Cooperative Effect of Hepatocyte Growth Factor and Fibronectin in Anchorage-independent Survival of Mammary Carcinoma Cells: Requirement for Phosphatidylinositol 3-Kinase Activity

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Abstract
Anchorage-independent survival and growth are critical characteristics of malignant cells. We showed previously that the addition of exogenous hepatocyte growth factor (HGF) and the presence of fibronectin fibrils stimulate anchorage-independent colony growth of a murine mammary carcinoma, SP1, which expresses both HGF and HGF receptor (Met; R. Saulnier et al., Exp. Cell Res., 222: 360–369, 1996). We now show that tyrosine phosphorylation of Met in carcinoma cells is augmented by cell adhesion and spreading on fibronectin substratum. In contrast, detached serum-starved cells exhibit reduced tyrosine phosphorylation of Met and undergo apoptotic cell death within 18–24 h. Under these conditions, the addition of HGF stimulates tyrosine phosphorylation of Met and restores survival of carcinoma cells. Soluble fibronectin also stimulates cell survival and shows a cooperative survival response with HGF but does not affect tyrosine phosphorylation of Met; these results indicate that fibronectin acts via a pathway independent of Met in detached cells. We demonstrated previously that inhibition of phosphatidylinositol (PI) 3-kinase activity blocks HGF-induced DNA synthesis of carcinoma cells (N. Rahimi et al., J. Biol. Chem., 271: 24850–24855, 1996). We now show in detached cells a cooperative effect of HGF and FN in the activation of PI 3-kinase and on the phosphorylation of PKB/Akt at serine 473. PI 3-kinase activity is also required for the HGF- and fibronectin-induced survival responses, as well as anchorage-independent colony growth. However, c-Src kinase or MEK1/2 activities are not required for the cell survival effect. Together, these results demonstrate that the PI 3-kinase/Akt pathway is a key effector of the HGF- and fibronectin-induced survival response of breast carcinoma cells under detached conditions and corroborate an interaction between integrin and HGF/Met signalling pathways in the development of invasive breast cancer.

Introduction
Basement membrane proteins and growth factors are important components of the tissue microenvironment that maintain survival and differentiation of normal epithelium (1). Disruption of basement membrane and aberrant expression of ECM proteins occur during development of invasive carcinomas (2–4) and are associated with loss of epithelial polarity, increased cell survival, motility, and invasion (5). This process, known as epithelial-mesenchymal transition, is characteristic of the malignant phenotype and is considered an indicator of poor prognosis in many types of carcinomas. However, the mechanisms that promote survival of carcinoma cells during the invasive stage of tumor progression are not clearly known.

We (6) and others (7, 8) have shown that increased expression of HGF and its receptor, Met, occurs in invasive human breast cancer, particularly at the migrating tumor front (6), and that high levels of HGF and Met expression correlate with poor survival of breast cancer patients (9, 10). In addition, overexpression of HGF or a constitutively active mutant form of Met (Tpr-Met) in transgenic mice (11, 12) or in transformed cell lines (13–15) promotes tumorigenic and metastatic properties. HGF is a multifunctional cytokine that stimulates mitogenic, motogenic, morphogenic and angiogenic functions in various cell types (reviewed in Ref. 16). Recent results also support a role of HGF as a survival factor during development of fetal liver (17), as well as in carcinoma cells treated with chemotherapeutic drugs (18, 19). Together, these findings imply that paracrine and autocrine activation of the HGF/Met signaling pathway may be an important

1 The abbreviations used are: ECM, extracellular matrix; HGF, hepatocyte growth factor; FN, fibronectin; PDGF, platelet-derived growth factor; PI, phosphatidylinositol; MAPK, mitogen-activated protein kinase; FAK, focal adhesion kinase; FBS, fetal bovine serum.
regulatory step in survival and growth of invasive breast carcinomas.

In addition to altered growth factor responsiveness, remodeling of the ECM microenvironment through degradation and atypical expression of ECM proteins, such as FN, occur during progression to invasive carcinomas (5) and may affect cell survival and growth phenotypes (20–22). Indeed, adhesion to FN via α5β1 and ligation of αvβ3 integrins have been shown to directly inhibit the death of Chinese hamster ovary cells (20) and human melanoma cells (23), respectively, under serum-starved conditions. Cell-ECM interactions have also been shown to collaborate with growth factor receptors (e.g., epidermal growth factor receptor, Her2/Neu, PDGF β-receptor, and insulin-like growth factor receptor) in many biological processes such as growth and differentiation of various cell types (reviewed in Refs. 24–27). However, the majority of these studies were carried out in monolayer cultures, and their relevance to invasive and metastatic carcinoma cells is not clearly established.

In the present work, we examined the cooperative role of FN and HGF in regulating the survival of mammary epithelial and carcinoma cells under anchorage-independent conditions, characteristic of the invasive phenotype. We showed previously that exogenous HGF and the presence of FN fibrils promote anchorage-independent colony growth of a mammary carcinoma cell line, SP1, which expresses HGF and tyrosine-phosphorylated Met (28, 29). We now report that under serum-starved detached conditions, SP1 cells show reduced tyrosine phosphorylation of Met and undergo cell death, whereas the addition of HGF promotes tyrosine phosphorylation of Met and cell survival. Soluble FN also promotes cell survival and shows a cooperative survival effect with HGF. A similar cooperative survival response to HGF and FN was evident in a nonmalignant mammary epithelial cell line, HC11; however, a greater dependence on FN was observed. Previously, we (30) and others (31) have shown that PI 3-kinase, which regulates a number of cellular functions including morphogenesis (32), motogenesis (33, 34), and cell survival (35, 36), is required for HGF-induced DNA synthesis in monolayer cultures. Our results now show that the PI 3-kinase/Akt pathway (37) is a common downstream regulator of the cooperative survival effect of HGF and FN in carcinoma cells. In contrast, c-Src kinase, which is involved in cell spreading, migration, and anchorage-independent growth (38), and MEK1/2 have no effect on survival of carcinoma cells. Our findings provide new evidence for cooperativity of HGF and FN in survival of detached carcinoma cells via a signalling pathway independent of the focal adhesion complex. These results may prove useful in developing improved treatments of invasive breast cancer.

Results

Serum-starved Epithelial and Carcinoma Cells Undergo Apoptotic Cell Death under Anchorage-independent Conditions. Nonmalignant (HC11) and malignant (SP1) epithelial cells survive under anchorage-independent condi-
tions in 7% FBS but die within 24–48 h after serum starvation (0% FBS; Ref. 29; data not shown). In contrast, both cell lines in monolayer culture survive in serum-starved conditions during the same time period. To examine the mechanism of cell death in cells maintained under serum-starved, anchorage-independent conditions, we used three different methods:

(a) Using acridine orange/ethidium bromide staining to assess nuclear morphology, we found that serum-starved, anchorage-independent cells develop irregularly shaped nuclei and condensation of chromatin within 24–48 h after serum starvation (Fig. 1, A and B, and data not shown). The majority of cells show uptake of ethidium bromide (red), indicating disruption of the plasma membrane, characteristic of late-stage apoptosis. In contrast, cells maintained in 7% FBS exclude ethidium bromide, indicating an intact plasma membrane, and show uniform green staining of nuclei with acridine orange. Similar results were found with two non-small cell lung carcinoma cell lines, A549 (which expresses Met but not HGF) and SK-Luci-6 (which expresses HGF but not Met; data not shown).

(b) Using an in situ end-labeling technique, we further demonstrated significant DNA fragmentation in serum-starved SP1 carcinoma cells, compared with cells in 7% FBS (Fig. 1, C and D).

(c) Using electron microscopy, we found that serum-starved SP1 carcinoma cells exhibit marked shrinkage and blebbing of the cell cytoplasm and dense condensation of the nuclear chromatin, in contrast with cells in 7% FBS (Fig. 1, E and F). Organelles and membranes in these cells also show good preservation.

Together, these characteristics indicate significant apoptotic cell death of serum-starved mammary epithelial and carcinoma cells in the absence of anchorage.

HGF and FN Stimulate a Cooperative Survival Response in Epithelial and Carcinoma Cells in the Absence of Anchorage. We have shown previously that HGF and FN promote anchorage-independent growth of SP1 cells (29). We therefore investigated whether soluble HGF and FN could provide survival signals to HC11 epithelial and SP1 carcinoma cells maintained in the absence of anchorage. Results from both cell staining and colorimetric assays show that soluble FN and HGF can promote survival of SP1 carcinoma cells in a dose-dependent manner (Fig. 2A). In contrast to FN, collagen type I and laminin do not promote survival of detached cells (data not shown). In addition, a cooperative increase in SP1 cell survival was observed in response to both HGF and FN at limiting concentrations, whereas a maximum cell survival with no demonstrable cooperative effect was observed in response to either HGF or FN at higher concentrations. A similar cooperative survival response to HGF and FN was observed in HC11 epithelial cells; however, these cells showed a greater dependence on FN for survival at all concentrations tested (Fig. 2B).

Soluble FN Acts Independently of Met Activation in the Survival Response of Detached Cells. The mechanism of soluble FN stimulation of cell survival may occur directly after integrin ligation and activation of downstream signaling mole-

We therefore examined the tyrosine phosphorylation level of Met in SP1 cells on FN substratum or in suspension with soluble FN. Our results showed that Met is constitutively phosphorylated at tyrosine residues in SP1 cells adhering to, and spreading on, FN substratum and shows a further increase after addition of HGF (Fig. 3A, and data not shown). However, the level of tyrosine phosphorylation of Met in detached cells is dramatically reduced in cells within 60 min after detachment and is restored within 15 min of incubation with HGF. The change in the level of tyrosine phosphorylation of Met in detached cells is not a result of protein loading because all lanes contained equal amounts of Met (Fig. 3B). In contrast, addition of soluble FN has no effect on the baseline level of Met tyrosine phosphorylation. Neutralizing anti-HGF IgG has no effect on FN-induced cell survival (data not shown). Together, these findings indicate that soluble FN acts independently of Met activation in the cell survival response. We have therefore examined the role of signaling molecules downstream of integrin and Met receptors in this system.

PI 3-Kinase Activity Is Required for HGF- and FN-induced Anchorage-independent Survival of SP1 Carcinoma Cells. We have shown previously that PI 3-kinase activity is required for HGF-induced proliferation of SP1 cells (30). PI 3-kinase is also involved in growth factor- and ECM-mediated survival of adherent cells in other systems (21, 35). We therefore examined the effect of HGF and FN on PI 3-kinase activity in SP1 cells in the absence of anchorage. Preliminary experiments showed that PI 3-kinase activity is reduced at least 2-fold in nonadherent, compared with adherent, SP1 cells (data not shown). Addition of HGF or FN alone at limiting concentrations marginally increases PI 3-kinase activity (Fig. 4, A and B), whereas addition of HGF and FN together at the same limiting concentrations results in a cooperative increase in PI 3-kinase activity. Higher concentrations of HGF or FN stimulate maximal PI 3-kinase activity (Fig. 4, C and D). In addition, a concomitant
cooperative increase in phosphorylation at serine 473 of PKB/Akt, which is an effector of cell survival downstream of PI 3-kinase (37), was also observed in response to HGF and FN at all of the concentrations tested (Fig. 5). Thus, HGF and FN stimulate PI 3-kinase activity and serine phosphorylation of PKB/Akt in a cooperative manner, corresponding to the cell survival response shown in Fig. 2.

To assess the role of PI 3-kinase in HGF- and FN-induced cell survival, we examined the effect of the PI 3-kinase inhibitor LY294002 (41), which was shown previously to block even very low basal levels of PI 3-kinase (42). Treatment with LY294002 inhibited HGF- and FN-induced survival (Fig. 6) in detached SP1 cells in a dose-dependent manner. A similar inhibition of serine phosphorylation of PKB/Akt was also observed (Fig. 5). As a control, we showed that LY294002 blocks PI 3-kinase activity in cells in monolayer culture with 7% FBS (Fig. 7A) and inhibits anchorage-independent colony growth in agar (Fig. 8) at the same concentrations that inhibit cell survival within 24 h. Treatment with LY294002 had no detectable effect on expression or tyrosine phosphorylation of Met in SP1 cells (Fig. 7B). Together, these results show that the PI 3-kinase pathway is an important regulator of the cooperative survival response of detached cells to HGF and FN.

c-Src Kinase and MEK1/2 Activities Are Not Required for HGF- or FN-induced Anchorage-independent Survival of SP1 Carcinoma Cells. We have reported previously that c-Src kinase activity is required for HGF-induced cell motility and anchorage-independent growth of SP1 cells (38). Furthermore, the Ras/MAPK pathway has been shown to regulate ECM-dependent cell survival (43). We therefore determined the role of c-Src kinase and MEK1/2 activities in survival of SP1 cells. SP1 cells transfected with a dominant-negative mutant of c-Src (SRC-RF) exhibited at least a 3-fold reduction in c-Src kinase activity compared with cells transfected with wild-type c-Src (SRC; Ref. 38). Under these conditions, the tyrosine phosphorylation levels of Met and phospholipase Cγ are unaffected (38). Our results now show no change in the survival response to HGF or FN of SP1 cells transfected with the dominant-negative SRC-RF mutant compared with wild-type SRC-transfected or untransfected cells (Table 1). Furthermore, we found that the MEK1/2 in-
hibitor PD98059 (44) had no effect on HGF- or FN-induced cell survival or serine phosphorylation of PKB/Akt at concentrations that completely blocked ERK1/2 activation (Fig. 9). In addition, the LY294002 inhibitor caused only a minimal reduction in ERK1/2 activity in SP1 carcinoma cells (Fig. 7C). The slight decrease observed could be attributable to a reduction in the basal levels of ERK1/2 activity observed with this drug in other systems (42).

Discussion

Stromal-derived ECM proteins (2–4, 21–23) and growth factors (45–47) provide a balance of signals that regulate cell survival, growth, and differentiation of nonmalignant and malignant epithelium. HGF and other associated growth factors stimulate normal mammary epithelial morphogenesis (reviewed in Ref. 16). However, during tumorigenesis, HGF stimulates phenotypic changes associated with epithelial-mesenchymal transition, invasion, angiogenesis, and metastasis (13–16). HGF has also been shown to be a key survival factor in carcinoma cells (17–19). The mechanisms that regulate the change in HGF response from a morphogenic to a tumorigenic phenotype in epithelial cells are not clear. In the present study, we have examined the cooperative role of FN and HGF in regulating the survival response of carcinoma cells under anchorage-independent conditions that mimic the invasive tumor phenotype.

We reported previously a murine mammary carcinoma cell line, SP1, that expresses HGF and tyrosine-phosphorylated Met (28) in monolayer culture, consistent with the presence of an HGF autocrine loop. Depending on culture conditions, both paracrine and autocrine effects of HGF have been observed in SP1 carcinoma cells. Under serum-starved conditions, paracrine stimulation with exogenous HGF was required for optimal migration through Transwell membranes (38) and colony growth in agar (29). In the present study, we have shown that in the absence of anchorage, serum-starved SP1 cells exhibit reduced tyrosine phosphorylation of Met and undergo apoptotic cell death, whereas the addition of HGF stimulates rephosphorylation of Met at tyrosine residues and increased cell survival. These findings implicate HGF as an important survival factor in carcinoma cells. However, the basal level of Met activation and function may be influenced by extracellular conditions, such as cell adhesion to various substrata (39, 40), cell density effects on autocrine HGF expression and secretion (48), or proteolytic processing of pro-HGF to biologically active forms (49). Our results also showed that the addition of soluble FN promotes survival of detached SP1 cells and shows a cooperative survival effect.
with HGF. The cooperative survival effect in carcinoma cells was most demonstrable at low concentration levels of HGF and FN and may be more relevant to in vivo tissue microenvironment. Matrix assembly of FN appears not to be required, because treatment with a Mr 70,000 NH2-terminal FN fragment, which inhibits FN fibril formation (50), had no effect on FN-induced cell survival (data not shown). In contrast, FN matrix assembly is required for colony growth of carcinoma cells (29). These results further support an antiapoptotic effect of HGF in breast carcinoma cells in the absence of anchorage and suggest that soluble FN can provide signals that enhance the survival effect of HGF. Soluble FN and HGF can also promote a cooperative survival effect in a nonmalignant mammary epithelial cell line, HC11, under detached conditions. However, Met expression is much lower in HC11 compared with SP1 cells (data not shown), whereas HC11 cells show an increased dependence on FN for survival. Thus, soluble FN and HGF may be important in promoting survival of epithelial cells during detachment and dissociation in early-stage carcinogenesis, whereas formation of a FN fibrillar matrix appears to be required for later stages of anchorage-independent growth of carcinoma cells, perhaps as scaffolding for colony formation.

Cell-ECM interactions have been shown to collaborate with growth factor receptors in many biological processes (reviewed in Refs. 24–26), including growth and differentiation of various cell types (24), activation of the Na+–H+ antiporter via protein kinase C (51), and activation of downstream signaling molecules, such as MAPK (52) and phospholipase Cγ (53). Our finding that HGF and FN stimulate a strong cooperative survival effect in SP1 cells implies a cooperative interaction between integrin and Met receptors. However, HGF was found to have no effect on expression of β1-integrins or adhesion to FN (data not shown). In addition, FN synthesis is greatly reduced in nonadherent, compared with adherent, SP1 cells (29). Thus, it is unlikely that HGF stimulates survival by up-regulating the FN adhesion system.

Our results further suggest that cell adhesion and spreading on FN substratum promote autoactivation of Met in SP1 cells, whereas sustained activation of Met in nonadherent cells requires paracrine stimulation with exogenous HGF. Similarly, Wang et al. (39) showed that cell adhesion elicits activation of Met in melanoma cells. In addition, Sundberg and Rubin (40) showed that stimulation of β1 integrins in fibroblasts induces PDGF-independent tyrosine phosphorylation of PDGF β-receptors. However, unlike adherent carcinoma cells, loss of anchorage has no detectable effect on Met activation in the presence of soluble FN, and anti-HGF neutralizing IgG does not affect FN-induced cell survival (data not shown). Together, these results imply that HGF and
FN stimulate cell survival via independent mechanisms, although a common downstream signaling pathway is likely involved in the cooperative effect.

We observed a cooperative increase in PI 3-kinase activity and phosphorylation at serine 473 of PKB/Akt in response to HGF and FN. We also showed that PI 3-kinase activity is required for HGF- and FN-induced PKB/Akt phosphorylation and cell survival. These findings indicate that the PI 3-kinase/Akt pathway is involved in regulating cell survival in this system. PI 3-kinase activity is also required for anchorage-independent colony growth and for HGF-induced proliferation in monolayer culture (33). Activation of PI 3-kinase has been shown to be sufficient for entry into S phase of the cell cycle and in the presence of serum, promotes oncogenic transformation (54). However, because PI 3-kinase is a major regulator of cell survival, the requirement of PI 3-kinase activity for HGF-induced DNA synthesis and colony growth as shown by us (30) and others (31) may be attributable, at least in part, to the role of PI 3-kinase in suppressing apoptosis.

Interestingly, inhibition of c-Src kinase activity by expressing the dominant-negative SRC-RF mutant in SP1 cells has no effect on the survival response to HGF or FN but blocks HGF-induced anchorage-independent growth and cell motility (38). It should be noted that c-Src family tyrosine kinases have been implicated in protection from Fas ligand-induced apoptosis in lymphocytic cells (55), and that expression of v-Src can promote survival in some cell types (43). However, in carcinoma cells, c-Src kinase function appears to be associated primarily with cell adhesion and coactivation of cytoskeletal molecules such as FAK and paxillin (56), which are important in cytoskeletal organization, cell shape, and locomotion. In addition, activation of MEK1/2 and ERK1/2 are not required for HGF- and FN-induced survival responses in detached cells. This observation is distinct from previous reports that cell adhesion and activation of the Ras/MAPK pathway stimulate a PI 3-kinase/Akt-dependent survival response (43). Our results therefore suggest that the PI 3-kinase/Akt pathway is a key effector of HGF- and FN-dependent cell survival of detached carcinoma cells and acts independently of c-Src and MEK1/2, which are involved primarily in cell adhesion-dependent survival and growth responses.
Our demonstration of a cooperative effect of HGF and FN in the activation of the PI 3-kinase/Akt pathway linked to anchorage-independent survival of carcinoma cells is novel. The nature of the cooperation between FN- and HGF-dependent induction of PI 3-kinase activity is not known. Possible mechanisms include increased binding of the p85 subunit to Met or interaction of p85 with signaling molecules associated with the cell adhesion complex (57). FAK is tyrosine phosphorylated in epithelial cells in response to ECM matrix proteins (58) and to HGF in carcinoma cells in monolayer culture (59) and is required for adhesion-dependent cell survival (60). However, soluble FN or HGF has no effect on tyrosine phosphorylation of FAK in detached SP1 cells (data not shown); this observation suggests that FAK is not a key regulator in the present system. This notion is further supported by our finding that c-Src is not involved in the survival response to HGF. We are currently investigating whether ILK, which is activated after cross-linking of β1 integrins in detached cells (61), is involved in the cooperative effect of HGF and FN on PKB/Akt activation and cell survival.

In summary, we have shown a cooperative effect of HGF and FN in anchorage-independent survival of mammary car-

### Table 1. c-Src kinase activity does not affect HGF- and FN-induced anchorage-independent survival of SP1 cells

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>SP1</th>
<th>SRC</th>
<th>SRC-RF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>22</td>
<td>6.1 ± 1.0</td>
<td>7.9 ± 1.0</td>
<td>4.1 ± 1.0</td>
</tr>
<tr>
<td>HGF</td>
<td>10 ng/ml</td>
<td>14.3 ± 2.3</td>
<td>12.5 ± 1.6</td>
<td>15.8 ± 3.8</td>
</tr>
<tr>
<td>HGF</td>
<td>20 ng/ml</td>
<td>18.2 ± 2.9</td>
<td>18.6 ± 2.4</td>
<td>14.2 ± 3.5</td>
</tr>
<tr>
<td>HGF</td>
<td>30 ng/ml</td>
<td>19.2 ± 3.1</td>
<td>20.1 ± 2.5</td>
<td>19.0 ± 4.6</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>7.0 ± 1.3</td>
<td>6.5 ± 0.1</td>
<td>7.0 ± 0.1</td>
</tr>
<tr>
<td>FN</td>
<td>5 μg/ml</td>
<td>8.8 ± 0.5</td>
<td>9.5 ± 0.1</td>
<td>10.0 ± 0.8</td>
</tr>
<tr>
<td>FN</td>
<td>10 μg/ml</td>
<td>15.6 ± 0.7</td>
<td>7.8 ± 0.1</td>
<td>10.0 ± 0.7</td>
</tr>
<tr>
<td>FN</td>
<td>20 μg/ml</td>
<td>27.2 ± 4.5</td>
<td>15.0 ± 1.8</td>
<td>21.7 ± 0.8</td>
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</table>

*Serum-starved SP1 cells were incubated in suspension for 24 h with the indicated reagents, and cell survival was assessed with the colorimetric enzyme assay as described in Fig. 6. Relative cell survival was calculated by normalization to results obtained from 7% FBS-treated groups. 

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**Fig. 8.** The PI 3-kinase inhibitor, LY294002, blocks growth of SP1 cells in agar. SP1 cells were cultured (10² cells/dish) in 60-mm culture dishes in soft agar (0.36%) with RPMI 1640 with 7% FBS and with the indicated amounts of the PI 3-kinase inhibitor, LY294002. The colony assay was performed as described previously (29). The cultures were incubated for 10 days, and then cells were fixed in methanol and stained with Giemsa. Colonies were counted manually. The results are expressed as the mean number of colonies of quadruplicates and are representative of two experiments; bars, SD.

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**Fig. 9.** The MAPK inhibitor PD98059 inhibits HGF-induced phosphorylation of MAPK but does not affect HGF- and FN-induced cell survival and phosphorylation of PKB/Akt at serine 473. A, SP1 cells were serum starved overnight, detached, and kept in suspension for 2 h. Cells were treated with HGF (20 ng/ml) or FN (20 μg/ml) as indicated for 40 min. Where PD98059 was used, it was added 10 min prior to HGF and FN treatment. Cells were then lysed, and extracts were analyzed by Western blotting with either anti-phospho-ERK1/2 or anti-ERK2 antibody. B, SP1 cells were assayed for survival under detached conditions as described in Fig. 6; bars, range of duplicates in one experiment. C, SP1 cell lysates from A were analyzed by Western blotting with anti-serine 473 phospho-Akt antibody. The same blot was stripped and reprobed with anti-Akt pan antibody.
cinoma cells, and that the PI 3-kinase/Akt pathway is a key regulator of this process. These findings are particularly important because they suggest that the PI 3-kinase/Akt signaling pathway is a potential target for inhibiting HGF-induced survival of carcinoma cells during detachment from the primary tumor site and metastasis (62). In addition, cell adhesion enhances Met activation in carcinoma cells, suggesting interaction between integrin- and Met-dependent signaling pathways. Further studies are in progress to determine the role of the cell adhesion complex in HGF-induced survival and growth. Together our results suggest that cooperative signaling via Met and integrin receptors may provide a selective survival advantage in invasive breast cancer.

Materials and Methods

Reagents. PI was purchased from Sigma (Oakville, Ontario, Canada). [γ-32P]ATP and enhanced chemiluminescence (ECL) reagents were purchased from DuPont NEN Life Science Products (Boston, MA). Rabbit antir PI 3-kinase IgG (specific for the p85 subunit) was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Mouse anti-phosphotyrosine (PY20) monoclonal antibody was purchased from Transduction Laboratories (Lexington, KY). Rabbit antianti Met and anti-ERK2 IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The PI 3-kinase inhibitor LY294002 (41) and MEK1/2 inhibitor, PD98059 (44), were purchased from Calbiochem (San Diego, CA). Rabbit anti-phospho-Akt (ser473) and pan Akt antibodies were from New England Biolabs (Beverly, MA). The phosphospecific ERK1/2 antibody was a gift from Erik Schaefer (BioSource International, Camarillo, CA).

Tissue Culture and Cell Lines. The SP1 tumor is a spontaneous nonmetastatic murine mammary intraductal adenocarcinoma isolated from an 18-month-old CBA/J female retired breeder in the mouse colony at Queen’s University (63, 64). The established SP1 cell line was frozen at −70°C to maintain stocks. HC11 murine mammary carcinoma cells were obtained from Dr. D. Medina (Baylor College of Medicine, Houston, TX). Maintenance medium for SP1 cells was RPMI 1640 (Life Technologies, Inc., Burlington, Ontario, Canada) supplemented with 7% FBS (Life Technologies). HC11 cells were cultured in RPMI 1640 supplemented with 10% FBS, 5 μg/ml insulin (Life Technologies), and 10 ng/ml EGF (Sigma). Cells were kept in culture for no more than 3 months before thawing a fresh stock and were tested periodically for Mycoplasma.

Transfection of Wild-type and Mutant c-Src cDNAs. cDNAs encoding wild-type c-src (SRC) and a dominant-negative double mutant of c-Src (SRC-RF), with loss-of-function mutations in the kinase domain (K295R) and a regulatory tyrosine residue (Y527F), ligated into the pRc/CMV plasmid (Invitrogen, San Diego, CA) carrying the neomycin resistance marker, were obtained from Dr. J. Brugge (BioSource International, Camarillo, CA).

Apoptosis Assay. The in situ end-labeling procedure described previously (66) was used to detect DNA fragmentation in carcinoma cells. Paraformaldehyde-fixed cells on glass slides were immobilized in 0.1 M PBS for 15 min and in buffer A [50 mM Tris-HCl, 5 mM MgCl2, 10 mM β-mercaptoethanol, and 0.005% BSA (Sigma), pH 7.5] for an additional 15 min, all at room temperature. The cells were then incubated for 70 min at 37°C in a humidified chamber in a solution of buffer A containing 0.01 μM each of dATP, dCTP, dGTP, biodin-16-dUTP (Boehringer Mannheim, La- val, Quebec, Canada), and 20 units/ml Escherichia coli DNA polymerase I (Promega, Madison, WI). As negative controls, the biotinylated UTP or DNA polymerase I was omitted from the above incubation solution for some groups. The reaction was terminated by two 15-min washes in 0.1 M PBS, 0.05% Tween 20 at 4°C. The cells were then incubated in pre-mixed Vectastain avidin and biotinylated horseradish peroxidase complex (ABC; Vector Laboratories, Inc.; 1:100) for 2 h at room temperature, followed by three 15-min washes in 0.1 M PBS, 0.05% Tween 20. Staining was then developed with 0.025% diaminobenzidine and 0.05% H2O2 in 0.1 M PBS for 12 min at room temperature. The slides were air-dried overnight, and the cells were then lightly counterstained with hematoxylin and coverslip in Permount (Fisher Scientific, Nepean, Ontario, Canada).

Survival Assay. Prestained SP1 cells were seeded at a density of 2 × 104 cells in 1.5 ml of RPMI 1640 containing 0.5 mg/ml BSA and reagents as indicated into 0.6% agar-coated, 35-mm Corning non-tissue culture plates. After 24 h incubation at 37°C, the cells were collected and centrifuged in Eppendorf tubes at 1000 rpm for 5 min and stained for live/dead cells with a 1:1 mixture of acridine orange (Sigma) and ethidium bromide (Sigma), each at 4 μg/ml (67). A Leitz fluorescence microscope equipped with epi-illumination was used to count live/dead cells. Nuclei of viable cells stained uniformly green with acridine orange, which intercalates with DNA. Early apoptotic cells, in which membranes are disrupted, stained red with ethidium bromide, also with patches of condensed chromatin in nuclei. In a parallel analysis, an enzyme survival assay, as described by Khwaja et al. (43), was also carried out. For the enzyme assay, cells were replated into a 96-well plate with 7% FBS/RPMI medium and incubated at 37°C for 4 h. A colorimetric method based on the conversion of [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphen- yl)-2-(4-sulfonyl)-2H-tetrazolium] to formazan (CellTiter aqueous kit; Promega Corp., Madison, WI) was used to measure cell survival.

Colony Assay. Colony assays were performed as described previously by Saulnier et al. (29). Briefly, a solution of 1.2% Bactoagar (Difco Lab) was mixed (1:1) with 2 × RPMI 1640, supplemented with 7% FBS, and layered onto 60-mm tissue culture plates and allowed to solidify. SP1 cells (101–2×104 cells) were mixed in a 0.36% Bactoagar solution prepared in the same way and layered on top of the 0.6% Bactoagar. Plates were incubated at 37°C in 5% CO2 for 8–10 days. Colonies were fixed with 100% methanol, stained with Giemsa (4%, v/v; BDH, VWR Scientific, Mississauga, Ontario, Canada), and counted manually.

Immunoprecipitation and Western Blotting. SP1 cells were grown to confluence and serum starved for 24 h. Cells were rinsed with cold PBS buffer and lysed in a buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP40, 1 mM Na3VO4, 50 mM NaF, 1 mM EDTA, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Lysates were centrifuged for 10 min at 14,000 rpm in an IEC/Micromax centrifuge at 4°C. The supernatants were measured for protein content with a biocinchoninic acid protein assay (Pierce, Rockford, IL), and were adjusted to equal protein concentrations. Equal volumes of each supernatant were incubated with or rabbit antibodies for 1 h. Immunoprecipitates were collected on protein A-Sepharose (Amersham-Pharma- cia Biotech, Baie d’Urfe, Quebec, Canada) for 1 h, washed three times with lysis buffer, separated on 8% SDS-PAGE under reducing conditions, and transferred to a nitrocellulose membrane by electroelution. The membrane was blocked with 3% skimmed milk or 1% BSA in TBST buffer (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.1% Tween 20) for 15 min and probed with the appropriate primary antibodies for 1 h at room temperature or overnight at 4°C. The membrane was washed three times with TBST for 5 min each, incubated with horseradish peroxidase-labeled secondary donkey antirabbit IgG (Amersham, Oakville, Ontario, Canada) or sheep antiserum IgG (Amersham) for 15 min, and washed three times for 10 min each with TBST. Immune complexes were detected with ECL.

PI 3-Kinase Assay. PI 3-kinase assays were performed essentially as described previously (30, 33). In brief, approximately 1 × 105 SP1 cells were seeded in 10-cm plates and serum starved for 24 h. Cells were detached by incubation with PBS containing 0.5 mM EDTA and 0.5 mM EGTA for 5 min at 37°C. Suspended cells were preincubated at 37°C for 15 min to stabilize baseline activity and stimulated by the addition of reagents as indicated. The cells were then washed with PBS supplemented with 1 mM CaCl2 and 1 mM MgCl2 , and lysed in cold lysis buffer [137 mM NaCl, 20 mM Tris-HCl (pH 7.0), 0.92 mM CaCl2, 0.49 mM MgCl2, 10% glycerol, 1% NP40, 100 μM Na3VO4, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride]. Clarified cell extracts were normalized for protein concentration and precipitated with anti-phosphotyrosine monoclonal antibody. In some experiments, anti-PI 3-
kinase IgG was used with similar results. The immunoprecipitates were washed two times with PBS/1% NP40, two times with PBS, two times with 0.1 M Tris (pH 7.0) and 0.5 M LiCl, once with TNE [10 mM Tris (pH 7.4), 100 mM NaCl, and 1 mM EDTA], and once with 20 mM HEPES (pH 7.4). Immune complexes were suspended in 50 μl of 20 mM HEPES (pH 7.4) with 20 μg of sonicated PI and were incubated on ice for 10 min. The reaction was initiated by addition of 30 μg MgCl2 and 25 μg [γ-32P]ATP. After incubation for 20 min at room temperature, reactions were stopped by the addition of 100 μl of 1 N HCl, and the lipids were extracted by addition of 200 μl of CHCl3/CH3OH (1:1) and were resolved by silica gel plate (Whatman Ltd., Maidstone, England) chromatography in CHCl3/CH3OH/H2O (9:7:2) solvent. The TLC plate was dried, and labeled lipids migrating as phosphatidylinositol 3-phosphate were measured with a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

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References


Epidermal growth factor, but not hepatocyte growth factor, suppresses phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival.  


