Soluble form of complement C3b/C4b receptor (CR1) results from a proteolytic cleavage in the C-terminal region of CR1 transmembrane domain

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The complement C3b/C4b receptor (CR1) is an integral protein, anchored in the plasma membrane through a hydrophobic domain of 25 amino acids, but is also found in the plasma in soluble form (sCR1). A recombinant, soluble form of CR1 has been demonstrated to reduce complement-dependent tissue injury in animal models of ischaemia/reperfusion. In view of the important pathophysiological relevance of sCR1, we have investigated the mechanisms governing CR1 release by using various mutated and chimaeric receptors transiently expressed in COS cells. Pulse–chase experiments revealed that (1) sCR1 is produced by a proteolytic process, (2) the cleavage site lies within the C-terminus of CR1 transmembrane domain, (3) the proteolytic process involves a fully glycosylated CR1 form and (4) this process takes place in late secretory vesicles or at the plasma membrane.

INTRODUCTION

The human C3b/C4b receptor (CR1) is a single-chain glycoprotein composed of an extracellular domain of 1930 residues, organized into 30 short repeats of a consensus element, a transmembrane domain of 25 residues and a 43-residue cytoplasmic domain [1,2] (Figure 1). CR1 is expressed in a large variety of cells including erythrocytes, polymorphonuclear leucocytes (PMN), monocytes, B-lymphocytes, some T-lymphocytes, mast cells and glomerular podocytes [3–6]. The main function of CR1 is the inhibition of complement activation by interfering with both the classical and alternative pathways [7]. CR1 also mediates other key immunological functions such as the transport of C3b-coated immune complexes in erythrocytes, activation of phagocytosis of C3b-bearing particles by neutrophils and monocytes, induction of interleukin 1 secretion by monocytes and enhancement of B-cell differentiation [8–11]. A membrane-bound CR1 derived from glomerular podocytes has been identified in human urine but its function is still unknown [12].

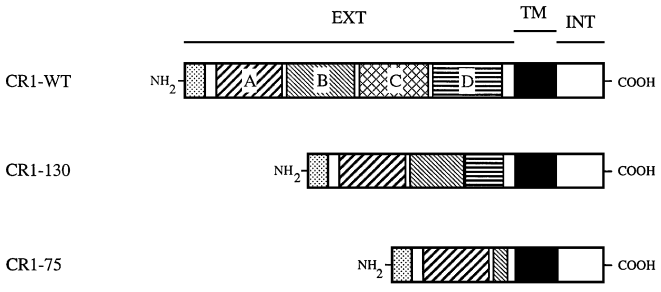

Figure 1 Structure and release of the wild-type and truncated CR1 receptors

The extent of CR1 release, expressed as a percentage of total CR1 (M + L), was evaluated as follows: CR1-WT, 46.5 ± 3.6; CR1-130, 37.5 ± 2.6; CR1-75, 17.9 ± 3.8% (ImageQuant, Molecular Dynamics). Abbreviations: EXT, external domain; TM, transmembrane domain; INT, cytoplasmic domain.

A soluble form of CR1 (sCR1), of which a large fraction might originate from PMN [13], has been identified in human plasma [14]. A recombinant sCR1 lacking the transmembrane and cytoplasmic domains has been engineered [15]. This sCR1 is a potent inhibitor of complement activation, decreasing complement-dependent tissue injury in ischaemia/reperfusion and in adult respiratory distress syndrome [15–17]. In view of these pathophysiological properties of sCR1 and its potential pharmacological interest, it is important to characterize sCR1 and to define the mechanism(s) responsible for its release into the plasma.

Given the hydrophobicity of the transmembrane domain, it seems unlikely that sCR1 is derived from the shedding of a membrane-bound CR1. Moreover, on the basis of studies performed on COS cells transfected with the cDNA encoding the full-length human CR1 and which also release sCR1, it was evident that both soluble and membrane-bound CR1 did not result from alternative splicing of the mRNA [18]. A proteolytic process was suggested but was difficult to establish because both CR1 and sCR1 exhibited indistinguishable electrophoretic mobilities. By using an ELISA, Danielsson et al. [13] provided results indicating that sCR1 was recognized by an antibody directed against the N-terminal extracellular sequence of CR1 but not by an antibody against a peptide corresponding to the C-terminal sequence of the CR1 intracellular domain. These results, based on a loss of antibody recognition, led Danielsson and co-workers to suggest that sCR1 derives from the loss of the intracellular portion of CR1 presumably by proteolytic cleavage. However, this lack of reactivity could also have another origin than the loss of a CR1 fragment (i.e. a change in the three-dimensional structure of the molecule).

In the present study we have reconsidered this question by using COS cells transiently expressing various truncated or chimaeric CR1 constructs designed to discriminate better between sCR1 and CR1. Then we have dissected CR1 in the search for a more precise definition of the intramolecular cleavage site. Finally, we have used conventional inhibitors of the secretory

Abbreviations used: BFA, brefeldin A; CR1, complement C3b/C4b receptor type I; DMEM, Dulbecco's modified Eagle's medium; endo F, endoglycosidase F; endo H, endoglycosidase H; HIR, human insulin receptor; sCR1, soluble CR1; PMN, polymorphonuclear leucocytes; TGFα, transforming growth factor α; Tm, transmembrane; uPA, mouse urokinase-type plasminogen activator; WT or wt, wild-type.

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pathway to identify the intracellular compartment(s) in which the cleavage occurs. Our results demonstrate directly that (1) sCR1 is generated by proteolytic cleavage; (2) the site of proteolysis lies within the C-terminus of the transmembrane region of CR1 and (3) the proteolytic process takes place in a late secretory compartment or at the plasma membrane.

MATERIALS AND METHODS

Chemicals and cell culture

COS cells were grown at 37 °C under air/CO_2 (19:1) in plastic flasks in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL)/10% (v/v) fetal calf serum/100 i.u./ml penicillin G/100 μg/ml streptomycin sulphate. The monoclonal antibody 3D9, directed against CR1, has been described previously [19]. T4-DNA ligase, Vent (exo-) polymerase and restriction enzymes were purchased from New England Biolabs (Allschwil, Switzerland), Tran^{35}S-label® from ICN (Costa Mesa, CA, U.S.A.) and Protein A–Sepharose from Pharmacia (Dübendorf, Switzerland). Endoglycosidase F (endo F) and endoglycosidase H (endo H) were obtained from Boehringer Mannheim (Servion, Switzerland). NH_4Cl, chloroquine, methylamine, leupeptin and aprotinin were obtained from Sigma (St. Louis, MO, U.S.A.). Bafilomycin A was purchased from Anawa (Wangen, Switzerland) and brefeldin A (BFA) from Epicenter Technologies (Madison, WI, U.S.A.). Batimastat was a gift from Dr. M. Pepper (Geneva, Switzerland).

Mutagenesis

Cloning procedures were performed as described in Sambrook et al. [20] unless stated otherwise. The sequence of all mutations was verified on both strands by the dideoxy-mediated chain termination method.

The entire coding sequence of the human CR1 gene (A allele) was cloned into a pCDM8-derived expression vector containing an ampicillin resistance gene (p^{r}CR1) and was under the control of the CMV/T7 promoter. CR1-130 was obtained by deletion of 2560 bp, spanning in part the B–D long homologous repeats of the extracellular domain on the cDNA sequence, between the two *Sma*I sites at positions 2331 and 4890 (Figure 1). CR1-75 was obtained by deletion of 4053 bp extending from the ninth short consensus repeat in the B long homologous repeat to the 30th repeat located just before the transmembrane region, beyond the D long homologous repeat, between the *Hin*cII and *Msc*I restriction sites.

CR1-tailless and CR1-LDL (Cyto) were obtained as described previously [21]. To replace the intracytoplasmic region of CR1 with that of the human insulin receptor (HIR), a unique *Afl*II site was introduced at the border between the transmembrane and intracytoplasmic domains [21]. The cDNA encoding the intracytoplasmic tail of HIR was amplified by PCR with a 5′ primer (5′-ATT CCT TAA GAA GAG GCA GCC AGA TGG-3′) designed to introduce an *Afl*II site (underlined) into the HIR sequence, and a 3′ primer (IR Bgl) containing a *Bgl*II site (underlined): 5′-GAA AGA TCT CTG AAC TCC-3′. pSP64-HIR, a gift from Dr. G. Bell (Chicago, IL), was used as template. The resulting fragment was cloned into p^r CR1 (wt), via the *Afl*II and *Bgl*II sites to generate p^{r}CR1-HIR (cyto).

The same procedure was followed to give rise to CR1-HIR (Tm+Cyto) (in which Tm represents transmembrane). The primers IR-15 (5′-CAA AAA TTA TCA TCG GC-3′) and IR Bgl were used to amplify the cDNA encoding the transmembrane and the intracytoplasmic domains of HIR. This fragment was digested with *Bgl*II and cloned into p^{r}CR1 (wt) via the *Msc*I and *Bgl*II sites. To obtain CR1-LDL (Tm+Cyto), LDL-Tm (5′-ATG CGT CGA CGC CCA GTA GCG TGA GG-3′) and LDL-Bgl (5′- GCG AGA TCT CAG GAA GG-3′) were used as primers and pLDLR3 (a gift from Dr. D. Russell, Dallas, TX, U.S.A.) as template. The PCR product was digested with *Bgl*II and cloned into p^{r}CR1 (wt) digested with *Msc*I and *Bgl*II.

To fuse the transmembrane and intracytoplasmic domains of CR1 to the mouse urokinase-type plasminogen activator (uPA), a unique *Nco*I site was created in both cDNA sequences by PCR. For uPA, M13 reverse (24-mer) (Promega, Madison, WI, U.S.A.) and uro-CR1 (5′-GGC ATC CCA TGG CGA AGG CCA GAC CTT TC-3′) were used as primers and pDB1519 as template [22]. The fragment obtained was digested with *Xba*I and *Nco*I. CR1 cDNA was amplified with CR-UK (5′-GGC ATC CCA TGG CCA AAT GTA-3′) and CR-B (5′-CAT CTT CAA GTG CAC ATG TC-3′) and digested with *Nco*I/*Bgl*II. Both fragments were incorporated into the pDB1519 plasmid digested with *Xba*I and *Bgl*II.

Zymography

The insertion of the C-terminal tail of CR1 downstream of the uPA sequence was also verified by zymography as previously described [22]. Briefly, samples of conditioned media and cell extracts were subjected to SDS/PAGE [10% (w/v) gel] under non-reducing conditions. Gels were layered on a 2% (w/v) casein/1.25% (w/v) agar underlay, containing 40 μg/ml human plasminogen and 0.1 M Tris/HCl, pH 8.0, and incubated at 37 °C in a humidified chamber until lytic bands appeared.

COS transfection and metabolic labelling

COS cells grown in 60 mm Petri dishes were transiently transfected with 10 μg of plasmid by calcium phosphate precipitation and washed with fresh DMEM 24 h later. Metabolic labelling was performed 48 h after transfection as follows: cells were incubated in methionine-free Eagle's medium (Gibco BRL) for 1 h, pulse-labelled for 2 h in 1.2 ml of the same medium supplemented with 70 μCi of Tran^{35}S-label®, washed three times with PBS and chased for 3 h in 1.2 ml of complete DMEM/10% (v/v) fetal calf serum. After the chase, conditioned medium was harvested and centrifuged at 100000 ***g*** to remove cell debris. Monolayers were washed with PBS and lysed for 30 min at 4 °C in 1.2 ml of 50 mM Hepes/1% (v/v) Triton X-100/0.1% SDS/1% (w/v) sodium deoxycholate/0.02% sodium azide/21 μM leupeptin/1 mM EDTA/0.2 mM sodium iodoacetate (pH 7.0) containing 23 m-units of aprotinin.

Immunoprecipitation

Aliquots of both conditioned media and cell lysates were precleared overnight at 4 °C with Protein A–Sepharose with constant swirling. After a centrifugation at 1000 ***g*** for 5 min at 4 °C, 2 μg of 3D9, a monoclonal antibody that recognizes an epitope located near the N-terminus of CR1, was added to the supernatants and incubated for at least 2 h. The antigen–antibody complexes were recovered by the addition of Protein A–Sepharose. After 1 h the immunoprecipitates were washed twice with solution A [50 mM Hepes (pH 7.4)/120 mM NaCl/1% Triton X-100/0.1% SDS/1% sodium deoxycholate], once with solution B [PBS (pH 7.4)/0.5% BSA/1% Triton X-100/0.5% sodium deoxycholate/2 M KCl], once with solution C [0.6 M NaCl/0.1% SDS/0.05% Nonidet P40 (pH 8.6)] and twice with solution A. Finally, samples were solubilized in Laemmli buffer and analysed by SDS/PAGE under reducing conditions. Gels

were subjected to autoradiography for at least 3 days. Autoradiographic films were analysed by densitometry (ImageQuant, Molecular Dynamics).

Glycosidase digestion

Immunoprecipitates were boiled for 5 min in the presence of 0.5% SDS/0.1 M 2-mercaptoethanol and then incubated overnight at 30 °C either with 20 m-units/ml endo H in 50 mM sodium citrate (pH 5.5)/0.04% PMSF or with 2 m-units/ml endo F in 50 mM sodium acetate (pH 6.0)/1.67% Nonidet P40.

RESULTS

CR1 biosynthesis in transfected COS cells

COS cells transiently transfected with the cDNA encoding the CR1 were pulse-labelled with Trans^{35}S label and chased in complete DMEM. After removal of the conditioned medium, cells were extracted in the presence of protease inhibitors and CR1 was immunoprecipitated from both samples. As previously reported [18], CR1 was recovered not only from the cell extract but also from the culture medium (Figure 2, lanes 1 and 2), in which $46.5 \pm 3.6\%$ (mean $\pm$ S.E.M.; $n = 8$) of total CR1-WT (where WT stands for wild-type) were recovered after 3 h of chasing. This sCR1 was not sedimentable at 100000 ***g***, indicating that sCR1 does not derive from transmembrane CR1 associated with cell debris.

Proteolytic cleavage of CR1

On 7.5% (w/v) polyacrylamide gels, the apparent molecular masses of both CR1 forms were hardly distinguishable. To visualize this size difference better, we generated two shorter forms of CR1 by deletion of 13 (CR1-130) or 21 (CR1-75) of the 30 short consensus repeats present in the cDNA sequence encoding the extracellular domain of CR1 (Figure 1). These truncations do not affect the antibody recognition site. Cells transfected with these truncated receptors expressed two forms of cell-associated CR1 (141 and 150 kDa for CR1-130, and 86 and 97 kDa for CR1-75) and one form of sCR1 of intermediate size (145 and 92 kDa respectively) (Figure 2, lanes 3–6). Time course experiments with increasing chase periods revealed that

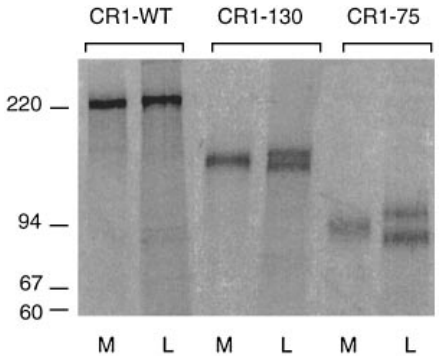

Figure 2 Biosynthetic labelling of COS transfected with wild-type and truncated CR1 cDNA

Transfected COS cells were pulse-labelled for 2 h with 70 μCi/ml Tran^{35}S Label. At the end of the chase period (3 h at 37 °C), conditioned medium was collected and cells were solubilized in the presence of protease inhibitors. CR1 was immunoprecipitated in both samples (M, medium; L, lysate) with a monoclonal anti-CR1 antibody and detected by autoradiography after SDS/PAGE. The positions of molecular mass markers (in kDa) are shown at the left.

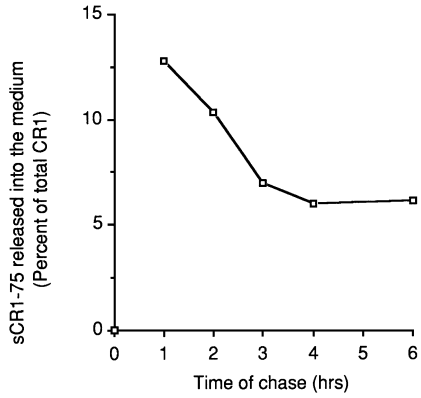

Figure 3 Time course of CR1-75 release into the medium

Five monolayers, pulse-labelled for 2 h, were chased for 1 h periods. At different times after labelling, the culture media were changed 1 h before the collection time. Cell lysates and media, recovered at the indicated time points, were processed to determine the percentage of sCR1 released during each period.

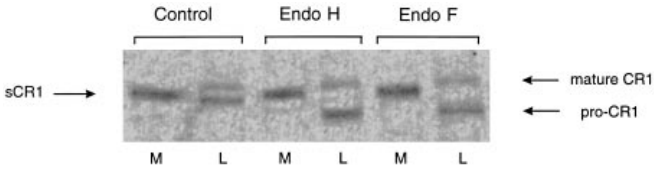

Figure 4 Glycosylation of CR1-130

At the end of a pulse–chase experiment, CR1 was immunoprecipitated from medium (M) and cell lysate (L) and the samples were subjected to digestion with endo H (20 m-units/ml) or endo F (2 m-units/ml). The samples were then run on a 7.5% (w/v) SDS/polyacrylamide gel and detected by autoradiography.

the two forms of cell-associated CR1 corresponded to two maturation steps of the same precursor (results not shown). Quantitative analysis indicated that the $t_{\frac{1}{2}}$ for maturation was approx. 2 h. Thus sCR1 was approx. 4 kDa smaller than the mature CR1-75 and CR1-130, suggesting that the proteolytic process occurs in the transmembrane or intracytoplasmic domain.

As shown in Figure 1, whereas half the total amount of CR1-WT was released into the medium, $37.5 \pm 2.6\%$ ($n = 10$) and $17.9 \pm 3.8\%$ ($n = 5$) of CR1-130 and CR1-75 respectively were recovered in the medium after 3 h of chasing, indicating that the length of the extracellular domain modulated the extent of CR1 release. To establish the time course of this release, cells transfected with CR1-75 and pulse-labelled for 2 h were chased for up to 6 h. Culture medium was changed after appropriate intervals to measure the percentage of sCR1 released during successive periods of 1 h. Densitometric scanning of the autoradiograms (Figure 3) indicated that most of the sCR1 was recovered during the first 2 h of chasing. At later time points CR1 was released into the medium at a slower rate.

To discover whether CR1 was released after complete maturation, immunoprecipitates were treated either with endo H, an enzyme that removes N-linked oligosaccharides of the high-mannose type without affecting those of the complex type, or with endo F, which acts on both N-linked oligosaccharides. As reported previously [23,24], the precursor form was sensitive to endo H and endo F and migrated at a lower molecular mass

Table 1 Sequences of the wild-type and mutated forms of CR1

Amino acids from CR1 are in bold; those from exogenous proteins are in italic. Soluble CR1 is expressed as a percentage of CR1 released as measured by densitometric analysis of autoradiographic films (ImageQuant, Molecular Dynamics).

Name	Extracellular domain	Transmembrane domain	Cytoplasmic domain	Soluble CR1 (%)*
CR1 WT	...[1963]**WDPPLAKCTSRAHD**	[1977]**ALIVGTLSGTIFFILLIIFLSWIIL**[2001]	**KHRKGNNAHENPKEVAIHLHSQGGSSVHPRTLQTNEENSRVLP**[2044]	46.5
uPA-CR1	*LAFAM***AKCTSRAHD**	**ALIVGTLSGTIFFILLIIFLSWIIL**	**KHRKGNNAHENPKEVAIHLHSQGGSSVHPRTLQTNEENSRVLP**[2044]	50.0
CR1 ΔSRAHDALIVG	**WDPPLAKCT**[1971]--	--[1982]**TLSGTIFFILLIIFLSWIIL**	**KHRKGNNAHENPKEVAIHLHSQGGSSVHPRTLQTNEENSRVLP**[2044]	49.1
CR1-2010	...**WDPPLAKCTSRAHD**	**ALIVGTLSGTIFFILLIIFLSWIIL**	**KHRKGNNAH**[2010]	46.2
CR1-HLA-HLA	...**WDPPLAKCTSRA***Q*	*PTIPIVGIIAGLVLFGAVITGAVVAAVMW*	*RRKSSDRKGGSYSQAASSDSAQGSDVSLTACKV*	2.5
CR1-HIR-HIR	...**WDPPLAK**	*IIIGPLIFVFLFSVVIGSIYLFL*	*RKRQPDGPLGPLYASSNPEYLSASDVFPCSVYVPDEWEVSREKITLLRELGQGS...*	5.3
CR1-LDLr-LDLr	...**WDPPL***DASTPSSVR*	*ALSIVLPIVLLVFLCLGVFLLW*	*KNWRLKNINSINFDNPVYQKTTEDEVHICHNQDGYSYPSRQMVSLEDDVA*	4.2
CR1-CR1-HIR	...**WDPPLAKCTSRAHD**	**ALIVGTLSGTIFFILLIIFLSWIIL**	*KKRQPDGPLGPLYAS SNPETLSASDVFPCSTVPDEWEVSREKITLLRELGQGSF...*	6.4
CR1-CR1-LDLr	...**WDPPLAKCTSRAHD**	**ALIVGTLSGTIFFILLIIFLSWIIL**	*KNWRLKNINSINFDNPVYQKTTEDEVHICHNQDGYSYPSRQMVSLEDDVA*	6.1
CR1-CR1-HIR (Exon 16)	...**WDPPLAKCTSRAHD**	**ALIVGTLSGTIFFILLIIFLSWIIL**	*KKRQPDGPLGPLYASSNPETLSASDV*	52.5
CR1-LDLr-CR1	...**WDPPL***DASTPSSVR*	*ALSIVLPIVLLVFLCLGVFLLW*	**KHRKGNNAHENPKEVAIHLHSQGGSSVHPRTLQTNEENSRVLP**[2044]	9.1

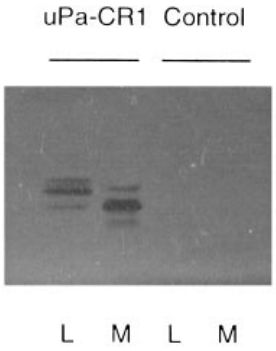

Figure 5 Secretion of uPA-CR1 as detected by zymography

Conditioned media (M) and cell lysates (L) from COS cells untransfected (Control) or transfected (uPA-CR1) with the cDNA encoding the chimaeric protein uPA-CR1 (uPA fused to CR1 transmembrane and cytoplasmic domains) were run on a 10% (w/v) polyacrylamide gel under non-reducing conditions. The gel was then layered on a casein/plasminogen/agar underlay and incubated for a few hours at 37 °C. Lysis bands were detected under dark-field illumination.

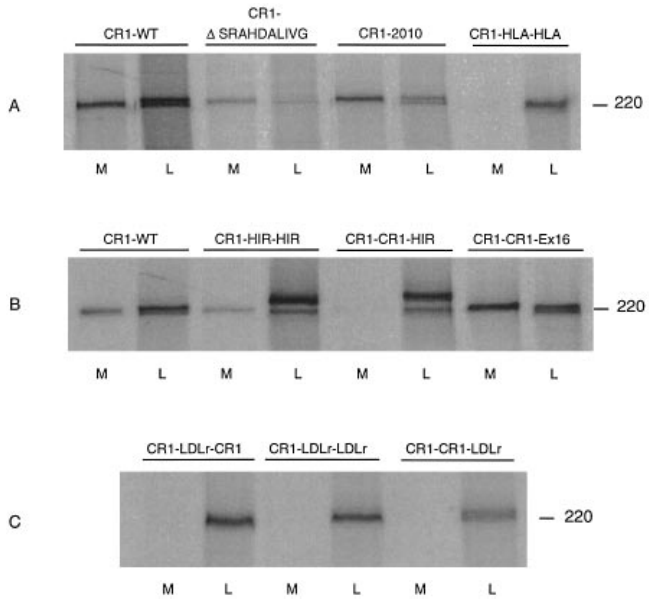

Figure 6 Biosynthetic labelling of COS overexpressing various mutated and chimaeric CR1 receptors described in Table 1

Transfected COS cells were pulse-labelled for 2 h. At the end of the chase period, conditioned medium was collected and cells were solubilized in the presence of protease inhibitors. Receptors were immunoprecipitated with a monoclonal anti-CR1 antibody in both samples (M, medium; L, lysate) and detected by autoradiography after SDS–PAGE.

(Figure 4). In contrast, the mature form was resistant to endo H, confirming that the two forms of membrane-associated CR1 corresponded to two different glycosylation states. Surprisingly, the mature form was also resistant to endo F, suggesting the presence of large multi-antennary structures. The fact that sCR1 was also insensitive to both endo H and endo F implies that its release occurs after complete receptor glycosylation.

Taken together, our results demonstrate that sCR1 is generated by proteolytic cleavage of the mature form of CR1 associated with the cell surface.

We then examined whether this proteolytic process was related to a loss of oligosaccharides from the extracellular domain, leading to an unmasking of the cleavage site. For that purpose we designed a chimaeric protein (urokinase-CR1) in which the sequence of the transmembrane and intracytoplasmic domains of CR1 were fused to the C-terminus of uPA (Table 1). Because mouse uPA is not N-glycosylated, the production of a secreted form with a higher electrophoretic mobility than the cellular form could be attributed only to proteolytic cleavage. Expression of uPA-CR1 was detected by zymography. As shown in Figure 5, we obtained three different forms of uPA-CR1, both in the medium and in the cell extract. These different forms probably reflect proteolysis within the non-catalytic portion of uPA. In any event it is clear that the extracellular forms had a lower molecular mass than the cell-associated forms, arguing that the proteolytic process does not result from a loss of oligosaccharides.

Further pulse–chase experiments were performed in the presence of various protease inhibitors to assess the role of extracellular proteases. For that purpose, trypsin inhibitors such as aprotinin (5 μg/ml) and soybean trypsin inhibitor (100 μg/ml), a serine/threonine protease inhibitor such as leupeptin (5 μg/ml) or an aspartate protease inhibitor such as pepstatin (1 μg/ml) were added at the beginning of the chase period. Under these conditions, none of these inhibitors either delayed the precursor maturation or inhibited CR1 release (results not shown). Likewise, α-2 macroglobulin (50 μg/ml), a protein that inhibits nearly all endoproteinases, and batimastat (1 or 10 μM), a broad-spectrum metalloproteinase inhibitor, were ineffective. CR1 release also remained unaffected when either 1 mM EDTA or a serum-free medium was used during the chase. On the basis of these results, neither soluble proteases released by the cells nor proteases present in the serum seem to be responsible for CR1 proteolytic cleavage. In addition, leupeptin, when used under conditions allowing a better penetration into the cells [i.e. at a higher concentration (50 μg/ml) and added 2 h before the pulse until the end of the chase] [25], did not impair CR1 release (results not shown). This suggests that the endogenous serine/threonine proteases are not involved in this process.

Cleavage site of CR1: intramolecular site

On the basis of the molecular mass difference between mature cell-associated CR1 and sCR1 and on the secretion of the chimaeric urokinase-CR1 (Tm + Cyto), which includes only nine residues of the extracellular CR1 domain, the proteolytic cleavage site must be located either within or near the transmembrane domain. To gain further insight into the site of CR1 cleavage, further chimaeric and mutated receptors were constructed (Table 1) and tested for their ability to produce a secreted form. All these receptors were expressed at the cell surface, as judged by ^{125}I-antibody-binding experiments (results not shown). A CR1 mutant, in which 10 residues (SRAHDALIVG) overlapping the boundary between the extracellular and transmembrane domains of CR1 were deleted, was secreted to the same extent as entire CR1 (Figure 6A). In addition, a receptor truncated at position 2010, after the first nine residues of the cytoplasmic domain, was also released into the medium. Taken together, these results indicate that the cleavage site must be situated either in the extracellular domain, upstream of Thr-1971, or between the C-terminal half of the transmembrane domain and the juxtamembrane domain, between Thr-1982 and His-2010 (Table 1). The former hypothesis seems unlikely for two reasons. First, the chimaeric receptor composed of uPA fused to the transmembrane and intracytoplasmic domains of CR1 (uPA-CR1) was also secreted (Figure 5). Secondly, the chimaeric receptors CR1-HLA-HLA (Figure 6A), CR1-HIR-HIR (Figure 6B) and

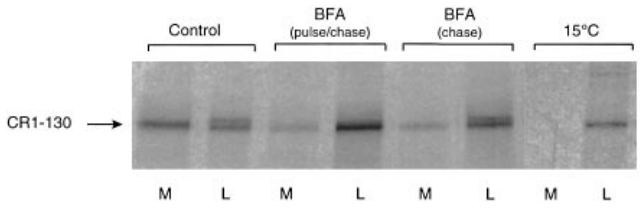

Figure 7 Intracellular site of CR1 cleavage: role of the endoplasmic reticulum

COS cells transfected with CR1-130 cDNA were pulse-labelled for 2 h in the absence or presence of BFA (6 μg/ml). BFA was present throughout the pulse–chase experiment or just during the chase period. In the last experiment, cells were chased at 15 °C. CR1 from medium (M) and cell lysates (L) were immunoprecipitated, separated on an SDS/polyacrylamide gel and detected by autoradiography.

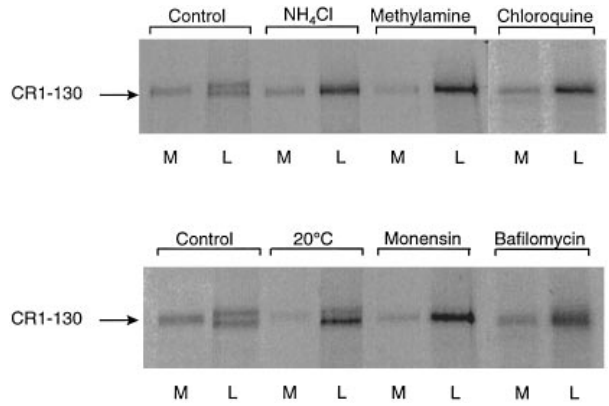

Figure 8 Intracellular site of CR1 cleavage: role of the acidic compartments

At the end of the pulse-labelling, COS cells, transfected with CR1-130 cDNA, were chased in the absence or presence of 50 mM NH_4Cl, 50 mM methylamine, 60 μM chloroquine, 1 μM monensin or 50 mM bafilomycin. In one experiment, after a pulse at 37 °C, cells were shifted to 20 °C during the chase period. CR1 from medium (M) and cell lysates (L) were immunoprecipitated, separated on an SDS/polyacrylamide gel and detected by autoradiography.

CR1-LDLr-LDLr (Figure 6C), formed by the extracellular domain of CR1 fused to the transmembrane and cytoplasmic domains of human major histocompatibility complex, human insulin receptor or human LDL receptor respectively, were essentially not recovered in the medium even though they all contained the extracellular domain of CR1.

To determine the role of the cytoplasmic domain of CR1 in its release, the cytoplasmic tail of CR1 was replaced by that of the insulin or the LDL receptor (CR1-CR1-HIR/LDLr). These chimaeric receptors remained exclusively cell-associated, suggesting that the cleavage site resides in the juxtamembrane region of CR1 (Figures 6B and 6C). However, the absence of secretion of the CR1-LDLr-CR1 argues against this conclusion and suggests that cleavage occurs in the transmembrane domain. In an attempt to resolve this paradox, another CR1-CR1-HIR with a shorter cytoplasmic domain [truncated after the 22nd residue of the juxtamembrane domain (exon 16)] was constructed. In contrast with the full-length receptor, this shorter receptor was released into the medium. Taken together, these results demonstrate that the site of CR1 cleavage lies within the C-terminal portion of the transmembrane region and that the sequence of the intracytoplasmic domain is important in allowing the access of protease(s) to the cleavage site.

Cleavage site of CR1: intracellular compartment

Along the biosynthetic pathway, CR1 must travel through the endoplasmic reticulum, the Golgi apparatus and secretory vesicles before reaching the plasma membrane. The possible involvement of each of these major compartments in CR1 cleavage was investigated. First, to determine the role of the endoplasmic reticulum and the Golgi apparatus, cells were pulse–chased in the presence of BFA, a fungal metabolite known to induce the redistribution of *cis*-, medial- and *trans*-Golgi proteins to endoplasmic reticulum [26]. BFA treatment prevented the acquisition of endo-F-resistant oligosaccharides by pro-CR1 and thus decreased the amount of CR1 released (Figure 7). The effects were less pronounced when BFA was added only during the chase period, indicating that BFA itself did not affect secretion. In addition, if the endoplasmic reticulum–Golgi transport was blocked by incubating cells at 15 °C during the chase, both processing and release were inhibited (Figure 7). These results indicate that the proteolytic cleavage of CR1 does not occur in the endoplasmic reticulum, but only after further transport through the secretory pathway.

We then investigated the involvement of the *trans*-Golgi compartment and of the lysosomes. Cells were chased in the presence of lysosomotropic agents such as NH_4Cl, methylamine and chloroquine, which have been shown to interfere with the secretion and post-translational processing of secretory proteins [27]. As depicted in Figure 8 (upper panel), all three weak amines prevented the complete maturation of newly synthesized CR1, but they did not completely abolish the generation of a soluble protein. Similar results (Figure 8, lower panel) were obtained with the carboxylic ionophore monensin and with bafilomycin A, a specific inhibitor of the vacuolar H^+-ATPases [28]. However, the sCR1 released under those conditions seems to be smaller than that obtained in the absence of any treatment, suggesting that the small proportion of CR1 reaching the cell surface, but improperly glycosylated, is immediately released into the medium. To ensure that the presence of sCR1 in the medium did not result from cell death, cells overexpressing CR1-HLA-HLA, which normally do not release CR1, were treated with the same drugs. Even under those conditions, sCR1 was not detected (results not shown). Thus alkalinization in the *trans*-Golgi cisternae and acidic secretory vesicles seems to inhibit the processing but not the proteolytic cleavage of the immature receptors reaching the plasma membrane. In contrast, when cells were biosynthetically labelled at 37 °C and then shifted to 20 °C, a temperature at which the exit of membrane proteins from the *trans*-Golgi network is greatly reduced, the CR1 release in the medium was affected drastically (Figure 8, lower panel).

These results imply that the proteolytic processing of CR1 occurs at a post-*trans*-Golgi step of the biosynthetic pathway, during the transfer to the plasma membrane or at the cell surface, and does not involve an acidic protease.

DISCUSSION

As do many integral membrane proteins, CR1 possesses a soluble form circulating in the plasma. In view of the pathophysiological relevance of sCR1, the mechanism(s) governing its formation are of great interest. Multiple isoforms of the same protein can be generated either by alternative pre-mRNA splicing of a common transcript [29], by post-translational hydrolytic cleavage of the extracellular domain of the corresponding proteins [30,31] or through a shedding of the intact protein due to its defective retention in the plasma membrane [32]. For CR1,

on the basis of restriction fragment length polymorphism and Southern blot analysis, previous studies have excluded alternative mRNA splicing [33,34]. In contrast, results of an ELISA assay making use of an antibody directed against CR1 cytoplasmic domain have shown a loss of reactivity of sCR1 compared with membrane-bound CR1 [13]. Although in that study a size difference between soluble and membrane-bound CR1 was hardly detectable by SDS/PAGE, the authors suggested on the basis of this loss of antibody recognition that sCR1 was released by proteolytic cleavage.

The first aim of the present study was to make a direct challenge on the hypothesis of CR1 proteolytic cleavage. For that purpose, COS cells, previously shown to be able to release CR1 in a similar manner to PMN [18], were used. Compared with PMN, COS cells have major advantages: (1) they survive in culture conditions, (2) they can be transfected with exogenous cDNA and (3) the behaviour of exogenous proteins can be analysed independently of the presence of endogenous CR1. A series of mutated CR1 molecules were designed to challenge the hypothesis of a proteolytic process and then to locate the putative cleavage site.

The use of two mutated receptors with partial deletions of the extracellular domain allowed us to demonstrate unambiguously that sCR1 results from a proteolytic cleavage because the electrophoretic mobility of the soluble and cellular forms of those mutated CR1 forms were clearly different. In addition, our results show that this proteolytic process is not preceded by a loss of oligosaccharides because a chimaeric molecule (uPA-CR1) composed of the transmembrane and intracellular domains of CR1 fused to the extracellular domain of uPA, a protein devoid of N-glycosylation sites, generated a soluble form in the same proportion as full-length CR1. Thus CR1 behaves similarly to a series of membrane-anchored growth factors, cytokine receptors, ectoenzymes, cell adhesion proteins and the β-amyloid precursor, soluble forms of which are released in an active form into the extracellular medium by proteolytic cleavage [30,35–37]. Moreover, under our experimental conditions, in partial contradiction of previous results [38], CR1 release seems to be an intracellular event because various protease inhibitors of different classes, including aprotinin, leupeptin, soybean trypsin inhibitor, pepstatin, α2-macroglobulin, batimastat and EDTA, failed to prevent the generation of sCR1, whereas the biosynthesis of the receptor was unaffected. These observations also argue against the release of the entire CR1 molecule followed by extracellular proteolytic cleavage. Furthermore it seems that the endogenous serine/threonine proteases are not involved in CR1 release because leupeptin, when used under conditions allowing its entrance into the cells, did not impair the process (results not shown).

Given the small size difference between the soluble and the cell-associated forms of the two truncated CR1 proteins, the intramolecular cleavage site was likely to be located close to or within the transmembrane domain of the molecule. The integration of the results obtained with a series of additional mutated and chimaeric CR1 proteins led us to conclude that the cleavage occurs in the C-terminal portion of the transmembrane domain. First, an extracellular or N-terminal transmembrane localization of the cleavage site, as observed with transforming growth factor α (TGFα) and L-selectin [30,31], was excluded on the basis of the cleavage of CR1 ΔSRAHDALIVG, a mutated CR1 with a deletion of 10 residues spanning the junction between the extracellular and the transmembrane domains, and of the chimaeric receptor uPA-CR1, which does not contain the extracellular CR1 domain. The lack of inhibitory effects of a series of protease inhibitors also supports this conclusion. Secondly, the release of sCR1 was shown not to rely on a cytoplasmic determinant as has been described for TGFα [39] because: (1) CR1-2010, which lacks the last 34 C-terminal residues, is released into the medium; (2) CR1-LDLr-CR1 was retained on cell surface despite the presence of the entire CR1 cytoplasmic domain; and (3) the cytoplasmic C-terminal valine residue, which has a key role in the release of pro-TGFα ectodomain, is present in CR1-HLA-HLA and CR1-CR1-HIR (exon 16) but the former receptor remains cell-associated whereas the latter is released into the medium. Thirdly, the retention of chimaeric receptors with an exogenous transmembrane domain (CR1-HLA-HLA, CR1-HIR-HIR, CR1-LDLr-LDLr and CR1-LDLr-CR1) favours a localization of the hydrolytic process in the transmembrane domain.

The absence of sCR1 production from COS cells expressing CR1-CR1-HIR or CR1-CR1-LDLr is in apparent contradiction of the above-mentioned conclusions. The three-dimensional structure of the HIR and LDLr cytoplasmic domains might, however, influence protease action either directly, by modifying the conformation of the C-terminal portion of the transmembrane domain, thereby preventing recognition of the cleavage site by the proteolytic enzyme, or indirectly, by decreasing access of the protease to the cleavage site by steric hindrance. The release of a soluble form of CR1-CR1-HIR exon 16, which contains only the first 22 residues of the insulin receptor cytoplasmic domain, supports this interpretation and demonstrates that the CR1 transmembrane domain can be sufficient to promote an efficient release of sCR1. Finally, the extracellular domain of CR1 could also have a role in the protein release because the mutant with the shorter extracellular domain is poorly released into the medium (Figure 1). Similarly to the intracellular domain, the large extracellular domain of CR1 might impose a particular three-dimensional structure on the transmembrane domain, allowing a good accessibility of the cleavage site to the endopeptidase.

Most of the integral membrane proteins that exist both as membrane-bound and soluble isoforms are cleaved within their extracellular domain, in a region close to the membrane. This membrane-proximal cleavage seems to be a specific mechanism to modulate rapidly the cell surface expression of various proteins [40,41]. For instance, L-selectin and TGFα are cleaved approx. 10 residues upstream of the transmembrane domain, mainly in response to leucocyte activation [30,31]. Here we report the case of an integral membrane protein that is released into the medium after a proteolytic cleavage within the hydrophobic transmembrane domain. To our knowledge the secretion of β-amyloid peptide from β-amyloid precursor protein is the only example of such an intramembrane cleavage [42–44].

Using conventional inhibitors of the secretory pathway and of lysosomal function, we showed that the proteolytic process must take place either during the transit of mature CR1 between the *trans*-Golgi network and the plasma membrane or at the plasma membrane itself. Several lines of evidence support this conclusion: (1) BFA, added during the chase period, delayed CR1 maturation but did not abolish mature CR1 release, suggesting that the protease has access to CR1 that has reached the cell surface but not CR1 retained in pre-Golgi compartments; (2) the inhibition of sCR1 production by a temperature block at 15 °C, together with the resistance of sCR1 to endo-H, indicates that the proteolytic cleavage does not occur in the endoplasmic reticulum and that CR1 must reach at least the Golgi apparatus before being exported to the extracellular medium; (3) CR1 release was greatly decreased when exit of membrane proteins from the *trans*-Golgi network was blocked by lowering the temperature to 20 °C during the chase period; (4) the proteolytic

process does not take place in an acidic compartment such as late Golgi cisternae, acidic secretory vesicles or lysosomes because alkalinization of these intracellular compartments with various primary amines or bafilomycin A failed to prevent CR1 release; (5) a role for lysosomes in CR1 release can also be excluded because the addition of leupeptin under conditions that blocked lysosomal degradation had no effect on the generation of sCR1 (results not shown). In comparison, the cleavage of the C-terminus of β-amyloid peptide occurs intracellularly, either in the endosomal/lysosomal compartment or along the secretory pathway [42–44].

In conclusion, we propose the following sequence of events leading to sCR1 release into the extracellular medium. After glycosylation in the endoplasmic reticulum and trimming of N-oligosaccharides in the *cis*- and *medial*-Golgi cisternae, CR1 would exit from the Golgi apparatus before or after the passage through the *trans*-Golgi network and enter secretory vesicles in transit to the plasma membrane. Then it would undergo a proteolytic cleavage within its transmembrane domain either in these secretory vesicles or once anchored on the plasma membrane. During the catalytic activity the protease would locally modify the lipid bilayer structure and allow the shedding of the truncated CR1. Further experiments are required to characterize this protease and to examine whether it is possible to modulate this proteolytic process in cells expressing CR1 *in vivo*.

We thank L. B. Klickstein for the generous gift of a few CR1 mutants. This work has been supported by grants 3100-043409-95 and 3100-037692-93 from the Swiss National Science Foundation.

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Received 16 May 1997/26 August 1997; accepted 5 September 1997