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Preparation and Characteristics of Ca^{2+}-Dependent Monoclonal Antibodies to Recoverin

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Abstract—Thirty-four primary hybridoma clones were prepared which expressed monoclonal antibodies to the Ca^{2+}-binding protein recoverin. Among the resulting monoclonal antibodies, two Ca^{2+}-dependent clones (mAb3 and mAb19) recognizing recoverin were detected by solid-phase immunoenzyme assay. In the presence of Ca^{2+}, antibodies of the mAb3 and mAb19 clones bound to recoverin several times better than in the absence of Ca^{2+}. The mAb3 and mAb19 antibodies recognized epitopes located inside the sequences Pro61-Met91 and Pro57-Tyr64 of the recoverin molecule, respectively. The possible mechanism of the Ca^{2+}-dependent recognition of recoverin by the prepared monoclonal antibodies is discussed.

Key words: recoverin, Ca^{2+}-binding proteins, phototransduction, monoclonal antibodies, recoverin epitopes

Recoverin is a Ca^{2+}-binding protein with molecular mass 23.3 kD [1, 2] that acts as a Ca^{2+}-dependent regulator of rhodopsin kinase-mediated phosphorylation of the visual pigment rhodopsin [3-5]. The primary structure of recoverin [1] suggests that four possible Ca^{2+}-binding regions of the EF-hand type should exist. In fact, only EF2 and EF3 are capable of binding the ligand because EF1 and EF4 lack in positions 1 and 3 of their Ca^{2+}-binding loops residues of acidic amino acids which are important for coordination of Ca^{2+} [6]. EF1 has no necessary conformation because Pro40 is present in position 4 of the twelve-membered Ca^{2+}-binding loop. EF4 contains a salt bond between side groups of Lys161 and Glu171, which correspond to positions 2 and 12 in the Ca^{2+}-binding loop [4]. Thus, EF1 and EF4 are most likely required to provide for the necessary three-dimensional structure of recoverin, whereas EF2 and EF3 are functionally active Ca^{2+}-binding regions of this protein [6].

The N-terminus of recoverin is acylated with residues of fatty acids C14:0, C14:1(5-*cis*), C14:2(5-*cis*, 8-*cis*), and C12:0 [7], and this is responsible for its Ca^{2+}-dependent interaction with photoreceptor and phospholipid membranes [8, 9]. In the presence of Ca^{2+}, the myristoyl residue is exposed into solution from the hydrophobic pocket of the recoverin molecule, whereas in the apoform the myristoyl group is submerged in the hydrophobic pocket [10].

These data as well as results obtained by other physicochemical methods have shown conformational changes in the molecule on binding Ca^{2+} [8, 9, 11]. It seems that the epitope structure of recoverin includes regions, which change their accessibility depending on the presence of Ca^{2+}. In fact, preparation of a hybridoma expressing monoclonal anti-recoverin antibodies has been reported [12], and the affinity of these antibodies for the antigen significantly decreased when the reaction mixture contained EGTA, which is a chelating agent of Ca^{2+}. The authors conclude that the sequence 64-70 located in the second Ca^{2+}-binding domain is the principal immunodominant region of recoverin.

The present work was designed to determine the epitope structure of the Ca^{2+}-binding protein recoverin. Thirty-four primary hybridoma clones were prepared, two of which expressed monoclonal antibodies capable of interacting with recoverin depending on Ca^{2+}.

MATERIALS AND METHODS

Reagents. Tris, dithiothreitol, $MgCl_2$, $CaCl_2$, myristic acid, EGTA, isopropylthio-β-D-galactoside, Freund's complete adjuvant, and Freund's incomplete adjuvant

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were obtained from Sigma (USA); ammonium persulfate, acrylamide, methylene bis acrylamide, TEMED, Coomassie Brilliant Blue Servablue G from Serva (Germany); phenyl-Sepharose from Pharmacia (Sweden); bovine serum albumin, Tween 20 from Ferak (Germany); goat antimouse antibodies conjugated with horseradish peroxidase from Amersham (England); HA-type nitrocellulose membranes with 0.22-μm pores from Millipore (USA).

Myristoylated recombinant recoverin and its mutant form of more than 95% purity were prepared as described in [13]. The protein concentration was determined spectrophotometrically at 280 nm using the molar absorption coefficient of 36,400 [14].

Preparation of monoclonal antibodies [15]. Mice were immunized subcutaneously with 50 μg recombinant recoverin per animal in Freund's complete adjuvant. After 14-20 days, the mice were re-immunized intraperitoneally two days in succession with a fivefold dose of the antigen, and on the fourth day, B-cells of the spleen from the recoverin-immunized mice were fused with mouse myeloma cells sp2.0. From the resulting primary hybridoma clones the recoverin-positive clones were selected by ELISA. These clones were thrice recloned by the final dilution method. Cultural media of the clones were twice reprecipitated with ammonium sulfate at 50% saturation and stored at 4°C. Before use, the solution was centrifuged, and the precipitate was dissolved in the appropriate buffer.

Chemical fragmentation of the recoverin molecule [16, 17]. In all experiments 100 μg purified recoverin at the concentration of 1 mg/ml was hydrolyzed. The resulting peptide mixture was lyophilized and stored at –20°C.

Hydrolysis at methionine residues. Lyophilized preparation of recoverin was dissolved in 70% formic acid and supplemented with dry cyanogen bromide in 200-fold excess relative to the methionine content in the protein. Hydrolysis was performed for 24 h at 25°C. Under these conditions, the hydrolysis at two methionine residues went virtually to completion with production of peptides $P_{1\text{-}91}$, $P_{92\text{-}131}$, and $P_{132\text{-}201}$, the mobilities of which could be determined electrophoretically. Trace amounts of peptides $P_{1\text{-}131}$ and $P_{92\text{-}201}$ were determined by immunoblotting, and antibodies of both clones failed to recognize the peptide $P_{92\text{-}201}$.

Hydrolysis at the cysteine residue. Lyophilized preparation of recoverin was dissolved in buffer containing 40 mM Tris-HCl (pH 8.0), 6 M urea, and 2 mM EDTA. To the protein solution (1 mg/ml), equimolar quantity of dithiothreitol (DTT) was added, and the mixture was incubated for 30 min at 37°C. Then a tenfold molar excess 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) relative to cysteine of the protein was added and the mixture was incubated for 30 min at 37°C. Then a 20-fold excess of KCN relative to DTNB was added, and the mixture was incubated overnight at 37°C.

Hydrolysis at the Asp–Pro bond. Lyophilized preparation of recoverin was dissolved in 70% formic acid in the presence of 6 M guanidine chloride. The reaction was performed at 37°C for 110 h. Under these conditions, the Glu–Pro bond located on the C-terminus of the recoverin molecule could be partially hydrolyzed. Note that such nonspecific hydrolysis resulted in peptides $P_{1\text{-}188}$, $P_{61\text{-}188}$, and $P_{189\text{-}201}$. Antibodies of both clones recognized the peptides $P_{1\text{-}188}$ and $P_{61\text{-}188}$; therefore, on analyzing the overlapping peptides only the peptide $P_{61\text{-}188}$ was used. The hydrolyzate was neutralized with 30% NaOH.

Electrophoresis. Peptides resulting from hydrolysis of recoverin were subjected to SDS-PAGE by the Laemmli method [18] in the presence of 15% separating gel and 5% concentrating gel. To distinctly separate the peptides, the volume of the concentrating gel was increased twofold and before application the hydrolyzates were diluted five-tenfold in the sample buffer. Standard molecular weight markers were from Oncogen Research Products kit as follows: ovalbumin (43 kD), carboanhydrase (29 kD), β-lactoglobulin (18.4 kD), lysozyme (14.3 kD), inhibitor of bovine trypsin (6.2 kD), and insulin (β-chain, 3.4 kD).

Molecular weights of polypeptides produced by hydrolysis of recoverin were determined from dependence of the polypeptide mobility on $\ln(M_r)$. Molecular weights of the polypeptides determined by electrophoretic mobilities and calculated by the amino acid sequences were virtually the same. Because the hydrolyzate contained a very small quantity of the product of hydrolysis in formic acid at the Glu–Pro bond ($P_{61\text{-}188}$), the mobility of this peptide was determined by immunoblotting.

Immunoenzyme assay and immunoblotting. Immunoenzyme assay was performed under standard conditions [19] in Costar 96-well plates (USA) with a high sorption capacity. Each well of the plate was filled with 200 μl of recoverin at the concentration of 1 μg/ml in 20 mM Tris-HCl buffer (pH 8.5). In experiments with Ca^{2+}, recoverin and all solutions contained 2 mM $CaCl_2$; in experiments without Ca^{2+}, all reagents contained EGTA. Vacant binding sites were blocked with 0.5% BSA. Sheep immunoglobulins conjugated with horseradish peroxidase were used as secondary antibodies. Staining was performed for 30 min by addition of 200 μl of 50 mM citrate buffer (pH 6.0) containing 0.03% H_2O_2 and 5-aminosalicylic acid (1.5 mg/ml). The staining intensity was determined at 450 nm.

For competitive immunoenzyme assay of recoverin (1 and 0.5 μg/ml) was incubated with monoclonal antibodies of the clones mAb3 and mAb19 in the presence of 2-, 10-, and 50-fold excess synthetic recoverin peptides with amino acid sequences 38-43 and 66-72.

Immunoblotting was performed as described in [19] with some modifications. The peptides were transferred onto nitrocellulose membrane in 0.3% Tris-HCl buffer (pH 7.5) and 1.44% glycine in 20% ethanol for 2 h at 150 mA current.

RESULTS AND DISCUSSION

Hybridomas were prepared from B-cells of the spleen of recoverin-immunized mice and cells of mouse myeloma sp2.0. From the primary clones, 34 recoverin-positive clones were selected by solid-phase immunoenzyme assay. Because the binding of Ca^{2+} by recoverin is accompanied by conformational changes in the structure of its molecule, among the resulting hybridomas cell lines could be present which express Ca^{2+}-dependent monoclonal antibodies to recoverin. In fact, two hybridoma lines were found which produced monoclonal antibodies of the IgG1 isotype (further abbreviations mAb3 and mAb19 are used) with different affinity for recoverin in the presence and absence of calcium ions (Fig. 1a). Antibodies of these clones recognized both denatured recoverin by immunoblotting and native recoverin by ELISA.

The observed Ca^{2+}-dependence of the mAb3 and mAb19 binding to recoverin can be explained by both the effect of calcium as it is on the antigen–antibody interaction and conformational changes in the recoverin molecule affecting the structure of recoverin epitopes recognizable by the clones mAb3 and mAb19.

It has been shown that the mutant recoverin E121Q fails to bind Ca^{2+}, but this substitution is not associated with changes in the protein structure [11]. Figure 1b shows the interaction of recoverin E121Q with antibodies mAb3 and mAb19. The figure shows that the antibodies of these two clones bind similarly to the mutant E121Q in both the presence and absence of Ca^{2+} and comparably to their binding to the wild type recoverin in the absence of Ca^{2+}.

Thus, in the presence of Ca^{2+} antigenic determinants of recoverin recognizable by the antibodies mAb3 and mAb19 became more available, obviously due to changes in their spatial orientation on transformation of the recoverin apoform to the Ca^{2+}-bound form.

To determine the location of recoverin epitopes for the clones mAb3 and mAb19, we used overlapping peptides prepared by chemical hydrolysis of the whole recoverin molecule by the unique cysteine residue, two methionine residues, and the Asp–Pro bond.

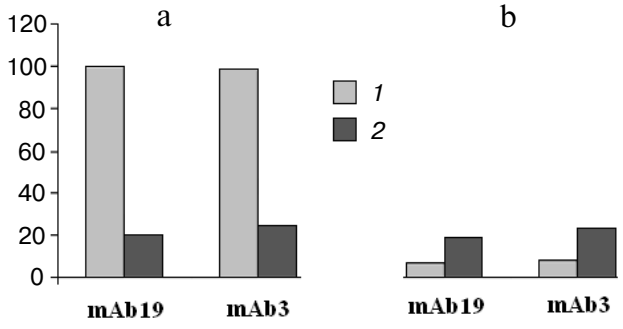

Fig. 1. Binding of antibodies of the clones mAb3 and mAb19 to wild type recoverin (a) and its mutant E121Q (b) in the presence (*1*) and absence (*2*) of Ca^{2+}. The binding of antibodies to the wild type recoverin in the presence of Ca^{2+} is taken as 100%.

Interaction of monoclonal antibodies with peptides produced by chemical cleavage of recoverin

Sites of hydrolysis	Resulting peptides	Apparent molecular weight, kD	Interaction with	
			mAb-3	mAb-19
	$P_{1–60}$	6.6	–	+
$Asp_{60}–Pro_{61}$	$P_{61–188}$	14.3	+	+
$Glu_{188}–Pro_{189}$	$P_{61–201}$	15.5	+	+
	$P_{1–91}$	10	+	+
	$P_{1–131}$	14.4	+	+
Met_{91}, Met_{131}	$P_{92–131}$	4.4	–	–
	$P_{132–201}$	7.7	–	–
	$P_{92–201}$	12	–	–
Cys_{38}	$P_{1–38}$	4.2	–	–
	$P_{39–201}$	17.9	+	+

By data of immunoblotting, mAb3 and mAb19 bound to the same peptides produced by hydrolysis of recoverin at cysteine and methionine residues (table). The clones mAb3 and mAb19 were different in recognizing the peptides only in the case of hydrolysis at the Asp60–Pro61 bond (Fig. 2). Antibodies of the mAb3 clone interacted only with C-terminal fragments of recoverin (Pro61-Leu202, Pro61-Glu188), whereas mAb19 bound to both N-terminal (Gly1-Asp60) and C-terminal (Pro61-Leu202, Pro61-Glu188) fragments of the recoverin molecule. Such a binding of mAb19 seemed to be due to hydrolysis by the Asp–Pro bond inside the antigenic determinant for mAb19 and binding of the antibodies to both sides from the site of the Asp60–Pro61 bond hydrolysis. Considering that the minimal length of the epitope recognizable by the antibodies was four amino acid residues and the Asp60–Pro61 bond was located inside the epitope recognized by mAb19, the antigenic determinant for mAb19 should be located inside the sequence Pro57-Tyr64. Figure 3 presents the general scheme of overlapping peptides produced by hydrolysis. The antigenic determinant for mAb3 is evidently located inside the sequence Pro61-Met91. Note that the two sites are in the second EF-hand domain of recoverin, which is a structural region responsible for the binding of Ca^{2+}.

Monoclonal antibodies, which recognize recoverin in the presence of Ca^{2+}, have been described earlier [12]. By pin-ELISA, the authors found that recognizable epitopes were located inside the regions of residues 38-43 and 64-70 of the amino acid sequence of recoverin. We have studied by competitive immunoenzyme assay the ability of mAb3 and mAb19 to recognize these fragments of recoverin using synthetic peptides corresponding to amino acid

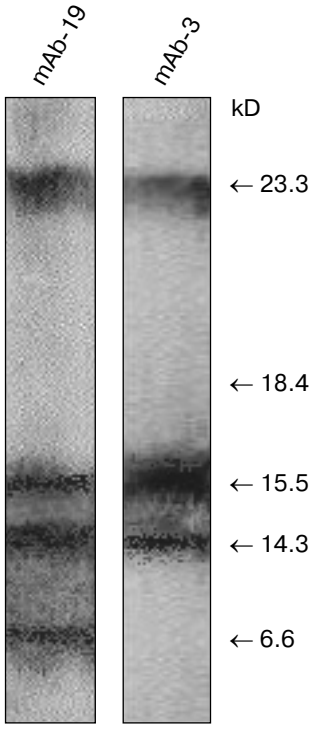

Fig. 2. Immunoblotting of polypeptides produced by hydrolysis of recoverin by the Asp–Pro (Glu–Pro) bond. Antibodies of clone mAb3 recognize only the C-terminal polypeptides. Antibodies of the clone mAb19 recognize both the C- and N-terminal polypeptides.

sequences 38-43 and 64-70 of recoverin (the peptides were kindly presented by Prof. M. Akhtar, Department of Biochemistry and Molecular Biology, Southampton University, Great Britain). These synthetic peptides did not compete for the sites of the recoverin molecule responsible for binding of clones mAb3 and mAb19.

Thus, we have prepared for the first time and characterized mAb3 and mAb19, which were not described earlier. The epitope for mAb3 is located inside the sequence Pro61-Met91 of recoverin and the epitope for the clone mAb19 occurs in the sequence Pro57-Tyr64.

The data on three-dimensional structure of the apoform and Ca^{2+}-bound form of recoverin [20, 21] suggest that the Pro57-Tyr64 fragment is unstructured, whereas on transition to the apoform this region of recoverin acquires α-helical structure (Fig. 4a); the Pro61-Met91 fragment in the Ca^{2+}-bound form of recoverin has a loop–α-helical structure and in the Ca^{2+}-free form of recoverin this is converted to β-structure–α-helix (Fig. 4b). We have shown that mAb3 and mAb19 recognize the recoverin molecule in the presence of detergent; thus, considering the spatial structure of recoverin, it is suggested that the Ca^{2+}-dependent binding of monoclonal antibodies to recoverin should be determined by conformational changes on transition of recoverin from the apoform to the Ca^{2+}-bound form.

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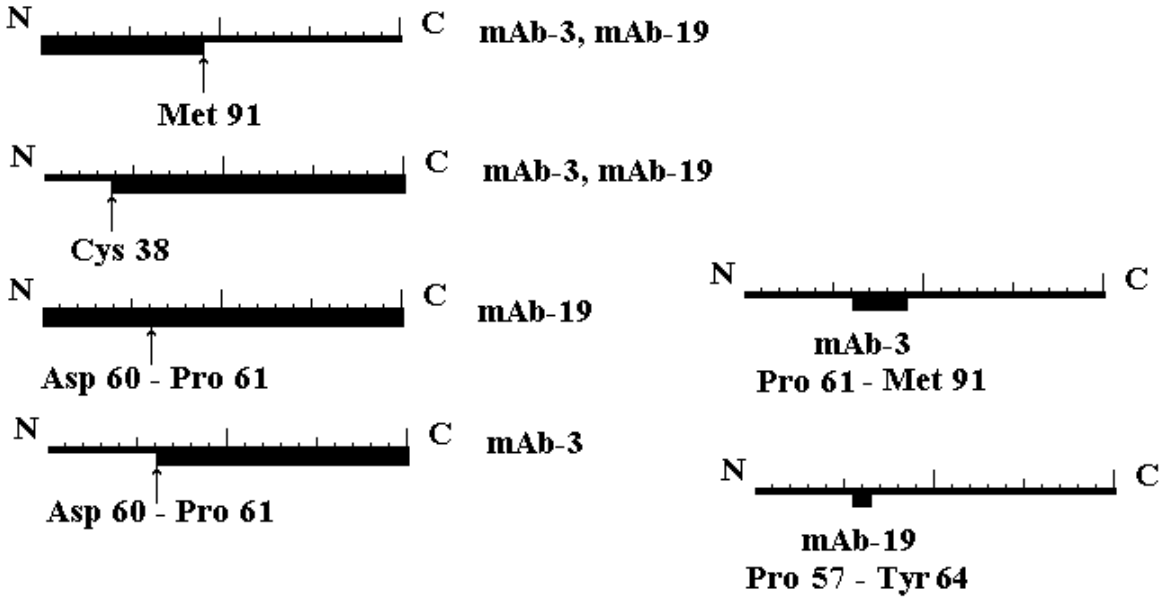

Fig. 3. Scheme for determination by chemical fragmentation of sites responsible for interaction with recoverin of antibodies of the clones mAb3 and mAb19. Arrows indicate the site of hydrolysis and the thickened lines show regions of the polypeptide chain recognized by the antibodies.

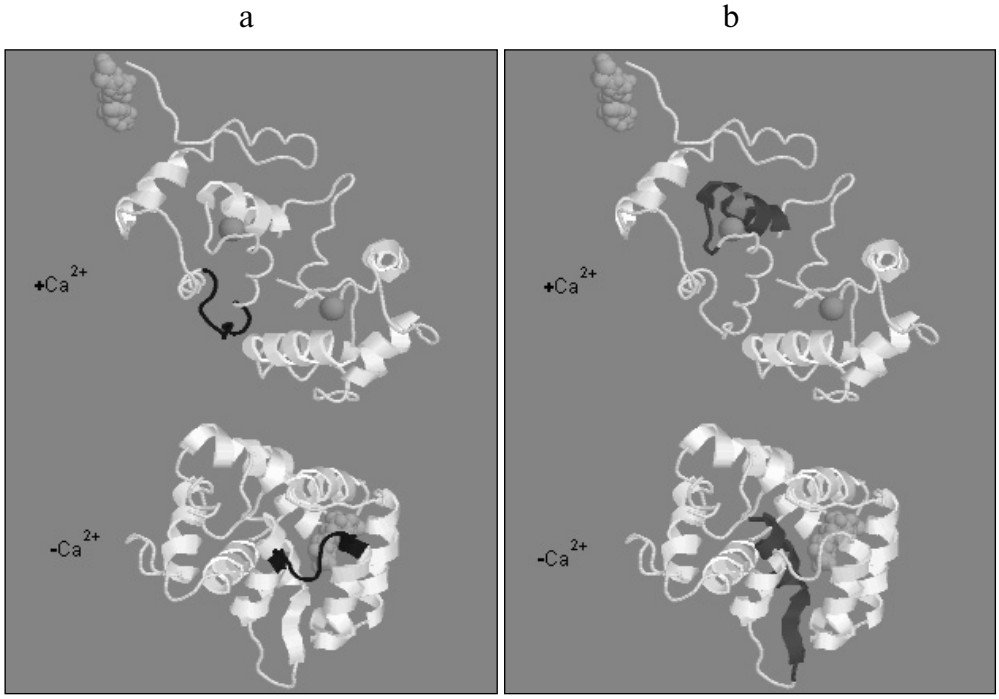

Fig. 4. Three-dimensional structure of myristoylated recoverin in the presence (structure 1JSA of the PDB databank) and in the absence of Ca^{2+} (structure 1IKU of the PDB databank) [20, 21]. In the presence of two calcium ions, the myristoyl residue is exposed from the hydrophobic pocket. In the absence of Ca^{2+}, the myristoyl group is submerged into the hydrophobic pocket. a) The binding site for mAb19 is shown; b) the binding site for mAb3 is shown.

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