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Dependence on Vitamin K-dependent Protein S for Eukaryotic Cell Secretion of the β-Chain of C4b-binding Protein*[S]

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The anticoagulant vitamin K-dependent protein S (PS) circulates in plasma in two forms, 30% free and 70% being bound to the complement regulatory protein C4b-binding protein (C4BP). The major C4BP isoform consists of 7 α-chains and 1 β-chain (C4BPβ$^+$), the chains being linked by disulfide bridges. PS binds to the β-chain with high affinity. In plasma, PS is in molar excess over C4BPβ$^+$ and due to the high affinity, all C4BPβ$^+$ molecules contain a bound PS. Taken together with the observation that PS-deficient patients have decreased levels of C4BPβ$^+$, this raises the question of whether PS is important for secretion of the β-chain from the cell. To test this hypothesis, HEK293 cells were stably and transiently transfected with β-chain cDNA in combinations with cDNAs for PS and/or the α-chain. The concentration of β-chains in the medium increased after co-transfection with PS cDNA, but not by α-chain cDNA, suggesting secretion of the β-chains from the cells to be dependent on concomitant synthesis of PS, but not of the α-chains. Thus, β-chains that were not disulfide-linked to the α-chains were secreted in complex with PS, either as monomers or dimers. Pulse-chase demonstrated that the complexes between PS and β-chain were formed intracellularly, in the endoplasmic reticulum. In conclusion, our results demonstrate that successful secretion of β-chains depends on intracellular complex formation with PS, but not on the α-chains. This provides an explanation for the decreased β-chain levels observed in PS-deficient patients.

Protein S (PS)[2] is an anticoagulant vitamin K-dependent protein functioning as a cofactor to activated protein C in the degradation of coagulation factors Va and VIIIa (1). Recent studies have also identified PS as a cofactor to tissue factor pathway inhibitor (2, 3). Tissue factor pathway inhibitor regulates the tissue factor-dependent initial step of coagulation by inactivating coagulation factors VIIa and Xa. In human plasma, 60–70% of PS circulates bound to the complement regulator C4b-binding protein (C4BP) (4), and this fraction has impaired anticoagulant functions (5). PS is a 635-amino-acid-long single chain molecule with several distinct domains. The N-terminal Gla domain is followed by a thrombin-sensitive region, four epidermal growth factor (EGF)-like domains, and the large C-terminal sex hormone-binding globulin (SHBG)-like region, which comprises two laminin G-type domains. The binding site in PS for C4BP is located in the SHBG region of PS, and both laminin G domains are required (6). The binding site in PS has not been narrowed down further, even though there are several studies suggesting different regions of the SHBG domain to be of importance (7, 8).

C4BP acts as a cofactor for the down-regulation of the complement cascade and shares structural features with several other proteins participating in this system. It is composed of multiple disulfide-linked subunits (α- and β-chains), each containing repeats of complement control protein (CCP) domains (9). There are several isoforms of C4BP in human plasma; the most common (approximately 80%) consists of 7 α-chains and 1 β-chain (7α1β), which create a molecule 570 kDa in size. Each α-chain is made up of 8 CCP domains, whereas the β-chain has 3 CCPs. The PS binding is totally dependent on the presence of the β-chain, the PS binding site being located mainly in the N-terminal CCP (10–12). The binding site has been further narrowed down by site-directed mutagenesis, highlighting the importance of four amino acids in the N-terminal CCP domain (13).

Both PS and C4BP are primarily produced in the liver. However, PS is additionally synthesized in many different cells, and the recent liver specific knock out of PS in mice revealed the importance of PS synthesis in endothelial cells (14). In contrast, there are no reports of C4BP expression in endothelial cells. In the liver, the genes for α- and β-chains are regulated differently. During the acute phase, α-chain expression increases more than that of the β-chain (15), leading to formation of the 7α0β isoform of C4BP, which does not bind PS. This form can also be obtained in eukaryotic expression systems, when the cDNA for the α-chain (*C4BPA*) is used to transfect the cells (16, 17). Even though this recombinant C4BP lacks the β-chain, it still has normal complement regulatory functions as cofactor to factor I in the degradation of C4b (16, 18). Co-transfection experiments

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Experimental Procedures, Table S1, and Figs. S1–S4.

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[2] The abbreviations used are: PS, protein S; C4BP, C4b-binding protein; C4BPβ$^+$, β-chain containing C4BP; CCP, complement control protein; IP, immunoprecipitation; qRT-PCR, quantitative RT-PCR; SHBG, sex hormone-binding globulin.

of COS1 cells with *C4BPA* and β-chain cDNA (*C4BPB*) generated small amounts of β-chain-containing C4BP, although the majority of the β-chain was retained inside the cells (19). The observation that the C4BP chains are differently regulated (15, 20, 21) has led to the hypothesis that the β-chain-containing C4BP isoform (denoted C4BPβ$^+$) has specific functions. It binds PS, and it has been shown that the Gla domain of PS can localize the PS·C4BP complex to anionic phospholipid membranes, *e.g.* exposed on apoptotic cells where the complex possibly can regulate complement activity (22–24). Two reports suggest that the β-chain can be expressed in absence of α-chains, in human ovary fibroblasts (25) and joints affected by rheumatoid arthritis (26), but the functional significance of this is unknown.

The binding between C4BP β-chain and PS is of very high affinity (0.1 nM) (27), and because PS circulates in molar surplus over C4BPβ$^+$, PS occupies all β-chains in human plasma. In 1995, it was reported that PS-deficient patients, in particular when treated with the vitamin K antagonist warfarin, not only had decreased levels of PS but also of C4BPβ$^+$ even though C4BP is not vitamin K-dependent (28). Based on this observation, and the difficulties we and others have experienced in expressing the β-chain CCPs in eukaryotic cells (10, 19), we hypothesized that PS is required for the secretion of the β-chain. Using recombinant eukaryotic expression systems we now demonstrate that the secretion of the β-chain is stimulated by co-expression of PS, the two proteins forming a complex already intracellularly.

EXPERIMENTAL PROCEDURES

Plasma PS (29), recombinant PS (30), and plasma C4BPβ$^+$ (31, 32) were purified, as previously described. For calculation of molarities, the following molecular masses were used: 570 kDa (C4BPβ$^+$), 45 kDa (β-chain), 70 kDa (PS), 40 kDa (PS-CTERM), and 30 kDa (mini protein S) (33). Data are presented as the mean value ± S.D., and Student's *t* test (Prism 4; GraphPad Software, Inc., La Jolla CA) was used for statistical calculations.

Cloning and Mutagenesis—The vector pBudCE4.1 (Invitrogen) was used for double expression of two separate proteins, *e.g.* PS plus β-chain or β-chain plus α-chain. For single protein expression, the vector pcDNA3 from Invitrogen was used. Mini protein S (miniPS) refers to a PS variant lacking the entire SHBG region. It comprises residues 1–242 and was created by replacing the codon for Val^{243} (amino acid residue number in mature PS) with a stop codon in the *PROS1* cDNA (33). N-terminally truncated PS-CTERM, human PS ranging from the second half of the third EGF-like domain through the SHBG domain, was obtained by PCR technique. Cloning and mutagenesis details can be seen in the supplemental Experimental Procedures.

qRT-PCR—Quantitative determination of mRNA levels in the cell lines was performed with the OneStep qRT-PCR kit (Invitrogen), as described in the provided instructions. Briefly, adherent cells were loosened and washed with phosphate-buffered saline (PBS) (Invitrogen). About 10,000 cells were then solubilized and treated with DNase. RNA concentration in the cell lysate was determined by using the $A_{260\ nm}$ in a NanoDrop2000 device (NanoDrop, Wilmington, DE). A PCR mix with ~10–40 ng of total RNA was prepared, and the method allowed for one-step PCR with a primary reversed transcriptase step for creating cDNA, followed by detection of amplified genes of interest. An internal standard (*GAPDH*) was always co-analyzed with the gene of interest (*C4BPA*, *C4BPB*, or *PROS1*). Primer/probe mixes for detection were purchased from Applied Biosystems. The reaction was performed with the ABI PRISM 7900HT system (Applied Biosystems). For quantification, the standard curve method was used; all samples were run in three replicates, and the results are described as the ratio between the gene of interest and *GAPDH*.

Cell Culture—Human umbilical cord endothelial cells and human microvascular endothelial cells (34) were cultured in Medium200 (Invitrogen) supplemented with a low serum growth supplement kit (Invitrogen), 50 units/ml penicillin, and 50 μg/ml streptomycin (Invitrogen). HEK293, HepG2, Hep3B, and MIHA (35) cell lines were cultured in complete medium, DMEM (Invitrogen) supplemented with 4 mM L-glutamine (Invitrogen), 50 units/ml penicillin, 50 μg/ml streptomycin (Invitrogen), 10% fetal bovine serum (Invitrogen), and 10 μg/ml vitamin K (Konakion Novum, F. Hoffmann-La Roche AG, Basel, Switzerland). The immortalized endothelial-like cell line EA.hy926 (36) was cultured in complete medium supplemented with 0.1 mM hypoxanthine (Sigma-Aldrich) and 16 μM thymidine (Sigma-Aldrich). Transfected cell lines were maintained by the addition of the appropriate antibiotics. Cells transfected with pcDNA3 constructs were cultured with the addition of 0.5 μg/ml Geneticin (G418; Invitrogen), and pBudCE4.1 cells were cultured in the presence of 100 μg/ml Zeocin (Invitrogen). For serum-free expression, OptiMEM (Invitrogen) supplemented with penicillin, streptomycin, and vitamin K was used. HEK293 cells were stably or transiently transfected as described in the supplemental Experimental Procedures.

Pulse-Chase—Cells stably expressing *C4BPA/C4BPB* (pBudCE4.1) and *PROS1* (pcDNA3) were seeded in 9-cm dishes and cultured to 90% confluence in complete medium. The medium was replaced with methionine/cysteine- and serum-free DMEM (Invitrogen) supplemented with 4 mM L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, and 10 μg/ml vitamin K, and the cells were incubated for 60 min. The cells were labeled with [^{35}S]methionine/cysteine (Tran^{35}S-label; MP Biomedicals, Solon, OH), 0.4 mCi/dish for 30 min and then chased by replacing the medium with OptiMEM containing 50 units/ml penicillin, 50 μg/ml streptomycin, 10 μg/ml vitamin K, and 2 mM cold methionine and cysteine. In some experiments, 5 μg/ml brefeldin A was added to the chase medium. The cells were chased for different time points before harvesting the medium and lysing the cells. The cells, placed on ice, were washed with PBS and solubilized with the addition of 0.8 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 1% Triton X-100 (Scharlau Chemie, Barcelona, Spain), 0.02% NaN_3, 1× protease inhibitor mixture (Sigma-Aldrich)). Proteins in lysates and medium were subjected to immunoprecipitation, further described in the supplemental Experimental Procedures.

Determination of Protein Concentrations—In-house developed ELISAs were performed in Maxisorp 96-well plates (NUNC), coated with a catcher antibody in 75 mM carbonate buffer overnight at 4 °C. The wells were then washed with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20 (TBS-T) before blocking with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2% BSA (TBS-2%BSA) for a minimum of 1 h at room temperature. Samples were diluted in TBS supplemented with 0.2% BSA and 2 mM $CaCl_2$ (TBS-0.2%BSA) and incubated overnight at 4 °C. A washing step was followed by incubation with a biotinylated antibody in TBS-0.2%BSA for at least 2 h. HRP-labeled streptavidin-biotin complexes (Vectastain; Vector Laboratories, Burlingame, CA) were added and incubated for 0.5 h after another wash. The plates were developed by the addition of 1,2-phenylenediamine dihydrochloride (DAKO) after a final washing step. The reaction was stopped by the addition of 0.5 M H_2SO_4, and the color intensity was measured at 490 nm in a Tecan Infinite 2000 (Tecan Group Ltd., Männedorf, Switzerland) microplate spectrophotometer. The individual protein concentrations were measured by using different sets of in-house antibodies, as described in the supplemental Experimental Procedures.

Stepwise Purification and Characterization of Recombinant PS and C4BP—About 900 ml of OptiMEM collected from cells stably transfected with *PROS1* and *C4BPB* in pBud, and *C4BPA* in pcDNA were used to analyze the different components. Aliquots were removed in each step for analysis with ELISA or SDS-PAGE and Western blotting techniques. The flow scheme of the purification can be seen in supplemental Fig. S1. Gla-containing proteins, *e.g.* PS and PS-complexed proteins, were absorbed to barium citrate and eluted with EDTA (29). To collect C4BP α-chain-containing proteins, the barium citrate-eluted proteins were applied to a HiTrap NHS activated 5-ml column (GE Healthcare) coupled with mAb MK104. Next, the flow-through fraction was applied to a 1-ml HiTrap column coupled with mAb MK36, directed against the C4BP β-chain. Finally, the flow-through from the MK36 column was applied to a 5-ml column coupled with MK54 (recognizing PS). In brief, all purification steps were performed by equilibration of the column with 10 column volumes of TBS, the samples were applied, and the columns were washed with 10 column volumes of TBS. Bound proteins were eluted with 0.1 M glycine-HCl, pH 2.7, and immediately neutralized with the addition of 1 M Tris-HCl, pH 9.0. The activity of the purified C4BP variants as factor I cofactors in degradation of C4b was tested as described (37). The purified proteins were frozen and stored at −20 °C until analysis.

RESULTS

In search for a model system of PS and C4BP synthesis, three liver cell lines, MIHA, HepG2, and Hep3B, were analyzed for PS and C4BP α- and β-chain (mRNA and protein) expression because the liver is known to synthesize both PS and C4BP (Fig. 1). The three cell lines were found to contain approximately the same amounts of mRNAs for the three protein chains. Unexpectedly, in MIHA and HepG2 cells, the molar concentrations of fully assembled C4BP (measured using α-chain ELISA) were 6-fold (MIHA) and 9-fold (HepG2) lower than those of the

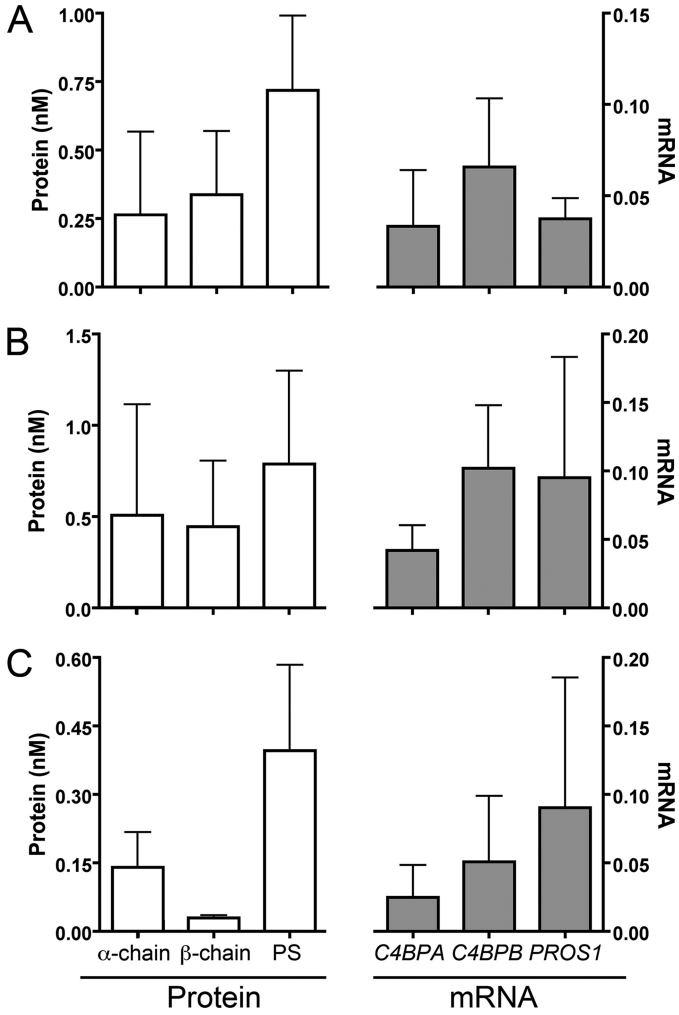

FIGURE 1. **Properties of liver cell lines HepG2, MIHA, and Hep3B.** Protein concentrations (*open bars*) of C4BP α-chain, C4BP β-chain and PS, and mRNA levels (*gray bars*) of *C4BPA*, *C4BPB* and *PROS1* were determined in HepG2 (*A*), MIHA (*B*), and Hep3B (*C*) cells. The protein concentrations were measured in cell medium collected from confluent cells. The mRNA content in the cells was detected by qRT-PCR and presented as the ratio between the gene of interest and the housekeeping gene (*GAPDH*). The *bars* correspond to the mean ± S.D. (*error bars*) value of at least three independent experiments.

β-chain concentrations, suggesting equimolar α- and β-chain expression and that fully assembled 7α1β C4BP is poorly produced. In MIHA and HepG2 cell media, the molar concentrations of β-chain protein were about half of that of PS. Hep3B was the only liver cell line tested that produced C4BP α- and β-chains at the expected 7:1 ratio, suggesting that these cells could potentially be used as means for analyzing the importance of PS for expression of C4BP β-chain. However, the expression levels of C4BP α- and β-chains in Hep3B cells were very low, which precluded further analysis. Moreover, Hep3B cells that were cultured for several passages changed phenotype as the α-chain expression decreased. PS is known to be synthesized by endothelial cells, and we therefore analyzed human umbilical cord endothelial cells, human microvascular endothelial-1 cells, and EA.hy926 cells for expression of C4BP α- and β-chains as well as for PS. All three cell lines contained mRNA for PS whereas no mRNAs for α- and β-chains were detected. Moreover, there was no detectable C4BP in the culture

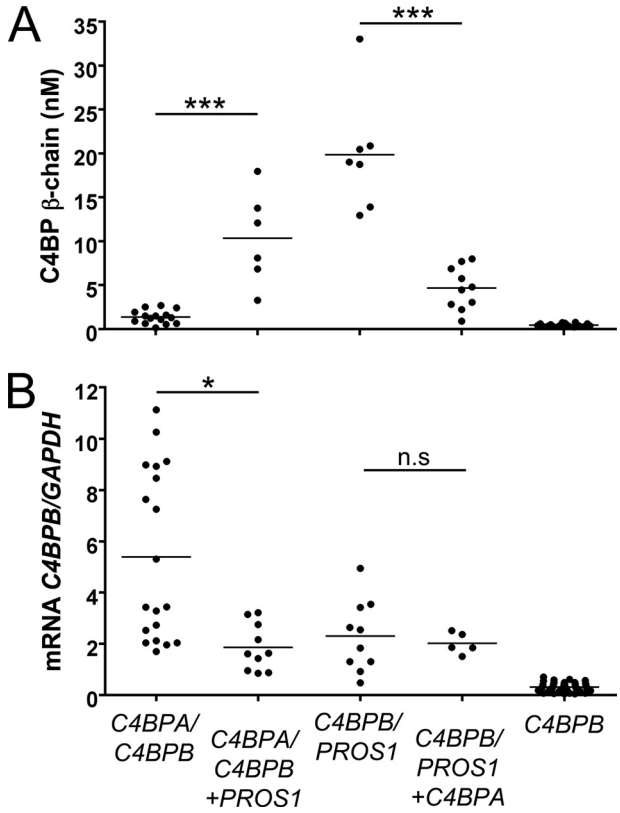

FIGURE 2. **Co-expression of *PROS1* and *C4BPB* increases the expression of C4BP β-chain.** HEK293 cells were stably transfected with the constructs indicated below each data set; *C4BPA/C4BPB* and *C4BPB/PROS1* were in the vector pBudCE4.1, whereas *C4BPA*, *C4BPB*, and *PROS1* were in pcDNA3. Multiple clones were picked and continuously cultured. *A*, concentrations of C4BP β-chain, PS (supplemental Fig. S2*A*), and C4BP α-chain (supplemental Fig. S2*B*) were measured in medium collected from confluent cells. *B*, mRNA levels of *C4BPB*, *PROS1* (supplemental Fig. S3*A*), and *C4BPA* (supplemental Fig. S3*B*) were measured by qRT-PCR. The statistical significance of differences is indicated by *horizontal lines*; *n.s.*, not significant. *, $p < 0.05$; ***, $p < 0.001$.

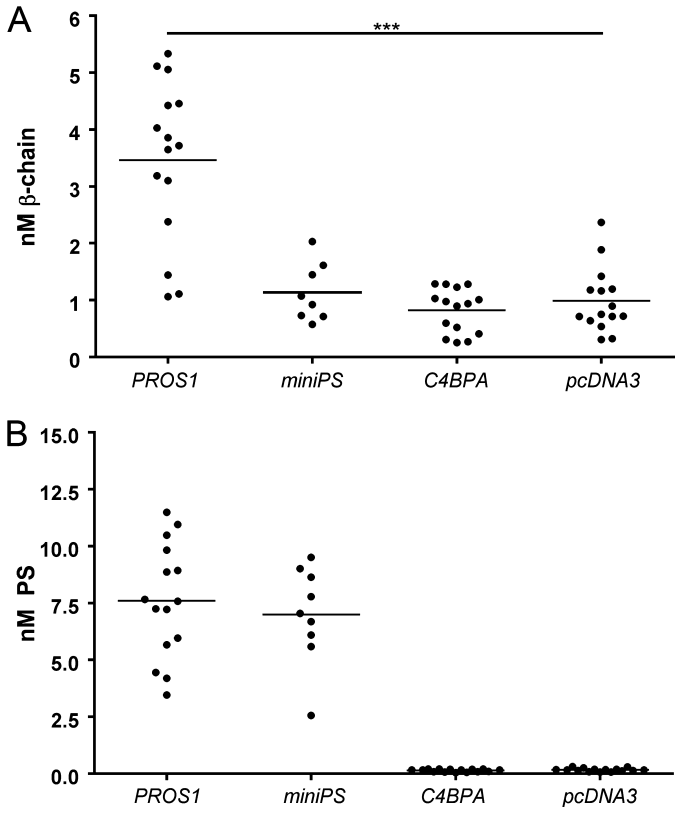

FIGURE 3. **C4BP β-chain expression is stimulated by simultaneous synthesis of PS whereas miniPS and C4BP α-chain have no effect.** HEK293 cells stably transfected with *C4BPB* in pcDNA3 were transiently transfected with *PROS1*, miniPS, cDNA, *C4BPA*, or empty pcDNA3 vector. Concentrations of C4BP β-chain (*A*), PS (*B*), and C4BP α-chain (supplemental Fig. S4) in the cell medium were measured 48 h after transfection. ***, $p < 0.001$.

medium, as measured by ELISA. Based on these results, we concluded that neither liver nor endothelial cell lines were useful for studies of β-chain synthesis. Therefore, we instead turned to recombinant expression systems to develop a model system.

PS Stimulates C4BP β-Chain Secretion—In an effort to express the C4BP β-chain, we stably transfected HEK293 cells with *C4BPB* in pcDNA3. Low levels of β-chain, 0.43 ± 0.16 nM (mean ± S.D.), were detected in the cell medium (Fig. 2*A* and supplemental Table S1). To elucidate whether PS can stimulate β-chain expression, as suggested by the observation that patients with severe PS deficiency also have low levels of C4BPβ$^+$ (28), *C4BPB* and *PROS1* were introduced into the pBudCE4.1 vector to allow simultaneous expression in HEK293 cells. In parallel, a *C4BPA* plus *C4BPB* pBudCE4.1 vector construct was used. The *C4BPA* plus *C4BPB* yielded concentrations of β-chain in the cell medium (Fig. 2*A*) of 1.37 ± 0.76 nM, whereas the vector containing both *C4BPB* and *PROS1* yielded 19.85 ± 6.59 nM β-chain in the cell medium. This difference was not due to differences in mRNA levels because relatively similar *C4BPB/GAPDH* mRNA ratios were obtained with the two vector constructs (Fig. 2*B*). In fact, the *C4BPB/PROS1* cells that produced the highest levels of β-chain had significantly lower *C4BPB* mRNA content than the *C4BPA/C4BPB* cells ($p = 0.0364$).

To elucidate whether it was possible to obtain PS in complex with a fully assembled C4BP containing both α- and β-chains, PROS1 in pcDNA3 was used to transfect the cells that stably expressed C4BP α- and β-chains (*C4BPA* plus *C4BPB* in pBudCE4.1). The introduction of PS to the C4BP α/β-expressing cells resulted in an increase of the expression level of β-chain from 1.37 ± 0.76 nM to 10.33 ± 5.30 nM. In contrast, transfection of *C4BPA* in pcDNA3 to cells stably expressing β-chain and PS (*C4BPB* plus *PROS1* in pBudCE4.1) resulted in a decrease of β-chain from 19.85 ± 6.59 nM to 4.65 ± 2.42 nM (Fig. 2*A* and supplemental Table S1). The mechanisms through which the introduction of *C4BPA* into the *C4BPB/PROS1* cells results in decreased secretion of the β-chain are not obvious. Possibly, the high α-chain expression results in efforts of the cells to incorporate the β-chain into a fully assembled C4BP, a process that could make the complex formation with PS less efficient.

SHBG Region of PS Stimulates C4BP β-Chain Expression—The results discussed above were obtained with stably transfected cells that had undergone a selection process, which possibly could bias the results. Therefore, we also tested a transient transfection system in which cDNA for PS, miniPS, and C4BP α-chain were introduced into cells stably expressing the C4BP β-chain (Fig. 3). Introduction of *PROS1* yielded β-chain levels

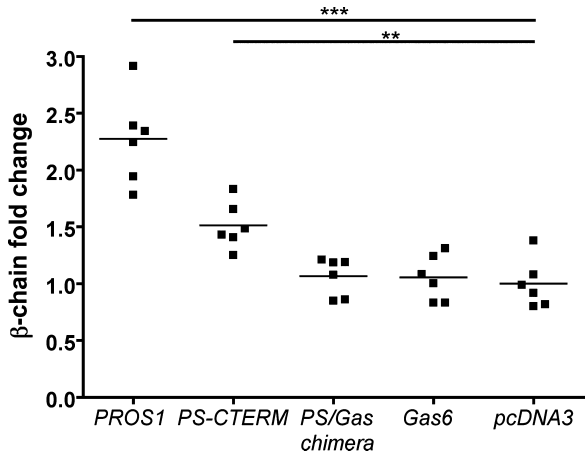

FIGURE 4. **C4BP β-chain expression stimulated by simultaneous synthesis of PS with intact C terminus.** HEK293 cells stably transfected with *C4BPA/C4BPB* in pBudCE4.1 were transiently transfected with wild type *PROS1*, PS-CTERM, *Gas6*, a PS/Gas6 chimeric protein containing the Gas6 C terminus, or empty pcDNA3 vector. The concentrations of total C4BP and C4BP β-chain were measured in cell medium 48 h after transfection. The *dots* represent the fold change in β-chain concentration compared with pcDNA3, after normalization to total C4BP concentration in the same sample. Results that differed significantly from the pcDNA3 sample are marked with ** ($p < 0.01$) and *** ($p < 0.001$).

in the cell medium of 3.46 ± 1.42 nM, whereas mock transfection resulted in 0.99 ± 0.57 nM β-chain in the medium, the difference being highly significant. Transfection with miniPS cDNA or *C4BPA* did not increase the expression level of β-chain (1.13 ± 0.51 nM and 0.82 ± 0.39 nM, respectively). To control the transfection efficiency, the concentrations of PS and miniPS and C4BP α-chain obtained in the cell medium were measured and found to be ~7.6, 7.0, and 3.4 nM, respectively (Fig. 3*B*). Compared with the concentration of the low, but detectable endogenous expression of PS in the HEK293 cells (0.17 ± 0.08 nM), the recombinant PS and miniPS concentrations were 45- and 41-fold higher, respectively.

We next studied the effects of PS on β-chain expression using cells stably expressing both C4BP α- and β-chains from the pBudCE4.1 vector. pcDNA with cDNA coding for PS, PS-CTERM (a PS construct starting in the third EGF domain and extending through the SHBG-region), Gas6, or PS/Gas6 (a chimeric protein being PS from Gla-EGF4 domains and Gas6 in the two laminin G domains) were transiently transfected to this stable cell line. When *PROS1* was used, a 2.3-fold increase in β-chain was observed, whereas neither Gas6 nor the PS/Gas6 chimera affected the β-chain levels (Fig. 4). The PS-CTERM caused a 1.5-fold increase in β-chain concentration into the cell medium. Control experiments, in which recombinant PS up to 1 μg/ml (about 10-fold higher concentrations than obtained with transient transfection) was added to the *C4BPB/C4BPA* cell culture medium, did not affect the expression level of β-chain. Thus, the PS-mediated stimulation of β-chain expression was only seen when PS and the β-chain were co-expressed.

Purification and Characterization of Recombinant C4BP Containing Both α- and β-Chains—To characterize the recombinant C4BP and PS expressed from the stably transfected *C4BPA/C4BPB/PROS1* cells, the medium was subjected to barium citrate absorption, a procedure known to pull down all Gla-containing proteins, *e.g.* PS. C4BP in complex with PS

TABLE 1

Isolation of β-chain-containing C4BP by barium citrate absorption, indicating complete complex formation with protein S but not with C4BP α-chains

Proteins in cell medium collected from HEK293 cells stably transfected with *C4BPB/PROS1* in pBudCE4.1 and *C4BPA* in pcDNA3 were subjected to barium citrate absorption, and the concentration of C4BP, C4BP β-chain, and PS was measured in the obtained fractions.

Sample	Total C4BP		C4BP β-chain		PS	
	μg	%	μg	%	μg	%
Starting medium (900 ml)	3534	100	40	100	787	100
Barium citrate supernatant	3107	88	ND[a]	0	ND	0
Barium citrate absorbed fraction	471	13	38	93	747	94

[a] Not detectable.

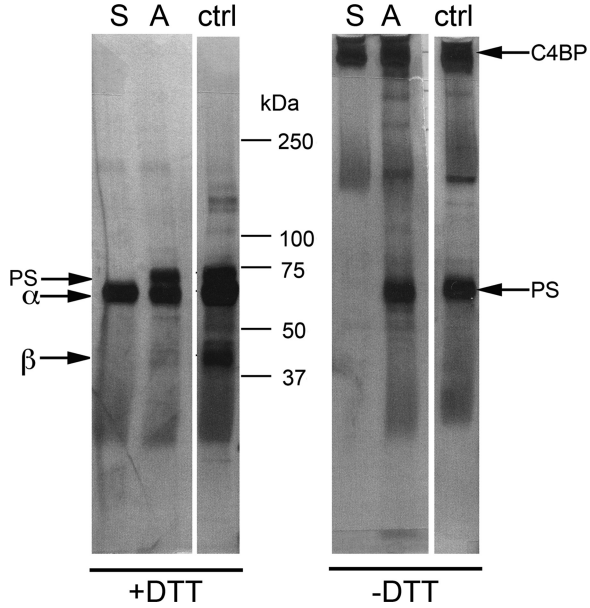

FIGURE 5. **SDS-PAGE analysis of proteins from barium absorption.** Samples (2 μg of protein/*lane*) were applied to a 4–15% gradient gel, electrophoretically separated, and silver stained. Proteins were eluted from the α-chain monoclonal column MK104 after application of barium citrate supernatant (*S*) or absorbed proteins (*A*) and can be compared with plasma-purified C4BP/PS (*ctrl*).

binds to the barium citrate precipitate and can thus be separated from C4BP that only contains α-chains. In this step, 93% of the β-chains were recovered from the barium citrate precipitate, suggesting that they were co-absorbed with PS (PS yield was 94%). This indicated that all β-chains were in complex with PS (Table 1 and Fig. 5). To characterize the β-chain-containing protein further, we applied the proteins that were eluted from the barium precipitate to a C4BP α-chain affinity column (MK104), and the flow-through fraction was tested for the presence of C4BP β-chain. Approximately half (47%) of the β-chains were not co-purified with the α-chains, indicating that only about half of the expressed β-chains were incorporated into the C4BP complex. The eluted proteins visualized in Fig. 5 (A samples) showed that PS was co-purified with C4BP, again indicating that the C4BP contained β-chain and that the PS·C4BP complex was fully assembled. The β-chains that did not bind to the α-chain-specific column were applied on a β-chain-specific mAb column (MK36), and the bound and eluted proteins were found to contain both β-chains and PS (Fig. 6, *A* and *C*). When the barium citrate supernatant was

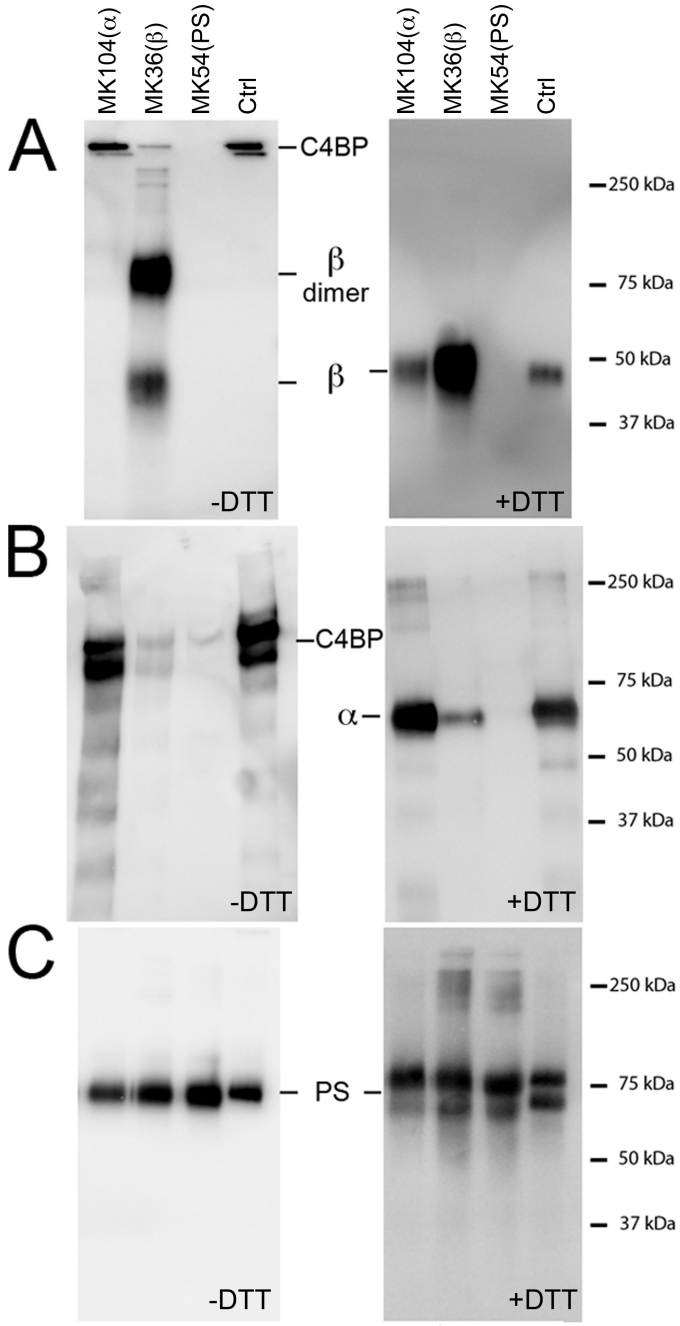

FIGURE 6. **Western blot analysis of proteins eluted in stepwise purification.** Eluted samples (100 ng of protein/*lane*) from the columns MK104 (binding C4BP α-chain), MK36 (binding C4BP β-chain), and MK54 (binding PS) or control (*Ctrl*) PS·C4BP purified from plasma, were applied to a 5% (*B*, *left panel*) or 10% SDS-PAGE, electrophoretically separated and transferred to a PVDF membrane. The proteins were detected with in-house mouse mAb against C4BP β-chain (*A*), in-house rabbit polyclonal antibody recognizing C4BP α-chains (*B*), and in-house mouse mAb against PS (unreduced sample) and a commercial rabbit polyclonal antibody (Dako) recognizing reduced PS (*C*). Adequate HRP-conjugated secondary antibodies were used to enable visualization with the ECL technique. Samples in the *right panel* were reduced by the addition of DTT.

purified on the α-chain affinity column (MK104), only α-chains were visible (*lanes* marked with *S* in Fig. 5) on silver-stained SDS-PAGE. The purified C4BPβ^+ and C4BP-containing α-chains only had normal complement regulatory function, as tested by C4b degradation assay.

Complex between C4BP and PS Is Formed Already Inside the Cells—We were also interested in where the complex between PS and C4BP is assembled. Cells expressing *C4BPA/C4BPB* (pBudCE4.1) and *PROS1* (pcDNA3) were pulsed with radiolabeled amino acids and chased for different time points. Separate *C4BPA/C4BPB* cells and *PROS1* cells were used as controls. Immunoprecipitation (IP) of cell lysates and medium with an antibody against PS revealed that C4BP α-chains and β-chain were co-immunoprecipitated with PS in both medium and lysate, demonstrating that the PS·C4BP complexes formed already in the cells (Fig. 7). When an antibody against C4BP was used in IP, also PS was co-immunoprecipitated. All three isoforms of C4BP (α7β1, α7β0, and α6β1) could be seen after separation on a 5% SDS-polyacrylamide gel after IP with the C4BP antibody; however, the PS antibody IP only pulled down the isoforms containing β-chain (Fig. 7, *top panel*). Control experiments, in which lysates of *C4BPA/C4BPB* cells were mixed with lysates of *PROS1* cells before the IP, yielded no co-immunoprecipitation (results not shown), demonstrating that the complexes were not formed during the IP procedure. Cross-reactivity of the antibodies was excluded by control experiment with cells expressing either C4BP α-chains or PS only. Unspecific bands were identified by IP with control rabbit antibodies.

To elucidate in which cell compartment the β-chain is incorporated into the C4BP molecule and where the PS·C4BP complex is formed, we performed a pulse-chase experiment in presence of brefeldin A, an inhibitor of the transport between Golgi apparatus and endoplasmic reticulum. Brefeldin A efficiently inhibited the secretion of assembled C4BP. However, high molecular mass C4BP could be recovered from the cell lysates by IP with an anti-PS antibody, suggesting that the incorporation of β-chain and the complex formation take place in endoplasmic reticulum.

DISCUSSION

The molecular mechanisms that govern the assembly and secretion of C4BP, with the six to eight α-chains being disulfide-linked to the single β-chain, are essentially unknown. Moreover, the importance of PS for the C4BP subunit assembly and subsequent secretion is not elucidated. We now demonstrate that recombinant overexpression of α-chains plus β-chains together with PS results in the efficient assembly and secretion of C4BP containing α-chains only, whereas the incorporation of β-chains into the C4BP molecule is a relatively inefficient process. Moreover, co-expression of PS with the C4BP β-chain, with or without the α-chains, generates intracellular complexes between PS and the β-chain, a process that is important for the efficient secretion of the β-chain from the cells.

The interaction between PS and the β-chain of C4BP is of very high affinity, and all β-chains in plasma are occupied with PS (27). The free form of PS is thus the molar surplus of PS over the β-chain concentration. In individuals with heterozygous PS deficiency, the free PS decreases, whereas the bound PS can be normal or slightly decreased. However, no unoccupied β-chains exist in plasma. During warfarin treatment, the plasma concentration of PS decreases further, and of particular interest for the present study, the C4BPβ^+ concentration also

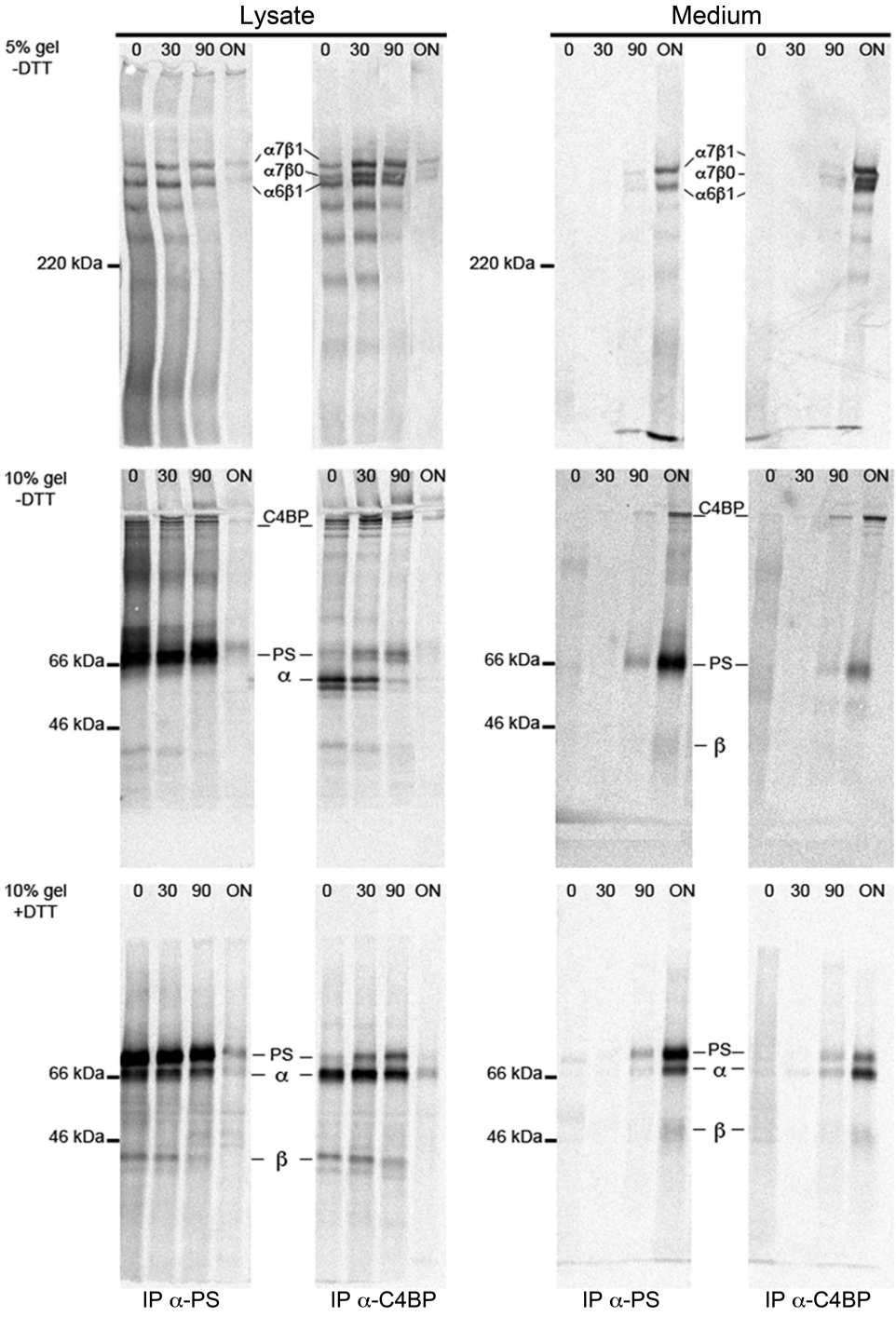

FIGURE 7. **Complex between C4BP β-chain and PS is formed inside the cells.** HEK293 cells expressing PS, C4BP β-chain, and α-chain were metabolically labeled for 30 min (0 sample) and chased for 30 min, 90 min, or overnight (*ON*). At the depicted time points the cells were lysed, and the cell medium was harvested. Proteins in the cell lysates and medium were immunoprecipitated with a rabbit polyclonal antibody binding to either PS or C4BP, and proteins were separated with SDS-PAGE (10% or 5% separation gel) and transferred to a PVDF membrane. The bands on the membrane were visualized with a PhosphorImager FLA-3000.

decreases, even though C4BP is not a vitamin K-dependent protein (28). This suggests that PS is either important for the synthesis and secretion of C4BPβ^+ or for keeping C4BPβ^+ in the circulation. The present study was undertaken to elucidate these questions further.

The liver is the main site for production of both C4BP chains as well as of PS. However, PS is also expressed by many other cell types, including endothelial cells (38), vascular smooth muscle cells (39), megakaryocytes (40), osteoblasts (41), neural tumor cells (42), and Leydig cells in the testis (43). In contrast, fully assembled C4BP containing both α- and β-chains is only synthesized in the liver. The ideal model for studying the expression of C4BP and PS would be liver cell lines of human origin. Unfortunately, most other species cannot be used because PS·C4BP complexes, similar to those present in humans, are only present in primates. Mice and rabbits lack the β-chain, whereas rats lack a cysteine in the β-chain, which results in a β-chain that binds PS with low affinity (44, 45). Unfortunately, the three human liver cell lines HepG2, Hep3B, and MIHA were found not to be useful models of C4BP and PS expression. Both HepG2 and MIHA cells expressed surprisingly little α-chains, precluding studies of the 7α1β isoform of C4BP. In Hep3B cells, the α-chain expression decreased during passage of the cells. The instability in the α-chain synthesis combined with low β-chain expression made them unsuitable for studies of PS·C4BP expression. We therefore used recombinant techniques for the further investigation.

Despite considerable efforts, we have previously been unsuccessful in attempts to express recombinant β-chains alone or together with the α-chains (10). To our knowledge, there are no reports on the expression of recombinant C4BP β-chains alone, even though this may happen *in vivo*. Thus, fibroblasts in human ovaries and in rheumatoid joints have been shown to produce C4BP β-chain, yet no synthesis of α-chain could be detected (25, 26). In HEK293 cells that we transfected with *C4BPB* cDNA alone, very low levels of β-chain were released into the cell medium. However, co-expression with PS resulted in the efficient secretion of PS·β-chain complexes, demonstrating PS to be important for the secretion of the β-chain. It is noteworthy that the α-chains were not important for the β-chain expression and moreover that the β-chains could form disulfide-linked dimers. The question of whether PS is strictly required for β-chain expression

could not be fully resolved with the *C4BPB*-transfected HEK293 cells because the HEK293 cells were found to express low levels of endogenous PS, which may stimulate β-chain secretion. Attempts to knock down the endogenous PS expression using several siRNA and transfection reagents from different vendors were unfortunately unsuccessful (results not shown). We have been unsuccessful in identifying a suitable human cell line that expresses no detectable PS to confirm the absolute requirement of PS for β-chain expression. The use of cell lines of other species is precluded because highly sensitive and specific methods to quantify PS from non-human sources are not available. In a previous report, COS1 cells, which are derived from monkey kidneys and possibly produce traces of PS, were transiently transfected with *C4BPB*. It was observed that most of the β-chain was retained intracellularly, which agrees with our conclusion that PS is important for the secretion of the β-chain (19).

To identify the structural requirements of PS for stimulation of β-chain expression, we transiently transfected wild type PS and miniPS (Gla-EGF4) into HEK293 cells with stably integrated *C4BPB*. MiniPS lacks the binding site for C4BP β-chain and should according to hypothesis not stimulate β-chain expression. This was also found to be the case, as PS, but not miniPS, increased the β-chain levels in the collected cell medium. Similar conclusions were obtained when stable *C4BPA/C4BPB* cells were transiently transfected with cDNA for either wild type PS, PS-CTERM, Gas6, or the PS/Gas6 chimera. Thus, neither Gas6, nor the PS/Gas6 chimera increased β-chain expression, whereas wild type PS and PS-CTERM increased β-chain expression.

Purification and characterization of PS and C4BP proteins synthesized by a stable cell line containing all three genes (*C4BPA*/*C4BPB*/*PROS1*) gave insights into the assembly of the C4BP chains and the interaction with PS. An important observation was that all secreted β-chains were in complex with PS, but only approximately 50% of the β-chains were incorporated into a fully assembled C4BPβ$^+$ molecule. Thus, PS is important for the secretion of the β-chain regardless of whether the β-chains are bound to the α-chains or not. Moreover, close to 90% of the α-chains were recovered in C4BP molecules lacking the β-chain, suggesting that assembly of C4BP α-chains is highly efficient, whereas the incorporation of β-chains into the C4BP molecule is relatively inefficient. The C termini of both α- and β-chains are involved in the covalent interactions that form a fully assembled C4BPβ$^+$. Two cysteines present in each of the chains are involved in the disulfide bridging of the chains, but the details of the cysteine pairing are unknown. The C4BP molecules formed by the α-chains alone appeared correctly assembled. The β-chain could be secreted as part of intact C4BPβ$^+$ molecules, form disulfide-linked dimers, or be secreted as monomers, all forms requiring PS for efficient secretion. Possibly the HEK293 cells lack chaperons that in the liver are important for the incorporation of the β-chain and the assembly of the C4BPβ$^+$ molecules.

PS is now shown to be important for the secretion of the β-chains, but the exact mechanisms remain to be elucidated. The PS-β-chain interaction is hydrophobic in nature (46). It is possible that by binding to the β-chain PS hides the hydrophobic binding patch on the β-chain, which otherwise might serve as a signal for retention in the cell. Results of the pulse-chase experiments, demonstrating that the PS·β-chain complex formation already inside the cells was required for secretion of the β-chain, argue in favor of this mechanism. Earlier experiments have shown that the α-chains are assembled in the ER (17), and our results indicate that it is likely that also the β-chain is incorporated in this step.

In conclusion, this work has shown that it is possible to express recombinant C4BP β-chain and that PS is important for efficient β-chain secretion. Moreover, the expression of C4BP β-chain is independent of C4BP α-chains. The details of the cellular mechanisms behind the effects of PS remain to be determined. The recombinant proteins can in the future be used not only to study cell biology in protein expression, but can also be used to further study the different functions of PS and C4BP and the complex PS·C4BP.

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REFERENCES

1. Dahlbäck, B., and Villoutreix, B. O. (2005) *FEBS Lett.* **579,** 3310–3316
2. Ndonwi, M., Tuley, E. A., and Broze, G. J., Jr. (2010) *Blood* **116,** 1344–1351
3. Hackeng, T. M., Maurissen, L. F., Castoldi, E., and Rosing, J. (2009) *J. Thromb. Haemost.* **7,** Suppl. 1, 165–168
4. Dahlbäck, B., and Stenflo, J. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78,** 2512–2516
5. Maurissen, L. F., Thomassen, M. C., Nicolaes, G. A., Dahlbäck, B., Tans, G., Rosing, J., and Hackeng, T. M. (2008) *Blood* **111,** 3034–3041
6. Evenäs, P., García de Frutos, P., Linse, S., and Dahlbäck, B. (1999) *Eur. J. Biochem.* **266,** 935–942
7. Linse, S., Härdig, Y., Schultz, D. A., and Dahlbäck, B. (1997) *J. Biol. Chem.* **272,** 14658–14665
8. Fernández, J. A., Griffin, J. H., Chang, G. T., Stam, J., Reitsma, P. H., Bertina, R. M., and Bouma, B. N. (1998) *Blood Cells Mol. Dis.* **24,** 101–112; discussion 113
9. Hillarp, A., and Dahlbäck, B. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87,** 1183–1187
10. Härdig, Y., and Dahlbäck, B. (1996) *J. Biol. Chem.* **271,** 20861–20867
11. van de Poel, R. H., Meijers, J. C., and Bouma, B. N. (1999) *J. Biol. Chem.* **274,** 15144–15150
12. van de Poel, R. H., Meijers, J. C., Dahlbäck, B., and Bouma, B. N. (1999) *Blood Cells Mol. Dis.* **25,** 279–286
13. Webb, J. H., Villoutreix, B. O., Dahlbäck, B., and Blom, A. M. (2001) *J. Biol. Chem.* **276,** 4330–4337
14. Burstyn-Cohen, T., Heeb, M. J., and Lemke, G. (2009) *J. Clin. Invest.* **119,** 2942–2953
15. García de Frutos, P., Alim, R. I., Härdig, Y., Zöller, B., and Dahlbäck, B. (1994) *Blood* **84,** 815–822
16. Härdig, Y., García de Frutos, P., and Dahlbäck, B. (1995) *Biochem. J.* **308,** 795–800
17. Kask, L., Hillarp, A., Ramesh, B., Dahlbäck, B., and Blom, A. M. (2002) *Biochemistry* **41,** 9349–9357
18. Blom, A. M., Kask, L., and Dahlbäck, B. (2003) *Mol. Immunol.* **39,** 547–556
19. Sánchez-Corral, P., Criado García, O., and Rodríguez de Córdoba, S. (1995) *J. Immunol.* **155,** 4030–4036
20. Arenzana, N., Rodríguez de Córdoba, S., and Rey-Campos, J. (1995) *Biochem. J.* **308,** 613–621
21. Criado García, O., Sánchez-Corral, P., and Rodríguez de Córdoba, S. (1995) *J. Immunol.* **155,** 4037–4043

22. Kask, L., Trouw, L. A., Dahlbäck, B., and Blom, A. M. (2004) *J. Biol. Chem.* **279,** 23869–23873
23. Webb, J. H., Blom, A. M., and Dahlbäck, B. (2002) *J. Immunol.* **169,** 2580–2586
24. Webb, J. H., Blom, A. M., and Dahlbäck, B. (2003) *Blood Coagul. Fibrinolysis* **14,** 355–359
25. Criado-García, O., Fernaud-Espinosa, I., Bovolenta, P., Sainz de la Cuesta, R., and Rodríguez de Córdoba, S. (1999) *Eur J. Cell Biol.* **78,** 657–664
26. Sánchez-Pernaute, O., Esparza-Gordillo, J., Largo, R., Calvo, E., Alvarez-Soria, M. A., Marcos, M. E., Herrero-Beaumont, G., and de Córdoba, S. R. (2006) *Ann. Rheum. Dis.* **65,** 1279–1285
27. Giri, T. K., Linse, S., García de Frutos, P., Yamazaki, T., Villoutreix, B. O., and Dahlbäck, B. (2002) *J. Biol. Chem.* **277,** 15099–15106
28. Zöller, B., García de Frutos, P., and Dahlbäck, B. (1995) *Blood* **85,** 3524–3531
29. Dahlbäck, B. (1983) *Biochem. J.* **209,** 837–846
30. Malm, J., Cohen, E., Dackowski, W., Dahlbäck, B., and Wydro, R. (1990) *Eur J. Biochem.* **187,** 737–743
31. Dahlbäck, B. (1983) *Biochem. J.* **209,** 847–856
32. Hillarp, A., and Dahlbäck, B. (1988) *J. Biol. Chem.* **263,** 12759–12764
33. Van Wijnen, M., Stam, J. G., Chang, G. T., Meijers, J. C., Reitsma, P. H., Bertina, R. M., and Bouma, B. N. (1998) *Biochem. J.* **330,** 389–396
34. Ades, E. W., Candal, F. J., Swerlick, R. A., George, V. G., Summers, S., Bosse, D. C., and Lawley, T. J. (1992) *J. Invest. Dermatol.* **99,** 683–690
35. Wu, J. C., Merlino, G., and Fausto, N. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91,** 674–678
36. Edgell, C. J., McDonald, C. C., and Graham, J. B. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80,** 3734–3737
37. Dahlbäck, B., and Hildebrand, B. (1983) *Biochem. J.* **209,** 857–863
38. Fair, D. S., Marlar, R. A., and Levin, E. G. (1986) *Blood* **67,** 1168–1171
39. Benzakour, O., and Kanthou, C. (2000) *Blood* **95,** 2008–2014
40. Ogura, M., Tanabe, N., Nishioka, J., Suzuki, K., and Saito, H. (1987) *Blood* **70,** 301–306
41. Maillard, C., Berruyer, M., Serre, C. M., Dechavanne, M., and Delmas, P. D. (1992) *Endocrinology* **130,** 1599–1604
42. Phillips, D. J., Greengard, J. S., Fernandez, J. A., Ribeiro, M., Evatt, B. L., Griffin, J. H., and Hooper, W. C. (1993) *J. Neurochem.* **61,** 344–347
43. Malm, J., He, X. H., Bjartell, A., Shen, L., Abrahamsson, P. A., and Dahlbäck, B. (1994) *Biochem. J.* **302,** 845–850
44. Hillarp, A., Wiklund, H., Thern, A., and Dahlbäck, B. (1997) *J. Immunol.* **158,** 1315–1323
45. He, X., and Dahlbäck, B. (1994) *Thromb. Haemost.* **71,** 446–451
46. Blom, A. M., Covell, D. G., Wallqvist, A., Dahlbäck, B., and Villoutreix, B. O. (1998) *Biochim. Biophys. Acta* **1388,** 181–189