The function of conserved cysteine residues in the extracellular domain of human receptor-activity-modifying protein 1

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Abstract The receptor-activity-modifying protein (RAMP) 1 is a single-transmembrane-domain protein associated with the calcitonin-like receptor (CLR) to reveal a calcitonin gene-related peptide (CGRP) receptor. The extracellular region of RAMP1 contains six conserved cysteines. Here, Cys^{27} in myc-tagged human (h) RAMP1 was deleted (hRAMP1Δ1), and Cys^{40}, Cys^{57}, Cys^{72}, Cys^{82} and Cys^{104} were each replaced by Ala. In COS-7 cells expressing hCLR/myc-hRAMP1Δ1 or -C82A, cell surface expression, [^{125}I]hαCGRP binding and cAMP formation in response to hαCGRP were similar to those of hCLR/myc-hRAMP1. Cell surface expression of myc-hRAMP1-C72A was reduced to 24 ± 7% of myc-hRAMP1, and that of -C40A, -C57A and -C104A was below 10%. [^{125}I]hαCGRP binding of hCLR/myc-hRAMP1-C72A was 13 ± 3% of hCLR/myc-hRAMP1 and it was undetectable in hCLR/myc-hRAMP1-C40A-, -C57A- and -C104A-expressing cells. Maximal cAMP stimulation by hαCGRP in hCLR/myc-hRAMP1-C40A- and -C72A-expressing cells was 14 ± 1% and 33 ± 2% of that of the hCLR/myc-hRAMP1 with comparable EC_{50}. But cAMP stimulation was abolished in cells expressing hCLR/myc-hRAMP1-C57A and -C104A. In conclusion, CGRP receptor function was not affected by the deletion of Cys^{27} or the substitution of Cys^{82} by Ala in hRAMP1, but it was impaired by the substitution of Cys^{40}, Cys^{57}, Cys^{72} and Cys^{104} by Ala. These four cysteines are required for the transport of hRAMP1 together with the CLR to the cell surface.

Key words: Calcitonin gene-related peptide; Cysteine; Disulfide bridge; Receptor-activity-modifying protein

1. Introduction

Receptor-activity-modifying proteins (RAMP) 1, -2 and -3 of man, rat, mouse and pig are single-transmembrane-domain proteins required for the functional expression of the calcitonin-like receptors (CLR) [1–4]. CLR/RAMP1 and -2 heterodimers are receptors for calcitonin gene-related peptide (CGRP) and adrenomedullin (AM), respectively, and the CLR/RAMP3 complex is an AM/CGRP receptor [5]. RAMP1, -2 and -3 exhibit 30% amino acid sequence similarity. The extracellular domains of between 90 and 100 amino acids define in part CGRP and AM selectivity of the CLR/RAMP heterodimers [6].

RAMP2 and -3, unlike RAMP1, are *N*-glycosylated. RAMP1, therefore different from RAMP2 and -3, requires the CLR for the transport to the cell surface. Intracellular retention of human (h) RAMP1 in the absence of the CLR was overcome when the nine-amino acid intracellular tail was shortened by eight amino acids, including a putative SKRT retention signal conserved in RAMP1 [7]. Moreover, the transport of mouse RAMP1 to the cell surface was revealed in the absence of the CLR when *N*-glycosylation sites were introduced into its extracellular domain [8].

RAMP1 contains six cysteines in the N-terminal extracellular domain in positions 27, 40, 57, 72, 82 and 104. They are conserved in RAMP3. RAMP2 has only four cysteine residues in the extracellular domain corresponding to Cys^{40}, Cys^{57}, Cys^{72} and Cys^{104} of RAMP1. In hRAMP2 all four cysteines are required for its expression at the cell surface [9]. In human embryonic kidney (HEK) cells expressing hRAMP1 alone, hRAMP1 dimers sensitive to dithiothreitol treatment were recognised [10]. This suggested that intermolecular disulphide bridges between cysteine residues were formed in the absence of the CLR. Interestingly, the RAMP1 dimer/monomer ratio decreased in the presence of the CLR. However, intermolecular disulphide bridges in CLR/RAMP1 heterodimers have so far not been identified [11]. Substitution of the sequence Cys^{27}–Ala^{50}, including the second cysteine in position 40 of hRAMP1, by the corresponding Asn^{36}–Pro^{78} sequence of hRAMP2 abolished cell surface expression and function of the chimeric hRAMP2/1 coexpressed with the CLR [12]. The N-terminus of hRAMP1 is therefore required for its delivery to the cell surface together with the CLR.

In the present study, the functional role of the extreme N-terminus of hRAMP1 and of the six cysteine residues was investigated. Deletions of Cys^{27}, Cys^{27}–Asn^{31}, Cys^{27}–Arg^{37} and Cys^{27}–Asp^{47}, including Cys^{40}, removed one, five, 11 and 21 N-terminal amino acids adjacent to the presumed signal sequence in hRAMP1. Moreover, except for Cys^{27} the cysteines were individually replaced by alanines. Cys^{40}, Cys^{57}, Cys^{72} and Cys^{104} are required for the cotransport with the CLR to the cell surface, as well as for [^{125}I]hαCGRP binding and CGRP-evoked stimulation of cAMP formation in CLR/hRAMP1-expressing cells.

2. Materials and methods

2.1. Materials

Human αCGRP(1–37) was purchased from Bachem (Bubendorf,

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Abbreviations: CGRP, calcitonin gene-related peptide; CLR, calcitonin-like receptor; RAMP, receptor-activity-modifying protein

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Switzerland). Restriction enzymes were obtained from Promega (Madison, WI, USA). ^{125}I, ECL Western blot detection reagents and Hybond ECL nitrocellulose membranes were from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, UK). Cell culture products were supplied by Invitrogen (Carlsbad, CA, USA) and linear polyethyleneimine (PEI) (25 kDa) by Polysciences (Warrington, PA, USA). Mouse antibodies to myc and to V5 epitopes were from Invitrogen and Cy3-labelled sheep anti-mouse antibodies from Sigma (St. Louis, MO, USA). Other chemicals and reagents were purchased from Sigma and Merck at the highest grade available.

2.2. DNA constructs

The cDNA encoding myc-hRAMP1, cloned into pcDNA3 (Invitrogen), was provided by S. Foord (GlaxoSmithKline, Stevenage, Hertfordshire, UK). Myc-hRAMP1 had the amino acids 1–26 of the predicted signal sequence of hRAMP1 replaced by the CD33 signal sequence [13] and fused to the myc epitope tag [Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu-Leu] for cell surface detection. Gly-Ser were encoded by a *Bam*HI restriction site that was used for construction. Thus, the hRAMP1 amino acid sequence in myc-hRAMP1 starts at Cys^{27} of wild-type hRAMP1. Along these lines, the neural network and the hidden Markow model, using the SignalP V2.0 software (www.cbs.dtu.dk/services/SignalP-2.0), predicted signal sequence cleavage in wild-type hRAMP1 between Cys^{27} and Glu^{28}. For further truncation of the N-terminus of hRAMP1, deletions of Cys^{27} (myc-hRAMP1Δ1) and of Cys^{27}–Asn^{31} (myc-hRAMP1Δ5), Cys^{27}–Arg^{37} (myc-hRAMP1Δ11) and Cys^{27}–Asp^{47} (myc-hRAMP1Δ21) were introduced into myc-hRAMP1 with a polymerase chain reaction (PCR)-based approach. Briefly, 5′-primers with a *Bam*HI restriction site at the 5′-end were designed to anneal at the desired positions in the hRAMP1 coding sequence. The 3′-primer with a *Xho*I restriction site at the 5′-end was complementary to the DNA sequence encoding the six amino acids of the C-terminus of hRAMP1 and the stop codon. The PCR products amplified with cloned *Pfu* DNA polymerase (Promega) in 25 cycles were digested with *Bam*HI and *Xho*I restriction enzymes, gel-purified and cloned into *Bam*HI/*Xho*I-digested pcDNA3 in frame with the coding sequence for the CD33 signal peptide (CD33) and the myc epitope tag encoded upstream of the *Bam*HI restriction site. All the DNA fragments encoding CD33-myc fused to mutated hRAMP1 were sequenced in both directions.

Mutagenesis with the QuikChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA), replacing cysteines 40, 57, 72, 82 and 104 in the hRAMP1 extracellular domain by alanine, revealed myc-hRAMP1-C40A, -C57A, -C72A, -C82A and -C104A. The mutations were introduced into the CD33-myc-hRAMP1 coding sequence subcloned into *Eco*RV/*Xho*I-digested pBluescript SK vector (Stratagene). PCR amplification with pairs of primers containing the nucleotide substitutions was carried out with cloned *Pfu* DNA polymerase (Promega). The products obtained after 30 cycles were gel-purified, digested with *Dpn*I restriction enzyme and ligated. The CD33-myc-hRAMP1 coding sequences with the introduced mutations were verified by sequencing in both directions, and subsequently excised from the SK vector with *Eco*RV and *Xho*I restriction enzymes and cloned into *Eco*RV/*Xho*I-digested pcDNA3.

The DNA encoding the hCLR with an N-terminal V5 epitope tag [Gly-Lys-Pro-Ile-Pro-Asn-Pro-Leu-Leu-Gly-Leu-Asp-Ser-Thr] (V5-hCLR) was constructed as described [7].

2.3. Cell culture and transfection

COS-7 cells were grown at 37°C in a humidified atmosphere of 95% air and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (4.5 g/l glucose) and Ham's F12 (1:1) medium containing 2 mM glutamine and 10% foetal calf serum. Simian virus 40 T-antigen-transformed human embryonic kidney (TSA) cells were grown under the same conditions in cell culture medium supplemented with 400 μg/ml geneticin. The cells were grown to 40–60% confluence and transfected with indicated concentrations of myc-hRAMP1 and V5-hCLR expression constructs and linear PEI [14]. Briefly, 0.2 μg/cm^2 plasmid DNA was diluted with 6.25 μl/cm^2 cell culture medium. This solution was then combined with 0.5 μg/cm^2 PEI in 6.25 μl/cm^2 cell culture medium, and vortexed immediately. After incubation for 15 min at room temperature the volume of the DNA/PEI mixture was adjusted to 125 μl/cm^2 with cell culture medium and added to the cells. The cells were incubated at 37°C for 16–24 h. The volume was then increased to 250 μl/cm^2 with cell culture medium, and the cells were incubated for another 48 h.

2.4. [^{125}I]hαCGRP binding and cAMP stimulation

[^{125}I]hαCGRP was prepared by a modified chloramine T method and subsequent purification by high performance liquid chromatography [15,16]. Binding was carried out in 24-well plates at 15°C for 2 h. The cells were incubated with 1700 Bq/well (60 pM) [^{125}I]hαCGRP in 200 μl/well DMEM/Ham's F12 supplemented with 0.1% bovine serum albumin (BSA) and 2 mM glutamine (binding medium) in the absence and presence of 1 μM non-labelled hαCGRP. The cells were washed once with 500 μl binding medium, lysed with 500 μl 0.5% sodium dodecyl sulfate (SDS), and the lysates were counted in a γ-counter (Kontron, Switzerland). Binding in the presence of 1 μM hαCGRP, always lower than 20%, was considered non-specific and was subtracted from total binding for the calculation of specific binding.

Cyclic AMP stimulation was performed in binding medium supplemented with 1 mM isobutylmethylxanthine (IBMX) for 15 min at 37°C as described [15].

2.5. Immunocytochemistry

COS-7 cells were seeded on cover slides in 24-well plates and 48 h later transfected with the V5-hCLR and the indicated wild-type and mutant myc-hRAMP1 expression constructs. Cell surface and total expression of wild-type and mutant myc-hRAMP1 was estimated 48 h after transfection by myc-immunofluorescent staining of intact and saponin-permeabilised cells, respectively. Briefly, the cells were fixed with 4% formalin in phosphate-buffered saline (PBS) for 20 min at room temperature, washed with PBS and incubated with immunostaining medium (DMEM/Ham's F12 (1:1), 0.1% BSA) in the absence (surface staining) or presence (total staining) of 0.1% saponin for 30 min at room temperature. The cells were then incubated for 2 h at room temperature with mouse antiserum to myc (diluted 1:300) in 200 μl immunostaining medium in the absence or presence of saponin. The cells were washed three times with the immunostaining medium and then incubated with Cy3-labelled sheep anti-mouse antiserum (1:200 final dilution) (Sigma) for 30 min. After three additional washes the slides were mounted with Immu-Mount (Shandon Scientific, Pittsburgh, PA, USA). The cells were viewed with an Eclipse E600 Nikon microscope (Küsnacht, Switzerland) equipped with a Plan Fluor 20×/0.5 DLL objective, a G-2A filter and a Kappa DX20 CCD camera (Gleichen, Germany) connected to the microscope with a Nikon 0.45× projection lens. Immunofluorescence of intact and saponin-permeabilised cells was recorded with a Nikon U-III multipoint sensor system and respective reciprocal exposure times were taken as a measure for cell surface and total expression of wild-type and mutant myc-hRAMP1.

2.6. Western blot analysis

TSA cells grown in 100-mm tissue culture dishes were transiently transfected with indicated combinations of V5-hCLR and wild-type and mutant myc-hRAMP1 expression constructs. Forty-eight hours after transfection the cells were detached with 0.05% EDTA in PBS and washed with PBS. 2×10^7 cells were lysed in 200 μl 50 mM HEPES (pH 7.5), 140 mM NaCl, 0.5% Triton X-100, 1 mM phenylmethylsulphonyl fluoride, 3 μg/ml aprotinin, 3 μg/ml leupeptin. The cell lysates were cleared by centrifugation for 5 min at 20 000×*g* and 70 μl 4×SDS–polyacrylamide gel electrophoresis (PAGE) loading buffer was added. Proteins in cell lysates were separated by SDS–PAGE (15% gel) and electrotransferred to nitrocellulose Hybond® ECL® membranes (Amersham, Piscataway, NJ, USA) in a Trans-Blot cell (Bio-Rad, Hercules, CA, USA) overnight at 10 V and 4°C. Immunoblots were blocked with 5% low-fat milk and the epitope-tagged proteins were visualised by enhanced chemiluminescence with horseradish peroxidase (HRP)-labelled monoclonal myc (1:2000) and V5 antibodies (1:5000) (Invitrogen) using the VersaDoc® Imaging System (Bio-Rad). Actin as a reference protein for the amount of loaded cell lysates was visualised with monoclonal antibodies to actin (1:3500) (Chemicon, Temecula, CA, USA) and secondary HRP-conjugated sheep antibodies to mouse immunoglobulins (1:5000) (Amersham).

2.7. Data analysis

In binding inhibition and cAMP stimulation experiments half-maximal inhibition concentrations (IC_{50}) and effective concentrations (EC_{50}) of hαCGRP were calculated by non-linear regression analysis using the FigP 6.0 software (Biosoft, Cambridge, UK). Results are means ± S.E.M. Comparison to controls was carried out using the

Student's *t*-test. *P* values of <0.05 were considered statistically significant.

3. Results

3.1. [^{125}I]hαCGRP binding and cAMP formation of the hCLR coexpressed with hRAMP1 deletion or cysteinelalanine substitution mutants

The deletions introduced into the extreme N-terminus of myc-hRAMP1 removed Cys^{27} (myc-hRAMP1Δ1) or the sequences Cys^{27}–Asn^{31} (myc-hRAMP1Δ5), Cys^{27}–Arg^{37} (myc-hRAMP1Δ11) or Cys^{27}–Asp^{47} (myc-hRAMP1Δ21) including the second cysteine in position 40. Substitution of individual cysteine residues in the extracellular domain of hRAMP1 revealed myc-hRAMP1-C40A, -C58A, -C72A and -C104A. Myc-hRAMP1 and the mutants were transiently coexpressed with the hCLR in COS-7 cells. Specific [^{125}I]hαCGRP binding in cells expressing the hCLR with intact myc-hRAMP1 was 10.4 ± 0.7% of added radioligand, set to 100% and defined as maximal binding (Fig. 1). [^{125}I]hαCGRP binding was indistinguishable from maximal binding in cells expressing the hCLR together with myc-hRAMP1Δ1 or -Δ5, but it was reduced to 15 ± 1% ($n=4$; $P<0.001$) of maximal [^{125}I]hαCGRP binding with myc-hRAMP1Δ11 and indistinguishable from non-specific [^{125}I]hαCGRP binding with myc-hRAMP1Δ21 also lacking Cys^{40}. Specific [^{125}I]hαCGRP binding was similarly suppressed with individual Cys^{40}, Cys^{57} or Cys^{104} to Ala substitutions in the extracellular domain of myc-hRAMP1. Substitution of Cys^{72} or Cys^{82} to Ala decreased [^{125}I]hαCGRP binding to 13 ± 3% ($n=5$, $P<0.001$) and 64 ± 6% ($n=6$, $P<0.005$) of maximal binding. [^{125}I]hαCGRP binding in hCLR/myc-hRAMP1-, -Δ1-, -Δ5- and -C82A-expressing cells was inhibited by hαCGRP at indistinguishable IC$_{50}$ (Table 1).

In COS-7 cells coexpressing the hCLR with intact myc-hRAMP1 hαCGRP stimulated cAMP formation with an EC$_{50}$ of 0.19 ± 0.04 nM and the 49 ± 7-fold increase over basal levels with 1 μM hαCGRP was considered maximal and set to 100% (Fig. 2, Table 1). The cAMP response was the same in cells coexpressing the hCLR with myc-hRAMP1-C82A and with myc-hRAMP1Δ1 and -Δ5. Interestingly, further truncation by 11 amino acids in myc-hRAMP1Δ11 maintained the maximal stimulation of cAMP by hαCGRP, but the EC$_{50}$ was increased nine-fold. Decreased affinity for hαCGRP explains the observed reduced [^{125}I]hαCGRP binding in hCLR/myc-hRAMP1Δ11-expressing cells. Conversely, substitution of Cys^{40} and Cys^{72} to Ala minimally affected the EC$_{50}$ of hαCGRP but the maximal cAMP response in hCLR/myc-hRAMP1-C40A- or -C72A-expressing cells decreased to 14 ± 1% and 33 ± 2%, respectively. Moreover, the stimulation of cAMP formation by hαCGRP was abolished in cells expressing the hCLR with the myc-hRAMP1 Cys^{27}–Asp^{47} deletion or the Cys^{57} or Cys^{104} to Ala substitution mutants. Taken together, the deletion of up to five amino acids in the extreme N-terminus including Cys^{27} or the substitution of Cys^{82} in the extracellular domain of hRAMP1 did not affect the CGRP receptor function of the hCLR. More extended deletions in the N-terminal region of up to 21 amino acids, including Cys^{40}, or the substitution of Cys^{40}, Cys^{57}, Cys^{72} and Cys^{104} in the extracellular domain of hRAMP1 impaired or abolished the CGRP receptor function of the coexpressed hCLR.

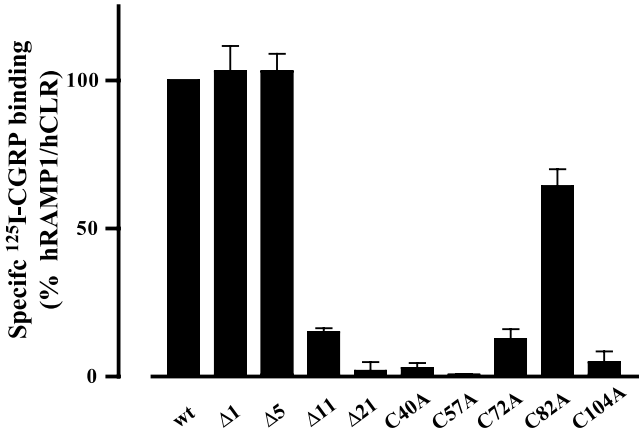

Fig. 1. [^{125}I]hαCGRP binding to intact and mutant myc-hRAMP1/hCLR in COS-7 cells. COS-7 cells were co-transfected with hCLR and indicated myc-hRAMP1. The cells were incubated for 2 h at 15°C with 60 pM [^{125}I]hαCGRP (1.5×10^{14} Bq/mmol) in the absence (total binding) or presence (non-specific binding) of 1 μM hαCGRP. Specific [^{125}I]hαCGRP binding (total minus non-specific binding) in cells cotransfected with the hCLR and myc-hRAMP1 (wt) was 1.3 ± 0.1 fmol/200 000 cells ($n=13$) and was set to 100%. The results are means ± S.E.M. of at least four independent experiments.

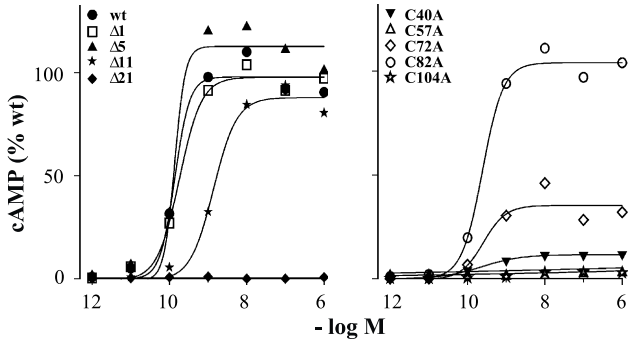

Fig. 2. Stimulation of cAMP formation by hαCGRP. COS-7 cells were transfected with constructs for expression of the hCLR together with myc-hRAMP1 (wt) or the indicated deletion (left) or Cys to Ala substitution (right) mutants. The cells were incubated for 15 min at 37°C with hαCGRP in the presence of IBMX. In cells expressing hCLR/myc-hRAMP1 10^{-6} M hαCGRP stimulated cAMP formation 49 ± 7-fold ($n=9$) and was set to 100%. Basal cAMP levels ranged from 1 to 2 pmol/200 000 cells. The results are means of at least three independent experiments.

Table 1
[^{125}I]hαCGRP binding inhibition and stimulation of cAMP formation by hαCGRP in COS-7 cells expressing the hCLR together with intact myc-hRAMP1 and the indicated mutants

	IC$_{50}$ (nM)	EC$_{50}$ (nM)
myc-hRAMP1	19 ± 4 (6)	0.19 ± 0.04 (8)
-Δ1	16 ± 3 (4)	0.26 ± 0.07 (5)
-Δ5	16 ± 4 (3)	0.20 ± 0.06 (4)
-Δ11	n.d.	1.53 ± 0.24 (4)
-Δ21	n.d.	n.s. (3)
-C40A	n.d.	0.77 ± 0.12 (3)
-C57A	n.d.	n.s. (3)
-C72A	n.d.	0.27 ± 0.03 (3)
-C82A	18 ± 1 (3)	0.27 ± 0.03 (3)
-C104A	n.d.	n.s. (3)

Results are means ± S.E.M. with numbers of experiments in parentheses; n.d., not determinable at up to 1 μM hαCGRP; n.s., no cAMP stimulation at up to 1 μM hαCGRP.

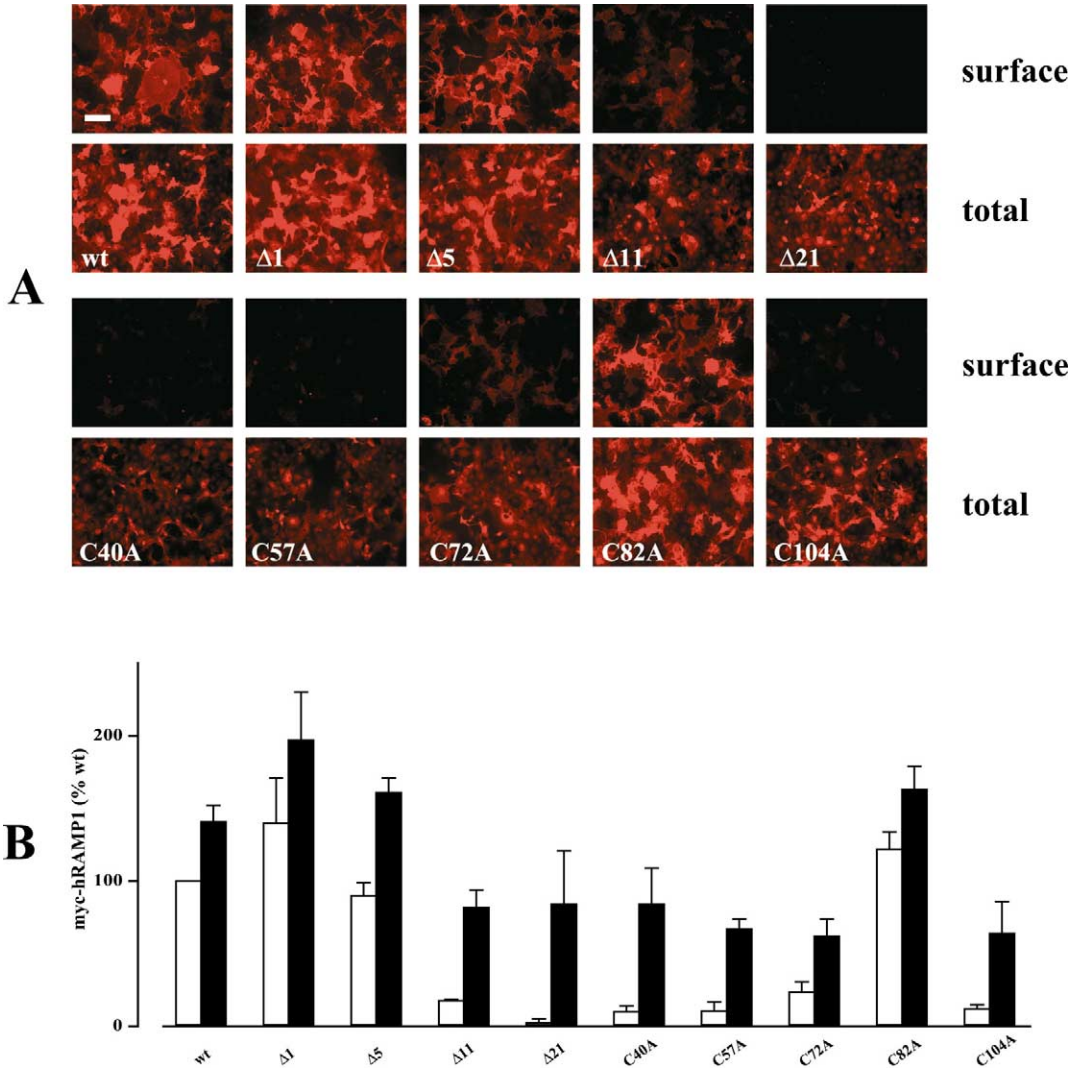

Fig. 3. Cell surface and total expression of intact and mutant myc-hRAMP1 in COS-7 cells revealed by myc immunofluorescent staining. Cells cotransfected with the hCLR and the indicated myc-hRAMP1 expression constructs were fixed with 4% formalin. Intact (surface) or saponin-permeabilised (total) cells were stained with mouse anti-myc serum and Cy3-labelled antibodies to mouse IgG. Cy3 fluorescence was recorded at a constant exposure time of 550 ms. A: Scale bar 100 μm. Representative experiment carried out three times. B: Cell surface (open bars) and total (closed bars) Cy3 immunofluorescence in A was quantified as described in Section 2. Inverse exposure times in control pcDNA3-transfected cells, reflecting background fluorescence, ranged from 0.1 to 0.2 min^{-1} in non-permeabilised and from 0.2 to 0.4 min^{-1} in permeabilised cells and were subtracted from individual measurements. The values obtained for wild-type (wt) myc-hRAMP1 in non-permeabilised cells ranged from 1.9 to 3.5 min^{-1} and were set to 100%. The results are means ± S.E.M. of three independent experiments.

3.2. *Total and cell surface expression of hRAMP1 deletion and cysteine/alanine substitution mutants*

Impaired expression and cell surface delivery of mutant myc-hRAMP1 were considered as mechanisms for defective receptor function of the coexpressed hCLR. Expression of myc-hRAMP1 and the mutants was estimated by myc immunofluorescence staining of saponin-permeabilised COS-7 cells coexpressing the V5-hCLR (Fig. 3) and by myc immunochemiluminescence on Western blots of extracts of corresponding cells (Fig. 4). Myc immunofluorescence of permeabilised cells and Western blot analysis of total cell extracts revealed comparable expression levels of myc-hRAMP1 and of all the mutants. The apparently faster migration on SDS–PAGE of myc-hRAMP1Δ5 as compared to myc-hRAMP1Δ11 cannot be explained. Extensive DNA sequencing of the corresponding constructs confirmed their predicted amino acid sequence.

The expression of myc-hRAMP1 and of its mutants at the cell surface was visualised and quantified by myc immunofluorescence staining of intact cells (Fig. 3). Indistinguishable cell surface expression was observed for myc-hRAMP1 and the -Δ1, -Δ5 and -C82A mutants, but the levels of myc-hRAMP1Δ11 and -C72A were only 18 ± 1% and 24 ± 7% of myc-hRAMP1 and those of myc-hRAMP1Δ21, -C40A, -C57A and -C104A were below 10%. Thus individual substitution by alanine of the second, third, fourth and the sixth cysteine in the extracellular domain of hRAMP1 impaired or abolished its transport to the cell surface in the presence of the hCLR. Deletions of 11 or 21 amino acids at the N-terminus of hRAMP1 had similar effects.

3.3. *Suppression of hCLR glycosylation by hRAMP1 mutations*

RAMP1-dependent posttranslational modification of core to mature *N*-glycosylated hCLR in mammalian cells has

been well documented. The corresponding increase in size of the V5-hCLR from 50–60 kDa in the absence of myc-hRAMP1 to 73 kDa in its presence was confirmed in the present study (Fig. 4). Maturation of the V5-hCLR was not affected by the myc-hRAMP1Δ1, -Δ5, -Δ11 and -C82A mutations, but mature *N*-glycosylation of the V5-hCLR was suppressed by the myc-hRAMP1Δ21, -C40A, -C57A, -C72A and -C104A mutations. Interestingly, mature *N*-glycosylation of the V5-hCLR required the expression of the myc-hRAMP1 mutants at the cell surface. Even diminished cell surface expression levels of myc-hRAMP1Δ11 and C72A were sufficient for at least partial mature *N*-glycosylation of the coexpressed V5-mCLR. Thus, mature *N*-glycosylation of the hCLR in mammalian cells appears to require its cotransport with hRAMP1 to the cell periphery.

4. Discussion

The extracellular domains of RAMP1 and -3 consist of approximately 90 amino acids. They have in common six conserved cysteine residues. RAMP2 lacks the first and the fifth cysteine of RAMP1 and -3 and the remaining four conserved cysteines are required for delivery to the cell surface and for AM receptor function of hCLR/hRAMP2 heterodimers [9]. Cell surface and functional expression of mouse RAMP3 in the presence of mouse CLR, on the other hand, was not greatly affected by individual substitutions of cysteines by serines when expressed in *Xenopus* oocytes [17]. Substitution of the N-terminal 24 amino acids of hRAMP1 by the corresponding amino acids of hRAMP2 abolished CGRP-stimulated cAMP formation [12]. This suggests that the extreme N-terminus of hRAMP1, which includes the first and the second cysteines, is required for CGRP receptor function of the hCLR/hRAMP1 complex.

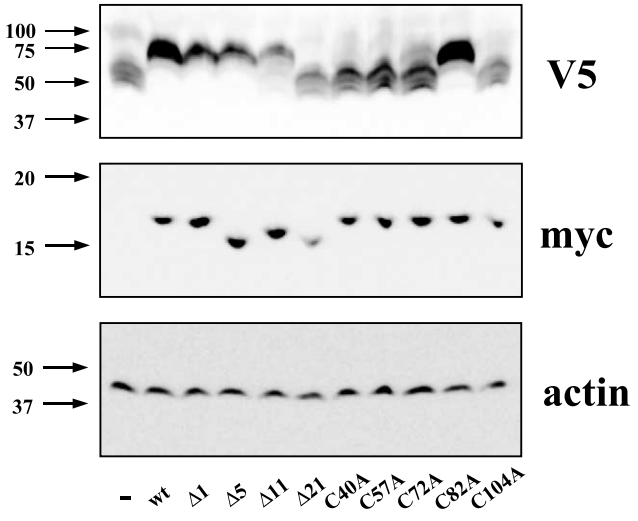

Fig. 4. Expression levels of V5-hCLR and myc-hRAMP1 and its mutants. Lysates of TSA cells transfected with V5-hCLR alone or cotransfected with V5-hCLR and myc-hRAMP1 or mutant expression constructs were separated by SDS–PAGE (15%) and the proteins were electroblotted to nitrocellulose. The V5-CLR and myc-hRAMP1 (wt) and the indicated mutants were visualised with HRP-labelled antibodies to V5 or myc. Actin as a reference for protein loading was estimated with mouse antibodies to actin and secondary HRP-conjugated antibodies to mouse IgG. Arrows indicate the position of protein size markers. Representative experiment carried out three times.

Here, the extreme N-terminus of myc-hRAMP1 including the first and the second cysteines, Cys^{27} and Cys^{40}, was progressively truncated. Moreover, Cys^{40}, Cys^{57}, Cys^{72}, Cys^{82} and Cys^{104} were each replaced by alanine. The effects of the mutations on the expression of myc-hRAMP1 at the cell surface and on CGRP receptor function of the coexpressed hCLR have been analysed. The deletion of Cys^{27} or of five amino acids from Cys^{27} to Asn^{31} of hRAMP1 maintained CGRP receptor function of the hCLR. This is in accordance with results obtained with a Δ28–33 deletion mutant of hRAMP1 [18]. Additional deletion of 11 or 21 amino acids, with the latter also removing the second conserved cysteine in position 40, impaired or abolished the expression of myc-hRAMP1 at the cell surface and, as a result, CGRP receptor function of the hCLR. This confirms the functional defect of a hRAMP2/1 chimera with 24 N-terminal amino acids of hRAMP2 replacing the corresponding sequence of hRAMP1 [12]. Altogether, the results indicate that hRAMP1 function tolerates only minimal N-terminal truncation.

The loss of function of myc-hRAMP1Δ21 suggested that the second cysteine in position 40, unlike Cys^{27}, may be important for myc-hRAMP1 function and forms a disulphide bridge with one of the four more C-terminal conserved cysteines. This was investigated by individual substitutions of Cys^{40}, Cys^{57}, Cys^{72}, Cys^{82} and Cys^{104} by Ala. The Cys^{82} to Ala substitution, much like the Cys^{27} deletion, maintained the myc-hRAMP1 function, but the Cys^{40}, Cys^{57}, Cys^{72} and Cys^{104} to Ala substitutions severely impaired or suppressed delivery of myc-hRAMP1 to the cell surface and as a consequence CGRP receptor function of the coexpressed hCLR. Interestingly, Cys^{40}, Cys^{57}, Cys^{72} and Cys^{104}, unlike Cys^{27} and Cys^{82} of hRAMP1, correspond to the four conserved cysteines in hRAMP2 shown to be required for AM receptor function of the coexpressed hCLR. This suggests that correct folding of the extracellular domain of hRAMP1 and -2 in the functional complex with the hCLR includes the formation of disulphide bridges between pairs of cysteine residues. At present the disulphide pairing cannot be assigned. From the observed residual cAMP stimulation of hCLR/myc-hRAMP1-C40A and -C72A heterodimers and the inactive hCLR in the presence of myc-hRAMP1-C57A or -C104A the formation of disulphide bonds between Cys^{40} and Cys^{72} and between Cys^{57} and Cys^{104} in hRAMP1 may tentatively be implied.

The suppressed transport of myc-hRAMP1Δ21, -C40A, -C57A and -C104A, moreover, impairs posttranslational mature *N*-glycosylation of the initially core-glycosylated hCLR. The hCLR on its way to the cell surface appears to bypass the machinery for mature glycosylation in the Golgi in the absence of wild-type hRAMP1 or in the presence of defective hRAMP1 mutants.

In conclusion, hRAMP1 can only be minimally truncated at its extreme N-terminus and four out of six cysteine residues also conserved in the extracellular domain of hRAMP2 are required for the functional interaction with the hCLR to form a hCLR/hRAMP1 CGRP receptor complex. The four conserved cysteine residues are essential for cell surface delivery of hRAMP1. Two disulphide bridges in the extracellular domain of hRAMP1 are possibly formed during maturation of the hCLR/hRAMP1 CGRP receptor.

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