Involvement of the recoverin C-terminal segment in recognition of the target enzyme rhodopsin kinase

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NCS (neuronal Ca²⁺ sensor) proteins belong to a family of calmodulin-related EF-hand Ca²⁺-binding proteins which, in spite of a high degree of structural similarity, are able to selectively recognize and regulate individual effector enzymes in a Ca²⁺-dependent manner. NCS proteins vary at their C-termini, which could therefore serve as structural control elements providing specific functions such as target recognition or Ca²⁺ sensitivity. Recoverin, an NCS protein operating in vision, regulates the activity of rhodopsin kinase, GRK1, in a Ca²⁺-dependent manner. In the present study, we investigated a series of recoverin forms that were mutated at the C-terminus. Using pull-down assays, surface plasmon resonance spectroscopy and rhodopsin phosphorylation assays, we demonstrated that truncation of recoverin at the C-terminus significantly reduced the affinity of recoverin for rhodopsin kinase. Site-directed mutagenesis of single amino acids in combination with structural analysis and computational modelling of the recoverin–kinase complex provided insight into the protein–protein interface between the kinase and the C-terminus of recoverin. Based on these results we suggest that Phe from the N-terminal helix of rhodopsin kinase and Lys from the C-terminal segment of recoverin form a cation–π interaction pair which is essential for target recognition by recoverin. Taken together, the results of the present study reveal a novel rhodopsin-kinase-binding site within the C-terminal region of recoverin, and highlights its significance for target recognition and regulation.

Key words: calcium-feedback mechanism, cation–π interaction, neuronal calcium sensor (NCS) protein, rhodopsin kinase (RK), ternary protein complex, visual phototransduction.

INTRODUCTION

Various aspects of neuronal function are regulated by changes in intracellular Ca²⁺ concentration. The intensity and duration of Ca²⁺ signals can trigger the activation of different Ca²⁺ pathways leading to specific physiological effects [1]. Eventually the large diversity of Ca²⁺-regulated events is the result of the action of Ca²⁺-sensor proteins, which transform Ca²⁺ signals into a wide range of cellular responses. The specificity of such transduction pathways depends on the affinity of the Ca²⁺ sensor for Ca²⁺, on the intracellular localization of the Ca²⁺ sensor and on the ability of the Ca²⁺ sensor to interact with effector enzymes [2]. Ca²⁺-sensor proteins contain specific structural elements, such as EF-hand motifs or C2-domains that recognize and bind Ca²⁺ in a highly sensitive and specific manner [3,4]. Binding of Ca²⁺ triggers conformational changes in the protein, enabling the protein to specifically modulate the activity of intracellular effector proteins [several Ca²⁺ sensors can modulate the activity of the target proteins in Ca²⁺-free (apo-) form] [5].

Although some of the known Ca²⁺ sensors are ubiquitous, the expression of others is restricted to certain tissues or cell types. The most common ubiquitous Ca²⁺ sensor is calmodulin which is widely expressed and regulates a large variety of targets. NCS (neuronal Ca²⁺ sensor) proteins have a more restricted expression pattern and repertoire of target proteins. This protein family can be divided into five groups based on structural and functional similarities: recoverins, visinin-like proteins, frequenins, GCAPs (guanylate cyclase-activating proteins) and KChIPs (K⁺-channel-interacting proteins). NCS proteins are highly homologous and the three-dimensional structures of some prototypical NCS proteins show that they consist of two domains with two EF-hand-type Ca²⁺-binding motifs each [5–7].

A distinctive feature of NCS proteins is that, in spite of a high degree of homology and structural similarity, each Ca²⁺ sensor can selectively regulate an intracellular target by sensing a specific narrow range of Ca²⁺ [5–7]. A fundamental question with respect to these observations is which structural elements account for the unique Ca²⁺-sensitive target recognition events. Visual inspection of the primary structural alignment of NCS proteins reveals hypervariable amino acid sequence segments in the C-terminus of NCS proteins that are located after the fourth EF-hand.

Recoverin is a small NCS protein that is involved in Ca²⁺-dependent feedback mechanisms of photoreceptor cells by regulating RK (rhodopsin kinase) activity. RK and recoverin form a ternary complex with rhodopsin, thereby preventing rhodopsin phosphorylation [8,9]. A first structural analysis of the binary RK–recoverin complex has revealed that the N-terminus
of RK forms an amphipathic α-helix, which interacts with a hydrophobic groove on the exposed surface of recoverin [9,10]. The C-terminal region of recoverin (residues 190–202) was not resolved in this complex and therefore was out of scope of the intermolecular contacts revealed in this study described above. In previous biochemical and X-ray crystallographic studies we have already demonstrated that the C-terminal segment in recoverin is an internal modulator of Ca$^{2+}$ sensitivity controlling the Ca$^{2+}$-myristoyl switch mechanism of recoverin and therefore the Ca$^{2+}$-dependence of recoverin binding to membranes and the range of Ca$^{2+}$ concentrations for recoverin regulation of RK activity [11]. Investigation of the involvement of the recoverin C-terminus in direct binding to RK has not so far been undertaken.

In the present study we address the question of whether the C-terminus of recoverin could directly participate in the interaction with RK and, if so, what is the functional impact of this interaction on the physiological role of recoverin as an inhibitor of RK.

**EXPERIMENTAL**

**Materials**

$^{45}$CaCl$_2$ was purchased from PerkinElmer and [γ-$^{32}$P]ATP was from Hartmann Analytic. CM5 BIAcore sensor chips, BIACore coupling reagents, GST (glutathione transferase), goat anti-GST antibody and CNBr-activated Sepharose were from GE Healthcare. Application of SPR technology in our laboratory was performed using BIAcore 2000 (GE Healthcare). All other reagents were obtained from Sigma, Merck, Fluka and Serva, and were at least analytical grade.

**Cloning, heterologous expression and purification of recoverin forms**

The truncated mutants of recoverin denoted Rc$_{2-196}$, Rc$_{2-192}$, Rc$_{2-188}$, Rc$_{2-186}$, and point mutants denoted Rc$_{C188}$A, Rc$_{Q191}$A, Rc$_{K192}$A and Rc$_{V193}$A, were obtained from a full-length recoverin cDNA in a pET-11d plasmid using standard site-directed mutagenesis procedures. For the production of each mutant DNA, a pair of oligonucleotide primers was employed in PCR. Primers containing the bacteriophage T7 promoter sequence were used in the forward direction and a primer containing a stop codon and BamHI restriction site instead of the codon of the target amino acid was used in the reverse direction. The PCR fragments were inserted in a pET-11d plasmid between the NcoI and BamHI restriction sites. The screening for the mutant clones was performed using BglII restriction analysis. The integrity of the insert was confirmed by sequencing using the Sanger method.

**Heterologous expression of myristoylated recoverin forms in E. coli**

Heterologous expression of myristoylated recoverin forms was confirmed by sequencing using the Sanger method. The integrity of the insert was confirmed by sequencing using the Sanger method. The phenyl-Sepharose-binding assay was performed according to a previously published procedure [17]. Briefly, 2 μM WT recoverin or mutants were mixed with 100 μl of phenyl-Sepharose (GE Healthcare) and incubated at 37 °C (Eppendorf thermomixer, 1000 rev./min) for 15 min in 20 mM Heps (pH 7.5), 150 mM NaCl, 20 mM MgCl$_2$, 1 mM DTT, and 2 mM EGTA or 2 mM CaCl$_2$ (total volume of 1000 μl). The mixture was centrifuged for 15 min (14000 g, table-top centrifuge Eppendorf model 5415), and the protein concentration in the supernatant was determined using a Bradford protein assay (Bio-Rad Laboratories).

**Pull-down assay**

Interaction of recoverin forms with N-RK was tested using analytical affinity chromatography (pull-down assay). A 10 μg amount (in 50 μl) of the N-RK fragment carrying a GST tag was immobilized on glutathione–Sepharose by incubating it for 1 h at 4 °C (Eppendorf thermomixer, 1200 rev./min) with 30 μl of a 75 % (v/v) suspension of glutathione–Sepharose in 20 mM Tris/HCl buffer (pH 7.5). The N-RK coupled to Sepharose was washed twice with 1 ml of a buffer containing 20 mM Tris/HCl buffer (pH 7.5), 100 mM NaCl, 1 mM DTT, 0.05 % Tween 20 and 2 mM CaCl$_2$, or 5 mM EGTA to remove any non-bound protein. The last supernatant was removed and 10 μg of the corresponding recoverin form was applied to washed beads. The suspension was incubated for 1 h at 4 °C in the above-mentioned buffer in a total volume of 80 μl. After washing three times with 1 ml of the same buffer, the beads were treated with 30 μl of SDS sample buffer and eluted proteins were analysed by Western blotting using an anti-recoverin antibody [18].

**SPR (surface plasmon resonance)**

SPR measurements were performed on a BIAcore 2000 (GE Healthcare). Application of SPR technology in our laboratory and the analysis of data have been described in detail previously [19–21]. Specific adjustments of the procedure for the analysis of N-RK constructs are published in a recent paper [8].
The docked solutions are characterized by a score index \( ZDs \) that is a convenient empirical descriptor of the affinity in protein–protein interactions occurring without major structural rearrangements as it is linearly correlated with \( \Delta G \) [22]. Therefore we employed this to predict the effect of recoverin truncations on its affinity for the N-RK peptide.

\[
ZD - s = \frac{\sum ZD^i}{M}
\]

in which the cluster groups \( M \) native-like solutions. Such a \( ZD-s \) index is a convenient empirical descriptor of the affinity in protein–protein interactions occurring without major structural rearrangements as it is linearly correlated with \( \Delta G \) [22]. Therefore we employed this to predict the effect of recoverin truncations on its affinity for the N-RK peptide.
of recoverin mutants below a protein length of 188 amino acids demonstrate essential structural disturbance in the protein. Therefore the respective recoverin mutants were excluded from the further analysis.

45Ca2+ and ROS membrane binding

A step-wise truncation of the C-terminus can impair the affinity of recoverin for Ca2+. Direct binding of Ca2+ was measured using the 45Ca2+-binding assay and yielded the higher Kd values the more amino acids were cut (Table 2). Whereas Rc2–196 was similar to the WT, Rc2–188 exhibited a 2-fold lower affinity. Similarly to WT recoverin, the binding of Ca2+ to these mutants was cooperative as indicated by Hill coefficients between 1.55 and 1.9. The change in the affinity for Ca2+ in truncated recoverin mutants suggested alterations in the Ca2+-myristoyl switch mechanism of the protein underlying its binding to membranes. One may expect a shift in Ca2+-dependence of the binding to a higher free Ca2+ concentration. Indeed, as revealed from analysis of the binding of truncated recoverin forms to native washed ROS membranes, the value of half-maximal free Ca2+ concentration for binding to membranes of Rc2–196 was similar to that of WT, whereas Rc2–188 exhibited a 1.5-fold shift to higher free Ca2+ concentration (Table 2). Taken together these results showed that the C-terminus is important for regulating the Ca2+ sensitivity of recoverin, thereby confirming previous conclusions on the mutant Rc2–190 [11]. Importantly, the changes in Ca2+ sensitivity were not related to any mutation-induced protein-stability changes, which are absent in our case (Figure 2 and Table 2).
### Table 2 The effect of recoverin C-terminal truncation on the thermal stability of the protein, its Ca\(^{2+}\)-binding parameters, Ca\(^{2+}\)-dependent interaction with ROS membranes and the affinity for N-RK

<table>
<thead>
<tr>
<th>Recoverin form</th>
<th>Thermal denaturation*</th>
<th>Binding of Ca(^{2+}) §</th>
<th>Binding to membranes∥</th>
<th>Affinity for N-RK∥</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T 1/2 (°C): apo form</td>
<td>K_d (μM)</td>
<td>Hill coefficient</td>
<td>EC_{50} (Ca(^{2+})) (μM)</td>
</tr>
<tr>
<td>WT</td>
<td>65.1</td>
<td>19.2 ± 0.4</td>
<td>1.78</td>
<td>3.5 ± 0.2</td>
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<td>2–196</td>
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<td>3.3 ± 0.2</td>
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<tr>
<td>2–192</td>
<td>65.8</td>
<td>26.2 ± 1.0</td>
<td>1.90</td>
<td>4.1 ± 0.3</td>
</tr>
<tr>
<td>2–190</td>
<td>66</td>
<td>36.8 ± 3.5</td>
<td>1.55</td>
<td>5.9 ± 0.6</td>
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<tr>
<td>2–188</td>
<td>64.6</td>
<td>41.9 ± 3.0</td>
<td>1.63</td>
<td>5.3 ± 0.5</td>
</tr>
<tr>
<td>2–187†</td>
<td>61.3</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>2–186†</td>
<td>56.6</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2–184†</td>
<td>43.3</td>
<td>–</td>
<td>–</td>
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</tr>
</tbody>
</table>

*Thermal denaturation of recoverin forms was monitored by tryptophan fluorescence of the protein.
†Recoverin forms with disturbed structural stability were excluded from further testing.
‡Ca\(^{2+}\)-binding parameters were measured by 45Ca\(^{2+}\)-binding assay.
∥The affinity of recoverin forms to N-RK were studied using SPR spectroscopy. The binding sensorgrams were recorded upon consecutive injections of Ca\(^{2+}\)-loaded recoverin forms at increasing concentrations over a sensorchip surface coated with N-RK via an anti-GST antibody. The equilibrium binding constants (K_d) are based on half-maximal concentration of recoverin required for saturation (Figure 3C).

### Interaction with RK

The N-terminal part of RK forms an amphipathic helix that interacts with an exposed hydrophobic groove in recoverin [9,10]. We tested the interaction of truncated recoverin mutants with the N-terminal domain of RK using a pull-down assay and SPR spectroscopy (Figure 3). GST-fusion constructs of the N-terminal domain of RK (N-RK) were immobilized on glutathione–Sepharose and incubated with recoverin WT and mutants in the presence and absence of Ca\(^{2+}\). The Ca\(^{2+}\) concentration was saturating in all cases. All recoverin forms showed a Ca\(^{2+}\)-induced interaction with N-RK, but mutants with a truncated C-terminus bound less strongly to N-RK, especially Rc2–188 and Rc2–190 (Figure 3A). We confirmed this result by immobilizing N-RK on a sensorchip surface and flushing 14 μM recoverin WT and mutants over the N-RK-coated surface. Positive amplitudes indicated binding of recoverin to N-RK and gave a first hint with regard to relative binding affinities. Mutants showed lower binding amplitudes than did WT (Figure 3B), which is in agreement with the decreased intensity of the protein bands shown in Figure 3(A). For further quantifying these results, we performed titration series with all recoverin mutants on N-RK-coated sensorchips. Recoverin forms were injected into the flow system at concentrations between 0.1 and 220 μM (Figure 3C). Apparent affinity constants (K_d) in Table 2) correspond to the concentration at which the response amplitudes were half-maximal. The difference in the apparent K_d values for WT and Rc2–188 was 7.8-fold; the mutants Rc2–196, Rc2–192 and Rc2–190 differed by factors of 3.4, 4.1 and 6.8 respectively (Table 2). We next asked whether the decrease in binding affinity might have a functional consequence in regulating RK activity. At saturating Ca\(^{2+}\) concentrations, WT recoverin prevented RK from phosphorylating rhodopsin by forming a ternary complex. Kinase activity was less inhibited by truncated mutants (Figure 3D). For example, the inhibitory response curve with Rc2–188 was shifted to higher concentrations of recoverin. A comparison of all tested mutants at a fixed recoverin concentration (14 μM) revealed an almost gradual decrease in inhibitory efficiency, when the C-terminus was more and more reduced (Figure 3D, inset). The shift in the inhibitory response curve is consistent with a lower affinity of the mutants for RK (Figures 3C and 3D). The lower affinity, however, did not result from a changed or disturbed exposition of hydrophobic regions in recoverin, since the mutants showed the same Ca\(^{2+}\)-dependent binding to phenyl-Sepharose as WT recoverin (results not shown). In summary, we conclude from our results that the C-terminus of recoverin not only participates in regulating its Ca\(^{2+}\) sensitivity, but also is involved in direct interaction with RK and thereby controls the inhibitory effect of recoverin.

### Modelling of the recoverin–N-RK protein complex structure

To investigate whether an involvement of the C-terminus of recoverin in the interaction with the N-terminal region of RK is feasible from a structural point of view, we built a model of the recoverin–N-RK complex focusing on the interaction interface involving the C-terminus of recoverin. The available three-dimensional NMR structure of recoverin in complex with the N-terminal fragment of rhodopsin kinase (N-RKc-25) was used as a template [9]. Furthermore, the missing C-terminal fragment of the recoverin NMR structure was taken from the crystallographic structure of non-myristoylated recoverin having this element resolved [14]. The model obtained of the complex revealed a position of the C-terminal a-helix of recoverin (highlighted in red, Figure 4A) in close proximity with the N-terminal kinase peptide (highlighted in blue, Figure 4A). Although the main part of the N-RK peptide is deeply buried inside a hydrophobic groove of recoverin, the first amino acids of the N-RK helix form a contact interface with the C-terminal region of recoverin (Figure 4A).

The resulting model was used to perform rigid-body docking simulations of the interaction process of N-RK with the truncated recoverin forms that we employed in pull-down and SPR experiments. Applied docking simulations suggested significantly different roles for the various portions of the C-terminus of recoverin in modulating RK recognition. Although the relative population of native-like solutions was the highest for WT recoverin (5.4%; see Table 1), it remained constant to ~5.0% for all of the subsequent truncations up to the shortest stretch (Rc6–190), for which it reduced to 3.4%. The best-scored solutions, in line, were found to be native-like in each independent docking run up to the Rc6–190 form, and they were ranked even lower for
Figure 3 Effect of recoverin C-terminal truncations on its binding to N-RK

(A) Pull-down assay demonstrating the dependence of recoverin binding to N-RK on the length of the recoverin C-terminus. All recoverin forms containing a truncated C-terminus showed a lower binding to N-RK than WT. The N-terminal domain of RK was immobilized on glutathione–Sepharose and used to pellet apo or Ca²⁺-loaded forms of recoverin. Bound proteins were eluted from the sorbent by SDS/PAGE sample buffer and analysed by SDS/PAGE and Western blotting. The recoverin content in each case was detected using anti-recoverin antibodies. (B) A representative overlay of SPR sensorgrams showing real-time binding of C-terminal-truncated recoverin mutants to N-RK. N-RK was anchored on the sensorchip surface via its GST tag using anti-GST antibodies covalently coupled to the dextran matrix. Ca²⁺-loaded forms of recoverin were injected over the N-RK surface to obtain binding curves. The concentration of recoverin during runs in each case was 14 μM. The reference signal from a control surface with immobilized GST was subtracted. (C) Steady-state affinity analysis of C-terminal-truncated recoverin binding to N-RK by SPR spectroscopy. Binding of truncated recoverin forms was recorded at different concentrations of recoverin and the amplitudes of binding signals at equilibrium were determined, normalized and shown as a function of recoverin concentration. The concentration of recoverin was varied from 0.1 to 220 μM. (D) Inhibition of RK by WT and Rc²–188. RK activity was measured by an in vitro phosphorylation assay in the presence of 200 μM free Ca²⁺. The concentration of WT and mutant Rc²–188 was varied within 0–0.1 mM and 0–1.0 mM respectively. Phosphorylation of rhodopsin in the presence of other truncated forms of recoverin is shown in the inset and was measured at 14 μM recoverin.

The Rc⁸–188 variant. Both the statistics and the average scores of the ensembles of reconstituted complexes (Table 1) are significantly affected by the truncation, especially for the 8–190 and 8–188 variants, hence suggesting a major role for the 189–192 residues.

A highly significant correlation ($R = 0.97$) was found between the average scores of ensembles of reconstituted complexes (ZD-s) and the experimental affinity measured by SPR (Figure 4B; for clarity we have plotted the $K_d$ instead of the $\ln K_d$ on the x-axis). The ZD-s index is known to linearly correlate with the free energy of binding of protein–protein complexes that form in water-soluble and in membrane environments without major conformational changes in either protein [22,23,25,29,30]. Hence such correlation corroborates the reliability of the three-dimensional model of the complex and suggests that the effect of truncation is not to perturb the remaining structure of recoverin, which appears to be stable independent of the truncated portion, a hypothesis indirectly confirmed by our thermal denaturation studies. Moreover, such correlation further suggests that the binding with the N-RK peptide may occur in a rigid-body-like manner.

### Protein–protein interface of the recoverin–N-RK complex

The structural analysis of the model suggested that a stretch of residues included in the 190–193 sequence of the recoverin
C-terminus might interact directly with residues from the N-terminal amphipathic helix of RK, presumably with Phe3. We constructed the corresponding point mutants, expressed them heterologously in *E. coli* and purified them to apparent homogeneity (Figure 5). The recoverin point mutations did not affect the thermal stability of the protein, neither in the apo nor in homogeneity (Figure 5). The recoverin point mutants bound to membranes as well as the WT protein (RcP190G Rct191A Rct192A Rct193G Rct194G). All recoverin point mutants bound to membranes in the presence of Ca2+ (Table 3), and the binding was only slightly different for the RcP190G mutant ($K_d$ was 28.5 $\mu$M in comparison with 19.2 $\mu$M for WT) and the cooperativity was lower for the RcP190G mutant ($n = 0.97$).

**DISCUSSION**

The turn off of a photoreceptor light response depends critically on the timely phosphorylation of rhodopsin by RK [31]. This step is under Ca2+-dependent control of the NCS protein recoverin operating in a ternary complex [8,9]. Since the Ca2+-dependent regulation of RK activity is of critical importance for the sensitivity regulation of a photoreceptor cell [32], we focused in the present study on the structural basis of this regulatory step. The C-terminus of recoverin has been described in a previous publication as an internal modulator of Ca2+ sensitivity [11]. In the present study we extend this work by showing that the C-terminus of recoverin (Phe188–Leu202) not only influences the binding of Ca2+, but also is critical for the affinity of recoverin binding to RK (for a summary of these data see Table 2). Using this approach we discovered a novel interaction site at the recoverin–RK protein–protein interface.

Since our conclusions are based on experiments employing truncated recoverin forms, it is important to verify that the decrease in affinity for Ca2+ and N-RK was not caused by defective

**Figure 5** SDS/PAGE analysis of purified C-terminal point mutants of recoverin

The C-terminal sequence of recoverin is shown above the gel with amino acid substitutions highlighted in bold.
folding of recoverin mutants. Using thermal denaturation studies we showed that mutants RC2–196, RC2–192, RC2–190 and RC2–188 have the same structural stability as recoverin WT and in the same time they exhibited lower affinity towards N-RK binding (Table 2 and Figure 2A). However, further shortening of the recoverin C-terminus in mutants RC2–187, RC2–186 and RC2–184 revealed progressive destabilization of the recoverin tertiary structure. In our previous publication [11], we indicated critical residues Phe188 and Ile186 forming a link to the cluster of non-polar residues in the central part of the recoverin structure (EF-hands 3 and 4). These contacts contribute to the hydrophobic core of recoverin, and disturbing them in the mutants RC2–187, RC2–186 and RC2–184 destabilized the protein structure. Of lesser importance for these intramolecular interactions are residues following Phe188 at the C-terminus. However, the results of the present study indicate their important role for the intermolecular interaction of recoverin with RK.

The Ca\(^{2+}\)-induced conformational change in recoverin exposes a number of non-polar amino acids in the N-terminal region that are involved in the interaction of recoverin to RK, and a recent NMR study using a 25 amino acid peptide of the N-terminus of RK (RK1–25) revealed that the RK peptide adopts an amphipathic helix fitting into the hydrophobic groove of recoverin [9,10]. Other members of the NCS protein family such as KChIP1 [26], GCAP2 [27] and yeast frequentin [33] also harbour hydrophobic grooves considered to be important for target interaction. Previous studies have also shown an involvement of the C-terminal part of NCS proteins in their binding to target [26,34–37]. Using SPR measurements and pull-down assays, we found that the same is true for the C-terminus of recoverin. Importantly, the effects of reduced affinity of recoverin C-terminal mutants to RK revealed are not the result of an alteration in the Ca\(^{2+}\) sensitivity of recoverin, since all of the experiments were performed at saturating Ca\(^{2+}\) concentrations when recoverin is already in the

### Table 3 The effect of single amino acid substitution in the C-terminal of recoverin on thermal stability of the protein, its Ca\(^{2+}\)-binding parameters, Ca\(^{2+}\)-dependent interaction with ROS membranes and the affinity for N-RK

<table>
<thead>
<tr>
<th>Recoverin form</th>
<th>Thermal denaturation*</th>
<th>Binding of Ca(^{2+})**</th>
<th>Binding to membranes§</th>
<th>Affinity for N-RK†</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>T(_{1/2}) (°C): apo form</td>
<td>K(_d) (µM)</td>
<td>Hill coefficient</td>
<td>EC(_{50}) (Ca(^{2+})) (µM)</td>
</tr>
<tr>
<td>WT</td>
<td>65.1</td>
<td>19.2 ± 0.4</td>
<td>1.78</td>
<td>3.5 ± 0.2</td>
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<td>4.9 ± 0.3</td>
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<tr>
<td>V193G</td>
<td>66.5</td>
<td>20.3 ± 0.8</td>
<td>1.59</td>
<td>4.2 ± 0.3</td>
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</table>

*Thermal denaturation of recoverin forms was monitored by tryptophan fluorescence of the protein.  
**Ca\(^{2+}\)-binding parameters were measured by \(^{45}\)Ca\(^{2+}\)-binding assay.  
§Binding of recoverin forms to ROS membranes were measured at different \([\text{Ca}^{2+}]\) using an equilibrium centrifugation assay.  
†The affinity of recoverin forms to N-RK were studied using SPR spectroscopy. The binding sensorgrams were recorded upon consecutive injections of \([\text{Ca}^{2+}]\)-loaded recoverin forms at increasing concentrations over a sensorchip surface coated with N-RK via an anti-GST antibody. The equilibrium binding constants (K\(_d\)) are based on half-maximal concentration of recoverin required for saturation (Figure 6B).
Ca\textsuperscript{2+}-loaded state. Moreover, the recoverin point mutants studied have almost the same Ca\textsuperscript{2+} sensitivity as WT recoverin, but lower affinity for RK (Table 3).

The C-terminal \(\alpha\)-helix of recoverin following the kink caused by Pro\textsuperscript{193} provides additional contacts to the RK peptide, as it is apparent from the visual inspection of the three-dimensional model of the recoverin–N-RK complex (Figure 4A). Furthermore, our modelling approach highlighted the possible involvement of four amino acids in the C-terminus (Pro\textsuperscript{190}–Val\textsuperscript{193}) (Figure 4A). Thus the N-terminal peptide of RK is retained in the complex not only by the hydrophobic groove of recoverin, but also by additional contacts within the C-terminus of recoverin that provide more tight interaction and proper positioning of the target peptide. Our rigid-body docking simulations revealed a sufficient decrease in kinase peptide binding energy upon step-wise truncation of recoverin C-terminal \(\alpha\)-helix, which is in agreement with the \(K_d\) values on interaction of truncated recoverin forms to immobilized N-RK (Table 2 and Figure 4B).

The major effect observed experimentally with the point mutant RcK\textsuperscript{192A} [3.2-fold lower affinity for N-RK (Table 3) and 8.6-fold less inhibitory efficiency (Figure 6C)] was also in agreement with structural analysis. Lys\textsuperscript{192} was predicted to form a cation–\(\pi\) interaction pair with the aromatic ring of Phe\textsuperscript{1} in N-RK following the minor rearrangement of both residue side groups for optimal contact (Figure 7). Figure 7 also highlights a salt bridge between Lys\textsuperscript{192} and Glu\textsuperscript{188} at the recoverin C-terminus. Such an electrostatic interaction is expected to stabilize the orientation of the subsequent \(\alpha\)-helix, hence providing an optimal orientation of the whole helix and creating the conditions for tight binding of Phe\textsuperscript{1} through the cation–\(\pi\) interaction with Lys\textsuperscript{192}. We confirmed these structural interpretations by measuring the affinity of the corresponding point mutant N-RK\textsuperscript{PSA} for recoverin that resulted in a similar affinity, like we obtained for the truncated recoverin mutant Rc\textsuperscript{188} and N-RK (Figure 6B). These results were also broadly consistent with previous work using several point mutants of a 15 amino acid peptide of N-RK [10]. For example, mutations of Phe\textsuperscript{1} and Leu\textsuperscript{6} cause a complete loss of recoverin binding in pull-down assays. Whereas the residue Leu\textsuperscript{6} forms the tight contact with residues of the hydrophobic groove in recoverin [9], the interaction partner for residue Phe\textsuperscript{1}, until the start of the present study, was unknown.

Site-specific mutations of the three other amino acid residues in the recoverin C-terminus (P190G, Q191A and V193G) revealed a 2–3-fold lower N-RK affinity for Rc\textsuperscript{190G} and Rc\textsuperscript{193G}, but no effect of mutation at Gin\textsuperscript{191}. Since proline is known to determine the direction of an \(\alpha\)-helix when located at the beginning of it, it probably helps to maintain the correct orientation of the recoverin C-terminus to the N-RK residues. A mutation to glycine would allow more flexibility and thus a less optimal interface. Val\textsuperscript{193} is faced almost at the opposite site of the helix with respect to the RK peptide and therefore is very likely to not be located in the interface region. Glycine is known as an helix breaker and its placement in position 193 instead of valine could therefore perturb the secondary structure of the whole C-terminal \(\alpha\)-helix, thereby decreasing the protein affinity for N-RK (Table 3).

Taken together, the results of the present study demonstrate an important role of C-terminal segment of recoverin for RK targeting. We found that the binding surface for RK is not limited by the hydrophobic groove within the N-terminal lobe of recoverin, but also includes the last \(\alpha\)-helix of C-terminal lobe. Involvement of distinct regions including the C-terminus in target binding or recognition is not uncommon among NCS proteins as this has been described for KChIP1 [26] and GCAP1 [34–37]. It will be of interest to see whether the cation–\(\pi\) interactions that have been observed in ligand–receptor interactions, as well as in enzymatic catalysis [38], also contribute significantly to NCS protein-related target recognition.

AUTHOR CONTRIBUTION

Evgeni Zernii and Konstantin Komolov were involved in experimental design, data collection, treatment and analysis, manuscript preparation and the overall design of the study. Sergei Perymakov was involved in experimental design, data treatment and analysis, and manuscript preparation. Tatiana Kolpakova was involved in data collection, and data treatment and analysis. Daniele Dell’Orco was involved in modelling and docking simulations, and manuscript preparation. Annika Poetzsch, Ekaterina Knyazeva, Ilya Grigoriev and Ekaterina Knyazeva were involved in data collection, and data treatment and analysis. Eugene A. Pernyakov was involved in experimental design and manuscript preparation. Ivan I. Serin was involved in experimental design, data collection, treatment and analysis, and the overall design of the study. Pavel P. Philippov was involved in data treatment and analysis, and manuscript preparation. Karl-Wilhelm Koch was involved in the experimental design, data treatment and analysis, manuscript preparation and the overall design of the study.

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