GABP Mediates Insulin-increased Prolactin Gene Transcription*

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The insulin-response element from the prolactin gene is identical to the Ets-binding site, and dominant-negative Ets protein inhibits insulin-increased prolactin gene expression. Immunoblotting identified the Ets-related transcription factor GABP in nuclear extracts from GH cells. Expression of GABPα and GABPβ1 squelches insulin-increased prolactin gene expression. GABPα and GABPβ1 bind the insulin-response element of the prolactin promoter, and anti-GABPα and anti-GABPβ1 antibodies supershift a species seen with nuclear extracts from GH cells. GABPα immunoprecipitated from insulin-treated, 32P-labeled GH cells was phosphorylated 3-fold more than GABPα from control cells. The ratio of phosphorylated GABPβ1 to total GABPβ1 in response to insulin. Mitogen-activated protein (MAP) kinase activity is increased 10-fold in insulin-treated GH4 cells. MAP kinase immunoprecipitated from control cells does not phosphorylate GABPα while MAP kinase immunoprecipitated from insulin-treated cells shows substantial phosphorylation of GABPβ1. These studies suggest that GABP mediates insulin-increased transcription of the prolactin gene. GABP may be regulated by MAP kinase phosphorylation.

The activation of gene transcription by hormones that function through protein-tyrosine kinase receptors is not well understood in comparison with that mediated by other classes of hormones. The receptors for the steroid-thyroid hormones are understood in comparison with that mediated by other classes of hormones to each of these classes of hormones is dependent on the presence in the gene of the appropriate DNA sequence to which the activated transcription factor binds. Neither the hormone-responsive DNA element nor the transcription factors activated by protein-tyrosine kinase receptors are known.

Recently, we have identified an insulin-response element in the prolactin promoter that is identical to the binding site for the Ets-related transcription factors (4). This element also mediates the insulin sensitivity of the thymidine kinase and somatostatin promoters in both HeLa and GH4 cells and confers insulin responsiveness to the mammary tumor virus promoter when it is added to that promoter at ~88. Further, the increase in the transcription of these genes in insulin-treated cells was inhibited by expression of a dominant-negative Ets protein (5). These studies identify the predominant Ets-related protein of GH4 cells, GABPα, and suggest that GABP mediates insulin-increased prolactin gene expression. Phosphorylation of GABP by MAP1 kinase may regulate its activity.

EXPERIMENTAL PROCEDURES

Materials—[32P]dCTP, 3000 Ci/mmol; [32P]JATP, 3000 Ci/mmol, and [14C]chloramphenicol, 50 mCi/mmol, were obtained from ICN Biochemicals Corporation. [32P]Pi-Po4 was from DuPont NEN. All enzymes and linkers were obtained from either New England Biolabs or from New Ringer Mannheim and, unless otherwise indicated, were used under conditions recommended by the suppliers. Oligonucleotides were purchased from Operon. Duplex poly(dI-dC) was obtained from Pharmacia Biotech Inc. Antibodies to MAP kinase (anti-Erk-1 and anti-Erk-2), antibody to the DNA binding domain of cEts-1 (pan-Ets), and horseradish peroxidase-conjugated goat anti-rabbit secondary antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies directed against GABPα and GABPβ1 were the generous gift of Dr. S. L. McKnight (Tularik, South San Francisco, CA). Reagents used for gel electrophoresis were purchased from Fisher Scientific. Protein A agarose, acetyl-CoA, and silica gel plates were obtained from Sigma, Dulbecco’s modified Eagle’s medium containing 4.5 g/liter glucose (DMEM) was from Life Technologies, Inc., and iron-supplemented calf serum was obtained from Hyclone Laboratories. Triton X-100, reagents for enhanced chemiluminescence, and BCA reagent were from Pierce. All other reagents were of the highest purity available and were obtained from Sigma, Behring Diagnostics, Bio-Rad, Eastman, Fischer, or Boehringer Mannheim.

Plasmids—The construction of pPrl-CAT plasmids containing ~173/+75 of prolactin 5′-flanking DNA was described (6). The human insulin expression vector, pRT3H1R2, was the gift of Dr. J. Whittaker (Stony Brook, NY). The plasmids CMV-GABPα and CMV-GABPβ were provided by Dr. C. Thompson (Carnegie Institute, Baltimore, MD). The Elk-1 and SRF expression vectors were the gift of Dr. R. Tremain. The GST-GABPα expression plasmid was constructed by ligating a blunt-ended BamHI/XhoI fragment from the GABPα DNA (plasmid F27, Dr. S. L. McKnight) into the blunt-ended EcoRI site of pGEX 2T (Pharmacia). The fusion protein consists of the GST protein (26 kDa) and GABPα(76–336) (30 kDa) that contains the three potential MAP kinase phosphorylation sites.

Western Immunoblot Analysis—GH4 cells were harvested after 48 h with or without 1 μg/ml insulin, and nuclei were prepared as described (7). A nuclear extract was prepared by disrupting the nuclei with 400 mg KCl in a buffer containing 15% glycerol, 25 mM Tris, pH 8, 10 mM β-mercaptoethanol, 0.5 mM EDTA, and 0.05% Triton X-100. SDS-polyacrylamide gel electrophoresis was performed using 12% gels (8). The proteins were then blotted to nitrocellulose membranes (Miron Separations) in Towbin’s buffer (25 mM Tris base, 192 mM glycine, and 20% methanol). Immunoblotting using enhanced chemiluminescence was performed as described by the manufacturer (Pierce).

Analysis of Prolactin Promoter Responsiveness Using Transient Transfection—Electroporation experiments and CAT assays were performed as described (9). GH4 cells were placed for 24 h in DMEM containing 10% hormones-depleted serum (0) and harvested with an EDTA solution, and 20–40 × 10^6 cells were used for each electroporation. Trypan blue exclusion before electroporation ranged from 95 to

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1 The abbreviations used are: MAP, mitogen-activated protein; DMEM, Dulbecco’s modified Eagle’s medium containing 4.5 g/liter glucose; GST, glutathione S-transferase; CAT, chloramphenicol acetyltransferase; EGF, epidermal growth factor; SRF, serum response factor.
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Assay of DNA-Protein Binding by Gel Electrophoresis—An oligonucleotide to the prolactin promoter sequence -106/-87 was prepared, purified on polyacrylamide gels, and end-labeled with [32P]dCTP. The assay was performed in a buffer containing 25 mM HEPES, pH 8.0, 50 mM MgCl2, 1 mM EGTA, 10% glycerol, 1 mM Na3VO4, 50 mM Na4P2O7, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin. Immuno-precipitations were performed for 90 min at 4 °C in this buffer using 200 μg of protein. Antibody to MAP kinase was obtained from Santa Cruz, and antibody to GST (Dr. S. L. McKnight). The position of migration of molecular weight markers ovalbumin (46,000 kDa) and bovine serum albumin (66,000 kDa) is indicated. The arrow on the left indicates the position of GABPα while the arrow on the right indicates GABPβ.

RESULTS AND DISCUSSION

Immunoblot analysis of GH4 cell nuclear extracts was performed to determine which of the Ets-related transcription factors might mediate the effects of insulin on prolactin gene expression (Fig. 1). A pan-Ets antibody was used to visualize Ets-related proteins in GH4 cell nuclear extracts. This antibody was raised against the conserved DNA binding domain of Ets-1, and it demonstrates broad cross-reactivity with Ets family proteins. One band of approximately 51 kDa was visible using either 1 or 3 μg of nuclear extract. This is the same size as the previously identified Ets-related protein GABPα. GABPα is a subunit of the heteromeric transcription factor, GABP. The other subunit is GABPβ, a notch-related protein (12). GABPα was originally identified as the transcription factor that binds to a purine-rich cis-regulatory element required for VP16-mediated activation of herpes simplex virus immediate early gene (13). A separate set of filters was therefore analyzed using antibodies against GABPα or GABPβ. One band, identical in size to that seen using the pan-Ets antibody, is seen with anti-GABPα. Anti-GABPβ antibody reveals two bands. The lower, more intense band migrates with an apparent molecular mass of 43 kDa and thus likely represents GABPα.

Fig. 2. Effect of expression of GABP on insulin-increased prolactin-CAT transcription. In control transfections, GH4 cells were cotransfected with 15 μg of Prl(-173/-75) CAT (10) and with 5 μg of an expression vector for the human insulin receptor, pRT3HR2 (J. Whittaker, Stony Brook) alone or with 10 μg of a cytomegalovirus expression vector pRK5. Vectors expressing GABPα and/or GABPβ (C. Thompson, Carnegie Institute) or Elk-1 and SRF (R. Treisman, Imperial Cancer Research Fund, London, United Kingdom) under control of the cytomegalovirus promoter were included at 5 μg in the designated experiments. The average percent acetylation/mg of protein in control and insulin-treated cultures was determined, and the insulin incubations were compared with control levels to determine the -fold stimulation by insulin (Fold-Control). The results are from three separate experiments done in duplicate.
shown that GABP is a complex formed with a bacterially expressed protein since it is inhibited by low levels of non-radioactive competitor (10). Gel-mobility shift experiments performed with bacterially expressed GABP showed three retarded bands in the absence of DNA and GABP a and GABP b dimer (12). Only the bacterial protein band was seen with bacterially produced GABP b1 (lane 3). This was expected since previous studies had shown that GABP b1 is not a DNA binding protein (12). When GABP a and GABP b1 were added together (lane 4), the bands corresponding to GABP a were no longer visible and an abundant, more slowly migrating band was seen. This band corresponds to an abundant band seen with nuclear extracts from GH cells. The identity of this band as consisting of GABP a and GABP b1 is confirmed in the experiment shown in Fig. 3 (right).

Lanes 1 and 2 show the gel-mobility shift pattern with nuclear extract alone (lane 1) and nuclear extract with normal rabbit serum (lane 2). Antibodies to GABP a, lane 3, GABP b1 (lane 4), or antibodies to both GABP a and GABP b1 (lane 5) shifted this complex to one with a slower migration.

Since insulin receptor is a tyrosine-protein kinase that is activated by insulin binding, it is thought that activation of gene transcription by insulin may be the end product of a phosphorylation cascade. Therefore, we examined the phosphorylation of GABP in response to insulin in 32P-labeled GH cells. The phosphorylation of GABP a was increased 3-fold in 1 h in insulin-treated cells as compared with control cells (Fig. 4A). GABP b co-immunoprecipitated with GABP a in this experiment shows no increase in response to insulin. Immunoprecipitation with anti-GABP b1 confirms this observation. GABP b1 phosphorylation was not significantly increased by insulin treatment (20% above control) while the co-immunoprecipitated GABP a is increased 3-fold by insulin.

MAP kinase activation was shown to be required for several types of insulin responses in numerous systems (15). Further, Elk-1, an Ets-related transcription factor, was shown to be activated by MAP kinase phosphorylation (16). Pointed-P2, an Ets-related protein from Drosophila, is phosphorylated by MAP kinase in the sevenless signal transduction pathway (17). Our studies suggest that insulin activation of prolactin gene expression in GH cells is MAP kinase-dependent since all factors that inhibit insulin-increased prolactin gene expression also inhibit MAP kinase activation. Therefore, MAP kinase activation by insulin might phosphorylate GABP a. The representative increase in MAP kinase activity in cell lysates from insulin-treated cells is shown (Fig. 4B). Multiple experiments show an increase of 10 ± 0.8-fold in MAP kinase activity in insulin-treated cells. GABP a contains three potential MAP kinase phosphorylation sites near the DNA binding domain. There-
GABP was shown to be important for enhancement of transcription of the herpes simplex virus immediate early gene, but its physiological role in uninfected cells is unknown. Our studies show that GABP mediates the insulin response of the prolactin gene. Since GABP is widely distributed, these results could be significant to understanding insulin regulation of other genes. Analysis of 22 insulin-responsive promoters has identified potential Ets-response elements in all of these. For some of these, the Ets-response element is in a region defined by deletion analysis to be important for the effects of insulin (5). The insulin-mediated increase in the transcription of all three genes, prolactin, somatostatin, and thymidine kinase, that we have studied is inhibited by dominant-negative Ets protein. This indicates that GABP may be implicated in the regulation of other insulin-responsive genes.

Ets-related transcription factors such as GABP are often found in large complexes with other transcription factors. For example, Ets-1 and Sp-1 interact to synergistically activate the human T-cell lymphotrophic virus long terminal repeat (18). Although this report demonstrates that GABP is necessary to the insulin effect, it may not be sufficient. The insulin responsiveness of the prolactin gene can be eliminated by mutation of two Ets motifs at -96/-87 and -76/-67 of the prolactin promoter (4). These mutations have little effect on basal prolactin gene transcription. However, mutation of -101/-92 of the prolactin promoter eliminates the effect of insulin and reduces basal prolactin gene expression by >100-fold. Clearly, another protein(s) interacts at this sequence and is important both for basal prolactin gene expression and the effect of insulin. It is likely that GABP is complexed with this protein(s) in the prolactin promoter and that this complex is important to the increase in prolactin gene expression seen in insulin-treated cells.

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