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Identification of Ets- and Notch-Related Subunits in GA Binding Protein

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Recombinant cDNA clones that encode two distinct subunits of the transcription factor GA binding protein (GABP) have been isolated. The predicted amino acid sequence of one subunit, GABPα, exhibits similarity to the sequence of the product of the *ets*-1 proto-oncogene in a region known to encompass the Ets DNA binding domain. The sequence of the second subunit, GABPβ, contains four 33–amino acid repeats located close to the NH_2-terminus of the subunit. The sequences of these repeats are similar to repeats in several transmembrane proteins, including Notch from *Drosophila melanogaster* and Glp-1 and Lin-12 from *Caenorhabditis elegans*. Avid, sequence-specific binding to DNA required the presence of both polypeptides, revealing a conceptual convergence of nuclear transforming proteins and membrane-anchored proteins implicated in developmentally regulated signal transduction processes.

HERPES SIMPLEX VIRUS 1 (HSV1) immediate early (IE) genes are induced at the outset of lytic infection by a virion-associated protein termed VP16 (*1*). At least two classes of cis-regulatory elements are required in HSV1 IE genes for induction by VP16. The most essential VP16 cis-response element is characterized by the nonanucleotide sequence 5′-TAATGARAT-3′ (*2, 3*). VP16 binds tightly to this DNA sequence in a complex with the cellular transcription factor Oct-1 (*4*). A second cis-regulatory element required for VP16-mediated induction of HSV1 IE genes consists of three imperfect repeats of the purine-rich hexanucleotide 5′-CGGAAR-3′ (*3, 5*). A protein complex capable of avid interaction with the purine-rich repeats (GA repeats) has been identified in soluble preparations of rat liver nuclei (*3*). This GA binding protein (GABP) consists of two separable subunits. Purified samples of either subunit do not interact with the GA repeats, yet regain potent DNA binding activity when mixed (*6*).

In an effort to resolve the molecular basis of subunit interdependency of GABP activity, we isolated recombinant cDNA clones of their encoding genes. GABP (20 μg) was purified from rat liver nuclear extracts and cleaved with trypsin. Proteolyzed fragments were separated by high-performance liquid chromatography (HPLC), recovered, and subjected to gas-phase amino acid sequencing (Fig. 1). Partial sequences were derived from 13 tryptic peptides. Degenerate oligonucleotides capable of encoding 4 of the 13 peptide sequences were synthesized and used as hybridization probes to screen an adipocyte cDNA library (7). One recombinant bacteriophage that contained a cDNA insert of 2 kb gave a positive signal when hybridized with each of the four oligonucleotide probes.

The insert of this recombinant was sequenced and found to contain an open reading frame that encoded a protein of 454 amino acids (Fig. 2A). The predicted molecular size of this polypeptide (51.3 kD) corresponded to the size of the α subunit of GABP (GABPα) purified from rat liver nuclei (*6*) (Fig. 1). Inspection of the deduced amino acid sequence revealed segments that corresponded to 8 of the 13 peptides isolated by trypsin digestion of intact GABP. On the basis of the latter two observations, we tentatively identified this 454-residue polypeptide as GABPα.

Degenerate oligonucleotides capable of encoding two of the tryptic peptide sequences not present in GABPα were synthesized and used as hybridization probes to search for a cDNA clone that encoded the β subunit of GABP (GABPβ) (*8*). Five recombinant bacteriophages were identified by their capacity to hybridize with both oligonucleotide probes. One of the cDNA clones differed at the 3′ end from the other four. The largest cDNA insert of the four (2.6 kb) and the variant (1.4 kb) were sequenced. Both DNA sequences revealed long open reading frames specifying very similar polypeptides (Fig. 2B). One cDNA encoded a protein of 382 amino acids, the other encoded a protein of 349 residues. Starting at their respective NH_2-termini, the two proteins exhibited identical sequences for 333 amino acids. At this point the sequences of the two proteins diverged such that the longer one contained an additional 50 residues before its COOH-terminus, and the other an additional 15 residues before its terminus. The divergent COOH-terminal segments bore no apparent amino acid sequence similarity. The open reading frames of both polypeptides contained segments that corresponded to the two tryptic peptides used to design hybridization probes. Moreover, the predicted molecular sizes of the two

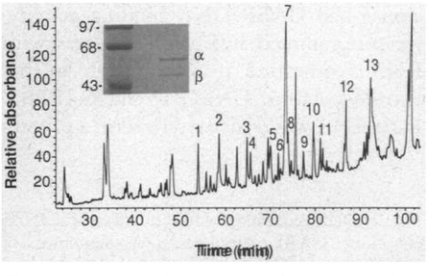

Fig. 1. Amino acid sequences of tryptic peptides derived from GA binding proteins. GABP (20 μg) was purified to homogeneity (inset) as described (*6*), except that boiled, salmon sperm DNA (20 μg/ml) was included as a nonspecific competitor in the DNA affinity chromatographic step. Approximately 500 pmol of protein was lyophilized, reduced, acetylated, and subject to cleavage by trypsin (Boehringer Mannheim). The resulting peptides were separated by reversed-phase HPLC (*22*). Amino acid sequence analysis was performed on a Vydac C18 column (*23*). The amino acid sequences derived from peaks 1 to 13 were 1, SLFDQGVIEK; 2, ?AWALEGY; 3, DEIS?VGDEGEFK; 4, ELESLNQEDFFQR; 5, LQESLDAHEIELQDIQL?P?R; 6, DQISIVGDEGEFK; 7, MAELV; 8, YVQASQLQQM- NEIVT-IDQP; 9, TPLHMWASEGHA; 10, GEILWS; 11, LIEIEIDGTEK; 12, ILMANGAPFTTD; 13, TGNNGQIQL?QFLLEL?TDR. Molecular size markers in kilodaltons are given at the left of the inset.

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polypeptides (41.3 and 37 kD) corresponded to the size of the GABPβ subunit purified from rat liver nuclei (*6*) (Fig. 1). We therefore provisionally designated the 41-kD polypeptide as GABPβ1 and the 37-kD polypeptide as GABPβ2.

Northern (RNA) blot assays were used to determine the sizes and tissue distributions of mRNA encoding GABPα, GABPβ1, and GABPβ2 (Fig. 3, A and B). The cDNA corresponding to GABPα identified three mRNAs of roughly 5.0, 2.8, and 2.6 kb, which were expressed in a variety of tissues. The GABPα cDNA characterized in our study, which was slightly less than 2.0 kb (Fig. 2A), represents a partial copy of any of the three mRNAs. Two mRNAs measuring 2.7 and 1.5 kb were identified in Northern blots probed with GABPβ1 cDNA. Like the GABPα mRNAs, those encoding GABPβ1 had a wide tissue distribution. Because the cDNAs that encoded GABPβ1 and GABPβ2 measured 2.6 and 1.4 kb, respectively, they probably represent nearly full-length copies of the respective mRNAs. Moreover, because the nucleotide sequences of the two cDNAs are identical from their respective 5′ termini to the point of divergence 1.1 kb internal to the start of the mRNA, they likely represent alternatively spliced transcripts derived from the same gene. In accord with this interpretation is the presence of a potential splice donor site (AG dinucleotide) immediately preceding the point of divergence.

To test whether these recombinant DNA clones had GABP DNA binding activity, we programmed reticulocyte lysates with RNA synthesized from the cDNAs that encode GABPα, GABPβ1, and GABPβ2. Each RNA was translated to form a protein product of the expected size. Individual lysates or mixtures thereof were tested for DNA binding to a fragment from the HSV1 ICP4 promoter that contained three GA repeats. Mixtures of protein and DNA were subjected to electrophoresis on nondenaturing polyacrylamide gels to separate free DNA from that complexed with pro-

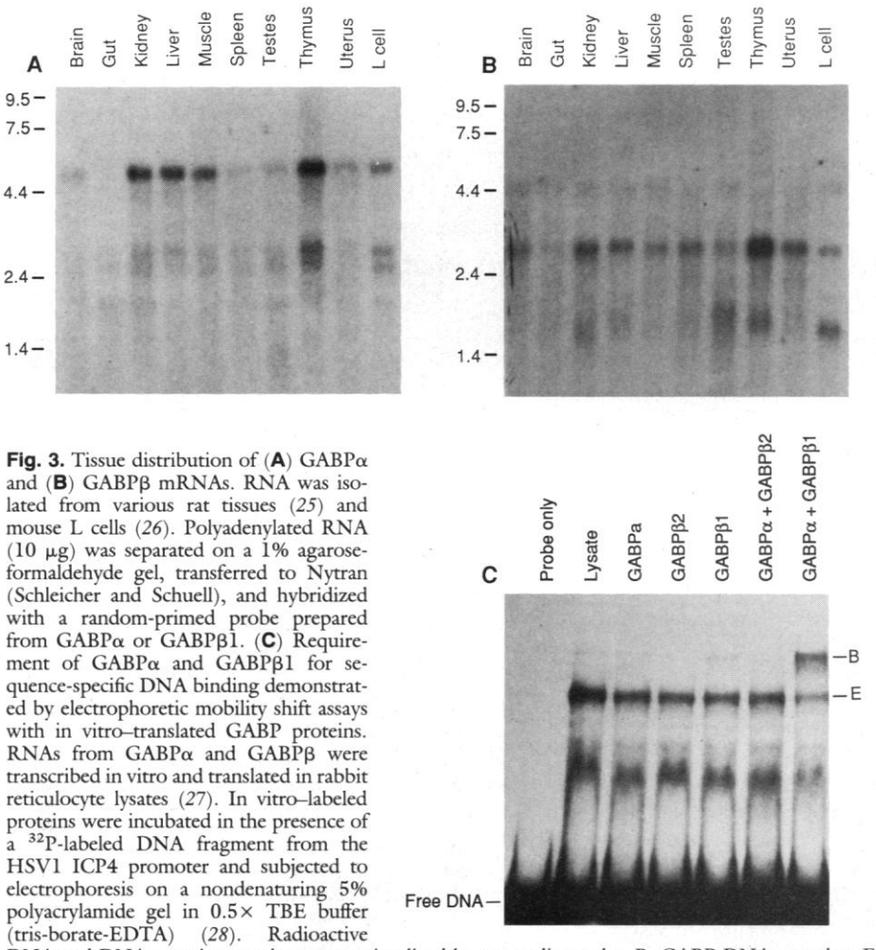

Fig. 3. Tissue distribution of (**A**) GABPα and (**B**) GABPβ mRNAs. RNA was isolated from various rat tissues (*25*) and mouse L cells (*26*). Polyadenylated RNA (10 μg) was separated on a 1% agarose-formaldehyde gel, transferred to Nytran (Schleicher and Schuell), and hybridized with a random-primed probe prepared from GABPα or GABPβ1. (**C**) Requirement of GABPα and GABPβ1 for sequence-specific DNA binding demonstrated by electrophoretic mobility shift assays with in vitro–translated GABP proteins. RNAs from GABPα and GABPβ were transcribed in vitro and translated in rabbit reticulocyte lysates (*27*). In vitro–labeled proteins were incubated in the presence of a ^{32}P-labeled DNA fragment from the HSV1 ICP4 promoter and subjected to electrophoresis on a nondenaturing 5% polyacrylamide gel in 0.5× TBE buffer (tris-borate-EDTA) (*28*). Radioactive DNA and DNA-protein complexes were visualized by autoradiography. B, GABP-DNA complex; E, endogenous reticulocyte lysate protein–DNA complex.

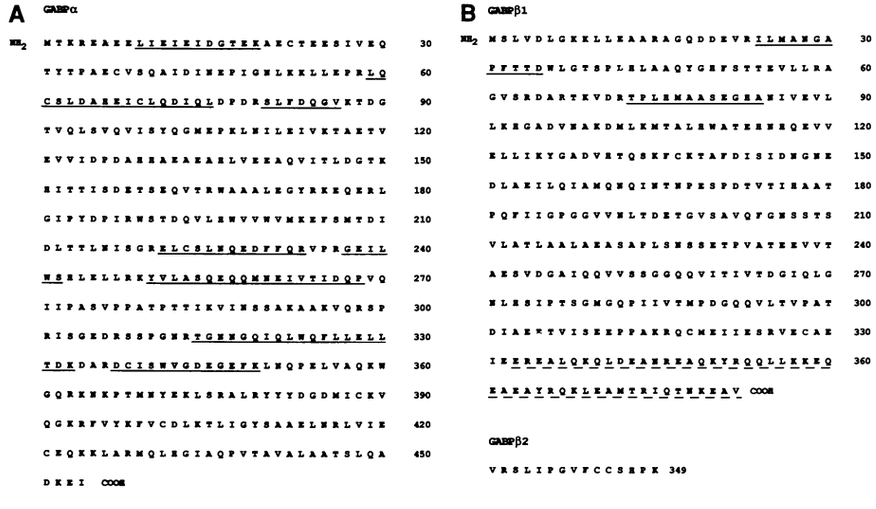

Fig. 2. Deduced amino acid sequences of cDNAs encoding GABP subunits. (**A**) Sequence of GABPα and (**B**) sequences of GABPβ1 and GABPβ2. An unamplified cDNA library prepared from mouse adipocyte mRNA (*7*) was screened with a mixture of degenerate oligonucleotides derived from the amino acid sequences of peptides 3, 4, 5, and 8 (Fig. 1). GABPβ1 and GABPβ2 were isolated by screening a cDNA library (*8*) from mouse 8.5-day embryos with degenerate oligonucleotides corresponding to peptides 9 and 12 (Fig. 1). DNA sequencing was by dideoxy chain termination (*24*) with Sequenase (U.S. Biochemicals) as suggested by the manufacturer. The nucleotide sequences have been deposited in GenBank under the following accession numbers: α, M74515; β1, M74516; and β2, M74517. Peptides identified by amino acid sequencing of purified GABP are underlined. The dashed lines indicate the sequence in GABPβ1 not found in GABPβ2. The sequence for GABPβ2 is shown from the point at which it diverges from GABPβ1. Abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

tein. Reticulocyte lysate that had not been programmed with RNA contained endogenous protein capable of forming an oligonucleotide-protein complex that migrated more rapidly than the oligonucleotide-protein complex formed by GABP. Other than this background activity endogenous to the reticulocyte lysate, specific protein-DNA complexes were not observed when lysates programmed with GABPα, GABPβ1, or GABPβ2 were tested in electrophoretic mobility shift assays. Likewise, no DNA binding activity was observed with lysate that had been used to cotranslate RNAs encoding GABPα and GABPβ2. However, cotranslation of RNAs encoding GABPα and GABPβ1 caused the lysate to display DNA binding activity that could be distinguished from background (Fig. 3C). The interdependency of GABPα and GABPβ1 observed in these assays is consistent with earlier observations on the interdependency of subunits purified from rat liver nuclei (*6*). The nature of this interdependency is described by Thompson *et al.* (*9*).

Having obtained the presumptive amino acid sequences of the two subunits of GABP, we searched the GenBank database to determine whether either subunit might be related to previously described proteins. Significant amino acid sequence similarity was observed between GABPα and the product of the *ets-1* (*10, 11*) proto-oncogene. The Ets-related region of GABPα is located close to the COOH-terminus of the subunit. Biochemical studies of Ets-1 (*11*), as well as the related proteins PU.1/Spi-1 (*12*) and E74A (*13*), have demonstrated direct, sequence-specific DNA binding. These proteins, as well as the products of several additional eukaryotic genes, share sequence similarity in an 85–amino acid region required for DNA binding termed the ETS domain (*14*). The region of GABPα that is related to this family of proteins coincides with the ETS domain (Fig. 4).

The amino acid sequences of GABPβ1 and GABPβ2 contain four repeats of a related amino acid sequence at the NH_2-termini of both subunits (Fig. 4). The first two repeats were 32 amino acids in length and the second two contained 33 amino acids. Similar repeats occur in the Notch protein of *D. melanogaster* (*15*) and the Lin-12 and Glp-1 proteins of *C. elegans* (*16, 17*). These 33–amino acid repeats were first recognized in studies of the yeast protein SWI6, which regulates gene expression involved in mating type switching (*18*). Similar repeats have been identified in ankrin, a multifunctional protein associated with the membrane of red blood cells (*19*), several vaccinia virus encoded proteins of unknown function (*20*), and the transcription factor NFκB (*21*).

The observations outlined in this study confirm earlier results showing that GABP is composed of two distinct subunits (*6*). However, the biological function of this DNA binding complex remains obscure. GABP may facilitate transcriptional activation of HSV IE genes in addition to its participation in the regulation of cellular gene expression.

Because the two subunits of GABP exhibit primary sequence motifs typical of proteins normally found in different cellular compartments, the question arises as to whether transcriptional regulatory proteins, such as members of the Ets family, might interact with membrane-bound proteins that contain the 33–amino acid repeats present in GABPβ. Notch, Glp-1, and Lin-12 might sequester at the plasma membrane a molecule that is important for signal transduction, possibly a transcription factor, which could be released in response to extracellular signaling events.

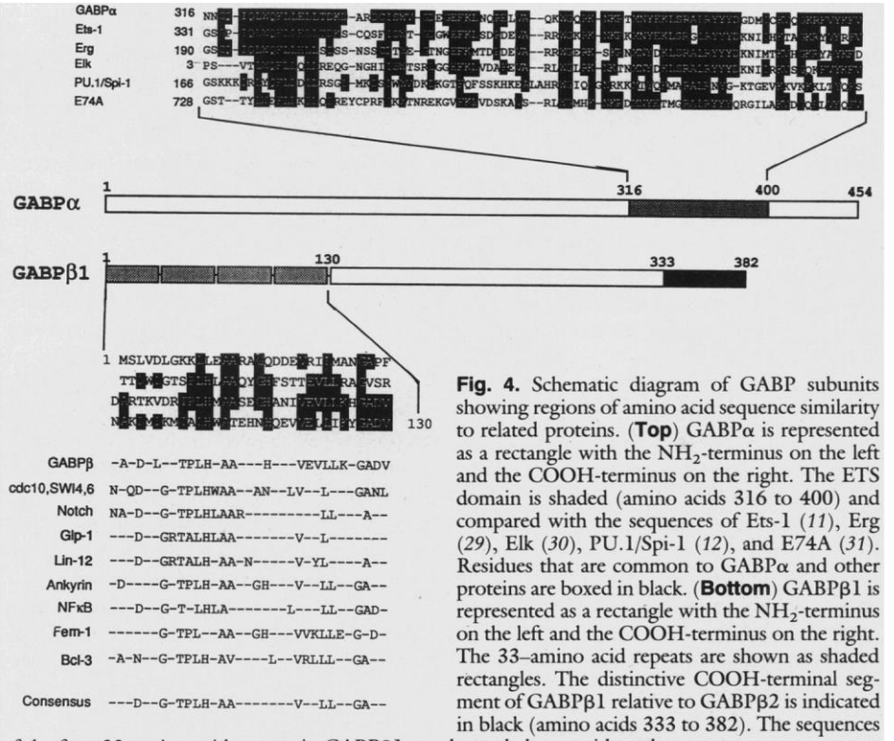

Fig. 4. Schematic diagram of GABP subunits showing regions of amino acid sequence similarity to related proteins. (**Top**) GABPα is represented as a rectangle with the NH_2-terminus on the left and the COOH-terminus on the right. The ETS domain is shaded (amino acids 316 to 400) and compared with the sequences of Ets-1 (*11*), Erg (*29*), Elk (*30*), PU.1/Spi-1 (*12*), and E74A (*31*). Residues that are common to GABPα and other proteins are boxed in black. (**Bottom**) GABPβ1 is represented as a rectangle with the NH_2-terminus on the left and the COOH-terminus on the right. The 33–amino acid repeats are shown as shaded rectangles. The distinctive COOH-terminal segment of GABPβ1 relative to GABPβ2 is indicated in black (amino acids 333 to 382). The sequences of the four 33–amino acid repeats in GABPβ1 are shown below; residues that are common to two or more repeats are boxed in black and used to derive the GABPβ consensus sequence. Similar criteria were used to derive consensus sequences for the 33–amino acid repeats of cdc10/SWI4,6 (*32, 33, 18*), Notch (*15*), Glp-1 (*17*), Lin-12 (*16*), ankyrin (*19*), NFκB (*21*), Fem-1 (*34*), and Bcl-3 (*35*). The repeats from cdc10, SWI4, and SWI6 were combined to determine the consensus. The consensus for ankyrin was taken from (*19*). The overall consensus was defined as residues present in at least six of the individual consensus repeat sequences.

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GABPβ1. The fifth cDNA clone was approximately 1.4 kb and differed from the other four at its 3′ end; this cDNA clone encoded GABPβ2. Four additional cDNA clones corresponding to GABPβ2 were subsequently identified.
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Design and Synthesis of a Mimetic from an Antibody Complementarity-Determining Region

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A technique for producing non-peptide compounds (mimetics) of designed specificities was developed that permitted the synthesis of a conformationally restricted molecule that mimicked the binding and functional properties of monoclonal antibody (MAb) 87.92.6, which recognizes the reovirus type 3 cellular receptor. Binding of either MAb 87.92.6, peptide analogs, or 87.1-mimetic to the cellular receptor inhibited cellular proliferation. The mimetic was a synthetic β-loop structure that mimics the second complementarity-determining region of the MAb. These studies may lead to strategies for the synthetic design of antibody complementarity regions, ligands, and other pharmacologically active agents that are water soluble, resistant to proteolysis, and nonimmunogenic.

ANTIBODIES USE THE COMPLEMENTARITY-determining regions of their hypervariable domains to bind antigens with high affinity and specificity. Synthetic peptides derived from complementarity-determining region (CDR) sequences have properties similar to the intact antibody (*1*). These peptides can inhibit idiotype–anti-idiotype interactions, bind specific antigens, interact with cellular receptors, and stimulate biological processes. For example, MAb 87.92.6 binds to the cell surface receptor for reovirus type 3 (Reo3R). Cyclic peptide analogs derived from the second CDR of MAb 87.92.6 bind with higher affinity to the Reo3R than the corresponding linear peptide analogs do (*2*). However, because of their proteinaceous nature, the peptide analogs tend to be water insoluble, are highly immunogenic, do not readily cross the blood-brain barrier, can adopt various conformations, and are subject to proteolysis (*2*). The usefulness of peptide analogs in vivo is therefore limited.

To synthesize organic compounds with biological activity and without the limitations of peptides, we first determined the relevant contact residues and conformation involved in MAb 87.92.6 binding. Then we developed a method for the synthesis of molecules with that conformation. Computer modeling of the structure of active and inactive cyclic peptide analogs of the second CDR of 87.92.6 allowed the elucidation of charged amino acid residues that were accessible and relevant in binding to the Reo3R (*2–4*). An approach to synthesize conformationally restricted cyclic organic peptides was developed (Fig. 1) (*5, 6*), and we used this strategy to produce a small organic molecule that contains the deduced relevant contact residues from the second CDR of MAb 87.92.6. This compound structurally mimics the 87.92.6 second CDR, and the technique has allowed the first synthesis of a β loop with the structural properties of an antibody. This organically synthesized structure is named the 87.1-mimetic and has a formula mass of 624 g/mol. The 87.1-mimetic is water-soluble and conformationally restricted. Because of its synthetic nature and size, it is resistant to protease activity and should be nonimmunogenic. Furthermore, the mimetic is expected to be permeable to the blood-brain barrier because of its solubility and small size.

To determine whether the 87.1-mimetic has the binding and functional properties of MAb 87.92.6, we compared the mimetic to the MAb and to other compounds. We have shown that the mouse immunoglobulin G

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