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The Central Segment of Herpes Simplex Virus Type 1 Glycoprotein C (gC) Is Not Involved in C3b Binding: Demonstration by Using Monoclonal Antibodies and Recombinant gC Expressed in *Escherichia coli*

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SUMMARY

Three monoclonal antibodies (MAbs) have been raised against cell membrane-derived herpes simplex virus type 1 glycoprotein C (gC-1). By using different DNA constructs of gC-1 expressed in *Escherichia coli* the sites recognized by these antibodies could be assigned to a peptide in the more hydrophobic and probably non-glycosylated middle third of the gC-1 molecule. This peptide segment corresponds to a 571 bp segment on the gC-1 gene located between the *Nco*I and the *Nru*I restriction sites. None of the three MAbs interfered with the binding of the human serum complement component C3b to gC, which has been shown to be rather glycosylation-dependent. Since the data of other groups have suggested that antibodies directed against all regions of gC-1 inhibit C3b binding to gC-1, whereas our results suggest that the central part of gC-1 is not actually involved in C3b-binding activity, it can be inferred that the N- and C-terminal segments are involved in C3b binding by gC-1.

Herpes simplex virus type 1 (HSV-1) glycoprotein C (gC-1) has been shown to act as a receptor for component C3b of the complement system (Friedman *et al.*, 1984). gC-1 shows binding specificities and functional properties very similar to known human complement receptors (Kubota *et al.*, 1987; Fries *et al.*, 1986). Purified gC-1 possesses decay accelerating activity for the alternative pathway C3 convertase similar to that of the other known regulators of the C3 convertase (Fries *et al.*, 1986). Although cells infected with HSV type 2 (HSV-2) do not have the ability to form rosettes with C3b-coated erythrocytes, it was recently shown that gC-2 binds to Sepharose-bound iC3 and provides protection against complement-mediated cell lysis (McNearney *et al.*, 1987).

The glycoprotein C gene of HSV-1 has been mapped in the *Hin*dIII L fragment of the viral genome and the DNA has been sequenced (Frink *et al.*, 1983; Draper *et al.*, 1984). From these data, it has been deduced that gC-1 has an open reading frame of 511 amino acids, with a hydrophobic N-terminal putative signal sequence and a hydrophobic membrane-anchoring sequence, and exhibits nine potential *N*-linked glycosylation sites. The M_r of gC-1 measured by SDS–PAGE is approximately 130K, whereas the M_r calculated from the amino acid sequence is only about 55K. This is evidence that gC-1 is post-translationally modified, a mechanism which has been demonstrated for attachment of *N*-linked oligosaccharides (Campadelli-Fiume *et al.*, 1982; Wenske *et al.*, 1982), *O*-linked oligosaccharides (Johnson & Spear, 1983; Olofsson *et al.*, 1983) and sulphation (Hope *et al.*, 1982). The heavy glycosylation has been shown to influence the immunoreactivity with monoclonal antibodies (MAbs) (Sjöblom *et al.*, 1987) and seems to be crucial for C3b receptor activity (Smiley *et al.*, 1985; Smiley & Friedman, 1985).

By using MAbs specific to gC-1, two major antigenic sites designated I and II have been identified (Marlin *et al.*, 1985). Antigenic site II, which is composed of three subsites, is

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probably located in the amino-terminal half of gC-1 (Holland *et al.*, 1984; Marlin *et al.*, 1985), which also contains eight of the nine potential *N*-glycosylation sites. Antigenic site I is probably located between amino acid residues 297 and 359 in the carboxy-terminal half (Homa *et al.*, 1986). All antibodies directed against these different sites, tested so far, have been shown to block C3b binding (Friedman *et al.*, 1986).

The MAbs tested in our study were raised in BALB/c mice according to standard methods (Fazekas de St. Groth & Scheidegger, 1980). BALB/c mice were immunized with gC-1 preparations obtained from infected BHK (baby hamster kidney) cells by NP40 extraction [0·5 h on ice in 1% NP40 in phosphate buffered saline (PBS), 1 mM-PMSF]. NP40 extracts were further purified on a preparative SDS–polyacrylamide gel and the 130K fraction containing gC-1 was used for immunization. Hybridoma cells were screened for antibody production by the ELISA method with NP40 extracts of HSV-1-infected (strain Wal) Vero cells as the antigen and also by immunofluorescence with methanol-fixed Vero cells infected with either HSV-1 or HSV-2. The same antigen preparation as that used for the ELISA was used for immunizing a rabbit to obtain a polyvalent antiserum to gC-1. The gC-1-specific MAb B1C1 was kindly supplied by Dr S. Olofsson (Olofsson *et al.*, 1983). HSV-1 strain Wal (Schröder *et al.*, 1981) and HSV-2 strain D 316 (Görtz *et al.*, 1984) have been described. BHK cells were grown in GMEM supplemented with 10% tryptose phosphate broth and 10% foetal calf serum (FCS) and were infected at a high multiplicity (m.o.i. > 10 p.f.u./cell). Infected cells were harvested after 2 days. The plaque assay and immunofluorescence were done with Vero cells (African green monkey) and grown in M199 supplemented with 5% FCS. Cells and media were purchased from Flow Laboratories.

Three stable hybridoma cell lines producing MAbs specific to gC-1, termed HC1, HC2 and HC3, were obtained. All of the MAbs recognized gC-1, seen as a band of 130K on Western blots using NP40 extracts for the antigen as described above. In immunofluorescence studies [done as described earlier by Petzer *et al.* (1988)] they recognized only HSV-1-infected Vero cells which could be used fixed or unfixed with ice-cold acetone or methanol, respectively. HSV-2-infected Vero cells were not recognized. All antibodies are of the IgG subtype 2b and are directed against different epitopes because they did not inhibit each other in ELISA studies [as described primarily by Petzer *et al.* (1988)].

In order to localize the peptide structures within the gC-1 molecule that are recognized by the MAbs, we have expressed defined polypeptide segments of the envelope protein in *Escherichia coli*. It has been observed that gC-1 peptides are unstable in *E. coli* and sometimes have toxic effects on the microbial cell, when expressed as nearly unfused polypeptides by vectors using the strong *lac* or *tac* promoters (Amann *et al.*, 1984*a*, *b*). Therefore, the gC-1 stretches were expressed as hybrid proteins either fused to the amino-terminal sites of β-galactosidase to create a tripartite hybrid protein with the form cI::gC-1::β-galactosidase, or flanking the carboxy-terminus of β-galactosidase to produce β-galactosidase::gC-1 fusions. The vector used to express the tripartite fusion proteins was pMF2 (Amann *et al.*, 1984*a*), which has the facility for the insertion of DNA fragments into the unique *Hin*dIII and *Bam*HI sites. The translational reading frames of the inserted gC-1-encoding DNA fragments were in alignment with cI and *lacZ* coding sequences and therefore yielded enzymically active β-galactosidase proteins. The vectors constructed in this way are represented by the pBO series (Fig. 1). As it is not possible to express the carboxy-terminal region of gC-1 including the translational stop codon in the form of a tripartite fusion protein, we also made fusions to the carboxy-terminus of β-galactosidase by using the pUR vector series (Rüther & Müller-Hill, 1983). These vectors facilitate the in-frame ligation of DNA fragments into the polylinker downstream of the *lacZ* coding sequences. The expression vectors directing the synthesis of β-galactosidase::gC-1 fusion proteins are represented by the pBD series (Fig. 2). Because the presentation of the expressed gC-1 antigenic sites may be influenced by their location within the fusion protein, we made amino-terminal as well as carboxy-terminal fusions of gC-1 segments to β-galactosidase.

The construction of the vectors pBO1, pBO2, pBO3 and pBO4 has been described in detail by Amann *et al.* (1984*a*). pBO5 was made as follows. pMB78 (Amann *et al.*, 1984*a*) was linearized with *Eco*RI, treated with DNA polymerase I Klenow fragment in the presence of dNTPs; the *Bam*HI DNA linker, 5′ CGGGATCCCG 3′, was added and the DNA was digested

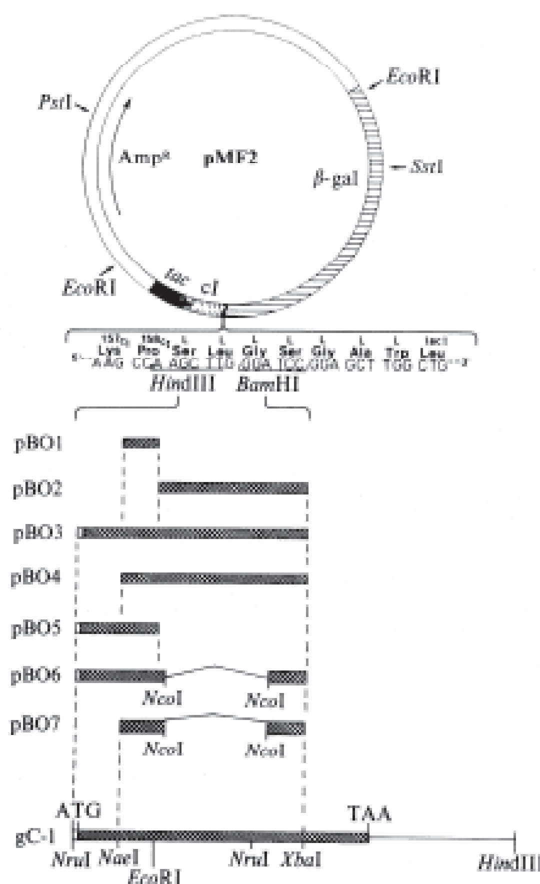

Fig. 1 Structure of the pBO vector series. The basis vector of the pBO series is pMF2 (Amann *et al.*, 1984*a*). Shaded bars denote gC-1 coding DNA sequences; open segment represents a non-translated 5′ region of gC-1 mRNA; thin line, HSV-1 DNA; Amp^R, ampicillin resistance gene; β-gal, β-galactosidase encoding region; black arrow, *tac* promoter; ^157^cI, amino acid 157 of cI; L, linker-coded amino acid; lacI, first amino acid (Leu) of lacI portion of the fusion protein (some amino acids of lacI have been fused to the enzymically active β-galactosidase protein in pLG400, the ancestor of pMF2) (Guarente *et al.*, 1980). The *Nru*I site marked within the coding region of gC-1 represents two *Nru*I sites, which are separated by only 13 bp. The thin connecting lines in pBO6 and pBO7 represent deletions of the DNA segment between the two *Nco*I sites.

with *Bam*HI and *Hin*dIII; the gC-1-encoding DNA fragment was isolated and ligated into pMF2 (Amann *et al.*, 1984*a*), which resulted in pBO5. The vector pBO6 is a derivative of pBO3 with an internal deletion of a *Nco*I fragment in the coding region of gC-1. Plasmid pBO3 was digested with *Nco*I, the 4200 bp and the 3400 bp DNA fragments were isolated and ligated to create pBO6. Plasmid pBO7 was made similarly, starting with pBO4 as the parental plasmid.

To construct pBD11, the 200 bp *Bam*HI DNA fragment from pBO1 was isolated and blunt-ended with the polymerase I Klenow fragment in the presence of dNTPs; then the *Hin*dIII DNA linker 5′CAAGCTTG 3′ was added, the DNA cut with *Hin*dIII and ligated into pUR278 (Rüther & Müller-Hill, 1983) linearized with *Hin*dIII. The vector pBD14 was made in the same manner, but in this case the origin of the gC-1-encoding DNA was the *Bam*HI fragment isolated from pBO2. Vector pBD18 was created by ligating the 1200 bp *Hin*dIII–*Xba*I DNA fragment from pMB59 (Amann *et al.*, 1984*a*) into pUR278 cut with *Xba*I and *Hin*dIII. The plasmid pBD19 was made by the simultaneous ligation of the 1200 bp *Hin*dIII–*Xba*I DNA fragment from pMB59 and the 1400 bp *Xba*I–*Hin*dIII from pMB89 (Amann *et al.*, 1984*a*) into pUR278, linearized with *Hin*dIII. To create pBD20, the 1600 bp *Nru*I–*Hin*dIII DNA fragment from pMB59 was isolated and ligated into pUR289 (Rüther & Müller-Hill, 1983), which had been cut with *Bam*HI. This was followed by incubation with the polymerase Klenow fragment to fill in the 5′ overhang and digestion with *Hin*dIII. The construction of all the plasmids was verified by restriction enzyme analyses. To prove that the expression vectors direct the synthesis of the expected fusion proteins, we analysed the bacterial lysates by immunoprecipitation of the

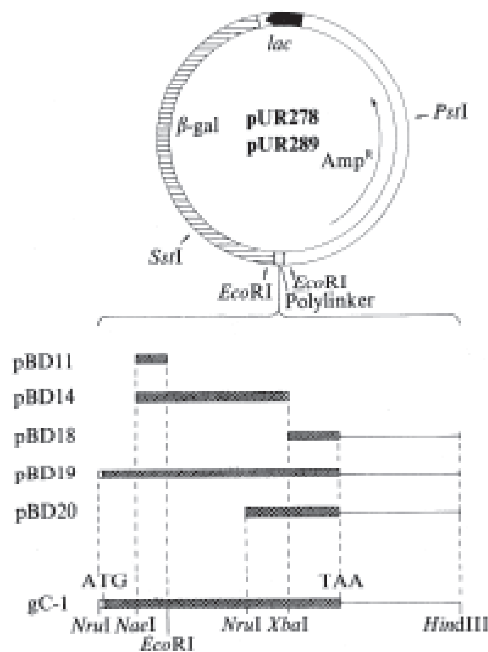

Fig. 2. Structure of the pBD vector series. The basis vectors of the pBD series are pUR278 and pUR289, which differ from the DNA sequence within the polylinker region (Rüther & Müller-Hill, 1983). Symbols as in Fig. 1. Black arrow; *lac* promoter; stippled area: polylinker downstream of the β-galactosidase-encoding sequence.

hybrid proteins with anti-HSV-1 goat antiserum and by staining SDS–polyacrylamide gels with Coomassie Brilliant Blue as described (Amann *et al.*, 1984*a*). The restriction map and the DNA sequences of the gC-1 gene (Fig. 1 and Fig. 2) are based on the data of HSV-1 strain F, the published DNA sequence of strain KOS (Frink *et al.*, 1983) and the corrected version presented by Draper *et al.* (1984).

The isolation of plasmid DNA, preparation of DNA fragments, DNA synthesis reactions, DNA ligations, transformation of *E. coli* and screening of plasmid-containing colonies were carried out as described by Maniatis *et al.* (1982). The DNA encoding the gene for gC was isolated from HSV strain F (Amann *et al.*, 1984*a*). For cloning and expression procedures, *E. coli* BMH 71-18 (Koenen *et al.*, 1982) was used. Restriction enzymes, alkaline phosphatase, the polymerase I Klenow fragment and ligase were purchased from New England Biolabs and from Boehringer Mannheim, and were used according to the manufacturers' instructions. *Hin*dIII and *Bam*HI DNA linkers were obtained from New England Biolabs.

Immunoblotting experiments of the fusion proteins produced from the described recombinant constructs are shown in Fig. 3 and Fig. 4. The MAb HC1 recognized the fusion proteins encoded by the expression vectors pBO3, pBO4, pBD14 and pBD19. The hybrid proteins encoded by pBO1, pBO5, pBO6, pBO7 and pBD11, pBD18 and pBD20 did not react with HC1. The two other MAbs, HC2 and HC3, showed the same recognition pattern (data not shown). The fusion protein encoded by pBO2 is extremely unstable and degrades very rapidly (Amann *et al.*, 1984*a*). This can be seen on the blots developed with the polyclonal rabbit serum raised against the gC antigen preparation mentioned above. All gC-containing fusion proteins were recognized by the polyvalent rabbit serum except the fusion proteins encoded by the vectors pBO1 and pBD11. These fusion proteins probably contain only a few antigenic epitopes as they are short, and in addition they may be less immunogenic than epitopes located in other regions of the gC molecule as the antigenicity may be influenced by the carbohydrate chains.

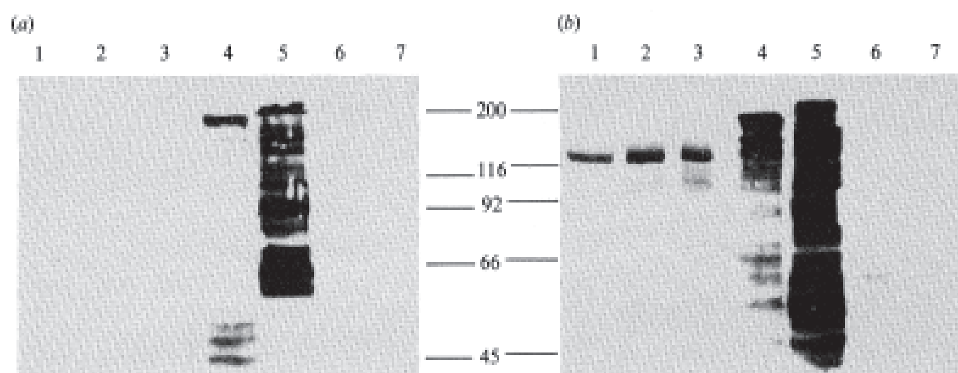

Fig. 3. Immunoblot of fusion proteins produced by the pBO vector series: pBO7 (lane 1); pBO6 (lane 2); pBO5 (lane 3); pBO4 (lane 4); pBO3 (lane 5); pBO2 (lane 6); pBO1 (lane 7). Blot (*a*) was developed with monoclonal antibody HCl, (*b*) with polyvalent rabbit serum to gC.

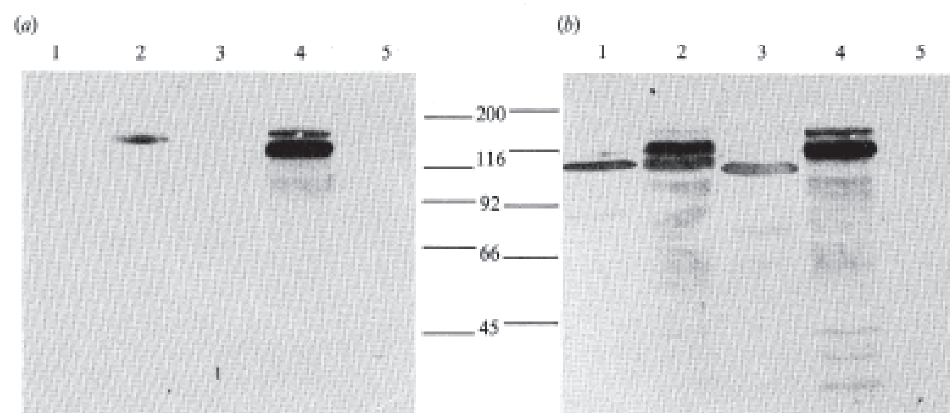

Fig. 4. Immunoblot of fusion proteins produced by the pBD vector series: pBD20 (lane 1); pBD19 (lane 2); pBD18 (lane 3); pBD14 (lane 4); pBD11 (lane 5). The same antibodies as those used in Fig. 3 were used.

From the recognition pattern of the MAbs with the various recombinant gC polypeptides in the Western blot analyses, we conclude that their binding domains are located within the peptide stretch between amino acids 163 and 352. This sequence of 189 amino acids is encoded by a 571 bp DNA fragment cleaved by *Nco*I and *Nru*I at nucleotide positions 630 and 1201, respectively, within the gC gene, referring to the numbering of Frink *et al.* (1983).

In looking for the functional importance of this part of gC-1 we were interested to see whether our MAbs would be able to inhibit the binding of C3b to gC-1 in a rosette inhibition assay using complement-coated erythrocytes and HSV-1-infected Vero cells. C3b-coated erythrocytes were prepared as described (Schmitt *et al.*, 1981). Briefly, sheep erythrocytes (E) were sensitized with subagglutinating rabbit anti-sheep erythrocyte serum (Amboceptor, Behring). The resultant amboceptor-coated erythrocytes (EA) were then incubated in a stepwise manner with purified complement components C1, C4, C2 and finally C3 (as described by Hammer *et al.*, 1981). The final preparation consisting of EAC1423b was stored on ice for up to 3 weeks. Complement-coated erythrocytes lacking C3b (i.e. EAC14 cells) served as a control.

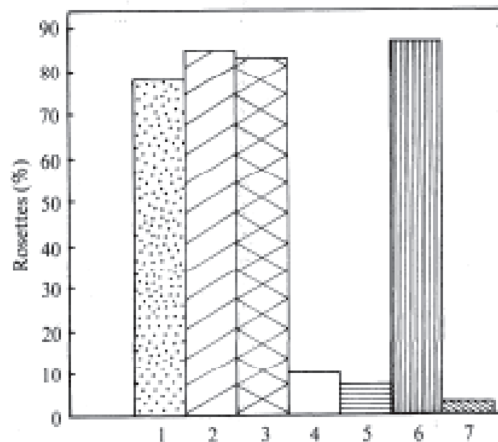

Fig. 5. Rosette inhibition assay. HSV-1-infected cells were preincubated with antibodies: column 1, monoclonal antibody HC1; column 2, HC2; column 3, HC3; column 4, monoclonal antibody B1C1 (Olofsson *et al.*, 1983). Column 5 represents polyvalent antiserum to C3c and column 6 no antibody. In column 7, EAC14 was used instead of EAC1423b.

Rosette inhibition assays were performed in 1 ml Eppendorf tubes. Vero cells (1×10^5), which had been infected with HSV-1 strain Wal 48 h previously were mixed with 1×10^7 C3b-coated erythrocytes and incubated for 30 min at 37 °C. At least three erythrocytes adherent to a Vero cell were required for the identification of a rosette. In order to block the C3b binding, Protein A-purified MAbs against gC as well as ascites fluid (diluted 1:100 in PBS) were preincubated with the infected cells for 30 min before adding the EAC3bs.

The addition of the MAbs HC1, HC2 and HC3 did not reduce the percentage of rosettes formed by HSV-1-infected Vero cells and C3-coated erythrocytes. However, in comparison, MAb B1C1 (Olofsson *et al.*, 1983) markedly reduced rosette formation up to a dilution of 1:1000. Polyvalent antiserum against the C3 molecule (anti-C3c rabbit serum, Dako) was used as a positive control and also led to an unequivocal reduction of the number of rosettes. In contrast, cells incubated with sheep erythrocytes lacking the complement component C3b (EAC14) did not show any rosette formation (see Fig. 5). Also, Vero cells infected with HSV-2 instead of HSV-1 did not rosette with EAC3b (not shown).

In summary, we can say that our MAbs which specifically react with HSV-1 in ELISA, immunofluorescence assays and also with denatured gC-1 as the antigen in immunoblotting procedures do not interfere with the functional activity of gC. We made use of defined polypeptide segments of gC-1 expressed in *E. coli* to study the binding of these three MAbs. Recombinant gC antigens, in contrast to cell-derived gC, are not post-translationally modified, either by *N*- or by *O*-glycosylation, sialylation or sulphation. So far, only antibodies directed against the peptide backbone of gC have been reported, but MAbs have been identified whose binding sites are influenced by carbohydrate chains (Sjöblom *et al.*, 1987). The region to which the antibodies HC1, HC2 and HC3 bind could be mapped in a peptide sequence of gC-1 encoded by a DNA fragment of approximately 571 bp located between the left *Nco*I site and the *Nru*I site. This means that the epitopes recognized by the three antibodies are located in the middle third of the gC-1 molecule. This part is probably non-glycosylated (Dowbenko & Lasky, 1984) and should represent continuous epitopes rather than conformational or non-sequential antigenic sites, because the MAbs recognize denatured structures. As glycosylation-dependent epitopes of gC-1 have been shown to be important for C3b binding, it was not surprising that none of the three antibodies interfered with the C3b binding. More evidence for the importance of carbohydrate-dependent epitopes in the interaction of gC-1 with complement is provided by the finding that purified fusion proteins of the pBD series did not bind to purified C3 in ELISA studies and that the MAb B1C1 (Olofsson *et al.*, 1983), which blocked the C3b binding

efficiently, did not recognize the recombinant non-glycosylated gC-1 polypeptides (not shown). In addition, MAbs to gC-1 that have been shown to be directed against the heavily glycosylated amino-terminal third of gC-1 (Marlin *et al.*, 1985) and against a site closer to the carboxy terminus between amino acids 297 and 359 (Homa *et al.*, 1986), did not recognize the *E. coli*-expressed gC molecules (M. Bröker, unpublished results). It may be assumed that the binding site for complement component C3b is more likely to be a conformational site represented by the amino-terminal and/or carboxy-terminal parts of gC-1 than a single epitope which can be reduced to an amino acid sequence.

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