Human protein S (PS) potentiates the anticoagulant activity of human but not bovine activated protein C (APC), whereas bovine PS is a cofactor to APC from both species. The structural requirements for the specificity of the APC cofactor function of human PS are located in its thrombin-sensitive region (TSR) and the first epidermal growth factor (EGF1)-like module. To elucidate which residues in these two modules determine the specificity of the APC cofactor activity, 41 human PS mutants were expressed. All mutants were cofactors to human APC and some also to bovine APC. Residues in TSR (positions 49 and 52) and EGF1 (residues 97 and 106) together determined the specificity of the APC cofactor function, whereas substitution of individual residues did not change specificity. Bovine PS, and mutants expressing cofactor activity to bovine APC, stimulated phospholipid binding of bovine APC. In contrast, human PS and mutants lacking cofactor activity to bovine APC failed to support binding of bovine APC to phospholipids. These data indicate that residues in TSR and EGF1 cause the specificity of the APC cofactor activity and support the concept that key residues in these two modules interact with APC on the phospholipid surface.

Protein S (PS) is a vitamin K-dependent plasma protein that functions as an anticoagulant cofactor to activated protein C (APC) in the degradation of the activated forms of coagulation factor V and factor VIII (1, 2). The biological importance of PS as an anticoagulant is clearly demonstrated by the massive thrombotic complications that affect infants with homozygous PS deficiency and by an increased risk of venous thrombosis in individuals with heterozygous PS deficiency (3–6).

The clear association between thrombosis and PS deficiency stands in sharp contrast to the weak APC cofactor function of PS in vitro. Thus, PS was reported to yield only a 2-fold increase in the rate of APC-mediated degradation of factor Va in a purified system (7). However, more recent results suggest that PS expresses rather specific APC cofactor function, because in the degradation of factor Va by APC, PS was found to stimulate only the APC-mediated cleavage at Arg\textsuperscript{306} and not the cleavage at Arg\textsuperscript{306} \textsuperscript{8}. That the protein C system indeed is more complicated than previously known became even more evident when factor V was found to function in synergy with PS as cofactor to APC (9, 10). Degradation of factors Va/VIIIa thus depends on the assembly of multimolecular complexes on the phospholipid membrane via molecular mechanisms that are still incompletely understood.

PS has a high affinity for negatively charged phospholipid (11), a property that is important for its APC cofactor activity because PS increases the affinity of APC for negatively charged phospholipid vesicles (12), endothelial cells (13), platelets (14, 15), and platelet microparticles (16). Recently, the interaction between APC and PS on the phospholipids was found to relocate the active site of APC about 10 Å closer to the membrane surface (17). The PS-induced conformational changes may be important for selectivity of the APC function. PS is also reported to have APC-independent anticoagulant function, which depends on direct interactions of PS with phospholipid membranes as well as with factor Va and factor Xa, and which leads to inhibition of factor X and prothrombin activation (18–20). However, the biological significance of the APC-independent functions of PS is still uncertain.

In human plasma, approximately 60% of PS is noncovalently complexed to C4b-binding protein (C4BP), a regulator of the classical complement pathway (21). Upon complex formation, PS loses its anticoagulant function (22) but retains its ability to bind negatively charged phospholipids (23).

The primary structures of human, monkey, bovine, rabbit, porcine, mouse, and rat PS have been elucidated (24–29). Mature PS is a mosaic protein composed of multiple modules. Starting from the NH$_2$ terminus, it contains a \(\gamma\)-carboxyglutamic acid (Gla)-rich module, a thrombin-sensitive region (TSR), four EGF-like modules, and a sex hormone-binding globulin-like module. The Gla module binds calcium and negatively charged phospholipids. The integrity of the TSR has been shown to be essential for the APC cofactor activity (30). Probing the structure-function relationships with well characterized monoclonal antibodies has suggested the TSR and EGF1 mod-
ules to be important for expression of full APC cofactor activity (31). Fab' fragments of monoclonal antibodies directed against these two modules completely inhibited the APC cofactor activity, whereas antibodies against other modules had no or very little effect. Additional support for the involvement of EGF1 in the PS-APC interaction has recently been gained from two studies. Thus, a thrombotic patient with type II protein S deficiency (functional defect) was found to carry an intron mutation in the PS gene resulting in exon 5 skipping and as a consequence a truncated PS molecule lacking EGF1 (32). The truncated PS was present at normal concentration in the patient plasma suggesting that the EGF1 deletion does not influence the synthesis of the protein. In the second report, a recombination PS fragment comprising EGF1–4 was found to inhibit the APC cofactor activity of protein S, whereas an EGF2–4 construct had no such inhibitory activity (33).

The anticoagulant activity of bovine APC is reported to be species-restricted, which has been found to be due to the interaction between APC and PS (22, 34). Thus, bovine APC is inefficient as anticoagulant in human plasma unless bovine PS is also present. The interpretation of this is that bovine APC and human PS are unable to form an anticoagulant complex on the phospholipid surface. Human APC on the other hand is found to function both in human and bovine plasma, suggesting human APC to interact with both human and bovine PS (22). The structural determinants in human PS responsible for the species-restriction have been demonstrated to be located in both the TSR and EGF1 modules (35). Thus, human/bovine recombinant PS chimeras containing both these modules of bovine origin were found to function like bovine PS, whereas those chimeras having only one of the two modules of bovine origin did not. With respect to specificity of the APC cofactor function, porcine PS functions like bovine PS, whereas monkey PS is similar to its human counterpart (25). Rabbit PS is not an efficient cofactor to bovine APC but is stimulating the anticoagulant properties of human APC (27). By sequence comparison, a number of amino acid residues have been suggested to play a role for the species restriction. The candidate residues are Arg49, Gln52, Thr53, Gln61, Pro77, Ser81, Ser92, Lys97, Ser99, Lys102, and Pro106 (25, 35).

Elucidation of the molecular basis of the specificity of the APC-PS interaction may provide valuable information as to which residues are functionally important in the interaction between APC and PS. The aim of this work was to identify amino acid residues in the TSR and EGF1 regions that are involved in determining the specificity of the APC cofactor function. This was achieved by expressing human recombinant PS mutants in which individual amino acids in the TSR and EGF1 were replaced with amino acid residues present at corresponding positions in bovine PS. The mutations were mapped on a preliminary model structure for the Gla-TSR-EGF1 region of human PS in order to further analyze our results.

**EXPERIMENTAL PROCEDURES**

**Materials**—Kits for DNA sequencing and the T7-Gen in vitro mutagenesis were from U. S. Biochemical Corp.; Q-Sepharose Fast Flow was obtained from Amersham Pharmacia Biotech, and hygromycin B was from Calbiochem. Saline of phosphate buffer without calcium and carbonate, Lipofectin, Opti-MEM medium, and Dulbecco's modified Eagle's medium were from Life Technologies, Inc. Polystyrene latex beads were from Sigma. The chromogen substrate 1-Palmitoyl-2-[1-14C-oleoyl]PC was from NEN Life Science Products. Lipids, Inc. 1-Palmitoyl-2-[1-14C-oleoyl]PC was from NEN Life Science Products. The human PS deficiency plasma was prepared by incubating normal human plasma with immobilized polyclonal anti-human PS at 4°C overnight as described (22). Bovine PS-deficient plasma was prepared in a similar way but using immobilized polyclonal anti-bovine PS.

**Proteins**—PS and activated protein C were prepared from human and bovine plasma using methods previously described (31). The monoclonal antibodies that reacted with human PS have been previously characterized (31).

**cDNA Clones**—Full-length human and bovine PS cDNA were previously isolated and characterized (26, 36). The original bovine PS cDNA clone was missing exon 2, a BclI-EcoRI EGF-like module. To express wild-type bovine PS, site-directed mutagenesis was used to introduce the missing codon. BamHI sites were introduced at both 5′- and 3′-end of the coding regions of full-length human and bovine CDNA clones as described previously (35). The numbering of the nucleotides of human and bovine PS cDNA used in this paper is based on the sequence which are available in the GenBank Data Bank having accession numbers M15036 and M13044, respectively.

**In Vitro Mutagenesis**—Three human/bovine PS chimeras were prepared in which the bovine TSR, EGF1, or both modules were introduced into human PS, i.e., the bovine modules replaced corresponding human modules. Mutant 1 containing the bovine EGF1 module was prepared from the previously described chimeras V (35). This chimeras contained the Gla, TSR, and EGF1 modules of bovine origin with the rest being of human origin. A cleavage site for HincII (position 454) located between TSR and EGF1 in both human and bovine PS cDNA was used to create the mutant 1. BamHI-XbaI (XbaI cleaves at 1481) fragments of both human PS cDNA and chimeras V were cleaved with HincII. The isolated larger HincII-XbaI fragment from chimera V was ligated with the small BamHI-HincII fragment from human PS cDNA and the 3′ XbaI-BamHI fragment to create full-length cDNA in BamHI-cleaved pUC18. This full-length construct, which contained EGF1 of bovine origin in a human PS background, was transferred to the expression vector pGT-h as described below. Mutant 6, which contained both TSR and EGF1 of bovine origin, was constructed in a similar way utilizing an NcoI cleavage site which was present in both human (position 372) and bovine (position 261) PS cDNA between the Gla and TSR modules. In short, chimeras V was cut with NcoI and BamHI, and the large fragment was isolated and ligated to the small BamHI-NcoI fragment obtained from the human cDNA clone. Mutant 5, which contained only the TSR of bovine origin, was made from mutant 6 and human PS cDNA. BamHI-XbaI fragments of both these cDNAs were cut with HincII and the BamHI-HincII fragment from mutant 6 was ligated to the BamHI-XbaI fragment from human PS together with the 3′-part XbaI-BamHI fragment to create full-length molecules. As a result, the mutant contained the TSR of bovine origin, and the rest was human PS.

Twelve EGF1 mutants and three TSR mutants were prepared. The sequences of the oligonucleotides used in the mutagenesis are given in Table I. Mutants containing multiple mutations were made using combinations of the single mutants. All EGF1 mutations were made using the BamHI-XbaI fragment, whereas a BamHI-HindIII fragment (HindIII cleaves at position 559) was used for the TSR mutations. These fragments of human PS cDNA were subcloned into M13mp18, and single-strand templates were prepared using standard methods (37). Site-directed mutagenesis was accomplished by the "gapped duplex" method (T7-Gen In Vitro Mutagenesis kit from U. S. Biochemical Corp.) using M13mp18 or M13mp19 carrying the PS cDNA were annealed with each specific oligonucleotide, subjected to the second strand synthesis with T7 DNA polymerase, and ligated with T4 DNA ligase. The DNA was used to transfect competent E. coli S. typhimurium (mcrA– merB–) cells. Single-stranded DNA from individual resultant plagues was isolated and sequenced by the dideoxy chain termination method using M13 or PS-specific primers (40). The mutated PS cDNA inserts were isolated from double-stranded phage DNA (HincII-XbaI for EGF1 mutants and BamHI-HindIII for TSR mutants), and the appropriate fragments of human PS cDNA were used to construct full-length human PS cDNA in pUC18. With the help of the HindIII site between TSR and EGF1, combinations between the TSR and EGF1 modules were prepared using standard restriction enzyme cleavage and fragment ligation procedures, as outlined above. Mutant cDNAs were isolated after BamHI digestion and subcloned into the Bluescript site of the expression plasmid pGT-h, which was a kind gift from Dr. B. W. Grinnell (Lilly)(41). The resultant PS cDNA expression plasmids were prepared by CsCl gradient ultracentrifugation (37) and used to transfect 293 cells.

**Cell Culture and Expression**—The adenovirus-transfected human kidney cell line 293 was grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin and streptomycin, 10 μg/ml vitamin K1. Transfection was performed using the Lipofectin method (42). DNA (2–4 μg) was diluted to 0.1 ml with sterile water. Lipofectin was added (1 μg/μl), and samples were left at room temperature for 10–15 min. Cell monolayers (40–50% confluent in a 5-cm Petri dish) were washed twice in serum.
Species Specificity of Protein S Cofactor Function

Oligonucleotides are numbered to indicate mutated amino acids. The underlined positions denote nucleotide replacements.

| hPS52 | 5′-GAA TAA CCC AGT TGG AAA AGA GCG AAG |
| hPS49/52 | 5′-AGG AGT GAA TAA CCC AGT TGG AAA AGA GCG AAG |
| hPS61 | 5′-AGG AGT GAA TAA CCC AGT TGG AAA AGA GCG AAG |
| hPS67 | 5′-AGG AGT GAA TAA CCC AGT TGG AAA AGA GCG AAG |
| hPS71 | 5′-GGA TGG CAG AGG ATT ACA AIA GTC TGG TGG-3′ |
| hPS81 | 5′-GGA TGG CAG AGG ATT ACA AIA GTC TGG TGG-3′ |
| hPS92 | 5′-CCA TCT TTA CAG GTC ATA SAT CCA CAT TAC-3′ |
| hPS97 | 5′-TAA AAG AAG CCT GTC CAT CTT TGC AG-3′ |
| hPS97/99 | 5′-TAA AAG AAG CCT GTC CAT CTT TGC AG-3′ |
| hPS103/106 | 5′-CTT GTC CAA CCT GAT TTA CAA GTG C-3′ |
| hPS106 | 5′-CTT GTC CAA CCT GAT TTA CAA GTG C-3′ |

Measure of Interaction of Polystyrene Latex Beads

Adsorption of Phospholipid Vesicles to Polystyrene Latex Beads—Phospholipid vesicles composed of 20% phosphatidylserine and 80% phosphatidylcholine (plus a tracer of [14C]phosphatidylcholine) were used as described by Smirnov et al. (49). In brief, the lipids were suspended (1 mg/ml) by vortexing in 0.15 M NaCl, 0.02 mM Tris-HCl, pH 7.4 (TBS), containing 0.02% sodium azide and sonicated for 20 min in an ice-water bath under argon flow. After sonication, the suspension was centrifuged at 8000 × g for 15 min and passed through a 100-nm nucleopore membrane in an extruder (Avestin) to obtain homogenous vesicles. The vesicles were used immediately or stored at −80 °C. The vesicles were adsorbed to latex beads as described by Smirnov et al. (50). In brief, polystyrene latex beads (100 μl) were pelleted in an Eppendorf tube by centrifugation (8000 × g for 1 min), washed three times with TBS, and resuspended in 100 μl of TBS containing 5 mM CaCl2. The suspension was continuously mixed at room temperature with shaking for 2 h. After washing twice with TBS, the beads were suspended in 1 ml of TBS. The concentration of phospholipid was determined by counting the [14C]phosphatidylcholine tracer which was included in the phospholipid mixture (Beckman model LS 6000 SE scintillation counter). The beads were used immediately or stored at +4 °C for up to 3 weeks.
RESULTS AND DISCUSSION

Comparison of TSR and EGF1 Sequences in Different Species and Site-directed Mutagenesis Strategy—Bovine PS supports the activity of both human and bovine APC, whereas human PS is restricted in its activity and only expresses activity to human APC. Sequence differences in the TSR and EGF1 regions between human and bovine PS explain this difference in specificity (22, 34, 35). Species restriction of the APC-PS interaction is not only expressed by the human-bovine pair but also by other species combinations. Thus, monkey PS (mPS) is functionally similar to hPS (neither of them stimulate bovine APC), whereas porcine PS (pPS) is similar to bPS (stimulates both human and bovine APC) (25). Comparison of PS amino acid sequences from these four species (human, monkey, bovine, and porcine) reveals a limited number of amino acid differences in the TSR and EGF1 which possibly are involved in the species restriction of the PS function (Fig. 1). In the TSR, there are three amino acid residues that are identical in hPS and mPS and different from the bovine/porcine PS sequences. Position 49 in hPS and mPS is occupied by Arg, whereas bPS and pPS have Gln; at position 52, hPS and mPS have Gln, whereas bPS and pPS have Arg; at position 61, hPS and mPS have Gln, whereas bPS and pPS have Arg; at position 81, hPS and mPS have Gln, whereas bPS and pPS have Arg; at position 99, hPS and mPS have Gln, whereas bPS and pPS have Arg; at position 97, hPS and mPS have Glu, whereas bPS and pPS have Gln, whereas Leu is found in both bPS and pPS. In the EGF1 module, the following six positions have a consensus sequence for the human/mouse pair that is different from that of the bovine/porcine one: Ser81 → Asn, Tyr90 → Phe, Ser92 → Thr, Lys97 → Gln, Thr103 → Ile, and Pro106 → Ser. In addition, at positions 77 and 99 there are some differences between the four sequences which, however, do not obey the human/mouse and bovine/porcine pairing rule. Thus, at position 77, Pro is found in hPS, mPS, and pPS, whereas bPS has Ser, and at position 99 hPS and mPS have Ser, and bPS has Thr, and pPS has Met.

Expression and Characterization of Recombinant Wild-type and Mutant PS—In addition to the discussed four primary structures, amino acid sequences of rabbit, rat, and mouse PS are known, and the sequences of the TSR and EGF1 of these species are also given in Fig. 1. It has been demonstrated that rabbit PS potentiates the anticoagulant activity of human APC, whereas rabbit PS is a poor cofactor to bovine APC. In this context it is interesting to note that the rabbit sequence is identical to the bovine/porcine pair at positions 77, 81, 97, and 103. However, it is identical to the human/mouse pair in having a Pro at position 106. Moreover, it is identical to hPS, mPS, and pPS in having a Pro at position 77, a position at which there is a Ser in bPS. Thus, at position 77, Pro is found in hPS, mPS, and pPS, whereas bPS has Ser, and at position 99 hPS and mPS have Ser, and bPS has Thr, and pPS has Met.
bovine chimeras for TSR and EGF1 individually, as well as for the two in combination. In addition, site-directed mutagenesis was used to create a number of mutants (Table II) in order to elucidate the importance of the individual amino acids in the two modules for the specificity of PS. It was not possible to create all different combinations of the eight different amino acids that were likely to be involved (positions 49, 52, and 61 in TSR and positions 81, 90, 92, 97, 103, and 106 in EGF1), and our strategy was to generate a limited number of mutations in the two modules. Forty one different recombinant mutants were expressed in addition to the wild-type human and bovine PS.

The mutant recombinant proteins were expressed as stable cell lines in human 293 cells as described previously for the human/bovine chimeras (35). The level of PS expression was estimated with an ELISA and found to vary between 0.6 and 1.2 mg/10^6 cells/24 h. The mutants were purified and characterized. In unreduced SDS-PAGE, the proteins migrated as

| Protein S mutants | Asp38 | Val46 | TSR | Ala75 | EGF1 | Asp116 |
|-------------------|-------|-------|-----|-------|------|--------|
| 1                 |       |       |     |       |      |        |
| 2                 |       |       |     |       |      |        |
| 3                 |       |       |     |       |      |        |
| 4                 |       |       |     |       |      |        |
| 5                 |       |       |     |       |      |        |
| 6                 |       |       |     |       |      |        |
| 7                 |       |       |     |       |      |        |
| 8                 |       |       |     |       |      |        |
| 9                 |       |       |     |       |      |        |
| 10                |       |       |     |       |      |        |
| 11                |       |       |     |       |      |        |
| 12                |       |       |     |       |      |        |
| 13                |       |       |     |       |      |        |
| 14                |       |       |     |       |      |        |
| 15                |       |       |     |       |      |        |
| 16                |       |       |     |       |      |        |
| 17                |       |       |     |       |      |        |
| 18                |       |       |     |       |      |        |
| 19                |       |       |     |       |      |        |
| 20                |       |       |     |       |      |        |
| 21                |       |       |     |       |      |        |
| 22                |       |       |     |       |      |        |
| 23                |       |       |     |       |      |        |
| 24                |       |       |     |       |      |        |
| 25                |       |       |     |       |      |        |
| 26                |       |       |     |       |      |        |
| 27                |       |       |     |       |      |        |
| 28                |       |       |     |       |      |        |
| 29                |       |       |     |       |      |        |
| 30                |       |       |     |       |      |        |
| 31                |       |       |     |       |      |        |
| 32                |       |       |     |       |      |        |
| 33                |       |       |     |       |      |        |
| 34                |       |       |     |       |      |        |
| 35                |       |       |     |       |      |        |
| 36                |       |       |     |       |      |        |
| 37                |       |       |     |       |      |        |
| 38                |       |       |     |       |      |        |
| 39                |       |       |     |       |      |        |
| 40                |       |       |     |       |      |        |
| 41                |       |       |     |       |      |        |
| wt:hPS            |       |       |     |       |      |        |
| wt:bPS            |       |       |     |       |      |        |
single chain bands, like the wild-type human and bovine PS and the respective plasma-derived proteins (not shown). As previously noted, the human PS and the mutants had slightly higher molecular weight than the bovine PS, which was due to differences in number of carbohydrate side chains in human (three carbohydrate sites) and bovine (two carbohydrate sites) PS (24, 26, 52). After reduction, the human and bovine wild-type proteins and some of the recombinants migrated as closely spaced doublets, just like the human and bovine plasma-derived PS preparations. The purified wild-type and mutant recombinant PS were fully post-translationally modified as judged by their Gla and Hya/Hyn residue contents, which were found to be similar to those of the plasma-derived proteins (results not shown). Human and bovine plasma-derived PS contained 2.4 and 3.9 mol of Hya/Hyn per mol of protein, respectively. Recombinant PS mutants yielded values close to that of plasma-derived human PS suggesting that the mutations in the TSR and EGF1 modules in human PS did not affect the hydroxylation reaction. Amino-terminal protein sequence analysis yielded the expected sequences of recombinant wild-type and mutant PS (data not shown). All recombinant PS variants were found to bind to immobilized human C4BP with high affinity (data not shown), half-maximum binding occurred at 5–20 nM PS, suggesting the different recombinant proteins to be correctly folded.

**APC Cofactor Activity of Recombinant Wild-type and Mutant PS**—The APC cofactor activity of the recombinant proteins was tested in an APTT-based assay using human or bovine PS-deficient plasma and human or bovine APC. The use of bovine and human PS-deficient plasma yielded similar results demonstrating that the species of factor V(a) did not influence the results. Only the results obtained with human PS-deficient plasma will be shown. In the absence of added PS, both human and bovine APC yielded approximately 10 s prolongation of the clotting time. In the absence of APC, the addition of protein S did not prolong the clotting time. In the presence of APC, wild-type human and bovine PS (and the plasma-derived proteins) yielded results in accordance with those on record, i.e., both human and bovine PS potentiated the anticoagulant activity of human APC, whereas only bovine PS functioned as cofactor to bovine APC. In this system, bovine PS was found to be more potent than its human counterpart even in the presence of human APC. The plasma-derived human and bovine PS gave results similar to those of their recombinant counterparts (results not shown).

The APC cofactor functions of the 41 mutants as well as the wild-type human and bovine recombinant PS and their plasma-derived counterparts were tested in the APTT-based assay at seven different PS concentrations (0–10 μg/ml final concentration) using both human and bovine APC. Fig. 2 illustrates the activities of some of the mutants. In order to extract the relevant information from the resulting 630 data points and simplify the interpretation, the following approach was adopted. The results obtained with the wild-type human PS were used as reference in regression analysis of the clotting results obtained with the different mutants. The slopes of the regression equations indicated the relative potency of the different mutants (i.e., their activities were in this way related to that of the human wild-type PS). Thus, PS mutants with potent APC cofactor activity resulted in steep slopes with high slope coefficients. The regressions slope coefficients obtained using both human and bovine APC are plotted in histogram form in Fig. 3.

Mutant 1, in which the whole EGF1 was of bovine origin, was slightly more active than wt-hPS both in the presence of human and bovine APC, but just like hPS it was a poor cofactor to bovine APC. The same was true for mutant 5 which had its TSR of bovine origin. When TSR and EGF1 were both of bovine origin (mutant 6), the recombinant product behaved like wt-bPS and functioned as a potent cofactor to both human and bovine APC. Like wt-bPS, this mutant expressed higher cofactor activity to bovine and human APC as did wt-hPS, suggesting sequence differences between the human and bovine TSR and EGF1 modules to be the cause for this difference.

To elucidate which amino acid residues were important in the TSR for the species restriction of hPS, different TSR mutants were introduced in a recombinant human PS having its EGF1 of bovine origin (mutants 2–4 and 6). Individual replacements of Gln52 with Arg (mutant 3) or Gln61 with Leu (mutant 4) did not lead to enhanced activity to bovine APC. However, mutant carrying the two replacements R49G and Q52R was functionally similar to bovine APC and expressed as high cofactor activity to bovine and human APC as did wt-bPS.

To determine which amino acid residues in the EGF1 module were important for the species restriction of hPS, three different groups of EGF1 mutants carrying different mutations in the TSR were expressed and characterized. In the first TSR group (GRA→), the TSR had the R49G, Q52R, and T53A replacements; the second group (→L) had only the Q61L replacement; and the third group had the full bovine TSR (GRAL) with all four replacements (R49G, Q52R, T53A, and Q61L). All the recombinant mutants of the →L group behaved like hPS and expressed low activity against bovine APC, even though there were some minor differences in activity between the

**Fig. 2. APC cofactor activity of protein S mutants.** Increasing concentrations of selected PS mutants (final concentrations of 0–10 μg/ml) were included together with human APC (A) or bovine APC (B) (both at final concentration of 0.3 μg/ml) in an APTT-based assay using PS-deficient human plasma, and the clotting time was measured. The values are the means of triplicate measurements. The clotting time without added APC was 34 s. ◊, wt hPS; Δ, wt bPS; +, mutant 1; ×, mutant 7; ○, mutant 8; ■, mutant 9; ▲, mutant 10; ○, mutant 32; □, mutant 33; ●, mutant 40.
different EGF1 mutants. Some were distinctly more active than wild-type human PS. The results obtained with the GRA- and GRAL group mutants were qualitatively similar although the GRAL group mutants were consistently more potent than corresponding mutants in the GRA-group. The presentation will focus on the GRAL group, but the conclusions are also valid for the GRA-group. In the GRA- and GRAL groups, three individual replacements in the EGF1 were expressed, the S81N (mutants 16 and 39), the K97Q (mutants 7 and 30), and the P106S (mutants 8 and 31). The S81N replacement did not affect the species restriction, whereas both the K97Q and the P106S replacements resulted in molecules functioning like bovine PS. In particular the P106S replacement in the GRA-group was noteworthy, because the introduction of these few amino acid replacements in the human PS molecule led to a dramatic change in PS functional activity. Not only was the APC cofactor specificity changed from human to bovine type, but, in addition, the resulting molecule was distinctly more active to human APC than the wt-human PS, approximately 200% activity as compared with the wt-human PS (Fig. 3). Addition of the P77S (mutants 18 and 41) replacement or the S81N (mutants 17 and 40) replacement to the P106S containing mutants did not increase the APC cofactor activity further. Likewise, addition of the S92T replacement to the K97Q mutant (mutants 9 and 32) did not increase the cofactor activity to bovine APC further, whereas when the S99T replacement was introduced into the K97Q mutant (mutants 10 and 33) this led to an increased activity of the recombinant proteins up to the level of wt-bPS. The combination of S92T, K97Q, T103I, and P106S replacements (mutants 11 and 34) was not better than...
the K97Q replacement alone and even somewhat less active than the P106S mutants. Among the most potent recombinants were mutants 13–14 and 36–37 which expressed potent APC cofactor activity to both bovine and human APC. In common, these PS mutants had the S92T, K97Q, T103I, and P106S replacements and in addition either the S81N (mutants 13 and 36) or the S99T (mutants 14 and 37) replacements. The most potent mutants (15 and 38) “super PS” had all six replacements (S81N, S92T, K97Q, S99T, T103I, and P106S).

From the presented results it can be concluded that the species restriction of APC cofactor activity depends on a combination of amino acid replacements in both the TSR and the EGF1 and that a limited number of amino acid replacements leads to a change in specificity. As the experimental approach was very time consuming and laborious, we were unable to test a large number of possible mutations or other combination of replacements, e.g., the individual replacements S92T, Y90F, S99T, and T103I were not tested.

Increased Binding of Bovine APC to Phospholipid in the Presence of Bovine Protein S and Some Human Protein S Mutants—As described above, bovine protein S and several of the human protein S mutants expressed cofactor activity to bovine APC. A likely explanation for this phenomenon was that these protein S variants stimulate binding of bovine APC to the phospholipid. To investigate this possibility, binding of bovine APC (fluorescein-labeled in its active site) to phospholipid vesicles was measured using FACScan flow cytometry. In the absence of protein S, binding of bovine APC to the vesicles was characterized by a $K_d$ of 626 nM (Table III). Bovine protein S yielded a 3.5-fold stimulation of the binding of bovine APC ($K_d$ was 177 nM). In contrast, human protein S did not affect the binding of bovine APC. Nine of the mutants were tested for their ability to stimulate binding of bovine APC to the phospholipid. Mutant 1 (human TSR + bovine EGF1) did not stimulate binding of bovine APC. In contrast, mutant 2 (bovine EGF1 and the double mutant R49Q, Q52R in the TSR) yielded a 3-fold stimulation of bovine APC binding. The individual Q52R and Q61L mutations in TSR combined with bovine EGF1 (mutants 3 and 4) were not sufficient to stimulate binding of bovine APC. In mutants 9, 10, and 11, the TSR carried the triple mutation R49Q, Q52R, T103A (GRA) in combination with point mutations at one or more of residues at positions 92, 97, 99, 103, and 106. These mutants all stimulated the binding of bovine APC. The mutant 35 yielded a 1.9-fold increase in bovine APC binding, which was somewhat lower than expected.

The results on record, i.e., those reported here taken together with results obtained with well characterized monoclonal anti-

### TABLE III

| Protein S mutant | $K_d$ (nM) | Enhancement |
|------------------|-----------|-------------|
| wt-hPS           | 647       | 1.0         |
| mut-hPS          | 177       | 3.5         |
| Mutant 1         | 645       | 1.0         |
| Mutant 2         | 210       | 3.0         |
| Mutant 3         | 549       | 1.1         |
| Mutant 4         | 564       | 1.1         |
| Mutant 9         | 235       | 2.7         |
| Mutant 10        | 111       | 5.7         |
| Mutant 11        | 153       | 4.1         |
| Mutant 22        | 631       | 1.0         |
| Mutant 35        | 333       | 1.9         |
| Control-PS       | 628       | 1.0         |

### Species Specificity of Protein S Cofactor Function

As described above, bovine protein S and several of the human protein S mutants expressed cofactor activity to bovine APC. A likely explanation for this phenomenon was that these protein S variants stimulate binding of bovine APC to the phospholipid. To investigate this possibility, binding of bovine APC (fluorescein-labeled in its active site) to phospholipid vesicles was measured using FACScan flow cytometry. In the absence of protein S, binding of bovine APC to the vesicles was characterized by a $K_d$ of 626 nM (Table III). Bovine protein S yielded a 3.5-fold stimulation of the binding of bovine APC ($K_d$ was 177 nM). In contrast, human protein S did not affect the binding of bovine APC. Nine of the mutants were tested for their ability to stimulate binding of bovine APC to the phospholipid. Mutant 1 (human TSR + bovine EGF1) did not stimulate binding of bovine APC. In contrast, mutant 2 (bovine EGF1 and the double mutant R49Q, Q52R in the TSR) yielded a 3-fold stimulation of bovine APC binding. The individual Q52R and Q61L mutations in TSR combined with bovine EGF1 (mutants 3 and 4) were not sufficient to stimulate binding of bovine APC. In mutants 9, 10, and 11, the TSR carried the triple mutation R49Q, Q52R, T103A (GRA) in combination with point mutations at one or more of residues at positions 92, 97, 99, 103, and 106. These mutants all stimulated the binding of bovine APC. The mutant 35 yielded a 1.9-fold increase in bovine APC binding, which was somewhat lower than expected.

The results on record, i.e., those reported here taken together with results obtained with well characterized monoclonal anti-

### TABLE III

| Protein S mutant | $K_d$ (nM) | Enhancement |
|------------------|-----------|-------------|
| wt-hPS           | 647       | 1.0         |
| mut-hPS          | 177       | 3.5         |
| Mutant 1         | 645       | 1.0         |
| Mutant 2         | 210       | 3.0         |
| Mutant 3         | 549       | 1.1         |
| Mutant 4         | 564       | 1.1         |
| Mutant 9         | 235       | 2.7         |
| Mutant 10        | 111       | 5.7         |
| Mutant 11        | 153       | 4.1         |
| Mutant 22        | 631       | 1.0         |
| Mutant 35        | 333       | 1.9         |
| Control-PS       | 628       | 1.0         |

### Species Specificity of Protein S Cofactor Function

As described above, bovine protein S and several of the human protein S mutants expressed cofactor activity to bovine APC. A likely explanation for this phenomenon was that these protein S variants stimulate binding of bovine APC to the phospholipid. To investigate this possibility, binding of bovine APC (fluorescein-labeled in its active site) to phospholipid vesicles was measured using FACScan flow cytometry. In the absence of protein S, binding of bovine APC to the vesicles was characterized by a $K_d$ of 626 nM (Table III). Bovine protein S yielded a 3.5-fold stimulation of the binding of bovine APC ($K_d$ was 177 nM). In contrast, human protein S did not affect the binding of bovine APC. Nine of the mutants were tested for their ability to stimulate binding of bovine APC to the phospholipid. Mutant 1 (human TSR + bovine EGF1) did not stimulate binding of bovine APC. In contrast, mutant 2 (bovine EGF1 and the double mutant R49Q, Q52R in the TSR) yielded a 3-fold stimulation of bovine APC binding. The individual Q52R and Q61L mutations in TSR combined with bovine EGF1 (mutants 3 and 4) were not sufficient to stimulate binding of bovine APC. In mutants 9, 10, and 11, the TSR carried the triple mutation R49Q, Q52R, T103A (GRA) in combination with point mutations at one or more of residues at positions 92, 97, 99, 103, and 106. These mutants all stimulated the binding of bovine APC. The mutant 35 yielded a 1.9-fold increase in bovine APC binding, which was somewhat lower than expected.

The results on record, i.e., those reported here taken together with results obtained with well characterized monoclonal anti-

### TABLE III

| Protein S mutant | $K_d$ (nM) | Enhancement |
|------------------|-----------|-------------|
| wt-hPS           | 647       | 1.0         |
| mut-hPS          | 177       | 3.5         |
| Mutant 1         | 645       | 1.0         |
| Mutant 2         | 210       | 3.0         |
| Mutant 3         | 549       | 1.1         |
| Mutant 4         | 564       | 1.1         |
| Mutant 9         | 235       | 2.7         |
| Mutant 10        | 111       | 5.7         |
| Mutant 11        | 153       | 4.1         |
| Mutant 22        | 631       | 1.0         |
| Mutant 35        | 333       | 1.9         |
| Control-PS       | 628       | 1.0         |
APC interaction as seen from the analysis of mutant 4. This suggests that the Gln 61 TSR "face" does not directly contact protein C. This is supported by the second set of mutants (—L) that behaved like human PS. Moreover, human and bovine PS have the same residues in this region from Ser62 to Ile 76. It would be expected that to display species-specific interaction; evolutionary pressure would have introduced several point mutations on this side of the loop. The fact that the GRAL group was more potent than the GRA suggests that residue 61 could have an indirect effect in the interaction with APC.

In EGF1, Ser 81 is solvent-accessible and can be easily replaced by Asn without inducing structural strains. This residue does not seem to play an important role in the interaction with APC (mutants 17 and 39). It is located (Fig. 4) on the EGF1 side opposite from the cluster of residues that produce important changes in the specificity (Lys97, Ser99, and Pro106, see below). Pro77 is several Angstroms below Lys 97 (Fig. 4). The data from mutants 18 and 41 suggest that Pro77 is not important for the APC-PS interaction. This is confirmed when looking at the model and the results from mutants 17 and 40 which contain the S81N mutation. Ser81 is on the same side as Pro 77 and on the other face as our expected binding site area (Fig. 4).

Ser92 is relatively close to residue Pro106, and therefore the Ser92 region could contact protein C (Fig. 4). Less conservative amino acid substitution than a Ser to Thr would be needed to test this hypothesis.

The K97Q replacement (e.g. mutants 7 and 30) seems important for the specificity of the APC-PS interaction. This residue is solvent-accessible in the model structure and is located in the loop connecting the EGF’s major β-sheet (Fig. 4). Residue 97 could thus have direct contact with APC.

The mutation S99T (mutants 10 and 33) further supports that the region of residue 97 is important for the interaction with APC since residues 97 and 99 are very close in space (Fig. 4). The replacement P106S is important for the specificity of the APC-PS interaction. This residue is located in a loop, at the opposite end of the EGF module when compared with residue 97 but on the same face (Fig. 4). In this region, several surrounding side chains may be involved in the interaction with APC. These involve Lys105, Tyr90, Met91, Thr103, and Glu109. Such solvent-exposed charged and hydrophobic/aromatic residues can in fact offer a suitable surface area for protein-protein interaction. Moreover, the T103N substitution, leading to a type II deficiency (57), would suggest that this region is of importance for macromolecular interaction since this replacement can be easily accommodated in the structure. Moreover,
bovine PS has an isoleucine there, thus an extra surface-exposed hydrophobic residue, consistent with a possible binding site.

The data obtained from the above mutations suggest that APC could wrap around PS, making contact with the area posed hydrophobic residue, consistent with a possible binding site. Dr. Naomi Esmon is gratefully acknowledged. Dr. Bergisa Hildebrand, Lise Borge, and Ingrid Dahlquist is gratefully acknowledged. The data obtained from the above mutations suggest that APC could wrap around PS, making contact with the area posed hydrophobic residue, consistent with a possible binding site. Dr. Naomi Esmon is gratefully acknowledged. Dr. Bergisa Hildebrand, Lise Borge, and Ingrid Dahlquist is gratefully acknowledged.

Acknowledgments—The technical assistance of Astra Andersson, Bergisa Hildebrand, Lise Borge, and Ingrid Dahlquist is gratefully acknowledged. Dr. Naomi Esmon is gratefully acknowledged for help with the active site labeling of bovine APC and the preparation of the liposome-coated latex solution.

REFERENCES

1. Dahlback, B., and Stenflo, J. (1994) in The Molecular Basis of Blood Diseases (Stamatoyanopoulos, G., Nienhuis, A. W., Majerus, P. W., and Varmus, H., eds) 2nd Ed., pp. 599–628, W. B. Saunders Co., Philadelphia, PA
2. Eason, C. T., and Schwartz, H. P. (1995) Trends Cardiovasc. Med. 5, 141–148
3. Comp, P. C., Nixon, R. R., Cooper, M. R., and Eason, C. T. (1984) J. Clin. Invest. 74, 2082–2088
4. Schwartz, H. P., Fischer, M., Hopmeier, P., Batard, M. A., and Griffin, J. H. (1984) Blood 64, 1257–1260
5. Dahlback, B. (1995) Thromb. Res. 77, 1–43
6. Mahasandana, C., Suwatt, V., Marlar, R. A., Manco-Johnson, M. J., Jacobson, L. J., and Hathaway, W. E. (1989) Lancet 335, 61–62
7. Bakker, H., Tans, G., Janssen-Claessens, T., Thomassen, M. C. L. G. D., Hemker, H. C., Griffin, J. H., and Rosing, J. (1992) Eur. J. Biochem. 206, 171–178
8. Rosing, J., Hoekema, L., Nicolaes, G. A. F., Thomassen, M. C. L. G. D., Hemker, H. C., Van der Kaa, D., Schwarz, H. P., and Tans, G. (1995) J. Biol. Chem. 270, 27852–27858
9. Dahlback, B., and Hildebrand, B. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 1396–1400
10. Shen, L., and Dahlback, B. (1994) J. Biol. Chem. 269, 18735–18738
11. Nelsestuen, G. L., Kiel, S., and Discipio, R. G. (1984) Biochemistry 23, 1313–1318
12. Walker, F. J. (1981) J. Biol. Chem. 256, 11128–11131
13. Stern, D. M., Nemothrot, P. P., Harris, K., and Eason, C. T. (1986) J. Biol. Chem. 261, 713–718
14. Suzuki, K., Nishioka, J., Maruyama, I., and Hashimoto, S. (1984) J. Biochem. (Tokyo) 96, 455–460
15. Harris, K. W., and Eason, C. T. (1985) J. Biol. Chem. 260, 2007–2010
16. Dahlback, B., Wiedmer, T., and Sims, P. J. (1992) Biochemistry 31, 12769–12777
17. Yegneswaraman, S., Wood, G. M., Eason, C. T., and Johnson, A. E. (1997) J. Biol. Chem. 272, 28003–28009
18. Heeb, M. J., Mesters, R. M., Tans, G., Rosing, J., and Griffin, J. H. (1993) J. Biol. Chem. 268, 2672–2677
19. Heeb, M. J., Rosing, J., Bakker, H. M., Fernandez, J. A., Tans, G., and Griffin, J. H. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 2729–2732
20. Hackeng, T. M., van’t Veer, C., Meijers, J. C. M., and Buma, B. N. (1994) J. Biol. Chem. 269, 21051–21058
21. Dahlback, B., and Stenflo, J. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 2512–2516
22. Dahlback, B. (1986) J. Biol. Chem. 261, 12022–12027
23. Schwab, R., Dahlback, B., Hillarp, A., and Nelsestuen, G. (1990) J. Biol. Chem. 265, 16074–16081
24. Lundwall, A., Daekowski, W., Cohen, E. H., Shaffer, M., Mahr, A., Dahlback, B., Stenflo, J., and Wydro, R. M. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 6716–6720
25. Greengard, J. S., Fernandez, J. A., Radtke, K.-P., and Griffin, J. H. (1995) Biochim. Biophys. Acta 1217, 325–328
26. Dahlback, B., Lundwall, A., and Stenflo, J. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 1499–1492