CD59 is a 77-amino acid membrane glycoprotein that plays an important role in regulating the terminal pathway of complement by inhibiting formation of the cytolytic membrane attack complex (MAC or C5b-9). The MAC is formed by the self assembly of C5b, C6, C7, C8, and multiple C9 molecules, with CD59 functioning by binding C5b-8 and C5b-9 in the assembling complex. We performed a scanning alanine mutagenesis screen of residues 16–57, a region previously identified to contain the C8/C9 binding interface. We have also created an improved NMR model from previously published data for structural understanding of CD59. Based on the scanning mutagenesis data, refined models, and additional site-specific mutations, we identified a binding interface that is much broader than previously thought. In addition to identifying substitutions that decreased CD59 activity, a surprising number of substitutions significantly enhanced CD59 activity. Because CD59 has significant therapeutic potential for the treatment of various inflammatory conditions, we investigated further the ability to enhance CD59 activity by additional mutagenesis studies. Based on the enhanced activity of membrane-bound mutant CD59 molecules, clinically relevant soluble mutant CD59-based proteins were prepared and shown to have up to a 3-fold increase in complement inhibitory activity.

Complement is an important component of host defense and is an effector mechanism for both innate and adaptive immune responses. Complement also plays important roles in enhancing the induction of both humoral and cellular immunity, regulating tolerance to self-antigens, and in the clearance of immune complexes and apoptotic cells. These effects of complement are mediated either directly or indirectly by bioactive cleaved protein fragments or by a terminal cytolytic protein assembly, termed the membrane attack complex (MAC or C5b-9). Generation of the MAC during the complement cascade is initiated by cleavage of C5, which yields C5b and results in the sequential binding of C6, C7, C8, and multiple C9 molecules. Necessarily, complement effector mechanisms are under tight control to prevent damage to host cells, and MAC formation is under the control of CD59, a widely distributed membrane-insertion and pore formation. Under certain disease conditions, such as autoimmune disease and inflammatory conditions, inappropriate or excessive complement activation occurs and complement control mechanisms, including CD59 function, are broken down or overcome. The MAC has been implicated as a key player in causing tissue injury in many of these pathological states (reviewed in Refs. 1–5). From a clinical standpoint, understanding the molecular interaction between CD59 and its complement ligands may assist with the design and engineering of effective recombinant soluble CD59-based therapeutics to limit MAC-dependent disease pathology. CD59 expression has also been implicated in tumorigenesis and in providing cancer cells with protection from monoclonal antibody immunotherapy (6–8), and has been shown to be up-regulated in some cancers indicating a role in immune resistance (9–12). Thus, knowledge of the CD59 binding interface may also assist with the rational design of molecules that inhibit CD59 function with a goal of enhancing cancer cell susceptibility to antibody therapy.

The determination of CD59 three-dimensional structure by NMR revealed a single cysteine-rich domain composed of two β-sheets running anti-parallel to each other and a short helix (13, 14). Previous studies of the CD59 binding interface indicate that its C8 (C5b-8) and/or C9 (C5b-9) binding site is located in the vicinity of a hydrophobic groove on the membrane distal face of the protein centered around residue Trp40 and close to the helix (15, 16). Mutational strategies used to putatively identify the CD59 binding interface have been based on either the rational selection of single residues or the production of chimeric proteins containing functional and nonfunctional domains. The selection of residues for site-specific mutation has primarily been based on their predicted positions at the protein surface, or the identification of evolutionarily conserved residues (15–20). The chimeric approach has involved swapping CD59 domains from different species that are known to function in a species selective manner (21, 22), or by swapping domains between human CD59 and mouse Ly6E, a structural, but not functional analog of CD59 (15).

The only full atom structural data currently available for CD59 comes from several NMR models (PDB 1cdq, 1cdr, 1cds, 1erg) (13, 14). However, those models have a number of potential defects, including a deformed α-helix (Phe72–Glu78) and partial separation of the C6-C13 loop from the rest of the structure, producing a continuous channel through the protein. Structure 1erh does not share the channel but is missing residues Phe72–Asn77. Additionally upon visual inspection, the current models show side chain packing significantly less compact than would be expected of typical high resolution crystallographic structures. Such defects make interpreting mutational data from a structural standpoint difficult, as one cannot be sure of the spatial relationships between
Mapping the CD59 Binding Interface

side chains. The ability to perform energy-based geometrical analysis or docking is similarly compromised.

Here we report scanning alanine mutagenesis of residues 16–57, in which the binding interface was previously identified by analysis of Ly6/CD59 chimeric proteins, and in which site-specific mutations identified as being important for function are located. We also report a significantly improved NMR model for structural understanding of CD59. Based on the scanning mutagenesis data, refined models and additional mutations, we provide new insight into the CD59-C8/C9 binding interface and engineer soluble CD59 molecules with significantly improved complement inhibitory activity.

EXPERIMENTAL PROCEDURES

Cell Line and DNA—CHO cells were used for CD59 expression using F12K medium (Invitrogen) supplemented with 10% fetal calf serum (Mediatech, Herndon, VA). Plasmid PCDNA3/CD59 (15) was used as the starting DNA for all mutagenesis experiments. The plasmid carries human CD59 cDNA sequence including sequence coding for the addition of a GPI anchor. To facilitate investigation of CD59 expression, an epitope tag consisting of amino acids NANPNANPNA was inserted immediately behind the first amino acid of the N terminus of CD59 (15). The presence of this tag has been shown to have no effect on the function of CD59. pCDNA3 carries a G418 resistance gene and 500 µg/ml G418 (Invitrogen) was supplemented for selection and cultivation of transfected CHO cells and populations.

Antibodies, Sera, and Reagents—Rabbit anti-CD59 polyclonal antibody was prepared by standard techniques (23). Anti-CD59 mAbs YTH53.1, IF5, and anti-tag mAb 2A10 were expressed in our laboratory. The remaining anti-CD59 mAbs were from Dr. V. Horejsi, Academy of Sciences of the Czech Republic, Prague (p282 and MEM43.5), Dr. S. Meri, University of Helsinki, Finland (HCl) and from BD Biosciences, San Jose, CA (MEM43). The anti-CD59 mAbs YTH53.1, MEM43, HCl, P282, MEM43.5 (15, 16), and IF5 (17) have been previously characterized and all recognize conformation epitopes on CD59. All fluorescein isothiocyanate-conjugated secondary antibodies were from Sigma-Aldrich. Rabbit anti-CHO cell membrane antisera was prepared by standard techniques (23). Normal human serum (NHS) was prepared from blood of healthy volunteers. All other reagents were from Sigma.

Mutagenesis and Transfection—Site-directed mutagenesis was carried out by PCR using the QuikChange® Site-directed Mutagenesis kit from Stratagene (La Jolla, CA). Two mutagenesis primers spanning the desired mutation site(s) were used for mutagenesis of each mutant. Mutated DNA samples were sequenced to verify the mutations. Wild-type and mutant CD59 expression plasmids were transfected into CHO cells using Lipofectamine according to the manufacturer’s instruction (Invitrogen). Twenty-four hours after transfection, G418 was added into F12K medium for selection of stably transfected populations of cells.

Cell Sorting and Antibody Staining—CHO cell populations expressing similar levels of CD59 or mutant CD59 were isolated by cell sorting using a FACS-Vantage flow cytometer (BD Biosciences) as described (15). All cells lines were sorted for 2–4 rounds by means of an antibody to the epitope tag (mAb2A10) in order to acquire population of cells expressing similar levels of CD59. The binding of anti-CD59 antibodies to CD59 and mutant CD59 expressed on CHO cells was performed by flow cytometry using standard methodology as described (15).

Expression and Purification of CR2-CD59 Fusion Proteins—A cDNA construct was prepared by joining the complement receptor 2 (CR2) sequence encoding the 4 N-terminal SCR units (residues 1–250 of mature protein, SwissProt accession no. P20023) to sequences encoding extracellular region of CD59 as described (24). The CR2-CD59 construct in expression vector pBMC2-CD59 (24), was used as the starting template for preparation of CR2 mutant CD59 fusion proteins. Mutant CR2-CD59 containing plasmids were constructed using the QuikChange® site-directed mutagenesis kit (see above). The CR2-CD59 proteins were expressed after transient transfection of CHO cells by the Lipofectamine method. Wild-type protein was expressed in suspension culture of transfected CHO cells and recombinant proteins were purified from CHO cell supernatant by anti-CD59 (mAb YTH53.1) affinity chromatography as described (24). Purity of eluted proteins was determined (>95%) by 10% SDS-PAGE and by Western blotting.

Complement-mediated CHO Cell Lysis Assays—CHO cells at 60–80% confluency were detached with 5 mM EDTA in phosphate-buffered saline, washed once with phosphate-buffered saline and resuspended to 10^6/ml in Dulbecco’s modified Eagle’s medium. Cells were sensitized with 5% rabbit anti-CHO membrane serum and 10% NHS diluted in Dulbecco’s modified Eagle’s medium was then added (final volume, 400 µl). Following incubation at 37 °C for 60 min, cell viability was determined by adding propidium iodide (PI) (5 µg/ml) and measuring the proportion of PI-stained (dead) cells by flow cytometry. Cells lysed with 0.01% saponin were used as 100% lysis controls, and samples with NHS heated at 56 °C for 30 min were used for background lysis. To test the complement inhibitory function of wt and mutant CR2-CD59 fusion proteins, the proteins were diluted in Dulbecco’s modified Eagle’s medium and added to NHS prior to addition to sensitized CHO cells. Cell viability was determined by both trypan blue exclusion (both live and dead cells counted) and PI staining. Both methods gave similar results.

NMR Model Refinement—Starting with coordinates from the NMR structure of CD59 (14, 25) (PDB code 1cds), the model was refined using a Biased Probability Monte Carlo (BPMC) minimization procedure implemented in the ICM software package (26). The force field included terms for van der Waals interaction, hydrogen bonding, torsion angle energy, and generalized Born electrostatics (27, 28). Distance and variable constraints were imposed for the original NOE data (kindly provided by Dr. Neuhaus, MRC Laboratory of Molecular Biology, Cambridge, UK) as well as the five disulfide bonds. For each of 12 separate refinement simulations, backbone torsion angles were randomly randomized ±30 degrees and side chain torsion angles were fully randomized ±180 degrees. In the first half of the simulation, van der Waals repulsive energy was initially limited and all torsion angles were free for perturbation. In the second half, van der Waals repulsive energy was returned to normal and only side chain torsion angles were free for perturbation. All torsion angles were minimized after each step of the BMPC procedure. Simulations were limited to 8 million energy evaluations each and averaged 10,200 Monte Carlo steps.

RESULTS

Effect of Mutations on CD59 Function—Previous studies have mapped the binding interface of human CD59 to the vicinity of a hydrophobic groove around Trp40 on the membrane-distal face of the molecule. Rational mutagenesis studies within this region indicated that Trp40, Arg53, Leu54, and Gln56 are involved in ligand binding, with Asp74 being crucial for the structure and accessibility of the binding interface (15, 16). We performed a systematic mutagenesis screening study together with functional analyses and computer modeling to better define the binding site. Thirty-four alanine substitutions were made in a mutagenesis screen of residues 17–57 (except for cysteine residues and
existing alanine residues), representing the primary sequence known to contain the binding interface (15).

Wild-type and mutant proteins were recombinantly expressed on the surface of CHO cells, and cell populations expressing similar levels of protein were isolated by cell sorting by means of an epitope tag. Only four of the alanine substitutions significantly reduced the inhibitory activity of CD59 as measured by the complement susceptibility of CHO cells expressing the mutant proteins (Fig. 1). For mutations showing decreased inhibitory activity, we verified expression and folding on the CHO cell surface using a panel of anti-CD59 antibodies that recognize conformational epitopes (TABLE ONE) (15–17). The F42A and H44A substitutions resulted in proteins with significantly reduced inhibitory activity ($p < 0.05$), but with relative anti-CD59 antibody binding affinities comparable to wt CD59 (as determined by flow cytometry; refer to TABLE ONE), the one exception being HC1 binding to H44A. D24A and W40A displayed evidence of mis-expression/folding, showing less than 10% reactivity with all antibodies. However, in previous mutational studies, D24R and W40E have been expressed with intact protein topology and with total loss of CD59 activity (16).

Surprisingly, 11 mutations significantly increased complement inhibitory activity (Fig. 1). Of these mutants, five (D22A, F23A, T29A, T51A, L54A) had a marked increase in activity ($60–80\%, p < 0.001$) and six mutants (S20A, S21A, L27A, R37A, R53A, N57A) showed a more moderate increase in inhibitory activity ($p < 0.05$ or $p < 0.01$). Interestingly, a number of the mutated residues were internal and localized to the $\beta$-sheet (L27A, T29A, N37A), possibly mediating their effect through induced conformational changes of the binding interface or through an increase in protein stability. The solvent-exposed mutants that increased activity were localized to two compact locations, the Ser$^{20}$–Phe$^{23}$ loop and the Asn$^{48}$–Asn$^{57}$ helix/loop region. (Fig. 2).

Because of the potential significance of CD59 as a therapeutic agent for preventing tissue injury in some pathological conditions, we combined single residue mutations in an attempt to further increase the complement inhibitory activity of CD59. Six mutant CD59 proteins were prepared that contained alanine substitutions at two different positions (nos. 1–6 in Fig. 3). Five of the double-mutant proteins (29A21A, 29A23A, 51A20A, 51A23A, and 51A29A) enhanced CD59 inhibitory activity and showed additive activity compared with the single residue mutations. The most potent inhibitor was the 51A20A mutant, which showed a 165% increase in inhibitory activity compared with the wild-type protein ($p < 0.001$) (Fig. 3). This double mutation exhibited twice the inhibitory activity of the highest single mutation, T29A. In contrast, one double alanine mutant (20A23A), together with other mutant proteins containing multiple alanine or glycine substitutions with more than one substitution in the Ser$^{20}$–Phe$^{24}$ loop, displayed significantly decreased inhibitory activity compared with the wild-type protein ($p < 0.05$ or $p < 0.001$) (Fig. 3). A single 23G substitution also resulted in decreased CD59 activity. A potential explanation lies in the fact that residues Ser$^{20}$, Ser$^{21}$, and Asp$^{22}$ form a polar loop with almost entirely solvent-exposed side chains. (Fig. 4). The backbone of these residues is partially supported by the bulky phenyl group of Phe$^{23}$. Without support of the phenyl group, the loop appears more prone to collapse triggered by substitution of the polar residues with more hydrophobic alanine or glycine. Such a local conformational change is evidenced by the partial disruption of YTH53.1, 1F5, and HC1 antibody reactivity to 20A23A and 51A20–23A (TABLE ONE). F23G, on the other hand, shows no disruption in antibody binding. The difference in CD59 activity of the F23A and F23G mutants is probably attributable to the structural importance of the additional carbon atom in F23A.

Because the absence of $N$-glycosylation by an N18Q substitution was previously shown to enhance CD59 activity (15, 29), a 29A18Q mutant was also prepared, but was found to display reduced activity.

Refinement of CD59 Structure—To produce better coordinates for structural analysis, we refined the existing CD59 model with a full

![FIGURE 1. Effect of alanine substitutions on CD59 complement inhibitory activity. CD59 activity was determined by assaying complement sensitivity of CHO cells expressing similar levels of wild-type or mutant CD59. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Mean ± S.D., n = 5–9.](attachment:image.png)
atomic force field and original experimental NOE constraints. After initially randomizing backbone and side-chain torsion angles, 12 simulations converged to an average pairwise all-atom RMSD of 1.6 Å and Ca RMSD of 0.9. The flattened α-helix was straightened to a more canonical form and the C6-C15 loop shifted to fill the previous channel. Notably, the energy penalty imposed by the NOE distance and variable constraints dropped by 2105 kcal/mol from the 1cds coordinates to the initial structures, and in particular the fact that the force field in the present calculations includes explicit terms to represent hydrogen bonds and electrostatic forces. Of course, given the nature of the constraints, the refined model is one of many possible models compatible with these constraints. This model is available upon request from the authors.

Analysis of CD59 Structure—To characterize the refined structure, we used a recently developed method for identifying potential small molecule binding sites in proteins (30). This identified a 150 Å³ pocket spanning an area between the N5-P9 loop and the hydrophobic groove centered around Trp⁹⁰ (Fig. 5). In addition to binding small molecules, the pocket may serve as peptide or loop binding groove that may be important to CD59 activity. Because of proximity to the predicted binding site and single alanine mutation data. 

### TABLE ONE

| Mutant | Poly | YTH53.1 | 1F5 | P282 | MEM43 | HC1 | MEM43.5 |
|--------|------|---------|-----|------|-------|-----|--------|
| WT     | 100  | 100     | 100 | 100  | 100   | 100 | 100    |
| 24A    | 5.7 ± 1.7 | 5.0 ± 1.8 | 2.8 ± 0.2 | 5.6 ± 0.5 | 3.5 ± 2.5 | 2.2 ± 0.2 | 1.9 ± 0.6 |
| 40A    | 4.3 ± 0.1 | 1.0 ± 0.1 | 2.2 ± 1.3 | 3.3 ± 0.1 | 6.2 ± 8.3 | 1.2 ± 0.8 | 0.7 ± 0.4 |
| 42A    | 99.7 ± 0.5 | 99.8 ± 0.4 | 99.8 ± 0.3 | 100 ± 0.1 | 99.7 ± 0.4 | 98.4 ± 2.3 | 99.9 ± 0.2 |
| 44A    | 82.7 ± 6.4 | 58.7 ± 5.1 | 74.0 ± 3.3 | 95.6 ± 4.9 | 88.8 ± 13.8 | 30.3 ± 10 | 85.9 ± 8.8 |
| 52A    | 97.6 ± 1.4 | 99.3 ± 0.1 | 99.0 ± 0.2 | 100 ± 0 | 99.2 ± 0.8 | 90.7 ± 2.9 | 99.2 ± 0.1 |
| 23G    | ND²  | 99.3 ± 0.1 | 97.5 ± 0.2 | 99.2 ± 0.1 | 98.8 ± 0.1 | 91.3 ± 1.5 | 98.9 ± 0.1 |
| 20A23A | ND   | 35.7 ± 1.1 | 83.1 ± 14 | 99.6 ± 0.1 | 98.1 ± 0.2 | 63.1 ± 21 | 97.7 ± 1.0 |
| 51A20–23A | ND | 18.3 ± 1.2 | 64.4 ± 26 | 96.7 ± 0.1 | 92.3 ± 0.4 | 43.5 ± 25 | 98.1 ± 1.5 |
| 5R     | 40.7 ± 8.0 | 47.9 ± 11 | 14.8 ± 5.3 | ND | ND | 6.3 ± 8.5 | 8.2 ± 3.9 |
| 9R     | 100  | 100     | 99.8 | ND | ND | 98.7 | 98.4 |

* Percent of relative mean fluorescence by flow cytometry compared to wt CD59. 
² ND, not determined.
ever, other substitutions of Tyr61 (17), as well as several other mutations, had no effect on the complement inhibitory activity of CD59, and it is unlikely that the “back” face extensively interacts with C8/C9. A new method for predicting protein-protein interaction sites (31) revealed a new set of residues potentially involved in protein-protein interaction. Three of these residues (Phe42, Asn48, Asn57) were shown to be involved in the CD59-C8/C9 binding interface based on our single alanine mutation data. In addition, this method revealed new residues potentially involved in another interaction. This site is on the flap of CD59 (Fig. 6) opposite the C8/C9 interface shown on Fig. 2 and may represent an interaction with another essential protein partner, possibly one of the other identified ligands for CD59 (see “Discussion”), or a membrane. It is worth mentioning that the prediction signal indicating the involvement of the newly characterized residues (e.g. Leu75, Thr60, Tyr61, Tyr62) in an intramolecular interaction is very strong, and a Y61G substitution was previously shown to abrogate CD59 activity (17). However, other substitutions of Tyr61 (17), as well as several other mutations within this region (in the current study and reported previously (16)) had no effect on the complement inhibitory activity of CD59, and it is unlikely that the “back” face extensively interacts with C8/C9.

Effect of CD59 Mutations on CR2-CD59 Fusion Protein Activity—We have previously shown that for CD59 to function effectively, it must be bound close to the site of complement activation and MAC formation (24, 32). Soluble CD59 is only a poor inhibitor of complement (MAC formation), and we demonstrated that the functional activity of CD59 could be marked enhanced by targeting CD59 to the site of complement activation (24, 32). By linking the extracellular region of CD59 to a C3 binding region of complement receptor 2 (CR2), we previously demonstrated a significant increase in the ability of CD59 to protect target cells from complement-mediated lysis (24). With the idea of developing an improved therapeutic agent by further enhancing the activity of CD59, two soluble CD59 molecules containing mutations that increased activity (when linked via GPI anchor to the cell surface) were linked to a CR2 targeting moiety. We prepared CR2-CD59 fusion proteins containing wt CD59 or CD59 containing either a T51A mutation or a combined mutation of T51A/S20A. The proteins were assayed for their ability to protect sensitized CHO cells from complement-mediated lysis, and both mutant CR2-CD59 proteins displayed significantly enhanced complement inhibitory activity compared with the wt CR2-CD59 protein, with CR2-CD59 (T51A/S20A) displaying an approximate 3-fold increase in activity (Fig. 7).

DISCUSSION

One result of the work presented here is the addition of two new residues, Phe42 and His44, to the list of amino acids likely involved in the binding interface of human CD59. Mutations of these residues to alanine resulted in proteins with significantly reduced complement inhibitory function, yet intact overall structure. Both residues are in close proximity to the proposed binding interface. Of relevance to this data, glycation of CD59 decreases its function and has been shown to occur in diabetic patients because of hyperglycemia. It has been shown that His44, together with Lys31, which is adjacent to the functionally important Trp35, form a preferential glycation motif in human CD59 (33–35). Our results also suggest that residues 20–22 are involved in the activity of human CD59. Based on the NMR structure of soluble CD59, Fletcher et al. (14) suggested that the S20-D24 loop is part of the binding inter-
Mapping the CD59 Binding Interface

The result seems to indicate some role of distant Pro9 in mediating allosteric. One, P9R, led to a modest, but significant reduction in CD59 function. To determine whether C8/C9 interacted more extensively with that pocket, we produced three additional mutations in the unexplored area. Interestingly, three activity-enhancing mutations (L27A, T29A, N37A) are located at the center of the protein in two strands of the β-sheet, and may confer increased activity through induced conformational changes. One potential mechanism for the increased activity may be the spatial relationship of these residues with Trp40. Previous studies have demonstrated the importance of Trp40 in CD59 activity (16). Our study shows that Trp40 is almost entirely surrounded by residues (Leu27/H9251, Arg23, and Leu34, when mutated to alanine, may increase Trp40 mobility and accessibility, bringing about increased CD59 binding and inhibitory activity.

Through computational geometrical analysis of the refined structure, we found a potential small molecule binding site around the previously observed hydrophobic groove (Fig. 5). Such geometry is favorable for the development of small-molecule antagonists of CD59-mediated complement inhibition. Novel small molecule inhibitors may be useful for improving the effectiveness of antibody based anti-cancer therapies. Whatever the evolutionary reasons for the sequence composition of CD59, we have demonstrated the capacity to engineer CD59 constructs with increased activity through single alanine mutations alone. We have also shown the negative synergy of multiple highly localized mutations, with effects possibly mediated through local conformational changes. On the other hand, combined point mutations in different locations preserved the overall conformation and produced synergistic increases in C8/C9 binding affinity. Use of this principle in future mutational studies may well enable further increases in CD59 inhibitory activity.

The experimental results of this study were limited to the specific interaction of CD59 with the C8/C9 proteins. However, as outlined above, CD59 has other functions beyond complement inhibition and may therefore have other protein interaction interfaces. In that regard, a particularly interesting observation we made is the “back” patch on the CD59 surface (Cys45, Asn46, Phe47, Thr60, Tyr61, Tyr62, Leu75) with a strong protein interface signal. These residues are good candidates for involvement in the interaction of CD59 with its other ligands.

In conclusion, through our extensive mutagenesis screen, we are now able to more precisely define the binding interface of CD59. With the inclusion of Asp23, Phe23, Phe42, and His44, the area of the binding interface is much broader than previously thought. We also identify a potential small molecule binding site directly interfering with the binding interface, which makes the rational design of efficient CD59 inhibitors more feasible. Such studies will be further aided by our improved NMR model. Inhibitors of CD59 function, if appropriately targeted, may be effective at enhancing antibody immunotherapy. Finally, we show that mutations that enhance the activity of GPI-linked membrane CD59 also significantly enhance the activity of soluble CD59 constructs (CR2-CD59 fusion proteins), thus establishing the potential for engineering a more effective CD59-based therapeutic to treat inflammatory conditions.

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