Dual Regulation of MMP-2 Expression by the Type 1 Insulin-like Growth Factor Receptor

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The matrix metalloproteinase (MMP)-2 has been recognized as a major mediator of basement membrane degradation, angiogenesis, tumor invasion, and metastasis. The factors that regulate its expression have not, however, been fully elucidated. We previously identified the type I insulin-like growth factor (IGF-I) receptor as a regulator of MMP-2 synthesis. The objective of the present study was to investigate the signal transduction pathway(s) mediating this regulation. We show here that in Lewis lung carcinoma subline H-59 cells treated with IGF-I (10 ng/ml), the PI 3-kinase (phosphatidylinositol 3'-kinase)/protein kinase B (Akt) and C-Raf/ERK pathways were activated, and MMP-2 promoter activity, mRNA, and protein synthesis were induced. MMP-2 induction was blocked by the PI 3-kinase inhibitors LY294002 and wortmannin, by overexpression of a dominant-negative Akt or wild-type PTEN (phosphatase and tensin homologue deleted on chromosome 10), and by rapamycin. In contrast, a MEK inhibitor PD98059 failed to reduce MMP-2 promoter activity and actually increased MMP-2 mRNA and protein synthesis by up to 30%. Interestingly, suppression of PI 3-kinase signaling by a dominant-negative Akt enhanced ERK activity in cells stimulated with 10 ng/ml but not with 100 ng/ml IGF-I. Furthermore, at the higher (100 ng/ml) IGF-I concentration, C-Raf and ERK, but not PI 3-kinase activation, was enhanced, and this resulted in down-regulation of MMP-2 synthesis. This effect was reversed in cells expressing a dominant-negative ERK mutant. The results suggest that IGF-I can up-regulate MMP-2 synthesis via PI 3-kinase/Akt/mTOR (the mammalian target of rapamycin) signaling while concomitantly transmitting a negative regulatory signal via the Rap/ERK pathway. The outcome of IGF-IR (the receptor for IGF-I) activation may ultimately depend on factors, such as ligand bioavailability, that can shift the balance preferentially toward one pathway or the other.

The breakdown of the extracellular matrix by proteinases is an essential step in the processes of cancer invasion and metastasis. Malignant progression is frequently associated with up-regulated production and/or activity of one or several matrix metalloproteinases (MMP), a family of extracellular matrix-degrading enzymes that have been implicated in tumor invasion by in vitro and in vivo studies. The 72-kDa type IV collagenase, MMP-2, is thought to play a critical role in tumor cell invasion by facilitating the degradation of basement membrane type IV collagen, by exposing cryptic, growth-affecting sites on extracellular matrix proteins, and by increasing the bioavailability of matrix-associated growth factors. Elevated levels of MMP-2 have been reported in various human malignancies including breast, oral, gastric, bladder, and pancreatic cancers (3–7). Moreover, this enzyme was identified as critical for the process of angiogenesis (8) and used as a target for anti-cancer therapy (9).

Similarly to other members of the metalloproteinase family, MMP-2 synthesis and function are regulated at multiple levels including transcriptional activation, post-transcriptional processing, and positive or negative regulation of proteolytic activity by membrane type-MMPs (MT-MMPs) and a family of endogenous inhibitors collectively known as tissue inhibitors of metalloproteinases (TIMPs) (10). MMP-2 is secreted as an inactive, 72-kDa zymogen and is activated by proteolytic cleavage extracellularly. This process involves MT1-MMP that binds MMP-2 on the cell membrane in a multimeric complex with TIMP-2 (11). Other cell surface-binding sites such as the integrin αvβ3 have also been implicated (12). The transcriptional regulation of MMP-2 is not fully understood. Among those factors implicated in its regulation are transforming growth factor-β (13), two extracellular matrix proteins, laminin and vitronectin (14, 15), intracellular calcium levels (16), IL (interleukin)-8 (17, 18), and IGF-I (19). Although the MMP-2 promoter lacks well characterized regulatory elements, several transcription factors including Sp1, Sp3, and AP-2 were recently implicated in its transcriptional activation in astrogliaoma cells (20).

The receptor for the type I insulin-like growth factor (IGF-IR) and its ligands IGF-I and IGF-II play critical roles in the regulation of cellular proliferation, apoptosis, and transforma-
IGFs have increasingly been recognized as important mitogens for many tumor types and overexpression and/or constitutive activation of IGF-IR in non-malignant cells resulted in the acquisition of a transformed phenotype and tumorigenic potential (21). In vivo, IGF-I-dependent tumor cell growth can be regulated in a paracrine manner by serum or stromal cell-derived IGFs, or it may be regulated through an autocrine loop. Indeed, increased expression of IGF-IR, IGF-I, and IGF-II or a combination thereof has been documented in many animal and human malignancies (22). In addition, prospective clinical studies identified serum levels of circulating IGF-I and II as potential risk factors for carcinomas of the breast, prostate, and colon (reviewed in Ref. 23). In several animal tumor models, inhibition of IGF-IR or IGF-I expression by various strategies including antisense oligodeoxynucleotides, antisense RNA, or IGF-I peptide analogues caused a suppression of tumor cell growth in vivo (23, 24), identifying this receptor/ligand system as a target for cancer therapy (21).

Ligand-dependent activation of the intrinsic IGF-IR tyrosine kinase results in the phosphorylation of several substrates including members of the insulin receptor substrate family and Shc (25, 26). Ligand binding to the IGF-IR can trigger multiple signaling pathways including the Ras/Raf/ERK pathway implicated for cancer therapy (21). tide analogues caused a suppression of tumor cell growth and GI activities, and antisense oligodeoxynucleotides, antisense RNA, or IGF-I peptide analogues caused a suppression of tumor cell growth in vivo (23, 24), identifying this receptor/ligand system as a target for cancer therapy (21).

We previously identified the IGF-IR as a regulator of carcinoma cell invasion and MMP-2 synthesis and activity (31). Here we analyzed the signal transduction pathways that link MMP-2 production to the IGF-IR axis and investigated the role of other inter-pathway cross talk.

**EXPERIMENTAL PROCEDURES**

**Cells and Chemicals**—The origin and invasive/metastatic phenotypes of Lewis lung carcinoma sublines, H-59 and M-27, were described in detail previously (22). M-27G19-19 cells were produced by stable transfection of M-27 cells with the pCl-neo vector expressing the full-length human IGF-IR receptor cDNA (33). All cell lines were cultured in RPMI 1640 supplemented with 10% fetal calf serum. Transfected cells were maintained in medium containing 200 μg/ml G 418. All cell lines were routinely tested for infectious agents and were found to be free of infection during the course of this study. Recombinant hIGF-I was from United States Biological. For analysis of MMP-2, 50-fold concentrated conditioned media were used, and 50 and 100 μg of protein (as specified) were loaded per lane and separated by electrophoresis on a 10% SDS-polyacrylamide gel. The separated proteins were transferred onto nitrocellulose membranes and probed with a monoclonal antibody to MMP-2 (developed by Fujio Co, for Medicorp, Montreal, Quebec, Canada) or with a rabbit anti-serum raised against human heparin (a kind of gift from Dr. John Mort, Shriners Hospital, McGill University, Montreal, Quebec, Canada (38)). A peroxidase-conjugated affiniPure goat anti-mouse IgG or donkey anti-rabbit IgG was used as secondary antibody. For detection of phospho-Akt, ERK, C-Raf, and tubulin, the cells were incubated with 10ng/ml phospho-specific antibodies (obtained from New England Biological (Swampscott, MA), LY294002, PD98059, and rapamycin were from Calbiochem, and wortmannin was from Sigma.

**Reverse Transcription PCR (RT-PCR)**—Total cellular RNA was extracted using TriZol (Invitrogen). RT-PCR was performed using the ThermoScript<sup>RT</sup>-PCR system, Platinum TaqDNA polymerase (both from Invitrogen), and the following primers: for MMP-2, sense, 5′-GAG TGG GTA CTA TAT CTT-3′ and antisense, 5′-GCC GTC TCT CTC AAA GTT GT-3′ (expected product 665 bp); and for GAPDH, sense, 5′-GGT GAA GGT CGG TGT GAA CGG ATT T-3′ and antisense, 5′-AAT GCC AAA GTT GTC ATG GAT GAC C-3′ (expected product 520 bp). The cDNA was amplified using 30 cycles for MMP-2 (25 cycles for GAPDH) of each 30 s at 94 °C, 30 s at 58 °C, and 30 s at 72 °C, and this was followed by a 10-min incubation at 72 °C. The amplified DNA fragments were analyzed by electrophoresis on a 1.5% agarose gel.

**Real-time Quantitative PCR**—PCR was performed with the LightCycler<sup>®</sup> FastStart DNA Master SYBR Green I (Roche Applied Science) in a total volume of 20 μl containing reaction mixture containing 0.5 μM of each oligonucleotide (described above), 3 μM MgCl<sub>2</sub>, and 2 μl of cDNA. Amplification and detection were performed in a LightCycler<sup>®</sup> instrument (Roche Applied Science) as follows: 20 μl of the reaction mixture were initially incubated for 10 min at 95 °C to denature the DNA and activate the Fast Start TaqDNA; amplification was then performed for 43 cycles of denaturation (95 °C, 10 s, ramp rate 90 °C/s), annealing (58 °C, 5 s, ramp rate 90 °C/s), and extension (72 °C, 30 s). A single fluorescence reading was taken at each extension step. The crossing points, marking the cycle when the fluorescence of a given sample significantly exceeded the baseline signal, were recorded and expressed as a function of the cycle number. The crossing points were plotted against known concentrations determined on the basis of a pre-established standard curve. The identity of the amplification products was confirmed by determining the melting curve. The melting point of the MMP-2 amplification product (665 bp) was 88 ± 0.5 °C.

**Plasminogen and Transfection—**Wild-type and dominant-negative Akt (DN-Akt) constructs were gifts from Dr. David Kaplan (University of Toronto, Toronto, Ontario, Canada). The DN-Akt cDNA encoding a catalytically inactive mutant was constructed by a lysine-methionine substitution (residue 179) within the ATP-binding domain (34). The PTEN cDNA was cloned into the NotI site of the pCEP4 vector (35). It was a gift from Dr. Nahum Sonenberg (McGill University, Montreal, Quebec, Canada). The expression vectors encoding the hemagglutinin-tagged wild-type ERK1 or a dominant-negative mutant, in which threonine 185 was replaced by alanine have been characterized elsewhere (36). Transient transfections of H-59 cells seeded in 10-cm culture dishes, at a density of 10<sup>5</sup> cells/dish, were performed using the LipofectAMINE Plus reagent (Invitrogen) and 5 μg of DNA/dish, as suggested by the manufacturer. The transfected cells were incubated first for 5 h in RPMI 1640 medium containing 10% fetal calf serum and then for 18 h in serum-depleted medium, prior to stimulation with IGF-I.

**Chloramphenicol Acetyltransferase (CAT)-**Reporter Assay—Tumor cells were co-transfected with a CAT vector expressing the CAT reporter gene downstream of a 680-bp (nucleotides −390 to +290) fragment of the human MMP-2 promoter (18) and a pSV40-Luciferase expression plasmid for normalization. The transfected cells were then cultured for 18 h in medium containing 200 μg/ml G 418. All cell lines were cultured in serum-free medium with or without the indicated concentration of recombinant IGF-I, except during the course of the study. Recombinant IGF-I was from United States Biological. For analysis of MMP-2, 50-fold concentrated conditioned media were used, and 50 and 100 μg of protein (as specified) were loaded per lane and separated by electrophoresis on a 10% SDS-polyacrylamide gel. The separated proteins were transferred onto nitrocellulose membranes and probed with a monoclonal antibody to MMP-2 (developed by Fujio Co, for Medicorp, Montreal, Quebec, Canada) or with a rabbit anti-serum raised against human heparin (a kind of gift from Dr. John Mort, Shriners Hospital, McGill University, Montreal, Quebec, Canada (38)). A peroxidase-conjugated affiniPure goat anti-mouse IgG or donkey anti-rabbit IgG was used as secondary antibody. For detection of phospho-Akt, ERK, C-Raf, and tubulin, the cells were incubated with 10ng/ml phospho-specific antibodies (obtained from New England Biological (Swampscott, MA), LY294002, PD98059, and rapamycin were from Calbiochem, and wortmannin was from Sigma).

**Western Blot Assay—**Subconfluent monolayers (5 × 10<sup>5</sup> cells) were washed extensively to remove serum, and the cells were cultured for 48 h at 37 °C in serum-free medium with or without the indicated concentration of recombinant IGF-I. Cell lysates were prepared using the Reporter lysis buffer (Promega), and CAT activity was measured using the **[H]**Chloramphenicol assay (37). Transfection efficiencies were normalized relative to luciferase activity as measured using an MLX microtiter plate luminometer (LKB Instruments).

**In Vitro Kinase Assay—**Stimulation with IGF-I and cell lysis was performed as described above. C-Raf activity was measured by the Raf (Thr<sup>290</sup>, ERK)1/2 (p42/p44 MAPK), phospho-ERK1/2, and phospho-C-Raf (Ser<sup>259</sup>) (all from Cell Signal Technology), C-Raf and phospho-C-Raf (Thr<sup>294/291</sup>) (both from Santa Cruz Biotechnology), and tubulin and hemagglutinin (from Sigma). Horseradish peroxidase-conjugated affiniPure donkey anti-rabbit, donkey anti-goat, or goat anti-mouse IgG antisera were used as required, as secondary antibodies. For detection of phospho-Akt, Akt, C-Raf, and tubulin, the cells were incubated with 10ng/ml phospho-specific antibodies (obtained from New England Biological (Swampscott, MA), LY294002, PD98059, and rapamycin were from Calbiochem, and wortmannin was from Sigma).

**MMP Assay—**MMP-2 activity was assayed by zymography, as we described previously (39). Briefly, concentrated conditioned media (50×) were electrophoresed on a 10% SDS-polyacrylamide gel containing 1 mg/ml gelatin. The gels were stained with Coomassie Blue R250 and destained with 10% acetic acid-20% methanol until the desired color intensity was obtained. The gelatinolytic activity on the blue background was identified by radiodensitometry using photographic negatives of the gels.
at 30°C. From each reaction mixture, 25μl were spotted onto the center of a 2-cm² P81 filter paper, and the papers were washed with 0.75% phosphoric acid and transferred into the scintillation mixture for counting. Reaction mixtures containing no enzyme were used as controls.

Statistical Analysis—The Student's t test was used for analysis of the CAT assay and real-time PCR data.

RESULTS

IGF-I Induces MMP-2 mRNA and Protein Synthesis—The effect of IGF-I on MMP-2 expression was measured at the mRNA and protein levels. An RT-PCR analysis showed that in response to 10 ng/ml IGF-I, MMP-2 mRNA expression in both H-59 and M-27IGF-IR, but not in control, M-27 cells began to rise at 2 h, reached maximal levels at 8 h, and persisted for up to 24 h (Fig. 1A). A quantitative real-time PCR analysis confirmed these results, showing a 4-fold increase in MMP-2 transcription at 8 h (Fig. 1B). The increase in MMP-2 mRNA expression was reflected in increased protein production and enzyme activity, as determined by Western blotting (Fig. 1C, I) and gelatin zymography (Fig. 1C, II) respectively, performed on medium conditioned by H-59 cells. Gelatin zymography revealed two bands corresponding to the native (72-kDa) and activated (64-kDa) forms of MMP-2 (Fig. 1C, II). In contrast, tumor cell treatment with 100 ng/ml IGF-I reduced MMP-2 mRNA expression by 70%, as measured by real-time PCR (Fig. 1B), and this resulted in a corresponding reduction in protein synthesis (Fig. 1C).

MMP-2 promoter activation by IGF-I was subsequently analyzed in H-59 cells that were transiently transfected with a pCAT basic vector in which a 680-bp fragment of the MMP-2 promoter (nucleotides -390 to +290) was cloned upstream of the CAT reporter gene. This promoter region was selected because it contains the Sp1- and AP-2-binding sites that were previously identified as critical for MMP-2 gene transcription (20). To control for transfection efficiency, the cells were co-transfected with a second plasmid vector expressing the luciferase gene under the control of a constitutive RSV promoter. CAT activity in the transfected cells increased 2.4-fold relative to controls when the cells were treated with 10 ng/ml IGF-I for 6 h (Fig. 2, p < 0.01). The activity remained elevated relative to controls for up to 24 h, declining to basal levels by 36 h (data not shown). A similar IGF-I-induced increase in CAT activity was seen in M-27IGF-IR cells (p < 0.01), but no response was observed in control, M-27 cells (p > 0.05, Fig. 2).

The PI 3-Kinase/Akt and MEK/MAPK Pathways Transit Opposing Signals for MMP-2 Synthesis in Response to IGF-I—
Signal transduction by the IGF-IR is known to involve both the ERK and the PI 3-kinase pathways (25, 26). To identify the pathway(s) regulating MMP-2 induction by IGF-I, we first tested the effects of pharmacological inhibitors of these pathways on MMP-2 expression, using a combination of RT-PCR, Western blotting, and the CAT-MMP-2 promoter assay. Results in Fig. 3 show that treatment of H-59 cells with the PI 3-kinase inhibitors LY294002 (20 μM) and wortmannin (5 μM), under conditions that had no deleterious effect on cell viability (38), blocked IGF-I-induced MMP-2 mRNA synthesis (Fig. 3A) and reduced MMP-2 protein synthesis by 82 and >90%, respectively, relative to non-treated, IGF-I-stimulated cells. Moreover, in H-58 cells that were transiently transfected with plasmid vectors expressing a dominant-negative (DN) Akt mutant or wild-type PTEN, IGF-I-mediated MMP-2 induction was completely blocked, whereas it increased by 3–3.6-fold in non-transfected or wild-type Akt-transfected cells (Fig. 3, C and E), confirming the involvement of the PI 3-kinase/Akt pathway in the induction. The decrease in MMP-2 induction in DN-Akt transfected cells was seen despite increased levels of phospho-ERK in these cells (Fig. 3D). In addition, treatment with PD98059 (20 μM) increased MMP-2 mRNA and protein levels, as measured by real-time PCR (Fig. 3A) and by Western blot analysis (Fig. 3B). Moreover, cell pretreatment with LY294002 and wortmannin, but not with PD98059, blocked IGF-I-induced MMP-2 promoter activation, as measured by the CAT reporter assay (Fig. 3F). Taken together, the data indicate that IGF-I can up-regulate MMP-2 synthesis via PI 3-kinase/Akt signaling while also transmitting a negative regulatory signal via C-Raf/ERK signaling.

Inhibition of IGF-I-mediated MMP-2 Protein but Not mRNA Synthesis by Rapamycin—The mammalian target of rapamycin (mTOR) is a substrate downstream of Akt that is involved in the regulation of translation initiation (40). To evaluate its involvement in IGF-I-induced MMP-2 synthesis, the cells were treated with rapamycin at non-toxic concentrations prior to stimulation with IGF-I (10 ng/ml). This treatment blocked the increase in MMP-2 protein synthesis (a decrease of 70% and 85% in cells treated with 20 and 100 ng/ml rapamycin, respectively, relative to non-treated cells, Fig. 4A) while not affecting MMP-2 mRNA synthesis (Fig. 4B).

Changes in Signaling in Response to High Dose IGF-I, Evidence for Inter-pathway Cross-talk—Because IGF-I at a concentration of 100 ng/ml decreased, rather than enhanced, MMP-2 synthesis (Fig. 1, B and C), it was of interest to compare the signals generated at this dose to those seen at the MMP-2-inducing concentration of 10 ng/ml. We observed that in cells treated with 10 ng/ml IGF-I, the PI 3-kinase/Akt/Raf/ERK pathways were both activated. Increased Akt phosphorylation was observed by 2 min following the treatment, returning to basal levels by 20 min. Phospho-ERK levels began to increase at 5 min, were maximal at 20 min, concomitantly with maximal C-Raf kinase activity, and returned to basal levels at 40–60 min (Fig. 5, A and B). This pattern was altered at 100 ng/ml IGF-I. At this concentration, there was no measurable increase in phospho-Akt levels, whereas C-Raf activation and ERK phosphorylation were both enhanced and more sustained and could be detected for up to 1 and 2 h, respectively, after IGF-I treatment (Fig. 5, B and I).

C-Raf was recently identified as an Akt substrate in several cell lines including human breast carcinoma line MCF-7 (29, 41, 42). Akt phosphorylates C-Raf on Ser\(^{259}\), resulting in C-Raf inactivation, probably as a result of its binding to protein 14–3–3 (30). C-Raf kinase activity, on the other hand, depends on phosphorylation of Tyr\(^{340/341}\) (43). Because we previously observed an increase in ERK phosphorylation in cells treated with PI-3K inhibitors (39), and in the present study, an increase in phospho-ERK was seen in cells transiently transfected with DN-Akt (Fig. 3D), it was of interest to investigate whether IGF-I in fact triggered cross-pathway phosphorylation in our cells. When C-Raf activation was measured in cells treated with 10 or 100 ng/ml IGF-I, we found that at the lower IGF-I concentration, C-Raf was transiently phosphorylated on Tyr residues 340 and 341, and this coincided with an initial decrease in phospho-Ser\(^{259}\) levels and C-Raf activation. However, an increase in phospho-Ser\(^{259}\) was subsequently seen following Akt activation, causing a decline in C-Raf activity to below baseline levels by 60 min and leading to MMP-2 induction. On the other hand, treatment with 100 ng/ml IGF-I resulted in a decrease in phospho-Akt levels by 20 min, and this coincided with a sustained decrease in phospho-Ser\(^{259}\). C-Raf levels were more sustained, and C-Raf activity (Fig. 5, A and B) increased by 3–4-fold. This increase in phospho-ERK production was observed by 2 min following the treatment, returning to basal levels by 20 min. Phospho-ERK levels began to increase at 5 min, were maximal at 20 min, concomitantly with maximal C-Raf kinase activity, and returned to basal levels at 40–60 min (Fig. 5, A and B). This pattern was altered at 100 ng/ml IGF-I. At this concentration, there was no measurable increase in phospho-Akt levels, whereas C-Raf activation and ERK phosphorylation were both enhanced and more sustained and could be detected for up to 1 and 2 h, respectively, after IGF-I treatment (Fig. 5, B and I).

Our results show that IGF-I can regulate MMP-2 synthesis at several levels. It can activate the MMP-2 promoter and also enhance protein synthesis through activation of mTOR, both via the PI-3K/Akt pathway. At the same time, it can also transmit a down-modulating signal via the Raf/MEK/ERK pathway. The net effect of these opposing regulatory signals appears to depend, among others, on the ligand load. At a concentration of 10 ng/ml, which is optimal for mitogenesis, cellular proliferation, and motility (33), the PI-3K/Akt pathway is activated, leading to Raf kinase inactivation and a short-lived ERK response, and this appears to shift the balance in favor of MMP-2 induction. At the higher dose, however, activation of the ERK pathway appears to dominate, and this leads to a down-regulation of MMP-2 synthesis (diagrammatic representation in Fig. 6). These results are consistent with a recent report of MMP-2 down-regulation in mesangial cells.

**FIG. 2.** **MMP-2 promoter activity is induced by IGF-I.** Tumor cells were co-transfected with the pCAT basic vector expressing a CAT reporter gene downstream of a 680-bp fragment of the human MMP-2 promoter and the pSV40-Luciferase plasmid expressing the luciferase gene under the control of an RSV promoter. CAT activity was induced by incubating serum-deprived cells with 10 ng/ml IGF-I for 6 h and analyzed using [\(\text{H}^{3}\)]chloramphenicol. The results are expressed as CAT: luciferase activity ratios. Con, non-stimulated cells.

**DISCUSSION**

Our results show that IGF-I can regulate MMP-2 synthesis at several levels. It can activate the MMP-2 promoter and also enhance protein synthesis through activation of mTOR, both via the PI-3K/Akt pathway. At the same time, it can also transmit a down-modulating signal via the Raf/MEK/ERK pathway. The net effect of these opposing regulatory signals appears to depend, among others, on the ligand load. At a concentration of 10 ng/ml, which is optimal for mitogenesis, cellular proliferation, and motility (33), the PI-3K/Akt pathway is activated, leading to Raf kinase inactivation and a short-lived ERK response, and this appears to shift the balance in favor of MMP-2 induction. At the higher dose, however, activation of the ERK pathway appears to dominate, and this leads to a down-regulation of MMP-2 synthesis (diagrammatic representation in Fig. 6). These results are consistent with a recent report of MMP-2 down-regulation in mesangial cells.
treated with 100 ng/ml IGF-I (44). They are at variance with the recent report by Moelling et al. (29) where PI-3 kinase signaling in MCF-7 cells was shown to predominate at 100 ng/ml IGF-I, causing an inhibition of Raf/ERK signaling at this concentration, but not at 10 ng/ml IGF-I. The mechanisms responsible for the preferential activation of one pathway or the other at different ligand concentrations are not presently clear. However, differences in cell surface receptor numbers and the expression of downstream substrates are likely to play a role (40). In this context, it is of interest to note that in humans, serum concentrations for total IGF-I can vary from 50 to 250 ng/ml with reported averages of 100–150 ng/ml (45, 46).

PI 3-kinase has been identified as the major transducer of the IGF-IR signal in other cellular systems, and its activity was shown to be critical for IGF-I-induced cell survival, mitogenesis, and transcriptional and translational regulation of protein synthesis (16, 47–52). In some systems, these processes were found to be equally dependent on ERK signaling (47, 50), whereas in others, the PI 3-kinase pathway or ERK signaling played more dominant roles (48, 53). The degree to which different cells utilize these pathways to convey IGF-IR signals may be cell context-dependent and could be determined, among others, by the levels and accessibility of downstream substrates such as insulin receptor substrate-1 or Shc (40, 53, 54) and by the repertoires and activation levels of other receptor tyrosine kinases, such as the epidermal growth factor receptor, expressed by the cells (56).

The transcription factors involved in positive and negative IGF-I-dependent MMP-2 regulation in our model remain to be identified. Recently, it has been shown that the transcription factors Sp1, Sp3, and AP-2 were required for constitutive MMP-2 gene expression (20). The present results show that IGF-I could activate an MMP-2 promoter fragment spanning nucleotides −390 to +290, a region that contains the Sp1 and AP-2-binding sites (20), suggesting that these elements may be

![Figure 3](http://www.jbc.org/)

**Fig. 3.** The PI 3-kinase/Akt and Raf/ERK pathways transmit opposing signals for MMP-2 expression. Serum-starved H-59 cells were pretreated (or not) for 5 h with 20 μM LY294002, 5 nM Wortmannin, or 20 μM PD98059 and then stimulated with 10 ng/ml IGF-I, in the presence or absence of the inhibitors. Total RNA was extracted 18 h later and analyzed by RT-PCR (A, I) or by real-time PCR (A, II). Conditioned media were harvested 48 h later, concentrated (50×), and analyzed by Western blotting using an anti-MMP-2 antibody (B). The fold change in the intensity of the bands was calculated on the basis of densitometry and is expressed relative to control (Con, non-stimulated) cells, which were assigned a value of 1 (B, bottom panel). H-59 cells were transiently transfected (or not, Con) with a plasmid vector expressing a wild-type or dominant-negative Akt (C and D) or wild-type PTEN (E) prior to incubation with 10 ng/ml IGF-I for 48 h. Concentrated conditioned media were analyzed by Western blotting using an anti-MMP-2 antibody. The effect of PI 3-kinase inhibitors on MMP-2 promoter activation by IGF-I (F) was analyzed as described in the legend for Fig. 2. Cells were pretreated with the inhibitors for 5 h and stimulated (or not, Con) with 10 ng/ml IGF-I for 6 h. The results are expressed as CAT: luciferase activity ratios.
FIG. 4. Rapamycin blocks MMP-2 protein synthesis but not mRNA induction. Serum-starved H-59 cells were rapamycin-treated for 5 h and incubated with 10 ng/ml IGF-I for 48 (A) or 16 (B) h. The concentrated conditioned media were analyzed by Western blotting using an anti-MMP-2 antibody (A), or total RNA was extracted and analyzed by RT-PCR (B). Con, non-stimulated cells.

FIG. 5. The IGF-I-induced signal is dose-sensitive and controlled by cross-pathway phosphorylation. Serum-deprived H-59 cells were incubated with IGF-I as indicated. The cell lysates were analyzed by Western blotting (A and B, I), essentially as described in the legend for Fig. 3. C-Raf activity (B, II) was measured by the Raf-1 immunoprecipitation kinase cascade assay kit, as detailed under “Experimental Procedures.” C, serum-starved H-59 cells transiently expressing hemagglutinin (HA)-tagged dominant-negative or wild-type (WT) ERK-1 were treated for 48 h with 100 ng/ml IGF-I. Concentrated conditioned media (50X, CM) and cell lysates were analyzed by Western blotting using 100 μg of protein/lane. Control, non-transfected cells.
involved in the IGF-I response. Recently, we also found that deletion of the PEA3-binding site enhanced IGF-I-mediated MMP-2 promoter activity, suggesting that this element may be exerting a negative regulatory effect on MMP-2 gene expression. Interestingly, other studies have recently shown that the activity of the Ets transcription factor PEA3 is regulated by two distinct MAPK pathways, namely the ERK and the Jun kinase (JNK) pathways (57). In turn, PEA3 was shown to suppress HER-2/neu expression and to inhibit tumorigenesis in HER-2/neu-overexpressing cancer cells (58). When taken together with the present findings, these observations suggest that PEA3 may be the element that exerts a negative regulatory effect on MMP-2 synthesis downstream of ERK, whereas Sp1 may be involved in positive regulation (59). Although recent reports identified the forkhead transcription factors AFX (Foxo4) (60) and Foxo1 (Fkhr) as potential transcriptional activators downstream of PI-3K/Akt (20), their role in IGF-I signaling is unclear. In this respect, it should be noted that although we observed an increase in MMP-2 transcription in the presence of the MEK inhibitor PD98059 (Fig. 3a), this inhibitor did not significantly alter the level of promoter activation by IGF-I (Fig. 3f). This difference may be related to the use of a partial promoter construct (nucleotides −390 to +290) in the CAT reporter assay. This portion of the promoter, although it contains the AP-2 and Sp1 elements implicated in positive regulation of MMP-2 transcription (20), is lacking 3 out of 4 PEA3-binding sites (20) that may exert the negative regulatory effects. The possibility that the observed increase in MMP-2 mRNA levels in the presence of a MEK inhibitor is due, at least in part, to increased mRNA stability cannot at present be ruled out.

We recently reported that in the present tumor model, IGF-I can also induce MT1-MMP expression in a PI 3-kinase/Akt signaling-dependent manner, but it does not affect TIMP-2 expression (39). The concomitant activation of MMP-2 and MT1-MMP synthesis by IGF-I, in the absence of a corresponding increase in TIMP-2 levels, could shift the balance in favor of increased production of catalytically active MMP-2 and is likely responsible for the increased invasion observed in these cells (33, 39). Because MMP-mediated extracellular matrix remodeling and growth are tightly linked (55, 62), the coordinated regulation of MMP-2 and MT1-MMP by IGF-I may also contribute to the central role that the IGF-IR plays in embryogenesis and development.

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