Identification of Proximate Regions in a Complex of Retinal Guanylyl Cyclase 1 and Guanylyl Cyclase-activating Protein-1 by a Novel Mass Spectrometry-based Method*

Dmitri M. Krylov and James B. Hurley‡

From the Department of Biochemistry and Howard Hughes Medical Institute, University of Washington, Seattle, Washington 98195

A key challenge in studying protein/protein interactions is to accurately identify contact surfaces, i.e. regions of two proteins that are in direct physical contact. Aside from x-ray crystallography and NMR spectroscopy few methods are available that address this problem. Although x-ray crystallography often provides detailed information about contact surfaces, it is limited to situations when a co-crystal of proteins is available. NMR circumvents this requirement but is limited to small protein complexes. Other methods, for instance protection from proteolysis, are less direct and therefore less informative. Here we describe a new method that identifies candidate contact surfaces in protein complexes. The complexes are first stabilized by cross-linking. They are then digested with a protease, and the cross-linked fragments are analyzed by mass spectrometry. We applied this method, referred to as COSUMAS (contact surfaces by mass spectrometry), to two proteins, retinal guanylyl cyclase 1 (RetGC1) and guanylyl cyclase-activating protein-1 (GCAP-1), that regulate cGMP synthesis in photoreceptors. Two regions in GCAP-1 and three in RetGC1 were identified as possible contact sites. The two regions of RetGC1 that are in the vicinities of Cys411 and Cys780 map to a kinase homology domain in RetGC1. Their identities as contact sites were independently evaluated by peptide inhibition analysis. Peptides with sequences from these regions block GCAP-1-mediated regulation of guanylyl cyclase at both high and low Ca2+ concentrations. The two regions of GCAP-1 cross-linked to these peptides were in the vicinities of Cys77 and Cys105 of GCAP-1. Peptides with sequences derived from these regions inhibit guanylyl cyclase activity directly. These results support a model in which GCAP-1 binds constitutively to RetGC1 and regulates cyclase activity by structural changes caused by the binding or dissociation of Ca2+.

RetGC1 and RetGC2, are membrane guanylyl cyclases that synthesize cGMP within the outer segments of rod and cone photoreceptor cells. They are structurally similar to membrane guanylyl cyclases that are regulated by extracellular peptides (1), but they are instead regulated by intracellular Ca2+ (2–6).

Photexcitation stimulates hydrolisis of cGMP in photoreceptors which in turn lowers the intracellular free Ca2+ concentration. Low levels of Ca2+ stimulate guanylyl cyclase activity in photoreceptor homogenates (7), and two small Ca2+-binding proteins called GCAPs (guanylyl cyclase-activating proteins) have been identified that impart Ca2+ sensitivity to cGMP synthesis by photoreceptor membranes (3, 8–10). GCAPs regulate RetGCs via the intracellular domain of the enzyme (5, 6). At physiologically low Ca2+ concentrations GCAPs stimulate RetGCs, and at high concentrations they inhibit them (11, 12). A structure of the Ca2+-free state of GCAP-2 has been determined by NMR spectroscopy (13), and the highly conserved catalytic domain of guanylyl cyclases has been modeled by homology methods (14, 15).

Several observations have suggested that there are direct physical interactions between RetGCs and GCAPs. One line of evidence for this interaction comes from two peptide binding and inhibition studies (16, 17). In the first study, Sokal and coworkers (16) reported a site of contact involving the RetGC1 sequence 461GTFKRMHMPFVPRIIRG776 within the intracellular catalytic domain. In the second study, Lange and coworkers (17) identified two different regions in RetGC1, 492LHIQMSGPNKILTDIDT511 and 553LYEGDWWLKKFPDDRHIAT571, as sites of contact with GCAP-1. These sequences lie within the intracellular region between the membrane and the kinase homology domain of RetGC1. Both studies relied on peptide binding and inhibition analyses to identify contact surfaces. Their results, therefore, are valid only with the assumption that peptides behave in solution as they do in the context of the full-length folded protein. An additional caveat is that interpretation of peptide inhibition requires appropriate controls, for instance peptides of identical amino acid composition with scrambled sequence. These were absent from one of these studies (16). Regions of GCAPs that are important for regulation of RetGCs have been studied primarily by mutagenesis of GCAP-1 and GCAP-2 (11, 12, 18–21). However, none of those studies provided direct evidence for sites of interaction with RetGCs.

Additional information about interactions between RetGCs and GCAPs comes from proteolysis of RetGC chimeras (22) and stabilization of the kinase homology domain by GCAPs (23). Those studies identified a region of RetGC1 that is stabilized and protected from proteolysis by GCAPs. The protected region was within residues 436–736 of the kinase homology domain of RetGC1. Stabilization and protection from proteolysis occurred independently of Ca2+ suggesting that GCAPs bind constitutively to RetGCs.

In the study described in this report we investigated GCAP/
RetGC1/GCAP1 Interaction

RetGC1 interactions by developing a novel method for mapping sites of proximity of proteins in a complex. The first step in this method is to stabilize proximate regions of whole native proteins by cross-linking. The proteins are then digested by trypsin. The resulting cross-linked fragments are separated by reverse phase chromatography and analyzed by mass spectrometry. Tandem mass spectrometry (ms/ms) analysis can be used to sequence trypptic fragments (24–27). We applied ms/ms analysis to the cross-linked peptides and developed software that determines the masses of all possible cross-linked trypptic fragments. The software also selects likely candidate peaks from a complex mass spectrum. Once these candidates are identified they are analyzed by ms-ms sequencing to confirm their identity. The software scores and evaluates candidate matches between predicted peptide conjugates and observed ms/ms spectra. This new method identifies residues of interacting proteins that are physically separated from each other by approximately the length of the cross-linker. Amino acid residues surrounding the cross-linked residues are considered candidates for contact surfaces that can be verified independently by biochemical methods.

We applied the method (referred to as COSUMAS) to the RetGC1/GCAP1 interaction and found two contact surfaces that had not been previously identified using other methods. The identity of these structures as contact regions was verified by peptide inhibition experiments.

**EXPERIMENTAL PROCEDURES**

**Cross-linking**—Cross-linking was performed on a mixture of washed bovine rod outer segment (ROS) membrane preparations (18) and bacterially expressed GCAP-1 (18). GCAP-1 was metabolically labeled by including 5 mCi of 35S protein labeling mix (PerkinElmer Life Sciences) in the growth medium 30 min prior to inducing expression. It was purified away from bacterial proteins by size exclusion chromatography using a HiPrep™ 26/60 Sephacryl S-100 high resolution column from Amersham Pharmacia Biotech. Cross-linking was performed for 15–30 min in the following buffer: 120 mM NaCl, 5 mM MgCl₂, 20 mM Tris, pH 7.5, 100 μM ATP. The volume of the reaction was 2.5 ml, rhodopsin concentration was 62 μM, and GCAP-1 concentration was 2 μM. The cross-linking agent bismaleimide hexane (bMH)/purified from Pierce Chemical Co.) was used at 30 μM. The reaction was stopped by adding free cysteine to a 100 mM final concentration. The membranes were pelleted by centrifugation at 80,000 rpm in an Optima™TLX ultracentrifuge using a TLA 100.1 rotor (Beckman), washed in 6 M deionized urea four times, and finally washed once in cross-linking buffer to remove soluble GCAP-1.

**Tryptic Digestion**—Tryptic digestion was performed at a 1:100 enzyme to rhodopsin weight ratio for 2 h at room temperature. 1-l-Trifluoroacetic-2-phenylthio-ethylmethyl ketone-treated trypsin was purchased from Sigma Aldrich.

**Mass Spectrometry**—All mass spectrometry was performed on a Finnigan LCQ Deca ion trap instrument with electrospray interface. Samples were analyzed by direct infusion at 10 μl/min with automatic gain control on. Typically mass spectra were collected in a 200–2000 m/z window for 1 min. The scans were averaged and visualized by the Qual Browser software from Thermo Quest. Ms/ms spectra on selected peaks were collected similarly with the following parameters: isolation width for the parent ion of 1.5–3 m/z, activation amplitude 25–70%, activation coefficient, “Q” 0.250, activation time 30 ms.

**Data Analysis**—Data were analyzed with a UNIX-based software package. The programs were written in Perl and linked by CGI. Mass spectra were fed into the programs as simple text files, and the output was formatted as html. The program is accessible by contacting the corresponding author.

**Peptide Synthesis and Purification**—All peptides were synthesized on an Applied Biosystem automated peptide synthesizer Synergy 432A. Applied Bio system amino acid cartridges and organic solvents were used. Peptides were washed and cleaved from the matrix, lyophilized, and dissolved in water or a Me2SO/H2O mixture. The presence of the correct molecular weight was verified by mass spectrometry. Ten milligrams of each peptide synthesis product were loaded onto a Waters 626 HPLC system with a Dynamax-300A C18 83-213-C5 column purchased from Rainin Instrument Co., Inc. A 45-min gradient of 10–80% acetonitrile was run in 0.05% trifluoroacetic acid with a flow of 2.5 ml/min. Absorbance at 214 nm was monitored in select fractions that were infused directly into the mass spectrometer. Those that contained only the correct mass predicted for the given peptide were selected. These fractions were lyophilized for 24 h in a Speedvac, and the dry residue was dissolved in water. Residual trifluoroacetic acid effects on the pH in the inhibition assay were controlled by neutralization based on direct measurements of the pH. Peptide concentrations were measured by amino acid analysis (AAA Laboratory, Mercer Island, WA) and spectrophotometrically at 214 nm.

**Cyclase Assay**—Guanylyl cyclase was assayed as described previously (10). A GCAP-1 concentration of 1 μM was determined to be subsaturating for stimulation of RetGC1 for this series of assays. That concentration of GCAP-1 was used in all guanylyl cyclase assays.

**RESULTS**

**Cross-linking RetGC1/GCAP1 Complexes**—A homobifunctional cross-linking agent, bMH, was selected to stabilize RetGC1/GCAP1 complexes. bMH reacts specifically with sulfhydryl groups of cysteine residues (28–30). It can cross-link two of them if the sulfhydryls are within the length that separates the reactive maleimide groups of bMH. Thus two cysteines belonging to different polypeptide chains can be covalently linked only if they are spatially proximate in the

**TABLE I**

| Washes             | cpm    |
|--------------------|--------|
| Total cpm in experiment | 5,911,071 |
| Urea washes of crosslinked ROS | 5,116,200 |
| I                  | 376,350 |
| II                 | 78,050  |
| III                | 4,805   |
| IV                 | 545     |
| V (low salt buffer) | 465,250 |
| Membranes after washes | 268,640 |
| Soluble fraction after trypsin | 285,640 |

**FIG. 1.** Western blots of cross-linked GCAP-1 and RetGC1. The left half of the panel shows washed rod outer segments combined with heterologously expressed GCAP-1. No cross-linker was added prior to blotting. The presence of a band of 30-kDa apparent molecular mass recognized by the anti-RetGC1 antibody was observed in several experiments. The identity of this band is unknown. The right half of the panel shows washed rod outer segments combined with heterologously expressed GCAP-1. No cross-linker was added prior to blotting. The volume of a band of 30-kDa apparent molecular mass recognized by the anti-RetGC1 antibody was observed in several experiments. The identity of this band is unknown.
protein complex. A preparation of washed bovine ROS mixed with recombinant GCAP-1 was used for cross-linking. Cross-linked proteins were separated by SDS-polyacrylamide gel electrophoresis, and the ability of bMH to stabilize RetGC1/GCAP complexes was assessed by Western blotting with anti-RetGC1 and -GCAP-1 antibodies. As shown in Fig. 1, a band of 230-kDa apparent molecular mass was recognized by both anti-RetGC1 and anti-GCAP-1 antibodies. This cross-reacting band was absent without prior cross-linking. It was also not observed when ROS and GCAP-1 were cross-linked separately (data not shown). It was therefore considered to be a covalent complex of RetGC1 and GCAP-1. The intensity of the GCAP-1 signal in the band was found to be slightly stronger in the presence of 10 μM Ca²⁺ than in 1 mM EGTA (data not shown). All subsequent cross-linking was therefore carried out in the presence of >10 μM Ca²⁺. No difference was observed in the cross-linking patterns under light conditions, making bMH the preferred cross-linker for this experiment.

**Finding candidate peaks for cross-linked peptides.** HPLC fraction 33 was analyzed by direct infusion into the mass spectrometer. The mass window was 200–2000 m/z; two regions of this range are shown in the figure. Data were collected for 1 min, and the scans were averaged to obtain the spectrum. Computer analysis of the spectrum identified a match between m/z values of the observed peaks and the mass of the GCAP-1 peptide AIRAINPCSDSTMAEEFTDVTFSK plus the mass of the RetGC1 peptide IRIGLHSGPCVAGVVGLTMPR with an additional 276 mass units from the cross-linker bMH. In particular, predictions for (M + 6H)/6 m/z equaled 858. A peak at this value is observed in the experimental spectrum. Likewise, prediction of (M + 4H)/4 m/z found a match of 1286 m/z in the spectrum.

**Assigning peaks in the ms/ms spectrum of the 858 parent ion.** Only major peaks are labeled. The total number of peaks in the spectrum is 191. We identified 91 of them as Y, B, or A ions resulting from fragmentation of the sequence AIRAINPCSDSTMAEEFTDVTFSK-IRIGLHSGPCVAGVVGLTMPR linked via cysteines by a cross-linker of 276 mass units (bismaleimide hexane). Some of the daughter ions underwent an apparent loss of H₂O or NH₃. GCAP-1 sequences are in italics; RetGC sequences are underlined.

**Reliability of ms/ms identification of cross-linked peptides.** To obtain a measure of reliability of the computer algorithm for matching ms/ms predicted and experimental spectra 10 cross-linked peptides whose parental masses did not match 858 were run against the same ms/ms spectrum of the 858 parental mass. The number listed next to each sequence is the number of ions in the predicted ms/ms spectrum matching the m/z values in this experimental data set. Y, B, and A ions were considered with or without H₂O or NH₃ loss.
versus dark conditions (data not shown), and all subsequent experiments were carried out in the light.

To monitor how much GCAP-1 became associated with membranes after cross-linking we used 35S-labeled GCAP-1. Unlike RetGC1, GCAP-1 is not an integral membrane protein, but it can become tightly attached to the membrane if it is chemically cross-linked to a transmembrane protein. ROS membranes were incubated with 35S-labeled GCAP-1 and bMH and then washed four times with 6 M urea. The amounts of radioactive label in four consecutive washes and in the final membranes were then measured. Labeled GCAP-1 that remained on the membranes after the washes amounted to 9% of the total GCAP-1 that had been added (Table I). In a control experiment bMH was not added, and only two urea washes were performed. In that case only 3% of the labeled GCAP-1 remained associated with the membranes after the washes (data not shown). We treated the washed cross-linked membranes with trypsin to release fragments of GCAP-1 and RetGC1 that had become cross-linked. Trypsin treatment released 57% of the bound label from the cross-linked membranes.

## Analysis of Cross-linked RetGC1/GCAP-1 Complexes

Following the trypsin treatment the soluble fraction was separated from the membranes, and the cross-linked tryptic fragments were resolved on a reverse phase C18 column. Scintillation counting of the HPLC fractions revealed that 19 of the 100 fractions contained significant amounts of radioactive label. Because only GCAP-1 was labeled in the experiment, HPLC fractions containing the label must contain GCAP-1 proteolytic fragments. The 19 fractions carrying significant amounts of radioactive label were analyzed on an electrospray mass spectrometer by direct infusion. This analysis produced spectra of varying complexity; the number of peaks that rose higher than expected were identified as fragments of the cross-linked peptides. Table II shows a list of all identified cross-linked peptides. An exhaustive analysis of all HPLC fractions with significant amounts of radioactive label produced this list of hits confirmed by ms/ms. For each observed pair of cross-linked cysteines the following are listed: sequence of cross-linked tryptic peptides, charge state observed, and percentage of ions in the experimental ms/ms spectrum that were identified as fragments of the candidate sequence. GCAP-1 sequences are in italics; RetGC sequences are underlined. Each cross-linked peptide pair matches a peak in the mass spectrum. Multiple charge states for the same ion were considered within the same HPLC fraction.

### Table II

Sequences of synthesized peptides

The left column lists cysteines in GCAP-1 and RetGC1 that were identified by COSUMAS. The right column lists the sequences of peptides derived from the corresponding flanking regions in GCAP-1 and RetGC1. These sequences were selected for testing in a peptide inhibition assay. Sequences flanking the cysteines identified by COSUMAS at positions 17, 105, and 124 in GCAP-1 and 622, 741, 780, 984, and 997 in RetGC1 were aligned with (i) homologous regions in Ca2+-binding proteins for GCAP-1 and (ii) guanylyl and adenylyl cyclases for RetGC1 (alignment not shown). Sequences that were unique for GCAP-1 and RetGC1 were selected for synthesis.

| Cysteines | Sequences |
|----------|-----------|
| GCAP-1   | Cys<sup>17</sup>, Cys<sup>105</sup>, Cys<sup>124</sup>, RetGC1, Cys<sup>622</sup>, Cys<sup>741</sup>, Cys<sup>780</sup>, Cys<sup>984</sup>, Cys<sup>997</sup> |
| GCAP-1   | Cys<sup>17</sup>, Cys<sup>105</sup>, Cys<sup>124</sup>, RetGC1, Cys<sup>622</sup>, Cys<sup>741</sup>, Cys<sup>780</sup>, Cys<sup>984</sup>, Cys<sup>997</sup> |
| GCAP-1   | Cys<sup>17</sup>, Cys<sup>105</sup>, Cys<sup>124</sup>, RetGC1, Cys<sup>622</sup>, Cys<sup>741</sup>, Cys<sup>780</sup>, Cys<sup>984</sup>, Cys<sup>997</sup> |
| GCAP-1   | Cys<sup>17</sup>, Cys<sup>105</sup>, Cys<sup>124</sup>, RetGC1, Cys<sup>622</sup>, Cys<sup>741</sup>, Cys<sup>780</sup>, Cys<sup>984</sup>, Cys<sup>997</sup> |
| GCAP-1   | Cys<sup>17</sup>, Cys<sup>105</sup>, Cys<sup>124</sup>, RetGC1, Cys<sup>622</sup>, Cys<sup>741</sup>, Cys<sup>780</sup>, Cys<sup>984</sup>, Cys<sup>997</sup> |
| GCAP-1   | Cys<sup>17</sup>, Cys<sup>105</sup>, Cys<sup>124</sup>, RetGC1, Cys<sup>622</sup>, Cys<sup>741</sup>, Cys<sup>780</sup>, Cys<sup>984</sup>, Cys<sup>997</sup> |
| GCAP-1   | Cys<sup>17</sup>, Cys<sup>105</sup>, Cys<sup>124</sup>, RetGC1, Cys<sup>622</sup>, Cys<sup>741</sup>, Cys<sup>780</sup>, Cys<sup>984</sup>, Cys<sup>997</sup> |
| GCAP-1   | Cys<sup>17</sup>, Cys<sup>105</sup>, Cys<sup>124</sup>, RetGC1, Cys<sup>622</sup>, Cys<sup>741</sup>, Cys<sup>780</sup>, Cys<sup>984</sup>, Cys<sup>997</sup> |
| GCAP-1   | Cys<sup>17</sup>, Cys<sup>105</sup>, Cys<sup>124</sup>, RetGC1, Cys<sup>622</sup>, Cys<sup>741</sup>, Cys<sup>780</sup>, Cys<sup>984</sup>, Cys<sup>997</sup> |
activity was measured in the presence of 1 μM GCAP-1 and 1 mM Ca²⁺. The following peptides derived from RetGC1 sequence were tested for their abilities to block inhibition of basal cyclase activity by GCAP-1 in the presence of Ca²⁺: A, 731SLGIIMQEVVCSRAPYAML770; B, CIEPLQSLAIRSVAMEGMVY (scrambled); C, 775QAPMEC-IQLMKQCW789; D, MLPQMACAIQWEQKC (scrambled); E, 877IRIG-LHSGPCVAQVVG906; F, 977DILLQRDHIKWFKSSG990. Closed circles represent the scrambled control peptides. 100% on the y axis refers to the Ca²⁺-dependent inhibition of cyclase activity in the presence of 1 μM GCAP-1 compared with basal activity in the absence of GCAP-1. Basal activity in the absence of GCAP-1 was taken to be 0%. The data shown are the averages of duplicate determinations from one or more experiments for each peptide.

significantly above the background varied from dozens to hundreds per HPLC fraction (data not shown).

A computer program was designed to enumerate all possible combinations of tryptic peptides from RetGC1 and GCAP-1 linked via a cysteine residue. The algorithm considers complete as well as partial digestion products. It predicts the masses of cross-linked peptides including the additional mass of the cross-linker. It considers all possible charge states for a given ion and compares the predicted masses with the peaks observed experimentally. An example is shown in Fig. 2. Candidate peaks for cross-linked peptides were identified using this program, and each was subjected to an ms/ms experiment to obtain partial sequence data. They were further analyzed by another computer program that compares predicted and experimental ms/ms spectra for a given sequence and provides a way to score the match between the two. Candidate ions were confirmed only if 1) at least two charge states of the same candidate cross-linked peptide were found in the same HPLC fraction, and 2) all major peaks in the ms/ms experimental spectrum were accounted for by the computer prediction, and 3) the number of peaks that matched expected masses was significantly higher than that produced by matching the experimental spectrum to a prediction from a random sequence of the same length. In cases where all three of these criteria were not completely satisfied we used an additional criterion: fragmentation at three or more consecutive residues. An ms/ms spectrum and its match with a predicted spectrum for the parent ion of 858 m/z is shown in Fig. 3. The same spectrum was matched with 10 other peptide sequences whose masses did not equal the parental mass of this ms/ms experiment (Fig. 4). The number of ions matching predictions varied from 38 to 81 with the average of 52.8. A peptide with the correct parental mass produced 91 identified ions.

From an exhaustive analysis of all candidate peaks a set of cross-linked RetGC1-GCAP-1 peptides emerged (Fig. 5). Based on these data cysteine pairs were identified in RetGC1 and GCAP-1 that become cross-linked and are thus likely to be on these data cysteine pairs were identified in RetGC1 and GCAP-1 that become cross-linked and are thus likely to be in contact surfaces. From an exhaustive analysis of all candidate peaks a set of cross-linked RetGC1-GCAP-1 peptides emerged (Fig. 5). Based on these data cysteine pairs were identified in RetGC1 and GCAP-1 that become cross-linked and are thus likely to be in contact surfaces. From an exhaustive analysis of all candidate peaks a set of cross-linked RetGC1-GCAP-1 peptides emerged (Fig. 5). Based on these data cysteine pairs were identified in RetGC1 and GCAP-1 that become cross-linked and are thus likely to be in contact surfaces. From an exhaustive analysis of all candidate peaks a set of cross-linked RetGC1-GCAP-1 peptides emerged (Fig. 5). Based on these data cysteine pairs were identified in RetGC1 and GCAP-1 that become cross-linked and are thus likely to be in contact surfaces.
domain were very effective at interfering with regulation by GCAP-1. We analyzed effects of these peptides on both GCAP-1-mediated inhibition in the presence of Ca\(^{2+}\) and activation in the absence of Ca\(^{2+}\). One peptide, 731SLGIIMQEVVCRSA-PMLE\(^{750}\), produced a half-maximal effect at 40 \(\mu M\) in Ca\(^{2+}\) (Fig. 6A) and at 30 \(\mu M\) in EGTA (Fig. 7A). A control peptide with the same amino acid composition but randomized residue order, CIEPLQSLAIRSVMEMGYV, caused only a minor effect in Ca\(^{2+}\) (Fig. 6B) and no detectable effect in EGTA (Fig. 7B). Another peptide corresponding to a different kinase homology domain sequence, 775QAPMCEILMLKQCWA\(^{789}\), produced a half-maximal effect at 80 \(\mu M\) in Ca\(^{2+}\) (Fig. 6C) and at 75 \(\mu M\) in EGTA (Fig. 7C). The corresponding randomized control peptide, MLPPMQACAIQEWQC, did not produce significant effects on RetGC activity in the same concentration range (Figs. 6D and 7D). The two peptides corresponding to a RetGC1 catalytic domain sequence, 965MRHMPEPVRIRIGLH\(^{980}\) and 970IRGLHSHPGCVAVG\(^{990}\), did not block regulation of RetGC activity by GCAP-1 either in Ca\(^{2+}\) or EGTA (only Ile\(^{975}\)-Gly\(^{980}\) is shown in Figs. 6E and 7E). This result is not consistent with a previous report (16) that a similar peptide from this region, Gly\(^{961}\)-Gly\(^{978}\), blocks activation by GCAP-1.

A peptide derived from the sequence adjacent to Cys\(^{622}\) in RetGC1, 629DLLAQRDKLDMWKSS\(^{645}\), blocked GCAP-1-mediated cyclase activation half-maximally at 270 \(\mu M\) in EGTA (Fig. 7F). Its randomized analog, LSDQIAMKLDFSLDK, also exhibited some inhibitory effect in EGTA but at higher concentration (Fig. 7G). The same peptide, 629DLLAQRDKLDMWKSS\(^{645}\), did not interfere with inhibition of cyclase activity by Ca\(^{2+}\)-GCAP-1 (Fig. 6F).

Peptides with GCAP-1 sequences were analyzed for effects on basal RetGC activity in the absence of GCAP-1 as well as for their ability to block regulation of RetGC by GCAP-1. A peptide with the sequence 8SVEELSTTECHQYKKFMTE\(^{77}\) reduced cyclase activity to less than 50% of its basal level with a half-maximal effect at 100 \(\mu M\) (Fig. 8A). However, the control peptide with the same residues in random order, HKVEYMSWEKFSSTLSCETEQ, also caused some inhibition at a similar concentration (Fig. 8B). A GCAP-1 peptide with the sequence 8EQKLRWFYKLYLDGNGCID\(^{107}\) also inhibited RetGC1 basal activity (Fig. 8C), whereas the control randomized peptide NFLIEDRVLKCKGDGWYY did not (Fig. 8D). A GCAP-1 peptide with the sequence 111LLTIIRAIRAINPCSDLT130 did not affect cyclase activity at concentrations up to 1 mM (data not shown).

**FIG. 8. Effects of free GCAP-1 peptides on cyclase activity in ROS.** A, “SVEELSTTECHQYKKFMTE\(^{77}\)”; B, HKVEYMSWEKFSSTLSCETEQ (scrambled); C, 8EQKLRWFYKLYLDGNGCID\(^{107}\); D, NFLIEDRVLKCKGDGWYY (scrambled). Closed circles represent the peptides with native sequences, and open circles represent the scrambled control peptides. Basal guanyl cyclase activity in the absence of peptides and GCAP-1 was taken to be 100%. The values shown are the average of duplicate data points obtained from two independent experiments.

**FIG. 9. Contacts between GCAP-1 and RetGC1.** Only the cytoplasmic part of RetGC1 is shown. Interactions that have been confirmed by peptide inhibition as contact surfaces are connected by solid lines. The boxes outline peptides that were analyzed. Interactions that were identified by COSUMAS but that were not confirmed in our peptide inhibition experiments are connected by dashed lines.

**DISCUSSION**

In this study we provide evidence for multiple contact surfaces involved in RetGC1/GCAP-1 interaction (Fig. 9). The evidence comes from two independent experimental methods. A novel mass spectrometry-based method, COSUMAS, identified cysteines in the two proteins that are sufficiently close to each other to be cross-linked by bMH. This method revealed previously unrecognized structures in the kinase homology domain of RetGC1 that are spatially proximate to bound GCAP-1. The involvement of these regions was confirmed by the second method, which was analyses of synthetic peptides derived from the RetGC1 sequence for the ability to interfere with regulation by GCAP-1. Peptides derived from the corresponding GCAP-1 sequence also were analyzed for effects on RetGC activity. The specificity of the effect of each peptide was controlled in our study by analyzing peptides with the same amino acid composition but randomized sequences.

**GCAP-1**

**RetGC1 (intracellular)**

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Sequences from RetGC1 That Affect the RetGC1/GCAP-1 Interaction—RetGC1 sequences that have the strongest inhibitory effect on regulation by GCAP-1 are 731SLHGMQEVVCRSAPYAML750 and 777QAPMECILIQMFKWC789. These peptides are derived from the kinase homology domain of RetGC1, and they interfere with GCAP-1 regulation at both high and low Ca\textsuperscript{2+} concentrations. It is likely that these effects derive from the peptides competing with full-length RetGC1 for a binding site on GCAP-1. This suggests that interactions between GCAP-1 and the kinase homology domain occur independently of Ca\textsuperscript{2+}. A Ca\textsuperscript{2+}-independent interaction of GCAP-1 with RetGC1 is also consistent with the following previous observations. (i) GCAP-1 regulates RetGC activity in the presence as well as absence of Ca\textsuperscript{2+} (11, 31). (ii) GCAP-1 stabilizes RetGC1 activity in the presence as well as absence of Ca\textsuperscript{2+} (23). (iii) GCAP-2 protects the kinase homology domain from proteolysis in the presence as well as absence of Ca\textsuperscript{2+} (22).

Another region of the kinase homology domain of RetGC1, a region adjacent to Cys\textsuperscript{622}, 623DILLAQRDIDLDWMKFSS\textsuperscript{645}, was also considered as a candidate for a contact surface based on our mass spectrometry data. This peptide, however, exhibited an effect on cyclase stimulation only in EGTA (Fig. 7F). Because the interaction was detected using COSUMAS in the presence of Ca\textsuperscript{2+} this interaction may occur in the presence of Ca\textsuperscript{2+} only when these amino acids are in the context of the folded RetGC1 protein. The free peptide may have a weaker affinity for GCAP-1 in the presence of Ca\textsuperscript{2+} than in its absence. Alternatively, COSUMAS may have detected a specific spatial proximity that is a point of contact at low but not at high Ca\textsuperscript{2+} concentrations.

The COSUMAS method also suggested that a region in the catalytic domain of RetGC1 is proximal to GCAP-1. This appears consistent with a previous finding (16) derived from peptide binding and inhibition experiments. However, neither of the two overlapping peptides we synthesized from this region affect regulation of cyclase by GCAP-1 either at high or low Ca\textsuperscript{2+} levels. Further studies will be required to confirm whether this region includes sites that interact directly with GCAP-1.

Sequences from GCAP-1 That Affect RetGC1/GCAP-1 Interaction—COSUMAS identified two regions in GCAP-1 that cross-linked with RetGC1: 5VEELSSTETCHQWYKVKFMTE\textsuperscript{577} and 89EQKLWRFYKLYDVDGNGCID107. The corresponding sequences from RetGC1 that affect the RetGC1/GCAP1 interaction of the two overlapping peptides we synthesized from this region appear consistent with a previous finding (16) derived from the kinase homology domain of RetGC1, and they support previous evidence that these two proteins interact directly without an adapter protein. The data presented here also independently confirm previous suggestions that the kinase homology domain of RetGC1 serves as a docking site for both Ca\textsuperscript{2+}-bound GCAP-1 and Ca\textsuperscript{2+}-free GCAP-1. Finally, this study specifically localizes some of the sites of interaction between RetGC1 and GCAP-1 providing fundamental information about molecular mechanisms by which cGMP is regulated in photoreceptors.

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RetGC1/GCAP1 Interaction
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