Structural requirements for palmitoylation of surfactant protein C precursor

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Pulmonary surfactant protein C (SP-C) propeptide (proSP-C) is a type II transmembrane protein that is palmitoylated on two cysteines adjacent to its transmembrane domain. To study the structural requirements for palmitoylation of proSP-C, His-tagged human proSP-C and mutant forms were expressed in Chinese hamster ovary cells and analysed by metabolic labelling with [^{3}H]palmitate. Mutations were made in the amino acid sequence representing mature SP-C, as deletion of the N- and C-terminal propeptide parts showed that this sequence by itself could already be palmitoylated. Substitution of the transmembrane domain by an artificial transmembrane domain had no effect on palmitoylation. However, an inverse correlation was found between palmitoylation of proSP-C and the number of amino acids present between the cysteines and the transmembrane domain. Moreover, substitution by alanines of amino acids localized on the N-terminal side of the cysteines had drastic effects on palmitoylation, probably as a result of the removal of hydrophobic amino acids. These data, together with the observation that substitution by alanines of the amino acids localized between the cysteines and the transmembrane domain had no effect on palmitoylation, suggest that the palmitoylation of proSP-C depends not on specific sequence motifs, but more on the probability that the cysteine is in the vicinity of the membrane surface. This is probably determined not only by the number of amino acids between the cysteines and the transmembrane domain, but also by the hydrophobic interaction of the N-terminus with the membrane. This may also be the case for the palmitoylation of other transmembrane proteins.

Key words: CHO cell, mutagenesis, proSP-C, protein acylation, SP-C.

INTRODUCTION

Protein palmitoylation is a post-translational modification in which palmitate, a 16-carbon fatty acid, is attached to the thiol group of a cysteine residue through a thioester linkage. Palmitoylated proteins can be grouped into four categories [1]: category 1 palmitoylated proteins are membrane proteins that are palmitoylated on one or several cysteine residues located adjacent to or just within the membrane domain, category 2 proteins include members of the Ras family that are palmitoylated in the C-terminal region after isoprenylation of their C-terminal CAAX box, category 3 proteins are palmitoylated at cysteine residues near the N- or C-terminus, and category 4 palmitoylated proteins are doubly fatty acylated with both myristate and palmitate. The palmitoylation of the second and fourth groups and also the process of N-myristoylation can be predicted on the basis of primary sequence [1]. However, for the first category of palmitoylated proteins, no consensus signal for palmitoylation has been identified. Although only distinct cysteines are palmitoylated, their position varies considerably in different acyl proteins. The only common characteristic of the palmitoylated cysteine residues is their location on the cytoplasmic side near transmembrane domains, or in the transmembrane domain itself [2,3]. By computer-assisted alignment of known palmitoylated transmembrane proteins Grosenbach et al. [4] came up with the motif $TMDX_{1-12}AAC(C)A$ (in which TMD stands for transmembrane domain and A for aliphatic amino acid) as a conserved acceptor motif for the palmitoylation of integral membrane proteins. Schweizer et al. [5], who studied the structural requirements for palmitoylation of the transmembrane protein p63, came to the conclusion that palmitoylation occurs without a primary sequence motif: only the six-amino acid spacing between the cysteine to be palmitoylated and the transmembrane segment allowed efficient palmitoylation, whereas the identities of neither the amino acids surrounding this cysteine, nor the transmembrane domain, were critical for palmitoylation. However, Ponimaskin and Schmidt [6], who investigated the palmitoylation of influenza virus haemagglutinin, argue that specific structural features must be required in addition to being in the vicinity of a transmembrane domain for a cysteine to become palmitoylated. In order to obtain more insight into the structural requirements for palmitoylation of cysteine residues of transmembrane proteins, we studied the palmitoylation of pulmonary surfactant protein C (SP-C) propeptide (proSP-C).

proSP-C, the precursor protein of SP-C, has a molecular mass of 21 kDa and adopts a type II orientation in the membrane [7,8]. Mature SP-C is formed by several proteolytic steps at both the N-terminal and the C-terminal ends of proSP-C and is stored in the lamellar body of alveolar type II cells before being secreted into the alveolar lumen [9–11]. It is a small (4.2 kDa) and very hydrophobic protein and its primary amino acid sequence is very conserved among species [12]. SP-C is one of the four specific proteins in pulmonary surfactant. The main function of this lipid–protein complex is to reduce the surface tension at the air–water interface of the lung alveoli by forming a surface-active film, thereby preventing alveoli from collapsing and facilitating the action of breathing (for review, see [9,13]). SP-C is important for promoting the spreading of the surfactant lipids to the air–water interface [9,13].

SP-C isolated from broncho-alveolar lavage fluid is dipalmitoylated [12]. Palmitoyl chains are attached to cysteine residues 28 and 29 of proSP-C (amino acids 5 and 6 in the mature SP-C

Abbreviations used: CHO, Chinese hamster ovary; SP-C, pulmonary surfactant protein C; proSP-C, SP-C propeptide.

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sequence) before its processing to mature SP-C distal to the *trans*-Golgi network [14,15]. The palmitoyl chains are thought to serve as anchors between two lipid layers, with the palmitoyl chains in one bilayer and the transmembrane domain of the peptide in the other. In this way lipids that have been squeezed out during compression of the surface film are kept near the surfactant monolayer for rapid insertion upon the next inhalation [16,17]. proSP-C typically contains two prolines, which flank the palmitoylated cysteine residues 28 and 29, and two positively charged amino acids, lysine and arginine, at positions 34 and 35 (11 and 12 in the mature SP-C sequence), which are followed by a transmembrane domain consisting of 23 hydrophobic amino acids [12]. In a previous study [18] we found that the two juxtamembrane basic residues, lysine and arginine, of proSP-C are not required structurally for palmitoylation, but that they influenced palmitoylation of proSP-C indirectly by changing the subcellular localization of the protein. The result of this is that proSP-C without the basic residues and the palmitoyltransferase were not in the same compartment. In this study we report that the amino acids located at the N-terminal side of the cysteines are important for palmitoylation. In addition, by varying the number of amino acids we observed that there is a negative correlation between the distance from the palmitoylated cysteines to the transmembrane domain of proSP-C and the level of palmitoylation. Furthermore, we found that the amino acids between the transmembrane domain and the palmitoylated cysteines, and the amino acids in the transmembrane domain itself, could be substituted without influencing the palmitoylation of proSP-C.

EXPERIMENTAL

Materials

Ham's F12 medium and enzymes used in molecular cloning were obtained from Life Technologies (Gaithersburg, MD, U.S.A.), [^{3}H]palmitate and ^{125}I-labelled goat anti-mouse IgG were from NEN Life Science Products (Boston, MA, U.S.A.) and oligonucleotides were from Amersham Pharmacia Biotech (Roosendaal, The Netherlands). Anti-Xpress antibody, used for detection of the His tag, was purchased from Invitrogen (Carlsbad, CA, U.S.A.).

Recombinant-DNA procedures

All basic DNA procedures were performed as described in [19]. The cloning of pHisproSP-C was described before [18]. Mutants lacking the N-terminal or C-terminal propeptide fragments, or both, were made by amplification of pHisproSP-C by PCR using a combination of the following primers: 5′-CGGGATCCATGGATGTGGGCAGCAAAGAGGTC-3′ (N-terminus forward), 5′-CGGAATTCCCGGAGGCGTCCTAGATGTAG-3′ (C-terminus reverse), 5′-CGGGATCCACCATGTTTGGCATTCCCTGCTGCCCA-3′ (mature forward) and 5′-CGGAATTCTCAGAGACCCATGAGCAGGGCTCCCAC-3′ (mature reverse) as forward and reverse primers. The PCR product was cloned into pcDNA3.1His (Invitrogen) using the *Bam*HI and *Eco*RI sites present in the primers. The constructs were designated ΔN-proSP-C, ΔC-proSP-C and mature SP-C for those lacking the N-terminal, C-terminal and both propeptide fragments, respectively.

The chimaeric constructs LAAAL2-, LAAAL3-, LAAAL4-, LAAAL5-, LAAAL6- and LLALL4-proSP-C were produced by standard PCR protocols using the overlap-extension technique [20]. In these constructs the transmembrane domain of proSP-C, amino acids 36–58, was replaced by artificial transmembrane domain sequences consisting of repetitions of the sequence Leu-Ala-Ala-Ala-Leu (LAAAL) or Leu-Leu-Ala-Leu-Leu (LLALL).

Plasmids containing DNA for *Pseudomonas aeruginosa* phage Pf3 coat protein [21] mutated to contain artificial transmembrane segments, used for the construction of the chimaeric constructs, were prepared as follows. In a single reaction with the QuikChange™ site-directed mutagenesis kit (Stratagene, Cedar Creek, TX, U.S.A.), a mutant was constructed in which residues 18–40 (the transmembrane segment and its flanking residues) were deleted and *Sfi*I and *Blp*I restriction sites were introduced around the deletion. Synthetic oligonucleotides were ligated between these two sites, coding for a 10-residue hydrophobic segment (LLALLLLALL or LAAALLAAAL) with the appropriate flanking sequences. The resulting LAAAL2 and LLALL2 mutants were lengthened in five-residue steps by subsequent rounds of cassette mutagenesis. For this purpose, the vector was digested with *Nhe*I and *Eco*47III (for the LLALL mutant) or *Not*I and *Nhe*I (for the LAAAL mutants), removing five codons. Consequently, oligonucleotides coding for 10 amino acids were ligated into the vector. In this manner, mutants encoding Pf3 coat protein with LLALL or LAAAL sequences of different lengths were prepared. These constructs (LAAAL2–6 and LLALL4), together with plasmid pHisproSP-C [18] were used as templates to create the chimaeric constructs. For each of the constructs one forward and one reverse primer was required for each of the two overlaps (termed N-side and C-side), which are listed in Table 1. For all chimaeric constructs the forward primer 5′-CGGGATC-

Table 1 List of primers used for the cloning of the SP-C chimaeric constructs

$n = 3$–6.

Transition	Side	Primer sequence (5′ to 3′)	Forward/reverse
proSP-C to LAAAL*n*	N-side	CCAGTGCACCTGAAACGCTTGGCGGCCGCTTTGCTAG	Forward
proSP-C to LAAAL*n*	C-side	GTGTTTCTGGCTCATGTGCAAAGCAGCAGCTAGGAG	Reverse
LAAAL*n* to proSP-C	C-side	CTCCTAGCTGCTGCTTTGCACATGAGCCAGAAACAC	Forward
LAAAL*n* to proSP-C	N-side	CTAGCAAAGCGGCCGCCAAGCGTTTCAGGTGCACTGG	Reverse
proSP-C to LAAAL2	N-side	CAGTGCACCTGAAACGCTTGGCGGCCGCTTTCGTA	Forward
proSP-C to LAAAL2	C-side	GTGTTTCTGGCTCATGTGCAAAGCAGCAGCTAG	Reverse
LAAAL2 to proSP-C	C-side	CTAGCTGCTGCTTTGCACATGAGCCAGAAACAC	Forward
LAAAL2 to proSP-C	N-side	TAGCAAAGCGGCCGCCAAGCGTTTCAGGTGCACTG	Reverse
proSP-C to LLALL4	N-side	CCAGTGCACCTGAAACGCTTGCTAGCTCTTCTCCTC	Forward
proSP-C to LLALL4	C-side	GTGTTTCTGGCTCATGTGCAAAAGCGCAAGGAGGAG	Reverse
LLALL4 to proSP-C	C-side	CTCCTCCTTGCGCTTTTGCACATGAGCCAGAAACAC	Forward
LLALL4 to proSP-C	N-side	GAGGAGAAGAGCTAGCAAGCGTTTCAGGTGCACTGG	Reverse

CATGGATGTGGGCAGCAAAGAGGTC-3′ and the reverse primer 5′-CGGAATTCCCGGAGGCGTCCTAGATGTAG-3′ were used to amplify proSP-C or the chimaeric constructs and to insert restriction sites. The PCR products were cloned into pcDNA3.1His (Invitrogen) using the *Bam*HI and *Eco*RI sites present in the primers. Mutations in pHisproSP-C were made with the QuikChange™ site-directed mutagenesis kit as described by the manufacturer using appropriate pairs of complementary oligonucleotide primers which encoded the desired mutation. All constructs were confirmed by DNA sequencing.

Cell culture and transfection

Chinese hamster ovary (CHO)-K1 cells (CRL-9618; A.T.C.C., Manassas, VA, U.S.A.) were cultured in Ham's F12 medium supplemented with 7.5 % fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin in a humidified 5 % CO_2 atmosphere at 37 °C. CHO cells, grown in 35 mm dishes, were transiently transfected with 1 μg of DNA using Lipofectamine Plus (Life Technologies) as recommended by the manufacturer.

Metabolic labelling with [^{3}H]palmitate

At 24 h after the introduction of plasmid DNA, transiently transfected CHO cells were rinsed with PBS and labelled in 800 μl of serum-free Ham's F12 medium containing 250 μCi of [^{3}H]palmitate (30–60 Ci/mmol) for 2 h at 37 °C. Labelled cells were washed twice with ice-cold PBS and lysed with SDS sample buffer (62.5 mM Tris/HCl/2 % SDS/10 % glycerol/0.003 % Bromophenol Blue, pH 6.8) for analysis by SDS/PAGE. Use of a reducing agent in the sample buffer was avoided, as thioester linkages of palmitate with the cysteine residues are labile upon treatment with reducing agents [22].

Quantification of [^{3}H]palmitate incorporation

Samples were boiled for 2 min and separated on two identical SDS/polyacrylamide gels (12 %) [23]. One gel was fixed with 10 % acetic acid/50 % methanol, impregnated with 1 M sodium salicylate to enhance the ^{3}H signal, dried and exposed to Kodak X-Omat AR films (Kodak, Rochester, NY, U.S.A.) to visualize radiolabelled proteins. Quantification of fluorograms was carried out by densitometry using an imaging densitometer GS-700 (Bio-Rad) and Molecular Analyst 1.5 software (Bio-Rad). By making a series of dilutions, up to 8-fold, of a sample containing ^{3}H-labelled wild-type proSP-C, we established that there was a linear relationship between the amount of ^{3}H-labelled proSP-C applied to the gel and the ^{3}H signal obtained from the fluorogram. The second gel was subjected to immunoblot analysis using the anti-Xpress antibody directed against the His tag. ^{125}I-Labelled goat anti-mouse IgG was used as a secondary antibody for detection. Quantification was performed using the Fujix BAS 1000 bio-imaging analyser system (Fuji Photo Film, Düsseldorf, Germany), BAS-MP imaging plates (Fuji Photo Film) and Aïda 2.11 software (Raytest Isotopenmessgeräte, Straubenhardt, Germany). By making a series of dilutions, we established that the signal was linear at least in the range of 50–200 % of the expression level of wild-type proSP-C. The variation in expression levels of the various mutants fell within this range. The amount of [^{3}H]palmitate incorporated was expressed relative to the amount of proSP-C protein expressed.

Hydroxylamine treatment

A cell-lysate sample of CHO cells transiently expressed with wild-type proSP-C and labelled with [^{3}H]palmitate was separated in duplicate lanes on a SDS/polyacrylamide gel as described above. After fixation, one part of the gel was treated with 1 M Tris/HCl (pH 7.0) as a control, while the other part was treated overnight at room temperature with 1 M hydroxylamine (pH 7.0) [22]. After this the standard procedure for fluorography was performed.

Immunocytochemistry

Immunocytochemistry was carried out as described previously [18]. CHO cells were grown on glass coverslips. After transfection (24 h), cells were fixed with 2 % formaldehyde in PBS and permeabilized with 0.1 % Triton X-100 in PBS. After blocking with PBS containing 0.1 % BSA, the cells were incubated with the mouse anti-Xpress antibody for 2 h at room temperature, washed three times for 5 min each with PBS containing 0.1 % BSA, incubated with Alexa Fluor™ 488 rabbit anti-mouse IgG (1:1000; Molecular Probes, Leiden, The Netherlands) overnight at 4 °C, and washed five times. PBS/glycerol (1:9, v/v) was used as an anti-fading agent.

Alkaline membrane-extraction assay

Integral association of proSP-C mutants with subcellular membranes was assessed by Na_2CO_3 extraction using the method of Fujiki et al. [24]. Cells were scraped with 200 μl of PBS and pelleted in an Eppendorf centrifuge. The cells were taken up in 150 μl of 0.1 M Na_2CO_3 (pH 11), incubated for 30 min at 4 °C and centrifuged in a Beckman Airfuge for 30 min at 100000 ***g*** to pellet the membranes. The pellet, containing the extracted membranes with integral membrane proteins, was taken up in SDS sample buffer with 2 % β-mercaptoethanol. The supernatant, containing the soluble proteins, was precipitated with 10 % trichloroacetic acid and also taken up in SDS sample buffer with 2 % β-mercaptoethanol. Fractions were analysed for proSP-C by SDS/PAGE and immunoblotting using mouse anti-Xpress antibody as a primary antibody and peroxidase-conjugated goat anti-mouse IgG (Nordic, Tilburg, The Netherlands) as a secondary antibody.

Statistics

To test whether the differences in the palmitoylation levels of wild-type and mutants were significant, we performed a two-tailed paired Student's *t* test, with the wild type as a control.

RESULTS

The propeptide parts of proSP-C are not necessary for palmitoylation of SP-C

To see whether the propeptides of proSP-C are essential for palmitoylation of SP-C, or whether the signals for palmitoylation are situated closer to the palmitoylated cysteines, we made the following proSP-C mutants: one which lacked the N-terminal propeptide part of proSP-C (ΔN proSP-C), one which lacked the C-terminal propeptide part (ΔC proSP-C) and mature SP-C (mSP-C), which lacked both propeptide parts (Figure 1A). All three mutants had a His tag attached to the N-terminus, as did wild-type proSP-C. The proSP-C mutants were transiently expressed in CHO cells and incubated with [^{3}H]palmitate. Clear incorporation of [^{3}H]palmitate was seen in all three mutants (Figure 1C). As described previously [18], the identity of the labelled bands as proSP-C was verified for wild-type proSP-C by immunoprecipitation, whereas mock transfections did not yield radiolabelled bands in this region. Furthermore, the loss of ^{3}H

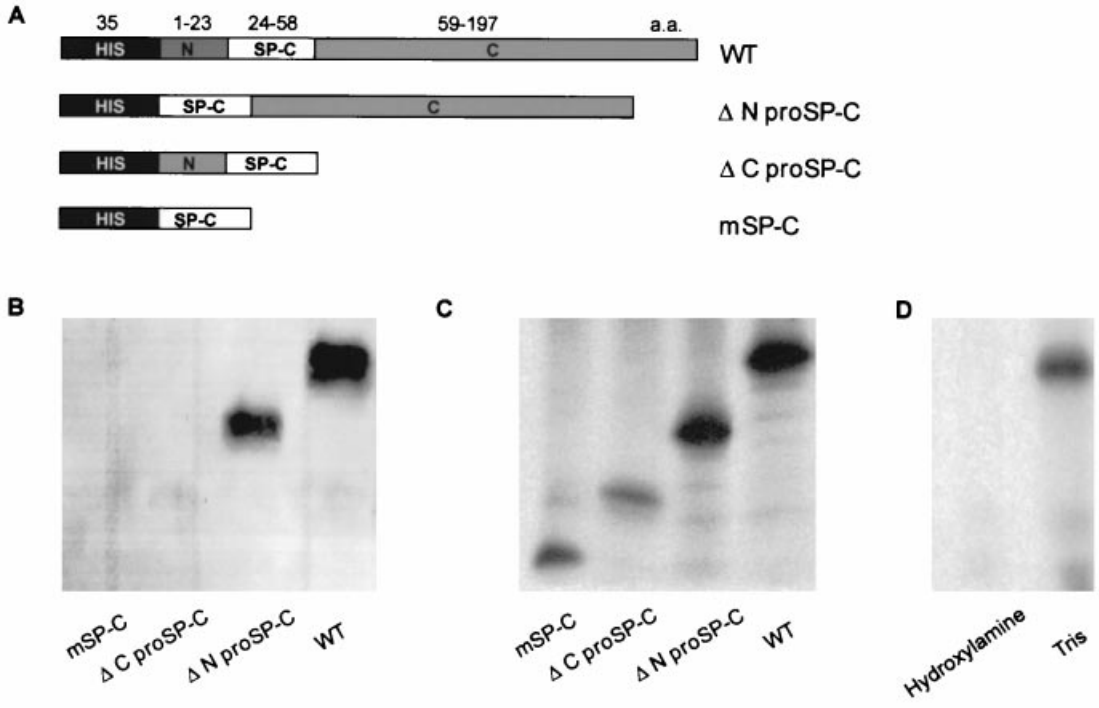

Figure 1 Palmitoylation of SP-C is possible without its propeptide parts

(**A**) Schematic representation of His-tagged proSP-C and the propeptide mutants. His-tagged proSP-C (WT) is represented by a bar that is subdivided into the His tag (black), the part of proSP-C encoding mature SP-C (white) and the N- and C-terminal propeptides (grey). The length in amino acids of the different parts is indicated. CHO cells transiently transfected with the wild type, ΔN proSP-C, ΔC proSP-C and mSP-C were labelled with [^{3}H]palmitate for 2 h. Cell lysates were subjected to SDS/PAGE and subsequently to immunoblotting to compare the amount of protein expressed (**B**) or to fluorography for the detection of ^{3}H radioactivity (**C**). On the immunoblot (**B**), mature SP-C could not be detected, presumably because of the less efficient transfer of this hydrophobic peptide to the nitrocellulose. (**D**) Duplicate lanes of wild-type proSP-C separated on a SDS/PAGE gel were treated overnight with either 1 M Tris/HCl (pH 7.0) or 1 M hydroxylamine (pH 7.0), followed by fluorography.

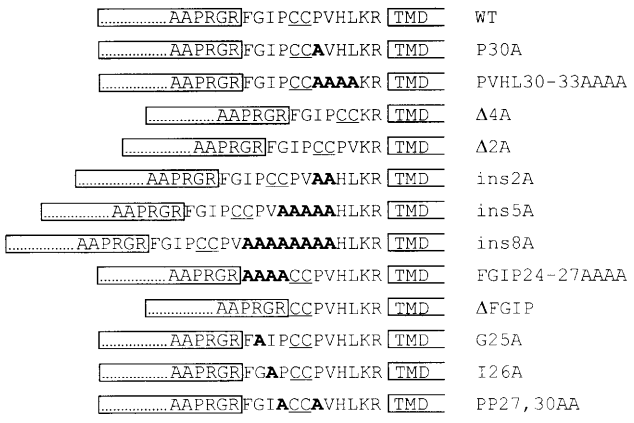

Figure 2 Schematic illustration of the different proSP-C mutants

Only the amino acids of the mature SP-C sequence are shown. The N-terminal propeptide part is represented by a box in which the first 17 amino acids are shown as by dots and the last six by the one-letter amino acid code. The half boxes represent the transmembrane domain (TMD) of proSP-C together with the C-terminal propeptide part. Substitutions and insertions are indicated in bold and the cysteines are underlined.

signal upon treatment of the [^{3}H]palmitate-labelled proSP-C with neutral hydroxylamine showed that the palmitate was attached via a thioester bond (Figure 1D). On the basis of the amount of proSP-C expressed (Figure 1B), which varied among the different constructs, we estimated that the incorporation of palmitate for the various constructs was comparable. From this we conclude that the amino acid sequence making up the mature SP-C contains the signal(s) for palmitoylation. We therefore subjected this sequence to site-directed mutagenesis.

The amino acids localized between the palmitoylated cysteines and the transmembrane domain do not contain a signal for palmitoylation of proSP-C

First we investigated whether the amino acid sequence between the palmitoylated cysteines and the transmembrane domain in proSP-C contains a signal for efficient palmitoylation. In a previous study [18] we showed that Lys-34 and Arg-35, which are adjacent to the transmembrane domain, are not involved directly in palmitoylation, but influence the cellular localization of the protein. Pro-30, which is located next to the palmitoylated cysteines, was substituted by an alanine residue (P30A; Figure 2). Palmitoylation of P30A was not significantly different from that of the wild type, as can be seen on the fluorogram in Figure 3(A) and in Table 2, which shows the results of the quantification. Next, all four amino acids, Pro-30, Val-31, His-32 and Leu-33, present between the cysteines and Lys-34, were substituted by alanines (PVHL30-33AAAA; Figure 2). This substitution had no significant effect on the palmitoylation level of proSP-C either (Figure 3 and Table 2). These data demonstrate that proSP-C is efficiently palmitoylated independently of the nature of the four

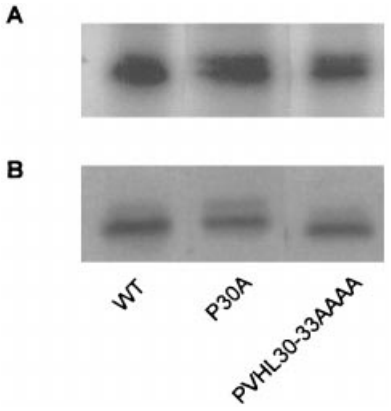

Figure 3 proSP-C is efficiently palmitoylated independently of the nature of the four amino acids present between the cysteines and the juxtamembrane lysine and arginine

CHO cells transiently transfected with WT, P30A and PVHL30-33AAAA were labelled with [^{3}H]palmitate for 2 h. Cell lysates were subjected to SDS/PAGE and subsequently to fluorography for the detection of ^{3}H radioactivity (**A**) or to immunoblotting with anti-Xpress antibody followed by a ^{125}I-labelled secondary antibody to determine the amount of proSP-C expressed (**B**). The band representing 28 kDa His-tagged proSP-C on a representative fluorogram out of three independent experiments and the accompanying immunoblot is shown.

Table 2 Palmitoylation of the various proSP-C mutants

CHO cells transiently transfected with wild-type proSP-C and various mutants of proSP-C were labelled with [^{3}H]palmitate for 2 h. Cell lysates were subjected to SDS/PAGE and fluorography for the detection of ^{3}H radioactivity. The amount of [^{3}H]palmitate incorporated was expressed relative to the amount of proSP-C (see the Experimental section). The value obtained for wild-type proSP-C was taken as 100%. Data are means ± S.D. from at least three separate experiments. $^*P < 0.05$ and $^{**}P < 0.01$. For details of the mutants see the text and Figure 2.

Mutant	Degree of palmitoylation (% of wild-type proSP-C)
P30A	131 ± 25
PVHL30-33AAAA	105 ± 18
FGIP24-27AAAA	37 ± 17**
ΔFGIP	104 ± 31
G25A	92 ± 44
I26A	64 ± 20*
PP27,30AA	133 ± 40
LAAAL2	0**
LAAAL3	136 ± 25
LAAAL4	107 ± 38
LAAAL5	113 ± 28
LAAAL6	28 ± 10**
LLALL4	131 ± 54

amino acids present between the cysteines and the juxtamembrane lysine and arginine residues.

Correlation between the spacing of the palmitoylated cysteines from the transmembrane domain and the palmitoylation level of proSP-C

As the nature of the amino acids between the cysteines to be palmitoylated and the transmembrane domain was unimportant for palmitoylation, we investigated whether the spacing between the cysteines to be palmitoylated and the transmembrane domain was critical for palmitoylation of proSP-C. Two amino acids, His-32 and Leu-33 (Δ2A) or all four amino acids (Δ4A) present between the cysteines and the juxtamembrane lysine and arginine were deleted (see Figure 2). When cells expressing these constructs were analysed by labelling with [^{3}H]palmitate, both Δ2A and Δ4A mutants showed a significant increase in palmitoylation compared with the wild type (Figure 4).

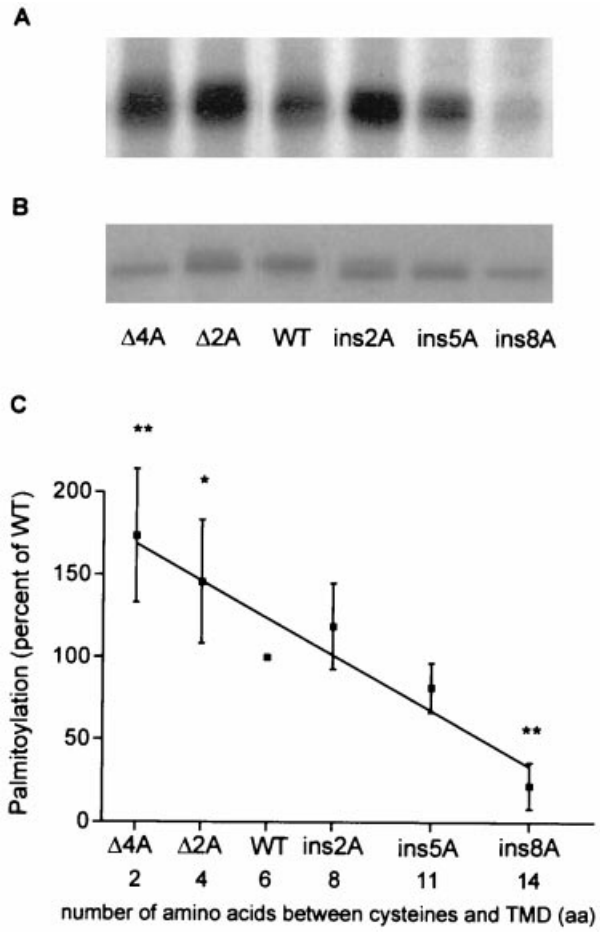

Figure 4 The palmitoylation level of proSP-C is negatively correlated with the spacing of the cysteines from the transmembrane domain

CHO cells transiently transfected with the wild type (WT), Δ4A, Δ2A, ins2A, ins5A and ins8A were labelled with [^{3}H]palmitate for 2 h. Cell lysates were subjected to SDS/PAGE and subsequently to fluorography for the detection of ^{3}H radioactivity (**A**) or to immunoblotting with anti-Xpress antibody followed by a ^{125}I-labelled secondary antibody to determine the amount of proSP-C expressed (**B**). The band representing 28 kDa His-tagged proSP-C on a representative fluorogram (**A**) and the accompanying immunoblot (**B**) is shown. (**C**) The amount of [^{3}H]palmitate incorporated was expressed relative to the amount of proSP-C (see the Experimental section). The value obtained with the wild type was taken as 100%. Data are means ± S.D. from four separate experiments; $^*P < 0.05$ and $^{**}P < 0.01$. TMD, transmembrane domain.

Another set of mutants was created in which the spacing of Cys-28 and Cys-29 from the transmembrane domain was increased by inserting two (ins2A), five (ins5A) or eight (ins8A) alanine residues between Val-31 and His-32 (see Figure 2). The incorporation of [^{3}H]palmitate into ins8A was drastically lowered compared with wild type, whereas palmitoylation of ins2A or ins5A was not significantly different from that of the wild type (Figure 4). The distance of the palmitoylated cysteines from the transmembrane domain and incorporation of palmitate was inversely correlated (correlation coefficient, 0.91), suggesting that the spacing of the cysteines from the transmembrane domain

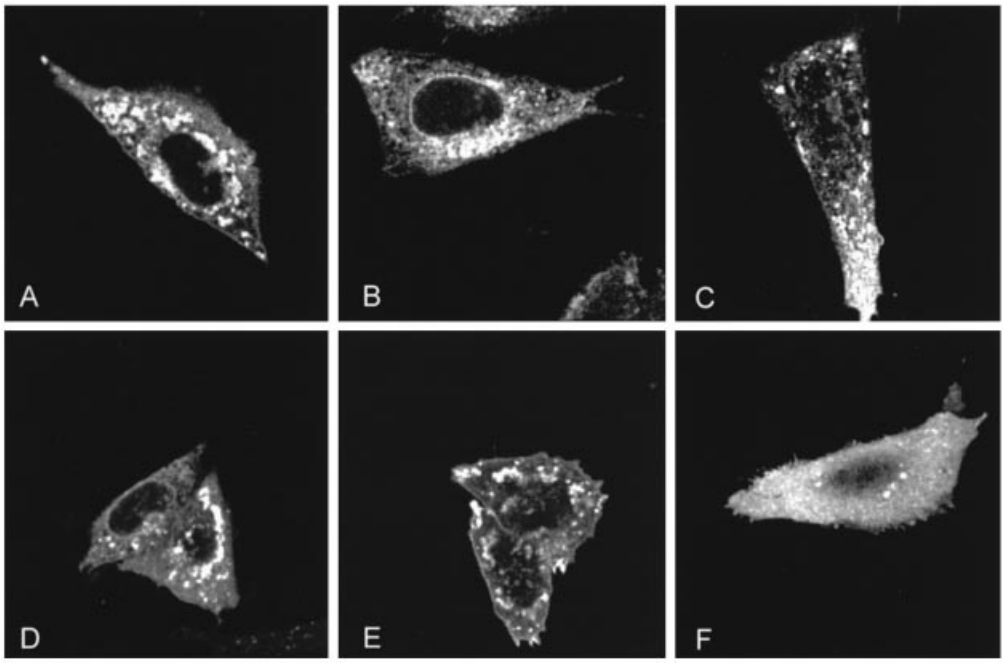

Figure 5 Subcellular localization of wild-type and various mutants of proSP-C

At 24 h after transfection of CHO cells with wild-type proSP-C (**A**), Δ4A (**B**), ins8A (**C**), FGIP24-27AAAA (**D**), I26A (**E**) or LAAAL2 (**F**), the cells were fixed and immunostained for the histidine tag by use of an anti-Xpress antibody. His-tagged Alexa 488-specific fluorescence is depicted.

is critical for efficient palmitoylation of proSP-C: the closer the cysteines are to the transmembrane domain, the better the palmitoylation.

The cellular localization of the mutant constructs was checked by immunocytochemistry and was found to be similar to that of the wild type (see Figures 5A–5C for the localization of wild-type proSP-C and the Δ4A and ins8A mutants). Therefore, we could exclude the possibility that palmitoylation had changed because of different localization in the cell. Some mutants, such as P30A, PVHL30-33AAAA, Δ2A and ins2A, were present as doublets on both the fluorogram and immunoblot (Figures 3, 4A and 4B). These doublets probably reflect differences in the level of a previously observed post-translational modification of proSP-C [25,26]. Both bands of the doublets were counted in the quantification of palmitoylation in these mutants.

Influence of the amino acids located at the N-terminal side of the palmitoylated cysteines on palmitoylation of proSP-C

As the nature of the amino acids located between Cys-28 and Cys-29 and the transmembrane domain appeared not to be essential for palmitoylation, we investigated the importance of the amino acids located on the N-terminal side of the cysteines. We substituted the four amino acids located on the N-terminal side of Cys-28 and Cys-29, which are the N-terminal amino acids of mature SP-C, by alanine residues (FGIP24-27AAAA; Figure 2). This construct was transiently expressed in CHO cells. Analysis of palmitoylation showed that the incorporation of [^{3}H]palmitate was decreased to approx. 40 % (Table 2). To investigate the importance of amino acids 24–27 for palmitoylation further, we deleted the four N-terminal amino acids (ΔFGIP; Figure 2). This amounts to their substitution by the last four amino acids of the N-terminal propeptide (Pro-Arg-Gly-Arg, amino acids 20–23). This mutant showed no significant change in palmitoylation level compared with the wild type (Table 2). Next we studied the importance of the single amino acids in this N-terminus by having them substituted individually by an alanine (F24A, G25A and I26A), except for Pro-27, which was substituted together with Pro-30 (PP27,30AA; Figure 2). The effect of changing Phe-24 to alanine on palmitoylation could not be determined, as the expression level of this mutant was too low for proper quantification. This low expression of F24A was not due to low transfection efficiency, as assessed by co-transfection of a plasmid containing the *lacZ* gene and determination of the β-galactosidase activity (results not shown). Mutants G25A and PP27,30AA showed no detectable differences in palmitoylation compared with the wild type (Table 2). In contrast, I26A showed a significant decrease in palmitoylation, amounting to approximately half the decrease found for FGIP24-27AAAA (Table 2). The localization of FGIP24-27AAAA and I26A was determined and found to be similar to that of wild-type proSP-C (Figures 5D and 5E). Therefore, Ile-26 is important for palmitoylation of proSP-C, whereas Gly-25 and Pro-27 appear not to affect it.

The transmembrane domain of proSP-C contains no information for palmitoylation

Given the correlation between the degree of proSP-C palmitoylation and the distance from the palmitoylation site to the transmembrane domain, we next tested whether this domain contains information for proSP-C palmitoylation. To this end, chimaeric constructs were made in which the transmembrane domain of proSP-C, amino acids 36–58, was replaced by artificial transmembrane domain sequences. These artificial transmembrane domains were 4-fold repetitions of the sequence Leu-Ala-Ala-Ala-Leu (LAAAL4-proSP-C) or Leu-Leu-Ala-Leu-Leu (LLALL4-proSP-C). The palmitoylation levels of LAAAL4-

and LLALL4-proSP-C were comparable with that of the wild type. We also studied the influence of the length of the transmembrane domain by varying the number of LAAAL repetitions (Table 2). The palmitoylation levels of LAAAL3- and LAAAL5-proSP-C were also comparable with that of the wild type. Palmitoylation of LAAAL6-proSP-C was reduced to about 25%, whereas no palmitoylation could be detected for LAAAL2-proSP-C. By immunocytochemistry and the alkaline membrane-extraction assay (results not shown) we found that all transmembrane-domain mutants were exclusively membrane-associated, except for LAAAL2-proSP-C, which was also found in the cytosol (Figure 5F). These results demonstrate that the transmembrane domain of proSP-C can be replaced by an artificial transmembrane domain without influencing the palmitoylation. The transmembrane domain of proSP-C therefore appears not to contain information required for palmitoylation.

DISCUSSION

There have only been a few articles published on the structural requirements for palmitoylation of integral membrane proteins. As these papers do not provide a conclusive model, we decided to study the structural requirements of proSP-C, a relatively simple integral membrane protein, with the purpose of increasing the knowledge concerning palmitoylation of integral membrane proteins. Previous work has shown that Cys-28 and Cys-29 are the only cysteines that are palmitoylated in proSP-C [18]. Furthermore, it was found that the two juxtamembrane positive charges at positions 34 and 35 had no direct effect on palmitoylation of proSP-C [18]. In the present study we have analysed the structural requirements for palmitoylation of proSP-C more comprehensively. We concentrated on the amino acid sequence constituting mature SP-C, as this sequence by itself can already be palmitoylated, which is in accordance with the palmitoylation of mature SP-C found in the baculovirus expression system in insect cells [27].

proSP-C was found to be palmitoylated efficiently and independently of the nature of the amino acids present between the cysteines and the transmembrane domain, and could also be palmitoylated in the presence of a transmembrane domain with an artificial amino acid sequence of a length that is in the order of the thickness of the membrane, suggesting that these parts of proSP-C contain no signal sequence for palmitoylation. Only when the artificial sequence was too short, so that it could not induce membrane association, or very long, was palmitoylation reduced. Schweizer et al. [5] also found that a chimaera of the p63 protein, in which the transmembrane domain of p63 had been substituted by the transmembrane domain of the plasma-membrane protein dipeptidyl peptidase IV, was not affected with respect to palmitoylation. In contrast, Ponimaskin and Schmidt [6] found a dramatic decrease in palmitoylation when they replaced the transmembrane domain of haemagglutinin with the transmembrane domain of Sendai virus F protein, and concluded that the transmembrane domain contains information for palmitoylation.

The substitution by alanine residues of the four amino acids localized on the N-terminal side of the palmitoylated cysteines of proSP-C had drastic effects on the palmitoylation level of proSP-C. By consecutively substituting these four amino acids we determined that the reduction in palmitoylation is caused mainly by the substitution of Ile-26 to alanine. The role of Phe-24 could not be assessed as the expression level of the mutant of this latter residue was too low for proper quantification. Surprisingly the deletion of the four amino acids had no effect on the palmitoylation. When the four amino acids Phe-Gly-Iso-Pro were deleted, the amino acids neighbouring the cysteine were replaced by the last four amino acid of the N-terminal propeptide, Pro-Arg-Gly-Arg (amino acids 20–23). This sequence differs from the original quartet by the presence of positively charged arginines instead of the hydrophobic residues phenylalanine and isoleucine. A possible explanation for the absence of an effect on palmitoylation is that in this area amino acids have to be present that can interact with the membrane, such as hydrophobic or positively charged amino acids. This was not the case in the substitution mutant, in which we substituted the amino acids at positions 24–27 by alanines. The primary sequence of mature SP-C is very conserved among different species [12] and SP-C from all species was found to be palmitoylated. In the amino acids located on the N-terminal side of the cysteines, some variations compared with the human sequence are found in other species, especially at positions 24 and 25. In some species, at position 24 a leucine residue is found instead of a phenylalanine residue, and at position 25 an arginine residue instead of a glycine residue is found [28]. These variations found in other species support our suggestion that hydrophobic and/or basic residues on the N-terminal side of the cysteines are favourable for palmitoylation, possibly through interaction of this domain with the membrane. This preference for basic and hydrophobic amino acids was also found in an *in vitro* study for non-enzymic palmitoylation using small peptides of the β_2-adrenergic receptor [29].

The distance of the cytosolic palmitoylated cysteine to the transmembrane domain in integral membrane proteins is not the same for every protein. For instance, this distance is one amino acid for the second palmitoylation site of CD4 [30], six amino acids for p63 [5], some subtypes of influenza virus haemagglutinin [31] and proSP-C [12], 10 amino acids for endothelin-converting enzyme-1 [32], 12 amino acids in the case of α2A-adrenergic receptor [33] and β2-adrenergic receptor 12 [34], and 33 for the 46 kDa mannose 6-phosphate receptor [35]. In a number of cases, including those of the transferrin receptor [36] and CD4 [30], the palmitoylation sites are localized within the transmembrane domain. Grosenbach et al. [4] came up with $TMDX_{1\text{-}12}AAC(C)A$ (in which TMD stands for transmembrane domain and A for aliphatic amino acid) as a general consensus sequence for the localization of cysteines in palmitoylated proteins. Moreover, for the p63 protein it was shown that the level of palmitoylation is critically dependent on its six-amino-acid spacing: changing the length of the spacer by one amino acid more or less caused a compete removal of palmitoylation [5]. We also observed that palmitoylation was influenced by changing the spacing between the transmembrane domain and cysteines. Varying the length of the spacer between the cysteines and the transmembrane domain showed that there was an inverse correlation between the length of this spacer and palmitoylation of proSP-C (Figure 4C), but the effect of a change in spacing by one amino acid was much less pronounced than was found for p63. A noteworthy difference between the studies on palmitoylation of p63 [5] and the present studies on palmitoylation of proSP-C is that the palmitoylation of p63 is markedly stimulated after addition of brefeldin A to the cells, whereas this is not the case for the palmitoylation of proSP-C [18]. Our study is more in line with the consensus sequence of Grosenbach et al. [4], according to which palmitoylation can occur efficiently over a range of approx. 1–12 amino acids present between the cysteines and the transmembrane domain.

Our observations, together with the studies of others [4–6], bring us to the following model. First of all, the transmembrane domain should orient the protein in the membrane in such a way that the cysteines to be palmitoylated are present at the cytoplasmic side of the membrane, thus enabling interaction with the

palmitoyltransferase, which is very likely to be membrane associated. In addition, the protein has to be present in the same membrane as its palmitoyltransferase [18]. Furthermore, palmitoylation of proSP-C seems to be determined by the probability of the cysteines interacting with the membrane-associated palmitoyltransferase. The chance of becoming palmitoylated is highest when the cysteine is in an optimal position relative to the enzyme. We have to consider that not only is the number of amino acids between the cysteines and the transmembrane domain important, but that the actual distance from the cysteine to the membrane is also dependent on the presence of hydrophobic or positively charged amino acids close to the cysteines. By interaction of hydrophobic and/or positively charged amino acids of the protein with the membrane, the conformation can change and lead to the formation of a sort of loop. The result of this is that the chance of the cysteine interacting with the membrane-associated palmitoyltransferase is higher than expected solely on the basis of the number of amino acids present between the cysteines and the transmembrane domain. This model can explain the large diversity in the number of amino acids found between the palmitoylated cysteines and transmembrane domains of palmitoylated proteins. Without the presence of such a loop, the introduction of one additional amino acid would have a much more pronounced effect on the position of the cysteine relative to the membrane.

However, being in the vicinity of the transmembrane domain seems not to be the only prerequisite for a cysteine to become palmitoylated. When cysteines were inserted by mutagenesis into a non-acylated fusion glycoprotein of Sendai virus at a spacing of six and 10 amino acids from the transmembrane domain, this was not sufficient to cause palmitoylation of this cysteine [37]. Furthermore, there are integral membrane proteins, like the small hydrophobic protein of human respiratory syncytial virus, with a cysteine at a relatively short distance from the transmembrane domain, that are not palmitoylated [38]. Therefore the possibility exists that there can be amino acids which have a negative effect on palmitoylation, as suggested in the recent *in vitro* study of Bélanger et al. [29].

In conclusion, palmitoylation of proSP-C depends more on the probability of the cysteine being in the vicinity of the membrane surface than on specific sequence motifs. This may also be the case for the palmitoylation of other transmembrane proteins.

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