Regulation of the Src-PP2A Interaction in Tumor Necrosis Factor (TNF)-related Apoptosis-inducing Ligand (TRAIL)-induced Apoptosis*

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Background: The mechanisms of TRAIL resistance in cancer cells are not fully understood.

Results: TRAIL activates Src, which in turn phosphorylates caspase-8. Src inhibition increases TRAIL-induced apoptosis. TRAIL also relieves PP2A-mediated Src inhibition, leading to Src activation.

Conclusion: The interaction between Src and PP2A plays an important role in TRAIL resistance.

Significance: Inhibition of Src survival signaling may enhance TRAIL-induced apoptosis in cancer cells.

TNF-related apoptosis-inducing ligand (TRAIL) selectively induces apoptosis in transformed and tumor cells but not in normal cells, making it a promising agent for cancer therapy. However, many cancer cells are resistant to TRAIL, and the underlying mechanisms are not fully understood. Here, we show that the regulation of the PP2A and Src interaction plays a critical role in TRAIL resistance. Specifically, we show that TRAIL treatment activates the tyrosine kinase Src, which subsequently phosphorylates caspase-8 at tyrosine 380, leading to the inhibition of caspase-8 activation. We also show that upon TRAIL treatment, Src, caspase-8, and PP2A/C (a catalytic subunit of the PP2A phosphatase) are redistributed into lipid rafts, a microdomain of the plasma membrane enriched with cholesterol, where PP2A dephosphorylates Src at tyrosine 418 and in turn inhibits caspase-8 phosphorylation. Furthermore, we find that TRAIL treatment causes PP2A/C degradation. These data suggest that the balance between Src-mediated caspase-8 phosphorylation and the inactivation of Src-mediated caspase-8 phosphorylation by PP2A determines the outcome of TRAIL treatment in breast cancer cells. Therefore, this work identifies a novel mechanism by which the interaction between PP2A and Src in the context of caspase-8 activation modulates TRAIL sensitivity in cancer cells.

TRAIL3 (Apo2 ligand) is a member of the TNF family (1, 2). TRAIL selectively induces apoptosis in transformed or tumor cells but not in normal cells, making it a promising agent for cancer therapy (1, 2). There are four membrane-bound receptors for TRAIL, including DR4 (3), DR5 (4–7), TRID (TRAIL receptor without an intracellular domain) (5, 8), and TRUNDD (TRAIL receptor with a truncated death domain) (9). Both DR4 and D5R contain a conserved death domain motif and are pro-apoptotic receptors (10), whereas TRID lacks an intracellular domain and TRUNDD has a truncated death domain, thereby acting as decoy receptors to antagonize TRAIL-induced apoptosis by competing for ligand binding (11). Binding of TRAIL to DR4 or DR5 leads to formation of the death-inducing signaling complex (DISC) by recruiting FADD and caspase-8, resulting in activation of caspase-8, which in turn activates caspase-3, -6, and -7, thus cleaving the death substrates and causing cell death. Activation of caspase-8 by TRAIL signaling can cleave the proapoptotic Bcl-2 family member BID to promote cytochrome c release, which amplifies the apoptotic signal. The TRAIL pathway provides a number of potential opportunities to understand death signaling and to develop therapeutic target because the TRAIL ligand itself and TRAIL receptor-specific agonistic antibodies effectively kill transformed and cancer cells but not most normal cells (12–14). However, not all cancer cells are susceptible to TRAIL, and there appears to be a growing list of possible mechanisms by which cancer cells can evade TRAIL-induced apoptosis, including the expression of proteins that can interfere with caspase-8 activation such as FLIP and the overexpression of Bcl-2 and Bcl-XL (15–21). Furthermore, it has been shown that TRAIL treatment can activate the survival pathways such as the Src pathway, which can counteract TRAIL-induced apoptosis, leading to TRAIL resistance (22–24). However, the mechanisms of TRAIL resistance are not fully understood.

Src is a member of the non-receptor tyrosine kinase family that is involved in the regulation of a host of cell processes, including cell proliferation, survival, and drug resistance. Src is activated via its interaction with a number of proteins, including growth factor receptors, integrin cell adhesion receptors, and steroid hormone receptors (25). The role of Src activation...
in cell survival is well established, but its involvement in the regulation of TRAIL-induced apoptosis has recently been emerging. It has been shown that Src-mediated AKT survival signaling contributes to TRAIL resistance in breast cancer cells (26). It has also been shown that the activation of the Src-STAT3 pathway is responsible for TRAIL-induced invasion in resistant non-small cell lung cancer cells (24). In addition, a study showed that the activation of Src plays a role in TRAIL resistance in colorectal cancer cells (27). All of these studies indicate that the activation of Src-mediated downstream survival signaling contributes to TRAIL resistance. However, it is not known whether Src is able to directly inhibit the activation of caspase-8, a member of the apoptotic machinery.

Protein phosphatase 2A (PP2A) is the major serine-threonine phosphatase that regulates a number of cell signaling pathways (28). PP2A is a trimeric holoenzyme that consists of a catalytic subunit, a structural subunit, and a regulatory subunit (29). A structural subunit and a catalytic subunit form the PP2A core enzyme, which further interacts with a regulatory subunit to assemble a heterotrimeric PP2A holoenzyme (30). PP2A enzymatic activity depends on PP2A/C, whereas the regulatory subunits determine substrate specificity and subcellular localization (29–31). PP2A plays an important role in the regulation of cell signaling; however, its role in the regulation of TRAIL remains to be determined.

In this study, we found that TRAIL activates Src, which in turn phosphorylates and blocks caspase-8 activation, leading to TRAIL resistance. We also showed that TRAIL treatment induces degradation of the catalytic subunit of protein phosphatase 2A (PP2A/C). We provided evidence that PP2A acts as a negative regulator of Src and that degradation of PP2A/C relieves Src-mediated caspase-8 phosphorylation and inactivation. Thus, our data suggest that in response to TRAIL treatment, the balance between Src-mediated caspase-8 phosphorylation and PP2A-mediated Src inhibition determines whether breast cancer cell undergo apoptosis or cell survival.

**EXPERIMENTAL PROCEDURES**

**Tissue Culture and Reagents**—Human breast cancer MDA231 cells and human embryonic kidney HEK293T cells were cultured in DMEM with 10% FBS and 1% penicillin and streptomycin. The human breast cancer MCF7 cells were obtained from Karmanos Cancer Institute and maintained in DMEM/F-12 supplemented with 5% FBS and 1% penicillin and streptomycin. Human breast cancer cell line SUM159 was maintained in F12 supplemented with 5% FBS, 1% penicillin and streptomycin, 10 μg/ml insulin, and 0.5 μg/ml hydrocortisone. Human breast cancer cell line BT549 was maintained in RPMI with 10% FBS. Recombinant human TRAIL/Apo2L was obtained from Peprotech, Inc. (Rocky Hill, NJ). Actin antibody was purchased from Sigma. Mouse antibodies to HA and ubiquitin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-phosphor-Src (Tyr-418) was obtained from Invitrogen. Rabbit anti-PP2A/C, FADD, phosphotyrosine, phospho-Src (Tyr-527), and Src antibodies and mouse anti-caspase-8 were obtained from Cell Signaling Technology (Danvers, MA). Mouse anti-caveolin-1 antibody was purchased from BD Biosciences. Mouse anti-Src was from Millipore (Billerica, MA). Anti-phospho-caspase-8 (Tyr-380) antibody was obtained from Assay Biotechnology (Sunnyvale, CA). Rabbit anti-DR5 antibody was obtained from Imgenex (San Diego, CA). The PP2A inhibitor LB-100 was provided by Lixte Biotechnology Holdings (East Setauket, NY) and described previously (32).

**Plasmids and Transfection**—pcDNA-PP2A/C-HA (PP2A/C-HA) was kindly provided by Dr. Xingming Deng (Emory University). pcDNA3-caspase-8-HA (caspase-8-HA) was kindly provided by Dr. Emad Alnemri (Thomas Jefferson University). pcDNA3.1-Src and active form Src were provided by Dr. Julie Boerner. Lipofectamine 2000 (Invitrogen) was used to transfect plasmids. Transfected cells were harvested 48 h post-transfection.

**Co-immunoprecipitation and DISC-IP**—For co-immunoprecipitation, cells were lysed with lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin, and 1 mM PMSF) on ice for 30 min. Soluble cell lysates (800 μl) were incubated with IP antibody at 4 °C overnight. Complexes were pulled down by Protein A/G agarose (Santa Cruz Biotechnology). The pellets were washed four to five times with lysis buffer and then subjected to Western blot analysis.

**Lipid Raft and Non-raft Fractionation and Cholesterol Analyses**—Lipid raft and non-raft soluble fractions were separated by discontinuous sucrose density gradients of Triton X-100 cell lysates from untreated and TRAIL-treated cells. In brief, BT549 cells (4 × 107 cells) from two 15-cm culture dishes were left untreated or treated with TRAIL (150 ng/ml) for 30 min and then lysed on ice for 30 min in 0.8 ml of MNX buffer (1% Triton X-100 in 25 mM MES, 150 mM NaCl, pH 6.5) supplemented with 1 mM of phenylmethylsulfonyl fluoride and protease inhibitor mixture, and then homogenized with douncer for 50 strokes. The homogenates were mixed with 0.8 ml of 90% sucrose in MNX buffer and placed on the bottom of a centrifuge tube. The samples were overlaid on 1.6 ml of 35% sucrose and 1.6 ml of 5% sucrose and centrifuged at 38,000 rpm with the SW55Ti rotor with Optima L-90K Ultracentrifuge (Beckman) for 16 h at 4 °C. Ten fractions of 0.4 ml were collected from the top to the bottom of the gradient and analyzed by Western blot analysis. To identify lipid raft fractions, cholesterol content in the fractions was determined with the cholesterol assay kit (Cayman Chemicals, Ann Arbor, MI). Cholesterol concentration in each fraction was indicated as μg/ml per microgram of protein. The protein concentration in each fraction was determined by the Bio-Rad protein assay kit (Bio-Rad). The fractions were also examined by Western blots with Src, PP2A/C, caspase-8, DR5, and FADD antibodies. Caveolin-1 antibody was used as a lipid raft marker.

**Cell Viability Assay**—Cell viability assay was described previously (33). Briefly, cells were seeded in six-well plates and treated with TRAIL, Src inhibitor dasatinib, or a combination of the two. 24 h later, cell viability was determined using trypan blue exclusion.

**Statistical Analysis**—Statistical analysis was performed using Student’s t test. The data were presented as the mean ± S.D., and p ≤ 0.05 was considered significant.
TRAIL Treatment Activates Src, and Inhibition of Src Sensitizes Breast Cancer Cells to TRAIL-induced Cell Death—To study the mechanism of TRAIL resistance, we treated breast cancer cell lines with TRAIL and then examined phosphorylation of Src at Tyr-418 and Tyr-527. Of note, phosphorylation of Tyr-418 leads to Src activation, whereas phosphorylation of Tyr-527 leads to Src inactivation. Fig. 1, A–C, show that TRAIL treatment increased the levels of phosphorylated-Src at Tyr-418, whereas the levels of p-Src at Tyr-527 remained unchanged. Because Src-mediated cell signaling is considered a survival pathway, these data suggest that TRAIL treatment activates the Src-mediated survival pathway that may counteract TRAIL-induced apoptotic cell death in breast cancer cells. To demonstrate Src indeed plays a role in TRAIL resistance, we treated breast cancer cell lines with TRAIL and then examined phosphorylation of Src at Tyr-418 and Tyr-527. Of note, phosphorylation of Tyr-418 leads to Src activation, whereas phosphorylation of Tyr-527 leads to Src inactivation. Fig. 1, A–C, show that TRAIL treatment increased the levels of phosphorylated-Src at Tyr-418, whereas the levels of p-Src at Tyr-527 remained unchanged. Because Src-mediated cell signaling is considered a survival pathway, these data suggest that TRAIL treatment activates the Src-mediated survival pathway that may counteract TRAIL-induced apoptotic cell death in breast cancer cells. To demonstrate Src indeed plays a role in TRAIL resistance, we treated breast cancer cell lines with TRAIL and then examined phosphorylation of Src at Tyr-418 and Tyr-527. Of note, phosphorylation of Tyr-418 leads to Src activation, whereas phosphorylation of Tyr-527 leads to Src inactivation. Fig. 1, A–C, show that TRAIL treatment increased the levels of phosphorylated-Src at Tyr-418, whereas the levels of p-Src at Tyr-527 remained unchanged. Because Src-mediated cell signaling is considered a survival pathway, these data suggest that TRAIL treatment activates the Src-mediated survival pathway that may counteract TRAIL-induced apoptotic cell death in breast cancer cells. To demonstrate Src indeed plays a role in TRAIL resistance, we treated breast cancer cell lines with TRAIL and then examined phosphorylation of Src at Tyr-418 and Tyr-527. Of note, phosphorylation of Tyr-418 leads to Src activation, whereas phosphorylation of Tyr-527 leads to Src inactivation. Fig. 1, A–C, show that TRAIL treatment increased the levels of phosphorylated-Src at Tyr-418, whereas the levels of p-Src at Tyr-527 remained unchanged. Because Src-mediated cell signaling is considered a survival pathway, these data suggest that TRAIL treatment activates the Src-mediated survival pathway that may counteract TRAIL-induced apoptotic cell death in breast cancer cells.

TRAIL Treatment Induces Caspase-8 Phosphorylation—To understand the mechanism of how Src activation contributes to TRAIL resistance, we focused on the effect of Src on caspase-8 activation because Src has been shown to phosphorylate and inactivate caspase-8 (21), and caspase-8 is a critical mediator of TRAIL-induced apoptosis. To determine whether Src-mediated caspase-8 phosphorylation is responsible for TRAIL resistance, we first asked whether caspase-8 was phosphorylated in TRAIL-treated cells whose Src is phosphorylated. To this end, BT549 cells were treated with TRAIL or left alone. Cell lysates were prepared and subjected to IP with caspase-8 antibody or IgG, and the level of caspase-8 was detected by Western blot analysis. Fig. 2 (left panel) shows that a tyrosine-phosphorylated protein was detected by IP with caspase-8 antibody but not with control IgG. Importantly, we were able to detect phosphorylated caspase-8 at Tyr-380 in TRAIL-treated BT549 cells (Fig. 2, right panel). Because caspase-8 phosphorylation at Tyr-380 is mediated by Src (21), simultaneous activation of Src and phosphorylation of caspase-8 suggest that TRAIL-induced Src activation may be responsible for caspase-8 phosphorylation at Tyr-380. Src Is Associated with Caspase-8 to Phosphorylate Caspase-8 at Tyrosine 380—Because both Src activation and caspase-8 phosphorylation were detected simultaneously in TRAIL-treated cells, we wanted to know whether these two events were connected. To this end, BT549 cells were treated with TRAIL or left untreated, and the resulting cells were used to perform IP with caspase-8 or Src antibody. Fig. 3A shows that caspase-8 antibodies pulled down endogenous Src and vice versa in untreated cells and that TRAIL treatment promoted their association. These results suggest that endogenous Src physically associates with endogenous caspase-8 and that TRAIL treat-
Src and TRAIL Resistance

To determine the importance of their association in terms of caspase-8 phosphorylation, we transfected a plasmid that expresses an active form of Src into BT549 cells. Forty-eight hours post-transfection, we performed IP Western to detect Src phosphorylation. Fig. 3 shows that the active form Src (p-Src-Tyr-418) was directly immunoprecipitated by a caspase-8 antibody in cells treated with TRAIL. This result suggests that TRAIL treatment activates Src and in turn phosphorylates caspase-8 at Tyr-380.

Src Associates with the Catalytic Subunit of PP2A Phosphatase and Caspase-8 and TRAIL Treatment Promotes Their Association—To understand the mechanism of Src activation in TRAIL-treated cells, we tested the involvement of PP2A phosphatase in Src activation. We chose PP2A because PP2A has been shown to target the holoenzyme to dephosphorylate Src at serine 12, a site that is important for c-Src activation upon cell stress (28). To this end, HEK293T cells co-transfected with pcDNA3-Src, PP2A/C-HA (catalytic subunit of PP2A), and caspase-8-HA were left untreated or treated with TRAIL and then harvested for Western IP. Fig. 4A shows that Src associated with PP2A/C in the absence of TRAIL treatment, which was increased further upon TRAIL stimulation. Importantly, TRAIL treatment promoted the association between endogenous Src and PP2A/C protein in BT549 cells (Fig. 4C). Furthermore, we found that PP2A/C antibody could bring down Src and vice versa (Fig. 4B). We also found that PP2A/C associated with caspase-8 upon TRAIL treatment (Fig. 4B). Because PP2A/C, caspase-8, and Src can associate and phosphorylation at Tyr-380 by Src inactivates caspase-8 (21), our results suggest that PP2A may inhibit Src-mediated caspase-8 phosphorylation.
TRAIL Stimulation Promotes an Association among Src, PP2A/C, and Caspase-8 in Lipid Rafts—Our hypothesis was that PP2A regulates Src activity to impact caspase-8 phosphorylation and activation, but several attempts failed to detect an association of Src with the DISC (data not shown), suggesting that Src is not part of the DISC complex. However, recent studies indicated that lipid rafts, microdomains of the plasma membrane enriched in cholesterol and sphingolipids, can serve as platforms for triggering death ligand-mediated apoptosis, including TRAIL-mediated apoptosis and that distribution of the components of the DISC in lipid rafts is important for death ligand-induced apoptosis (34–36). To determine an association of Src with PP2A/C and other DISC components including caspase-8 and FADD with lipid rafts, BT549 cells in the presence and absence of TRAIL treatment were subjected to discontinuous sucrose density gradients of Triton X-100 cell lysates.

**FIGURE 4.** Src interacts with PP2A/C and caspase-8, and TRAIL treatment promotes their interaction. A, Src interacts with PP2A/C and caspase-8 (Casp8). 293T cells were co-transfected with pcDNA3-Src (Src), caspase-8-HA, and PP2A/C-HA. After 48 h, cells were left untreated or treated with TRAIL (100 ng/ml) for 30 min, lysed, immunoprecipitated with IgG or Src (rabbit) antibody and blotted with caspase-8, PP2A/C, and Src (mouse) antibodies. B, PP2A/C interacts with Src and caspase-8. HEK293T cells were transfected and treated with TRAIL as described in A. Cells were lysed, immunoprecipitated with IgG, PP2A/C antibody, or caspase-8 antibody, and blotted with Src, PP2A/C, and caspase-8 antibodies. The lower panel shows input. C, endogenous Src associates with endogenous PP2A/C. BT549 cells (2.0 × 10⁷) were treated with TRAIL (100 ng/ml) for 30 min. Cell lysates were prepared, immunoprecipitated with Src antibody, PP2A/C antibody, or IgG, and blotted with PP2A/C or Src antibody. The lower panel shows input.

**FIGURE 5.** TRAIL treatment leads to redistribution of Src, PP2A/C, and caspase-8 from non-rafts to lipid rafts. A, PP2A/C and caspase-8 (Casp8) localize in non-rafts, whereas Src localizes in both non-rafts and lipid rafts in the absence of treatment. BT549 cells were subjected to discontinuous sucrose density gradients of Triton X-100 cell lysates to isolate lipid raft and non-raft fractions. Fractions 1 to 10 were subjected to Western blot analysis for the levels of Src, PP2A/C, caspase-8, DR5, and FADD. Lipid raft fractions 4 and 5 were shown by the lipid raft marker caveolin-1 (Caveo-1). B, Src, PP2A/C, and caspase-8 were redistributed from non-rafts to lipid rafts upon TRAIL treatment. BT549 cells treated with TRAIL (150 ng/ml) for 30 min were subjected to discontinuous sucrose density gradients of Triton X-100 cell lysates for separation of lipid raft and non-raft fractions. Fractions 1 to 10 were detected by Western blot analysis for the presence of Src, PP2A/C, caspase-8, DR5, and FADD. Lipid raft fractions 4 and 5 were identified by Western blot analysis using lipid raft marker caveolin-1. C, identified lipid raft fractions by cholesterol assay. Cholesterol content in the fractions was determined with the cholesterol assay kit. Cholesterol concentration in each fraction was indicated as μg/ml per microgram of protein. Concentration of cholesterol and protein were measured in fractions 1 to 10 in BT549 cells treated with TRAIL (150 ng/ml) for 30 min.
lysates to isolate lipid raft and non-raft fractions. As shown in Fig. 5A, Src, caspase-8, and FADD were detected in non-rafts, whereas Src and caveolin-1, a lipid raft marker, were detected in lipid rafts. In contrast, TRAIL treatment resulted in the redistribution of PP2A/C, caspase-8, and FADD from non-rafts to lipid rafts, and the level of Src was increased in lipid rafts compared with that in cells without TRAIL treatment (Fig. 5B). We confirmed that purity of non-rafts and lipid rafts in TRAIL-treated cells based on the concentrations of cholesterol and protein levels (Fig. 5C). Because lipid rafts are critical for death ligand-induced apoptosis, an association of Src with PP2A/C and caspase-8 suggests that Src may phosphorylate and inhibit caspase-8 activation in lipid rafts.

**PP2A Inhibits TRAIL-induced Src Activation, but TRAIL Induces PP2A/C Degradation**—Because Src associates with PP2A/C in lipid rafts, we sought to determine the effect of PP2A on Src phosphorylation. To this end, HEK293T cells co-transfected with pcDNA3-Src and PP2A/C-HA were left untreated or treated with TRAIL, followed by immunoprecipitation with PP2A/C antibody and immunoblotting with phospho-Src (Tyr-418) and PP2A/C antibodies, respectively. Fig. 6A shows that PP2A/C antibody pulled down p-Src (Tyr-418) in cells treated with TRAIL as compared with untreated cells. This result suggests that PP2A/C may directly associate with phospho-Src (Tyr-418) upon TRAIL treatment and that PP2A may play a role in Src dephosphorylation at Tyr-418. To further confirm this, we treated BT549 cells with the PP2A inhibitor LB-100 to inhibit PP2A phosphatase activity and then examined the levels of Src phosphorylation by IP with Src antibody. Fig. 6B shows that the inhibition of PP2A phosphatase activity by LB-100 increased Src phosphorylation, suggesting that PP2A may be involved in negatively regulating Src activity. Interestingly, TRAIL treatment led to the decreased levels of PP2A/C protein in BT549 (Fig. 6C) and SUM159 cells (data not shown). Importantly, we showed that TRAIL treatment induced PP2A/C ubiquitination (Fig. 6D). Thus, this result suggests that TRAIL treatment may lead to PP2A/C ubiquitination-mediated degradation and in turn release PP2A-mediated inhibition of Src activation.

**Inhibition of PP2A Activity Sensitizes Resistant Cells to TRAIL-induced Death**—Because the activation of Src sensitizes breast cancer cells to TRAIL-induced apoptosis, we asked whether such resistance mechanism occurs in human breast cancer cells that are intrinsically resistant to TRAIL. To this end, we treated MCF7 and MDA231 cells with TRAIL and showed that MCF7 cells are resistant to TRAIL, whereas MDA231 cells are sensitive to TRAIL (Fig. 7A). We found that TRAIL-resistant MCF7 cells express a higher level of P-Src
(Tyr-418) than TRAIL-sensitive MDA231 cells, whereas the similar levels of p-Src (Tyr-527) were detected between these two cell lines (Fig. 7B). Furthermore, we showed that LB100 sensitizes MCF7 cells to TRAIL-induced death (Fig. 7C). In fact, cell survival rate after the combination treatment with TRAIL and LB100 remained similar between these two cell lines (Fig. 7C). These data suggest that the activation of Src has pathophysiological relevance in human cancer cells and that targeting PP2A sensitizes resistant cancer cells to TRAIL-induced apoptosis.

**DISCUSSION**

It has been shown that breast cancer cells often have increased Src activation (37). It has also been shown that most breast cancer cells are TRAIL resistant, but the underlying mechanism is not fully understood. Here, we showed that in breast cancer cells, TRAIL treatment activates Src and that inhibition of Src activation by its pharmacological inhibitor dasatinib enhances TRAIL-induced apoptosis, suggesting that the activation of Src may contribute to TRAIL resistance in breast cancer cells.

Upon stimulation by death ligands including TRAIL, the activation of caspase-8 is critical for initiating apoptosis. A previous study indicated that Src can phosphorylate and inactivate caspase-8 (21). Because the inhibition of Src activity enhanced TRAIL induced apoptosis, we tested the effect of Src activation by its pharmacological inhibitor dasatinib on caspase-8 phosphorylation. Indeed, we showed that TRAIL treatment induced caspase-8 phosphorylation on Tyr-380 in cells whose Src was phosphorylated. Furthermore, we found that Src interacts with caspase-8 and that TRAIL treatment facilitates the interaction between caspase-8 and activated Src.

Taken together, these considerations strongly suggest that TRAIL-induced survival signaling can be in part due to Src-mediated caspase-8 phosphorylation, leading to the inability of caspase-8 to be cleaved and activated.

The role of Src activation in TRAIL resistance has been implicated, and the underlying mechanism has been suggested the involvement of the activation of the Src-mediated survival pathways, including the Src-AKT, Src-STAT3, and Src-EGFR pathways (24, 26, 27). In our study, we clearly showed that Src can phosphorylate caspase-8, a critical component of the core apoptotic machinery, thereby impairing caspase-8 activation. Therefore, the mechanisms of Src-mediated TRAIL resistance can be attributed to both the activation of Src-mediated downstream survival signaling (e.g. AKT and STAT3) and Src-mediated caspase-8 phosphorylation.

Cell survival signaling involving the Src survival pathway can be negatively regulated by several phosphatases, including PP2A (38). It has been shown that PP2A can dephosphorylate Src (28). Furthermore, a previous study showed that Src is involved in the activation of the PI3K-AKT pathway during TRAIL treatment (39). Although PP2A is a serine-threonine phosphatase, overexpression of PP2A/C has been shown to dephosphorylate Src at Tyr-418 (38), thus inhibiting its activity. These considerations led us to study the relationship between Src and PP2A/C. By performing co-immunoprecipitation experiments, we confirmed that Src associates with PP2A/C under untreated conditions, which can be enhanced in response to TRAIL treatment. Because the inhibition of PP2A phosphatase activity by a pharmacological inhibitor LB-100 increased Src phosphorylation at Tyr-418 fol-
Following TRAIL treatment, we believed that PP2A plays a negative role in Src activation. Importantly, we showed that PP2A/C is degraded in response to TRAIL treatment. Thus, our results suggest that degradation of PP2A/C relieves PP2A-mediated Src inhibition, leading to Src activation, and subsequent phosphorylation and caspase-8 inactivation.

It is well established that caspase-8 is activated in the DISC that consists of other proteins such as FADD that are critical for caspase-8 activation. Because our data suggested that targeting caspase-8 phosphorylation plays a role in Src-mediated TRAIL resistance, we speculated that Src may phosphorylate caspase-8 in the DISC. However, several attempts failed to detect Src in the DISC (data not shown), suggesting Src is not part of the DISC. Instead, we detected that Src co-exists with caspase-8, PP2A/C, and DR5 in lipid rafts where the DISC can be formed and apoptosis can be initiated (35, 36). Therefore, co-existing in lipid rafts suggests that Src is able to phosphorylate caspase-8. Consistently, TRAIL treatment caused caspase-8 phosphorylation, which is supported by the fact that in addition to triggering apoptosis, TRAIL signaling can activate survival pathways to counteract TRAIL-induced apoptosis in lipid rafts. Based on our data, we propose that PP2A regulates TRAIL signaling through two opposing pathways (Fig. 8). On the one hand, following TRAIL treatment, caspase-8 is recruited to the DISC where it is activated, leading to apoptosis. On the other hand, TRAIL-induced Src activation causes caspase-8 phosphorylation, which inhibits apoptosis. The latter appears to be more dominant in resistant cancer cells. In lipid rafts, PP2A inhibits Src-mediated caspase-8 phosphorylation to counteract Src-mediated survival signaling. Therefore, the balance between PP2A/C degradation and inhibition of Src-mediated caspase-8 phosphorylation by PP2A may dictate the outcome whether cells undergo apoptosis or survival.

In summary, we show that TRAIL treatment induces Src activation and promotes an association between caspase-8 and Src, the latter phosphorylates and inactivates caspase-8, leading to TRAIL resistance. Paradoxically, we also show that TRAIL treatment can cause degradation of PP2A/C, the catalytic subunit of a negative regulator PP2A for Src, which provides a regulatory pathway to keep Src in activated status. In this regard, Src-mediated TRAIL resistance in breast cancer cells depends on the balance between Src-mediated caspase-8 phosphorylation and PP2A-mediated Src inactivation. Thus, our data suggest that the regulation of the Src-PP2A interaction is critical for TRAIL-induced apoptosis in human breast cancer cells.

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