Insulin-mediated activation of activator protein-1 through the mitogen-activated protein kinase pathway stimulates collagenase-1 gene transcription in the MES 13 mesangial cell line

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Abstract

The initial stages of diabetic nephropathy are characterized, in part, by expansion of the mesangial matrix and thickening of the glomerular basement membrane which are caused by increased extracellular matrix (ECM) protein synthesis and reduced degradation, a consequence of decreased matrix metalloproteinase (MMP) activity. These changes have been largely attributed to the effects of hyperglycemia such that the potential contribution of impaired insulin action to alterations in the ECM have not been studied in detail. We have shown here that insulin stimulates collagenase-1 fusion gene transcription in the MES 13 mesangial-derived cell line. Multiple collagenase-1 promoter elements are required for the full stimulatory effect of insulin but the action of insulin appears to be mediated through an activator protein-1 (AP-1) motif. Thus, mutation of this AP-1 motif abolishes insulin-stimulated collagenase fusion gene transcription and, in isolation, this AP-1 motif can mediate a stimulatory effect of insulin on the expression of a heterologous fusion gene. This suggested that the other collagenase-1 promoter elements that are required for the full stimulatory effect of insulin probably bind accessory factors that enhance the effect of insulin mediated through the AP-1 motif. In MES 13 cells, the AP-1 motif is bound by Fra-1, Fra-2, Jun B and Jun D. Stimulation of collagenase-1 fusion gene transcription by insulin requires activation of the mitogen-activated protein kinase (MEK) pathway since inhibition of MEK-1 and -2 blocks this effect. The potential significance of these observations with respect to a role for insulin in the pathophysiology of diabetic glomerulosclerosis is discussed.

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Introduction

The abundance of extracellular matrix (ECM) protein is determined by the balance of ECM protein synthesis and degradation. The latter is primarily mediated by a family of ECM proteinases, the matrix metalloproteinases (MMPs) (Borden & Heller 1997, Brinckerhoff & Matrisian 2002, Visse & Nagase 2003), whose actions can be modulated through the regulation of their abundance or activity. MMP activity is regulated by the

tissue inhibitors of MMPs, which bind covalently to MMPs and prevent both the activation of MMPs and the ability of MMPs to bind substrates (Borden & Heller 1997, Brinckerhoff & Matrisian 2002, Visse & Nagase 2003).

MMP gene expression is regulated by multiple factors (Borden & Heller 1997, Brinckerhoff & Matrisian 2002, Visse & Nagase 2003). The molecular mechanisms that mediate this regulation have received considerable attention primarily because of the potential role of increased MMP

activity in determining the invasiveness of tumors (Vihinen & Kahari 2002, Yoon *et al.* 2003). However, several of the complications associated with both type I and type II diabetes mellitus, including nephropathy (Lenz *et al.* 2000, Mason & Wahab 2003), retinopathy (Sivak & Fini 2002), periodontal disease (Birkedal-Hansen 1993, Reynolds & Meikle 1997) and various aspects of cardiac disease (Creemers *et al.* 2001) are also characterized, in part, by alterations in the amount and composition of ECM protein.

The early stages of diabetic nephropathy are characterized by glomerular hypertrophy, expansion of the mesangial matrix and thickening of the glomerular basement membrane, resulting in a reduction of the available filtration surface area and the glomerular filtration rate (Osterby 1996). This expansion of the mesangial matrix and the thickening of the glomerular basement membrane can be attributed to a stimulation of ECM protein synthesis and a decrease in ECM degradation, a secondary consequence of reduced MMP activity (Lenz *et al.* 2000, Mason & Wahab 2003). In the streptozotocin rat model of type I diabetes, the expression of genes encoding three prominent MMPs, MMP-1 (collagenase-1; referred to henceforth as collagenase), MMP-2 and MMP-9 is decreased in the glomerulus (Nakamura *et al.* 1994, McLennan *et al.* 2002). The decrease in collagenase gene expression is reversed by insulin treatment (Nakamura *et al.* 1994), but the effect of insulin treatment on MMP-2 and MMP-9 gene expression has not been explored. Moreover, it is unclear whether this change in collagenase expression represents a direct effect of insulin as opposed to an indirect effect mediated through changes in glucose concentration. Nevertheless, a direct stimulatory effect of insulin on collagenase gene transcription has been observed in NIH 3T3 (Medema *et al.* 1991), CHO.T (Rutter *et al.* 1995) and HeLa cells (Chapman *et al.* 1999).

Insulin regulates the transcription of more than 100 genes through *cis*-acting elements collectively referred to as insulin response sequences or elements (IRSs/IREs) (O'Brien & Granner 1996, 2004, O'Brien *et al.* 2001). Unlike cAMP, which regulates gene transcription predominantly through one *cis*-acting element (Waterman 1994, Montminy 1997, Daniel *et al.* 1998, Mayr & Montminy 2001), it is apparent that a single consensus IRS does not exist (O'Brien & Granner 1996, 2004, O'Brien *et al.*

2001). Instead, seven distinct classes of IRS have currently been defined, in addition to several IRSs that appear to be unique to individual genes (O'Brien & Granner 2004). One of these classes of IRS has the sequence T(G/A)TTT(T/G) and mediates the insulin-dependent transcriptional inhibition of several hepatic genes such as those encoding phosphoenolpyruvate carboxykinase (PEPCK), insulin-like growth factor (IGF)-binding protein-1, tyrosine aminotransferase, apolipoprotein CIII and the glucose-6-phosphatase catalytic subunit (O'Brien & Granner 2004). This class of IRS, designated the PEPCK-like motif (O'Brien *et al.* 2001, O'Brien & Granner 2004), binds the transcription factor FKHR (FOXO1a), although recent data indicate that this is not the only insulin-regulated factor that can bind this element (Vander Kooi *et al.* 2003). The other six classes of IRS all mediate stimulatory effects of insulin on gene transcription. They are the activator protein-1 (AP-1) motif, the Sp1 binding motif, the serum response element, the Ets motif, the thyroid transcription factor-2 motif, and the sterol response element-binding protein motif (O'Brien & Granner 2004). However, unlike the PEPCK-type IRS that confers a selective effect of insulin (O'Brien & Granner 2004), the six positive IRSs can mediate the effects of multiple other hormones on gene transcription (Wasylyk *et al.* 1993, Treisman 1995, Ortiz *et al.* 1997, Miyazaki *et al.* 1999, Wisdom 1999, Horton *et al.* 2002, Samson & Wong 2002, Shaulian & Karin 2002, Shimano 2002, Oikawa & Yamada 2003).

The AP-1 motif mediates the action of insulin on the transcription of the hepatitis B virus X gene and the genes encoding collagenase and malic enzyme (Medema *et al.* 1991, Rutter *et al.* 1995, Choi *et al.* 1998, Streeper *et al.* 1998, Chapman *et al.* 1999). Heterodimers between members of the Fos (c-Fos; FosB; Fra-1; Fra-2) and Jun (c-Jun; Jun B; Jun D) transcription factor families form a major component of the AP-1 transcription factor complex (Shaulian & Karin 2002). The mechanism of insulin signaling through the AP-1 motif has not been studied in detail but it appears to involve effects of insulin on both the mass and/or the phosphorylation state of the AP-1 complex (O'Brien & Granner 1996). However, the mechanism of insulin signaling through AP-1 appears to vary with the cell type studied. For example, the stimulation of Fra-1 gene expression by insulin is

seen in some (Mamounas *et al.* 1991, Griffiths *et al.* 1998) but not all cell types (Mohn *et al.* 1990). Similarly, the protein kinases JNK-1 and JNK-2, which phosphorylate and activate c-Jun (Shaulian & Karin 2002), are activated by insulin in rat 1 fibroblasts (Miller *et al.* 1996) but not in CHO.T cells (Griffiths *et al.* 1998). In CHO.T cells, activation of the mitogen-activated protein (MAP) kinase pathway is required for insulin signaling through the AP-1 motif, an effect mediated through increased Fra-1 gene transcription and phosphorylation (Hurd *et al.* 2002) whereas, in adipocytes, this insulin-stimulated induction of Fra-1 gene expression also requires activation of the FRAP/mTOR pathway (Culbert & Tavaré 2002).

In the studies presented here, we have analyzed the effect of insulin on collagenase fusion gene transcription in murine mesangial-derived MES 13 cells. Insulin stimulates collagenase fusion gene transcription in MES 13 cells and this effect requires an AP-1 motif in the collagenase promoter, as it does in HeLa cells (Chapman *et al.* 1999). However, unlike HeLa cells, a distal collagenase promoter region is necessary for the maximal stimulatory effect of insulin on collagenase fusion gene transcription in MES 13 cells. Moreover, the factors binding the collagenase AP-1 motif in MES 13 and HeLa cells are distinct. Inhibition of MEK-1 and MEK-2 blocks the stimulatory effect of insulin on collagenase gene transcription in MES 13 cells, suggesting that activation of the MAP kinase pathway is necessary for mediating this effect of insulin. The potential significance of these observations with respect to a role for insulin in the pathophysiology of diabetic glomerulosclerosis is discussed.

Materials and methods

Materials

[α - 32 P] dATP (>3000 Ci/mmol) and [3 H]acetic acid, sodium salt (>10 Ci/mmol) were obtained from Amersham and ICN respectively. Insulin was purchased from Collaborative Bioproducts (Bedford, MA, USA). The MEK-1 and -2 inhibitor U0126 was purchased from Calbiochem (San Diego, CA, USA) or Promega. Specific antisera to c-fos (sc-52), Fra-1 (sc-183), Fra-2 (sc-604), c-jun (sc-45), Jun B (sc-46) and Jun D (sc-74) were all obtained from Santa Cruz Biotechnology Inc.,

Santa Cruz, CA, USA. All oligonucleotides were synthesized by the Vanderbilt University Medical Center Diabetes Core laboratory.

Plasmid construction

The construction of a human collagenase–chloramphenicol acetyltransferase (CAT) fusion gene containing promoter sequence, relative to the transcription start site, from –518 to +64 in the pCAT(An) expression vector (Jacoby *et al.* 1989), has previously been described (Chapman *et al.* 1999). The pCAT(An) vector has polyadenylation signals located 5' of the polylinker to prevent read-through transcription (Jacoby *et al.* 1989). Control experiments demonstrated that there was no basal CAT expression and no effect of insulin when the pCAT(An) vector, minus the collagenase promoter, was transiently transfected into MES 13 cells (data not shown). A series of truncated collagenase–CAT fusion genes, with the 5' endpoints shown in Fig. 5, were generated using the –518/+64 pCAT(An) construct as a template as previously described (Chapman *et al.* 1999). Similarly, a construct designated –79 AP-1 SDM, containing a site-directed mutation (SDM) of the collagenase AP-1 motif in the context of the –79 to +64 promoter fragment (Fig. 5), was generated as previously described (Chapman *et al.* 1999).

A three-step PCR strategy (Higuchi *et al.* 1988) was used to introduce a site-directed mutation into the collagenase AP-1 motif within the context of the –518 to +64 collagenase promoter fragment. Briefly, two complementary PCR primers were designed to mutate four nucleotides within the collagenase AP-1 motif. The sequence of the sense strand oligonucleotide was as follows: 5'-GGATGT TATAAAGCAactaTCAGACAGCCTCTGGCTT TC-3'. The location of the AP-1 core sequence is underlined and the mutated nucleotides are in lower case. This sense strand oligonucleotide was used in conjunction with a 3' PCR primer to generate the 3' half of the collagenase promoter, whereas the complementary antisense strand oligonucleotide was used in conjunction with a 5' PCR primer to generate the 5' half of the collagenase promoter. These 5' and 3' primers were designed to maintain the 5' and 3' junctions of the collagenase promoter fragments to be the same as those in the wild-type –518 to +64 collagenase–CAT fusion gene construct. The PCR

products from these two reactions were then combined and used themselves as both primer and template in a second PCR to generate a small amount of the full-length, mutated collagenase promoter fragment. Finally, the 5' and 3' PCR primers were then used to amplify this fragment. This fragment was ligated into the pGEM7 vector (Promega) and completely sequenced using the USB (Cleveland, OH, USA) Sequenase kit to ensure the absence of polymerase errors. The promoter fragment was then re-isolated and ligated into the pCAT(An) vector and the resulting construct was designated -518 AP-1 SDM (Fig. 6).

For primary human mesangial cell transfection experiments, the CAT reporter gene was replaced with the more sensitive firefly luciferase reporter gene. This was achieved by re-isolating the wild-type and AP-1 mutated -518 to +64 collagenase promoter fragments from the pCAT(An) expression vector using HindIII and XhoI and ligation into the polylinker of pGL3 MOD (Martin *et al.* 2003).

A plasmid, designated XMB, containing a minimal *Xenopus laevis* 68 kDa albumin promoter ligated to the CAT reporter gene was generated as previously described (Chapman *et al.* 1999). Double-stranded complementary oligonucleotides, representing the wild type (-78 AAGCATGAGT CAGACA -63; AP-1 motif underlined) or mutated (-78 AAGCAactTCAGACA -63; mutated bases in lower case letters) collagenase AP-1 motif were synthesized with HindIII compatible ends and ligated into HindIII-cleaved XMB in multiple copies as previously described (Chapman *et al.* 1999).

A fragment of the human MMP-9 promoter representing the sequence located between -600 to +6 was isolated using PCR in conjunction with the following 5' (5'-CCCAAGCTTGAATTCC CCAGCCTTGCTAGCAG-3') and 3' (5'-CC GCTCGAGTGTCTGACTGCAGCTGCTGTT GTG-3') primers. HindIII and XhoI sites used for cloning purposes are underlined. This fragment was ligated into the pGEM-T vector and completely sequenced using the USB Sequenase kit to ensure the absence of polymerase errors. This promoter fragment was then re-isolated and ligated into the pGL3 MOD expression vector.

All plasmid constructs were purified by centrifugation through cesium chloride gradients (Sambrook *et al.* 1989).

Cell culture and transient transfection

Murine MES 13 kidney mesangial-derived cells were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 medium containing 10% (v/v) fetal bovine serum and re-plated the day before use into six-well culture plates. Attached cells were then transiently transfected using the calcium phosphate-DNA co-precipitation method as previously described (Streeter *et al.* 1998, Chapman *et al.* 1999). Collagenase-CAT and MMP-9-luciferase fusion gene constructs (2 µg/well) were co-transfected with expression vectors encoding βgalactosidase (0.5 µg/well) and the insulin receptor (0.8 µg/well). After an overnight incubation in DMEM/F-12 containing 10% (v/v) fetal bovine serum, the medium was removed and the cells rinsed in PBS before incubation for a further 18–24 h in serum-free DMEM supplemented with or without insulin at the concentrations indicated in the Figure legends prior to harvesting. In one experiment (Fig. 8), attached MES 13 cells were co-transfected with a collagenase-CAT fusion gene construct (2 µg/well) along with expression vectors encoding βgalactosidase (0.5 µg/well) and the insulin receptor (0.8 µg/well), using the lipofectamine reagent (Gibco-BRL, Invitrogen, Carlsbad, CA, USA) exactly as previously described (Bischof *et al.* 2001). The lipofectamine:DNA ratio was 5:1. After lipofectamine-mediated transfection, cells were then incubated for 18–24 h in serum-free DMEM in the presence or absence of 100 nM insulin prior to harvesting.

Low passage primary human mesangial cells and mesangial cell growth medium (MsGM) were purchased from Cambrex Bio Science Inc. (Walkersville, MD, USA). Cells were grown in complete MsGM and re-plated the day before use into six-well culture plates. Attached cells were transiently co-transfected with collagenase-luciferase fusion gene constructs (14 µg/well) along with an expression vector encoding *Renilla* luciferase (0.7 µg/well) and either an expression vector encoding the insulin receptor or the empty pcDNA3 vector (2.8 µg/well), using the lipofectamine reagent (Gibco-BRL) exactly as previously described (Bischof *et al.* 2001), except that the lipofectamine:DNA ratio was 0.7:1. After lipofectamine-mediated transfection, cells were then incubated for 18–24 h in basal MsGM prior to harvesting.

Liver-derived H4IIE cells and cervix-derived HeLa cells were cultured in DMEM supplemented with either 2.5% (v/v) newborn bovine serum and 2.5% (v/v) fetal bovine serum or 10% (v/v) newborn bovine serum respectively.

CAT, β galactosidase and luciferase assays

Transfected MES 13 cells were harvested by trypsin digestion and sonicated in 150 μ l 250 mM Tris (pH 7.8) containing 2 mM phenylmethylsulfonyl fluoride (PMSF). CAT and β galactosidase assays were performed as previously described (O'Brien *et al.* 1995a,b). To compare the relative basal CAT expression obtained with the various reporter gene constructs described, CAT activity in samples from control cells was corrected for the β galactosidase activity in the same sample. Transfected primary mesangial cells were harvested using passive lysis buffer (Promega) and both firefly and *Renilla* luciferase activity were then assayed using the dual luciferase assay kit (Promega) as previously described (Martin *et al.* 2002). To correct for variations in transfection efficiency, the results are expressed as a ratio of firefly to *Renilla* luciferase activity. Several independent preparations of each plasmid construct were analyzed in duplicate in multiple transfections, as specified in the Figure legends.

Protein extraction, SDS-PAGE and immunodetection

Whole cell extracts were generated from HeLa, H4 IIE, MES 13 and primary mesangial cells. Cells were rinsed in PBS and then harvested by scraping and subsequent centrifugation. The cell pellets were resuspended in 500 μ l Triton X-100 extraction buffer (1.0% Triton X-100, 300 mM NaCl, 20 mM Tris, pH 7.3 and 10 mM EDTA) supplemented with 1 $\times$ Roche complete EDTA-free protease inhibitor cocktail. The cell suspensions were then forced through a 27 gauge needle and incubated on ice for 1 h. Protein concentrations were then determined using the Bio-Rad protein assay system. Twenty micrograms of protein were then resolved on a 7.0% SDS-polyacrylamide gel and subsequently transferred to a polyvinylidene fluoride membrane by semidry blotting in 25 mM Tris, 192 mM Glycine and 20% methanol as previously described (Hutton *et al.*

1988). Immunoblot analysis was then performed using an insulin receptor β -subunit antiserum (Santa Cruz; sc-711) as the primary antibody and a goat anti-rabbit immunoglobulin G antiserum conjugated to horseradish peroxidase (Calbiochem) as the secondary antibody. Antibody binding was detected using the enhanced chemiluminescence detection kit (Amersham Biosciences) according to the manufacturer's instructions.

Gel retardation assay

Labeled probes

Oligonucleotides representing the sense and anti-sense strands of the wild-type collagenase AP-1 motif (–78 AAGCATGAGTCAGACA –63) were synthesized with BamHI compatible ends, gel purified, annealed and then labeled with [α - 32 P]dATP using the Klenow fragment of *Escherichia coli* DNA polymerase I to a specific activity of approximately 2.5 μ Ci/pmol.

Nuclear extract preparation

MES 13 cells were transiently transfected with an expression vector encoding the human insulin receptor using the calcium phosphate–DNA co-precipitation method, as described above. After an overnight incubation in DMEM/F-12 containing 10% (v/v) fetal bovine serum, the medium was replaced with serum-free DMEM supplemented with or without 100 nM insulin. After a 5 h incubation, nuclear extracts were then prepared by the method of Andrews & Faller (1991) except that cells were lysed using Nonidet (NP)-40 to isolate nuclei (Marzluff 1990). Briefly, cells were lysed in 10 mM Tris–HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 0.5% NP-40, 0.5 mM dithiothreitol (DTT) and 0.5 mM PMSF and the isolated nuclei were extracted using 20 mM HEPES, pH 7.8, 0.4 M NaCl, 0.75 mM spermidine, 0.15 mM spermine, 0.2 mM EDTA, 2 mM EGTA, 25% glycerol, 2 mM DTT and 1 mM PMSF. The protein concentration of the nuclear extracts was determined using the Bio-Rad assay.

Standard AP-1 binding assay

Labeled AP-1 oligonucleotide ($\sim$ 7.5 fmol, $\sim$ 30 000 c.p.m.) was incubated with MES 13 nuclear extract (0.5–2 μ g) in a final reaction volume

of 20 μ l containing 20 mM HEPES, pH 7.8, 100 mM NaCl, 0.38 mM spermidine, 0.08 mM spermine, 0.1 mM EDTA, 1 mM EGTA, 2 mM DTT, 12.5% glycerol (v/v) and 1 μ g poly(dI-dC) $\times$ poly(dI-dC). After incubation for 10 min at room temperature, the reactants were loaded onto a 6% polyacrylamide gel and electrophoresed at room temperature for 90 min at 150 V in a buffer containing 25 mM Tris-HCl, pH 7.8, 190 mM glycine and 1 mM EDTA. Following electrophoresis, the gels were dried, exposed to Kodak XAR5 film, and binding was analyzed by autoradiography.

Competition experiments

For competition experiments (Fig. 10) the indicated unlabeled double-stranded oligonucleotides (100-fold molar excess) were mixed with the labeled oligomer prior to addition of nuclear extract (0.5 μ g). Binding was then analyzed as described above.

Gel supershift

Gel supershift assays (Fig. 11) were carried out by incubating MES 13 nuclear extract (2 μ g) with the indicated antisera (1 μ g) for 10 min at room temperature, prior to the addition of the labeled AP-1 oligonucleotide probe and binding buffer and incubation for an additional 10 min at room temperature. Binding was then analyzed as described above.

Results

Insulin stimulates collagenase–CAT fusion gene transcription in MES 13 cells

To begin to study the regulation of collagenase gene transcription by insulin in mesangial cells, a collagenase–CAT fusion gene construct, containing collagenase promoter sequence from –518 to +64, was transiently transfected into MES 13 cells. These cells represent a mesangial cell line model and are derived from glomerula explants of mice transgenic for the transforming gene encoding large T-antigen, which is present in the early region of simian virus 40 (SV40) (MacKay *et al.* 1988). MES 13 cells exhibit several of the biochemical and morphological features of normal mesangial

Figure 1 Concentration dependence of insulin-stimulated collagenase–CAT fusion gene transcription in MES 13 cells. MES 13 cells were transiently co-transfected using calcium phosphate co-precipitation, as described in Materials and methods, with a collagenase–CAT fusion gene (2 μ g), containing promoter sequence from –518 to +64, and expression vectors encoding β galactosidase (0.5 μ g) and the insulin receptor (0.8 μ g). Following transfection, cells were incubated for 18–24 h in serum-free media, in the presence or absence of the indicated concentration of insulin. The cells were then harvested and both CAT and β galactosidase activities were assayed as described in Materials and methods. Results are presented as the ratio of CAT: β galactosidase activity in insulin-treated versus control cells and are expressed as fold induction. Results represent the means $\pm$ S.E.M. of three experiments using several independent preparations of the fusion gene construct, assayed in duplicate.

cells in culture (MacKay *et al.* 1988, Robey *et al.* 1999, Maile *et al.* 2000, Coy *et al.* 2002). As seen in Fig. 1, insulin markedly stimulated collagenase–CAT fusion gene transcription in this cell line with a maximal effect at a concentration of 100 nM. Insulin also stimulated MMP-9–luciferase fusion gene transcription in MES 13 cells (Fig. 2) though the magnitude of the insulin effect ($\sim$ 3-fold) was much less than that seen on collagenase–CAT fusion gene transcription ($\sim$ 20-fold; Fig. 1).

As reported previously for HeLa cells (Chapman *et al.* 1999), this effect of insulin on collagenase–CAT fusion gene transcription in MES 13 cells was only observed following co-transfection with an expression vector encoding the insulin receptor (Fig. 3A). In the absence of insulin, co-transfection with the insulin receptor alone was insufficient to activate collagenase–CAT fusion gene transcription

Figure 2 Insulin stimulates MMP-9-luciferase fusion gene transcription in MES 13 cells. MES 13 cells were transiently co-transfected using calcium phosphate co-precipitation, as described in Materials and methods, with an MMP-9-luciferase fusion gene (2 µg), containing promoter sequence from -600 to +6, and expression vectors encoding βgalactosidase (0.5 µg) and the insulin receptor (0.8 µg). Following transfection, cells were incubated for 18–24 h in serum-free medium in the presence or absence of 100 nM insulin. The cells were then harvested and both luciferase and βgalactosidase activity were assayed as described in Materials and methods. Results are presented as the ratio of luciferase:βgalactosidase (βGal.) activity and are expressed as arbitrary units. Results represent the means ± S.E.M. of three experiments with all samples assayed in duplicate.

in MES 13 cells, suggesting a low level of signaling through the basal receptor (Fig. 3B).

The requirement for insulin receptor co-transfection to manifest an insulin response suggests that MES 13 cells no longer express endogenous insulin receptors because insulin receptors are normally present in mesangial cells (Abrass *et al.* 1988, Arnqvist *et al.* 1988) and their numbers increase in the diabetic state (Oemar *et al.* 1991). Similarly, human cervix, from which HeLa cells are derived, also expresses insulin receptors (Sheets *et al.* 1985), suggesting that endogenous insulin receptor expression is also lost in HeLa cells. Surprisingly, Fig. 4 shows that endogenous insulin receptors are expressed in both MES 13 and HeLa cells. The antiserum used in this analysis is specific for the insulin receptor and does not cross-react with IGF-I or insulin-related receptors. The requirement for co-transfection of an insulin receptor expression vector to study insulin-regulated collagenase gene expression in MES 13 and HeLa cells suggests that, even though these cells express endogenous insulin receptors, they are

Figure 3 Insulin-stimulated collagenase-CAT fusion gene transcription in MES 13 cells requires the insulin receptor. MES 13 cells were transiently co-transfected using calcium phosphate co-precipitation, as described in Materials and methods, with a collagenase-CAT fusion gene (2 µg), containing promoter sequence from -518 to +64, an expression vector encoding βgalactosidase (0.5 µg) and either with (+IREV) or without (-IREV) an expression vector encoding the insulin receptor (0.8 µg). Following transfection, cells were incubated for 18–24 h in serum-free medium in the presence or absence of 100 nM insulin. The cells were then harvested and both CAT and βgalactosidase activity were assayed as described in Materials and methods. In (A) results are presented as the ratio of CAT:βgalactosidase activity in insulin-treated versus control cells and are expressed as fold induction. In (B) results are presented as the ratio of CAT:βgalactosidase (βGal.) activity in control cells and are expressed as arbitrary units. Results represent the means ± S.E.M. of three experiments using several independent preparations of the fusion gene construct, assayed in duplicate.

Figure 4 MES 13 cells express endogenous insulin receptors. Insulin receptor expression in MES 13, H4IIE and HeLa cells, as well as in primary mesangial (Mes.) cells, was analyzed by immunoblot analysis as described in Materials and methods.

non-functional. Figure 4 also demonstrates that insulin receptors are expressed in primary mesangial cells as well as in rat H4IIE hepatoma cells. Insulin-regulated gene expression has been extensively studied in H4IIE cells without a requirement for co-transfection of an insulin receptor expression vector (O'Brien & Granner 1996).

Multiple promoter elements are required for the stimulatory effect of insulin on collagenase fusion gene transcription in MES 13 cells

Although in the streptozotocin rat model of type I diabetes, the glomerula expression of collagenase, MMP-2 and MMP-9 are all decreased (Nakamura *et al.* 1994, McLennan *et al.* 2002), the former MMP is associated with degradation of type 1 collagen whereas type 4 collagen is the principal collagen to accumulate in the glomerulus in diabetic nephropathy (Mauer 1994). Type 4 collagen is a substrate for MMP-2 and MMP-9 (Nagase & Woessner 1999, Brinckerhoff & Matrisian 2002). This suggests that MMP-2 and MMP-9, rather than collagenase, could play a major role in the regulation of ECM accumulation in diabetic nephropathy. However, in this study we chose to focus on identifying the promoter elements through which insulin stimulates collagenase fusion gene expression in MES 13 cells, given that the magnitude of the effect of insulin on collagenase (Fig. 1) was much greater than that on MMP-9 (Fig. 2).

To identify the promoter elements that mediate the stimulatory effect of insulin on collagenase fusion gene transcription in MES 13 cells, a series of 5' truncated collagenase–CAT fusion genes were

Figure 5 Progressive deletion of the collagenase promoter sequence between –518 and –80 reduces the stimulatory effect of insulin on collagenase–CAT fusion gene transcription. MES 13 cells were transiently co-transfected using calcium phosphate co-precipitation, as described in Materials and methods, with a series of collagenase–CAT fusion genes (2 µg), with 5' deletion end-points as shown on the abscissa, and expression vectors encoding βgalactosidase (0.5 µg) and the insulin receptor (0.8 µg). Following transfection, cells were incubated for 18–24 h in serum-free media in the presence or absence of 100 nM insulin. The cells were then harvested and both CAT and βgalactosidase activities were assayed as described in Materials and methods. Results in (A) are presented as the ratio of CAT:βgalactosidase activity in insulin-treated versus control cells and are expressed as fold induction. Results in (B) are presented as the ratio of CAT:βgalactosidase (βGal.) activity in control cells and are expressed as a percentage relative to the –518 5' deletion construct. Results represent the means ± S.E.M. of three experiments using several independent preparations of each fusion gene construct, in which each construct was assayed in duplicate.

transiently transfected into MES 13 cells and the effect of insulin on CAT expression directed by these constructs was analyzed. Figure 5A shows

that progressive deletion of the collagenase promoter sequence between -518 and -79 resulted in a progressive decrease in insulin-stimulated collagenase–CAT fusion gene transcription (Fig. 5A) with little change in basal fusion gene transcription (Fig. 5B). This result suggested that either multiple independent IRSs are present in the collagenase promoter region between -518 and -79 or that this promoter region contains binding sites for multiple accessory factors that enhance the action of insulin mediated through a more proximal IRS. Indeed, we have previously shown that an AP-1 motif, located between -73 and -67 , is important for the stimulation of collagenase–CAT fusion gene transcription by insulin in HeLa cells (Chapman *et al.* 1999). The -79 to $+64$ collagenase promoter region does confer an ~ 4 -fold stimulation of CAT expression by insulin but when this AP-1 motif was mutated in the context of the -79 to $+64$ collagenase promoter fragment it had no effect on the insulin response (Fig. 5A; -79 AP-1 SDM), though basal fusion gene expression decreased slightly (Fig. 5B).

This result would appear to indicate that the collagenase AP-1 motif is not important for insulin-stimulated collagenase–CAT fusion gene transcription in MES 13 cells. However, it is becoming increasingly apparent that the analysis of *cis*-acting elements in the context of truncated promoter fragments can be misleading, such that the importance of individual elements may only be apparent in the context of an otherwise intact promoter (Fry & Farnham 1999, Falvo *et al.* 2000). To address this possibility, the collagenase AP-1 motif was mutated in the context of the -518 to $+64$ collagenase promoter region. Figure 6 shows that mutation of the AP-1 motif in this context dramatically reduced basal collagenase–CAT fusion gene expression (Fig. 6A) and abolished the stimulatory effect of insulin (Fig. 6B). In contrast to the conclusion reached through the analysis of the truncated collagenase promoter (Fig. 5), this result indicated that the collagenase AP-1 motif is critical for both basal and insulin-stimulated collagenase–CAT fusion gene transcription in MES 13 cells.

Given this marked difference in results depending on whether the collagenase AP-1 motif was studied in the context of the $-518/+64$ or $-79/+64$ collagenase promoter fragments, we repeated this analysis in HeLa cells. We had previously shown that, in contrast to MES 13 cells

Figure 6 An AP-1 motif is required for basal and insulin-stimulated collagenase–CAT fusion gene transcription in both MES 13 cells (shaded bars) and HeLa cells (solid bars). MES 13 cells and HeLa cells were transiently co-transfected using calcium phosphate co-precipitation, as described in Materials and methods, with expression vectors encoding βgalactosidase (0.5 μg) and the insulin receptor (0.8 μg), and either a collagenase–CAT fusion gene (2 μg) containing promoter sequence from -518 to $+64$ (-518 wild type (WT)) or the same fusion gene with an SDM of the AP-1 motif (-518 AP-1 SDM). Following transfection, cells were incubated for 18–24 h in serum-free media in the presence or absence of 100 nM insulin. The cells were then harvested and both CAT and βgalactosidase activities were assayed as described in Materials and methods. In (A and B) results are presented as the ratio of CAT:βgalactosidase (βGal.) activity in either control or insulin-treated cells respectively, and are expressed as arbitrary units. Results represent the means $\pm$ S.E.M. of three experiments using several independent preparations of each fusion gene construct, in which each construct was assayed in duplicate.

(Fig. 5B), deletion of the collagenase promoter region between -518 and -79 resulted in a progressive decrease in basal collagenase–CAT

fusion gene expression in HeLa cells (Chapman *et al.* 1999). Moreover, again in contrast to MES 13 cells (Fig. 5B), mutation of the collagenase AP-1 motif in the context of the -79 to $+64$ collagenase promoter fragment almost completely abolishes basal collagenase–CAT fusion gene expression in HeLa cells (Chapman *et al.* 1999). Nevertheless, despite these differences between MES 13 and HeLa cells, mutation of the collagenase AP-1 motif in the context of the -518 to $+64$ promoter fragment dramatically reduced basal collagenase–CAT fusion gene expression (Fig. 6A) and abolished the stimulatory effect of insulin (Fig. 6B) in both MES 13 and HeLa cells. This result would appear to indicate that the collagenase AP-1 motif is critical for both basal and insulin-stimulated collagenase–CAT fusion gene transcription in both MES 13 and HeLa cells.

The collagenase AP-1 motif is essential for high basal fusion gene expression in primary human mesangial cells

Although MES 13 cells exhibit several of the biochemical and morphological features of normal mesangial cells in culture (MacKay *et al.* 1988, Robey *et al.* 1999, Maile *et al.* 2000, Coy *et al.* 2002), the need to express insulin receptors in these cells so as to study insulin action indicates that they do not entirely mimic the behavior of mesangial cells *in vivo*. We therefore investigated the regulation of collagenase fusion gene expression in primary human mesangial cells. Figure 7 shows that the collagenase AP-1 motif is also critical for basal collagenase–luciferase fusion gene transcription in primary human mesangial cells as it is in MES 13 and HeLa cells. Thus, mutation of the collagenase AP-1 motif in the context of the -518 to $+64$ promoter fragment dramatically reduced basal collagenase–luciferase fusion gene expression (Fig. 7). In contrast to MES 13 cells, no collagenase–luciferase fusion gene expression or SV40–*Renilla* luciferase expression was detected when these primary human mesangial cells were transfected using the calcium phosphate–DNA co-precipitation method (data not shown). While expression was detected using the lipofectamine transfection method (Fig. 7) there was no effect of insulin on collagenase–luciferase fusion gene expression, even in the presence of co-transfected insulin receptors.

We believe that this lack of insulin-regulated

Figure 7 The collagenase AP-1 motif is also required for high basal collagenase–luciferase fusion gene transcription in primary human mesangial cells. Primary human mesangial cells were transiently co-transfected using lipofectamine, as described in Materials and methods, with either a collagenase–luciferase fusion gene (14 µg) containing promoter sequence from -518 to $+64$ (-518 WT) or the same fusion gene with an SDM of the AP-1 motif (-518 AP-1 SDM) along with an expression vector encoding *Renilla* luciferase (0.7 µg) and the empty pcDNA3 vector (2.8 µg/well). Following transfection, cells were incubated for 18–24 h in serum-free medium. The cells were then harvested and both firefly and *Renilla* luciferase activity were assayed as described in Materials and methods. Results are presented as the ratio of firefly:*Renilla* luciferase activity and are expressed as arbitrary units. Results represent the means $\pm$ S.E.M. of three experiments each using an independent preparation of each fusion gene construct, assayed in triplicate.

collagenase–luciferase fusion gene expression reflects a technical problem with the use of lipofectamine for transfection rather than an inherent difference between MES 13 cells and primary human mesangial cells. Thus, the magnitude of insulin-stimulated collagenase–CAT fusion gene transcription was much greater when MES 13 cells were transiently transfected using the calcium phosphate co-precipitation method than when transfected using lipofectamine (Fig. 8A). An analysis of basal collagenase–CAT fusion gene transcription in MES 13 cells revealed that the use of lipofectamine markedly enhanced basal fusion gene transcription (Fig. 8B). This observation suggested that the reason why insulin fails to stimulate collagenase–CAT fusion gene transcription after lipofectamine-mediated transfection is that transcription of the fusion gene is already fully

activated. Therefore, insulin treatment has little additional effect. By contrast, basal collagenase–CAT fusion gene transcription was relatively low after calcium phosphate-mediated transfection (Fig. 8B) but insulin treatment enhanced expression to a level similar to that obtained following lipofectamine-mediated transfection (Fig. 8C). We therefore hypothesized that insulin-regulated collagenase–luciferase fusion gene expression would be detected in primary human mesangial cells if it were possible to transfect these cells using the calcium phosphate co-precipitation method.

The collagenase AP-1 motif confers a stimulatory effect of insulin on the expression of a heterologous promoter

Although the preceding experiments demonstrated that the AP-1 motif is required for the action of insulin on collagenase fusion gene transcription (Fig. 6), they do not address the issue as to whether the AP-1 motif is a direct target of insulin signaling in MES 13 cells. It is possible that the AP-1 motif just acts as an accessory element to enhance the effects of insulin mediated through another element in the collagenase promoter. To address this question, multiple copies of a double-stranded oligonucleotide representing the collagenase AP-1 motif were ligated into the polylinker of a heterologous vector, designated XMB, that contains a minimal *Xenopus laevis* 68 kDa albumin promoter ligated to the CAT reporter gene (Chapman *et al.* 1999). The resulting construct, designated A(WT) XMB, and the XMB vector were transiently transfected into MES 13 cells (Fig. 9). Neither basal CAT expression nor insulin-induced CAT expression were detected with the XMB vector alone (Fig. 9). In contrast, the multimerized AP-1 motif was able to mediate an induction of fusion gene expression by insulin (Fig. 9). Mutation of the AP-1 motif in this context reduced basal fusion gene expression (Fig. 9B) and abolished the induction of XMB fusion gene

Figure 8 The magnitude of insulin-stimulated collagenase–CAT fusion gene transcription in MES 13 cells is influenced by the transfection method employed. MES 13 cells were either transiently co-transfected using calcium phosphate (Ca-PO₄) co-precipitation or using lipofectamine (Lipofect.), as described in Materials and methods, with a collagenase–CAT fusion gene (2 µg) containing promoter sequence from –518 to +64, and expression vectors encoding βgalactosidase (0.5 µg) and the insulin receptor (0.8 µg). Following transfection, cells were incubated for 18–24 h in serum-free medium in the presence or absence of 100 nM insulin. The cells were then harvested and both CAT and βgalactosidase activity were assayed as described in Materials and methods. In (A) results are presented as the ratio of CAT:βgalactosidase activity in insulin-treated versus control cells and are expressed as fold induction. In (B and C) results are presented as the ratio of CAT:βgalactosidase (βGal.) activity in either control or insulin-treated cells respectively, and are expressed as arbitrary units. Results represent the means ± S.E.M. of three experiments using several independent preparations of the fusion gene construct, assayed in duplicate.

Figure 9 The collagenase AP-1 motif can confer a stimulatory effect of insulin on the expression of a heterologous *Xenopus*–CAT fusion gene. MES 13 cells were transiently co-transfected using calcium phosphate co-precipitation, as described in Materials and methods, with expression vectors encoding β galactosidase (0.5 μ g) and the insulin receptor (0.8 μ g), and XMB vector constructs (2 μ g) in which oligonucleotides representing the wild-type or mutated collagenase AP-1 motif, designated A(WT) and A(MUT) respectively, had been ligated into the HindIII site of the *Xenopus* albumin promoter in multiple (four to five) copies. Following transfection, cells were incubated for 18–24 h in serum-free medium in the presence or absence of 100 nM insulin. The cells were then harvested and both CAT and β galactosidase activity were assayed as described in Materials and methods. In (A) results are presented as the ratio of CAT: β galactosidase activity in insulin-treated versus control cells and are expressed as fold induction. In (B) results are presented as the ratio of CAT: β galactosidase (β Gal.) activity in control cells and are expressed as arbitrary units. Results represent the means $\pm$ S.E.M. of three experiments using several independent preparations of each fusion gene construct, assayed in duplicate.

expression by insulin (Fig. 9A), just as it did in the context of the collagenase–CAT fusion gene (Fig. 6). This result suggested that the collagenase AP-1 motif is a direct target of insulin signaling in MES 13 cells.

Protein binding to the collagenase AP-1 motif

The gel retardation assay was used to analyze the specific binding of MES 13 nuclear extract proteins to the collagenase AP-1 motif (Fig. 10). A single major protein–DNA interaction was detected when a labeled double-stranded oligonucleotide representing collagenase promoter sequence from –78 to –63, which contains the AP-1 motif between –73 and –67, was incubated with MES 13 nuclear extract (Fig. 10; see arrow). Competition experiments, in which a 100-fold molar excess of unlabeled DNA was included with the labeled probe, were used to correlate protein binding with the action of insulin on collagenase gene transcription. A 100-fold molar excess of the unlabeled –78/–63 oligonucleotide competed effectively against the labeled probe for binding of the protein(s) present in the major complex (Fig. 10). By contrast, an oligonucleotide representing the same –78 to –63 collagenase sequence, but with a mutation in the AP-1 core sequence that abolished insulin signaling through this element (Figs 6 and 9), failed to compete with the labeled probe for protein binding, even in a 100-fold molar excess (Fig. 10). This indicated that formation of this major complex represents a specific protein–DNA interaction that correlates with the effect of insulin mediated through this sequence in the context of the collagenase promoter (Fig. 6) and the heterologous XMB vector (Fig. 9).

Although AP-1 motifs can bind members of the Fos, Jun, Maf and activating transcription factor-2 (ATF) transcription factor families (Shaulian & Karin 2002), we have previously shown that, when using HeLa (Streeper *et al.* 1998, Chapman *et al.* 1999) and H4IIE (Ayala *et al.* 2002) nuclear extract in gel retardation assays, the collagenase AP-1 motif primarily bound Fra-2 and Jun D. The effect of antibodies to members of the Fos and Jun family of proteins on the formation of the specific complex detected with the collagenase AP-1 motif when using MES 13 nuclear extract was therefore investigated. As can be seen in Fig. 11, addition of antibodies recognizing either Fra-1 or Jun D and,

Figure 10 A specific protein–DNA complex was detected in gel retardation assays using the collagenase AP-1 motif as the labeled probe. Protein binding to a labeled oligonucleotide probe, representing the WT collagenase AP-1 motif (–78 AAGCATGAGTCAGACA –63; AP-1 motif underlined), was analyzed using MES 13 cell nuclear extracts and the gel retardation assay, as described in Materials and methods. The labeled probe were incubated in the absence (–) or presence of a 100-fold molar excess of unlabeled oligonucleotides representing either the WT or mutated (MUT; –78 AAGCAactaTCAGACA –63; mutated bases in lower case letters) collagenase AP-1 sequence prior to the addition of nuclear extract. A representative autoradiograph is shown.

to a lesser extent, Fra-2 and Jun B, all resulted in a selective retardation in the migration of the major complex, a so-called supershift. Since Fos proteins do not homodimerize (Angel & Karin 1991, Shaulian & Karin 2002), this suggested that the major complex binding the collagenase AP-1 motif in MES 13 cells is a heterodimer of Fra-1 and Jun D. There was no apparent change in the abundance or in the nature of the factors present in this complex when nuclear extracts were prepared from MES 13 cells that had been incubated for 5 h in the presence of insulin (Fig. 11).

Figure 11 Fra-1 and Jun D are the predominant AP-1 factors binding to the collagenase AP-1 motif in MES 13 nuclear extract. MES 13 cells were transiently transfected using calcium phosphate co-precipitation, as described in Materials and methods, with an expression vector encoding the insulin receptor (0.8 µg). Nuclear extracts were then prepared from MES 13 cells incubated for 5 h in serum-free media (C) or serum-free media supplemented with 100 nM insulin (I). Nuclear extract (2 µg) was preincubated in the absence of antibody (None) or in the presence of 1 µg of the indicated antisera for 10 min at room temperature, prior to the addition of a labeled oligonucleotide probe, representing the collagenase promoter sequence between –78 and –63, and binding buffer and incubation for an additional 10 min at room temperature. Protein binding was then analyzed using the gel retardation assay as described in Material and methods. In the representative autoradiograph shown, only the retarded complexes are visible and not the free probe, which was present in excess.

A MEK-1 and -2 inhibitor blocks the stimulation of collagenase gene transcription by insulin in MES 13 cells

Since insulin treatment did not seem to increase the mass of protein bound to the AP-1 motif, this suggested that the stimulatory effect of insulin on collagenase gene transcription in MES 13 cells may be mediated through post-translational modification of some or all of the proteins binding to the AP-1 motif. Rutter *et al.* (1995) have previously shown that, in CHO.T cells, overexpression of either a dominant negative form of MEK-1, the dual specificity protein kinase that phosphorylates and activates ERK1 and ERK2, or CL100, a MAP

Figure 12 MAP kinase activation is required for the stimulatory effect of insulin on collagenase–CAT fusion gene transcription in MES 13 cells. MES 13 cells were transiently co-transfected using calcium phosphate co-precipitation, as described in Materials and methods, with expression vectors encoding β galactosidase (0.5 μ g) and the insulin receptor (0.8 μ g), and with a collagenase–CAT fusion gene (2 μ g) containing either promoter sequence from –518 to +64 or from –158 to +64. Following transfection, cells were incubated for 18–24 h in serum-free media, in the presence or absence of 100 nM insulin and in the presence or absence of 10 μ M of the MEK-1 and -2 inhibitor U0126 as indicated. The cells were then harvested and both CAT and β galactosidase activities were assayed as described in Materials and methods. Results are presented as the ratio of CAT: β galactosidase activity in insulin-treated versus control cells, in the presence or absence of U0126, and are expressed as fold induction. Results represent the means $\pm$ S.E.M. of three experiments using several independent preparations of each fusion gene construct, in which each construct was assayed in duplicate.

kinase phosphatase, inhibits the effect of insulin on collagenase fusion gene transcription. To determine whether activation of the MAP kinase pathway is necessary for stimulation of collagenase–CAT fusion gene transcription by insulin in MES 13 cells, we adopted an alternative approach and investigated the effect of U0126, a specific inhibitor of the dual specificity kinases MEK-1 and MEK-2 (Favata *et al.* 1998) (Fig. 12).

In vitro, U0126 inhibits MEK-1 with an IC_{50} of 0.07 μ M and MEK-2 with an IC_{50} of 0.06 μ M (Duncia *et al.* 1998). However, *in situ*, U0126 undergoes isomerization and cyclization reactions that form various products, all of which exhibit less affinity for MEK (Duncia *et al.* 1998). Thus, U0126 maximally inhibits phorbol ester-stimulated, AP-1-

dependent fusion gene expression in COS-7 cells *in situ* at ~ 20 μ M (Favata *et al.* 1998). Similarly, U0126 maximally inhibits lysophosphatidic acid stimulation of hexokinase II activity in MES 13 cells *in situ* at ~ 10 μ M with an IC_{50} of 0.4 μ M (Coy *et al.* 2002). MES 13 cells were transiently transfected with either the –518/+64 or the –158/+64 collagenase–CAT fusion gene constructs and the effect of insulin on CAT expression was assayed in cells incubated in the presence or absence of 10 μ M U0126 (Fig. 12). As seen in Fig. 12, the robust insulin-stimulated expression of CAT directed by both the –518/+64 and the –158/+64 fusion gene constructs was completely blocked in the presence of U0126. These results suggested that activation of the MAP kinase pathway is necessary for insulin-stimulated collagenase–CAT fusion gene transcription in MES 13 cells.

Discussion

The studies presented here sought to analyze the regulation of collagenase fusion gene transcription by insulin in the murine mesangial-derived MES 13 cell line. We have shown that insulin markedly stimulates collagenase fusion gene transcription in MES 13 cells (Fig. 1) and this effect requires an AP-1 motif in the collagenase promoter (Fig. 6), as it does in HeLa cells (Chapman *et al.* 1999). Some of the factors binding the AP-1 motif in MES 13 cells (Fig. 11) are distinct from those that bind the AP-1 motif in HeLa cells (Streeter *et al.* 1998, Chapman *et al.* 1999), though in both cases the action of insulin requires activation of the MAP kinase pathway (Fig. 12 and data not shown). Which of these AP-1-bound factors actually mediates the effect of insulin in MES 13 cells remains to be determined. Although this AP-1 motif can mediate an effect of insulin on heterologous fusion gene expression in MES 13 cells (Fig. 9), multiple other promoter elements are required for maximal insulin-stimulated collagenase fusion gene transcription and the AP-1 motif is unable to mediate an insulin response in their absence (Fig. 5). Since mutation of the AP-1 motif in the context of the otherwise intact collagenase promoter abolishes insulin-stimulated collagenase fusion gene transcription (Fig. 6), this suggested that these other promoter elements bind

accessory factors that enhance insulin signaling through the AP-1 motif. Such an arrangement, referred to as a hormone response unit, is common with respect to cAMP- and glucocorticoid-regulated gene transcription (Lucas & Granner 1992, Roesler & Park 1998). Although multiple other promoter elements are also required for maximal insulin-stimulated collagenase fusion gene transcription in HeLa cells (Chapman *et al.* 1999), the accessory element(s) located between -518 and -158 (Fig. 5) is uniquely important in MES 13 cells. Analysis of this promoter region using the MatInspector software (Quandt *et al.* 1995) identified multiple potential *cis*-acting elements. These include binding sites for chicken ovalbumin upstream promoter-transcription factor and hepatocyte nuclear factor-1. Both of these factors are expressed in the kidney (Baumhueter *et al.* 1990, Suzuki *et al.* 2000) and both have been previously shown to physically interact with members of the AP-1 transcription factor family (Leu *et al.* 2001, Lin *et al.* 2002).

The Diabetes Control and Complications Trial demonstrated that tight glycemic control markedly reduced the development of complications associated with type I diabetes, including diabetic nephropathy (Crofford 1995). Tight control was achieved through an increased frequency of insulin administration (Crofford 1995). Since the subjects undergoing intensive insulin therapy and standard insulin therapy both received a similar total amount of insulin (Crofford 1995), whereas hyperglycemia and complications were reduced in the intensive insulin therapy group, it might appear that hyperglycemia is the primary risk factor for the development of diabetic complications. However, although both groups received a similar total amount of insulin it is apparent, from the improved glycemic control, that insulin was clearly more efficacious in the intensive insulin therapy group. As such, it is not possible to exclude a role for reduced insulin action in the development of diabetic complications. *In situ* studies clearly demonstrate that high glucose levels do decrease MMP gene expression in cultured mesangial cells (Lenz *et al.* 2000). Moreover, the mechanisms by which hyperglycemia leads to decreased mesangial MMP gene expression have been intensively investigated (for reviews see Sheetz & King 2002, Mason & Wahab 2003). However, based on the results presented here, we hypothesize that, in type

I diabetes, reduced insulin action in mesangial cells may also directly contribute to the decreased collagenase gene expression characteristic of diabetic glomerulosclerosis. This hypothesis may have a broader significance since AP-1 motifs are important for the expression of the genes encoding several matrix metalloproteinases other than collagenase (Vincenti 2001). These include both MMP-2 and MMP-9 whose glomerula expression, like that of collagenase, is also decreased in type I diabetes (Nakamura *et al.* 1994, McLennan *et al.* 2002). Although the synthesis of MMP-2 was widely considered, in contrast to collagenase and MMP-9, to be independent of AP-1 (Benbow & Brinckerhoff 1997, Westermarck & Kahari 1999), it has recently been shown that MMP-2 is regulated through a non-consensus AP-1 motif that binds members of the AP-1 transcription factor family (Bergman *et al.* 2003). Interestingly, if insulin does directly regulate collagenase gene expression in mesangial cells *in vivo* then collagenase expression would actually be predicted to be increased in individuals who are hyperinsulinemic as a consequence of insulin resistance, since the insulin resistance associated with muscle, adipose tissue and liver may not be manifest in the kidney (Reaven 1997). Moreover, even in tissues where insulin resistance develops, it appears that insulin signaling through the PI3-kinase pathway is differentially affected (Cusi *et al.* 2000) rather than the MAP kinase pathway through which insulin regulates collagenase gene expression (Fig. 12).

In summary, the studies presented here have demonstrated that insulin stimulates collagenase fusion gene transcription in mesangial-derived MES 13 cells through an AP-1 motif. Future studies will investigate whether insulin also regulates endogenous collagenase gene expression in primary mesangial cells. It will be of particular interest to use these cells to compare the regulation of MMP gene expression by insulin and IGF-I. IGF-I has been reported to inhibit, rather than stimulate, MMP gene expression in primary mesangial cells (Lupia *et al.* 1999). Thus, it is possible that the regulation of MMP gene expression in mesangial cells represents one of the rare examples of the differential regulation of gene expression by insulin and IGF-I that exist despite the similarities in their signaling pathways (Siddle *et al.* 2001, Kim & Accili 2002).

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