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 α1 (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-2 (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 in v-Src-transformed cells. Cancer Res; 71(4); 1229–34. ©2011 AACR.

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 α1 (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 SIRPα1 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 the SIRP family of proteins (4), is a receptor-type transmembrane glycoprotein with 3 immunglobulin (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 2 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-MAP kinase (MAPK) pathway (9–11). In v-src-transformed cells, SHP-2 is required for the activation of antiapoptotic protein kinase Akt (12). A recent report (13) showed that mutated form of SHP-2 worked as an oncogenic phosphatase in Hodgkin 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 in 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 in earlier publications (16, 17). Anti-SHP-2 and Erk2 antibodies were purchased from Santa Cruz Biotechnology. Anti-Src, anti-phospho-Src, and antivinculin antibodies were obtained from Cell Signaling. Anti-SIRPα antibody was generated as described previously (2). Stress fiber formation was performed by rhodamine-conjugated phalloidin (Sigma-Aldrich).

Plasmid and establishment of cell lines
SR3Y1 was transfected with Rat SIRPα1 cDNA ligated with pBabe puro expression vector and drug-resistant colonies were selected as previously described (2). To establish SHP-2 knockdown cells, oligonucleotides encoding shRNA specific for SHP-2 (5′-CGCCACCGTGGCGAAAT-3′) and Luciferase (5′-CTTACGTCAGATCTCGA-3′) were cloned into pSilencer2.1 (Ambion). Cells were transfected with plasmid and drug-resistant colonies were obtained. To establish V3Tet that expresses SIRPα1 by the removal of tetracycline, SIRPα1 cDNA was cloned into pTRE2pur vector (Clontech) and transfected to V3 (Balb3T3 transformed with v-src) together with pTet-Off vector (Clontech; ref. 16). Drug-resistant colonies that expressed SIRPα1 by the removal of tetracycline were selected. Human breast carcinoma cells, Hs578T, and Hs578T expressing human SIRPα1, Hs578T/SIRPα, cells were cultured as described in ref. 3.

siRNA transfection
Transfection of siRNA was described previously (18). siRNAs were obtained from Sigma. The sequence of mouse SHP-2 siRNA is 5′-GUGACAUCGACGUUCCUAAA-3′, human SHP-2 is 5′-CGCUCUAUGACUAUCGCUATT-3′, and Luciferase siRNA is 5′-CUUACGCUGAGAUCCGATT-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 hours. 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 hours were subjected to a terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay according to the manufacturer’s protocol (Promega). 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 hours. A pancaspase inhibitor, Z-VAD-FMK, caspase-3 inhibitor, Z-DEVD-FMK, caspase-8 inhibitor, Z-IETD-FMK, and caspase-9 inhibitor, Z-LEHD-FMK, from BioVision Inc. were used.

Rho activation assay
Rho activity was measured as described previously (4). Cells were lysed and incubated with GST-Rhotekin-RBD

Results 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, 2 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 Fig. 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 Luciferase shRNA. Because 3 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 (Fig. 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 (Fig. 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 Fig. 2B, SR/SIRP/Luc showed increased rate of condensation and fragmentation of DNA, that is, 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 (Fig. 2C).

Figure 2. Requirement of SHP-2 in SIRPα1-induced anoikis of SR3Y1. A, each cell line was incubated attached or suspended for 48 hours 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 hours and subjected to TUNEL assay. 4′,6-Diamidino-2-phenylindole Dihydrochloride (DAPI) was used to stain the nucleus. A pancaspase inhibitor, Z-VAD-FMK, was used to assess the involvement of caspase. C, SR/SIRP/Luc cells were cultured in suspension for 48 hours 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).
Because 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 Fig. 3A, actin stress fibers and focal adhesions were restored to some extent in SR/SIRP/Luc, although cells overlapped each other. In contrast, SR/SIRP/shSHP-2 had disorganized actin stress fibers and loss of focal adhesions.

Because Rho, a small GTPase, is required for the organization of stress fibers, we next examined the Rho activity by pull-down assay (Fig. 3B and 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 that of 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 SIRPα expression was reversibly controlled by tetracycline. V3Tet, v-Src-transformed Balb3T3 in

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, 20 μm). B and C, cells were subjected to Rho activation assay as described in Materials and Methods.
which SIRPα1 was expressed only in the absence of tetracycline, was established. As shown in Fig. 4A, expression of SIRPα1 was clearly induced in the absence of tetracycline but suppressed by the presence of tetracycline. Whereas 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 Fig. 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 Fig. 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 SIRPα1/SHP-2 signaling regulates anoikis in human carcinoma cell line with activated c-Src. Hs578T and Hs578T/SIRPα1 were used for this study. Hs578T and Hs578T/SIRPα1 were transfected with Luciferase or SHP-2 siRNA and 24 hours later, cells were cultured in suspension for 72 hours, and the ratio of dead cells was assayed by trypan blue exclusion test. As shown in Table 4D, Hs578T/SIRPα1, but not Hs578T showed anoikis. In addition, silencing of SHP-2 expression in Hs578T/SIRPα1 inhibited anoikis. Thus, SIRPα1/SHP-2 signaling appears to restore anoikis and SIRPα1/SHP-2 Signaling.
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 morphologic recovery induce by SIRPα. 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 SIRPα-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 SIRPα may be required for the suppression of aberrant growth of cells. Based on present results, authors are tempted to discuss that the SIRPα/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 transduces its signal to activate anoikis in transformed cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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