Elk-1, C/EBPα, and Pit-1 Confer an Insulin-responsive Phenotype on Prolactin Promoter Expression in Chinese Hamster Ovary Cells and Define the Factors Required for Insulin-increased Transcription

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The transcription factor(s) that mediate insulin-increased gene transcription are not well defined. These studies use phenotypic conversion of Rat2 and Chinese hamster ovary (CHO) cells with transcription factors to identify components required for regulation of prolactin promoter activity and its control by insulin. The pituitary-derived GH4 cells contain all of the transcription factors required for insulin-increased prolactin-chloramphenicol acetyltransferase (CAT) expression while HeLa cells require only Pit-1, a pituitary-specific factor. However, Rat2 and CHO cells require additional factors. We had determined previously that the transcription factor that mediates insulin-increased prolactin gene expression was likely an Ets-related protein. Elk-1 and Sap-1 were the only Ets-related transcription factors tested as chimeras with LexA DNA-binding domain that were able to mediate insulin-increased expression of a LexA-CAT reporter plasmid. Elk-1 and Sap-1 are expressed in GH4 and HeLa cells but Rat2 and CHO cells express Sap-1, but not Elk-1. Expression of Elk-1 made Rat2 cells (but not CHO cells) insulin responsive. C/EBPα also binds to the prolactin promoter at a sequence overlapping the binding site for Elk-1. Expression of both C/EBPα and Pit-1 in CHO cells is required for high basal transcription of prolactin-CAT. Expression of Elk-1 converts CHO cells into a phenotype in which prolactin gene expression is increased by insulin treatment. Finally, antisense mediated reduction of Elk-1 in GH4 cells decreased insulin-increased prolactin gene expression and confirmed the requirement for Elk-1 for insulin-increased prolactin gene expression. Thus, both C/EBPα and Pit-1 were required for high basal transcription while insulin sensitivity required Elk-1.

It is important to identify the insulin responsive transcription factor(s) that mediate insulin regulation of gene transcription. This will allow the definition of signaling pathways to those factors. It may also suggest strategies for replacing insulin in these processes. The proximal prolactin promoter contains sequences that mediate cell-specific expression and respond to numerous stimuli including insulin, epidermal growth factor (EGF), and agents that elevate cAMP. The transcription factor Pit-1 mediates cell type-specific prolactin gene transcription by binding to several sites in the proximal and distal prolactin promoter (1, 2). Pit-1 is a Pou domain protein that is found in pituitary lactotrophs, somatotrophs, and thyrotrophs. Insulin, EGF and cAMP act at an overlapping element at −100 to −66 that is also critical for high basal transcription of the prolactin gene (3, 4). This control region contains a cAMP responsive sequence TGACGCA. Overlapping this element is an Ets-factor binding sequence CCGAAA that is repeated at −71 to −68. This element was shown to mediate the effects of insulin, insulin-like growth factor-1, and EGF (3, 4).

Furthermore, mutation of this multiresponse element causes a profound reduction in basal prolactin gene expression (3). This result from elimination of C/EBPα binding that is necessary for high-level basal prolactin gene expression (5). The other transcription factors that interact at this complex element have not been identified, but it is likely that at least one Ets-related factor functions through this element. Expression of the DNA-binding domain of Ets-2 acts as a dominant negative inhibitor of transcription mediated by Ets-related factors. Expression of this inhibitor in GH cells partially blocks both insulin and EGF-increased prolactin-CAT expression (4, 6). Ets-related factors have been shown to bind to this promoter region (6, 7).

The Ets-related proteins are a large family of transcription factors with a highly conserved DNA-binding domain (8). This domain has a basic region and a cysteine-rich region similar to the binding domains found in Myc and Myb. These proteins specifically interact with sequences containing the core trinucleotide GGA. Although this sequence is essential for Ets-factor binding, flanking sequences are also important and may determine the different sequence specificity of these proteins (9). The Ets-binding domain is also crucial to protein-protein interactions that determine the biological function of the DNA-protein interaction (10). The activity of Ets-related factors was shown to be altered by phosphorylation (11). C/EBPs belong to the bZip family of transcription factors. These have a conserved COOH-terminal domain containing a basic DNA-binding domain and a leucine zipper that mediates protein-protein interaction. At least 6 different genes have been identified that produce C/EBP-related proteins, c/ebpα, c/ebpβ, c/ebpγ, c/ebpδ, c/ebpε, and c/ebpζ (12–15). Several of these have alternate splice variants. Thus, C/EBPα is found in alternately translated 42- and 30-kDa forms. The 42-kDa form

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is initiated from the first start codon and activates gene transcription while the 30-kDa protein is initiated from the third start codon and has been shown to be inhibitory (16). These proteins can bind to DNA only as homo- or heterodimers and heterodimerization between stimulatory and inhibitory bZIP proteins has been shown to be important in regulating their activity. The consensus DNA binding sequence for bZIP proteins is T/T/G/INGNAA/T/G (17). C/EBPs are expressed only in terminally differentiated tissues such as liver and adipocytes. C/EBPs have been linked to numerous genes that are regulated by insulin and/or cAMP such as phosphoenolpyruvate carboxykinase (14) and the acetyl-CoA carboxylase gene (18).

Physical interactions between transcription factors are known to be important for a number of processes. The recruitment of CBP/p300 by phosphorylated CREB was shown to account for the activity of CREB (19). The activity of the nuclear receptor superfamily of transcription factors is modulated by a number of co-repressors and co-activators all of which physically interact (20). C/EBPs directly interacts with the DNA-binding domain of c-Myc (21). The Ets-related factors Elk-1 and Sap-1 were identified as proteins interacting with serum response factor on the c-fos promoter (22, 23).

Previous studies identified Ets-related proteins in GH4 cells and nuclear extracts from GH4 cells (3) and suggested that an Ets-related transcription factor mediated insulin increased prolactin gene expression (6, 7). Therefore, plasmids that expressed LexA fusion proteins of a variety of Ets-related factors were prepared. Insulin-increased CAT expression was observed only with the fusion proteins containing the ternary complex factors Elk-1 and Sap-1 (22). The observation that knockdown of Elk-1 reduced insulin-increased prolactin gene expression commenced with Elk-1 reduction indicated that Elk-1 was required for insulin sensitivity. This was supported by transformation of Rat-2 and CHO cells to insulin-sensitive phenotype by Elk-1 expression (Sap-1 is naturally expressed in those cell lines). However, insulin-sensitive transcription in CHO cells also required C/EBPα. Thus, both Elk-1 is required for insulin-increased prolactin gene expression under conditions where Pit-1 and C/EBPα support basal transcription of the gene.

**EXPERIMENTAL PROCEDURES**

**Materials**—Tnt lysates for transcription translation were purchased from Promega. Glutathione-agarose, acetly-CoA, and silicon gel plates were obtained from Sigma. Reagents used for gel electrophoresis were purchased from Fisher Scientific. A filter binding kit for preparation of mRNA was purchased from Almacron. The production of the correct sized fusion proteins in yeast and/or bacteria was verified by Western blotting with antibodies to LexA, Elk-1, Sap-1, and C/EBPα.

**Transient Gene Transfection Facilitated by Electroporation**—Electroporation experiments and CAT assays were performed as described (25). GH4 cells were placed in Dulbecco's modified Eagle's medium that contained 10% hormone-depleted serum for 24 h, 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 99%. The voltage of the electroporation was 1550 volts. This gives trypan blue exclusion of 70 to 80% after electroporation. The transfected cells were then plated in multiwells dishes (Falcon Plastics) at 5 × 10^6 cells/9-cm² tissue culture well in Dulbecco's modified Eagle's medium with 10% hormone-depleted serum. Cells were reseeded at 24 h with Dulbecco's modified Eagle's medium with 10% hormone-depleted serum. L+[35S]Methionine-labeled proteins were prepared using TNT reticulocyte lysates (Promega). L-[35S]Methionine-labeled protein expression vectors containing various fractions of the wild type protein are listed in the appropriate figure legends. The CDNA for Elk-1 and Sap-1 were also cloned into pcDNA3 to make plasmids pcDNA-Elk and pcDNA-Sap. The plasmid pGEX-2T-C/EBPα expressing a glutathione S-transferase (GST)-C/EBPα fusion protein was constructed by inserting the NorI fragment of C/EBPα into the vector pGEX-kg. The NorI insert includes amino acids 1 to 353 of C/EBPα. GST-Elk(1-105/350) and GST-Sap(95/340) were made by cloning a PCR fragment into the EcoRI site of pGEX-2T (Amersham Pharmacia Biotech). The production of the correct sized fusion proteins in yeast and/or bacteria was verified by Western blotting with antibodies to LexA, Elk-1, Sap-1, and C/EBPα.

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 transfected into the cells. The β-galactosidase activity in the cell lysates was determined using o-nitrophenyl-β-D-galactopyranosidase. The transfection efficiency did not vary significantly among transfections performed at the same time. The percent acetylation was then corrected for minor variations in β-galactosidase activity by converting the percent acetylation to percent acetylation/A_590 β-galactosidase activity/mg of protein. The fold stimulation or inhibition was then determined. Statistical analysis was performed on all experiments and p values are presented for relevant comparisons.

**Protein-Protein Interaction Assay Using GST Fusion Proteins**—Glutathione-agarose beads (Sigma) were mixed with bacterial lysates containing GST fusion protein or GST. The beads were then washed extensively to remove unbound proteins. The amount of GST fusion protein or GST on the beads was estimated by dye binding after SDS-polyacrylamide gel electrophoresis and equal amounts of glutathione-agarose bound GST fusion protein or GST were used in each incubation. 5 × [35S]Methionine-labeled proteins were prepared using TNT reticulocyte lysates (Promega). Approximately equal amounts of [35S]labeled protein (as estimated from gel band using ImageQuant software, Amersham Pharmacia Biotech) were used in each incubation. The incubations were performed out for 1 h in 300 μl of a binding buffer containing 50 mM HEPES (pH 7.9), 6% glycerol, 5 mM EDTA, 5 mM MgCl₂, 0.05% Triton X-100, and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride. The beads were collected by centrifugation and washed three times with incubation buffer. The beads were then resuspended in SDS sample buffer and the bound proteins were analyzed by SDS-polyacrylamide gel electrophoresis.

**RT-PCR of Elk-1 and Sap-1 mRNA**—mRNA was prepared using a...
filter binding protocol ( Trevigen). 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 then used with primers for the mRNA for glyceraldehyde-3-phosphate dehydrogenase in a single tube RT-PCR assay (Tetra Link, Amberst, NY). The glyceraldehyde-3-phosphate dehydrogenase primers were 459GATTGTCACTGGTCAACTCC 945 and 820GCCAAGAAGTCGGTGGATAAGAAC 843. Elk-1 PCR was carried out for 30 cycles at 94 °C for 20 s, 55 °C for 20 s, and 68 °C for 20 s. In all of the RT-PCR experiments, the number of PCR cycles was adjusted to achieve linearity. For glyceraldehyde-3-phosphate dehydrogenase, 25 cycles were used to maintain linearity and samples varied less than 25%. The RT-PCR product from this reaction was quantitated using a Fluoromager 575 and ImageQuant software (Amersham Pharmacia Biotech). Equal amounts of mRNA (based on glyceraldehyde-3-phosphate dehydrogenase signal) were then used for assay of C/EβPa, Elk-1, Pit-1, and Sap-1 mRNAs. Elk-1 primers were 190TCTGTGCCTTCCTGTCATTATGG 212 and 1392GCCCAGACAGAGTGAATGGCCCATGACTG 1364. Sap-1 PCR was carried out for 30 cycles at 94 °C for 20 s, 55 °C for 20 s, and 68 °C for 20 s. Sap-1 primers were 891GCTTTTGCCACCACACCATTTCG 917 and 663CCTCCGTTTCCTCTTCCTTTCG 683. Pit-1 PCR was carried out for 30 cycles at 94 °C for 20 s, 55 °C for 20 s, and 68 °C for 5 s. Pit-1 primers were 190TCTGTGCTCCTGCCTATTG 212 and 664CCTCCGTTCCTCTCCTCCCTGG 685. Pit-1 PCR was carried out for 25 cycles at 94 °C for 20 s, 55 °C for 20 s, and 68 °C for 30 s.

RESULTS

LexA Fusion Proteins of Elk-1 and Sap-1 Mediate Insulin-Induced Transcription—Previous studies (3, 6, 7) suggested that insulin-increased prolactin gene expression was mediated by an Ets-related transcription factor. Therefore, LexA fusion proteins were constructed with a variety of Ets-related transcription factors to determine whether any of these could mediate the effects of insulin. LexA was chosen instead of the more common Gal4 since the Gal4 operator contains an Ets-response element and is strongly activated by insulin in GH4 cells.2 GH4 cells were co-transfected with the Lex6X-CAT reporter plasmid and various LexA-Ets fusion protein vectors and incubated with or without insulin for 24 h (Fig. 1). The LexA fusion constructs of Elk-1 (LexA-Elk-(105/428)) and Sap-1 (LexA-Sap-(90/431)) were insulin responsive. Insulin increased Lex6X-CAT expression 9–10-fold in GH4 cells transfected with LexA fusion proteins containing the C terminus of either Elk-1 or Sap-1 (Fig. 1). EGF also increased Lex6X-CAT expression 8-fold in LexA-Elk-transfected GH4 cells, but no increase in Lex6X-CAT expression was observed in LexA-Sap-transfected cells treated with EGF. The plasmids expressing LexA-GABPs (1–320), LexA-Ellf-(1/195), LexA-Ellf-(292/536), and LexA-Ets1-(1/332) were not insulin or EGF responsive (Fig. 1). Finally, a LexA-SRF fusion protein was also tested since Elk and Sap were shown to cooperate with SRF in growth factor and serum stimulation of the c-fos promoter (22, 27). However, neither insulin nor EGF increased Lex6X-CAT expression in the presence of LexA-SRF fusion protein.

Rat2 Cell Conversion into Cells with Insulin-Responsive Prolactin Promoter Expression by Expression of Elk-1 and Pit-1—Prolactin-CAT expression is insulin sensitive in GH4 and HeLa cells, but not in Rat2 cells. Transcription from the prolactin promoter is increased by cAMP in Rat2 cells. This implies that the lack of insulin responsiveness is not due to a general inactivation of the prolactin promoter in Rat2 cells and suggested that the insulin-sensitive transcription factor might not be present in Rat2 cells. RT-PCR was used to estimate levels of expression of Elk-1 and Sap-1 in the GH4, HeLa, Rat 2, CHO, and 3T3 cells (a positive control) since levels of Elk-1 and Sap-1 were too low to be detected by Western blotting in our cultures. All of the cell lines have measurable, but variable amounts of Sap-1 (Fig. 2, bottom). However, Elk-1 was not detectable in lysates from Rat2 cells or CHO cells (Fig. 2, top). Therefore, Rat2 cells were transfected with an expression vector for Elk-1 (MLV-Elk-1) along with the prolactin-CAT reporter, RSV-Pit-1 (to increase transcription from the prolactin promoter), and the insulin receptor. Insulin increased prolactin-CAT expression 4–5-fold in Elk-1 transfected Rat2 cells (Fig. 3). This compares to the 8–10-fold increase in prolactin-CAT expression in cAMP-treated GH4 cells. Sap-1, SRF, Ets1, E1Fl, and GABP were also expressed under the same conditions to determine if this effect was specific to Elk-1. Insulin did not increase prolactin-CAT expression with any of these transcription factors. Thus, Elk-1 specifically mediated the insulin-increased expression of prolactin-CAT.

C/EβPa, Elk-1, and Sap-1 Interact in Vitro—However, prolactin-CAT expression was not increased in CHO cells (that also lack Elk-1) under the same conditions (see Fig. 5, below). This implied that another transcription factor was required for insulin-increased prolactin-CAT expression in addition to Elk-1 and Pit-1. It was possible that this factor was C/EβPa since C/EβPa binds to the prolactin promoter at a site overlapping the insulin response element (5). A physical interaction between Elk-1/Sap-1 and C/EβPa was established by the association of 35S-Sap-1 or 35S-Sap-1 translated in vitro with GST/C/EβPa (Fig. 4, top). Elk-1 (lane 2) binds to C/EβPa with an apparent lower affinity than Sap-1 (lane 5) since an equal number of counts/min of 35S-labeled protein was used in each case. However, binding of SRF (lane 8) used as a control, was not detected.

Elk-1 and Sap-1 also interact with each other (Fig. 4, bottom, lanes 2, 6, and 10) and with GST-Sap (95–340) (Fig. 4, bottom, lanes 3, 7, and 11). None of

Fig. 1. Effect of other LexA-transcription factor fusion proteins on Lex6X-CAT expression. GH4 cells were electroporated with 10 µg of Lex6X-CAT (26), 5 µg of an expression vector for the human insulin receptor, pRTH3HR2 (J. Whittaker), and 2 µg of Rous sarcoma virus-β-galactosidase. Vectors (10 µg) that express the LexA DNA-binding domain (1/202) fused to a portion of the cDNA for a transcription factor fused COOH-terminal to and in-frame with the LexA DNA-binding domain were also included in each electroporation. The transcription factors used were Ets1, Elk-1, Sap-1, GABPα, and SRF. A vector containing only the LexA DNA-binding domain was used as a control. After 24 h, the medium was exchanged and 1 µg/ml insulin, or 40 ng/ml EGF 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 percent 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.

2 K. K. Jacob and F. M. Stanley, unpublished observation.
into CHO cells transfected with Elk-1 and Pit-1 (Fig. 5). A 2-fold increase in CAT activity in cultures electroporated with Pit-1 and C/EBPα was set as 1 since CAT activity in cultures without both Pit-1 and C/EBPα were too low to measure (data not shown). The results are from three separate experiments done in duplicate.

FIG. 2. Relative Elk-1 and Sap-1 mRNA levels in various cell types. The cell types indicated in the figure were cultured in growth medium until nearly confluent. They were washed with normal saline solution and solubilized in lysis buffer. The mRNA was then prepared. RT-PCR was performed first using primers for glyceraldehyde-phosphate dehydrogenase to standardize the level of mRNA between samples (data not shown). Standardization of the mRNA using RT-PCR of GAP-DH agreed closely with measurement of the optical density at 260 nm. The RT-PCR was then repeated using primers specific for Elk-1 and Sap-1 (top) and 18S (bottom). The PCR products were resolved on 1% agarose gel electrophoresis using ethidium bromide to stain the DNA. The image was then developed and quantitated using a FluorImager (Amersham Pharmacia Biotech). The input lanes contain 10% of the total 35S added in the incubations. Top, 35S-labeled Elk-1, Sap-1, or SRF were incubated with a GST-C/EBPα fusion protein or with GST that had been purified using GST-agarose (Sigma). The agarose beads were washed extensively and eluted in SDS sample buffer. The labeled proteins were resolved on a 10% SDS-polyacrylamide gel and visualized using a PhosphorImager (Amersham Pharmacia Biotech). The input lanes contain 10% of the total 35S added in the incubations. Bottom, 35S-labeled Elk-1, Sap-1, or SRF were incubated with GST-Elk-(105–350) or Sap-(89–340) fusion proteins or with GST that had been purified using glutathione-agarose. The samples were washed, resolved, and visualized as above.

the 35S-labeled proteins associated with the GST control (Fig. 4, bottom, lanes 4, 8, and 12).

CHO Cells Are Converted into Cells with Insulin-responsive Prolactin Promoter Transcription by Expression of Elk-1, Pit-1, and C/EBPα—Insulin did not increase prolactin-CAT expression in CHO cells electroporated with Elk-1 and Pit-1 (data not shown). This was unlike the response of Rat2 cells (Fig. 3) and suggested that at least one other transcription factor (in addition to basal factors) was required for insulin-increased prolactin-CAT expression. The interactions between Elk and Sap and C/EBPα suggested that C/EBPα might be the factor that is low or missing in CHO cells. Expression of the prolactin-CAT reporter plasmid was undetectable in CHO cells transfected with either C/EBPα or Pit-1 alone while co-transfection with both Elk-1 and C/EBPα resulted in high level expression from the prolactin promoter. However, insulin treatment had no effect unless Elk-1 was co-transfected with C/EBPα and Pit-1 (Fig. 5). Only insulin increased prolactin promoter activity when any of the other Ets-related transcription factors (the <2-fold increase seen in Sap-1 electroporated cultures was not significant). Thus, CHO cells can be transformed into an insulin-sensitive phenotype through co-expression of the transcription factors Elk-1 and C/EBPα.

Knockdown of Elk-1 in GH4 Cells—Knockdown experi-
Elk and C/EBP Transformation of CHO Cells

Fig. 6. Insulin-increased prolactin-CAT expression is inhibited by antisense Elk-1. Antisense oligonucleotides were delivered into GH4 cells by scraping the cells from the dish in the presence of 1 μM antisense oligonucleotide. Pores open in adherent cells when they are scraped from the plastic dish and small molecules in the medium can enter the cell for a short period after scraping (28). The GH4 cells were then plated in growth medium. The cells were harvested after 24 h and electroporated with 10 μg of pPr(−173/+75)CAT (28), 5 μg of an expression vector for the human insulin receptor, pRTHIR2 (J. Whitaker), and 2 μg of Rous sarcoma virus-β-galactosidase and with or without 1 μM antisense oligonucleotide. 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 percent acetylation, 10 μg of protein in control and insulin-treated cultures was determined, adjusted for β-galactosidase expression, and the CAT activity from cells incubated with insulin were compared with control levels to determine the fold-stimulation (Fold-Control). The results are from three separate experiments done in duplicate.

ments could confirm that Elk mediates insulin-increased prolactin gene expression. Twelve different Elk-1 antisense oligonucleotides were designed to the 5′ region of the mouse Elk-1 gene flanking the first ATG. One of these, CATCAC- TAGGGAAGCACTCACGCCATT, was able to consistently reduce levels of Elk-1 mRNA without affecting C/EBPα, Pit-1, or Sap-1 mRNA. This oligonucleotide was delivered into GH4 by scraping the cells from the dish in the presence of 1 μM antisense oligonucleotide. Pores open in adherent cells when they are scraped from the plastic dish and small molecules in the medium can enter the cell for a short period after scraping (28). GH4 cells were also scraped in the presence of a control antisense oligonucleotide and without any oligonucleotide. The cells were harvested after 24 h and electroporated as described above with the prolactin-CAT reporter plasmid in the presence of 1 μM antisense oligonucleotide. Six wells were set up from each electroporation. Two each for determination of CAT activity in control and insulin-treated cultures and two wells for preparation of mRNA for comparative determination of the level of mRNA for Elk-1 and other transcription factors. The cells were treated as described under “Experimental Procedures.” Insulin-increased prolactin-CAT expression is reduced to 40% of control levels by treatment of GH4 cells with the antisense Elk-1 oligonucleotide (Fig. 6) while the level of CAT expression in the cells treated with the control antisense oligonucleotide is not significantly different from the control.

Antisense oligonucleotides can have many non-antisense effects (29). Therefore, it was important to determine the levels of Elk-1 in antisense-treated GH4 cells. Parallel wells were harvested in lysis buffer and mRNA was prepared. RT-PCR was then performed using primers to C/EBPα, Elk-1, Pit-1, and Sap-1. Comparative mRNA levels for control cells and cells treated with antisense Elk and the control antisense oligonucleotide are shown (Fig. 7). The antisense oligonucleotide to Elk-1 reduced Elk-1 mRNA levels to 30% of control (Fig. 7), while the control antisense oligonucleotide was without effect. The antisense oligonucleotides did not affect expression of C/EBPα, Sap-1, or Pit-1 mRNA.

DISCUSSION

Insulin-increased prolactin gene expression requires Elk-1—Fusion proteins containing the C terminus of either Elk-1 or Sap-1 mediated insulin increased prolactin-CAT expression (Figs. 1). These results were specific for Elk-1 and Sap-1 since LexA fusion proteins with other Ets-transcription factors, Elf-1, Ets-1, and GABPs (Fig. 1) did not mediate this response. This suggested that either Elk-1 or Sap-1 could mediate insulin-responsive gene transcription. Insulin-increased prolactin gene transcription is not observed in Rat-2 and CHO cells. The Rat-2 and CHO cells have endogenous Sap-1, but Elk-1 was not detectable in these cell lines. The expression of Elk-1 in Rat-2 and CHO cells, along with transcription factors necessary for establishing basal transcription of the prolactin gene, converts them into an insulin-sensitive phenotype. Finally, Elk-1 knockdown in GH4 cells reduced insulin-increased prolactin-CAT expression commensurate with reduced Elk-1 expression. These studies establish that Elk-1 is necessary for insulin-increased prolactin gene expression.

Sap-1 alone is not able to mediate insulin-responsive gene transcription since Rat2 and CHO cells expressed Sap-1 (Fig. 2) and yet were not insulin responsive (Figs. 3 and 5). This was surprising since either Elk-1 or Sap-1 could function independently in the activation of the serum response element of the c-fos promoter (22, 23, 27, 30). It is possible that Sap-1 levels in Rat-2 and CHO cells were below some threshold necessary to mediate insulin responses. However, this appears unlikely as insulin-increased prolactin-CAT expression was not observed in Rat-2 or CHO cells even when Sap-1 was exogenously expressed (Figs. 3 and 5). Thus, the role of Sap-1 in insulin-increased prolactin gene expression remains unclear. Sap-1 might play no role in insulin-increased transcription. Alternatively, it is possible that Elk-1/Sap-1 heterodimerization might be required for insulin-increased transcription. The interaction between Elk-1 and Sap-1 in the GST pull-down experiments suggested that Elk-1 and Sap-1 might heterodimerize in vivo to mediate effects on gene transcription. Elk-1 and Sap-1 were not known to form dimers in solution, but the phosphorylation dependent formation of a dimer on the c-Fos serum response
element was reported (31). Since the c-Fos serum response element has only a single, weak Ets-response element, this is likely a protein-protein interaction. The GST pull-down experiments (Fig. 4) demonstrated physical association between Elk-1/Etk-1, Elk-1/Sap-1, and Sap-1/Sap-1. The associations observed in our GST pull-down experiments are clearly independent of phosphorylation. The prolactin promoter has two Ets-response sequences. The CCGAA sequence at −96/−92 is the strongest while an AGGA sequence is at −76/−73. Thus, it appears likely that two Ets-related transcription factors can bind simultaneously to the prolactin promoter. Protein-protein interactions may stabilize this interaction and select for the heterodimer over the monomer. However, it has not yet proved possible to knockdown Sap-1 in GH4 cells. The use of antisense oligonucleotides designed against the sequence of human Sap-1 was unsuccessful. This is likely due to a substantial difference in the non-coding region of the Sap-1 gene from human and mouse. The sequences of the human Elk-1 and the mouse Elk-1 differ greatly in the 5′-untranslated region and the same is likely to be true of Sap-1. However, the sequence of the mouse and/or rat Sap-1 gene is unknown. Finally, no Sap-1 minus cell line has been identified.

C/EBPa Is an Accessory Factor for Elk-1 in Insulin-increased Transcription—Insulin does not increase prolactin-CAT expression in CHO cells that transiently express Elk-1 (data not shown) although insulin-increased Lex6X-CAT transcription is observed in CHO cells expressing LexA-Elk-1 fusion proteins (data not shown). This suggested that the insulin-signaling pathway was intact in CHO cells but that some other transcription factor required for insulin-sensitive prolactin-CAT expression was not expressed. The observation that C/EBPa was a physiological regulator of prolactin gene expression whose binding site overlaps the insulin response element (5) suggested that C/EBPa might be this required factor. C/EBPa associates with Elk-1/Sap-1 in vitro as indicated by association in GST pull-down assays (Fig. 4). However, the physiological significance of this observation is presently unclear. The C/EBPa binding element of the prolactin promoter is weak compared with the consensus C/EBP-response element. Thus, it is possible that interaction with Elk-1/Sap-1 stabilizes the weak association with the prolactin DNA. This seems unlikely since deletion of the Ets-binding element did not reduce basal prolactin gene expression as would be expected if this also weakened the association of C/EBPa with the prolactin promoter (3).

Expression of C/EBPa along with Pit-1 and Elk-1 in CHO cells converted CHO cells to an insulin responsive phenotype. Previous experiments showed that insulin-increased prolactin-CAT expression was inhibited by expression of Chap 10/Gad 53. This factor binds to C/EBPa and inactivates it. Thus, both knock-in and knock-down experiments now establish that C/EBPa is an accessory factor required for insulin-increased prolactin-CAT expression.

Several reports have suggested that C/EBP family members can mediate effects of CAMP (32, 33). It is possible that C/EBPa may mediate CAMP-increased prolactin gene expression since mutation of the C/EBP response element eliminates CAMP responsiveness. However, C/EBPa expression in CHO cells does not make the prolactin promoter CAMP responsive in those cells. This could be due to failure of CAMP signaling pathways to link to C/EBP in CHO cells or to lack of a co-regulator recruited by C/EBPa. Finally, the CAMP response might be mediated by another factor that associates in the complex of transcription factors that bind to this element.

It remains to be determined how the factors that interact in the promoter region of the prolactin gene increase transcription in response to insulin. The carboxyl terminus of Elk-1 contains numerous phosphorylation sites and experiments with LexA-Elk-1 fusion proteins indicated that this region of Elk-1 is essential for insulin-increased LexA-CAT expression mediated by these fusion proteins. This modification of Elk-1 might be required to recruit a co-activator complex to the promoter. It was reported that Elk-1 can recruit CBP to the Fos promoter (34). However, experiments using expression of E1a mutants did not support a role for CBP in prolactin promoter activation by insulin. Alternatively, phosphorylated Elk-1 might interact with the basal transcription factors such as TFIIB to stabilize the initiation complex. Further research will attempt to further define how insulin-modified Elk-1 activates transcription.

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