Biochemical and Biophysical Research Communications **290,** 532–538 (2002)
doi:10.1006/bbrc.2001.6223, available online at http://www.idealibrary.com on IDEAL®

Differential Effect of Brefeldin A on the Palmitoylation of Surfactant Protein C Proprotein Mutants

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Received December 4, 2001

The surfactant protein C precursor (proSP-C) is palmitoylated on two cysteines adjacent to its transmembrane domain. We showed previously that palmitoylation of proSP-C occurs in a postendoplasmic reticulum compartment and is not affected by the Golgi-disturbing agent brefeldin A (BFA). In contrast, the investigations presented here showed that BFA almost completely abolished palmitoylation of proSP-C mutants that contained alterations in the region between the palmitoylated cysteines and the transmembrane domain, including a Pro 30 to Leu mutant associated with interstitial lung disease. This differential effect of BFA was not caused by differences in the palmitoylation kinetics between wild-type proSP-C and the mutants and was not mimicked by nocodazole and monensin. However, differences between the mutants and wild-type proSP-C in the relative degree of processing suggest that BFA may unmask a difference in routing. This would imply that the amino acids just N-terminal of the transmembrane domain may be important for a proper sorting of proSP-C.

Key Words: **brefeldin A; palmitoylation; surfactant protein C; proSP-C; CHO cells; sorting.**

Surfactant protein C (SP-C) is one of the four specific proteins in pulmonary surfactant. The precursor protein of SP-C (proSP-C) has a molecular mass of 21 kDa and adopts a type II orientation in the membrane (1, 2). proSP-C is transported through the biosynthetic pathway from the Golgi complex via multivesicular bodies to the lamellar bodies of the alveolar type II cells, where mature SP-C is stored before being secreted into the alveolar lumen (3, 4). The signal for this sorting is thought to reside in the N-terminal propeptide of proSP-C (2, 4). Processing to mature SP-C begins as early as the medial Golgi via several proteolytic steps at both the C-terminal and the N-terminal ends of proSP-C (4, 5). Mature SP-C is a small (4.2 kDa) and very hydrophobic protein, and its primary amino acid sequence is highly conserved among species (6). SP-C isolated from broncho-alveolar lavage is dipalmitoylated (6). Palmitoyl chains are attached to cysteine residues 28 and 29 of proSP-C before its processing to mature SP-C (7, 8). proSP-C contains two prolines which flank the palmitoylated cysteines and two positively charged amino acids, lysine and arginine, at positions 34 and 35 of which are followed by a transmembrane domain consisting of 23 hydrophobic amino acids (6).

Previously (9, 10) we studied the structural requirements for palmitoylation of proSP-C by making different mutations in the mature SP-C sequence by site-directed mutagenesis. These studies made us propose that the palmitoylation of proSP-C depends more on the conformation of its cytosolic domain than on its primary structure and occurs probably in the endoplasmic reticulum–Golgi intermediate compartment (ERGIC) or cis-Golgi (8–10). In our search for amino acids sequences in proSP-C that are important for its palmitoylation, we noticed that some mutants were not palmitoylated because they were retained in the endoplasmic reticulum (ER) (9). Addition of brefeldin A (BFA) rescued the palmitoylation of these ER retention mutants, probably by transferring the palmitoyl acyltransferase (PAT) from an ERGIC/Golgi compartment to the ER. BFA is a fungal metabolite that has profound and dramatic effects on the secretory pathway in mammalian cells and therefore it has become a valuable tool to analyze protein transport in the secretory

Abbreviations used: SP-C, surfactant protein C; proSP-C, SP-C precursor protein; ERGIC, endoplasmic reticulum–Golgi intermediate compartment; ER, endoplasmic reticulum; BFA, brefeldin A; PAT, palmitoyl acyltransferase; NDGA, nordihydroguaiaretic acid.

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0006-291X/02 $35.00

pathway. It blocks membrane export out of the ER *in vivo* (11, 12) and inhibits vesicle formation both *in vivo* (13) and *in vitro* (14). At the same time, BFA induces Golgi-derived tubular networks, which are responsible for the microtubule-dependent retrograde transport to the ER (14, 15), leading to complete loss of Golgi structure and the redistribution of the Golgi and ER to one compartment.

After our observation that BFA could restore palmitoylation of proSP-C mutants retained in the ER, we routinely determined the incorporation of palmitate in the presence and absence of BFA in subsequent experiments, in order to discriminate between a direct or indirect (ER retention) effect of various mutations in proSP-C on its palmitoylation. Surprisingly, some mutants, all with mutations in the region between the cysteines and the transmembrane domain, showed a dramatic decrease in palmitate incorporation in the presence of BFA. Subsequent studies suggested that BFA treatment may unmask a difference in routing of the proSP-C mutants to an ER compartment that apparently does not mix with the PAT-containing Golgi compartment after BFA addition.

MATERIALS AND METHODS

Recombinant DNA procedures. All basic DNA procedures were performed as described (16). The cloning of pHisproSP-C and the construction of most proSP-C mutants was described before (9, 10). Mutants P30L and P30S were made with the QuikChange site-directed mutagenesis kit (Stratagene, Cedar Creek, TX) as described by the manufacturer using pHisproSP-C as a template and appropriate pairs of complementary oligonucleotide primers which encoded the desired mutation. The mutants were confirmed by DNA sequencing.

Cell culture and transfection. CHO-K1 cells (Chinese hamster ovary, CRL-9618, American Type Culture Collection, Manassas, VA) were cultured in Ham's F12 medium (Life Technologies, Gaithersburg, MD) supplemented with 7.5% fetal calf serum, 100 units/ml penicillin, 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 DNA using Lipofectamine Plus (Life Technologies) as recommended by the manufacturer.

Metabolic labeling with [^{3}H]palmitate. At 24 h after the introduction of plasmid DNA, transiently transfected CHO cells were rinsed with phosphate-buffered saline (PBS) and labeled in 800 μl serum free Ham's F12 medium containing 250 μCi [^{3}H]palmitate (NEN Life Science Products, Boston, MA) (30–60 Ci/mmol) for 2 h at 37°C. BFA (10 μg/ml in ethanol), cycloheximide (50 μg/ml in ethanol), monensin (10 μM in ethanol), NDGA (30 μM in ethanol), cerulenin (10, 25, and 100 μg/ml in ethanol) and nocodazole (20 μg/ml in DMSO) were added to the labeling mixture where indicated. In the case of incubations with cycloheximide or monensin, cells were preincubated for 1 or 2 h, respectively, before labeling with [^{3}H]palmitate. Labeled cells were washed twice with ice-cold PBS and lysed with SDS–PAGE sample buffer (62.5 mM Tris, 2% SDS, 10% glycerol, 0.003% bromophenol blue, pH 6.8) for analysis on SDS–PAGE.

Quantification of [^{3}H]palmitate incorporation. After separation of the samples on two identical 12% SDS polyacrylamide gels (17), the fluorogram and immunoblot of these gels were quantified as described previously (9). The amount of [^{3}H]palmitate incorporated was expressed relative to the amount of SP-C protein expressed.

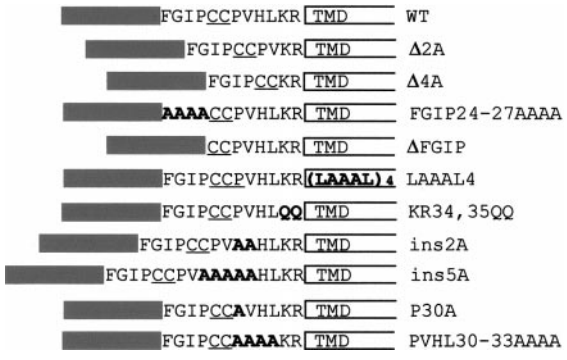

FIG. 1. Schematic illustration of the mutants described. Only the amino acids of the mature SP-C sequence are shown. The N-terminal propeptide part is represented by a gray box. The half boxes represent the transmembrane domains of proSP-C together with the C-terminal propeptide part, $(LAAAL)_4$ represents an artificial transmembrane domain sequence, which is a fourfold repetition of the sequence leu-ala-ala-ala-leu, together with the C-terminal propeptide part of proSP-C. Substitutions and insertions are indicated in bold and the cysteines are underlined.

Immunocytochemistry. Immunocytochemistry was carried out as previously described (9). CHO cells were grown on glass coverslips. At 24 h after transfection, cells were fixed with 2% formaldehyde in PBS and permeabilized with 0.1% Triton X-100 in PBS. For visualization of His-proSP-C, mouse anti-Xpress antibody (Invitrogen, Carlsbad, CA) was used in combination with Alexa Fluor 488 rabbit anti-mouse IgG (1:1000, Molecular Probes, Leiden, The Netherlands).

RESULTS

Brefeldin A Reduces the Palmitoylation Level of a Specific Group of proSP-C Constructs, Which All Have Mutations Located between the Cysteines and the Transmembrane Domain

As explained in the introduction we routinely determined the incorporation of palmitate in the presence and absence of BFA to discriminate between a direct or indirect (ER retention) effect of various mutations in proSP-C on its palmitoylation. A schematic representation of the proSP-C mutants described in this article is given in Fig. 1. The presence of BFA caused no or only minor changes in palmitoylation compared to the situation without BFA, for wild-type proSP-C, for the deletion mutants δ2A and δ4A, in which two or all four, respectively, amino acids present between the cysteines and the juxtamembrane lysine and arginine were deleted, for the N-terminal mutants in which the four amino acids located at N-terminal side of cysteines 28 and 29 were substituted by alanine residues (FGIP24-27AAAA) or were deleted (δFGIP) and for a chimer in which the transmembrane domain of proSP-C was replaced by an artificial transmembrane domain sequence (LAAAL4-proSP-C) (Fig. 2). As already described before (9), the palmitoylation level of the mutant in which the positively charged lysine and

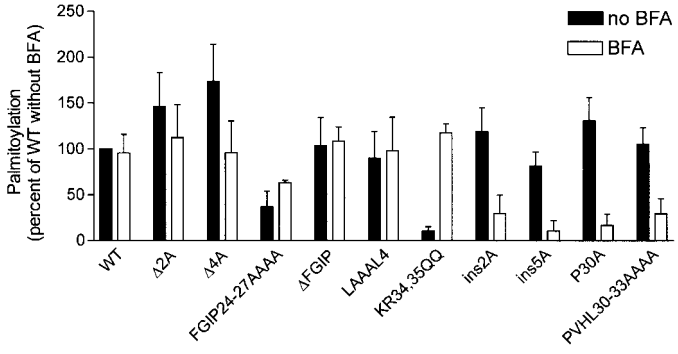

FIG. 2. Differential effect of BFA on the palmitoylation of wild-type and mutant proSP-C. CHO cells transiently transfected with WT and proSP-C mutants were labeled with [^{3}H]palmitate for 2 h in the presence (open bars) or absence (black bars) of 10 μg/ml BFA. Cell lysates were subjected to SDS–PAGE 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. The value obtained with WT was taken as 100%. Data are means ± SD of three separate experiments.

arginine residues were substituted to glutamine residues (KR34,35QQ, previously named KR^{11,12} QQ) was increased back to wild-type levels when BFA was added (Fig. 2). In contrast, dramatic decreases in the amount of palmitate incorporated in the presence of BFA compared to control condition without BFA were found for mutants with increased distance of the cysteines from the transmembrane domain by insertion of two (ins2A) or five (ins5A) alanine residues, for the mutant in which proline 30, located next to the palmitoylated cysteines, was replaced by alanine (P30A), and for the mutant in which all four amino acids present between the cysteines and lysine 34 were substituted by alanines (PVHL30-33AAAA) (Fig. 2). Remarkably, inhibition of palmitoylation by BFA was only observed for those mutants that were modified in the region between the cysteines and the transmembrane domain. For further study of the underlying mechanism of this differential effect of BFA on the palmitoylation of the different proSP-C mutants, we chose P30A as a representative, because the mutation made in this mutant is a relatively simple single amino acid substitution and because this mutant showed the most dramatic decrease in palmitoylation upon addition of BFA. Furthermore, a substitution of this proline to leucine (P30L) may be of clinical interest, as it has been associated with interstitial lung disease (18).

The BFA-Induced Decrease in Palmitoylation Is Not Alanine Specific

The mutants showing a decrease in palmitoylation upon the addition of BFA all contained additional alanines or contained alanines instead of the original amino acids. To see whether the decrease in palmitoylation in the presence of BFA was specific for the alanine mutations, we substituted proline 30 also into a serine (P30S) and leucine (P30L), with the latter mimicking the mutation associated with interstitial lung disease. P30S and P30L behaved in the same way as P30A in the presence and absence of BFA (Fig. 3), indicating that the BFA-induced decrease in palmitoylation is not alanine specific.

The Effect of BFA on Palmitoylation Is Reversible

Next, we tested whether the BFA effect on palmitoylation was mediated by its effect on the secretory pathway. The effects of BFA on the secretory pathway have been described to be reversible after removal of the drug (19). To investigate whether this was also the case for the BFA effect on palmitoylation of P30A proSP-C, the cells expressing P30A were washed after incubation with BFA and labeled with [^{3}H]palmitate. Palmitoylation of P30A indeed returned to control level after wash out of BFA (not shown).

Kinetics of Palmitoylation of Wild-Type and P30A proSP-C

The difference between the palmitoylation of wild-type proSP-C and the P30A mutant in the presence of BFA might be due to a difference in the rate of palmitoylation or depalmitoylation of wild-type proSP-C compared to the P30A mutant. A difference in palmitoylation kinetics might not be detectable in the relatively long incubation time with [^{3}H]palmitate in the absence of BFA, but might become apparent after BFA treatment, resulting in relatively lower amounts of PAT activity in the ER compared to the level of PAT activity in the post-ER/Golgi compartment in the native situation. However, wild-type and P30A proSP-C showed comparable kinetics for palmitate incorporation (Fig. 4A) and decrease of incorporated palmitate in a pulse-chase experiment (Fig. 4B). The presence of BFA during the chase period of the pulse-chase experiment did not affect the rate of decrease of [^{3}H]palmitate incorporation, neither for wild-type nor for P30A proSP-C (Fig. 4B). Furthermore both wild-type and P30A proSP-C were not repeatedly de- and repalmitoy-

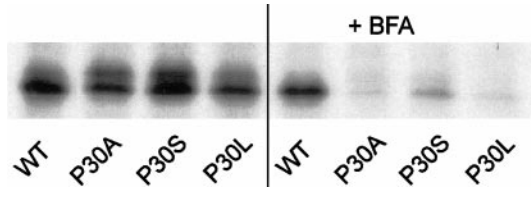

FIG. 3. The BFA induced decrease in palmitoylation is not alanine specific. CHO cells transiently transfected with WT, P30A, P30S and P30L were labeled with [^{3}H]palmitate for 2 h in the presence or absence of 10 μg/ml BFA. Cell lysates were subjected to SDS–PAGE and fluorography for the detection of [^{3}H] radioactivity. A representative fluorogram of three separate experiments is shown.

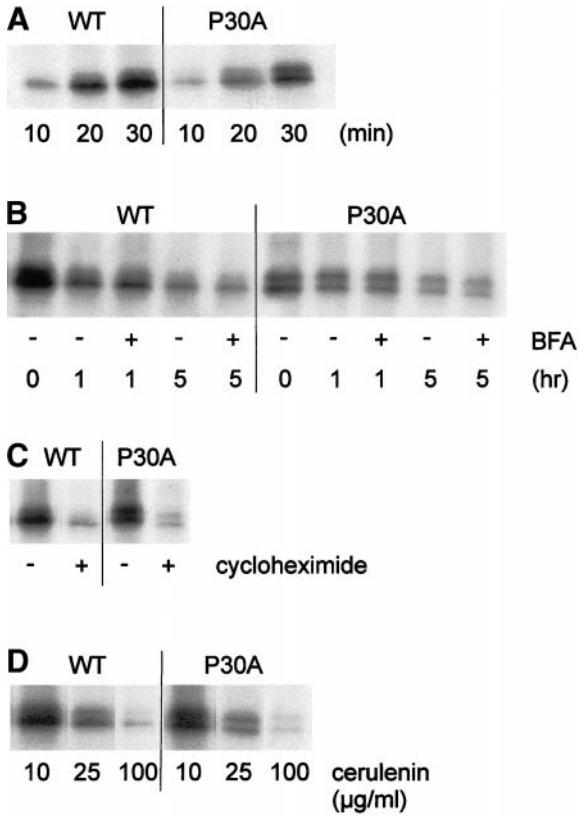

FIG. 4. Kinetics of palmitoylation of proSP-C and P30A. CHO cells transiently transfected with WT and P30A proSP-C were incubated with [^{3}H]palmitate for (A) 10, 20, and 30 min; (B) 30 min and chased with 0.2 mM palmitate for 1 and 5 h in the presence or absence of 10 μg/ml BFA; (C) 2 h in the presence or absence of 50 μg/ml cycloheximide, after a preincubation of 1 h without [^{3}H]palmitate; and (D) 2 h in the presence of 10, 25, and 100 μg/ml cerulenin. For analysis, see Fig. 3.

lated after synthesis, as cycloheximide, an inhibitor of protein synthesis, inhibited the incorporation of [^{3}H]palmitate in both P30A and wild-type proSP-C almost completely (Fig. 4C). Addition of an inhibitor of palmitoylation might also reveal differences in palmitoylation kinetics. However the palmitoylation of wild-type and P30A proSP-C were equally sensitive to cerulenin (Fig. 4D). Thus, P30A and wild-type proSP-C are predominantly irreversible palmitoylated after translation with similar kinetics, are inhibited by the same inhibitor, cerulenin, and have a similar degradation pattern, suggesting that they are both palmitoylated by a similar PAT.

Effects of Other Secretory Pathway-Disrupting Agents

The effect of other secretory pathway disrupting agents on palmitoylation was tested to study whether these compounds also had a differential effect on the palmitoylation of P30A and wild-type proSP-C. Monensin is a Na^+/K^+ proton ionophore that disrupts ion gradients across biological membranes and induces a dose-dependent arrest of intracellular transport at the level of the Golgi (20). Addition of monensin to the cells had no effect on palmitoylation of either wild-type or P30A proSP-C compared to control conditions (Fig. 5A). Nordihydroguaiaretic acid (NDGA), a compound that disrupts the secretory pathway by stimulating a retrograde movement of Golgi stack and TGN membranes back to the ER (21, 22), decreased the palmitoylation level of both wild-type and P30A proSP-C to almost zero compared to control condition (Fig. 5B). Nocodazole, a microtubuli depolymerizing agent that inhibits retrograde transport of vesicles form the Golgi to the ER, caused a partial decrease in palmitoylation of both wild-type proSP-C and P30A (Fig. 5C). Addition of nocodazole in the presence of BFA blocked not only the palmitoylation of P30A, but also that of wild-type proSP-C (Fig. 5C), which can be explained by the inhibition of the retrograde transport of PAT from the Golgi to proSP-C, which is retained in the ER by the effect of BFA on the anterograde transport.

Wild-Type and P30A proSP-C Have Almost the Same Subcellular Localization

Next we determined if differential sorting within the ER or Golgi may be an explanation for the differential BFA effect on palmitoylation. In Fig. 6, the confocal images of wild-type and P30A proSP-C are shown. No major difference in localization between the two could be seen: both are distributed throughout the whole cell in punctate structures. When the cells expressing P30A and wild-type proSP-C were also imaged after an incubation of 2 h with BFA, the distribution throughout the cell observed for both constructs was very similar, which was more dispersed compared to control conditions (Fig. 6).

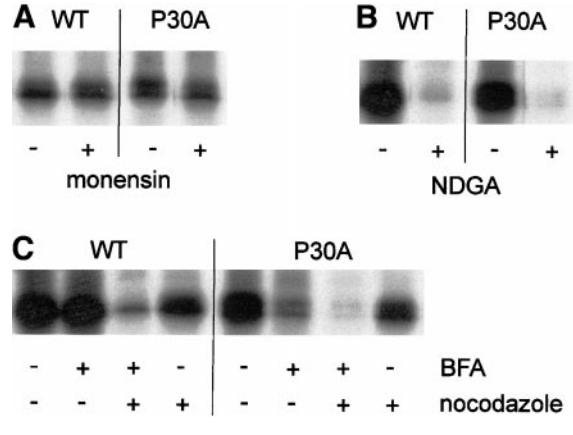

FIG. 5. Effects of monensin, NDGA, and nocodazole on palmitoylation. CHO cells transiently transfected with WT and P30A proSP-C were labeled with [^{3}H]palmitate for 2 h in the presence or absence of (A) 10 μM monensin, after a preincubation of 2 h; (B) 30 μM NDGA; and (C) 10 μg/ml BFA and/or 20 μg/ml nocodazole. For analysis, see Fig. 3.

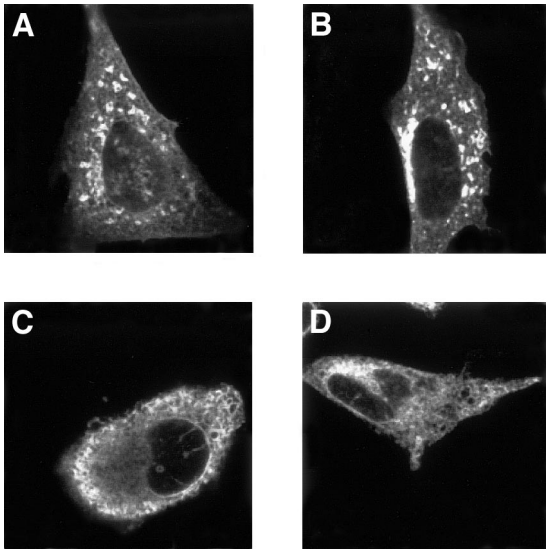

FIG. 6. Subcellular localization of wild-type and P30A proSP-C. At 24 h after transfection of CHO cells with WT (A, C) or P30A (B, D), the cells were incubated for 2 h in the presence (C, D) or absence (A, B) of 10 μg/ml BFA, fixed, and immunostained for his tag by use of an anti-Xpress antibody. His tag-Alexa 488 specific fluorescence is depicted.

Difference in Posttranslational Modification between Wild-Type and P30A proSP-C

proSP-C is expressed in CHO cells and type II cells as a doublet of 21 and 21.5 kDa (23, 24). Keller and colleagues (24) showed that the 21.5-kDa form represents a posttranslational modified form of proSP-C. On the fluorogram we see (Figs. 3 and 4) that the 21.5-kDa modified proSP-C form was present in a relatively higher amount in P30A compared to wild-type proSP-C. After incubation in the presence of BFA the 21.5-kDa form of wild-type proSP-C was not observed (Fig. 3), suggesting that the 21.5-kDa form is formed by a modification in a compartment distal to the ER/ERGIC. Although the palmitoylation of P30A decreased dramatically upon the addition of BFA, the ratio between the two forms did not seem to change compared to incubation under control conditions. In the pulse-chase experiment, with a chase in the presence of BFA, the ratio of the two proSP-C forms was not affected for either wild-type or P30A proSP-C (Fig. 4B). In the other mutants that showed a decrease in palmitoylation upon the addition of BFA, the 21.5-kDa modified form was also present in a relatively higher amount compared to wild type (not shown).

DISCUSSION

Brefeldin A has a negative effect on palmitoylation of various proSP-C mutants, which all have mutations in the region between cysteines 28 and 29 and the transmembrane domain (e.g., P30A proSP-C), while wild-type proSP-C and several other proSP-C mutants are not affected by the addition of BFA. The palmitoylation of some mutants of proSP-C, which are retained in the ER, can even be restored upon BFA addition (9).

A decrease in palmitoylation of proteins upon addition of BFA is not a common feature: palmitoylation of most proteins is not influenced by addition of BFA (25, 26), or even increased, as seen for p63 protein (27) and a 62-kDa protein (28). Only a few reports describe a decrease in palmitoylation upon the addition of BFA, for instance for the peripheral proteins SNAP-25 and GAP-43 (25). In those cases palmitoylation was also inhibited by other secretory pathway disrupting compounds, such as monensin, although this inhibition was less striking than the inhibition found for BFA. It was suggested that SNAP-25 and GAP-43 require an intact secretory transport for palmitoylation and have a pathway for palmitoylation that differs from that of the proteins that are not inhibited by BFA, such as several proteins that are both myristoylated and palmitoylated (25, 29). The palmitoylation of the membrane protein caveolin-1 was also blocked in the presence of BFA. Since the addition of monensin or cycloheximide also inhibited palmitoylation of the protein, it was postulated that the palmitoylation of caveolin-1 occurs distal to the Golgi (30).

Besides BFA also the effects of other inhibitors of the secretory pathway were tested on the palmitoylation of wild-type and P30A proSP-C. Monensin had no effect on palmitoylation of either P30A or wild-type proSP-C, while NDGA decreased palmitoylation of both constructs to the same extent (Fig. 5). Nocodazole caused a partial decrease in palmitoylation for both constructs (Fig. 5C). Thus, monensin, NDGA and nocodazole had no differential effect on palmitoylation of P30A and wild-type proSP-C. Therefore the BFA induced decrease in palmitoylation of P30A can not be explained by a general block in secretory pathway, but appears to be BFA specific. Since proSP-C is palmitoylated early in the secretory pathway (ERGIC or early Golgi), upstream from the site were monensin interrupts the secretory pathway, it is not surprising that palmitoylation of proSP-C is not inhibited by monensin, as is the case for the palmitoylation of caveolin-1, which takes place at the plasma membrane. In contrast to BFA, NDGA did not only influence the palmitoylation of P30A but also the palmitoylation of wild-type proSP-C, while both compounds cause the redistribution of Golgi proteins into the ER (14, 15, 21, 22). However, there are some differences described between the effects on the secretory pathway of the two compounds. It is possible that in the presence of NDGA (unlike in the presence of BFA) PAT is not transported back to the ER or not to the same subdomain of the ER where proSP-C is retained with the result that palmitoylation of proSP-C cannot occur.

A possible explanation for the differential effect of BFA on palmitoylation of various proSP-C mutants could be the presence of different PATs with different substrate specificities: a relatively nonspecific one located distal to the cis-Golgi, that it is not recycled back to the ER upon addition of BFA and, in addition, one very specific for wild-type proSP-C, that is retrieved into the ER after BFA treatment, does not tolerate mutations in the region between the cysteines and the transmembrane domain, and thus palmitoylates only wild-type and some other mutants, like KR34,35QQ and LAAAL4-proSP-C, but not P30A proSP-C. However, the effects of the addition of monensin or cycloheximide on the palmitoylation of P30A proSP-C do not favor this hypothesis. Addition of monensin had no influence on palmitoylation of P30A, while a blockage of palmitoylation is expected in the case of a nonspecific PAT localized distal to the Golgi, especially since cycloheximide addition indicated that mainly newly formed protein is palmitoylated (Fig. 4C). Furthermore, P30A and wild-type proSP-C had the same palmitoylation kinetics, which favors the idea that P30A and wild-type proSP-C are palmitoylated by the same PAT.

Another explanation is that P30A and wild-type proSP-C are sorted to different ER subdomains, and that the subdomain to which P30A is sorted is not accessible to BFA-retrieved PAT upon BFA addition. Under normal conditions, in the absence of BFA, the effect of a different sorting may not influence palmitoylation when both constructs are further transported to PAT containing compartments. This hypothesis is supported by the difference in ratio between the 21- and 21.5-kDa forms of proSP-C. Keller *et al.* (24) investigated the nature of the 21.5-kDa form and found that it was not susceptible to thioester bond-cleaving reagents, and therefore concluded that the modification of the 21-kDa form into the 21.5-kDa form is not caused by palmitoylation. This is in accordance with the fact that we found incorporation of palmitate in both bands. Furthermore the modification probably does not involve oligosaccharides as it was also not susceptible to N- and O-glycanases (24). Moreover, when the C-terminal 22 amino acids of proSP-C were deleted, causing retention in the ER, the modification did not take place *in vivo*, although this construct was still modified in the *in vitro* translation system. This might indicate that a first transport step is a prerequisite for the modification *in vivo* (24). Furthermore, the 21.5-kDa modified form is not seen in the presence of BFA, indicating that it is formed in a post-ER compartment or is broken down in the presence of BFA very quickly. After labeling with palmitate the 21.5-kDa modified form is more abundantly present in the case of P30A proSP-C compared to wild type, suggesting a faster modification of the 21-kDa form or a slower degradation of the modification, which is in agreement with a difference in sorting between wild-type and P30A proSP-C, although a direct effect of the mutation on the 21.5-kDa modification cannot be excluded.

If the differential effect of BFA on P30A and wild-type proSP-C is due to a subtle difference in sorting, this differential BFA effect on palmitoylation may be used to detect differences in sorting of proSP-C mutants. Moreover, the existence of this difference in sorting between P30A and wild-type proSP-C would imply that the region between cysteine 29 and the transmembrane domain is important for sorting of proSP-C, together with amino acids 10–18 of the N-terminal propeptide for which it has been described that they have a role in sorting of proSP-C (4). The importance of the region between cysteine 28 and the transmembrane domain for correct processing of proSP-C is supported by the fact that in a patient with interstitial lung disease who had a proline 30 to leucine substitution in proSP-C (18), only very low SP-C levels could be detected (Lawrence M. Nogee, personal communication).

ACKNOWLEDGMENTS

The investigations described in this article were supported in part by The Netherlands Foundation for Chemical Research (CW) with financial aid from The Netherlands Organization for Scientific Research (NWO). The authors also received financial support from the European Commission (Contract QLK2-CT-2000-00325).

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