A novel cyclosporin A binding glycoprotein of 21 kDa was isolated from human milk by several steps of cation exchange chromatography. The corresponding gene was cloned from human T cells, expressed in *Escherichia coli* and the recombinant protein purified. We report the existence of a novel secreted cyclophilin-like protein (SCYLP) in the extracellular environments. We have fortuitously purified this protein in our attempts to isolate novel growth factors from human milk. We present results concerning cloning, complete nucleotide sequence, purification of native protein and recombinant protein expressed in *Escherichia coli*, their binding to CsA, and the associated proline isomerase activity.

**EXPERIMENTAL PROCEDURES**

**Materials**

Cyclosporin A (CsA),* Sephadex G-50 superfine, SP-Sephadex C-25, S-Sepharose Fast Flow, and Mono S HR 5/5 column were purchased from Pharmacia (Uppsala, Sweden). Nucleic acid modifying enzymes were from Boehringer Mannheim. Succinyl-Ala-Ala-Pro-Phe-p-nitroanilide was provided by Bachem AG (Bubendorf, Switzerland). Recombinant human cyclophilin was prepared from an *E. coli* strain engineered to express this protein.1

**Methods**

*Oligonucleotide Screening—All recombinant nucleic acid methods were as described (10). Two sets of oligonucleotides, GAYGARAA-RAARAARGGICCIAARGT (corresponding to amino acids 2-10 in Fig. 2) and CAATGAGACCATTTTCCTAATTGCT (amino acids 16-24 in Fig. 2), were subcloned into plasmid vectors, and the longest insert was sequenced. The insert was subjected to bombardment with the sequencer kit from U. S. Biochemical Corp.

*Construction of Recombinant Plasmid for Expression in *E. coli*—A recombinant plasmid, PPGF-72, capable of expressing the mature SCYLP was generated by inserting the nucleotide sequence 118–893 as shown in Fig. 2, into an *E. coli* expression vector, pKK233-2 (supplied by Pharmacia, Sweden, Ref. 11) between the restriction enzyme sites *Nei* and *Hind*III. The insert was generated by polymerase chain reaction and engineered such that the ATG codon within the *Nei* site of the plasmid specified the initiating methionine followed by mature protein. This plasmid specified the expression of recombinant mature SCYLP under the control of Tc promoter.

*Purification of SCYLP from Human Milk—Human milk proteins were obtained from a milk bank pool collected from mothers at all stages of lactation and stored frozen before use. Unless stated otherwise all subsequent operations were conducted at 4 °C. SCYLP was prepared from human skim milk and isolated by sequential ion exchange chromatography and gel filtration chromatography as described below. Thawed human milk (20 liters) was centrifuged at 10,000 × *g* for 1 h. 50 g of dry SP-Sephadex C-25 were added to the skim milk, and the mixture was stirred overnight. The resin was extensively washed with 0.22 M sodium acetate, pH 7.0, and eluted with solutions containing increasing sodium acetate concentrations (0.22, 0.3, 0.6, and 2.5 M). The fraction eluted with sodium acetate 0.22 M was applied to 5 × 10-cm column of S-Sepharose Fast Flow equilibrated with 0.22 M sodium acetate, pH 7. The column was washed extensively with the equilibration buffer, and the proteins were eluted successively with 500 ml of a solution containing 0.4, 0.6, and 1.5 M sodium acetate. In the first experiments, separation was monitored by absorbance at 280 nm and by the mitogenic activity using [*H]*thymidine incorporation in 3T3 fibroblast cells (12). From there on, purification was routinely performed and monitored by absorbance at 280 nm and by enzyme-linked immunosorbent assay (for purification monitored at 490 nm) and dot-blot immunosassays (data not shown) using polyconal antiserum against the COOH-terminal peptide (CGKIEVEKPFIAKE) of SCYLP. Pooled immu-

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*The abbreviations used are: CsA, cyclosporin A; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SCYLP, secreted cyclophilin-like protein; MES, 4-morpholineethanesulfonic acid.*

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1. K. Stedman, unpublished data.
Peptide of SCYLP.

Stored by absorbance at 280 nm. Ten individual fractions (numbers 1-3) were chromatographed on a fast protein liquid chromatography Mono S column equilibrated with 50 mM sodium phosphate buffer, pH 6.6 and a possible adhesion site (**).

Purification of SCYLP from human milk. In the routine assays, presence of SCYLP was detected by using a polyclonal antiserum against the COOH-terminal peptide of SCYLP. Pooled immunoreactive material was resuspended in 50 mM sodium phosphate buffer, pH 6.6, and a possible adhesion site (**). The arrow shows the peak. The position of cleavage from the precursor. A potential glycosylation site (**) is indicated. Nucleotide sequence dataaaa corresponds to a polyadenylation signal.

**Fig. 1.** Purification of SCYLP from human milk. In the routine assays, presence of SCYLP was detected by using a polyclonal antiserum against the COOH-terminal peptide of SCYLP. Pooled immunoreactive fractions isolated from human milk as described under "Methods," by batch chromatography on SP-Sephadex C-25 and S-Sepharose were further purified by chromatography on Sephadex G-50 superfine column (3.5 × 100 cm), equilibrated, and eluted with 0.1 M ammonium bicarbonate and lyophilized. The lyophilized immunoreactive material was resuspended in 50 mM sodium phosphate buffer, pH 6.6, and passed through an ultrafiltration HV membrane (Millipore). Purification of the filtrate was further performed on a fast protein liquid chromatography Mono S HR 5/5 column (Pharmacia) as described in the legend of Fig. 1. The pooled immunoreactive fractions were stored at 4 °C.

**Fig. 2.** Nucleotide sequence of SCYLP insert and the derived amino acid sequence of the protein. The underlined amino acids were initially identified by sequencer analysis of the intact native protein or of its tryptic fragments. The arrow shows the position of cleavage from the precursor. A potential glycosylation site (**) is indicated. Nucleotide sequence dataaaa corresponds to a polyadenylation signal.

**Amino Acid Sequence Analysis**—The amino acid sequence analysis of native SCYLP was carried out by automated Edman degradation using an Applied Biosystems 470A Sequencer equipped with a model 120A on-line phenylthiohydantoin analyzer. The digest of the native protein with trypsin (1%, w/v) was separated on an Aquapore RP-300 column using a 130 A high performance liquid chromatography system (Applied Biosystems).

**Enzymatic Activity**—The peptidyl-prolyl-cis/trans-isomerase activity was assessed according to the method of Fischer et al. (5) with the exception that the reaction was allowed to proceed for 90 s at 30 °C. The peptidyl-cis/trans-isomerase activity was measured by the liberation of the fluorophore from the substrate, succinyl-Ala-Ala-Pro-Phe-m-nitroanilide (50 μM final concentration), was assayed at 410 nm. To assay the inhibition of
enzyme activity by CsA, the enzyme was preincubated with CsA for 15 min at 37 °C before the addition of substrate.

CsA Binding—An automated LH-column binding assay as described by Handschumacher et al. (2) was used. The indicated amount of protein was incubated for 15 min at 37 °C with 100,000 dpm of [3H]CsA (cyclosporin A, specific activity of 12.12 μCi/μg) in a final volume of 100 μl. 50 μl of this reaction mixture was injected into the column. Binding was calculated from the total amount of label injected versus the amount bound to the fraction isolated in the void volume of the column.

RESULTS AND DISCUSSION

In the first experiments, human milk was separated into various components with the aim of isolating novel growth factors as assayed by [3H]thymidine incorporation into 3T3 fibroblasts. Various chromatography steps led to the isolation of three fractions (Fig. 1A). Fraction 2, still possessing mitogenic activity, was further fractionated on a fast liquid chromatography Mono S column. Ten-well defined fractions were obtained (Fig. 1B) and analyzed. Fraction 7 was not mitogenic and amino acid sequencing by Edman degradation permitted identification of 48 out of 52 amino-terminal residues. A computer search of the National Biomedical Research Foundation data base showed that the residues from 9 to 52 were 50% identical to the NH2-terminal 44 residues of human cyclophilin (14, 15). Oligonucleotide probes corresponding to the amino acid stretches least homologous to human cyclophilin were synthesized and employed to screen a T-cell cDNA library. We have reasoned that, similar to cyclophilin, which was originally isolated from thymus but subsequently detected in virtually all tissues (16), this highly related SCYLP might be ubiquitous and expressed in particular by T cells in virtually all tissues (16), this highly related SCYLP might be ubiquitous and expressed in particular by T cells.

Amino acid sequence comparison between mature SCYLP and human cyclophilin. The GENALIGN program of Intelligent Software was used. Gaps are introduced to maximize similarities. CON shows the identical residues.

| SCYLP 1 | adekkpgkphTVkkyFD1igEdGrV1qfLFgktVPFRTvDNVfALATGEGFCYKSHKFRHVR1kDF |
| CYP 1   | mnpTV | FDDAvddgEPlgVseFLFAdXPFKTeaHFCALTEGEGFGRGFCFRHVR1kDF |

Fig. 3. Amino acid sequence comparison between mature SCYLP and human cyclophilin.
same gene (23). In Saccharomyces cerevisiae, a cDNA sequence probably corresponding to a non-cytoplasmic form of the protein has also been found recently (24). In addition, the nisA gene product of Drosophila melanogaster specifies a membrane-associated cyclophilin (25) that is synthesized with a signal sequence necessary for translocation. Last, a periplasmic proline isomerase that is homologous to cyclophilin, although insensitive to CsA, has been isolated from E. coli (26). The protein folding activity displayed by peptidyl-prolyl isomerases must be part of a fundamental biochemical mechanism, since two structurally unrelated abundant proteins, cyclophilin and FK-506 binding protein, the first of which was shown to be ubiquitous and extremely conserved in the phylogeny (16), share this activity. In the case of cyclophilin, the existence of a family of proteins responding to changing cell physiological conditions was postulated as numerous cyclophilin related sequences were detected in the human genome (15). Even though most of those were later found to be pseudogenes (27), it is still possible that additional variants exist besides the cytosolic cyclophilin and the secreted form described here. The precise role of these polypeptides in vivo awaits further investigation. Purified SCYLP is not mitogenic to 3T3 fibroblasts, but it is tempting to think that its folding function may activate an associated growth factor. A homologue of SCYLP from chickens (108 out of 184 amino acids are identical) has been independently isolated and is described in the accompanying manuscript of Caroni et al. (28).

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