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Immunology

Sites within the complement C3b/C4b receptor important for the specificity of ligand binding

(short consensus repeat/site-directed mutagenesis/complement receptor type 1/CD35)

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ABSTRACT **Cysteine-rich repeating units of 40–70 amino acids are building blocks of many mammalian proteins, including 12 proteins of the complement system. Human complement receptor type 1 (CR1) is composed of 30 such tandemly arranged motifs, designated short consensus repeats (SCRs), which constitute the entire extracellular portion of this protein. Klickstein *et al.* [Klickstein, L. B., Bartow, T. J., Miletic, V., Rabson, L. D., Smith, J. A. & Fearon, D. T. (1988) *J. Exp. Med.* 168, 1699–1717 (abstr.)] localized a C4b binding domain to SCR-1 and/or SCR-2 and a C3b binding domain to SCR-8 and/or SCR-9. These SCRs bind different ligands, although SCR-1 and SCR-8 are 55% homologous and SCR-2 and SCR-9 are 70% homologous. To examine if one or two SCRs are required for ligand binding and to define sites within the SCRs that determine specificity of binding, mutagenesis analysis of a truncated, secreted form of CR1, called CR1-4 by Hourcade *et al.* [Hourcade, D., Meisner, D. R., Atkinson, J. P. & Holers, V. M. (1988) *J. Exp. Med.* 168, 1255–1270], was undertaken. The latter, composed of the first eight and one-half amino-terminal SCRs of CR1, efficiently bound C4b but not iC3. SCR-1 and SCR-2 were necessary for this interaction. Analysis of the mutant CR1-4 proteins, in which amino acids in SCR-1 and SCR-2 were substituted a few at a time with the homologous amino acids of SCR-8 and SCR-9, led to the identification of one amino acid in SCR-1 and three amino acids in SCR-2 important for C4b binding. Furthermore, five amino acids at the end of SCR-9, if placed in the homologous positions of SCR-2, conferred iC3 binding and are likely essential for ligand binding activity of SCR-8 and SCR-9. This iC3 binding occurred only if SCR-1 was present, indicating that two contiguous SCRs are necessary for this interaction. These results provide identification of amino acids within SCRs that are important for ligand binding.**

Cysteine-rich repeating motifs of 40–70 amino acids are a structural feature common to many mammalian proteins (1). One such unit of ≈60 amino acids, termed a short consensus repeat (SCR), is found in 12 complement-related proteins as well as in many other proteins, including the interleukin 2 receptor, factor XIIIb of the coagulation system, lymphocyte homing receptors, and several viral proteins (1, 2). It features four invariant cysteines and 17 other amino acids conserved in 40–100% of cases (2). More than 100 examples of this motif have been identified (2).

The recently defined regulators of the complement activation family of proteins include two receptors (CR1 and CR2) and four inhibitors [factor H, C4b binding protein (C4bp), membrane cofactor protein (MCP), and decay accelerating factor (DAF)]. These proteins interact with activation products of the complement protein C3 and/or C4 (1). Each one is almost exclusively composed of these tandem SCR arrays (1). SCRs are also found at the amino terminus of proenzymes factor B and C2, in C1r and C1s, and in the terminal complement components C6 and C7 where they interact with C3 and C4 derivatives or their homologue C5 (1). Further, several viral proteins interact with C3b or C4b, possess SCR-like motifs, and probably function as virulence factors through their ability to regulate the complement system (1, 3).

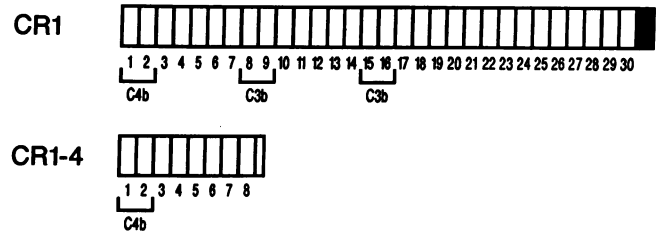

FIG. 1. Schematic representation of CR1 and CR1-4 and identification of the repeats involved in C3b and C4b binding. The SCRs are numbered, and assignment of the ligand binding SCRs for CR1 is from ref. 8 and for CR1-4 is based on this report. The solid black area denotes the carboxyl-terminal portion of CR1 that contains the transmembrane domain and cytoplasmic tail.

Human complement receptor type 1 (CR1) or the C3b/C4b receptor (CD35) is expressed on erythrocytes and most peripheral blood leukocytes (4–6). It binds C3b/C4b-bearing immune complexes, serves as a cofactor for factor I-mediated cleavage of C3b and C4b, and accelerates decay of the C3 convertases (4, 5, 7). The extracellular portion of the most common polymorphic size variant of CR1 is composed entirely of 30 SCRs (8–10). Klickstein *et al.* (8) determined that a C4b binding domain of CR1 is in SCR-1 and/or SCR-2 and C3b binding domains are in SCR-8 and/or SCR-9 and in an identical region encompassing SCR-15 and/or SCR-16.

We undertook experiments to define CR1 amino acids that play critical roles in ligand binding. A truncated, secreted form of CR1 was chosen as a model because of its smaller size and relative ease of manipulation and because it carries only one major binding site (see Fig. 1). Its mutagenesis analysis led to identification of several amino acids in SCR-1 and SCR-2 important for C4b binding and one area in SCR-9 important for iC3 binding (iC3 is C3 with a broken thioester bond and with a similar reactivity to C3b). Part of this work has been published in abstract form (11, 12).

MATERIALS AND METHODS

Subcloning into Mammalian Expression Vector. CR1-4 encodes the first eight and one-half SCRs of CR1 (9). It is

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Abbreviations: SCR, short consensus repeat; mAb, monoclonal Ab; iC3, form of C3 in which the thioester bond is broken and with similar functional activity to C3b; iC3-S and C4b-S, iC3 and C4b coupled to cyanogen bromide-activated Sepharose; MCP, membrane cofactor protein; DAF, decay accelerating factor; C4bp, C4b binding protein; BSA, bovine serum albumin.

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preceded by sequences coding for an incomplete signal peptide, lacking codons for the first 25 amino acids. Therefore, as an expression vector plasmid, pBC12-RF (a gift from B. Cullen, Duke University Medical Center, Durham, NC), derived from pBC12BI (13), was used because it provides codons for the initial methionine and three initial amino acids of the rat preproinsulin II signal peptide.

CR1-4 was subcloned into pBC12-RF, using standard procedures (14), to yield pCR1-4. In pCR1-4 codons for four amino-terminal amino acids of the vector rat preproinsulin II signal peptide are followed by a GGC codon (the result of the cloning procedure) for glycine and then the CR1-4 sequences.

Construction of Mutants. Mutants of CR1-4 were constructed by oligonucleotide-directed mutagenesis (15). ΔSCR-1 and ΔSCR-2 are derivatives of CR1-4 from which SCR-1 (amino acids 1–60 of mature CR1) or SCR-2 (amino acids 61–122) was deleted. Conditions for making the deletions were as described (16), except that T4 DNA polymerase was used instead of Klenow polymerase and S1 nuclease treatment was omitted. The substitution mutants, in which amino acids from SCR-1 or SCR-2 were changed to their counterparts from SCR-8 or SCR-9, are illustrated in Fig. 2. Mutants were checked by sequencing using the dideoxy method (17).

Transfection and Labeling. COS cells were maintained in Dulbecco's modified Eagle's medium (DMEM). Transfection was performed using lipofectin reagent (BRL) (18). Forty-eight hours after transfection, medium was removed, and the cells were washed and subsequently labeled for 16 hr using cysteine-free and serum-free DMEM supplemented with 70 μCi (1 Ci = 37 GBq) of [^{35}S]cysteine per ml.

Each mutant was transfected at least three times and the protein product was characterized by immunoprecipitation and affinity chromatography each time.

Immunoprecipitation. Immunoprecipitation was performed using anti-CR1 monoclonal antibody (mAb) E11 (19) or 3D9 (20) and rabbit anti-mouse IgG (Calbiochem) as a second antibody. Immunoprecipitates were analyzed by electrophoresis on SDS/7.5% polyacrylamide gels.

Affinity Chromatography. Affinity chromatography columns were prepared by coupling iC3 or C4b, obtained as described (21, 22), to cyanogen bromide-activated Sepharose (Sepharose 6B, Sigma) at a ligand concentration of 1 mg/ml. iC3-S and C4b-S are iC3 and C4b coupled to cyanogen bromide-activated Sepharose. Affinity chromatography was performed essentially as described (21, 23), using 0.3 ml of packed beads for 1 ml of the starting material. For incubation with Sepharose, medium was routinely diluted 12-fold to a final NaCl concentration of 12.5 mM. Eluates were analyzed by SDS/PAGE, followed by autoradiography. Quantitation of the relative intensity of bands on the autoradiographs was performed with an Ultrascan XL laser densitometer (LKB). Additionally, in some experiments the level of unbound and eluted CR1 protein was measured by ELISA.

ELISA Determinations. mAbs 3D9 and E11 were purified from ascites fluid by ammonium sulfate precipitation. Horseradish peroxidase (Boehringer Mannheim)-conjugated E11 was prepared by a modification of the procedure of Nakane and Kawaoi (24). Microtiter wells (Nunc, MaxiSorp) were coated with 100 μl of 3D9 per well at 2.5 μg/ml in phosphate-buffered saline (PBS) for 12–18 hr at 4°C. Remaining protein-reactive sites on the plastic were blocked with 300 μl of 1% bovine serum albumin (BSA)/0.1% Tween 20 for 1 hr at 37°C. After four washes with 0.05% Tween 20 in PBS, standards, samples, or controls (100 μl) were diluted in sample buffer [PBS containing 2% BSA and 0.2% Nonidet P-40 (NP-40) (Sigma)]. Conjugated E11 (50 μl of a 100 ng/ml solution in PBS containing 2% BSA and 0.2% NP-40) was added to each well and plates were then incubated overnight at 4°C. Wells were washed five times (with wash buffer). *o*-Phenylenediamine [100 μl of 0.2% (Sigma) in citrate-buffered 0.02% hydrogen peroxide (pH 5.0)] was added to each well and incubated for 30 min at room temperature. Color development was stopped by the addition of 100 μl of 1 M H_2SO_4 and the absorbance was read at 490 nm in a microplate reader (Dynatech).

RESULTS

pCR1-4, a plasmid containing a cDNA clone encoding the first eight and one-half amino-terminal SCRs of CR1 (9), was transfected into COS cells, resulting in the synthesis of a secreted, truncated form of CR1 termed CR1-4, with M_r 78,000 (Fig. 3). The CR1-4 protein was recognized by two mouse anti-CR1 mAbs, E11 (Fig. 3, lane 2) and 3D9 (not shown). Functional activity was assessed by the capacity to bind to C4b-S or iC3-S. Eluates of C4b-S contained CR1-4, whereas eluates of iC3-S contained no detectable protein (Fig. 3, lanes 1 and 3) or only trace amounts in some experiments. The quantity of the protein eluted from C4b-S was routinely between 60% and 80% of the amount that was immunoprecipitated. The efficiency of C4b binding by CR1-4 was ionic strength dependent, similar to CR1 and related C3b/C4b binding proteins (25, 26), with most efficient binding at low ionic strength (not shown). Binding was inhibited in a concentration-dependent fashion by fluid-phase C4b but

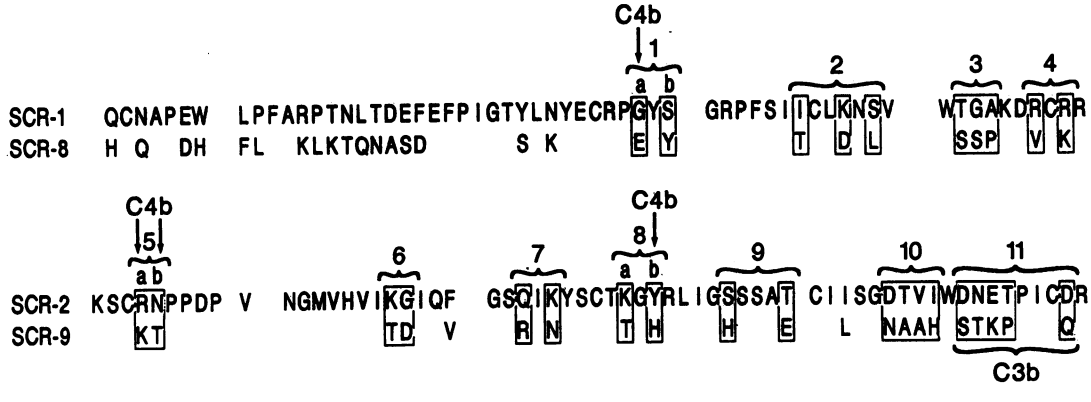

FIG. 2. Alignment of CR1 amino acid sequences in SCR-1 with those in SCR-8 and in SCR-2 with those in SCR-9 and identification of the mutations introduced in SCR-1 or SCR-2 of CR1-4. The sequences in braces and the corresponding number above each represent the 11 initial mutants. The amino acids within these numbered regions in SCR-1 or SCR-2 that were changed to their counterparts in SCR-8 or SCR-9 are boxed. In the case of the mutants 1, 5, and 8, their derivatives with a single amino acid substitution are marked with the letters "a" or "b". Amino acids indicated by the arrows are important for C4b binding. "C3b" indicates a site in SCR-9 with five amino acids, which, if placed in homologous positions in SCR-2, confers iC3/C3b binding.

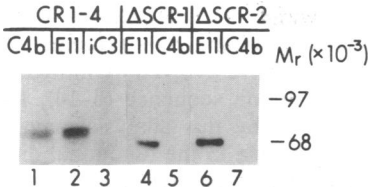

FIG. 3. CR1-4 protein binds C4b; this interaction requires SCR-1 and SCR-2. Analysis of culture medium of COS cells, transfected with CR1-4 (lanes 1–3) or its deletion mutants, ΔSCR-1 (lanes 4–5) and ΔSCR-2 (lanes 6–7), by immunoprecipitation with E11 (lanes 2, 4, and 6) and by affinity chromatography using C4b-S (lanes 1, 5, and 7) or iC3-S (lane 3).

not by iC3 or BSA (not shown). Further, mAb 3D9, which blocks C4b- and C3b-mediated rosette formation (20, 27), abrogated binding to C4b-S (not shown). Thus, CR1-4 specifically bound C4b but not iC3.

Two CR1-4 deletion mutants, ΔSCR-1 and ΔSCR-2, lacking SCR-1 and SCR-2, respectively, were constructed. Both mutant proteins were recognized by E11 (Fig. 3, lanes 4 and 6) and 3D9 (not shown) and, as anticipated, migrated slightly faster than CR1-4 on SDS/PAGE. Neither protein bound to C4b-S (Fig. 3, lanes 5 and 7), indicating that SCR-1 and SCR-2 are necessary for C4b binding.

The next set of experiments were aimed at identifying regions within a SCR important for C4b and iC3 binding. The strategy is illustrated in Fig. 2 and outlined below. In CR1, there is 55% and 70% homology between SCR-1 and SCR-8 and between SCR-2 and SCR-9, respectively. However, SCR-1 and SCR-2 are essential for C4b binding (ref. 8 and preceding results), whereas SCR-8 and SCR-9 are necessary for C3b binding (8). Therefore, specificity of ligand binding must be determined by *differences* between amino acids in the corresponding positions of SCR-1 and SCR-8 and those of SCR-2 and SCR-9. Thus, mutants were produced in which amino acids in SCR-1 were substituted a few at a time with the corresponding amino acids of SCR-8 and, similarly, those in SCR-2 were changed to their counterparts in SCR-9. Such alterations were introduced in the second half of SCR-1 and throughout the entire length of SCR-2, regions suggested by previous work (8) to encompass major determinants of binding specificity.

The mutant proteins were tested for binding to C4b-S. Most of them were indistinguishable from the parent (not shown). However, two mutant proteins, no. 1 with two amino acid substitutions in SCR-1 and no. 8 with two substitutions in SCR-2, did not bind to C4b-S (Fig. 4*A*, lanes 2 and 6). Next, mutants with one of the two possible amino acid substitutions were tested. Mutations 1a and 8b eliminated binding to C4b-S (Fig. 4*A*), whereas mutations 1b and 8a were without effect (not shown).

A third mutant, no. 5, with two amino acid alterations, reproducibly demonstrated 60–80% reduction of binding compared to that of CR1-4 (Fig. 4*B*). Mutants 5a and 5b, each with one amino acid substitution, both showed decreased binding to C4b-S in comparison to CR1-4. To more precisely quantitate the binding, after incubation with C4b-S, residual protein was measured using a capture ELISA. In the case of CR1-4, 16–25% of the protein remained unbound in three experiments, whereas in the case of mutant 5, 59–79% did not bind; the unbound fraction for mutants 5a and 5b was 42–57% and 54–66%, respectively.

The mutant proteins were also examined for binding to iC3-S. All but one of them, like the parent CR1-4, did not bind (not shown). However, mutant 11, with five amino acids at the end of SCR-2 substituted with their counterparts from SCR-9 (Fig. 2), bound to iC3-S as well as to C4b-S (Fig. 4*C*, lanes 1 and 3). Mutants with a single amino acid substitution

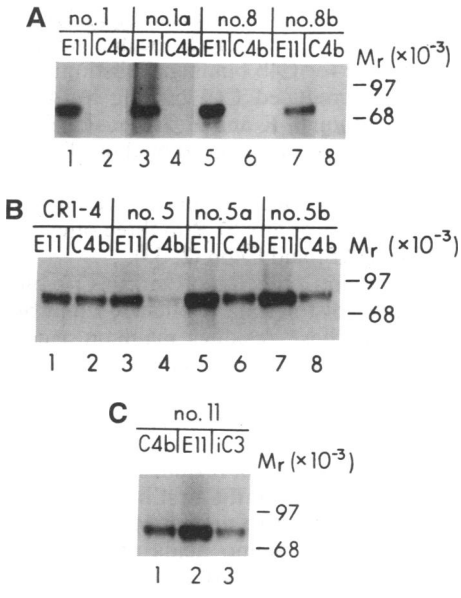

FIG. 4. Analysis of culture medium of COS cells transfected with amino acid substitution mutants of CR1-4. (*A*) Mutants 1, 1a, 8, and 8b do not bind to C4b-S. (*B*) Mutants 5, 5a, and 5b have reduced C4b-S binding capacity. (*C*) Mutant 11 binds to iC3-S as well as to C4b-S.

within group 11 or with substitutions of KP for ET did not bind to iC3-S. If SCR-1 was deleted from mutant 11, iC3 binding was abolished (not shown).

DISCUSSION

The previously described cDNA clone CR1-4 (9) encompassing the first eight and one-half amino-terminal SCRs was predicted to code for a truncated, secreted protein because it lacks sequences coding for a transmembrane domain and cytoplasmic tail. In the present study, COS cells transfected with CR1-4 cDNA secreted a protein of M_r 78,000 that was immunoprecipitated by two mAbs that recognize distinct epitopes of CR1. CR1-4 efficiently bound C4b but not iC3, consistent with data of Klickstein *et al.* (8), who localized a C4b binding site to SCR-1 and/or SCR-2.

Utilizing the transfected CR1-4 protein as a model system, we have determined that *both* SCR-1 and SCR-2 are essential for C4b binding because derivatives lacking SCR-1 or SCR-2 did not bind C4b. These results are similar to the requirement for two SCRs for C3d binding in CR2 (28) and for C3b binding in CR1 (see below).

To identify amino acids critical for C4b binding in SCR-1 and SCR-2 and for iC3 binding to SCR-8 and SCR-9, mutagenesis analysis of CR1-4 was performed such that amino acids in SCR-1 and SCR-2 were substituted by their counterparts in SCR-8 and SCR-9. This strategy is based on the fact that although there is 55% homology between SCR-1 and SCR-8 and 70% homology between SCR-2 and SCR-9, their binding specificity is distinct and therefore must be determined by amino acids that are *different* in the homologous positions in the corresponding SCRs.

Substitution of a single amino acid in SCR-1, G, or in SCR-2, Y (mutants 1a and 8b of Fig. 2), abrogated C4b binding. These amino acids, located three or four residues after the second cysteine in the respective SCR, are conserved as a GY (or a GF) pair in many SCRs (2), including some SCRs in three other C4b binding proteins, MCP, DAF, and C4bp. Their role may be to maintain conformation necessary for C4b binding. If so, it is interesting that iC3 binding occurs regardless of whether these pairs are disrupted (in SCR-8 and SCR-9) or retained (in

mutant 11). Furthermore, we have identified two neighboring amino acids, R and N, in SCR-2, located next to the first cysteine, important for C4b binding. Substitution of *each* of these amino acids reduced C4b binding by 40–65%. It is surprising that, although R and K are positively charged, a change of R to K markedly reduced binding. The amino acids at these positions are highly variable in the general SCR population (2). In particular, R and N are not found in the homologous positions of any SCR in three other C4b binding proteins, MCP, DAF, and C4bp (2, 33).

In summary, amino acids important for C4b binding were identified in three distinct areas of SCR-1 and SCR-2. One possibility is that these residues maintain a conformation necessary for ligand binding and that their change leads to the distortion of the secondary structure, thereby indirectly effecting binding. A second possibility is that, among the identified residues, there are amino acids that could interact directly with the ligand. Also, residues that play a role in C4b binding could be present in the first half of SCR-1, a region that has not been subject to mutagenesis analysis.

Substitution of five amino acids at the end of SCR-2 DNETPICD with the residues of SCR-9 STKPPICQ resulted in the acquisition of iC3 binding *without alteration of C4b binding*. Therefore, it is possible that these amino acids at the end of SCR-9 are a part of the C3b binding domain in native CR1. This result is also consistent with the lack of iC3 binding by CR1-4 (missing the second half of SCR-9). Among the five amino acid substitutions in mutant 11 are such potentially important alterations as changes of negatively charged amino acids to uncharged (D to S and D to Q) or to positively charged (E to K) residues and loss of a potential glycosylation site, NET. Apparently, more than one of them are essential, since substitution of any single amino acid (or KP for ET) does not result in detectable iC3 binding. Of note, the averaged SCR secondary structure prediction (2) using the method of Garnier (29) for these first four substituted positions is an aperiodic "coil" conformation that is surrounded on either side by turns. This is quite different from the structure predicted for most of an SCR, which is dominated by β strands interspersed by turns.

The C3b binding site in CR1 may be different from the sites in other related proteins, factor H and factor B, since a similar sequence is not found in the homologous positions in their SCRs (2). This is of interest because it has been suggested that these proteins bind to the same region of C3 (30–32). On the other hand, there is a sequence at the end of SCR-2 in MCP (SGKPPICD) that resembles the sequence of SCR-9 in CR1 (STKPPICQ) and this repeat is involved in C3b binding and cofactor activity (E. Adams and J.P.A., unpublished data).

Since mutant 11 bound iC3 only in the presence of SCR-1, for iC3 binding as for C4b binding, *two* SCRs are required. The implication of this observation is that, in mutant 11, SCR-1 compensates at least partially for the function of SCR-8 in CR1. Further studies are necessary to establish if this is a full functional compensation and consequently may lead to the identification of other amino acids in SCR-1 and/or SCR-8 important for iC3 binding. Similarly, it remains to be seen if in SCR-9 other amino acids, in addition to the site already identified, play a role in iC3 binding.

Although C4b and iC3 binding sites share requirements for two SCRs, there are some significant differences. Not only are distinct sets of two SCRs necessary for C3b vs. C4b binding (ref. 8; this report) but amino acids important for iC3 and C4b binding are also located in *different* positions in their respective SCRs. Further, it is sufficient to change five amino acids in SCR-2 into their counterparts from SCR-9 to confer iC3 binding ability on SCR-1 and SCR-2 and yet not alter C4b binding. These data argue strongly for the evolution of two distinct binding sites, one for C4b and one for C3b, within CR1 and likely within the CR1-related regulators of the complement activation family of proteins. Since the two binding sites of CR1 have evolved by intragenic duplication from a common peptide sequence (8–10), the amino acid differences identified by this analysis may have played a critical evolutionary role in the functional differentiation of the C3b/iC3 and C4b binding sites of contemporary CR1 from a common ancestral sequence.

This analysis provides identification of iC3 and C4b binding sites within SCRs. These results should prove valuable in the assessment of ligand binding sites of other SCR-containing proteins and in their three-dimensional analysis. The strategy utilized is especially applicable to other complement proteins bearing SCRs, many of which, like CR1, bind C3b and/or C4b.

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