A *Schistosoma* protein, Sh-TOR, is a novel inhibitor of complement which binds human C2

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Abstract Human complement regulatory (also called inhibitory) proteins control misdirected attack of complement against autologous cells. Trypanosome and schistosome parasites which survive in the host vascular system also possess regulators of human complement. We have shown Sh-TOR, a protein with three predicted transmembrane domains, located on the *Schistosoma* parasite surface, to be a novel complement regulatory receptor. The N-terminal extracellular domain, Sh-TOR-ed1, binds the complement protein C2 from human serum and specifically interacts with the C2a fragment. As a result Sh-TOR-ed1 pre-incubated with C2 inhibits classical pathway (CP)-mediated haemolysis of sheep erythrocytes in a dose-dependent manner. In CP-mediated complement activation, C2 normally binds to C4b to form the CP C3 convertase and Sh-TOR-ed1 has short regions of sequence identity with a segment of human C4b. We propose the more appropriate name for TOR of CRIT (complement C2 receptor inhibitory trispanning).

Key words: Complement; C2; Receptor; *Schistosoma*

1. Introduction

Complement (C) activation can be regulated by a number of membrane-associated or soluble (secreted) C regulatory proteins. Activation of the classical pathway (CP) is regulated by the blocking of the CP C3 convertase, C4b2a. This action is mediated by regulators of complement activation (RCA) [1] or complement control proteins (CCPs), which essentially inhibit the activation of C on the surface of host cells. In the fluid phase these include factor I, [2] and C4b binding protein [3,4]. The membrane-bound RCAs (mRCA) [5] control convertase on host cells and these include decay-accelerating factor, DAF (CD55) [6] and complement receptor type I (CR1) or C3b/C4b receptor (CD35) [7]. DAF [8,9] and CR1 [10–12] inhibit the binding of C2 to C4b and promote the dissociation of C2a from C4b in the C3 convertase. Membrane cofactor protein, MCP (CD46), and CR1 also promote the catabolism of C4b by the plasma serine protease, factor I [13,14]. The amplification steps of complement (C3 and C5 convertases) are thus targeted by three membrane-bound regulators, MCP, DAF and CR1. Such mRCAs are more highly expressed in certain carcinoma cells and continuous cell lines [15–17] and it is believed that this overexpression protects such cells from complement-dependent cytotoxicity (CDC).

Homologues of human C regulators have been found in other species. In particular, the surfaces of certain human parasites which spend at least a part of their life cycle in the host vascular system have such proteins, possibly to protect them against host CDC. Schistosomes, for example, inhibit C activation by adsorbing host DAF from erythrocytes [18] whilst trypanosomes express CD59 and DAF homologues with decay-accelerating activity for both classical and alternative pathway convertases [19] or for the alternative pathway only [20].

This paper describes Sh-TOR [21], which is found on the surface of the *Schistosoma* parasite, as a receptor to which human complement C2 can bind specifically. This results in the inhibition of the CP of C activation, probably by interfering with the formation of the C3 convertase, C4b2a. Receptor affinity chromatography with the Sh-TOR ligand binding region extracellular domain 1 (ed1) was used to isolate the ligand from normal human serum (NHS). This was shown by N-terminal sequencing and Western blot analysis to be complement C2. The C regulatory function of Sh-TOR is suggested by a haemolytic assay in which the Sh-TOR-ed1 peptide, which contains the C2 binding region, causes inhibition of lysis.

2. Materials and methods

2.1. Antibodies

Horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Bio-Rad. The HRP-conjugated rabbit anti-goat antibody was purchased from Sigma. Rabbit anti-Sh-TOR-ed1 was described before [21]. Rabbit polyclonal anti-(human C2) and anti-(human factor B) antibodies were produced in the MRC Immunochemistry Unit by immunisation with purified proteins. The anti-C2 monoclonal antibody HYB50-5 [22] was a gift from Dr C. Koch, State Serum Institute, Copenhagen.

2.2. Receptor affinity chromatography

The Sh-TOR-ed1 peptide (NH_2-MSPSLVSDTQKHERGSHEVKIKHFSPY-COOH) was coupled to epoxy-activated Sepharose 6B (3 mg/ml and 2 ml final gel volume) at pH 9. Excess active groups were blocked with ethanolamine. After two successive washes in borate buffer (pH 8) and acetate buffer (pH 4), the affinity matrix was placed in a column (5 cm×1 cm diameter) and equilibrated with phosphate-buffered saline (PBS) at 20°C. Then 5 ml of human serum diluted with one volume of PBS was passed through. The column was washed with PBS until the A_{280} of the eluate was < 0.005. Bound material was eluted with 0.1 M glycine–HCl (pH 2.8). 1 ml fractions were collected, neutralised with 50 μl 1 M Tris–HCl (pH 8.5) and analysed by SDS–PAGE.

2.3. SDS–PAGE and Western blotting

Protein assay by dye binding (Bio-Rad protein assay kit) was carried out to ensure even loadings for SDS–PAGE, which together with Western blot analysis of proteins was done as described previously [21].

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2.4. Protein sequencing

Protein(s) obtained by 'receptor affinity chromatography' were resolved by SDS–PAGE with 2 mM mercaptoacetic acid in the upper electrode buffer, and transferred to ProBlott™ (Applied Biosystems). The protein visible with Coomassie blue was subjected to N-terminal sequencing.

2.5. Haemolytic assay

Inhibition of the CP of C activation was determined as follows. Various concentrations of Sh-TOR-ed1 peptide in 50 μl freshly made $DGVB^{2+}$ [23] were pre-incubated with 100 μl of NHS (diluted 1/40 in $DGVB^{2+}$) for 30 min at room temperature and added to 50 μl EA (sheep erythrocytes, 2×10^8/ml, coated with antibodies). The mixtures were incubated at 37°C for 30 min and cells centrifuged. The controls, both made up to 200 μl with $DGVB^{2+}$ consisted of cells plus 50 μl of the maximum concentration of peptide (10 μM) and cells plus 100 μl of NHS (1/40 dilution). A 100% lysis control (lysed by water) was also included. Per cent haemolysis was calculated from the A_{412} of the supernatant [23].

A haemolytic assay in which C2 concentration was limiting was performed similarly. Essentially the same concentrations of peptide (in 50 μl $DGVB^{2+}$) were pre-incubated with 2 μg C2. This was then added to 100 μl of C2-deficient serum (Sigma) (diluted 1/80), before adding 50 μl of EA. After 1 h at 37°C, haemolysis was determined by haemoglobin release. C2 used in this assay was prepared by immunoaffinity chromatography (N. Wong, T.P. Hickling, A. Laich, B. Moffatt, MRC Immunochemistry Unit, unpublished).

3. Results

3.1. Identification of a ligand for Sh-TOR by affinity chromatography

Fig. 1 shows the membrane topology of Sh-TOR as predicted by computer analysis (PHDhtm) [24]. The surface exposure of the N-terminal segment was confirmed by use of anti-Sh-TOR-ed1 antibody in fluorescent microscopy of RBL-2H3 cells transfected with Sh-TOR [21] and by showing that none of the predicted six N-linked glycosylation sites in the Sh-TOR-id2 region is utilised [21]. In the *Schistosoma* parasite, Sh-TOR was located to the surface tegument of the adult worm and is believed to be in contact with host blood plasma. The protein has nine tyrosine residues in the predicted cytoplasmic domain, and was shown to be phosphorylated on tyrosine [21]. This, together with its predicted membrane topology and predicted three transmembrane domains, meant that it was probably a new orphan receptor for some protein ligand in the host blood plasma. Sh-TOR has two extracellular and therefore putative ligand binding domains, extracellular domains 1 and 2 (ed1 and ed2). In order to identify a possible ligand of the receptor, ed1 was used in affinity chromatography to see whether it would selectively bind any human serum proteins.

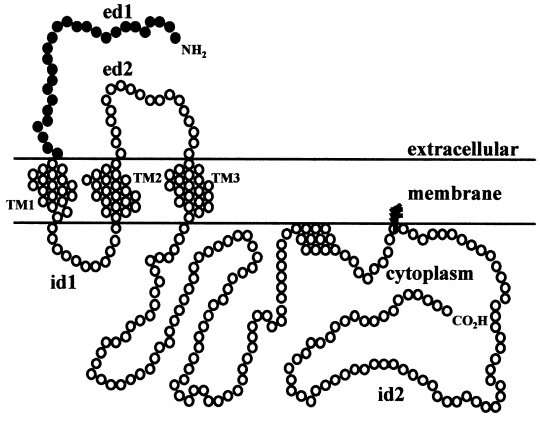

Fig. 1. Schematic representation showing predicted membrane topology of Sh-TOR [21] including three predicted transmembrane (TM) domains, cytoplasmic tail, and a predicted myristoylation site. The extracellular domains (ed1 and ed2) are indicated. The ed1 region is depicted by filled circles, a peptide based on this sequence having been used in affinity purification of the C2 ligand from NHS. The intracellular domains (id1 and id2) are also indicated.

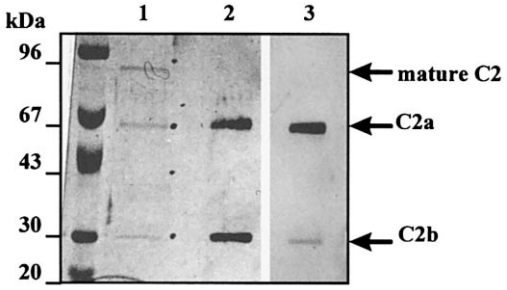

Fig. 2. Identification of ligand for Sh-TOR by receptor affinity chromatography using the immobilised ed1 putative ligand binding domain. Lane 1, Coomassie-stained SDS–PAGE of a low pH eluate from a chromatography carried out on 5 ml of NHS. Lane 2 is a Western blot of the same sample, probed with a polyclonal anti-C2. Lane 3 is a Western blot as for lane 2 probed with a monoclonal antibody HYB50-5 recognising C2a.

NHS (5 ml) was passed through a column of epoxy-activated Sepharose 6B covalently coupled with the 27 amino acid long Sh-TOR-ed1 peptide (which is highlighted in Fig. 1). Fractions were collected from a low pH elution and 10 μl aliquots resolved by SDS–PAGE. As shown in Fig. 2, the Coomassie-stained gel revealed three protein bands, of (approximate) molecular weights 94 kDa, 67 kDa and 30 kDa. Protein sequencing of the 30 kDa band revealed the N-terminal sequence: NH_2-APSCPQNV. A search of the data base resulted in the assignment of this sequence as human complement C2b (30 kDa) part of C2 which in fully glycosylated form is 102 kDa. Purified C2 is highly susceptible to proteolysis by plasma proteases yielding C2a and C2b [25]. In a standard C2 purification method [26], protease inhibitors would be included in the starting serum but the affinity purification carried out here was at 25°C and in the absence of protease inhibitors. Immunoblotting with a polyclonal anti-C2 antibody specifically recognised 67 kDa and 30 kDa bands (Fig. 2, lane 2). The monoclonal antibody against C2a strongly recognised the 67 kDa band, C2a. That both C2a and C2b were identified on elution from the column suggested that proteolysis probably occurred after binding of the intact C2 to the Sh-TOR-ed1 affinity column. When the mixture of C2a and C2b was passed back through the column, after washing, only C2a was specifically retained by the ed1 column and subsequently eluted (not shown).

Complement proteins C2 of the CP and factor B of the alternative pathway show 39% identity at the amino acid level [27]. Western blot analysis was also carried out using anti-factor B antibodies. No positive interaction was found (not shown).

3.2. Inhibition of classical pathway-mediated lysis of sheep erythrocytes by Sh-TOR-ed1

To determine whether the interaction of C2 with Sh-TOR-

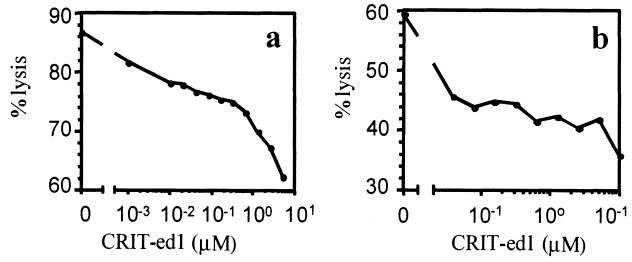

Fig. 3. Inhibition of human C by Sh-TOR-ed1. a: Effect of increasing Sh-TOR-ed1 peptide on the C-mediated killing of sensitised sheep erythrocytes in the presence of NHS as a source of C. b: Effect of increasing Sh-TOR-ed1 peptide, pre-incubated with C2, on haemolysis, in the presence of C2-deficient serum (Sigma).

ed1 could affect the normal interaction of C2 with other C proteins, the effect of Sh-TOR-ed1 peptide on C-dependent lysis of antibody-sensitised sheep erythrocytes was tested. There was a dose-dependent inhibition of lysis, with inhibition evident at 10^{-3}–10^{-2} μM ed1. The maximum concentration of Sh-TOR-ed1 peptide tested (~7 μM) gave approximately 30% inhibition of lysis (Fig. 3a).

As an additional confirmation that Sh-TOR-ed1 specifically binds to human complement C2, C2-deficient human serum with enough C2 added to reconstitute haemolytic activity was used in the same assay. Pre-incubation of Sh-TOR-ed1 peptide with C2 before addition to the C2-deficient serum was found to inhibit haemolytic activity, once more in a dose-dependent manner (Fig. 3b), with a maximum 43% inhibition of lysis.

These results suggest that Sh-TOR binds specifically to human complement C2 and that it inhibits the CP of C activation, presumably by inhibiting the formation of the CP C3 convertase (C4b2a). No comparable inhibitor of C2 has been described previously.

3.3. Amino acid sequence homology of Sh-TOR and Sm-TOR with C4b

The function of C2 in the CP is mediated by its binding with C4b but the region on C4b to which C2 binds has not yet been identified. The TOR-ed1 sequences of trematodes (Sh-TOR-ed1 and *Schistosoma mansoni* or Sm-TOR-ed1) were compared with that of human C4b to look for any homology which might be related to C2 binding. The result in Fig. 4 shows a similar region, in the β-chain of human C4b. Mouse C4b sequence is also included for comparison because mouse and human C2 are 76.2% identical (or 80.3% similar) at the amino acid level. The Sh-TOR-ed1 sequence from residue Ser^{2} to Tyr^{27} and C4b β-chain from residue Ser^{225} to Tyr^{251} share 35% homology or 42% taking into account conservative amino acid replacement.

4. Discussion

We have presented evidence that Sh-TOR encodes a functional receptor. The main criterion, from this study, is its affinity for the human C2 protein. From previous work [21], Sh-TOR was found to be recognised by an antibody raised against the N-terminal, predicted extracellular domain, and to be of an equivalent size to that predicted from the open reading frame [21]. Sh-TOR, which is phosphorylated on tyrosine, was found principally in the tegument of the *Schistosoma* adult worm [21]. Having assigned to Sh-TOR the function of receptor for C2, we postulate that it constitutes a novel C regulator or inhibitor. If so, it presumably does not act systemically, but rather acts at the interface between tegument and plasma. Its efficiency as an inhibitor would in this case depend on both affinity and its density (concentration) on the tegument surface.

That Sh-TOR prevents the formation of the classical C3 convertase by preventing C2 from binding to C4b was suggested in this work by showing that the 27 amino acid long Sh-TOR-ed1 can bind C2 in an affinity purification of human C2 from NHS. This was confirmed by then using a soluble form of Sh-TOR, in the form of the Sh-TOR-ed1 peptide, to inhibit in a dose-dependent manner the haemolytic activity of C2 from the CP of C activation. It appears likely that Sh-TOR-ed1 competes with C4b for binding to C2.

That the inhibitory concentration of Sh-TOR-ed1 in haemolytic assays of about 10 μM (40-fold higher than the concentration of C2 in NHS) is not any lower may be due to peptide sequestration by low affinity interactions with other plasma proteins or to an unusual conformational structure of the ligand binding domain presented in the form of a synthetic peptide. We would thus expect that if at least ed1 were presented in a more 'native' conformation, an inhibitory concentration closer to the serum concentration of C2 would have been obtained. At this stage it is not known what contribution, if any, ed2 may play in C2 binding to Sh-TOR. Besides the extracellular loops being involved in the binding of C2 to Sh-TOR, it is conceivable that certain amino acids within the cavity formed by the three transmembrane α-helices may also play a part. Further studies to reveal which particular residues in ed1 are critical for C2 binding should now be possible. The finding that only C2a bound and was subsequently eluted when a mixture of C2a and C2b was applied to the Sh-TOR-ed1 affinity column, shows that Sh-TOR-ed1 binds specifically the C2a region of C2.

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MoC4bβ(S223)  S N R S T H F E V K K Y V L P N F E V K I T P W K P Y
HuC4bβ(S225)  S N S S T Q F E V K K Y V L P N F E V K I T P G K P Y

Sh-TOR-ed1    S P S . L V S D T Q K H E R G S H E V K I K H F S P Y
Sm-TOR-ed1    S P S . L V S Y T Q K N E R G S H E V K I K H F S P Y

Consensus     S - S - - - - - - - K - - - - - - E V K I - - - - P Y
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Fig. 4. Sequence alignment between S-TOR-ed1 and C4b. Amino acid sequence alignment of the N-terminal ed1 regions of *Schistosoma haematobium*-TOR and *Schistosoma mansoni*-TOR with a region from the β-chain of human and mouse C4b (numbering according to GenBank release P01028 and P01029 respectively) with which they show homology. Residues within dark grey boxes are identical and those within light grey boxes are similar. A full stop indicates a gap introduced to make the alignment. A consensus sequence is also given.

Human C4b binds C2 and although the C2 binding site on C4b has not yet been identified, the two C4b binding sites on C2 have been described. C2b, the domain which binds with C4b initially, consists of three Sushi (SCR) repeats or CCP repeats of 62, 57 and 55 amino acid residues, all three of which constitute the Mg^{2+}-independent C4b binding site [28,29]. After C1s cleaves C2 to release C2b, C2a remains attached to C4b with a higher binding avidity. C2a is bound via the MIDAS (metal ion-dependent adhesion site) which lies within the VWFA domain of C2a [30,31]. In view of the C2 binding site on C4b not having been identified so far, it is interesting that we report within the β-chain of C4b a region homologous to the C2 binding ed1 domain of Sh-TOR.

As a result of the data presented, it seems appropriate to rename *Schistosoma* trispanning orphan receptor (TOR) as S-CRIT (complement C2 receptor inhibitory trispanning). Structural studies to understand in detail the Sh-CRIT-ed1 interaction with C2 are now under way.

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