP-2 expression in Hs578T/ SIRPα inhibited anoikis. Thus, SIRPα1/SHP-2 signaling appears to restore anoikis not only in v-Src-transformed cells but also in human breast carcinoma cells.

In this report, we demonstrated that forced expression of SIRPα in v-src-transformed cells restored suspension-specific apoptosis, anoikis. Moreover, our results clearly demonstrated that SHP-2 was required for the suspension-specific apoptosis as well as morphological recovery induce by SIRPα1. SHP-2 has been regarded as a cancer-promoting molecule (10). Indeed, gain-of-function mutations of SHP-2 have been reported to associate with some human cancers (11). In contrast to these reports, our findings strongly suggest that SHP-2 is also involved in the SIRPα1-mediated tumor suppression. Our results and previous observations suggest that SHP-2 may have ambivalent functions in terms of cell transformation. Proper control of SHP-2 function by SIRPα1 may be required for the suppression of aberrant growth of cells. Based on present results, authors are tempted to discuss that the SIRPα1/ SHP-2 signaling system might work as a sensor for anoikis that antagonizes transforming capability of the Src kinase not only in v-src-transformed cells but also in breast carcinoma cells with activated c-Src. Further studies are required to elucidate how the SIRPα/SHP-2 system recognizes cell attachment and transduce its signal to activate anoikis in transformed cells.

Acknowledgments

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References


**Figure Legend**

**Figure 1.** Requirement of SHP-2 in SIRPα-mediated suppression of anchorage independent growth. A, Expressions of indicated proteins were examined by immunoblotting. Erk2: a loading control. B, Each cell line was cultured in the soft agar for 1 week and the number of colonies was counted. Three independent experiments were performed and the number of colonies is represented as mean±SD.

**Figure 2.** Requirement of SHP-2 in SIRPα1-induced anoikis of SR3Y1. A, Each cell line was incubated attached or suspended for 48 h and trypan blue exclusion test was performed. Three independent experiments were performed and ratios of dead cells are indicated (mean±SD) (*P<0.01). B, Each cell line was cultured in suspended condition for 48 h and subjected to TUNEL assay. DAPI was used to stain the nucleus. A pan caspase inhibitor, Z-VAD-FMK, was used to assess the involvement of caspase. C, SR/SIRP/Luc cells were cultured in suspension for 48 h with or without the indicated caspase inhibitors and subjected to TUNEL assay to determine the ratios of apoptotic cells. Three independent experiments were performed to determine the relative ratio of apoptosis (mean±SD). (*P<0.01).

**Figure 3.** Requirement of SHP-2 in the recovery of stress fiber formation by
SIRPα1. A, Cells cultured on glass coverslips were stained for actin stress fiber and vinculin (Scale bar = 20μm). B and C, Cells were subjected to Rho activation assay as described in Materials and Methods.

Figure 4. The SIRPα1/SHP-2 signaling system in a tetracycline-dependent SIRPα1-inducible system and in human breast carcinoma cells.
A, Expressions of indicated proteins in V3Tet with or without tetracycline and siRNA treatment was examined by immunoblotting. B, V3Tet cultured in the presence or absence of tetracycline was transfected with Luciferase or SHP-2 siRNA, and cultured with or without tetracycline in the agar for one week. Three independent experiments were performed and the numbers of colony were indicated (mean±SD) (*P<0.01). C, V3Tet cultured in the presence or absence of tetracycline was transfected with siRNAs. Three days later, cells were fixed and actin stress fiber formation was examined (bars= 20μm). D, Hs578T and Hs578T/SIRPα were transfected with Luciferase or SHP-2 siRNA. Then cells were subjected to the suspension culture for 72 h. Trypan blue exclusion test was performed to evaluate the ratio of dead cells. Three independent experiments were performed and the ratio of dead cells is indicated on the graph (mean±SD) (*P<0.01).
Figure 1

A

3Y1  | SR3Y1  | SR/shLuc  | SR/shSHP-2 | SR/SIRPα/shSHP-2 | SR/SIRPα/shLuc
---|---|---|---|---|---
C1 | C2 | C3 | C1 | C2 | C3 | C1 | C2 | C3

SIRPα1
SHP-2
pY416-Src
Src
Erk2

B

Colony number per field

3Y1  | SR/shLuc  | SR/shSHP-2 | SR/SIRPα/shSHP-2 | SR/SIRPα/shLuc
---|---|---|---|---
0  | 50 | 0  | 0  | 0
Figure 2

A

![Graph showing changes in percentage (%) with different treatments](image)

B

![Immunofluorescence images](image)

C

![Graph showing changes in TUNEL positivity with different treatments](image)
Figure 3

A

Phalloidin  Vinculin  Merge

3Y1

SR/shLuc

SR/shSHP-2

SR/SIRPα/shSHP-2

SR/SIRPα/shLuc

B

3Y1  SR/shLuc  SR/shSHP-2  SR/SIRPα/shSHP-2  SR/SIRPα/shLuc

Active Rho  Total Rho  SHP-2  β-actin
Figure 4

A

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B

Colony number per field

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Balc3T3 | V3Tet

C

V3Tet (tet +) | V3Tet (tet -)

Luc/siRNA

SIRPα1 | SHP-2 | pY416-Src | Src | Erk2

D

Ratio of dead cells

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Hs578T | Hs578T/SIRPα1

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