The complement system is an important part of the innate immune response to infection but may also cause severe complications during inflammation. Small molecule antagonists to complement receptor 3 (CR3) have been widely sought, but a structural basis for their mode of action is not available. We report here on the structure of the human CR3 ligand-binding I domain in complex with simvastatin. Simvastatin targets the metal ion-dependent adhesion site of the open, ligand-binding conformation of the CR3 I domain by direct contact with the chelated Mg\(^{2+}\) ion. Simvastatin antagonizes I domain binding to the complement fragments iC3b and C3d but not to intracellular adhesion molecule-1. By virtue of the I domain’s wide distribution in binding kinetics to ligands, it was possible to identify ligand binding kinetics as discriminator for simvastatin antagonism. In static cellular experiments, 15–25 μM simvastatin reduced adhesion by K562 cells expressing recombinant CR3 and by primary human monocytes, with an endogenous expression of this receptor. Application of force to adhering monocytes potentiated the effects of simvastatin where only a 50–100 nm concentration of the drug reduced the adhesion by 20–40% compared with untreated cells. The ability of simvastatin to target CR3 in its ligand-binding-activated conformation is a novel mechanism to explain the known anti-inflammatory effects of CR3 (4), and the severity of acute ischemic stroke correlates with the level of CR3 expressed on monocytes (5). Inhibition of CR3 expressed in microglial cells limits synaptic loss in animal models of Alzheimer disease (6). Taken together, the significant involvement of CR3 in multiple pathogenic mechanisms points to this receptor as a promising target for anti-inflammatory therapy.

The major ligand-binding site in CR3 is the α\(_M\) subunit-inserted domain (α\(_M\)l), so named because it is inserted, in sequence, between the N and C termini of the larger, seven-bladed β-propeller domain (7). For both CR3 and the other I domain-carrying integrins, these domains have been useful in characterizing ligand interactions (8). Recent structural studies have shown that integrin I domains are tethered on the top of the head piece, formed by the α subunit β-propeller domain and domains of the β subunit (9). In the case of CR3, there is evidence that parts of the β-propeller domain may contribute to the recognition of certain large ligands, such as the complement fragment iC3b (10, 11). The flexible attachment of the I domain to the rest of the integrin ectodomain allows greater accessibility for ligand recognition, which makes it a major determinant for binding (9, 10). The I domains adopt at least
two conformations, the open and the closed, based on x-ray crystallographic studies of the $\alpha_{M1}$ (12, 13). Mutagenesis later confirmed that the open conformation binds ligands with considerably higher affinity than the closed conformation (8).

The mode of CR3 ligand recognition is complex and includes the binding to several protein and non-protein biomacromolecules (14). Experimental investigations point to a simple carboxylic acid as minimal ligand for the $\alpha_{M1}$ (15). In its carboxylate form, it becomes a part of the Mg$^{2+}$ coordination sphere in the metal ion-dependent adhesion site (MIDAS) of the $\alpha_{M1}$ open conformation (12, 13). In consequence, $\alpha_{M1}$ binds many ligands through a heterogeneous ensemble of stronger and weaker interactions (15–18). This is possible through a relatively high affinity for carboxylates, further regulated by the presentation of these carboxylates in folded macromolecules (15, 16). Simple equilibrium-based analyses will, however, only reveal binding heterogeneity under relatively extreme circumstances, because they are typically dominated by the strongest interactions (19, 20), as was also found by Bajic et al. (16) in characterization of the $\alpha_{M1}$ binding to C3d. By contrast, the binding kinetics are a rich source of information on this property (19). As shown recently by Zhang et al. (17), at least for small ligands, such ensembles of weak and strong interactions can be characterized both from injection of the $\alpha_{M1}$ over ligand-coated surfaces or, in the opposite orientation, with the $\alpha_{M1}$ immobilized to capture injected ligands. This is strong evidence that the $\alpha_{M1}$ has an intrinsic ability to form multiple types of contacts with polycarboxylate ligands, such as most proteins. Even the ligand iC3b, which supports the strongest reported binding by the $\alpha_{M1}$ at $K_D$ $\sim$0.5 $\mu M$, presents, in addition, weaker, but stoichiometrically more abundant, interactions at $K_D$ $\sim$200–300 $\mu M$ (16). This weaker affinity agrees quantitatively with the affinity of the $\alpha_{M1}$ for soluble glutamate (15). Although well established biophysical principles explain how such weak interactions support cell adhesion in the context of the membrane (21), their potential as antagonistic targets remains undefined.

With respect to inhibiting the function of $\beta_2$ integrins, several small molecule antagonists have been identified (22). Type-1 statins, such as lovastatin and simvastatin, inhibit binding of lymphocyte function-associated antigen (LFA-1; also known as integrin $\alpha_L\beta_2$ or CD11a/CD18) to intercellular adhesion molecule (ICAM)-1 (23, 24). In vivo experiments showed that lovastatin significantly changes the leukocyte distributions in several lymphoid tissues, including those of the mucosal immune system (25). These findings have a clinical correlate. Statins lower plasma cholesterol levels and are among the most widely used drugs. In treated patients, however, these compounds exhibit pleiotropic effects, including an anti-inflammatory capacity not directly related to the cholesterol-lowering activity (26).

Simvastatin is administered as a lactone prodrug and converted to a hydroxy acid form through hydrolysis (Fig. 1A). As noted by Kallen et al. (23), the ability of $\alpha_{M1}$ to form a contact with $\gamma$-carboxylate of glutamate (12, 13) encouraged the hypothesis that integrin I domains would bind statins through similar contact between the hydroxylated statin and the MIDAS of the I domain (23). Surprisingly, as revealed by both x-ray crystallography and nuclear magnetic resonance spectroscopy, statin prodrug bound the $\alpha_1$ I domain ($\alpha_1$I) of LFA-1 far from the MIDAS, underneath the $\alpha_7$ helix (the L site). This locked the domain in the closed, low affinity ligand binding conformation. Nevertheless, whether and how integrins may bind the hydroxy acid form of statins through the I domain MIDAS remains unclear, and the ability of statins to inhibit CR3 functions is controversial. Indeed, simvastatin showed no antagonistic effects on CR3 binding of ICAM-1 (24). More recently, however, simvastatin was found to interfere with CR3-mediated adhesion by neutrophil granulocytes in several experimental and clinical settings (27–29).

Using x-ray crystallography and molecular dynamics (MD) simulations, we reveal that the carboxylate in the hydroxy acid form of simvastatin engages in a ligand receptor-like complex with the MIDAS of the $\alpha_{M1}$. In surface plasmon resonance (SPR)-based assays, simvastatin inhibited the binding of $\alpha_{M1}$ to iC3b and C3d but not ICAM-1. We found that simvastatin inhibits preferentially $\alpha_{M1}$ interactions typified by slowly forming bonds to iC3b or C3d. We furthermore show that simvastatin blocked the adhesion of cell surface CR3 to complement-coated substrate. Our study provides a chemical rationale for simvastatin as CR3 antagonist and novel ways of understanding how antagonists may modulate the ligand binding kinetics of targeted proteins.

**Results**

*The Crystal Structure of the $\alpha_{M1}$-Simvastatin Complex Reveals the Molecular Basis for Simvastatin Binding—* To examine the molecular basis for the effect of simvastatin on CR3, we determined the crystal structure of the CR3 I domain bound to simvastatin at 2.0 Å resolution (Fig. 1 and Table 1). In the crystal, the $\alpha_{M1}$ with a Mg$^{2+}$-bound MIDAS, adopted the open, ligand binding conformation (Fig. 1B). The initial electron density indicated that one molecule of simvastatin in its hydroxy acid form (Fig. 1A) acted as a ligand to the MIDAS Mg$^{2+}$ ion, using its carboxylate group to complete the coordination sphere of the Mg$^{2+}$ ion (Fig. 1, B and C). The major contacts between simvastatin and $\alpha_{M1}$ involve polar contacts of the simvastatin carboxyl moiety with MIDAS residues Ser-142, Ser-144, and Thr-209. The carboxylate moiety is firmly anchored to the $\alpha_{M1}$ via its metal ion coordination. One of the simvastatin carboxyl oxygens is directly hydrogen-bonding to the hydroxyl of Thr-209. Two MIDAS-surrounding residues, Phe-246 and Arg-208, may contribute to the binding by forming $\pi-\pi$ stacking interactions with the unsaturated decalin moiety of simvastatin. However, their orientation is not optimal for the binding. Furthermore, although the electron density for the simvastatin ring system is strong, it cannot be perfectly described by the compound in a single conformation. Hence, the fitted simvastatin ligand is likely to approximate its average conformation in the crystal rather than representing a detailed structure of a single, stable conformation. A neighboring I domain molecule in the crystal unit cell may provide an additional buttress to the simvastatin decalin moiety but is evidently insufficient to maintain the molecule in a unique conformation, as indicated by the electron density, as noted from Fig. 1, B and C, and the associated B-factor values (Table 1).
Molecular Dynamics Simulations Support the Flexible Nature of the \( \alpha_{	ext{M}} \)-Simvastatin Complex—Considering that the crystal structure of the simvastatin complex was derived from soaking with the compound and the apparent lack of a single, predominant binding conformation of the unsaturated decalin moiety, we sought to validate the stability of the observed complex through MD simulations (Fig. 2). Three independent 100-ns simulations were performed using the crystal structure as the starting model. In these simulations, simvastatin remained firmly attached to the MIDAS through the carboxylate coordination of \( \text{Mg}^{2+} \) (Fig. 2A). This was further emphasized by the small variations in the bond length from the \( \text{Mg}^{2+} \) ion to the carboxylate oxygen of simvastatin with fluctuations in bond length of a maximum of 0.15 Å (Fig. 2B). As in the crystal structure, Ser-142, Ser-144, and Thr-209 formed hydrogen bonds to the simvastatin carboxylate group. These were maintained as stable contacts during the simulation, also with minimal fluctuations in bond length (Fig. 2B). In addition to these contacts, the carboxylate was soliciting non-MIDAS residues for hydrogen bonding, namely the backbone carbonyl oxygens of Gly-143, Gly-207, and Phe-246 (Fig. 2C). Importantly, besides the polar interactions, simvastatin was able to establish hydrophobic contacts with the side chains of Arg-208.

### TABLE 1

X-ray crystallography data collection and refinement statistics

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**FIGURE 1. The structure of the \( \alpha_{	ext{M}} \)-simvastatin complex.** A, structures of simvastatin in the lactone prodrug and hydroxy acid forms. The unsaturated decalin ring is indicated in cyan, and the carboxylate of the hydro-acid form is shown in red. B, simvastatin binds at the top of the I domain coordinating the MIDAS-bound Mg\(^{2+}\) ion. The 2\( \text{mF}_{o} - \text{DF}_{c} \) electron density map contoured at 1.0 \( \sigma \) is shown for simvastatin. C, stereo view of the simvastatin binding interface. I domain residues within 4 Å of the simvastatin molecule are shown as sticks. Water molecules (shown in cyan) contribute to the interaction.
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FIGURE 2. MD simulations of the αMl binding of simvastatin. A, from the MD simulations, the occupancy of simvastatin as calculated by the VolMap plugin for the Visual Molecular Dynamics (65) is indicated as the covered volume (in gray) together with one simvastatin molecule (shown as sticks) at the position found in the crystal structure. B, occurrence of contacts between αMl residues and the simvastatin molecule during three independent MD simulations (Simul.). Primary structure-linked residues are indicated with bars. Residues forming parts of the primary Mg2+ coordination sphere are indicated in boldface type. Residues in contact with simvastatin are indicated in italic type. C, bond lengths from Mg2+ to oxygens in Ser-142, Ser-144, Thr-209, and simvastatin throughout the simulation in three MD simulations of the structure of the αMl domain in complex with simvastatin.

and Phe-246, although the latter was in contact with simvastatin only for 30–60% of the time during the simulations (Fig. 2C). Leu-206, located on the opposite side of the MIDAS relative to Phe-246, was also a possible interacting partner, contacting simvastatin for 60–80% of the time during the simulations (Fig. 2C).

Simvastatin Antagonism Targets αMl Ligand Binding Kinetics—The crystal structure and MD simulations suggested that simvastatin may act as an αMl ligand binding antagonist. To test this hypothesis, we prepared surfaces carrying ligands for the αMl in a setting suitable for SPR monitoring of the interactions.

As noted previously, the ligand binding kinetics of αMl provide information on its interactions with various ligands. The experimental data were fitted using the EVILFIT algorithm (19, 30). This analysis was used to characterize the interaction of αMl with native and denatured fibrinogen (15); myelin basic protein and glatiramer acetate (18); the C3 fragments C3b, iC3b, and C3d (16); and the antimicrobial peptide LL-37 (17). Briefly, the algorithm solves the problem of calculating the minimal distribution of association (k_a) and dissociation (k_d) rates, which is required to account for the experimentally observed binding to ligand-coated surfaces. Therefore, rather than the usual characterization of protein-ligand interaction by a single pair of k_a and k_d, the algorithm returns a two-dimensional distribution of these constants. For easier interpretation of results with regard to affinity, results are shown as a distribution of k_d and the equilibrium constant K_D, the latter calculated from K_D = k_d/k_a (19). Results are typically shown in a three-dimensional coordinate system, where the x and y coordinates indicate the K_D (in M) and k_d (s⁻¹), respectively. For each pair of K_D and k_d, defining a single 1:1 interaction, the z-coordinate indicates the abundance of such a 1:1 interaction with the visual aid of contour maps (20). In this analysis, I domains, which bind their ligand through a well defined, single type of 1:1 interaction, produce narrow distributions of binding constants with the appearance of a single, symmetric “island” in the contour map (15). By contrast, binding to most ligands by the αMl produces a more composite and wide distribution of binding constants, often appearing as an “archipelago” in contour maps (16–18).

Initially, the αMl was injected in nine concentrations from 20 to 10,000 nm over surfaces coated with iC3b, C3d, or ICAM-1 (Fig. 3, A–C). To capture all relevant binding events within the range of the instrument, the algorithm probed interactions on a grid with 10 uniformly separated grid points in the interval 10⁻⁹ M < K_D < 10⁻² M and 10 grid points in the interval 10⁻⁶ s⁻¹ < k_d < 10⁹ s⁻¹. The distribution of interactions for iC3b (Fig. 3A) and C3d (Fig. 3B) was essentially identical to our previous report (16, 17). The ligands iC3b and ICAM-1 shared a population of binding sites with k_d between 10⁻⁴ and 10⁹ s⁻¹ (Fig. 3A and C). In addition, the iC3b presented another type of interaction with k_d between 10⁻⁴ and 10⁻⁴ s⁻¹ and K_D between 10⁻⁵ and 10⁻² M (Fig. 3A), discernible, albeit more weakly, also for C3d (Fig. 3B) but not found for ICAM-1 (Fig. 3C).

To investigate the influence of simvastatin on αMl ligand binding, αMl with simvastatin and DMSO or, as a reference, with DMSO alone was injected over the surfaces mentioned above. The SPR signal decreased with increasing simvastatin concentrations, both for iC3b and C3d but not for ICAM-1 (Fig.
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From this comparison, it seemed possible that only some types of interactions by \( \alpha_{\text{M}} \) were susceptible to simvastatin inhibitions, whereas others were exempt. A simple, essentially model-independent testing of this hypothesis was made by comparing the relative inhibition at two time points. In the \( \alpha_{\text{M}} \) sensorgrams, the signal at \( t = 234 \) s (\( S_{243s} \)) reflects combined contributions from high affinity and a spectrum of lower affinity interactions. By contrast, the signal at 210 s after the end of the injection period (i.e. \( t = 450 \) s (\( S_{450s} \))) corresponds to the late dissociation phase. Hence, only interactions with a halftime of dissociation (\( t_{1/2} \)) on the order of 50 s or longer, and hence a dissociation rate of \( k_d = \ln 2/t_{1/2} = 10^{-2} \) s\(^{-1}\) or less, would be expected to influence the signal (\( S_{450s} \)). As shown by the normalized inhibition (Fig. 3, G–I), simvastatin acted potently on such slowly dissociating interactions (\( S_{450s} \)) with iC3b (Fig. 3G) and C3d (Fig. 3H). The combined interactions (\( S_{243s} \)) were less affected. In the case of ICAM-1 ligand, only the combined interactions were robustly measureable and showed little, if any, inhibition (Fig. 3I).

To conduct a more comprehensive analysis of the simvastatin antagonism, we used the EVILFIT methodology described above. We extracted the binding kinetics for \( \alpha_{\text{M}} \) binding to flow cell-immobilized iC3b (A), C3d (B), and ICAM-1 (C). For iC3b and C3d (A and B), \( \alpha_{\text{M}} \) were injected in nine concentrations from 20 to 10,000 nm (ascending order of sensorgrams). For each ligand, the results shown were from one of two similar binding experiments. Fits to the experimental data were made with EVILFIT (30) as described elsewhere (20). The goodness of fit is indicated in A–C from a comparison between the data (solid red line) and the model (solid black curve) and the model (solid red line). For ICAM-1, sensorgrams for iC3b, C3d, and ICAM-1 measured by SPR.

FIGURE 3. Effects of simvastatin on the \( \alpha_{\text{M}} \) binding to iC3b, C3d, or ICAM-1 measured by SPR. A–C, SPR sensorgrams for the \( \alpha_{\text{M}} \) binding to flow cell-immobilized iC3b (A), C3d (B), and ICAM-1 (C). For iC3b and C3d (A and B), \( \alpha_{\text{M}} \) were injected in nine concentrations from 20 to 10,000 nm (ascending order of sensorgrams). For each ligand, the results shown were from one of two similar binding experiments. Fits to the experimental data were made with EVILFIT (30) as described elsewhere (20). The goodness of fit is indicated in A–C from a comparison between the data (solid black curve) and the model (solid red line). For ICAM-1, sensorgrams for iC3b, C3d, and ICAM-1 measured by SPR. For ICAM-1, sensorgrams from one of two similar binding experiments. Fits to the experimental data were made with EVILFIT (30) as described elsewhere (20). The goodness of fit is indicated in A–C from a comparison between the data (solid black curve) and the model (solid red line). For ICAM-1 binding, we used the EVILFIT methodology described above.
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![Graph](image-url)

**Figure 4.** SPR sensorgrams (*solid black lines*) for injections of 10 μM αmβ2 in the presence of 0–100 μM simvastatin in flow cells coupled with iC3b, C3d, or ICAM-1. For each sensorgram, the ligand binding kinetics were extracted by fitting with the EIVLFIT algorithm. In each panel, the goodness of fit is indicated with *opaque gray lines* (model) and the root mean square deviation (in resonance units (RU)), comparing experimental data and the model, as shown in italic type.

s⁻¹, are indicated as Bin 2. From integration of the signals in the bins, it appeared that only Bin 2 interactions in iC3b and C3d were susceptible to simvastatin antagonism. By contrast, for all ligands, including ICAM-1, Bin 1 interactions were unaffected (Fig. 5, A and B).

**Antagonistic Effects of Simvastatin on the Binding of CR3 to iC3b**—To investigate the effect of simvastatin on CR3 binding to one of its natural substrates, complement iC3b, we employed three different types of cell adhesion or contact assays.

In the electric cell-substrate impedance sensing (ECIS) assay, cells are applied in tissue culture wells with small, planar gold film electrodes deposited on the bottom surface (31). In this system, the alternating current impedance increases with cellular adhesion and thereby offers a label-free means of following this process over time (32). The adhesion of recombinant K562 cells or primary human monocytes was compared, in the absence or in the presence of simvastatin. The recombinant cells expressed ~76% of the CR3 level found on monocytes, as judged from the mean fluorescence intensity when staining both cell types for CD11b (data not shown).

The binding to iC3b substrate by CR3/K562 cells was probed in the presence of 6.25–50 μM simvastatin with clear signs of inhibition at 25 and 50 μM (Fig. 6A), whereas simvastatin had no influence on the parental K562 cell line (Fig. 6B). Simvastatin also inhibited the adhesion by primary human monocytes to the substrate, either with an added activating antibody (Fig. 6C) or without such an addition (Fig. 6D). Half-maximum inhibition for the CR3-supported adhesion was reached with ~25 μM simvastatin (Fig. 6E) when taking into account the background binding observed for parental K562 cells (Fig. 6B). In the case of the monocytes, which formed stronger contacts with the iC3b-coated substrate, half-maximum inhibition was reached at ~35 μM (Fig. 3E). For both CR3/K562 cells and monocytes, the inhibition persisted for hours.

Simvastatin also inhibited the ability of monocytes to bind iC3b-coated fluorescent particles, as investigated by flow cytometry (Fig. 7, A and B). The cellular gating was carefully adjusted to accommodate the small changes in forward and side scatter induced by the addition of simvastatin (Fig. 7A). As also noted in experiments with other bead-coupled CR3 ligands (18), distinct peaks in the fluorescence histograms suggested a quantized association or phagocytosis of the beads. This was used to distinguish contacts with more than one bead from the total of bead-contacted monocytes. In both types of measures
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**FIGURE 5.** Distribution of αm ligand binding interactions in the presence of simvastatin. A, from fitting of the sensorgrams, as shown in Fig. 4, the distribution in kinetics of the αm ligand binding was determined as described (19, 20, 30) for iC3b, C3d, and ICAM-1. Results are shown in two-dimensional grids as explained under “Results” in the section “Simvastatin Antagonism Targets αm Ligand Binding Kinetics,” and in the legend to Fig. 3, A–C. To quantify the development in the distribution of binding kinetics, two bins (Bin 1 and Bin 2) were defined, guided by the major types of interactions observed for iC3b and C3d. B, from integration of signals (∫∫S(kRU), in kilo resonance units (kRU)) in the bin areas, it was possible to plot these values as a function of the applied simvastatin concentration. Data represent the average of the two experiments, with error bars indicating the minimum and maximum levels.

**FIGURE 6.** Simvastatin inhibition of cellular CR3 adhesion to iC3b measured by ECIS. A and B, the ECIS cell adhesion assay was applied to surfaces coupled with iC3b, using either K562 cells with a recombinant expression of CR3 (A) or the parental cells (B), which do not express CD18 integrins. C and D, similar studies were made with primary human monocytes, either in the presence of the function-activating antibody KIM185 to CD18 (C) or without such antibody (D). In A–D, the shown cell index recordings are representative of three independent experiments. E and F, the normalized inhibition was calculated from the cell index without the addition of simvastatin for CR3/K562 and the parental K562 cells (E) and monocytes (F). In each panel, the mean values for three experiments are indicated along with error bars showing the S.E.

and unlike in the purely static cell adhesion assays (Fig. 6), an inhibitory influence was discernible even at ~12 μM simvastatin (Fig. 7C).

To directly test whether force load on adhering cells would alter the potency of simvastatin-mediated inhibition, we employed a previously described centrifugation-based cell...
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Statin potentiated the detachment of adhering cells as a function of increasing centrifugal force, clearly shown in the presence of 20 μM simvastatin compared with the detachment without such an addition (Fig. 8A). With weak centrifugal force (10 × g), only a modest difference was found in the adhesion by simvastatin-treated and -untreated cells when analyzed according to Equation 1. By contrast, with increased centrifugal forces (30–50 × g), almost complete inhibition (94%) of the binding was obtained with statin, whereas the untreated controls showed robust binding. Nevertheless, even at low centrifugal force (10 × g) it was possible to detect an influence of simvastatin (Fig. 8A, B and C). As also shown in Fig. 8A, when using Equation 1, only a modest reduction in cell adhesion was obtained from application of 20 μM simvastatin at 10 × g. Lower concentration apparently did not reduce the binding (Fig. 8B). However, as shown from the inclusion of the raw signals $F_{\text{coated}}$ and $F_{\text{uncoated}}$ (Fig. 8B), the simvastatin concentration significantly impacted the background binding in uncoated wells. This creates a difficulty in comparing the cell binding through Equation 1, because there is a non-linear change in CA with changes in $F_{\text{uncoated}}$

$$
\frac{\partial CA}{\partial F_{\text{uncoated}}} = \frac{1}{F_{\text{uncoated}} - F_{\text{coated}}} \left( \frac{F_{\text{uncoated}} - F_{\text{coated}}}{F_{\text{uncoated}}^2} \right) \quad (\text{Eq. 2})
$$

We compared the influence of simvastatin through a simpler equation,

$$
\Delta F = F_{\text{uncoated}} - F_{\text{coated}} \quad (\text{Eq. 3})
$$

where $\Delta F$ is proportional to the iC3b-specific cell adhesion and changes proportionately with changes in either $F_{\text{uncoated}}$ or $F_{\text{coated}}$. Using Equation 3 with normalization against no treatment with simvastatin ($\Delta F_0$), it was clear that simvastatin lowered the iC3b-specific binding in a titratable fashion, at least in the interval 100–20,000 nm (Fig. 8C). In principle, inhibition was also observed at 50 nm, but the binding data were subject to more variation.

**Discussion**

We report here on structural and ligand binding kinetics aspects of competitive antagonism of CR3. Structural insight was gained from both x-ray crystallographic studies and MD simulations of the complex between $\alpha_{m}I$ and a small molecule antagonist, simvastatin. Alongside these findings, cellular and biochemical assays measured inhibition of CR3 ligand binding by simvastatin. Our report is the first to structurally characterize an $\alpha_{m}I$ ligand-binding antagonist in complex with $\alpha_{m}I$. As judged from the x-ray crystallographic data, the major site for interaction between simvastatin and the ligand-binding activated $\alpha_{m}I$ is the MIDAS. In this way, the $\alpha_{m}I$ antagonism differs markedly from how statins block binding by the $\alpha_{L}I$, where the primary interaction between statin and the I domain is distant from the MIDAS, in the so-called L site (24). In the structure of the $\alpha_{m}I$-simvastatin complex, the carboxylate moiety of simvastatin forms a contact with the MIDAS Mg$^{2+}$. This

adhesion assay (33). This methodology works by use of microtiter wells with a conical shape, usually referred to as v-wells, and fluorescent dye-labeled cells. Following centrifugation, the propensity of the cells to reach the bottom, or nadir, of the wells is inversely proportional to the ability to adhere to the sides of the wells. By comparison of the fluorescent signals obtained in the ligand-coated wells ($F_{\text{coated}}$) with wells without ligand coating ($F_{\text{uncoated}}$), the percentage of cell adhesion (CA) is established through the following equation (33).

$$
CA = 100\% \times \frac{F_{\text{uncoated}} - F_{\text{coated}}}{F_{\text{uncoated}}} \quad (\text{Eq. 1})
$$

FIGURE 7. Simvastatin inhibition of monocyte binding and phagocytosis of iC3b-coupled fluorescent beads. A, forward side scatter plots of monocytes either with no additions, with beads and DMSO vehicle, or with beads and 50 μM DMSO-dissolved simvastatin. B, histograms show bead fluorescence intensities either with no additions, with beads and DMSO vehicle, or with beads and 50 μM DMSO-dissolved simvastatin. Gates were placed to distinguish the total number of cells with beads (solid line) or cells with multiple (>1) beads (hatched line). C, normalized inhibition of bead adhesion or phagocytosis. The percentage of monocytes positive for bead contact (Total Beads $^*$) was calculated in the absence or presence of simvastatin. By normalizing to the value of positive cells in the absence of simvastatin (0% inhibition), the percentage of inhibition was calculated for each concentration of simvastatin. A similar calculation was also made for the gate only including cells with multiple beads (Multiple Beads $^*$). The mean values for three experiments are indicated along with error bars showing the S.E.
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affinity, even in the high affinity ligand binding conformation (15), which may explain why the \( \alpha_L \) fails to bind statins through the MIDAS.

In addition, simvastatin also forms hydrogen bonds with main chain carbonyl oxygens, as well as with the functional groups of the side chains of MIDAS-forming residues. In MD simulations, the unsaturated decalin moiety of simvastatin was found to be highly mobile. Consistent with these data, simvastatin probably also adopted multiple conformations in the crystals. Although Leu-209 and Phe-246 are not in the immediate vicinity of the simvastatin in the crystal structure, the MD studies suggested that these residues may form transient contacts to the simvastatin decalin moiety. No significant electron density was observed within the putative \( \alpha_L \) L site.

To efficiently study antagonistic effects, we used an \( \alpha_M \) mutated to break an isoleucine-based allosteric switch and permit the \( \alpha_M \) \( \alpha7 \) helix to preferentially occupy its open conformation position (34, 35). It is widely acknowledged in the literature that such stabilized, or locked, integrin I domains have been useful in ligand binding studies, including both x-ray crystallographic studies and biochemical investigations of ligand affinity (8). Nevertheless, the induced conformational rearrangements in these constructs are likely to destroy the putative L site (i.e. the site of contact between statin and the \( \alpha_L \)). Hence, we cannot exclude the possibility that statins also might bind this site in the closed conformation of \( \alpha_M \). Clearly, the tight link between conformation and affinity presents a challenge with regard to choosing appropriate \( \alpha_M \) constructs in studies with antagonists. There can be little doubt, however, about the relevance of the open \( \alpha_M \) L conformation in these studies. In a pro-inflammatory environment, a large population of CR3 molecules is in their ligand-binding conformation (36). In the past, the influence of statins on conformationally activated \( \beta2 \) integrins or their I domains was never tested. We now show that simvastatin is able to antagonize receptors in this biologically relevant conformation.

It is increasingly evident that binding kinetics are important determinants of antagonist efficacy (37). Our study adds to this concept by showing that the complex \( \alpha_M \) ligand binding kinetics are affected in a non-trivial way by the presence of an antagonist. As noted above, equilibrium-based analyses of \( \alpha_M \) ligand binding are a poor source of information on its multiple interactions with ligands. This compromises the use of the classic Schild regression (38) for understanding the mode of simvastatin antagonism to \( \alpha_M \) ligand binding. We now propose extracting the relevant information directly from the observed association and dissociation phases, using a methodology previously described by Schuck and co-workers (19, 30). We noted that only some of the interactions in the ensemble, namely those referred to as Bin 2, were affected by simvastatin, whereas those belonging to Bin 1 were not. Mass transport effects influencing these data are unlikely because of the goodness of fit for the applied model of the \( \alpha_M \) L site. Hence, the mean values for three independent experiments are indicated together with error bars showing the S.E. Data in B are from one experiment representative of a total of three independent experiments.

contact agrees well with what was reported in the past, namely that \( \alpha_M \) reacts with simple carboxylic groups of free glutamate and even glutamate side chains in the context of the \( \alpha_M \) itself (13, 15). The \( \alpha_L \) binds such moieties with considerably weaker affinity, even in the high affinity ligand binding conformation (15), which may explain why the \( \alpha_L \) fails to bind statins through the MIDAS.
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nism to α₁L ligand binding (23, 24). We suggest that at least two structurally different mechanisms can account for the observed selectivity. First, it is known from RGD-binding integrins, such as the β₃ integrins, that small RGD motif-containing compounds may exert both ligand binding antagonistic and agonistic effects. Binding of these ligand mimetics induces changes in the tertiary structure of the β₃ chain I-like domain (40, 41), which persist after dissociation of the compound (42). I domains are structurally highly similar to I-like domains (43). It has already been reported that the α₁L may exist in several distinct tertiary structures with low, intermediate, or high ligand-binding affinities (44). By acting as a ligand mimic with several MIDAS contacts, we propose that simvastatin imprints the conformation of the simvastatin-dissociated α₃L to permit the Bin 1, but not Bin 2, interactions. Second, as an alternative explanation, it is possible that some protein ligands (i.e. in our study iC3b and C3d) form types of contacts with the α₃L that permit the simvastatin to write itself inside the complex and gain access to the MIDAS, causing the complex to dissociate. In other ligands (i.e. ICAM-1), the contacts formed with α₃L do not permit such intrusion by simvastatin and are hence exempt from simvastatin antagonism toward α₃L binding. Unlike for the ability of small drugs to make conformational imprints in integrin I-like domains, to our knowledge, there are currently no experimental data available to support this model. In any event, both mechanisms proposed here add a chemical rationale to an earlier report on the inability of simvastatin to block CR3 binding of ICAM-1 (24) while still exerting antagonism toward complement binding.

The ability of simvastatin to inhibit intact CR3 was shown in three distinct types of cellular assays; however, all used the same ligand, iC3b. Two of these, namely the ECIS and bead-based assays, involved no external force load on the cell-ligand contact, whereas the v-well assay works by application of centrifugal force. The ECIS assays, which measure time-resolved cell-substrate contacts, revealed a persistence of the simvastatin antagonism to CR3-mediated adhesion, lasting for hours, both for recombinant cell lines and primary monocytes. Under conditions similar to those in our cell culture experiments, it was previously found that the simvastatin lactone prodrug quickly hydrolyzed to the hydroxy acid form (45). Consequently, the persistent antagonizing effect on CR3 adhesion suggests that the hydroxy acid form of simvastatin is the antagonist rather than the lactone prodrug.

To enable a study with CR3 uniformly activated and with a clear link to conformational change, we used the KIM185 antibody to activate CR3 (46) through an allosteric mechanism, which ultimately involves the α₃L in the open conformation (47). Also, in this case, simvastatin inhibited cell adhesion. Natural targets for CR3 binding, such as complement-opsonized microbes, were mimicked with iC3b-coated beads. Although we did not formally distinguish between engulfed beads and those merely attached to the membrane, it is noteworthy that simvastatin was able to block particle interactions with these phagocytic cells. The simvastatin concentration required to obtain robust (~20%) inhibition in both of these assays was on the order of ~25 μM. Half-maximum inhibition (50%) was obtained with ~50 μM, almost identical to what was reported in the past for inhibition of LFA-1-mediated adhesion to ICAM-1 (23). In the cell adhesion assay based on centrifugal force, simvastatin increased the sensitivity to cell detachment. This was clearly demonstrated from the almost equal cell adhesion following centrifugation at 10 × g, irrespective of simvastatin addition, versus near ablation of cell adhesion at 30–50 × g for cells treated with 20 μM simvastatin. In contrast to the two static adhesion assays mentioned above, further comparisons with the force-based assay revealed that such differences could be found even at a concentration of 100 nM statin, which reduced the cell adhesion by ~20% compared with cells treated only with the vehicle DMSO.

Considerable evidence exists that simvastatin acts as an immunosuppressant independently of its cholesterol lowering capacity (26). However, with this and other pleiotropic effects, a general concern is the plasma concentration of statins found to be in the low nanomolar range (48, 49). Such concentrations would seem to preclude any influence of statin on, for instance, LFA-1, expressed by leukocytes in blood, because past measurements made in both cellular and purely molecular assays reported effects only in the micromolar range (23). We are now able to point to at least two principles that may alter the view of the β₃ integrin sensitivity to simvastatin inhibition and hence the immunomodulatory capacity of statins.

First, a special feature of integrin ligand binding is the dynamic influence of force loads on the adhesive bonds to substrates, which involves significant conformational alterations in the integrin ectodomains (50). Weitz-Schmidt (51) suggested that such forces increase the integrin susceptibility to statin antagonism of ligand binding, but experimental evidence has been lacking. Our findings now support this idea. Adding to the role of integrin conformation, it seems likely that force load on the structurally labile integrin ectodomain supports conformational changes, which potentiate the antagonism of simvastatin. This also points to a limitation in quantifying the simvastatin ligand-binding antagonism, however, because available methodologies do not capture the full plethora of forces affecting CR3 substrate adhesion in vivo.

Second, the oral delivery and later absorption over the intestinal epithelium is a topic often neglected in the discussion of the pharmacokinetics of statins vis-à-vis an influence on the immune system. Statins reach the mucosal immune system before the liver or even the albumin-rich plasma, both factors being critical to the low availability of statins in blood. Although enzymes responsible for degradation of simvastatin are also expressed in the intestines, the resulting concentration is likely to be far higher in important secondary lymphoid tissue, such as the Peyer patches and lamina propria, and in the environment of intraepithelial leukocytes, compared with the concentration in plasma. Little is known, however, about the actual statin concentration in these tissues. An important study by Bergman et al. (52) showed that rosuvastatin accumulates in the bile in concentrations ~5,000-fold higher than in plasma, clearly implicating the statins as potentially active in the intestinal environment. Indeed, experimental evidence already shows that the leukocyte distribution is altered in the Peyer patches following lovastatin administration (25); simvastatin directly affects the intraepithelial lymphocytes (53). With the emerging
focus on the role of the mucosal immune system in inflammatory diseases (54), an important part of the statin immunomodulation is likely to relate to the action of these compounds in the gut-associated lymphoid tissues.

**Experimental Procedures**

*Cells and Biologic Reagents—*Codon-optimized C3d cDNA sequence (encoding protein residues 993–1288 of human C3, mutant C1010A) and a human αM (subunit αM residues 127–321), mutated as C128S/I316G (34), were expressed in BL21 (DE3) *Escherichia coli* cells and purified to >95% purity as judged by SDS-PAGE, as shown by Bajic *et al.* (16) in Fig. 5E of that paper. The same constructs were used for structural, biochemical, and cellular studies. iC3b was purchased as 1.0 mg/ml stock solution (A115; CompTech), and ICAM-1 was bought from R&D Systems (ADP4). Simvastatin (S6196) was purchased from Sigma-Aldrich. The monoclonal KIM185 antibody (46) recognizes the fourth, membrane-proximal cysteine-rich domain of the β2 subunit (47) and activates integrin β2 ligand binding (46, 47). Manufacture and purification of KIM185 antibody was performed by GenScript.

K562 cells with an engineered expression of CR3 (CR3/K562) and their parental cell line have been described elsewhere (55). Before use in the experiments, the cells were cultivated in RPMI 1640 medium (BE12-702F, Lonza) supplemented with 10% (v/v) FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 292 μg/ml l-glutamine (PenStrep Glutamine, Life Technologies, Inc.), and 10 mM HEPES, pH 7.5, in an incubator (Thermo-Fisher) set at 37 °C, 5% (v/v) CO2, and 80% relative humidity. For maintenance of recombinant CR3 expression, 4 μg/ml puromycin was added to the culture medium.

Human monocytes were prepared from the buffy coat of centrifuged donor blood, kindly supplied by the Blood Bank at Aarhus University Hospital Skejby. The buffy coat was deated by Ficoll-Paque™ PLUS (GE Healthcare) gradient centrifugation, followed by negative isolation of monocytes by use of Invitrogen’s Untouched™ human monocyte kit (REF11350D, LifeTech). Cells were stored as frozen in RPMI 1640 medium (BE12-702F, Lonza) supplemented with 20% (v/v) FCS and 10% (v/v) DMSO (Sigma-Aldrich). The cell viability was >90% after thawing.

**Crystallization and Structure Determination—**The I domain was purified in 20 mM HEPES, pH 7.5, 200 mM NaCl. Five mM MgCl2 was added to the protein solution before crystallization. The protein crystallized by vapor diffusion at 19 °C. Crystals were grown in a few days by mixing the CR3 I domain sample (1 μl at 12 mg/ml) with an equal volume of reservoir solution (0.2 M sodium malonate, pH 7.0, 20% (w/v) PEG 3350). Rod-shaped crystals appeared after a couple of days. The crystals were then annealed to 1–5 h in mother liquor containing 1 mM simvastatin. Before data collection, the crystals were soaked in 1 M sodium PEG 3350, 30% (v/v) glycerol, and flash-frozen in liquid nitrogen. The data were collected at 1.0 Å with a rotation of 0.5°/image on the I911-3 beamline at MAX-lab, Lund, Sweden. Data reduction was carried out with XDS (56). The structure was solved with molecular replacement in PHASER (57), using the previously published structure of αM (Protein Data Bank entry 1IDO (12)) as the search model. The initial electron density maps revealed the presence of a ligand in the MIDAS, after which one molecule of simvastatin was placed manually. Rebuilding with Coot (58) and refinement with phenix.refine (59) were carried out in an iterative manner, whereas model quality was assessed with MolProbity (60). All figures were prepared with the PyMOL Molecular Graphics System version 1.5.0.4 (Schrödinger LLC, New York).

**Molecular Dynamics Simulations—**MD simulations based on the αM crystal structure were prepared by adjusting atom types in the ligand by using Protein Preparation Wizard in the Schrödinger software package (22). In Protein Preparation Wizard, the protonation states of the four histidine residues were chosen based on surrounding residues. His-148 and His-210 were modeled as the Nδ-tautomer, whereas His-183 and His-295 were modeled as the flipped Nδ-tautomer. None of the histidines were close to the binding site of simvastatin. The system was solvated with the TIP3P water model (61) and subsequently neutralized with NaCl to a concentration of 150 mM, which produced a structure containing 28,801 atoms. All simulations were performed in Desmond (Desmond Molecular Dynamics System version 3.1, Schrödinger, LLC) using the OPLS force field (62) as embedded in the Schrödinger 2012 suite of programs (Protein Preparation Wizard; Epik version 2.3; Impact version 5.8; Prime version 3.1) and by applying the Multisim method. The simulations were made using a RESPA integrator (63), with bonded and nearly non-bonded interactions calculated every 2 fs, the non-bonded interactions truncated at a cut-off of 9 Å. Far interactions were calculated every 6 fs, using smooth particle mesh Ewald (64) to treat full electrostatics beyond 9 Å. Here, the Multisim method minimizes the system with restraints on solute, followed by a minimization without any restraints. First, a short simulation of 12 ps was run in the NVT ensemble at 10 K. Afterward, another short simulation was run in the NPT ensemble at 10 K. Short simulations were then run in the NPT ensemble for 12 ps, with restraints on solute heavy atoms at 310 K, followed by a similar simulation without restraints, which was run for 24 ps. Then the MD production runs were initiated and continued for 100 ns. Constant pressure of 1 atm was maintained by the Martyna-Tobias-Klein barostat with a relaxation time of 2 ps. A constant temperature of 310 K was achieved utilizing the Nosé-Hoover chain thermostat method when simulated in the NPT ensemble. Snapshots were saved every 4.8 ps, and snapshots for every 192 ps were used for analysis. The trajectories were analyzed using the program VMD (65).

**Surface Plasmon Resonance-based Assays—**Preparation of CM4 sensor chips (GE Healthcare) and recording of sensorsgrams was carried out on a BIAcore 3000 instrument (GE Healthcare). C3d, iC3b, and ICAM-1 were immobilized at 0.057, 0.053, and 0.011 pmol/mm², respectively, through amine coupling together with a reference prepared without protein as described (20). Surfaces were initially probed with αM diluted in running buffer (150 mM NaCl, 1 mM MgCl₂, 50 mM HEPES, pH 7.4) to concentrations of 20 nM, 39 nM, 78 nM, 156 nM, 312 nM, 625 nM, 1.25 μM, 2.5 μM, 5 μM, and 10 μM. The samples were injected over the reference and ligand-coupled surfaces with a flow rate of 10 μl/min and a contact time of 240 s, fol-
lowed by a dissociation phase of 210 s. The sensor chips were re-stored with 5.0 mM NaCl, 50 mM EDTA, 100 mM Hepes (pH 7.5). Following subtraction of the reference sensor signal, the resulting sensorgrams were carefully aligned with the BIAevaluation software (GE Healthcare) and analyzed using EVIL-FIT version 3 software (19, 20, 30) to determine the distribution of binding kinetics. The operator-set boundaries for the distributions were uniformly set to limit K_D values in the interval from 10^-9 to 10^-2 M, and k_d values in the interval from 10^-6 to 10^-3 s^-1. For experiments with simvastatin, the procedures for SPR measurements were the same as for characterization of the αs1 binding to ligands described above. In all experiments with simvastatin, the αs1 concentration was fixed at 10 μM. From stocks of simvastatin diluted in DMSO to 10 mg/ml (23.9 mM), a fresh dilution of simvastatin for each experiment was made to a final concentration of 200 μM, corresponding to a final concentration of 0.42% (v/v) DMSO. From this dilution, further serial dilutions of simvastatin were made to 100, 50, 25, 12.5, 6.25, and 3.13 μM simvastatin in running buffer supplemented with 0.42% (v/v) DMSO together with a reference containing no simvastatin. This buffer was also used as running buffer. The distribution in binding kinetics was extracted for each sensorgram resulting from application of simvastatin or the reference with no simvastatin.

**Electric Cell-substrate Impedance Sensing Cell Adhesion Assay**—ECIS measurements were made essentially as described (17). E-plate L 8 plates (ACEA Biosciences, Inc., San Diego, CA) were activated with 4 mg/ml dithiobis(succinimidyl propionate) for 0.5 h and then treated with 100 μl/well of 10 μg/ml iC3b dissolved in PBS or PBS only as coating reagents for 1 h at room temperature. The plates were then washed with PBS three times. Meanwhile, the monocytes were thawed, added to PBS supplemented with 20% (v/v) FCS and harvested by centrifugation. Freshly cultured K562 cells were simply harvested from culture medium. Cells were subsequently washed once and then resuspended in binding buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl_2, 1.8 mM CaCl_2) supplemented with 0.1 mg/ml human serum albumin and 5 mM D-glucose. Monocytes were used at 1 × 10^6 cells/ml whereas the K562 cells were used at 1.5 × 10^6 cells/ml. CR3 was activated by the addition of KIM185 antibody at 10 μg/ml. DMSO-dissolved simvastatin was applied in a fixed 100-fold dilution to the cell suspensions for reaching a final concentration of 6.25–50 μM. The mixtures were added to the E-plate L 8, which were subsequently mounted on an iCELLigence device (ACEA) for data collection over 3 h in an incubator at 37 °C and with 5% (v/v) CO_2. All measurements for both coated and control wells were done at least three times.

**Fluorescent Bead Binding Assay**—Samples of 50 μl of fluorescent beads (F-13081, Life Technologies) were activated by 1.5 mg/ml NHS and 0.3 mg/ml N,N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide in 50 mM MES, pH 6.1, for 0.5 h and then washed twice with 50 mM MES, pH 6.1. Immediately afterward, beads were incubated with 100 μg of iC3b diluted in 50 mM MES, pH 6.1, for 1 h. Finally, the beads were washed twice in 50 mM MES, pH 6.1, and diluted in binding buffer with human serum albumin and glucose as described above to 1 ml (5 × 10^8 beads/ml). Frozen monocytes were thawed as described for the ECIS assay. Simvastatin stock solutions were added into flow cytometry tubes, followed by the addition of 100 μl of monocyte suspension and a brief mixing. Quickly following this step, 35 μl of iC3b-coated beads were added and mixed well. The mixture was transferred to the incubator at 37 °C and with 5% (v/v) CO_2 for 15 min. Afterward, cells were washed with 2 ml of 1% (w/v) human serum albumin, 2 mM EDTA in PBS, pH 7.4, buffer and then with 2 ml of 0.5% (w/v) BSA in PBS, pH 7.4, buffer. Finally, the cells were fixed with 400 μl of 0.9% (v/v) formaldehyde in PBS, pH 7.4, and used in flow cytometry analysis with the BD Bioscience LSRII™ cell analyzer.

**Centrifugation-based Cell Adhesion Assay with Monocytes**—The centrifugation-based cell adhesion assay was conducted essentially as described (15, 33). Briefly, 96-well polystyrene microtiter plates with V-formed wells (Costar™, Corning) were coated in 100 μl/well of 1 μg/ml iC3b dissolved in coating buffer (150 mM NaCl, 20 mM Tris, pH 9.4) and incubated for 1 h at 37 °C. The plates were blocked in PBS with 0.05% (v/v) Tween 20, pH 7.2, at room temperature for 1 h. Meanwhile, monocytes were thawed and added to PBS supplemented with 20% (v/v) FCS. The cells were centrifuged and resuspended in RPMI 1640 supplemented with 2% (v/v) FCS. Cells were fluorescently labeled by incubation with 2,7-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxyethyl ester (catalog no. 14562, Sigma-Aldrich) at 37 °C and 5% (v/v) CO_2 for 15 min. The cells were washed twice and resuspended in the binding buffer described above supplemented with 2.5% (v/v) FCS to 1 × 10^6 cells/ml. Fifty microliters of binding buffer supplemented with 2.5% (v/v) FCS and 0, 2, 10, or 20 μM simvastatin were added to each well. Finally, to each well, 50 μl of the fluorescence-labeled monocyte suspension was added. Following incubation at 37 °C and 5% (v/v) CO_2 for 15 min, the plates were centrifuged for 5 min at 10 × g, 20 × g, 30 × g, and 50 × g. Following each centrifugation, signals were read in a fluorescence plate reader (Victor 3, Wallac Oy), and the fraction of binding cells was estimated by comparison with the signal from uncoated wells blocked with Tween 20. All measurements for both coated and control wells were done in triplicate and were averaged and used to calculate the cell binding.

**Author Contributions**—M. R. J. performed S. P. R. and cell adhesion experiments; G. B. performed the x-ray crystallographic studies; X. Z. performed the cell adhesion studies; and M. R. J., K. K. S., A. K. L., and H. D. performed the MD studies. B. S., G. R. A., and T. V.-J. designed and planned studies. M. R. J. and T. V.-J. wrote the paper. All authors contributed to the writing of the draft and final manuscripts.

**Acknowledgments**—We thank Drs. Thorsten J. Maier and Niels Jessen for advice and Bettina W. Grumsen and Anne Marie Bindszgaard for excellent technical assistance. Allocations of time for supercomputing were generously offered by the Center for Scientific Computing, Aarhus University.

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