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Cytoplasmic CREBα-like Antigens in Specific Regions of the Rat Brain

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We have investigated the expression of the α-region of CREB (amino acids 88-101) in the rat brain. This region is encoded by a separate exon and has been reported to enhance the transcriptional efficiency of CREB. Light microscopic immunohistochemistry showed labeled neurons in several brain regions, such as the spinal cord, pontine reticular formation, cerebellum, hypothalamus, hippocampus and olfactory cortex. The labeling was exclusively cytoplasmic, and electron microscopy revealed that the CREBα-immunoreactivity was localized to the Golgi apparatus and axon terminals. Immunoblotting after SDS-PAGE showed specifically detected proteins of 21 and 160 kD. We suggest that neuronal CREBα-proteins represent alternatively spliced isoforms of CREB that lack the nuclear translocation signal. These isoforms may be involved in the regulation of CREB-mediated transcription.

The cAMP response element (CRE) is the nuclear target for the CRE-binding protein, CREB (1, 2). CREB is constitutively expressed in certain neuronal populations. Although CREB may possess some basal transcription abilities (3, 4), its transcriptional activity is generally dependent on its phosphorylation at position Ser-133 (2, 5) via intracellular pathways such as the cAMP-dependent protein kinase (PKA) route (5, 6). Additional phosphorylation (7) as well as the interaction with co-activators (8) have also been shown to be necessary for transcriptional activation by CREB.

It was originally suggested that the catalytic subunit of PKA and CREB interacted at the protein sequence 88-101, named the α-region, of CREB, since it was shown that an alternatively spliced form of CREB, CREBΔ or CREB-327, which lacks the α-region, could not be efficiently phosphorylated by PKA in F9 cells (9). However, the difference in transcriptional abilities between CREBα (CREB-341) and CREBΔ has been questioned in work with other cell types (3, 10), and the functional role of the α-region is still not known (11). To further clarify the role of CREB in neuronal transcription, we have investigated the immunohistochemical expression of CREBα-like immunoreactivity in the rat brain.

MATERIALS AND METHODS

Male Sprague-Dawley rats (250-400g) received a brief ether anesthesia before they were given injections of lethal doses of sodium pentobarbital. The animals were then transcardially perfused with 100 ml 0.9% NaCl followed by 500 ml 4% paraformaldehyde (for light microscopy), or 2% paraformaldehyde and 0.1% picric acid (for electron microscopy) in phosphate-buffered saline (PBS, 0.1 M, pH 7.4). Alternatively, animals were decapitated and the brain was removed on a bed of ice, and immediately frozen at −70°C. All procedures were approved by the Animal Care and Use Committee at the University of Linköping.

The primary antibody, polyclonal antibody against the α-region of CREB (aa 88-101) raised in rabbits (9) (CREB-240; SC-58, Santa Cruz Biotechnology; lots L073 and I205), was diluted to concentrations ranging between 0.05-0.5 μg/ml in PBS (pH 7.4) with bovine serum albumin (0.2-2%) and normal swine serum (1:30). Sections cut at a freezing microtome at 20-40 μm were incubated in the primary antibody solution for 20-24 hours at room temperature, followed by secondary antibody (swine anti-rabbit; 1:100; Dakopatts, Sweden) and peroxidase anti-peroxidase antibody (1:150; Dakopats, Sweden) for one hour each. The sections were then developed for 5-8 minutes in 0.01% hydrogen peroxide in sodium acetate buffer (NaAc; pH 6.0) using 3,3′-diaminobenzidine tetrahydrochloride (DAB; 0.035%) and ammonium nickel sulphate (2.5%) as a chromogen (12), and mounted on gelatin-coated glasses. Adjacent sections were

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stained with thionin. In control experiments, the primary antibody was preincubated with a synthetic α-region peptide (1 μg/ml; SC-58P; Santa Cruz Biotechnology; lot B164) for four hours. The immunohistochemical procedures were then performed as described above.

Preembedding electron microscopic immunohistochemistry was performed as described previously (13). Sections were cut at 50 μm on a Vibratome. Immunohistochemistry was performed as described above, except that the sections were developed with 0.02% hydrogen peroxide in Tris-HCl buffer (0.1 M, pH 7.4) using 0.075% DAB as chromogen. The sections were then osmicated in 1% OsO_4 in phosphate buffer (0.1 M, pH 7.4) for 20 min, dehydrated in graded series of ethanol, flat-embedded in resin (Durcopan, Fluka) and polymerized at 56°C for 48 h. Ultrathin sections were collected on Formvar-coated slot grids and counterstained with uranyl acetate and lead citrate.

Immunoblotting was performed on whole cell homogenates of rat forebrain that were subjected to SDS-PAGE (10% acrylamide), and electrophoretically transferred to a polyvinylidene difluoride blotting membrane (Immobilon-P; Millipore) at 40 mA overnight (14). The membrane was saturated in 5% dry milk powder in 0.14 M NaCl, 3 mM KCl, 11 mM phosphate (pH 7.5), 0.1% (v/v) Tween 20, for one hour at 37°C, followed by incubation with primary antibody at 2.5 μg/ml for two hours in room temperature. Alternatively, 0.35 μg/ml of the synthetic peptide of the CREBα-region, or 0.5 mg/ml of glycogen phosphorylase b was added to the primary antibody solution. Bound CREB antigens were detected with horseradish peroxidase-conjugated anti-rabbit-IgG, adhering to the ECL protocol and reagents (Amersham).

RESULTS

Immunohistochemical investigations of CREBα-region-like immunoreactivity in the brain of adult male rats revealed specific labeling in several regions (Fig. 1). The reaction product was localized to the cytoplasm and axoplasm of the neurons, and no significant nuclear labeling was detected (Fig. 1A, C-F). Incubation of the primary antibody with the α-region peptide followed by immunohistochemical staining abolished all specific labeling (Fig. 1A-B).

CREBα immunoreactivity was consistently found in many different brain regions. Of somatosensory regions, the dorsal horn of the spinal cord and the trigeminal nucleus showed heavy axonal labeling and occasional cell body labeling, while the external cuneate nucleus showed heavy labeling of cell bodies. The somatosensory thalamus showed only axonal labeling. Regions associated with autonomic and nociceptive mechanisms, e.g., the nucleus of the solitary tract, the pontine parabrachial nucleus, the coerulear region (Fig. 1E), the periaqueductal gray matter, several hypothalamic nuclei including the ventromedial and paraventricular (Fig. 1F) nuclei, and the bed nucleus of the stria terminalis, showed consistently heavy labeling of cell bodies and axon terminals. Some regions involved in auditory processing also showed heavy labeling of cell bodies and axon terminals, e.g., the dorsal and ventral cochlear nuclei, and the medial geniculate nucleus. Of regions associated with the visual system, a specific pattern of CREBα immunoreactivity was seen in the superior colliculus. Hence, the intermediate layer of the superior colliculus showed heavy labeling of cell bodies, while the superficial layer showed heavy axonal but no cell body labeling. CREBα immunoreactivity was also seen in the oculomotor and Edinger-Westphal nuclei.

The densest CREBα immunoreactivity was detected in regions associated with learning and memory functions, such as the cerebellum (Fig. 1A), the hippocampus, the dentate gyrus, the mammillary complex, and the olfactory cortex (Fig. 1C). Also in these areas specific patterns were seen. For example, in the cerebellum a dense axonal labeling was seen in all layers, while labeling of cell bodies was prominent in the Purkinje cell layer, moderate in the molecular layer, and absent in the granular layer (Fig. 1A). CREBα immunoreactivity was also detected in circumventricular structures, the striatum and cortical layers IV-V (Fig. 1D).

The electron microscopic immunocytochemical staining demonstrated the intracellular localization of CREBα expression. Several brain stem regions were investigated, including the parabrachial nucleus and the locus coeruleus region. We found that CREBα immunoreactivity was confined to two loci, the Golgi apparatus and axon terminals (Fig. 2). No labeling was detected in any other loci and many unlabeled terminals were detected in close vicinity to densely labeled terminals (Fig. 2C-D), confirming the specific nature of CREBα expression.

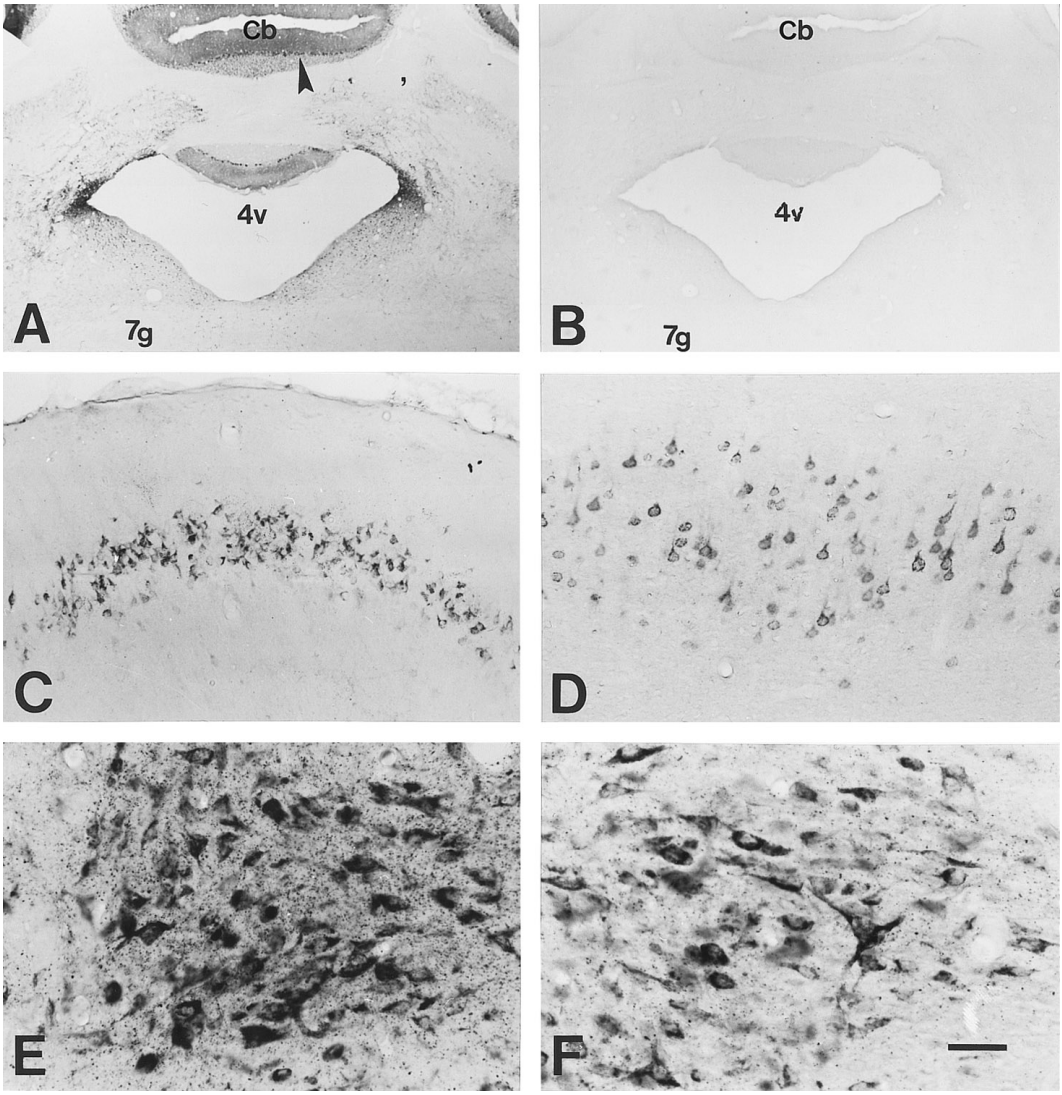

FIG. 1. Light micrographs of CREBα immunoreactivity in rat brain sections. (A-B) Adjacent pontine sections. The CREBα antibody was preincubated with the CREBα peptide before immunohistochemical procedures in (B). The arrowhead in (A) points at the Purkinje cell layer. 4v, fourth ventricle; 7g, genu of the facial nerve; Cb, cerebellum. (C-D) CREBα expression, predominantly in cell bodies, of the olfactory cortex (C) and neocortical layers IV-V (D). Note the unlabeled nuclei. (E-F) CREBα labeling in the coerulear region (E) and the paraventricular hypothalamic nucleus (F). The punctate labeling represents labeled axon terminals. Bar = 640 μm (A-B), 100 μm (C), 90 μm (D), 50 μm (E-F).

Occasionally, labeled terminals could be seen making synaptic contact with labeled cell bodies (Fig. 2B).

Immunoblot analysis after SDS-PAGE on brain tissue from the basal forebrain, including the hypothalamus, revealed two consistently detected bands that were blocked by simultaneous incubation with the α-region peptide (Fig. 3). One heavily stained protein migrated with an apparent molecular mass of 160 kD, but the most densely stained protein

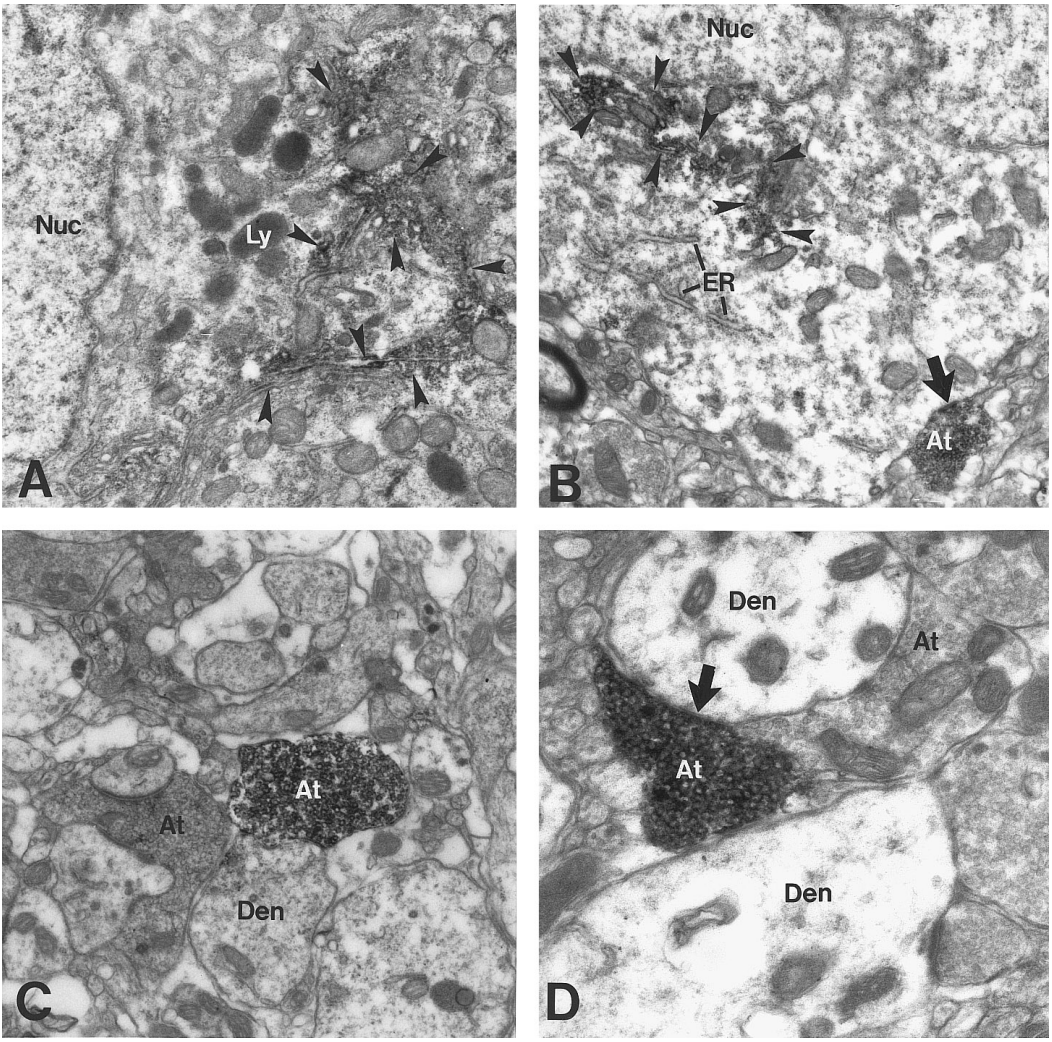

FIG. 2. Electron microscopic demonstration of the intracellular localization of CREBα immunoreactivity. (A-B) Localization of peroxidase reaction product to the Golgi apparatus. Arrowheads delineate the Golgi apparatus. Arrow points at a synaptic contact with a CREBα immunoreactive axon terminal. At, axon terminal; ER, endoplasmic reticuluum; Nuc, nucleus. (C-D) CREBα immunoreactive axon terminals. Note the unlabeled terminals in close vicinity to labeled terminals. Arrow points at an axo-dendritic synaptic contact. At, axon terminal; Den, dendrite.

migrated at 21 kD (Fig. 3, lane 1). The staining of both bands was completely abolished after incubation with the α-peptide (Fig. 3, lane 2). To further confirm the specificity of the immunodetection, competitive incubation of the antibody was made with rabbit skeletal muscle glycogen phosphorylase b, which contains a glutamine-rich region with similar characteristics to the α-region (9, 15), but which otherwise is unrelated to the CREB family. This procedure did not affect the staining of the 21 and 160 kD proteins (data not shown).

Search for pattern or sequence homologies (SWISS-PROT) with the CREBα-region did not reveal any significant homology with any known eukaryotic protein except mouse, rat and human CREB. A poor sequence homology (60% in a 10 amino acid overlap) was seen in a

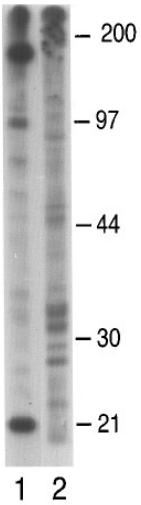

FIG. 3. Immunoblot of CREBα expression in the basal forebrain after SDS-PAGE. Lane 1, darkly stained proteins were detected at 21 and 160 kD. Lane 2, competition with the CREBα region peptide totally abolished labeling at 21 and 160 kD. The sizes of molecular weight markers are indicated alongside.

subunit of the protochlorophyllide reductase in the protozoa Chlamydomonas Reinhardt, but this homology was considered irrelevant.

DISCUSSION

Our results show that an antibody raised against the CREBα-region specifically identifies cytoplasmic antigens present in a subset of neurons of the rat brain. The antigens are localized to the Golgi apparatus and the axon terminals of the neurons.

Although no known sequence homologies with the CREBα-region exist, the possibility of sequence homology with unknown proteins, or with previously unknown epitopes induced by conformational changes of protein structure cannot be excluded. However, the labeling after immunoblotting was completely blocked by preincubation with the α-region peptide, but not with a protein sequence sharing general similarities. Further, putative low-affinity bindings should have been blocked by our use of high concentrations of normal serum and/or bovine serum albumin in the immunohistochemical procedures, and the labeling obtained in our material was very selectively localized with a high signal-to-noise ratio (Fig. 1 and 2). If the antibody detects another sequence than the CREBα-region, such a pre-existing or induced sequence must contain epitopes that are very similar to the CREBα-region.

The expression of CREBα has previously not been described in the intact brain. Previous immunochemical investigations of CREBα-expression have been performed on nuclear extracts from different cell types (c.f.(9)). Other studies have utilized antibodies that does not disinguish between CREBα and CREBΔ, or that only detect CREBΔ (9, 16). To reliably detect cytoplasmic neuronal CREBα antigens it is necessary to perform either immunohistochemistry or immunoblots after SDS-PAGE of whole-cell brain extracts with an antibody that is raised specifically for the α-region, the approach used in the present study.

Alternative CREB antigens in neuronal or neuronal-like cell lines have very recently been reported by others. For example, an antibody raised specifically against CREB phosphorylated at Ser-133 (16) detects not only CREB (43 kD), CREMt (45 kD) and ATF-1 (38 kD) (which all share the similar kinase-inducible domain), but also an antigen at 21-22 kD after blotting

of whole-cell extracts (Pende and Gallo, personal communications; Sarvey and Voulalas, personal communications). This 21-22 kD CREB antigen has not been reported after blotting of nuclear extracts (16, 17), which indicates that it is located in the cytoplasm. Intriguingly, this protein is of similar size as the CREBα 21 kD antigen reported in this study.

Earlier investigations of CREB-like immunoreactivity in the brain have exclusively demonstrated nuclear labeling (16-18) (Hermanson, unpublished observations), and the present study is the first demonstration of an antigen of the CREB family localized to the neuronal cytoplasm. However, there are previous demonstrations of different cytoplasmic isoforms of CREB in the developing testes (19-21). As a result of alternative splicing, the cytoplasmic isoforms lack the basic zipper (bZIP) region, which also possesses the signal that directs CREB to translocate to the nucleus (20,22). The α-region is included in some of the cytoplasmic CREB isoforms in the testes (11,22). Taken together with these previous data, our results suggest that the CREBα 21kD antigen is a truncated isoform of CREB. However, with respect to the sizes of the previously characterized truncated CREB isoforms (20, 22), it is likely that the CREBα 21kD antigen represents a novel isoform, thereby suggesting the presence of additional, yet unknown, stop codons, or post-translational processing.

Several studies have shown CREB antigens larger than 43 kD, and these antigens are generally considered to be protein complexes that contain CREB (23). Accordingly, it is possible that the CREBα 160 kD antigen is a protein complex containing the α-region.

Truncated isoforms of other members of the CREB family, e.g. CREM and ATF, have previously been detected in the rat brain (24-26), but no such isoforms have been reported to lack the nuclear translocation signal. Instead, these CREM and ATF isoforms compete with more potent transcriptional activators in DNA binding, thereby down-regulating transcriptional activity (24,27). The truncated cytoplasmic CREB isoforms in the testes have also been suggested to act as repressors (19). The CREB gene contains a CRE-site itself and CREB transcription may therefore be up-regulated via a positive feedback mechanism (28). Since the truncated CREB isoforms lack the nuclear translocation signal (20), they may interrupt such a mechanism by remaining in the cytoplasm after translation. The neuronal cytoplasmic CREBα antigens may consequently act, indirectly, as transcriptional repressors.

In addition to being involved in down-regulation of a putative positive feedback, other functional roles for the neuronal cytoplasmic CREBα antigens should be considered. There are several observations that support an interaction between nuclear and cytoplasmic events concerning PKA-elicited phosphorylation of CREB. For example, the kinetics of the phosphorylation of CREB is very precisely regulated. It has been shown that the nuclear entry of the catalytic subunit of PKA is rate-limiting for the CREB phosphorylation (29) and that down-regulation of PKA activity is necessary for sufficient repression of cAMP induced transcription via CREB (30). Since it has been shown that the α-region can bind PKA (9), cytoplasmic interaction with PKA may be a function of the CREBα antigens described in the present study.

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