Caspase-8 Association with the Focal Adhesion Complex Promotes Tumor Cell Migration and Metastasis

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Abstract

Caspase-8 is a proapoptotic protease that suppresses neuroblastoma metastasis by inducing programmed cell death. Paradoxically, caspase-8 can also promote cell migration among nonapoptotic cells; here, we show that caspase-8 can promote metastasis when apoptosis is compromised. Migration is enhanced by caspase-8 recruitment to the cellular migration machinery following integrin ligation. Caspase-8 catalytic activity is not required for caspase-8–enhanced cell migration; rather, caspase-8 interacts with a multiprotein complex that can include focal adhesion kinase and calpain 2 (CPN2), enhancing cleavage of focal adhesion substrates and cell migration. Caspase-8 association with CPN2/calpastatin disrupts calpastatin-mediated inhibition of CPN2. *In vivo*, knockdown of either caspase-8 or CPN2 disrupts metastasis among apoptosis-resistant tumors. This unexpected molecular collaboration provides an explanation for the continued or elevated expression of caspase-8 observed in many tumors. [Cancer Res 2009;69(9):3755–63]

Introduction

Caspase-8 is an apical protease and initiator of the extrinsic programmed death pathway. The caspase-8 zymogen is recruited to the death-inducing signaling complex following ligation of death receptors, such as Fas, where it undergoes activation. The loss of caspase-8 has been associated with increased malignancy of neuroendocrine tumors, including neuroblastoma (1–5). We reported previously that caspase-8–expressing neuroblastoma exhibits increased dependence on integrins and the extracellular matrix relative to caspase-8–lacking counterparts (6). Failure to maintain adequate integrin-mediated extracellular matrix contacts promoted caspase-8–dependent, and death receptor-independent, apoptosis among invasive cells (7). This process, which we termed "integrin-mediated death," acted to limit metastasis *in vivo*. In turn, the results from those studies have supported the implementation of new therapeutic regimens to caspase-8 expression within neuroblastoma tumor cells *in vivo* as a potential therapeutic approach.

However, it remains unclear whether up-regulation of caspase-8 would be universally beneficial for preventing metastasis. It is notable that a significant fraction of aggressive stage IV neuroblastoma (10-30%) maintains caspase-8 expression and that caspase-8 is not frequently inactivated among adult cancers, such as carcinoma (8). Inactivating mutations are surprisingly rare (8, 9), although it is important to note that such tumors, which develop over many decades, frequently have other lesions that interfere with the apoptotic cascade (10, 11). Because caspase-8 is an initiator caspase, downstream mutations common in some cancers could well promote cell survival irrespective of caspase-8 (12). Under such circumstances, caspase-8 may play alternative physiologic roles within the cell. Caspase-8 has been linked to proliferation (13–16) and to the migration in several primary and tumor cells (17–20). Such observations imply that clinical strategies to up-regulate caspase-8 might not be universally beneficial and may even contribute to tumor aggressiveness. A particular concern is the possibility that it could promote tumor cell dissemination among apoptosis-resistant tumor cells.

Although unligated or antagonized integrins promote caspase-8 activation (7), ligated integrins suppress caspase-8 activation (21). Ligated integrins promote assembly of the focal adhesion complex, a signaling complex anchored by the actin cytoskeleton (22). The focal adhesion complex contains an interacting matrix of numerous proteins, which includes nonreceptor tyrosine kinases, such as Src and focal adhesion kinase (FAK), adaptor and actin-binding proteins, including talin and paxillin, as well as cytosolic phosphatases and proteases (23, 24). In particular, the calpain proteases have been implicated in the cleavage of focal adhesion proteins that promotes focal adhesion turnover (25, 26). The high degree of complexity of the focal adhesion reflects its physiologic versatility in promoting signaling, survival, anchorage, and migration. Here, we have explored the interaction between the focal adhesion complex and caspase-8 in migration and metastasis. Surprisingly, the "normally proapoptotic" enzyme caspase-8 is found to be incorporated into focal adhesions and promotes not only cell migration but also metastasis of apoptosis-resistant cells.

Materials and Methods

Chemicals reagents, cDNA, and vectors. Calpastatin peptide and calpain inhibitor II ALLM were purchased from Calbiochem. Leupeptin, phenylmethylsulfonyl fluoride, fibronectin from bovine plasma, and laminin were purchased from Sigma. Collagen type I was from Upstate. Vitronectin generated from human placenta was the kind gift of Dr. David Cheresh. Complete mini protease inhibitor and Fugene transfection reagent were from Roche Diagnostics. cDNA of human calpastatin cloned into pCMV.SPORT6 vector was from the American Type Culture Collection (GenBank ID BC013579). pcDNA3.1 myc-His mammalian expression vector was from Invitrogen. Caspase-8, caspase-3, and calpain 2 (CPN2) lentiviral short hairpin RNAs (shRNA) were from Open Biosystems. Rat CPN2

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

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recombinant protein was from Calbiochem. 7-Amino-4-chloromethylcoumarin and *t*-BOC-L-leucyl-L-methionine amide were from Molecular Probes.

Antibodies. Anti-talin NH_2 terminus (MAB 1676 clone TA205), anti-CPN2, and anti-calpastatin were purchased from Chemicon. Anti-caspase-8 antibody was from BD Biosciences. Anti-phospho-p44/42 mitogen-activated protein kinase was from Cell Signaling. Anti-phospho-FAK (P-Y397) was from Biosource International. Anti-FAK (C-20) and anti-extracellular signal-regulated kinase (ERK) 2 (C-14) were from Santa Cruz Biotechnology. Anti-actin (clone AC-15) was from Sigma. Anti-myc antibody was from Invitrogen.

Cell lines. A549 lung carcinoma cells were acquired from the American Type Culture Collection. To create FAK-deficient and control cell lines, A549 cells were infected with a lentivirus encoding shRNA to FAK (Open Biosystems) or a control shRNA (plasmid 1864; Addgene). The neuroblastoma tumor lines NB7, NB5, and NB16 were established at St. Jude Children's Hospital. Mouse embryo fibroblasts from Src-deficient mice ($Src^{-/-}$) or FAK-deficient mice ($FAK^{-/-}$) mice were the kind gift of Dusco Ilic. $FAK^{-/-}$ fibroblasts reconstituted with GFP-FAK ($FAK^{-/-}$ + FAK*) cells were described previously (27). Cells or cell lines were maintained in either DMEM (A549 and mouse embryo fibroblast cells) or RPMI 1640 (NB7, NB5, and NB16) supplemented with 10% FCS, 1% glutamine, and minimal amino acids. Caspase-8–deficient NB7 neuroblastoma cells were reconstituted with caspase-8 by retroviral transduction with a caspase-8 expression construct as described previously (6). Knockdown of caspase-8 was accomplished by using adenovirus-delivered caspase-8 shRNA (6) or via stable lentiviral delivery of shRNA to caspase-8 as described previously (ref. 28; Open Biosystems). As a control shRNA, cells were infected with a lentivirus encoding a nonspecific shRNA sequence (plasmid 1864; Addgene). Knockdown of caspase-3 was similarly done using the lentivirus (Open Biosystems). Knockdown of CPN2 was done by using lentivirus (Open Biosystems) in which the puromycin resistance cassette of the pLKO.1 vector was replaced with a neomycin resistance cassette to permit double antibiotic selection. Calpastatin cDNA cloned into pCMV.SPORT6 vector was amplified by PCR using SP6 and 5′-agtcatcttttggcttgg primers to remove the stop codon. The PCR product was subcloned into the mammalian expression vector pcDNA3.1 myc-His using *Eco*RV restriction site to allow expression of a COOH-terminal myc and polyhistidine-tagged protein. Plasmid was transfected into NB7 cells using the Fugene system (Roche) for 24 h.

Immunoprecipitation and immunoblotting. Cells were lysed in either NP-40 lysis buffer [150 mmol/L NaCl, 50 mmol/L Tris (pH 7.4), 1% NP-40] or radioimmunoprecipitation assay buffer [50 mmol/L Tris (pH 7.4), 100 mmol/L NaCl, 0.1 % SDS] supplemented with complete protease inhibitor mixture (Roche), 50 mmol/L NaF, and 1 mmol/L Na_3VO_4 and centrifuged at 13,000 × *g* for 10 min at 4°C. Protein concentration was determined by bicinchoninic acid assay (Pierce). For immunoprecipitation, 500 μg proteins were incubated with 2 μg anti-caspase-8 antibody (BD Biosciences) or anti-CPN2 (Chemicon) antibody overnight at 4°C. Complexes were precipitated with 30 μL protein A/G (Pierce). Beads were washed five times with TBS-0.1% NP-40, and bound proteins were eluted in boiling Laemmli buffer, resolved on 10% SDS-PAGE, and immunoblotted using specific antibodies for total FAK (anti-FAK C-20; Santa Cruz Biotechnology) or calpain (Chemicon) or caspase-8 (BD Biosciences). For the calpain-calpastatin interaction, NB7 cells were transfected with the calpastatin myc-His construct using Fugene system (Roche). myc-His-calpastatin was immunoprecipitated with an anti-myc antibody, and immunoprecipitates were then incubated for 90 min at 37°C with or without addition 100 ng or 1 μg recombinant human caspase-8 catalytic domain (inactive C360A mutant). Lysates were washed once and assessed by SDS-PAGE and Western blotting with anti-CPN2 or anti-calpastatin antibodies.

Adhesion-dependent signaling. Neuroblastoma cells were serum starved for 6 h, placed in suspension for 15 min in ice (representing the 0 time point), or plated into 100 mm non-tissue culture-treated dishes (1×10^6 per 100 mm dish) precoated with extracellular matrix proteins (2 μg/mL fibronectin, 2 μg/mL vitronectin, or 10 μg/mL collagen). Cells were allowed to attach for the times as indicated. At each time point, adherent cells were directly lysed in radioimmunoprecipitation assay buffer as described above. Where indicated, cells were incubated in the presence of ALLM (50 μmol/L) or calpastatin peptide (2 μmol/L) before (20 min) and during cell adhesion. PBS vehicle served as a control. The adenovirus mediating delivery of caspase-8 small interfering RNA, or an empty type 5 adenoviral vector (pAd Easy), was added to the cells 72 h before and during cell adhesion (6). Cells lysates were analyzed by Western blot as above described for the specific antibodies.

Focal adhesion enrichment and isolation. The assay was done after that described by Kaplan et al. (29). Briefly, cells were plated onto fibronectin-coated plates as described for the cell signaling assay. At the times indicated, adherent cells were preextracted with 0.5% Triton X-100 lysis buffer for 0.5 h; this fraction is clarified by centrifugation to remove nuclei and is called the cytosolic fraction (500 × *g*, 5 min). The focal adhesion enriched fraction was prepared from the remaining cell fraction bound (adhesion complex) on the plate, which was lysed in radioimmunoprecipitation assay buffer for 5 min on ice, and scraped off the dish. This fraction is clarified by centrifugation (10 min, 14000 × *g*) to remove contaminating nuclear material. Lysates from the two different fractions were analyzed by immunoblotting.

Tumor growth and metastasis. Neuroblastoma cells (5×10^6) suspended in 40 μL complete medium were seeded on 11-day-old chick chorioallantoic membrane. Tumors were left to develop for 8 days and then resected and weighed as determined previously (6). The metastasis assays were done by seeding 7×10^6 cells onto the surface of the chick chorioallantoic membrane and assessing the presence of metastases in lungs and bone marrow by amplification of a human-specific Alu sequence as reported previously (6).

Migration assays. Cell migration was done using a variant of the wounding as described previously (20) or a Transwell assay using modified Boyden chambers, 6.5 mm diameter, 8 μm pore size (Transwell; Costar), according to the protocol of the manufacturer. Briefly, the bottom sides of the inserts were coated with fibronectin (2 μg/mL). Cells (5×10^5) were plated in the top chamber of Transwell inserts and serum-free medium was added to the bottom chamber. After 4 h, stationary cells were removed from the top side of the membrane, whereas migrated cells in the bottom side of the inserts were stained with 0.1% crystal violet in 2% ethanol. Dye was eluted with methanol, and absorbance was measured at 600 nm.

Calpain activity assay in living cells. NB7 or NB7-C8 cells were plated in 96-well multiwell plate precoated with fibronectin (2 μg/mL) at concentration of 25,000 per well for 10 min. Calpain activity was evaluated after the times as indicated by incubating the cells with the cell-permeable calpain fluorogenic substrate *t*-BOC-L-leucyl-L-methionine amide (10 μmol/L) and measuring the fluorescence with the TECAN Geniios Pro fluorometer at excitation and emission wavelength of 350 and 460 nm, respectively. As a control, cells are incubated with the solubilizing vehicle (DMSO).

Immunofluorescence studies. Cells were permitted to attach to coverslips coated with fibronectin (2 μg/mL) for 1 h, such that they were confluent. Cell monolayer was then wounded with a pipette tip and cells were allowed to begin to migrate into the wound for 1 h. Alternatively, cells were plated at subconfluence and allowed to migrate randomly. In either case, cells were fixed with 4% paraformaldehyde for 10 min, permeabilized in PBS containing 0.1% Triton X-100 for 3 min, and blocked for 60 min at room temperature with 2% bovine serum albumin in PBS. Cells were then stained with monoclonal antibody to caspase-8 (BD Biosciences; 1:100) for 1 h. After washing several times in PBS/bovine serum albumin, the cells were exposed to secondary antibody specific for mouse [1:300; Alexa 488 (*green*) or 565 (*red*); Invitrogen]. Samples were mounted in Vectashield hard set mounting medium (Vector Laboratories) and imaged on a Nikon Eclipse C1 confocal microscope.

One-dimensional gel-based ABPP of focal adhesion and cytosolic fractions. Standard conditions for ABPP reactions were as follows: cytosolic and focal adhesion proteomes were adjusted to a final protein concentration of 0.6 mg/mL in 25 μL PBS (pH 7.4) and treated with 1 μmol/L AB19-bodipy-TMR (caspase-8 probe) or 1 μmol/L DCG-04-bodipy-TMR (calpain probe) for 30 min at 37°C as described previously (30, 31). Reactions were

quenched with 1 volume of standard 4× SDS-PAGE loading buffer (reducing) and separated by one-dimensional SDS-PAGE gels (10% acrylamide). Fluorescently labeled proteins were visualized in-gel with a Hitachi FMBio IIe flatbed fluorescence scanner (MiraiBio).

Statistical analysis. Where pertinent, results were compared using unpaired *t* tests of at least three independent experiments or ANOVA as indicated. $P < 0.05$ was considered significant. For the *in vivo* studies, statistical power was realized by evaluating cohorts including all animals from all experiments by χ^2 and Mann-Whitney statistical tests as described previously (6).

Results

Caspase-8 promotes metastasis among caspase-3-deficient cells. Caspase-8 has been implicated in the suppression of neuroblastoma metastasis via the induction of apoptosis among invasive cells (6), consistent with loss of caspase-8 in the majority of aggressive neuroblastoma (4). However, caspase-8 can promote cell migration via localization to the cell periphery and activation of small GTPases and calpain (17). These results suggest that caspase-8 may promote metastasis, particularly when apoptosis is compromised. To test this, we used a shRNA approach to suppress expression of caspase-3, a critical downstream effector of caspase-8–mediated killing (32) and other forms of apoptosis (Supplementary Fig. S1). Caspase-3 expression was suppressed in NB7 neuroblastoma cells reconstituted with caspase-8 (NB7-C8) and tumor growth and metastasis was assessed in the chorioallantoic membrane model that previously established a metastasis suppressor role for caspase-8 (6).

Caspase-3 knockdown (Fig. 1*A*) did not significantly affect neuroblastoma proliferation *in vitro*[6] or tumor growth *in vivo* (Fig. 1*B*). Loss of caspase-3 did not appreciably affect metastasis of NB7 neuroblastoma deficient for caspase-8 (Fig. 1*C*, compare *right open* and *left filled columns*) but rescued metastasis among tumors expressing caspase-8 (+; $P < 0.001$, compare *middle filled* and *open columns*). Surprisingly, a disproportionate increase in metastasis was observed; $Casp8^+Casp3^-$ cells disseminated more efficiently than neuroblastoma lacking both caspases ($P < 0.05$; Fig. 1*C*). As expected, the $Casp8^+Casp3^+$ tumors exhibited the lowest overall incidence of metastasis (Fig. 1*C, left open column*), confirming that caspase-8 blocks metastasis when an intact caspase cascade is present (6). Together, the results indicated that caspase-8, a putative metastasis suppressor, could act to promote tumor dissemination among populations of "apoptosis-compromised" cells.

Supporting this notion, caspase-8 promotes neuroblastoma migration under nonapoptotic condition *in vitro* (Fig. 2*A*; refs. 18–20). Similarly, shRNA-based knockdown of caspase-8 transgene expression in the NB7-C8 cells or knockdown of endogenous caspase-8 expression in A549 carcinoma cells decreases cell migration relative to cells treated with control shRNA (Fig. 2*B*). The caspase-8 shRNA had no effect on caspase-8–deficient cells (Fig. 2*B*). Collectively, the results support a general role for caspase-8 in supporting cell migration; accordingly, we find that caspase-8 is enriched in the leading edge among randomly migrating cells (Fig. 2*C*; ref. 20). These results were extended using confocal microscopy; caspase-8 was found to be enriched among cells entering a wound in both pseudopods (Fig. 2*D, top*) and lamella (Fig. 2*D, bottom*).

[6] Unpublished data.

Caspase-8–dependent alterations are observed in talin following substrate adhesion. Caspase-8 localizes to different cellular compartments and may be targeted based on post-translational modification (9, 33, 34). Caspase-8 can colocalize

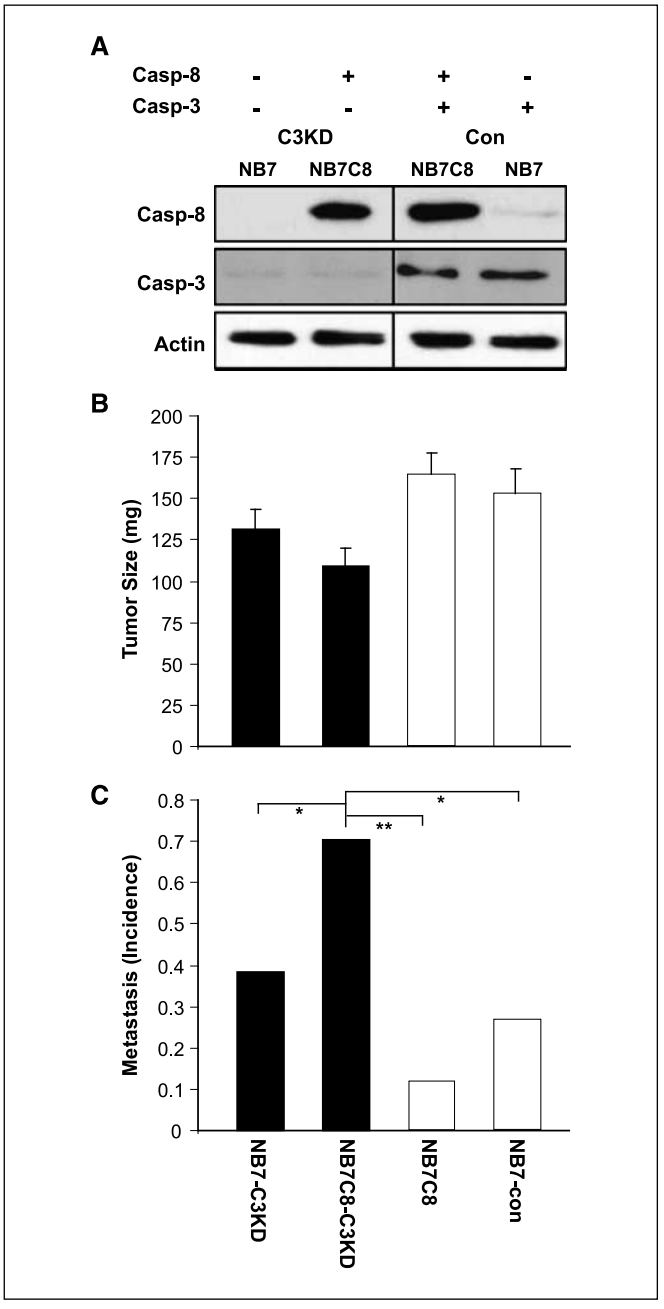

Figure 1. Effect of caspase expression on neuroblastoma progression. NB7 neuroblastoma cells reconstituted for caspase-8 and/or caspase-3 were seeded in the chorioallantoic membrane of chick embryos and tumors were allowed to develop for 8 d. *A,* immunoblot analysis of the expression of caspase-3 (*Casp-3*) and caspase-8 (*Casp-8*) and actin as control of proteins loading. *B,* tumors were resected from the chorioallantoic membrane and the wet weight was determined. Mean ± SE. *C,* total cumulative incidence of detection of human Alu sequence from genomic DNA isolated from lung and bone marrow of the tumor-seeded chick cohorts. DNA isolated from lung and bone marrow was used as a template for PCR-based detection of human cells using primers specific for the human Alu sequence. The limit of sensitivity of the assay is ~200 tumor cells. Incidence with which human genomic DNA was detected in the cohort, with statistical comparisons between cohorts representing three similar but separate experiments ($n = 38$, 40, 70, and 72) done via Mann-Whitney test. **, $P < 0.001$; *, $P < 0.05$.

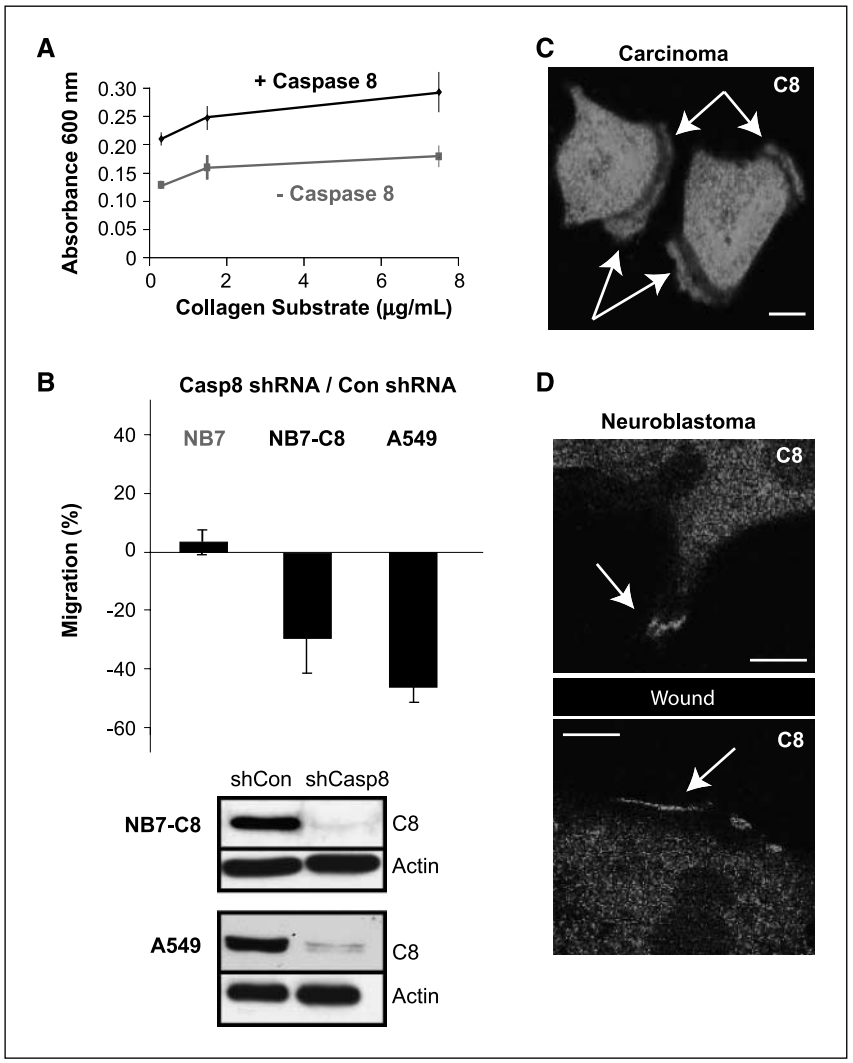

Figure 2. Caspase-8 influences cell migration. *A,* NB7 neuroblastoma cells deficient in caspase-8 (*- Caspase 8*) or reconstituted for caspase-8 expression (*+ Caspase 8*) were assessed for their ability to migrate on increasing concentrations of collagen substrate in a Transwell Boyden chamber system as described in Materials and Methods. Migrated cells were fixed and stained with crystal violet and extracted dye was quantitated (absorbance 600 nm). Mean ± SE from six determinations in a representative assay. *B,* NB7 cells expressing or lacking caspase-8 or A549 carcinoma cells expressing endogenous caspase-8 were infected with either control nonspecific shRNA lentivirus or lentivirus encoding shRNA to caspase-8 to knock down caspase-8 (immunoblots shown). Cells were evaluated for their capacity to migrate using a wound assay, and alterations in cell migration were plotted. NB7 did not significantly vary in migration when treated with the caspase-8 shRNA, whereas NB7-C8 and A549 exhibited decreased wound closure ($P < 0.05$ and $P < 0.01$, *t* test, respectively). *C,* localization of caspase-8 in A549 cells migrating randomly on a coverslip. Cells were fixed and permeabilized and caspase-8 localization was determined via immunofluorescence using a monoclonal antibody to caspase-8 (*green*). Note the localization at the peripheral ruffles of the moving cells (*arrows*). *D,* confocal microscopy assessment of caspase-8 in migrating cells. NB7-C8 cells seeded on fibronectin-coated coverslips (2 μg/mL) were wounded and allowed to migrate into the wound for 4 h. Confocal thin sections were captured at the cell-slide interface to assess caspase-8 localization at the front of migrating cells. *Bar*, 10 μm.

with integrins (7) and can be activated (promoting apoptosis) when integrins are antagonized or unligated (6, 7). Because integrin-mediated adhesion protects cells from caspase-8–mediated apoptosis (21), and caspase-8 is phosphorylated following attachment to fibronectin (20), we speculated that caspase-8 might also associate with nascent focal adhesions, thereby influencing cell migration. Therefore, NB7 cells deficient or reconstituted for caspase-8 were replated onto fibronectin substrate to activate integrin signaling, and the activation of integrin downstream signaling molecules was evaluated as a function of time by immunoblot analysis. However, we detected no differences in the activation of the integrin-proximal nonreceptor tyrosine kinase FAK or in the activation of the downstream target ERK (Fig. 3*A*).

In contrast, differences were evident in the appearance of the integrin-associated cytoskeletal protein talin during substrate adhesion to fibronectin (Fig. 3*A*) or collagen or vitronectin substrates (Supplementary Fig. S2*A*) in the caspase-8–expressing or caspase-8–deficient NB7 cells. In particular, caspase-8–expressing cells showed enhanced production of the NH_2-terminal talin fragment (35) during substrate attachment. This fragment contains the integrin-binding region of talin known as the FERM domain. Binding of the FERM domain to integrins enhances their binding to ligand, thus influencing cell migration (25, 36–38). Accordingly, knockdown of caspase-8 expression via shRNA blocked the production of the FERM domain fragment following substrate adhesion (Fig. 3*B*; Supplementary Fig. S2*B*) and inhibited cell migration (see Fig. 2*B*). Together, the results implicate caspase-8 as a talin-dependent regulator of cell motility. Nonetheless, caspase-8 did not appear to cleave talin directly, as we were unable to show cleavage of talin immunoprecipitates by recombinant caspase-8 *in vitro* (data not shown). This was not completely surprising because caspase-8 activity is influenced by steric or allosteric factors as well as post-translational modifications (18, 20, 39–41). For example, Src-mediated phosphorylation inhibits caspase-8 activation (41). Moreover, these results were consistent with our prior observations that a proteolysis-deficient mutant of caspase-8, in which the tyrosine in position 360 has been substituted to alanine (caspase-8 C360A), promotes caspase-8 targeting to the cell periphery and migration (20).

Talin is an integral member of the focal adhesion complex assembled following integrin ligation, and competition for available talin within a cell limits integrin activity (38). When focal adhesion-containing fractions were purified (29) from cells expressing or lacking caspase-8, we found no obvious differences in focal adhesion-associated talin holoprotein but selectively observed accumulation of the FERM domain fragment specifically within

focal adhesion of caspase-8–expressing cells (Fig. 3*C*). The caspase-8 zymogen was also observed (~56 kDa), whereas FAK and ERK accumulated independent of caspase-8 expression (Fig. 3*C*).

Caspase-8 promotes calpain activity within focal adhesions. To identify the protease responsible for mediating talin cleavage, we used an activity-based profiling approach, probing for the activity of caspases and calpains (42). The caspase-selective probes used (such as caspase-8–selective probe AB19-BTMX) detected no signal in either cytosolic or focal adhesion cell fractions (Supplementary Fig. S3). However, the activity-based probe DCG-04, a calpain selective probe, was incorporated strongly within focal adhesion fractions of NB7 cells reconstituted for caspase-8 expression, identifying a putative protease of ~72 kDa (Fig. 4*A*, *arrow*). Similar results were obtained in NB5 neuroblastoma cells expressing endogenous caspase-8, and the 72 kDa signal was eliminated by knockdown of caspase-8 expression (Supplementary Fig. S3). These results are in agreement with reports that (*a*)

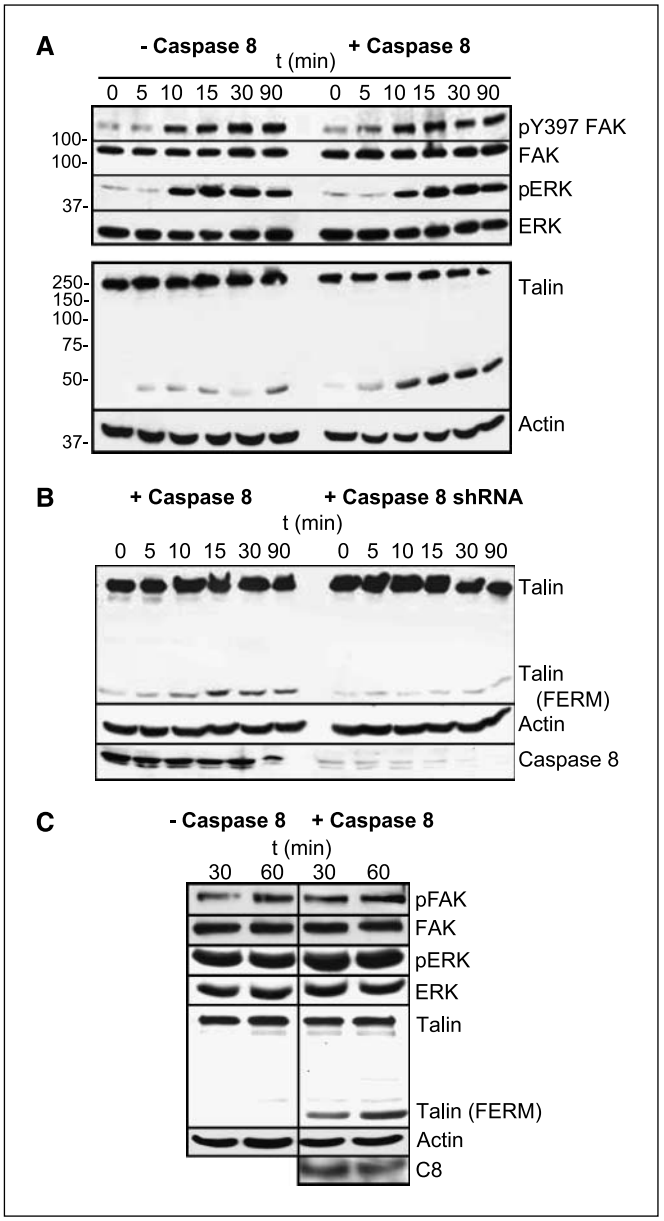

Figure 3. Effect of caspase-8 on integrin signaling. *A,* NB7 cells lacking or reconstituted for caspase-8 were plated on fibronectin-substrate (2 μg/mL) for the times and lysed in radioimmunoprecipitation assay buffer, and the expression of FAK, ERK, and phosphorylated forms of these proteins was evaluated by immunoblotting (*top*). Immunoblotting was similarly done to assess the presence of talin as well as the NH_2-terminal FERM cleavage product (*bottom*). *B,* immunoblot analysis of talin was done in NB7 caspase-8–reconstituted cells and shRNA knockdown cells. *C,* focal adhesion fractions were purified from NB7 neuroblastoma cells lacking or expressing caspase-8. Cells were allowed to attach for 30 or 60 min, and 25 μg protein was subjected to immunoblot analysis for the presence of FAK and pFAK, ERK and pERK, and talin.

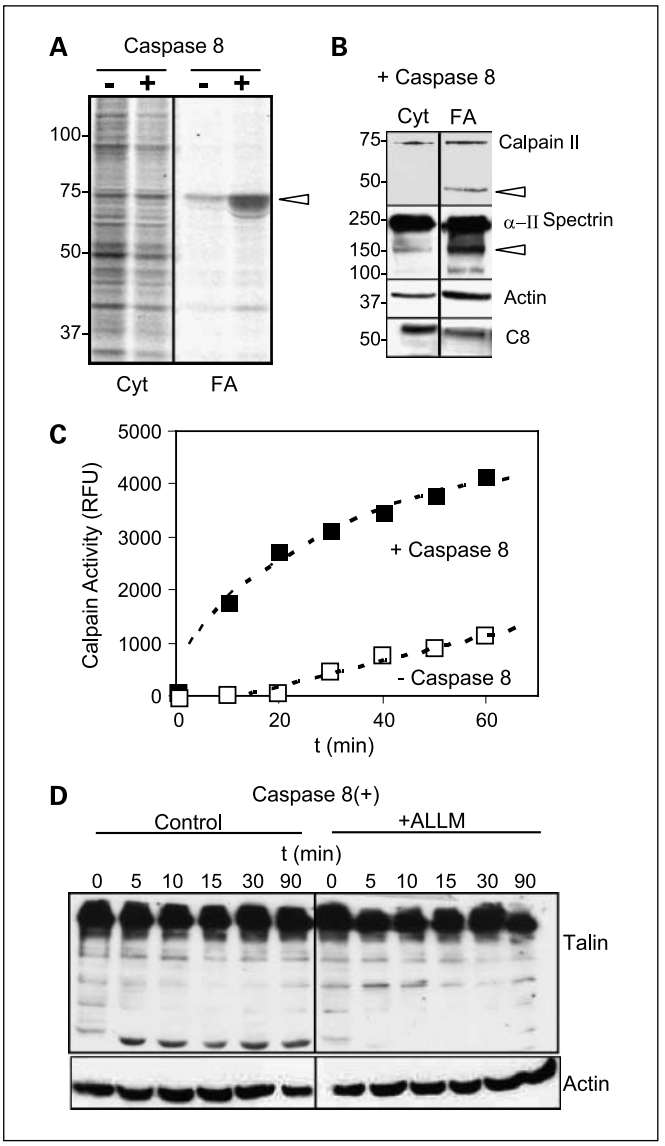

Figure 4. Calpain is activated selectively in caspase-8–expressing cells. *A,* isolated focal adhesion (*FA*) or cytosolic (*Cyt*) fractions were incubated with the cysteine protease probe DCG-04, which reacts with active calpain, and 20 μg of each lysate were then resolved by electrophoresis and DCG-04 reactive proteins resolve. *B,* focal adhesion and cytosolic fractions were assessed to detect calpain substrate cleavage. Calpain substrates tested included CPN2 (to evaluate production of the autocatalytic fragment) and the CPN2 substrate α-II spectrin. *C,* NB7 cells lacking (*open squares*) or reconstituted for caspase-8 (*filled squares*) were plated on fibronectin (2 μg/mL) in the presence of a calpain-activated fluorescent substrate (*t*-BOC-L-leucyl-L-methionine amide), and fluorescence was recorded as a function of time. Calpain activity induced by attachment from a representative experiment. *D,* immunoblot analysis of the effect of ALLM, an inhibitor of CPN2, on talin cleavage induced by substrate adhesion was done as described in Fig. 3 above.

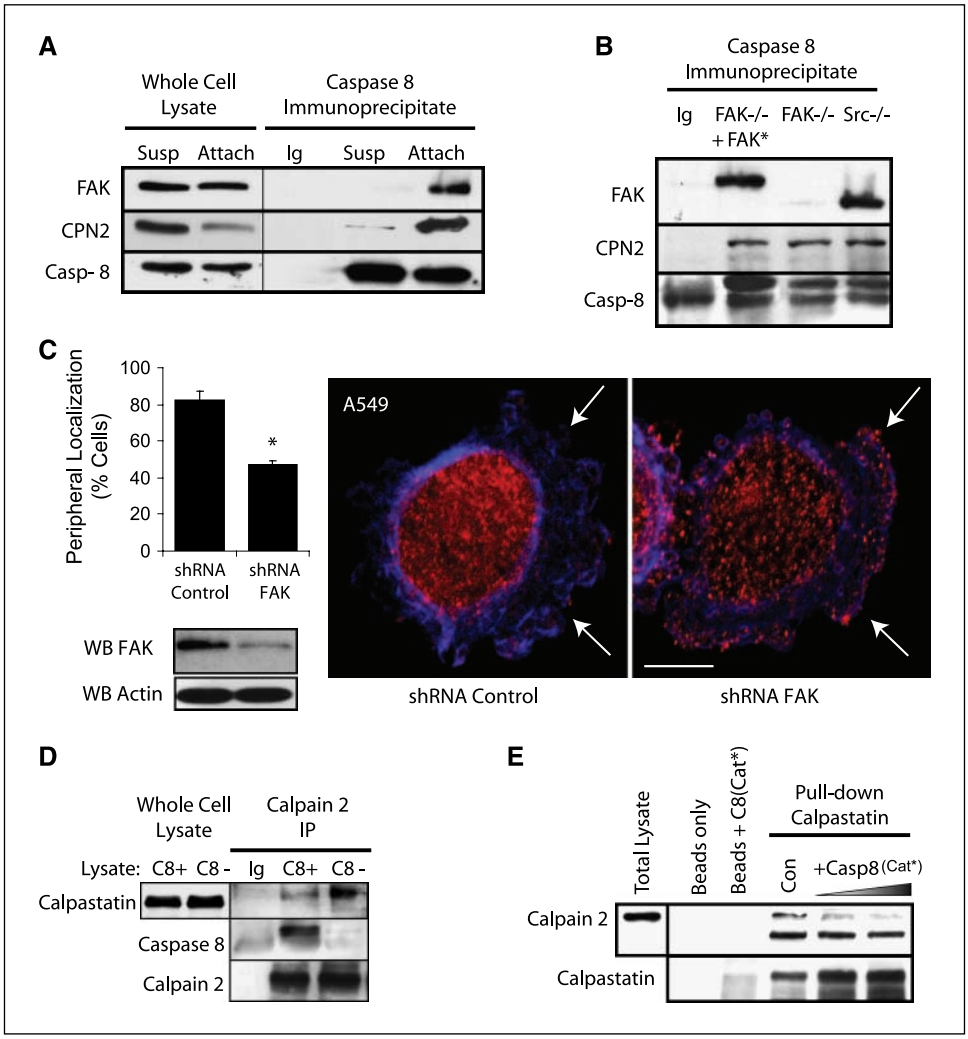

Figure 5. Caspase-8 is targeted to the periphery in a FAK-dependent manner. *A,* NB7 cells reconstituted for caspase-8 in suspension (*Susp*) or those allowed to attach to fibronectin (2 μg/mL; *Attach*) for 30 min were lysed and subjected to immunoprecipitation of caspase-8. Whole-cell lysate and nonspecific beads + antibody control was done in parallel, and all samples were subjected to immunoblot analysis for total FAK, caspase-8, and CPN2. *B,* mouse embryo fibroblasts from Src-deficient mice ($Src^{-/-}$), FAK-deficient mice ($FAK^{-/-}$), or $FAK^{-/-}$ fibroblasts reconstituted with GFP-FAK ($FAK^{-/-}$ + *FAK**) were allowed to attach to fibronectin substrate, lysed, and subjected to caspase-8 immunoprecipitation. Immunoprecipitates were analyzed for the presence of total FAK, caspase-8, and CPN2 (tagged FAK is resolved as a higher molecular weight species). *C,* A549 cells were transduced with lentivirus encoding a scrambled shRNA (*Control*) or a FAK shRNA (*FAK*) to knock down FAK (*left, immunoblot inset*). Cells were allowed to attach and spread on fibronectin-coated (2 μg/mL) surfaces and stained for actin (*blue*; *arrows,* actin ruffles) and caspase-8 (*red* to show caspase-8 distribution) and images by confocal microscopy. *Bar*, 10 μm. Spreading cells were scored for the presence of enriched caspase-8 by blinded observers (150 cells per group; *left*). $P < 0.012$. *D,* NB7 expressing or lacking caspase-8 were allowed to attach and lysed and CPN2 complexes were immunoprecipitated. Complexes were resolved by SDS-PAGE and assessed for the presence of calpastatin and caspase-8. *E,* His-calpastatin was overexpressed in NB7, and pull-downs were done with Nickel NTA resin. Lysates were incubated in the presence of GST (*Con*) or with 100 ng or 1 μg of the catalytic domain of caspase-8 (catalytically inactive C360A mutant), washed, and resolved. The presence of calpastatin and CPN2 was determined by immunoblotting.

CPN2-mediated talin cleavage regulates focal complex turnover and cell migration (35) and (*b*) calpains regulate caspase-8–mediated motility (19). Supporting the concept that CPN2 activity was elevated in focal adhesions, we observed cleavage of calpain substrates, such as α-II spectrin, and an autoprocessed form of CPN2 selectively in focal adhesions (*open arrowhead*) but not in the cytosolic fraction (Fig. 4*B*). Fluorometric substrate cleavage assay using live cells showed increased calpain activity selectively among NB7-C8 cells during substrate attachment (Fig. 4*C*). The peptidyl protease inhibitor, ALLM, can block cell migration via inhibition of CPN2 (ref. 43; data not shown). In agreement with the notion that talin cleavage facilitates migration, we find that ALLM treatment also suppressed substrate attachment-induced cleavage of talin (Fig. 4*D*). Similar results were seen with calpeptin, another peptidyl inhibitor of calpain (derived from the endogenous calpain inhibitor, calpastatin; Supplementary Fig. S4*A*). These results support the proposed role of calpain as an effector of caspase-8–mediated motility (19) and extend these results by localizing the activity to nascent focal adhesion/cytoskeletal complexes initiated by integrin-substrate ligation.

Caspase-8 associates with CPN2 and FAK. The focal adhesion targeting of CPN2 has been proposed to occur via a scaffolding function of FAK, with CPN2 binding near the FAK-Y397 phosphorylation site (24). Because caspase-8 associates with FAK-associated SH2-containing proteins such as Src and p85α (18, 20), we tested whether caspase-8 and CPN2 might be present within the same molecular complex. NB7 cells reconstituted for caspase-8 expression were kept in suspension or

permitted to attach to fibronectin (2 μg/mL) for 30 min and cell lysates were immunoprecipitated with anti-caspase-8 antibody (BD Biosciences) and subjected to immunoblot analysis for total FAK (anti-FAK C-20; Santa Cruz Biotechnology) or calpain (Chemicon) or caspase-8 (BD Biosciences).

Coprecipitation analysis revealed that CPN2 and caspase-8 associated with each other and with FAK selectively following substrate adhesion but not among suspended cells (Fig. 5*A*). The induced association following substrate adhesion suggested a role for the focal adhesion complex in assembling the caspase-8/CPN2 containing complex. Surprisingly, however, we found that caspase-8 and CPN2 could associate in $FAK^{-/-}$ cells, suggesting that FAK was not essential (Fig. 5*B*). Similarly, the kinase c-Src, which associates with both caspase-8 and FAK, was not necessary for caspase-8/CPN2 association or the formation of the FAK/caspase-8/CPN2 complex (Fig. 5*B*). However, FAK was important for caspase-8 distribution, because mouse embryo fibroblast cells lacking FAK had disrupted localization of caspase-8 in the periphery (Supplementary Fig. S5*A*), whereas reconstituted cells exhibited normal peripheral localization of caspase-8. Similarly, we assessed the distribution of caspase-8 among A549 cells in which FAK has been knocked down (~80-90%; Fig. 5*C, inset*). Among similarly spread cells (Supplementary Fig. S5*B*), we found that the distribution of caspase-8 in the membrane ruffles was compromised in FAK knockdown cells relative to control A549 (Fig. 5*C*), and this was not simply time-dependent, because $FAK^{-/-}$ cells do not show enhanced peripheral caspase-8 localization at later time points (data not shown; Supplementary Fig. S5*B*). Thus, FAK appears to play a role in localizing caspase-8 to the periphery among spreading cells.

We next examined how caspase-8 association with CPN2 or with focal adhesions might influence calpain activity. The principle regulator of calpain activity in living cells is calpastatin (37). Interestingly, calpastatin cleavage was enhanced in the focal adhesion fraction of NB7-C8 cells (Supplementary Fig. S4*B*). Although caspase-8 did not cleave calpastatin *in vitro* (data not shown), active calpains can cleave calpastatin, and the observed products were consistent with those previously described for calpain cleavage. Calpastatin binds calpain via its NH_2-terminal domain (44) and via three distinct conserved peptide sequences within its "calpastatin repeats," each of which is required for effective inhibition of the enzyme (45). Physiologically, the activation of calpain requires displacement of calpastatin and association with targeting or anchoring proteins (46). Therefore, CPN2 or calpastatin binding to caspase-8 might act to disrupt the calpastatin-CPN2 interaction.

To test this possibility, we examined immunoprecipitates of calpain from cells expressing or lacking caspase-8. Calpastatin was readily detected coprecipitating with CPN2 in lysates from cells lacking caspase-8 but was nearly absent in lysates derived from cells expressing caspase-8 (Fig. 5*D*). This suggested that caspase-8 prevented formation of a CPN2-calpastatin complex. To determine if this was a direct effect, we then added back recombinant caspase-8 (C360A mutant, inactive) to the precipitated calpastatin complexes. The addition of recombinant caspase-8 disrupted the preexisting calpain-calpastatin complex (Fig. 5*D, lanes 4* and *5*), indicating that caspase-8 antagonizes calpastatin-CPN2 interaction and further suggesting that caspase-8–enhanced migration and metastasis was effected by CPN2.

CPN2 is crucial for promotion of migration and metastasis by caspase-8. To address this, we first knocked down the expression of CPN2 (via shRNA) in caspase-8–deficient or caspase-8–expressing NB7 cells already bearing a C3 knockdown (creating a double-knockdown phenotype; Fig. 6*A*). Assessing these cells, we found that the $Casp8^{+}Casp3^{-}CPN2^{-}$ cells exhibited decreased talin cleavage following substrate attachment, similar to caspase-8–deficient cells, whereas $Casp8^{+}Casp3^{-}$ cells expressing a control shRNA exhibited talin cleavage following substrate attachment (Fig. 6*B*). To determine whether there was a selective effect on migration, we next assessed migration *in vitro* among the CPN2 knockdown cells (Fig. 6*B, left*). Interestingly, the knockdown of CPN2 had a greater effect on the migration on fibronectin substrate of caspase-8–expressing cells relative to caspase-8–deficient cells. This suggested that knockdown of CPN2 might also decrease tumor metastasis of caspase-8–expressing cells *in vivo*. Evaluating this possibility, we found that suppression of CPN2 decreased the incidence of metastasis selectively among caspase-8–expressing cells (Fig. 6*C, left*). Together, these results extend prior suggestions that

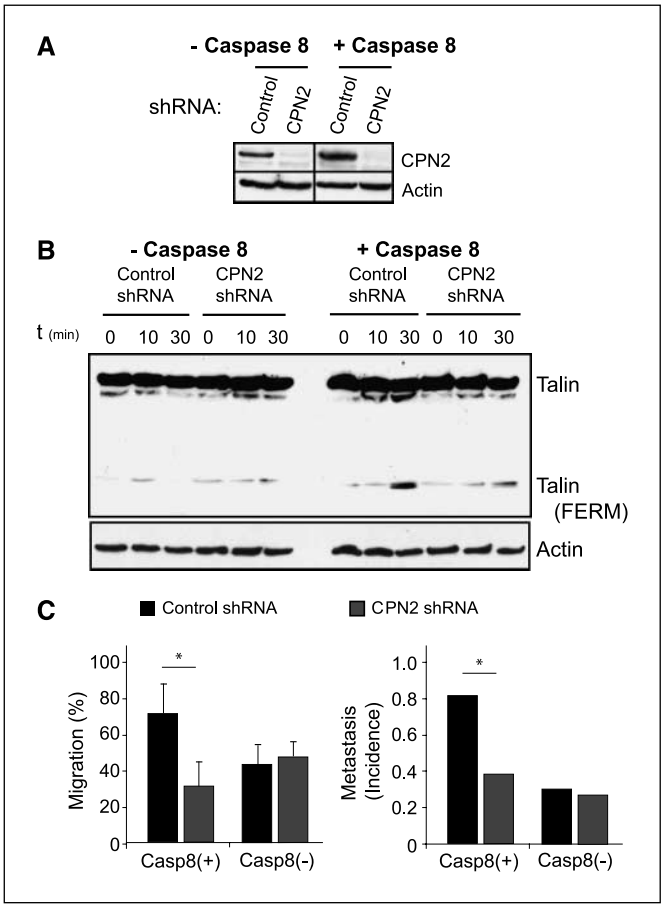

Figure 6. CPN2 knockdown compromises talin cleavage, migration, and metastasis. *A,* CPN2 was knocked down in the paired C3-silenced NB7 cells expressing or deficient for caspase-8. *B,* CPN2 knockdown cells were allowed to attach to fibronectin substrate and talin cleavage was assessed by immunoblotting. *C,* change in wound closure after 24 h of migration following calpain knockdown in NB7 cells expressing or deficient for caspase-8 was assessed via Boyden chamber assay for their ability to migrate on fibronectin substrate (2 μg/mL; *left*). Mean ± SE from six determinations in a representative assay. *, $P < 0.05$. Metastasis assays were done in the chick chorioallantoic membrane and relative change in metastasis incidence was plotted. Data are cumulative and represent the absolute incidence of metastasis for all animals used in the study (+ Caspase-8 $n = 54$ and - Caspase-8 $n = 36$). $P < 0.05$, inhibition in caspase-8–expressing cells (Mann-Whitney test).

caspase-8–induced migration was dependent on CPN2 (19) and show an important synergy with caspase-8 in metastasis *in vivo* among apoptosis-resistant tumors.

Discussion

Tumors can become apoptosis-resistant via many mechanisms, including the expression of mitochondrial gatekeeper proteins of the Bcl-2 family, overexpression of inhibitors of apoptosis, or lost expression of caspases, such as caspase-8, caspase-9, or caspase-3. Here, we examined the metastasis of neuroblastoma cells in which we compromised caspase-8–mediated killing by silencing the expression of the downstream effector caspase-3. The studies showed that disruption of caspase-3 in the apoptotic cascade could not only relieve the metastasis-suppressing activity of caspase-8 but also further revealed an unexpected metastasis-enhancing property due to caspase-8 expression. Examining the mechanism by which this occurred, we found that caspase-8 promoted cell migration independent of its proteolytic activity, via recruitment to a complex that contained FAK, and CPN2. Caspase-8 disrupted the interaction of calpastatin with calpain and permitted activation of CPN2. In turn, this promoted CPN2 cleavage of focal adhesion substrates and subsequent cell migration (43). Accordingly, knockdown of CPN2 inhibited caspase-8–initiated metastasis. Our results show that the recruitment of caspase-8 to the focal complex regulates both cell migration and calpain activity (19). The capacity of caspase-8 to increase migration and metastasis may be clinically relevant; these nonapoptotic roles of caspase-8 suggest caution be used in strategies that seek to amplify caspase-8 expression.

The lack of apoptosis induced by enriched peripheral caspase-8 may be due to allosteric limitations present within the tightly packed focal adhesion complex or may result from posttranslational modifications such as phosphorylation of caspase-8 on Y380 by Src (41). Indeed, these events may not be easily dissociable, because recruitment of caspase-8 to the periphery of cells attaching to substrate is abnormal in the absence of FAK (Fig. 5). Together with previous studies, our results suggest that extracellular matrix adhesion may trigger post-translational modification of caspase-8, permitting caspase-8 to play a nonapoptotic role as a promoter of cell migration. This is of particular interest, because Serial Analysis of Gene Expression analysis suggests that increased caspase-8 expression may occur in bladder, liver, ovarian, pancreatic, prostate, and (non-small cell lung carcinoma) lung cancers (47).

The capacity for caspase-8 to interact with a cytoskeletal complex and influence cell behavior may be noteworthy with respect to prior studies. Many "nonapoptotic" cellular processes that have been found to be disrupted in caspase-8–deficient animals, such as T-cell activation (48, 49), have well-documented requirements for talin and integrin (50). Other "nonapoptotic" caspase-8 activities, such as activation of nuclear factor-κB (51) or the small GTPase Rac (19), similarly link caspase-8 signaling to integrins and the cytoskeleton. Further, cell adhesion and cytoskeletal rearrangements are linked with resistance to apoptosis (34, 52, 53). Although resistance can be related to transcriptional events and downstream modulation of apoptosis-regulating proteins (at least in some cases), our results provide a basis for exploring how early signaling events elicited by integrin-ligand interactions can directly contribute to the regulation of caspase cascade initiation. The apparent linkage between apoptosis and cellular cytoskeletal dynamics appears to be physiologically convenient; integrins act as biosensors that physically interrogate the local microenvironment and thus are well-poised to help guide cell fate decisions.

It is also important to consider that caspases represent clinically relevant targets. Although current strategies are focused on stimulating or inhibiting the caspase catalytic activity, the potential for noncatalytic function is likely to be important in future therapeutic considerations. Our results would strongly suggest that retention of caspase-8 may be "contextually" advantageous to a tumor cell, particularly those bearing downstream disruptions within the programmed cell death pathway. With respect to this, it is possible that current clinical trials that seek to up-regulate caspase-8 expression might, under some circumstances, exacerbate disease and promote metastasis. In addition to placing patients at risk, this could act to mask efficacy within statistical cohorts. However, an increased understanding of the molecular mechanisms involved in regulating this process would be predicted to provide new targets for use in personalized, and combinatorial, therapeutic approaches.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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