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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Complement C2 Receptor Inhibitor Trispanning: A Novel Human Complement Inhibitory Receptor\textsuperscript{1,2}

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The complement system presents a powerful defense against infection and is tightly regulated to prevent damage to self by functionally equivalent soluble and membrane regulators. We describe complement C2 receptor inhibitor trispanning (CRIT), a novel human complement regulatory receptor, expressed on hemopoietic cells and a wide range of tissues throughout the body. CRIT is present in human parasites through horizontal transmission. Serum complement component C2 binds to the N-terminal extracellular domain 1 of CRIT, which, in peptide form, blocks C3 convertase formation and complement-mediated inflammation. Unlike C1 inhibitor, which inhibits the cleavage of C4 and C2, CRIT only blocks C2 cleavage but, in so doing, shares with C1 inhibitor the same functional effect, of preventing classical pathway C3 convertase formation. Ab blockage of cellular CRIT reduces inhibition of cytolysis, indicating that CRIT is a novel complement regulator protecting autologous cells. The Journal of Immunology, 2005, 174: 356–366.

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Received for publication August 12, 2004. Accepted for publication October 15, 2004.

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1 This work was supported by the Swiss National Foundation, the Novartis Research Foundation, the Stiftung für Medizinische und Biologische Forschung, and the Icelandic Research Council.

2 The complement C2 receptor inhibitor trispanning (CRIT) sequence data are available from European Molecular Biology Laboratory/GenBank under accession nos. AY464186 (Homo sapiens), Q93H42 (Rattus norvegicus), AY464185 (Trypanosoma cruzi), Q9BLM6 (Schistosoma haematobium), and AY464184 (Gadus morhua).

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5 Abbreviations used in this paper: RCA, regulator of complement activation; C1-INH, C1 inhibitor; CP, classical pathway; CRIT, complement C2 receptor inhibitor trispanning; DAF, decay-accelerating factor; AP, alternative pathway; ed, extracellular domain; id, intracellular domain; H17 or CRIT-H17, the 11-aa C terminus of ed1; NHS, normal human serum; Sh, Schistosoma hematobium; Tc, Trypanosoma cruzi; Ra, rat; Hu, human; C2\textsuperscript{\textasciitilde}, biotinylated C2; K\textsubscript{d}app, apparent dissociation constant; ORF, open reading frame; TM, transmembrane; GPCR, G protein-coupled receptor; SMC, smooth muscle cell; CR, complement receptor; MCP, membrane cofactor protein; vWFA, von Willebrand factor A.

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Biotinylation, FITC labeling, and radioiodination of C2

C2 (50 μg) was biotinylated by using NHS-LC-biotin (Pierce) according to previously described methods (10), but using a lower biotin-to-protein ratio of 50 μg of biotin per milligram of protein. After dialysis vs PBS or PBS containing MgCl2 (1 mM), the protein was assayed. For FITC labeling, C2 (10 μg) was incubated with FITC (Sigma-Aldrich) at room temperature (6 μl of 0.1 mg/ml) and 1 ml of carbonate buffer at room temperature for 2 h in the dark, followed by extensive dialysis against PBS. Radioiodination of C2 was conducted according to a standard protocol (12). Essentially, 100 μg of protein in 0.5 ml was added to 100 μg of IodoGen (Pierce). Iodination was conducted by adding 1 ml of NaI (Sigma-Aldrich). Unincorporated iodine was separated from iodinated C2 using a PD-10 gel filtration column (Amersham Biosciences) presaturated with 5 mg of BSA. The specific activity of 125I-labeled C2 was 8 × 104 cpm/mg.

Cell and human cells line

Lymphocytes were obtained from heparinized human blood diluted 1:1 with RPMI 1640 (using Histopaque-1077 (Sigma-Aldrich). Erythrocytes and platelets were isolated according to a standard protocol (13). The lymphocytes were washed and resuspended in RPMI 1640. Erythrocytes were recovered, and the pellet was washed with PBS. Monocytes were obtained from heparinized human blood buffy coat by density gradient centrifugation using Histopaque-1077 (Sigma-Aldrich). Monocytes were purified by adherence separation (14). Purified monocytes (2 × 106 cells/ml) were dispensed in 100-μl aliquots into the wells of a 96-well microtiter plate and stimulated, if necessary, with 10 U/ml final concentration of human IFN-γ (Sigma-Aldrich) and incubated overnight at 37°C in 5% CO2. To remove the fibrinolasts that proliferate on addition of IFN-γ, the monocytes were washed three times with RPMI 1640. The human carcinoma cell lines, Jurkat, Raji, THP-1, U937, ECV-304, HELA-S3, H1, MRC5, T47D, and rat cell line, C58(NT) (all American Type Culture Collection) were maintained in RPMI 1640 medium with 10% FCS, glutamine (2 mM), sodium pyruvate (250 mM), 5% glucose (250 mM), and streptomycin (100 μg/ml) at 37°C and 5% CO2.

SDS-PAGE, Western and lidg blotting

Protein assays by dye binding using a Bio-Rad protein assay kit were conducted to ensure even loadings for SDS-PAGE. SDS-PAGE and immunoblotting analysis of proteins was conducted as described before (10). Reduced samples (with 20 or 50 mM DTT) and nonreduced samples were prepared in SDS-PAGE loading buffer. Where necessary in experiments to study the covalent oligomerization of CRIT, cell lysis buffer included 10 mM EDTA and 1% (v/v) Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS in PBS) in the presence of protease inhibitors. After centrifugation (14 k rpm for 10 min), the supernatant was resup as above.

For lysates of mammalian cells, cells (typically 30 × 106) were washed twice in PBS and resuspended in 0.75 ml of lysis buffer (PBS with 1% (v/v) Nonidet P-40 and 5 mM EDTA (pH 8)). After adding protease inhibitors (0.2 mM PMSF, 4 mM aminoethylbenzenesulfonyl fluoride (Pefabloc), 1 mM NaF, 5 mM α-glycerophosphate, 4 mM benzamidine) (all Sigma-Aldrich), the cells were mixed gently and incubated on ice for 10 min. After low-speed centrifugation (250 × g for 5 min), the supernatant was resup as (25,000 × g; 25 min), and the supernatant was aliquoted and stored (−80°C).

CRIT expression in Escherichia coli was induced with isopropyl-β-D-thiogalactopyranoside for 1 h. After harvesting, the bacteria were resuspended in 1/10 the original volume of buffer (20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 2 mM EGTA, protease inhibitor mixture as for mammalian cell lysates) and kept on ice with 10 μg/ml lysozyme. 0.1% Triton X-100, 10 mM MgCl2, 50 μg/ml DNase, and 20 μg/ml RNase for 60 min at room temperature. Cell lysates were centrifuged (25,000 × g for 30 min), and supernatants were used freshy.
DNA isolation and Southern blotting

Rat genomic DNA was purchased (Stratagene). Human genomic DNA for Southern blotting was prepared from 500 µl of packed Jurkat cells (17). Southern blotting (17) using the full-length Sh-CRIT cDNA as a probe was conducted at low stringency by incubating at 45°C for 16 h, followed by two washes with 1× SSC and 0.1% SDS at room temperature, rinsing in 2× SSC. For higher stringency, blots were incubated at 65°C and then washed with 1× SSC and 0.1% SDS at 65°C. Blots were exposed to x-ray film with an intensifying screen for 48 h.

Cloning of CRIT homologs and in vitro transcription/translation

A rat homolog of Sh-CRIT was obtained by PCR from rat genomic DNA, using degenerate oligonucleotides based on the Sh-CRIT N-terminal and C-terminal nucleotide sequence and, where possible, based on a rat codon usage. The primers were as follows: RaF (sense), 5′-CGGATGTC/CCCCCAGCCGGCCCTCAAGTCG-3′, and RaR (antisense), 5′-GCCTGTTGCAAAGAAG/CCCTCGCAGTTCG-3′. The PCR was conducted at 94°C (30 s), 55°C (60 s), and 72°C (60 s) for 35 cycles. The purified product (using GeneClean (Bio 101)) was then cloned into pGEM-T (Promega), according to the manufacturer’s instructions, and fully sequenced using the manufacturer’s primers in both directions.

Southern blotting was prepared from 500 ng of genomic DNA and probed with the full-length Sh-CRIT cDNA. The blots were incubated at 65°C and then exposed to x-ray film with an intensifying screen for 48 h.

DNA sequencing

One sequencing was conducted on both strands of the pGEM-T clones using an ABI Prism BigDye terminators, version 3.0, cycle sequencing kit and detected on an ABI 3100 Avant Genetic Analyzer, automated DNA sequencer, according to the manufacturer’s protocols (Applied Biosystems). Vector-specific primers SP6 and T7 were used, as well as primers based on the established insert sequence. The primers were as follows: RaF (sense), 5′-TACGACTCACTATAGGG-3′; and RaR (antisense), 5′-CGGGTACCTATAGGAGGGTACCG-3′. Pfu ultra high-fidelity DNA polymerase (Stratagene) was used, and the amplified PCR product was digested with Hinfl and Pst1, and ligated into the pDNA3.1 vector precut with the same enzymes. The ligation was transformed into DH5α competent cells (17). In vitro transcription/translation from the pDNA3.1-HuCRIT template was conducted using the TNT-coupled reticulocyte lysate system (Promega) according to the manufacturer’s instructions. The trypanosome and cod homologs were obtained similarly by PCR using oligonucleotides based on the Sh-CRIT sequence.

Assay for cleavage of C2 by C1s

This was essentially conducted as described previously (10). To test for the effect of CRIT as expressed on the Jurkat cell surface (5×10⁵ cells) as well as the CRIT-ed1 synthetic peptide (105 µM), on C1s cleavage of C2, they were preincubated with C2 (2 µg) in 20 mM Tris at 20°C for 30 min. Then, 0.2 µg of C1s was added and incubated for 1 h at 37°C before analysis by immunoblotting.

Results

Human and parasite CRIT

CRIT is found on the larval and adult worm stage of the Schistosoma parasite (9). Under the previous designation of “trispanning orphan receptor,” it has also been found recently on schistosome eggs (18). Schistosomes live in veins near the urinary bladder or intestines of the host and the CRIT receptor for C2 is able to limit the membrane. Empirical evidence for the internal/external orientation of CRIT has been described before (9) and is supported by the fluorescence staining of CRIT-positive cells incubated with anti-CRIT-ed1 (Fig. 2A). Comparison of a nonreduced Jurkat extract (NR, lane 2) with a reduced extract (R, lane 2) shows, under reducing conditions, the disappearance of the dimeric form and a slight increase in intensity (which was frequently more prominent) of the monomeric form. The presence of the sulfhydryl trapping agent iodoacetamide (10 mM) in the cell lysis buffer did not prevent the appearance of the covalently linked dimeric CRIT (not shown), meaning that disulfides were not formed artifically as a result of preventing non-denaturing and oxidizing conditions during cell lysis. At this stage, we cannot categorically say whether homodimerization or heterodimerization is occurring. However, because dimers occurred in Jurkat cells that do not make C2, regardless of whether amino acid level (Fig. 1B) reveals near identical TM domains and ligand-binding ed1 in all species, the latter accounting for the cross-immunoreactivity, between schistosomes and humans in ed1. The high interspecies homology at 82% between full-length Hu- and Ra-CRIT also occurs in the G protein-coupled receptor (GPCR) family which can show 85–98% identity between species (22). At the nucleotide level, as shown in the phylogenetic tree in Fig. 1C, the parasite cluster (Sh-CRIT and Te-CRIT) is as closely related to Hu-CRIT as rat, suggesting that these human parasites acquired the CRIT gene from their host. The appropriation of host genes by parasites accounts for the many genes with high degrees of identity between parasite (especially schistosome) and host (23, 24), and such molecular mimicry in which host proteins are expressed by parasites helps disguise the parasite as “self” in terms of the host immune system. We found the nucleotide sequence of the Hu-CRIT cDNA to be identical with that of the genomic sequence, implying that the ORF of Hu-CRIT is encoded by a single exon, although we cannot yet rule out an intron in the untranslated regions. In this study, we present the Hu-CRIT gene structure only as the predicted ORF based on that of Schistosoma CRIT, thus excluding 5′ and 3′ noncoding regions and other structures such as promoters and transcription regulatory elements. The presence of a CRIT gene in cod of which we present a partial sequence in Fig. 1B, indicates that CRIT genes may have evolved from a common ancestral gene, at least present in the earliest teleosts.

CRIT membrane topology and sequence motifs

Fig. 1D shows how, for five of six topology algorithms, Hu-CRIT, representative of the other CRIT sequences, is expected to lie in the membrane. Empirical evidence for the internal/external orientation of CRIT has been described before (9) and is supported by the fluorescence staining of CRIT-positive cells incubated with anti-CRIT-ed1 (Fig. 2A). The majority of substitutions between the species occur within the first 60 residues of the 161-residue cytoplasmic tail, id2, proximal to the third TM domain. Within the remainder of the cytoplasmic tail, as illustrated in Fig. 1D, and shared by parasite and mammalian sequences, are three predicted tyrosine-based YXXΦ endocytosis motifs (where X is any amino acid and Φ is a strongly hydrophobic amino acid), two di-leucine endocytosis motifs, and, illustrated schematically in Fig. 1D, an amphipathic α helix (putative sorting signal toward degradation) (25). Also within the cytoplasmic tail, there is a consensus binding motif for the Src homology 2 domain of the Src family cytoplasmic tyrosine kinases (26), in particular, Fyn, Fps/Fes, and Syk, as well as consensus phosphorylation sites for various tyrosine kinases.

CRIT forms covalently linked dimers

Having first detected CRIT receptor by flow cytometry on the surface of Jurkat cells (Fig. 2A), we found, by immunoblotting of a cell lysate, that CRIT exists as a dimer under nonreducing conditions (B). Comparison of a nonreduced Jurkat extract (NR, lane 1) with a reduced extract (R, lane 2) shows, under reducing conditions, the disappearance of the dimeric form and a slight increase in intensity (which was frequently more prominent) of the monomeric form. The presence of the sulphydryl trapping agent iodoacetamide (10 mM) in the cell lysis buffer did not prevent the appearance of the covalently linked dimeric CRIT (not shown), meaning that disulfides were not formed artifically as a result of prevailing non-denaturing and oxidizing conditions during cell lysis. At this stage, we cannot categorically say whether homo- or heterodimerization is occurring. However, because dimers occurred in Jurkat cells that do not make C2, regardless of whether...
FIGURE 1. CRIT family genes and membrane topology. 

A. Southern blot of human genomic DNA digested with: lane 1, EcoRI, and lane 2, PstI, probed with Sh-CRIT full-length cDNA under low and high stringency conditions, respectively. 

B. Amino acid sequence alignment of CRIT from human (Hu), rat (Ra), T. cruzi (Tc), S. hematobium (Sh), and cod. Amino acid identity or similarity is indicated by black or gray shading, respectively. The three TM domains are indicated by a bar above the sequence. 

C. Unrooted phylogenetic tree based on analysis of nucleotides 1–846 of Hu-CRIT cDNA sequence with other CRIT cDNAs, using a topological algorithm (European Molecular Biology Laboratory-European Bioinformatics Institute Molecular Biology Server). Length of line segments, in brackets, is indicative of evolutionary distance. 

D. Schematic of representative Hu-CRIT, showing membrane topology and important sequence motifs, common to all sequences presented, including, in the cytoplasmic tail, three Tyr-XX-Ile/Leu endocytosis motifs, the third lying within an amphipathic helix (in which hydrophobic residues are within shaded circles), as well as two di-leucine endocytosis motifs and a serine/threonine rich region. The extracellular domains (ed1 and ed2) and intracellular domains (id1 and id2) are also indicated. The region to which the anti-CRIT-id2 Ab was designed is illustrated. 
they were maintained in complete or serum-free medium, it appears that the dimerization is constitutive and not dependent on ligand. Furthermore, heterodimerization could conceivably occur between CRIT and a molecule other than C2. To resolve this, studies are ongoing to cotransfect cells with CRIT possessing different tags to see whether (homo-)dimers can be detected with both tag-specific Abs. If homodimerization is occurring, then disulfide bonds could be formed between any of the three cysteines in TM1, one each in TMs 2 and 3 and three in the cytoplasmic tail.

**CRIT cellular and tissue distribution**

We expressed Hu-CRIT both in *E. coli* (Fig. 2C) and by in vitro transcription translation (D) as a ∼31- to 32-kDa protein. Full-length CRIT1–280 was also detected in Jurkat cell lysates using anti-ed1 or anti-ed2 as well as an Ab, anti-id2, against the cytoplasmic tail of CRIT, specifically against a peptide based on the region K195–A208. Anti-ed1 also revealed the rat CRIT homolog as a ∼31- to 32-kDa protein in the rat T cell line, C58(NT). Comparing CRIT sequences available, the predicted size of human CRIT is not dissimilar to CRIT from *T. cruzi, S. hematobium/S. mansoni/S. japonicum*, or rat. Compared with CRIT monomer electrophoresed under nonreducing conditions (Fig. 2D), 1 mM DTT decreased the electrophoretic mobility of CRIT monomer (as viewed on an 8% SDS-PAGE gel to maximize the resolution of proteins between 20 and 40 kDa), indicating a likely reduction of an intramolecular disulfide bond.

Western blotting (Fig. 2E) showed expression of CRIT in various human hemopoietic cell lines including Jurkat (T lymphocyte), Raji (B lymphocyte), THP-1 (myeloid), U937 (myeloid), and ECV304 (endothelial), as well as several nonleukocyte cells (Table I) such as T47D (epithelial breast ductal) and weakly on HeLaS3 (epithelial) and MRC5 (fibroblast). In addition, CRIT was detected by immunoblotting in human platelets, monocytes, dendritic cells, and lymphocytes, but not in erythrocytes, neutrophils, nor HS1 (liver fibroblast-like cell line). CRIT was also found (Fig. 2E and Table I) in testis, tonsil, kidney, thymus, and liver (latter not shown), but not in colon.

**Table I. Cell and tissue distribution of Hu-CRIT**

<table>
<thead>
<tr>
<th>Cell Type/Tissue</th>
<th>CRIT</th>
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<tbody>
<tr>
<td>T47D (epithelial breast ductal carcinoma)</td>
<td>+</td>
</tr>
<tr>
<td>MRC5 (fibroblast, passage 31)</td>
<td>+/-</td>
</tr>
<tr>
<td>HS1 (liver fibroblast)</td>
<td>-</td>
</tr>
<tr>
<td>HeLa S3 (cervix uteri epithelial carcinoma)</td>
<td>+/-</td>
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<tr>
<td>Monocytes</td>
<td>+</td>
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<tr>
<td>Macrophages</td>
<td>-</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>+</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>-</td>
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<tr>
<td>Dendritic cells</td>
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<td>Platelets</td>
<td>+</td>
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<tr>
<td>Erythrocytes</td>
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<td>Liver</td>
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Localization of CRIT in 38 normal, fresh (biopsy material) human tissues was determined by immunohistochemistry (summarized in Fig. 3) using anti-CRIT-ed2, which unlike anti-CRIT-ed1, worked well in staining of paraffin-embedded sections. We were able to establish the following distribution for the CRIT receptor: CRIT was found to be strongly to moderately positive in almost all tissues in smooth muscle cells (SMCs) and related cells (pericytes) in vessels. According to the literature, complement regulators DAF, CD59, membrane cofactor protein (MCP), and Crry are expressed at the mRNA level by vascular SMCs in rat (27), whereas C3 and C4 are found in human SMCs (28). Interestingly, CRIT stained strongly in glandular epithelial cells in the endometrium (Fig. 3A) during the proliferating but not secretory phase. Likewise, MCP, DAF, and CD59 are expressed on an endometrial epithelial cell line (29) and, together with complement receptor 1 (CR1), expressed in normal endometrial tissue in all phases of the menstrual cycle (30). Other CRIT-positive cells include pancreatic islet cells (Fig. 3B) (which, according to their percentage and distribution, are suggestively insulin-producing β cells), cells which also express CD59 and MCP (31). Keratinocytes in the esophagus (which express DAF, MCP, and CD59) (32) also stained positive for CRIT (Fig. 3C). In the esophagus, SMCs were also clearly CRIT positive (Fig. 3D). In the kidney, anti-CRIT-ed2 showed a strong reaction with podocytes in kidney glomeruli (Fig. 3E) (as

**FIGURE 3.** Cellular localization of CRIT by immunohistochemistry in various human tissues probed with anti-CRIT-ed2. Proliferating endometrium (A); pancreas (B); esophagus (C and D); kidney (E); venules/arterioles in kidney (F); testis (G and H). Scale bars, F and H, 100 μm; A–E and G, 50 μm. SS, Stratified spinosum; SG, stratified germinatum; SC stratified corneum; SM, smooth muscle; sert, Sertoli cells. Controls that consistently gave no staining (not shown) included omission of primary Ab (anti-ed2) as well as primary Ab absorbed with ed2 peptide. Identical staining patterns in kidney were obtained from at least 10 fresh tissue samples.
for CR1) (33) as well as endothelia and SMCs of blood vessels (F) but not in fibrocytes. However, DAF, MCP, and CD59 are expressed on glomerular epithelial, endothelial, and mesangial cells (34). In the testis (Fig. 3, G and H), there was a positive reaction of scattered and partly elongated cells attached to the basement membrane of the testicular tubules, which most likely are Sertoli cells. CRIT was also found in the myoepithelia of some exocrine glands (breast) and in stromal cells in breast and prostate gland. Finally, in the placenta, there were groups of decidual cells weakly positive for CRIT.

**CRIT binds complement C2**

We previously demonstrated C2 binding to Sh-CRIT-ed1 by affinity purification of C2 from NHS using the Sh-CRIT-ed1 peptide (19). Furthermore, by using C2 hemolytic assays in which limiting amounts of C2 (just sufficient to restore complement activity) were added to C2-deficient serum, we showed that preincubation of C2 with CRIT-ed1 peptide inhibited complement activation (10). We now confirm the C2 interaction with Hu-CRIT by ligand blotting. For this, total protein lysates of Jurkat cells, immunoblated onto nitrocellulose, were probed with C2bio (Fig. 4A). C2, like anti-ed1, bound to monomeric and dimeric CRIT. C2 still bound when preincubated with either CRIT-ed2 peptide or CRIT-H17S peptide. However, CRIT-ed1 peptide abrogated C2 binding. Identical results were obtained with 125I-labeled ligand. In work to be reported in detail elsewhere, we showed that a recombinant von Willebrand factor A (vWFA) domain of C2, binds CRIT via ed1, and that this binding can be blocked by prior incubation of vWFA with the C2 mAb Hyb-5050 (35). In this study, we show that the vWFA domain of C2 expressed and purified from *E. coli*, binds in vitro-expressed CRIT. After stabilizing the protein interaction by cross-linking, both unbound CRIT and CRIT-vWFA C2 are detected by anti-CRIT-ed1 in Western blots (Fig. 4B). The binding of biotinylated C2 or FITC-labeled C2 (not shown) to Jurkat cells via Hu-CRIT-ed1 was also shown by FACS analysis (Fig. 4C) and immunofluorescence microscopy. Binding could be eliminated by prior incubation of the cells with anti-CRIT-ed1 blocking Ab. Factor B and serum albumin showed no binding to the Jurkat cell surface.

The dependence on C2bio concentration for the ELISA signal of C2bio interacting with C4b or CRIT-ed1 and that this interaction is a saturable phenomenon are shown in the graph of binding data or inset. Nonlinear regression of the equation describing one-site binding of ligand to receptor that follows the law of mass action (*y* = *B*max *X/K*app + *X*) (Equation 1), where *B*max is the maximal binding, and *K*app is the concentration of ligand required to reach half-maximal binding) onto the data shown in Fig. 4D gave an estimate for *K*app of 0.0152 μM for C4b/C2bio. This is in close agreement with the *K*trans estimate of 0.015 μM for C4b/C2 by plasmin surface resonance (36) and indicates that the biotinylation of C2 had not compromised its protein-binding ability. In our study, the *K*trans from a Scatchard analysis plot of concentration of C2 bound (*y*bound) against concentration of free C2, was not calculated.

Although we have no data for the affinity of C2 for native CRIT, we have been able to estimate the affinity for the ligand-binding region, CRIT-ed1 in the form of a synthetic peptide. Comparison of data for C2bio binding CRIT-ed1 or C4b (Fig. 4D) indicates that an ELISA reading for CRIT-ed1/C2bio binding ~3-fold lower than for C4b/C2bio corresponds to a *K*app for CRIT-ed1/C2bio (0.022 μM) 2-fold lower than between C4b/C2bio (0.0152 μM) (*K*trans of 0.015 μM (36)) (Table II). According to our estimations, C2 has an equal to 2-fold lower affinity for CRIT than for C4b and therefore could not prevent assembly of the CP C3 convertase but is more likely reducing excessive activation on a target. The concentration of C2 (ligand) in plasma is 0.25 μM. This is ~10 times the average (using two different methods; Table II) equilibrium dissociation constant of C2-CRIT receptor complexes, *K*app of 0.0235 μM, implying (Equation 1) that C2 will occupy 90% of CRIT receptors at equilibrium.

Again, maximum blockade of the C4b/C2bio interaction was possible (Fig. 4E) by preincubation of C2bio with a concentration of ed1 (1.2 μM) ~80 times that of the *K*trans for C4b/C2 (0.015 μM) and ~51 times the *K*trans for CRIT-ed1/C2 (0.0235 μM). This is in agreement with predictions for ligand/receptor associations that the ligand concentration needs to be 99 times the *K*trans to occupy 99% of the receptors at equilibrium. C4b/C2bio binding could not be inhibited by preincubating C2bio with ed2 (Fig. 4E).

As an additional test of the binding of C2-ed1, it was possible to inhibit the binding of C2 to C4 in the assembly of the CP C3 convertase with increasing concentrations of CRIT- or C4-based peptides. Plotting percent inhibition of hemolysis, as a measure of the extent of binding of CRIT- or C4-based peptides to C2, resulted in Fig. 4F. Similar nonlinear regression calculations of the data gave estimates of *K*app for CRIT-ed1/C2 of 0.025 μM and C4b/C2bio/C2 (where C4b/C2bio is the C4 β-chain peptide described before (10)) of 0.011 μM. These data are in good agreement with other empirical data summarized in Table II, and are not compromised by the many proteins in NHS, because the CRIT-ed1/C2 vs C4b/C2 interactions are highly specific. CRIT-H17 representing the 11-aa C-terminal part of CRIT-ed1 has a 10-fold order of magnitude greater affinity for C2 than CRIT-ed1. Interestingly, in preliminary unreported results before the study of Inal and Schifferli (10), CRIT-H17 alone, but not CRIT-ed1, gave significant reductions in various parameters of complement-mediated inflammation in the reversed passive Arthus reaction in mice (11). In all current experiments, C2 binding to Hu-CRIT-ed1 was not Mg2+ dependent.

**CRIT inhibits CP-mediated complement cytolysis**

To ascertain whether CRIT is able to protect cells from complement-mediated attack, cells sensitized with Abs against human lymphocytes (or whole human serum) were incubated with increasing concentrations of anti-CRIT Ab, before exposure to NHS as a source of complement. A titration was first conducted with increasing NHS (Fig. 5A) to show that lysis was being achieved with this system and to find a convenient serum concentration (chosen to be 10%) to be able to see easily any increase in percent lysis of cells. The results (Fig. 5B) show a significant increase, from 8% lysis without Ab to 28% lysis with 12.5 μg/ml anti-CRIT-ed1 in the promonocytic cell line, U937. With the macrophage-like THP-1 cells (Fig. 5C), and monocytes (D), there were significant increases from 10 and 15% lysis, respectively, to 43 and 52%, respectively, with 10 μg/ml. As noted previously, macrophages are negative for CRIT (see Table I). Monocytes treated with IFN-γ, thereby expressing lower levels of CRIT, showed a nonsignificant increase in cytolysis from ~20% without Ab to 34% with 10 μg/ml anti-CRIT-ed1. To control for a possible activation of complement through the presentation of additionally bound anti-CRIT and so additional Fc by the neutralizing anti-CRIT Ab, we incubated cells with increasing anti-CRIT-ed1 but without prior sensitization with the complement-fixing antilymphocyte serum. Under these conditions shown in Fig. 5, B and C (▲), there was no increase in cytolysis with increasing
anti-CRIT-ed1 concentration, indicating that the blocking Ab did not activate complement.

C2 bound to CRIT is rendered resistant to C1s cleavage

We and others have shown previously that CRIT-ed1 inhibits the complement-mediated lysis of sheep erythrocytes (10, 19–21). Having also shown that cleavage of C2 by C1s was inhibited in the presence of ed1 peptide, we now wanted to see whether this inhibition could be provided by native CRIT as found on the surface of Jurkat cells. As illustrated in Fig. 5E, CRIT-ed1 peptide completely inhibited C1s cleavage of C2. This is in contrast to cleavage to C2a and C2b by C1s in the presence of sham peptide (C4βS).

FIGURE 4. Complement component C2 binds Hu-CRIT on the cell surface via extracellular domain 1. A, Ligand blotting showing binding of C2bio to the dimeric and monomeric forms of Hu-CRIT in Jurkat cells. Blocking is successful by preincubation of C2 with a 10,000 M excess (100 μM) of CRIT-ed1 peptide only. B, CRIT expressed in an in vitro reticulocyte lysate system binds to rC2 (vWFA domain). The proteins are then cross-linked as described before (10) and detected by immunoblotting (IB) with anti-CRIT-ed1. C, Binding of C2bio to Jurkat cell surface in FACS analysis. Binding is blocked by preincubating cells with anti-CRIT-ed1. Factor B and human serum albumin do not bind. D, ELISA for binding of C2bio to C4 or CRIT-ed1. E, Blockage of ELISA signal for C4b/C2bio interaction with CRIT-ed1, but not CRIT-ed2 peptide. F, Percent inhibition of hemolysis (as a measure of the extent of binding of CRIT- or C4-based peptides to C2) with increasing peptide concentration.
Interestingly, in the presence of Jurkat cells (and therefore CRIT), there is almost no cleavage of C2. When the receptor is blocked with anti-CRIT-ed1, the inhibition by CRIT of C1s-mediated cleavage of C2 is lifted.

**Discussion**

The complement system is a rapidly activated and self-amplifying system that, to avoid extensive consumption and self-depletion, requires control at several levels. Interestingly, there is often symmetry of function between inhibitors of complement in the fluid phase and on cell surfaces (37), although there is an exception: C1-INH is a major regulator of C4 and C2 cleavage in the fluid phase, preventing excessive formation of the C3 (C4b2a) convertase.

Hu-CRIT was cloned from both genomic and cDNA. Thus, although the 5′ and 3′ untranslated regions may yet be found to possess introns, the high level sequence identity suggests that the coding region of Hu-CRIT could be coded for within a single exon and therefore be intronless, as for example are many GPCR genes.

Whether the entire CRIT gene is shown to possess introns or not, we have presented evidence that it encodes a functional receptor (of a size equivalent to that predicted from the ORF) that has affinity for the human complement serum protein, C2. Using an Ab raised against the N-terminal extracellular domain, CRIT was found on the plasma membrane in a wide variety of human cell types. We show that the *Schistosoma* (and *Trypanosoma*) parasite and its human host share a receptor for C2, and we postulate that they share a complement regulatory function. The high sequence homology between the mammalian (human and rat)/parasite (*Schistosoma* and *Trypanosoma*) and cod CRIT genes is also indicative that they may have evolved from a common ancestral gene.

CRIT regulates the formation of the CP C3 convertase by reducing the association of C2 with C4b. The *K_{Dapp}* of CRIT-ed1/C2 is of the same order as the *K_{Dp}* for C4b/C2 (0.015 μM) (36) and also CR1 for C3b dimers (0.01–0.02 μM) (38, 39). Overall, CRIT-ed1 appears to compete with C4b for binding to C2 with an apparent moderate affinity, but we do not yet know whether CRIT accelerates the decay of the C4b2a convertase. In this study, we have found that anti-CRIT-ed1 blockade of the CRIT receptor increases the complement-mediated killing of two human myeloid cell lines (U937 and THP-1) and of monocytes. However, the relative contribution of CRIT to protecting against complement-dependent cytotoxicity in the THP-1 cells, for example, cannot be known, because certain other complement regulatory proteins are expressed, such as CD59 (40). Once C2 is bound to CRIT (unlike C2 bound to C4b), it cannot be cleaved by C1s to yield C2a and C2b and thus no longer partakes in C3 convertase formation. Of the complement regulators that control the C3 convertase (C4b2a) and C5 convertase (C4b2a3b), three of them, DAF, CR1, and C4BP, compete with C2 for binding to C4b and also bind to C3 or C5 convertases, causing C2 dissociation. These proteins are composed mainly or only of complement control protein repeats, which contain the C4b (and C3b) binding sites and are all encoded by genes in the RCA cluster on chromosome 1q31–32. CRIT, like CD59, is another member of the complement control family of proteins that lacks structural resemblance to the RCA group of proteins.

In humans, CRIT is widely distributed. It is present on many hematopoietic cells, but not neutrophils, and notably, like MCP, not on erythrocytes. CRIT is found in many tissues, and interestingly, like DAF (41), its expression appears to be hormonally regulated during the menstrual cycle. CRIT is also found on endothelial and epithelial cells. Besides its complement-regulating function concomitant with its wide distribution in the body, we speculate that its particular expression in highly differentiated cell types, such as

### Table II. Dissociation constants (K_{Dapp}) for C2 binding to CRIT-ed1/H17 and C4β212–232/C4b

<table>
<thead>
<tr>
<th>Assay</th>
<th>CRIT-ed1</th>
<th>CRIT-H17</th>
<th>C4β212–232/C4b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemolytic assay</td>
<td>0.025</td>
<td>0.0033</td>
<td>0.011</td>
</tr>
<tr>
<td>Surface plasmon resonance (36)</td>
<td>0.015 (C4b/C2) (K_{Dp})</td>
<td>0.015 (C4b/C2) (K_{Dp})</td>
<td></td>
</tr>
<tr>
<td>ELISA</td>
<td>0.022</td>
<td>0.0152</td>
<td></td>
</tr>
</tbody>
</table>

*Micromolar concentration. Mean of four independent experiments.*
glomerular podocytes (42), Sertoli cells (43), and keratinocytes (44, 45), could suggest additional roles.

Component results in the release of the anaphylatoxins C3a and C5a, which in turn bring about the proinflammatory responses of complement, including an increase in vascular permeability, mast cell degranulation, and smooth muscle contraction. C5a is then able to further increase the proinflammatory response by inducing chemotaxis of macrophages and neutrophils and by the activation of neutrophils. These inflammatory responses are a natural reaction of host tissue to injury. However, an unregulated inflammatory response may injure host tissues and is important in many complement-mediated diseases (46). As complement and its activation products, in particular, C5a, can aggregate, stimulate, and cause degranulation of neutrophils, any anti-inflammatory agents should, in certain diseases, preferably interrupt the complement cascade at an early stage. In myocardial infarction, for example, it might be important to block CP and/or lectin pathway but leave the AP and lytic pathway intact. By competing with C4b for binding to C2, and inhibiting its activation, CRIT prevents the formation of the CP C3 convertase and is thus an obvious target for diminishing the proinflammatory response of unregulated complement activation. A soluble form of CRIT in the form of the CRIT-ed1 peptide has been shown to inhibit CP-mediated hemolysis by human serum. Indeed, recombinant soluble forms of the membrane complement inhibitors have been proven to inhibit complement-mediated inflammation (46). If soluble CRIT in the form of the CRIT-ed1 peptide or the smaller CRIT-H17 derivative peptide is present, there is a reduction in inflammatory response. This reduction is due to a failure of C5a to activate neutrophils.

It is well documented that conformational changes can occur within the secondary structure of a receptor upon reduction (47, 48). Essentially, reduction of the intramolecular disulfide bond(s) of a protein, as we believe occurs with CRIT, results in its unfolding from a globular state to a more rod-like conformation, which migrates more slowly in an SDS-PAGE gel. Whether CRIT folding or dimerization affects its ability to bind C4b, for example with the TNFR, BAFF-R (49), will be the subject of future work.

If the dimerization of CRIT occurs through the formation of intermolecular disulfide bonds then candidate cysteine residues would be Cys31,40,41 of TM1, Cys69 of TM2, Cys110 of TM3, or Cys126,214,280 of the cytoplasmic tail. Disulfide linkages are not usually found in the cytoplasm because of the reducing environment there. Because there are no cysteines in the extracellular domains, it seems probable that the cysteines in the TM domains are involved in dimerization. Disulfide-bonded dimers, mediated through TM domains have been reported for some receptors such as CD44 (50) and the insulin receptor (51). Although reducing agents can dissociate receptor homodimers suggesting disulfide-bonded dimerization, other intermolecular hydrogen interactions, between TM domains, have been implied, at least for the GPCRs (52). We previously noted that the C2-binding CRIT-H17 motif [F/H]EVKX4/5P, which in the C4β chain occurs contiguously, separated by a β turn, in CRIT, occurs only once, and also speculated that CRIT might be found to exist as a dimer (10). Our current study has shown Hu-CRIT to form dimers (and that C2 binds to both CRIT monomers and dimers). If these dimers are shown to be homodimers, then it is conceivable that two CRIT-H17 motifs (forming part of two ed1 regions) could be brought into close proximity for C2 binding, as in the C4β chain. Whether dimeric CRIT binds C2 with greater affinity and represents the functional form of the receptor we cannot say, but already the work of Oh et al. (20) indicates that a recombinant homodimeric CRIT-H17 peptide has an increased binding capacity for C2 and 5-fold higher anti-complementary activity.

In addition to genetic abrogation and mutagenesis experiments in cells, which together with structural studies are revealing details of the CRIT-ed1 interaction with C2, it is envisaged that CRIT gene knockout experiments will place the relative contribution of CRIT toward complement regulation in context with other CP regulators.

Acknowledgments

At its inception, this work benefited from stimulating discussions with David Meyer, Andrew Falconar, and Robert Sim.


