Computational design of an integrin I domain stabilized in the open high affinity conformation

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We have taken a computational approach to design mutations that stabilize a large protein domain of ~200 residues in two alternative conformations. Mutations in the hydrophobic core of the αMβ2 integrin I domain were designed to stabilize the crystallographically defined open or closed conformers. When expressed on the cell surface as part of the intact heterodimeric receptor, binding of the designed open and closed I domains to the ligand iC3b, a form of the complement component C3, was either increased or decreased, respectively, compared to wild type. Moreover, when expressed in isolation from other integrin domains using an artificial transmembrane domain, designed open I domains were active in ligand binding, whereas designed closed and wild type I domains were inactive. Comparison to a human expert designed open mutant showed that the computationally designed mutants are far more active. Thus, computational design can be used to stabilize a molecule in a desired conformation, and conformational change in the I domain is physiologically relevant to regulation of ligand binding.

Integrins are heterodimeric adhesion receptors that mediate cell–cell and cell–substrate interactions[1]. The α-chain of integrin αMβ2 (Mac-1) contains an inserted (I) domain of ~200 residues that is implicated in binding to protein ligands[2–4]. Crystal structures of I domains reveal that they contain a dinucleotide binding fold, with a metal ion dependent adhesion site (MIDAS) on the upper face[4–11]. The metal ion is coordinated by an acidic residue from protein ligands and residues surrounding the metal ion also contact ligands[4,12–15]. The bottom face of the I domain is connected to a putative integrin β-propeller domain[16]. Two different crystal forms of the Mac-1 I domain, termed open and closed, are hypothesized to represent the I domain in active and inactive conformations[4,5]. Although experimental data support this idea[13,17], it has remained controversial because many other I domain structures, including those from other α-subunits, have failed to reveal a corresponding open conformation[6–11]. However, a recent cocrystal of the integrin α2 I domain bound to a triple-helical peptide derived from collagen reveals that it adopts an open conformation very similar to that described for αM[15]. Three residues that directly coordinate the metal differ between the closed and open structures, and other nearby residues shift in position. These movements appear to be structurally linked to a dramatic 10 Å movement in the C-terminal α-helix. The structurally homologous G protein α-subunit undergoes a similar change in metal coordination between the GDP-bound and GTP-bound forms, which is coupled to long range structural rearrangements[4].

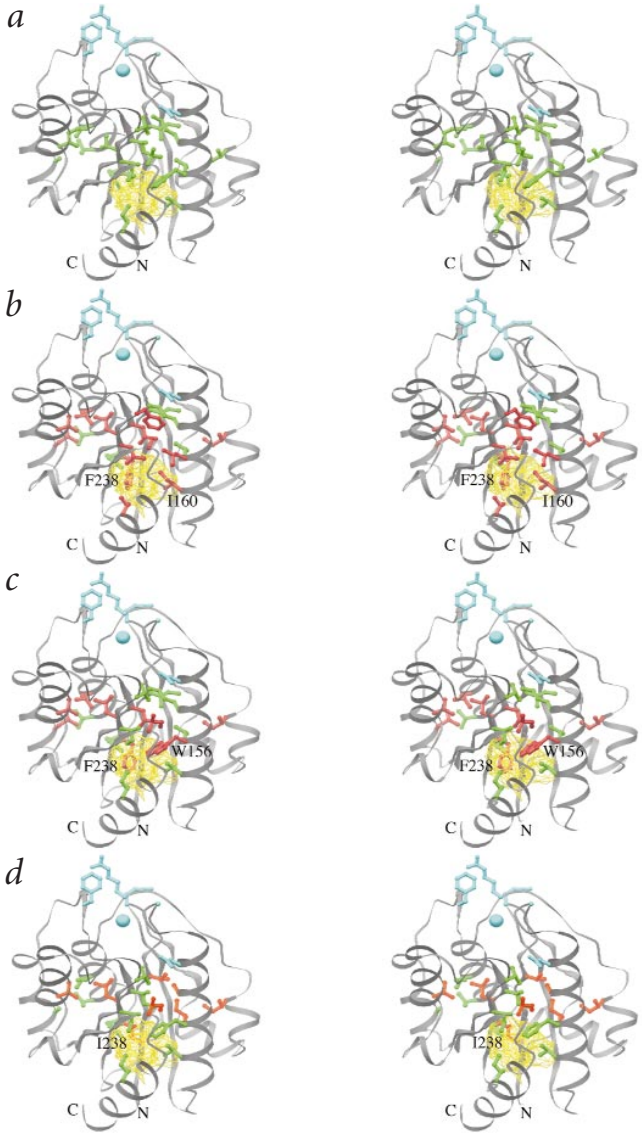

Fig. 1 Location of mutations in the Mac-1 I domain open structure. ***a***, Wild type (PDB code 1ido). Structures computed for ***b***, the ido1q mutant, ***c***, the ido1r mutant and ***d***, the ido2r mutant. Side chains of all residues that were mutated, including those in jlm2r are shown (green for wild type, red for mutant). The Mg^{2+} (large sphere) and the residues that are directly involved in the recognition of iC3b are blue[13,14]. A cavity of 202 $Å^3$ was detected in the wild type structure (1ido) but not in the designed mutants, using VOIDOO[41] with a probe of 1.4 Å, a van der Waals growth factor of 1.1 and a minumum of 5 voxels. The van der Waals surface of the cavity observed in the wild type structure (1ido) is shown in yellow in all four structures. The cavity is filled by mutations V238F and V160I in 1do1q (*b*); V238F and F156W in ido1r (*c*); and V238I in ido2r (*d*). Figure made with RIBBONS[42].

Mutations that stabilize one protein conformation relative to another have previously been found empirically, such as those in hemoglobin[18]. In addition, visual inspection by human experts has been used to predict mutations that stabilize the open conformer of the Mac-1 I domain[13]. Recently, advances in computational design have allowed mutations to be engineered that enhance the stability of small proteins on the order of 60 residues[19,20]. We have tested whether computational design could be successfully applied to larger protein domains, to stabilize one known conformer over another, and have compared our mutants to those designed by experts[13].

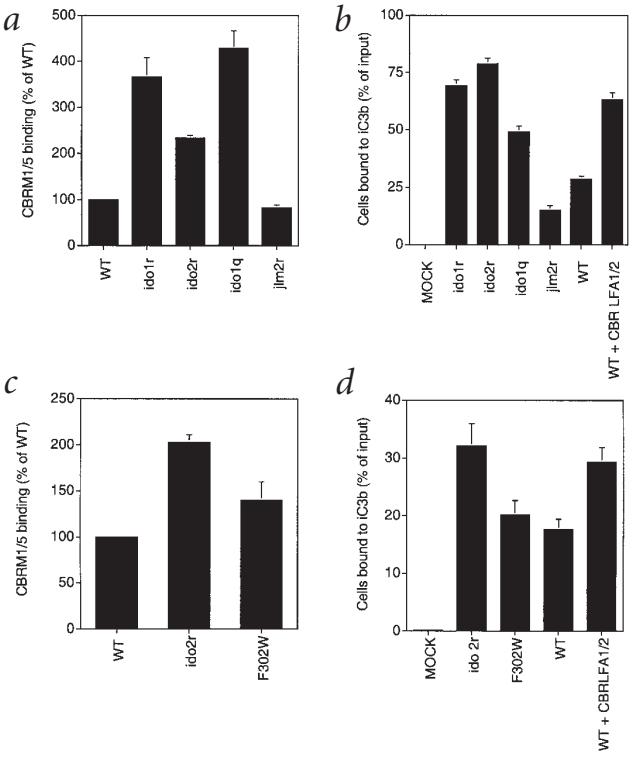

Fig. 2 Intact Mac-1 molecules with computationally designed I domains are more active than wild type in binding ligands when transiently expressed in 293T cells. Wild type αM or αM with mutant I domains were expressed transiently in 293T cells in association with wild type β2. ***a***, CBRM1/5 mAb binding. Binding of the activation dependent antibody CBRM1/5 was determined by flow cytometry as specific mean fluorescence intensity and expressed as a percentage of wild type. ***b***, Binding of transfectants to immobilized iC3b. Wild type Mac-1 in 293T cells has basal activity but can be further activated by the activating β2-chain mAb CBR LFA1/2. ***c***, Comparison of CBRM1/5 binding between the computationally designed mutant ido2r and the expert designed mutant F302W. ***d***, Comparison of ligand binding between ido2r and F302W. In (*a–d*), values are normalized to those of wild type by the binding of CBRM1/20 mAb, which recognizes the β-propeller domain of αM, and are expressed as mean ± S.E.M. of values in three (*a*,*b*) or two (*c*,*d*) independent experiments in duplicate.

Computational design

To stabilize the αM I domain in its open conformation (PDB code 1ido), or in its closed conformation (PDB code 1jlm), we utilized a computational protein design algorithm called ORBIT (for optimization of rotamers by iterative techniques) that selects amino acid sequences that are optimal for a particular target fold[19,21,22]. The algorithm considers pairwise interaction energies between amino acid side chains and between the side chains and the protein backbone. The interaction energies are calculated according to an empirical energy function that contains terms for van der Waals, electrostatics, hydrogen bonding, and solvation (see Methods). Amino acids are represented with a discrete set of allowed side chain conformations, called rotamers. The resulting rotamer-space optimization problem had a combinatorial complexity for the I domain mutants of up to 6×10^{100}, and was solved using the dead-end elimination theorem[23]. To prevent mutations that could directly affect binding of ligands such as iC3b, the design procedure was limited to residues in the protein's hydrophobic core. Core residues near the Mg^{2+} of the MIDAS, or that are partially exposed and near the bottom of the I domain where they may interact with the putative integrin β-propeller domain, were also kept as wild type. Out of 184 residues in the Mac-1 I domain, 40–45 hydrophobic core residues were included in the calculations.

Four mutant sequences each were computed based on the open and closed structures using two different solvation potentials and subsets of core residues. Three out of a total of four designed open (ido) mutants were well-expressed; all have unique amino acid substitutions (Table 1). Fewer substitutions were predicted for the closed (jlm) mutants, and only one of these, jlm2r, was constructed; it was well-expressed. All mutated side chains were buried in the core of the I domain and were distant from the MIDAS and from the residues critical for iC3b binding[13,14], which are located on the top of the I domain (Fig. 1). The mutated residues, therefore, do not directly affect binding of iC3b. The calculated energies of the selected sequences were determined in both the open (1ido) and closed (1jlm) backbones (Table 1). All of the mutant sequences had energies lower than that of wild type in the desired backbone configuration and higher than that of wild type in the undesired configuration. Thus, the designed open mutants both stabilized the αM I domain in the 1ido conformation and destabilized it in the 1jlm conformation[24]. Similar results were obtained regardless of the solvation potential used in the calculation. The calculated energy of wild type in the closed 1jlm structure was lower than that in the 1ido structure; the wild type sequence should thus favor the closed conformation (Table 1). This is consistent with the finding that in all crystal structures of αM, αL, α2 and α1 I domains determined to date, the I domain assumes a closed, 1jlm-like structure in the absence of a bound ligand or pseudo-ligand[5–11].

Ligand binding of designed I domains within αMβ2

The function of mutant I domains was first examined within intact Mac-1 αMβ2 heterodimers in transfected cells. Formation of αMβ2 heterodimers was confirmed by monoclonal antibody (mAb) CBRM1/20, which recognizes an epitope in the putative β-propeller domain of the αM subunit only after association with the β2 subunit[25,26]. mAb CBRM1/5, which binds to a region of the I domain near the MIDAS that undergoes conformational change upon activation and only recognizes activated αMβ2[17,27], was used as a probe for I domain activation. Binding of mAb CBRM1/5 was increased two- to four-fold in the three designed open mutants, and slightly decreased in the designed closed mutant (Fig. 2*a*). Ligand binding of the designed mutants was examined by adhesion of 293T cell transfectants to iC3b immobilized in microtiter plates. Wild type β2-integrins, including αMβ2 and αLβ2, are basally active in ligand binding in 293T cells[17,28]. Nonetheless, binding of all designed open mutants to iC3b was greater than that of wild type and was comparable to binding of wild type Mac-1 activated by mAb CBR LFA1/2 to the β2 subunit[29] (Fig. 2*b*). By contrast, the designed closed mutant bound to iC3b less well than wild type (Fig. 2*b*).

The function of designed I domains within intact αMβ2 was further investigated in stably transfected K562 cells (Fig. 3), in which αMβ2 shows little basal activity[17,28]. αMβ2 heterodimers containing wild type or the designed closed mutant I domain showed little expression of the CBRM1/5 activation epitope (Fig. 3*a*,*b*) or binding to iC3b (Fig. 3*c*). By contrast, αMβ2 containing designed open mutant I domains showed marked expression of the CBRM1/5 epitope (Fig. 3*a*,*b*) and binding to iC3b (Fig. 3*c*). Binding to iC3b was increased 10- to 13-fold relative to wild type and was similar to binding by wild type in the presence of the activating mAb CBR LFA-1/2 (Fig. 3*c*). Binding of the designed open mutants to iC3b appeared to be maximal, because it could not be further increased by the activating mAb CBR LFA-1/2 (Fig. 3*d*). The αMβ2 heterodimer containing the designed closed mutant

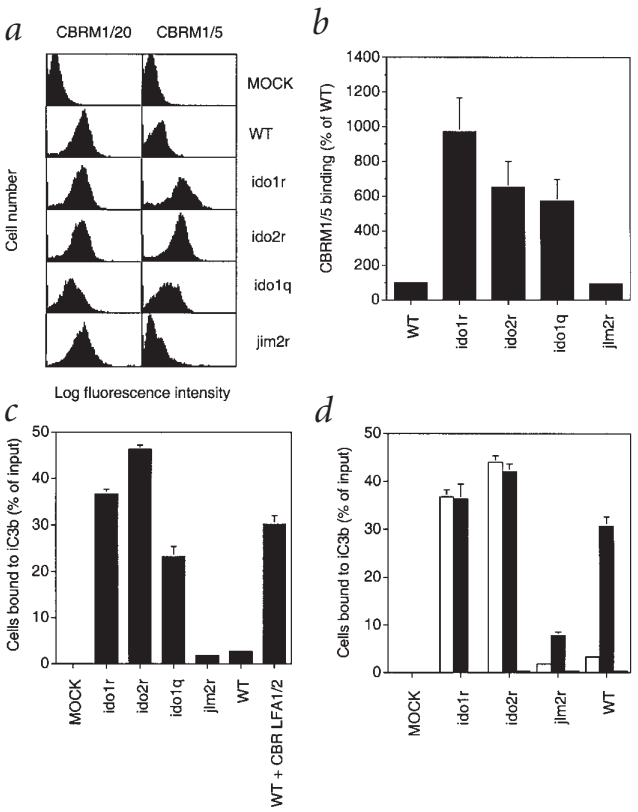

Fig. 3 αMβ2 heterodimers with computationally designed open and closed I domains are active in binding iC3b, and resistant to activation, respectively, when stably expressed in K562 cells. ***a***,***b***, Immunofluorescence flow cytometry. ***c***, Binding of transfectants to immobilized iC3b. Binding of wild type was tested in the presence and absence of CBR LFA1/2, an activating mAb that binds to the β2 subunit. ***d***, Effect of blocking or activating mAbs. Transfectants were incubated in the presence of X63 (control IgG, white bar), CBR LFA1/2 (central black bar) or CBRM1/5 (activation dependent and inhibitory I domain mAb, right black bar that may be too short to see). In (*b*–*d*), the values were normalized by the ratio of mutant/wild type CBRM1/20 mAb specific fluorescence intensity and expressed as mean ± S.E.M of three independent experiments.

I domain was resistant to activation; binding to iC3b activated by CBR LFA-1/2 mAb was reduced 85% for jlm2r compared to wild type (Fig. 3*d*). All mutant and wild type heterodimers bound CBR LFA-1/2 mAb equally well (data not shown). All binding was specific, since it was completely inhibited by mAb CBRM1/5 (Fig. 3*d*).

Function of designed I domains in isolation

The I domain of integrins plays a major role in binding ligand, as shown by muations in the I domain and mAbs to the I domain that block ligand binding; however, other domains appear to cooperate in creating the ligand binding interface[30,31]. For example, when the isolated I domain of integrin αLβ2 is expressed on the cell surface, ligand binding is much weaker than with an intact integrin and is only detected when the I domain is expressed at very high levels[32]. Since shape shifting in I domains is proposed to be related to conformational movements elsewhere in integrins[16], it was important to examine ligand binding by the mutant I domains in isolation from other integrin domains. Therefore, mutant αM I domains were expressed transiently on the surface of 293T cells or stably on K562 cells after fusion to the αM N-terminal signal sequence and a C-terminal platelet derived growth factor receptor (PDGFR) transmembrane domain. Wild type I domain and designed closed I domain showed essentially no binding to iC3b (Fig. 4*a*,*b*). By contrast, designed open mutant I domains expressed at the same level on the cell surface showed robust binding to iC3b (Fig. 4*a*,*b*). This binding was enhanced by the addition of Mn^{2+} (Fig. 4*b*), but completely inhibited by EDTA, showing that ligand binding was dependent on the MIDAS (Fig. 4*c*).

Comparison with an expert designed mutant

In a comparison of the closed and open conformations of the Mac-1 I domain, Phe 302 was found to be buried in the closed form but completely exposed in the open form[4]. To stabilize the open form, Phe 302 was mutated to Trp, Arg, or Tyr[13]. It was reported that the F302W mutant was activated in ligand binding and the F302R mutant was not expressed, while the F302Y mutant resembled wild type in ligand binding. Thus, two of three expert designed mutants were expressed, one of which was activating, compared to three of four computationally designed mutants expressed, all three of which were activating.

For the purposes of direct comparison, αMβ2 heterodimers containing I domains with the F302W mutation or computationally designed mutations were expressed in 293T cell transfectants. CBRM1/5 mAb bound to the F302W heterodimer at a level intermediate between wild type and ido2r heterodimers (Fig. 2*c*). αMβ2 heterodimers with the F302W mutation bound to iC3b only slightly better or similarly to wild type, in contrast to the marked enhancement seen with designed open mutants or activation with CBR LFA-1/2 mAb (Fig. 2*d*). The F302W mutation was found to increase transfectant binding to iC3b by two-fold in a different assay configuration[13].

Isolated I domains containing the F302W mutation were slightly more active than wild type in binding the ligand iC3b; however, the computationally designed open I domains were 10-fold more active than the F302W mutant in binding iC3b (Fig. 4*a*). The affinity of the F302W mutant I domain for iC3b was previously measured with BIAcore in the presence of Mg^{2+} (ref. 13), although in contrast to the present work, binding was not measured in the presence of EDTA. Scatchard analysis showed that F302W and wild type I domains bound with equal affinity to iC3b, based on the total amount of protein in I domain preparations[13]. However, mass transport limited binding suggested that only a subset of proteins in these preparations bound ligand, and that this active subset was 2.3-fold larger in the F302W mutant than in the wild type I domain preparation. Therefore, it was claimed that the F302W mutation stabilized the I domain in the active conformation[13]. However, this argument is a conundrum because it can only be compatible with the findings of equal overall affinity of the two preparations if the active conformer in the F302W preparation binds ligand with 2.3-fold lower affinity than the active conformer in the wild type preparation.

Conclusion

We computationally designed mutations that energetically favor either the open or closed conformation of the αM I domain. All three open mutants bound the ligand iC3b much better than wild type, either in αMβ2 heterodimers or as isolated I domains. Neither wild type nor closed mutant isolated I domains bound ligand. Furthermore, in αMβ2 heterodimers, the closed mutation inhibited activation of ligand binding (Figs 2*b*, 3*d*). These findings show that the open and closed conformations have high and low affinity for ligand, respectively, and demonstrate that computational design can be used to shift the equilibrium between different conformations of a protein structure. Lack of binding to

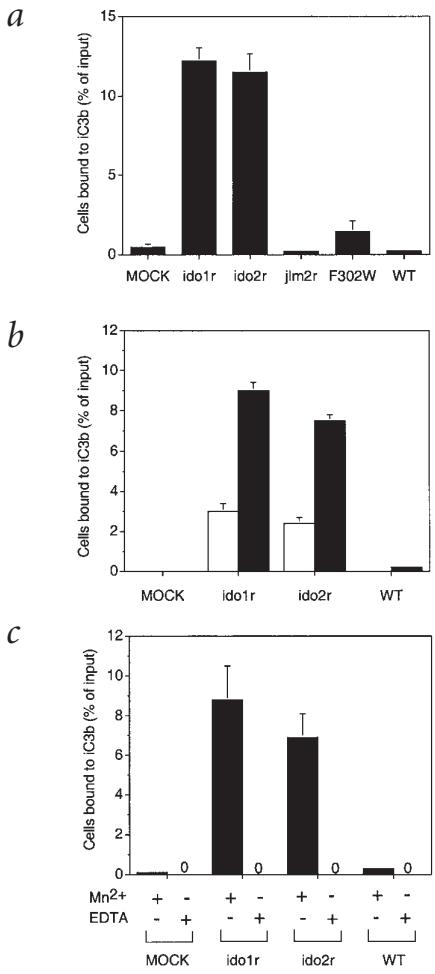

Fig. 4 Isolated computationally designed open I domain mutants bind ligands, whereas wild type and designed closed I domains do not. ***a***, Binding of 293T transient transfectants to iC3b. Binding was performed in L15 medium/fetal bovine serum (FBS), which contained Mg^{2+} and Ca^{2+}, with 1 mM Mn^{2+}. ***b***, Binding of K562 transfectants to iC3b, and its enhancement with Mn^{2+}. Binding was performed in L15 medium/FBS, which contained Mg^{2+} and Ca^{2+}, without (white bar) or with (black bar) 1 mM Mn^{2+}. ***c***, Binding of K562 transfectants to iC3b in the presence or absence of divalent cation. Binding was performed in HEPES/NaCl/glucose supplemented with 1 mM Mn^{2+} (black bar) or 2 mM EDTA (white bar; values are zero in all columns). Immunofluorescent staining with mAb to a c-*myc* tag (Invitrogen) and CBRM1/1 and CBRM1/2 mAb to the I domain showed that expression of the 1r and 2r mutants was 90% and 93% of wild type, respectively.

iC3b by the isolated wild type I domain is consistent with its lower computed energy in the closed as opposed to the open conformation, and the finding that all I domain structures determined in the absence of bound ligand or pseudo-ligand assume the closed conformation[6–11]. Furthermore, our results demonstrate that the open conformation, now seen in two different types of I domain structures[5,15], is physiologically relevant for ligand binding by integrins on the cell surface.

What factors might explain the greater activity of the computationally designed mutants than the expert designed mutant in side by side comparisons? Among the factors that could contribute to global protein stabilization, packing of the hydrophobic core plays a dominant role[33,34]. A cavity in the hydrophobic core of the wild type 1ido structure (Fig. 1*a*) may accommodate repacking of the hydrophobic core along a shape shifting pathway that does not require unfolding. In the computationally designed mutants these cavities were filled by introducing larger side chains (Fig. 1*b–d*). The F302W mutation was designed to favor exposure of residue 302 in the open conformation. This mutation should disfavor burial of this residue in the closed conformation, but may not uniquely stabilize the open conformation because there may be many intermediates along the shape shifting pathway in which residue 302 is exposed. Thus, we believe that our computational approach of repacking the hydrophobic core may uniquely favor the open conformation more than the mutation of a residue that is surface exposed in the open conformation.

Computationally designed protein conformers open a wide range of opportunities in biology and medicine. Our work is with a domain of above average size (200 residues) and thus almost all domains are computationally within reach. Although shape shifting is unusual for adhesion molecules, many signaling molecules and regulatory enzymes undergo conformational change. Freezing in specific conformational states can enable their unique biological functions to be elucidated *in vivo* and *in vitro*. Such designer proteins may also have broad pharmaceutical applications. For example, high affinity I domains may be useful in screening for small molecule antagonists, or for antagonizing integrin function in inflammatory disease.

Methods

Computational modeling. Computational details, potential functions, and methods for defining core residues were as described[19,33,35–37]. A scale factor of 0.9 was used for all van der Waals radii[34]. 'Solvation potential 1' utilized 23.2 cal mol^{-1} $Å^{-2}$ to benefit hydrophobic burial[36], and a hydrophobic exposure penalty of the same magnitude to penalize residual hydrophobic exposure[34]. 'Solvation potential 2' (ref. 38) utilized 48.0 cal mol^{-1} $Å^{-2}$ for the hydrophobic burial benefit and an exposure penalty 1.6 times the magnitude of the burial benefit. Energies for polar surface area burial were not included in any of the calculations; however, a penalty of 2.0 kcal mol^{-1} was applied to the burial of polar hydrogens not involved in hydrogen bonds[37]. Out of 184 residues in the Mac-1 I-domain (Asp 132–Lys 315), 56 were initially defined as core residues. Residues 176 and 267, both hydrogen bonded tyrosines; and residues Phe 234, Ile 265, Val 296 and Leu 305, partially exposed and near the bottom of the I domain where they may interact with the β-propeller domain of integrins, were excluded from the calculations. For calculation q, the 42 residues Ile 135, Ala 136, Phe 137, Leu 138, Ile 139, Ile 145, Phe 150, Phe 156, Val 157, Val 160, Leu 164, Phe 171, Leu 173, Phe 186, Val 199, Ile 202, Ala 212, Ile 215, Val 218, Val 219, Leu 222, Phe 223, Ala 229, Ala 233, Leu 237, Val 238, Val 239, Ile 240, Val 255, Ala 259, Val 264, Val 268, Ile 269, Val 271, Ala 274, Leu 284, Ile 287, Ala 288, Val 299, Ala 304, Ile 308, and Leu 312 were allowed to become Ala, Val, Leu, Ile, Phe, Tyr, or Trp; and the three residues Met 153, Met 161, and Met 174 were allowed to be Ala, Val, Leu, Ile, Met, Phe, Tyr, or Trp. Residues Leu 170, Phe 189, Leu 198, Ile 236, and Phe 297 had some solvent exposure and were not allowed to mutate but were allowed to change rotamer. The same residues were used for calculation r, except that three residues in the C-terminal α-helix, Ala 304, Ile 308, and Leu 312, were not allowed to mutate but were allowed to change rotamer. In the jlm2r calculation, we used the same core residues as in the r calculation except for omitting Val 271 and Ala 274, which are surface exposed in the 1jlm structure.

Plasmid construction and mutagenesis. Recursive PCR with 10 overlapping oligonucleotides[38,39] was used to construct a 649 bp I domain mutant DNA fragment. Unique *Bam*HI and *Kpn*I sites were introduced at nucleotide positions 451 and 1,681, respectively, using silent mutations. The PCR products containing mutated I domain cDNA fragments were cut with *Bam*HI and *Kpn*I and swapped into

Table 1 Computationally designed mutants[1]

	WT	ido1q	ido1r	ido2r	jlm2r
Backbone		Energy[2] (kcal^{-1} mol^{-1})			
1ido	-1,037	-1,145	-1,138	-1,116	-678
1jlm	-1,059	+82,758	-840	-1,000	-1,086
Position			Residues		
139	I	-	-	V	-
153	M	-	-	A	-
156	F	L	W	-	-
157	V	-	-	I	-
160	V	I	-	-	-
199	V	I	I	I	-
215	I	L	L	-	V
219	V	-	-	-	I
223	F	-	-	-	L
238	V	F	F	I	I
239	V	L	L	L	-
240	I	L	L	-	-
259	A	L	L	-	-
269	I	L	-	-	-
271	V	F	-	-	-
287	I	V	V	V	-
299	V	A	I	I	-
308	I	V	-	-	-

[1]Mutants are named according to the structure that was stabilized (ido or jlm), the solvation potential used (1 or 2) and the definition of core residues (q or r).
[2]The lowest energy rotamer configuration was calculated for each sequence in the 1ido structure, and cross-calculated in the 1jlm structure, using both solvent potentials; all 50 core residues were used in order to make the q and r energies comparable. Results are shown for solvent potential 1 and were similar for potential 2. A severe clash of the side chain of Phe 271 with the backbone caused the high energy of the 1q sequence in the 1jlm structure; no movement of the backbone is allowed by the design method.

wild type αM cDNA. Human β2 subunit cDNA[40] was subcloned into pcDNA3.1(+). To construct isolated, cell surface I domains, the signal peptide and following 9 bp from the 5′ end of αM were ligated to the sequence Gly 127–Pro 348 containing the I domain. *Hin*dIII and *Sal*I sites were introduced immediately adjacent to the 5′ and 3′ ends of this fragment, respectively. The *Hin*dIII-*Sal*I fragment was subcloned in frame with and 5′ to a c-*myc* tag and the PDGFR transmembrane domain in vector pDisplay™ (Invitrogen) and further subcloned into pcDNA3.1/Hygro. All DNA amplification was carried out with *Pfu* DNA polymerase (Stratagene) and the final constructs were verified by sequencing.

Cell lines and transfection. 293T cells were maintained and transfected by calcium phosphate coprecipitation[17] by using 12 μg of α-subunit and 8 μg of β-subunit cDNA or 10 μg I domain–PDGFR cDNA. After 48 h, cells were detached with 5 mM EDTA in phosphate-buffered saline (PBS) and subjected to flow cytometry or adhesion assays.

For stable K562 cell lines, 2 μg of *Ssp*I linearized pcDNA3.1/Hygro(+) containing the αM cDNA was cotransfected with 20 μg of the *Ssp*I linearized wild type β2 cDNA in pcDNA3.1(+) by electroporation as described[17,28]. Immunofluorescence flow cytometry was as described[26].

Binding of transfectants to immobilized iC3b. Binding of fluorescently labeled cells to human complement component iC3b immobilized in flat bottomed 96-well plates was as described[17,28], except that plates were blocked with 2% nonfat milk in PBS; and binding assays using 293T cell transfectants were performed at room temperature.

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