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Models of insulin action on metabolic and growth response genes

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Abstract

In ongoing studies aimed at elucidating the mechanism of insulin action on the expression of genes that modulate glucose utilization and cell growth, we have focused on the inductive effect of insulin on transcription of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the early growth response gene, Egr-1. Insulin acutely stimulates the expression of both genes in 3T3 adipocytes; however, in primary adipocytes, chronic insulin exposure has opposing effects on the expression of these genes. GAPDH mRNA is decreased in the epididymal fat cells of diabetic animals and is increased over control levels when insulin is replaced, while Egr-1 mRNA levels are increased in diabetic animals. These observations, coupled with the finding that insulin-stimulated Egr-1 gene transcription is impaired in a Chinese hamster ovarian (CHO) cell line that displays normal metabolic responses but impaired ability to regulate DNA synthesis, support the conclusion that insulin regulation of Egr-1, a growth response gene, and GAPDH, a metabolic response gene, are mediated by distinct pathways. We present evidence that supports the role of protein phosphorylation in mediating the effect of insulin on activation of Egr-1 and GAPDH gene transcription. (Mol Cell Biochem **109**: 99–105, 1992)

Key words: phosphorylation, IRE-A, Egr-1, GAPDH, gene transcription, insulin

Introduction

Insulin induces glyceraldehyde-3-phosphate dehydrogenase, GAPDH, mRNA levels 8-fold in cultured 3T3-L1 adipocytes and 10-fold in fat or liver tissue isolated from rats fasted then refed a high-carbohydrate, low-fat diet overnight [1]. In contrast, expression of the GAPDH gene is not regulated in preadipocytes [2]. Insulin markedly stimulates expression of the early growth response gene, Egr-1, in fibroblasts, epithelial cells, and H35 hepatoma cells [3], which suggests that the signal transduction pathway involved in mediating the *growth* effect of insulin is intact in all three cell types.

In primary adipocytes, we show that GAPDH mRNA levels are markedly decreased in streptozotocin-treated diabetic animals and induced above basal levels upon replacement of insulin. In contrast, Egr-1 gene expression is induced during the induction of diabetes and is decreased below control values with chronic insulin therapy. This result supports the conclusion that insulin is not the predominant factor regulating expression of this gene *in vivo*.

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This observation raised the question whether the effect of insulin on the transcription of Egr-1 and metabolic genes is mediated via distinct pathways. This hypothesis was supported by the fact that Chinese hamster ovarian (CHO) cells which express an insulin receptor mutant capable of stimulating glycogen synthesis, a metabolic event, but not DNA synthesis [4], a growth-related event, also showed impaired insulin-stimulated Egr-1 gene transcription. Mutations in the juxtamembrane region of the receptor that impair tyrosine kinase activity and intracellular signalling, as measured by phosphorylation of pp 185 [5], also inhibit Egr-1 gene transcription. This result suggests that the ability of insulin to stimulate Egr-1 gene transcription is linked to its ability to stimulate protein phosphorylation.

The role of phosphorylation in mediating the effect of insulin on the GAPDH gene could not be studied in CHO cells because insulin regulation of GAPDH gene expression is limited to lipogenic tissues. A detailed description of the mechanism of insulin action on gene transcription will require that the trans-acting factors that regulate gene expression be defined, characterized, and cloned. Towards this end, we have identified a cis-acting sequence, IRE-A, in the upstream region of the GAPDH gene that confers insulin-responsive gene transcription to marker genes. Binding of a trans-acting factor, IRP-A, to this motif is enhanced by insulin. Insulin is known to regulate the activity of its target enzymes by promoting phosph-dephospho interconversions. For the most part, insulin has been shown to regulate the activity of metabolic enzymes by promoting a net decrease in phosphorylation; glycogen synthase is an example. We posed the question whether IRP-A binding activity would be enhanced by phosphatase treatment. We found that DNA binding was undetectable when the protein was dephosphorylated. This result suggests that acute regulation of IRP-A activity may be mediated by a net increase in protein phosphorylation.

Methods

Isolation of epididymal fat cell RNA

Fat cells were isolated as previously described [6] and total RNA extracted using the guanidium isothiocyante method [6]. Total RNA was matched by optical density and ethidium staining and subjected to Northern analysis. Northern analysis of cellular RNA was performed using a randomly primed Egr-1 cDNA probe [3] and a GAPDH-cDNA [2]. The autoradiographs were subjected to densitometry.

Mutant receptor lines

Stable lines of CHO cells transfected with i) normal human insulin receptors (HIR), ii) mutant HIR, or iii) the neomycin-resistance gene alone were grown to confluence in Ham's F12 medium supplemented with 10% fetal bovine serum, glutamine (2 mM), and G418 (400 μg/ml). The cells were incubated in serum-free Ham's F12 medium containing 10 mM Hepes and 0.1% crystalline bovine serum albumin overnight then stimulated with 15% FBS or 10 nM insulin for 30 min. Total RNA was isolated as previously described [2] and matched by optical density and ethidium bromide staining.

Isolation of nuclear extracts

3T3 adipocytes were grown to confluence and differentiated as previously described [2]. Nuclear extracts were isolated from differentiated 3T3-L1 cells exposed to insulin for 1 h (I) using a modification of the procedure of Dignam *et al.* [1].

UV cross-linking

Body-labelled bromouridinated IRE-A was prepared for UV cross-linking experiments as previously described [7]. A specific primer was used to synthesize the IRE-A [1] binding motif in the presence of 50 μM dCTP, dGTP, 5-bromo-2'-deoxyuridine triphosphate, and 5 μM [α^{32}P]-dATP. Nuclear extract (10 μg) was incubated with 1 ng of body-labelled probe in a buffer containing 20% glycerol (V/V), 150 mM NaCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 20 mM Hepes (pH 8.0) in a total volume of 20 μl. The mixture was incubated at 0° C for 90 min then irradiated with a Fotodyne UV lamp (maximum emission wave length 310 nm) for 15 min. At the end of the incubation, the mixture was brought up to 10 mM $CaCl_2$ and the DNA was digested for 30 min at 37° C with 1 μg of DNAse I. The mixture was subjected to electrophoresis on a 12.5% SDS polyacrylamide gel. The gel was dried and subjected to autoradiography.

Phosphatase treatment of nuclear extracts

Nuclear extracts isolated from insulin-treated 3T3 adipocytes were treated with active or heat-killed, agarose-bound bacterial alkaline phosphatase for 10 min at room temperature. The reaction mixture contained 2 units (0.4 mg) of alkaline phosphatase in a buffer containing 20% glycerol (V/V), 150 mM NaCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 1 mM $ZnCl_2$, and 20 mM Hepes (pH 8.0) in a total volume of 18 μL. Control incubations were performed in two ways: (1) alkaline phosphatase was added to the control tubes and immediately spun out from the incubation mixture or (2) parallel incubations were performed with phosphatase that had been heat-killed by boiling for 10 min at 100° C. After phosphatase treatment was complete, DNA binding activity was measured using the Southwestern assay described below.

Southwestern analysis

Insulin-treated 3T3 adipocyte nuclear extracts were subjected to electrophoresis on a 9%-SDS polyacrylamide gel. Proteins were electrophoretically transferred to nitrocellulose paper in 25 mM Trizma base/190 mM glycine. The nitrocellulose strips were blocked with 5% (w/vol) non-fat dry milk (Blotto), 30 mM Hepes (pH 7.2). Proteins bound to the filters were denatured with 7 M guanidine-HCl in 50 mM Tris HCl (pH 8.3), 50 mM DTT, 2 mM EDTA, 0.25% non-fat dry milk for 1 h at 25° C and then allowed to renature in 50 mM Tris HCl (pH 7.5), 100 mM NaCl, 2 mM DTT, 2 mM EDTA, 0.1% Nonidet P-40, and 0.25% skim milk [8]. The filter was exposed to a binding buffer that contained 1 μg/ml dI·dC, 25 mM NaCl, 5 mM $MgCl_2$, 0.5 mM DTT, 25 mM Hepes (pH 7.5), and 1×10^6 cpm/ml ^{32}P-DNA (IRE-A element) for 60 min at 25° C. After 60 min, nitrocellulose strips were washed 3 to 4 times with the binding buffer, dried, and subjected to autoradiography.

Results and discussion

Regulation of GAPDH and EGR-1 mRNA levels in primary adipocytes

In the streptozotocin-induced diabetic rat (80 mg/kg streptozotocin for 7 days), GAPDH mRNA levels are markedly decreased. When insulin is replaced by osmotic pump, (4 U insulin/day) GAPDH mRNA is increased 10-fold. In contrast, Egr-1 mRNA levels are increased 2-fold in diabetic rat epididymal fat cells and decreased below control with insulin replacement. These observations support the importance of GAPDH gene regulation by insulin *in vivo* and indicate that GAPDH is an excellent marker of the *metabolic* effects of insulin on gene expression. Egr-1, on the other hand, is predominantly regulated by factors other than insulin *in vivo*.

Regulation of Egr-1 by insulin: A model of its growth effects

The fact that insulin regulation of Egr-1 and GAPDH mRNA can be uncoupled during the induction and treatment of diabetes raised the question whether distinct signal transduction pathways were involved in regulation of cell growth and cell metabolism by insulin. The fact that the Egr-1 gene responds to insulin in diverse cell types made it possible to examine the effects of mutations in the autophosphorylation domain of the insulin receptor tyrosine kinase on the ability of insulin to stimulate Egr-1 mRNA in stable lines of CHO cells expressing normal and mutant HIR. A Tyr-to-Phe substitution at residue 960, F_{960}, has been previously shown to inhibit insulin-stimulated phosphorylation of endogenous substrates, thymidine incorporation into DNA, and activation of glycogen synthase [5]. A Tyr-to-Phe substitution at residue 1146, F_{1146}, inhibits the activation of thymidine incorporation but has no effect on activation of glycogen synthase [4]. A representative experiment in which the effect of insulin on Egr-1 mRNA was compared to that of serum in each cell line is shown (Table 1). The effect of insulin was 86% of the maximal serum stimulation in cells that expressed 80,000 normal human insulin receptors, CHO/HIR. In contrast, CHO cells that expressed 3,000 endogenous insulin receptors, CHO/NEO, and cells that expressed 80,000 F_{960} mutant receptors, CHO/IR_{F960}, or 80,000 F_{1146}, mutant receptors CHO/IR_{F1146}, achieved only 32%, 24%, and 35% of maximal stimulation, respectively. The effect of these mutations on insulin regulation of Egr-1 mRNA mirrors the effect of insulin on thymidine incorporation into DNA in these lines and strongly suggests i) that the signal transduction pathway used to stimulate acute changes in Egr-1 gene transcription and those utilized to modulate the effect of insulin on DNA synthesis are similar, and ii) that the signal transduction pathway

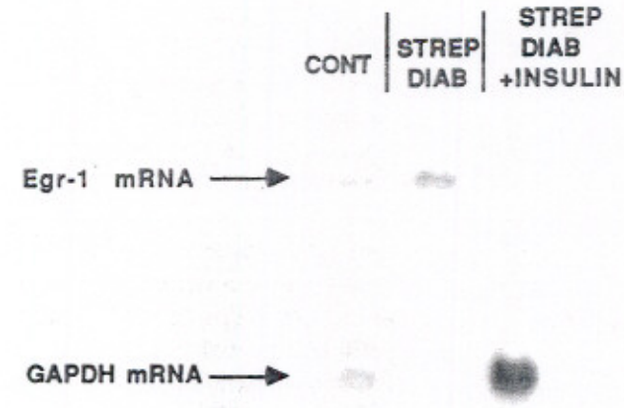

Fig. 1. Regulation of GAPDH and Egr-1 mRNA by insulin in epididymal fat cells.
Epididymal fat cells were isolated from i) control (CONT), ii) diabetic (STREP/DIAB), or iii) diabetic rats treated with a subcutaneous infusion of insulin 4 U/day for seven days (STREP/DIAB + INSULIN). RNA was isolated as previously described [6] and subjected to Northern analysis. The autoradiograph is shown.

utilized to stimulate Egr-1 gene transcription is distinct from that utilized to regulate metabolic events. Because both the F_{960} and F_{1146} mutations impair activation of the insulin receptor tyrosine kinase and stimulation of intracellular phosphorylation, these observations suggest that stimulation of protein phosphorylation is linked to regulation of two nuclear events, regulation of cell growth and Egr-1 gene transcription.

Future efforts will address the question whether the transcriptional effect of insulin on metabolically active genes is maintained in CHO/IR_{F1146} lines. It is conceivable that tissue specificity of the hormone response results from the component of trans-acting factors expressed in a given tissue and that all nuclear events are inhibited in lines expressing CHO/IR_{F1146}. Alternatively, the effect of insulin on metabolically active genes may be mediated by a distinct pathway, as is the ability of insulin to activate glycogen synthase.

Regulation of GAPDH gene transcription by insulin: A model of its metabolic effects

As shown in Fig. 1, we find that the effect of insulin on growth and metabolic genes can be disassociated, indicating that a distal component in the signal transduction pathway is regulated differently. Our goal in studying the GAPDH gene was to identify a metabolic gene regulated by insulin at the transcriptional level and proceed to identify the trans-acting factors that mediate insulin's action on genes that regulate glucose utilization. We found that activation of GAPDH gene expression in insulin-responsive tissues correlates with activation of an insulin-responsive DNA binding protein, IRP-A, that binds specifically to an element in the 5'-flanking region of the GAPDH gene to confer insulin-responsive gene expression to the chloramphenicol acetyl transferase gene. Within 60 min of exposure of 3T3 adipocytes or H35 hepatoma cells to insulin, the activity of this sequence-specific DNA binding protein is increased 4-fold [1] in the absence of new protein synthesis (unpublished observations). Thus it appears that the activity of these factors is regulated acutely by a post-translational modification. Defining the interconverting enzymes that modulate IRP-A binding activity in response to insulin should lead to an understanding of the mechanism by which insulin regulates metabolically active genes.

The initial interaction of insulin with its receptor results in autophosphorylation of the receptor tyrosine activity. Insulin-stimulated phosphorylation results in the activation of certain serine and threonine protein kinases as well [9–11]. In contrast, for the most part, insulin activates metabolic enzymes by promoting a net decrease in protein phosphorylation. Well-studied examples include activation of glycogen synthase, pyruvate dehydrogenase, acetyl-coA carboxylase, and inactivation of phosphorylase.

We posed the question whether dephosphorylation would lead to activation of the IRP-A binding activity. Control and insulin-treated extracts were incubated with alkaline phosphatase for 0 or 10 min at 4° C. The activity of the IRE-A DNA binding protein as assessed

Table 1. Fold effect of insulin on expression of Egr-1 mRNA in CHO cell lines transfected with normal or mutant insulin receptors

	NEO	HIR	F1146	F960
Insulin (10 nM)	2.0	4.3	2.0	2.1
Serum (15%)	6.0	5.0	5.6	8.6
% of Maximal Stimulation	32	86	35	24

Total RNA was isolated from cells stimulated with insulin 10 nM and serum as described in Methods. The fold effect of serum and insulin vs. control (corrected for GAPDH mRNA since the gene is not regulated in these cells) was calculated and plotted. Egr-1 is a serum regulated gene. Stimulation of Egr-1 mRNA by 10 nM insulin in each cell line was compared to the effect of 15% serum and is referred to as the '% of Maximal Stimulation' in the table.

by formation of a shifted complex on a gel shift assay was decreased 70% (data not shown) by treatment of control and insulin-stimulated extracts with alkaline phosphatase at 4° C for 10 min. A room temperature incubation with phosphatase completely eliminated IRE-A-binding activity. Parallel extracts treated with heat-killed alkaline phosphatase at room temperature for 10 min showed little change in binding activity.

The interaction of IRP-A with IRE-A DNA is inhibited by phosphatase inhibitors. This problem can be cirumvented by use of a Southwestern analysis in which the binding reaction is performed on nitrocellulose-bound proteins. In Fig. 2, IRP-A was incubated with agarose-bound alkaline phosphatase, subjected to SDS-PAGE, and transferred to nitrocellulose. The nitrocellulose-bound proteins were denatured and renatured, then incubated with labelled IRE-A DNA. In the absence of active phosphatase, a 57,000 molecular weight protein (that comigrates with the 48,000 dalton prestained molecular weight marker) bound IRE-A DNA avidly. Phosphatase treatment prior to SDS-polyacrylamide gel electrophoresis eliminated the ability of IRP-A to interact with the IRE-A DNA binding motif. The pattern of background proteins did not change with phosphatase treatment.

To confirm that the 57,000 protein detected on Southwestern analysis was the specific IRE-A DNA binding protein, we performed an experiment in which the IRE-A binding motif was cross-linked to crude extracts containing IRP-A and extracts partially purified by affinity chromatography. The pattern of proteins detected was similar in both (Fig. 3). When crude and affinity purified preparations of IRP-A were covalently linked to DNA, two proteins were detected, a prominent 57,000 molecular weight protein and a 116,000 molecular weight protein. The 57,000 molecular weight protein was competed by the wild-type IRE-A binding motif, not by mutant motifs with decreased affinity for the IRP-A protein [1]. Thus, the 57,000 dalton protein is the IRP-A protein. This assay also allowed us to eliminate the possibility that IRP-A was inactivated by proteolysis. When cross-linked extracts were incubated with agarose-bound alkaline phosphatase, no change in the migration of the cross-linked proteins was detected after alkaline phosphatase treatment (data not shown). This result indicates that the IRP-A protein was not less active because of degradation. Thus, we conclude that i) binding of the IRE-A protein to DNA is dependent on phosphorylation and

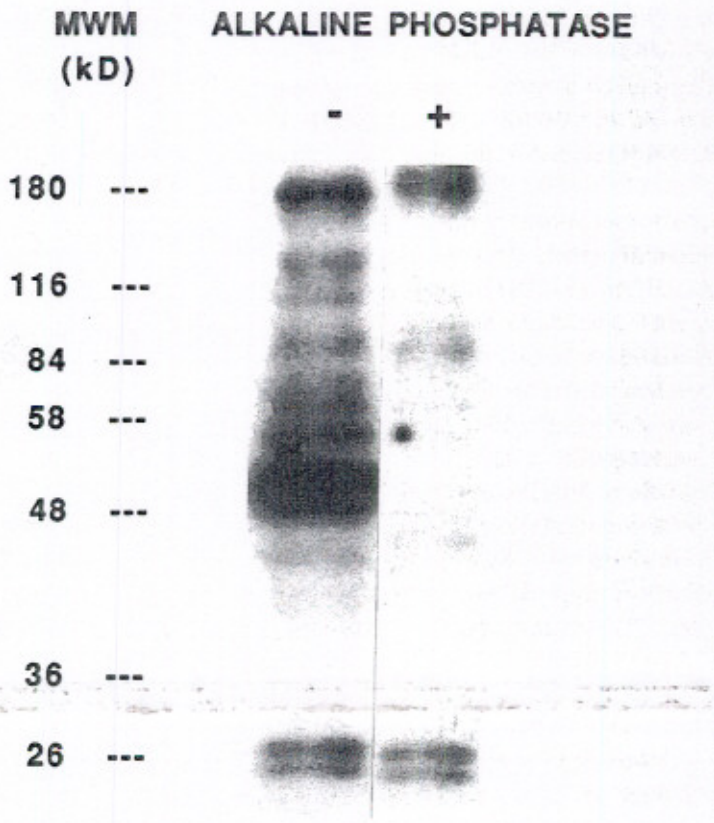

Fig. 2. Southwestern Analysis of Phosphorylated and Dephosphorylated IRP-A.
Crude nuclear extract isolated from 3T3 adipocytes was incubated with inactivated (−) or active (+) alkaline phosphatase and the proteins subjected to electrophoresis as described in Methods. The binding of IRP-A to DNA was assessed by Southwestern analysis as described in Methods. The autoradiograph is shown.

ii) that dephosphorylation is unlikely to be the mechanism by which insulin activates IRP-A.

Insulin regulates the activity of numerous rate-limiting metabolic enzymes by inhibiting protein phosphorylation. We have shown that DNA binding activity of an insulin-sensitive trans-acting factor is markedly decreased when IRP-A is dephosphorylated. While this finding clearly indicates that binding of IRP-A to DNA is dependent on phosphorylation, it is as yet unclear whether insulin alters the activity of this protein by stimulating protein phosphorylation. Several mechanisms are possible. Insulin could stimulate protein phosphorylation resulting in a direct increase in binding affinity of this protein for DNA. Alternatively, a protein-protein interaction may occur. A second possibility would be that insulin dephosphorylates this protein and promotes dissociation of the protein DNA complex allowing another protein, which acts as an activator, to

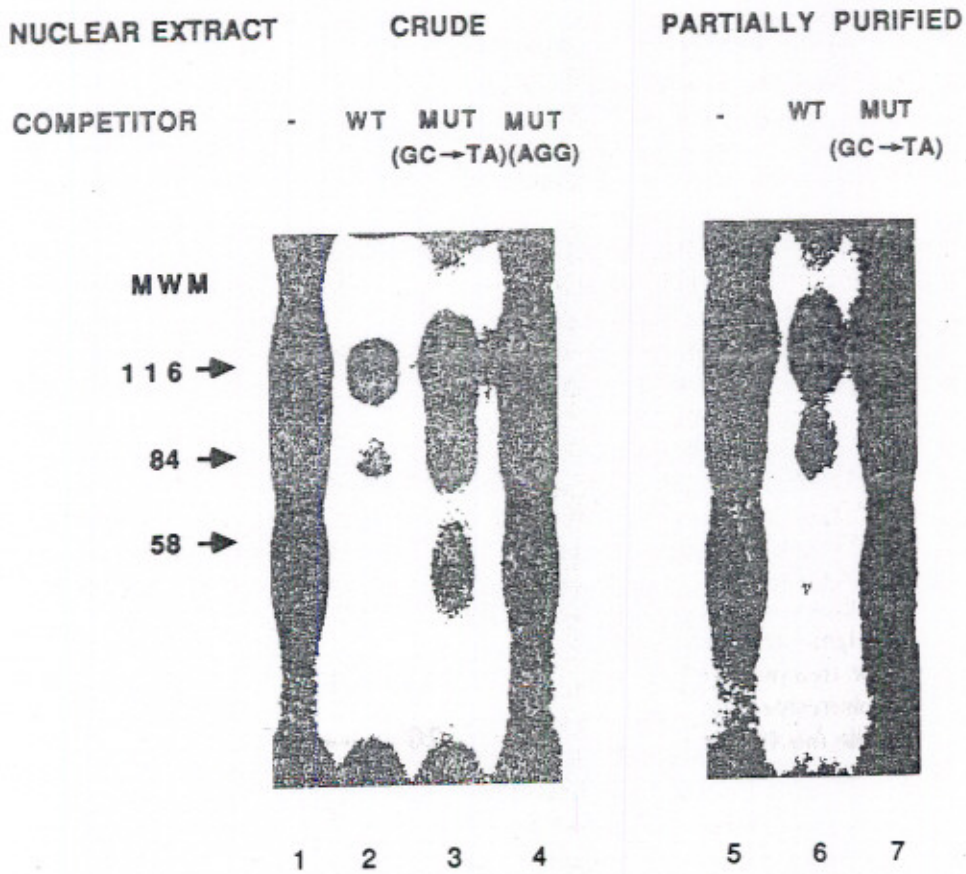

Fig. 3. Identification of a Specific IRE-A DNA Binding Protein. Crude adipocyte nuclear extract or partially purified adipocyte IRP-A was incubated with a bromouridinated IRE-A binding motif in the presence of i) unlabelled DNA, or ii) a 100-fold excess of wild-type motif (aactttcccgcctctcagcctttgaaag), or iii) a 100-fold excess of either a mutant IRE-A binding motif with the central nucleotides GC changed to TA, MUT (GC→TA) (aactttccctactctcagcctttgaaag) or a downstream repeat of IRE-A that does not bind the insulin-sensitive protein, MUT (AGG) (ccgcctcccgccaggctcagccagtcccag). Proteins were cross-linked and subjected to SDS-PAGE. The autoradiograph is shown. The migration of prestained molecular weight markers is indicated. These markers run approximately 10,000 daltons higher than unmodified markers. Lane 1: Nuclear extract cross-linked to wild-type (wt) IRE-A; Lane 2: Nuclear extract cross-linked to wt IRE-A in the presence of a 100-fold excess of wt IRE-A; Lane 3, 4: same as Lane 1, but competed with a 100-fold excess of the two mutants described above; Lane 5: same as Lanes 1–3 except the extract was partially purified by affinity chromatography on an IRE-A sepharose column prior to cross-linking with labelled IRE-A DNA.

occupy the site. These possibilities can be distinguished by *in vivo* phosphorylation studies. Whatever the result, elucidating the mechanism of insulin action on the GAPDH gene should provide insight into the mechanism by which insulin signals the nucleus to alter the expression of metabolically active genes.

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