Guanylyl cyclase activating protein 1 (GCAP1), after substitution of Ca\(^{2+}\) by Mg\(^{2+}\) in its EF-hands, stimulates photoreceptor guanylyl cyclase, RetGC1, in response to light. We inactivated metal binding in individual EF-hands of GCAP1 tagged with green fluorescent protein to assess their role in GCAP1 binding to RetGC1 in co-transfected HEK293 cells. When expressed alone, GCAP1 was uniformly distributed throughout the cytoplasm and the nuclei of the cells, but when co-expressed with either fluorescently tagged or non-tagged RetGC1, it co-localized with the cyclase in the membranes. The co-localization did not occur when the C-terminal portion of RetGC1, containing its regulatory and catalytic domains, was removed. Mutations that preserved Mg\(^{2+}\) binding in all three metal-binding EF-hands did not affect GCAP1 association with the cyclase in live cells. Locking EF-hand 4 in its apo-conformation, incapable of binding either Ca\(^{2+}\) or Mg\(^{2+}\), had no effect on GCAP1 association with the cyclase. In contrast to EF-hand 4, inactivation of EF-hand 3 reduced the efficiency of the co-localization, and inactivation of EF-hand 2 drastically suppressed GCAP1 binding to the cyclase. These results directly demonstrate that metal binding in EF-hand 2 is crucial for GCAP1 attachment to RetGC1, and that in EF-hand 3 it is less critical, although it enhances the efficiency of the GCAP1 docking on the target enzyme. Metal binding in EF-hand 4 has no role in the primary attachment of GCAP1 to the cyclase, and it only triggers the activator-to-inhibitor functional switch in GCAP1.

Guanylyl cyclase activating proteins (GCAPs)\(^2\) are Ca\(^{2+}\)/Mg\(^{2+}\)-binding proteins that regulate retinal guanylyl cyclase (RetGC), the enzyme that supplies photoreceptors with the phototransduction messenger, cGMP (1–4). Calcium, which enters outer segments of vertebrate photoreceptors through cGMP-gated Na\(^+\)/Ca\(^{2+}\) channels in the outer segment plasma membrane, is continuously removed from the outer segment by a light-independent Na\(^+\)/K\(^+\), Ca\(^{2+}\) exchanger (for review, see Refs. 5–8). In the dark, cGMP keeps a small percentage of the Na\(^+\)/Ca\(^{2+}\) channels open, and the hydrolysis of cGMP by a light-activated phosphodiesterase, PDE6, generates photoreponses in rods and cones. When light triggers cGMP hydrolysis, it also, through the closure of the channels, lowers the intracellular concentration of Ca\(^{2+}\) from ~250 nM in the dark to ~25 nM in the light (9–12). At the same time, free concentrations of Mg\(^{2+}\) in photoreceptors remain near 1 mM, regardless of illumination conditions (13). In response to the light-dependent decrease in free Ca\(^{2+}\) concentrations, GCAPs exchange Ca\(^{2+}\) in their EF-hands for Mg\(^{2+}\) (14, 15), which stimulates RetGC and thus prompts re-opening of the cGMP-gated channels and accelerates the recovery. Of the four EF-hand domains in GCAPs, only three are capable of Mg\(^{2+}\)/Ca\(^{2+}\) exchange, whereas the N-proximal EF-hand 1 domain deviates from the consensus sequence and cannot bind metal ions. Instead, this EF-hand is required for GCAPs interaction with their target enzyme, RetGC (16–19). We previously described mutations that can inactivate only Ca\(^{2+}\) or both Ca\(^{2+}\) and Mg\(^{2+}\) binding in all three metal-binding EF-hands of GCAP1 (15, 20). We have demonstrated that the apo form of GCAP1 does not stimulate RetGC1. We have also found that when neither Ca\(^{2+}\) nor Mg\(^{2+}\) is present in EF-hands 2 and 3, activation of RetGC is suppressed. Inactivation of Ca\(^{2+}\) coordination in EF-hand 4 prevented inhibition of RetGC1 by Ca\(^{2+}\) but had no effect on the cyclase activation by Mg\(^{2+}\)-liganded GCAP1 (15). Our previous data suggested that divalent cation binding in EF-hand 2 and, to a lesser extent, in EF-hand 3, was required for GCAP1 to stay in complex with RetGC1 in either Mg\(^{2+}\)- or Ca\(^{2+}\)-liganded forms. Here, we evaluated this hypothesis by monitoring the binding of a green fluorescent protein (GFP)-tagged GCAP1 to functional RetGC1 in cultured cells. We demonstrate that elimination of both Ca\(^{2+}\) and Mg\(^{2+}\) binding in EF-hand 2 suppresses compartmentalization of GCAP1 with RetGC1, whereas inactivation of EF-hand 3 reduces the efficiency of the GCAP1 attachment to RetGC1, but does not prevent it. We also demonstrate that cation binding in EF-hand 4 has no role in GCAP1 association with the cyclase.

EXPERIMENTAL PROCEDURES

Recombinant GCAP1 and Its Mutants—GFP-tagged bovine GCAP1 was expressed in HEK293 cells from a Clontech...
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pQBI25-fN3 vector. The GCAP1 cDNA was inserted into the BamHI/EcoRI site, in-frame with the Superglo GFP-coding sequence of the vector and downstream of the CMV promoter, as follows. The GCAP1 sequence was PCR-amplified by Pfu polymerase (Stratagene) from a DNA clone for transgenic expression of wild type bovine GCAP1 in mouse rods (9) using a forward primer, 5'-GGGCGAGGCCGGGTCGGTTGGCCAGG-3', and a reverse primer, 5'-GGGCGAGGCCGGGTCGGTTGGCCAGG-3', by adding the Kozak motif and the required restriction sites to the GCAP1 cDNA in the resultant plasmid, GCAP1-GFP/pQBI25fN3. Expression constructs for EF-hand mutants were made by substitution of the Bpi/SfiI fragment in the GCAP1-GFP/pQBI25fN3 construct with the corresponding fragment from cDNA coding for the EF-hand mutations described previously (15, 20).

To produce GFP-tagged GCAP1 and its mutants in E. coli, the GCAP1-GFP cDNA fragment was inserted into the pET11d vector (Novagen/Calbiochem) in two steps. We first PCR amplified with Pfu polymerase two fragments from the GCAP1-GFP pQBI25fN3, and the expression pET11d vector for wild type GCAP1 (21, 22). The two fragments were amplified separately, using two pairs of primers: 5'-GGGCGAGGCCGGGTCGGTTGGCCAGG-3' (pair 1), and 5'-GGGCGAGGCCGGGTCGGTTGGCCAGG-3' (pair 2), respectively. The two isolated fragments were spliced together by a second round of PCR, using the first and the last primers only, thus resulting in the BamHI site being placed near the 3'-end of the fusion cDNA. The resultant fusion cDNA was inserted into the SacII/BamHI sites of the modified pET11d vector harboring bovine GCAP1 cDNA, thus placing the GFP sequence in-frame with the GCAP1, followed by the stop codon and the transcription termination site of the pET11d vector. Wild type GCAP1 and its mutants all had a recognition site for a yeast cytoplasmic domain of the cyclase. We used this construct, dsRed-RetGC1, as a control for the specificity in co-localization experiments. The reading frame in the dsRed-BoGCAP1, followed by the stop codon and the transcription termination site of the pET11d vector, was preceded by an additional nucleotide, which truncated the amino acid sequence by shifting the reading frame after the BstEII restriction site in the dsRed cDNA, GGTGACC, was inactivated via a silent substitution, GGTGACT, using PCR splicing by overlap extension. The BstEII restriction site to the GCAP1 cDNA in the resultant plasmid, GCAP1-GFP pQBI25fN3, and the expression pET11d vector constructs were verified by automated DNA sequencing.

Transfection of RetGC1 and GCAP1 into HEK293 Cells—HEK293 cells were grown at 37 °C, 5% CO2, in high glucose Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). To express RetGC1 for the functional assay in vitro, HEK293 cells were transfected with 40 μg/100-mm culture dish of pRCCMV plasmid containing wild type RetGC1 or dsRed-RetGC1 using Ca2+-phosphate method (Promega Profection protocol), and the membranes were harvested as previously described (24). We also developed a stable neomycin-resistant line expressing GCAP1-GFP selected in the presence of Geneticin (Invitrogen).

Co-expression of RetGC1 and GCAP1 in HEK293 Cells and Confocal Laser Scanning Microscopy—Cells were grown in standard glass coverslip chambers (four 2-cm2 chambers per slide) in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, and were transfected with a mixture of expression constructs using the Ca2+-phosphate method. Routinely, a mixture of 3 μg of pRCCMV plasmid containing wild type RetGC1 cDNA or dsRed-RetGC1 and about 0.02 μg of the GCAP1-GFP pQBI25fN3 plasmid was used per chamber. In 24–32 h, the live cells were either viewed directly or fixed for 15 min with freshly prepared 4% paraformaldehyde in a standard Tris-buffered saline (TBS) at room temperature for subsequent anti-RetGC1 staining with an antibody. The cells were gently washed twice with 1 ml of TBS, blocked by 1% goat serum, 1% bovine serum albumin in TBS containing 0.05% Triton X-100, for 30 min, incubated with anti-RetGC1 antibody in TBS, 0.3% Triton X-100 for 30 min, washed three times with the Triton X-100/TBS for 5 min, and then incubated with Alexa Fluor 568-conjugated goat anti-rabbit antibody (Invitrogen) for 30 min. The cells were then washed twice with TBS and covered with Vectashield solution (Vector Laboratories). Where indicated, the DNA in nuclei of the cells was counterstained for 10 min with 1 μm TO-PRO-3 iodide stain (Invitrogen) containing 20 μg/ml RNase A (added to the first wash following the incubation with the secondary antibody), and then washed as described above. Where indicated, live cells expressing GCAP1-GFP were treated for 30 min with a fluorescent ER-TrackerTM Red marker (E34250, Invitrogen) to counterstain the endoplasmic reticulum or with a CellMaskTM Deep Red stain (C10046, Invitrogen) to counterstain the plasma membrane, all according to the manufacturer's protocols.

The cells were viewed using an inverted Olympus IX81 microscope/FV1000 Spectral laser confocal system, and images were collected and analyzed using Olympus Fluoview FV10-ASW software. Fluorescence from different spectral markers in the same sample was recorded in a sequential mode, at 40 μs/pixel, typically averaged from three to four repeats, and where indicated, were superimposed on a confocal transmitted light differential interference contrast image of the same cells. The far-red fluorescence emitted from the TO-PRO-3 iodide or the CellMask Deep Red plasma membrane stain was pseudo-colored blue or red, respectively.

Anti-RetGC1 Antibody—Anti-RetGC1 antibody was produced in a rabbit against recombinant fragments of a human RetGC1, Met725–Ser1652 (antibody GC1Cat) or Arg1580–Asn1615 (antibody
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FIGURE 1. Properties of dsRed-RetGC1 and GCAP1-GFP. A, activation of non-tagged RetGC1 in HEK293 cell membranes by purified non-tagged (□) or GFP-tagged (○) GCAP1; B, Ca\(^{2+}\) sensitivity of the non-tagged RetGC1 reconstituted with the non-tagged or GFP-tagged GCAP1; C, activation of dsRed-RetGC1 in HEK293 cell membranes by the purified non-tagged or GFP-tagged GCAP1; D, Ca\(^{2+}\) sensitivity of the dsRed-RetGC1 in HEK293 cell membranes reconstituted with the non-tagged or GFP-tagged GCAP1. RetGC1 activation by GCAP1 was measured in the presence of 2 mM EGTA, 1 mM free Mg\(^{2+}\), and increasing concentrations of wild type GCAP1 or GCAP1-GFP. The data were fitted by the equation, \(A = A_{\text{max}} \times ([\text{GCAP}]^{n} + [\text{GCAP}]^{n})^{1/2}\), where \(A\) is the activity of RetGC1 in the assay, \(A_{\text{max}}\) is the maximal activity of RetGC1, [GCAP] is the concentration of GCAP, \(K_{n}\) is the concentration of GCAP1 required for half-maximal activation of RetGC1, \(n\) is the cooperativity coefficient. \(Ca^{2+}\) sensitivity of the recombinant RetGC1 regulation by GCAP1 was assayed at various free \(Ca^{2+}\) concentrations in the presence of 10 \(\mu\)M recombinant GCAP1. The data were fitted as fractional activity of RetGC1 by the function, \(Y = 100\%/[1 + ([Ca]^{1/\gamma})([Ca]^{1/\gamma})]^{1/2}\); \([Ca]^{1/\gamma}\) is the free \(Ca^{2+}\) concentration required for half-maximal inhibition of RetGC1, \(\gamma\) is the cooperativity coefficient. The values from the fit are summarized in supplemental Table S1. For other conditions of the assay see “Experimental Procedures.”

GC1KHD), expressed in E. coli from pET15b vector (Novagen/Calbiochem). The IgG fraction was purified using Protein A-Sepharose (GE Healthcare). The antibodies specifically stained the ~115-kDa band on immunoblots of HEK293 cells transfected with RetGC1 expressing constructs, but not from non-transfected control cells, and specifically stained membranes in RetGC1-transfected, but not in non-transfected cells.

Immunoblotting—The HEK293 cells were washed in TBS, dissolved in a Laemmli SDS-PAGE sample buffer, and aliquots were separated in 4–12% PAGE (Invitrogen). Following electrophoretic transfer to Immunoblot P membrane (Millipore), proteins were probed with the rabbit polyclonal anti-RetGC1 and anti-GCAP antibodies and developed using a Pierce Femto SuperSignal luminescent peroxidase substrate, according to the manufacturer’s protocol. The signal intensity on x-ray film was quantified by densitometry as previously described (9).

RetGC Assay—Guanylyl cyclase activity was assayed as previously described (15). Briefly, the assay mixture (25 \(\mu\)l) contained 30 mM MOPS/KOH, pH 7.2, 60 mM KCl, 4 mM NaCl, 1 mM dithiothreitol, 1 mM free Mg\(^{2+}\), 2 mM Ca/EGTA buffer, 0.3 mM ATP, 4 mM cGMP, 1 mM GTP, 4 mM creatine phosphate, 0.5 units of creatine phosphokinase, 1 \(\mu\)Ci of [\(\alpha^{32}\P\)]GTP, 0.1 \(\mu\)Ci of [\(8-\text{H}\)]mGMP, GCAP1, and HEK293 cell membranes. The reaction mixture was incubated for 40 min at 30 °C, stopped by heating for 2.5 min at 95 °C, and aliquots were analyzed by TLC on fluorescent plastic-backed polyethyleneimine-cellulose plates (E. Merck) as described previously (2, 24). The data shown are representative from three to four independent experiments producing virtually identical results.

Statistics—In co-localization experiments, five groups of GCAP1-GFP (wild type and four mutants) expressed in HEK293 cells were compared by one-way analysis of variance and post hoc processed by Bonferroni all-pairs comparison test using a GraphPad QuickCalc calculator (GraphPad Software, Inc., San Diego, CA) and Synergy Kaleidagraph software. The same software was used for a non-paired \(t\) test to compare the data for the D64N mutant versus the wild GCAP1-GFP type expressed in the absence of the cyclase.

RESULTS

Study of direct binding of GCAPs to the cyclase is very challenging. In contrast to some other proteins, direct binding of GCAPs to RetGC1 could not be reliably assessed using the membrane binding in vitro assay due to the effect of nonspecific interactions (16, 25, 26), and a detergent-solubilized RetGC1 is not sensitive to GCAPs (27). Therefore, we decided to tag GCAP1 and its mutants with the enhanced GFP and test their co-localization with the target enzyme in live cells expressing either non-modified or fluorescently tagged dsRed-RetGC1.

The Activity of GFP-tagged GCAP1 and dsRed-tagged RetGC1—It was important for the entire line of the study to establish whether or not the fluorescent tags can affect the activity of GCAP1 or RetGC1. We found that the main regulatory properties of GCAP1 were unaltered by its fusion with GFP (Fig. 1, A and B), nor were altered by its tag the properties of the dsRed-tagged RetGC1 (Fig. 1, C and D). There was no difference between the wild type RetGC1 activation by either non-tagged or the GFP-tagged GCAP1 or dsRed-RetGC1 activation by the non-tagged or GFP-tagged GCAP1, except for a minor
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FIGURE 2. The effect of various substitutions in EF-hands of GCAP1-GFP on RetGC1 activation. Mutations that either preserved or prevented Mg$^{2+}$ binding were introduced in the individual EF-hands of GCAP1-GFP and the activity of RetGC1 was measured in the presence of 2 mM EGTA, 1 mM free Mg$^{2+}$, and increasing concentrations of GCAP1-GFP mutants as described under “Experimental Procedures.” Symbols are: □ wild type EF-hands; ▼ D64N; △ D100N/D102G; ▲ D144N/D148G; ○, E75Q/E111Q/E155Q. For the fit, see the legend to Fig. 1, A and C. The values for $A_{max}$ and $K_{1/2}$ are summarized in supplemental Table S2.

Decrease in the apparent affinity for dsRed-RetGC1 activation by both the tagged and non-tagged GCAP1. Ca$^{2+}$ sensitivity of both forms of RetGC1 reconstituted with either tagged or non-tagged GCAP1 was the same (see the supplemental Table S1 for more detail).

Properties of GFP-tagged GCAP1 EF-hand Mutants—In our previous study, we identified mutations in individual EF-hands that disabled either Ca$^{2+}$ binding or both Ca$^{2+}$ and Mg$^{2+}$ binding altogether (20). Disabling of metal binding in different EF-hands had profoundly different effects on RetGC1 activation and inhibition (15): inactivation of only Ca$^{2+}$ binding in all three EF-hands by E75Q/E111Q/E155Q substitutions prevented the cyclase inhibition by Ca$^{2+}$, but not affecation of RetGC1 by the triple mutant in the presence of Mg$^{2+}$. A complete inactivation of both Ca$^{2+}$ and Mg$^{2+}$ binding in EF-hand 4 by a double substitution, D144N/D148G, did not affect it either. However, complete inactivation of Ca$^{2+}$/Mg$^{2+}$ binding in EF-hands 2 and 3 by D64N and D100N/D102G mutations, respectively, suppressed activation of the cyclase. The effect was most dramatic in the case of EF-hand 2 (15). We have verified that the corresponding GFP-tagged GCAP1 mutants displayed virtually the same properties as their non-tagged counterparts (Fig. 2 and supplemental Table S2): (a) disabling the EF-hand 2 drastically increased the $K_{1/2}$ of GCAP1 for RetGC1; (b) inactivation of EF-hand 3 reduced the apparent affinity of GCAP1 for RetGC1, however, to a lesser extent than EF-hand 2; (c) prevention of both Ca$^{2+}$ and Mg$^{2+}$ binding in EF-hand 4 had no effect on RetGC1 activation. Therefore, it was justified to use the fluorescent protein-tagged GCAP1 mutants and RetGC1 in co-expression and co-localization analyses to directly assess their binding to each other.

Localization of GCAP1 and RetGC1 in HEK293 Cells—GCAPs have the ability to weakly bind to lipid membranes (16, 25, 26, 28), but when expressed in HEK293 cells, GCAP1 was uniformly distributed throughout the cell, including the nucleus (Fig. 3A and supplemental Fig. S1A). Only the nucleoli appeared to have lower density of the fluorescent GCAP1 in the nucleus, and only vacuoles were devoid of fluorescence in the cytoplasmic portion of the cells. This pattern remained in all GCAP1-GFP expressing cells, regardless of the overall intensity of the fluorescence (i.e. the levels of GCAP1 expression). We also found no difference in GCAP1-GFP distribution between the cells transiently expressing GCAP1-GFP versus a stable line of GCAP1-GFP expressing cell (data not shown). The uniform cellular distribution of GCAP1 drastically changed when the cells expressed both GCAP1-GFP and RetGC1 (Fig. 3A, panels b and c); GCAP1 fluorescence was depleted from the nuclei and was only observed in the cytoplasm of the cells, where it demonstrated a membrane association pattern, indistinguishable from that of RetGC1 (Fig. 3A, panels a – d, and supplemental Fig. S1B). The intrinsic fluorescence of GCAP1-GFP and immunofluorescence of RetGC1 both displayed a typical shape of “donuts” and “tennis rockets”: the empty nucleus was surrounded by well defined membrane fluorescence, extended along the endoplasmic reticulum (ER) (Fig. 3, B and C, and supplemental Fig. S2). It should be noted that although RetGC1 expressed in HEK293 cells was active and responsive to GCAP in vitro (Figs. 1 and 2), most of the cyclase was localized to the membranes of the ER (Fig. 3A, panel c and supplemental Fig. S2), and so was GCAP1-GFP, co-expressed with the cyclase. Only a minor portion of the GCAP1 fluorescence appeared to coincide with the marker for the plasma membrane, whereas most of it followed the profile matching that of the ER marker (Fig. 3, B and C).

We have also observed co-localization of the GFP-tagged wild type GCAP1 with RetGC1 in non-fixed live cells expressing dsRed-tagged RetGC1 (Fig. 4A, panels a – c). Both fluorescent tags in the non-fixed cells co-localized in the same pattern that was observed in the fixed cells immunostained for RetGC1 (Fig. 3). That argued that co-localization in Fig. 3 was not an artifact of fixation. The distribution of both GCAP1-GFP and dsRed-RetGC1 had the characteristic absence of the signal in the nucleus and a sharp increase in the fluorescence of the surrounding ER membranes (Fig. 4A, panels a – c, the cell marked with an arrow). Such a pattern was not observed in control experiments when we co-expressed the GFP-tagged GCAP1 with the extracellular portion of the cyclase, incapable of binding GCAP1 (29–32). The fluorescently tagged fragment, ΔdsRed-RetGC1, retained the leader peptide sequence for guiding it to the ER (Fig. 4A, panel e), but the distribution of GCAP1-GFP and ΔdsRed-RetGC1 was drastically different (panels d – f); the GCAP1-GFP fluorescence was spread throughout the cell, including the nucleus (same as GCAP1 expressed in the absence of RetGC1 in Fig. 3), and the cyclase fragment was only detected in the ER. Fig. 4B, panels a – d, further demonstrates that the diffuse distribution of GCAP1-GFP co-expressed with the truncated cyclase was not due to much...
higher GCAP1 expression relative to the ΔdsRed-RetGC1 fragment. We could not use anti-retGC1 antibody for quantitative immunoblotting, because the truncated cyclase lacks the intracellular domains, against which the anti-RetGC1 antibodies were raised. Yet, we took advantage of the same fluorescent tag being present in both the truncated fragment and the non-truncated cyclase. We used the same excitation laser intensity and the photomultiplier gain settings to compare the fluorescence profiles of GCAP1-GFP co-expressed with either dsRed-RetGC1 or ΔdsRed-RetGC1 in the neighboring chambers on the same coverslip. In that case, the relative intensities of the red versus green fluorescence in one group of the cells could be directly compared with the intensities of the corresponding fluorescent tags in the other group (Fig. 4B, panels b and d). The representative results shown in this figure illustrate that GCAP1-GFP failed to co-localize with the ΔdsRed-RetGC1 fragment, even when GCAP1-GFP levels were lower, and the levels of ΔdsRed-RetGC1 were higher than in the cells expressing the non-truncated cyclase. Therefore, the characteristic pattern of GCAP1 co-localization with RetGC1 in Figs. 3 and 4 could only be attributed to a direct binding of GCAP1 to the “intra-cellular” domains of RetGC1, missing in the truncated cyclase.

Differential Binding of GCAP1 Mutants to RetGC1 in HEK293 Cells (Fig. 5)—We were unable to modulate the binding of GCAP1 to RetGC in live cells by removing Mg\(^{2+}\) from the cells by chelating agents, because that affected their shape and adhesion to the plate and thus hampered the analysis. Instead, we used the GFP-tagged mutants of GCAP1, which either lost their ability to bind Ca\(^{2+}\), yet retained their high-affinity Mg\(^{2+}\) binding, or could bind neither of the two ions (15, 20). All GCAP1-GFP mutants, expressed in the absence of the cyclase, displayed a diffuse pattern, the same as the wild type GCAP1-GFP in Fig. 3A (data not shown).

Consistent with its unchanged ability to activate RetGC1 in the presence of Mg\(^{2+}\) (Fig. 2), GFP-tagged E75Q/Δ111Q/Δ155Q GCAP1, which does not bind Ca\(^{2+}\), but binds Mg\(^{2+}\) in all three metal binding EF-hands (15, 20), co-localized with RetGC1, just like the wild type (Fig. 5A). The green fluorescence of GCAP1 matched immunofluorescence of RetGC1 in the ER and did not overlap with the TO-PRO-3-stained nuclei. We used an excess of the cyclase-expressing vector over that of each GCAP1 mutant (this will also be discussed in detail further below). Therefore, the cells, which would express GCAP1, but had no detectable expression of RetGC1, were present at low frequency. However, in Fig. 5A, for comparison with the other marked cells, there is shown a cell (cell number 3) that did not express RetGC1. That cell had a uniform GCAP1-GFP distribution, in sharp contrast to the typical “donuts and tennis rackets” pattern of the surrounding cells. Co-localization of the E75Q/Δ111Q/Δ155Q mutant with RetGC1 in HEK293 cells was consistent with the ability of the Mg\(^{2+}\)-liganded GCAP1, the physiological form of GCAP1 in light-adapted photoreceptor cells (20), to activate the cyclase (Fig. 2). Inactivation of Mg\(^{2+}\) binding in the individual EF-hands had profoundly different effects on the association of the GCAP1 mutants.
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FIGURE 4. GCAP1-GFP co-localizes in live cells with dsRed-RetGC1, but not with truncated cyclase fragment, lacking the intracellular domains. A, GCAP1-GFP was co-expressed with dsRed-RetGC1 (a–c) or ΔdsRed-RetGC1 (d–f); a and d, GFP fluorescence; b and e, dsRed fluorescence (excitation at 543 nm); c, merged a and b; f, merged d and e. B, direct comparison of fluorescence intensities, F, profiles of GCAP1-GFP co-expressed with dsRed-RetGC1 (a–b) or ΔdsRed-RetGC1 (c–d); a and b, representative fluorescence intensity profiles recorded from the two cells expressing GCAP1-GFP (green) and dsRed-RetGC1 (red), shown in the two left panels, along the lines superimposed on the merged fluorescence images shown as the insets in the right two panels; c and d, two fluorescence intensity profiles recorded from the cell expressing GCAP1-GFP (green) and ΔdsRed-RetGC1 (red) shown in the two left panels, along the lines superimposed on the merged fluorescence images, shown as the insets in the right two panels. The images in panels a and c were recorded in the same experiment, at the same laser excitation and photomultiplier gain settings, from the cells in the neighboring chambers of the same coverslip; bar, 10 μm.

In contrast to the wild type GCAP1, distribution of the D64N mutant in the presence of RetGC1 remained rather diffuse (Fig. 5B). The intensity of the GCAP1-GFP fluorescence was only slightly elevated outside the nuclei (panels a and c), regardless of the overall intensity of the GCAP-GFP expressed in the cells (compare cells 1–4 in panel a). In all cells of this group, the GCAP1 fluorescence was nearly the same in the nuclei as in the cytoplasm, strikingly different from the localization of RetGC1 (panels b and c).

When Mg<sup>2+</sup> binding in EF-hand 3 was abolished (D100N/D102G) (Fig. 5C), co-localization of the mutant with the cyclase resembled the wild type, rather than the D64N mutant, except that the relative brightness of the GCAP1-GFP fluorescence in the ER versus the nucleus was typically lower than in wild type. Although not as striking as in wild type, its pattern was still different from the diffuse fluorescence in the cells that did not express RetGC1 (compare cell 1 with 2). Complete inactivation of Ca<sup>2+</sup> and Mg<sup>2+</sup> binding in EF-hand 4 had no detectable effect on co-localization of GCAP1 with RetGC1 (Fig. 5D): the D144N/D148G GCAP1 fluorescence was depleted from the nucleus and co-localized with RetGC1 immunofluorescence in membrane structures, the same as in the wild type GCAP1-GFP.

Among all the mutants that we tested in Fig. 5, D64N showed the weakest co-localization with the cyclase. This pattern, however, was not due to an excessive level of the D64N expression compared with the wild type GCAP1. As an example, in Fig. 6 we compared the profiles of the D64N and the wild type GCAP1 versus that of RetGC1 directly, by their relative fluorescence intensities (Fig. 6). The overall GFP fluorescence integrated for the entire cell was almost twice as high as in the wild type GCAP1 than in the D64N mutant (Fig. 6, A and B, panels a), and the overall immunofluorescence of RetGC1 was the same in both cells (Fig. 6, A and B, panels b). Yet, the distribution of the wild type GCAP1 exactly matched that of the RetGC1, whereas the distribution of the D64N remained much more diffuse (compare the profiles in Fig. 6, A and B, panels d).

To quantify the patterns of the GFP-GCAP1 co-localization with RetGC1, we measured the ratio between the maximal GFP fluorescence intensity in the whole cell versus its maximal fluorescence intensity inside the nucleus and plotted the data in Fig. 7A. We excluded from the analysis cells that were collapsing, dividing, detaching from the glass, or had exceedingly bright fluorescence of the entire cell, beyond the linear range of the photomultiplier. The distribution ratio varied widely between the cells within each group. That was not surprising, given the expected variability in the levels of GCAP1 and RetGC1 co-expression between different cells. However, after a sufficiently large number of randomly quantified cells, we were able to evaluate the statistical difference between the four GCAP1 mutants and the wild type by one-way analysis of variance (also see supplemental Fig. S3), post hoc processed by Bonferroni test for all pairs comparison. There was a significant difference from the wild type both for the D64N mutant and the D100N/D102G mutant (CI = 99%, p < 0.0001), as well as...
between the two mutants themselves \((p = 0.0003)\). The fluorescence distribution ratio (mean \(\pm S.E\)) for the EF-hand 3 mutant fell nearly 2-fold \((6.6 \pm 0.4, n = 115)\), and for the EF-hand 2 mutant at least 6-fold \((2.2 \pm 0.2, n = 59)\) compared with the wild type \((13.5 \pm 1.6, n = 65)\). The difference between the wild type and E75Q/E111Q/E155Q \((11.9 \pm 1.0, n = 62)\) or the wild type and D144N/D148G \((13.7 \pm 1.1, n = 56)\) mutants was not significant \((Fig. 7B)\). The D64N GCAP1 fluorescence distribution in the presence of RetGC1 \((2.2 \pm 0.2, n = 59)\) was also very close to the wild type expressed in the absence of RetGC1 \((1.01 \pm 0.03, n = 41, Fig. 7, A and B)\), yet the small difference between the two remained statistically significant based on a non-paired \(t\) test \((p < 0.05)\).

The more diffuse pattern of the D64N and D100N/D102G mutants could not result from saturation of the cyclase with the mutant GCAP1. In addition to the comparison of the fluorescence profiles in Fig. 6, such a possibility was also ruled out by the results shown in Fig. 7, \(c\) and \(d\). The ER:nucleus GCAP1-GFP fluorescence ratio rises as the GCAP1-GFP expression levels drops relative to that of RetGC1 \((Fig. 7C)\). To prevent RetGC1 from saturation by GCAP1-GFP, we used in our experiments the GCAP1-GFP:RetGC1 DNA ratio of 1:150 for the wild type and all GCAP1 mutants. The D100N/D102G mutant expression efficacy had to be at least 8–16 times higher than the wild type to match the distribution shown in Fig. 7, \(A\) and \(B\) (the D64N mutant would have to be expressed at even higher levels, still). Yet, neither the D64N nor the D100N/D102G mutants were more abundant relative to the cyclase than the wild type GCAP1-GFP \((Fig. 7D)\). We also estimated the average density of expression of RetGC1 and GCAP1-GFP in cell culture by immunoblot \((Fig. 7D)\). Assuming that the immunoreactivity of the recombinant RetGC1 KHD fragment used for calibration was the same as the native cyclase (purified native human RetGC1 is unavailable), there was about 1.7, 0.7, 1.2, and 1.1 fmol of GCAP1/cm\(^2\) of the wild type, D64N, D100N/D102G, and D144N/D148G mutants, respectively, versus about 37 fmol of RetGC1/cm\(^2\). This analysis does not reflect cell to cell variations.
DISCUSSION

RetGC regulation by GCAPs is essential for recovery of photoreceptors from excitation. When the free Ca\(^{2+}\) concentrations fall in the light, and Ca\(^{2+}\) becomes replaced by Mg\(^{2+}\) in EF-hands of GCAPs, they acquire their activator conformation and accelerate the cyclase (7, 12, 15, 33). The three-dimensional structure of GCAPs in their inhibitory, Ca\(^{2+}\)-loaded, state has been established (34–36), but mechanistic details of the cyclase activation in the light by the Mg\(^{2+}\)-liganded GCAPs remain elusive. It has also been established that both the activator and inhibitor forms of GCAPs interact with the cyclase in vitro and in vivo (24, 30, 37–39), yet the mechanism, through which GCAPs change between their two functional states in response to the Ca\(^{2+}/Mg\(^{2+}\) exchange, remains unclear. Both processes must involve docking of GCAP on the cyclase and a conformational change that likely translates into the cyclase dimerization and activation (40, 41).

The replacement of Mg\(^{2+}\) by Ca\(^{2+}\) in the three metal-binding EF-hands, which occurs in rods and cones in the dark, switches the cyclase off, but does not prevent its interaction with the cyclase (14, 15, 20). Our previous study (15, 20) indicated that binding of Mg\(^{2+}\) in the light or Ca\(^{2+}\) in the dark in two of the three metal ligand-binding EF-hands was likely required to maintain GCAP1 in complex with RetGC1. We also suggested that EF-hand 4 was required for turning RetGC1 off upon binding of Ca\(^{2+}\), rather than for the activation of cyclase in the light. Inactivation of Mg\(^{2+}\) binding in EF-hands 2 and 3 drastically increases GCAP1 \(K_{i}\) for activation of RetGC1, measured in the conditions that mimic light-adapted rods (Ref. 15 and Fig. 2). Although other potential explanations for this phenomenon could not be ruled out at the time of the original observation, we hypothesized that the divalent cation binding in EF-hand 4 and, to a lesser extent, in EF-hand 3, but not in EF-hand 4, was responsible for the docking of GCAP1 on the cyclase (15). Here, we present direct evidence in support of that hypothesis by studying the effects of the mutations in EF-hands on GCAP1 on its association with RetGC1 using live cultured cells.

Co-localization of GCAP1 with RetGC1 in vivo can be reliably assessed in a semi-quantitative fashion using co-transfection of HEK293 cells with fluorescently tagged GCAP1 and RetGC1. When RetGC1 is expressed in excess over GCAP1, this method reveals a clear co-localization pattern of RetGC1-bound GCAP1 (Figs. 3 and 7).

The experiments, described in Figs. 3 and 4, demonstrate that GCAP1 changes its cellular distribution, from soluble to membrane-associated, when it is co-expressed with the cyclase. The robust shift of the GCAP1-GFP fluorescence from the nucleus to the ER is easily detectable by the confocal microscopy in either fixed cells stained with TO-PRO-3 (insets). Fluorescence intensity profiles were recorded along the lines shown in the insets. The images were taken and the fluorescence intensity profiles in panels A and B were measured in the same experiment, at the same laser excitation and photomultiplier gain settings, from the cells in the neighboring chambers of the same coverslip.
free Mg\(^{2+}\) in the cytoplasm (42, 43), which resembles the conditions in photoreceptor cells exposed to light (10–13). The free Mg\(^{2+}\) concentration in HEK293 is therefore sufficient to maintain a cation-loaded state of GCAP1 (14, 15, 20). That allows GCAP1 to bind to RetGC1 in membranes of HEK293 cells when both proteins are co-expressed (Figs. 3–5). Consistent with the high intracellular Mg\(^{2+}\) concentrations in HEK293 cells, inactivation of Ca\(^{2+}\) coordination in all three metal-binding EF-hands does not affect its co-localization with the cyclase, as long as Mg\(^{2+}\) binding is preserved (E75Q/E111Q/E155Q mutant in Figs. 5 and 7).

The role of the individual EF-hands in regulation of RetGC1 activity is shown in Fig. 8). The D64N mutation in EF-hand 2, which blocks not only Ca\(^{2+}\), but also Mg\(^{2+}\) coordination, ham-
pers co-localization of GCAP1 and RetGC1 (Figs. 6 and 7). This result directly supports our hypothesis (15, 20) that EF-hand 2, together with a non-cation binding EF-hand 1, creates the GCAP1 structure that fits into the docking site on the cyclase. The role of the metal ligand binding in EF-hand 2 is to maintain the proper conformation of this part of the GCAP1 molecule, rather than to provide a switch between the activator and inhibitor conformations of GCAP1.

The EF-hand 3 strongly contributes to the maximal level of RetGC1 activation (Fig. 2) and Ca²⁺ sensitivity (15, 16, 20). In addition to that, cation binding in this EF-hand also contributes to the attachment of GCAP1 to the cyclase (Figs. 5 and 7). It contributes less than EF-hand 2, and not necessarily through a direct interaction with the cyclase. For example, ligand binding in EF-hand 3 may affect EF-hand 2, due to their proximity (36).

In contrast to the other EF-hands, metal ligand binding in EF-hand 4 has no major effect on GCAP1 association with the cyclase. Binding of Mg²⁺ in this EF-hand is involved in neither the docking process (Figs. 5 and 7) nor the RetGC1 activation in the light (15, 20). This EF-hand needs metal ligand binding only to function as a negative regulator of RetGC1 activity, and only in response to the binding of Ca²⁺ in the dark (4, 15, 20).

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