Purification and cDNA Cloning of ARPP-16, a cAMP-regulated Phosphoprotein Enriched in Basal Ganglia, and of a Related Phosphoprotein, ARPP-19*  

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ARPP-16 (cAMP-regulated phosphoprotein of \(M_r = 16,000\)) is a substrate for cAMP-dependent protein kinase and is enriched in the basal ganglia. ARPP-16 has been purified to homogeneity from the soluble fraction of bovine caudate nuclei. An additional substrate for cAMP-dependent protein kinase of \(M_r = 19,000\) (ARPP-19) was found to cross-react with rabbit antiserum prepared against purified ARPP-16. Immunological analysis indicated that ARPP-16 was enriched in the basal ganglia while ARPP-19 was present in similar levels in all brain regions studied and was also present in non-neuronal tissues. ARPP-19 was also purified to homogeneity from bovine caudate nucleus cytosol.

Using oligonucleotide probes based on the partial amino acid sequence of purified ARPP-16, cDNA clones for ARPP-16 and ARPP-19 were isolated from a bovine caudate nucleus cDNA library and sequenced. The predicted amino acid sequences of the two proteins were identical except that ARPP-19 had an additional 16 amino acids at the NH₂-terminal. The two cDNA clones share an identical 3'-untranslated region of 756 nucleotides. The cDNA clone for ARPP-16 contained 896 additional nucleotides located 3' to this common sequence. The 5'-untranslated regions of the two clones were entirely different. The results suggest the possibility that ARPP-16 and ARPP-19 are produced by alternative transcription and splicing.

Regulation of phosphorylation of specific substrate proteins by cAMP-dependent protein kinase appears to be a general mechanism by which neurotransmitters produce physiological effects in specific target neurons. We have previously described the enrichment of several such substrates in the basal ganglia (Walaas et al., 1983). These include proteins of \(M_r = 21,000, 32,000, 40,000,\) and 90,000, as estimated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). One of these proteins, DARPP-32 (dopamine and cAMP-regulated phosphoprotein of \(M_r = 32,000\)), is specifically enriched in neurons possessing \(D_1\)-dopamine receptors and is believed to mediate some of the actions of dopamine in these neurons by its ability to inhibit protein phosphatase-1 (for review, see Hemmings et al., 1987). In this paper, we describe the identification of ARPP-16, a protein of \(M_r = 16,000\) which is also highly enriched in the caudate putamen. Antiserum raised against purified ARPP-16 revealed the presence of a related phosphoprotein, ARPP-19, which was found in a variety of tissues, unlike ARPP-16. ARPP-16 and ARPP-19 have been purified and characterized biochemically, and their cDNA clones have been isolated and sequenced. Their nucleotide sequences suggest that mRNA species for ARPP-16 and ARPP-19 may be produced by a differential splicing mechanism.

**Experimental Procedures**

**Materials**

The catalytic subunit of cAMP-dependent protein kinase was purified from bovine heart as described (Kaczmarek et al., 1980) and was stored at 4 °C in 150 mM KPO₄ (pH 6.8). *Escherichia coli* DNA polymerase I (Klenow fragment), deoxy- and dideoxynucleotide triphosphates, DNA ligase, E. coli polynucleotide kinase, T₄ polynucleotide kinase, and restriction endonucleases were obtained from Boehringer Mannheim, New England Biolabs, or Pharmacia LKB Biotechnology Inc. 1-Tosylamide-2-phenylethylchloromethyl ketone (TPCK)-trypsin was obtained from Worthington, and thermolysin was obtained from Calbiochem-Behring. All other chemicals were of reagent grade and were obtained from standard commercial suppliers. Fresh bovine brains were obtained from a local slaughterhouse and transported on ice to the laboratory. The caudate nuclei were removed, frozen in liquid nitrogen, and stored at −70 °C until use. [γ-³²P]ATP and [α-³²P]ATP were obtained from Du Pont-New England Nuclear.

**Phosphorylation Assays**

Endogenous phosphorylation was assayed at 30 °C in a reaction mixture (final volume 0.1 ml) containing 50 mM HEPES (pH 7.5), 10 mM magnesium acetate, 1 mM EGTA, 2 μM [γ-³²P]ATP, 50 μg of cytosolic protein, in the absence or presence of 10 μM 6-bromo-cAMP. After 60 s of preincubation at 30 °C, reactions were initiated by the addition of cyclic AMP (cAMP).

**Acknowledgements**

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**References**

1. The abbreviations used are: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ARPP-16, cAMP-regulated phosphoprotein of \(M_r = 16,000\); ARPP-19, cAMP-regulated phosphoprotein of \(M_r = 19,000\); DARPP-32, dopamine- and cAMP-regulated phosphoprotein of \(M_r = 32,000\); TPCK, 1-Tosylamide-2-phenylethylchloromethyl ketone; HEPES, N₂ hydroxyethylpiperazone N₆ 2 ethanesulfonic acid; NEPHGE, non-equilibrium pH-gradient gel electrophoresis; HPLC, high performance liquid chromatography; FPLC, fast performance liquid chromatography; EOTA, [ethylenediamine (toxethyleneminitrilo)] tetraacetic acid; bp, base pair(s).

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addition of [γ-32P]ATP. Reactions were terminated after 60 s by the addition of 20 μl of SDS-containing stop buffer (final concentrations, 1% SDS, 60 mM Tris-HCl (pH 6.8), 5% (v/v) glycerol, 0.2 M β-mercaptoethanol, trace of pyronin Y). The samples were boiled for 2 min and were subjected to SDS-PAGE using 13.5% polyacrylamide gels according to the method of Laemmli (1970). Gels were stained in Coomassie Brilliant Blue, destained, dried, and subjected to autoradiography.

To monitor purification, ARPP-16 was assayed by its ability to accept the γ-phosphate from [γ-32P]ATP in a reaction catalyzed by the purified cAMP-dependent protein kinase, essentially as described for DARPP-32 by Hemmings et al. (1984). Reactions were carried out at 30 °C for 30 min in the reaction mixture described above using 3 to 6 μg/ml catalytic subunit of cAMP-dependent protein kinase and 50 μM [γ-32P]ATP (200-500 cpm/pmol). Reactions were terminated by the addition of SDS-stop solution, and samples were subjected to SDS-PAGE and autoradiography. Non-equilibrium pH-gradient gel electrophoresis (NEPHGE) was carried out using the method of O'Farrell et al. (1977) as described by Walas et al. (1989). Isoelectric points of ARPP-16 and ARPP-19 were estimated by comparing their migration with that of synapsin I (pl 10.2) and cytochrome c (pl 10.6). Protein determination was performed using the method of Bradford (1976) or the method of Peterson (1977) using bovine serum albumin as standard.

Two-dimensional Tryptic Phosphopeptide Mapping
Purification and cDNA Cloning

Step 1: Homogenization—Frozen bovine caudates (280 g) were homogenized in 5 volumes (1400 ml) of 10 mM KPO4, 0.1 M EDTA, 0.5 mM EGTA, 15 mM β-mercaptoethanol, 0.1 M phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 4 μg/ml pepstatin (buffer A), using a Polytron homogenizer (Kinematica GmbH, Brinkmann Instruments) for 2 × 1 min with a 1-min interval. The homogenate was centrifuged at 30,000 × g for 20 min at 4 °C, and the supernatant was collected. The pellet was then homogenized in 3 volumes (840 ml) of buffer A as described above and centrifuged at 1,600 × g for 20 min. The two supernatants were combined and centrifuged at 25,000 × g for 3 h. ARPP-16 was purified from the resulting supernatant.

Step 2: DEAE-cellulose and CM-cellulose Chromatography—A 250-mL DEAE-cellulose column (5 × 15 cm, DE52, Whatman) and a 70-mL CM-cellulose column (2.5 × 5 cm, CM52, Whatman) were connected in series and equilibrated with buffer A. The supernatant was loaded at 135 ml/h, and the columns were washed with 800 ml of buffer A. Flow-through from the DEAE-cellulose column was loaded to the CM-cellulose column was eluted from the latter column with a 500-ml linear gradient of 0 to 5 M NaCl in buffer A, and 7-ml fractions were collected. ARPP-16 was assayed as described under "Experimental Procedures." ARPP-16 eluted at a conductivity of 900 to 1100 μS/cm. The peak fractions were pooled and dialyzed against 3 changes of 4 liters of 10 mM NH4HCO3, and lyophilized.

Step 3: FPLC Using Mono S Chromatography.—The dried sample was dissolved in 3.5 ml of 20 mM KPO4 (pH 7.0) and centrifuged at 10,000 × g for 10 min. Aliquots of the supernatant were injected onto a Mono S HR 5/5 column (Pharmacia) equilibrated in 20 mM KPO4, pH 7.0, and the column was washed with 10 ml of 20 mM KPO4 (pH 7.0). Protein was eluted with a linear gradient of 0.3 to 0.5 M NaCl in 20 mM KPO4, and 1-ml fractions were collected and assayed for ARPP-16. The pooled fractions of ARPP-16, which eluted between 0.33 and 0.36 M NaCl, were pooled.

Step 4: HPLC Using Reverse Phase C18 Chromatography.—The pooled FPLC fractions were injected onto a Vydac C18 reverse phase column (0.4 × 25 cm, 5-μ particle size, 300-Å pore size) equilibrated in 0.1% trifluoroacetic acid (v/v). The column was developed isocratically using 0.1% trifluoroacetic acid (v/v) and 15% acetonitrile (v/v) for 5 min, followed by a linear gradient of 15 to 30% acetonitrile for 30 min. Pure ARPP-16 eluted at 26% acetonitrile.

Antibodies
Polyclonal antibodies were raised in two female New Zealand white rabbits by injecting purified ARPP-16 (0.14 mg) conjugated to bovine thyroglobulin with glutaraldehyde and mixed with Freund's complete adjuvant, as described for Hemmings and Greengard (1986).

Immunoprecipitation of Phosphorylated Tissue Extracts
Crude cytosol was prepared and endogenously phosphorylated as described above. Immunoprecipitation was carried out as described by Hemmings and Greengard (1986) using formalin-fixed Staphylococcus aureus Cowan cells (Pansorbin, Calbiochem-Behring).

Immunoblotting
Proteins were separated by SDS-PAGE as described by Laemmli (1970) using 13.5% acrylamide and transferred to nitrocellulose (pore size, 0.2 μm; Schleicher & Schuell) as described by Towbin et al. (1979). Immunoblotting was performed at room temperature as described (Hemmings and Greengard, 1986), using non-fat dry milk as blocking agent and [125I]-protein A (Amersham). [125I]-Labeled bands were visualized by autoradiography. Quantitation was performed by cutting the nitrocellulose pieces using the autoradiogram as a guide and counting in a γ counter. Purified ARPP-16 and ARPP-19 (2-50 ng) were used on all gels for standardization.

Determination of Sedimentation Coefficient of ARPP-16
Linear sucrose density gradient ultracentrifugation was performed by the method of Martin and Ames (1961) as described by Hemmings et al. (1984) except that carbonic anhydrase, soybean trypsin inhibitor, and cytochrome c (Sigma) were used as marker proteins.

Partial Amino Acid Sequencing of ARPP-16
Since direct NH2-terminal sequencing suggested that ARPP-16 has a blocked NH2 terminus (data not shown), the sequence of several chymotryptic and cyanoctrom peptide fragments was determined in order to serve as a basis for synthesizing oligonucleotide probes for cDNA cloning. The chymotryptic digestion was carried out on 4.3 ml of ARPP-16 that had been heated at 65 °C for 10 min in 50 μl of 8 M urea, 0.4 M NH4HCO3. Using a 1:25 protease/substrate (w/w) ratio, the digestion was allowed to continue for 24 h at 37 °C until the reaction was stopped by injecting the mixture directly onto a Waters Associates high performance liquid chromatography (HPLC) system. For the cyanoctrom bromide digestion, 2.0 ml of ARPP-16 was dried in vacuo prior to dissolving in 0.1 ml of 70% formic acid containing 0.02% phenol. Comparative chymotryptic HPLC peptide mapping of ARPP-16 that was blocked at 110 °C in 6 N HCl containing 0.05% trifluoroacetic acid (v/v) in H2O. The peptides were eluted by increasing the concentration of solvent B (0.05% trifluoroacetic acid in H2O) from 0 to 63% in 63 min, 37–75% B (63–95 min), 75–98% B (95–105 min). Of the two major cyanoctrom bromide and eight major chymotryptic peptides isolated that appeared to be homogeneous based on their 210 nm absorbance profiles (data not shown), a total of six were subjected to amino acid sequencing on a Model 470A Applied Biosystems Protein/Peptide Gas Phase Sequencer equipped with a Model 120 on-line HPLC.

Comparative HPLC Chymotryptic Peptide Mapping of ARPP-16 and ARPP-19
Amino acid compositions for ARPP-16 and ARPP-19 were determined on a Beckman 121M amino acid analyser after hydrolyzing aliquots of these proteins for 24 h at 110 °C in 6 N HCl containing 0.02% phenol. Comparative chymotryptic HPLC peptide mapping of ARPP-16 and ARPP-19 was carried out as described above on 500-pmol aliquots of each digest. The NH2-terminal ARPP-19 peptide used for amino acid analysis and mass spectrometry (that corresponds
to residues 2-32, see “Results”) was isolated from a larger scale ARPP-19 chymotryptic digest carried out on 9.3 nmol of protein. Positive ion fast atom bombardment mass spectrometry of the NH2-terminal ARPP-19 chymotryptic peptide was carried out by the Yale Comprehensive Cancer Center Mass Spectrometry Facility.

**Bacterial Strains and Plasmids**

The E. coli K-12 strains used were WA 802 (Wood, 1966; Nelson et al., 1981) and JM 105 (Messing et al., 1981). Plasmids included psv7186 (Pharmacia), pGEM4 (Promega Biotec), and pTK501 (Kurihara et al., 1988). The WA 802 strain harboring the plasmid was grown in LB medium containing 50 μg/ml ampicillin (Maniatis et al., 1982).

**Preparation of Oligonucleotide Hybridization Probes**


** Colony Hyrbridization**

A bovine caudate nucleus cDNA library (Kurihara et al., 1988) was screened at a density of 2500 to 6500 colonies/82-mm Petri dish. The DNA from lysed bacteria was transferred to nitrocellulose filters as described by Maniatis et al. (1982) and Grunstein and Hogness (1975) with minor modifications (Kurihara et al., 1988).

**Subcloning and Sequencing of cDNA Fragments**

Restriction fragments of clone 1 and clone 6 were inserted into the appropriate sites in M13 mp18, and mp18 phages (Messing, 1983) and the sequences were determined from both the 5' and 3' ends by the dideoxy chain termination method (Sanger et al., 1977). In some cases, sequences were confirmed using the method of Maxam-Gilbert (Maxam and Gilbert, 1980).

**RESULTS**

**Identification of ARPP-16 in Bovine Caudate Nucleus Cytosol**—Crude cytosol fractions from bovine cerebral cortex and caudate nucleus were incubated with Mg2+ and [γ-32P]ATP in the absence and presence of 8-bromo-cAMP. The pattern of protein phosphorylation was analyzed by SDS-PAGE and autoradiography (Fig. 1). As described previously (Walnea et al., 1983), several substrates for cAMP-dependent protein kinase were found to be enriched in the caudate nucleus. The phosphorylation of a protein of apparent Mr = 16,000 (termed ARPP-16), enriched in the caudate nucleus, was found to be stimulated by cAMP. The phosphorylation of a protein of apparent Mr = 19,000 (termed ARPP-19), which was found at similar levels in both cerebral cortex and caudate nucleus, was also stimulated by cAMP. The isoelectric properties of these two proteins were analyzed by NEPHGE (Fig. 2A). ARPP-16, which was more abundant in the caudate nucleus than in the cerebral cortex, migrated as a very basic protein in this gel system (pI 10.5). ARPP-19 migrated as a less basic protein (pI 9.2).

**Purification and Characterization of ARPP-16 from Bovine Caudate Nucleus**—ARPP-16 was purified from bovine caudate nucleus as described under “Experimental Procedures.” A summary of the purification of ARPP-16, obtained from a typical preparation, is shown in Table I. Using the linear sucrose density gradient centrifugation procedure of Martin and Ames (1961), the sedimentation constant of ARPP-16 (1.3 S) was below that of cytochrome c (1.9 S, Mr = 12,500). Assuming a globular conformation for ARPP-16, this sedimentation coefficient corresponds to a Mr of less than 10,000. The amino acid composition of purified ARPP-16, determined as described under “Experimental Procedures,” is shown in Table II.

**Distribution of ARPP-16 in the Brain**—Rabbit antiserum was raised against purified ARPP-16 and used for immunoprecipitation experiments. Following in vitro phosphorylation by the catalytic subunit of cAMP-dependent protein kinase and [γ-32P]ATP and immunoprecipitation, ARPP-16 was detected in highest levels in the caudate nucleus, in lower levels in the cerebral cortex, but not in other tissues including hippocampus, thalamus, cerebellum, and hypothalamus (Fig. 3).

Furthermore, immunoprecipitation experiments indicated that the anti-ARPP-16 cross-reacted with another phosphoprotein of Mr = 19,000 (termed ARPP-19) (See Fig. 1). This protein, which was also phosphorylated by cAMP-dependent protein kinase, was found in all brain regions analyzed (Fig. 3) as well as in various non-neuronal tissues (Girault et al., 1990). Two-dimensional tryptic (Fig. 4, compare A with B) or thermolytic (Fig. 4, compare C with D) phosphopeptide maps prepared from ARPP-16 and ARPP-19 phosphorylated by cAMP-dependent protein kinase were identical.

**Purification of ARPP-19 from Bovine Caudate Nuclei**—ARPP-19 was purified from bovine caudate nucleus cytosol using essentially the same procedures as for ARPP-16, i.e. DEAE-cellulose and CM-cellulose, FPLC Mono S, and HPLC (G1α reverse phase chromatography). ARPP-19 eluted at slightly lower ionic strength than ARPP-16 following CM-cellulose and FPLC Mono S chromatography and eluted from the G1α reverse phase HPLC column as a single peak at 25% acetonitrile. A summary of the purification of ARPP-19, obtained from a typical preparation, is shown in Table IB. The isoelectric properties of purified ARPP-16 and ARPP-19 were analyzed by NEPHGE and compared with those of the phosphorylated proteins identified following endogenous phosphorylation of bovine caudate nucleus cytosol (Fig. 2). The dephospho-forms of both proteins migrated with slightly lower electrophoretic mobility than the phosphorylated forms.
FIG. 2. Two-dimensional electrophoresis of soluble proteins from bovine caudate nuclei. A, endogenous phosphorylation of soluble proteins from bovine caudate nuclei was performed in the presence of 8-bromo-cAMP as described in the legend to Fig. 1. Samples were separated by NEPHGE; acidic (left), basic (right), and the phosphoproteins were visualized by autoradiography. Arrows indicate phosphorylated ARPP-16 and ARPP-19. B, purified ARPP-16 (1 μg) and ARPP-19 (1 μg) were mixed and separated by NEPHGE. Proteins were visualized by Coomassie Blue staining. Arrows indicate dephosphorylated ARPP-16 and ARPP-19. Dephosphorylated ARPP-16 and ARPP-19 both migrated with more basic isoelectric points than the respective phosphorylated proteins.

more basic isoelectric points than their respective phosphoforms.

cDNA Cloning and Sequencing of ARPP-16 and ARPP-19—A cDNA library of bovine caudate mRNA was prepared using a modified Okayama-Berg vector (Okayama and Berg, 1982; Kurihara et al., 1988). Transformed E. coli colonies were screened by in situ colony hybridization (Grunstein and Hogness, 1975; Maniatis et al., 1982) using a mixture of two 5’ end-labeled oligonucleotides (A-1 and A-3). Six positive clones were picked from 50,000 examined, and their restriction maps were constructed. Based on restriction mapping and Southern hybridization (data not shown), these six clones were classified into two species represented by clone 1 and clone 6 which contained a 1.2- and a 2-kilo base pair insertion, respectively. Various restriction fragments were isolated, subcloned into M13mp18 and M13mp19, and their nucleotide sequences were determined. Restriction maps and sequence strategies are shown in Fig. 5.

Fig. 6A shows the entire nucleotide sequence of the cDNA of clone 1. A 5’ leader sequence of 111 bp is followed by a coding region of 288 bp which encoded the 96-amino acid ARPP-16 protein. Following the coding region is a noncoding region of 1562 bp followed by the poly(A) tail. The poly(A) signal of AATAAA is found 14 bases upstream from the start of the poly(A) tail (Proudfoot and Brownlee, 1976). As shown by the dotted underlining in Fig. 6A, 83% of the proposed ARPP-16 sequence was confirmed by direct amino acid sequencing of chymotryptic and cyanogen bromide peptides that were isolated as described under “Experimental Procedures.” The experimentally determined amino acid composition of ARPP-16 (Table II) was in excellent agreement with that predicted on the basis of the cDNA sequence. An NH₂-terminal methionine is apparently present in the isolated ARPP-16, albeit with a blocking group attached. This was consistent with the failure of intact ARPP-16 to yield to direct NH₂-terminal sequencing and with the isolation of a cyanogen bromide peptide corresponding to residues 2-51 that was successfully sequenced. An acetylated methionine was identified at the NH₂-terminal by matrix-assisted UV laser desorption mass spectrometry (Beavis and Chait, 1989). The measured M₁ of 10,708 ± 1 agreed closely with the predicted value calculated from the cDNA sequence assuming an NH₂-terminal acetylated methionine (M₁ = 10,707.2).

Fig. 6B shows the entire nucleotide sequence of the cDNA of clone 1. It contains a 5’ leader sequence of 96 bp followed by a 336-bp coding region and a 756-bp noncoding region before the poly(A) tail. There is no AATAAA sequence before the poly(A) tail. The sequence with the thick underline is identical with a large region of the clone 6 cDNA (also thick underlined) except for one base 59 bp upstream from the poly(A) tail. The amino acid sequences deduced from the base sequence of clones 6 and 1 are compared in Fig. 7A. The only difference between the two is a 16-amino acid stretch at the NH₂ terminus encoded by clone 1. This finding prompted us

<table>
<thead>
<tr>
<th>Total protein</th>
<th>ARPP-16</th>
<th>ARPP-19</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg</td>
<td>%</td>
<td>μg</td>
</tr>
<tr>
<td>A. Purification of ARPP-16</td>
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<td></td>
</tr>
<tr>
<td>Supernatant</td>
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<tr>
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<tr>
<td>HPPLC starting material</td>
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<td>ARPP-16</td>
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<tr>
<td>B. Purification of ARPP-19</td>
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<td></td>
</tr>
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<td>Supernatant</td>
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<tr>
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</tr>
<tr>
<td>ARPP-19</td>
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Purification and cDNA Cloning of ARPP-16

**TABLE II**

Amino acid composition of ARPP-16 and ARPP-19

Hydrolysis was carried out for 24 and 66 h at 110 °C in 6 N HCl. The values for serine and threonine are extrapolated to zero time.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>ARPP-16 (Chemically determined)</th>
<th>ARPP-16 (Deduced from base sequence)</th>
<th>ARPP-19 (Chemically determined)</th>
<th>ARPP-19 (Deduced from base sequence)</th>
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<tbody>
<tr>
<td>Asx (Aspartic acid + Asparagine)</td>
<td>8.93</td>
<td>9 (7 + 2)</td>
<td>8.77</td>
<td>9 (7 + 2)</td>
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<tr>
<td>Threonine</td>
<td>5.82</td>
<td>6</td>
<td>5.87</td>
<td>6</td>
</tr>
<tr>
<td>Serine</td>
<td>4.62</td>
<td>5</td>
<td>7.53</td>
<td>7</td>
</tr>
<tr>
<td>Glx (Glutamic acid + Glutamine)</td>
<td>11.14</td>
<td>11 (5 + 6)</td>
<td>16.89</td>
<td>17 (10 + 7)</td>
</tr>
<tr>
<td>Proline</td>
<td>9.24</td>
<td>9</td>
<td>8.87</td>
<td>10</td>
</tr>
<tr>
<td>Glycine</td>
<td>7.25</td>
<td>7</td>
<td>10.27</td>
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</tr>
<tr>
<td>Alanine</td>
<td>8.14</td>
<td>8</td>
<td>11.68</td>
<td>12</td>
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<td>Cysteine ND*</td>
<td></td>
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<td>0</td>
</tr>
<tr>
<td>Valine</td>
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<td>3</td>
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<tr>
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<td>3.16</td>
<td>3</td>
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<tr>
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<tr>
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<tr>
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<td>15.65</td>
<td>16</td>
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<td>Arginine</td>
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<td>4</td>
</tr>
<tr>
<td>Tryptophan ND</td>
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</tr>
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</table>

**Fig. 3.** Immunoprecipitation of ARPP-16 and ARPP-19 from endogenously phosphorylated cytosol prepared from various bovine brain regions. Crude cytosol was prepared from various bovine brain regions and endogenous proteins phosphorylated in the presence of 8-bromo-cAMP and [γ-32P]ATP. The phosphorylated proteins were immunoprecipitated with rabbit serum raised against purified ARPP-16. The immunoprecipitated proteins were then separated by SDSPAGE using 13.5% polyacrylamide gels, and the phosphoproteins were visualized by autoradiography. P, preimmune serum; I, anti-ARPP-16 serum. CORT, cortex; CAUD, caudate; HIPPO, hippocampus; CEREB, cerebellum; HYPOTH, hypothalamus.

* ND, not determined.

**Fig. 4.** Two-dimensional thin layer tryptic and thermolytic phosphopeptide mapping of phosphorylated ARPP-16 and ARPP-19. ARPP-16 and ARPP-19, phosphorylated in crude cytosol of bovine caudate nuclei by the addition of 8-bromo-cAMP, were digested using TPCK-trypsin or thermolysin as described under “Experimental Procedures.” The digests were applied to thin layer cellulose chromatography plates (origin at lower right, O) and separated in two dimensions, first by electrophoresis in the horizontal dimension (cathode, right; anode, left), followed by ascending chromatography in the vertical dimension. Phosphopeptides were visualized by autoradiography. A and B, comparison of the tryptic phosphopeptide maps of ARPP-16 (A) and ARPP-19 (B). C and D, comparison of the thermolytic phosphopeptide maps of ARPP-16 (C) and ARPP-19 (D).

NH₂-terminal methionine is cleaved off. FAB mass spectrometry of this peak gave an average \( M_r \) of 3,403.8. This value agrees exactly with the calculated \( M_r \) for the amino acid sequence spanning residues 2 to 32 as deduced from the base sequence, assuming that the peptide begins with acetylserine. Moreover, rabbit antiserum to a synthetic peptide coupled to bovine thyroglobulin, corresponding to residues 2 to 15 deduced from the sequence of clone 1, recognized ARPP-19 but
not ARPP-16 on immunoblots (data not shown). The relationship of clone 6 and clone 1 sequences is diagrammatically represented in Fig. 7B. We conclude that clone 6 and clone 1 represent the cDNAs of ARPP-16 and ARPP-19 mRNAs, respectively. Based on the amino acid sequences deduced from their nucleotide sequence, the molecular weights of ARPP-16 and ARPP-19 are 10,665 and 12,353, respectively (not including the blocking group).

**DISCUSSION**

We have purified ARPP-16, a neuronal substrate for cAMP-dependent protein kinase that is specifically enriched in the basal ganglia. ARPP-16 was purified 8,400-fold with a 7.1% recovery to apparent homogeneity from bovine caudate nucleus cytosol. It is estimated that this protein accounts for 0.012% of total cytosolic protein in bovine caudate nucleus. It is estimated that this protein had the same amino acid sequence as ARPP-16 except that ARPP-19 contained an additional 16 amino acid residues at its NH2 terminus. Five of the sixteen residues are glutamic acid, which makes ARPP-19 significantly less basic (pl of 9.2) than ARPP-16.

Analysis of the blocked NH2-terminal peptide of ARPP-19 by FAB mass spectrometry suggested that it starts with acetylserine. This indicates that the 1st residue, methionine, is post-translationally cleaved off and the 2nd residue, serine, is then acetylated. In contrast, the amino acid composition of ARPP-16 indicated that the 1st methionine residue is not removed. Bois et al. (1986) have analyzed the effects of the second codon on removal of the NH2-terminal methionyl residue and found that the presence of a glutamyl residue at position 2 prevents the cleavage of the NH2-terminal methionyl residue. In agreement with their observations, ARPP-16 contains a glutamyl residue at position 2.

Comparison of the cDNA sequences for ARPP-16 and ARPP-19 suggest the possibility that differential splicing is responsible for generating the two species of mRNA. In fact, a consensus sequence of a splice junction (Mount, 1982) corresponding to the 3' end of an exon can be found at position 106 to 108 (CAG) of clone 6 and 138 to 140 (AAG) of clone 1. Clone 6 and clone 1 share an essentially identical sequence of 1044 bp that follows the consensus sequence. This sequence begins at the amino terminus of the coding region of clone 6. In clone 1 it is directly followed by a poly(A) tail, while clone 6 contains an additional 806-bp sequence between the 1044 bp homologous region and the poly(A) tail. A single base difference between the two sequences at the 56th position upstream of the poly(A) tail of clone 1 may be due to a genetic polymorphism or to a cloning artifact.

The expression of ARPP-16 and ARPP-19 proteins has been shown to differ markedly (Fig. 3 and Girault et al., 1990). While ARPP-19 was found in every tissue and every vertebrate species studied, ARPP-16 was only detected in a restricted set of neurons in birds and mammals which receive a dopaminergic innervation. Moreover, the levels of ARPP-19 were highest in the embryo and decreased during development, while ARPP-16 appeared in the caudate putamen and cerebral cortex during the postnatal period (Girault et al., 1990). These results suggest that the expression of ARPP-16 and ARPP-19 is regulated in different ways. Interestingly, the 5' leader sequences of the two cDNAs are totally different. In particular, the 5' leader sequence of ARPP-16 is extremely AT-rich (75%) over a range of 111 bp while the 5' leader sequence of ARPP-19 is GC-rich. The unusual sequence in the ARPP-16 cDNA may reflect a special function of the DNA in this region, such as DNA bending or protein binding (Koo et al., 1986; Gartenberg and Crothers, 1988). Preliminary results from genomic Southern hybridization suggest the differential expression of ARPP-16 and ARPP-19 involves a combination of alternative promoters and splicing rather than the conventional differential splicing of an identical primary transcript. Such a case leading to a ubiquitous and an erythroid-cell-specific form of a gene product has been described for human porphobilinogen deaminase (Chretien et al., 1988). Two isoforms of this enzyme, which are identical except at their NH2 termini, are encoded by two mRNAs differing solely

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1A. Horiuchi, unpublished results.
### Purification and cDNA Cloning of ARPP-16

#### A

The sequences of ARPP-16 and ARPP-19 do not display phosphoprotein, stathmin, has also been shown to be enriched in their 5' ends. The two distinct mRNAs are generated through differential splicing of two primary transcripts which arise from different promoters. The sequences of ARPP-16 and ARPP-19 do not display any homology with other known proteins including myelin basic protein which is also a very basic, low molecular weight protein enriched in the brain. 

#### B

The amino acid sequences determined by direct amino acid sequencing of ARPP-16 are shown with the thick underline represents the identity between clone 6 and clone 1. An arrow indicates the only nucleotide urzedlined. The nucleotide sequences used to generate the synthetic oligonucleotide probes (A-1 and A-3) are denoted by a single thin underline. The potential splice junctions are boxed. AATAAA is double underlined. The complete nucleotide sequences of bovine ARPP-16 cDNA (clone 6) (A) and ARPP-19 cDNA (clone 1) (B) and the amino acid sequences encoded therein. The number on the left of each line is the number of the leftmost nucleotide on that line. The number on the right corresponds to the amino acid number.

### FIG. 6

Complete nucleotide sequences of bovine ARPP-16 cDNA (clone 6) (A) and ARPP-19 cDNA (clone 1) (B) and the amino acid sequences encoded therein. The number on the left of each line is the number of the leftmost nucleotide on that line. The number on the right corresponds to the amino acid number. The thick underline represents the identity between clone 6 and clone 1. An arrow indicates the only nucleotide urzedlined. The nucleotide sequences used to generate the synthetic oligonucleotide probes (A-1 and A-3) are denoted by a single thin underline. The potential splice junctions are boxed. AATAAA is double underlined. A, clone 6, B, clone 1.
actions of dopamine in these cells. It will be of interest to determine the specific role of ARPP-16 in the actions of dopamine or vasoactive intestinal peptide, as well as to determine the mechanisms leading to the specific expression of the protein in these neurons.

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