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Obtaining and characterization of EF-hand mutants of recoverin

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Abstract Several EF-hand recoverin mutants were obtained and their abilities to bind to photoreceptor membranes and to inhibit rhodopsin kinase were determined. The mutants with the 'spoiled' 2nd, 3rd or (2nd+3rd) EF-hand structures did not act upon the kinase activity in the μM range of Ca^{2+} concentrations. Mutations of the 4th EF hand, which 'repaired' its Ca^{2+}-binding activity, resulted in recoverin with three 'working' Ca^{2+}-binding sites. The latter mutant inhibited rhodopsin kinase even more effectively than the wild-type recoverin, containing two working Ca^{2+}-binding structures.

Key words: Photoreception; Phosphorylation; Rhodopsin; Rhodopsin kinase; Recoverin; Calcium-binding protein; Retinal rod cell

1. Introduction

Recoverin (the initial name 'p26' [1]) is a protein from bovine retinal rod cells [1–3] which belongs to the EF-hand superfamily of Ca^{2+}-binding proteins. The actual in vivo function of recoverin continues to be a subject of discussion (see [4] for review), though according to a number of in vitro data obtained in rod outer segment (ROS) suspensions and reconstituted systems [5–9] it inhibited rhodopsin kinase in a Ca^{2+}-dependent manner that pointed at the kinase as a cell target of recoverin. Amino terminus of recoverin is heterogeneously fatty acylated (mainly myristoylated) [10] and this modification enhances the recoverin inhibitory efficiency with respect to rhodopsin kinase and confers cooperativity with respect to Ca^{2+} on this inhibitory effect [11]. One more important feature of the recoverin structure is that its molecule is composed of two domains, each having two potential Ca^{2+}-binding sites of the EF-hand type; of four EF-hand sites, only two (the 2nd and the 3rd) EF-hands are capable of binding Ca^{2+} whereas the remaining two sites (the 1st and the 4th ones) do not have such an ability [12,13]. Since up to now the contribution of the specific EF-hand structures to the inhibitory activity of recoverin with respect to rhodopsin kinase is fully unknown, we obtained several EF-hand recoverin mutants and determined their ability of binding to photoreceptor membranes and of inhibiting rhodopsin phosphorylation catalyzed by rhodopsin kinase Fig. 1.

A preliminary form of this work was presented at the 5th European Symposium on Calcium Binding Proteins in Normal and Transformed Cells held in Münster, 1998 [14].

2. Materials and methods

[γ-^{32}ATP] was purchased from the Physicoenergetical Institute (Russia), all the other chemicals were obtained from Sigma and Amersham.

Rod outer segments (ROS) prepared from frozen bovine retinae under dim red light [15] were frozen in liquid nitrogen and stored at −70°C. Rhodopsin concentration was determined by the difference in optical densities of the samples at 500 nm before and after illumination in the presence of cetyltrimethyl ammonium bromide taking ε = 42 000 [16]. Recoverin concentrations were estimated by absorbance at 280 nm taking ε = 36 400 [17]. SDS-PAGE was performed as described in [18]. Free Ca^{2+} concentrations ($[Ca^{2+}]_f$) in the $CaCl_2$-EGTA buffer were calculated according to [19] and determined using a Ca^{2+}-sensitive electrode [20].

Site-directed mutagenesis was carried out as described in [21]. The oligonucleotides presented in Table 1 were used as antisense primers during the obtaining of site-directed mutants. Full length bovine cDNA [22] was inserted into M13mp19 plasmid (Biolabs) between the *Hin*cII and *Bam*HI sites. Sculptor in vivo mutagenesis system (Amersham) was used to obtain −EF2, −EF3 and −EF2,3; +EF4 was obtained using megaprimer PCR strategy [23]. The *Nco*I-*Hin*cII fragments of the mutants were cloned into the *Nco*I-*Bam*HI digested pE11d plasmid (Biolabs).

Expression of recombinant forms of recoverin was performed in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside as described earlier [11]. Briefly, the expression vectors, pET11-rec and its mutants were transfected to *E. coli* BL21DE3 (Biolabs) with or without pBB131, an expression vector of *N*-myristoyl transferase. −EF2, −EF3 and +EF4 were purified according to [11]; in the case of −EF2,3 gel filtration of Superone 12 (Pharmacia) was used instead of chromatography on phenyl-Sepharose.

Ca^{2+}-dependent binding of recoverin to ROS membranes was determined according to [24]; the procedure is briefly described in the legend to Fig. 2. Rhodopsin phosphorylation was assayed as described in [25] at 25°C in the reaction mixture (50 ml) containing 20 mM Tris-HCl (pH 7.4), 140 mM NaCl, 2 mM $MgCl_2$, 1 mM [γ-^{32}ATP] ($2–5\times10^4$ cpm/nmol), 100 μM GTP, 10 μM fully bleached rhodopsin in the content of ROS membranes, 0.02 E rhodopsin kinase, 15 μM wild-type recombinant myristoylated recoverin (WT-recoverin) or its mutants, and Ca^{2+} in the content of the $CaCl_2$-EGTA buffer (as indicated). The reaction was initiated by a light flash and the addition of ATP 30 s after the flash. After 30 min aliquots were taken from the reaction mixture and mixed with the SDS-PAGE sample buffer to stop the reaction. After SDS-PAGE of the samples, zones of rhodopsin were cut out and ^{32}P incorporation was estimated by Cherenkov counting in plastic tubes; the counting level was 3000–11 000 cpm per sample from which the 'dark' level (80–320 cpm) was subtracted. The data presented in Fig. 3 are means ± S.D. of duplicate assays carried out on three occasions.

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Abbreviations: ROS, rod outer segments; $[Ca^{2+}]_f$, free Ca^{2+} concentration; WT-recoverin, wild-type recombinant myristoylated recoverin; −EF2, −EF3 and −EF2,3, myristoylated recoverin mutants with the 'spoiled' 2nd, 3rd and (2nd+3rd) EF-hands, respectively; +EF4, myristoylated recoverin mutant with the 'repaired' 4th EF-hand

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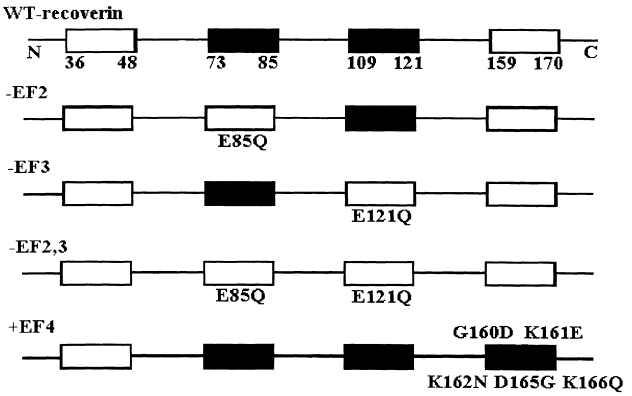

Fig. 1. Schematic sketch of EF-hand domains of WT-recoverin and its EF-hand mutants capable (filled boxes) and incapable (empty boxes) of binding Ca^{2+}.

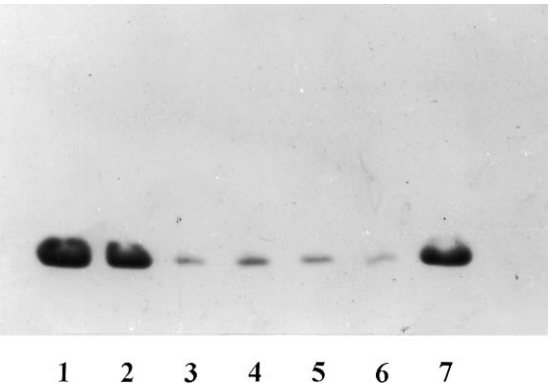

Fig. 2. Binding of WT-recoverin and its EF-hand mutants to photoreceptor membranes in the presence of Ca^{2+}. 15-μM recoverin preparations and urea-washed ROS membranes were mixed and incubated at 25°C for 15 min in 50 mM Tris-HCl (pH 7.5), containing 70 mM NaCl and 175 μM $[Ca^{2+}]_f$. Membranes were separated by centrifugation (275 000 × *g*, 10 min), extracted with 50 mM Tris-HCl (pH 7.5), containing 10 mM EGTA and portions of the extracts were analyzed by SDS-PAGE. Track 1: A total amount of myristoylated recoverin of a wild type in the sample analyzed before preincubation in the presence of membranes. Tracks 2–7 demonstrate an amount of the protein bound to membranes; 2: myristoylated recoverin of a wild type; 3: non-myristoylated recoverin of a wild type; 4: −EF2; 5: −EF3; 6: −EF2,3; 7: +EF4.

3. Results and discussion

EF-hand structural motif consists of two perpendicularly placed α-helices and an interhelical loop, which together form a Ca^{2+}-binding site in the EF-hand superfamily proteins (see [26] for review). In the recoverin EF-hand structure six amino acid residues are involved in the binding of Ca^{2+}; five of them are in the loop and the sixth one, namely glutamate, is present in the Z-position of the 2nd helix of the helix-loop-helix motif [13]. Using site-directed mutagenesis, we obtained four EF-hand mutants of recoverin. In three mutants substitutions of glutamate for glutamine in the Z-position of the 2nd and/or the 3rd EF-hand sites were made to 'spoil' Ca^{2+}-binding properties of the corresponding sites. The aim of substitutions in the fourth mutant was to give to its 4th EF-hand site the canonical EF-hand properties and thus to confer on this site a Ca^{2+}-binding capacity.

The 'spoiled' recoverin mutants were the following: (i) E85Q in the 2nd EF hand (it was symbolized as '−EF2'); (ii) E121Q in the 3rd EF hand ('−EF3'); and (iii) simultaneously E85Q in the 2nd and E121Q in the 3rd EF hands ('−EF2,3'). The fourth mutant ('+EF4') had simultaneously five substitutions in the 4th EF hand site: G160D, K161E, K162N, D165G and K166Q that 'repaired' it and conferred on it an ability to bind Ca^{2+}. Thus, in contrast to the wild-type recoverin, containing two 'natural' Ca^{2+}-binding sites (the 2nd and 3rd EF-hands), +EF4 had one additional Ca^{2+}-binding site (the 4th EF-hand).

Estimation of the Ca^{2+}-binding capacity of the mutants, using blots probed with 10 mM $^{45}Ca^{2+}$ [2], revealed that it was decreased in the order: +EF4≥WT-recoverin > −EF2 > −EF3 > −EF2,3 (data not shown). The latter three mutants were bound weakly to ROS membranes (Fig. 2) and did not inhibit rhodopsin phosphorylation catalyzed by rhodopsin kinase (Fig. 3). In contrast to the mutants with 'spoiled' EF hands, the behavior of +EF4 in the experiments on binding to ROS membranes (see Fig. 2) and on measuring the inhibitory efficiency (see Fig. 3) was analogous to that of WT-recoverin in the μM range of Ca^{2+} concentrations. Moreover, this recoverin form containing three working EF-hands was an even more effective inhibitor of rhodopsin phosphorylation; in the presence of +EF4 the plot 'relative activity of rhodopsin kinase vs. $[Ca^{2+}]_f$' was shifted to lower Ca^{2+} concentrations in comparison with the same plot but in the presence of WT-recoverin.

It is yet unclear, whether the inefficiencies mentioned of −EF2, −EF3 and −EF2,3 resulted from the point mutations as such or from perturbations, caused by the mutations, in the α-helices and possibly in the overall structure of the recoverin molecule. To answer this question, an investigation of the structures of the recoverin mutants is in progress.

Quite recently the obtaining and properties of two EF-hand mutants of *S*-modulin (a frog analog of recoverin), similar to our −EF2 and −EF3, were described [27]. These *S*-modulin mutants (E85M and E121M), just as E85Q and E121Q of recoverin, did not inhibit rhodopsin phosphorylation but, in contrast to our data, E85M, which corresponded to our −EF2, was able to bind to ROS membranes.

According to our data, the appearance of the 3rd active Ca^{2+}-binding site in the 4th EF hand of +EF4 did not destroy the recoverin ability to bind to ROS membranes and even enhanced its inhibitory efficiency with respect to rhodopsin

Table 1
Oligonucleotides used in the site-directed mutagenesis of recoverin

Symbols	Mutants	Oligonucleotide primers
−EF2	E85Q	5′-GCGATGGTACCTTGGACTTCAAGCAGTATGTC-3′
−EF3	E121Q	5′-GCAAGAACCAGGTGCTCGAGTTTGTCACGGAC-3′
−EF2,3	E85Q, E121Q	As in −EF2 and −EF3 simultaneously
+EF4	G160D, K161E, K162N, D165G, K166Q	5′-GGATTCTTGACGAGAATGATGATGGTCCACTTACAG-3′

The mutation sites are underlined.

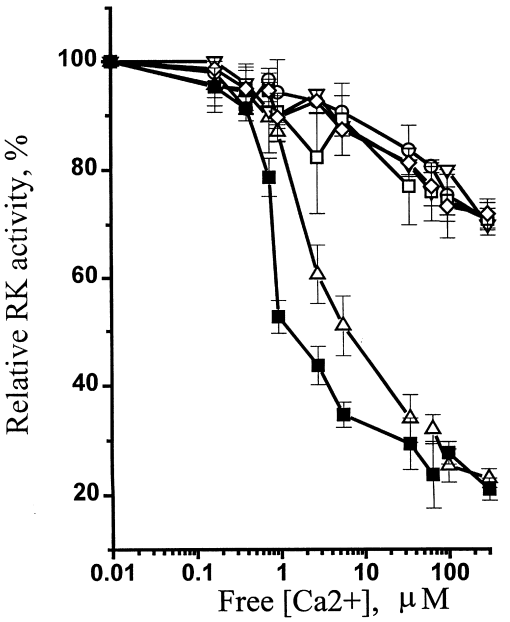

Fig. 3. Effects of WT-recoverin and its EF-hand mutants upon the relative rhodopsin kinase (RK) activity at different $[Ca^{2+}]_f$ values. The ^{32}P incorporation in the absence of recoverin is taken as 100%. 15-μM myristoylated recoverin forms were added; WT-recoverin (△); −EF2 (□); −EF3 (▽); −EF2,3 (◇); +EF4 (■); without recoverin (○).

kinase. The Hill coefficients, calculated from the plots presented in Fig. 3, were about 1.6 for WT-recoverin and 1.9 for +EF4, i.e. in the experimental conditions used the 'artificial' EF-hand site did not show a clearly defined cooperative interaction with two 'natural' Ca^{2+}-binding sites of recoverin. In future it is of interest to compare functional activities of +EF4 with those of related EF-hand proteins, for example GCAPs [28,29], molecules which contain 3 Ca^{2+}-binding sites.

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