Structure/Function Relationships of CREB/ATF Proteins

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Signal-transduction pathways converge ultimately at the level of transcriptional activation to produce specific patterns of gene expression in response to environmental stimuli. The initiation of transcription mediated by these signaling pathways is regulated by the coordinate expression and/or activation of specific transcription factors that bind to the control regions of genes. Specific insights into the mechanisms underlying transcriptional activation have recently arisen from studies of the structure and functions of these transcription factors. The CREB/ATF family of transcriptional transactivating proteins has only recently been discovered and appears to provide a link between the regulation of gene expression in response to activators of cellular signaling pathways and the regulation of gene expression by viral transactivating proteins. In addition, these proteins may be involved in the normal regulation of growth and differentiation. Understanding the nature and importance of the role(s) of these proteins in the normal regulation of growth and differentiation will have profound influences on the understanding of the aberrant regulation of these processes during oncogenesis. J Invest Dermatol 98:21S–28S, 1992

Hormonal activation of signal-transduction pathways results in the transcriptional stimulation of many cellular genes [1–5]. Studies of genes responsive to activators of the protein kinases A and C have identified similar DNA regulatory elements that mediate responses to these agents [6–19]. The octameric cyclic AMP (cAMP)-response element (CRE, 5'-TGACGTCA-3') differs from the heptameric phorbol ester (TPA) response element (TRE, 5'-TGAGTCA-3') by only a single base insertion or deletion. Multiple studies have demonstrated that the protein products of two protooncogenes, jun and fos, interact and mediate transcription through sequence-specific binding to the TRE motif [20–23]. The cloning of human placental CRE-binding protein (CREB) [24] marked the beginning of a long and continuing series of reports describing the cloning of related CREB and activating transcription factor (ATF) proteins, which interact and mediate transcription through sequence-specific binding to the CRE motif [24–30]. To date, the only members of this family that have been shown to mediate transcriptional responses to the activation of protein kinase A are the original CREB-327 isolate [24], an alternatively spliced variant of this factor isolated subsequently [30], CREB-341, and, most recently, an ATF-1/GAL4 fusion protein was shown to mediate a small transcriptional effect in response to co-transfection with the catalytic subunit of protein kinase A [31]. A specific member of the ATF group of transcriptional activators, ATF-2, has been shown to mediate adeno-viral EIA-stimulated transcription via sequence-specific binding to CRE motifs present in the adenovirus genome [32]. Therefore, the CREB/ATF proteins have been demonstrated to mediate transcriptional responses to both cellular signaling and viral stimulatory pathways through the common mechanism of sequence-specific binding to the CRE motif.

Recently, in collaboration with Dr. Aleem Siddiqui's laboratory, we have identified a new mechanism of viral-stimulated gene expression mediated by CREB/ATF proteins [33]. We demonstrated that the interaction of either CREB or ATF-2 with the hepatitis B virus (HBV) X protein produces a DNA-binding complex with a unique sequence specificity not exhibited by any of these proteins alone. This complex (either CREB:pX or ATF-2:pX) binds specifically to a motif in the HBV enhancer 1 with the sequence 5'-TGACGTCA-3'. More recently, Sheng et al [34] have demonstrated activation of CREB transcriptional activity by phosphorylation of CREB by calcium-calmodulin-dependent kinases I and II, suggesting that membrane depolarization and increases in intracellular Ca++ can also activate CREB-mediated transcription. Taken together, these data suggest that the CREB/ATF proteins represent an important family of transcriptional regulatory proteins that normally mediate transcriptional responses to extracellular events transduced to the nucleus via intracellular signaling pathways, but that these proteins are also critical components of the aberrant gene expression programs initiated and maintained by viral infections of normal cells.

The cellular control mechanisms that regulate growth and differentiation are influenced by hormone and growth-factor stimulated signal-transduction pathways, through the actions of CREB/ATF
proteins, and others. These pathways converge at the level of the nucleus to influence the expression of genes that dictate cell phenotype. It is thus critical that we understand the normal regulation of gene expression through these pathways, such that we may better understand the aberrant regulation of these processes during oncogenesis.

RESULTS AND DISCUSSION

Dominant Negative CREB/ATF Proteins Early studies on the functional domains of the first CREB cDNA isolate suggested that CREB, like many other previously characterized eukaryotic transactivator proteins, was a functionally bipartite protein. Several laboratories have demonstrated that the carboxy-terminal 64 amino acids of the CREB protein can function alone to bind DNA in a sequence-specific manner [35,36]. Chimeric activator fusion proteins containing the DNA-binding and dimerization domains of GAL4 and different regions of CREB sequences amino-terminal to the binding and dimerization domains of CREB were shown to be responsive to activation by protein kinase A [37]. This allowed the mapping of a phosphorylation domain in CREB that is absolutely required for the activation of this protein by A kinase. Early studies (Fig 1) of the overexpression of wild-type CREB in JEG-3 cells demonstrated that the levels of endogenous protein kinase A were insufficient to activate enough of the overexpressed CREB to see an increase in transcription of a reporter plasmid driven by the human alpha gonadotropin gene promoter, which has been shown previously in many laboratories to be extremely responsive to the activation of PKA [10,11,14,16]. This data was significant in that the results implied that the overexpressed CREB was binding to the promoter sequences of the reporter construct in an inactivated state and blocking the binding of any activated (PKA-phosphorylated) CREB. This was further emphasized when the co-transfection of the catalytic subunit of protein kinase A was all that was required to restore transcriptional responses to a level much greater than those noted for the cells that had not been transfected with the CREB expression vector (Fig 1). This was our first example of a dominant negative effect of CREB. Because all cells have different levels of endogenous protein kinase A, this does not represent the best method of producing a dominant negative response.

To carry these results further, we overexpressed true dominant negative versions of CREB and ATF-2 by expressing just the DNA-binding and dimerization domains of these proteins. The rationale behind this approach is that by expressing just the DNA-binding domain (in the absence of the transactivating domain) in sufficient quantities, we could potentially saturate the CREB/ATF binding sites on endogenous promoters and inhibit the binding of endogenous (activated) CREB/ATF proteins to these sites, and subsequently inhibit the expected transcriptional stimulation. We therefore deleted the amino-terminal sequences of CREB that are involved in transcriptional activation by PKA. We also deleted the amino-terminal sequences of ATF-2 that have been shown to be responsible for mediating the interaction of ATF-2 with E1a, which subsequently activates transcription. The results of transient co-transfection studies utilizing these plasmids are shown in Fig 2. Note that transcriptional stimulation in response to added 8-bromo-cAMP, or co-transfected PKA catalytic subunit, were completely abolished by the overexpression of these dominant negative proteins.

Constitutively Active CREB/ATF Proteins After realizing the utility of using the dominant negative CREB/ATF proteins for probing the functional aspects of CRE-mediated transcriptional activity, we then decided to produce and analyze the functional consequences of constitutively active CREB/ATF proteins. To this end, we obtained an expression vector that encodes a constitutively active ATF-2 protein from Dr. Michael Green's laboratory. This plas-
mid directs the expression of a fusion protein containing the full-length ATF-2 protein fused amino-terminal to the activating sequences of HSV VP-16 (amino acids 413–490). We have modified this vector to include only the sequences of ATF-2 from 254–505 fused to the same region of HSV VP-16. Transient co-transfection experiments demonstrate that the constitutively active ATF-2 induces alpha gonadotropin reporter gene activity three- to fivefold in the absence of exogenously added 8-bromo-cAMP in HeLa cells (Fig 3). This response is not as great as the magnitude of the response seen when the catalytic subunit of PKA is co-transfected with the same reporter plasmid in these cells (up to fiftyfold). However, we feel that the use of this constitutively active transcription factor of a defined class is a much more focused approach to the elucidation of the mechanism of action of PKA-stimulated growth and differentiation programs.

Similarly, we have constructed an expression plasmid to overproduce the constitutively active version of CREB. We have utilized only the DNA-binding and dimerization domains of CREB [254–327]. Using polymerase chain reaction (PCR), we have amplified this segment of the CREB cDNA incorporating a ribosome binding site before an initiator methionine and deleting the stop codon so that we could fuse the coding sequences of VP-16 (amino acids 413–490) in frame. Co-transfection of this plasmid with the human alpha gonadotropin reporter plasmid shows a consistently higher level of activation of the reporter than the corresponding ATF-2 protein (Fig 3). We believe that this result may be related to the affinity of the CREB fusion protein for the alpha gonadotropin CRE, but this may also be related to the levels of this protein expressed in comparison to the other. We are currently evaluating these possibilities.

Figure 3. Overexpression of constitutively active CREB or ATF-2 proteins in HeLa cells stimulates transcription from a human alpha-gonadotropin reporter (Luciferase) plasmid. Luciferase activity (in light units) for transfected reporter plasmid alone, or the same reporter plasmid cotransfected with expression plasmids encoding either ATF-2/VP-16 or CREB/VP-16 chimeric activators is presented.

Figure 4. Overexpression and purification of recombinant constitutively active CREB and ATF-2 with the pRSET system. Proteins in aliquots of total bacterial lysates or the different fractions denoted above each lane (and described in the text) were separated by SDS-PAGE and visualized by coomassie staining. The migration of prestained molecular size markers is denoted in the first lane of each panel.
for introducing these proteins directly into cells. We are currently assessing the utility of pinocytosis, electroporation, and folate receptor-mediated endocytosis for the direct introduction of recombinant proteins into mammalian cells. We have analyzed the time course of uptake and nuclear localization of the dominant negative CREB and ATF-2 proteins into FRTL-5 cells in culture. The FRTL-5 rat thyroid cell line is a unique model system in which to study the influence of increased intracellular cAMP levels on the processes of growth. These cells have an absolute requirement for TSH (acting through the adenylyl cyclase/protein kinase A pathway) in order to grow. Therefore these cells provide an ideal system in which the effects of constitutively active and dominant negative CREB/ATF proteins can be studied to elucidate the role of these proteins in the processes of growth. In preliminary studies, we found that these proteins were efficiently introduced into FRTL-5 cells by pinocytosis, are taken into cells immediately, translocate to the nucleus of several cells within 2 h, are found in the nucleus of all cells within 6 h, remain in the nucleus at 12 h, but by 24 h have translocated back out of the nucleus to the cytoplasm where they seem to be found in discreet structures (possibly lysosomes). At 24 and 48 h after addition, there is no protein found in the nucleus. These results are quite substantial in that we can introduce these proteins acutely into 100% of the FRTL-5 cells in culture and, further, these results attest to the feasibility of this approach for studying the effects of a dominant negative CREB and ATF-2 proteins on the regulation of growth in these cells.

The ability of the dominant negative CREB protein to influence transcriptional activation has been demonstrated previously by transient co-transfection of an expression vector encoding this protein into HeLa cells with the alpha gonadotropin reporter plasmid (see Fig 2). To investigate whether dominant negative CREB protein introduced into cells by pinocytosis would fulfill the same function, we introduced this protein into HeLa cells that had integrated a stable alpha gonadotropin reporter plasmid (Fig 5). Introduction of the dominant negative CREB protein into these cells resulted in a slight decrease in basal promoter activity, and almost completely abolished the response of this promoter to addition of 8-Br-cAMP. These results were consistent with those demonstrated in the co-transfection experiments, and demonstrate that the introduced protein is functionally competent. In fact, preliminary studies suggest that introduction of the functionally competent dominant negative CREB protein into FRTL-5 cells inhibits progression of these cells through the cell cycle in an acute fashion. Analyses of the percentages of cells in the various stages of the cell cycle by flow cytometry suggest that addition of the dominant negative CREB protein to the cells significantly inhibits progression of these cells through the cell cycle. These results provide the first direct evidence for the involvement of CREB in cell cycle progression and further attest to the importance of this diverse family of transcriptional regulatory proteins in the regulation of cellular phenotype.

Recombinant Proteins as Molecular Probes of CREB/ATF Functions and Family Diversity The CREB/ATF family of transcriptional activators provides a unique system to study protein/protein interactions because they bind their cognate DNA elements as homo- and/or hetero-dimers [19,24,28,30]. This dimerization is mediated through a structural motif, called a leucine zipper, at the carboxy-terminus of these proteins. The leucine zipper motif was originally described [38] as a periodic array of leucine residues spaced seven amino acids apart, placing these hydrophobic residues on the face of an idealized alpha helix. The leucine zipper motif is a common characteristic shared by the new class of transcription factors including myo, fos, C/EBP, GCN4, c-jun, jun-B, jun-D, fra, CREB/ATF proteins, and others [24,28,39]. The leucine zippers in these proteins can mediate specific complex formation between heterologous proteins by the parallel interaction of these helical domains in the structure of a coiled coil [40]. To investigate whether we could utilize the strong protein/protein interactions that are formed between homologous and/or heterologous leucine zipper proteins to detect specific dimerization

Use of Bacterially Expressed CREB/ATF Proteins to Investigate Functional Activity Recently, my co-workers and I have developed a new bacterial expression system that we have utilized to produce large quantities of the constitutively active and dominant negative CREB and ATF-2 proteins (manuscript in preparation). This system represents a significant improvement over the original T7 expression vectors introduced by Studier and co-workers by allowing efficient purification and detection of recombinant proteins through a multifunctional leader peptide incorporating a polyhistidine sequence, an epitope-tag, an enterokinase cleavage site, and a tyrosine residue for radio-iodination. Figure 4 depicts a typical purification of two separate proteins (the constitutively active CREB and ATF-2 proteins) expressed with our pRSET system. The efficient production and purification of up to 250 mg of recombinant protein from 1 l of bacterial culture can be performed in 2 d. For purification, constitutively active CREB and ATF-2 proteins were grown in 1-I cultures. The cell pellets were lysed in buffer A, and applied to the nickel NTA column in this buffer. A pH 8.0 gradient was used to selectively elute bacterial and recombinant proteins. Buffer A = 6 M guanidine HCl, 100 mM NaH2PO4, 10 mM TRIS, pH 8.0; buffer B = buffer A with 8 M urea substituted for 6 M guanidine HCl; buffer C = buffer B, pH 6.3; buffer D = buffer B, pH 5.9; buffer E = buffer B, pH 4.5. Note that these columns can also be run under non-denaturing conditions if the biologic activity of the recombinant proteins cannot be reconstituted after denaturation/renaturation. Utilizing this system, we have been able to purify up to 220 mg of constitutively active ATF-2, and 90 mg of constitutively active CREB from 1-I cultures. These large amounts of purified proteins have been utilized to facilitate studies of the roles of CREB/ATF proteins in transcriptional activation relating to growth and differentiation.

Phenotypic Consequences of Direct Introduction of Recombinant Proteins into Mammalian Cells We have utilized the large amounts of purified recombinant proteins we have already produced for a variety of different types of studies aimed at addressing the functional properties of these important transcriptional regulatory proteins. For instance, we have used the dominant negative version of CREB to investigate the feasibility of several techniques
Figure 6. Schematic and practical representation of the Far-Western assay for the detection of protein/protein interactions. A) (left panel) schematic diagram depicting and describing the basic protocol for performing the Far-Western assay. B) (right panel) autoradiograms depicting the experimental paradigm described in the cartoon presented in A. The Far-Western assay in B was performed as described in A.

between a labeled protein in solution and an immobilized dimerization partner protein on a nitrocellulose filter, we first attempted to detect these interactions with total bacterial extracts containing CREB or ATF-2. A schematic representation of the Far-Western assay for detecting protein/protein interactions is presented in Fig 6A. Briefly, extracts containing recombinant CREB or ATF proteins are separated by SDS-PAGE, and transferred to nitrocellulose membranes. After blocking non-specific protein binding, a labeled CREB or ATF protein probe is then incubated with the immobilized proteins on the nitrocellulose filter. After subsequent washing, the immobilized proteins that interact with a labeled protein probe are detected by autoradiography. An example of the assay diagrammed in Fig 6A is presented in Fig 6B. The specificity of protein/protein interaction for the recombinant CREB and ATF-2 proteins is demonstrated. Two microliters of total protein from extracts of the appropriate BL21 host bacterial strain alone, or this same strain expressing CREB (254-327) or ATF-2 (350-505) peptides, were separated on 20% SDS-polyacrylamide gels and transferred to nitrocellulose. The blots were then incubated at room temperature in BLOTTO [41] with gentle shaking for 2 h, before adding 10 ml of [125I]-labeled CREB or ATF-2 at 1 X 10^6 cpm/ml in BLOTTO. The blots were probed with labeled protein for 12-18 h at room temperature, and subsequently washed in TNE-50 [41] 4 X 30 min before autoradiography. Note that labeled ATF-2 forms interactions only with ATF-2 peptide in ATF-2-containing extracts, labeled CREB forms interactions only with the CREB peptide in CREB-containing extracts. This figure demonstrates two important points. First, we are able to detect protein/protein interactions between our labeled proteins in solution and the immobilized nuclear factors on the nitrocellulose filters and, second, that these interactions are specific.

We have since utilized this assay to detect multiple proteins that interact with CREB and ATF-2 in extracts of HeLa [35], and multiple other cell lines. Similarly, we have utilized this assay to clone cDNA encoding several of these proteins. During the course of these studies we have demonstrated that ATF-2 will form specific heterodimers with fos and jun proteins and others, and that CREB can also form heterodimers with certain forms of fos and jun proteins, as well as ATF-1 [35] and unpublished data). More recently we have demonstrated specific high-affinity interaction of CREB and ATF-2 with topoisomerase II (unpublished data). Finally, in collaboration with Dr. Aleem Siddiqui’s laboratory, we have also demonstrated heterodimerization of CREB and ATF-2 with the hepatitis B virus X protein [33]. These interactions are of particular note because the DNA-binding specificity of the heteromeric complex is unique from the DNA-binding specificity of any of the proteins alone. This data suggests a new mechanism of transcriptional activation in response to viral stimulation that is mediated by the CREB/ATF proteins. Therefore, the utility of this assay is just now being employed and refined in our laboratory, and those of others.

The Far-Western assay has also been used in our laboratory to investigate the repertoire of CREB/ATF proteins present in different tumor specimens and cell lines derived from other tumors. The repertoire of CREB/ATF interactive proteins present in a variety of cell lines derived from lung tumors is demonstrated in Fig 7 (these studies were done in collaboration with Dr. Al Malkinson and Carol Lange-Carter from CU, Boulder). The nuclear proteins from these cell lines were separated on 10% SDS-PAGE and transferred to nitrocellulose membranes. The blots (containing 50 µg nuclear protein/lane) were probed with the labeled CREB or ATF-2 peptides as described above. These blots are presented to demonstrate the utility
Figure 7. Multiple proteins in extracts from cell lines derived from lung tumors interact with labeled CREB (254–327) and ATF-2 (350–505) probes by Far-Western assay. The extracts were separated by SDS-PAGE on 10% gels, transferred to nitrocellulose membranes, and subjected to Far-Western analyses as described in the cartoon depicted in Fig 6A. Note that the repertoire of CREB and ATF-2 interactive proteins present varies with each cell line, yet there are many interactive species in these extracts derived from cell lines in culture.

Figure 8. The repertoire of CREB/ATF interactive proteins detected by Far-Western assays of extracts derived from a variety of melanoma tumors is very limited. The Far-Western Assays were performed as described previously with extracts produced from primary (back) or metastatic (brain and liver) human melanomas as denoted above each lane. Note the defined repertoire of CREB/ATF interactive proteins detected in these extracts compared to the HeLa extracts included for comparison, or the extracts derived from the lung tumor cell lines depicted in Fig 7.
of the assay for the detection of the defined repertoire of CREB/ATF interactive proteins and to allow the visualization of how the repertoire of CREB/ATF proteins varies between cell types or phenotypic states.

A more graphic representation of the differences we can detect in the profile of CREB/ATF proteins in different cell lines and tumor specimens is evidenced in Fig 8. These blots depict Far-Western assays utilized to detect the repertoire of CREB/ATF interactive proteins present in a variety of human malignant melanoma tumors. The results are quite dramatic when one compares the profile of proteins detected in the melanoma tumors with the profile of proteins detected in the lane representing the proteins from HeLa nuclear extracts (the lanes are labeled at the top), or the diverse repertoire of proteins detected in the lung tumor cell lines in Fig 7. In these extracts from melanoma tumors, a single 120-kD protein binding to ATF-2 is demonstrated. We have used unique systems such as this to increase our chances of subsequently cloning the unique or tissue specific proteins in these extracts that interact with the CREB and/or ATF-2 probes. For instance, we have successfully isolated several overlapping cDNA from a human melanoma library that potentially encode the 120-kD protein detected in Fig 8 by directly screening this library with labeled ATF-2.

In summary, we have taken advantage of the functionally bipartite nature of the CREB/ATF proteins to produce reagents utilizing recombinant techniques to probe the structure and functions of the members of this diverse family of transcriptional regulatory proteins. We have utilized the strategy of producing and studying the functions of constitutively active and dominant negative CRE-binding proteins in order to simplify this very complex system. By utilizing the constitutively active and dominant negative CRE-binding proteins, we have been able to study the combined effects of these CREB/ATF proteins on functional processes without regard to input of individual members of this diverse family, and without regard to the signaling or viral stimulatory pathways that influence the activity of the individual members of this family. Once we have defined the cellular functions of the members of this family as a whole, we can begin to investigate the specific functions of the individual members of this family.

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