Critical Role for Caspase-8 in Epidermal Growth Factor Signaling

Darren Finlay, Amy Howes, and Kristiina Vuori
Cancer Center, Burnham Institute for Medical Research, La Jolla, California

Abstract
Caspase-8 has a well-defined canonical role as an apical protease of the extrinsic apoptosis pathway. Evidence is growing, however, that the protein has numerous other nonapoptotic functions. We have previously shown that caspase-8 is required for efficient adhesion-induced activation of the extracellular signal–regulated kinase (Erk)-1/2 pathway. We now show that caspase-8 is also necessary for the efficient activation of downstream events associated with epidermal growth factor (EGF) signaling. This promotion of EGF-induced Erk1/2 activation is independent of the proteolytic activity of caspase-8 and can be recapitulated using only the pro-domains of the protein. In addition, we identify specific residues within the caspase-8 “RXDLL motif” that are essential for Erk pathway activation. Furthermore, these residues are also involved in forming a complex with the tyrosine kinase Src. Caspase-8 null cells and cells reconstituted with caspase-8 harboring point mutations of these critical amino acids also show defective EGF-induced migration as compared with cells reconstituted with the wild-type protein. In sum, we provide the first evidence for caspase-8 as an essential component of growth factor signaling and suggest that this may be due to its association with Src. As the EGF/Src pathway activity has been shown to promote oncogenic events, our findings that caspase-8 is necessary for these activities may help explain why it is rarely deleted or silenced in tumors.

Introduction
Caspase-8 is a well-characterized protease of the “extrinsic” apoptotic pathway known to be important in death receptor–mediated killing. Recruitment of caspase-8 to activated death receptors results in its dimerization, activation, subsequent auto-processing, and initiation of the “effector” caspase activity associated with classic apoptosis (1–4). This death receptor–mediated apoptosis has been the focus of various attempts to induce cell death in tumors. Indeed, tumor necrosis factor–related apoptosis-inducing ligand has been shown to induce apoptosis in a variety of tumor, but not normal, cells (5, 6). Whereas resistance to death receptor ligands currently limits their efficacy, deletion or silencing of essential proteins of the cascade, such as caspase-8, occurs only extremely infrequently in cancers (7). Caspase-8 has in fact been shown to have increased expression in lung cancers (8), supporting the hypothesis that the protein may be involved in other nonapoptotic but potentially promutagenic events.

Mounting evidence to support alternative nonapoptotic functions for caspase-8 has emerged in recent years (reviewed in refs. 9–11). Several reports have shown a role for caspase-8 in hematopoietic cell proliferation and “maturation” (e.g., refs. 12–14), whereas other laboratories have shown caspase-8 to be essential for activation of nuclear factor κB (15–17). Further data describing a role for caspase-8 in adhesion and cell motility have recently accumulated. Involvement of caspase-8 in cell motility has been described by several independent laboratories (18–21). We recently showed an essential role for caspase-8 in promoting cell adhesion–induced activation of the extracellular signal–regulated kinase (Erk)-1/2 pathway through association with Src (7). Intriguingly, this activation is independent of the catalytic activity of caspase-8 and can be recapitulated in caspase-8 null cells using only the NH2-terminal “death effector domains” (DED). Indeed, the DEDs alone are capable of forming a protein complex with Src and act indistinguishably from full-length caspase-8 in these biochemical and physiologic analyses.

Here we show that caspase-8 is also critical for epidermal growth factor (EGF)–induced activation of the Erk pathway. Again the DEDs alone of caspase-8 are sufficient for this activation, and we identify residues within the so called “RXDLP motif” that are essential for the promulgation of EGF signaling. We show that caspase-8 is required for EGF-induced cell migration and that point mutants of the RXDLP motif show impaired motility similar to that of caspase-8 null cells. In sum, we provide the first evidence that caspase-8 is an essential component of growth factor signaling pathways and that its effects in this regard may be due to the ability of DEDs to associate with a protein complex containing Src. That caspase-8 is involved not only in adhesion-induced but also in growth factor–induced signaling may help explain why it is so seldom silenced or deleted in tumors. In addition, these findings suggest that potentially driving caspase-8 from nonapoptotic to more canonical proapoptotic signaling may be an important therapeutic intervention point in cancers.

Materials and Methods
Reagents. Unless otherwise specified, all reagents were from Sigma-Aldrich. AG1478, PP1, and SU6656 were from Calbiochem. Matrigel is from BD Biosciences. Zeocin and Primocin are from InvivoGen. EGF was always used at a concentration of 100 ng/mL and platelet-derived growth factor (PDGF) was used at 50 ng/mL.

Cell culture, DNA transfections, and stable cell line generation. 293T and HeLa cells were cultured in standard DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin/t-glutamine (Omega Scientific, Inc.). MDA-MB-231 cells were cultured in F12/DMEM (1:1) with 10% FBS and penicillin/streptomycin/t-glutamine, whereas SKOV3 cells were grown in McCoy’s 5A medium with 10% FBS, nonessential amino acids (Hyclone), and penicillin/streptomycin/t-glutamine. SH-SY5Y cells were cultured in α-MEM plus 10% FBS and penicillin/streptomycin/t-glutamine. Cultures were grown in McCoy’s 5A medium with 10% FBS, nonessential amino acids (Hyclone), and penicillin/streptomycin/t-glutamine. Cultures were grown in McCoy’s 5A medium with 10% FBS, nonessential amino acids (Hyclone), and penicillin/streptomycin/t-glutamine.

Caspase-8–deficient NB7 cells were a kind gift from Dr. Jill Lahti (St. Jude Children’s Research Hospital, Memphis, TN) and were maintained in RPMI 1640 supplemented with 10% FBS, penicillin/streptomycin/t-glutamine, and 100 μg/mL Primocin (InvivoGen). Plasmids encoding shRNA mirs were obtained from OpenBiosystems. Caspase-8 cDNA and a c-Src expression

www.aacrjournals.org

Note: Supplemenetary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Kristiina Vuori, Cancer Center, Burnham Institute for Medical Research, La Jolla, CA 92037. Phone: 858-648-3129; Fax: 858-795-5272; E-mail: kvuori@burnham.org.

©2009 American Association for Cancer Research.
doi:10.1158/0008-5472.CAN-08-3731

Published OnlineFirst May 26, 2009; DOI: 10.1158/0008-5472.CAN-08-3731

Downloaded from cancerres.aacrjournals.org on July 28, 2017. © 2009 American Association for Cancer Research.
plasmid were kind gifts from Drs. Guy Salvesen and Sara Courtneidge, respectively (both at Burnham Institute for Medical Research, La Jolla, CA). Plasmid DNA was transfected using Fugene 6 (Roche Applied Science) as per manufacturer’s instructions, and point mutants were generated using a QuikChange XL Mutagenesis kit (Stratagene). For stable cell line production, the cDNAs of interest were cloned into MSCV-IRES-zeo plasmid were kind gifts from Drs. Guy Salvesen and Sara Courtneidge, respectively (both at Burnham Institute for Medical Research, La Jolla, CA). Plasmid DNA was transfected using Fugene 6 (Roche Applied Science) as per manufacturer’s instructions, and point mutants were generated using a QuikChange XL Mutagenesis kit (Stratagene). For stable cell line production, the cDNAs of interest were cloned into MSCV-IRES-zeo plasmid were kind gifts from Drs. Guy Salvesen and Sara Courtneidge, respectively (both at Burnham Institute for Medical Research, La Jolla, CA). Plasmid DNA was transfected using Fugene 6 (Roche Applied Science) as per manufacturer’s instructions, and point mutants were generated using a QuikChange XL Mutagenesis kit (Stratagene). For stable cell line production, the cDNAs of interest were cloned into MSCV-IRES-zeo plasmid were kind gifts from Drs. Guy Salvesen and Sara Courtneidge, respectively (both at Burnham Institute for Medical Research, La Jolla, CA). Plasmid DNA was transfected using Fugene 6 (Roche Applied Science) as per manufacturer’s instructions, and point mutants were generated using a QuikChange XL Mutagenesis kit (Stratagene). For stable cell line production, the cDNAs of interest were cloned into MSCV-IRES-zeo plasmid were kind gifts from Drs. Guy Salvesen and Sara Courtneidge, respectively (both at Burnham Institute for Medical Research, La Jolla, CA). Plasmid DNA was transfected using Fugene 6 (Roche Applied Science) as per manufacturer’s instructions, and point mutants were generated using a QuikChange XL Mutagenesis kit (Stratagene). For stable cell line production, the cDNAs of interest were cloned into MSCV-IRES-zeo plasmid were kind gifts from Drs. Guy Salvesen and Sara Courtneidge, respectively (both at Burnham Institute for Medical Research, La Jolla, CA). Plasmid DNA was transfected using Fugene 6 (Roche Applied Science) as per manufacturer’s instructions, and point mutants were generated using a QuikChange XL Mutagenesis kit (Stratagene). For stable cell line production, the cDNAs of interest were cloned into MSCV-IRES-zeo plasmid were kind gifts from Drs. Guy Salvesen and Sara Courtneidge, respectively (both at Burnham Institute for Medical Research, La Jolla, CA). Plasmid DNA was transfected using Fugene 6 (Roche Applied Science) as per manufacturer’s instructions, and point mutants were generated using a QuikChange XL Mutagenesis kit (Stratagene). For stable cell line production, the cDNAs of interest were cloned into MSCV-IRES-zeo plasmid were kind gifts from Drs. Guy Salvesen and Sara Courtneidge, respectively (both at Burnham Institute for Medical Research, La Jolla, CA). Plasmid DNA was transfected using Fugene 6 (Roche Applied Science) as per manufacturer’s instructions, and point mutants were generated using a QuikChange XL Mutagenesis kit (Stratagene). For stable cell line production, the cDNAs of interest were cloned into MSCV-IRES-zeo plasmid were kind gifts from Drs. Guy Salvesen and Sara Courtneidge, respectively (both at Burnham Institute for Medical Research, La Jolla, CA). Plasmid DNA was transfected using Fugene 6 (Roche Applied Science) as per manufacturer’s instructions, and point mutants were generated using a QuikChange XL Mutagenesis kit (Stratagene). For stable cell line production, the cDNAs of interest were cloned into MSCV-IRES-zeo plasmid were kind gifts from Drs. Guy Salvesen and Sara Courtneidge, respectively (both at Burnham Institute for Medical Research, La Jolla, CA). Plasmid DNA was transfected using Fugene 6 (Roche Applied Science) as per manufacturer’s instructions, and point mutants were generated using a QuikChange XL Mutagenesis kit (Stratagene). For stable cell line production, the cDNAs of interest were cloned into MSCV-IRES-zeo plasmid were kind gifts from Drs. Guy Salvesen and Sara Courtneidge, respectively (both at Burnham Institute for Medical Research, La Jolla, CA). Plasmid DNA was transfected using Fugene 6 (Roche Applied Science) as per manufacturer’s instructions, and point mutants were generated using a QuikChange XL Mutagenesis kit (Stratagene). For stable cell line production, the cDNAs of interest were cloned into MSCV-IRES-zeo plasmid were kind gifts from Drs. Guy Salvesen and Sara Courtneidge, respectively (both at Burnham Institute for Medical Research, La Jolla, CA). Plasmid DNA was transfected using Fugene 6 (Roche Applied Science) as per manufacturer’s instructions, and point mutants were generated using a QuikChange XL Mutagenesis kit (Stratagene). For stable cell line production, the cDNAs of interest were cloned into MSCV-IRES-zeo plasmid were kind gifts from Drs. Guy Salvesen and Sara Courtneidge, respectively (both at Burnham Institute for Medical Research, La Jolla, CA). Plasmid DNA was transfected using Fugene 6 (Roche Applied Science) as per manufacturer’s instructions, and point mutants were generated using a QuikChange XL Mutagenesis kit (Stratagene). For stable cell line production, the cDNAs of interest were cloned into MSCV-IRES-zeo plasmid were kind gifts from Drs. Guy Salvesen and Sara Courtneidge, respectively (both at Burnham Institute for Medical Research, La Jolla, CA). Plasmid DNA was transfected using Fugene 6 (Roche Applied Science) as per manufacturer’s instructions, and point mutants were generated using a QuikChange XL Mutagenesis kit (Stratagene). For stable cell line production, the cDNAs of interest were cloned into MSCV-IRES-zeo plasmid were kind gifts from Drs. Guy Salvesen and Sara Courtneidge, respectively (both at Burnham Institute for Medical Research, La Jolla, CA). Plasmid DNA was transfected using Fugene 6 (Roche Applied Science) as per manufacturer’s instructions, and point mutants were generated using a QuikChange XL Mutagenesis kit (Stratagene). For stable cell line production, the cDNAs of interest were cloned into MSCV-IRES-zeo plasmid were kind gifts from Drs. Guy Salvesen and Sara Courtneidge, respectively (both at Burnham Institute for Medical Research, La Jolla, CA). Plasmid DNA was transfected using Fugene 6 (Roche Applied Science) as per manufacturer’s instructions, and point mutants were generated using a QuikChange XL Mutagenesis kit (Stratagene). For stable cell line production, the cDNAs of interest were cloned into MSCV-IRES-zeo plasmid were kind gifts from Drs. Guy Salvesen and Sara Courtneidge, respectively (both at Burnham Institute for Medical Research, La Jolla, CA). Plasmid DNA was transfected using Fugene 6 (Roche Applied Science) as per manufacturer’s instructions, and point mutants were generated using a QuikChange XL Mutagenesis kit (Stratagene). For stable cell line production, the cDNAs of interest were cloned into MSCV-IRES-zeo plasmid were kind gifts from Drs. Guy Salvesen and Sara Courtneidge, respectively (both at Burnham Institute for Medical Research, La Jolla, CA). Plasmid DNA was transfected using Fugene 6 (Roche Applied Science) as per manufacturer’s instructions, and point mutants were generated using a QuikChange XL Mutagenesis kit (Stratagene). For stable cell line production, the cDNAs of interest were cloned into MSCV-IRES-zeo plasmid were kind gifts from Drs. Guy Salvesen and Sara Courtneidge, respectively (both at Burnham Institute for Medical Research, La Jolla, CA). Plasmid DNA was transfected using Fugene 6 (Roche Applied Science) as per manufacturer’s instructions, and point mutants were generated using a QuikChange XL Mutagenesis kit (Stratagene). For stable cell line production, the cDNAs of interest were cloned into MSCV-IRES-zeo plasmid were kind gifts from Drs. Guy Salvesen and Sara Courtneidge, respectively (both at Burnham Institute for Medical Research, La Jolla, CA). Plasmid DNA was transfected using Fugene 6 (Roche Applied Science) as per manufacturer’s instructions, and point mutants were generated using a QuikChange XL Mutagenesis kit (Stratagene). For stable cell line production, the cDNAs of interest were cloned into MSCV-IRES-zeo plasmid were kind gifts from Drs. Guy Salvesen and Sara Courtneidge, respectively (both at Burnham Institute for Medical Research, La Jolla, CA). Plasmid DNA was transfected using Fugene 6 (Roche Applied Science) as per manufacturer’s instructions, and point mutants were generated using a QuikChange XL Mutagenesis kit (Stratagene). For stable cell line production, the cDNAs of interest were cloned into MSCV-IRES-zeo plasmid were kind gifts from Drs. Guy Salvesen and Sara Courtneidge, respectively (both at Burnham Institute for Medical Research, La Jolla, CA). Plasmid DNA was transfected using Fugene 6 (Roche Applied Science) as per manufacturer’s instructions, and point mutants were generated using a QuikChange XL Mutagenesis kit (Stratagene). For stable cell line production, the cDNAs of interest were cloned into MSCV-IRES-zeo plasmid were kind gifts from Drs. Guy Salvesen and Sara Courtneidge, respectively (both at Burnham Institute for Medical Research, La Jolla, CA). Plasmid DNA was transfected using Fugene 6 (Roche Applied Science) as per manufacturer’s instructions, and point mutants were generated using a QuikChange XL Mutagenesis kit (Stratagene). For stable cell line production, the cDNAs of interest were cloned into MSCV-IRES-zeo
Results and Discussion

Caspase-8 is essential for EGF-induced activation of the Erk1/2 pathway. We have previously shown a role for caspase-8 in promoting cell adhesion and downstream activation of the Erk1/2 pathway (7), and others have shown a role for caspase-8 in adhesion and motility (refs. 19–21 and reviewed in ref. 22). Surprisingly, while investigating EGF-induced motility, we discovered that caspase-8 also plays a critical role in growth factor signaling (Fig. 1). NB7 neuroblastoma cells deficient in caspase-8 (refs. 7, 23; Fig. 1A, lanes 1–4) show defective EGF-induced activation of the Erk1/2 pathway as compared with cells reconstituted with wild-type caspase-8 (Fig. 1A, lanes 5–8) over the course of 1 hour at all EGF concentrations tested (not shown). This is not a cell-specific effect because SH-SY5Y neuroblastoma cells with no detectable caspase-8 expression also show impaired activation of

Figure 2. The caspase-8 RXDLL motif is critical for EGF-induced Erk pathway activation. A, alignment of the NH2-terminal protein sequences of caspase-8 and caspase-10. B, multispecies alignment of a 30-amino-acid region containing the RXDLL motif (top). Cladogram of the alignment presented above (bottom). C, immunoblot analysis of EGF-induced Erk pathway activation in NB7 cells lacking caspase-8 reconstituted with empty vector (E.V.), with caspase-8 (W.T.), or with R71A, D73A, or L74A point mutants of caspase-8 (lanes 1–5, respectively). D, immunoblot analysis of EGF-induced Erk pathway activation in NB7 cells reconstituted with wild-type caspase-8 (Casp8; lanes 1–3) or with R71A point mutant of caspase-8 (Casp8R71A; lanes 4–6) in the presence and absence of AG1478 (10 μmol/L; top) or PP1 (1 μmol/L) or SU6656 (10 μmol/L; bottom).
the Erk pathway in response to EGF as compared with cells reconstituted with the protein (Supplementary Fig. S1). We note that EGF also induces phosphorylation of Akt at serine 473 but that this activation is independent of the presence or absence of caspase-8, suggesting that the phosphatidylinositol 3-kinase pathway is not affected. The activation of the Erk pathway in response to EGF (100 ng/mL, 3 minutes) is not dependent on the proteolytic activity of caspase-8 because expression of a catalytically inactive point mutant of caspase-8 (C360A; ref. 24) promotes activity comparable to that of the wild-type protein (Fig. 1B). A schematic diagram of caspase-8 indicating domains and residues of interest is also shown. We note that targeted depletion of caspase-8 (>90%) using shRNA-mirs is insufficient to inhibit EGF-induced Erk signaling, implying a signaling role that can be effected by relatively low levels of the protein (Supplementary Fig. S2). As we have previously shown that adhesion-induced activation of the Erk pathway is also dependent on caspase-8, these findings suggest that the protein may have a more generalized, nonapoptotic role as a critical factor in several signaling events. To investigate if other growth factors were dependent on caspase-8 for efficient Erk pathway signaling, caspase-8–deficient NB7 cells (NB7, Fig. 1C, lane 1) or NB7 cells reconstituted with wild-type caspase-8 (NB7 + Casp8, lane 2) or a catalytically inactive point mutant of caspase-8 (NB7 + Casp8C360A, lane 3) were treated with 50 ng/mL PDGF for 10 minutes and analyzed by immunoblotting. Again, the caspase-8 null NB7 cells showed impaired activation of the Erk, but not Akt, pathway as compared with cells expressing wild-type or a proteolytically inactive form of caspase-8. Caspase-8 was also found to be essential for tumor necrosis factor α–induced activation of the Erk pathway in these cells (data not shown).

Our prior studies suggested that caspase-8 effected adhesion-mediated activation of the Erk pathway by facilitating Src family kinase activity through association with its DED NH2-terminal pro-domain. In these studies, caspase-8 was further shown to maintain Src in a detergent-soluble fraction, presumably to facilitate appropriate cellular localization (7). Here, NB7 cells reconstituted with only the DEDs of caspase-8 show EGF-induced Erk pathway activation at least comparable to that of cells expressing the caspase-8C360A protein (Fig. 1D, lanes 3 and 2, respectively). Thus, these findings suggest that the DEDs of caspase-8 play an essential role in facilitating both EGF-induced (Fig. 1) and adhesion-induced (7) Erk pathway activation.

The caspase-8 RXDLL motif is critical for EGF-induced Erk pathway activation. Because the DEDs of caspase-8 are sufficient to promote both the adhesion- and EGF-induced activation of the Erk1/2 pathway, it was of interest to identify specific residues that may be involved. Although NB7 cells do not express caspase-8, they do express its related homologues, FADD, c-FLIP, and caspase-10...
The presence of caspase-10 in humans seems to result from a recent evolutionary duplication of caspase-8 due to its close homology (Fig. 2A) and absence from some other mammalian species such as mouse (25). Whereas caspase-10 has been shown to be interchangeable with caspase-8 in some apoptotic death receptor events (26), the impaired EGF signaling in caspase-8 null cells occurs in the presence of caspase-10, suggesting a divergence of function between the two proteins. Further to these findings, Muppidi and colleagues (27) identify specific residues within the adaptor protein FADD that are conserved in many DED-containing proteins and are essential for its self-association. Interestingly, this “conserved RXDLL motif” is not conserved in caspase-10 (Fig. 2A).

We noticed that the RXDLL motif of caspase-8 is widely conserved across many divergent species from human to platypus or zebrafish (Fig. 2B), suggesting an important role. To investigate the role of the RXDLL motif, we generated NB7 cells reconstituted with caspase-8 wild-type or point mutants of this region and tested their respective ability to transduce EGF signaling. Whereas the mutation of arginine 71 to alanine (R71A) showed Erk pathway activation comparable to wild-type protein, the D73A and L74A mutants showed extremely impaired signaling similar to the caspase-8 null cells (Fig. 2C). We used further pharmacologic analysis of the responsive cell lines (NB7 + Casp8 and NB7 + Casp8R71A) to investigate canonical signaling. EGF-induced activation of the Erk pathway was blocked by the EGF receptor inhibitor AG1478 in both cell lines (Fig. 2D, top). In addition, EGF activation of the Erk pathway was also impaired by Src kinase inhibitors PP1 or SU6656 (Fig. 2D, bottom).

The caspase-8 RXDLL motif is essential both for association with Src and for EGF-induced cell motility. The observation that Src family members may be involved in EGF-mediated signaling events (Fig. 2D) is consistent with our previous findings that caspase-8 can associate with a protein complex containing Src (7). We previously showed that endogenous caspase-8 and Src are associated in U87 glioblastoma cells and that the DEDs alone of these proteins were sufficient yet critical for this protein complex formation. Furthermore, others have shown that Src can phosphorylate caspase-8 (on Y380), suggesting that at least a temporal association indeed takes place (28). Using transient expression studies, we show that c-Src coimmunoprecipitates with wild-type and the R71A mutant of caspase-8. Consistent with the impaired Erk signaling, however, both the D73A and L74A mutants fail to associate with c-Src under the same experimental conditions (Fig. 3A, left). We also confirm that the catalytic activity of caspase-8 is not required for this association as the C360A mutant is also coimmunoprecipitated with c-Src. Interestingly, we show that this association is not affected by a dual C360A/Y380F mutation of caspase-8 (Fig. 3A, right). These coimmunoprecipitations were also confirmed in the reverse direction. We show that the wild-type caspase-8 or the R71A mutant coimmunoprecipitates with endogenous Src in NB7 cells, whereas the D73A and L74A point mutants are defective in this respect (Fig. 3B, left). Consistent with our previous finding (Fig. 3A), nonproteolytic (C360A) and the C360A/Y380F dual mutant of caspase-8 also coprecipitate with endogenous Src (Fig. 3B, right).

To investigate if this ability to associate with Src and promote Erk pathway signaling is involved in EGF-induced cell motility, we used migration assays as described by Barbero and colleagues (21). Caspase-8 null NB7 cells and NB7 cells reconstituted with the wild-type protein or with the R71A, D73A, or L74A mutants (Fig. 2) were analyzed for cell motility as described. NB7 cells reconstituted with wild-type or R71A caspase-8 showed enhanced EGF-induced cell motility compared with caspase-8 null NB7 cells or cells expressing the D73A or L74A mutants (Fig. 3C). Representative images of the cell migration in wound healing assays used to generate quantitative data are depicted in Fig. 3D. Furthermore, the caspase-8 null NB7 cells seeded in Matrigel failed to migrate and/or invade the matrix and remained instead as individual cells. Cells reexpressing caspase-8, however, showed enhanced invasive properties by migrating through the Matrigel and showing an elongated phenotype (Supplementary Fig. S3).

Caspase-8 seems to modulate EGF signaling through Src. Because caspase-8 associates with a protein complex containing Src, it was of interest to further elucidate mechanistically if it could modulate Src signaling. We show that EGF promotes the association of caspase-8 and Src (Fig. 4A) and, furthermore, that the Src coupled with caspase-8 in response to EGF is catalytically active (Fig. 4B). More striking evidence is provided by our observation that cells lacking caspase-8 show impaired EGF-induced Src activation as compared with cells reconstituted with caspase-8C360A (Fig. 4C). Also we present data showing that the presence or absence of caspase-8 has no effect on the phosphorylation of the Erk signaling pathway.

**Figure 4.** Caspase-8 seems to modulate EGF signaling through Src. A, anti-Src immunoblot of anti-HA immunoprecipitates from NB7 + Casp8C360A-HA cells serum starved for 24 h and then treated with either vehicle or EGF (100 ng/mL) for 3 min. Anti-Src and anti-caspase-8 immunoblots of the total cell lysates (TCL) used for immunoprecipitations are shown at the bottom. B, anti-P-Y416Src immunoblot of anti-HA immunoprecipitates from NB7 + Casp8C360A-HA cells serum starved for 24 h and then treated with either vehicle or EGF (100 ng/mL) for 3 min. Anti-Src and anti-caspase-8 immunoblots of the total cell lysates used for immunoprecipitations are shown at the bottom. C, anti-P-Y416Src immunoblot of anti-Src immunoprecipitates from NB7 + E.V. or NB7 + Casp8C360A-HA cells serum starved for 24 h and then treated with EGF (100 ng/mL) for 3 min. Anti-Src and anti-caspase-8 immunoblots of the total cell lysates used for immunoprecipitations are shown at the bottom. D, immunoblot analysis of vehicle (lanes 1 and 3) or EGF (100 ng/mL, 3 min)–induced EGFR activation in NB7 cells lacking caspase-8 (lanes 1 and 2) or same reconstituted with wild-type protein (lanes 3 and 4).
EGFR in response to EGF (Fig. 4D). Taken together, these data suggest that caspase-8 modulates the observed effects in growth factor signaling, not at the level of the growth factor receptor itself but through Src activation. Presently, it remains to be determined if caspase-8 can modulate EGF signaling in a Src-independent manner, or whether the role of caspase-8 in EGF-induced Erk activation is limited to cells in which EGF-induced Erk activation is Src dependent.

Caspase-8 tyrosine 380 may be involved in negative regulation of EGF signaling. Recent studies of the role of caspase-8 in cell adhesion and motility, and in particular, a potential involvement of phosphorylation at Y380, have yielded some seemingly contradictory results. Caspase-8 has been shown to be phosphorylated by Src on this residue in response to EGF or adhesion to fibronectin (28). Whereas we have observed no differences in the capability of the caspase-8C360A/Y380F dual mutant to affect cell adhesion, other groups have shown a role for the Y380 site in cell motility studies that used the caspase-8Y380F mutant. To assess the potential involvement of caspase-8 Y380 in EGF-induced signaling, we first analyzed Erk and Akt pathway activities in cells lacking caspase-8 and in cells that had been reconstituted with either the NH2-terminal DEDs alone (this region lacks the Y380 site) or the COOH-terminal “caspase domain” alone (this region contains the Y380 site; Fig. 5A, lanes 1–3, respectively). As noted earlier, the DEDs alone are sufficient to permit activation of the Erk pathway in response to EGF to a similar extent as the wild-type protein (Figs. 1D and 5A, lane 2). Cells expressing the caspase domain only mutant in turn show impaired Erk activation that is comparable to that observed in the caspase-8 null cells (Fig. 5A, lane 3). Thus, these results suggest that the COOH terminus of caspase-8 (which contains the Y380 site) does not directly contribute to the Erk pathway activation by EGF, and that the DED domains of caspase-8 are both necessary and sufficient to mediate Erk activation. Of note, we see no impairment of activation of the Akt pathway in the absence or presence of caspase-8 (Fig. 1A) or in cells expressing either the DEDs alone or caspase domain alone forms of caspase-8 (Fig. 5A).

As shown previously, the proteolytically inactive C360A mutant of caspase-8 promotes EGF-induced activation of the Erk pathway to the same extent as the wild-type protein (Figs. 1B and 5B). Unexpectedly, we found that the caspase-8C360A/Y380F dual mutant fails to allow efficient EGF-induced Erk pathway activation to take place. Taken in isolation, this result could imply a direct role for the Y380 residue in caspase-8–dependent Erk signaling. However, this result is quite surprising in light of the fact that the caspase domain alone form does not contribute to Erk activation, and that the DEDs alone form fully restores Erk activation downstream of EGF (Fig. 5A). More intriguingly, we found that although wild-type caspase-8 expression, or expression of the caspase domain alone form of caspase-8, has no effect on Akt pathway activation (see above), cells expressing the C360A/Y380F dual mutant show impaired Akt signaling (Fig. 5B, lane 3). In other words, expression of the caspase-8C360A/Y380F mutant seems to have a negative effect on both Erk and Akt signaling. Thus, we postulate that an “inhibitory signaling moiety” may be associated with the C360A/Y380F protein and, as a corollary, with the wild-type caspase-8 protein when not phosphorylated at Y380. Accordingly, we suggest that EGF facilitates phosphorylation of caspase-8 on Y380, resulting in a loss of inhibition of the Erk and Akt signaling pathways by the Y380 site. On caspase-8 phosphorylation on Y380, the DED-associated Src complex then additionally potentiates the activity of the Erk pathway (but not of the Akt signaling pathway).

At present time, it remains to be determined how the unphosphorylated Y380 site (or the Y380F mutant site) could exert its negative effects. The phosphorylation status of this site does not seem to affect the association of the DED domains with the Src protein complex (Fig. 3A and B). Because the most attractive candidates for negative regulation of classic kinase cascades are phosphatases, we decided to ascertain if general phosphatase activity...
was involved in our model of negative signaling by the caspase-8 C360A/Y380F protein. To test this hypothesis, we repeated the previous experiment but assayed Erk pathway activity in the cells expressing the C360A/Y380F mutant in the presence or absence of the phosphatase inhibitor sodium orthovanadate. We show that the ablation of phosphatase activity restores the Erk signaling of the C360A/Y380F mutant to levels comparable to, but not exceeding, those of the C360A mutant (Fig. 5C). We also show that this is a true caspase-8 response because treatment of NB cells lacking caspase-8 with sodium orthovanadate either alone or in combination with EGF has no effect on Erk pathway activation (Supplementary Fig. S4). Thus, we not only provide preliminary evidence of an inhibitory moiety potentially associated with caspase-8 Y380 but also suggest that phosphatase activity may be involved. Future proteomic analyses of the components of the caspase-8 signaling complex should allow for identification of the phosphatase potentially involved, thus facilitating more robust confirmation. A very basic diagram displaying the proposed mechanism of our model system as discussed is shown in Fig. 5D. We note, however, that the system is likely to be more complicated and may vary based on temporal, dynamic, and/or contextual factors. Indeed, others have shown that phosphorylation of caspase-8 may also allow binding of the p85 subunit of phosphatidylinositol 3-kinase, potentially displacing an inhibitory molecule or complex (20).

In sum, we provide further evidence to suggest that caspase-8 is involved in nonapoptotic and indeed potentially antiapoptotic roles. We show that caspase-8 is essential for EGF (and PDGF)-induced activation of the Erk, but not Akt, pathway. We further show that the protein effects these actions via its DED pro-domain and identify residues within the RXDLL motif that are critical. We postulate that this is presumably through an ability to associate with a protein complex containing Src because point mutants unable to do so show impaired EGF-induced signaling and cell migration. We further show that cells lacking caspase-8 show impaired activation of Src in response to EGF. We also provide the first preliminary evidence that phosphorylation of caspase-8 at Y380 might relieve inhibitory signaling rather than positively potentiating the EGF response directly per se. Because the EGF-Src axis has been strongly implicated in oncoprogenic progression (e.g., reviewed ref. 29), further research should confirm this unexpected layer of complexity and potentially identify novel therapeutic intervention points. Indeed, EGFR and Src kinase activities have been the focus of the development of novel antitumor treatments (e.g., reviewed in refs. 30, 31). Thus, these findings and the recent observations from other laboratories (7, 19–21) may explain why caspase-8 is rarely absent in cancers.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

Received 10/1/08; revised 3/23/09; accepted 4/7/09; published OnlineFirst 5/26/09.

**Grant support:** Grants from the NIH (K. Vuori). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**References**


Critical Role for Caspase-8 in Epidermal Growth Factor Signaling

Darren Finlay, Amy Howes and Kristiina Vuori


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-08-3731

Cited articles
This article cites 31 articles, 16 of which you can access for free at:
http://cancerres.aacrjournals.org/content/69/12/5023.full#ref-list-1

Citing articles
This article has been cited by 4 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/69/12/5023.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.