Caspase-8 Interacts with the p85 Subunit of Phosphatidylinositol 3-Kinase to Regulate Cell Adhesion and Motility

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Abstract

Cell migration plays an important role in tumor cell invasion and metastasis. Previously, we reported that caspase-8 contributes to cell migration and adhesion, a novel nonapoptotic function of an established apoptotic factor. Herein, we report that pro-caspase-8 is capable of restoring cell migration/adhesion to caspase-8-null cells, establishing the first biological function of a pro-caspase. The catalytic activity of caspase-8 was not required for cell motility. Stimulation of motility with epidermal growth factor induced the phosphorylation of caspase-8 on tyrosine-380 and the interaction of caspase-8 with the p85α subunit of phosphatidylinositol 3-kinase. Tyrosine-380 was required for the restoration of cell motility and cell adhesion in caspase-8-null cells, demonstrating the importance of the caspase-8–p85 interaction for these nonapoptotic functions. These results suggest that caspase-8 phosphorylation converts it from a proapoptotic factor to a cell motility factor that, through tyrosine-380, interacts with p85, an established cell migration component. [Cancer Res 2007;67(24):11505–9]

Introduction

Cell migration plays a critical role in tumor cell invasion and metastasis (1). Previously, we reported that caspase-8 promotes cell migration, cell adhesion, and Rac activation in normal and tumor cell lines (2). Caspase-8 has also been implicated in the proinvasive effects of EASL in tumor cells (3). Although the genesis of certain tumor types such as neuroblastoma and small-cell lung cancer involves the loss of caspase-8 expression, which suppresses anoikis in neuroblastomas (4), expression is maintained or increased in most tumor types (Supplementary Fig. S1). Thus, caspase-8 may contribute to metastasis in the more typical anoikis-resistant tumor cell context. This mechanism could have important ramifications for cancer therapy.

Phosphatidylinositol 3-kinase (PI3K) is an important component of the cell migration apparatus. PI3K supports Rac/cdc42 activation both by production of lipid products that activate or localize guanine exchange factors (GEF; ref. 5), as well as through direct interactions of Rac, cdc42, and Rac-GEFs with the p85 regulatory subunit (6, 7), which are involved in cdc42-mediated c-Jun-NH2-kinase activation (8). PI85 function is important for cell migration and motility. This study presents data that caspase-8 interacts with the p85 subunit of PI3K, promoting cell adhesion and motility.

Materials and Methods

Cell lines. NB7 cells were obtained and cultured as described previously (2). Stable derivatives expressing caspase-8 mutants were constructed by packaging the appropriate retrovirus construct in the vector pBABE-puro in gp2+293 cells in the presence of pAmpho (Invitrogen), infection of viral stock, selection with 0.5 μg/mL puromycin, and Western blotting of mixed populations; similar expression levels were obtained for all constructs used in this study (data not shown). A431 epidermal carcinoma cells were obtained from American Type Culture Collection and maintained in DMEM containing 10% fetal bovine serum, penicillin, streptomycin, and glutamine.

DNA constructs, transfections, and pull downs. Details of the construction of our expression constructs are provided in the Supplementary Materials and Methods section. For pull-down experiments on transfected S-tagged constructs, cell lysates were incubated with S-protein agarose, washed, and analyzed as described in Supplementary Materials and Methods.

Endogenous coimmunoprecipitation. A431 cells treated with epidermal growth factor (EGF) were lysed and immunoprecipitated with p85 antibodies as described in Supplementary Materials and Methods.

Rac activation assay. Rac activity was assayd by incubation of cell lysates with glutathione S-transferase-PAK (CRIB domain) constructs as described in Supplementary Materials and Methods.

SH2 domain array screening. A peptide spanning human caspase-8 Y380 ([biotin-K]GGGDSSEPQ[phospho-Y]LEMDLSSPQT) was synthesized (Bachem) and reacted with an SH2 domain array (Panomics), detecting with streptavidin–horseradish peroxidase, according to the manufacturer’s protocol, except that detection was with enhanced chemiluminescence reagent (Pierce).
Adhesion and motility assays. Adhesion measurements using electrical cell substrate impedance sensing (ECIS) and motility measurements using single-cell motility were done as indicated and assayed for motility or adhesion. A, pro-caspase-8 rescues adhesion. Cells were assayed for kinetics of adhesion to fibronectin using ECIS; the slopes of the linear portion of the ECIS tracing are plotted; note that the fold stimulation of adhesion is similar to that of wild-type caspase-8 reported previously (2). Cas8DA, the uncleavable caspase-8 mutant, D374A/D384A; vector, pBABE-puro. B, the catalytic activity of caspase-8 is not required and the phosphorylation site Y380 is critical for cell adhesion. An ECIS adhesion assay comparing the wild-type (wt), catalytically inactive (C360A), or the phosphorylation site-defective mutant of inactive (Y380F/C360A) caspase-8 is shown. P values for the difference between the indicated samples are shown. C, pro-caspase-8 promotes cell motility, which is dependent on Y380. A single cell-motility assay is presented as a radial plot indicating distance (not direction) of migration. DA, the uncleavable mutant D374A/D384A; Y380F/DA, the phosphorylation site-defective/uncleavable double mutant; vector, pBABE-puro. D, inactive caspase-8 promotes cell motility, which is dependent on Y380. C360A, catalytically inactive caspase-8; Y380F/C360A, the phosphorylation site–defective/catalytically inactive double mutant caspase-8.

Figure 1. Caspase-8 enhancement of cell adhesion/motility is dependent on tyrosine-380, but independent of catalytic activity or autoproteolytic processing. For these experiments, the caspase-8-null cell line NB7 was infected with retroviral caspase-8 expression constructs as indicated and assayed for motility or adhesion. A, pro-caspase-8 rescues adhesion. Cells were assayed for kinetics of adhesion to fibronectin using ECIS; the slopes of the linear portion of the ECIS tracing are plotted; note that the fold stimulation of adhesion is similar to that of wild-type caspase-8 reported previously (2). Cas8DA, the uncleavable caspase-8 mutant, D374A/D384A; vector, pBABE-puro. B, the catalytic activity of caspase-8 is not required and the phosphorylation site Y380 is critical for cell adhesion. An ECIS adhesion assay comparing the wild-type (wt), catalytically inactive (C360A), or the phosphorylation site–defective mutant of inactive (Y380F/C360A) caspase-8 is shown. P values for the difference between the indicated samples are shown. C, pro-caspase-8 promotes cell motility, which is dependent on Y380. A single cell-motility assay is presented as a radial plot indicating distance (not direction) of migration. DA, the uncleavable mutant D374A/D384A; Y380F/DA, the phosphorylation site–defective/uncleavable double mutant; vector, pBABE-puro. D, inactive caspase-8 promotes cell motility, which is dependent on Y380. C360A, catalytically inactive caspase-8; Y380F/C360A, the phosphorylation site–defective/catalytically inactive double mutant caspase-8.

Results and Discussion

Caspase-8 enhancement of cell adhesion/motility is dependent on tyrosine-380, but independent of catalytic activity or autoproteolytic processing. We reported previously that caspase-8 enhanced EGF-activated cell motility and cell-matrix adhesion in embryonic and adult fibroblasts, as well as several mammary and lung tumor lines, demonstrating the effect of caspase-8 in diverse cell contexts (2); to facilitate the analysis of caspase-8 mutants, the caspase-8-null NB7 neuroblastoma cell line was used primarily in the current study. The cleavage of caspase-8 was not observed in cells undergoing stimulated motility or cell adhesion (data not shown). We assayed an uncleavable caspase-8 mutant that persists in the full-length pro-caspase form even in death ligand–stimulated cells and therefore fails to support apoptosis, D374A/D384A (10), for its effects on cell adhesion and motility. When expressed in the caspase-8-null neuroblastoma cell line NB7, this mutant restored both cell adhesion kinetics and single-cell random motility as efficiently as wild-type caspase-8 [compare Fig. 1 with (Supplementary Fig. S6 of ref. 2)]. This result suggested that the pro-caspase-8 protein, the predominant species observed in nonapoptotic cells,
functions as a cytoskeletal control element that affects motility and adhesion. Moreover, the inactivation of caspase-8 catalytic activity (C360A) had no substantial effect on either its motility or adhesion effects (Fig. 1), suggesting a novel noncatalytic mechanism.

Pro-caspase-8 is phosphorylated in cells with hyperactive c-Src or stimulated with EGF (11), conditions that stimulate cell motility. The phosphorylation site, Y380, is uniquely present in pro-caspase-8 but absent in processed caspase-8 products. In light of the function of pro-caspase-8 shown above, we hypothesized that this phosphorylation promoted a protein-protein interaction that contributed to the cytoskeletal effects of caspase-8. To test this hypothesis, a Y380F (phosphorylation site-deficient) mutant was expressed in NB7 cells in the context of uncleavable (D374A/D384A) or catalytically inactive (C360A) forms of caspase-8. These mutants were defective in rescuing both cell motility and cell adhesion (Fig. 1). This result shows that Y380 plays a substantial role in these nonapoptotic functions of caspase-8. This effect of the phosphorylation site Y380 is clearly distinct from its role in suppressing caspase-8 processing in that the point mutant Y380F exhibited a strong effect in a caspase-8 mutant that was uncleavable. These results suggested that the phosphorylation at Y380 promoted a noncatalytic activity (C360A).

Figure 2. Caspase-8 interacts with p85. A, a phosphorylated peptide spanning caspase-8 Y380 reveals candidate interaction partners including p85. B, caspase-8 interaction with p85: dependence on Src and Y380. COOH-terminally S-tagged constructs of catalytically inactive caspase-8 containing no other mutations (wt), or the mutations Y380F or Y334F as indicated were cotransfected with the indicated c-Src constructs (K295M, catalytically inactive src; Y527F and dl92, activated mutant src) and HA-tagged p85. Lysates were precipitated with S-protein agarose and probed for HA-p85, caspase-8-S-tag, or phosphotyrosine (the band corresponding to caspase-8 is shown). To confirm similar c-Src expression levels for the four constructs used, separate wells were transfected, thus avoiding antifade spillover bands due to the chromatography of caspase-8 with c-Src (data not shown). IB, immunoblotting. C, the COOH-terminal SH2 domain of p85 is required for the caspase-8 interaction. Caspase-8-S-tag was cotransfected with p85-6Xhis (wild-type or ΔSH2-COOH terminal) and S-protein agarose pull downs were analyzed for p85 as above. D, endogenous caspase-8–p85 interaction. Top, lysates from EGF-treated A431 cells were immunoprecipitated (IP) with antibodies against p85, p85-Ab1 (Upstate Biotechnology), or p85-Ab2 (Upstate Biotechnology) or an equal amount of control rabbit IgG. Immunoprecipitates were probed for caspase-8; the Amido Black–stained bands of equal amounts of p85 versus control IgG precipitated. Note that a diffuse band of immunoglobulin heavy chain (not shown) migrated only slightly faster than the caspase-8 band, causing the smear of black density seen at the lower portion of each caspase-8 band. Middle, A431 cells were serum starved, and either induced with EGF (4 min) or uninduced, before the immunoprecipitation with p85-Ab1. For clarity (i.e., to avoid spillover signal from the IgH chain band), a light exposure is shown. Bottom, EGF stimulates caspase-8 phosphorylation. A cell line that expresses S-tagged caspase-8 at a total caspase-8 level of ~150% of the endogenous level was generated (Materials and Methods). This cell line, or, as a control, normal A431 cells were serum starved and treated with EGF (5 min). Cell lysates were affinity-purified with S-protein agarose and the phosphorylated caspase-8 band was detected on a Western blot by probing with phosphotyrosine antibody.
Caspase-8 interacts with the p85 subunit of PI3K. Y380 occurs in the peptide sequence YELM, which was predicted by Scansite\(^1\) to interact with p85 SH2 domains with 99.5\% probability. To test potential interactions with SH2 domain proteins in an unbiased manner, a SH2 domain array (Panomics) was screened in an unbiased manner, a SH2 domain array (Panomics) was screened for phospho-Akt and total Akt ratios derived by densitometry; an additional experiment produced similar results. The average fold increase of Y380 versus F380 at 25 min was 1.67 ± 0.14. D374A/D384A, uncleavable caspase-8 mutant; D374A/D384A/Y380F, uncleavable/phosphorylation site-defective caspase-8 mutant. B, caspase-8 does not contribute to Akt phosphorylation. NB7 cells with the indicated caspase-8 constructs were serum-starved, stimulated with EGF, and assayed for Akt serine-473 phosphorylation by Western blotting. Densitometry of phospho-Akt and total Akt were used to derive ratios, which were plotted here; a lack of effect of caspase-8 on Akt phosphorylation was also observed with matrix reattachment and serum re-addition (data not shown).

In light of the Scansite prediction and these data, we tested the potential of these proteins to interact with an inactive mutant of caspase-8—to prevent apoptosis induction—in transfected cells. P85 strongly interacted with caspase-8 (Fig. 2B). This interaction depended on the integrity of the Y380 site but was independent of another phosphorylation site, Y334, identified independently by proteinomics (12). The interaction was also dependent on cotransfected, activated c-src (Y327F or Δ92-95) or c-fyn (Y531F), which were more effective than wild-type c-src or kinase-inactive c-src (K295M). The phosphorylation of caspase-8 by these src constructs correlated with the magnitude of the caspase-8–p85 interaction. Deletion of the COOH-terminal SH2 domain of p85, the adaptor protein Nck-2, c-fyn, SHP-2, and Ras-GAP as potential interactors (Fig. 2A).

To test whether caspase-8 interacted endogenously with p85, total protein from EGF-treated A431 cells was immunoprecipitated with polyclonal p85\(\alpha\) antibodies directed against the NH\(_2\)-terminal SH2 domain or the full-length protein, followed by Western blotting for caspase-8 using a highly specific monoclonal antibody. Caspase-8 coimmunoprecipitated with p85 (Fig. 2C). The reverse coimmunoprecipitation of p85 with caspase-8 was observed as well, but a variable background of p85 binding to protein-A beads even under stringent detergent conditions in the presence of protein blocking agents made this direction less reliable (data not shown). These data indicated that caspase-8 interacts with p85\(\alpha\) in EGF-stimulated cells. The endogenous caspase-8–p85 interaction was stimulated by EGF, which correlated with the induction of caspase-8 phosphorylation (Fig. 2D).

Caspase-8 stimulates the activation of Rac in response to matrix reattachment of mouse embryo fibroblasts (2). In light of the important role of p85 in Rac activation, we hypothesized that the effect of caspase-8 on Rac was mediated by p85. To test this, NB7 cells with Y380 (wild-type) or F380 (non-tyrosine-phosphorylated) forms of caspase-8 were assayed for Rac activation after reattachment to fibronectin. Consistent with the effect of the Y380F mutation on cell adhesion and motility, this mutation also suppressed Rac activation relative to wild-type caspase-8 (Fig. 3A). One potential mechanism for the activation of Rac through p85 is the stimulation of PI3K activity, generating lipid products that activate Rac-GEFs. Phosphorylation of Akt on serine-473, an indicator of PI3K activity, was not, however, affected by caspase-8, after EGF stimulation (Fig. 3B) or serum re-addition (data not shown). It is therefore more likely that caspase-8 affects Rac activation mediated by p85 through an alternative mechanism that remains to be elucidated.

In principle, caspase-8 could affect motility and adhesion through multiple mechanisms that may or may not depend on its catalytic activity. Pro-caspase-8 can acquire catalytic activity by homodimerization mediated by FADD protein or by...

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\(^1\) http://scansite.mit.edu
Caspase-8 could regulate PI3K-mediated cell migration (14–16) through at least two pathways. First, caspase-8 could affect PI3K catalytic activity, which generates lipid products (PIP2 and PIP3) that activate GEFs (5). Second, caspase-8 could affect p85 Bcr-homology domain interaction with Rac/cdc42, and/or the NH2-terminal SH2 domain interaction with a Rac-GEF complex (6, 7, 15, 17). Although the data do not exclude the possibility that caspase-8 mediates a localized PI3K activation, which could be detected by other experimental approaches, the lack of effect of caspase-8 on Akt phosphorylation supports the second mechanism. The details of this mechanism are being elucidated currently, as is the possibility that p85 serves to recruit caspase-8 to EGF receptor complexes, affecting signaling through this receptor.

Consistent with our observation that caspase-8 is usually maintained in human tumors, a six-nucleotide polymorphic variant in the caspase-8 promoter region that attenuates transcription is associated with lowered cancer incidence (18). The contribution of caspase-8 to cell migration through p85 suggests that caspase-8 may contribute to tumor progression in this manner.

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References

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