Caspase-8 Promotes Cell Motility and Calpain Activity under Nonapoptotic Conditions

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Abstract

Significant caspase-8 activity has been found in normal and certain tumor cells, suggesting that caspase-8 possesses an alternative, nonapoptotic function that may contribute to tumor progression. In this article, we report that caspase-8 promotes cell motility. In particular, caspase-8 is required for the optimal activation of calpains, Rac, and lamellipodial assembly. This represents a novel nonapoptotic function of caspase-8 acting at the intersection of the caspase-8 and calpain proteolytic pathways to coordinate cell death versus cell motility signaling. (Cancer Res 2006; 66(8): 4273-8)

Introduction

Many cell types, including breast and pancreatic cancer cells, have significant caspase-8 activity that could potentially affect cell behavior under nonapoptotic conditions (1, 2). This increased activity may be caused by elevated soluble FASL in some cancers, especially those treated with chemotherapeutic agents (3, 4). Heterodimerization with overexpressed tumor cell c-FLIP also activates caspase-8 (5, 6), elevating basal activity. At present, the possible significance of this constitutive caspase-8 activity is unknown. Diverse nonapoptotic functions are emerging for caspases, including differentiation, proliferation, and promotion of the immune response (7–11), suggesting that constitutive caspase activity may be relevant to normal and tumor biology.

Cell migration is a critical contributor to tumor invasion and metastasis (12). Interestingly, roles for certain caspases in cell migration have been suggested. The *Drosophila* caspase, DRONC, a homologue of mammalian caspase-2 and caspase-9, modulates developmental cell migration due to an interaction of its endogenous inhibitor, DIAP, with the Rac pathway (13). *Caspase-8* gene knockouts are early-embryonic lethal in mice (14), mainly because they fail to assemble a functional circulatory system, suggesting a defect in endothelial cell migration; other possible cell migratory defects occur in the context of tissue-specific knockouts (15). Moreover, several adhesion/motility–related structural and signaling proteins are cleaved by caspases during apoptosis (16–32). Corresponding cleavage of these substrates during nonapoptotic processes, such as cell migration, may provide a mechanism for caspases to control the turnover or alter the activation status of these substrates.

Calpains are implicated in several aspects of cell migration, including adhesion turnover, lamellipodial extension/retraction cycles, detachment of the trailing edge of the cell, and activation of rac (reviewed in ref. 33). Knockout of the mouse *calpain-4* gene, which encodes a regulatory subunit that is required for the *in vivo* stability of the ubiquitous calpain-1 and calpain-2, generates an embryonic-lethal phenotype (34) that is similar in timing and cause of death to that of *caspase-8* knockout mice. (Note, however, that the substantial amount of cell migration occurring before day 10 of development in these and *caspase-8*-knockout mice occurs normally, indicating that this cell migration is independent of these proteases, perhaps due to the compensation by or predominance of other migration signaling pathways in early embryonic cell types responding to a unique configuration of growth factors/chemoattractants.) Consistent with their cell migration function, calpains provide an important tumor-promoting function (33, 35–37) and tumor cells often have increased calpain activity (35, 36, 38–41). A role of caspase-8 and/or a functional relationship between caspase-8 and calpains in tumor progression has yet to be addressed.

In this report, we show that caspase-8 regulates cell motility and calpain activity. Cells that lack caspase-8 have reduced cell motility and calpain activity as well as other manifestations of calpain deficiency, such as reduced Rac activation, lamellipodial assembly, and fidelity of cytokinesis. This reveals a potential nonapoptotic role of caspase-8 in tumor progression.

Materials and Methods

Cell Lines

Mouse embryonic fibroblasts (MEF) derived from *caspase-8*-null, *caspase-3*-null, or isogenic wild-type control mice were previously characterized (14, 42). WTNR6 cells (43) were provided by Alan Wells. To generate derivatives expressing the cowpox virus–derived caspase-8 inhibitory protein crmA (44), the hemagglutinin-tagged *crmA* gene was retrovirally transduced using the vector MSCV-IRES-puro in the gp2+293 cell packaging system (Invitrogen, Carlsbad, CA). NB7 cells that were infected with empty green fluorescent protein retrovirus or rescued with human caspase-8 retrovirus (45) were provided by Jill Lahti. MDA-MB231 (American Type Culture Collection, Rockville, MD) were transduced with *crmA* as described above. Cells were maintained in DMEM (MEF, MDA-MB231), advanced MEM (WTNR6), and RPMI 1640 (NB7), supplemented with 10% fetal bovine serum (FBS) and 1× penicillin-streptomycin-glutamine (Invitrogen).

Cell lines with inducible caspase-8 expression were generated using the Rheoswitch system (www.neb.com). *Caspase-8*$^{-/-}$ MEFs (W102) were coelectroporated with a 4:1 ratio of the Rheoswitch activator/repressor–expressing plasmid, NEBR-R1:pcDNA3.1hyg, and colonies were selected

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

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in 500 μg/mL hygromycin. Colonies were expanded and screened for expression of the VP16 moiety of the hybrid activator protein by Western blotting and for induction of luciferase activity after transient transfection of luciferase/pNEBR-X1. Wild-type mouse caspase-8 cDNAs were subcloned into the pNEBR-X1 target plasmid and coelectroporated in a 4:1 ratio with pBABE-puro. Colonies were selected in 5 μg/mL puromycin and screened for caspase-8 expression by Western blotting with the anti-mouse–caspase-8 antibody 1G12 (Alexis, San Diego, CA) after induction with the compound RSL1. A Western blot of the induction time course is shown in Fig. 1. The level of caspase-8 expression at 24 hours of induction was ~30% to 50% of the level in wild-type MEFs (data not shown).

Production of Calpastatin–TAT Peptide

Double-stranded oligonucleotides corresponding the active calpain-inhibitory domain of calpastatin (46) were subcloned into the vector pET28b-TATv1 (provided by Steve Dowdy). The fusion protein was expressed in *Escherichia coli* BL21 with 2 hours of isopropyl-L-thio-B-D-galactopyranoside induction; bacteria were lysed in buffer Z [6 mol/L urea/100 mmol/L NaCl/20 mmol/L HEPES (pH 8)] and purified on a nickel-chelate resin (Qiagen, Valencia, CA) in the same buffer containing 20 mmol/L imidazole. The protein was eluted using a 40 mmol/L interval imidazole step gradient, and pooled fractions were chromatographed on a BioRex70 column in 50 mmol/L NaCl/20 mmol/L HEPES and eluted with 1 mol/L NaCl/20 mmol/L HEPES; pooled fractions were dialyzed against 0.5 mol/L NaCl/20 mmol/L HEPES and frozen.

Cell Motility Assays

Method 1. Electrical cell-substratum impedance sensing. Cells were plated on the chambers (~1 cm^2) of a single-electrode electrical cell-substratum impedance sensing (ECIS) arrays (Applied Biophysics, Troy, NY; www.biophysics.com) that were precoated with fibronectin. After growing the cells to confluence, equal coverage of the electrodes was checked by measuring the initial impedance value (averages were 1,924 ohms for caspase-$8^{-/-}$ cells and 2,598 for caspase-$8^{+/+}$ cells). The cells attached to the small electrode disc (0.25-mm diameter) were selectively ablated by applying a 4 V pulse for 10 seconds, causing the impedance drop seen at 0 hours. The impedance increase due to the surrounding cells migrating back onto the electrode was then monitored at 40 kHz frequency over the indicated time course; migration was in the presence of 10% serum and was carried out in triplicate chambers with one additional "unwounded" chamber serving as a negative control. Data were exported to Excel for calculation of slopes.

Method 2. Conventional wound healing. Cells were grown to confluence on fibronectin-coated coverslips or 35 mm Mattek dishes, serum-starved overnight (0.5% serum), and the monolayer was wounded with a pipette tip; epidermal growth factor (EGF; 10 nmol/L) was then added. The wound was photographed at zero time and at the indicated time points using vertical and horizontal alignment marks made on the plastic well with a syringe needle. Three wound areas were traced and measured using the Axiovision software, averaged, and represented here as the percentage wound closure. Alternatively, 1 hour after attachment, a wound was generated, unattached cells were washed off, and videomicroscopy was done on the dish, which was incubated on a heated stage in CO_2-independent medium/CCM1 medium (1:1). In experiments using the Rheoswitch-inducible cell lines, cells were induced for 24 hours in the presence of 500 nmol/L RSL1 before wounding.

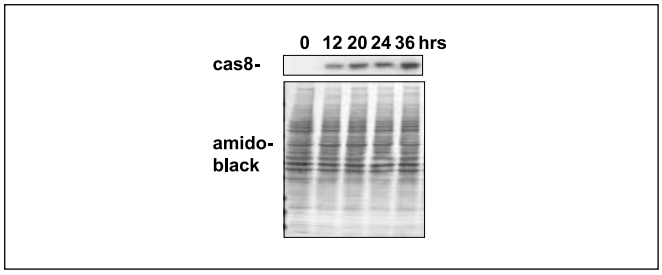

Figure 1. Inducible expression of caspase-8.

Method 3. Single-cell motility assays. Cells were plated onto fibronectin at low density for 1 hour in the absence of serum, restimulated with serum, and recorded by videomicroscopy for 10 hours. The paths traveled by seven individual cells were traced and the total distance traveled by each cell was computed using the Axiovision software (interactive measurement module).

Method 4. Transwell/chemotaxis assays. Cell migration assays were done with modified Boyden chambers, 6.5 mm diameter, 8 mm pore size (Transwell from Costar Corp., Acton, MA) according to the protocol of the manufacturer. Briefly, after serum starvation of the MEF cells, they were harvested with trypsin and quenched with Soybean trypsin inhibitor (Calbiochem, San Diego, CA). The lower compartment of the migration chamber was filled with 3T3-fibroblast conditioned medium and 6×10^4 cells were suspended in DMEM/10 mmol/L HEPES/0.5% bovine serum albumin (BSA)/1% penicillin-streptomycin and added to the upper compartment of the migration chamber. Migration chambers were incubated for 4 hours at 37°C in 5% CO_2. After removal of stationary cells from the upper side of the membrane, migrated cells were fixed in 3.7% paraformaldehyde in PBS and stained with 1% crystal violet in 2% ethanol. Membranes were extensively washed, the dye was eluted with 10% acetic acid, and absorbance was measured at 600 nm. Results are expressed as percentage of migrated cells relative to a control in which total adherent cell absorbance was measured.

Calpain Assays

Method 1. Plate-reading fluorimetry using succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin substrate. Cells were seeded into 96-well plates (10^4 per well) and allowed to attach overnight (47). When EGF stimulation was used, the cells were serum starved (0.5% FBS) for 8 to 12 hours before treatment. Five minutes before addition of EGF, the medium was aspirated and replaced with 100 μL phenol red–free medium containing 0.5% FBS with 50 μmol/L succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (Suc-LLVY-AMC; Calbiochem). At time zero, 10 nmol/L EGF was added. The plate was then loaded into the prewarmed (37°C) plate reader (Gemini XPS, Molecular Devices, Sunnyvale, CA). When the calpstatin-TAT (CPS-TAT) inhibitor was used, the cells were incubated with the inhibitor (2 μmol/L) for 1 hour before addition of the substrate and the assay was conducted in the presence of the inhibitor. Fluorescence was assessed using SOFTmax-pro software at an excitation wavelength of 360 nm and an emission wavelength of 465 nm. Readings were taken every 3 minutes for a period of 45 minutes. Columns in graphs represent maximum relative fluorescence units, whereas error bars are the SD values (of either of the samples with or without CPS-TAT) of two to four duplicate measurements after subtraction of activity obtained in the presence of CPS-TAT. Line graph represents kinetic readings of triplicate measurements with SDs. The lines were curve-fitted after time zero values were normalized.

Method 2. Fluorescence microscopy using *t*-butoxycarbonyl-Leu-Met-chloromethylaminocoumarin substrate. *Caspase-$8^{-/-}$* versus *caspase-$8^{+/+}$* MEFs were plated on glass-insert 35 mm dishes (Mattek), serum-starved overnight, and loaded with 10 μmol/L *t*-butoxycarbonyl-Leu-Met-chloromethylaminocoumarin (Boc-LM-CMAC) for 16 minutes on a heated microscope stage (47, 48). EGF (10 nmol/L) was added and time-lapse photographs were taken every minute for 16 minutes using the 4′,6-diamidino-2-phenylindole (DAPI) filter for the fluorescent signal and phase contrast. The original raw images had different overall brightness levels, which were normalized using the numerical values of a small sample of background area, generating images that subjectively resembled those observed under the microscope; thus, these images are nonquantitative.

Rac Activity Assays

Cells were incubated overnight in DMEM with 0.5% FBS, detached by trypsinization and neutralized with an equal volume of 2.5 mg/mL soybean trypsin inhibitor, or detached by treatment with TrypLe Express

(Invitrogen) and neutralized by 10-fold dilution in serum-free medium, followed by centrifugation. The cells were then resuspended in serum-free DMEM containing 20 mg/mL BSA and plated onto a series of 60 mm dishes that had been precoated with 20 μg/mL fibronectin (Calbiochem) and blocked with 3% BSA. At the indicated times, cells were scraped into 0.4 mL cold lysis buffer [0.5% Triton X-100, 50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 10% glycerol, 10 mmol/L $MgCl_2$ + 1× complete Roche protease inhibitors, EDTA-free] and precleared. Ten micrograms of bacterially expressed GST-PAK (CRIB domain) protein were added and the samples were incubated on ice for 1 hour, followed by addition of 30 μL of a 50% slurry of glutathione-Sepharose (Pharmacia, Piscataway, NJ; equilibrated in lysis buffer containing 20 mg/mL BSA) and further incubation for 1 hour in the cold room on a rotation device. Beads were washed thrice in lysis buffer and analyzed on Western blots for Rac using monoclonal antibodies (mAb) from BD PharMingen, peroxidase-labeled antimouse antibody (Invitrogen), and Pierce West Pico chemiluminescence kit (Pierce, Rockford, IL).

Immunofluorescence

Cells grown on coverslips were fixed with cold methanol for the tubulin/pericentrin staining or with 4% paraformaldehyde for the cortactin/actin staining, followed by 5-minute permeabilization with 0.1% Triton X-100 for the latter. Cells were stained with anti-α-tubulin mouse mAb (DM1A; Calbiochem), antipericentrin polyclonal antibody (Covance, Berkley, CA), anticortactin mAb (provided by Scott Weed) followed by Alexa-488- or Alexa-594-labeled secondary antibodies (Pierce) and mounting with Vectashield containing DAPI (Vector Labs, Burlingame, CA) using AxioVision software and imported into Photoshop, where individual color intensities and contrast were adjusted for clarity of presentation; image intensities are subjectively accurate but nonquantitative.

Results and Discussion

Caspase-8 regulates cell motility. We assayed MEFs obtained from caspase-8 knockout mice (14) for motility using an ECIS instrument (49). Caspase-8-null cells were motility deficient (Fig. 2*A*), in agreement with conventional wound healing (Fig. 2*B*) and chemotaxis/transwell assays (Fig. 2*C*). To exclude the possibility that these MEFs might differ in motility due to caspase-8-independent epiphenomena, we expressed the viral caspase-1/8 inhibitor protein crmA (44) in the wild-type MEFs and in WTNR6 fibroblasts; crmA inhibited motility efficiently in both (Supplementary Figs. S1 and S2). To further exclude epiphenomena, reexpression of inducible *caspase-8* gene in caspase-8-null MEFs stimulated cell motility (Supplementary Fig. S3). The knockout of the caspase-8 effector, caspase-3, did not suppress cell motility (Supplementary Fig. S4). This result suggests that caspase-8 may act directly rather than through caspase-3 to cleave substrates critical for cell motility, although other effector caspases cannot be excluded.

Although different cell types undoubtedly use diverse cell motility pathways, similar results were obtained in NB7 neuroblastoma cells (45) and MDA-MB-231 mammary carcinoma cells (Supplementary Fig. S5-S7; note that the effect in NB7 cells was observed in single-cell motility and ECIS assays but not in a standard wound-healing assay, presumably reflecting greater sensitivity of detection and resistance to cell-to-cell interaction effects in the assays used; data not shown). Thus, caspase-8 may promote motility in tumor cells, potentially enhancing tumor metastasis in contexts where cell migration is rate limiting (e.g., ref. 37). In certain specific tumor types, such as neuroblastoma, where *caspase-8* is silenced or deleted, reexpression of caspase-8 can, paradoxically, suppress metastasis by conferring anoikis sensitivity (50). However, most metastatic tumor cells express

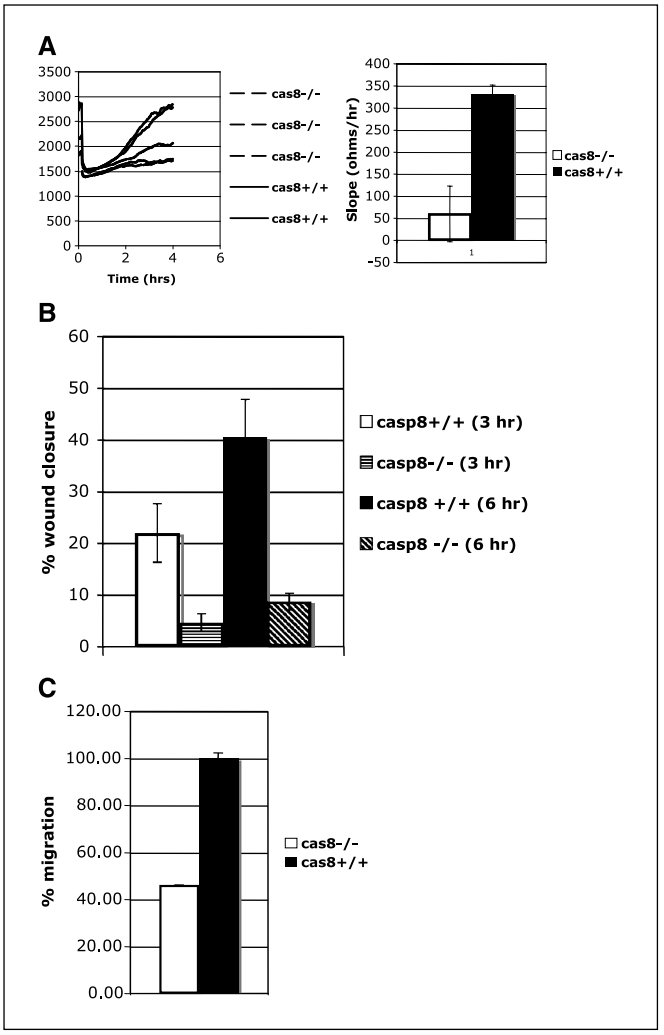

Figure 2. Caspase-8 promotes cell motility. *A,* ECIS analysis of caspase-$8^{-/-}$ versus caspase-$8^{+/+}$ MEFs. *Left,* impedance versus time plot. *Right,* slopes calculated from the linear part of the graphs in the ECIS tracing (after subtraction of slopes measured in "unwounded" wells). *B,* conventional wound healing assay, comparing caspase-$8^{-/-}$ versus caspase-$8^{+/+}$ MEFs. *C,* directed migration/chemotaxis assay, comparing caspase-$8^{-/-}$ versus caspase-$8^{+/+}$ MEFs, normalized for total number of adherent cells.

active caspase-8 but are, by definition, resistant to anoikis. This resistance is presumably due to alterations in numerous other pathways (e.g., bcl-2 family members, integrin signaling molecules, and autocrine matrix generation). Thus, in most human tumors, the presence or absence of caspase-8 is not the major parameter of anoikis sensitivity. In summary, although caspase-8 is metastasis suppressive in neuroblastoma, it is potentially metastasis promoting in other tumor types because it stimulates cell migration.

Caspase-8 promotes calpain activation. Cell motility is dependent on calpain activity in numerous cell systems. In particular, the dependence of cell motility on calpain has been shown in MEFs using genetic knockout of calpain-4 (51) and in WTNR6 cells by using antisense RNA (43).

We tested the effect of caspase-8 on cell motility in these calpain-dependent cell types. Caspase-8 promoted calpain activity in both of these cell lines as well as in a tumor cell line (Fig. 3*A-C*; Supplementary Fig. S8). To exclude epiphenomena, inducible

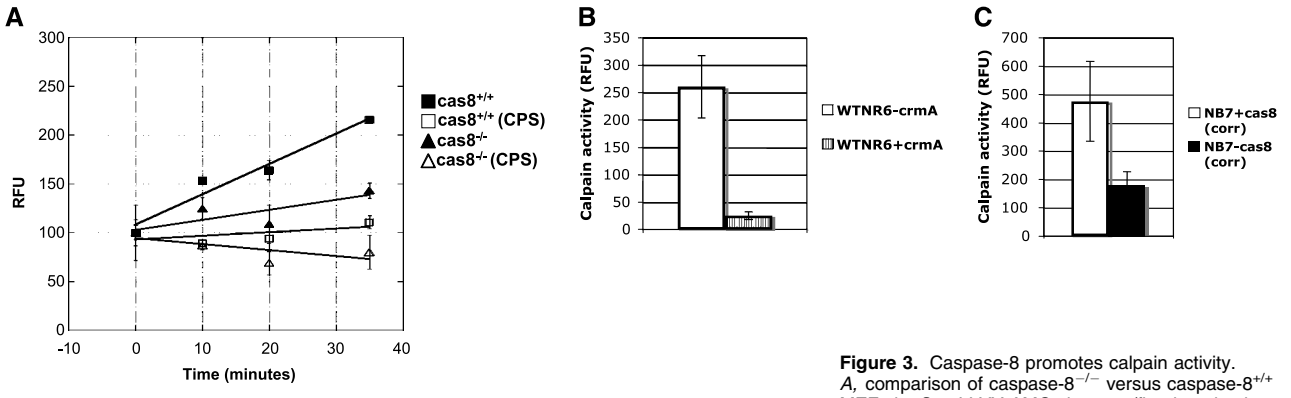

Figure 3. Caspase-8 promotes calpain activity. *A,* comparison of caspase-$8^{-/-}$ versus caspase-$8^{+/+}$ MEFs by Suc-LLVY-AMC cleavage/fluorimetric plate reader assay. *RFU,* relative fluorescence units. *B,* comparison of WTNR6 cells with or without crmA by Suc-LLVY-AMC cleavage assay. *C,* comparison of NB7 cells with vector alone or with caspase-8 (*NB7+cas8*) using the Suc-LLVY-AMC cleavage assay. *D,* comparison of caspase-8-null MEFs stably transfected with Rheoswitch-inducible caspase-8 or with Rheoswitch vector alone after 24 hours of induction.

reexpression of caspase-8 in caspase-8-null MEFs stimulated calpain activity (Fig. 3*D*). By contrast, caspase-3 knockout had no significant effect on calpain activity (Supplementary Fig. S9). These data indicate that caspase-8 promotes calpain activity and cell motility in cell types whose motility is calpain dependent. Although these data suggest that caspase-8 promotes motility through calpain activation, additional effects of caspase-8 on calpain-independent aspects of cell motility cannot be excluded.

Calpain activity is often elevated in transformed cells (33) where it promotes cell motility/invasion/metastasis (35, 37). Our results suggest that caspase-8 promotes calpain activity, thereby augmenting these malignant properties of tumor cells.

Caspase-8 affects cellular processes downstream of calpain activation. To confirm that caspase-8 regulates the calpain pathway, we examined several calpain-dependent processes for caspase-8 dependence: Rac activation, lamellipodial assembly, cell adhesion, and accurate nuclear segregation. First, Rac activation is calpain dependent in certain cell systems (52). Consistent with this, caspase-$8^{-/-}$ cells activated Rac inefficiently compared with control cells (Fig. 4). This suggests that caspase-8 promotes rac activation and subsequent cytoskeletal remodeling through calpains. Second, the genetic knockout of calpain-4 or small interfering RNA (siRNA) depletion of calpain-2 inhibits lamellipodia formation in MEFs (51, 53). Accordingly, caspase-8-deficient MEFs were defective in generating lamellipodia (Fig. 5*A*). Interestingly, the caspase-8-null MEFs exhibited numerous long, thin projections resembling calpain-4-knockout MEFs (51) or calpain-2 knockdown cells (53). Conversely, the induced reexpression of caspase-8 in caspase-8-null MEFs restored their ability to form lamellipodia (Fig. 5*B*). Third, calpains are involved in both adhesion complex assembly and disassembly (52, 54), suggesting that cell adhesion per se could be affected either positively or negatively in a context-dependent fashion. Caspase-8 promoted cell adhesion in MEFs and NB7 cells (Fig. 5*C*). These results suggested that caspase-8 also promotes cell adhesion, although cell type specificity of this effect was observed in that caspase-8 reduced cell adhesion in two epithelial cell lines (data not shown).

Fourth, calpain activity is required for accurate alignment of chromosomes on the metaphase plate; siRNA depletion of calpain causes misalignment and increases the frequency with which cytokinesis is aborted, resulting in multinucleate cells (55). Consistent with this, caspase-8-null MEFs displayed an elevated frequency of multinucleate cells (Supplementary Fig. S10). This

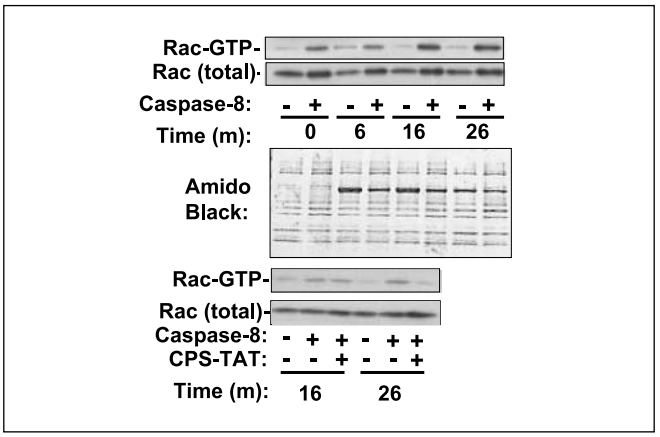

Figure 4. Caspase-8 promotes Rac activity. Caspase-$8^{-/-}$ or caspase-$8^{+/+}$ MEFs were assayed at the indicated times after attachment to fibronectin for activated or total Rac; the Amido Black–stained filter shows equal loading (the BSA band arises from the carrier protein used in the cell attachment incubation buffer; the zero time sample is free of BSA because cells were immediately lysed after trypsinization.) *Bottom,* a similar experiment was done with or without the addition of CPS-TAT protein to the indicated samples at time zero. (Note that import normally takes 5 to 10 minutes, consistent with the lack of effect at the first time point).

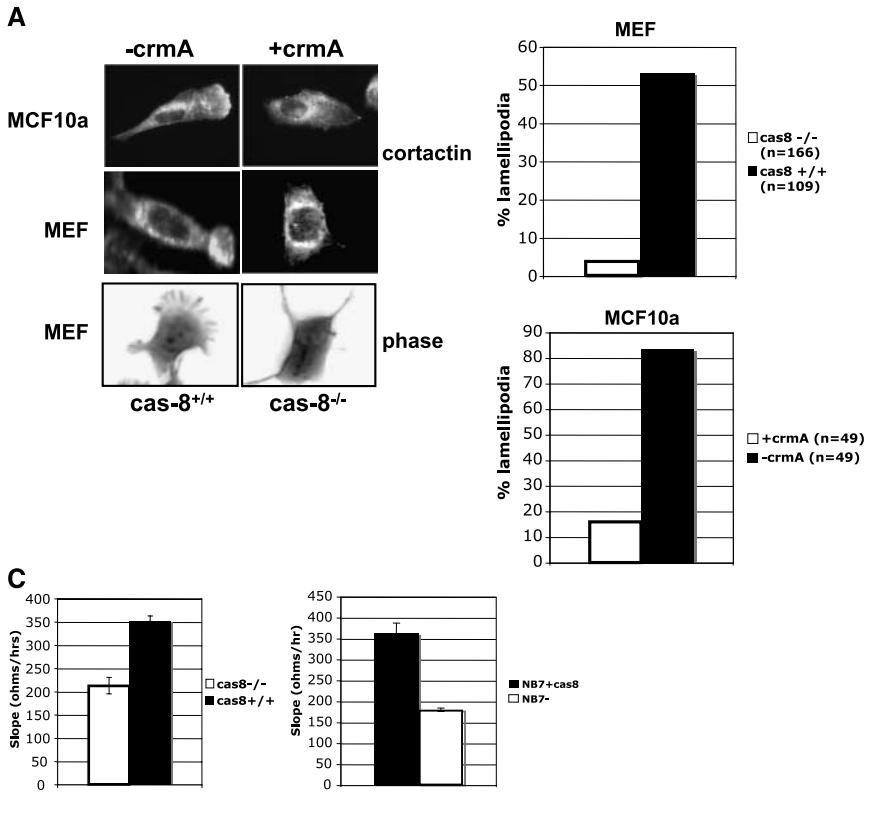

Figure 5. Caspase-8 affects cellular processes downstream of calpain activation: lamellipodial assembly, cell adhesion, and normal nuclear segregation. *A,* caspase-8 promotes the accumulation of lamellipodia. *Top* and *middle,* MCF10a cells with or without crmA were plated at low density in medium containing EGF, fixed, and stained with antibodies against the lamellipodia marker cortactin (*green*), rhodamine-phalloidin (*red*), and DAPI (*blue*). *Bottom,* the "attachment-control" MEFs that were assayed for chemotactic migration in Fig. 1*C* were stained with crystal violet and photographed. Frequencies of lamellipodia were scored and represented graphically (the phase images were used to generate the MEF graph). MEFs, $P < 0.003$; MCF10a, $P < 0.001$. *B,* caspase-8 promotes the accumulation of lamellipodia: caspase-8-inducible cell lines. *C,* caspase-8 promotes cell adhesion to matrix in MEF cells and NB7 cells.

latter result may connect the paradoxical caspase-8 down-regulation that is observed in restricted tumor types (45) with chromosomal instability (56).

Elucidation of the mechanism by which caspase-8 activates calpains is in progress. Caspase-8 could affect the phosphorylation of calpain-2 (48), the levels of the endogenous inhibitor calpastatin (46), or the availability of calcium or phospholipids for interaction with calpains (33); caspase-8 had no effect on the expression of calpain-1 or calpain-2 (data not shown).

The elevated caspase-8 activities in many tumor cell lines (2) may be causally related to their increased calpain activity, motility, and invasiveness. In this connection, inhibition of calpains suppresses invasion in certain tumor models (35, 37), whereas activation of several signaling pathways, including caspase-8, by FASL promotes invasion (57), suggesting that caspase-8 inhibition may provide the basis for a novel antimetastatic therapy in tumor types that are anoikis-resistant due to other gene alterations.

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