Novel Noncatalytic Role for Caspase-8 in Promoting Src-Mediated Adhesion and Erk Signaling in Neuroblastoma Cells

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Abstract

Neuroblastomas are extremely aggressive, although heterogeneous, cancers with a poor prognosis upon metastasis. Some evidence has suggested a correlative silencing of caspase-8 with MYCN amplification in neuroblastoma. A prognostic effect of this silencing, however, has been disputed. We report here hitherto undescribed roles for caspase-8 in the modulation of cell adhesion and subsequent activation of the Erk signaling pathway. Re-expression of caspase-8 in neuroblasticoma cells lacking endogenous caspase-8 expression was found to promote cell adhesion to extracellular matrix and to activate adhesion-dependent signaling pathways, such as the Erk kinase cascade. This function of caspase-8 occurred irrespective of its proteolytic activity. Additionally, a pool of caspase-8 was shown to co-localize with the Src tyrosine kinase at the cellular periphery. Furthermore, our studies showed that caspase-8 forms a physical protein complex with Src via its death effector domains (DED) and maintains the complex in a detergent-soluble fraction. We also show that the DEDs of caspase-8 alone are necessary and sufficient to recreate the adhesive and biochemical phenotypes observed with the full-length protein, suggesting that caspase-8 may exert these effects via its association with Src. This protein complex association of caspase-8 and Src, and concomitant downstream signaling events, may help reconcile why a potential tumor suppressor such as caspase-8 is rarely absent in cancers.

Introduction

Neuroblastoma arises from progenitor cells of the peripheral nervous system and is the most common, and deadly, childhood solid tumor (1). MYCN amplification occurs in a subset of neuroblastoma and has been associated with a particularly unfavorable outcome. Some evidence exists for an association of MYCN amplification and deletion or silencing of caspase-8 (2), whereas other data show no such relationship (3). Indeed, although caspase-8 has been proposed to act as a metastasis suppressor (4), loss of caspase-8 has no correlative role in neuroblastoma progression or prognosis (3).

Caspase-8 was first described as a FADD-homologous interleukin 1β converting enzyme (ICE)-like protease, or FLICE, that becomes recruited to the Fas death receptor complex upon activation of Fas death pathways (5). Its role as the crucial apical or initiator caspase in the transduction of cell death signals through Fas/CD95/Apo-1 has since become well established and defined (5–8). Evidence persists, however, that caspases may serve other diverse cellular functions (9). Of note, it has also been reported that many human tumors are refractory to the cytotoxic potential of activation of the Fas/FADD/caspase-8 pathway by death-inducing ligands (10–12). However, mutations or silencing of caspase-8 occurs only rarely in cancer (2, 3, 13), implying a potential benefit to maintaining caspase-8 integrity during tumor development. Caspase-8 has also been shown to activate the antiapoptotic nuclear factor-κB (NF-κB) signaling pathway by a number of laboratories (14–16). Interestingly, these data show that this activation of NF-κB is independent of the protein’s catalytic activity and can be induced by a catalytically inactive caspase-8 mutant. Paradoxically, classic enzymatic activation of caspase-8 results in the proteolytic cleavage of proteins involved in NF-κB signal transduction, thus demonstrating that caspase-8–induced apoptosis and cell survival signaling via NF-κB are uncoupled.

Here, we have examined the role of caspase-8 in cell adhesion and related biochemical signaling events in neuroblastoma cells. We find that caspase-8 promotes a Src-dependent adhesive phenotype and subsequent activation of the Erk signaling pathway that is independent of the protein’s proteolytic activity. We also show that caspase-8 forms a protein complex involving Src, and that this association is mediated by the protein’s death effector domains (DEDs). Indeed, the DEDs of caspase-8 alone are necessary and sufficient to recreate the adhesive phenotype and Erk activation observed with the wild-type protein. Thus, our studies reveal a novel noncatalytic role for caspase-8 in promoting Src-mediated signaling and adhesion in neuroblastoma cells.

Materials and Methods

Reagents. Unless otherwise specified, all reagents were from Sigma-Aldrich. Caspase-inhibitory peptides and PP1 were from Calbiochem. Rh-caspase-8 was a gift from Dr. Guy Salvesen (Burnham Institute for Medical Research, La Jolla, CA). Zeocin and primocin were obtained from InvivoGen.

Preparation of cellular protein extracts. Cells were treated as described for experimental conditions. Media supernatants were then removed by vacuum, and the cells were washed in ice-cold PBS. The cells were then scraped in 1 mL of ice-cold PBS and pelleted by centrifugation at 20,000 × g for 2 min at 4°C. Pellets were then resuspended in an appropriate volume of cell lysis buffer containing 40 mmol/L Tris (pH 8.0), 200 mmol/L NaCl, 0.2% Triton X-100, 10% glycerol, 2 mmol/L β-glycerophosphate, 500 μmol/L NaF, and a protease inhibitor cocktail (Roche Diagnostics) and incubated on ice for 15 min with occasional agitation. The suspension was then cleared by centrifugation at 20,000 × g for 15 min at 4°C to yield supernatants comprising the total cell extract. Protein concentrations were determined using a modified method of Bradford (17). Where specified, cells were lysed directly on plate with 1× Laemmli sample buffer to harvest both detergent-soluble and detergent-insoluble proteins (18).
Immunoblot analysis. Protein extracts were resolved on SDS-polyacrylamide gels and were then electrophoretically transferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore) by standard methodology. Membranes were blocked for 1 h in TBSTw [20 mmol/L Tris-HCl (pH 7.6), 150 mmol/L NaCl, 0.05% Tween] containing 5% nonfat dried milk [or 5% bovine serum albumin (BSA) for anti-phosphoryrosine studies] and were incubated rocking overnight at 4°C with the appropriate primary antibody: anti-extracellular signal-regulated kinase (anti-Erk, 1:2,000), anti–phospho-Erk (1:2,000; from New England Biolabs); anti-Src (mAb 327, 1:2,000), anti–α-tubulin (DM1A, 1:4,000; both from Calbiochem); anti-hemaggulutinin (anti-HA; 12CA5, 1:2,000; 12CA5-HRP, 1:1,000 or 3F10, 1:5,000; Roche Applied Science); anti-FAK (CL 77, 1:2,000; Cell Signalling Technology Inc.); anti–P-Y397 FAK (44-624, 1:2,000; Biosource International); anti–P–Y (4G12, 1:2,000; anti–c–Src (EC10, 1:2,000; Upstate); or anti–caspase-8 (C15, 1:2,000; kind gift from Marcus Peter, University of Chicago, Chicago, IL). After incubation for 1 h with anti-rabbit immunoglobulin G (IgG), anti-mouse IgG, or anti-rat IgG secondary antibodies conjugated to horseradish peroxidase (Jackson Immunoresearch Laboratories Inc.), bands were visualized using enhanced chemiluminescence (SuperSignal West Pico Chemiluminescent substrate, Pierce).

Immunofluorescence. Cells to be studied were cultured on LabTek chamber slides under the relevant experimental conditions. Cells were washed with ice-cold PBS and fixed by incubation in 4% paraformaldehyde in PBS. Slides were washed thrice in excess PBS and subjected to permeabilization in 0.2% Triton X-100 in PBS for 20 min at room temperature. Slides were again washed thrice in excess PBS before a 30-min blocking step in 5% BSA, 5% normal goat serum in PBS. Cells were then incubated with primary antibody (as specified) diluted in blocking solution (1:250) for 90 min at room temperature. Slides were then washed twice in excess PBS before incubation with secondary antibody [anti-mouse A488; anti-mouse A594; anti-rat A488; anti-rabbit A488; or anti-rabbit A594 as required (all 1:1,000)] and, where stated, rhodamine-conjugated phallolidin (1:500; all from Molecular Probes) in blocking solution for 30 min at room temperature. Slides were again washed thrice in excess PBS, and most of the remaining fluid was blotted away before mounting of the slides in 20 μL of Vectashield with 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Images were taken on an inverted TE300 Nikon wide-field and fluorescence microscope using Spot RT Acquisition and Processing Software (Diagnostic Instruments Inc.).

Cell culture, DNA and small interfering RNA transfections and stable cell line generation. 293T, U87, and SK-N-AS cells were cultured in standard DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin/l-glutamine (Omega Scientific Inc.). MCF-10 cells were cultured in DMEM/F12 (1:1) supplemented with 5% horse serum, l-glutamine (Omega Scientific Inc.) and 100 μg/mL insulin, and 5 ng/mL epidermal growth factor (EGF). 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/l-glutamine (Omega Scientific Inc.) and 100 μg/mL primocin (InvivoGen). Caspase-8 RNA and a c-Src expression plasmid were kind gifts from Drs. Gay Salvesen and Sara Courtneidge, respectively (both at Burnham Institute for Medical Research, La Jolla, CA). Caspase-8 small interfering RNA (siRNA) was synthesized by Dharmacon and is as described previously (19). siRNA transfections were carried out using LipofectAMINE 2000 (Invitrogen), and plasmid DNA was transfected by using Fugene 6 (Roche Applied Science). Both procedures were completed as per the manufacturers’ instructions. For stable cell line production, the cDNAs of interest were cloned into MSCV-ires-zp2 plasmids as standard. The subsequent DNA was checked by sequencing and transfected into Phoenix-A packaging cells as above. Viral supernatants were removed (at 48 and 72 h), the debris was pelleted by centrifugation, and polybrene was added to a final concentration of 0.8 mg/mL before being added to the cells to be infected. Cells were cultured in viral supernatants as such for 48 h before selection with 10 mg/mL zeocin for 14 days. Cell lysates were then assayed for protein expression using the immunoblotting techniques described above.

Cell adhesion assays. Briefly, 100,000 serum-starved cells were resuspended in 1 mL serum-free blocking medium (+0.5% BSA) and seeded for 30 min onto one well in a 12-well dish that had been coated with fibronectin (0.5 μL; 10 μg/mL) and blocked. Nonadherent cells were then removed by three washes with PBS, and the remaining cells were stained with 0.1% crystal violet for 10 min at room temperature. The crystal violet–stained cells were then washed four times in highly distilled water (3 mL each), the stain was solubilized in 1 mL of 10% acetic acid, and the absorbance was read at 570 nm. Data shown are means (± SE) of triplicates from a representative experiment independently carried out at least twice (usually two to four times).

Results
Caspase-8 promotes cell adhesion. Intuitively, a cell death–promoting protein such as caspase-8 would be an ideal candidate for deletion or silencing in tumors (20). Caspase-8 is, however, only deleted in a small proportion of tumors (2, 3, 13), implying that caspase-8 could have additional and perhaps tumorogenesis-promoting roles in cancer cells. Immunoblot analysis of various cancer cell lines confirmed this observation (Fig. 1, A, lanes 1–6), whereas a neuroblastoma-derived cell line (NB7) lacking caspase-8 (2) was included as a negative control (Fig. 1, A, lane 7). Immunoblots for caspase-3 and α-tubulin are shown for comparison. In light of these findings and the previously discussed nonapoptotic roles of caspase-8, it was therefore of interest to identify other potential functions of caspase-8 in cancer cells. In the course of these and other studies, we qualitatively observed that cells lacking caspase-8 showed less efficient adhesion to standard tissue culture dishes as compared with cells transfected to express caspase-8. This serendipitous finding prompted a thorough investigation of a potential role for caspase-8 in cell adhesion and related processes. Cell adhesion assays were carried out with neuroblastoma cells lacking caspase-8 but infected with either empty vector (NB7), wild-type caspase-8 (NB7 + Casp8) or proteolytically inactive caspase-8 (NB7 + Casp8C360A; ref. 2; Fig. 1A, lanes 7–9). The caspase-8–deficient NB7 cells showed a reduced adhesion to fibronectin as compared with cells that have caspase-8 stably reconstituted (Fig. 1B; P ≤ 0.01, Student’s t test). Interestingly, the cells expressing the catalytically inactive form of caspase-8 (21) adhered better to fibronectin than the caspase-8 null cells (P ≤ 0.01, Student’s t test), and in a manner comparable to the wild-type caspase-8–expressing cells (Fig. 1B), indicating that this pro-adhesive phenotype is an atypical role for caspase-8 and occurs independent of its catalytic activity.

To confirm this novel finding in another cell line, siRNA specific for caspase-8 (19) was transfected into MCF10A cells as described in Materials and Methods. Depletion of caspase-8, which was confirmed by immunoblotting, resulted in a loss of adhesion as compared with control-transfected cells (Fig. 1C, P ≤ 0.001, Student’s t test). The inhibition of adhesion in cells with caspase-8 levels reduced by siRNA was also confirmed in U87 and SK-N-AS cells (Supplementary Fig. S1). Due to the novel nature of a role for caspase-8 in cell adhesion and our evidence that this effect is independent of the protein’s well-defined proteolytic activity, cell adhesion assays were carried out in the presence or absence of small peptide inhibitors of caspase activity. Treatment of MCF10A cells with the caspase-8 selective z-IETD-fmk inhibitor or with the broad-range caspase inhibitor z-VAD-fmk (both 40 μmol/L) had no effect on cell adhesion as compared with vehicle-only–treated control cells (Fig. 1D), again demonstrating that the pro-adhesive effect of caspase-8 is independent of the protein’s catalytic activity.
A pool of caspase-8 is located at the cellular periphery in actin-rich protrusions. As we showed a role for caspase-8 in cell adhesion, it was of interest to investigate the subcellular localization of the protein. Previous immunostaining studies into the localization of caspase-8 have proved troublesome due to the lack of availability of a suitable antibody that can specifically bind the protein’s native form. To assess therefore the cellular localization of caspase-8, the cDNA was cloned with a COOH-terminal triple HA tag and point mutants generated in the construct as specified in Materials and Methods. Using anti-HA antibodies, no detectable staining is visible in the control cells transfected with empty vector. Cells transfected with the HA-tagged caspase-8 (Casp8) or with a catalytically inactive point mutant of caspase-8 (Casp8C360A) showed distinctive protein staining (Fig. 2A). Although most caspase-8 seems to be cytosolic and perinuclear, there is a clear pool of protein localized at the cellular periphery (Fig. 2A, arrows). Consistent with our previous findings, this localization is irrespective of the protein’s catalytic activity as the caspase-8 C360A mutant shows an almost identical staining pattern. This staining pattern was confirmed using an antibody against endogenous caspase-8 in NB7 and NB7 + Casp8 cells (Supplementary Fig. S3). Rhodamine-labeled phalloidin staining of the filamentous actin in cells transfected with HA-tagged caspase-8 is also shown. Caspase-8 is shown to be colocalized in areas of actin abundance at the cellular periphery regardless of whether the wild-type or inactive point mutant is used (Fig. 2B).

Caspase-8 promotes adhesion-induced activation of Erk 1/2 via Src. Activation of the Erk pathway following integrin-mediated adhesion to the extracellular matrix (ECM) is well documented (22–25). We show here that caspase-8–deficient NB7 cells show impaired phosphorylation of Erk 1/2 following adhesion to fibronectin over the course of 60 min, and that re-expression of caspase-8 in these cells results in an increase in activation of the pathway as judged by phospho-immunoblot analysis (Fig. 3A). Again, this caspase-8–potentiated phosphorylation of Erk 1/2 occurs even in the presence of the small peptide inhibitors of caspase-8, indicating that this response is independent of the enzyme’s proteolytic activity (Fig. 3B). This observation is also confirmed using NB7 cells reconstituted with the catalytically inactive form of caspase-8. Here, we show that the activation of the Erk pathway upon adhesion to fibronectin is restored in NB7 cells with either the wild-type or the catalytically inactive caspase-8 re-expressed (Fig. 3C). ECM-integrin signaling, and in particular, its activation of the Erk 1/2 pathway, has been previously shown to be facilitated by the protein tyrosine kinase Src (24, 26). It was therefore of interest to ascertain this kinase’s role, if any, in the caspase-8–promoted, cell adhesion–induced activation of Erk 1/2. As previously shown, NB7 cells reconstituted with either the wild-type or the C360A point mutant form of caspase-8 were allowed to adhere to fibronectin for 60 min. Preincubation of either cell type with the Src family kinase inhibitor PP1 (1 μmol/L) over the course of the experiment completely abrogated the activation of the pathway as determined by phospho-immunoblotting (Fig. 3D), implying a canonical role for Src kinase in the caspase-8–mediated adhesive phenotype.

Figure 1. Caspase-8 promotes cell adhesion. A, immunoblot analysis of caspase-8, caspase-3, and α-tubulin expression in various human cancer cell lines. U87, glioblastoma; LNCaP, prostatic adenocarcinoma; PC3, prostate carcinoma; H460, large cell lung carcinoma; MCF7 and MDA-MB-231, mammary gland adenocarcinomas; and NB7, neuroblastoma. B, cell adhesion assay (fibronectin; for 30 min) of caspase-8–deficient NB7 cells (+empty vector) or NB7 cells reconstituted with wild-type (NB7 + Casp8) or a proteolytically inactive point mutant (NB7 + Casp8C360A) of caspase-8. Columns, averages of triplicates from a representative experiment; bars, SE; n = 4. C, cell adhesion assay (fibronectin; for 30 min) of MCF10A cells treated with vehicle (+ Veh.); caspase-8 inhibitor (+z-IETD-fmk, 40 μmol/L), or a broad range caspase inhibitor (+z-VAD-fmk, 40 μmol/L). Columns, averages of triplicates from a representative experiment; bars, SE; n = 2. D, cell adhesion assay (fibronectin; for 30 min) of MCF10A cells transfected with a control siRNA or a caspase-8–depleting siRNA. Columns, averages of triplicates; bars, SE. Bottom, immunoblot analysis of caspase-8 and α-tubulin expression in MCF10A cells used above.
Caspase-8 has no effect on FAK activation but co-localizes with Src and maintains Src in a detergent-soluble cellular fraction. Due to the observations that classic biochemical ECM adhesion markers such as Erk 1/2 phosphorylation were impaired in caspase-8–deficient cells (Fig. 3A–C), and that caspase-8–mediated Erk pathway activation required the activity of Src in our model system (Fig. 3D), it was of interest to investigate the involvement of other related and potentially associated signaling molecules. Once more, NB7 cells lacking caspase-8 or cells reconstituted with either the wild-type or the C360A mutant caspase protein were allowed to adhere to fibronectin for 60 min, and the subsequent lysates were analyzed by immunoblotting as described. Although phosphorylation of Erk 1/2 was promoted by caspase-8 as shown previously (Fig. 4A, fourth panel), a distinct reduction in the protein level of soluble Src was observed in cells lacking caspase-8 (Fig. 4A, third panel). Interestingly, treatment of the cells with the protein synthesis inhibitor cycloheximide had no differential effect on Src levels irrespective of caspase-8 status (data not shown), indicating that the differences observed are not due to an effect on protein expression. Not all classic integrin-signaling pathways were affected by the presence or absence of caspase-8. For example, FAK has been proposed to function as a dual-kinase complex, together with Src upon integrin-mediated cell attachment (27). Immunoblot analysis of the autophosphorylation site (Tyr397) of FAK shows that it is phosphorylated post-ECM adhesion, but...
that there are no differences in the level of auto-activation irrespective of caspase-8 levels (Fig. 4A, top), indicating that a direct involvement of FAK in caspase-8–mediated adhesive signaling is unlikely. In sum, some classic ECM adhesive effects upstream of Src are unperturbed by caspase-8 protein levels (such as FAK activation); however, Src protein amount itself and downstream readouts of adhesion, such as Erk 1/2 activation, are promoted by caspase-8.

The striking differences in the level of Src protein observed in the cells with or without caspase-8 after adhesion to fibronectin for 60 min (Fig. 4A, third panel) prompted us to investigate whether this disparity was a localization phenomenon (due to a detergent-insoluble localization) or due to variations in the total Src protein levels in the cells. To answer this question, NB7 cells lacking caspase-8 or reconstituted with wild-type or catalytically inactive caspase-8 were grown to confluence, and SDS lysates were directly made using the method of Laemmli (18). These SDS-lysed total cell lysates were then subjected to SDS-PAGE and immunoblot analysis as previously described. Interestingly, the total level of Src protein was unchanged in these lysates irrespective of the caspase-8 status of the cells (Fig. 4B), indicating that the differences observed in Fig. 4A (third panel) were likely due to Src protein localization into a Triton X-100–insoluble (0.2% as described) fraction in the absence of caspase-8, but retention in the soluble fraction in the presence of caspase-8. Invariably, this retention of Src in a detergent-soluble fraction was independent of the proteolytic activity of caspase-8.

Consistent with these observations, recent findings by Cursi et al. (28) show that caspase-8 may be a Src kinase substrate. It was therefore of interest to investigate a potential interaction between the two proteins in our models. 293T cells were transiently transfected with expression vectors for c-Src and caspase-8 C360A or empty vector as a control. Forty-eight hours later, the cells were analyzed by immunofluorescence as described previously. c-Src was found to be localized in cellular protrusions (Fig. 4C) and to be co-localized with caspase-8 in these protrusions upon cotransfection (Fig. 4C, bottom), implying a potential interaction between the two. The proteolytically inactive C360A mutant of caspase-8 was used in these studies to prevent any anomalies due to apoptosis induction. We failed to observe any effect by the putative phosphorylation of caspase-8 by Src on Tyr380 (28) in regulating the adhesive phenotype. Thus, NB7 cells reconstituted with a Casp8C360AY380F dual mutant showed adhesion comparable to those reconstituted with the Casp8C360A mutant (Supplementary Fig. S4).

The role of caspase-8 in adhesion-induced activation of the Erk pathway was also confirmed in MCF10A cells using siRNA (Fig. 4D). Caspase-8 depletion resulted in less efficient Erk pathway signaling and a concomitant reduction in the levels of soluble Src, thus confirming our previous observations in NB7 cells (Fig. 4A).

**Caspase-8 forms a complex with Src in vivo via its DEDs.**

To further examine the interplay between Src and caspase-8, co-immunoprecipitations were done. Endogenous Src was found in a complex immunoprecipitated by an anti–caspase-8 antibody, but not control IgG antibody in U87 cells (Fig. 5A, top). This association of Src and caspase-8 was confirmed in NB7 cells. Again, endogenous Src could be co-immunoprecipitated from NB7 + Casp8C360A-HA cells using an anti-HA antibody (Fig. 5A, bottom).

Based on these discoveries, it was of interest to ascertain what domains of caspase-8 might be involved in associating with a Src-containing complex. Various caspase-8 deletion mutants were created with a COOH-terminal triple HA tag and cloned into mammalian expression vectors using standard biochemical techniques. These truncation mutants are depicted schematically in Fig. 5B. The mutant versions of caspase-8 were co-transfected into 293T cells with a c-Src expression plasmid. Any c-Src–containing complexes were subsequently immunoprecipitated as described, and the final pellets were analyzed by immunoblotting (Fig. 5C). Consistent with our earlier data, full-length Casp8C360A is found in a complex with c-Src (Fig. 5C, lane 2). Mutants lacking either one or both of the DEDs of the protein (Δ1-99Casp8 and Δ1-210Casp8) were unable to associate with a c-Src–containing complex (lanes 3 and 4, respectively); however, the DED-alone mutant lacking the
Figure 5. Caspase-8 forms a complex with Src in vivo via its DEDs. A, top, anti-Src immunoblot of U87 cell extracts immunoprecipitated with anti–green fluorescent protein (anti-GFP; as a control) or anti–caspase-8 antibodies. Bottom, anti-Src immunoblot of anti-HA immunoprecipitation of NB7 lane (lane 1) or NB7 + Casp8C360A (lane 2) cell extracts. Anti-α-tubulin immunoblot of the total cell lysate (TCL) is shown, as indicated. B, schematic representation of various truncation mutants of caspase-8 that were used in this study. C, anti-HA immunoblot of anti-c-Src immunoprecipitates from cells transfected with c-Src and either empty vector (lane 1) or expression vectors for Casp8C360A-HA (lane 2), Δ1-99Casp8C360A-HA (lane 3), Δ1-210Casp8C360A-HA (lane 4), Δ210-479Casp8-HA (lane 5). An anti-HA immunoblot of the total cell lysates (TCL) used for immunoprecipitation is shown in the bottom with theoretical molecular weights marked. D, anti-c-Src immunoblot of anti-HA immunoprecipitates from cells transfected with c-Src and either empty vector (lane 1) or expression vectors for Casp8C360A-HA (lane 2) or Δ210-479Casp8-HA (lane 3). The total cell lysates used for the immunoprecipitation are immunoblotted for c-Src (middle) and HA (bottom) as described.
total Erk 1/2 protein levels and expression of the HA-tagged proteins are shown for comparison (Fig. 6B, middle and bottom, respectively). Thus, we show that the DEDs of caspase-8 alone are sufficient to promote the adhesive phenotype, and that this adhesion is reflected by activation of the Erk 1/2 signaling pathway.

In summary, we provide the first evidence that caspase-8 has a role in promoting Src-mediated cell adhesion and concomitant Erk 1/2 pathway activation. These effects are all independent of the proteolytic activity of caspase-8. In addition, caspase-8 is found co-localized with and in a protein complex containing Src. Finally we show that this association is mediated by the protein’s DEDs, and that the DEDs alone are both sufficient and necessary to recreate the phenotypes observed.

Discussion

Neuroblastomas are the most common extracranial childhood tumors (1), and although caspase-8 silencing has been suggested to play a correlative role (2), no prognostic effect has been shown (3). Caspase-8 has a well-established role in death receptor–mediated apoptosis (5–8). Compelling evidence exists, however, for diverse nonapoptotic roles (9, 29, 30). Indeed, although caspase-8 knock-out mice are embryonically lethal (31), humans with a catalytically inactivating point mutation of the protein develop normally with only a lymphoproliferative disorder associated with improper immune cell apoptosis (32). This seeming incongruity can be reconciled by theorizing that caspase-8 has an essential non-apoptotic role during development. Recent work from Richard Flavell’s laboratory (33) shows that mice with homozygous deletions in both effector caspase-3 and caspase-7 only die postnatally. Based on these findings, Lakhani et al. (33) state that the embryonic lethality observed in caspase-8 null mice may not only be due to apoptosis defects but also likely proceed through alternative pathways.

In this article, we show that neuroblastoma cells lacking in caspase-8 show reduced adhesion to fibronectin with concomitant reduction in associated biochemical responses. Our data show that the protein’s role in promoting cell adhesion is independent of its protease activity. We also observe reduced activation of the Erk 1/2 pathway upon cell attachment in the absence of caspase-8 (Fig. 3), and these effects can be restored upon re-expression of either the wild-type or a catalytically inactive point mutant of the protein. That this adhesion-induced phosphorylation of Erk 1/2 is dependent on Src kinase activity is well characterized (Fig. 3D). Interestingly, we found that caspase-8 is also required for efficient EGF-, platelet-derived growth factor- and tumor necrosis factor-α (TNF-α)–mediated activation of the Erk 1/2 pathway (data not shown), suggesting a more generalized role for caspase-8 in cell surface receptor signaling. To further elucidate the biochemical events responsible for the phenotypes observed, an investigation into the potential involvement of adhesion-related signaling molecules was undertaken. Although some investigators have suggested that FAK may act as a protease targeting moiety (34), we observed no caspase-8–related differences in the adhesion-induced autophosphorylation of FAK (Fig. 4A), indicating a more downstream role for caspase-8 in our model. We further show, rather unexpectedly, that the levels of detergent-soluble Src are seemingly proportional to caspase-8 expression (Fig. 4A and B). Immunofluorescence studies of the subcellular localization of caspase-8 show co-staining with that of Src (Fig. 4C), whereas recent discoveries by Cursi et al. (28) provide compelling evidence that caspase-8 is, in fact, a Src kinase substrate. In light of these data, we investigated a potential interaction between the two molecules. Our data provide evidence that endogenous caspase-8 and Src are within the same protein complex (Fig. 5A). Most intriguingly of all, we reveal that this complex formation is solely dependent on the DEDs of caspase-8 (Fig. 5C and D). We must point out, however, that this association is almost certainly not due to a direct interaction because of our failure to observe an interaction with purified proteins in vitro (data not shown). Proteomics studies to elucidate other associated molecules involved will form the basis of future work in this respect. Other investigators have also shown roles for DED-containing proteins in the modulation of signaling of...
integrins (35), NF-κB (14), Rsk2 (36), and, indeed, directly with Erk 1/2 (37, 38). Recently, Yao et al. (39) have shown that the first DED of caspase-8 alone can associate with Erk 1/2. In another study, Luschen et al. (40) show that TNF-induced activation of Erk 1/2 is dependent solely on the DEDs of caspase-8, thus providing more evidence to support our hypotheses for a more generalized role for DED-containing proteins in various nonapoptotic cellular processes.

Our data also establish that the DEDs of caspase-8 alone are sufficient yet necessary for the formation of a protein complex comprising Src (Fig. 5). We show that the DEDs alone of caspase-8 are capable of reestablishing the adhesive phenotype absent in cells deficient in the protein. These findings are supported by the restoration of adhesion-induced Erk 1/2 activation by the DEDs alone of the protein. Thus, the data provide evidence to adduce the theory that DEDs of caspase-8 may have a more generalized role in protein-protein interactions with a complex involving Src. This association seems to facilitate and thereby promote a more efficient cell adhesive phenotype. The phenomena we observe are always independent of the proteolytic activity of caspase-8, whereas the association with Src, and other alternate roles, may help to explain why caspase-8, as an intuitively potent tumor suppressor, is rarely absent in cancers.

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