Expression of Functional Human C1 Inhibitor in COS Cells*

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Full length human C1 inhibitor cDNA was cloned into a vector suitable for transient expression in COS-1 cells. Transfected COS cells secreted an immunoreactive protein of \( M_r \approx 110,000 \) that appeared to be functionally equivalent to the plasma-derived protein as established by the following criteria: 1) ability to form sodium dodecyl sulfate-stable complexes with C1s, factor XIIa, and kallikrein; 2) inhibition of C1s-mediated C4 consumption; and 3) susceptibility to inactivation by the nontarget protease elastase. Quantitation of secreted recombinant C1 inhibitor by radioimmunoassay indicated that 72 h after transfection the level was approximately 2.2 \( \mu \text{g} \)/ml. Treatment of transfected cells with tunicamycin resulted in secretion of a protein of \( M_r \approx 90,000 \) that was also capable of complex formation with C1s.

C1 inhibitor is a plasma glycoprotein of \( M_r \approx 105,000 \) that belongs to the superfamily of serine protease inhibitors (serpins). Other members of this family include α1-antitrypsin, α1-antiplasmin, antithrombin III, and plasminogen activator inhibitor types I and II (1, 2). C1 inhibitor inhibits activated components of the classical pathway of complement (C1r and C1s (3)) and the intrinsic coagulation system (factor XIIa, factor XIIa, and kallikrein (4, 5)). In addition, interaction of C1 inhibitor with the fibrinolytic components plasmin (6) and tissue plasminogen activator (7) has been described. Genetic deficiency of C1 inhibitor, resulting from either quantitative or structural alterations, is the cause for the disease hereditary angioedema (8).

Analogous to the other serpins, C1 inhibitor inhibits a target proteinase by offering it a peptidyl bond, believed to lie on an exposed stressed loop in the molecule (9, 10), that matches the substrate specificity of the proteinase. This peptidyl bond, identified as Arg-Thr (11), resists complete cleavage which results in the formation of an SDS-resistant complex between inhibitor and proteinase. During this process a COOH-terminal peptide of \( M_r 5000 \) originating from the inhibitor is generated (11).

Several nontarget proteinases, notably elastase, are able to catalytically cleave and thereby inactivate C1 inhibitor near the reactive site (12). This phenomenon is hypothesized to play a role in local inflammatory reactions as well as in pathological circumstances, e.g. septicemia or endotoxemia (10, 12).

Partial (13–15) and complete (16) C1 inhibitor cDNA clones have been reported. These results have confirmed protein sequencing data (11, 17) and show that C1 inhibitor is a relatively distant member of the serpin family (18). The nonhomologous NH₂-terminal part of the protein (residues 1–120) contains most of the attachment sites for the carbohydrates that account for 49% of the molecular weight (16).

To study the structure and function of C1 inhibitor in more detail, we constructed a full length cDNA and expressed it in a heterologous system. This paper describes the production by COS-1 cells of recombinant C1 inhibitor that is functionally active as assessed by its ability to form complexes with C1s, factor XIIa, and kallikrein and to inhibit cleavage of C4 by fluid phase C1s.

EXPERIMENTAL PROCEDURES

Enzymes and Reagents

Restriction endonucleases, T₄ DNA ligase, T₄ DNA polymerase, and polynucleotide kinase were purchased from Bethesda Research Laboratories. *Escherichia coli* DNA polymerase (Klenow fragment) and calf intestine alkaline phosphatase were purchased from Boehringer Mannheim (Mannheim, Federal Republic of Germany). All enzymes were used according to the manufacturer’s instructions. [γ⁻³²P]ATP (specific activity 111 TBq/mmol) for kinasing oligomers and α⁻³²P-CTP (specific activity 15.2 TBq/mmol) for sequencing were obtained from the Radiochemical Centre (Amersham, United Kingdom). Porcine pancreatic elastase (type II) was purchased from Sigma. Blood samples were obtained from healthy volunteers by venipuncture and collected in siliconized Vacutainer tubes (Becton Dickinson, Plymouth, United Kingdom) containing EDTA and Polynurese (10 mM and 0.05% (w/v) final concentrations, respectively). The tubes were centrifuged for 10 min at 1300 × g and the plasma samples were stored immediately at −70°C.

The C1 inhibitor, C1s, factor XIIa, and kallikrein preparations used in this study were purified as described previously (19, 24).

Cloning of Human C1 Inhibitor cDNA

cDNA clones encoding C1 inhibitor were isolated from a human liver cDNA library in pUC9 (kindly provided by Dr. H. Pannekoeck, Dept. of Molecular Biology of this Institute) by hybridization with radiolabeled specific probes. These probes, consisting of 20 nucleotides, are complementary to the C1 inhibitor cDNA sequence as published by Bock et al. (16) and were synthesized on an Applied Biosystems DNA Synthesizer Type 381 A (Warrington, United Kingdom). 50,000 independent colonies were screened with probe I (nucleotides 34–53, according to Bock et al. (16)) and 35,000 colonies were screened with probe II (nucleotides 1422–1441) following published procedures (20). Thirty-two positive clones were obtained and further analyzed by restriction mapping and Southern blotting; however, none of these contained the complete coding region. Two overlapping partial clones were therefore used to construct the full length cDNA: clone 30 (containing nucleotides 17–724) was digested with PstI and AcaI, and clone E3 (nucleotides 521–1811) was digested with AcaI and HindIII, and the desired fragments were ligated into PstI and HindIII-cleaved pUC18 (see also Fig. 1). Subcloned fragments of the partial clones and the full length construct in M13mp vectors were

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1 The abbreviations used are: SDS, sodium dodecyl sulfate; IMDM, Iscove’s modified Dulbecco’s medium; RIA, radioimmunoassay; PBS, phosphate-buffered saline; mAbs, monoclonal antibodies.
sequenced (21) and were in complete agreement with Bock et al. (16).

The cDNA clone isolated by us codes for methionine as residue 458 (16).

**Insertion of cDNA in a Eukaryotic Expression Vector**

The vector used was pSVL (Pharmacia, Uppsala, Sweden). This plasmid consists of the SV40 origin of replication, the SV40 late promoter, and the VP1 intron in front of a polylinker followed by the SV40 late polyadenylation signal, fused to a pBR322 fragment containing the origin of replication and the ampicillin resistance gene.

A pAAH-HaeII C1 inhibitor cDNA fragment containing the complete coding region was blunt-ended with T4 DNA polymerase and inserted in pSVL, digested with Smal, and treated with alkaline phosphatase. The ligation mixture was used to transform E. coli strain JM101 to ampicillin resistance. Correct orientation of the insert was confirmed by restriction analysis of the cDNA relative to the SV40 sequences was verified by restriction analysis. The resultant plasmid was termed pCI1 inhibitor.

**COS-1 Cell Expression**

The SV40-transformed COS-1 monkey cells (kindly provided by Dr. M. Verbeet, Dept. of Molecular Biology of this Institute) were grown in Iscove's modified Dulbecco's medium (IMDM) containing 10% fetal calf serum, 10% penicillin and streptomycin supplemented with 10% heat-inactivated fetal calf serum (Flow Laboratories, Irvine, United Kingdom).

**Analysis of Recombinant C1 Inhibitor**

Radioimmunoassays (RIAs)—The radioimmunoassay for functional C1 inhibitor is based on the principle that functional C1 inhibitor will bind to activated C1s. Purified activated C1s were coupled to Sepharose 4B (Pharmacia, Uppsala, Sweden) and suspended in phosphate-buffered saline, pH 7.4 (PBS), 10 mM EDTA, 0.1% (w/v) Tween-20; 0.3 ml of this suspension (containing approximately 1.5 μg of C1s) was incubated with samples for 5 h. After extensive washing complexed C1 inhibitor was detected by overnight incubation with rabbit polyclonal 125I-anti-C1-inhibitor antibodies (24). The bound radioactivity was quantitated on an LKB 1260 multigamma II gamma counter. Serial dilutions of pooled human plasma were used as a reference.

RIAs for total C1 inhibitor antigen and inactive modified C1 inhibitor (inactivated C1 inhibitor) were performed in a similar manner using monoclonal antibodies (mAbs) coupled to Sepharose (0.3 ml of Sepharose suspension containing approximately 1 μg of mAb). mAb RII binds functional, complexed, and inactivated C1 inhibitor equally well and mAb KII is specific for inactivated C1 inhibitor. A more detailed description of these mAbs is given elsewhere (24). All RIAs were performed in duplicate.

Western Blot—Samples of conditioned medium (0.5 ml) or 0.5 μg of purified C1 inhibitor were incubated overnight with 2.5 μl of mAb RII-Sepharose. After washing bound C1 inhibitor was dissociated by addition of 100 μl of nonreducing sample buffer and heating in boiling water for 5 min. After centrifugation for 5 min at 2700 × g, samples were subjected to electrophoresis on a 5% SDS-polyacrylamide gel (25). In the case of detection of complexed C1 inhibitor, samples were incubated 3.5 h at 37 °C with C1s (5 μg) prior to incubation with RII-Sepharose. Culture supernatant of transfected cells treated with tunicamycin (1.5 μl) was incubated with 10 μg of C1s to account for the larger volume. Complexes with β-factor XILs and kallikrein were detected by first absorbing these proteinases onto Sepharose-bound mAbs; mAb F3, directed against the light chain of factor XILs and mAb K15, directed against prekallikrein as well as kallikrein. Sepharose-bound mAb and proteinase were incubated overnight in such manner using 0.1% (w/v) sodium dodecyl sulfate (SDS) in buffer B (50 mM Tris-HCl, pH 8.0, 10% glycerol, 0.1% sodium dodecyl sulfate) for 16 h before addition of polyclonal 125I-anti-C1-inhibitor antibodies (6 × 106 cpm; i.e. approximately 200–300 ng of antibodies) and further incubated overnight, washed with PBS, 0.1% (w/v) Tween, and autoradiographed.

**RESULTS AND DISCUSSION**

Expression of Human C1 Inhibitor in COS-1 Cells—The construction of the full length C1 inhibitor cDNA from two partial cDNA clones and the subsequent insertion of the full length cDNA in the expression vector pSVL is shown schematically in Fig. 1. In the construct, the C1 inhibitor cDNA is under transcriptional control of the SV40 “late” promoter, SV40 enhancer, and SV40 late polyadenylation signal. The plasmid was transfected into COS-1 cells, and serum-free conditioned medium was harvested after 72 h and analyzed.

**Fig. 1. Schematic presentation of partial C1 inhibitor cDNA clones, construction of full length cDNA, and expression vector.** I and II refer to the positions of the primers used for screening. Short and long black bars represent cDNA clones 30 and 53, respectively. Restriction sites used for subcloning are: P, PstI; Hi, HindIII (both originating from the polyn linker in pUC9); A, AatII; Av, AvoI; HaII, S, Smal. The arrow marks the position of the reactive site on the C1 inhibitor (C1-INH) protein. Vector sequences: LP, SV40 late promoter, VPItron, VPI intron, p4, SV40 late polyadenylation signal. The vector part of the figure is not drawn to scale.
by RIAs specific for total C1 inhibitor antigen and functional C1 inhibitor. The level of C1 inhibitor antigen and activity in the medium was compared with that of pooled fresh plasma (see Fig. 2). C1 inhibitor levels varied slightly between two different transfection experiments and ranged between 1/160 and 1/160 of values found in plasma. Since the concentration of C1 inhibitor in human plasma is of the order of 275 μg/ml (29), we concluded that the transfection medium contained 1.8–2.75 μg/ml recombinant C1 inhibitor (rC1 inhibitor). The functional index, i.e. the ratio between levels of functional versus antigenic C1 inhibitor, of rC1 inhibitor was equal to that of C1 inhibitor in plasma. Thus, probably all of the rC1 inhibitor produced was in the functional uncomplexed form. This was confirmed by the fact that rC1 inhibitor did not bind to mAb KII (not shown), specific for the inactivated form of C1 inhibitor (24). mAb KII is able to detect inactivated C1 inhibitor in normal plasma, representing about 2% of total C1 inhibitor (30 and Footnote 2).

Western Blotting—A further comparison between plasma C1 inhibitor and the recombinant product was made using gel electrophoresis and Western blotting. Comparable amounts of purified C1 inhibitor and rC1 inhibitor were immunoprecipitated with mAb RII. The results of a representative experiment in Fig. 3A show that the recombinant protein migrated as a slightly diffuse band with an estimated M, of ~110,000 compared with plasma C1 inhibitor of M, 105,000. Given the fact that C1 inhibitor has an extraordinarily high carbohydrate content of 49% (16), the slight heterogeneity of the recombinant product may be explained by assuming that COS-1 cells are not able to reproduce the exact pattern of glycosylation and/or processing of the oligosaccharide chains (31). Treatment of transfected cells with 10 μg/ml tunicamycin, which inhibits N-glycosylation, leads to a reduction in mass of rC1 inhibitor to 90 kDa (Fig. 3, lane 3). Since the primary protein structure accounts for only 53 kDa of the mass (16), it can be estimated that N-glycosylation accounts for 15 kDa and that O-glycosylation is responsible for the remaining 37 kDa. Treatment with tunicamycin reduced the expression level approximately 3-fold, measured 48 h after transfection.

Complex Formation—The ability of rC1 inhibitor to form SDS-resistant complexes with target proteinases was investigated by incubation with C1s, factor XIIa, and kallikrein.

Footnotes

2 J. H. Nuijens et al., manuscript in preparation.

Heterologous Expression of Functional C1 Inhibitor

FIG. 2. Radioimmunoassays for total C1 inhibitor (C1-INH) antigen (A) and functional C1 inhibitor (B). Two-fold serial dilutions of pooled human plasma (●) and conditioned medium of COS-1 cells transfected with pc1 inhibitor (○) were compared using Sepharose-bound mAb RII and C1s. • samples of medium from pSVL-transfected COS-1 cells; ---, exhibited background values.

FIG. 3. Western blot of immunoprecipitated purified plasma C1 inhibitor (0.5 μg in 0.5 ml of PBS, 0.1% (w/v) Tween 20, lanes 1 and 4), 0.5 ml of pC1 inhibitor transfection medium (lanes 2 and 5), and 1.5 ml of medium from tunicamycin-treated transfected cells (lanes 3 and 6). Complexes were formed by the addition of purified C1s (lanes 4–6). C1 Inhibitor antigen on the blot was detected with 125I-anti-C1-inhibitor antibodies, as described under "Experimental Procedures." B, Western blot of 0.5 μg of purified plasma C1 inhibitor (lanes 1 and 3) and 0.5 ml of pC1 inhibitor transfection medium (lanes 2 and 4) complexed with β-factor XIIa absorbed onto mAb F3-Sepharose (lanes 1 and 2) and kallikrein bound to mAb K15-Sepharose (lanes 3 and 4).

FIG. 4. Demonstration of C1 inhibitor (C1-INH) functional activity by using a C4-dependent hemolytic assay. The functional activity of purified plasma C1 inhibitor (●) and rC1 inhibitor (○) was assessed by means of a hemolytic assay that utilizes the ability of C1 inhibitor to inactivate C1s and thus prevents C1s-induced consumption of C4. Values are expressed as percentage inhibition of C1s, where 100% equals the amount of lysis in the absence of C1s and 0% corresponds with the amount of lysis when no C4 was added. Shown are the mean values of duplicate samples. The amount of rC1 inhibitor present in the transfection medium was established by RIAs, as described under "Experimental Procedures" and Fig. 2.

Complexes with C1s were formed by the addition of 5 μg of purified C1s, followed by immunoprecipitation with mAb RII. The recombinant product interacted with C1s in a similar manner to purified plasma C1 inhibitor (Fig. 3A, lanes 4 and 5). In Fig. 3A, lane 6, it is shown that the 90-kDa rC1 inhibitor produced during tunicamycin treatment of transfected cells is also capable of forming a complex with C1s, confirming earlier observations (32) that removal of carbohydrate groups does not influence C1 inhibitor activity.

A different approach was used to demonstrate complex formation with factor XIIa and kallikrein. The factor XIIa preparation was partially purified (19) and contained mostly...
Duplicate samples of purified plasma Cl inhibitor and conditioned medium (containing approximately 100 ng of rCl inhibitor) were incubated with elastase-Sepharose. After centrifugation the supernatant was tested for the presence of inactivated (r)Cl inhibitor, using mAB KII Sepharose as outlined under "Experimental Procedures." Values are expressed as percent binding of 125I-f anti-Cl-inhibitor-antibodies.

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<tr>
<th>Incubation with elastase-Sepharose</th>
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<tr>
<td>Cl inhibitor (100 ng)</td>
<td>5.8</td>
<td>22.0</td>
</tr>
<tr>
<td>rCl inhibitor (50 μl)</td>
<td>4.9</td>
<td>19.8</td>
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β-factor XIIa, but was not completely free of contaminants. Therefore the β-factor XIIa was further purified by adsorption onto a mAB F3) coupled to Sepharose. MAB F3 is directed against the light chain of factor XIIa and does not interfere with complex formation. The Sepharose-bound β-factor XIIa was subsequently used to generate complexes with C1 inhibitor (Fig. 3B, lanes 1 and 2). Analogously, the kallikrein preparation was incubated with mAB K15, directed against prekallikrein as well as kallikrein, and the Sepharose suspension was incubated with Cl inhibitor samples (Fig. 3B, lanes 3 and 4). The results in Fig. 3B clearly show that rCl inhibitor has a similar capacity as plasma-derived Cl inhibitor to form SDS-resistant complexes with β-factor XIIa and kallikrein.

Inhibition of the Enzymatic Activity of Cts—It is generally accepted that complex formation between Cl inhibitor and a target proteinase abolishes the enzymatic activity of the latter. Although it is shown in the previous experiments that rCl inhibitor is capable of complex formation, an additional experiment was performed to ensure that incubation with rCl inhibitor indeed prevents the enzymatic activity of Cts. We used an approach similar to Randazzo et al. (27) who measured C1 inhibitor activity by means of Cts-dependent destruction of hemolytic C2, but instead of C2, we used C4. Samples of medium from COS-1 cells transfected with Cl inhibitor gave essentially the same results as purified C1 inhibitor (Fig. 4), thus indicating that the recumbent protein is an inhibitor of the enzymatic activity of Cts.

Inactivation by Elastase—The susceptibility of rCl inhibitor to catalytic inactivation by nontarget proteinases was evaluated in comparison with purified plasma Cl inhibitor. Samples were incubated with Sepharose-coupled elastase (5.6 ng of elastase), and the appearance of the inactivated form of Cl inhibitor was monitored by mAB KII (Table I). Under the conditions used, approximately 15% of starting material is converted to the inactivated form, as calculated from a reference curve with known amounts of inactivated Cl inhibitor (not shown). It appears that Cl inhibitor is inactivated by elastase to the same extent as plasma-derived Cl inhibitor. This indicates that the region around the active site is in the same configuration in both molecules and that the neodeterminant exposed after cleavage that is specifically recognized by mAB KII (24) is also present on rCl inhibitor.

Concluding Remarks—This study shows that the serine proteinase inhibitor with the highest carbohydrate content, Cl inhibitor, can be expressed in COS-1 cells in a functional form. This provides a powerful tool for future studies on this important inhibitor of the contact system of coagulation and the classical pathway of complement by means of specifically engineered mutants of Cl inhibitor. Construction of such mutants is currently being undertaken in our laboratory.

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REFERENCES

TABLE I

Heterologous Expression of Functional Cl Inhibitor 11779

Inactivation of rCl inhibitor by elastase

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