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Communication

Identification and Characterization of a κB/Rel Binding Site in the Regulatory Region of the Amyloid Precursor Protein Gene*

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Several observations support the hypothesis that pathogenetic mechanisms of β amyloid formation in Alzheimer's disease may involve alterations in amyloid precursor protein (APP) gene expression. In this regard, molecular dissection of the APP gene transcriptional regulation is of primary importance. We report evidence that members of the family of transcription factors NFκB/Rel can specifically recognize two identical sequences located in the 5′-regulatory region of APP. These sequences, which we refer to as APPκB sites, interact preferentially with p50-containing members of the family. In particular, p50 homodimers and p50/p65 and p50/c-Rel heterodimers act as transcriptional activators at the APPκB site. Finally, the nuclear complex specifically binding to the APPκB sites proves to be an integral part of neurons and lymphocytes.

Molecular genetic studies in familial forms of Alzheimer's disease (AD)[1] and in Down's syndrome have clearly pointed out that brain deposition of β amyloid peptide in senile plaques and cerebrovasculature plays a central role in the pathogenesis of AD (see Ref. 1 for review). The molecular nature and origin of the β peptide have also been clarified. β amyloid is a small polypeptide fragment of 39–42 amino acids which is generated by proteolytic cleavage of a family of alternatively spliced transmembrane proteins, the amyloid precursor proteins (APP), whose functional significance is still controversial. The APP gene, localized on chromosome 21, is expressed in brain and all major tissues.

In the past few years, *in vitro* studies on processing of endogenous or transfected (wild-type and mutated) APP molecules have emerged as major experimental endeavors (1). This kind of approach has been extremely important for unraveling some of the molecular mechanisms involved in the production of the β amyloid and, likely, in the disease. Nevertheless, the possibility that pathogenetic mechanisms of plaque formation in AD may also involve alterations in APP gene expression as a required step should not be underestimated. Several observations support this hypothesis. Among them: (i) augmented expression of the APP gene transcripts in Down's syndrome and in specific areas of the brain of AD patients (2–5); (ii) *in vitro* degeneration of postmitotic neurons overexpressing full-length APP (6); (iii) marked increase of the APP gene expression after head trauma, a well-recognized enviromental risk factor for AD (7).

Increased and/or dysregulated expression of the APP gene may potentially result from disturbances at any step in the transcriptional regulatory pathway, like altered activation or expression of specific transcription factors and/or mutations in the 5′-regulatory region of the gene. It appears important therefore to elucidate the molecular mechanisms of APP gene regulation by identifying pathophysiologically Relevant *cis*-elements and transcriptional regulators interacting with these sites. Despite cloning of its promoter region (8), little is known about the participants in the transcriptional control of the APP gene. In particular, although several potential recognition sequences for transcriptional control proteins have been identified (9–14), only for AP-1 has a direct role in the control of the APP gene expression been established (15).

The present paper reports evidence that members of the NFκB/Rel family of transcriptional control proteins may represent critical regulators for modulation of the APP gene expression. Regulatory proteins belonging to this family have been widely characterized as very pleiotropic factors, able to respond to a wide variety of signals and to control expression of a large number of genes mainly implicated in defensive responses, such as immune and inflammatory reactions (16, 17). On the contrary, there has been only modest progress in determining their contribution to regulated expression of genes whose products are functionally Relevant in the central nervous system (18–20).

EXPERIMENTAL PROCEDURES

Cell Culture—H9 and HeLa cell lines were grown, respectively, in Dulbecco's modified Eagle's medium and minimum essential medium supplemented with 10% fetal bovine serum.

The embryonal carcinoma F9 was grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml).

Synthetic DNA Oligonucleotides—Oligonucleotides corresponding to the two putative κB sites in the 5′-regulatory region of the APP gene and their respective complementary strands were synthesized using a DNA synthesizer (Applied Biosystems) and purified by denaturing gel electrophoresis. For gel shift analysis, double-stranded oligonucleotides were end-labeled with [γ-^{32}P]ATP (ICN, >7000 Ci/mmol) and T4 polynucleotide kinase (Boehringer Mannheim) to obtain a specific activity of more than 10^8 cpm/μg. Sequences were as follows: IL-2κB (−212 to −195 of the interleukin 2 gene enhancer region), 5′-CCAAGAGGGATTTCACCTAAATCC-3′; APP1 (−2257 to −2234 of the APP 5′-regulatory region), 5′-TAGAGACGGGGTTTCACCGTGTTA-3′; APP2 (−1844 to −1821 of the APP 5′-regulatory region), 5′-AGAGATGGGGGTTTCACCATGTTG-3′; OCTA (octamer protein binding site from the interleukin 2 gene enhancer region), 5′-TATGTGTAATATGTAAAACATTTTGACACC-3′; mutAPP, 5′-TAGAGACGctcTTTCACCGTGTTA-3′.

Both strands of each oligonucleotide probe have a 5′-TCGA-3′ overhang at their 5′ end to facilitate cloning.

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[1] The abbreviations used are: AD, Alzheimer's disease; APP, amyloid precursor protein; CAT, chloramphenicol acetyltransferase; IL, interleukin.

Nuclear Extracts and Electrophoretic Mobility Shift Assay—Nuclear extracts from rat brain areas were prepared essentially as described (21). Nuclear extracts from cell lines and from A.E7 cells were prepared according to a small scale protocol (22). Protein concentration was assessed by Bio-Rad Bradford assay according to the manufacturer's instructions. DNA binding reactions were initiated by combining 2 μg of nuclear extracts with 20,000 cpm (0.1 ng) of γ-^{32}P-labeled oligonucleotides in 1 × lipage buffer (10 mM Tris-Cl, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 10% glycerol) containing 0.5 μg of poly(dI·dC) in a total volume of 10 μl. In competition experiments, 5- and 80-fold molar excess (0.5 and 8 ng) of unlabeled competitor oligonucleotides were added together with ^{32}P-labeled probes. Reactions were carried out for 20 min at room temperature, and protein-DNA complexes were resolved on nondenaturing 4% polyacrylamide gels in 1 × Tris-glycine-EDTA buffer. Gels were then dried and subjected to autoradiography at room temperature. In supershift experiments, 0.5–1 μl of preimmune or immune serum was incubated with 2 μg of nuclear extracts for 15 min at room temperature, before addition of the other components of the reaction mixture and incubation for an additional 20 min. Polyclonal rabbit anti-p50 antibodies were prepared against bacterially produced and affinity-purified recombinant mouse p50 as described in Ref. 23. Anti-p50B antibodies were purchased from Santa Cruz Biotechnology (Genzyme, Italy).

Plasmid Constructs—Oligonucleotides representing the two putative κB sites from the APP gene regulatory region, APP1 and APP2, were subcloned into the *Sal*I restriction site of PBLCAT2 (25). The κB/Rel expression plasmids, pSG-p50, pSG-p65, and pSG-Rel, have been described previously (24) and kindly provided by Dr. Pierre Jalinot.

Transfection of Cell Lines—F9 cells (5 × 10^5 cells/60-mm dishes) were transiently transfected with 10 μg of supercoiled DNA using the calcium phosphate technique. 4 h after calcium phosphate-DNA addition to cell medium, cells were washed twice with serum-free medium followed by culturing in complete medium. After 36–48 h, cells were washed in phosphate-buffered saline, lysed by three cycles of freezing and thawing in 250 mM Tris-Cl, pH 7.5. Supernatants obtained by centrifugation for 5 min at 12,000 rpm were sampled to determine protein content and assayed for chloramphenicol acetyltransferase (CAT) activity. Transfection efficiency throughout the experiments was monitored by cotransfection with pSVβgal plasmid.

RESULTS

We identified a sequence corresponding to 5′-GGGGTTTCAC-3′, repeated in positions −2250 to −2241 and −1837 to −1828 in the 5′-regulatory region of the APP gene and remarkably similar to the consensus sequence for NFκB/Rel transcription factors. The possibility that the sequence is actually a binding site for κB/Rel proteins and is operative in the central nervous system was investigated. Double-stranded oligonucleotides comprising the sequence and designated as APP1 (−2257 to −2234) and APP2 (−1844 to −1821) were tested, in gel shift analysis, for binding of nuclear proteins in extracts from rat cerebellum. As shown in Fig. 1, *lanes 1* and *2*, both oligonucleotides detected a complex with identical retarded migration. To verify if the complex interacting with the sequence from the APP gene was a κB-Related protein, APP1 and APP2 oligonucleotides were tested for their ability to compete with a characterized κB sequence for binding of members of the family of transcription factors. An established model for κB-mediated gene transcription is the murine CD4$^+$ T cell clone A.E7, where IL-2 gene regulation has been studied extensively (23). A κB site in the IL-2 gene enhancer region (IL-2κB site) interacts with at least two dimeric complexes with different subunit compositions, both constitutively present in A.E7 nuclear extracts, p50/p65 (NFκB) and p50/p50 (NFκC) dimers (Fig. 1, *lane 9*). Interplay between these two complexes ensures fine control of IL-2 gene regulation in nontransformed lymphocytes (23). APP1 and APP2 oligonucleotides displaced both NFκB and NFκC complexes from binding to the IL-2κB site probe in a concentration-dependent manner (*lanes 3–6*). As a control, the same amount of an oligonucleotide sequence for octamer proteins (OCTA) failed to compete for interaction between the IL-2κB sequence and the two κB complexes (*lanes 7* and *8*). Furthermore, the APP1 and APP2 oligonucleotide sequences, when used as probes and incubated with A.E7 extracts, recognized a major complex (*lanes 10* and *11*) whose binding activity was disrupted by three base changes in the core κB-like sequence within the APP1 oligonucleotide (*lane 12*). Nuclear extracts were obtained also from rat cortex and hippocampus and tested for binding activity to the APP1 (and APP2, not shown) oligonucleotide. As depicted in Fig. 2, a single nuclear complex comigrating with the one from cerebellum was intercepted by the probe. More importantly, the rat brain complex binding the APP1 sequence comigrated with the complex from A.E7 cell extracts. To confirm specificity of DNA-protein interaction, binding of the constitutive nuclear activity from rat brain could be abolished by competition with the unlabeled APP1 sequence (20-fold excess) and by the same mutations in the APPκB sequence which abolished binding in A.E7 extracts. A representative experiment of gel shift analysis with cerebellum nuclear extracts is shown in Fig. 2, but comparable results were observed with extracts from cortex and hippocampus (not shown). The results clearly demonstrated that the sequence from the APP regulatory region could indeed interact with κB complexes. Moreover, they strongly suggested that the specific complex recognized in rat brain extracts from the APP sequence was either identical with or very similar to p50 homodimers.

To better characterize binding specificity of the APP sequence, the APP1 oligonucleotide was incubated in the presence of 100 ng of recombinant p50 protein (*bact. p50*): a specific complex was obtained (Fig. 3*A*, *lane 2*) which comigrated with the one identified by the IL-2κB oligonucleotide probe (*lane 1*). The same results were obtained using the APP2 oligonucleotide (data not shown). Finally, the APPκB complex identified in rat cerebellum nuclear extracts (as well as in extracts from other

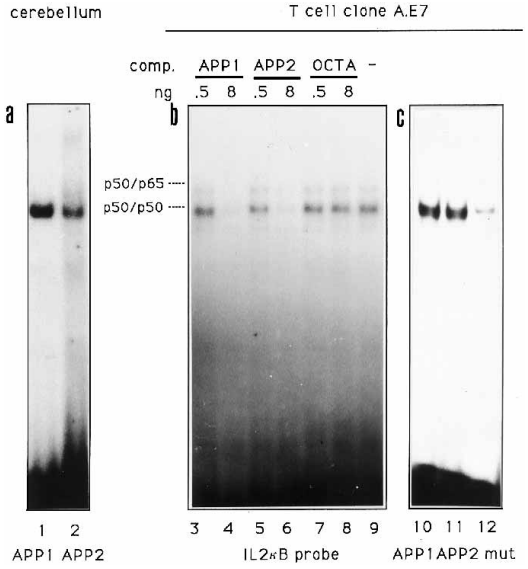

FIG. 1. **The sequences from the APP gene 5′-regulatory region bind a specific complex constitutively present in rat cerebellum extracts and Related to members of the NFκB/Rel family of transcription factors.** *a*, *lanes 1* and *2*, electrophoretic mobility shift assay with 2 μg of nuclear extracts from rat cerebellum and γ-^{32}P end-labeled oligonucleotide probes APP1 and APP2. *b*, *lanes 3–9*, the sequences APP1 and APP2, unlike an oligonucleotide for octamer binding proteins (OCTA), can compete binding of p50/p65 heterodimers (or NFκB, upper migrating complex) and p50 homodimers (or NFκC, lower migrating complex) to the κB sequence from the IL-2 enhancer region. Nuclear extracts from the murine CD4$^+$ T cell clone A.E7 were used. *c*, *lanes 10–12*, the APP1 and APP2 oligonucleotides incubated with A.E7 nuclear extracts bind a complex comigrating with NFκC. Binding specificity was proven using an APP oligonucleotide sequence with three base changes in the core κB site (*mut*).

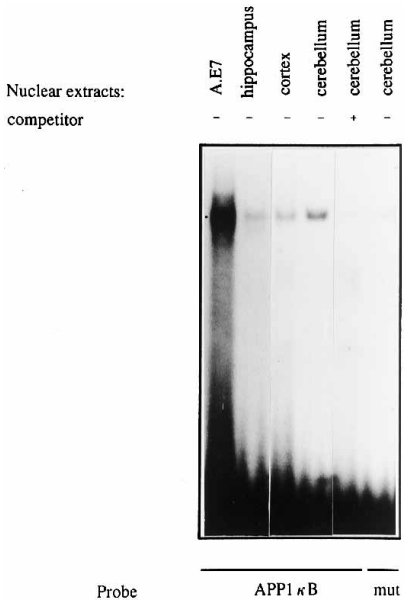

FIG. 2. **A constitutive nuclear factor from several rat brain regions binds specifically to the APP1 sequence and comigrates with the APP1 binding complex from A.E7 cell extracts.** Electrophoretic mobility shift assay obtained by incubating nuclear extracts from A.E7, rat hippocampus, cortex, and cerebellum with the wild type ^{32}P-labeled APP1 oligonucleotide sequence or a mutated version, with three base changes in the core κB-like sequence (*mut*), in the absence (−) or presence (+) of a 20-fold excess unlabeled APP1 oligonucleotide as competitor, to prove specificity of DNA-protein interaction.

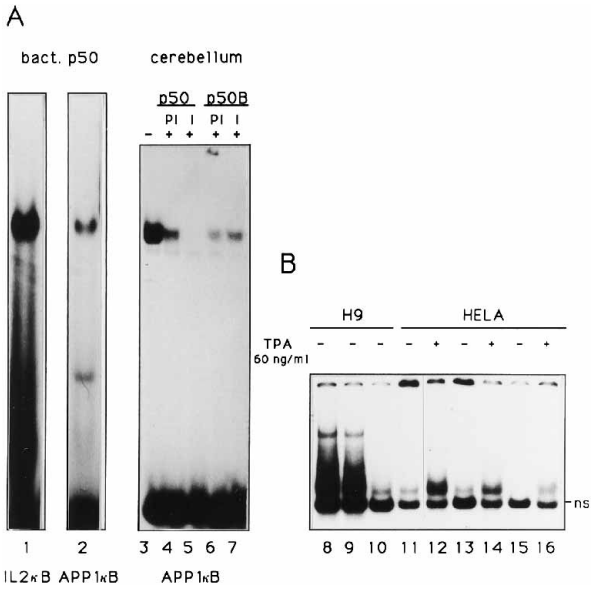

FIG. 3. **The nuclear complex binding the sequence from the APP gene contains the p50 subunit of the NFκB/Rel family.** *A*, *lanes 1* and *2*, recombinant p50 protein binds indistinguishably oligonucleotide probes containing κB sites from the IL-2 gene enhancer region (*IL2κB*) and the APP gene (*APP1κB*). *Lanes 3–7*, supershift of the APPκB binding complex from rat cerebellum by 1 μl of anti-p50 antiserum but not by the same amount of anti-p50B or of their respective preimmune sera. *I* = immune serum, *PI* = preimmune serum. *B*, *lanes 8–15*, APPκB binding activity corRelates with APP gene expression in H9 and HeLa cell lines. Electrophoretic mobility shift assay with H9 nuclear extracts incubated with γ-^{32}P end-labeled oligonucleotide probes APP1 (*lane 8*), APP2 (*lane 9*), or an APP sequence with three base changes (*lane 10*). Electrophoretic mobility shift assay with nuclear extracts from HeLa cells either untreated (−) (*lanes 11*, *13*, and *15*) or treated with 12-*O*-tetradecanoylphorbol-13-acetate (*TPA*) (60 ng/ml for 12 h) (+) (*lanes 12*, *14*, and *16*) in the presence of γ-^{32}P-labeled APP1 (*lanes 11* and *12*), APP2 (*lanes 13* and *14*), or mutated oligonucleotide (*lanes 15* and *16*). *ns* indicates a nonspecific band which appeared in all nuclear extracts of human origin.

brain regions, data not shown) could be recognized by a polyclonal antibody against p50 (Fig. 3*A*, *lane 5*) but not by a polyclonal antibody against p50B (*lane 7*), a highly Related NFκB/Rel family member, or the corresponding preimmune sera (*lanes 4* and *6*).

CorRelation between APPκB binding activity and gene expression was analyzed in cell lines expressing different levels of APP (Fig. 3*B*). Nuclear extracts from H9 cells, a human T cell line which expresses high levels of APP (26), displayed extremely high amounts of APP1 and APP2 κB binding activity (*lanes 8* and *9*, respectively). HeLa cells, which express lower levels of APP mRNA compared to H9, displayed low amounts of APP1 (*lane 11*) and APP2 (*lane 13*) κB binding activity. Furthermore, treatment with 12-*O*-tetradecanoylphorbol-13-acetate (60 ng/ml for 12 h), which has been proven to augment APP mRNA levels in HeLa cells (27), increased both APP1 (*lane 12*) and APP2 (*lane 14*) binding activity. In both cell lines, specificity of protein complexes was confirmed using a version of the APPκB sequence with three base changes (*lanes 10*, *15*, and *16*). In conclusion, the nuclear complex specifically binding to the APPκB sites proved to be an integral component in the examined cell lines. Its basal activity corRelated with levels of APP gene expression and was induced in response to signals that augment gene transcription.

Transcriptional responses at κB sites have been shown to involve complex molecular mechanisms, being the result of the different combinatorial possibilities among different members of the regulatory family, to form homo- and heterodimers (16, 17). F9, a mouse embryonal carcinoma in which endogenous κB binding activity is very low (28), appeared to be a suitable model for dissecting functional significance of the APPκB binding site and contribution of different subunit members of the κB/Rel-Related family. Expression vectors for three κB-Related proteins, p50, c-Rel, and p65 (24) were cotransfected in F9 cells with a reporter plasmid obtained by cloning the APPκB sequence at the *Sal*I site of a PBLCAT2 vector (25). As shown in Fig. 4, expression of p50, by itself or in combination with p65 and c-Rel, led to an increase in CAT activity, while expression of c-Rel, p65, and p65/c-Rel was devoid of effect on the reporter gene transcription. Comparable results were obtained with a reporter plasmid obtained by inserting, at the *Sal*I site of PBLCAT2, the APP2 oligonucleotide sequence (not shown). Since none of the κB/Rel-Related proteins had any effect on the vector itself, which lacks κB binding sites, we concluded that the observed effect on CAT gene transcription resulted from specific binding to the inserted sequence from the APP gene.

DISCUSSION

The APP promoter contains numerous potential recognition sequences for known transcription factors (9–15). However, except for the AP1 site (15), none of these regulatory regions has been implicated directly in the control of the APP gene expression. We report the identification of two identical sequences in the 5′-regulatory region of the APP gene which are specific binding sites for regulatory members of the NFκB/Rel family of transcription factors (16, 17). The two nuclear factor binding domains, which we referred to as APPκB sites, show high affinity for p50-containing members of the family, which appear to be constitutively expressed in the CNS. Furthermore, the APPκB binding activity corRelates with APP gene expression in cell lines of various origin in both resting and stimulated conditions. Analogously to previously characterized κB sequences, in transfection assays we observed that κB-Related complexes with different subunit composition displayed different transcriptional activities at the APPκB site. Cotransfection

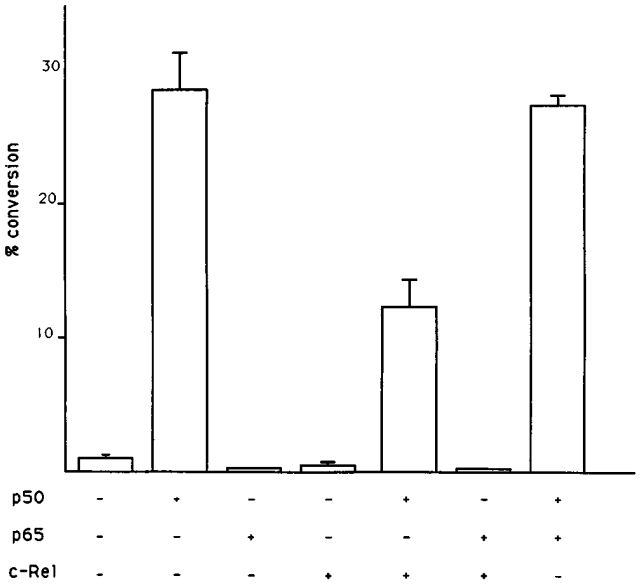

FIG. 4. **Transcriptional activities of different members of the NFκB/Rel family at the APPκB site.** Undifferentiated F9 cells were transfected with 10 μg of the reporter plasmid APPκBCAT (obtained by cloning the APP1 oligonucleotide sequence at the *Sal*I site of PBLCAT2) either in the absence or presence of various combinations of the expression vectors for κB/Rel proteins: pSG-p50, pSG-p65, pSG-Rel, pSG-p50 plus pSG-Rel, pSG-p65 plus pSG-Rel, pSG-p50 plus pSG-p65. In cotransfection experiments, 2 μg of expression vector were used and the total amount of DNA was adjusted to 15 μg with pSG5. Transfection experiments were repeated three times in duplicate with at least two independent plasmid preparations. CAT activities are expressed as % chloramphenicol conversion.

experiments showed that p50-containing complexes, unlike c-Rel/c-Rel, c-Rel/p65, and p65/p65 dimers, behave as transactivators when interacting with the APPκB sequence. Interestingly, differences in transcriptional activity were observed with the different p50-containing complexes. Although the reason for these differences has not been addressed directly, this is in agreement with previous elegant demonstrations by other groups (17). It appears that whether complexes with different subunit composition serve as weak or strong activators (or even repressors, in some cases) is determined by their conformation on DNA or whether their transcriptional activation domains are accessible to components of the general transcription machinery.

An interesting observation made by several groups is that the APP gene is rapidly transcribed in brain in response to a number of circumstances, ranging from head trauma, focal ischemia, neurotoxicity, and heat shock (29–32). Although very different, these situations can be grouped together under the generic term of stress conditions. The finding that κB/Rel-Related proteins may be implicated in the control of the APP gene transcriptional control is intriguing. In fact, these regulatory proteins are utilized in most cell types for genetic interpretation of cellular events underlying responses to stress, since among their target genes are those encoding for cytokines, and they are themselves activated by cytokines (16). Intracerebral responses to damage are often mediated by cytokines, like interleukin 1 (IL-1), interleukin 6, tumor necrosis factor α, so that in analogy to what happens in the liver, the existence of a "brain acute phase response" has been suggested (33). In this regard, it should be underlined that the levels of one of the best characterized inducers for κB/Rel proteins, IL-1, is augmented in the brains of patients with AD and Down's syndrome (34). Furthermore, it has been shown that IL-1 induces an increase in APP transcript levels in endothelial (9) and in neuronal cells (35). PReliminary results indicate that the APPκB sites are indeed responsive to IL-1β in primary neuronal cultures.[2]

We speculate that the APP gene may be one of a set of κB site-containing genes coordinately modulated in brain in response to situations that require a defensive response.

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[2] M. Grilli, unpublished observation.

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