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c-Cbl acts as a mediator of Src-induced activation of the PI3K-Akt signal transduction pathway during TRAIL treatment

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ABSTRACT

We have previously observed that TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) induces acquired TRAIL resistance by increasing Akt phosphorylation and Bcl-xL expression. In this study, we report that Src, c-Cbl, and PI3K are involved in the phosphorylation of Akt during TRAIL treatment. Data from immunoprecipitation and immunoblotting assay reveal that Src interacts with c-Cbl and PI3K. Data from immune complex kinase assay demonstrate that Src can directly phosphorylate c-Cbl and PI3K p85 subunit protein. Data from gene knockdown experiments with an RNA interference (RNAi) technique show that c-Cbl is involved in the interaction between Src and PI3K p85 during TRAIL treatment, playing an important role in TRAIL-induced Akt phosphorylation. Taken together, c-Cbl may act as a mediator to regulate the Src-PI3K-Akt signal transduction pathway during TRAIL treatment.

1. Introduction

TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) is a type II integral membrane protein belonging to the TNF family. Like Fas ligand (FasL) and TNF-α, the c-terminal extracellular region of TRAIL (amino acids 114–281) exhibits a homotrimeric subunit structure [1]. However, unlike FasL and TNF-α, TRAIL induces apoptosis in a variety of tumor cell lines more efficiently than in normal cells [2]. The apoptotic signal of TRAIL is transduced by binding to the death receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5), which are members of the TNF receptor superfamily. Ligation of TRAIL to its receptors results in trimerization of the receptor and clustering of the receptor's intracellular death domain (DD), leading to the formation of the death-inducing signaling complex (DISC). Trimerization of the receptors leads to the recruitment of an adaptor molecule, Fas-associated death domain (FADD), and subsequent binding and activation of caspase-8 and -10. Activated caspase-8 and -10 then cleave caspase-3, which in turn leads to cleavage of the death substrate. Despite TRAIL's potential as an anticancer agent both *in vitro* and *in vivo*, some cancer cells that were originally sensitive to TRAIL-induced apoptosis can become resistant after repeated exposure (acquired resistance) [3,4], suggesting that the physiological role of TRAIL is more complex than merely activating caspase-dependent apoptosis of cancer cells [5]. For example, it was reported that TRAIL stimulated the anti-apoptotic PI3K/Akt pathway in endothelial cells [5,6] and fibroblast cells [7] as well as that TRAIL-induced PI3K/Akt and NF-κB activation in Jurkat T leukemia cells [8]. These results imply that, depending on circumstances, TRAIL can function as a cytokine of either cell death or cell survival, similar to NF-κB [9]. In the pathway of cell survival, Akt has been known to be activated by phosphorylation at threonine 308 and serine 473 in response to various growth factors through a pathway that requires PI3K-dependent generation of PI(3,4,5)P3 [10]. PIP3 facilitates the recruitment of Akt to the plasma membrane through binding with the pleckstrin homology (PH) domain of Akt. At the plasma membrane, Akt is activated by phosphoinositide-dependent kinase-1 (PDK-1) at threonine 308 and becomes fully activated after phosphorylation within the carboxy-terminus at serine 473 [11,12].

Previously, we reported that DU-145 prostate cancer cells develop acquired TRAIL resistance after TRAIL treatment, and that phosphorylated Akt (pAkt) and its downstream member Bcl-xL are involved in the process of acquired resistance [4]. However, how Akt phosphorylation is increased during development of acquisition of TRAIL resistance has not yet been clearly understood. The main point is that, as stated by Trauzold et al. [13], TRAIL and TRAIL death receptors do not only stimulate

Abbreviations: c-Cbl, Casitas B-lineage lymphoma; DD, death domain; DISC, death-inducing signaling complex; DMEM, Dulbecco's modified Eagle's medium; DR4, TRAIL-R1; DR5, TRAIL-R2; DTT, dithiothreitol; EGF, epidermal growth factor; EGFR, EGF receptor; EGTA, ethylene glycol tetraacetic acid; FADD, Fas-associated death domain; FasL, Fas/APO-1 ligand; FBS, fetal bovine serum; GST, glutathione-S transferase; PAGE, polyacrylamide gel electrophoresis; PARP, poly (ADP-ribose) polymerase; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PDK-1, phosphoinositide-dependent kinase-1; PH, pleckstrin homology; PMSF, phenylmethylsulfonyl fluoride; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; RIP, receptor-interacting protein; RNAi, RNA interference; SDS, sodium dodecyl sulfate; TNF, tumor necrosis factor; TRADD, TNF receptor-associated protein with death domain; TRAF2, TNF receptor-associated factor 2; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

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apoptosis but also engage non-apoptotic signaling pathways leading to activation of survival-related signals. One of the well known nondeath signaling pathways induced by TRAIL is through TNF receptor-associated protein with death domain (TRADD), receptor-interacting protein (RIP) and TNF receptor-associated factor 2 (TRAF2) [14], which are nondeath signaling modulatory adaptors that interact with the ligand's homo-trimerized receptors, and lead to the activation of kinase cascades resulting in activation of NF-κB and the mitogen-activated protein kinases [15]. However, activation of Akt, upstream of NF-κB [16,17], has not been well defined.

Recently, we observed that acquired TRAIL resistance is developed through degradation of TRAIL receptors as well as increased Bcl-xL expression, demonstrating that degradation of TRAIL receptors is mediated by c-Cbl (Casitas B-lineage lymphoma) during TRAIL treatment [4,18]. However, the mechanism of increased Akt phosphorylation during TRAIL treatment which is another cause of acquired resistance has not yet been clearly explained. Here, we demonstrate that c-Cbl is also responsible for TRAIL-induced Akt phosphorylation through Src-PI3K activation. Src is activated during TRAIL treatment, followed by phosphorylation of PI3K and c-Cbl. c-Cbl may act as a scaffolding molecule for phosphorylation of PI3K by Src.

We recently observed that c-Cbl functions in the degradation of TRAIL receptors as an E3 ligase [18]. In addition to this E3 ligase activity, many cellular events mediated or regulated by c-Cbl protein are dependent on its adaptor functions, suggesting c-Cbl's diverse and sometimes opposing roles in the regulation of signal transduction in response to different stimulation [19]. For example, association of the distal proline-rich sequences of c-Cbl and the SH3 domain of the p85 subunit of PI3 kinase is responsible for the activation of PI3-kinase by EGF stimulation [20–22].

In this paper we demonstrate that c-Cbl plays an important role in the TRAIL-induced activation of the Src-PI3K-Akt signal transduction pathway.

2. Materials and methods

2.1. Cell culture and survival assay

Human prostate adenocarcinoma DU-145 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA) and 26 mM sodium bicarbonate for monolayer cell culture. The cells were maintained in a humidified atmosphere containing 5% CO_2 and air at 37 °C.

2.2. Reagents and antibodies

Anti-caspase 8, anti-phosphoS473-Akt, anti-Akt, anti-phospho-Tyr416-Src, anti-Src, anti-phosphoTyr731-c-Cbl, and anti-c-Cbl were purchased from Cell Signaling (Beverly, MA, USA). Anti-phosphoTyr508 PI3K p85, anti-PI3K p85, anti-Src antibody, and protein G and protein A-agarose were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). PP-2 was purchased from Calbiochem (San Diego, CA, USA). Anti-actin was purchased from ICN (Costa Mesa, CA, USA), and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Monoclonal anti-PARP was purchased from Biomol International, L.P. (Plymouth Meeting, PA, USA). Monoclonal anti-HA (clone 3F10) was purchased from Roche Applied Science (Indianapolis, IN, USA). Monoclonal anti-actin was purchased from ICN (Costa Mesa, CA, USA).

2.3. RNA interference by siRNA c-Cbl or by siRNA caspase-8

To construct siRNA of c-Cbl, pSilencer 2.1-U6 hygro vector (Ambion, Inc., Austin, TX, USA) was used for expressing siRNA for c-Cbl. The insert for hairpin siRNA into pSilencer was prepared by annealing two oligonucleotides. For human c-Cbl siRNA, the top strand sequence was 5′-GATCCGATGGAGACACTTGGAGAATTCAAGAGATTCTCCAAGTGTCTC-CATCTTTTTTGGAAA-3′, and the bottom strand sequence was 5′-AGCTTTTCCAAAAAAGATGGAGACACTTGGAGAATCTCTTGAATTCTC-CAAGTGTCTCCATCG-3′. The annealed insert was cloned into pSilencer 2.1-U6 hygro digested with BamH I and Hind III. The correct structure of pSilencer 2.1-U6 hygro-c-Cbl was confirmed by nucleotide sequencing. The resultant plasmid, pSilencer-c-Cbl, was transfected into DU-145 cells. The interference of c-Cbl protein expression was confirmed by immunoblot using anti-c-Cbl antibody (Cell Signaling). To construct siRNA of caspase-8, we used the same methods described previously for si c-Cbl except the insert for hairpin siRNA into pSilencer. For human caspase-8 siRNA, the top strand sequence was 5′-GATCCAGGGAACTT-CAGACACCAGTTCAAGAGACTGGTGTCTGAAGTTCCCTTTTTTTGGAAA-3′, and the bottom strand sequence was 5′-AGCTTTTCCAAAAAAAGGG-AACTTCAGACACCAGTCTCTTGAACTGGTGTCTGAAGTTCCCTG-3′ was used for annealing.

2.4. Protein extracts and polyacrylamide gel electrophoresis

Cells were lysed with 1× Laemmli lysis buffer (2% sodium dodecyl sulfate, 10% glycerol, 0.002% bromophenol blue, 62.5 mM Tris, pH 6.8) and boiled for 10 min. Protein content was measured with BCA Protein Assay Reagent (Pierce, Rockford, IL, USA). The samples were diluted with 1× lysis buffer and β-mercaptoethanol was added to be 350 mM, then equal amounts of protein were loaded on 10% or 15% sodium dodecyl sulfate (SDS)-polyacrylamide gels. SDS-PAGE analysis was performed according to Laemmli using a Hoefer gel apparatus.

2.5. Immunoblot analysis

Proteins were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membrane. The nitrocellulose membrane was blocked with 5% nonfat dry milk in PBS-Tween-20 (0.1%, v/v) at 4 °C overnight. The membrane was incubated with primary antibody (diluted according to the manufacturer's instructions) for 2 h. Horseradish peroxidase conjugated anti-rabbit or anti-mouse IgG was used as the secondary antibody. Immunoreactive protein was visualized by the chemiluminescence protocol (ECL, Amersham, Arlington Heights, IL, USA).

2.6. In vivo binding

To examine the interaction between Src and c-Cbl or phosphorylated PI3K p85 and the interaction between PI3K p85 and c-Cbl, DU-145 cells in 100-mm culture plates were treated with TRAIL (100 ng/ml) for various times (0–4 h). For immunoprecipitation, cells were lysed in buffer containing 150 mM NaCl, 20 mM Tris–HCl (pH 7.5), 10 mM EDTA, 1% Triton X-100, 1% deoxycholate, 1 mM phenylmethylsulfonyl fluoride

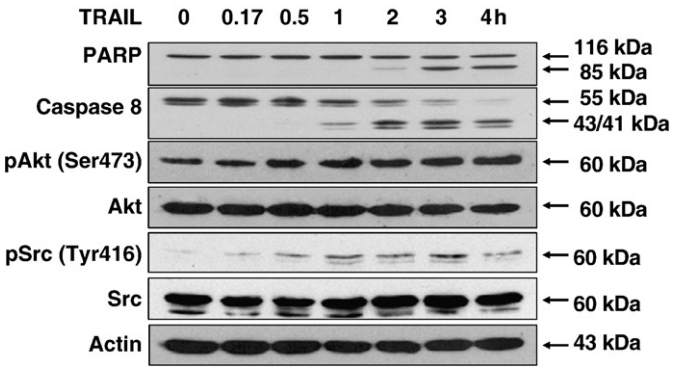

Fig. 1. TRAIL-induced Akt and Src activation. DU-145 cells were treated with 100 ng/ml TRAIL for various times (0–4 h). Cells were lysed, and lysates were analyzed for the detection of PARP, caspase 8, phosphorylated Akt, Akt, phosphorylated Src and Src. Actin was used to confirm the equal amount of proteins loaded in each lane.