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Dear Editor,

Accessibilities of *N*-terminal myristoyl chain and cysteines in guanylyl cyclase-activating protein-2 (GCAP-2) studied by covalent labeling and mass spectrometry

The guanylyl cyclase-activating protein 2 (GCAP-2) has been identified in mammalian photoreceptor cells where it controls retinal rod-outer-segment guanylyl cyclase (ROS-GC) activity in a Ca^{2+}-dependent manner.[1–4] ROS-GCs convert GTP into cGMP – the second messenger involved in photo-transduction. Their GCAP-mediated Ca^{2+}-dependent regulation plays a central role in light adaptation and in modulating the photoreceptor light response.[5] GCAP-2 belongs to the neuronal calcium sensor family and is myristoylated at its *N*-terminus.[6] The three-dimensional structure of GCAP-2 has so far only been determined for non-myristoylated Ca^{2+}-loaded protein.[7] In previous studies, it was concluded that the lack of myristoylation does not significantly affect the ability of GCAP-2 to stimulate ROS-GC[6] and that the myristoyl group controls the Ca^{2+} sensitivity of GCAP-1, but not that of GCAP-2.[8]

In the present work, we investigate the accessibilities of the thiol groups in GCAP-2, both in the Ca^{2+}-bound and Ca^{2+}-free state, with the aim of rapidly monitoring the conformational changes in GCAP-2 that are associated with Ca^{2+}-binding. To investigate the Ca^{2+}-dependent accessibility of the *N*-terminal myristoyl group, a thiol group was introduced at the terminus of the fatty acid side chain. The reactivities of all thiol groups (Cys-35, Cys-111, Cys-131, and thiolauroyl group) were determined by modification with iodoacetamide (IAA) followed by pepsin digestion and electrospray ionization (ESI)-LTQ-Orbitrap tandem mass spectrometry (MS/MS).

Myr-GCAP-2 was co-expressed in *E. coli* with yeast *N*-myristoyltransferase I (NMT1) to selectively introduce the myristoyl group at the *N*-terminus of GCAP-2 and was purified as described previously.[9,10] To create thiolauroylated (thiolaur-)GCAP-2, we added 12-thiolauric acid during coexpression in a similar manner as described for GCAP-2 myristoylation.[11]

Identical ratios were found for thiolauroylated/non-acylated GCAP-2 compared to those obtained for myristoylated (myr-) GCAP-2, with ca. 75–80% of the total GCAP-2 species being thiolauroylated. The thiolaurylation of GCAP-2 was verified by ESI-LTQ-Orbitrap mass spectrometry yielding an average mass of 23810.9, which is in agreement with the calculated molecular weight of thiolauroylated GCAP-2 (Fig. 1). The identity of thiolaur-GCAP-2 was additionally confirmed by peptide fragment fingerprint analysis using a double digestion with trypsin and GluC. The properties of thiolaur-GCAP2 were confirmed to be essentially identical to myr-GCAP-2 in terms of secondary structure (Supplementary Fig. S1, Supporting Information) and folding kinetics (Supplementary Fig. S2, Supporting Information).

Thiol groups of myr- and thiolaur-GCAP-2 were modified with IAA in the presence and absence of Ca^{2+}. The cysteine residues Cys-35, Cys-111, and Cys-131 as well as the *N*-terminal thiolauroyl residue are presented in the 3D structure of GCAP-2 (Fig. 2). IAA (4 mM) was added to myr- and thiolaur-GCAP-2 solutions containing 125 μM $CaCl_2$. After 2 and 9 min, respectively, reaction mixtures were divided into two fractions. $CaCl_2$ was removed by adding 2 mM EGTA to one fraction, while the other fraction was used as a reference sample for monitoring the accessibilities of SH groups in the presence of Ca^{2+}. Performing the experiments in this manner poses the advantage to directly observe Ca^{2+}-dependent structural changes in GCAP-2. Aliquots of 20 μL were taken at different time points and the reactions were quenched by addition of 0.4% (v/v) trifluoroacetic acid (TFA). After IAA modification, GCAP-2 samples were digested with pepsin (Sigma Aldrich) for 30 min at 37 °C. Peptide mixtures were analyzed by nano-HPLC/nano-ESI-MS/MS on an UltiMate nano-HPLC system (Dionex/LC Packings) coupled to an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific) equipped with a nano-ESI source (Proxeon). Selected ion chromatograms of the identified cysteine-containing peptides were extracted from raw data by considering the exact precursor ion mass (±1 ppm) and retention time (Xcalibur 2.0.7, Thermo Fisher Scientific). At least four different cysteine-containing GCAP-2 peptides were detected throughout all measurements. The chromatographic peak areas were integrated and the rate of cysteine alkylation was determined by comparing peak areas for unmodified and alkylated peptides. Semi-quantitative analysis of Ca^{2+}-dependent IAA-modified peptides is presented for each SH group in Fig. 3. The experiments were repeated three or four times, depending on the peptide.

All thiol-containing peptides showed an increasing fraction of IAA-modified peptides with increasing reaction times. Investigation of myr-GCAP-2 resulted in identical cysteine modifications as for thiolaur-GCAP-2 (Supplementary Fig. S3, Supporting Information). As MS is not a quantitative method, a direct comparison of kinetics between the individual peptides with different ionization properties is not feasible. Therefore, different pseudo-rates of modifications are presented for each GCAP-2 peptide – even if they contain the same cysteines, namely Cys-35, Cys-111 or Cys-131. Upon Ca^{2+} dissociation, all peptides containing Cys-35 and Cys-111 showed an increased fraction of IAA modification compared to the Ca^{2+}-loaded form. In contrast, we did not observe Ca^{2+}-dependent changed IAA modifications, neither for Cys-131-containing peptides, nor for peptides with the *N*-terminal thiolauroyl group (Fig. 3). Equal pseudo-modification rates in the presence and absence of Ca^{2+} clearly indicate that Ca^{2+}-induced conformational changes do not alter the accessibility of the *N*-terminal fatty acid side chain in GCAP-2.

Our data are in good agreement with observations of Hughes *et al*. who proposed a permanently solvent-accessible myristoyl group based on nuclear magnetic resonance (NMR)

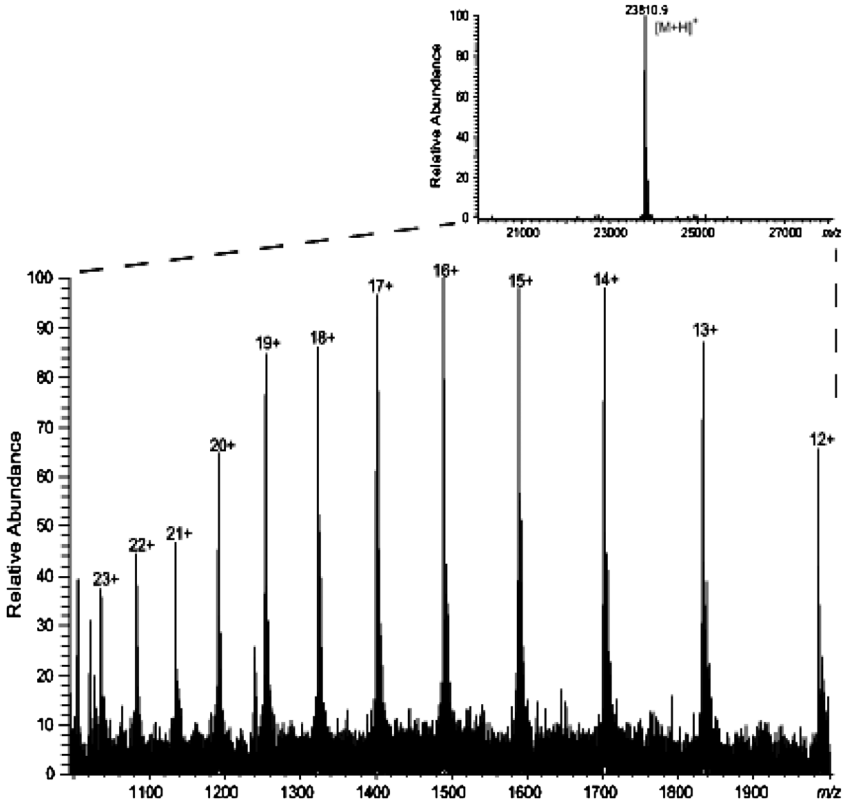

Figure 1. ESI-LTQ-Orbitrap mass spectrum of purified thiolaur-GCAP-2; inset: deconvoluted mass spectrum of thiolaur-GCAP-2, calculated average molecular weight: 23810.78 u.

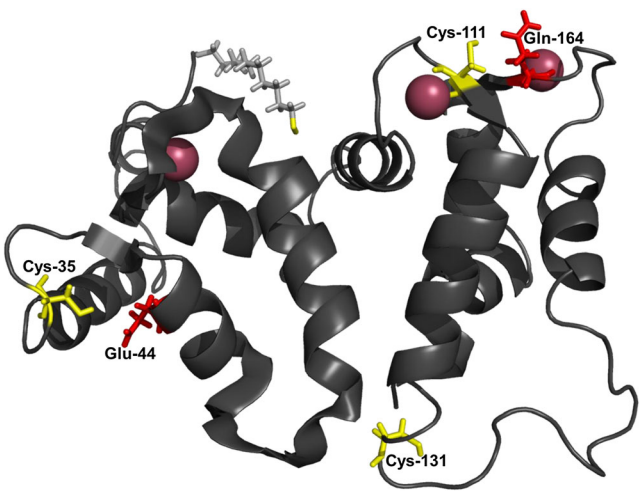

Figure 2. Structure of GCAP-2 (pdb entry 1JBA) with cysteines at positions 35, 111, and 131 and the *N*-terminal thiolauroyl group shown as yellow sticks. Potential H-bond donors (Glu-44 and Gln-164) are shown as red sticks; Ca^{2+} ions are shown as magenta spheres. The thiolauroyl group was manually added to the *N*-terminus of GCAP-2; visualization was performed with PyMol.

studies.[12] We recently conducted 3D structural studies of *N*-terminally myristoylated GCAP-2 by chemical cross-linking and mass spectrometry, showing a higher flexibility of the protein in the Ca^{2+}-free state, but we could not draw conclusions on the orientation of the myristic acid chain of GCAP in those studies.[11,13]

Previous biochemical studies with Ellman's reagent (5,5′-dithio-bis(2-nitrobenzoic acid)) had revealed that only two cysteines in GCAP-2, Cys-35 and Cys-111, change their SH reactivities in the presence of Ca^{2+}, while the reactivity of Cys-131 was unaffected by Ca^{2+}.[14] This is confirmed by our labeling experiments. Strikingly, both Ca^{2+}-sensitive cysteines (Cys-35 and Cys-111) are localized in the EF-hands of GCAP-2 (Fig. 2): It can be speculated that the SH group of Cys-35 forms an H-bond to Glu-44 in the Ca^{2+}-bound state. Cys-111 is part of EF-hand 3 in GCAP-2, with its thiol group forming an H-bond to Gln-164 (Fig. 2).[7] The H-bond formed between Cys-111 and Gln-164 might contribute to mediating the cooperativity in Ca^{2+} binding between the tandem arrays. We assume that upon Ca^{2+} release, the H-bond between both residues is weakened leading to an accelerated modification rate. Cys-131 is apparently solvent-exposed, both in the presence and in the absence of Ca^{2+}. It is localized in a flexible loop connecting EF-hand 3 and 4 and, apparently, no H-bond is formed to another amino acid. Thus, it is not surprising that the modification rate of Cys-131 is not changed upon Ca^{2+} release.

In conclusion, we show that we were able to gain information on the accessibilities of cysteines and a modified fatty acid side chain in a protein within a very short time. The same information is otherwise only amenable by conducting time- and material-consuming NMR or mutagenesis studies.

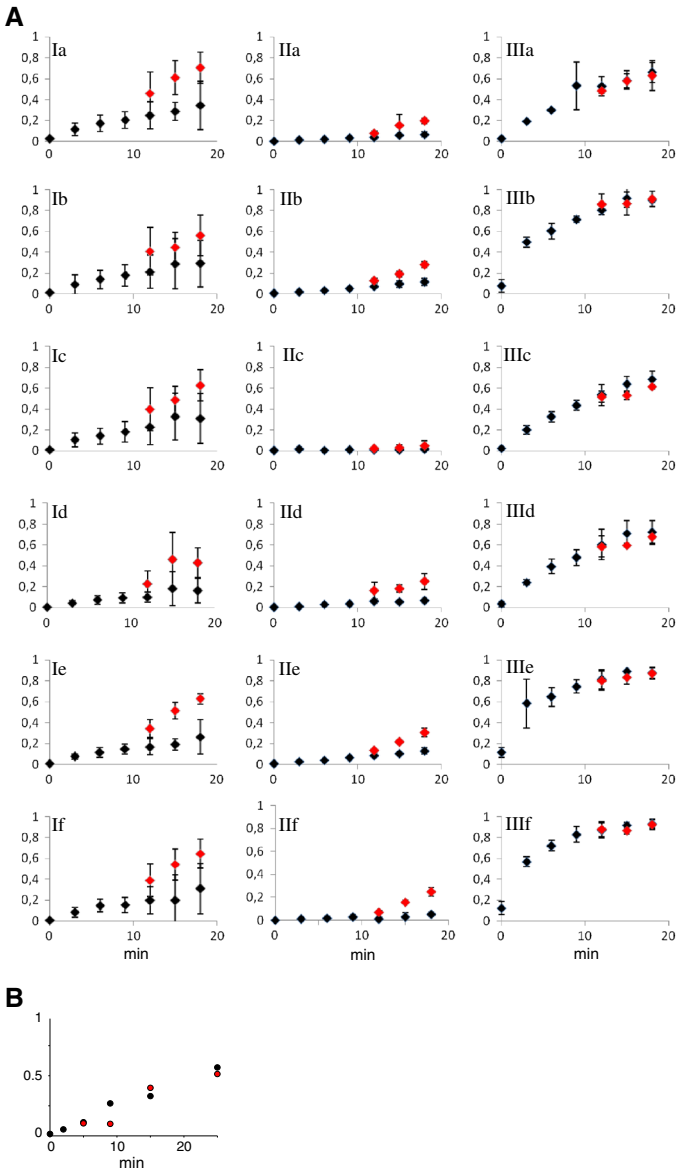

Figure 3. IAA modification of different SH-containing peptides at several time points after IAA addition in the presence of Ca^{2+} (black). After 9 min (A) or 2 min (B), the sample was split and Ca^{2+} was removed by adding an excess of EGTA (red). (A) ***(I) Cys-35*** (Ia: WYKKFLEECPSGTLF (doubly charged, *m/z* 924.4555/952.9662), Ib: YKKFLEECPSGTLF (doubly charged, *m/z* 831.4158/859.9165), Ic: YKKFLEECPSGTLF (triply charged, *m/z* 554.6130/573.6201), Id: WYKKFLEECPSGTL (doubly charged, *m/z* 850.9213/879.4320), Ie: LEECPSGTLF (singly charged, *m/z* 1095.5027/1152.5242), and If: LEECPSGTLF (doubly charged, *m/z* 548.2550/576.7657)); ***(II) Cys-111*** (IIa: KIYDKDRNGCIDRQEL (doubly charged, *m/z* 983.4942/1012.0049), IIb: KIYDKDRNGCIDRQEL (triply charged, *m/z* 655.9985/675.0057), IIc: KIYDKDRNGCID (doubly charged, *m/z* 720.3510/748.8617), IId: KIYDKDRNGCIDRQELLD (doubly charged, *m/z* 1097.5497/1126.0604), IIe: KIYDKDRNGCI-DRQELLD (triply charged, *m/z* 732.0355 / 751.0427), and IIf: KIYDKD-RNGCIDRQELL (doubly charged, *m/z* 1040.0362/1068.5469)); ***(III) Cys-131*** (IIIa: KLKKACSVE (doubly charged, *m/z* 503.2917/531.8024), IIIb: KLKKACSVEVE (doubly charged, *m/z* 617.3472/645.8579), IIIc: SIYKLKKACSVE (doubly charged, *m/z* 684.8814/713.3921), IIId: SIYKLKKACSVE (triply charged, *m/z* 456.9234/475.9305), IIIe: SIYKLKKACSVEVE (doubly charged, *m/z* 798.9369/827.4476), and IIIf: SIYKLKKACSVEVE (triply charged, *m/z* 532.9604/551.9675)). (B) ***12-thiolauric acid*** ((●) mercaptododecanyl-GQQ (singly charged, *m/z* 531.3085/588.3300)).

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