Recombinant Human Complement Subcomponent C1s Lacking β-Hydroxyasparagine, Sialic Acid, and One of Its Two Carbohydrate Chains Still Reassembles with C1q and C1r To Form a Functional C1 Complex†

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ABSTRACT: In contrast to the human serum protein which is approximately one-half *erythro*-β-hydroxyasparagine at asparagine 134 [Theilens et al. (1990) *Biochemistry 29*, 3570–3578], recombinant C1s expressed by insect cells after infection with recombinant baculovirus entirely lacks posttranslational modification at asparagine 134. It is also incompletely glycosylated, lacking, at least, sialic acid. Site-directed mutagenesis of one of the two sites of carbohydrate attachment (Asn 159 to Gln 159) yields a faster migrating recombinant C1s still abundantly secreted. Furthermore, the mutated protein displays good hemolytic activity when reassembled with C1q and either human serum or recombinant C1r, demonstrating that these posttranslational modifications are not critical for any of the multiple interactions between C1s and C1q, C1r, C2, and C4 required for reassembly of the C1 complex, activation, and initiation of the classical complement pathway. The 4.0S recombinant C1s dimerizes to yield 5.6S $C1s_2$ in the presence of Ca^{2+} and forms the 9.1S C1s–C1r–C1r–C1s tetramer upon the addition of human serum C1r and the 15.6S C1 complex upon the addition of C1q to the tetramer. The recombinant C1s and human serum C1s have identical N-terminal amino acid sequences, indicating proper recognition by the insect signal peptidase. The recombinant C1s is secreted and isolated as the unactivated zymogen, and it may be activated by human serum $C\bar{1}r$ which cleaves at Arg^{422}–Ile^{423} to yield the characteristic heavy and light chains. A very tight complex is formed between C1-inhibitor and the light chain of recombinant $C\bar{1}s$. To summarize, recombinant C1s expressed in insect cells is not β-hydroxylated, lacks sialic acid, and has been mutated to remove one-half of the carbohydrate, yet reassembles with the remaining subcomponents to form a functional C1 complex.

The first component of complement (C1) plays an important role in the immune defense by recognizing immune complexes. C1 is composed of five subcomponent glycoproteins: one C1q, two C1r, and two C1s.[1] Immune complex recognition is accomplished through the C1q subcomponent binding multivalently to a cluster of IgG or a single IgM antibody attached to the same surface (Metzger, 1974, 1978; Borsos & Rapp, 1965; Borsos et al., 1981; Schumaker et al., 1976). Distortion of the regularly arranged cone of C1q arms upon binding to a cluster of antibody Fc probably provides the signal resulting in the autoactivation of C1 (Hoekzema et al., 1988; Kilchherr et al., 1986); this distortion is proposed to bring the C1r and C1s catalytic domains into contact to induce the conformational change required for autoactivation of the $C1r_2$ dimer (Villiers et al., 1985; Weiss et al., 1986; Schumaker et al., 1986; Arlaud et al., 1987a) generating the $C\bar{1}r_2$ enzyme. Autoactivation is followed by cleavage of proenzyme C1s by $C\bar{1}r$. The activated $C\bar{1}s$ enzyme is highly specific for cleavage and activation of C2 and C4, initiating the classical complement cascade (Schumaker et al., 1990). Detailed models for the structure of C1 and the function of its subcomponents have been proposed [reviewed in Arlaud et al. (1987a) and Schumaker et al. (1987)].

Human C1s is synthesized as a 688-residue polypeptide from which a 15-residue signal sequence is cleaved to yield the mature zymogen (Tosi, 1987; MacKinnon, 1987); here the convention of numbering the N-terminal Glu of the mature protein as residue 1 is employed; the polypeptide then extends to C-terminal Asp 673. Upon activation, zymogen C1s is cleaved between Arg 422 and Ile 423 to yield two disulfide-linked chains, the larger N-terminal chain (A chain) and the smaller C-terminal chain (B chain) (Spycher et al., 1986). The B chain contains the active site and is homologous to the catalytic domains of the common serine proteases. Activated C1s is irreversibly inactivated by C1-inhibitor; when analyzed by reducing SDS gel electrophoresis, the C1-inhibitor remains

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[1] Abbreviations: VBS, veronal-buffered saline; VBS-Ca^{2+}, veronal-buffered saline containing 2.5 mM calcium; TBS, Tris-buffered saline; AcNPV, *Autographa californica* nuclear polyhedrosis virus; Sf9, *Spodoptera frugiperda* insect cells (army worm); HPLC, high-pressure liquid chromatography; PNGase F, peptide:*N*-glycosidase F; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EAC4, sheep erythrocytes coated with antibody and complement component C4. The nomenclature of complement components is that recommended by the World Health Organization; activated components are indicated by an overhead bar, e.g., $C\bar{1}s$.

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tightly bound to the B chain (Chesne et al., 1982). Human serum C1s has two N-linked carbohydrate chains, both of which are attached to the A chain at Asn 159 and Asn 391. Approximately one-half of Asn 134 in human serum C1s is found as *erythro*-β-hydroxyasparagine (Thielens et al., 1990); this partial posttranslational modification may be compared with the homologous residue 150 in human serum C1r which is completely converted to *erythro*-β-hydroxyasparagine (Arlaud et al., 1987b).

The mature 673-residue C1s polypeptide has a molecular weight of 74 900 (Tosi et al., 1987); in addition, two carbohydrate chains, each considered to contribute about M_r 2000, yield a total M_r of about 78 900 and a carbohydrate content of about 5% (Thielens, 1990). Sim and co-workers (Sim et al., 1977) reported that the carbohydrate of human serum C1s was composed of equal amounts of mannose, galactose, *N*-acetylglucosamine, and sialic acid, suggesting that C1s carbohydrates belong to the complex type. Sedimentation coefficients, $s^0_{20,w}$, of 4.3 S for the C1s monomer in the presence of EDTA (Tschopp et al., 1980) and 5.6 S for the C1s dimer which forms in the presence of Ca^{2+} (Arlaud et al., 1977) have been reported. The sedimentation coefficient for the $C1r_2C1s_2$ tetramer was reported to be 8.7 S (Tschopp et al., 1980), and for C1 it was 15.9 S (Siegel et al., 1981).

Rapid activation of native or reassembled C1 occurs spontaneously in solution upon incubation at 37 °C (Ziccardi, 1982; Bianchino et al., 1988), as measured by cleavage of C1r and C1s on western blots. Activation is markedly retarded by the presence of C1-inhibitor (Ziccardi, 1982b; Bianchino et al., 1988), probably through inhibition of a side reaction involving $C\bar{1}$-catalyzed activation of unactivated C1 (Tseng et al., 1991).

Previously, recombinant C1r was expressed in the baculovirus insect cell system and shown to be hemolytically active when reassembled with human serum C1q and C1s (Gal et al., 1989). In the present paper, the physical, chemical, and biological characterization of recombinant human C1s is reported. Site-directed mutagenesis is employed to remove one of the two carbohydrate chains. This protein is shown to be hemolytically active when reassembled with C1q and either human serum or recombinant C1r.

Experimental Procedures

Buffers, Media, Enzymes, and Materials. VBS was 5 mM sodium barbital, 150 mM NaCl, and 1% BSA, pH 7.4. VBS-Ca^{2+} was VBS containing 2.5 mM Ca^{2+}. Tris-buffered saline (TBS) was 10 mM Tris-HCl and 150 mM NaCl, pH 7.4. The column buffer was 75 mM NaCl, 5 mM EDTA, and 10 mM imidazole, pH 6.1. The elution buffer was 500 mM NaCl, 5 mM EDTA, and 10 mM imidazole, pH 6.1. The washing buffer was 1 M NaCl and 0.05 M EDTA, pH 8.6. TNM-FH medium was prepared as described (Summers & Smith, 1987). Serum-free medium was EX-CELL 400 insect cell medium (JRH Biosciences, Woodland, CA) with 1× antibiotic–antimycotic (GIBCO-BRL, Grand Island, NY). Restriction endonucleases and T4 ligase with their supplementary buffers were purchased from New England BioLabs, Beverly, MA, or GIBCO-BRL. [^{32}P]GTP and ^{125}I were obtained from Amersham, Arlington Heights, IL. The oligonucleotide-directed in vitro mutagenesis kit 2.0 was from Amersham. Corning tissue culture plasticware was obtained from Fisher Scientific, Santa Clara, CA. Sheep erythrocytes were purchased from Organon Teknika Corp., Westchester, PA. Human C1q, C1r, and C1s (hC1q, hC1r, hC1s) were purified from human serum as described (Lane et al., 1991; Arlaud et al., 1979). Complement components C2 and C4 and anti-sheep erythrocyte hemolysin (IgM) were obtained from Cordis Laboratories, Miami, FL. Guinea pig complement was supplied by JEM Research Products, Bethesda, MD. Human plasmin was obtained from Kabi Vitrum, Stockholm, Sweden. Porcine elastase (type IV) and neuraminidase from *Clostridium perfringens* (type X) were from Sigma. Peptide:*N*-glycosidase F was purified from cultures of *Flavobacterium meningosepticum* according to the method of Tarentino et al. (1985), modified as described by Aude et al. (1988).

Cells and Viruses and C1s cDNA. The insect cells, *Spodoptera frugiperda* (Sf9), and wild-type (WT) *Autographa californica* nuclear polyhedrosis virus (AcNPV) were generously provided by Professor Lawrence Feldman and Professor Owen Witte, UCLA, and maintained and stored as described (Summers et al., 1987). A full-length C1s cDNA-containing Blue Script plasmid, pBS-C1s, was assembled from previously described clones (Tosi et al., 1987).

Isotopic Labeling. The ^{32}P-labeled C1s cDNA probe was prepared to a specific activity greater than 300 μCi/μg using the random priming technique (Feinberg & Vogelstein, 1983, 1984), according to instructions provided by the manufacturer of the random priming kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). Swine anti-goat IgG (Boehringer Mannheim Biochemicals) was radioiodinated by Enzymobeads (Bio-Rad, Richmond, CA) to a specific activity of approximately 560 μCi/mg. The unreacted ^{125}I was removed by centrifugation three times through a column of Sephadex G50 (Penefsky, 1977).

Isolation of Recombinant C1s. Monolayers of Sf9 cells were grown to confluence in TMN-FH medium supplemented with fetal bovine serum in 100-mm tissue culture plates. The cells were then washed with serum-free medium once and infected with AcNPV-C1s at a ratio of 1 to 3 pfu per cell (Summers & Smith, 1987). After 3 days of incubation in serum-free medium (10 mL/dish) at 27 °C, cell-free culture medium was collected by low-speed centrifugation. The medium was loaded at 12 mL/h onto a DEAE-Sephacel column (1 cm × 9 cm) preequilibrated with column buffer. Subsequently, the column was washed with 12 mL of column buffer and then eluted with a linear salt gradient prepared from 25 mL of column buffer and 25 mL of elution buffer, running from 75 to 500 mM NaCl at 10 mL/h; C1s fractions were identified by SDS–PAGE of each fraction. The column was regenerated by washing with 50 mL of washing buffer and reequilibrated with 50 mL of column buffer. The C1s pool was diluted with 3 volumes of water and reloaded onto the regenerated DEAE column. The C1s eluted a second time with a linear salt gradient, was concentrated, and changed to low salt buffer (25 mM Hepes, pH 7.0), using a Centricon-30 filter (Amicon, Beverly, MA). Protein concentration was determined by absorbance measurements, using an extinction coefficient at 280 nm of ϵ (1%, 1 cm) = 14.5 (Thielens, 1990).

SDS–PAGE and Western Blots. Thirty microliters of cell culture medium or 20 μL of C1s fractions was boiled with sample buffer (0.25 Tris-HCl, 40% glycerol, 0.04% bromphenol blue, 8% SDS with 50 mg/mL DTT, pH 6.8). SDS–PAGE and Coomassie blue staining were performed as described (Lane et al., 1991). Detailed procedures for the western blot and its quantitation have been published (Bianchino et al., 1988; Hosoi et al., 1987; Towbin et al., 1979; Gershoni & Palade, 1983). Goat anti-human C1s IgG and goat anti-human C1q IgG were used as first antibodies.

Isolation and Characterization of Peptides Derived from the α2 Fragments of hC$\bar{1}$s and rC$\bar{1}$s. Recombinant C1s (0.4 mg/mL) and hC1s (1.0 mg/mL) in 145 mM NaCl and 50

mM triethanolamine hydrochloride (pH 7.4) were activated by incubation with hC$\bar{1}$r (5% w/w) for 75 min at 37 °C and then digested with plasmin (5% w/w) for 90 min at 37 °C. Purification of the proteolytic fragments γ-B, α1, and α2 was realized by high-pressure hydrophobic interaction chromatography on a TSK-Phenyl 5PW column (Beckman) as described by Thielens et al. (1990), except that elution was carried out by both decreasing the concentration of $(NH_4)_2SO_4$ from 1.5% to 0% and increasing the concentration of acetonitrile from 0% to 5% (v/v). The isolated α2 fragments obtained from both proteins were reduced, alkylated by iodo-[1-^{14}C]acetic acid, and then digested with elastase as described previously (Thielens et al., 1990). Peptides were first separated by reversed-phase HPLC on a Nova-PAK C18 column (Waters Associates) with solvent system 1 consisting of 0.1% NH_4HCO_3 and acetonitrile and then further purified on the same column with solvent system 2 consisting of 0.1% trifluoroacetic acid and acetonitrile.

Amino acid analysis of the peptides was performed by reversed-phase HPLC of amino acid phenylthiocarbamyl derivatives with a Pico-Tag amino acid analysis system (Waters Associates). The phenylthiocarbamyl derivative of *erythro*-β-hydroxyaspartic acid was resolved from that of glutamic acid by lowering the pH of the starting buffer from 6.4 to 5.3. N-Terminal sequence determination was performed in an Applied Biosystems Model 470A gas-phase protein sequencer, as described previously (Thielens et al., 1990).

Enzymatic Activity. C$\bar{1}$s was obtained by incubation of the proenzyme with 5% (w/w) hC$\bar{1}$r for 150-min in TBS. A stock solution of 1 mM *N*-carbobenzoxy-L-tyrosine *p*-nitrophenyl ester (Sigma, St. Louis, MO) was prepared in reagent grade acetone, and 6–20 μL was added to 1 mL of 0.09 M NaCl and 5 mM Tris-HCl, pH 8.1, containing 5 μg of protein. The production of *p*-nitrophenol was measured as the difference between the reading at 410 nm and that at 560 nm; readings were repeated at 50-s intervals for 300 s at 20 °C in a Hewlett-Packard 8452A diode array spectrophotometer. Each data point was corrected by subtracting the corresponding reading from an associated control experiment containing no enzyme to correct for spontaneous hydrolysis of the substrate. The molar extinction coefficient of *p*-nitrophenol at pH 8.1 was taken as 1.66×10^4 M^{-1} cm^{-1}. K_m and V_{max} were calculated from a Lineweaver–Burk plot.

Site-Directed Mutagenesis of C1s. The full-length C1s cDNA was cloned into M13mp19 between the *Sma*I and *Pst*I sites. The mutagenesis was performed with the Amersham oligonucleotide-directed mutagenesis kit following the manufacturer's instruction, using the oligonucleotide CGGAGTTCAATGCAGTG which changes the Asn 159 codon AAT to a Gln codon CAA. The mutated C1s sequence was cloned into pAcC6 between the *Pst*I and blunt-ended *Bam*HI sites. The same procedures were used for expression and isolation of the mutant recombinant C1s, designated as $rC1s_{NQ}$ for the Asn to Gln change.

Results

Construction of the Recombinant Virus and Its Isolation and Purification. The construction of the pAcC6 transfer vector and the subsequent screening and purification of pure recombinant baculovirus are described in the legends to Figures 1 and 2. Purified recombinant virus, AcNPV-C1s, was propagated at 27 °C and stored at 4 °C as virus stocks containing about 10^7 pfu/mL. The correct recombinantion of C1s cDNA into the polyhedrin gene was further confirmed by a southern blot of the DNA isolated from AcNPV-C1s-infected Sf9 cells (data not shown).

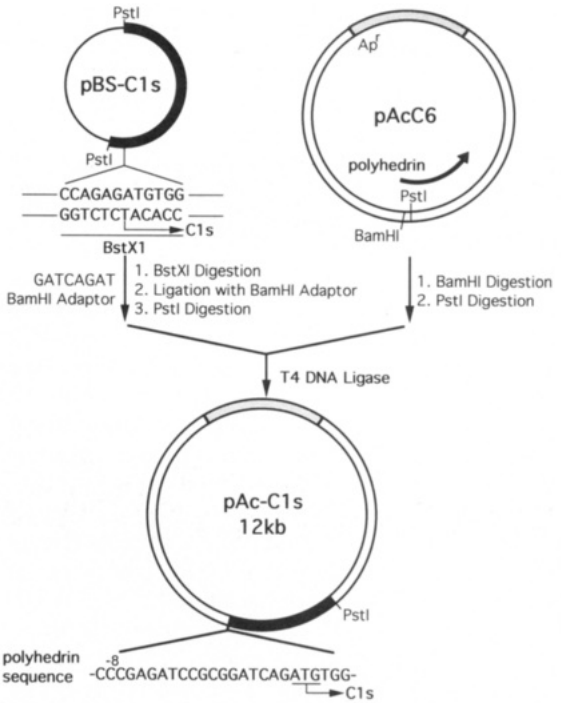

FIGURE 1: Construction of the transfer vector pAc-C1s. The cDNA for human C1s that contained the 2067-bp coding region and the 186-bp 5′- and 293-bp 3′-untranslated regions was subcloned into the *Pst*I site of the Blue Script vector (pBS-C1s plasmid). The cDNA contained a convenient *Bst*XI restriction site coinciding with the first codon of C1s. To eliminate possible interference of the 5′-untranslated sequence on the expression of C1s, *Bst*XI was employed to excise the 5′ end; however, this treatment cut within the first codon. Ligation of an octameric oligonucleotide GATCAGAT restored the ATG and converted the *Bst*XI end into a *Bam*HI "sticky" end. *Pst*I digestion at the 3′ end released the full-length C1s cDNA, which was then ligated into the transfer vector pAcC6, obtained from Cetus (Emeryville, CA), between the *Bam*HI and *Pst*I sites. The resulting recombinant vector pAc-C1s contained C1s cDNA under the control of the strong viral polyhedrin promoter. The region around the ATG start codon was sequenced directly from the plasmid (Chen & Seeburg, 1985) to confirm proper insertion and ligation. This recombinant plasmid pAc-C1s was used to generate recombinant baculovirus as described (Summers & Smith, 1987). Standard recombinant DNA procedures were employed in these studies (Sambrook et al., 1989).

Expression of Recombinant C1s. When a cell monolayer was infected with the pure recombinant virus, AcNPV-C1s, the Sf9 cells swelled but, in contrast to WT AcNPV infected cells, did not contain occlusion bodies. Mock-infected cells grew normally. Three days post infection, cell culture medium was collected and analyzed for the presence of C1s protein. Western blots demonstrated that cells infected with recombinant virus isolated and purified from four independent clones secreted human recombinant C1s into the media; control cells infected with WT AcNPV or mock-infected cells did not secrete C1s (data not shown). When analyzed directly by SDS–PAGE, the recombinant C1s (rC1s) was the only visible band stained by Coomassie blue, although other bands including the 64-kDa viral envelope protein were visible on silver-stained gels (data not shown). The rC1s appeared slightly smaller than human serum hC1s on gels (see below); moreover, neither A nor B chains were detected on reducing gels (data not shown), suggesting that rC1s was secreted as a zymogen.

Purification of rC1s from the Cell Culture Medium. Usually ten 100-mm Petri dishes of Sf9 cell monolayers were infected at a multiplicity of 1–3 pfu/cell. The serum-free culture medium was collected 3 days post infection, loaded directly onto a DEAE-Sephacel column, and subsequently

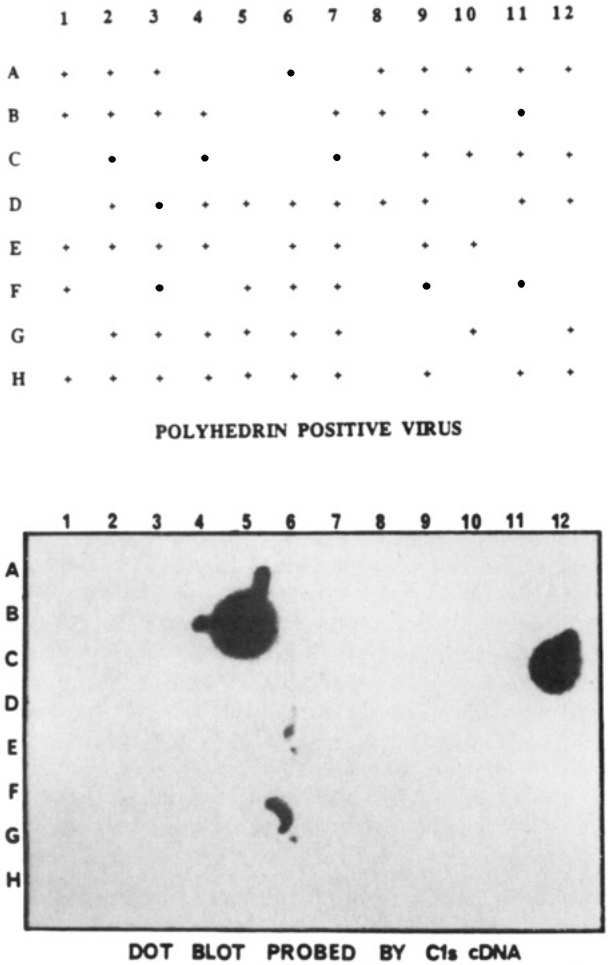

FIGURE 2: A typical result of the second screening for recombinant virus. Mixtures of plasmid pAc-C1s and purified viral DNA were used to cotransfect S59 insect cells; typically, 6 days later all the cells contained visible occlusion bodies, demonstrating that the infection was complete. The culture medium was collected and titered; the titer was usually in the order of 10^7 pfu/mL. Series dilution infection of cells in 96-well plates and dot blot hybridization were used to follow the purification of the recombinant virus (Summers & Smith, 1987; Pen et al., 1989). An initial screen involved the inoculation of Sf9 cells grown in 96-well plates at a virus density of about 20 pfu/mL; one or two wells containing cells with C1s cDNA were always found, demonstrating a recombination efficiency of 1–2 recombinant particles for every 2000 plaque forming units. The medium from positive wells was titered for wild-type particles and then employed in a second screening at about 1 pfu/well. The Sf9 cells in a 96-well plate were infected with viruses at about 1 pfu/well and incubated at 27 °C for 6 days. In the top panel, the (+) symbol marks wells containing wild-type virus, as visualized by polyhedrin occlusion bodies inside cells. The lower panel shows an autoradiograph of a dot blot of cell lysates from corresponding wells. The blot was hybridized with labeled C1s cDNA probe. Recombinant virus in well C12 was contaminated with wild-type virus and required an additional screening. Those wells which were occlusion body negative and C1s cDNA positive contained pure recombinant virus, AcNPV-C1s. Recombinant virus in well B5 was pure.

eluted with a salt gradient. Most contaminating proteins were eluted before the rC1s, and the major peak containing rC1s emerged at about 0.3 mM, close to the ionic strength where hC1s was eluted. The column was regenerated, and the diluted rC1s was reloaded and reeluted to yield an average (from 27 purifications) of 0.75 mg of rC1s obtained from 100 mL of cell media. The rC1s has been routinely concentrated by centrifugal filtration (Centricon) to concentrations of 14 mg/mL in 25 mM Hepes, pH 7.0; unlike the human serum C1s, the rC1s aggregated in distilled water at high protein concentrations.

Table I: Sedimentation Coefficients of Recombinant C1s and Its Complexes[a]

	sedimentation coeff ($s_{20,w}$ in svedbergs)				
solvent	rC1s	hC1s	$rC1s_2hC1r_2$	rC1	hC1
TBS + 2.5 mM Ca^{2+}	5.6	5.8			
	5.5	5.7			
TBS + 0.2 mM Ca^{2+}	5.7	5.7	9.0	15.6	16.1
	5.4	5.7	9.2		
TBS + 0.3 mM EDTA	4.0	4.0			
		4.4			

[a] The model E analytical ultracentrifugation was run as described (Poon et al., 1985). Sedimentation coefficients are reported as $s_{20,w}$. A wavelength of 236 nm was employed for most of the experiments, and because of the high extinction coefficient at this wavelength, the protein concentrations were about 0.1 mg/mL; thus, reported sedimentation coefficients were close to the infinite dilution values. For reassembled C1, physiological concentrations of each component were employed, yielding a final protein concentration of 0.16 mg/mL. For these studies, TBS buffer was employed, with Ca^{2+} or EDTA, as specified above.

Physical Characterization of the Recombinant C1s. The physical properties of recombinant C1s were very similar but not identical to those of human serum C1s: it sedimented as a single sharp boundary in the analytical ultracentrifuge, dimerized upon the addition of Ca^{2+}, and formed a tetramer upon addition of a stoichiometric amount of $C1r_2$, and the tetramer assembled with C1q to yield a 15.6S C1 complex. Recombinant C1s (rC1s) and human serum C1s (hC1s) were compared in paired runs using two cells in the same rotor to eliminate variations in temperature and rotor speed and timing. The results are tabulated in Table I, where it may be seen that all values agree with those obtained in earlier studies, within the error limits.

Inspection of the values in Table I shows a small but reproducible decrease in the sedimentation rate of the rC1s when compared to hC1s; the differences are most pronounced for the four paired runs using either 2.5 or 0.2 mM Ca^{2+}. In these four paired runs, the rC1s was slower by −0.2, −0.2, −0.0, and −0.3 S, yielding an average difference of −0.18 ± 0.06 S (SEM, $n = 4$), significantly different from zero ($p = 0.05$). Since the sedimentation coefficient varies as $M^{2/3}$, this would suggest a molecular weight difference of 4000 ± 1300 for the C1s monomer. Some of this could be due to a change in frictional ratio, as well. Human serum C1s contains 673 amino acids and has a polypeptide molecular weight of 74 900. Since C1s is believed to contain about 4000 g/mol carbohydrate (Thielens, 1990), the total molecular weight of the glycoprotein is about 78 900. As will be shown below, incomplete glycosylation is the probable cause of the reduction in molecular weight detected by these sedimentation experiments.

Recombinant and Human C1s Migrate Differently on Gels Due to Differences in Their Carbohydrate Contents. SDS–PAGE analysis of the proenzyme forms of rC1s and hC1s indicated that they migrated with a small but significant difference in mobility, with apparent molecular weights of 80 000 and 84 000, respectively. Activation of rC1s by $hC\bar{1}r$ yielded, as expected, the A and B chains, but while the B chain (M_r 30 000) had virtually the same mobility as the $hC\bar{1}s$ B chain, the A chain (M_r 52 00) migrated significantly faster than the $hC\bar{1}s$ A chain (M_r 55 500) (Figure 3). Considering that the two glycosylation sites of hC1s are both located on the A chain, it appeared likely, therefore, that the observed differences in electrophoretic mobility were due to differences in the carbohydrate contents of the proteins. To further investigate this question, $hC\bar{1}s$ and $rC\bar{1}s$ were both treated with neuraminidase and with PNGase F, an endoglycosidase that hydrolyzes all classes of N-linked glycans. In agreement with

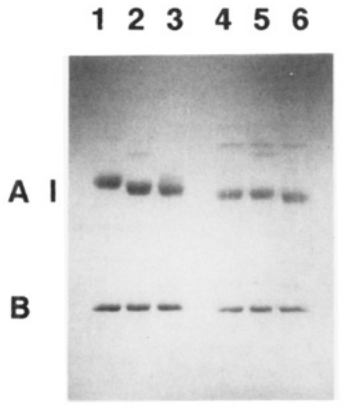

FIGURE 3: SDS–PAGE analysis of the deglycosylation of hC1s and rC1s by neuraminidase and PNGase F. hC1s (0.35 mg/mL) and rC1s (3.0 mg/mL), in 145 mM NaCl and 50 mM triethanolamine hydrochloride (pH 7.4), were incubated with neuraminidase (10 units/mg of protein) for 5 h at 25 °C or with 6% (w/w) PNGase F for 1 h at 30 °C. Electrophoresis of reduced and alkylated samples was performed on 10% polyacrylamide gels as described by Laemmli (1970), and Coomassie blue was used for protein staining. Lane 1, untreated hC1s; lane 2, hC1s treated with neuraminidase; lane 3, hC1s treated with PNGase F; lane 4, untreated rC1s; lane 5, rC1s treated with neuraminidase; lane 6, rC1s treated with PNGase F.

previous results (Aude et al., 1988), treatment of hC1s with PNGase F removed the carbohydrate chain attached to Asn 159, converting the A chain to its monoglycosylated form (M_r 53 500). The same treatment performed on rC1s caused a slight but significant shift in the mobility of the A chain, decreasing its M_r from 52 500 to 52 000 (Figure 3). Treatment with neuraminidase, in contrast, decreased the apparent M_r of the hC1s A chain from 55 500 to 54 000 but had no detectable effect on the mobility of the rC1s A chain. These observations strongly suggested, therefore, that, compared to hC1s, rC1s incompletely processed N-linked carbohydrate chains lacking, at least, the terminal sialic acid residues.

Isolation and Characterization of Peptides Derived from the α2 Fragment of rC1s. Recombinant C1s was activated with hC1r and then submitted to limited proteolysis by plasmin, as described under Experimental Procedures. SDS–PAGE analysis of the plasmin digest (data not shown) indicated that it contained the three major fragments, γ-B, α1, and α2, already described in the case of hC1s (Thielens et al., 1990). However, fragments γ-B and α2 obtained from rC1s migrated significantly faster than the corresponding fragments generated from hC1s, whereas the α1 fragments obtained in both cases had virtually the same mobility. Similarly, when the two plasmin digests were fractionated by high-pressure hydrophobic interaction chromatography (data not shown), no difference was observed in the elution positions of the α1 fragments, but again, fragments γ-B and α2 obtained from rC1s eluted later than their hC1s counterparts, indicating a more hydrophobic character. Considering that fragments γ-B and α2 each contain one of the two glycosylation sites of C1s (Aude et al., 1988; Thielens et al., 1990), these observations provided further support to the hypothesis of an incomplete processing of the carbohydrate chains of rC1s.

In hC1s, two asparagine residues located within the α2 fragment (positions 97–195) are posttranslationally modified: Asn 134 is partially converted to β-hydroxy-Asn, whereas Asn 159 carries an oligosaccharide chain (Thielens, 1990). With a view to check whether these modifications were also performed by the insect cells, tha α2 fragments obtained from both hC1s and rC1s were reduced and alkylated and then digested with elastase. The digests were first fractionated by reversed-phase HPLC with solvent system 1 (Figure 4). The

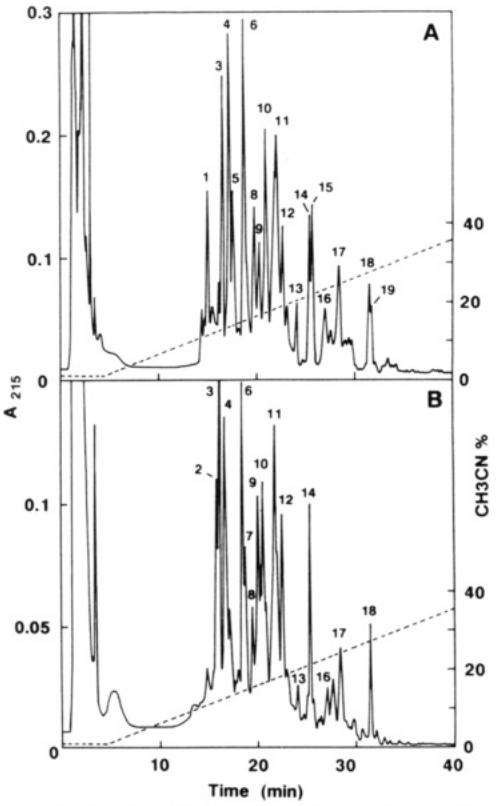

FIGURE 4: Initial fractionation by reversed-phase HPLC of the peptides generated by elastase cleavage of the reduced and alkylated α2 fragments from hC1s and rC1s. The α2 fragments obtained from plasmin cleavage of hC1s (A) and rC1s (B) were reduced and alkylated and then digested with elastase. The digests were fractionated on a Nova-PAK C18 column using solvent system 1 consisting of 0.1% NH_4HCO_3 and acetonitrile.

major peptides contained in each peak were further purified by reversed-phase HPLC with solvent system 2 and then characterized by amino acid and/or N-terminal sequence analysis. Three groups of peptides containing Asn 134 were isolated from each digest:

(i) Peptide Phe[131]-Cmc-Asn-Xaa[134]-Phe-Ile-Gly-Gly[138] eluted as a doublet (peaks 14 and 15) in the digest from hC1s (Figure 4A) but as a single peak (peak 14) in the digest from rC1s (Figure 4B). Amino acid and N-terminal sequence analyses indicated that the same variant, with asparagine at position 134, was present in both peaks 14, whereas the variant eluting in peak 15 contained *erythro*-β-hydroxyasparagine at this position. Analysis by mass spectrometry of the peptides contained in peaks 14 yielded similar spectra showing, in each case, a peak at m/z = 929.4, i.e., a value corresponding to that expected for the protonated [$(M + H)^+$] pseudo-molecular ion of the variant with an unmodified Asn. The peptide contained in pool 15, in contrast, yielded a peak at m/z = 945.4, consistent with the presence of hydroxyasparagine (data not shown).

(ii) Peptide Phe[131]-Cmc-Asn-Xaa[134]-Phe-Ile-Gly-Gly-Tyr-Phe[140] also eluted as a doublet (peaks 18 and 19) in the digest from hC1s (Figure 4A) and as a single peak (peak 18) in the digest from rC1s (Figure 4B). Again, it was shown unambiguously that both peaks 18 contained the variant with an unmodified asparagine, whereas peak 19 contained the variant with *erythro*-β-hydroxyasparagine.

(iii) The intermediate peptide Phe[131]-Cmc-Asn-Xaa[134]-Phe-Ile-Gly-Gly-Tyr[139], obtained in low yield, eluted as a single peak (peak 16) in both digests (Figure 4) and was found to contain asparagine at position 134.

These data clearly demonstrated, therefore, that, in contrast with hC1s, rC1s lacked posttranslational hydroxylation of asparagine 134.

During the search for peptides containing Asn 134, three major peptides containing the glycosylation site at position 159 were isolated from each digest. Peptide Asp[151]-Asp-Met-Lys-Asn-Cmc-Gly-Val-Asn(CHO)-Cmc-Ser-Gly-Asp-Val-Phe[165], contained in peak 1 of the digest from hC$\bar{1}$s (Figure 4A), was not found at the corresponding position in the digest from rC$\bar{1}$s but instead eluted in peak 2 (Figure 4B). In addition, two variants of another peptide, Leu[149]-His-Asp-Asp-Met-Lys-Asn-Cmc-Gly-Val-Asn(CHO)-Cmc-Ser-Gly-Asp-Val-Phe[165], were recovered from each digest: these eluted in peaks 5 and 6 of the digest from hC$\bar{1}$s (Figure 4A) but in peaks 7 and 9 of the digest from rC$\bar{1}$s (Figure 4B). The glycopeptides obtained from both hC$\bar{1}$s and rC$\bar{1}$s were submitted to N-terminal sequence analysis, and in each case, no residue was identified at position 159, indicating that Asn was linked to a carbohydrate. It became clear, therefore, that position 159 of rC1s was indeed occupied by an oligosaccharide chain which, compared to that present on hC1s, had a more hydrophobic character compatible with the lack of sialic acid. Tentative analysis by mass spectrometry of the glycopeptides isolated from both rC$\bar{1}$s and hC$\bar{1}$s was unsuccessful.

All other peptides isolated from the elastase digest of the α2 fragment of rC$\bar{1}$s had the same amino acid sequence as the corresponding peptides obtained from hC$\bar{1}$s, indicating that, apart from the posttranslational modifications of Asn 134 and Asn 159, both α2 fragments had identical primary structures. Moreover, reconstitution of the α2 fragment of rC$\bar{1}$s from its constituent peptides indicated that it extended from Ser 97 to Lys 195, as previously reported for hC$\bar{1}$s (Thielens et al., 1990).

N-Terminal Sequence Analysis of rC1s, rC$\bar{1}$s, and Fragments Obtained from Plasmin Cleavage. Edman degradation of the proenzyme form of rC1s yielded a single sequence Glu-Pro-Thr-Met-Tyr... identical to the N-terminal sequence of human serum C1s (Tosi, 1987), indicating proper recognition and cleavage by the insect cell signal peptidase. After activation by hC$\bar{1}$r, an additional sequence, Ile-Ile-Gly-Gly-Ser..., corresponding to the N-terminus of the hC$\bar{1}$s B chain, was obtained, indicating that hC$\bar{1}$r cleaves at the usual location (Arg[422]–Ile[423]) to yield the A and B chains (Spycher et al., 1986). N-Terminal sequence analysis of fragments γ-B and α1 yielded from limited proteolysis of rC$\bar{1}$s by plasmin also showed that these originated from cleavage of peptide bonds Lys[269]–Leu[270] and Lys[23]–Ser[24], respectively, as previously reported for the corresponding fragments from hC$\bar{1}$s (Thielens et al., 1990).

Activation of rC1s by hC$\bar{1}$r and Its Interaction with C1-Inhibitor. C1s is a proenzyme activated by C$\bar{1}$r at the zymogen cleavage site Arg[423]–Ile[424] (Tosi et al., 1987). Upon cleavage, C1s becomes an enzyme highly specific for cleavage of C4 and C2, the next components to become activated, initiating the complement cascade. The C$\bar{1}$s polypeptide still migrates as a single band on nonreducing gels, but if the disulfide bond holding together the polypeptide is cleaved, it separates into the characteristic A and B chains (sometimes called heavy and light chains) which migrate with apparent molecular weights of 55000 and 30000 on reducing gels, respectively. A covalent complex is formed between the B chain of activated C$\bar{1}$s and C1-inhibitor. These characteristic reactions of hC1s are also observed with the recombinant C1s (Figure 5).

In order to demonstrate the acquisition of C$\bar{1}$s enzymatic activity, recombinant C$\bar{1}$s was studied with a synthetic substrate, *N*-carbobenzoxy-L-tyrosyl-*o*-nitrophenol. Values of k_{cat} and K_m were 1.20 0.16 s^{-1} and 109 ± 28 μM, respectively, not significantly different from values of 2.51 ± 1.18 s^{-1} and 125 ± 47 μM obtained with hC$\bar{1}$s or values of 1.22 s^{-1} and 56 μM reported by Bing (1969) (data not shown).

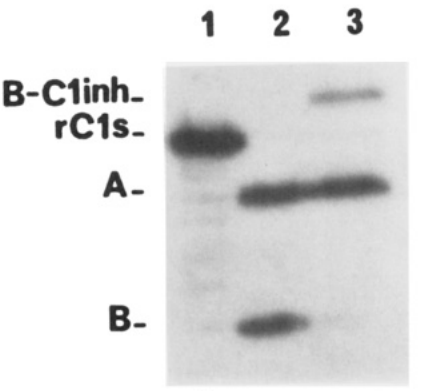

FIGURE 5: rC1s cleavage by hC$\bar{1}$r and formation of a complex with C1-inh through B chain. Purified rC1s was incubated with 5% w/w hC$\bar{1}$r in VBS-Ca^{2+} at 37 °C for 2 h (lane 2), and then C1-inh was added and the incubation continued at 37 °C for another hour (lane 3). Samples were analyzed by reducing SDS–PAGE followed by western blotting with anti-C1s antibodies. Lane 1 contains untreated, unactivated rC1s.

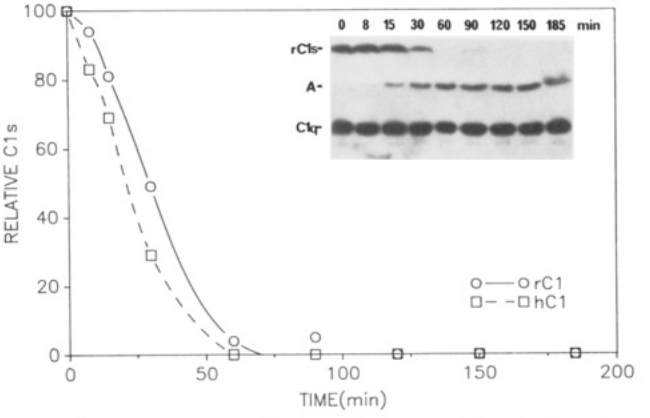

FIGURE 6: Spontaneous activation of the recombinant C1s when reassembled as C1 using human serum C1q and C1r. Physiological concentrations of rC1s, hC1q, and hC1r were mixed and incubated in VBS-Ca^{2+} at 37 °C. At the indicated time, an aliquot was removed for reducing SDS–PAGE analysis and western blot with anti-C1s and anti-C1q antibodies. The insert shows the autoradiograph of the western blot. C1s indicates the unactivated form; A is the A chain. The plot shows the values obtained upon scanning the autoradiograph for the quantity of rC1s remaining as a function of time (open circle). As a control, the spontaneous activation of C1 reassembled with human serum C1s was followed simultaneously (open squares).

Spontaneous Activation of C1. In the absence of C1-inhibitor, C1 undergoes rapid and complete spontaneous activation, which can be assayed by assessing the cleavage of C1r or C1s. In Figure 6 the spontaneous activation of C1 reassembled from hC1q, hC1r, and either hC1s or rC1s is shown. The results are similar, although the activation of rC1s appears slower than that of hC1s (with observed half-lives of 33 and 24 min^{-1} at 37 °C, respectively).

Acquisition of Hemolytic Activity. When reassembled with human serum C1q and C1r to form C1, rC1s yielded 16S C1 with about 70% of the hemolytic activity of the control C1 reassembled with C1s (Figure 7). Stoichiometric addition of $C1s_2$ to a equimolar mixture of C1q and $C1r_2$ yielded maximal activity (data not shown).

Recombinant C1r and C1s, Both Expressed in Baculovirus Infected Cells, Form a Hemolytically Active Tetramer.

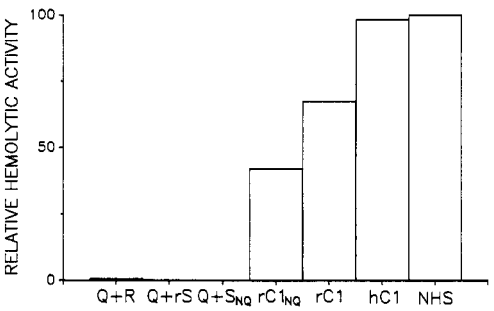

FIGURE 7: Comparison of hemolytic activity of reassembled C1 samples. Stoichiometric additions of C1q, $C1r_2$, $rC1s_2$, or $(rC1s_{NQ})_2$ or pairs of these subcomponents were added to yield final concentrations of 200 nM, and the mixtures were incubated on ice for 30 min at 37 °C to form C1. The C1-specific assay described by Rapp and Borsos (1970) was employed to determine the percentage of hemolytically active C1 in serum and mixtures containing a physiological concentration of reassembled C1 subcomponents. A two-step, 20000-fold dilution into VBS-Ca^{2+} was followed by addition of 100 μL of the diluted mixture to 100 μL containing 10^7 EAC4 cells/mL. C1 was allowed to adsorb to the EAC4 cells for 1 h at 30 °C. Then, functionally purified C2 was added and incubation continued for 30 min. Finally, EDTA-treated guinea pig serum was added to supply the remaining complement components, and lysis was achieved by incubation at 37 °C for 1 h. The degree of lysis, y, was determined at 412 nm, and the number of hemolytically active C1 molecules/EAC4 was calculated according to "one-hit" theory as $Z = -\ln(1 - y)$. Calibration plots of ln Z vs ln (dilution) were straight with slopes of about 1.3 as expected (Loos et al., 1973), and these were used to determine the relative concentrations of hemolytically active C1. Plotted on the vertical axis are relative values of Z, normalized to 100% for normal human serum (NHS).

Table II[a]

	% hemolysis	hemolytic sites/cell	fraction of C1 hemolytically active
C1q + 6× mock	0.1 ± 0.05	0.001	
C1q + 6× WT	0.1 ± 0.05	0.001	
C1q + 6× rC1r, rC1s	65 ± 6	1.05	0.45

[a] Sf9 cell cultures (10^7 cells in 10 mL of serum-free medium) were infected with either C1s or C1r cDNA-containing recombinant baculovirus. Simultaneous mock and WT infections were used as controls. Three-day post-infection culture media were analyzed by ELISA and shown to contain 5 μg/mL C1s or 5 μg/mL C1r, respectively, for the recombinant virus infected flanks, while no detectable signal was observed in the controls. Sixfold concentrated culture supernatants were combined with C1q, to give a reconstitution mixture containing 0.07 μM C1r, 0.07 μM C1s, and 0.17 μM C1q.

Previously, C1r has been expressed in the baculovirus insect cell system (Gal et al., 1989). When rC1s was mixed with $rC1r_2$ and human serum C1q, hemolytically active C1 was generated, as demonstrated in Table II.

Recombinant C1s, Lacking One of Its Two Carbohydrate Chains, Still Exhibits Good Hemolytic Activity. Site-directed mutagenesis, as described under Experimental Procedures, was employed to eliminate one of the two carbohydrate attachment sites in C1s by converting Asn 159 to Gln 159. The recombinant virus was constructed by techniques identical to those described above. The mutant C1s was purified by the same isolation procedure; the yield was approximately the same. To clearly demonstrate the molecular weight difference due to the missing carbohydrate chains, the mutated C1s was activated with human serum C1r and compared on reducing SDS–PAGE with the nonmutated recombinant C1s. The A chain of the mutated protein, which normally contains both attachment sites, migrated distinctly faster than its nonmutated counterpart; a double band was visible when a mixture of the two activated proteins was analyzed (data not shown). Hemolytic activity of the mutant C1s was 70% of that found for the nonmutated recombinant protein (Figure 7).

Discussion

Substantial quantities of human recombinant C1s have been expressed in the baculovirus insect cell system. The mature protein has the same N-terminus as human serum C1s, indicating that the insect cell signal peptidase recognizes and cuts at the same cleavage site as the human enzyme. The recombinant polypeptide folds within the insect cell to form a protein which appears to possess all of the multiple interaction sites found on the human serum C1s; thus, the monomeric recombinant C1s dimerizes in the presence of Ca^{2+} and forms the C1s–C1r–C1r–C1s tetramer upon addition of hC1r. The tetramer contains interaction sites for reassembly with C1q to form C1, some of these sites are located on the C1s moiety (Poon & Schumaker, 1991), and it is possible that additional sites on C1r are formed upon interaction of the α domain of C1s with C1r (Busby & Ingham, 1990). The recombinant C1s is recognized by human serum $C\bar{1}r$ which cuts at the usual cleavage site to yield enzymatically active $C\bar{1}s$. Plasmin also cleaves $rC\bar{1}s$ and $hC\bar{1}s$ at identical sites, as determined by sequencing, to yield the three major fragments, α1, α2, and γ-B. The catalytic site of the recombinant $C\bar{1}s$ recognizes and cleaves a conventional $C\bar{1}s$ peptide substrate with about the usual K_m and k_{cat}, and it also recognizes its specific inhibitor and forms a very tight complex between the B chain and C1-inhibitor. C1 reassembled with the recombinant C1s activates spontaneously, and it forms hemolytically active C1. This last observation implies that the recombinant C1s has re-formed its specific interaction sites which recognize C4 (Matsumoto et al., 1989) and C2 (Matsumoto & Nagaki, 1986). Taken together, these observations imply that the recombinant C1s polypeptide possesses a three-dimensional structure which must be very similar to that of the human serum protein.

The recombinant C1s is not identical to the human serum C1s, however; it sediments more slowly in the analytical ultracentrifuge and migrates more rapidly on gels, it lacks β-hydroxylation of Asn 134, and it may show a somewhat diminished hemolytic activity when reassembled to form C1. The lower molecular weights implied by the sedimentation and migration data are probably the result of incomplete glycosylation; the contrasting behavior of the recombinant and human serum proteins to neuraminidase implies that the recombinant C1s lacks sialic acid. Carbohydrate is present at Asn 159; however, the glycosylated peptide released by elastase digestion emerges in a fraction different from the corresponding peptide released from human serum C1s, implying that the carbohydrate must be different. The A chain of $rC\bar{1}s$ decreases in M_r only slightly upon treatment with PNGase F. It can be concluded from our data that the carbohydrate linked to Asn 159 lacks, at least, sialic acid and probably contains only a few saccharide units. The chromatographic behavior of fragment γ-B on the TSK-Phenyl column suggests that this is probably true for the carbohydrate attached to Asn 391.

Contrary to the accepted idea that insect cell-derived proteins only contain high-mannose-type oligosaccharides, processing to the complex type may occur under certain conditions, depending on the time of infection of the cells by the recombinant baculovirus (Davidson & Castellino, 1991). In the case of rC1s, processing may have yielded small, complex oligosaccharides lacking sialic acid.

rC1s retains the ability to dimerize in the presence of Ca^{2+} and to form the Ca^{2+}-dependent C1s–C1r–C1r–C1s tetramer; this implies that it retains the ability to bind Ca^{2+} ions. Given

that this function is mediated by the N-terminal α region and that this region contains both Asn 134 and Asn 159, this implies that hydroxylation of the former residue and proper glycosylation of the latter residue are not critical for Ca^{2+} binding. This confirms the previous observation that complete removal of the carbohydrate attached to Asn 159 does not impair the ability of fragment C1s α to mediate homologous (C1s–C1s) and heterologous (C1s–C1r) Ca^{2+}-dependent interactions (Thielens, 1990). However, the functional implication of the hydroxylation of Asn 134 in Ca^{2+} binding and Ca^{2+}-dependent interactions was not clear, up to now.

A mutant recombinant C1s lacking one of the two carbohydrate chains was secreted abundantly and reassembles with human serum C1r and C1q to form hemolytically active C1. Thus, this carbohydrate is not required for folding or export of the protein in insect cells, nor is it essential for reassembly or C1 function. Perhaps it plays some role in the removal of activated complexes from the plasma.

Recombinant C1s should prove to be very useful in future studies of C1s structure and function. Since it possesses a very similar three-dimensional structure and demonstrates the same multifunctional behaviors as the human serum protein, site-directed mutagenesis studies could identify critical residues for each function; indeed, hemolytic activity, which requires proper reassembly of a functional C1 and recognition of C2 and C4, should provide a sensitive assay for functionally significant mutations, allowing a rapid initial screen.

Recombinant C1s contains no trace of human C1r or other human serum proteins and should be the antigen of choice for the production of polyclonal antibodies for ELISA, western blots, and immunoprecipitation procedures.

Finally, it is hoped that recombinant C1s may be obtained in sufficient quantity and purity to allow successful crystallization of the intact or modified protein or, at least, of well-defined domains.

Acknowledgments

We thank A. Buzy and J. Ulrich for performing mass spectrometry analysis.

Registry No. C1s, 80295-35-8; C1, 80295-32-5; C1q, 80295-33-6; C1r, 80295-34-7; *erythro*-β-hydroxyasparagine, 20790-74-3.

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Influence of Phospholipid Asymmetry on Fusion between Large Unilamellar Vesicles†

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ABSTRACT: The ability of lipid asymmetry to regulate Ca^{2+}-stimulated fusion between large unilamellar vesicles has been investigated. It is shown that for 100-nm-diameter LUVs composed of dioleoylphosphatidylcholine, dioleoylphosphatidylethanolamine, phosphatidylinositol, and dioleoylphosphatidic acid (DOPC/DOPE/PI/DOPA; 25:60:5:10) rapid and essentially complete fusion is observed by fluorescent resonance energy transfer techniques when Ca^{2+} (8 mM) is added. Alternatively, for LUVs with the same lipid composition but when DOPA was sequestered to the inner monolayer by incubation in the presence of a pH gradient (interior basic), little or no fusion is observed on addition of Ca^{2+}. It is shown that the extent of Ca^{2+}-induced fusion correlates with the amount of exterior DOPA. Further, it is shown that LUVs containing only 2.5 mol % DOPA, but where all the DOPA is in the outer monolayer, can be induced to fuse to the same extent and with the same rate as LUVs containing 5 mol % DOPA. These results strongly support a regulatory role for lipid asymmetry in membrane fusion and indicate that the fusogenic tendencies of lipid bilayers are largely determined by the properties of the monolayers proximate to the fusion interface.

The asymmetric transbilayer distributions of lipids commonly observed in biological membranes may be expected to play a role in regulating membrane fusion in vivo. Model membrane systems composed of unsaturated phosphatidylethanolamine (PE) and phosphatidylserine (PS), approximating the inner monolayer composition of the erythrocyte membrane, for example, fuse readily in the presence of physiological stimuli such as Ca^{2+} (Hope et al., 1983). Alternatively, vesicles composed of phosphatidylcholine (PC) and sphingomyelin, the outer monolayer composition, are resistant to fusion. It may therefore be expected that membranes whose external monolayers contain fusogenic lipids such as PE and PS will fuse more readily than membranes with identical lipid compositions but where the fusogenic lipids are localized to the inner monolayer. These speculations are supported by several observations. For example, Sessions and Horwitz (1981, 1983) have shown that the external leaflet of the plasma membrane of myoblasts, which undergo fusion to form myotubes, contains more PE and PS than the outer monolayer of the erythrocyte. Further, the concentrations of these lipids in the outer monolayer increase prior to fusion (Santini et al., 1990). It may also be noted that erythrocytes which have lost lipid asymmetry fuse more readily than erythrocytes exhibiting asymmetric lipid distributions with PE and PS in the inner monolayer (Tullius et al., 1989).

The regulatory role of lipid asymmetry in fusion has proven difficult to investigate, due in part to the lack of an appropriate model system. However, recent work from this laboratory has shown that lipid asymmetry can be generated in large unilamellar vesicles (LUVs) by imposing transmembrane pH gradients (Hope et al., 1989; Redelmeier et al., 1990; Eastman et al., 1991). Here we utilize this phenomenon to investigate the role of lipid asymmetry in the regulation of Ca^{2+}-induced membrane fusion. It is shown that lipid asymmetry can profoundly regulate fusion phenomena between LUV systems and that the composition of the outer monolayer plays a dominant role in determining the rate and extent of fusion.

Materials and Methods

Lipids and Chemicals. Dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylethanolamine (DOPE), dioleoylphosphatidic acid (DOPA), bovine liver phosphatidylinositol (PI), *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidyl-

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