Antibody Imprint of a Membrane Protein Surface

PHAGOCYTE FLAVOCYTOCHROME b

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Structural features of the integral membrane protein flavocytochrome b (Cyt b) were discovered using an antibody “imprint” of the Cyt b surface. Amino acid sequences were selected from a random nonapeptide phage-display library by their affinity for the monoclonal antibody 44.1 binding site, which recognizes the native conformation of the p22phox subunit of Cyt b. Transferred nuclear Overhauser effect spectroscopy and rotating frame Overhauser effect spectroscopy NMR were used to study the antibody-bound conformation of a synthetic peptide derived from phage-displayed sequences. The NMR data supported the phage-display analysis suggesting the existence of a complex epitope and allowed the modeling of the close spatial proximity of the epitope components and from discontinuous regions of p22phox. Although these regions are separated by two putative membrane-spanning domains and are 150 residues apart in the sequence, they appear to combine to form a complex epitope on the cytosolic surface of the transmembrane protein. NMR constraints, measured from the antibody-bound conformation of a composite peptide mimic of the Cyt b epitope, and one constraint inferred from the phage-display results, were used to demonstrate the close proximity of these two regions. This information provides a low resolution view of the tertiary structure of the native discontinuous epitope on the Cyt b surface. Given additional antibodies, such imprint analysis has the potential for producing structural constraints to help support molecular modeling of this and other low abundance or noncrystallizable proteins.

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Epitope Conformation by Antibody Imprint Analysis

cagccgccccgacgcggcggggttattggttttgctgagta-3'. The oligonucleotides were phosphorylated and allowed to anneal under conditions previously described (11). The insert was then ligated to the M13K8 vector in a molar ratio of 1:2.5 for vector:insert, respectively. The ligation product was then transfected into electrocompetent MC1061 cells and plated on a lawn of K91 cells to produce isolated plaques. The relevant nucleotide sequence of resulting clones was determined (13) to verify the intended mutations.

ELISA—ELISA analysis was used to determine the ability of synthetic peptides to mimic the putative epitope regions of p22phox when bound by mAb 44.1. Seventy-five-μl of a wheat germ agglutinin-activated glucosamine solution (0.4 mg/ml/2 molar) was adsorbed to a Dynex Immunol 2 plate for 3 h at 4 °C. Unbound protein was removed with four rinses of wash buffer (2.7 mM KCl, 137 mM NaCl, 1.5 mM KH2PO4, 8 mM Na2HPO4, 0.9 mM CaCl2, 0.5 mM MgCl2 (pH 7.4)). Human neutrophil membranes served as a source of Cyt b, which were extracted with octyl glucoside according to a previous protocol (14), except the membrane resuspension buffer lacked dithiothreitol and EDTA. Thirty-μl of mAb 44.1 (final concentration 200 ng/ml) was then exposed to the immobilized Cyt b in the presence of various dilutions of synthetic peptides as shown in Fig. 2. The antibody remaining after four rinses of wash containing 0.1% Triton X-100 was measured with an antipeptide conjugated with peroxidase. The signals were developed with a standard ABTS-hydrogen peroxide reagent and measured at 405 nm.

NMR Analysis—NMR spectra were obtained on a Bruker DRX 500 instrument at 5 and 25 °C, in 50 mM phosphate buffer (pH 6.5 or 5.0), in the presence of 5% D2O, as described in the legend to Fig. 3. The TACRFGGGQVQPPP peptide was 1 molar, and the mAb 44.1 was either 47 or 84 μl. The interproton distances in the peptide complexed with mAb 44.1 were estimated by comparison with the cross-peak intensity of the F5:H8 protons, which are 1.8 Å apart.

RESULTS

To obtain structural imprint information about Cyt b from mAb 44.1, we extended phage-display epitope mapping of the mAb recognition site (7). From a pool of mAb 44.1-immunoreactive phage-displayed peptides, colony-lift screening (see “Experimental Procedures”) was used to select avidly binding sequences that most closely resemble the epitope bound by mAb 44.1. Of approximately 500 colonies of Escherichia coli infected by different mAb 44.1-selected phage clones, 22 (4%) were found to produce distinctly darker staining replica signals (represented by signals in Fig. 1A; I = darker, and II = lighter). The unique peptide displayed on phage associated with each colony type was deduced by nucleotide sequence analysis. Peptides expressed by the lightly staining I-type phage were found to belong to the PX3-VCX3-3 motif (where X3 ≠ R if X3 = Q) and contained from two to four residues matching the 183-PQVNPI188 sequence of p22phox, as shown previously (7). Peptides associated with the more strongly staining I-type colonies segregated into two groups according to their sequence (Table I). Sequences 1–3 in Table I (AQPQVRPIG, NMPQVRPID, and DRPQVRPIL) each contained the six-residue sequence, PQVRPI, matching five of six residues of the NMPQVRPID, and DRPQVRPIL) each contained the six-residue sequence, PQVRPI, matching five of six residues of the PQVRPI region of the epitope. To determine whether mAb 44.1 was able to recognize the epitope-mimicking regions expressed on the pIII protein of phage, 100 μl of Luria broth with 75 μg/ml kanamycin was inoculated with K91 cells infected with phage expressing specific peptide sequences. Phage were harvested, the proteins were separated by SDS-polyacrylamide gel electrophoresis, and Western blots were probing with mAb 44.1 as described previously (7). Unique regions expressed on the pIII proteins of phage shown were: ATAGRFGGGQVQPPP (lane a), EGFRFGGGQVQPPP (lane b), AQPRVRPG (lane c), and contained phage proteins from the M13KB vector (12) (lane d), which does not express a unique peptide. Results were confirmed by four separate analyses.

of p22phox, respectively (Table I). Two of these individual phage peptides: 4, EGRFGGGQVQPPP and 5, ISRFGGGQVQPPP, were identical in seven of the nine randomized positions and sequence 4 was found in 14 clones (64%) selected in this way.

The immunoreactivity of mAb 44.1 with phage-expressed peptides resembling each of the two different putative epitope moieties of p22phox was supported using semiquantitative Western blots probed by mAb 44.1. All lanes of the Western blot in Fig. 1B contain 1013 phage plaque-forming units differing only by the sequence of their displayed peptides. In lanes b and c, strong signals were observed at the molecular weight corresponding to the phage pIII protein bearing either the AQPRVRPG or EGFRFGGGQVQPPP peptides (1 and 4, respectively, Table I). These sequences were derived from darkly staining type I colonies. Phage displaying peptides that mimicked the putative TACRFGGQVQPPP epitope moiety alone were not selected from the library, presumably because this sequence has relatively low affinity for mAb 44.1 when it occurs without the PQVNPI region of the epitope. To determine whether mAb 44.1 can indeed recognize the TACRF sequence expressed on the pIII protein of the M13 phage, a clone displaying the ATAGRFGGGQVQPPP sequence (sequence 15, Table I) was created (see “Experimental Procedures”) and probed by mAb 44.1 as shown in lane a of Fig. 1B. A weak but detectable signal suggests at

FIG. 1. A, colony-lift immunological screening of phage-displayed peptides in infected E. coli (K91) cells. Phage previously selected with mAb 44.1(7) were used to infect 1 ml of mid-log phase K91 cells. Dilutions of the infected cells were performed to obtain isolated colonies when plated on Luria broth agar containing kanamycin at 75 μg/ml. The colonies resulting after overnight incubation at 37 °C were blotted on nitrocellulose discs and probed with mAb 44.1, producing signals of variable intensity that corresponded to the immunoreactivity of the phage-displayed peptides. I and II represent strong and light-staining classes of colonies, respectively. B, Western blot analysis of putative epitope regions recognized by mAb 44.1. To determine whether mAb 44.1 was able to recognize the epitope-mimicking regions expressed on the pIII protein of phage, 100 μl of Luria broth with 75 μg/ml kanamycin was inoculated with K91 cells infected with phage expressing specific peptide sequences. Phage were harvested, the proteins were separated by SDS-polyacrylamide gel electrophoresis, and Western blots were probed with mAb 44.1 as described previously (7). Unique regions expressed on the pIII proteins of phage shown were: ATAGRFGGGQVQPPP (lane a), EGFRFGGGQVQPPP (lane b), AQPRVRPG (lane c), and contained phage proteins from the M13KB vector (12) (lane d), which does not express a unique peptide. Results were confirmed by four separate analyses.

These sequences, revealed only by colony-lift screening, were included in the list of sequences given in Ref. 7 prior to our realization that they suggest a complex epitope bound by mAb 44.1.
least part of the ATAGRFGGG polypeptide is bound by mAb 44.1. No signal is observed in lane d containing the pII protein of the M13KBst (12) vector, showing that the constant region of the molecule is not recognized by mAb 44.1.

The ability of phage-discovered peptides to substitute for the native cytochrome b epitope was quantitated by their ability to compete for the antibody combining site in an ELISA shown in Fig. 2. Component peptides examined in ELISA analyses (ac-PQVNPI-amide and ac-ATAGRFTQW-amide, 11 and 12, respectively, in Table I) were identical to the PQVNPI and ATAGRFTQW regions of p22*.

The composite sequence of p22* consists of two epitope regions, representing both in sequence 9 in Table I, which is in a suitable range for study of the antibody-bound conformation of the peptide by transferred-NOESY (Tr-NOESY) NMR (15, 16). The free peptide evidently has little or no persistent structure in solution in the absence of the antibody, as evidenced by two-dimensional NOESY NMR shown in part in Fig. 3A, showing cross-peaks only within amino acid side chains and between nearest neighbor residues. Upon addition of antibody, the bound peptide develops NOESY cross-peaks only within the epitope on Cyt b, indicating the importance of arginine in this region of the epitope.

The consensus peptide (TAGRFGGGQVGPP, 10 in Table I) was tested as was the ac-GPPFGQTGAVGRG-amide (peptide 13, □), but not the irrelevant ac-KNNLIKDCGLF-amide (peptide 14, △) or scrambled ac-PGFPQGQTAQVGRG-amide (peptide 13, □). When the two putative epitope regions are fused in a single linear peptide (ac-ATAGRFGGGQVGPP-amide (peptide 9, △)), synergistic binding is evident, since the ability of peptide competitiveness is enhanced about 100-fold. The high (580 nM) competitiveness of ac-PQVRPI-amide (peptide 8, □) for the mAb 44.1 binding site illustrates the importance of the arginine residue near the Asn186 in the native conformation of the protein.

The epitope on Cyt b was immobilized on wheat germ agglutinin-coated Immulon 2 plates to allow detection by mAb 44.1 using ELISA. The binding of mAb 44.1 to the epitope on Cyt b can be competed by synthetic peptides corresponding to each of the two putative epitope regions ac-PQVNPI-amide (peptide 11, ○) ac-ATAGRFTQW-amide (peptide 12, □, △), and the phage-expressed consensus ac-TAGRFGGGQVGPP-amide (peptide 10, △), but not the irrelevant ac-KNNLIKDCGLF-amide (peptide 14, △) or scrambled ac-PGFPQGQTAQVGRG-amide (peptide 13, □) peptides. When the two putative epitope regions are fused in a single linear peptide (ac-ATAGRFGGGQVGPP-amide (peptide 9, △)), synergistic binding is evident, since the ability of peptide competitiveness is enhanced about 100-fold. The high (580 nM) competitiveness of ac-PQVRPI-amide (peptide 8, □) for the mAb 44.1 binding site illustrates the importance of the arginine residue near the Asn186 in the native conformation of the protein.
Fig. 3. A, a portion of the two-dimensional NOESY NMR spectrum of the free TAGRFGGGQVGPP synthetic peptide. B, a portion of the Ty-NOESY NMR spectrum of the peptide in exchange with the mAb 44.1-bound form at pH 6.5 and 5 °C. C, a portion of the two-dimensional Tr-ROESY NMR spectrum of the peptide in exchange with mAb 44.1-bound form at pH 6.5 and 5 °C. Spectra A and B were collected using a standard two-dimensional NOESY pulse sequence with a watergate solvent suppression and a 300 ms mixing time. Spectrum C was collected using a two-dimensional ROESY pulse sequence with 150-ms spin lock of 2.3 KHz and watergate solvent suppression. The spectra were processed to give 2K × 2K real points and are displayed at identical contour levels. In spectrum A of the free peptide, only cross-peaks of protons within an amino acid or between adjacent residues are observed and are labeled on the figure. In spectrum B, new cross-peaks are observed and labeled and arise from the peptide binding to the antibody. In spectrum C, magnetized transfers mediated by other protons subtract from the cross-peak intensities in the Tr-NOESY NMR experiments, as described in the text. The long range NOEs that support the bound conformation of the folding of the peptide onto itself are weakened compared with the corresponding peaks the Ty-NOESY, but are still present in the Tr-ROESY (spectrum C), and so these cross-peaks largely result from direct cross-relaxation between the bound peptide protons as discussed under “Results.”

Table II

| Residues involved in cross-peaks | NOE distances in angstrom |
|----------------------------------|---------------------------|
|                                  | Lower limit | Upper limit |
| **8 long range NOEs (1–8)**      |             |             |
| 1. PHE5:HEα VAL10:HGα            | 2.5         | 3.5         |
| 2. PHE5:HDα VAL10:HGα            | 2.5         | 3.5         |
| 3. PHE5:HN THR1:HN               | 2.5         | 3.5         |
| 4. PHE5:HDα THR1:HGα             | 2.5         | 3.5         |
| 5. PHE5:HEα THR1:HGα             | 2.5         | 3.5         |
| 6. PHE5:HN THR1:HGα              | 2.5         | 3.5         |
| 7. THR1:HN ARG4:HBα              | 2.5         | 3.5         |
| 8. ARG4:HBα ALA2:HA              | 1.8         | 2.5         |

α Indicates that pseudoatoms were used in the structure determination, since the two or three protons at the sites denoted were not resolved or not resolved separately.

**TABLE II**

*mAb 44.1-bound peptide NOE constraints*

mobilized residues, which are widely separated in the primary sequence of the peptide, supporting a folded conformation of the peptide when bound to mAb 44.1. Long range cross-peaks, which indicate the association between F5:ring and V10:Hγ protons in the peptide when bound to mAb 44.1, are denoted with an asterisk in the Ty-NOESY spectrum (Fig. 3B). Within the resolution limits of the Ty-NOESY experiments, no medium range i to i + 2, or i to i + 3 were observed (data not shown), arguing against helices or tight turns in the modeled structure. The short range i to i + 1 cross-peaks were included in simulated annealing calculations modeling the bound structure that suggested a folded peptide with a broad open bend centered around Gly7 and Gly8 and a close proximity (3–5 Å) between Phe5 and Val10. When the additional phage display constraint suggesting juxtaposition of Asn186 and Arg32 side chains of p22<sub>phox</sub> was included in the calculation, the peptide maintained its general folded conformation with minimal structural violations. A higher resolution model of this epitope will be the subject of a future report.
TAGRFGGGQVGPP synthetic peptide at pH 5.0 and 25 °C. B, the synthetic composite peptide ac-ATAGRFGGPQVNPI-amide (peptide 9, Table I), discovered by phage display and ELISA epitope analysis of mAb 44.1, can mimic the proposed p22^buxo^ fold in A. By removing the underlined Ala1 amino acid and replacing the underlined Pro2 with Gly, Asn3 with Gly, and Ile4 with Pro to produce the consensus ac-TAGRFGGGQVGPP-amide (peptide 10, Table I), the affinity of the antibody for the peptide was low enough to use Tr-NOESY NMR to measure the proximity of the FR and QVG segments. The NMR data support the folded conformation of the bound peptide and the close proximity between the two regions of p22^buxo^ Long range NOEs are represented by dotted lines between the residues indicated and are numbered to correspond to the respective NOEs listed in Table II.

Our colony-lift data suggested two widely separated regions of the p22^buxo^ polypeptide might both participate in the epitope bound by mAb 44.1, implying the antibody recognizes an epitope in the folded protein. The mAb 44.1 binds to Cyt b in permeabilized cells (7) and so recognizes the native Cyt b structure. The mAb 44.1 does not inhibit oxidase function (data not shown) and so does not perturb its structure in functionally important ways. ELISA analysis was used to measure the ability of synthetic peptides to compete with mAb 44.1 binding to octyl glucoside-solubilized Cyt b. This detergent-solubilized form of Cyt b retains its native heme spectrum (24) and can be reconstituted with exogenous FAD to produce a functional protein capable of generating superoxide by itself (25) or in complex with other neutrophil proteins (26). Our ELISA data provide evidence for synergistic binding affinities of mAb 44.1 to the composite peptide sequence (ATAGRFGGPQVNPI) relative to the component sequences (ATAGRFTQW and PQVNPI), supporting the existence of both protein segments in the mAb 44.1 epitope (Fig. 5).

DISCUSSION

Antibodies that bind native proteins contain topological information about the protein surface structure, in the form of a three-dimensional imprint. Because many epitopes bound by antibodies involve residues that are surface-accessible, but not necessarily contiguous in the polypeptide chain, their resolution can reveal important structural data about the protein. Phage-display peptide libraries have proven a useful source of epitope analogs (12) and therefore provide a tool in antibody binding site analysis. The ability to apply NMR to confirm epitope analogs and therefore provide a tool in antibody binding site analysis. The ability to apply NMR to confirm epitope analogs and therefore provide a tool in antibody binding site analysis. The ability to apply NMR to confirm epitope analogs and therefore provide a tool in antibody binding site analysis. The ability to apply NMR to confirm epitope analogs and therefore provide a tool in antibody binding site analysis. The ability to apply NMR to confirm epitope analogs and therefore provide a tool in antibody binding site analysis.
respectively) show that mediation of cross-relaxation between peptide protons by antibody protons is not the major source of cross-peak intensity in the peptide Tr-NOE5Y. A decrease in the Tr-ROE5SY cross-peak intensity between two protons relative to the TR-NOE5SY cross is likely to be due to antibody protons mediating NOE5SY cross-relaxation of the peptide protons (29). The ability to adjust the off-rate of the peptide with the antibody by lowering the pH and increasing the temperature allows the adjustment of the off-rate (Fig. 4, A and B) so that a structure with additional reliability can be determined. Such studies are under way using a fluorine-substituted peptide to obtain more accurate off-rate measurements (30).

Our results imply that the ATAGRF segment of the consensus ATAGRGFGGQPVNPI sequence is folded back upon the PQVNPI region to bring Phe$^5$ and Val$^{10}$ to less than 5 Å of one another, and hence place the positively charged arginine residue in close proximity to the carboxyl half of the peptide. The importance of an arginine in this vicinity would explain the occurrence of this residue in the epitope that exists in the native 183PQVNPI188 segment (7). The importance of an arginine in this region is further demonstrated by ELISA, in the 600-fold higher binding effectiveness of mAb 44.1 to PQVRPI compared with the PQVNI peptide (Fig. 2). Because arginine is not found in the native 183PQVNPI188 segment of p22$^{phox}$, the occurrence of this residue in the epitope could well be satisfied by the arginine in the 24852TAGRF33 region (29).

In summary, the data presented in this report indicate that regions of an integral membrane protein, separated by membrane-spanning domains, can be shown to be associated in an epitope that exists in the native form of the protein. Future applications of this approach promise to provide more detailed three-dimensional information about native conformations of integral membrane proteins.

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