A Forkhead/Winged Helix-related Transcription Factor Mediates Insulin-increased Plasminogen Activator Inhibitor-1 Gene Transcription*

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Plasminogen activator inhibitor-1 (PAI-1) is an important regulator of fibrinolysis by its inhibition of both tissue-type and urokinase plasminogen activators. PAI-1 levels are elevated in type II diabetes and this elevation correlates with macro- and microvascular complications of diabetes. Insulin increases PAI-1 production in several experimental systems, but the mechanism of insulin-activated PAI-1 transcription remains to be determined. Deletion analysis of the PAI-1 promoter revealed that the insulin response element is between −117 and −7. Mutation of the AT-rich site at −52/−45 abolished the insulin responsiveness of the PAI-1 promoter. This sequence is similar to the inhibitory sequence found in the phosphoenolpyruvate carboxykinase/insulin-like growth factor-I-binding protein I promoters. Gel-mobility shift assays demonstrated that the forkhead bound to the PAI-1 promoter insulin response element. Expression of the DNA-binding domain of FKHR acted as a dominant negative to block insulin-increased PAI-1-CAT expression. A LexA-FKHR construct was also insulin responsive. These data suggested that a member of the Forkhead/winged helix family of transcription factors mediated the effect of insulin on PAI-1 transcription. Inhibition of phosphatidylinositol 3-kinase reduced the effect of insulin on PAI-1 gene expression, a result consistent with activation through FKHR. However, it was likely that a different member of the FKHR family (not FKHR) mediated this effect since FKHR was present in both insulin-responsive and non-responsive cell lines.

PAI-1 is a major regulator of fibrinolysis. It inhibits both tissue-type and urokinase plasminogen activators and serves an essential role in wound healing where it is required to maintain the fibrin clot. Abnormal expression of PAI-1 is observed in obesity (1), inflammation (2), and diabetes (3) and increased PAI-1 has been correlated to the higher risk of cardiovascular disease seen in these syndromes (4).

PAI-1 was found to be expressed in virtually all of the tissue types studied. These included pancreas (5), liver (6), spleen (6), kidney (7), brain (8), adipocytes (1), synovial membranes (9), platelets monocytes and other blood cells (6), heart (6), and the smooth muscle and endothelial cells of the vasculature (1). Numerous studies suggest that PAI-1 is elevated due to stress or injury in these tissues (10–14). This could be secondary to an increase in cytokines or growth factors in these areas.

PAI-1 transcription was increased by numerous factors including platelet-derived growth factor (15), β-fibroblast growth factor (15), interleukin-1α, transforming growth factor β (16), angiotensin II (17), tumor necrosis factor-α (18), thrombin (19), and oxidation products (20), while interferon-γ (21) inhibited PAI-1 production. Several specific response elements were defined in the PAI-1 promoter. A paired Sp1 element at −73 and −42 mediated responses to glucose and angiotensin II (22, 23).

A domain at −59/−52 was reported to mediate the response of the PAI-1 promoter to D dimer, a proteolytic fragment of fibrin (24), and also to mediate effects from PKC and PKA (25, 26). The response element for transforming growth factor β was sought by a number of groups with conflicting results. One group found an element at −732/−721 that was transforming growth factor β responsive when 6 copies were cloned in front of a heterologous promoter (27). Another group found that duplicate E box sequences between −740/−528 mediated the response to transforming growth factor β (28). A glucocorticoid response element was identified at −1212 (29).

Two hypoxia response elements were identified at −175/−158 whose mutation eliminated the 3-fold response to hypoxia (30) and an E box at −165/−160 was shown to bind USF-1 and increase basal transcription of the PAI-1 promoter (31).

Insulin increases PAI-1 mRNA under a number of conditions. PAI-1 is increased in patients with type 2 diabetes (32). Insulin or proinsulin infusion can cause local elevation of PAI-1 detected by analysis of PAI-1 protein levels (33–35) or by in situ hybridization (36). Insulin also increases expression of the endogenous PAI-1 gene in HepG2 cells (37) and the transcription of a luciferase reporter plasmid under control of the PAI-1 promoter in human umbilical vein endothelial cells (HUVEC) in culture (38, 39). The insulin response element was suggested to be in the region −98/−62, but this was never confirmed by mutational analysis of the PAI-1 promoter (40). Thus, the location of the insulin response element of the PAI-1 promoter has not been well defined.

A number of potential transcription factor-binding sites in the proximal PAI-1 promoter were mutated to determine which of these mediated the insulin response. Mutation of a sequence resembling the negative insulin response elements found in the
phosphoenolpyruvate carboxykinase/IGF-I-binding protein promoters eliminated insulin activation of PAI-1. Gel mobility shifts demonstrated that GST fusion proteins with the Forkhead DNA-binding domain bound to this element, but did not bind to a non-functional mutant of this sequence. Expression of the DNA-binding domain of Forkhead acted as a dominant negative inhibitor of insulin-increased PAI-1 gene expression. Insulin-increased expression of a LexA-CAT reporter in cells expressing a LexA-Forkhead fusion protein. Finally, PI 3-kinase inhibition abrogated insulin-increased PAI-1 gene transcription. This is consistent with activation through a Forkhead-related protein and with data on insulin activation of PAI-1 (40).

EXPERIMENTAL PROCEDURES

Materials—[32P]dGTP, 3000 Ci/mmol, and [32P]ATP, 3000 Ci/mmol, were obtained from ICN Biochemicals Corp. All enzymes and linkers were obtained from either New England Biolabs or Roche Molecular Biochemicals and, unless otherwise indicated, were used under conditions recommended by the suppliers. Oligonucleotides were synthesized by Operon and reagents for PCR were obtained from Roche. Dulbecco’s modified Eagle’s medium containing 4.5 g/liter glucose and iron-supplemented calf serum were obtained from GIBCO-BRL Laboratories. Wortmannin (1000 mg/ml from Sigma and 100 mg/ml from Calbiochem) was used at concentrations ranging from 1 to 100 nM. All other reagents were of the highest purity available and were obtained from Sigma, Behring Diagnostics, Bio-Rad, Eastman, Fisher, or Roche Molecular Biochemicals.

Cell Culture—GH4 pituitary tumor cells, Rat2 cells, and HeLa cells were maintained in Dulbecco’s modified Eagle’s medium with 10% iron-supplemented calf serum. HepG2 cells and CHO cells were maintained in Ham’s F-12 medium with 5% iron-supplemented calf serum and 5% fetal calf serum. HUVEC cells generally provided by Dr. R. Levin (NYU School of Medicine) were maintained in medium 199 with 20% fetal calf serum. 3T3-L1 cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. 3T3-L1 cells were differentiated using insulin, dexamethasone, and methylisobutylxanthine as described (41).

Plasmids—The PAI-1 promoter reporter plasmid, p800neo-Luc, was the generous gift of Dr. D. Rifkin (NYU School of Medicine) (42). Chloramphenicol acetyltransferase (CAT) reporter plasmids were constructed from p800neo-Luc by polymerase chain reaction (PCR) as previously described (43). Deletion mutants of this plasmid were made by PCR using mutant primers as described (44). DNA sequencing confirmed the accuracy of all mutant sequences. The C terminus of FKHR was cloned by PCR from a Marathon cDNA library using nested primers and inserted into pcDNA-LexA to make LexA-FKHR (aamin acids 261–652). Dr. Kun-Ling Guan (University of Michigan Medical School) generously provided a FLAG-tagged full-length FKHR (in pcDNA3-FLAG) (45). This was used as a template for PCR of the FKHR DNA-binding domain that was then cloned into pGEX-kg to make a GST-FKHRdbd expression plasmid. The DNA-binding domain of this plasmid was then cut with BamHI and cloned into pCDNA3 to make pCDNA3-FKHRdbd. The coding sequence of the truncated c-fos expression plasmid RSV-RafC4 (Ulf Rapp) was copied by PCR and cloned into pCDNA3/V5HisB (Invitrogen, Carlsbad, CA). This plasmid expressed the truncated c-fos in frame with the V5 epitope tag. The plasmid was verified by sequencing and produced the correct size protein on Western blotting. The HA-tagged PTEN was from Dr. J. Schlessinger (Yale, New Haven, CT). The wild type and mutant plasmids expressing HA-Akt were from Dr. T. Franke (Columbia, New York, NY). GFP-tagged wild type and N17 Ras were from Dr. M. Philips (NYU, New York). The human insulin expression vector, p3THH22, was the gift of Dr. J. Whittaker (Hagedorn Institute, Copenhagen, Denmark). Levels of expressed proteins were determined by Western blotting to the specific protein or to epitope tags.

Transient Gene Transfection FACilitated by Electroporation—Electroporation experiments and CAT assays were performed as described (46). Each electroporation used 20 to 40 × 10⁶ GH4 cells to 10 μl DNA. Trypsinization before electroporation was performed by addition of 0.1% trypsin. The voltage of the electroporation was 1550 V. This gives trypsin blue exclusion of 70 to 80% after electroporation. The transfected cells were then plated in 6-spot multiwell dishes (Falcon Plastics) in Dulbecco’s modified Eagle’s medium with 10% hormone-depleted serum. Cells were refed at 24 h with Dulbecco’s modified Eagle’s medium with 10% hormone-depleted serum with or without insulin (1 μg/ml bovine insulin, Calbiochem). After 48 h, the wells were washed three times with normal saline and frozen. CAT activity was assayed essentially as described previously (47) except that [14C]chlordemphenicol was replaced with BODIPY chlorpromazine (Molecular Probes, Eugene, OR) and fluorescence intensity was measured using a Fluoromager 575 (Molecular Dynamics, Sunnyvale, CA) with ImageQuant software.

Control of transfection efficiency was performed using a Rous sarcoma virus-β-galactosidase expression plasmid. Briefly, 2 μg of Rous sarcoma virus-β-galactosidase expression plasmid was included in the experiments. The β-galactosidase activity in the cell lysates was determined using o-nitrophenyl-β-D-galactosyranoside. Transfection efficiency did not vary significantly among transfections performed at the same time. The color reaction was then corrected for minor variations in β-galactosidase activity by converting the % acetylation to % acetylation/β-galactosidase activity/mg of protein. The fold stimulation or inhibition was determined. Statistical analysis was performed on all experiments and p values are presented for relevant comparisons.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) of FKHR—mRNA was prepared using a filter binding protocol (Treven). The amount of mRNA was estimated from the absorbance at 260/280 nm. The ratio of the optical density at 260 nm to the optical density at 280 nm was generally 2 or greater. Approximately equal amounts of mRNA (~0.1 μg) were used with primers for the mRNA of glyceraldehyde-3-phosphate dehydrogenase in a single tube RT-PCR assay (Tetra Link, Ambion, NY). The RT-PCR product was quantitated using a DNA Fragment Mapper 575 and ImageQuant software (Amersham Biosciences). Equal amounts of mRNA (based on GAPDH signal) were then used for assay of FKHR mRNA. FKHR primers for RT-PCR were 5’-CGCTGT- CAGCACGCGTTATG-3’ (sense) and 5’-GAAAACCTGAGACCAGGGG- CTGTCGACGGGAC-3’ (antisense). Nested primers for PCR of the RT-PCR product were 5’-CCTCGCGGAGACCGCAGGACAGG-3’ (sense) and 5’-tacctcctcgagctgcatccc-3’ (antisense). The products were sequenced to verify that they were FKHR.

Assay of DNA-Protein Binding by Gel Electrophoresis—An oligonucleotide containing three iterations of the insulin response element of the PAI-1 promoter was prepared, annealed, purified on polyacrylamide-gels, and end labeled with [32P]dGTP using the Klenow fill-in reaction. The sequence of this oligonucleotide is 5’-AATCTATATTTCG- TCCGCTATTCTCCTGCTATTCTCCTGCC-3’. A mutant oligonucleotide with the sequence 5’-AATTCAGAGACCTACCTTG CTCAGCAGACGCTAGCCTTCAGCAGCTAGCCC-3’ was also prepared (mutated region is underlined). This mutation abolishes insulin responsiveness of the PAI-1 promoter. Labeled PAI-1-S-flanking DNA was then used in mobility shift experiments with unlabeled GST proteins as described (46). Two μg of GST-FKHRdbd or 2 μg of GST were incubated at 25 °C for 30 min with 30,000 cpm (10 to 20 fmol) of [32P]-labeled PAI. The protein-DNA complexes were then analyzed by electrophoresis on a 6% polyacrylamide gel in 25 mM Tris (Tris base), 25 mM boric acid, and 1 mM EDTA.

Western Immunoblot Analysis—GH4 cells were harvested in a lysis buffer consisting of 50 mM HEPES, pH 7.5, 1% Triton X-100, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EDTA. Cells were incubated at 25 °C for 30 min with 30,000 cpm (10 to 20 fmol) of [32P]-labeled PAI. The protein-DNA complexes were then analyzed by electrophoresis on a 6% polyacrylamide gel in 25 mM Tris (Tris base), 25 mM boric acid, and 1 mM EDTA.

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RESULTS

PAI-Luc Expression in Different Cell Types—It was necessary to find a tissue culture system that supported high levels of PAI-1 reporter expression to determine the insulin response element of the PAI-1 promoter. It was impossible to predict which cell type might express the highest levels of PAI-1 since PAI-1 is expressed in a wide variety of tissues (see above). Fig. 1 shows results of a study where the expression of p800PAI-1-luc was compared in HepG2 and 3T3 cells that were previously determined to express PAI-1 and GH4, HeLa, CHO, Rat2, and
The plates were harvested 48 h after electroporation by washing 3 times with normal saline and freezing. The average relative light units/10^6 cells was determined, adjusted for CAT expression, and the relative light units from cells incubated with hormones were compared with control levels to determine the fold-stimulation (Fold-Control). The results are from three separate experiments done in duplicate.

Deletion Analysis of the PAI-1 Promoter—Deletion mutants of the PAI-1 promoter were made to locate the insulin response element (Fig. 2). Deletion from −800 to −117 and deletion from −7 to +60 did not significantly alter insulin-increased PAI-1-CAT expression. However, deletion of the region between −117 to −52 reduced both basal (data not shown) and insulin-stimulated PAI-1-CAT expression significantly and deletion to −46 completely eliminated insulin-increased PAI-1-CAT expression. This suggested that at least part of the insulin response element was located between −117 and −46.

Mutation Analysis of the PAI-1 Promoter −117/−45—An examination of the PAI-1 promoter revealed a number of potential transcription factor-binding sites in proximal promoter between −117 and −7 (fig. 3). We made mutations to all of these potential elements in the PAI-1 promoter. The 2 distal Ets-related elements and the distal Sp1 element were mutated in the reporter plasmid PAI−247/−5260D Mut/CAT while the 3 proximal Ets-related elements and the proximal Sp1 site were mutated in the plasmid PAI−247/−52P mut/CAT. Neither of these mutations reduced insulin stimulation of PAI-1-CAT expression (Fig. 4). All of the potential Ets sites and both Sp1 sites were mutated in the reporter PAI−247/−60/P&D Mut) CAT. Insulin-increased PAI transcription was not affected using this mutation. The AP-1 site was mutated in the plasmid PAI−1(DAP-1)CAT. This mutation was likewise without effect. However, mutation of the PEPECK/IGF-1-binding protein-2-related sequence (Fig. 4, PAI−1−52−43Mut/CAT) completely eliminated insulin-increased PAI-1-CAT expression while it did not affect basal levels of PAI-1-CAT expression significantly (data not shown).

FKHR Binds to the PAI-1 Promoter—Previous studies demonstrated that FKHR could bind to the insulin response element of PEPECK/IGF-1-binding protein-1. Those studies used 3 copies of the insulin response element to demonstrate binding of GST-FKHR (45). An oligonucleotide containing 3 copies of the −56/−40 sequence from the PAI-1 promoter was used to determine whether FKHR could bind to the insulin response element of the PAI-1 promoter. A GST-FKHR fusion protein containing the DNA-binding domain of FKHR was incubated with a 32P-labeled oligonucleotide containing the PAI-1 insulin response element (Fig. 5, lane 2) or with competing unlabeled oligonucleotides (lanes 3–10). Lanes 3–6 contained increasing amounts of an oligonucleotide with a mutation of the insulin response element while lanes 7–10 contained increasing amounts of the wild type oligonucleotide. GST, which does not bind to the 32P-labeled oligonucleotide, served as a control (Fig. 5, lane 1). One band due to the association of the GST-FKHRdbd with the oligonucleotide is seen (right arrow). This interaction was not inhibited by any concentration of the mutant oligonucleotide while the lowest concentration of wild type oligonucleotide completely inhibited binding. Thus, FKHR binds to the insulin response element of the PAI-1 promoter.

Inhibition of Insulin-increased PAI-CAT Expression by Expression of the FKHR DNA-binding Domain—It was possible that overexpression of the FKHR DNA-binding domain might block insulin signaling since FKHR bound to the PAI-1 promoter IRE and the DNA-binding domain of the FKHR family is highly conserved. This was shown for the Ets family of transcription factors where expression of the conserved DNA-binding domain of Ets2 blocked the action of numerous Ets-related transcription factors (49). It was not necessary to determine the specific Ets-related transcription factors that activated a particular promoter. GH4 cells were transfected with the PAI-1/CAT reporter plasmid and with increasing amounts of an expression vector for FKHR DNA-binding domain. Expression of the FKHR dbd inhibited insulin-increased PAI-1-CAT expression in a dose-dependent manner while expression of the dbd of Ets-2 was without effect (Fig. 6). This implied that a FKHR-related factor mediated the effects of insulin on the PAI-1 promoter.

The FKHR C Terminus Is an Insulin Responsive Activation Domain—A vector was prepared to express a fusion protein consisting of the LexA DNA-binding domain and the C-terminal amino acids of FKHR (amino acids 211–652) to test whether insulin could directly activate FKHR. Expression of a
LexA-6X-CAT reporter plasmid was low in GH4 cells when only the LexA DNA-binding domain was expressed. Expression of LexA-FKHR-(261–652) resulted in a large increase in basal expression of the reporter plasmid. Incubation of these cells with insulin further increased the expression of CAT. A 6-fold increase in LexA-6X-CAT activity is seen in response to insulin in GH4 cells transfected with this LexA-FKHR-(261–652) and incubated with insulin for 24 h (Fig. 7). This demonstrated that the Ζ terminus of FKHR contained an insulin responsive transactivation domain and this is consistent with the stimulation of PAI-1-CAT by insulin. A LexA-Elk expression plasmid (amino acids 105–428 of Elk-1) that was used as a control did not increase basal expression of LexA-6X-CAT, but did produce a >10-fold increase in CAT expression in response to insulin. This agreed with previous findings (50). Thus, LexA-FKHR-(261–652) reacted differently from the response observed previously with LexA-Elk1-(105–428).

FKHR mRNA Expression in Various Cell Types—The insulin responsive transcription factor should be present in the cell types in which the PAI-1 promoter is insulin regulated, but not in non-responsive cell types. An RT-PCR approach was used to detect the mRNA for FKHR in the cells that were used for Fig. 1. These RT-PCR studies (Fig. 8) demonstrated that FKHR was expressed in all of the cell types that were examined in Fig. 1.
FIG. 6. The C terminus of FKHR is an insulin-activated transcriptional enhancer. GH4 cells were electroporated with 10 μg of a CAT reporter plasmid containing 6 copies of the LexA response element (LexA6X-CAT), 5 μg of pRT3HIR2, 2 μg of RSV-βGal. Each electroporation also contained 10 μg of an expression vector for the LexA DNA-binding domain, LexA-Erk (amino acids 256–652). After 24 h, the medium was exchanged and 1 μg/ml insulin was added to the appropriate cultures. The plates were harvested 48 h after electroporation by washing 3 times with normal saline and freezing. The average % acetylation/10 μg of protein in control and insulin- or EGF-treated cultures was determined, adjusted for β-galactosidase expression, and the CAT activity from cells incubated with hormones were compared with control levels to determine the fold-stimulation (Fold-Control). The results are from three separate experiments done in duplicate.

This suggested that FKHR could be the insulin-sensitive transcription factor, but if so, required other factors to act since both insulin-sensitive and insensitive cell types expressed FKHR mRNA.

Insulin-increased PAI-1 CAT Expression Is Dependent on Ras-Raf-PI 3-Kinase—Our studies of the prolactin promoter demonstrated that insulin signaled through Ras-PI 3-kinase to activate the transcription factor Elk-1 (50, 51). We sought to determine whether insulin activation of the PAI-1 and the prolactin promoters involved the same insulin signaling intermediates. Insulin-increased prolactin-CAT expression was inhibited by expression of a dominant negative Raf (Raf-C4) and is also inhibited by the PI 3-kinase inhibitor wortmannin, but not by the MAP kinase inhibitor PD98059 or the FRAP/mTOR inhibitor rapamycin (51). Insulin-increased PAI-1-CAT expression exhibits similar responses. Dominant negative Ras N17 and dominant negative Raf-C4 both inhibit insulin-increased PAI-1-CAT expression 50–70% (Fig. 9A). An expression vector for the PI 3-phosphatase PTEN also inhibits insulin-increased PAI-1-CAT expression to the same degree (Fig. 9A). These studies imply that insulin signals through Grb2-SOS to Ras and then to PI 3-kinase to increase PAI-1-CAT expression. These results are analogous to those obtained with the prolactin promoter (51). Inhibitor studies support these experiments (Fig. 9B). The PI 3-kinase inhibitors LY294002 and wortmannin blocked insulin-increased PAI-1-CAT expression while the Map kinase inhibitor PD98059, the p38 Map kinase inhibitor SB20358, and the inhibitor of FRAP/mTOR/p70S6K rapamycin are without effect. Expression of a dominant negative MKK4 (blocks phosphorylation of c-Jun N-terminal kinase) also has no effect on PAI-1-CAT expression (data not shown). Thus, these results were consistent with the conclusion that insulin signals through Grb2-SOS to Ras and then to PI 3-kinase to activate PAI-1 gene expression.

PI phosphorylation at the 3’ position provides binding sites for molecules containing PH domains. The docking of protein kinase B/Akt to the membrane results in its activation by PDK-1 that is also recruited to the membrane in response to elevated inositol 3,4,5-trisphosphate and protein kinase B/Akt-phosphorylated FKHR (52, 53). Protein kinase B/Akt might be the kinase that phosphorylated the insulin-responsive transcription factor that activated PAI-1 gene expression. However, the expression of several protein kinase B/Akt mutants that had previously acted as dominant negatives did not block insulin-increased PAI-1-CAT expression (Fig. 9C) despite efficient expression of the mutant proteins in GH4 cells (Fig. 9D).

DISCUSSION
Deletions and mutations of the PAI-1 promoter have identified the sequence −52/−43 as an insulin response element. Deletions from the 5’ direction retained insulin responsiveness until −52 was reached. This is at the 3’ end of the AP-1 site within the PAI-1 promoter, suggesting that the AP-1 element is
not the primary insulin response element. Deletion from the 3' direction to −7 also retained insulin responsiveness. Mutations of the Ets-related elements, of the AP-1 site, or of the Sp-1 sites of the PAI-1 promoter did not affect insulin signaling. Only mutation of the sequence 52/43 (TCTATTTCCT) eliminated insulin-increased PAI-1-Cat expression. Thus this sequence constitutes the primary insulin response element.

The insulin response element of the PAI-1 promoter resembles that found in genes negatively regulated by insulin such as phosphoenolpyruvate carboxylkinase (TGGTGTTTTGAC) (54) or IGF-I binding protein 1 (AACCTATTTTGAA) (55). This was surprising because all of the positive response elements that we had defined previously had been Ets-related-binding sites and Ets-related sites were present in the PAI-1 promoter (56).

However, specific mutation of these sites demonstrated that they were not the insulin response element of the PAI-1 promoter. It is possible that the context in which the Ets sites are located is important for their being able to function as insulin response elements. The genes in which we have definitively shown that the Ets site constitutes the insulin response element all have important CAAT/enhancer-binding protein sites. The PAI-1 promoter is without such a site and instead has Sp1 and API sites in the promoter. Alternately, the Sp-1 binding may interfere with Ets factor binding.

**Fig. 9.** Insulin-increased PAI-1-CAT requires Ras and PI 3-kinase. GH4 cells were electroporated with 10 μg of the PAI-1-CAT, 5 μg of pRT3HIR2, and 2 μg of RSV-βGal. After 24 h, the medium was exchanged and 1 μg/ml insulin was added to the appropriate cultures. The plates were harvested 48 h after electroporation. The average % acetylation/10 μg of protein in control and insulin- or EGF-treated cultures was determined, adjusted for β-galactosidase expression, and the CAT activity from cells incubated with hormones was compared with control levels to determine the fold-stimulation (Fold-Control). The results are from three separate experiments done in duplicate. A, the electroporations also included 10 μg of a dominant negative expression plasmid or a vector control as indicated in the figure. B, inhibitors, as indicated, were added when the medium was changed 24 h after electroporation. After 2 h with inhibitors, 1 μg/ml insulin was added to the appropriate cultures and the incubation was continued an additional 24 h. C, the electroporations also included 10 μg of an expression plasmid for wild type or mutants of protein kinase B/Akt or a vector control as indicated in the figure. D, Western blot analysis of the expression of various dominant negative plasmids used in panels A and C. Wild type and mutant Akt and wild type PTEN expression was determined using antibody to the HA tag. The expression of the truncated c-Raf, RafC4, was determined using antibody to the V5 epitope while expression of wild type and N17 Ras was determined with an antibody to green fluorescent protein.
Sp-1- and AP-1-binding sites are present in the PAI-1 proximal promoter. The Sp-1 sites are at −72/−67 and −45/−40 while the AP-1 site is at −61/−54. The AP-1 site was reported to mediate the effects of both cAMP and phorbol esters on PAI-1 transactivation (26). This site was not important for insulin responses since deletion to −52 had only a slight effect on the insulin response and specific mutation of this sequence in the context of the PAI-1 promoter −245/+72 did not decrease insulin-activated transactivation of the PAI-1 promoter. However, it is possible that phorbol esters and cAMP may modulate insulin action through effects mediated by this sequence. We have not yet examined these potential interactions that could be important for explaining how insulin and inflammatory responses combine to effect PAI-1 gene transcription.

Several experiments suggested that a winged helix/Forkhead-related transcription factor mediated the effects of insulin on the PAI-1 promoter. First, FKHR bound to the PAI-1 promoter (Fig. 5). Expression of the FKHR dbd (Fig. 6) eliminated insulin-increased PAI-1-CAT activity. Finally, a LexA construct containing the C terminus of FKHR was insulin responsive (Fig. 7). However, studies by others (53, 57, 58) demonstrated that FKHR phosphorylation in response to insulin caused its exclusion from the nucleus. This prevented FKHR from activating transcription and suggested that FKHR was not itself responsible for insulin-increased PAI-1 transcription. The identification of FKHR mRNA in insulin responsive, as well as non-responsive, cell types (Fig. 8) supported this. Thus, it seemed likely that some other member of the Forkhead family mediated insulin-increased PAI-1 gene expression. The FKHR-related family of winged helix transcription factors is large, containing more than 100 members to date (59). This group contains both cell type-specific as well as ubiquitous transcription factors. The insulin-responsive factor responsible for activating PAI-1 expression could be one of these or a novel factor containing the winged helix DNA-binding domain.

The insulin responsiveness of the LexA-FKHR construct indicated that this was plausible. LexA-CAT activity was increased 6-fold by insulin in GH4 cells transfected with LexA-FKHR. This was significantly less than the responses seen with LexA-Elk and LexA-Sap under similar conditions, but it is comparable with insulin-increased PAI-1-CAT expression. The LexA-FKHR construct used here did not include serine 253 that was phosphorylated in response to insulin in a PI 3-kinase/Akt-dependent manner (53). Phosphorylation of this serine led to nuclear export of FKHR and inhibition of the activity of the FKHR activated genes. The behavior of the LexA-FKHR fusion protein has important implications for understanding insulin-activated PAI-1 transcription. First, it shows that FKHR and presumably the FKHR related factor that mediates the insulin response can be activated by insulin. This is contrary to previous reports (see below). It also supports the conclusion from experiments with dominant negatives of PKB/Akt that PKB/AKT is not required for insulin-activated PAI-1 transcription.

Insulin activation of FKHR was not seen using Gal4-FKHR fusion proteins that contained part of the DNA-binding and the C-terminal activation domains of FKHR (58). It is possible that the discrepancy between our results arise from differences in the LexA and Gal4 DNA-binding and expression systems. Insulin may activate FKHR if its promoter binding is strong enough for it to be retained in the nucleus despite signals for its export. Whether insulin activates or represses a gene could depend on the strength of the response element. The LexA system may provide a stronger DNA binding/dimerization domain than Gal4 or the LexA response element of the reporter may be stronger than the Gal4 response element used in those studies (58). Alternately, an accessory protein may be required for nuclear export of the FKHR fusion proteins and this might not be present in the cell lines that we examined. Studies showing the importance of 14-3-3 proteins for nuclear export of FKHR support this possibility (48, 57).

The activation of PAI-1-CAT expression by insulin is inhibited by LY294002 and by overexpression of PTEN (Fig. 9, A and B). This implied that insulin signaled through PI 3-kinase activation. PI 3-kinase was shown to activate PKD-1, which activated PKB/Akt. This suggested that protein kinase B/Akt might phosphorylate the insulin-responsive transcription factor. Expression of mutated Akt that acted as a dominant negative in other systems failed to block insulin-increased PAI-1-CAT expression (Fig. 9C) implying either that PKB/Akt does not phosphorylate the insulin-responsive transcription factor or that the PKB/Akt phosphorylation is not functional in the cell lines tested. Insulin induced ATF-2 and p38 MAPK in a PI 3-kinase dependent manner (53). Phosphorylation of this serine leads to nuclear export of FKHR at a site(s) other than Ser253; but the export signal would not be activated while FKHR transcriptional activity increased, explaining how insulin could activate transcription through FKHR.

PAI-1-luciferase was expressed in all of the cell lines tested (Fig. 1), while insulin stimulation was only observed in GH4, HeLa, HepG2, 3T3, and HUVEC cells. The lack of insulin stimulation in Rat-2 and CHO cells might be secondary to an incomplete insulin-signaling pathway in those cells. The lack of an important downstream kinase would make the promoter unresponsive to insulin. However, previous experiments demonstrated that CHO and Rat-2 cells exhibited insulin-increased promoter expression using different reporter genes (50). Another explanation for the lack of insulin-responsive PAI-1-CAT expression is the lack of the insulin-responsive transcription factor. This could be an insulin modified member of the Forkhead family that activates at the sequence −52/−43. It is intriguing to speculate that this might be a cell type-specific factor since PAI-1-luciferase expression was not insulin activated in all cell lines tested. Finally, it might be an accessory factor for the insulin-modified transcription factor that is not present in all cell types.

It is important to conclusively identify the insulin-responsive transcription factor. The experiments presented above suggest that this factor may be a forkhead-related/winged helix transcription factor. The cell type experiments suggested that GH4 cells could be the most useful for isolating this factor. Experiments to determine the size of the factor that binds to the insulin response element of the PAI-1 promoter are currently in progress as a prelude to identifying it from an appropriate cDNA library.

Acknowledgments—We thank Dr. R. Brent (Harvard University, Cambridge, MA), Dr. T. Franke (Columbia University, New York, NY), Dr. Kun-Ling Guan (University of Michigan, Ann Arbor, MI), Dr. J. Schlessinger (Yale University, New Haven, CT), Dr. M. Philips (NYU, New York, NY), Dr. Ulf Rapp (Universitat Wurzburg, Wurzburg, Germany), Dr. L. Feig (Tufts, Boston, MA) and Dr. J. Whitaker (Hagedorn Institute, Copenhagen, Denmark) for plasmids used in these studies.

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