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 FASL 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-NH_2-kinase activation (8). P85 function is affected by the binding of tyrosine phosphorylated proteins to its SH2 domains, as well as src-mediated phosphorylation (9). The potential for caspases to interact functionally with PI3K has not been explored.

In apoptotic cells, caspase-8 undergoes two sequential autoproteolytic cleavage reactions that separate the large and small catalytic subunits as well as deleting a small linker peptide sequence (amino acids 374–384) and the prodomain (amino acids 1–216), releasing the mature tetramer form into the cytoplasm (10). However, the pro-caspase-8 form predominates in nonapoptotic cells. In principle, pro-caspase-8 versus processed caspase-8 could function as a migration/adhesion and apoptosis factors, respectively. In that scenario, pro-caspase-8 could potentially cleave key substrates for motility—remaining to be identified—or could function noncatalytically as an interaction partner for a motility factor. In the latter connection, it has been reported recently that pro-caspase-8 is phosphorylated on tyrosine-380 by c-src; this phosphorylation suppresses caspase-8 autocleavage and apoptosis (11). In principle, it could also facilitate the interactions of caspase-8 promigratory proteins containing phosphotyrosine binding domains.

In this report, we show that Y380-phosphorylated pro-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 transiently 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 assayed 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)GGGDSEEQP(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).

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

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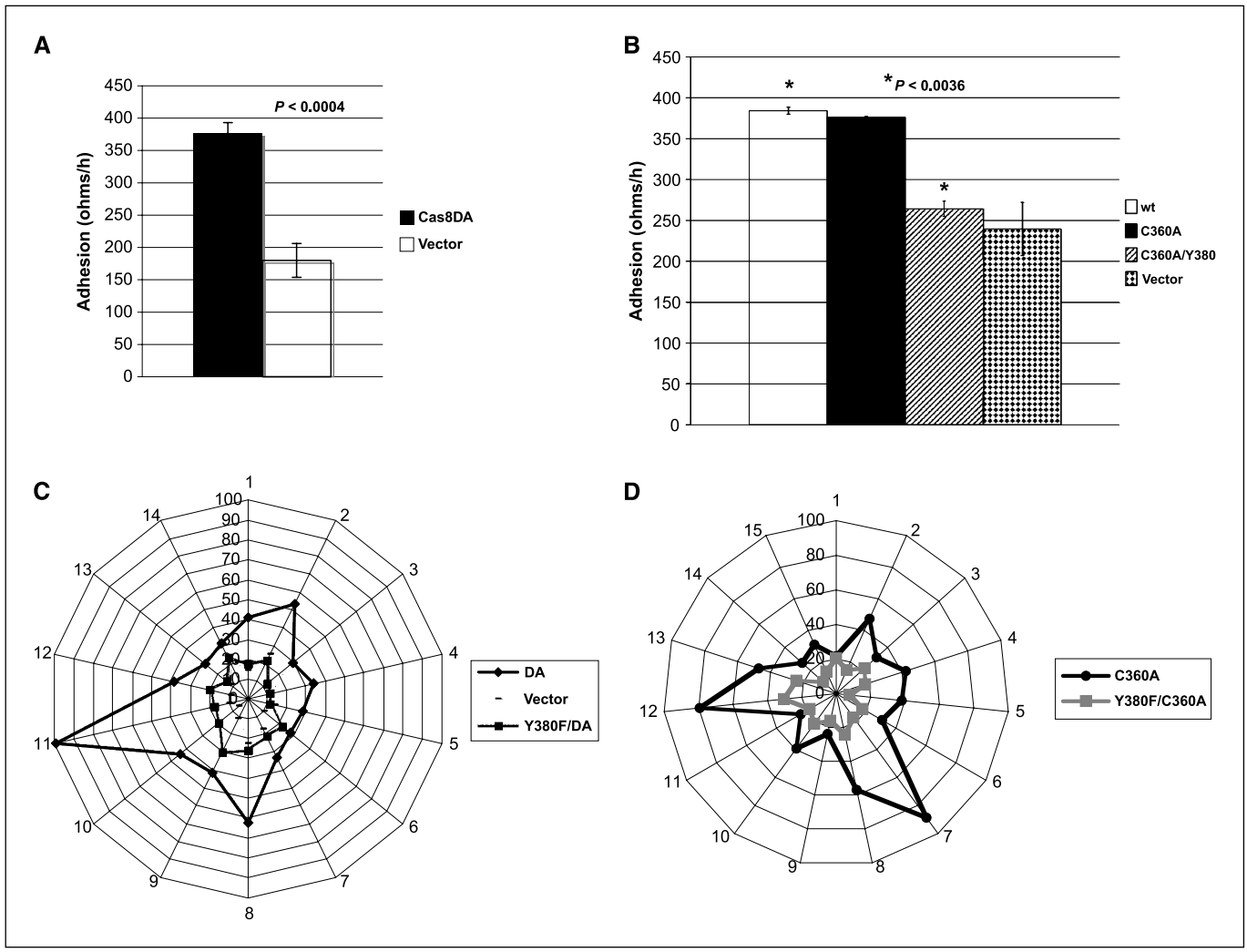

Figure 1. Caspase-8 enhancement of cell adhesion/motility is dependent on tyrosine-380, but independent of catalytic activity or autoproteolytic processing. For these experiments, the caspase-8-null cell line NB7 was infected with retroviral caspase-8 expression constructs as indicated and assayed for 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.

Adhesion and motility assays. Adhesion measurements using electrical cell substrate impedance sensing (ECIS) and motility measurements using single-cell motility were done as described previously (2).

Akt phosphorylation assays. Akt phosphorylation after EGF stimulation was monitored on Western blots using a phospho-Akt–specific antibody as described in Supplementary Materials and Methods.

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

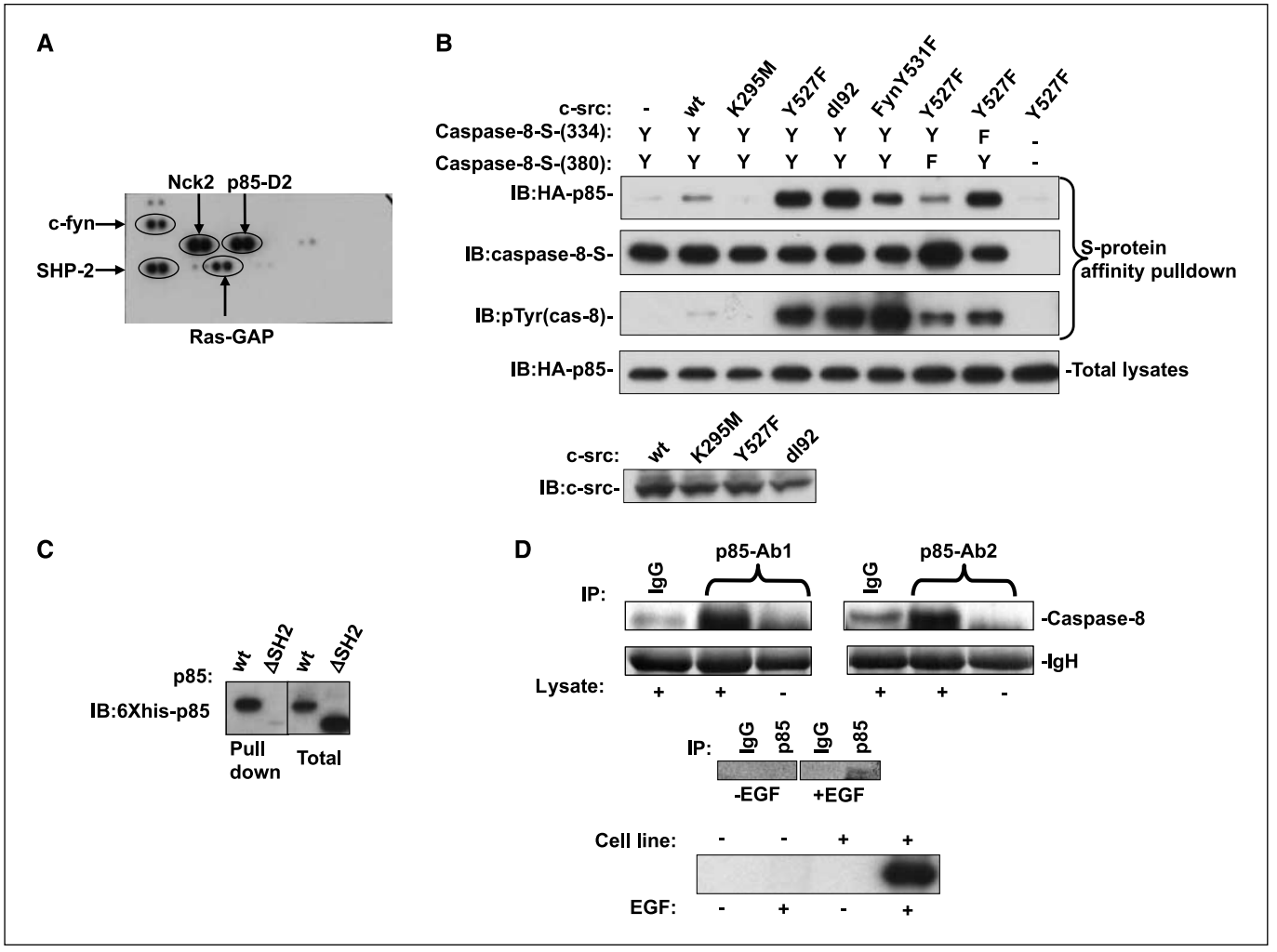

Figure 2. Caspase-8 interacts with p85. *A,* a phosphorylated peptide spanning caspase-8 Y380 reveals candidate interaction partners including p85. *B,* caspase-8 interaction with p85: dependence on src and Y380. COOH-terminally S-tagged constructs of catalytically inactive caspase-8 containing no other 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 artifactual spillover bands due to the comigration 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 p85α, 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 beneath the Western blot show that 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.

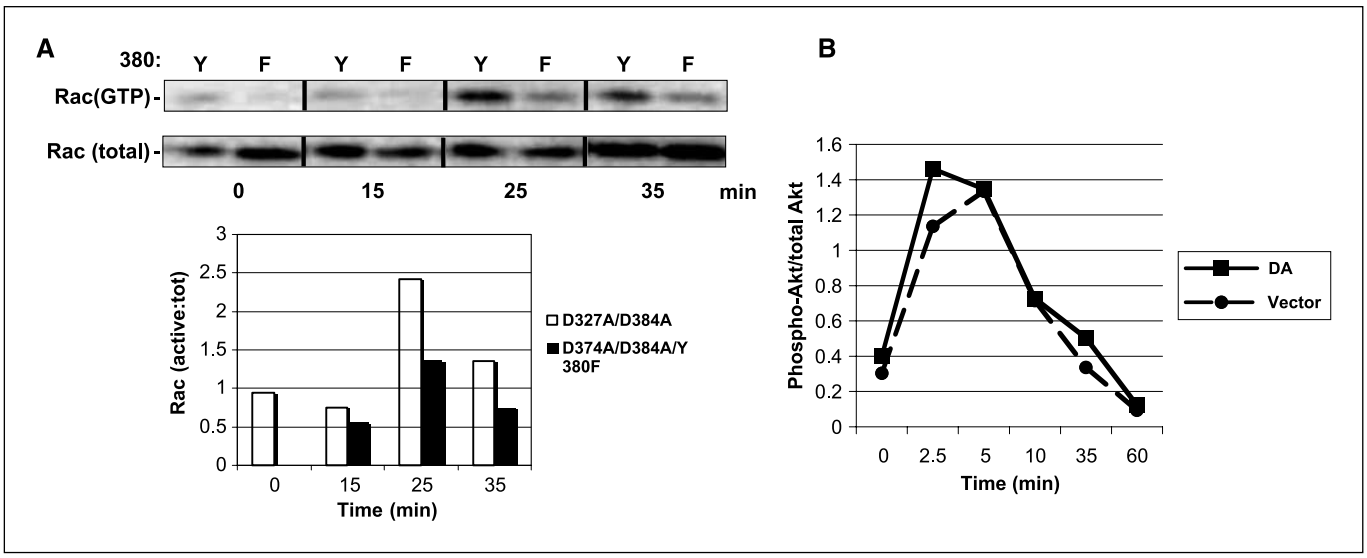

Figure 3. Caspase-8–p85 interaction contributes to Rac activation but does not enhance Akt activation. *A,* caspase-8–p85 interaction contributes to Rac activation. NB7 cells rescued with uncleavable caspase-8 containing Y380 (*Y*) or F380 (*F*) were trypsinized, attached to fibronectin-coated dishes for the indicated times, and lysates were assayed for total or activated Rac. *Bottom,* a plot of activated Rac to total Rac 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).

protein interaction that was involved in cell adhesion and/or motility.

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 with a phosphorylated, biotinylated peptide spanning this sequence. The array identified the COOH-terminal SH2 domain of p85, the adaptor protein Nck-2, c-fyn, SHP-2, and Ras-GAP as potential interactors (Fig. 2*A*).

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. 2*B*). This interaction depended on the integrity of the Y380 site but was independent of another phosphorylation site, Y334, identified independently by proteomics (12). The interaction was also dependent on cotransfected, activated c-src (Y527F 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 abolished the interaction (Fig. 2*C*), implicating this SH2 domain in the caspase-8 interaction. Other candidates identified from the Panomics screen (Nck-2, c-fyn, SHP-2) interacted more weakly or independently of phosphorylation (Supplementary Fig. S2). These data map the interaction on p85 to the COOH-terminal SH2 domain and on caspase-8 to Y380.

To test whether caspase-8 interacted endogenously with p85, total protein from EGF-treated A431 cells was immunoprecipitated with polyclonal p85α 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. 2*D*). 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 p85α in EGF-stimulated cells. The endogenous caspase-8–p85 interaction was stimulated by EGF, which correlated with the induction of caspase-8 phosphorylation (Fig. 2*D*).

The caspase-8–p85 interaction affects Rac activation. Caspase-8 stimulated 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. 3*A*). 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. 3*B*) 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

[1] http://scansite.mit.edu

heterodimerization with c-FLIP, and, in apoptotic cells, this proteolytic activity autocatalytically releases the active subunit tetramer form of caspase-8 into the cytoplasm (10, 13). A biological function for pro-caspase-8 other than serving as a precursor for mature caspase-8 has not yet been found. In this report, we show that neither caspase-8 processing nor catalytic activity is required for the nonapoptotic cell motility and matrix adhesion functions of caspase-8. This dissociates the apoptotic from the nonapoptotic functions of caspase-8, which are performed by the processed versus unprocessed forms of the protein, respectively. The observation that Y380 phosphorylation of caspase-8 by c-src interferes with processing (11), integrated with our current data, suggests a new mechanism for c-src to promote cell migration.

The interaction of phosphorylated caspase-8 with p85 and the importance of this interaction for cell motility, cell adhesion, and Rac activation provides one mechanism underlying the effects of caspase-8 on these processes. It is important to note, however, that additional mechanisms undoubtedly exist, as mouse caspase-8, which lacks a Y380 homologue, can also stimulate cell motility in certain mouse cell lines (2). Although the mouse homologue would not be predicted to be effective in the human cell system used here, there may be other human cell contexts in which other mechanisms involving catalytic activity play a more significant role; in this connection, mouse and human caspase-8 also enhanced EGF-mediated extracellular signal-regulated kinase activation (data not shown).

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 NH_2-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

1. Yamaguchi H, Wyckoff J, Condeelis J. Cell migration in tumors. Curr Opin Cell Biol 2005;17:559–64.
2. Helfer B, Boswell BC, Finlay D, et al. Caspase-8 promotes cell motility and calpain activity under nonapoptotic conditions. Cancer Res 2006;66:4273–8.
3. Barnhart BC, Legembre P, Pietras E, Bubici C, Franzoso G, Peter ME. CD95 ligand induces motility and invasiveness of apoptosis-resistant tumor cells. EMBO J 2004;23:3175–85.
4. Stupack DG, Teitz T, Potter MD, et al. Potentiation of neuroblastoma metastasis by loss of caspase-8. Nature 2006;439:95–9.
5. Rossman KL, Der CJ, Sondek J. GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors. Nat Rev Mol Cell Biol 2005;6:167–80.
6. Zheng Y, Bagrodia S, Cerione RA. Activation of phosphoinositide 3-kinase activity by Cdc42Hs binding to p85. J Biol Chem 1994;269:18727–30.
7. Innocenti M, Frittoli E, Ponzanelli I, et al. Phosphoinositide 3-kinase activates Rac by entering in a complex with Eps8, Abi1, and Sos-1. J Cell Biol 2003;160:17–23.
8. Taniguchi CM, Aleman JO, Ueki K, et al. The p85α regulatory subunit of phosphoinositide 3-kinase potentiates c-Jun N-terminal kinase-mediated insulin resistance. Mol Cell Biol 2007;27:2830–40.
9. Luo J, Cantley LC. The negative regulation of phosphoinositide 3-kinase signaling by p85 and it's implication in cancer. Cell Cycle 2005;4:1309–12.
10. Chang DW, Xing Z, Capacio VL, Peter ME, Yang X. Interdimer processing mechanism of procaspase-8 activation. EMBO J 2003;22:4132–42.
11. Cursi S, Rufini A, Stagni V, et al. Src kinase phosphorylates caspase-8 on Tyr380: a novel mechanism of apoptosis suppression. EMBO J 2006;25:1895–905.
12. Rush J, Moritz A, Lee KA, et al. Immunoaffinity profiling of tyrosine phosphorylation in cancer cells. Nat Biotechnol 2005;23:94–101.
13. Boatright KM, Renatus M, Scott FL, et al. A unified model for apical caspase activation. Mol Cell 2003;11:529–41.
14. Fukata M, Nakagawa M, Kaibuchi K. Roles of Rho-family GTPases in cell polarisation and directional migration. Curr Opin Cell Biol 2003;15:590–7.
15. Jimenez C, Portela RA, Mellado M, et al. Role of the PI3K regulatory subunit in the control of actin organization and cell migration. J Cell Biol 2000;151:249–62.
16. Brachmann SM, Yballe CM, Innocenti M, et al. Role of phosphoinositide 3-kinase regulatory isoforms in development and actin rearrangement. Mol Cell Biol 2005;25:2593–606.
17. Hill KM, Huang Y, Yip SC, Yu J, Segall JE, Backer JM. N-terminal domains of the class IA phosphoinositide 3-kinase regulatory subunit play a role in cytoskeletal but not mitogenic signaling. J Biol Chem 2001;276:16374–8.
18. Sun T, Gao Y, Tan W, et al. A six-nucleotide insertion-deletion polymorphism in the CASP8 promoter is associated with susceptibility to multiple cancers. Nat Genet 2007;39:605–13.