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ISOLATION AND CHARACTERIZATION OF RECOVERIN-LIKE Ca^{2+}-BINDING PROTEIN FROM RAT BRAIN

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SUMMARY: Rat brain was found, by immunoblot analysis, to have a protein of Mr 23,000 (P23k) that was clearly different from recoverin and was labeled with an antiserum raised against the NH_2-terminus of recoverin. P23k could not be detected by an antiserum raised against the COOH-terminus of recoverin. Blots with ^{45}Ca demonstrated that P23k bound Ca^{2+}. This calciprotein was further purified by Ca^{2+}-dependent hydrophobic interaction and ion-exchange chromatography. In SDS polyacrylamide gel electrophoresis, P23k had an apparent Mr of 21,000 in the presence of 10 μM Ca^{2+} and 23,000 in the absence of Ca^{2+} (0.1 mM EGTA). The isoelectric point of P23k was 5.6. Ca^{2+}-binding analysis indicated that P23k bound 2 moles of Ca^{2+} per mole of protein and had two binding sites with dissociation constants of 13 μM and 0.2 μM. Purified P23k bound to the crude membrane fractions from the cerebellum, cerebrum and retina in a Ca^{2+}-dependent manner. Partial amino acid sequence analysis of proteolytic fragments of P23k revealed the sequence homology between P23k and recoverin. These results suggested that P23k may act as a Ca^{2+}-sensitive regulator by forming a complex with its target on the membrane.

The calcium ion is a major second messenger whose intracellular receptors include a number of structurally related Ca^{2+}-binding proteins (1, 2). These calciproteins transduce the calcium messages into a variety of physiological responses. Recently, a new family of intracellular Ca^{2+}-binding proteins designated as recoverin and/or S-modulin, which serve to regulate light-adaptation, were found in retina and were isolated from a variety of vertebrate species (3, 4, 5, 6, 7, 8). Recoverin activates guanylate cyclase by detaching from the catalytic moiety when free calcium decreases to less than 100 nM (5). This activation is a key event in the resynthesis of cGMP and recovery of the dark state. On the other hand, S-modulin, a similar Ca^{2+}-binding protein, binds to the disk membrane at high Ca^{2+} concentrations and prolongs cGMP phosphodiesterase activation (6, 7). It is not known whether recoverin and S-modulin are the same protein, but the chromatographic and electrophoretic similarity between them suggests that they are members of a new family of

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Ca^{2+}-sensitive regulators that, through cooperative interactions, act as switches at submicromolar Ca^{2+} levels (5, 7). During a survey of recoverin-like immunoreactivity in various tissues, we found a protein of Mr 23,000 (P23k) that was clearly different from recoverin and was labeled with an antiserum raised against the NH_2-terminus of recoverin in mouse (9) and rat brain by immunoblot analysis. P23k was a Ca^{2+}-binding protein and had a Ca^{2+}-dependent membrane-binding property. Immunohistochemical studies provided the specific distribution of P23k in the cerebral cortex and cerebellum (9). The present study demonstrates the purification and characterization of P23k from rat brain.

MATERIALS AND METHODS

Tissue Extraction and Purification Procedure: Rat (Wister albino strain, 3 months old, 40 g) brain was homogenized in 5 volumes of buffer A [20 mM Tris·HCl, pH 7.4, 1 mM $CaCl_2$, 0.5 mM dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride (PMSF)] and centrifuged at 100,000 g for 60 min at 4°C. The supernatant was discarded, the pellet was then resuspended with 5 volumes of buffer B [20 mM Tris·HCl, pH 7.4, 1 mM ethylene glycol bis(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA), 0.5 mM DTT, 0.2 mM PMSF] and centrifuged at 100,000 g for 60 min at 4°C. The resultant EGTA extract was subjected to ammonium sulfate precipitation. The 50-80% ammonium sulfate fraction was dissolved with buffer C [buffer A containing 500 mM NaCl] and dialyzed for 24 hr at 4°C against buffer C. Then, insoluble materials were removed by centrifugation at 100,000 g for 60 min at 4°C, the clear fraction was applied to a column of phenylsepharose CL4B (1 x 15 cm, Pharmacia) equilibrated with buffer C. After washing with buffer D [buffer A containing 100 mM NaCl], retained proteins were eluted with buffer E [buffer B containing 100 mM NaCl] in a total volume of 10 ml. Then the fraction was dialyzed for 24 hr at 4°C against buffer B, and was applied to a Mono Q FPLC column (Pharmacia) equilibrated with buffer B. After elution of the non-bound proteins, the retained proteins were eluted with a linear gradient of NaCl, from 0 mM to 200 mM in a volume of 15 ml and from 200 mM to 500 mM in a total volume of 10 ml, at a flow rate of 0.2 ml/min. The peal fractions were collected and the presence of P23k was assessed by the ^{45}Ca blot method as described below.

One-Dimensional and Two-Dimensional Gel Electrophoresis, and Development of the ^{45}Ca blot and Immunoblot: One-dimensional gel electrophoresis was performed according to the method of Laemmli (10), and two-dimensional gel electrophoresis was performed according to the method of O'Farrell (11). Proteins were transferred onto Immobilon P membrane (MilliGen/Biosearch), and the ^{45}Ca blot was developed as described by Maruyama *et al* (12). The immunoblot was developed as described by Towbin *et al* (13). The polyclonal antibodies to bovine recoverin were used at a dilution of 1:800 as described previously (9).

Calcium Binding Assay: The calcium binding assay was performed by the modified method of Hernandez *et al* (14). In brief, the sample protein was dissolved with the reaction mixture and shaken for 24 hr at 25°C prior to filtration. The reaction mixture was composed of 60 mM KCl, 5 mM $MgCl_2$, 10 mM imidazole (pH 6.8), 100 μM $CaCl_2$ containing ^{45}Ca (10 μCi/ml: New England Nuclear, 56.5 mCi/mg) and various concentrations of EGTA for the Ca^{2+} buffering system. The free calcium concentrations were calculated according to Caldwell (15) using a pKd of 6.29 for Ca/EGTA at pH 6.8. After the reaction mixture was filtered through an Ultrafree filter (type C3GC, Millipore), the radioactivity of the filter was determined by using a liquid scintillation counter (Tri-Carb 2000, Packard). A blank value was obtained by following the same procedure without the protein samples.

Preparation of Membrane Fraction and Membrane Binding Assay: Rat brain was homogenized in 10 volumes of 0.32 M sucrose and centrifuged at 1,000 g for 15 min. The supernatant was then centrifuged at 17,000 g for 60 min. The precipitate was washed twice by resuspension in buffer B and re-centrifuged at 100,000 g for 60 min. The final precipitate was resuspended in buffer A or buffer B and used for the binding assay as a crude membrane fraction. The crude membrane fraction and purified P23k were incubated for 60 min at 25°C in the same buffer. After incubation the crude membrane fraction was pelleted by centrifugation at 100,000 g for 30 min, and using SDS-PAGE, the supernatants were analyzed for unbound P23k, and the pellets were analyzed for bound P23k.

Amino Acid Sequence Analysis: Approximately 2 nmoles of purified P23k was digested with endoproteinase Lys-C (enzyme/substrate ratio; 1/200, Wako) in 20 μl of 20 mM Tris (pH 9.0), 0.1% SDS for 6 hours at 37°C. After adding 100 μl of 2% formic acid, the proteolytic digests were directly fractionated on a Chemcosorb 5-ODS-H reversed phase column (4.6 x 250 mm, Chemco) using a linear gradient over 60 min of 0-80% acetonitrile in 0.1% TFA at a flow rate of 1 ml/min. The amino acid sequence was determined by using an automated gas phase protein sequencer (Model 477A, Applied Biosystems).

RESULTS AND DISCUSSION

Recoverin is a Ca^{2+}-binding protein of Mr 26,000 with a three EF-hand structure (5). The NH_2- and COOH-terminal portions of recoverin have a specific sequence motif that is different from those of other proteins with EF-hand structures. Since the sequence in these parts of recoverin and those of comparable proteins from other species have amino acid homology greater than 90 % (5, 8, 16, 17), NH_2- and COOH-terminal specific antisera were raised against the synthetic peptides coupled to the carrier protein, keyhole limpet hemocyanin [antiserum 1: NH_2-terminal specific antiserum, antiserum 2: COOH-terminal specific antiserum] (9). To survey the expression of recoverin and recoverin-like immunoreactivities in various tissues, immunoblot analysis was performed using these antisera. As shown in Figure 1, in the sample from the retina, a prominent band at 26 kD (P26k) was labeled by antiserum 1 and 2, and was found to be rat recoverin by further experiments. A minor band at 23 kD (P23k) could also be detected by antiserum 1 only. In the sample from the cerebral cortex and cerebellum, only the 23 kD band was intensely labeled by antiserum 1, while no protein bands could be detected by antiserum 2.

Blots with ^{45}Ca were performed on the same sample as described. As shown in Figure 2, P23k in the cerebellum was found to bind Ca^{2+}, however, P23k in the retina could not be detected. This might be due to the amount of P23k in the retina being very small. P26k in the retina also could not be detected in this experiment. This might be due to the affinity of P26k to Ca^{2+} being low comparing with that of P23k. The 30 kD band corresponded to calretinin (18), the 28 kD band to calbindin-D28k (19) and the 19 kD band to calmodulin (20), respectively. Under Ca^{2+}-free conditions these protein bands revealed a discrete higher apparent molecular weight indicating a less

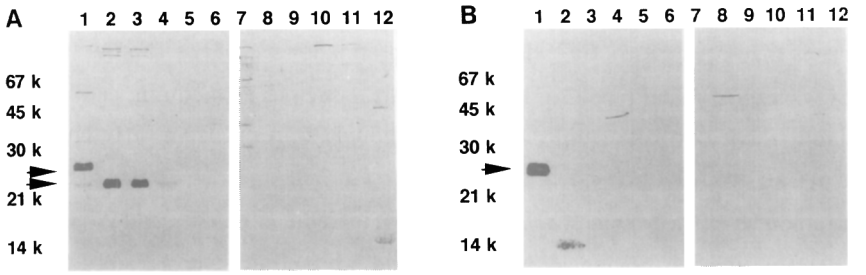


Fig. 1. Immunoblot analysis of the expression of recoverin and recoverin-like immunoreactivities in various tissues reacted with antiserum 1 (**A**), and with antiserum 2 (**B**). Lane 1: retina, lane 2: cerebral cortex, lane 3: cerebellum, lane 4: brain stem, lane 5: kidney, lane 6: liver, lane 7: skeletal muscle, lane 8: heart muscle, lane 9: intestine, lane 10: thyroid, lane 11: thymus, lane 12: spleen. Thirty μg protein of the extracts under low Ca^{2+}-conditions was subjected to SDS-PAGE (14% gel) and proteins were transferred onto Immobilon P membrane for staining with antisera, as described in the text.

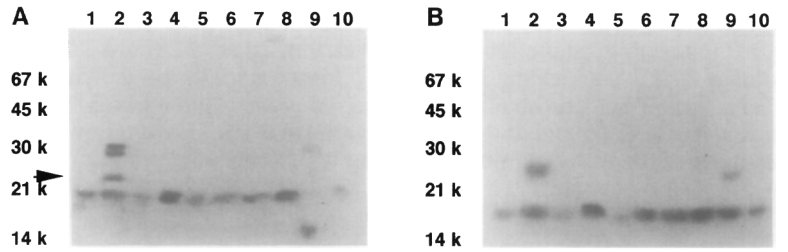


Fig. 2. ^{45}Ca Blot analysis of the extracts from various tissues under low (**A**) and high (**B**) Ca^{2+} conditions. Lane 1: retina, lane 2: cerebellum, lane 3: thyroid, lane 4: thymus, lane 5: spleen, lane 6: skeletal muscle, lane 7: heart muscle, lane 8: intestine, lane 9: kidney, lane 10: liver. Thirty μg protein of the extracts was subjected to SDS-PAGE (14% gel) under Ca^{2+}-free (**A**) and Ca^{2+}-loaded (**B**) conditions. The proteins were transferred onto Immobilon P for binding with ^{45}Ca, as described in the text.

compact and therefore less mobile molecule. P23k was extracted as soluble fractions under low Ca^{2+} conditions (1 mM EGTA), whereas in the presence of excess Ca^{2+}, this protein tended to remain bound to the membrane fractions and could not be extracted as soluble fractions. These results suggested that P23k might be a Ca^{2+}-binding protein with a similar sequence motif to the NH_2-terminal portion of recoverin and that it binds to the membrane in a Ca^{2+}-dependent manner.

We then aimed to purify P23k from rat brain. The EGTA extracts from rat brain were subjected to ammonium sulfate precipitation. P23k was recovered in the fraction containing 50-80% ammonium sulfate. The fraction was then applied to a column of phenylsepharose CL4B. After washing with high Ca^{2+} buffer (buffer D), the retained proteins were eluted with a low Ca^{2+} buffer (buffer E). Then, the fraction was applied to a Mono Q FPLC column, and the retained proteins were eluted with a linear gradient of NaCl. P23k was eluted at around 150 mM NaCl and was found to be of 70% purity. P23k was purified to a final yield of 0.002% of the proteins of total rat brain homogenate, and was subjected to the following analysis.

In one dimensional gel electrophoresis, P23k had an apparent Mr of 21,000 in the presence of 0.1 mM Ca^{2+}, and 23,000 in the absence of Ca^{2+} (0.1 mM EGTA) (data not shown). In two dimensional gels as shown in Figure 3, under Ca^{2+}-free conditions P23k migrates as one spot, the isoelectric point (pI) of which was 5.6. Under Ca^{2+}-loaded conditions, the pI of P23k shifted to a slightly basic position, and P23k appeared as a double spot. The implication of these electrophoretic analyses is that P23k shows Ca^{2+}-dependent conformational changes. Similar observations are reported for other EF-hand type Ca^{2+}-binding proteins such as calmodulin, calbindin-D28k and parvalbumin whose two bands on SDS-gels are thought to result from different shapes of the molecule in Ca^{2+}-loaded /or Ca^{2+}-free conditions (21, 22, 23).

The amounts of Ca^{2+} bound to P23k are shown in Table 1. P23k bound 2 moles of Ca^{2+} per mole of protein, whereas bovine serum albumin did not bind Ca^{2+} appreciably. Scatchard plots analysis for the Ca^{2+}-binding activity indicated two binding sites with dissociation constants of 13 μM and 0.2 μM. Ca^{2+}-dependent binding of purified P23k to the crude membrane fraction was also demonstrated. As shown in Figure 4, in the presence of excess Ca^{2+}, P23k tended to remain bound to the crude membrane fraction from the cerebellum, cerebral cortex and retina and could not

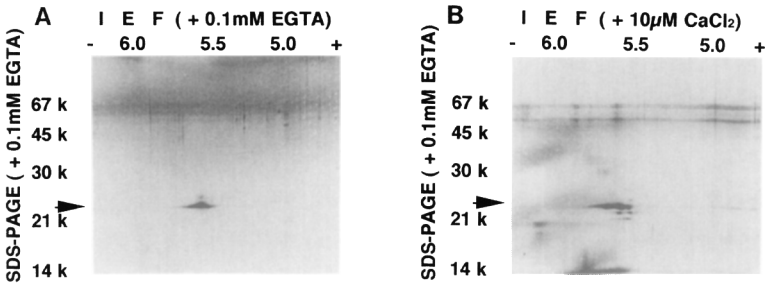


Fig. 3. Two dimensional gel electrophoresis of 0.4 μg purified P23k in Ca^{2+}-free (**A**) and Ca^{2+}-loaded (**B**) conditions. 100 μM of EGTA or 10 μM of Ca^{2+} was added to the IEF (first dimension) gel system, and the second dimension gel electrophoresis (14% gel) was performed under Ca^{2+}-free conditions. Note the double spot under Ca^{2+}-loaded conditions, in contrast to the single spot in Ca^{2+}-free conditions.

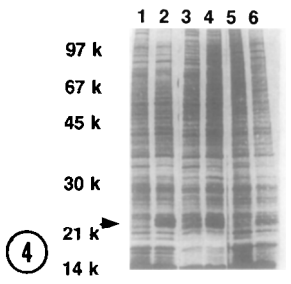
be extracted as a soluble fraction, whereas under low Ca^{2+} conditions P23k was extracted as a soluble fraction.

Sequential Edman degradation of intact P23k generated no PTH amino acids, indicating that P23k has a blocked N-terminus. As shown in Figure 5, the partial amino acid sequence of the endoproteinase Lys-C fragments of P23k indicated that P23k had EF-hand structures but had no sequence identity with known EF-hand type Ca^{2+}-binding proteins. However, P23k shows a 40% amino acid sequence homology with recoverin. The crossreactivity of antiserum 1 shown in immunoblot and immunohistochemical analyses, might be due to the amino acid sequence homology between the NH_2-terminal portion of recoverin and P23K. Recently, Terasawa *et al* isolated novel EF-hand type Ca^{2+}-binding proteins, designated neurocalcins, from bovine brain using W-77 affinity chromatography (25). Since P23k has an amino acid sequence homology of more than 90% with neurocalcin, P23k and neurocalcin might be related. The physiological roles of P23k and neurocalcins are not known yet, however, immunohistochemical studies have revealed that P23k is localized in the cell bodies of pyramidal neurons in the cerebral cortex and of granule cells in the cere-

Table 1. Ca^{2+}-binding relative to the Ca^{2+} concentration

	Calcium/Protein (mol/mol)	
Calcium (M)	P23k	BSA
1×10^{-7}	0.13	0.003
5×10^{-7}	0.22	—
1×10^{-6}	0.26	0.005
5×10^{-6}	0.79	—
1×10^{-5}	0.92	0.010
5×10^{-5}	1.68	—
1×10^{-4}	2.26	—

Proteins were incubated with ^{45}Ca in the reaction mixture at the Ca^{2+} concentration indicated as described in the text.



Recoverin	41	SGRITRQEFQTIYSKFFPEADPKAYAQHVF 61
P^{26}		SGRITRQEFQTIYS FFPEADPK YAQHVF
Visinin		DGRIRCDEFERIYGNFFPNSEPQGYARHVF
P23k		IYANFFPYGGAXK
Recoverin	101	KLEWAFSLYDVDGNGTISKNEVLEIVTAIF 121
P^{26}		KLEWAFSLYDVDGNG
Visinin		KLEWAFSLFDVDRNGEVSKSEVLEIITAIF
P23k		LNWAFXMYDLDGDGXI
Recoverin	141	LPEDENTPEK
P^{26}		LPEDENTPEK
Visinin		LPEDENSPQK
P23k		MPEDTFTP

Fig. 4. Ca^{2+}-dependent membrane binding property of P23k. Purified P23k was incubated with the crude membrane fraction for 60 min at 25°C under high (lane 1, 3, 5) or low (lane 2, 4, 6) Ca^{2+}-conditions. After centrifugation of the reaction mixtures, the supernatants were subjected to SDS-PAGE (10-20% gel). Lane 1, 2: membrane from cerebellum, lane 3, 4: membrane from cerebral cortex, lane 5, 6: membrane from retina.

Fig. 5. Comparison of the amino acid sequence of endoproteinase Lys-C fragments of P23k with homologous parts of the known sequences of bovine recoverin (5, 8) and comparable proteins; human P^{26} (18) and chick visinin (17). Underlines indicate the parts of the putative EF-hand structure. Amino acid numbers correspond to those of recoverin (5, 8).

bellum (9). In view of these findings, P23k is essentially a neuronal protein which is present throughout the cytosol of the cells when intracellular free calcium is being low and shows the membrane association when free calcium increases to micromolar levels. It seems very likely that this protein plays an important role just like recoverin (5) and S-modulin (6, 7) and that a target enzyme exists for P23k whose activity is regulated by this calciprotein in a Ca^{2+}-dependent manner. The complete amino acid sequence of P23k and the identity of this putative target enzyme are now under investigation.

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