Detailed Comparison of Two Molecular Models of the Human CD40 Ligand with an X-ray Structure and Critical Assessment of Model-based Mutagenesis and Residue Mapping Studies*

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Jürgen Bajorath‡

From MDS Panlabs, Computational Chemistry and Informatics, Bothell, Washington 98011-8805 and the Department of Biological Structure, University of Washington, Seattle, Washington 98194

The interactions between the B cell receptor CD40 and its ligand on T cells are critical for the integrity of immune responses. The human CD40 ligand gp39, a tumor necrosis factor-like protein, has been the subject of intense efforts to identify the receptor-binding site and to analyze naturally occurring mutations that compromise gp39 function in vivo. These investigations relied heavily on molecular models of gp39, built in the presence of only ~25% sequence identity to tumor necrosis factor. The x-ray structure of gp39 has made it possible to assess modeling accuracy and to evaluate the results of model-based mutagenesis analyses. Although the models display local errors, their accuracy was sufficient to predict the CD40-binding site, to map natural mutations, and to rationalize their effects. One of five gp39 residues critical for CD40 binding was displaced in the models, and 1 of 21 point mutants was incorrectly classified. Factors most important for the reliability of the molecular models and their successful applications were valid sequence alignments and the focus of experimental studies on regions of high prediction confidence. Analysis of mutagenesis experiments correlated with anti-gp39 monoclonal antibody binding studies to assess the conformational integrity of mutant proteins.

The CD40 ligand (CD40L, gp39) is a type II transmembrane protein predominantly expressed on the surface of activated T cells (1, 2). Interactions between gp39 and its receptor, CD40, a type I transmembrane protein on B cells (3, 4), are critical for T cell-dependent B cell proliferation and the regulation of the humoral immune response (5, 6). The critical role of CD40/gp39 receptor/ligand interactions for effective antibody production and isotype switching was firmly established by the discovery of naturally occurring mutations in gp39 that cause X-linked hyper-IgM (XHIM)1 syndrome (7). Patients with defective gp39 genes do not mount an effective humoral immune response and are severely immunocompromised (8). Thus, the study of CD40/gp39 interactions has been a focal point of intense efforts to better understand T cell-dependent B cell activation and the basis of productive immune responses (6).

CD40 and gp39 are members of the tumor necrosis factor (TNF) receptor (9, 10) and TNF (10) superfamilies, respectively. The extracellular region of gp39 displays ~25% sequence identity to TNF proteins (2, 7). To aid in the analysis of CD40-gp39 interactions, we generated, in the absence of an experimentally determined three-dimensional structure, two molecular models of gp39 by comparative modeling (11, 12). These models were used to compare x-ray structures of TNF-α (13) and TNF-β (14, 15). Initially, we constructed a TNF-α-based model of gp39 (AM) (7), while others reported a three-dimensional model of the murine CD40 ligand (16). The gp39 model was used to analyze the location of natural gp39 mutations isolated from XHIM patients (7) and to guide an initial mutagenesis effort to identify gp39 residues important for the interaction with CD40 (17). After an x-ray structure of the TNF receptor:TNF-β complex (15) became available, we also generated a TNF-β-based molecular model of gp39 (BM) (18). This model was used to continue the mutagenesis analysis of the CD40/gp39 interaction, to outline the receptor-binding site of gp39 (18), and to carry out a three-dimensional survey of 21 naturally occurring gp39 mutations (19).

The more recently determined x-ray structure of gp39 (20) has made it possible to assess the accuracy of our comparative structure prediction and to evaluate if the models were applied in a meaningful way and if the obtained results were valid. Since a substantial body of experimental analysis was based on the gp39 models (7, 17–19), a critical assessment of these studies should be of considerable interest in addition to the prediction exercise. Therefore, a detailed comparison of the gp39 models and x-ray structure and a reevaluation of our binding site analysis and residue mapping studies were carried out. The results are presented herein.

With the exception of a short β-strand at the edge of a sheet, the core regions and β-strands of gp39 were well predicted. Substantial conformational errors were observed in several loops. Modeling inaccuracies were the source of subsequent errors including the misinterpretation of 1 of 21 naturally occurring gp39 mutants and the relative displacement of a residue important for CD40 binding by 6–7 Å. However, the analysis shows that surface residues could be selected for mutagenesis with confidence and that residues important for CD40 binding were successfully predicted and experimentally confirmed. The CD40-binding site was correctly mapped to the interface between adjacent gp39 monomers; and the locations of natural gp39 mutations were well predicted, and their putative effects were rationalized in a meaningful way.

MATERIALS AND METHODS

Gp39 modeling, mutagenesis, and ligand binding experiments have been described in detail (7, 17, 18) and are briefly summarized herein. Methods applied to analyze and compare the models and x-ray structure are also described.

The paper is dedicated to the memory of my colleague and friend Dale E. Yelton.

‡ To whom correspondence should be addressed: MDS Panlabs, 11804 North Creek Pkwy. S., Bothell, Washington 98011-8805. Tel.: 425-487-8297; Fax: 425-487-8262; E-mail: j.bajorath@panlabs.com.
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Model Building—After generating structure-oriented sequence alignments of the extracellular region, gp39 models AM (7) and BM (18) were generated using TNF-α (13) and TNF-β (15) as structural templates, respectively. Regions predicted to be structurally conserved in TNF proteins and gp39 were included in the models. Nonconserved residue replacements were carried out using a side chain rotamer search procedure (21, 22). Conformations of regions including insertions or deletions and nonconserved loop conformations were modeled using systematic conformational search with CONGEN (23). Loop conformations with minimum solvent-accessible surface and low potential energy were selected following a previously described protocol (24). The molecular contacts of the initially assembled models were refined by limited energy minimization calculations (7, 18), and the stereochemical quality of the models was confirmed using PROCHECK (25). The sequence-structure compatibility of the refined models was assessed using three-dimensional profile (26) or energy profile (27) analysis. Similar procedures were applied to generate three-dimensional models of CD40 (18, 28) based on the x-ray structure of the TNF-bound TNF receptor (15).

Mutagenesis and Binding Experiments—Residues were selected for mutagenesis based on visual inspection of the molecular models. A focal point was the interface region between adjacent gp39 monomers in the homotrimer. Point mutants were constructed by overlap extension polymerase chain reaction (17) and expressed in soluble recombinant form as gp39-CD8 fusion proteins (17, 18). A panel of conformationally sensitive anti-gp39 monoclonal antibodies (mAbs) was generated and used to assess the gross structural integrity of mutant proteins (17, 18). Only mutants that consistently bound to these mAbs comparable to the wild type were considered structurally sound. These gp39 mutant proteins were tested for receptor binding in enzyme-linked immunosorbent assays using recombinant CD40-Ig fusion protein (17) and also in cell binding assays (17, 18).

Analysis of Naturally Occurring Mutations—Residues affected by naturally occurring gp39 mutations were mapped on the models using computer graphics (7, 19). These positions were then classified as described under “Results.” This classification made it possible to predict the effects of these mutations.

Structure Comparison—Coordinates of the gp39 monomer (20) were obtained from the Brookhaven Protein Data Bank (29) (PDB code 1ALY). The active homotrimeric form of gp39 (20) was generated from these coordinates by applying 3-fold symmetry around the crystallographic c axis (space group H3) using an awk script. The gp39 models and x-ray structure were compared using the Biopolymer module of InsightII (MSI, San Diego, CA) and ALIGN (30). Secondary structure calculations and contact analysis were performed using PROCHECK (25) and InsightII. The molecular models were compared with the x-ray structure using different superposition sets and after superposition of residues most conserved across the TNF family (10). Graphical representations were produced using InsightII and processed as RGB images or postscript files.

RESULTS

A detailed comparison of the gp39 molecular models (AM and BM) and x-ray structure is presented first, followed by an analysis of model-based mutagenesis experiments, binding site prediction, and residue mapping studies.

Three-dimensional Structure of the CD40 Ligand—As predicted (7), gp39 adopts the TNF fold (13, 14), which consists of a sandwich of two β-sheets with jelly roll topology (13). Like TNF, the extracellular region of gp39 forms a 3-fold symmetric homotrimer. Fig. 1 shows the gp39 x-ray structure.

Sequence Comparison—After initial detection of weak sequence similarity between gp39 and TNF molecules (2), a detailed comparison of TNF and gp39 sequences was carried out (7, 18) in light of TNF x-ray structures. Model building of gp39 was based on the results of these structure-oriented (topological) sequence alignments. Fig. 2 summarizes the results of TNF/gp39 sequence comparisons. Although the aligned regions in TNF and gp39 show only ~25% sequence identity, a number of key residues, which are conserved in TNF-α and TNF-β and are important for the structural integrity of these proteins, are conserved in gp39 (Fig. 2). Mapping of conserved residues on the (then publicly available) x-ray structure of TNF-α confirmed the conservation or conservative replacement of a number of core residues in gp39 (7). In addition, 22 of 29 residues at TNF-α dimer or trimer interfaces were conserved, at least in residue character (7, 13). On the basis of these findings, gp39 was predicted (i) to display a three-dimensional structure more similar to TNF than indicated by the relatively low level of similarity.
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Fig. 2. Alignment of sequences for model building. A summary of alignments of gp39 with human TNF-α and TNF-β is shown. The sequence alignments were based on structural considerations (e.g., conservation of core residues). Residue numbers are given for gp39, and β-strands are underlined. Conserved and largely conserved residues are shaded. Residues that were misaligned in AM and BM are shown in italics. Residues whose α-carbon positions were, after optimal superposition, in at least one of the two models, are equivalent for both AM versus TNF-α and BM versus TNF-β.

Alignment Accuracy—The gp39 molecular models included residues 121–261. After optimal superposition of AM, BM, and 1ALY, the accuracy of the initial alignments was determined (Fig. 2). With the exception of the region encompassing residues 145–151, which include a topological misalignment (see below), residues in β-strands and their periodicity (exposed and buried residues) were correctly predicted. The positions of six of eight insertions/deletions in gp39 relative to TNF were also predicted and experimentally determined gp39 monomer structures. AM is overall more similar to 1ALY than are BM, and TNF-β.

Template and Target Structures—Pairwise structure comparisons including TNF templates were also carried out. These calculations were performed using ALIGN, which determines optimal superpositions based on pairwise residue comparisons and deletes pairs from the comparison that exceed three times the r.m.s.d. for superposition of all residues. The average backbone r.m.s.d. for gp39 residues 121–261 was reduced to 2.6 Å, and the differences between AM and BM match the corresponding positions in 1ALY within 1.5 Å. Superposition of most conserved regions in proteins ensures that deviations in more variable regions are not reduced due to averaging over many residues. Table I summarizes r.m.s.d. values obtained for model/structure comparisons. The average backbone r.m.s.d. for gp39 residues 121–261 was reduced to ~1.8 Å when the six misaligned residues and the two loop regions with the largest errors were excluded from the comparison. The average backbone and all atom r.m.s.d. values for all residues in β-strands and ⍺-turns were ~1 and ~1.4 Å, respectively. The smallest r.m.s.d. values were observed, as to be expected, for the subset of the 20 most conserved residues in TNF and gp39. Fig. 3 shows the comparison of a dimer interface in gp39, obtained by superposition of the trimers. The arrangement of the monomers is very similar in the models and x-ray structure.

Main Chain Modeling—Fig. 3 shows a comparison of the predicted and experimentally determined gp39 monomer structures, obtained by superposition of the 20 most conserved residues (Fig. 2). The α-carbon positions of ~60% of residues included in AM and BM match the corresponding positions in 1ALY within 1.5 Å. Superposition of most conserved regions in proteins ensures that deviations in more variable regions are not reduced due to averaging over many residues (32). Table I summarizes r.m.s.d. values obtained for model/structure comparisons. The average backbone r.m.s.d. for gp39 residues 121–261 was reduced to ~1.8 Å when the six misaligned residues and the two loop regions with the largest errors were excluded from the comparison. The average backbone and all atom r.m.s.d. values for all residues in β-strands and ⍺-turns were ~1 and ~1.4 Å, respectively. The smallest r.m.s.d. values were observed, as to be expected, for the subset of the 20 most conserved residues in TNF and gp39. Fig. 4 shows the comparison of a dimer interface in gp39, obtained by superposition of the trimers. The arrangement of the monomers is very similar in the models and x-ray structure.

Fig. 3. Comparison of gp39 monomers. The molecular models AM (blue) and BM (green) were superposed on the x-ray structure (red). A stereo view of the α-carbon traces is shown. Loop regions with the largest errors in both models are shown in black. For structure comparison, the same color code is used in all figures.
TABLE I
Overall comparison of gp39 molecular models and x-ray structure

The TNF-α (AM) and TNF-β (BM)-based gp39 monomer models were superimposed on the x-ray structure (PDB code 1ALY) using different residue sets (subset). Resulting backbone (b_r.m.s.d.) and all atom (a_r.m.s.d.) root mean square deviations were calculated. Subset S1, all residues (121–261) included in the models; S2, S1 without residues 145–151 (region with a topological misalignment in AM and BM); S3, S1 without two mismodeled loops (residues 132–136 and 180–186); S4, S1 without residues 132–136, 145–151, and 180–186; S5, all residues in β-strands and β-turns of 1ALY; S6, 20 residues most conserved across the TNF family (see Fig. 1). r.m.s.d. values for α-carbon superpositions were similar to b_r.m.s.d. values: for example, for S1, AM/1ALY, 2.49 Å; and BM/1ALY, 2.70 Å.

| Subset | AM b_r.m.s.d. (a_r.m.s.d.) | BM b_r.m.s.d. (a_r.m.s.d.) |
|--------|----------------------------|---------------------------|
| S1     | 2.49 (3.46) 2.72 (3.74)    |                           |
| S2     | 2.43 (3.27) 2.64 (3.53)    |                           |
| S3     | 1.87 (2.80) 2.94 (3.07)    |                           |
| S4     | 1.73 (2.47) 1.86 (2.73)    |                           |
| S5     | 1.02 (1.71) 1.04 (1.79)    |                           |
| S6     | 0.91 (1.53) 1.00 (1.37)    |                           |

Loop Modeling—In AM, loops with insertions or deletions relative to TNF-α were modeled by ab initio conformational search, whereas the backbone conformations of TNF-α were retained for loops predicted to have the same length in gp39. In the construction of BM, the protocol was modified. Loops with the same length in TNF-β and gp39 but with different sequences were also modeled by conformational search. Two of 11 modeled loops in gp39, residues 142–146 and 149–151, could not be compared since the predicted and observed sizes of these loops differed by one residue each (see above). Table III summarizes the comparison of modeled and crystallographic loop conformations. Representative examples are shown in Fig. 5. Large backbone r.m.s.d. values of 2–3 Å relative to the experimentally determined conformations were observed for two loops in both models, residues 132–136 and 180–186, regardless of how the loops were modeled. Conformational deviations were largest for loop 180–186, which contains an α-helical turn in 1ALY. A large segmental shift was observed for loop 132–136 with relative α-carbon displacements of up to ~12 Å. Five of nine loop conformations in AM and/or BM were predicted with backbone r.m.s.d. values of ~1 Å or better. The best backbone r.m.s.d. values for loops modeled by conformational search were ~1 Å, whereas backbone conformations of β-turns, correctly predicted to be conserved in gp39 and TNF, agreed within experimental accuracy (i.e. 0.2–0.3 Å).

Side Chain Modeling—The side chain conformations of non-conserved residues were approximated by calculation of low energy combinations of standard rotamers (21, 22). Side chain conformations in structurally conserved regions were compared (33). For residues in AM and BM with α-carbon positions within 1.5 Å of the experimentally determined positions, ~54 and ~48% of the side chain rotamers (χ1 and χ2 angles) were correctly predicted, and differences between incorrectly predicted and observed conformations were often small.

Analysis of the CD40-binding Site—The gp39 molecular models were used, in combination with site-directed mutagenesis, to identify residues important for CD40 binding and to delineate the binding site. Five gp39 residues were identified that, when mutated, significantly reduced CD40 binding while displaying wild type-like mAb binding profiles (17, 18). Fig. 6 shows the comparison of predicted and experimentally determined positions of these residues. As predicted, all residues map to the protein surface and cluster at the interface between adjacent monomers, which suggested the presence of three equivalent binding sites in the trimer (18). Lys-143, Arg-203, and Gln-220 are very similar in the models and x-ray structure. Tyr-145 and Tyr-146 map to the loop and edge strand where the local alignment was shifted by one residue (Fig. 2). Whereas Tyr-145 at the loop/strand junction is not much displaced, the β-carbon of Tyr-146 in the strand is displaced by ~6.5 Å in the models due to the shift in strand register. This represents the largest error in the prediction of the binding site.

Classification of Naturally Occurring gp39 Mutants—The gp39 models were also used to predict the location of natural point mutations in gp39 (7), identified in defective gp39 genes of patients with XHIM syndrome, a severe form of immunodeficiency (8). A total of 21 point mutations were mapped on the models to better understand the distribution and putative effects of these mutations (7, 19). Taking the results of the mutagenesis analysis into account, it was possible to classify these mutations as likely to compromise (i) the packing of the hydrophobic core, (ii) the integrity of the dimer or trimer interface, and (iii) interactions within the binding site region. The reassessment of this analysis based on the gp39 x-ray structure revealed that only 1 of 21 mutations was misinterpreted. Arg-181 is part of the incorrectly modeled loop 180–186. This residue is largely buried in the structure and not exposed, as originally predicted. Interestingly, R181W was the only naturally occurring gp39 mutation in this survey not associated with XHIM syndrome (19).

Fig. 7 shows the location of the natural double mutant S128R/E129G, which renders gp39 inactive and causes XHIM syndrome (7). In the x-ray structure, Glu-129 forms an ionic interaction with Lys-143, a residue important for CD40 binding, and this interaction was suggested by both molecular models. Mutation of Lys-143 to alanine abolished CD40 binding, whereas mutation of Glu-129 to alanine did not affect the CD40/gp39 interaction (17, 18). It was concluded that Lys-143, but not Glu-129, was important for CD40 binding and that a salt bridge between Glu-129 and Lys-143 was not critical for the structural integrity of gp39. However, mutation of Glu-129 to glycine, mimicking the natural mutation, significantly reduced CD40 binding (17), presumably by causing a structural perturbation. The molecular models also suggested that mutation of Ser-128 (which is partially buried in 1ALY) to arginine would disrupt the structure of gp39 (17, 19).

Monoclonal Antibody Binding Studies—Generated mutant proteins were tested for binding to a panel of anti-gp39 mAbs, which did not show reactivity in Western blots and were thus conformationally sensitive (17, 18). We selected predicted surface residues for mutagenesis, and reexamination of the selected mutagenesis sites based on the x-ray structure confirmed these predictions. The majority of designed mutations did not result in significant loss of mAb binding, suggesting that the mutant proteins were conformationally sound (17, 18). Other mutations disrupted mAb binding. For example, the E129A mutant, which bound CD40 like wild-type gp39, was consistently recognized by mAbs, whereas the naturally occurring E129G mutant, which failed to bind CD40, showed significantly reduced CD40 binding (17). Similarly, both the S128R (see above) and S128R/E129G mutant proteins failed to bind mAbs (17). Thus, in retrospect, anti-gp39 mAb binding profiles were reliable tools to assess overall structural integrity of gp39 mutant proteins.

DISCUSSION

The studies on gp39 provide an example for extensive applications of molecular models in experimental studies. How reliable were the results, considering the inherent limitations of model building? The analysis presented here provides some insights into the opportunities and limitations of this approach.
The accuracy of the gp39 structural predictions can be compared with results obtained in the Critical Assessment of Techniques for Protein Structure Prediction Meetings CASP1 and CASP2 (34–36). CASP predictions have shown that reasonable protein models can be generated in the presence of sequence identity as low as 25% (35). The accuracy of the gp39 models is comparable to state-of-the-art models obtained by comparative modeling at this low level of sequence identity (main chain r.m.s.d. values of 2.2–2.5 Å) (34–36). As a reference, the backbone (all atom) r.m.s.d. for independently determined structures of the same protein is often 0.5 Å (~1 Å) (35). At sequence identity below 25%, modeling accuracy decreases significantly, as it becomes increasingly difficult to generate accurate sequence alignments of templates and targets (35–37).

### Table II

| Structure pair | Residue pairs | Cα r.m.s.d. Å |
|---------------|--------------|---------------|
| TNF-α/TNF-β   | 130          | 0.90          |
| TNF-α/1ALY    | 128          | 1.49          |
| TNF-β/1ALY    | 121          | 1.28          |
| AM/1ALY       | 124          | 1.29          |
| BM/1ALY       | 129          | 1.62          |
| AM/TNF-α      | 130          | 0.57          |
| BM/TNF-α      | 131          | 1.06          |
| AM/TNF-β      | 128          | 0.96          |
| BM/TNF-β      | 127          | 0.54          |
| AM/BM         | 132          | 1.04          |

### Table III

Comparison of modeled and crystallographic loop conformations

Loops in AM, BM, and 1ALY were superimposed, and backbone (b_r.m.s.d.) and all atom (a_r.m.s.d.) root mean square deviations were calculated.

| Residues | AM b_r.m.s.d. (a_r.m.s.d.) | BM b_r.m.s.d. (a_r.m.s.d.) |
|----------|-----------------------------|-----------------------------|
| 132–136  | 2.50 (3.50)"                | 2.24 (3.60)"                |
| 155–159  | 1.53 (3.62)"                | 1.05 (3.17)"                |
| 164–186  | 0.34 (1.97)"                | 0.34 (1.94)"                |
| 180–186  | 2.77 (4.86)"                | 3.21 (4.70)"                |
| 197–200  | 1.23 (2.21)"                | 1.00 (4.01)"                |
| 213–217  | 1.51 (1.96)"                | 2.31 (3.44)"                |
| 232–234  | 0.23 (0.90)"                | 0.23 (1.05)"                |
| 242–245  | 0.26 (0.70)"                | 0.91 (1.28)"                |
| 248–252  | 2.02 (3.10)"                | 1.75 (2.39)"                |

* Loop conformations in AM and/or BM modeled by conformational search.

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Overall accurate molecular models at <20% sequence identity have only been reported in a few cases such as the T cell costimulatory molecule CTLA-4 (37, 38). These results indicate that correct initial sequence alignments are extremely important for the quality of comparative models (35, 38). The topological TNF/gp39 sequence alignments were correct with the
The two gp39 models, AM and BM, were built using similar modeling protocols, but different structural templates. The differences between these models are relatively small, and their accuracy is comparable. For gp39 modeling, the choice of either TNF-α or TNF-β as structural template was not critical, and the generation of consensus template(s) would not have changed the results in a significant way. At the backbone level, both models are closer to their respective structural templates than to the gp39 x-ray structure. This framework bias toward structural templates (38) is generally observed in comparative models (34, 35, 39) and results from retaining structurally conserved regions of templates, which also diverge from targets with decreasing sequence similarity (40). However, the magnitude of this framework bias is usually smaller than other modeling errors (38) and does not often significantly affect applications of the models (41).

The largest errors in the gp39 models were detected in loop regions, which is typical for many models. Ab initio modeling of larger loops continues to be a significant problem (35, 38, 39). For gp39, errors of >2-Å backbone r.m.s.d. were observed for several loops in at least one of the models. In our studies, selection of loops (24) generated by conformational search did not produce accurate conformations. In two cases, simply retaining the TNF backbone conformations was not much more or less inaccurate than the selected loop. However, several loops were correctly predicted to be conserved in TNF and gp39 and thus accurately modeled.

Side chain modeling accuracy is also limited. Since side chain conformations are backbone-dependent (33, 36), meaningful comparison of predicted and observed side chains is only possible for structurally conserved regions. In gp39, ~50% of side chain rotamers were correctly predicted, similar to results obtained in more recent predictions (35). When side chain conformations are included in overall comparisons, r.m.s.d. values between models and experimentally determined structures generally increase by ~1 Å. Side chain modeling errors have several sources. Many surface residues may adopt more than one conformation, whereas the conformations of core residues are usually constrained. Prediction accuracy is generally higher for the latter residues (35). However, up to 45% of conserved residues in similar structures may have different rotamer conformations (39), which is probably a consequence of differences in core packing. This may cause modeling errors even in the case of conserved residues. Therefore, the use of backbone-dependent rotamer libraries (33) may only moderately increase modeling accuracy (35).

The gp39 models have been used in two major applications, the identification of residues critical for CD40 binding (17, 18) and the analysis of natural mutations associated with XHIM syndrome (7, 19). Despite their limitations, the gp39 models made it possible to select exposed residues for mutagenesis, to identify critical residues, to generate an approximate spatial outline of the CD40-binding site, and to obtain a reasonable classification of mutants and their putative effects. Only a few significant errors were found in these studies. In some cases, such as experiments involving Lys-143, Ser-128, and Glu-129, the results could be rationalized in detail, which is difficult and often impossible using models. Which factors were critical for the successful applications of the models? At least two aspects can be discussed. First, the initial sequence alignment was, despite the presence of a local register shift, sufficiently accurate to correctly model the majority of residue positions in β-strands. Residue mapping critically depends on the correct relative placement of residues. Second, the CD40-binding site maps to a region of high prediction confidence, i.e. the interface between gp39 monomers, which is well conserved in gp39 and TNF. Regions with the largest errors are distant from this site. These findings support the approach to identify regions of high and low prediction confidence in molecular models prior to their application and to focus model analysis on high confidence regions (42). For example, in the case of gp39, loops at the top of the trimer, where errors were most significant, could have been deleted from the model without significantly changing the results. However, had these low confidence regions included residues important for binding, a meaningful analysis of the binding site would not have been possible. Thus, it is important to understand which regions of the model can be accurately built and where majors errors are expected.

CONCLUSION

The comparison of gp39 molecular models and x-ray structure made it possible to assess modeling accuracy and the validity of model-based mutagenesis and mapping studies in detail. Despite their limitations, the gp39 models significantly aided in the design and rationalization of experiments, and important features of the gp39 structure and binding site could be captured. Since not many comparisons similar to the gp39 study have been reported, it is difficult to draw more general conclusions. In the presence of low sequence similarity, standard modeling protocols are difficult to apply and do not necessarily produce sound molecular models. In addition, structure or binding characteristics of target proteins may limit model applications. The results presented here suggest, however, that novel members of the TNF superfamily should be promising modeling targets since their core structures may be very similar to TNF and gp39, despite limited sequence similarity, and their binding sites may map to corresponding regions.

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