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Amino Acid Sequences of Two Immune-Dominant Epitopes of Recoverin Are Involved in Ca^{2+}/Recoverin-Dependent Inhibition of Phosphorylation of Rhodopsin

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Abstract—Antibodies AB^{60-72} and AB^{80-92} against two immune-dominant epitopes of photoreceptor Ca^{2+}-binding protein recoverin, 60-DPKAYAQHVFRSF-72 and 80-LDFKEYVIALHMT-92, which can be exposed in a Ca^{2+}-dependent manner, were obtained. The presence of AB^{60-72} or AB^{80-92} results in a slight increase in Ca^{2+}-affinity of recoverin and does not affect significantly a Ca^{2+}-myristoyl switch mechanism of the protein. However in the presence of AB^{60-72} or AB^{80-92} recoverin loses its ability to interact with rhodopsin kinase and consequently to perform a function of Ca^{2+}-sensitive inhibitor of rhodopsin phosphorylation in photoreceptor cells.

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Key words: Ca^{2+}-dependent antibodies, Ca^{2+}-myristoyl switch, cancer associated retinopathy, recoverin

Recoverin is a photoreceptor Ca^{2+}-binding protein with molecular mass of 23.3 kDa consisting of 201 residues [1]. Four potential sequences corresponding to Ca^{2+}-binding sites of EF-hand type were found; however, it has been determined experimentally that only two of them – the second (EF2) and the third (EF3) – are able to bind the cation [2]. The N-terminal glycine of recoverin is acylated with a residue of one of the fatty acids C14:0 (myristic acid), C14:1 (5-*cis*), C14:2 (5-*cis*, 8-*cis*), or C12:0 [3]. Based on numerous data obtained with the NMR technique, the following mechanism of recoverin function has been established. In recoverin apo-form the myristoyl group is submerged into a hydrophobic pocket inside the protein globule [4]. Binding of two calcium ions to recoverin leads to a number of conformational changes in the protein molecule [2], which results in firstly solvation of the myristoyl group enabling the protein to interact with natural photoreceptor membranes and artificial phospholipid membranes as well [5] (this mechanism was named Ca^{2+}-myristoyl switch of recoverin) and secondly exposure of a cluster of hydrophobic residues on the surface of the recoverin molecule that is responsible for interaction of the protein with its target – rhodopsin kinase [6]. The latter is required for recoverin functioning in the photoreceptor cell as a Ca^{2+}-dependent regulator of visual receptor rhodopsin phosphorylation by rhodopsin kinase [7, 8]. Since multiple phosphorylation is the mechanism for fast switching off of rhodopsin after transduction of the visual signal [9], recoverin function is narrowed down to regulation of lifetime of the photoactivated receptor [10, 11]. Furthermore, recoverin is thought to function additionally as a Ca^{2+}-buffer in photoreceptor cells [10].

Recoverin is normally expressed primarily in photoreceptor cells of the retina [1]. However, it has been established that malignant transformation of lung epithelial tissues (mainly in small cell lung cancer) leads to aberrant expression of recoverin in the tumor. Autoantibodies against recoverin appear in blood serum

Abbreviations: AB^{60-72} and AB^{80-92}, antibodies against peptide fragments of recoverin 60-DPKAYAQHVFRSF-72 and 80-LDFKEYVIALHMT-92, respectively; CAR, cancer associated retinopathy; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; ROS, rod outer segment; TCA, trichloroacetic acid.

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of patients with small cell lung cancer, which leads to development of paraneoplastic syndrome – cancer associated retinopathy (CAR) [12-14]. CAR is characterized by a quick decrease in visual acuity along with development of cancer tumor and, eventually, degeneration of photoreceptor cells of the retina [15]. In CAR pathogenesis the key role is generally played by autoantibodies against specific markers of the target tissue (so-called CAR-antigens) attacking the retinal tissue and determining formation of the clinico-immunological picture of the disease [14]. High affinity antibodies of IgG and IgE classes [16] targeting a number of retina-specific proteins were found in patients with small cell lung cancer [14]. Recoverin is one of the most common CAR antigens. It has been found that autoantibodies against recoverin are able to pass through the blood–retinal barrier and penetrate into photoreceptor cells triggering their apoptosis [17].

Mapping of immunodominant epitopes of the recoverin molecule has revealed that blood serum of patients with small cell lung cancer is dominated by antibodies against epitopes situated inside two recoverin sequences "64-70" and "81-90" [18]. Antibodies against the same recoverin sequences have also been found in blood serum of experimental animals with model retinopathy [19]. It is noteworthy that such antibodies are conformational, and formation of the complex between these antibodies and the antigen is Ca^{2+}-dependent (Ca^{2+}-bound form of recoverin interacts with antibodies but the apo-form does not) [19, 20]. Here we have obtained monospecific antibodies against "60-72" and "80-92" sequences of recoverin and examined their influence on functional properties of the protein, namely, its ability to bind calcium, Ca^{2+}-myristoyl switch functionality, and inhibition of rhodopsin kinase. Our data are of interest for understanding the molecular mechanisms of CAR development.

MATERIALS AND METHODS

Bovine rod outer segment (ROS) preparations and urea-washed ROS membranes were obtained according to previously published procedures [21, 22] and stored at –80°C. Recombinant myristoylated recoverin was obtained according to a previously developed procedure [23].

Rhodopsin kinase was purified from ROS according to a published method [24]. ROS from 100 retinas were homogenized in darkness in 10 ml of buffer A (20 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF)) using a Potter homogenizer, illuminated for 10 min by a 150-W white light source, and the ROS membranes were pelleted (30 min, 155,000*g*). The pellet was then resuspended in 10 ml of buffer A containing 300 mM NaCl, and the suspension was centrifuged (30 min, 155,000*g*), the rhodopsin kinase activity thereby being passed to the supernatant. The extraction procedure was then repeated twice, and the resulting extracts were mixed and diluted with buffer A to reach a final concentration of NaCl of 125 mM. The combined extract was loaded onto a heparin-agarose column (15 ml) with flow rate of 0.5 ml/min. The column was washed with 60 ml of buffer A containing 125 mM NaCl. The rhodopsin kinase was eluted with a linear gradient of 125-400 mM NaCl in buffer A. The fractions containing rhodopsin kinase activity were combined, diluted with buffer A to yield 100 mM NaCl, and applied to a 5 ml anion-exchange Mono-Q column (GE Healthcare, USA). The column was washed with 20 ml of buffer A containing 100 mM NaCl, and the rhodopsin kinase was eluted with a linear gradient of 100-400 mM NaCl in buffer A. The preparation of purified rhodopsin kinase was stored at 0°C for up to 72 h.

The synthesis of recoverin peptide fragments 60-DPKAYAQHVFRSF-72 and 80-LDFKEYVIALHMT-92 was performed by Sintez Peptidov ZAO (Russia).

Polyclonal monospecific antibodies against recombinant myristoylated recoverin were prepared by immunization of Chinchilla rabbits according to a standard procedure [25]. The antibodies against recombinant myristoylated recoverin were purified by immunoaffinity chromatography using recoverin immobilized on BrCN-activated Sepharose as a sorbent [25]. The resulting polyclonal monospecific antibodies against recoverin were concentrated on Millipore-3000 NMWL membranes (Millipore, USA) (2500*g*, 40 min) and stored at –20°C.

Preparations of antibodies AB^{60-72} and AB^{80-92} were obtained from polyclonal monospecific antibodies against recoverin by immunoaffinity chromatography using a sorbent with peptide–bovine serum albumin conjugates immobilized on BrCN-activated Sepharose. For the preparation of the conjugates the synthetic peptide fragments of recoverin 60-DPKAYAQHVFRSF-72 or 80-LDFKEYVIALHMT-92 were used. The protein concentrations and the chromatography procedure specifications were the same as in [25]. The resulting polyclonal monospecific antibodies were concentrated on Millipore-3000 NMWL membranes (2500*g*, 40 min) and stored at –20°C. The interaction between AT^{60-72} and AT^{80-92} and purified recoverin was analyzed by enzyme-linked immunosorbent assay (ELISA) and immunoblotting.

Binding of Ca^{2+} to recoverin was investigated using an ultrafiltration procedure as follows [23, 26]. Residual amounts of Ca^{2+} in all buffers and protein solutions were removed using Ca^{2+}-adsorbing resin Chelex-100 (Bio-Rad, USA). Recoverin (50 μM) was preliminarily incubated with control antibodies (polyclonal monospecific antibodies against glutathione S-transferase), AB^{60-72}, or AB^{80-92} (in all cases the antibody concentration was 100 μM). Recoverin samples (0.5 ml) dissolved in 20 mM Hepes buffer, pH 7.5, 100 mM NaCl, and 1 mM DTT with addi-

tion of 10 μl of 0.2 mM $^{45}CaCl_2$ (0.4-0.5 μCi) were transferred to the top reservoir of a Centricon-10 concentrator (retention of molecules above 10 kDa) (Millipore). The samples were centrifuged (1 min, 7000 rpm), and 15 μl aliquots from both top and bottom reservoirs of the concentrator were collected and applied for Cherenkov radioactivity counting. In the next step, 15 μl of 0.2 mM solution of nonradioactive $CaCl_2$ was added to the top reservoir with the retained protein, and the centrifugation was repeated. The procedure was repeated several times until the calculated value of $[Ca^{2+}]_{free}$ reached ~300 μM (using higher values of $[Ca^{2+}]_{free}$ resulted in increased experimental error). In the control experiments the nonspecific binding of radioactive Ca^{2+} to immunoglobulins was determined and considered in calculations of stoichiometry of Ca^{2+}-binding. The data were analyzed as follows: $Ca^{2+}_{free} = R_f/R_p \times Ca^{2+}_{total}$, where R_f is the radioactivity in the filtrate, R_p is radioactivity in protein sample, and Ca^{2+}_{total} and Ca^{2+}_{free} are the total and free Ca^{2+} concentration, respectively. The amount of Ca^{2+} bound per mol of the protein (N) was calculated according to the equation: $N = (Ca^{2+}_{total} - Ca^{2+}_{free})/P_{total}$, where P_{total} is total concentration of the protein in the sample.

Binding of recoverin to ROS membranes was performed according to the previously published procedure [5, 23]. Recoverin (30 μM) was preliminarily incubated with control antibodies, $AB^{60\text{-}72}$, or $AB^{80\text{-}92}$ (in all cases the antibody concentration was 60 μM). Urea-washed ROS membranes (50 μM rhodopsin) were mixed with recoverin in 20 mM Hepes buffer, pH 7.5, containing 150 mM NaCl, 20 mM $MgCl_2$, 1 mM DTT, and either 2 mM $CaCl_2$ or 2 mM EGTA in total volume 50 μl and incubated at 37°C for 15 min in thermomixer (1000 rpm). Membranes were separated by centrifugation (25 min, 16,000*g*), the supernatant was removed, and the pellet was resuspended in 25 μl of sample buffer and analyzed by SDS-PAGE. The amount of recoverin bound to membranes was determined by densitometric scanning of the protein bands in the polyacrylamide gel (the band staining is proportional to the amount of loaded protein).

Rhodopsin phosphorylation was assayed in the reconstructed system consisting of urea-washed ROS membranes, purified rhodopsin kinase, and recoverin [27]. Recoverin (30 μM) was preliminarily incubated with control antibodies, $AB^{60\text{-}72}$, or $AB^{80\text{-}92}$ (in all cases the antibody concentration was 60 μM). The assay was performed in the following mixture (total volume 50 μl): 20 mM Tris-HCl buffer, pH 7.5, containing 2 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, either 0.2 mM $CaCl_2$ or 2 mM EGTA, 1 mM [γ^{32}-P]ATP (specific activity 30-100 cpm/pM), 10 μM rhodopsin, 0.3 unit of rhodopsin kinase activity, and recoverin. The reaction mixture was prepared in darkness; the enzymatic reaction was started by illumination of the sample with a 150-W white light source. After 30 min of the incubation at 37°C, the reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid (TCA) and the samples were centrifuged (10 min, 5000*g*). The supernatant was removed, and the pellet was resuspended in 10% TCA. The TCA washing procedure was repeated 3-4 times until the absence of the radioactivity in supernatant. The inclusion of the ^{32}P in rhodopsin was determined by Cherenkov counting.

Binding of rhodopsin kinase to ROS membranes was determined as follows [28]. Recoverin (10 μM) was preliminarily incubated with control antibodies, $AB^{60\text{-}72}$, or $AB^{80\text{-}92}$ (in all cases the antibody concentration was 20 μM). The ROS membranes (10 μM rhodopsin) containing 0.3 unit of rhodopsin kinase activity were incubated for 10 min in darkness in 50 mM Tris-HCl buffer (pH 7.5) containing 100 mM NaCl, 2 mM $MgCl_2$, in the presence of either (1) 2 mM EGTA or (2) 200 μM Ca^{2+} or (3) 2 mM EGTA and 10 μM recoverin or (4) 200 μM Ca^{2+} and 10 μM recoverin. After the incubation the membranes were collected by centrifugation (16,000*g*, 10 min). The concentration of EGTA in supernatant was adjusted to 10 mM, and 100 μl aliquots of the supernatant were taken for the determination of the rhodopsin kinase activity and for subsequent calculation of the corresponding amount of the enzyme. The amount of rhodopsin kinase corresponding to total rhodopsin kinase activity in 100 μl of the reaction mixture before the experiment was taken as 100%.

Enzyme-linked immunosorbent assay (ELISA) and immunoblotting were performed according to the previously described procedures [20, 29]. The assay was performed in 96-well plates. Each well was filled with 200 μl of recoverin (1 μg/ml) in 20 mM Tris-HCl buffer, pH 8.5, containing either 2 mM $CaCl_2$ or 2 mM EGTA. Free binding sites were blocked with 0.5% solution of bovine serum albumin. Appropriate concentrations of $AB^{60\text{-}72}$, $AB^{80\text{-}92}$, and polyclonal monospecific antibodies against recoverin were picked up by a double dilution. Secondary antibodies were goat anti-rabbit IgG conjugated with horseradish peroxidase. Staining was performed 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) to each well and subsequent incubation for 30 min. Staining intensity was detected at 450 nm. In concurrent immunoassay recoverin (1 μg/ml) was incubated with $AB^{60\text{-}72}$ or $AB^{80\text{-}92}$ in the presence of 2-, 10-, or 50-fold excess of recoverin peptide fragments 60-0DPKAYAQ-HVFRSF-72 or 80-LDFKEYVIALHMT-92. Immunoblotting was performed under denaturing conditions as previously described [20, 29]. The purified recoverin was transferred onto nitrocellulose membrane and stained using $AB^{60\text{-}72}$, $AB^{80\text{-}92}$, or polyclonal monospecific antibodies against recoverin.

SDS-PAGE was performed according to Laemmli procedure [30]. The protein concentrations were determined by spectrophotometric Bradford protein assay [31].

RESULTS AND DISCUSSION

Preparation of antibodies against recoverin sequences "60-72" and "80-92" (AB^{60-72} and AB^{80-92}). Using polyclonal monospecific antibodies against recoverin as a starting material for affinity chromatography with immobilized synthetic peptide recoverin fragments 60-DPKAYAQHVFRSF-72 and 80-LDFKEYVIALHMT-92, antibodies against corresponding recoverin sites (AB^{60-72} and AB^{80-92}) have been obtained (antibodies against these exact sites of recoverin have been found in the blood of CAR patients as well as in serum of animals with an experimental model of retinopathy [19]). Results of enzyme-linked immunosorbent assay (Fig. 1a) and denaturing Western blotting (Fig. 1b) demonstrate that AB^{60-72} and AB^{80-92} recognize both denatured and native recoverin. Also, the enzyme-linked immunosorbent assay has revealed competition between the synthetic peptides 60-DPKAYAQHVFRSF-72 and 80-LDFKEYVIALHMT-92 and recoverin for binding with AB^{60-72} and AB^{80-92}, respectively, indicating specificity of complex formation between AB^{60-72} and AB^{80-92} and the corresponding antigen sites (Fig. 1a). Moreover, interaction of AB^{60-72} and AB^{80-92} with recoverin is Ca^{2+}-dependent (Fig. 1a): the presence of 2 mM Ca^{2+} in the reaction mixture results in 3-5-fold enhancement of the binding of the antibodies to recoverin. This result is in accordance with the previously described data that monoclonal antibodies with epitopes located within sequences 64-YAQHVFR-70 and 81-FKEYVIALH-90 interact with recoverin in Ca^{2+}-dependent fashion [19]. The observed Ca^{2+}-dependent interaction of AB^{60-72} and AB^{80-92} with recoverin can be attributed to the direct effect of calcium ions on antigen–antibody interaction as well as to indirect action via Ca^{2+}-dependent conformational adjustments in recoverin that result in changes of availability of the epitopes recognized by AB^{60-72} and AB^{80-92} or in changes of their structural properties.

To reveal which of these two options takes place, we have used the mutant form of recoverin – E121Q. Introduction of this mutation does not affect recoverin structure but eliminates Ca^{2+}-binding properties of recoverin [23]. As shown by enzyme immunoassay, AB^{60-72} and AB^{80-92} binding to E121Q is essentially independent of Ca^{2+} and comparable to the level of binding of these antibodies to the wild-type recoverin without Ca^{2+} (data not shown). Therefore, taking into consideration these results and our data on the ability of AB^{60-72} and AB^{80-92} to interact with recoverin under denaturing conditions, we conclude that Ca^{2+} binding is more likely to increase availability of the epitopes located within recoverin sequences 60-DPKAYAQHVFRSF-72 and 80-LDFKEYVIALHMT-92. Thus we have obtained Ca^{2+}-dependent polyclonal monospecific antibodies against recoverin that recognize epitopes within recoverin sequences 60-DPKAYAQHVFRSF-72 and 80-LDFKEYVIALHMT-92.

Examination of effects of AB^{60-72} and AB^{80-92} antibodies on Ca^{2+}-binding properties of recoverin. To characterize the Ca^{2+}-binding properties of recoverin depending on the presence of the AB^{60-72} and AB^{80-92} antibodies, we employed the radioactive calcium isotope $^{45}Ca^{2+}$ for measuring the amount of calcium that binds to the protein. In

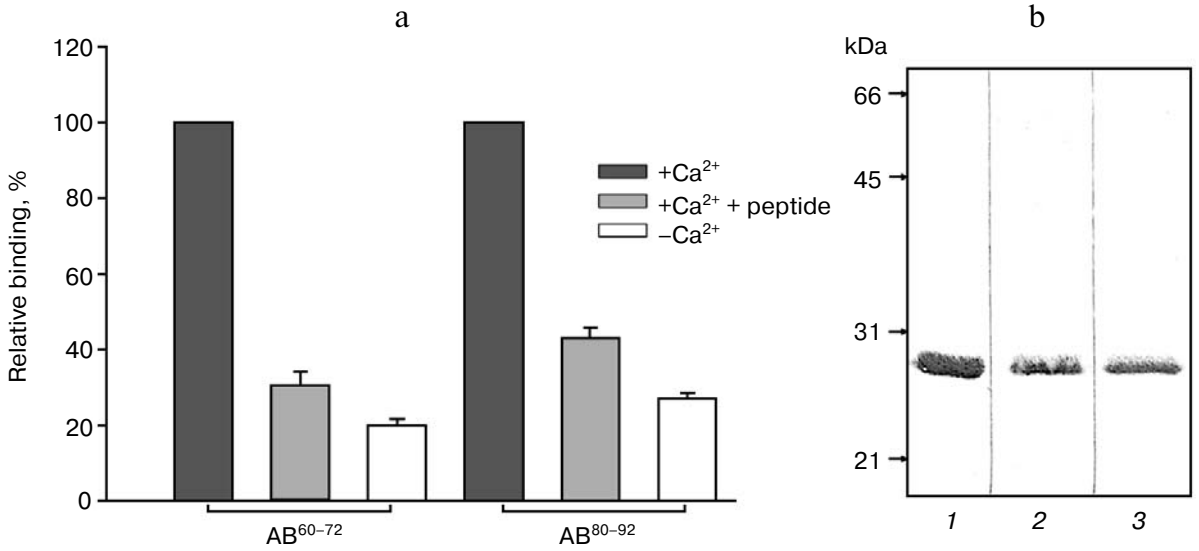

Fig. 1. a) Ca^{2+}-dependent binding of the AB^{60-72} and AB^{80-92} antibodies to recoverin (0.2 μg) according to the data from the enzyme-linked immunosorbent assay in the presence or absence of the corresponding synthetic peptides, 60-DPKAYAQHVFRSF-72 and 80-LDFKEYVIALHMT-92 (3 mM). Binding of AB^{60-72} and AB^{80-92} to recoverin in presence of 2 mM Ca^{2+} and in absence of the peptide was taken as 100%. b) Binding of the antibodies AB^{60-72} (lane *2*) and AB^{80-92} (lane *3*) to recoverin according to denaturing Western blot data. Lane *1*, recoverin stained with the starting preparation of polyclonal (monospecific) antibodies before AB^{60-72} and AB^{80-92} purification. Molecular mass standards are on the left side.

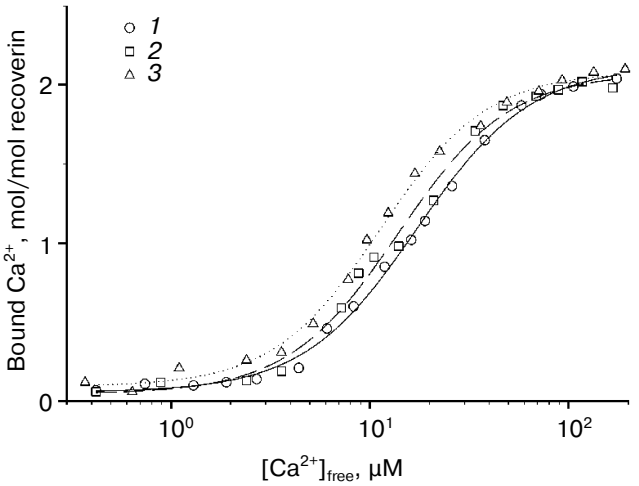

Fig. 2. Effects of AB^{60-72} and AB^{80-92} antibodies on Ca^{2+}-binding properties of recoverin. Recoverin (50 μM) pretreated with 100 μM solution of control antibodies (*1*), AB^{60-72} (*2*), or AB^{80-92} (*3*) was incubated in the presence of $^{40}Ca^{2+}/^{45}Ca^{2+}$ mixture containing 0.3 to 200 μM of free Ca^{2+} ($[Ca^{2+}]_{free}$) (see "Materials and Methods" section).

these experiments free concentration of calcium ions ($[Ca^{2+}]_{free}$) was varied in the range between 0.3 and 200 μM. According to our results (Fig. 2), in the absence of the antibodies recoverin binds two calcium ions cooperatively with a Hill coefficient of 2.05 and K_d of 17.5 ± 0.7 μM. These values are consistent with the previous estimates for recoverin [23, 26]. Stoichiometry and cooperativity of Ca^{2+} binding by recoverin is unaffected in the presence of AB^{60-72} and AB^{80-92} (Fig. 2). In this case a minor change of recoverin affinity to Ca^{2+} is observed: the dissociation constant of recoverin–Ca^{2+} complex is 11.8 ± 0.5 and 15.1 ± 0.3 μM in the presence of AB^{60-72} and AB^{80-92}, respectively.

A study of the effect of AB^{60-72} and AB^{80-92} antibodies on the Ca^{2+}-myristoyl switch of recoverin. To address the question of the effect of AB^{60-72} and AB^{80-92} antibodies on the Ca^{2+}-myristoyl switch of recoverin, we investigated the ability of recoverin to interact with ROS membranes in a Ca^{2+}-dependent manner in the presence or absence of these antibodies. In these experiments the amount of recoverin transferred to the membrane fraction after incubation with the ROS membranes (%) was determined. Recoverin binding to the membranes was found to be strongly dependent on the presence of calcium ions in the reaction mixture: the level of binding in suspension containing 2 mM Ca^{2+} (25 ± 1.2%) is 5-6-fold higher than in the presence of 2 mM EGTA (4.5 ± 0.6%). Addition of 2-fold excess of AB^{60-72} and AB^{80-92} (relative to recoverin) into the reaction mixture has no effect on the ability of recoverin to interact with the ROS membranes at both low and high calcium levels.

Based on the results it can be concluded that the AB^{60-72} and AB^{80-92} antibodies have no significant effect on the ability of recoverin to bind calcium ions as well as on its Ca^{2+}-dependent interaction with ROS membranes, and they therefore do not affect the Ca^{2+}-myristoyl switch mechanism of recoverin.

Effects of the AB^{60-72} and AB^{80-92} antibodies on Ca^{2+}-dependent inhibition of rhodopsin kinase by recoverin. Numerous *in vitro* and *in vivo* studies have demonstrated the ability of recoverin for Ca^{2+}-dependent inhibition of rhodopsin kinase activity, the enzyme responsible for multiple phosphorylation (and consequently desensitization) of the visual receptor rhodopsin [7-11]. We supposed that the antibodies under investigation in this work might compete with rhodopsin kinase for binding sites in the recoverin molecule. In such a case antibodies should impair the ability of recoverin to inhibit rhodopsin kinase. As shown in Fig. 3, in the presence of calcium ions the effectiveness of inhibition by recoverin is decreased by 3 or 1.7 times after addition of AB^{60-72} and AB^{80-92} antibodies to the reaction mixture, respectively. It is worth mentioning that in the absence of Ca^{2+} these antibodies had no effect on rhodopsin kinase activity with or without recoverin (data not shown). Based on these results one can suggest that the loss of inhibition of the rhodopsin kinase activity by recoverin in the presence of AB^{60-72} and AB^{80-92} is possibly due to the competition between the antibodies and the rhodopsin kinase for the binding sites in recoverin. To test this hypothesis further, we investigated the effect of AB^{60-72} and AB^{80-92} on formation of the recoverin–rhodopsin kinase complex directly.

Effects of AB^{60-72} and AB^{80-92} on interaction of recoverin with rhodopsin kinase. As previously established for *in vitro* conditions, there is a strong correlation between the

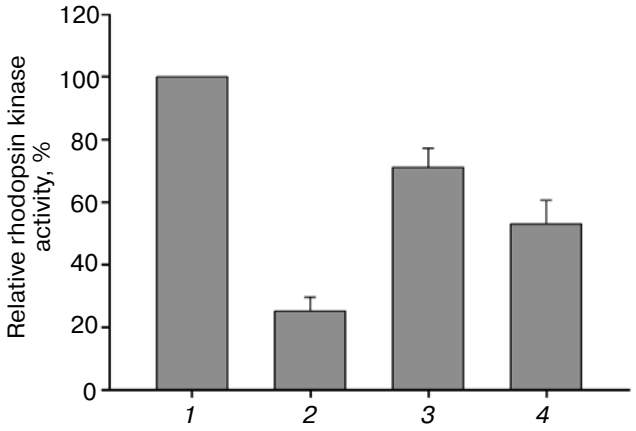

Fig. 3. Effects of AB^{60-72} and AB^{80-92} on Ca^{2+}-dependent inhibition of rhodopsin kinase by recoverin. Relative rhodopsin kinase activity in the rhodopsin phosphorylation reaction in ROS membranes suspension was estimated in the presence of Ca^{2+}: in the absence of recoverin but in the presence of the control antibodies (*1*); in the presence of 30 μM recoverin pretreated with 2-fold excess of the control antibodies (*2*), AB^{60-72} (*3*), or AB^{80-92} (*4*). Rhodopsin kinase activity without recoverin in the presence of the control antibodies was taken as 100% (see "Materials and Methods").

amount of rhodopsin kinase attached to the membrane and the amount of recoverin in the system, i.e. recoverin serves as an "anchor" that fixes rhodopsin kinase to the membrane in the presence of Ca^{2+} [28]. If the antibodies in question prevent interaction of recoverin with rhodopsin kinase upon binding to recoverin, then this fact should be reflected by the amount of rhodopsin kinase in the membrane fraction. Figure 4 demonstrates that without recoverin about 80% of rhodopsin kinase is in the soluble fraction. As expected, after addition of recoverin to the reaction mixture nearly all of the rhodopsin kinase (90%) is transferred to the membrane fraction. Addition of AB^{60-72} and AB^{80-92} to the reaction mixture results in a decrease of rhodopsin kinase in the membrane fraction to ~36% for AB^{60-72} and to ~62% for AB^{80-92} (in the absence of Ca^{2+} there is no such effect). It should be noted that these data are in agreement with the results obtained in the study of the effects of the investigated antibodies on rhodopsin kinase inhibition by recoverin.

In conclusion, AB^{60-72} and AB^{80-92} compete with rhodopsin kinase for formation of the complex with recoverin. Recently, data in the literature have emerged indicating that nine amino acid residues in the recoverin molecule (W31, F35, F49, I52, Y53, F56, F57, Y86, and L90) are involved in interaction with rhodopsin kinase [6]; all of these residues are situated in close proximity to 60-DPKAYAQHVFRSF-72 and 80-LDFKEYVIALHMT-92 with Y86 and L90 located within 80-LDFKEYVIALHMT-92. This may result in steric hindrances for recoverin interaction with rhodopsin kinase triggered by AB^{60-72} or AB^{80-92} binding to recoverin.

What is the role of the antibody-induced disturbance of recoverin function in CAR pathogenesis? Before this study was started, two principal models for CAR pathogenesis induced by anti-recoverin antibodies were discussed in the literature [32]. The first model suggested that binding of the antibodies to recoverin causes the loss of its calcium-binding ability, thereby affecting the Ca^{2+}-buffer role of recoverin in photoreceptor cells. This leads to alteration of calcium homeostasis in the photoreceptor cells, to an increase in cytoplasmic calcium levels and, consequently, to activation of the caspase cascade and apoptosis of photoreceptor cells in the retina. According to the second model, the cause of photoreceptor cells death can be hyperphosphorylation of rhodopsin induced by the failure of recoverin to inhibit rhodopsin kinase activity, which, as shown earlier, can trigger apoptosis of photoreceptors as well. Taking into consideration results obtained in the present work, the second model of CAR pathogenesis appears to be more favorable.

In conclusion, it should be noted that CAR is not the only instance of visual abnormality caused by recoverin failure to inhibit rhodopsin phosphorylation. In our previous works a similar effect was observed in the investigation of molecular mechanisms for an inherited visual decease – retinitis pigmentosa – evoked by the R135L mutation in the rhodopsin gene [33]. In particular, it has been shown that phosphorylation of R135L rhodopsin is less effectively inhibited by Ca^{2+}-bound recoverin than in the wild-type rhodopsin, which may result in hyperphosphorylation of the mutant receptor and apoptosis of photoreceptor cells.

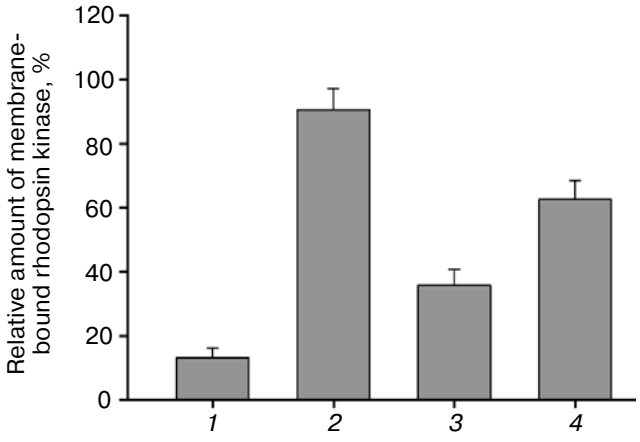

Fig. 4. Effects of AB^{60-72} and AB^{80-92} on interaction of recoverin with rhodopsin kinase. The amount of rhodopsin kinase bound to the membrane in the presence of Ca^{2+} was determined: in the absence of recoverin (*1*); in the presence of 10 μM recoverin pretreated with 20 μM of the control antibodies (*2*), AB^{60-72} (*3*), or AB^{80-92} (*4*). The total amount of rhodopsin kinase added to the reaction mixture was taken as 100% (see "Materials and Methods").

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