Cell Type-specific Protein-DNA Interactions at the cAMP Response Elements of the Prohormone Convertase 1 Promoter

EVIDENCE FOR ADDITIONAL TRANSACTIVATORS DISTINCT FROM CREB/ATF FAMILY MEMBERS*

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Erik Jansen, Torik A. Y. Ayoubi, Sandra M. P. Meulemans, and Wim J. M. Van de Ven‡
From the Laboratory for Molecular Oncology, Center for Human Genetics, University of Leuven and the Flanders Interuniversity Institute for Biotechnology, Herestraat 49, B-3000 Leuven, Belgium

The proximal promoter region of the neuroendocrine-specific human prohormone convertase 1 (PCI) gene contains two distinct cAMP response elements (CRE-1 and CRE-2). Both elements are essential in directing the cAMP-mediated hormonal regulation of PCI gene transcription. In this study, we have demonstrated that CRE-1 binds several trans-acting factors. In electrophoretic mobility shift assay experiments with nuclear extracts prepared from neuroendocrine AtT-20 and β-TC3 cells and non-neuroendocrine COS-1 cells, three specific protein-DNA complexes (I–III) were detected. Complexes II and III were shown to contain CREB-1 and ATF-1, respectively. The most slowly migrating complex I was only detected with the neuroendocrine cell lines and appeared to comprise a c-Jun-containing heterodimer. In addition, CRE-2 was shown to bind a protein that was only detected in nuclear extracts derived from the neuroendocrine cell lines. Antibody supershift experiments indicated that both the c-Jun-interacting protein in CRE-1 complex I and the CRE-2-interacting protein are distinct from known members of the basic domain, leucine zipper family of transcription factors. UV cross-linking experiments demonstrated that these potential novel proteins are ~100 and 60 kDa in size, respectively. Site-specific mutagenesis experiments demonstrated that the formation of both CRE-1 and CRE-2 complexes is correlated with the transcriptional activity of the proximal PCI promoter as has been shown in transient transfections with wild-type and mutant promoter constructs. In addition, it was shown that both CREB-1 and ATF-1 transactivate the human PCI promoter in transient transfection experiments.

A variety of regulatory peptides and proteins are generated from inactive precursors by endoproteolytic processing. This endoproteolytic cleavage, generally at sites consisting of paired basic amino acid residues, is a common post-translational modification of membrane and secretory proteins on the exocytic transport route. Such proteins include precursors of peptide hormones, neuropeptides, growth factors, coagulation factors, serum albumin, cell-surface receptors, adhesion molecules, and viral glycoproteins. All these proteins play important roles in a large variety of different biological processes, and their function depends on proteolytic cleavage of their respective precursor molecules (for reviews, see Refs. 1 and 2). In mammals, seven prohormone- and proprotein-processing enzymes, responsible for this cleavage, have been molecularly characterized.

The mammalian prototype of this enzyme family is furin (3, 4), which is involved in the cleavage of precursor molecules within the constitutive secretory pathway. The structure and expression of the FUR gene, encoding furin, have been analyzed in detail (5). It has been shown that the FUR gene is expressed in a wide variety of tissues and cell lines. Recently, we have shown that FUR gene transcription is regulated by multiple promoter regions (6). In addition to furin, the very recently cloned member of the prohormone convertase (PC) enzyme family, LPC (7), described as PC7 in Ref. 8, also exhibits rather a ubiquitous expression pattern and is capable of processing substrates within the constitutive secretory pathway.

In contrast to this, PCI (9, 10), also described as PC3 (11), and PC2 (9, 12) are neuroendocrine-specific (13) and have been shown to be involved in the tissue-specific processing of prohormones and neuropeptide precursors, e.g. proglucagon, proinsulin, prosomatostatin, proenkephalin, and pro-opiomelanocortin, within the regulated secretory pathway (14–20). In addition, it has been demonstrated that the cell type-specific processing of the multifunctional precursor protein pro-opiomelanocortin by PC1 and PC2 is down-regulated in antisense transfection experiments in pituitary corticotroph-derived AtT-20 cells (21).

Very recently, a novel plurihormonal syndrome, comprising impaired glucose tolerance, early-onset obesity, hypogonadism, and hypothalamic dysfunction, was found to be caused by a primary defect in prohormone processing. The complete failure of processing of a normal proinsulin molecule and a defect in pro-opiomelanocortin processing at the PCI cleavage sites strongly suggested aberrant PCI activity (22).

In previous experiments, we have cloned and sequenced the genomic DNA encompassing the 5′-flanking region of the human PCI gene, identified the transcription start sites, and localized transcriptional control elements (23). It was shown that neuroendocrine-specific human PCI gene expression and its hormonal regulation are directed by the proximal promoter region. Several positive regulatory elements were identified.

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‡ To whom correspondence should be addressed. Tel.: 32-16-345987/346080; Fax: 32-16-346073; E-mail: Wim.VandeVen@med.kuleuven.ac.be.

1 The abbreviations used are: PC, prohormone convertase; bp, base pair(s); CRE, cAMP response element; bZIP, basic DNA-binding and leucine zipper; ATF, activating transcription factor; CREB, CAMP response element-binding protein; EMSA, electrophoretic mobility shift assay; HTLV-I, human T cell lymphotropic virus type I.
within 224 bp of the proximal promoter region. This was found using AtT-20 cells and β-TC3 pancreatic insulinoma cells transfected with fusion genes containing the 5’-flanking region of the human PC1 gene linked to luciferase as reporter. Two structurally related cis-acting DNA elements located at −283 bp (CRE-1) and −263 bp (CRE-2) were found to specifically mediate cAMP-regulated expression of human PC1 promoter activity. CRE-1 matches the palindromic consensus CRE (TGACGTCGTA (24)), whereas CRE-2 is different (TGACGCTGT).

A number of nuclear proteins specifically bind to the CRE and CRE-like sequences (25), and these are all members of the bZIP superfamily of transcription factors. They are characterized by the presence of a basic domain required for DNA binding and an adjacent leucine zipper domain, which facilitates dimerization between family members (26). To identify the trans-acting factors involved in the hormone-mediated, transcriptional regulation of the human PC1 gene, we analyzed the nuclear proteins interacting with the CRE-1 and CRE-2 motifs of the proximal PC1 promoter. Our findings demonstrate that in addition to ATF-1 and CREB-1, a novel c-Jun-containing heterodimer binds to the CRE-1 site. Both ATF-1 and CREB-1 were shown to enhance PC1 promoter activity in transient transfection experiments. In addition, CRE-2 was found to specifically interact with a novel protein present in nuclear extracts derived from the neuroendocrine cell lines AtT-20 and β-TC3. No binding of CRE-2 was observed using nuclear extracts from the control non-neuroendocrine COS-1 cells.

**EXPERIMENTAL PROCEDURES**

Conditions for Cell Culture and Transfections—AtT-20 pituitary corticotroph cells (ATCC CRL1785), β-TC3 insulinoma cells (27), COS-1 kidney fibroblasts (ATCC CRL1650), and F9 teratocarcinoma cells (ATCC CRL1779) were cultured according to the suppliers’ protocols. The wild-type and mutant CRE-1- and CRE-2-containing PC1 promoter-luciferase reporter constructs have been described previously (23). An expression vector encoding the catalytic subunit of protein kinase A was kindly provided by Dr. R. A. Maurer. Expression vectors for ATF-1, CREB-1, and c-Jun were kindly provided by Drs. W. Schmid, G. Schütz, and B. Burgering, respectively. DNAs were purified using anion-exchange chromatography (NucleoBond AX, Macher Nagel, Duren, Germany). Unless otherwise indicated, cells were propagated in the prescribed media supplemented with 10% fetal calf serum. Cells were transfected using cationic liposomes (Lipofectamine, Life Technologies, Inc.) according to the manufacturer’s protocol. For each experiment, luciferase activity was determined in duplicate wells. The results are expressed as the mean of three independent transfection experiments. Cells were harvested at 24 h after the start of transfection, and luciferase reporter enzyme activity driven by the various human PC1 promoter fragments was determined with the luciferase assay system (Promega) using a Monolight 2010 luminometer (Analytical Luminiscence Laboratory).

**Nuclear Extract Preparation and EMSA Conditions**—Nuclear extracts were prepared according to Schreiber et al. (28). Protein concentrations in nuclear extracts were 5–10 mg/ml, as determined by the BCA protein assay (Pierce). Complementary oligonucleotide concentrations in nuclear extracts were 5–10 mg/ml, as determined by the BCA protein assay (Pierce). Complementary oligonucleotide concentrations in nuclear extracts were 5–10 mg/ml, as determined by the BCA protein assay (Pierce). Complementary oligonucleotide concentrations in nuclear extracts were 5–10 mg/ml, as determined by the BCA protein assay (Pierce).

**Protein-DNA Interactions at the Human PC1 Promoter**—Previous studies in our laboratory (23) have demonstrated the presence of two functional CREs located within the proximal promoter region of the human PC1 gene. These elements, CRE-1 (TGACGTCGTA) and CRE-2 (TGACGCTGT), have both been demonstrated to mediate the hormonal regulation of PC1 gene expression. Using site-specific mutagenesis, it was demonstrated that both CREs differentially contributed to the cAMP-mediated regulation of transcription. Although the palindromic consensus CRE-1 was demonstrated to be the most important element, site-specific mutagenesis of the CRE-2 sequence also clearly down-regulated the cAMP inducibility of PC1 gene transcription. In addition, cell type-specific effects were observed in neuroendocrine AtT-20 and β-TC3 cells versus non-neuroendocrine COS-1 cells.

To identify the trans-acting factors involved in the hormonal regulation of human PC1 gene transcription, we performed EMSA experiments with both CRE-1- and CRE-2-containing probes. As shown in Fig. 1 (lanes 1 and 4), in EMSAs using the CRE-1 probe, nuclear extracts derived from the neuroendocrine cell lines AtT-20 and β-TC3, respectively, gave rise to three retarded protein-DNA complexes (complexes I–III), whereas nuclear extracts prepared from the non-neuroendocrine COS-1 cells gave rise to only two complexes (Fig. 1, lane 2). These COS-1 complexes exhibited the same mobility as complexes II and III detected with the AtT-20 and β-TC3 nuclear extracts. Of interest is the difference in relative abundance of complex I versus complexes II and III when the AtT-20 extracts are compared with the β-TC3 extracts. In β-TC3 cells, the more slowly migrating complex I was the most abundant one, whereas in AtT-20 cells, complex I was as abundant as complexes II and III. As can be seen in Fig. 1 (lane 5), the CRE-2 probe gave rise to the formation of only a single retarded complex. Moreover, the mobilities of the CRE-1 complexes are clearly different from the mobility of the complex observed when the CRE-2 motif is used as a probe. Characterization of the CRE-2 complex is presented below.

To determine whether all three EMSA complexes observed with the PC1 CRE-1 sequence represent specific interactions of nuclear proteins with this probe, we tested several competitor oligonucleotides. As can be seen in Fig. 2A, in AtT-20 and COS-1 cells, the addition of increasing molar concentrations of unlabeled wild-type CRE-1 competes for formation of the retarded complexes. Moreover, no difference in competition pro-

**RESULTS**

**EMSA Complexes with the CRE-1 Motif of the Human PC1 Promoter**—Probes for UV cross-linking were prepared according to a published protocol (29). Briefly, the above-mentioned CRE-1 and CRE-2 oligonucleotides were labeled with [α-32P]dCTP (Du Pont NEN) on the antisense strand by primer extension reaction using Klenow enzyme (Boehringer Mannheim). The reaction was carried out in non-denaturing polyacrylamide gel. This bromodeoxyuridine substitution had no effect on the gel shift patterns (data not shown). CRE-2 nuclear extracts were incubated with the bromodeoxyuridine-substituted CRE-1 and CRE-2 probes in standard EMSA reaction mixes and analyzed on EMSA gels as described above. Subsequently, the gel was exposed to UV light for 15 min at 4°C in a UV Stratalinker 1800 (Stratagene), and the various cross-linked protein-DNA complexes were visualized by autoradiography of the wet gel for 2 h at 4°C. Gel slices containing the cross-linked complexes were excised from the gel, boiled in SDS sample buffer, resolved on a 10% SDS-polyacrylamide gel, and visualized by autoradiography. Colored Rainbow Markers (Amersham Corp.) were used as protein molecular mass markers.
In an in vivo situation. In an additional EMSA, we tested a probe containing a consensus AP-1-binding sequence (TGAGTCA). As can be seen in Fig. 3C, the addition of the c-Jun antibody gives rise to a superset of complex I in AtT-20 (lane 2) and β-TC3 (lane 5) nuclear extracts and does not interfere with the formation of complexes II and III. Therefore, complex I seems to contain c-Jun, a member of the bZIP transcription factor superfamily. It has been demonstrated that c-Jun can form heterodimers with members of the bZIP family and subsequently bind to CRE sequences (26). However, heterodimerization is highly selective due to the leucine zipper structure of each factor. Since all other antibodies tested did not interfere with the formation of complex I, this complex does not seem to represent either the well-described c-Jun/Fos (AP-1) heterodimer or a c-Jun/ATF-2 heterodimer. Both heterodimers have been shown to interact with palindromic CREs (26, 32). Another option would be that complex I represents the binding of c-Jun homodimers to the CRE-1 motif. However, in the presence of c-Fos, Jun/Fos heterodimers are preferred. Moreover, the binding of c-Jun homodimers to palindromic CREs is of lower affinity and therefore does not seem to reflect the in vivo situation. In an additional EMSA, we tested a probe containing a consensus AP-1-binding sequence (TGAGTCA). As can be seen in Fig. 3C, the addition of the AP-1 probe resulted in strong complex formation with proteins present in the AtT-20 and COS-1 nuclear extracts. The observed retarded complex migrates clearly due to the leucine zipper structure of each factor. Since all other antibodies tested did not interfere with the formation of complex I, this complex does not seem to represent either the well-described c-Jun/Fos (AP-1) heterodimer or a c-Jun/ATF-2 heterodimer. Both heterodimers have been shown to interact with palindromic CREs (26, 32). Another option would be that complex I represents the binding of c-Jun homodimers to the CRE-1 motif. However, in the presence of c-Fos, Jun/Fos heterodimers are preferred. Moreover, the binding of c-Jun homodimers to palindromic CREs is of lower affinity and therefore does not seem to reflect the in vivo situation. In an additional EMSA, we tested a probe containing a consensus AP-1-binding sequence (TGAGTCA). As can be seen in Fig. 3C, the addition of the AP-1 probe resulted in strong complex formation with proteins present in the AtT-20 and COS-1 nuclear extracts. The observed retarded complex migrates clearly differently from CRE-1 complex I. When the anti-c-Jun or anti-Fos antibodies were included (Fig. 3C, lanes 6 and 8), supershifts were observed. This indicates that both the c-Jun and Fos bZIP transcription factors are present in these nuclear extracts.
extracts and therefore do not underlie the differential complex I formation as observed with the CRE-1 probe. In conclusion, the results from the complex I antibody supershifts led us to hypothesize that complex I is composed of c-Jun, which binds to the PC1 CRE-1 site through heterodimerization with a novel, yet unidentified partner protein present in AtT-20 and β-TC3 cells, but not in COS-1 cells.

To obtain additional information on the presumed partner protein present in the heterodimeric c-Jun-containing PC1 CRE-1 complex I, we performed UV cross-linking experiments. The results are presented below.

**EMSA Complexes with CRE-2 of the Human PC1 Promoter**—In previous studies (23), we have demonstrated that the CRE-2 motif mediates activation of the human PC1 promoter in response to cAMP-mediated signal transduction. To identify the trans-acting factor(s) involved, we have performed EMSAs with the CRE-2 probe and nuclear extracts from the neuroendocrine AtT-20 and β-TC3 cells and the non-neuroendocrine COS-1 cells. As can be seen in Fig. 4A, CRE-2 complex formation was only detected in AtT-20 and β-TC3 cells (lanes 3 and 4), but not in COS-1 cells (lane 5). The absence of complex formation in COS-1 cells is not due to the quality of nuclear extracts since reference AP-1 probe (Fig. 3C, lane 4) and the CRE-1 probe (Fig. 4A, lane 1) clearly produced retarded protein-DNA complexes. As has also been shown above (Fig. 1), the mobility of the CRE-2 complex is clearly different from the mobilities of CRE-1 complexes I–III and is therefore likely to represent binding of nuclear protein(s) different from the ones identified in complexes I–III with the CRE-1 probe. To assess the specificity of CRE-2 complex formation, competition experiments were performed. As can be deduced from Fig. 4B (lanes 1–3), binding to the CRE-2 probe was efficiently competed for when unlabeled CRE-2 was added to the binding reaction. However, unlabeled CRE-2mut did not compete for binding of nuclear proteins to the wild-type CRE-2 probe (Fig. 4B, lanes 4–6). This is in agreement with our observation that labeled CRE-2mut did not bind nuclear proteins in EMSAs (data not shown). In a previous report (23), we have shown that in addition to CRE-1 mutagenesis, the conversion of the CRE-2 sequence into CRE-2mut clearly affects the cAMP-mediated transcriptional induction of the proximal PC1 promoter. This indicates that, in addition to the CRE-1-bound proteins, the CRE-2-interacting protein is also involved in regulating PC1 promoter activity in response to cAMP-mediated signal transduction.

It has been demonstrated in Fig. 2C that CRE-2 did not compete for binding of nuclear proteins to the labeled CRE-1 probe. In the reciprocal experiment, we tested whether the addition of excess unlabeled CRE-1 competitor could prevent CRE-2 complex formation. The results, as depicted in Fig. 4C, clearly indicate that CRE-1 did not compete for binding of nuclear factors to the labeled CRE-2 probe. This strongly suggests that the CRE-2-bound protein is distinct from the CRE-1-interacting transcription factors. Subsequently, we performed antibody supershift experiments with the same set of monospecific antibodies used in the CRE-1 EMSAs. In these immunoshift experiments, we did not observe any effect of the addition of the antibodies on CRE-2/protein complex formation with nuclear extracts derived from both AtT-20 and β-TC3 cells (data not shown). In conclusion, although we were not able to identify the CRE-2-bound protein(s), the EMSA results strongly suggest that the interacting protein is distinct from known members of the bZIP superfamily of transcription factors.

**Characterization of CRE-1- and CRE-2-binding Proteins by UV Cross-linking**—Information on the molecular mass of the PC1 CRE-1- and PC1 CRE-2-binding proteins was obtained by UV cross-linking experiments. In these experiments, AtT-20
nuclear extracts were incubated with CRE-1 or CRE-2 probes in standard EMSA reaction mixtures and size-fractionated on EMSA gels as described above. Subsequently, the gel was exposed to UV light, and the various cross-linked protein-DNA complexes were visualized by autoradiography of the wet gel. Gel slices containing cross-linked CRE-1 complexes I–III and FIG. 3. EMSA antibody supershift analysis of the PC1 CRE-1 complexes and control consensus AP-1 probe. A, COS-1 nuclear extracts incubated with the CRE-1 probe. Lane 1, no antibody added; lane 2, preincubation with the ATF-1 antibody; lane 3, preincubation with the CREB-1 antibody. B, AtT-20 (lanes 1–3) and β-TC3 (lanes 4 and 5) nuclear extracts incubated with the CRE-1 probe. Lanes 1, 3, and 4, no antibody was added; lanes 2 and 5, preincubation with the c-Jun antibody. C, CRE-1 and CRE-2 complex formation with AtT-20 nuclear extracts (lanes 1 and 2, respectively) compared with AP-1 complex formation with AtT-20 nuclear extracts (lanes 3 and 5–8) and COS-1 nuclear extracts (lane 4). Shown are antibody supershifts with the c-Jun antibody (lane 6) and the Fos antibody (lane 8) added to the EMSA reactions.

FIG. 4. Specificity of PC1 CRE-2 complex formation as compared with formation of the CRE-1 complexes. A, nuclear extracts prepared from COS-1 cells (lanes 1 and 5), AtT-20 cells (lanes 2 and 3), and β-TC3 cells (lane 4) were incubated with the radiolabeled CRE-1 probe (lanes 1 and 2) or the CRE-2 probe (lanes 3–5). B, shown are EMSA competition experiments with the CRE-2 probe. Nuclear extracts were prepared from AtT-20 cells, and unlabeled CRE-2 was added as competitor oligonucleotide in a 50-fold (lane 2) or 100-fold (lane 3) molar excess. In lanes 5 and 6, unlabeled CRE-2mut was added as competitor oligonucleotide in 100- and 1000-fold molar excesses, respectively. C, shown is the EMSA competition with the CRE-2 probe. AtT-20 nuclear extract was used, and unlabeled CRE-1 was added as competitor in a 100-fold (lane 2) or 1000-fold (lane 3) molar excess.
the CRE-2 complex were excised from the gel, boiled in SDS sample buffer, size-fractionated on a 10% SDS-polyacrylamide gel, and visualized by autoradiography.

As can be seen in Fig. 5 (lane 1), cross-linking of the nuclear proteins present in CRE-1 complex I to radiolabeled CRE-1 results in two protein-DNA complexes of ~50 and 110 kDa. Because the protein-DNA samples were boiled and the molecular mass of the single-stranded oligonucleotides was ~9 kDa, it can be deduced that the proteins present in CRE-1 complex I are ~40 and 100 kDa in size. The detection of a protein of ~40 kDa is in agreement with our previous observation in antibody supershift experiments that c-Jun (39 kDa) is present in complex I. Therefore, the novel heterodimerized partner of c-Jun as present in PC1 CRE-1 complex I is now determined to be ~100 kDa in size. In a similar way as for CRE-1 complex I, the proteins of CRE-1 complexes II and III, cross-linked to radiolabeled CRE-1, were determined to be 35 and 45 kDa in size (Fig. 5, lane 2). This coincides with the data obtained in the antibody supershift experiments, in which the presence of ATM-1 (36 kDa) and CREB-1 (43 kDa) in these complexes was clearly demonstrated. As can be seen in Fig. 5 (lane 3), the PC1 CRE-2 sequence is bound by a nuclear protein of ~60 kDa. This is, in addition to the distinct CRE-1 and CRE-2 EMSA patterns (Figs. 1, 3C, and 4A), another difference between the CRE-1 and CRE-2 DNA-binding proteins.

Functional Analysis of the CRE-1-binding Proteins—To investigate the functional significance of ATM-1, CREB-1, and c-Jun in the regulation of PC1 gene expression, we have performed transient transfection experiments in which PC1 promoter-reporter constructs containing wild-type, mutant CRE-1 (CRE-1mut), and mutant CRE-2 (CRE-2mut) sequences were tested for transcriptional activation by ATM-1, CREB-1, and c-Jun. Functional analysis was performed by introducing expression vectors encoding these factors into F9 teratocarcinoma cells along with the PC1 promoter-luciferase reporter constructs and an expression vector encoding the catalytic subunit of protein kinase A. F9 cells provide a good system to analyze protein kinase A-mediated transcriptional regulation of target promoters because of low levels of endogenous protein kinase A-responsive transcription factors (33, 34, 36).

As shown in Fig. 6A, the PC1 promoter construct containing wild-type CRE-1 and CRE-2 sequences was activated 3- and 12-fold by ATM-1 and CREB-1, respectively. Activation was shown to be protein kinase A-dependent since no significant change in promoter activity was observed when the cotransfected protein kinase A DNA was replaced by a similar amount of empty expression vector. This is in agreement with previous reports in which transcriptional activities of ATM-1 and CREB-1 were investigated and in which differential activation was observed (34, 36). In addition, as shown in Fig. 6A, protein kinase A only modestly stimulated the PC1 promoter, which coincides with previous observations that F9 cells have low levels of endogenous protein kinase A-responsive transcription factors (34, 36). In contrast to the results obtained with F9 cells, introduction of the protein kinase A expression vector, without cotransfecting ATM-1 or CREB-1, in AtT-20 and COS-1 cells resulted in a 15–20-fold induction of PC1 promoter activity (data not shown), which illustrates the high levels of protein kinase A-responsive, CRE binding activities in these cell lines.

In contrast to the observed activation by ATM-1 and CREB-1, we were not able to detect an effect of c-Jun on PC1 promoter activity in transient transfections of F9 cells, COS-1 cells, AtT-20 cells, and β-T3 cells.

In additional experiments, PC1 promoter constructs containing CRE-1mut or CRE-2mut sequences were assayed for transactivation by ATM-1 and CREB-1. The results (Fig. 6B) indicate that no activation by ATM-1 or CREB-1 is observed when CRE-1 is mutated. However, no significant difference in activation by ATM-1 and CREB-1 is seen when CRE-2 is mutated. This supports our previous observations (Fig. 3A) that ATM-1 and CREB-1 form part of the CRE-1 binding activities and that the CRE-2 binding activity is distinct from ATM-1/CREB-1 family members. In conclusion, the results from the transfection experiments indicate that ATM-1 and CREB-1 are involved in the regulation of PC1 gene expression. The functional significance of the c-Jun-containing complex, however, remains to be established.

DISCUSSION

In a previous report (23), we have shown that the proximal promoter region of the human PC1 gene confers both basal and hormone-regulated transcriptional activity. The results of site-specific mutagenesis experiments have demonstrated that two distinct, closely spaced elements (CRE-1 and CRE-2) within the proximal promoter region direct cAMP-mediated hormonal regulation of transcription of the PC1 gene. In our effort to identify the transcription factors involved, we have now focused our attention on the analysis of protein-DNA interactions at the PC1 CRE-1 and CRE-2 regulatory elements.

In this study, we have demonstrated that PC1 CRE-1 is bound by multiple trans-acting factors. In EMSA experiments with a CRE-1 probe, nuclear extracts from AtT-20, β-T3, and COS-1 cells gave rise to the formation of two common protein-DNA complexes (complexes II and III), which were demonstrated to contain transcription factors CREB-1 and ATM-1, respectively. In addition, it was demonstrated that site-specific mutagenesis of the PC1 CRE-1 element disrupted binding of both ATM-1 and CREB-1 to this regulatory element within the human PC1 promoter. This coincides with our previous observation (23) that the same mutation also results in a decreased cAMP-mediated activation of the human PC1 promoter, which is indicative of the involvement of ATM-1 and CREB-1 in the regulation of PC1 gene expression. Additional support for this hypothesis was provided by the results obtained in transient
transfection experiments in which both ATF-1 and CREB-1 were shown to enhance PC1 promoter activity, although to a different extent. Moreover, it was shown that CREB-1 was about four times more potent in the activation of the PC1 promoter. Similar observations with various promoters have been reported previously (36). This effect is exerted through CRE-1 since only CRE-1 mutagenesis abolished the transcriptional activation. Since both ATF-1 and CREB-1 activities have been shown to be differentially modulated by the protein kinase A- and protein kinase C-mediated phosphorylation of distinct amino acid residues (33–37), the interaction of both ATF-1 and CREB-1 with the PC1 CRE-1 sequence provides an integration point of several signal transduction pathways regulating the transcriptional activity of the human PC1 gene. Apart from the crucial function of CREB-1 in modulating the activity of several hormone-regulated neuroendocrine-specific genes, the involvement of both CREB-1 and ATF-1 has been demonstrated for several other promoters. Recently, both CREB-1 and ATF-1 have been shown to be involved in the cell type-specific CRE-mediated regulation of the neural and thyroid T cell-specific calcitonin gene (38), and both factors also represent the regulatory DNA binding activities within oxygen tension-regulated genes, e.g., the erythropoietin (EPO) gene (39).

In addition to the above-mentioned PC1 CRE-1 complexes II and III observed with AtT-20, β-TC3, and COS-1 nuclear extracts, another protein-DNA interaction was detected, described as the more slowly migrating complex I. This EMSA complex was only detected in AtT-20 and β-TC3 nuclear extracts, but not in COS-1 nuclear extracts. The antibody supershift experiments demonstrated that c-Jun is part of this protein complex that can recognize PC1 CRE-1. In this context, it is important to note that c-Jun has been demonstrated to form heterodimers with specific members of the bZIP superfamily of transcription factors since heterodimerization is highly selective due to the leucine zipper structure of each factor (26). Apart from the well described heterodimerization with c-Fos, resulting in the AP-1 complex, c-Jun has also been detected as a functional heterodimer with ATF-2 (40). These heterodimers, which are also capable to bind to palindromic CREs (26, 32), have been described to be essential in regulating the transcriptional activity of their respective target genes in response to various extracellular stimuli (41, 42). However, the results of our antibody supershift experiments indicate that c-Jun as present in PC1 CRE-1 complex I is bound to the DNA through interaction with an novel partner protein that is clearly distinct from the known members of the bZIP family. Additional information was obtained by UV cross-linking experiments, indicating that this protein is ~100 kDa in size. In this context, it is interesting to note that it was recently shown that a palindromic CRE in the murine prostaglandin synthase-2 (PGS2) promoter is essential for transcriptional activity. Antibody supershift experiments demonstrated that, in addition to ATF-1 and CREB-1, c-Jun participates in a heterodimeric protein-DNA complex at the PGS2 CRE (43). However, in this study,
the partner protein was not further characterized, although it was shown to be distinct from c-Fos, ATF-2, and ATF-3. Recently, searches for additional c-Jun-interacting factors have led to the identification of a 25-kDa protein, Jif-1 (44), which binds to the bZIP domain of c-Jun, inhibits DNA binding, and reduces transactivation by c-Jun.

In the case of palindromic CREs, it has been shown that c-Jun mediates transcriptional activation. Since the c-Jun-containing PC1 CRE-1 complex was only detected in AT-20 and β-TC3 cells, this may provide additional means for the regulation of PC1 promoter activity in these cell lines. In transient transfection experiments, however, we were unable to detect an effect of c-Jun on PC1 promoter activity. This may be due to special features of the c-Jun partner protein, e.g. required modifications, essential for transcriptional activity, but not provided in the transfection experiments. It may be essential for this protein to be present in a similar amount as exogenous c-Jun. Since the nature of the c-Jun partner protein is still unknown, evaluation of the functional significance of the c-Jun-containing CRE-1-binding complex requires additional investigations. Functional significance, suggested by our findings that this c-Jun-containing complex is the predominant protein-DNA complex detected in EMSAs with β-TC3-derived nuclear extracts. The observed differential abundance of the CRE-1 complexes may very well underlie a potential difference in the regulation of PC1 promoter activity in response to various extracellular stimuli. As ATF-1, CREB-1, and c-Jun represent the final targets of various intracellular signal transduction pathways, they could act as mediators of pathway crosstalk resulting in the versatility of the transcriptional response to signal transduction.

We have demonstrated before that CRE-1 mutagenesis only partially abrogated activation of the PC1 promoter and that site-directed mutagenesis of CRE-2 also resulted in decreased PC1 promoter activation. Moreover, only when both CRE-1 and CRE-2 were mutated was transcriptional activation completely abrogated (23). Therefore, it is interesting to identify the transcription factor(s) involved in PC1 promoter activation via CRE-2. In the present study, we have demonstrated in EMSAs that a nuclear factor that is specifically present in AT-20 and β-TC3 cells interacts with CRE-2. The antibody supershifts indicated that this protein is not a known member of the bZIP transcription factor family and is also not an immunologically related factor. Since CRE-2 mutagenesis abrogated complex formation in EMSAs and also decreased transcriptional activation in transient transfection analysis (23), this suggests that the CRE-2-bound protein is involved in the regulation of PC1 gene expression. By means of UV cross-linking experiments, we have determined that the molecular mass of this putative novel trans-acting factor is ~60 kDa.

A sequence homology search of known cAMP-responsive regions in various promoters revealed until now only one additional example of a functional regulatory element homologous to the core sequence of the PC1 CRE-2 motif. This motif is present in one of the three 21-bp repeats of the HTLV-I long terminal repeat. These three 21-bp repeats all contain a core TGACGT motif with small variations in flanking sequences and mediate transcriptional activation. Moreover, it was shown that the most 5'-located CRE (TGACGTCT) and the CRE (TGACGTTG) within the central repeat are indispensable for transcriptional activation by the HTLV-I-encoded Tax protein. HTLV-I is the etiologic agent of adult T cell leukemia and a degenerative neurologic syndrome. Tax is critical for modulating HTLV-I gene expression and is also involved in cellular transformation. Recently, it was demonstrated that this nuclear protein, which does not directly interact with DNA, activates transcription through interaction with cellular factors that are able to bind to the HTLV-I long terminal repeat, including members of the CREB/ATF family (45). In addition, it was shown that Tax activates transcription of the human immunodeficiency virus type I long terminal repeat and several cellular CRE-containing genes including interleukin-2 and proenkephalin (46, 47). Since this transcriptional activation is dependent on the presence of CREs, Tax may be a potential activator of human PC1 expression.

In summary, our data have shown that multiple factors interact with the CRE motifs within the proximal PC1 promoter. Our observations support a direct involvement of ATF-1 and CREB-1 in the regulation of PC1 gene expression. Gene regulation through CRE motifs is of particular interest since these motifs have been found in the promoter regions of various genes encoding hormones and peptides. Proper functioning of neuroendocrine cells requires the coordinate expression of hormones and their respective processing enzymes.

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