High Affinity Binding Protein for the Regulatory Subunit of cAMP-dependent Protein Kinase II-B

CLONING, CHARACTERIZATION, AND EXPRESSION OF cDNAs FOR RAT BRAIN P150

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Cyclic AMP-dependent protein kinase II-B appears to be adapted for function in the mammalian central nervous system via the properties of its regulatory subunit (RII-B). RII-B is selectively expressed in the central nervous system, tightly associated with cerebral cortex membranes, and avidly complexed by the bovine brain calmodulin-binding protein designated P75 (Sarcker, D., Erlichman, J., and Rubin, C. S. (1984) J. Biol. Chem. 259, 9840-9848). Complexes of RII-B and P75 polypeptides can be purified to near homogeneity from either membrane or cytosolic fractions of brain homogenates, suggesting that the binding protein plays a role in determining the central nervous system-specific properties of protein kinase II-B. To investigate the properties of a prototypic, nonabundant, RII-B-binding protein, we have cloned and characterized cDNAs for rat brain P150, a homolog of bovine brain P75. cDNAs were retrieved from a 3' agt11 expression library using 32P-labeled RII-B as a functional probe. cDNA inserts (800 and 1100 base pairs) subcloned into expression plasmids directed the production of partial P150 polypeptides in Escherichia coli that bind RII-B. Sequence analyses disclosed that P150 is a previously uncharacterized protein that contains multiple octapeptide repeats as well as unique sequences. Antibodies directed against 15-residue peptides corresponding to either repeated or unique sequences bound the polypeptides expressed in E. coli and a 150-kDa protein in rat brain membranes and cytosol. Moreover, the immunoprecipitated 150-kDa protein exhibited high affinity RII-B-binding activity. Finally, 3' deletion analysis demonstrated that a 15-amino acid segment of P150 is essential for binding with RII-B.

Several isozymes of cAMP-dependent protein kinase have been characterized (1, 2). The classical type I and type II-H enzymes account for most, if not all, of the cAMP-stimulated phosphotransferase activity in heart, skeletal muscle, liver, kidney, and a broad spectrum of other cells and tissues (2-4). A third isozyme, cAMP-dependent protein kinase II-B, is expressed primarily in brain, neuroendocrine tissues, ovarian granulosa cells, and Sertoli cells (4-7). Each isozyme is an R2C2 (R, regulatory subunit; C, catalytic subunit) tetramer that is activated and dissociated by cAMP: RzC2 + 4cAMP = RzC2 + 2C. The C subunits of the isozymes exhibit similar properties (8-10) indicating that the unique characteristics of cAMP-dependent protein kinase isozymes are conferred by their cAMP-binding subunits (reviewed in Refs. 1 and 2). The R subunit of protein kinase II-B (RII-B) has been distinguished from the R subunit of protein kinase II-H (RII-H) by the production of RII isoform-specific antibodies (4, 11, 12), by peptide mapping analysis (10, 13), and more recently, by primary sequence comparisons (14-16). Both RII isoforms are differentiated from RI subunits by their divergent physicochemical features and the properties listed above (1, 2, 8, 9).

Several studies suggest that protein kinase II-B is adapted for the regulation of metabolism and cell functions in the central nervous system. RII-B subunits appear to be highly conserved and are selectively expressed as the predominant R isoform in the brain tissue of a variety of mammals (5, 17, 18). No exception to these generalizations has been reported. In purified populations of neurons and astrocytes from rat cerebral cortex, 90% of RII subunits and >80% of total cAMP-binding activity are contributed by the RII-B isozyme (18). In contrast, RII-H appears to be functionally interchangeable with RI in many non-neural tissues. For example, in hearts from different species, the proportion of total R contributed by RII-H or RI varies from 10 to 90% (19). Nevertheless, activation of cardiac muscle adenylyl cyclase produces the same biochemical and physiological consequences in each case (19).

Other distinctive properties of protein kinase II-B are its intracellular compartmentalization and ability to form complexes with other proteins in cerebral cortex. More than 90% of protein kinase II-H fractionates as a soluble, cytoplasmic enzyme from most tissue homogenates, whereas 50-75% of the type II-B kinase is tightly associated with the particulate components of disrupted cerebral cortex (3, 4, 18, 20). RII-B, not C, is the subunit bound by components of the cytoskeleton.

The abbreviations used are: R, regulatory subunit of cAMP-dependent protein kinase; C, catalytic subunit of cAMP-dependent protein kinase; EGTA, [ethylenebis(oxethylenenitrilo)tetraacetic acid; SDS, sodium dodecyl sulfate; bp, base pairs; BSA, bovine serum albumin; PBS, phosphate-buffered saline (10 mM potassium phosphate, pH 7.4, containing 0.15 M NaCl).
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A substantial proportion of cytosolic RII-B also forms stable complexes with proteins that are enriched in cerebral cortex (11, 23).

Little is known about protein-protein interactions that govern the association of RII-B (protein kinase II-B) with cell organelles or cytosolic modulatory proteins. The derived amino acid sequence of RII-B (16) does not contain transmembrane or membrane anchor sequences. Furthermore, after an initial solubilization with Triton X-100, nonionic detergent can be removed, and membrane-derived RII-B can be purified by procedures applied to cytosolic RI and RII subunits. Thus, RII-B might be bound to organelles by a high affinity interaction with a specific "intracellular receptor" or anchor protein. Similar interactions might also explain the selective association of RII-B with certain cytosolic proteins.

We previously described (11) a bovine brain calmodulin-binding protein with a M, of 75,000 (P75) that is a candidate for such a role. P75 and RII-B copurify to near homogeneity, and the two proteins are not dissociated by heat, detergent, dilution, or high ionic strength, properties consistent with a high-affinity binding interaction. P75 is also selectively expressed in cerebral cortex and distributed between cytosol and membranes in parallel with the distribution of RII-B.

In order to evaluate the physiological significance of RII-B binding proteins, it is first necessary to characterize their structural and functional properties and develop tools such as specific antibodies to probe binding protein-RII-B interactions in vitro and in cells. We now report the isolation, characterization, and expression of cDNA clones for P150, the rat brain homolog of bovine P75.

EXPERIMENTAL PROCEDURES

Partial Purification of Cytosolic RII-B-binding Proteins—Samples (5 g) of rat, rabbit, bovine, and porcine cerebral cortex (Pel-freez, Rogers, AR) were homogenized in 30 ml of buffer A (25 mM potassium phosphate buffer, pH 7.3, containing 5 mM EDTA, 1 mM EGTA, 1 mg/ml aprotinin, 1 pg/ml leupeptin, and 1 pg/ml pepstatin) in a Waring blender. The homogenate was centrifuged at 40,000 × g for 30 min, and the supernatant solution was loaded onto a 1.5 × 8-cm column of DEAE-cellulose (Whatman DE53) which had been equilibrated with 25 mM potassium phosphate buffer, pH 7.0. After washing with 80 ml of buffer A and 50 ml of Buffer A plus 80 mM NaCl, a fraction enriched in CAMP-dependent protein kinase II-B and RII-binding activity was eluted with buffer A plus 0.4 M NaCl (4, 9, 11).

All operations were performed at 4 °C.

Preparation of Cytosol and Membranes from Rat Tissues—For a survey of P150 protein expression in rat tissues, organs were removed from two Wistar rats (200 g) and homogenized in (5 ml/g wet weight) buffer B (20 mM potassium phosphate buffer, pH 7.0, containing 0.25 M sucrose, 50 mM NaCl, 5 mM EDTA, 1 mM MgCl2, 1 mM spermin, 1 mM leupeptin, 1 mM pepstatin, and 10 mM benzamidine-HCl) in a Waring blender. Nuclei were pelleted by centrifugation at 700 × g for 5 min. The supernatant was further fractionated into "membranes" (pellet) and "cytosol" (supernatant) by centrifugation at 150,000 × g for 1 h. The membranes were resuspended in buffer A (5 ml/g of tissue in the original homogenate). All operations were performed at 4 °C. Protein concentrations were determined by the method of Lowry et al. (24) using bovine serum albumin (BSA) as a standard.

Western Blots—Samples were subjected to electrophoresis in 0.1% sodium dodecyl sulfate (SDS)-10% (or 8%) polyacrylamide gels as described previously (25). The resolved polypeptides were transferred to nitrocellulose filters (0.45 μm, Schleicher & Schuell) by the procedure of Burnette (11). Transfers were carried out at room temperature for 24 h at a constant current of 0.1 A (30 V) in transfer buffer containing 25 mM Tris base, 192 mM glycine, and 20% methanol at pH 8.3. Proteins bound to the nitrocellulose were stained with 0.05% Coomassie Brilliant Blue in 25% methanol, 10% acetic acid, and destained with 50% methanol, 3% acetic acid. Lanes and positions of molecular weight standards were then indicated with a marking pen.

Method for Labeling RII-B—Purified RII-B proteins were identified by using a modification of a procedure (26) previously used in this laboratory. Proteins transferred to nitrocellulose were incubated in (10 ml/lane) Blotto/BSA (10 mM potassium phosphate buffer, pH 7.4, 0.15 M NaCl, 5% (w/v) Carnation non-fat dry milk, 0.1% (w/v) BSA, 0.01% antifreeze (Sigma), and 0.02% NaN3 (27) for 16 h at 4 °C. The filters were then incubated with 32P-labeled RII-B (106 cpm/ml) in fresh Blotto/BSA (10 ml/lane) for 4–6 h at 22 °C. Next, the filters were washed with Blotto/BSA (25 ml/lane) for 15 min. This step was repeated three times. Subsequently the filters were washed twice with (10 ml/lane) 10 mM potassium phosphate buffer, pH 7.4, containing 0.15 M NaCl (PBS). Binding of 32P-labeled RII-B to proteins was visualized by autoradiography using Kodak XAR-5 film at -75 °C.

Radio labeling of RII-B—Bovine brain RII-B subunits, that were purified to near homogeneity (4), were labeled by incubation with [35S]methionine (25, 26) and [γ-32P]ATP (107, 27). Labelled RII-B subunit in a final volume of 0.5 ml of 25 mM potassium phosphate buffer, pH 7, containing 10 μM CAMP, 10 mM MgCl2, 0.5 mM dithiothreitol, and 0.1 μM [γ-32P]ATP (3.3 × 108 cpm/ml). After a 5-min incubation at 0 °C, the concentration of ATP was adjusted to 1.0 μM by addition of nonradioactive ATP. The 32P-labeled RII-B was incubated for 50 min on ice. Residual [γ-32P]ATP was separated from 32P-labeled RII-B by passing the reaction mixture through a Sephadex G-50 column equilibrated with PBS containing 1 mg/ml (w/v) BSA. The column was poured in a 10-ml disposable pipette and was developed with PBS. Typically the 32P-labeled RII-B was labeled to a specific activity of 1010 cpm/μg RII-B.

The 32P-labeled RII preparation contained a single radioabeled polypeptide with an apparent M, of 55,000. All of the polypeptide was immunoprecipitated with a monoclonal antibody (No. 110, Refs. 11 and 28) which binds RII-B, but does not cross-react with RII-H.

Sewing of AgtII Library—A rat brain cDNA library containing 107 independent clones in the expression vector Agt11 (Clontech, Palo Alto, CA) was screened utilizing a modification of the technique of Huynh et al. (29). Nitrocellulose filter lifts from 20 plates (50,000 plaques/plate) were prepared as for an antibody screening (29), but the filters were probed with 32P-labeled RII-B to detect high affinity RII-B-binding proteins as described above (overlay method). The same library and a rat brain cDNA library (5.5 × 106 independent clones) in Agt10 (30) were subsequently screened with the 32P-labeled cDNA insert from clone λII100R (see Fig. 3) according to the procedure of Young and Davis (29). cDNA inserts from plaque-purified clones were subcloned into the plasmid pGEM-blue (Promega) for sequence analysis.

Expression of RII-binding Polypeptides in Escherichia coli—The cDNA inserts of clones λ800R and λ1100R were directly subcloned into the EcoRI site of the bacterial expression vector pN-IA2 (51) to create pN-800R and pN-1100R, respectively. In this vector the inserted DNA is under the control of an efficient constitutive lac promoter and appropriate translation initiation and termination sequences derived from E. coli genes (31). E. coli LE392 was transformed with the plasmids. After growing the transformed bacteria to OD_{600} = 1, aliquots (0.3 ml) of the cell suspension were centrifuged at 15,000 × g for 1 min in a microcentrifuge, and the pelleted bacteria were resuspended by boiling in 50 μl of electrophoresis loading buffer (125 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.01% bromphenol blue, 2% mercaptoethanol). Polypeptides in the lysate were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose (see above) for further analysis. The cDNA insert from λ1100R was also subcloned into the unique BamHI site of the inducible expression vector pET-3b(32) to create pET-1100R. This was accomplished by treating the 1100R cDNA (excised with EcoRI) with T4 DNA polymerase to produce blunt ends, the addition of BamHI linkers, BamHI digestion, and ligation with BamHI-digested, dephosphorylated plasmid pET-3b. cDNA inserts from λII100R and pET-1100R were each induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside for 1 h after the culture reached OD_{600} = 0.5. The expressed product of pET-1100R was assayed by the overlay method (see above). Lysate proteins from 0.1-ml aliquots of the bacterial cell suspension were assayed for binding activity.

cDNA Sequence Analysis—Restriction fragments derived from

D. Sarkar and C. S. Rubin, unpublished observations.
cDNA inserts in the lgt11 recombinants were subcloned into pGEM blue and were sequenced by the dideoxynucleotide chain termination procedure of Sanger (33) using pGEM primers (Promega) and the modifications introduced by Hattori and Sakaki (34) for double-stranded DNA sequencing.

Production of Antiserum Directed against Synthetic Peptides—The peptides Val-Gly-Gln-Ala-Glu-Glu-Ala-Thr-Val-Gly-Gln-Ala-Glu-Glu (RB15) and Lys-Ser-Lys-Asn-Val-Pro-Lys-Gln-Phe-Leu-Ile-Ser-Met-Glu (RB16) predicted from cDNA sequence data were synthesized with an additional Cys residue at the C terminus. The peptides were synthesized on a 430A peptide synthesizer (Applied Biosystems) with t-butoxycarbonyl chemistry and polyamide resins (Applied Biosystems). Purity was assessed by monitoring the resolution profile from a reverse phase (C18) high performance liquid chromatography column after applying a 0–80% acetonitrile gradient in 0.1% trifluoroacetic acid and by amino acid analysis. Only peptides that contained 5% (w/v) BSA (10 ml/lane) for 90 min to probe PET-1100R were sequenced by the dideoxynucleotide chain termination method of Sanger (33) using pGEM primers (Promega) and 5% (w/v) BSA (10 ml/lane) PBS containing 5% (w/v) BSA and ²⁵¹-protein A (2 pg/ml). Samples of cytosol (6 pl) were mixed with 5 ml of buffer C (20 mM potassium phosphate, pH 7.0, containing 50 mM NaCl, 5 mM EDTA, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, and 10 mM benzamidine) in a motor-driven teflon-glass homogenizer at 4 °C for 30 s (five strokes). The homogenate was centrifuged at 4500 × g at 4 °C for 30 min, and the pellet was discarded. The supernatant solution was centrifuged at 150,000 × g for 1 h to pellet the remaining particulate material. The supernatant (cytosol) was subjected to immunoprecipitation analysis. Samples of cytosol (6 µl) were mixed with either anti-RB15 serum or preimmune serum in a final volume of 70 µl of buffer C supplemented with 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS. Incubations were performed at 4 °C for 20 h. Immune complexes were precipitated with 25 µl of a 50% v/v suspension of protein A-bearing heat-killed Staphylococcus aureus in 20 mM potassium phosphate, pH 7.0, containing 0.2 M NaCl (buffer D). The samples were incubated 90 min on ice with occasional agitation. Next, the suspension was centrifuged at 13,000 × g for 1 min in a microcentrifuge, and the supernatant solution was discarded. The pellet was washed by resuspension in 0.5 ml of buffer D and centrifugation as described above. After three additional washes, the pellet was resuspended in 50 µl of electrophoresis loading buffer, incubated at 100 °C for 3 min, and centrifuged 20 s at 13,000 × g. The supernatant solution was applied to an 8% polyacrylamide-0.1% SDS gel for RII-B overlay analysis.

3'-Deletion Analysis of Plasmid pL-800R—The constructs pl-740R, pl-620R, and pl-450R contain fragments of the cDNA insert from pl-800R (Fig. 2 above and Fig. 3) that were generated by using convenient restriction sites to eliminate 3' sequences of various lengths. pl-740 was constructed by cleaving pl-800 at the unique HpaI site indicated in Fig. 3 and then at a unique HindIII site located just 3' to the 3' EcoRI cloning site to remove a 60-bp fragment. The remaining plasmid DNA was ligated with T4 DNA ligase after generating flush ends with T4 DNA polymerase. pl-450 was constructed in a similar manner using the SpHi site indicated in Fig. 3. pl-600 was constructed by isolating the small SpHi-Ncol fragment (Fig. 3) from a sample of pl-800R which had been treated sequentially with Ncol, T4 polymerase, and SpHi. The SpHi-Ncol fragment was ligated to the large fragment from another sample of pl-800R which had been treated sequentially with HindIII, T4 polymerase, and SpHi.

Molecular Weight Standards for SDS-Polyacrylamide Gel Electrophoresis—The proteins used to calibrate the SDS-polyacrylamide gels, RNA polymerase (subunit M, values = 165,000, 155,000, 95,000, and 40,000), β-galactosidase (M, = 116,000), transferrin (M, = 77,000), and BSA (M, = 68,000) and were purchased from Sigma.

RESULTS
Identification of Unique Binding Proteins for RII-B in the Brains of Several Mammals—Before undertaking the characterization of a high affinity binding protein for RII-B, it was necessary to establish that such proteins occur in cerebral cortex in various species and to obtain an estimate of the number of such polypeptides.

Cytosolic proteins from rat, rabbit, porcine, and bovine brains were partially purified to obtain fractions enriched in cAMP-dependent protein kinase II-B (see "Experimental Procedures"). These fractions were expected to contain high affinity binding proteins complexed with RII-B subunits of the kinase (4, 11). The sample from bovine brain contained the expected 75-kDa binding protein (Fig. 1, lane 1 and Refs. 11 and 26). In addition, a single major RII-B-binding protein was detected among a large constellation of polypeptides in each of the other samples (Fig. 1, lanes 2–4). The binding proteins from pig (82 kDa) and rabbit (66 kDa) brain had sizes similar to bovine P75. In contrast, the rat brain binding protein exhibited a M, of 150,000 (Fig. 1, lane 4). Because the overlay assay (a) reproduces the tight association of RII-B with bovine P75 (26) observed during the copurification of these polypeptides (11) and (b) employs an RII-B concentration of only 1–2 nM, it seemed probable that the binding proteins in other species were homologs of P75. "P150" from rat brain was chosen for further study because of the availability of cell lines and animal systems for future experimental manipulations. Since bovine brain P75 was present at low abundance, highly susceptible to proteolysis and refractory to purification by conventional procedures, a recombinant DNA approach was chosen for the study of rat P150.

Fig. 1. Identification of RII-B-binding proteins from the brains of several species. Fractions enriched in protein kinase II-B were obtained as described under "Experimental Procedures." Samples (0.1 mg of protein) were denatured and electrophoresed in a 10% polyacrylamide-0.1% SDS gel (25), and the resolved proteins were transferred to a nitrocellulose filter as indicated under "Experimental Procedures." The filter was probed with 3²⁵¹-labeled RII-B 10⁴ cpm/µg protein, 10⁵ cpm/ml) as outlined under "Experimental Procedures." An autoradiogram is presented. The samples were derived from bovine (lane 1), rabbit (lane 2), porcine (lane 3), and rat (lane 4) brains.
Isolation and Expression of cDNA Clones Containing Sequences Corresponding to P150 mRNA—A rat brain cDNA expression library in λgt11 was screened by a modification of the immunoscreening procedure of Young and Davis (29), in which \( ^3P \)-labeled RII replaces IgG (see "Experimental Procedures"). Two positive clones were isolated from \( 2 \times 10^6 \) plaques by this functional assay (Fig. 2). The sizes of the cDNA inserts from the two clones were 800 and 1100 bp, indicating that only a limited portion of the P150 amino acid sequence was expressed in the \( \beta \)-galactosidase fusion proteins. The predicted size for a full-length P150 coding sequence is \(~4.0 \) kilobases.

The cDNAs were subcloned into the expression plasmid pIN-A12 (see "Experimental Procedures" and Ref. 31). Restriction maps and alignments of pl-800R and pl-1100R and other vectors used in these studies are presented in Fig. 3. Proteins in lysates of E. coli LE392 transformed with plasmids pl-800R and pl-1100R were resolved by SDS-polyacrylamide gel electrophoresis and assayed for RII-B-binding activity (Fig. 4). Both P68, \(^4\) encoded by the insert in pl-1100R, and P38,40, derived from pl-800R, displayed substantial RII-B-binding activity (Fig. 4, lanes 1 and 2, 7, 9, and 12). No binding proteins were detected in bacteria transformed with plasmids containing the 1100 bp (Fig. 4, lanes 3-5) and 800 bp (Fig. 4, lanes 8, 10, and 11) inserts in the reverse orientations or in nontransformed bacteria (Fig. 4, lanes 6 and 13). Nomenclature and sizes of various P150 cDNA clones and expressed products are provided in Table I.

Sequence Analysis of P150 cDNA—The third P150 clone with a cDNA insert of \(~1.4 \) kilobases (λ1400R) was obtained by resccreening the λgt11 library with the \(^3P \)-labeled insert from λ1100R. The nucleic acid and derived amino acid sequences of the cDNA insert of λ1400R are presented in Fig. 5, A and B. These data were replicated when the corresponding regions of clones λ800R and λ1100R were analyzed. A fourth cDNA (1 kilobase) obtained from a λgt10 library (see "Experimental Procedures" and Ref. 30) contained the sequence corresponding to nucleotides 245-1257 in λ1400R. The presence of a translation termination codon immediately after the last amino acid shown (Fig. 5B) indicates that this sequence represents the C-terminal portion of P150. A striking and remarkable feature of the open reading frame is the occurrence of 36 near-perfect repeats of an octapeptide sequence (rectangles in Fig. 5B). Thirty-two repeats are arranged in tandem (residues 92-347, Fig. 5B). A consensus octapeptide is shown in Fig. 5C. A computer comparison with protein sequence databases (39) revealed that P150 is a previously uncharacterized protein that is not highly related to other, well studied polypeptides.

The Cloned cDNAs Encode Epitopes Associated with a 150-kDa RII-B-binding Protein—Two 15-residue peptides which correspond to sequences predicted from the cDNA clones were synthesized, coupled to carrier proteins, and used as immunogens (36, 40). Peptide RB15 contains one complete repeat followed by the first 6 residues of a contiguous octapeptide, whereas RB16 corresponds to a unique primary sequence near the C terminus of P150 (Fig. 5, B-D). The specificity and potencies of the antisera were assayed by Western blot analyses in which each lane contained a sample of lysate protein from a culture of E. coli BL21 (DE3) which was transformed with the expression vector construct pET-1100R (see "Experimental Procedures" and Table I). pET-1100R contains the 1100-bp cDNA insert from λ1100R under the control of a strong and specific T7 RNA polymerase promoter. Upon induction of the T7 polymerase (inserted in the E. coli BL21 (DE3) genome) by isopropyl-\( \beta \)-D-thiogalactopyranoside, the bacteria synthesize large amounts (\(~5\%\) of total protein) of a polypeptide with an apparent \( M_r \) of 68,000 on SDS-polyacrylamide gels. Both antisera bind P68 (Fig. 6A, lanes 1, 4, and 5) whereas preimmune sera yielded no signals (Fig. 6A, lanes 3 and 6). When these antisera were used to probe Western blots of rat brain membrane proteins, a 150-kDa protein that co-migrates with RII-B-binding activity (binding activity not shown) was avidly complexed (Fig. 6B), indicating that the epitope sequences in peptides RB15 and RB16 are indeed present in P150. Moreover, anti-RB15 serum precipitated a 150-kDa polypeptide from brain cytosol that contained RII-B binding activity (Fig. 6C). Similar results were obtained with anti-RB16 serum. The anti-P150 IgGs were also employed to determine the distribution of P150 between soluble and particulate fractions in rat tissues (Fig. 7A). P150 is relatively abundant in both membrane and cytosol fractions of brain (Fig. 7A, lanes 1 and 2) and is also present in lung membranes (Fig. 7A, lane 3). The binding protein is not found in heart, skeletal muscle, intestine, kidney (Fig. 7A, lanes 5-
that direct the expression of a parent polypeptide (P38,40) with an
terminus (see text and "Experimental Procedures").

cDNA inserts form a series of 3' deletions with common 5' sequences
800R, pI-740R, pI-GOOR, and pI-450R, which were ligated into the
of the cDNA inserts from clones X800R, X1100R, and X1400R. pG-
blue. The positions of restriction sites for NcoI (Nc), NdeI (Nd),
and SphI (S), and HpaI (HI), HpaII (HII), Ral (R), HhaI (Hh),
and BamHI (B) are indicated. EcoRI sites at the 5' end of clones λ800R,
λ1100R, and λ1400R were introduced during the construction of the
library. The sizes and alignments of cDNA inserts in plasmids pl-
800R, pl-740R, pl-600R, and pl-450R, which were ligated into the
constitutive expression vector PIN-IA2 (31), are also presented. These
cDNA inserts form a series of 3' deletions with common 5' sequences
that direct the expression of a parent polypeptide (P38,40) with an
RII-B-binding site and a set of polypeptides truncated at the C

FIG. 3. Sizes and restriction maps of cDNA clones for P150.
The diagram shows the sizes, alignments, and partial restriction maps
of the cDNA inserts from clones λ800R, λ1100R, and λ1400R. pl-
900, pg-500, pg-400, pg-520, pg-450, and pg-350 were prepared by
subcloning appropriate restriction fragments into the plasmid pGEM
blue. The positions of restriction sites for NcoI (Nc), NdeI (Nd),
and SphI (S), and HpaI (HI), HpaII (HII), Ral (R), HhaI (Hh),
and BamHI (B) are indicated. EcoRI sites at the 5' end of clones λ800R,
λ1100R, and λ1400R were introduced during the construction of the
library. The sizes and alignments of cDNA inserts in plasmids pl-
800R, pl-740R, pl-600R, and pl-450R, which were ligated into the
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cDNA inserts form a series of 3' deletions with common 5' sequences
that direct the expression of a parent polypeptide (P38,40) with an
RII-B-binding site and a set of polypeptides truncated at the C terminus (see text and “Experimental Procedures”).

FIG. 4. Expression of polypeptide sequences that include an
RII-B-binding domain. cDNA inserts from λ1100R and λ800R
were subcloned into the EcoRI site of PIN-IA2 to create pl-1100R
and pl-800R, respectively. Cultures of E. coli LE392 transformed with
these constructs were grown to OD600 = 1. Bacterial pellets from 0.3-
ml aliquots of cell suspension were lysed by boiling in buffer contain-
ing 2% SDS and 1% mercaptoethanol, and the proteins were sepa-
rated by electrophoresis on a 10% polyacrylamide-0.1% SDS gel.
Resolved proteins were transferred to nitrocellulose and assayed for
32P-labeled RII-B-binding activity as described under “Experimental
Procedures.” An autoradiogram is presented. Lanes 1 and 2 received
lysate protein from bacteria containing pl-1100R. Lanes 7, 9, and 12
contained lysate protein from bacteria harboring pl-800R. Lanes 3-5
received lysate proteins from bacteria transformed with the 1100R
cDNA insert subcloned in the reverse orientation with respect to the
lpp promoter; lanes 8, 10, and 11 received lysate proteins from
transformed E. coli containing 800R cDNA in the reverse orientation.
Lanes 6 and 13 contained polypeptides from nontransformed E. coli.

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<th>Sizes of P150 cDNAs and their expressed protein products</th>
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<tr>
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DISCUSSION

RII subunit isoforms contain interaction sites that promote complex formation between dissociated RIIc-cAMP, or protein kinase II isozymes and either of two proteins that are enriched in bovine cerebral cortex, P75 and MAP-2 (11, 23, 26, 41). The observation that these interactions are selectively and specifically reproduced in a modified Western blot overlay procedure (26, 41) that employs 32P-labeled RII-B at a concentration of 1–2 nM facilitated the identification of analogous proteins in cerebral cortex from other species (Fig. 1). Unique polypeptides putatively related to bovine P75 were observed in cytosol from rabbit, porcine, and rat brains (Fig. 1, lanes 2–4) and from other species (not shown) after vigorous ho-

5 D. B. Bregman and C. S. Rubin, manuscript in preparation.
6 S. Glantz and C. S. Rubin, unpublished observations.
7 The 60 bp deleted in pl-740R comprise 45 bp of C-terminal coding sequence, a translation stop codon, and 12 bp of the 3'-untranslated sequence.
mogenization and partial purification by ion-exchange chromatography. Despite the functional similarity, the RII-B-binding proteins had apparent M, values ranging from ~66,000 to 150,000. MAP-2-like proteins did not co-purify with protein kinase II activity under standard extraction and purification procedures.

After establishing the generality of expression of RII-B-binding proteins in mammalian brains, we exploited the high modified nitrocellulose overlay technique (see "Experimental Procedures") to obtain two cDNA clones encoding proteins of ~66,000 to 150,000. MAP-2-like proteins did not co-purify with the cDNAs. The IgGs recognize P150 (Fig. 7B). Furthermore, P150 is a 150-kDa protein in Western blots of rat brain cytosolic and membrane proteins as well as the partial P68 and P40 segments predicted from the cDNA sequence (Fig. 5A). The IgGs recognize a 150-kDa polypeptide containing the binding activity (Figs. 6 and 7A). This biological activity. We demonstrated that the cDNAs from these clones encode authentic P150 sequences by preparing antisera directed against two synthetic peptides predicted from the cDNA sequence (Fig. 5D). The IgGs recognize a 150-kDa protein in Western blots of rat brain cytosolic and membrane proteins as well as the partial P68 and P40 sequences produced in E. coli and also immunoprecipitate a 150-kDa polypeptide containing the binding activity (Figs. 6 and 7A). The anti-P150 peptide sera do not bind MAP-2. Rat P150 appears to be a member of a P75/P150 family of brain proteins. The anti-RB16 serum directed against a 150-kDa polypeptide as well as the partial P68 and P40 sequences produced in E. coli and also immunoprecipitate a 150-kDa polypeptide containing the binding activity (Figs. 6 and 7A). The anti-P150 peptide sera do not bind MAP-2.
FIG. 6. Anti-RB15 and RB16 sera recognize epitopes in an RII-B-binding polypeptide expressed in E. coli and in a 150-kDa protein in rat brain membranes and cytosol. A, cultures of E. coli BL21 (DE3) transformed with pET-110B3R were grown to OD_{600} = 0.5 and induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside for 1 h. Lysate proteins from 0.1-ml aliquots of cells were resolved in each lane of a 10% polyacrylamide-0.1% SDS gel and transferred to nitrocellulose. Western blots were incubated for 90 min with anti-RB15 serum (lanes 1 and 2, respectively) and anti-RB16 serum (lanes 3 and 4, respectively). Lanes 5 and 6 were incubated with the corresponding preimmune sera (1:100). After incubation of the filters with 125I-protein A, washing, and drying (see "Experimental Procedures"), an autoradiogram was developed on Kodak XAR-5 film. B.12

FIG. 7. A, distribution of P150 between cytosol and membranes in rat tissues. Samples (60 μg of protein) of membranes and cytosol from rat tissue homogenates (see "Experimental Procedures") were subjected to electrophoresis on an 8% polyacrylamide-0.1% SDS gel, and the resolved proteins were transferred to a nitrocellulose filter. The filter was incubated with anti-RB15 serum diluted 1:1000 for 24 h at 22 °C and then with 125I-Protein A as described in Fig. 6 and under "Experimental Procedures." An autoradiogram is presented. Odd-numbered lanes received samples of membranes; even-numbered lanes contained cytosolic proteins. B, pancreas; C, liver; D, kidney; E, heart; F, skeletal muscle; G, colon; H, intestine; I, ileum; J, testis; K, spleen; L, lung; M, brain; N, brain. 32P-labeled RII-B overlay analysis as described in Fig. 6C.
of protein kinase II-B for brain-specific functions. Alternatively, the mechanically vigorous, low ionic strength homogenization procedures used to disrupt brain tissue might artifically release P150 from particulate structures when the complex architecture of the brain is destroyed. When cultured glial and neuronal tumor cells that express P150 are gently disrupted, virtually all of the P150 is found in noncytosolic compartments. Further studies will be required to determine the subcellular location of P150 in primary cultures of brain cells, neuronal and glial tumor lines, and rat brain sections.

A potential site for P150-mediated targeting of protein kinase II-B is the Golgi apparatus of neurons, as determined by the immunocytochemical studies of DeCamilli et al. However, direct evidence for the association of P150 with Golgi membranes is not yet available. The partial, derived P150 sequence does not include likely transmembrane or anchor sequences or sequences characteristic of particulate (e.g. cytoskeletal) proteins. Moreover, the P150 cDNAs encode soluble polypeptides in E. coli. The sequencing of full-length cDNAs (in progress) will permit a straightforward analysis at the level of primary structure. In complementary studies anti-P150 antibodies can be used to examine the subcellular location of P150 in primary cultures of brain cells, neuronal and glial tumor lines, and rat brain sections.

Finally, 3' deletion analysis demonstrates that the C-terminal 15-amino acid residues are essential for RII-B-binding activity (Fig. 8). Although these residues might participate in higher order interactions or serve to regulate RII-B-binding activity at another site, it is possible that a single stretch of C-terminal sequence constitutes the entire high affinity binding site. This possibility can be evaluated by in vitro mutagenesis and expression analysis. If the C terminus of P150 is shown to be the binding site it will be of considerable interest to use recombinant DNA methods to attach the C-terminal pentadecapeptide to C termini of other proteins that are targeted to the plasma membrane, lysosome, endoplasmic reticulum, etc. By using mammalian expression vectors, it should then be possible to examine the biochemical and regulatory consequences of systematically altering the intracellular localization of cAMP-dependent protein kinases.

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REFERENCES


