A Single Chain Fv Fragment of P-glycoprotein-specific Monoclonal Antibody C219

DESIGN, EXPRESSION, AND CRYSTAL STRUCTURE AT 2.4 Å RESOLUTION

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A construct encoding a single chain variable fragment of the anti-P-glycoprotein monoclonal antibody C219 was made by combining the coding sequences for the heavy and light chain variable domains with a sequence encoding the flexible linker (GGGGS)₃, an OmpA signal sequence, a c-myc identification tag, and a five-histidine purification tag. The construct was expressed in Escherichia coli and purified from the periplasmic fraction using a nickel chelate column and ion exchange chromatography. Three-step Western blot analysis showed that the construct retains binding affinity for P-glycoprotein. Crystals of 1.0 × 0.2 × 0.2 mm were grown in 100 mM citrate, pH 4.5, 21% polyethylene glycol 6000 in the presence of low concentrations of subtilisin, resulting in proteolytic removal of the linker and purification tags. The structure was solved to a resolution of 2.4 Å with an R factor of 20.6, an R̃ factor of 28.5, and good stereochemistry. This result could lead to a clinically useful product based on antibody C219 for the diagnosis of P-glycoprotein-mediated multidrug resistance. The molecule will also be useful in biophysical studies of functional domains of P-glycoprotein, as well as studies of the intact molecule.

The Fv portion of an antibody comprises the variable domains of a heavy and a light chain, and it is the smallest fragment that maintains the binding specificity and affinity of the entire antibody (1). This antibody fragment is produced most easily using recombinant DNA technology because, unlike Fab fragments (antigen-binding fragments), it cannot be readily produced proteolytically (2). Usually a single chain construct (scFv) is made by linking the heavy and light chains with a flexible linker (3, 4). Several linkers have been reported in the past, but the linker (GGGGS)₃ has become most popular (2, 5, 6). This linker spans the 35-Å distance between the carboxyl terminus of one variable domain and the amino terminus of the other without distorting the conformation of the antigen binding site (3). Because of their small size, scFv fragments could be useful in tumor imaging and therapeutic strategies (7–9).

The murine monoclonal antibody C219 was raised against plasma membranes of multidrug-resistant Chinese hamster ovary cells (10), and is directed against Chinese hamster P-glycoprotein (Pgp), an ATP-dependent transporter. Pgp is a member of a large family of ATP-driven transmembrane transporters called ABC transporters, present in both eukaryotes and prokaryotes (11). Overexpression of Pgp has been associated with multidrug resistance in some cancer cells undergoing chemotherapy (12). Based on amino acid sequence analysis, Pgp is thought to consist of two halves, each with six transmembrane helices and a cytoplasmic nucleotide binding domain (NBD). C219 recognizes a continuous peptide epitope present in both NBDs of Pgp (13). The sequence of the epitope has been determined by Georges et al. (13) to be VQEALD in Chinese hamster Pgp. C219 has been shown to react with all P-glycoproteins studied so far but not with other ABC transporters (13–15). The antibody therefore has been used widely for Pgp immunohistochemistry (14–16), and has also been used to characterize novel Pgp cDNAs (17). A potential disadvantage of using the whole C219 antibody in immunohistochemical studies or in immunotherapy might be that its epitope lies in the cytoplasmic portion of Pgp and is therefore relatively inaccessible. Furthermore, it has been shown that some commercial lots of the C219 monoclonal antibody contain antibodies to blood group A carbohydrate determinants (18) and that commercially available C219 may cross-react with unrelated proteins (19). The use of a recombinant version of C219 may improve the uptake into cells and at the same time rule out contamination with other antibody specificities. Furthermore, it would facilitate mutagenic studies aimed at improving the binding and selectivity of the molecule and could form the basis for developing immunotoxins against cells overexpressing Pgp.

Additionally, the scFv can be a useful tool in the process of crystallization of Pgp or of its separate NBDs. An atomic structure of P-glycoprotein would be of great interest in understanding the process of multidrug resistance, in particular, the atomic basis of substrate recognition and the mechanism by which ATP drives the transport process. Such a structure would also contribute valuable information on other members of the ABC superfamily. At present, structural information on ABC transporters is limited to a low resolution structure of Pgp determined by electron microscopy (20) and two recent theoretical models for NBDs (21, 22).

We have designed, cloned, and expressed a gene encoding a

1 The abbreviations used are: Pgp, P-glycoprotein; scFv, single chain variable fragment; ABC, ATP binding cassette; NBD, nucleotide binding domain; Vₓ, light chain variable domain; Vᵧ, heavy chain variable domain; RMS, root mean square; CDR, complementarity-determining region; R, residual; Fₒ, observed structure factor amplitudes; F̃, calculated structure factor amplitudes.
scFv variant of C219. The protein product was purified and crystallized, and the three-dimensional structure was determined to a resolution of 2.4 Å.

EXPERIMENTAL PROCEDURES

Isolation and Characterization of Monoclonal Antibody C219—A cDNA clone encoding the antibody was obtained and sequenced previously. The determination of its peptide epitope sequence has been described (13).

Construction of the Expression Vector and the scFv Construct—The expression vector pSJF2 was designed and donated by Dr. S. Foote (National Research Council of Canada, Ottawa, Ontario, Canada). The vector is based on pUC8 (23) and contains a multiple cloning site (National Research Council of Canada, Ottawa, Ontario, Canada). The expression vector pSJF2 was designed and donated by Dr. S. Foote (National Research Council of Canada, Ottawa, Ontario, Canada). The expression vector were grown in M9 medium supplemented with 12% (v/v) 10-phenyl-2-nitrophenyl-β-D-galactopyranoside to a final concentration of 0.1 mM, and the culture was grown for an additional 64 h at 24 °C.

Isolation of Periplasmic Fractions and Purification of scFv—Cells were harvested by centrifugation at 5000 × g and washed once in 10 mM Tris-HCl, pH 8.0, 0.9% NaCl and once in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 25% sucrose. Cells were osmotically shocked by rapid dilution in 10 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 100 µM ethylenediaminetetraacetic acid, 0.9% NaCl and 1 mM EDTA, 25% sucrose. Cells were osmotically shocked by rapid dilution in 10 mM Tris-HCl, pH 8.0, 5 mM MgCl2, and the periplasmic fraction was collected by centrifugation at 5000 × g. Osmoshock supernatant was concentrated using ultrafiltration in a stir cell with a 10-kDa cutoff (Amicon Inc., Beverly, MA) and dialyzed against 50 mM sodium phosphate buffer, pH 8.0. The protein was loaded onto a Ni2+-nitrilotriacetic acid agarose column (Qiagen, Chatsworth, CA); washed with 50 mM sodium phosphate buffer, pH 6.0, 300 mM NaCl, and 10% glycerol; and eluted with 50 mM sodium acetate buffer, pH 4.0, 300 mM NaCl, and 10% glycerol. The protein was further purified to homogeneity using anion exchange chromatography on a 1-ml Hi-TRAP Q-column (Pharmacia, Uppsala, Sweden).

Three-step Western Blot Analysis—SDS-polyacrylamide gel electrophoresis gels loaded with whole cell extracts of E. coli cells overexpressing a fusion of the carboxyl-terminal NBD of Pgp to maltose-binding protein (24) was electroblotted onto nitrocellulose. Blots were treated with scFv C219 as a primary antibody, mouse anti-c-myc as a secondary antibody, and peroxidase-conjugated goat anti-mouse as a tertiary antibody. Blots were stained with diaminobenzidine and compared with conventional Western blot analysis (data not shown).

RESULTS

A single chain variable domain construct of monoclonal antibody C219 was made, expressed in E. coli, and purified from the periplasmic fraction. Typically, the yield was 1.5–4.5 mg of protein per liter of cell culture. The protein was soluble and active, as judged from three-step Western blot analysis (Fig. 1). Preliminary crystallization conditions were identified by screening a standard set of solutions (CrystalScreen, Hampton Research). Initially, crystals appeared within 3 weeks and grew to a maximum size of 0.5 × 0.1 × 0.1 mm. SDS-polyacrylamide gel electrophoresis analysis of individual crystals indicated that the crystallizing species lacks the linker peptide, c-myc epitope, and His tag (data not shown). Degradation of scFv
proceeded slowly upon prolonged storage, probably due to proteolysis by contaminating proteases (Fig. 2). Therefore, in subsequent crystallization experiments, subtilisin was added directly to the hanging drops. Crystals then appeared within 3 days and grew to a maximum size of 1.0 × 0.2 × 0.2 mm. A preliminary x-ray diffraction evaluation of these crystals showed significant intensities to a resolution of 2.2 Å and permitted the assignment of the crystallographic unit cell to the orthorhombic space group P2_1_2_1_2_1 with dimensions a = 59.04 Å, b = 64.35 Å, and c = 154.11 Å. Based on a calculated molecular weight of 25,630, there are two scFv molecules in the crystallographic asymmetric unit (8 per unit cell), resulting in an expected unit cell volume to mass ratio (V_M) of 2.85 Å³/dalton (32) and a solvent content of about 58% in the crystal. The data collection statistics are summarized in Table I. Data were collected to 91.6% completeness, with an R_sym of 9.8 on intensities. Molecule replacement yielded two solutions, corresponding to the expected two molecules per asymmetric unit.

Refinement statistics are summarized in Table II. The final model contained 3600 non-hydrogen protein atoms and 102 water molecules, with a standard R factor of 20.6% and an R_free of 28.5%. Root mean square (RMS) deviations from ideality were 0.006 Å for bond lengths, 1.110° for bond angles, 30.26° for dihedral angles, and 0.572° for improper angles. The Ramachandran plot (Fig. 3) showed 81.4% of the 403 non-glycine, non-proline residues in the most favored regions, while none were found in disallowed regions (33). All other geometric parameters analyzed by PROCHECK had acceptable values for a 2.4-Å structure (31).

Each scFv consists of two immunoglobulin fold units, as is seen in the variable regions of antibodies, one each from the light (L) and heavy (H) chains. The two units interact noncovalently to form an internal β-barrel with the antigen binding site at one end.

As mentioned above, there are two scFv molecules in the asymmetric unit. The two molecules are related by a pseudo-2-fold axis approximately along the diagonal between the a and b axes. No noncrystallographic symmetry restraints were used during refinement. The variable domains of the individual light and heavy chains (V_L and V_H) were virtually identical (RMS deviations for the light and heavy chains were 0.44 and 0.52 Å, respectively), but in one of the molecules, the domain interface was “twisted” by about 1.4° along an axis approximately perpendicular to the pseudo-2-fold axis relating V_L to V_H. This led to a RMS deviation of 3.9 Å when the complete Fv fragments were superimposed. Fig. 4 shows a strong interaction between the heavy and light chains mediated by two glutamines (Gln-H39 and Gln-L44). In the second molecule in the asymmetric unit, the distance between these two residues was increased by about 1 Å because of the twist mentioned above, but the geometry was still suitable for the formation of two hydrogen bonds. Crystal contacts between the molecules are mainly formed by Van der Waals interactions, with the exception of a strong hydrogen bond (2.74 Å) between the side chain of Gln-H65 in molecule two and the main chain carbonyl of Thr-L12 in molecule one. Two of the CDR loops (L1 and H3) do form some weak crystal contacts between the two molecules, although both binding sites are largely accessible to the solvent channels.

DNA sequencing revealed a number of discrepancies with the deposited C219 sequences (heavy chain, GenBank accession no. Z33491; light chain, GenBank accession no. Z33492): Ile-L92 → Tyr, Met-L96 → Asn+Asp, Thr-H71 → Ile, and Ser-H120 → Pro. These discrepancies could be due to sequencing errors or amplification errors. Only one of these (Met-L96 → Asn+Asp) affected the binding region, CDR L3. Because this sequence (Asn, Asp) is commonly found in this loop in related light chain sequences as revealed by a BLAST search (data not shown), we ascribed this discrepancy to an error in the original deposited sequence. The final electron density is consistent with our DNA sequencing results and unambiguous where the sequence differs from the GenBank entry.

Analysis of the canonical structures of the six antigen bind-

Table I

| Resolution (Å) | I | I/σ | R_sym | Total | Unique | Completeness (%) | Redundancy |
|----------------|---|-----|-------|-------|--------|------------------|------------|
| Total          | 50.2–2.36 | 48.1 | 9.7   | 9.8%  | 81247  | 22616            | 91.6%      | 3.6        |
| Highest shell  | 2.55–2.36 | 19.7 | 2.8   | 27.7% | 8466   | 3671             | 76.1%      | 2.3        |

* R_sym = Σ[I − ⟨I⟩]/Σ[I], summed over all reflections, where ⟨I⟩ is the average intensity of I equivalent (scaled) measurements of a reflection.

**Observed unique reflections.

| Resolution (Å) | R<sup>i</sup> | R<sub>free</sub> |
|----------------|---------------|-----------------|
| Resolution, 6.00–2.36 Å | 20.58% | 28.45% |
| Resolution, 2.46–2.36 Å | 25.82% | 37.30% |
| RMS deviation bond lengths | 0.006 Å | |
| RMS deviation angles | 1.110° | |
| RMS deviation dihedral angles | 30.26° | |
| RMS deviation impropers | 0.572° | |

* R = Σ(Fo − Fc)/Σ(Fo).

Fig. 3. Ramachandran plot as produced by PROCHECK (31). ■, non-glycine residues; ▲, glycine residues; A, B, and L, most favored regions for α helices, β sheets, and loops, respectively; a, b, and l, additional allowed regions. −a, −b, and −l, generously allowed regions. 81.4% of the non-glycine, non-proline residues were found in most favorable regions.
**Fig. 4.** Detail of the electron density at the $V_L$-$V_H$ interface, contoured at 1σ. View is “up” toward the CDRs along the pseudo-2-fold axis. Two short-range hydrogen bonds between glutamates in the light and heavy chains are indicated in green. Figs. 4 and 5A were produced with SETOR (49).

**Fig. 5.** A, crystal packing around the antigen binding site. The light chain is indicated in yellow, the heavy chain in green. In blue, two symmetry-related heavy chains are shown. CDRs are indicated by number and are shown with residue side chains. B, molecular surface area of the antigen binding site. The molecular surface is colored for electrostatic potential (blue for positive charge and red for negative charge) on the left and hydrophobicity (green) on the right. The approximate location of the CDRs is indicated. Produced with GRASP (50).
ing loops (CDRs; Ref. 34) showed the following conformations:

L1, type 3; L2, type 1; L3, type 1; H1, type 1; and H2, type 3. Apart from loop H3, for which no canonical classes have been defined to date, the CDRs did not have novel main chain conformations.

Fig. 5A shows the antigen binding site in relation to neighboring molecules in the crystal. Although some of the CDRs, especially L1 and H3, are involved in crystal contacts, there appears to be space for the epitope peptide to bind without perturbing the crystal lattice, which would allow us to study the conformation of the peptide in the antigen binding site. Fig. 5B shows the molecular surface of the antigen binding site, colored for electrostatic potential on the left and exposed hydrophobic residues on the right. The antigen binding surface of the two molecules in the asymmetric unit are not significantly different, despite the small distortion mentioned above (data not shown). The antigen binding surface shows a charged well formed by Arg-H99 (CDR H3) and Asp-L97 (CDR L3), as well as an exposed hydrophobic patch formed by Val-H101 (CDR H3) and Phe-H33 (CDR H1).

Discussion

Although many similar single chain antibody fragments have been made in the past, only nine have been crystallized successfully to date (28). In the crystallization of scFv C219, once the crystals were found to contain molecules with a cleaved linker, a small amount of subtilisin was used as an additive to the crystallization droplet itself, rather than prereacted. Interestingly, in a previously reported scFv structure (Protein Data Bank entry 1MFA), no electron density was found for the (uncleaved) linker region, despite the high resolution of the diffraction data (38). The simple approach of adding subtilisin to the crystal drops may be applicable to other single chain products or to multidomain proteins in general.

The electron density for the third light chain CDR convincingly clarified the ambiguity in the primary sequence results. Clear, continuous electron density with the appropriate shape for two side chains (Asn and Asp), as opposed to a single Met, was evident at position 96. This region is very well defined, with B values well below average. Asp-L97 interacts with Arg-H99 on the antigen binding surface and may well contribute directly or indirectly to antigen binding.

The comparison of the two independent scFv molecules in the final model illustrates the flexibility in $V_{\gamma} - V_{\delta}$ interaction in antibodies. This type of flexibility has been observed commonly in antibodies and antibody fragments and can indeed be much more significant than that observed here (39, 40). In most cases, however, conformational changes of this nature have been reported upon binding of antigen. In this structure, the small interface twist is likely an adaptation to the crystal lattice. Nonetheless, it is an example of the inherent flexibility and adaptability of antibody molecules and may be an important factor in antigen binding.

The determination of the three-dimensional structure of the single chain variable fragment of C219 is a first step toward obtaining detailed information on the atomic structure of Pgp and its functional domains. The corresponding epitope peptide of Pgp has been synthesized (13) and shows a slight helix propensity in solution, as determined by circular dichroism. A helical wheel plot of the sequence shows an amphiphilic helix (Fig. 6), in which the residues implicated in C219 binding (13) are clustered on one side of the helix. This is in agreement with our model of ABC transporter NBDs (22). A prediction of the detailed mode of epitope binding by simulated docking, solely based on the structure of an unliganded antibody molecule, is very difficult if not impossible. However, based on the surface plots depicted in Fig. 5B and the helical wheel plot in Fig. 6, a preliminary, qualitative proposal can be made. The Asp in the epitope, identified as the most crucial determinant of binding, would be exposed on the surface of Pgp and interact with Arg-H99 in a cleft in the antigen binding site of the antibody, between H1 and L1. The Val and Leu in the epitope could then bind to a hydrophobic patch on the antibody, formed by Phe-H33 in H1 and Val-H101 in H3. Crystals of scFv in the presence of its peptide epitope have been obtained but are not yet of sufficient size and quality to allow structure determination. Obtaining suitable crystals of the scFv with bound peptide epitope would provide a confirmation of the helical nature of the epitope as observed in solution and a test of the binding mode proposed here.

Attempts to crystallize Pgp or its separate domains have not been successful so far. However, binding of the scFv to separately expressed and purified nucleotide binding domains of P-glycoprotein or to the intact molecule itself will be a valuable approach in attempts to enhance the chances of their crystallization. It has been shown that the crystallization of intrinsic membrane proteins can be facilitated by the use of Fv domains to form complexes that increase the extramembrane surface area available for making crystal contacts (41, 42).

As discussed in the Introduction, the antibody C219 has been used extensively in the study of the localization and properties of P-glycoprotein, as well as in the detection of P-glycoprotein-mediated drug-resistant cells. This detection may be important clinically in the evaluation of the prognosis and treatment of certain types of cancer (35–37). The scFv described here may also be a useful reagent in such detection assays and is a starting point for future variants produced by genetic engineering techniques that have improved properties for clinical application. In particular, the design and production of immunotoxins based on this construct could have a significant clinical relevance (43, 44). Although C219 is directed toward a cytoplasmic epitope, there are a number of efforts under way to direct compounds to the cytoplasm either by using translocation signals (e.g., charged peptide sequences (45)) or by targeting cell surface receptors (e.g., the insulin receptor (46)). An alternative approach relevant to gene therapy protocols is to express products intracellularly (47, 48). In either event, it would be of interest to develop products with higher affinity.
and specificity. The design of these variants will be facilitated by the atomic structural analysis of the scFv as presented here.

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