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Autonomous Tetramerization Domains in the Glycan-binding Receptors DC-SIGN and DC-SIGNR

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Multivalent binding of glycans on pathogens and on mammalian cells by the receptors DC-SIGN (CD209) and DC-SIGNR (L-SIGN, CD299) is dependent on correct disposition of the C-type carbohydrate-recognition domains projected at the C-terminal ends of necks at the cell surface. In the work reported here, neck domains of DC-SIGN and DC-SIGNR expressed in isolation are shown to form tetramers in the absence of the CRDs. Stability analysis indicates that interactions between the neck domains account fully for the stability of the tetrameric extracellular portions of the receptors. The neck domains are approximately 40% α-helical based on circular dichroism analysis. However, in contrast to other glycan-binding receptors in which fully helical neck regions are intimately associated with C-terminal C-type CRDs, the neck domains in DC-SIGN and DC-SIGNR act as autonomous tetramerization domains and the neck domains and CRDs are organized independently. Neck domains from polymorphic forms of DC-SIGNR that lack some of the repeat sequences show modestly reduced stability, but differences near the C-terminal end of the neck domains lead to significantly enhanced stability of DC-SIGNR tetramers compared to DC-SIGN.

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Keywords: DC-SIGN; glycan binding; receptor; oligomerization; polymorphisms

The dendritic cell receptor DC-SIGN (CD209) and the closely related sinusoidal endothelial cell receptor DC-SIGNR or L-SIGN (CD209L or CD299) bind to glycans on the surfaces of viruses, parasites and other microbial pathogens.[1-3] Under various circumstances that are not well understood, such interactions lead to pathogen neutralization or to enhancement of infection.[4] DC-SIGN also appears to have a role in adhesive interactions between dendritic cells and other cells of the immune system.[5,6]

Like many glycan-binding receptors in the C-type lectin family, DC-SIGN and DC-SIGNR are oligomeric type II transmembrane proteins.[7] The necks that lie between the membrane and the C-terminal carbohydrate-recognition domains (CRDs) in these proteins are believed to stabilize the receptor oligomers, to project the CRDs away from the cell surface, and to position the CRDs for appropriate multivalent interaction with glycan ligands. Sequence analysis and indirect structural information indicates that some of the necks in this family of receptors consist of extended coiled coils of α-helices that lead to formation of dimeric or trimeric receptors. Langerin,[8] LSECtin,[9] the Kupffer cell receptor,[10] and the asialoglycoprotein receptor[11] all appear to follow this general structural model.

The neck domains in the predominant forms of DC-SIGN and DC-SIGNR comprise 7.5 repeats of largely conserved 23 amino acid segments. The extracellular portion of each of these receptors forms stable tetramers while the isolated CRDs are monomeric, indicating that the neck domains are required for oligomerization.[7] However, several properties of DC-SIGN and DC-SIGNR suggest that they do not follow the simple helical stalk model. These receptors form tetramers rather than dimers or trimers, and the neck sequences are not consistent with formation of continuous coiled coils of α helices, because although roughly two-thirds of each repeat shows evidence of a heptad pattern of

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Abbreviation used: CRD, carbohydrate-recognition domain.

hydrophobic amino acids typical of coiled coils of helices, the remaining portions of the repeat sequences contain proline residues and lack the heptad motif (Fig. 1a). These properties prompted an investigation of the role of the neck domains in organization of DC-SIGN and DC-SIGNR tetramers.

In order to examine the ability of the neck domain of DC-SIGN to form oligomers in the absence of the CRDs, the extracellular portion of DC-SIGN was truncated two amino acids before the first cysteine residue of the globular CRD and a histidine purification tag was appended. Following expression in *Escherichia coli*, incubation overnight with 10 mM EDTA was required to release His_6-tagged protein from a nickel affinity column, so a shorter His_2 tag was substituted. This version of the protein was still efficiently retained on the nickel affinity column but could be eluted with 100 mM imidazole (band indicated by arrow in Supplementary Data Fig. 1). The efficiency of binding to the nickel affinity column suggested that the isolated neck domain was able to form stable oligomers and thus increase the clustering of histidine residues for binding to the column.

The oligomeric state of the neck domain was established by hydrodynamic analysis following further purification by ion-exchange chromatography (Fig. 1b). Sedimentation equilibrium experiments provided direct evidence that the neck domain is a tetramer with a molecular mass of 88,970 Da, compared to the predicted value of 88,850 Da (Fig. 1c). Sedimentation velocity analysis

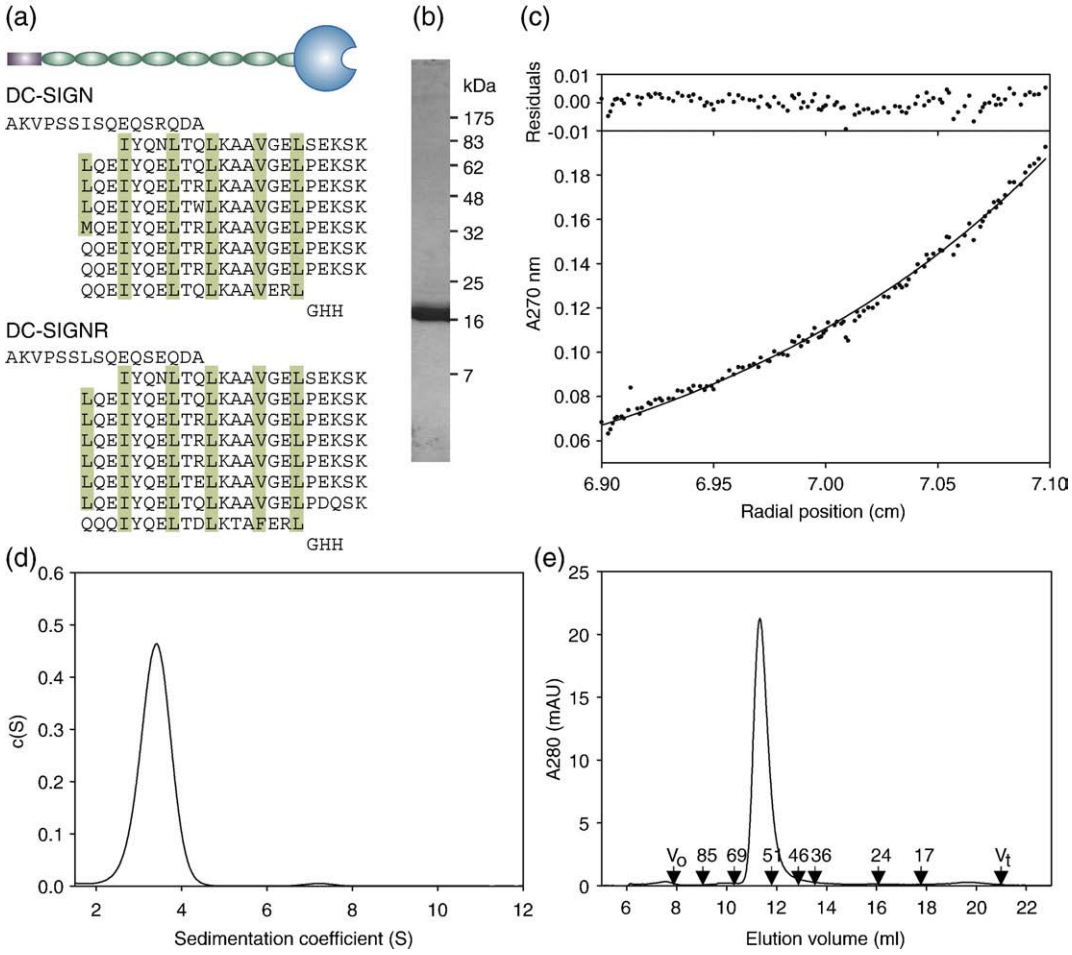

Fig. 1. Hydrodynamic analysis of the purified neck domain of DC-SIGN. (a) A summary of the structures of the extracellular portions of DC-SIGN and DC-SIGNR, including sequences of the neck domains. (b) SDS-PAGE (17.5% acrylamide gel) of the final purified DC-SIGN neck domain after anion-exchange chromatography. (c) Sedimentation equilibrium analysis performed in six-sector cells in an An60Ti rotor of a Beckman XL-A ultracentrifuge (Department of Biochemistry, University of Oxford). Data were analyzed using UltraSpin software developed by Dmitry Veprintsev (ultraspin.mrc-cpe.cam.ac.uk). A scan of a sample at 0.25 mg/ml after 24 h at 9000 rpm is shown. (d) Sedimentation velocity analysis showing distribution of estimated sedimentation coefficients for a sample at 0.5 mg/ml centrifuged at 30,000 rpm in two-sector cells. Data collected at 238 nm were analyzed using Sedfit software.[12] (e) Gel-filtration analysis performed on a Superdex 200 column (1 cm × 30 cm; GE Healthcare) in 100 mM NaCl, 10 mM Tris–HCl, pH 7.8, 2.5 mM EDTA at a flow rate of 0.5 ml/min. The positions of marker proteins are indicated by the Stokes radius: cytochrome *c*, 17 Å; bovine erythrocyte carbonic anhydrase, 23.9 Å; bovine serum albumin, 35.5 Å; yeast alcohol dehydrogenase, 45.5 Å; β-amylase, 51 Å; *E. coli* β-galactosidase, 69 Å; and thyroglobulin, 85 Å.

and gel filtration were used to confirm that the protein is a homogeneous, stable oligomer (Fig. 1d and e). Insertion of the deduced values of 3.4 S for the sedimentation coefficient and 3.8×10^{-7} cm^2/s for the diffusion coefficient into the Svedberg equation provided an independent estimate of 87,000 Da for the molecular mass. The low sedimentation and diffusion coefficients relative to those expected for a globular protein of this molecular mass suggest an elongated protein structure, which was modeled using a bead model in Hydro 8c.[13] A cylindrical structure of diameter 25 Å, corresponding to the approximate diameter of a four-stranded helical bundle[14] and length 350 Å gave predicted sedimentation and diffusion coefficients of 3.5 S and 3.9×10^{-7} cm^2/s, closely matching the measured values. These results demonstrate that the neck domain forms an extended structure.

The neck length value derived from the modeling exercise is considerably more than the length expected from a fully helical polypeptide of 195 residues, which would be approximately 300 Å. This result, combined with the presence of a heptad repeat sequence, suggested that the neck domain is extended and probably contains extensive α-helical structure. The circular dichroism spectrum of the neck domain, with minima at 208 nm and 222 nm, confirmed the presence of helical structure (Fig. 2a). However, the mean residue ellipticity value of 17,000 deg-cm^2/dmol at 222 nm is substantially less than the value of 39,500 deg-cm^2/dmol predicted for a fully helical polypeptide.[15] Fitting the spectrum with several different deconvolution programs[16] and with multiple different basis sets indicated consistently that the neck is approximately 40% helical.

Initial measurements of the stability of the neck domain were made by monitoring circular dichroism at 222 nm during heating (Fig. 2b). Fitting the resulting curve indicated that the midpoint of the denaturation curve occurs at 53.9 °C. Differential scanning calorimetry was used to obtain complementary information about the behavior of the isolated domains and the domains in the context of the intact extracellular portion of the receptor. In agreement with the circular dichroism measurements, calorimetry of the neck peptide indicated a melting temperature of 54.1 °C (Fig. 3a).

Comparing the calorimetry result for the isolated neck domain with profiles for the isolated CRD and the full extracellular region showed that the full profile represents the sum of the isolated domains (Fig. 3b). This result provides strong evidence that the domains are independent, as their stability is the same in isolation or in the context of the full extracellular domain. Since the isolated neck domain is a tetramer, the unfolding process would include disassembly of the tetramer. Because the neck contribution to the total unfolding profile of the extracellular portion of DC-SIGN is identical with the behavior of the isolated neck domain, these results indicate that the tetramer

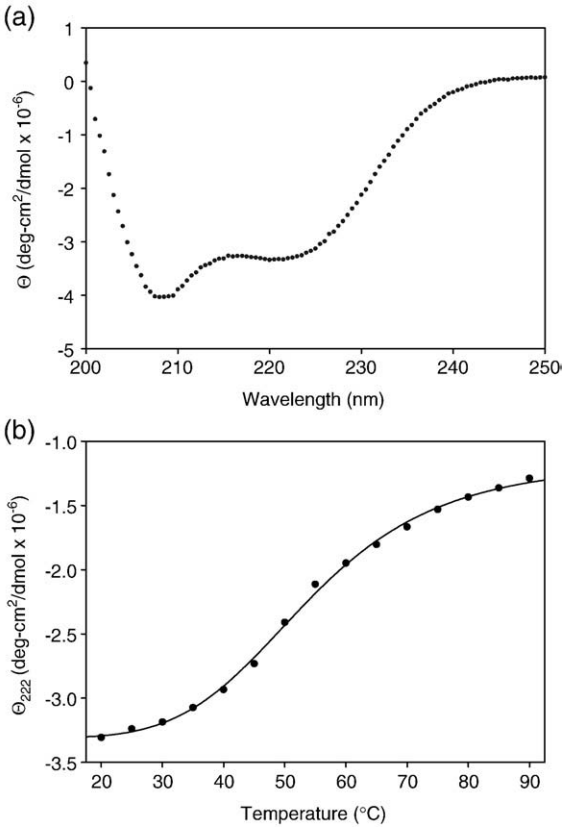

Fig. 2. Circular dichroism analysis of the neck domain of DC-SIGN. (a) The spectrum obtained at a protein concentration of 0.2 mg/ml at 20 °C in 125 mM NaCl, 25 mM Tris–HCl, pH 7.8, 5 mM $CaCl_2$. Circular dichroism was measured on a Chirascan spectropolarimeter from Applied Photophysics in a 0.1 cm quartz cuvette. (b) Denaturation of the neck domain of DC-SIGN was monitored by performing scans at intervals of 5 degC, after equilibration for 2 min at each temperature. Data were fit to a simple first-order curve using SigmaPlot.

stability is unchanged in the presence of the CRDs. Thus, these results show that the neck domain functions autonomously as a tetramerization domain. This conclusion is consistent with the partial view of the oligomer structure provided by crystallographic analysis of a truncated fragment from DC-SIGN, which showed CRDs in multiple orientations attached to the end of the neck domain through potentially flexible polypeptide linkers.[17] The present results provide experimental evidence for the independent functioning of the neck domain and the CRDs in the context of the full extracellular portion of DC-SIGN, supporting the picture obtained with the severely truncated fragment.

Similar experiments were undertaken to investigate the degree of independence of the neck domain and CRDs of DC-SIGNR. Both the CRD and the neck domain of DC-SIGNR are more stable than the corresponding regions of DC-SIGN, with the neck domain showing a 25 degC shift in denaturation temperature, so the CRDs of DC-SIGNR denature

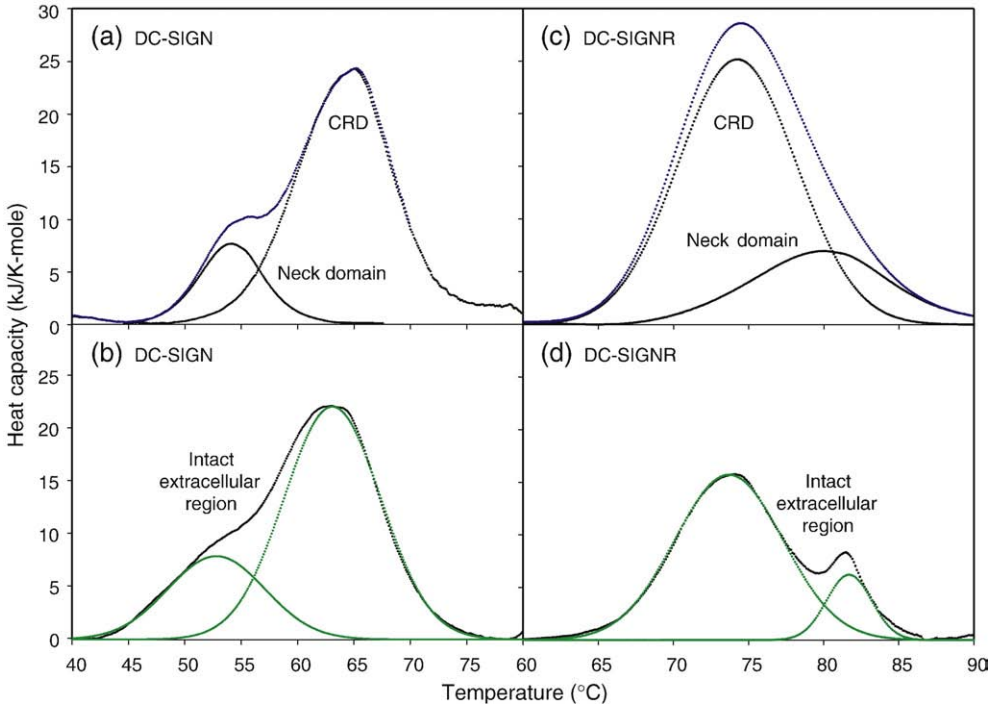

Fig. 3. Differential scanning calorimetry of fragments of the extracellular portions of DC-SIGN and DC-SIGNR. (a and c) Separate data for the neck domains and CRDs are shown as black lines, with the predicted combined results shown as a blue line. (b and d) Data for the intact extracellular domains are shown as a black line, fit to two Gaussian curves shown as green lines. Extracellular domain fragments and CRDs were expressed as described.[7,16] Differential scanning calorimetry was performed in a Nano-III instrument from Calorimetry Sciences Corporation. Samples dialyzed into or dissolved directly in 125 mM NaCl, 25 mM Hepes, pH 7.8, 5 mM $CaCl_2$, were degassed for 15 min before equilibration in the calorimeter. The volume of the sample loop was 300 μl. Multiple scans from 20 °C to 35 °C were performed until a flat baseline was obtained, after which complete scans to high temperature were performed. Protein concentrations were assayed using ninhydrin. Data were fit with SigmaPlot as described.[18]

before the neck domain (Fig. 3c). Independent behavior of the neck domain and CRDs is evident in the profile for the intact extracellular domain of DC-SIGNR, although the presence of the denatured CRDs leads to decreased solubility, which results in rapid precipitation when the neck domain denatures, so that the neck denaturation cannot be fully resolved (Fig. 3d).

The independent behavior of the neck domains and CRDs in DC-SIGN and DC-SIGNR contrasts with the intimate association of these regions in other glycan-binding receptors in which the necks consist of simple coiled coils of helices. Mannose-binding protein serves as a model for such proteins.[18] The close association of the CRDs and the neck observed in the crystal structure of the trimeric terminal fragment of mannose-binding protein is reflected in the fact that this fragment denatures in one step.[19] The extracellular domain of langerin displays similar denaturation behavior, rather than the separate denaturation observed for DC-SIGN and DC-SIGNR.[20]

Variants of DC-SIGNR containing more or fewer neck repeats are common in humans.[21] Genetic evidence has linked the presence of different neck length variants with susceptibility to infection by human immunodeficiency virus, SARS coronavirus and other pathogens, focusing attention on the importance of the neck domain.[22,23] Previous studies of the extracellular portions of polymorphic forms of DC-SIGN indicated that versions with shorter forms of the neck do not form completely stable tetramers.[17] Since the results presented here indicate that oligomer stability results from the properties of the neck domains, it should be possible to recapitulate these differences with fragments consisting of only the neck domains of polymorphic forms. Versions of the DC-SIGNR neck domain with 6.5 and 5.5 repeats were expressed with His_2 tags and their ability to form tetramers was compared to the 7.5 repeat form by gel filtration (Fig. 4a). The results confirm that the two longer neck domains form stable tetramers, while the 5.5 repeat version is a mixture of tetramers and smaller species. These properties mirror the behavior of the extracellular portions of DC-SIGNR containing these neck domains with appended CRDs and are thus consistent with the idea that the oligomerization properties are determined by the neck domain. The shorter neck domains show a progressive decline in stability measured by differential scanning calorimetry (Fig. 4b). Thus, in spite of the fact that the 6.5 repeat form is able to form tetramers that are stable at physiological temperatures, loss of even one repeat from the neck domain does reduce its overall stability.

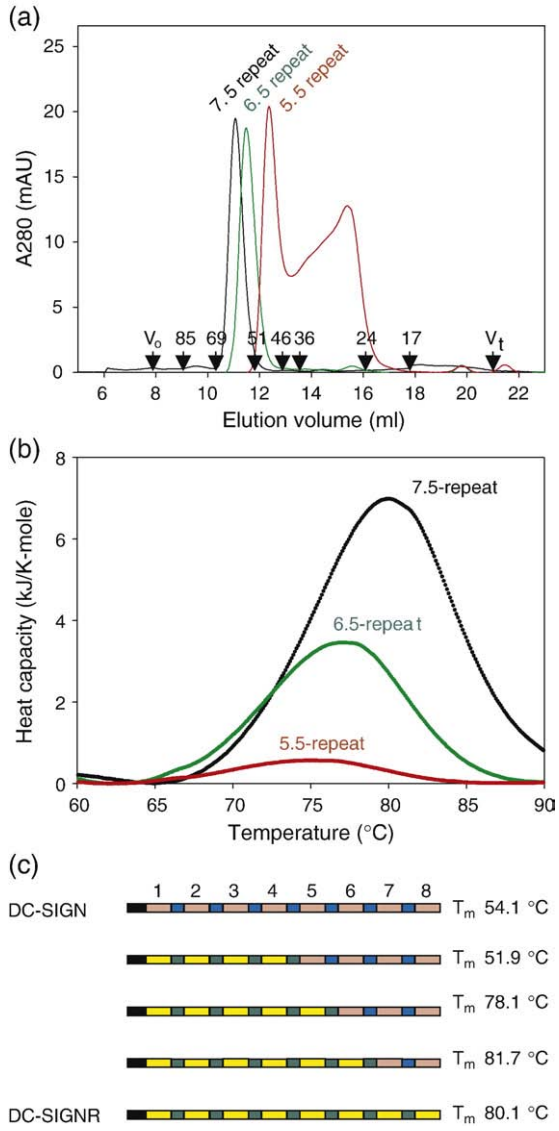

Fig. 4. Analysis of polymorphic forms of DC-SIGNR neck domains and DC-SIGN/DC-SIGNR hybrids. (a) The most common length variants of the DC-SIGNR neck domain were compared by gel-filtration analysis and (b) differential scanning calorimetry. (c) Comparison of denaturation temperatures for the neck domains of DC-SIGN and DC-SIGNR as well as hybrids between the two determined by differential scanning calorimetry.

Overall, the sequences of DC-SIGN and DC-SIGNR are 88% identical, with 93% identity in the neck domains, 79% identity in the CRDs, and 63% identity in the cytoplasmic tail and transmembrane domains. It is interesting that apparently minor sequence differences in each of these regions have important functional consequences, leading to differences in oligomer stability demonstrated here as well as in sugar-binding specificity and intracellular trafficking.[24] The basis for the striking difference between the stabilities of the neck domains from DC-SIGN and the 7.5 repeat form of DC-SIGNR was investigated by creating a series of hybrid neck domains containing portions of each of the proteins. Each of the hybrid constructs formed tetramers, as judged by gel-filtration analysis, but differential scanning calorimetry revealed a range of stabilities (Fig. 4c). The greater stability associated with DC-SIGNR maps in the C-terminal half of the neck domain, as the major increase in stability occurs with introduction of the fifth repeat of DC-SIGNR into DC-SIGN. The DC-SIGNR sequence in this repeat differs from the corresponding sequence in DC-SIGN only by the presence of leucine rather than glutamine at the first position in the heptad repeat. Thus, the difference between the two proteins must reflect subtle differences in a relatively short region of the neck domain. The difference emphasizes that although the two receptors diverged relatively recently in human evolution, they have evolved to fulfill different roles. The differences in neck domain stability probably reflect differences in the packing of the four receptor subunits, particularly toward the C-terminal end, which in turn may affect the disposition of the CRDs and thus alter the way that these receptors interact with ligands on the surfaces of pathogens.

The linking of the neck domains and CRDs in DC-SIGN and DC-SIGNR through flexible tethers that allow the domains to function independently may underlie the difficulty of obtaining structural information for the full extracellular domains of these receptors. Fortunately, the present studies suggest that it may be possible to obtain useful structural information for the neck domains in isolation.

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2009.02.046

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