Anti-apoptotic signaling by hepatocyte growth factor/Met via the phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase pathways

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Hepatocyte growth factor (HGF) is a ligand of the receptor tyrosine kinase encoded by the c-*Met* protooncogene. HGF/Met signaling has multifunctional effects on various cell types. We sought to determine the role of HGF/Met in apoptosis and identify signal transducers involved in this process. In experiments with human SK-LMS-1 leiomyosarcoma cells, we show that the Akt kinase is activated by HGF in a time- and dose-dependent manner by phosphatidylinositol 3-kinase (PI3-kinase). Akt is also activated by active tumorigenic forms of Met, i.e., ligand-independent Tpr-Met, a truncated and constitutively dimerized form of Met, and a mutationally activated version of Met corresponding to that found in human hereditary papillary renal carcinoma. In NIH 3T3 cells transfected with wild-type Met, HGF inhibits apoptosis induced by serum starvation and UV irradiation. HGF-induced survival correlates with Akt activity and is inhibited by the specific PI3-kinase inhibitor LY294002, indicating that HGF inhibits cell death through the PI3-kinase/Akt signal transduction pathway. Furthermore, transiently transfected Tpr-Met activates Akt (both Akt1 and Akt2) and protects cells from apoptosis. Mitogen-activated protein kinase (MAPK) also is activated by HGF and rescues cells from apoptosis, although the cytoprotective effect is less marked than for PI3-kinase/Akt. Blocking MAPK with the specific MAPK kinase inhibitor PD098059 impairs the ability of HGF to promote cell survival. Similar results were obtained with NIH 3T3 cells expressing the fusion protein Trk-Met and stimulated with nerve growth factor, the Trk ligand. These results demonstrate that HGF/Met is capable of protecting cells from apoptosis by using both PI3-kinase/Akt and, to a lesser extent, MAPK pathways.

Hepatocyte growth factor (HGF) is a mesenchymal-derived cytokine that acts as mitogen, motogen, and morphogen in various target cells, including smooth muscle cells (1–4). The pleiotropic activities of HGF are mediated through its receptor, a transmembrane tyrosine kinase encoded by the protooncogene c-*Met* (5–8). In addition to mediating a variety of normal cellular processes, HGF/Met has been implicated in the generation and spread of tumors (9). c-*Met* was originally isolated as the cellular counterpart of a transforming fusion gene, *Tpr-Met* (5, 6). The resulting chimeric proteins homodimerize and transphosphorylate, thereby generating molecules possessing constitutive kinase activity and transforming ability. Activating c-*Met* mutations cause human hereditary papillary renal carcinomas (10). These *Met* mutants are oncogenic when transfected into NIH 3T3 mouse fibroblasts (9). HGF and Met are coexpressed, often overexpressed, in a significant number of human tumors, suggesting that HGF acts as a paracrine and/or autocrine growth factor (11).

Both pro- and anti-apoptotic effects of HGF have been reported (12–16). Studies of HGF null (−/−) mice indicate that HGF is essential in embryonic development (12). The major effect is severe reduction in the size of the liver, with dissociation of parenchymal cells showing signs of apoptosis. In this study, we sought to determine the role of HGF/Met in apoptosis and identify signal transduction mediators involved in HGF/Met signaling. We show that HGF/Met signaling protects cells from apoptosis induced by serum starvation and UV irradiation through both PI3-kinase/Akt and mitogen-activated protein kinase (MAPK) pathways. These findings imply that activation of PI3-kinase/Akt and MAPK pathways, and the resulting inhibition of apoptosis, are critical events in tumorigenesis driven by an activated HGF/Met circuitry.[‖]

Materials and Methods

Reagents. Recombinant human HGF was purified from the supernatant of transfected NIH 3T3 cells that overproduce this growth factor. HGF concentrations are presented as scatter units/ml; one unit is equivalent to ≈2 ng of protein. Nerve growth factor (NGF) and histone 2B were obtained from Boehringer Mannheim. Wortmannin (WM), PD098059, and transcriptional inhibitor PHAS-1 were from Calbiochem. Hoechst 33342 was from Molecular Probes. LY294002 and all other fine chemicals were from Sigma.

Cell Lines and cDNA Constructs. NIH 3T3 cells (CRL1658) were obtained from American Type Culture Collection and cultured in DMEM/10% calf serum. SK-LMS-1 is a human leiomyosarcoma cell line that overexpresses Met. Murine wild-type (WT)-*Met*, *Met-mut*, *Tpr-Met*, and *Trk-Met* cDNAs were subcloned into pMB1 expression vector (9). *Met-mut*, a mutationally activated version of *Met*, contains a double mutation (M1268T + L1213V) which renders the Met kinase highly active and tumorigenic. Tpr-Met, a truncated and constitutively dimerized form of the Met receptor, is ligand independent. The Trk-Met fusion consists of the extracellular domain of Trk (NGF receptor) fused to the intracellular domain of Met. To construct hemagglutinin (HA) epitope-tagged human *AKT1* and *AKT2* expression plas-

Abbreviations: HGF, hepatocyte growth factor; WM, wortmannin; MAPK, mitogen-activated protein kinase; PI3-kinase, phosphatidylinositol 3-kinase; NGF, nerve growth factor; WT, wild-type; HA, hemagglutinin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

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mids, a HA epitope tag was inserted after the first methionine residue of full-length human *AKT1* and *AKT2* cDNAs and cloned into pcDNA3 vector (Invitrogen). Authenticity was verified by nucleotide sequencing. Construction of murine HA-*Akt* expression plasmids was reported (17). A dominant-negative *Akt1* mutant (HA-Akt1AA) was created by replacing Thr-308 and Ser-473 with Ala residues; dominant-negative *Akt2* (HA-Akt2E299K) was made by replacing Glu-299 with Lys (17).

***In Vitro* Kinase Assays.** After transfection with HA-AKT1 or HA-AKT2 constructs, SK-LMS-1 cells were serum starved for 16 h and treated with or without WM for 30 min before HGF stimulation. AKT activity was determined according to Klippel *et al.* (18). Briefly, cells were lysed in ice-cold lysis buffer [20 mM Tris·HCl (pH 7.5)/150 mM NaCl/10% glycerol/1% Nonidet P-40/10 mM NaF/1 mM Na_3VO_4/1 mM sodium pyrophosphate/25 mM β-glycerophosphate/2 mM EDTA/1 mM DTT/0.5 mM PMSF/5 μg/ml aprotinin/2 μg/ml leupeptin], insoluble material was pelleted by centrifugation, and supernatants were immunoprecipitated by using anti-HA antibody, HA11 (Babco, Richmond, CA). For measurement of endogenous Akt activity, 300 μg of total protein was incubated with sheep polyclonal anti-Akt1 or anti-Akt2 antibody (Upstate Biotechnology, Lake Placid, NY). Protein A and protein G (1:1) Sepharose beads were added, and the mixture was incubated for 2–3 h and then washed. Immunoprecipitates were divided equally in two aliquots: one for kinase assay, and the other to assess the amount of protein in the precipitate by Western blotting. Akt kinase reactions were carried out in the presence of 20 mM Hepes (pH 7.5)/10 mM $MgCl_2$/10 mM $MnCl_2$/1 mM DTT/5 μM ATP/5 μCi of [γ-^{32}P]ATP by using histone 2B (100 ng/μl) as substrate. Samples were separated on SDS/PAGE and the dried gel was exposed to autoradiography.

For MAPK assays, cells were stimulated and lysed as described above. Immunoprecipitation was performed by using an anti-p44/ERK1 polyclonal antibody (Santa Cruz Biotechnology). The immunocomplex was washed extensively with lysis buffer and once with kinase buffer [25 mM Hepes (pH 7.5)/2 mM $MnCl_2$/20 mM $MgCl_2$]. MAPK reactions were carried out at 30°C for 20 min in kinase buffer supplemented with 5 μM ATP and 5 μCi of [γ-^{32}P]ATP, by using PHAS-1 (50 ng/μl) as substrate.

Western Blotting. SDS/PAGE gels of cell lysates or immunoprecipitates were probed with the following antibodies: HA11, polyclonal anti-Akt1 (New England Biolabs) or polyclonal anti-ERK1 (Santa Cruz Biotechnology) as indicated in the figure legends. Detection was performed by using enhanced chemiluminescence (Amersham International).

Apoptosis Assay. Cells were serum starved for 16 h, the medium was removed, and then cells were irradiated with UV at 100 J/m^2 by using an UV-crosslinker (Stratagene). After irradiation, cells were fed with serum-free medium and supplemented with inhibitors and/or HGF as required. After incubation for another 24 h, both floating and adherent cells were pooled and stained with Hoechst 33342. Apoptotic cells with characteristic nuclear fragmentation were counted in at least 300 cells in randomly chosen fields and expressed as a percentage of the total cell number.

Apoptosis after transient expression of Tpr-Met in NIH 3T3 cells was determined by immunofluorescence and Hoechst 33342 staining. At 24 h after transfection, the cells were serum starved overnight, UV irradiated at 100 J/m^2, and incubated in serum-free medium for another 24 h. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. Expression of Tpr-Met was examined by staining with rabbit anti-human Met antibody (Santa Cruz Biotechnology), which shows no crossreaction with endogenous Met of murine NIH 3T3 cells (data not shown), followed by FITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch). Apoptotic cells were assessed by Hoechst 33342 staining.

Cell viability was evaluated by using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma) reduction conversion assay (19). Briefly, after serum starvation and UV irradiation, cells grown in 96-well plates were incubated for 24 h with serum-free medium, supplemented with inhibitors and/or HGF as required. Then, 40 μl of MTT at 5 mg/ml was added to each well, and incubation was continued for 2 h. The formazan crystals resulting from mitochondrial enzymatic activity on MTT substrate were solubilized with 100 μl of 20% (wt/vol) SDS/50% (vol/vol) *N*,*N*-dimethylformamide (pH 4.7), and incubated overnight. Absorbance was measured at 590 nm by using a MRX microplate reader (Dynatech). Cell survival was expressed as absorbance relative to that of untreated controls.

Caspase-3 activity was assayed as described by Enari *et al.* (20). Briefly, both floating and attached cells were pooled and lysed by repeated freezing and thawing in lysis buffer. After centrifugation and protein quantitation, 50 μg of supernatant was diluted in 1 ml of reaction buffer [10 mM Pipes (pH 7.4)/2 mM EDTA/0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate/5 mM DTT) containing the fluorogenic substrate Ac-DEVD-AFC in the presence or absence of a specific inhibitor of caspase-3, Z-DEVD-CMK (Bio-Rad). Fluorescence of the cleaved substrate was determined with a spectrofluorimeter (Perkin–Elmer, LS-3) at an excitation wavelength of 400 nm and emission wavelength of 520 nm. Caspase-3 activity was determined by subtracting the values in the presence of inhibitor.

Results

AKT1 and AKT2 Are Activated by HGF Through PI3-Kinase. To investigate Akt involvement in HGF signaling, HA-AKT1 was transiently transfected into SK-LMS-1 cells. After serum starvation and HGF stimulation, activation of AKT1 by HGF occurred in a dose- and time-dependent manner. AKT1 activation was detected at HGF concentrations as low as 1 unit/ml (Fig. 1*A*). Maximum activity was seen within 2 min, and activation was observed for at least 2 h (Fig. 1*B*). Activation of AKT1 by HGF was abolished by the PI3-kinase inhibitor WM (Fig. 1*C*), indicating that AKT1 activation by HGF is mediated by PI3-kinase. Similar results were obtained in cells transfected with HA-AKT2 (Fig. 1*E*).

AKT1 and AKT2 Are Activated by Ligand-Independent Active Forms of Met. SK-LMS-1 cells were cotransfected with HA-*AKT1* and either pMB1 vector, WT-*Met*, or active forms of *Met*. After overnight serum starvation, cells were lysed, immunoprecipitated with HA antibody, and assayed for activation of AKT. AKT1 was strongly activated by Tpr-Met and, to a lesser extent, Met-mut (Fig. 1*D*). AKT1 activity induced by WT-Met was slightly elevated as compared with vector alone. Similar results were obtained with AKT2 (Fig. 1*F*).

HGF Protects Cells from Apoptosis via Activation of PI3-Kinase/Akt. Because Akt is a multifunctional mediator of growth factor-induced survival (21–23), we hypothesized that enhanced cell survival may be one of the biological consequences of PI3-kinase/Akt activation by HGF. To address this possibility, we examined the effect of HGF on apoptosis in NIH 3T3 cells transfected with WT-*Met*. After overnight serum starvation, followed by UV irradiation, many nuclei exhibited chromatin condensation and nuclear fragmentation, characteristic of apoptosis (Fig. 2*A*). In contrast, when HGF was added to the serum-free medium immediately after UV irradiation, the percentage of apoptotic cells was significantly reduced. Pretreatment with LY294002, a synthetic specific inhibitor of PI3-kinase,

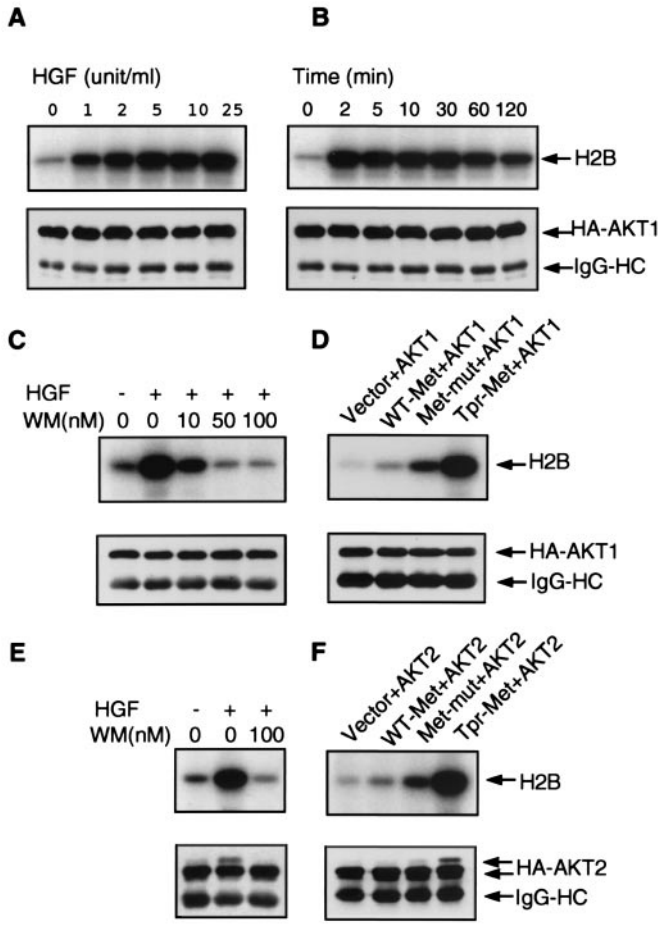

Fig. 1. Activation of AKT1 and AKT2 by HGF or active forms of Met. A human HA-AKT1 expression plasmid was transfected into SK-LMS-1 cells. Cells were serum starved for 16 h, followed by stimulation at increasing concentrations of HGF for 10 min (*A*) or with 10 units/ml HGF for various times (*B*). (*C*) AKT1 activity is stimulated by 10 units/ml HGF and inhibited by WM. (*D*) SK-LMS-1 cells were transiently cotransfected with an HA-AKT1 plasmid together with pMB1 vector, WT-*Met*, *Met-mut*, or *Tpr-Met*. AKT1 is strongly activated by Met-mut and Tpr-Met. (*E*) AKT2 activity is also stimulated by HGF and inhibited by WM. (*F*) AKT2 is activated by Met-mut and Tpr-Met. (A–*F Upper*) Akt kinase activity. Expressed HA-AKT1 or HA-AKT2 was immunoprecipitated with an anti-HA antibody, and kinase assays were performed as described in *Materials and Methods*. IgG-HC, immunoglobulin heavy chain. (A–*F Lower*) Western blot analysis of immunoprecipitates by using anti-HA antibody, demonstrating equivalent loading in each lane. Slower mobility bands in *E* and *F* are presumed to be the fully phosphorylated activated form of HA-AKT2 (17).

before HGF stimulation abolished the protective effect of HGF (Fig. 2*B*).

Tpr-Met Protects Cells from Apoptosis via Activation of Akt. Tpr-Met possesses constitutive kinase activity and transforming ability. As shown in Fig. 1, Tpr-Met strongly stimulates both HA-AKT1 and HA-AKT2 activities in human SK-LMS-1 cells. Tpr-Met also activates HA-Akt1 and HA-Akt2 in NIH 3T3 cells (Fig. 3*A*). We next examined if Tpr-Met could protect cells from apoptosis. NIH 3T3 cells were transiently transfected with *Tpr-Met* followed by immunofluorescence and Hoechst 33342 staining. Unlike untransfected cells, cells expressing Tpr-Met were resistant to apoptosis induced by serum starvation and UV irradiation (Fig. 3*C Left*). To determine if the protective effect is Akt dependent, NIH 3T3 cells were cotransfected with plasmids encoding Tpr-Met, HA-Akt1AA, and HA-Akt2E299K at a ratio of 1:1.5:1.5.

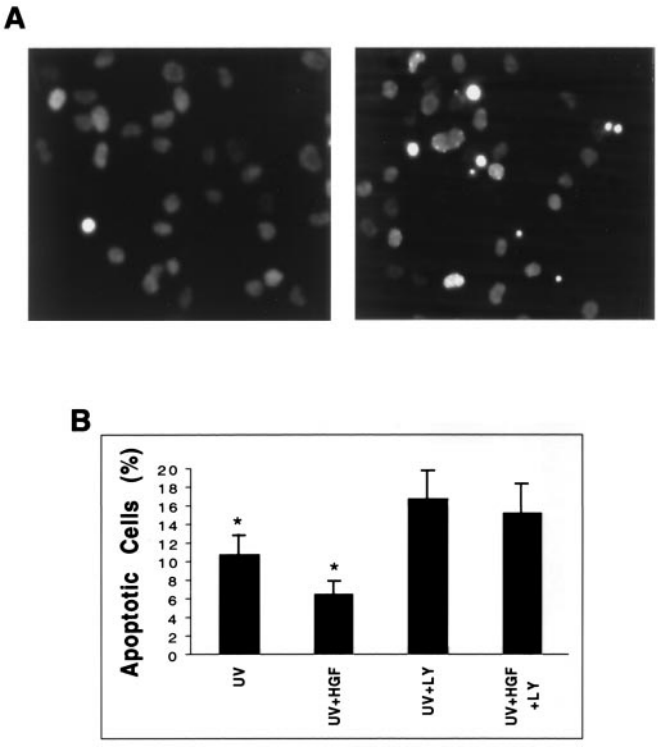

Fig. 2. Anti-apoptotic signaling by HGF in NIH 3T3 cells transfected with WT-*Met*. (*A*) Cells were serum starved overnight, and after treatment with UV irradiation, fresh serum-free medium, supplemented with (*Left*) or without (*Right*) HGF, was added to the cells. Cells were incubated for another 24 h and harvested for evaluation of apoptosis by Hoechst 33342 staining. Normal nuclei show faint delicate chromatin staining, nuclei at the early stage of apoptosis display increased condensation and brightness, and nuclei at the late stage of apoptosis exhibit chromatin condensation and nuclear fragmentation. (*B*) Apoptotic cells with characteristic chromatin condensation and nuclear fragmentation were counted and expressed as a percentage of the total cell number. Bar = mean ± SD of three independent experiments. *, $P < 0.01$.

The dominant-negative forms of Akt blocked the anti-apoptotic activity of Tpr-Met (Fig. 3*C Right*).

Cytoprotection by HGF/Met via Activation of PI3-Kinase/Akt and MAPK Pathways. To further analyze the effect of HGF/Met signaling on cell survival, we conducted a MTT assay, which is based on the ability of viable cells to convert MTT, a soluble tetrazolium salt into an insoluble formazan precipitate (19). After UV irradiation, NIH 3T3 cells stably transfected with WT-*Met* were treated with or without HGF. HGF was found to enhance the viability of serum-starved cells treated with UV irradiation, and the effect of HGF was abolished by LY294002.

The cytoprotective effect of HGF was also demonstrated by caspase-3 assays. Caspase-3 plays a pivotal role in the apoptotic cell death pathway, and activation of caspase-3 is an early event in apoptosis (24). As shown in Fig. 4*A*, caspase-3 activity was decreased by HGF treatment, and the effect of HGF on inhibition of caspase-3 activity was abolished by LY294002. This result is consistent with the finding that LY294002 can abolish the protective effects of Akt, as determined by Hoechst staining (Fig. 2*B*), and diminished cell viability, as demonstrated by MTT assay (Fig. 4*A*). However, in the presence of LY294002, HGF still provides some protection from apoptosis compared with that observed in cells treated with the inhibitor alone (Fig. 4*A*). These results suggest that HGF triggers some other anti-apoptotic mediator(s), in addition to PI3-kinase.

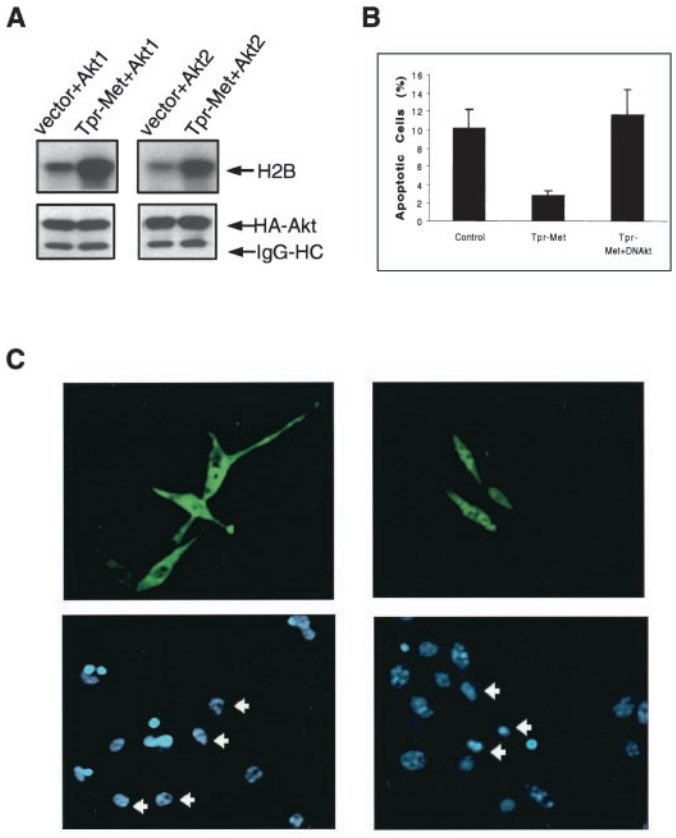

Fig. 3. Expression of Tpr-Met protects cells from apoptosis in an Akt-dependent manner. (*A*) Akt1 and Akt2 are activated by Tpr-Met in NIH 3T3 cells. (*Upper*) Akt kinase activity. (*Lower*) Western blot analysis of immunoprecipitates by using anti-HA antibody, demonstrating equivalent loading in each lane. (*B*) Untransfected cells and cells transiently transfected with Tpr-Met or Tpr-Met plus dominant-negative mutants Akt1AA and Akt2E299K were serum starved and UV irradiated. Cells were then fixed and stained with anti-Met antibody followed by FITC-conjugated goat anti-rabbit IgG. The frequency of apoptotic cells was assessed by Hoechst 33342 staining and expressed as percent of total cell number. Bar = mean ± SD of three independent experiments. Cells expressing Tpr-Met were resistant to apoptosis induced by serum starvation and UV irradiation; dominant-negative forms of Akt1 and Akt2 blocked the anti-apoptotic effect of Tpr-Met. (*C*) Representative staining of transfectants of Tpr-Met (*Left*) and Tpr-Met plus Akt1AA and Akt2E299K (*Right*). (*Upper*) Green fluorescence of anti-Met staining. (*Lower*) blue fluorescence of Hoechst 33342 staining of apoptotic and nonapoptotic cells of the same field. Arrows indicate nuclei of Tpr-Met-positive cells shown in corresponding *Upper* panels.

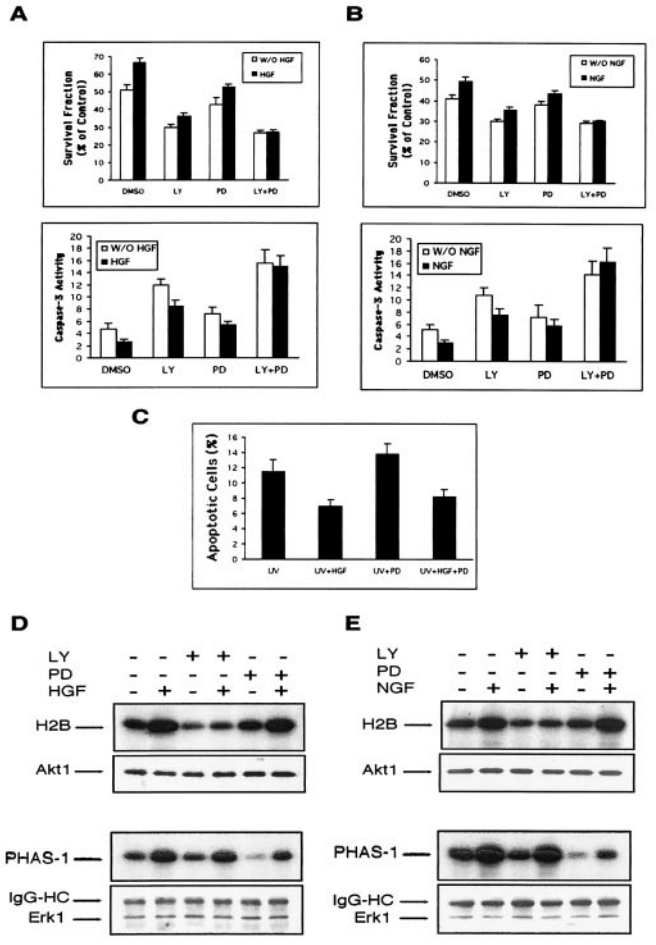

Fig. 4. Effect of LY294002 and PD098059 treatment on apoptosis and signaling of NIH 3T3 cells transfected with WT-*Met* or *Trk-Met*. (*A* and *B*) Both PI3-kinase/Akt and MAPK activities protect cells from apoptosis. After serum starvation and UV irradiation, WT-*Met* cells in *A* were incubated in the absence (−) or presence (+) of HGF (10 units/ml) plus LY294002 (20 μM) or PD058059 (30 μM) as indicated for 24 h. Trk-Met cells in *B* were treated in the same way as WT-Met cells, except that HGF was replaced with NGF (20 μg/ml). Cell survival in both cell lines was assessed by the MTT assay and caspase-3 activity assay. In the MTT assay, bars = mean ± SD of eight replicate wells; the experiment was conducted three times with similar results. In the caspase-3 activity assay, bars = mean ± SD of three independent experiments. (*C*) Apoptosis in NIH 3T3 cells transfected with WT-*Met*. Percentage of apoptotic cells was determined by Hoechst 33342 staining as described in Fig. 2. (*D* and *E*) PI3-kinase/Akt and MAPK signaling by HGF/Met. NIH 3T3 cells transfected with WT-*Met* in *D* were incubated in serum-free medium for 16 h and stimulated with or without HGF (10 units/ml) for 10 min. LY294002 (20 μM) or PD098059 (30 μM) was added 30 min before stimulation. Trk-Met cells in *E* were treated in the same way as WT-Met cells, except that HGF was replaced with NGF (20 μg/ml). Lysates were immunoprecipitated with antibodies to Akt1 or ERK1 and assayed for kinase activity. (*D* and *E Upper*) Histone 2B or PHAS-1 phosphorylation by Akt1 or MAPK activity, respectively. (*D* and *E Lower*) Western blot analysis of immunoprecipitates by using rabbit polyclonal anti-Akt1 or anti-ERK1 antibodies, demonstrating equivalent loading in each lane.

Because the MAPK pathway is required for HGF-induced scattering in Madin–Darby canine kidney (MDCK) cells (25) and is involved in insulin-like growth factor 1-associated inhibition of apoptosis in PC12 cells (26) and hematopoietic cells (27), we tested whether this pathway also plays a role in anti-apoptotic signaling by HGF. We found that HGF stimulates MAPK activity in NIH 3T3 cells transfected with WT-*Met*, and the elevated MAPK activity is decreased below the basal level in unstimulated cells by preincubation with the specific MAPK kinase inhibitor PD098059 (Fig. 4*D*).

As with the PI3-kinase inhibitors, PD098059 not only abolished the protective effect of HGF but also enhanced the degree of apoptosis as measured by caspase-3 activity, and decreased cell viability as determined by the MTT assay, whether used alone or in combination with HGF (Fig. 4*A*). When used in combination with PD098059, HGF still provides some protection from apoptosis compared with that observed in cells treated with PD098059 alone.

Because both LY294002 and PD098059 inhibited the anti-apoptotic effect of HGF, we next examined the effect of combined treatment with both inhibitors. As shown in Fig. 4*A*, treatment with LY294002 plus PD098059 led to a decrease in survival as determined by the MTT assay, and to an additive increase in caspase-3 activity, obliterating the HGF protective effect on apoptosis. The additive, but not synergistic, effects in survival and caspase-3 activity by LY294002 and PD098059

suggest that there was no crosstalking between PI3-kinase/Akt and MAPK pathways in WT-*Met*-transfected NIH 3T3 cells. In agreement with this conclusion, whereas LY294002 abolished HGF-induced Akt activation, it did not inhibit HGF-induced MAPK activation. Similarly, PD098059 inhibited HGF-induced MAPK activation, but had no effect on Akt activity (Fig. 4*D*).

To further examine the effect of PD098059 in this system, we performed an additional apoptosis assay, namely Hoechst 33342 staining. The results, shown in Fig. 4*C*, indicate that PD098059 potentiates UV-induced apoptosis and inhibits rescue of UV-induced apoptosis by HGF. However, the effects of PD098059 were less marked than those observed with LY294002 (Fig. 2), suggesting that the primary pathway involved in HGF-induced anti-apoptotic signaling by Met occurs via PI3-kinase/Akt.

To further explore the anti-apoptotic mechanisms of HGF/Met signaling, we analyzed regulation of cell death and signal transduction in NIH 3T3 cells stably transfected with *Trk-Met*. Because the intracellular domain of Met is fused to the extracellular domain of Trk, Trk-Met cells are responsive to NGF stimulation. After Trk-Met cells were serum starved and UV irradiated, LY294002 or PD098059 was added in the presence or absence of NGF, and survival and caspase-3 activity were measured. The results obtained with Trk-Met cells stimulated with NGF were very similar to those obtained with WT-Met cells stimulated with HGF (Fig. 4*B*).

In parallel, we conducted immunocomplex kinase assays to examine Akt and MAPK activities in Trk-Met cells. The results were essentially identical to those obtained in WT-*Met*-transfected NIH 3T3 cells. As shown in Fig. 4*E*, Met signaling in Trk-Met cells leads to activation of both PI3-kinase/Akt and MAPK. Treatment with LY294002 and PD098059 indicates that there is no crosstalking between these two pathways. Thus, anti-apoptotic signaling by HGF/Met is mediated by both the PI3-kinase/Akt and MAPK pathways.

Discussion

On HGF binding, a multidocking site at the C terminus of c-Met is generated by phosphorylation of two Tyr residues (28). These phosphotyrosines mediate high-affinity interactions with various src homology region 2-containing cytoplasmic effectors, including Gab1, PI3-kinase, Shc, and Grb2. These effectors then transduce extracellular signals elicited by HGF to downstream targets. We demonstrate here that HGF activates Akt kinases in both SK-LMS-1 leiomyosarcoma and NIH 3T3 fibroblast cells. In addition, we show that activation of AKT1 and AKT2 by HGF is completely abrogated by WM and LY294002, indicating that HGF activates Akt in a PI3-kinase-dependent manner.

Different studies suggest that HGF triggers either anti-apoptotic (12–14) or proapoptotic (15, 16) signals and the mechanisms by which HGF exerts these opposite effects are not fully understood. The contrary actions of HGF may be dependent on both the cell context and the expression of coreceptors and downstream pro- or anti-apoptotic effectors involved in HGF/Met signaling. The present study demonstrates that HGF can protect cells from apoptosis by means of both PI3-kinase/Akt and MAPK signaling. Our data indicate that HGF rescues cells from apoptosis and that the specific PI3-kinase inhibitor LY294002 sensitizes cells to apoptosis and abolishes HGF protection. Inhibition of apoptosis by HGF correlates with kinase activity of the anti-apoptotic mediator Akt. Other recent investigations have also suggested that HGF exerts its anti-apoptotic actions via the PI3-kinase/Akt pathway (29, 30). However, unlike the present report, these studies did not use dominant-inhibitory forms of Akt to demonstrate definitively a requirement for Akt activity for HGF/Met effects on cell survival. Our experiments with Tpr-Met show that this ligand-independent activated Met receptor protects cells from apoptosis. Cotransfection with dominant-negative *Akt* constructs demonstrated the necessity of Akt activation for the anti-apoptotic effects of Tpr-Met. In agreement with the important role of HGF in the biology of smooth muscle cells (3, 4), recent preliminary studies from our laboratory indicate that HGF inhibits apoptosis induced by exposure to high doses of doxorubicin in SK-LMS-1 leiomyosarcoma cells (data not shown).

Akt exerts its anti-apoptotic effects in a variety of ways, including phosphorylation and activation of IκB kinase (31, 32). This results in IκB degradation and allows NF-κB to enter the nucleus and activate transcription of anti-apoptotic genes. Another mechanism whereby Akt functions to promote survival is through phosphorylation of Bad (33). The phosphorylated Bad then associates with 14-3-3, which sequesters Bad from Bcl-X_L, thereby preventing cell death. However, because Bad is expressed only in a limited range of tissues and cell lines (34), this may not be the primary mechanism used by Akt to prevent cell death. It has been shown previously that procaspase-9 is activated by the cytochrome *c*–Apaf-1 complex after apoptotic stimulation. Active caspase-9 can then directly cleave and activate procaspase-3, which plays a critical role in the initiation of apoptosis. Recently, Akt was found to phosphorylate procaspase-9 and inhibit its protease activity (35), thus suppressing activation of procaspase-3 and promoting cell survival. Our results show that caspase-3 activity has a reverse correlation with Akt activity. Caspase-3 activity is blocked when Akt is activated by HGF/Met signaling, suggesting that the anti-apoptotic effect elicited by HGF/Met is related to the caspase cascade. In addition to caspase-3 activity, analysis of another more general indicator of cell viability, i.e., mitochondrial function as measured by MTT assay, revealed that HGF enhances cell survival and that this effect can be abolished by LY294002.

MAPK is another mediator of HGF/Met signaling and is activated through the Gab1/Grb2/SOS/Ras pathway. MAPK activation is required for HGF-elicited cell scattering and tubulogenesis (25, 36). MAPK is also involved in insulin-like growth factor 1-induced protection of differentiated PC12 cells from apoptosis by growth factor withdrawal (26) and hematopoietic cells from ceramide-induced apoptosis (27). Our findings demonstrate that MAPK is markedly activated by HGF and that HGF induction of MAPK activity is suppressed by PD098059 treatment. Elevated MAPK activity is concomitant with decreased caspase-3 activity and increased cell viability, suggesting that the MAPK pathway, like the PI3-kinase/Akt pathway, is involved in anti-apoptotic signaling elicited by HGF. It should be noted, however, that MAPK activity appears to have less effect on cell survival than PI3-kinase/Akt activity (Fig. 4 *A–C*).

It has been reported that WM inhibits MAPK activation induced by various factors such as platelet-derived growth factor (18, 37, 38). Thus, it is possible that there was crosstalk between the PI3-kinase and MAPK pathways in our cells, and that the effect of PI3-kinase inhibitor WM on HGF prevention of apoptosis is, in part, by indirect inhibition of MAPK. To exclude this possibility, we used LY294002 to replace WM as the PI3-kinase inhibitor. Because LY294002 had no inhibitory effect on HGF-induced MAPK activity, and PD098059 did not affect HGF-induced Akt activation under our experimental conditions, we conclude that HGF exerts its anti-apoptotic effects by independent use of both PI3-kinase/Akt and MAPK pathways. In agreement with this possibility, LY294002 and PD098059 had an additive, but not synergistic, effect on cell viability and caspase-3 activation and abolished the anti-apoptotic effect of HGF/Met signaling. In conclusion, this study implicates apoptosis inhibition via PI3-kinase/Akt and MAPK as a critical component of Met oncogenesis.

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