Detection of P-glycoprotein isoforms by gene-specific monoclonal antibodies
(epitope mapping/peptides/immunohistochemical staining/multidrug resistance)

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Communicated by Keith R. Porter, August 31, 1989

ABSTRACT P-glycoprotein is a highly conserved membrane protein shown to be overexpressed in many multidrug-resistant tumor cell lines. P-glycoprotein is encoded by a small gene family in mammalian cells. Class I and II isoforms cause multidrug resistance, whereas class III does not. In this report, we have characterized three P-glycoprotein-specific monoclonal antibodies (mAbs) by high-resolution epitope mapping with a series of hexapeptides. mAb C494 is gene specific, binding to a sequence present only in the class I isoform of hamster and human. The mAb C32 recognizes a sequence conserved in hamster class I and II isoforms but not in class III isoforms. In contrast, the mAb C219 recognizes a highly conserved amino acid sequence found in all P-glycoprotein isoforms characterized to date. These mAbs were used to reveal differential expression and specific localization of the three P-glycoprotein isoforms in hamster tissues by immunohistochemical staining and competition with epitope-specific peptides. Colonic epithelial cells expressed predominantly the class I isoform in a polarized manner, adrenal cortical cells expressed predominantly the class II isoform, whereas a small percentage of skeletal muscle fibers expressed the class III isoform of P-glycoprotein. These findings suggest that the P-glycoprotein isoforms have distinct physiological roles associated with specialized cell functions.

The development of multidrug-resistant tumor cells during malignant progression may be a major factor contributing to nonresponse in chemotherapeutic treatment of cancer. The increased expression of the membrane P-glycoprotein (Pgp; Mr, 170,000) is the most consistent change seen in multidrug-resistant cells in vitro, and gene transfer studies have shown this change to cause multidrug resistance (MDR) (1–3). The role of Pgp as an energy-dependent efflux pump was proposed from its primary sequence and structural similarity to many membrane-associated transport proteins—most notably, the bacterial transport protein hemolysin B (4–6). The presence of Pgp or its mRNA transcript has been demonstrated in a variety of human malignant tumors, and Pgp may play a role in limiting a patient's response to chemotherapy (7–11). Pgp is also found in certain normal tissues, including large intestine, adrenal glands, kidney, liver, and brain (12–18); such localization has led to the speculation that this glycoprotein is involved in normal detoxification and transport of lipophilic molecules.

Recent data indicate that Pgp is encoded by a family of three genes in rodent and two genes in human (19). A comparison of the amino acid sequences among the different gene family members, or isoforms, indicates a similar overall structure (20). However, transfection studies that used full-length cDNAs have suggested that only some Pgp isoforms confer a MDR phenotype on otherwise drug-sensitive cells (19, 21). Thus, the class I and II isoforms have been directly implicated in drug resistance, whereas the function of the class III isoform is not known (19). A study of the differential expression of Pgp isoforms in normal mouse tissues where gene-specific cDNA probes and Northern (RNA) blot analysis were used has been recently described (22). However, localization of Pgp expression was not possible in that study.

Immunohistochemical staining of tissue for Pgp expression has the advantages of single cell localization and detection of polarized distribution. However, until now the question of differential gene expression could not be addressed with the available monoclonal antibodies (mAbs). In the present study, we report on the epitope mapping of three Pgp-specific mAbs to a resolution of three amino acids. In addition, we demonstrate the differential expression of Pgp isoforms in hamster tissues by using these reagents and their epitope-specific peptides in a competitive immunohistochemical staining assay.

MATERIALS AND METHODS

Materials. Prederivatized plastic pins and polypolypropylene trays were obtained from Cambridge Research Biochemicals (Valley Stream, NY). Active esters of 9-fluorenylmethoxycarbonyl (Fmoc) amino acids were supplied by MilliGen (Millipore), phenylacetalidomethyl polystyrene (Pam) resins were from Applied Biosystems, and t-butoxycarbonyl (Boc) amino acids were from Institut Armand Frappie Biochemicals (Montreal). Other reagents used were of highest chemical grade.

Solid-Phase Peptide Synthesis. Overlapping hexapeptides were synthesized on polyethylene pins as described by Geyser et al. (23). The peptides were assembled on the pins in the C-to N-terminus direction by using 9-fluorenylmethoxycarbonyl-protected amino acids (24). Larger quantities of the characterized linear epitopes of mAbs C219, C494, and C32 were synthesized by classical solid-phase methods (25, 26).

ELISA. Peptides coupled to solid-support polypropylene pins were incubated in phosphate-buffered saline (PBS) (pH 7.4) for 30 min at room temperature. Pins were then soaked for 1 hr in a blocking buffer [1% (wt/vol) ovalbumin, 1% (wt/vol) bovine serum albumin, 0.1% (vol/vol) Tween 20 in PBS] to reduce nonspecific adsorption of antibodies. The pins were incubated overnight at 4°C in wells containing 100-μl aliquots of primary antibody solutions (0.5–2.0 μg of dissolved mAb per ml of blocking buffer). After 1-hr incubation with peroxidase-conjugated goat anti-mouse antibody, pins were washed four times with PBS solution containing 0.05% (vol/vol) Tween 20. The binding of antibody to peptide was detected by incubating the pins for 30 min with a freshly prepared solution of 2,2'-azinobis(3-ethylbenothiazoline-6-sulfonic acid) in 1 M citric acid (pH 4.0). Measurements of color development were made at 405–630 nm by

Abbreviations: Pgp, P-glycoprotein; MDR, multidrug resistance; mAb, monoclonal antibody.
using the microplate reader (EL30; Bio-Tek, Burlington, VT).

**Immunohistochemical Staining.** Frozen sections of normal Chinese hamster tissues were fixed in cold acetone (10 min at 4°C) and then stained for Pgpl by using an avidin–biotin–peroxidase complex technique. The primary antibody (C219, C32, or C494) was used at 10 μg/ml in 1% bovine serum albumin/PBS. One hour before incubation of tissue sections with the primary antibody, the antibody solution was preincubated with a 100-fold molar excess of either the peptide encoding the antibody epitope or an irrelevant peptide. Competitive binding of the primary antibody to the peptide and to Pgpl present in tissues proceeded for 1 hr at room temperature in a humidified chamber. The sections were washed with PBS and incubated sequentially with biotinylated horse anti-mouse antibody and with avidin–biotin–peroxidase complex (Vector Laboratories), according to manufacturer’s instructions. The binding of the antibody to tissues was detected by 5-min incubation with 3,3′-diaminobenzidine tetrahydrochloride (1 mg/ml; Sigma) and hydrogen peroxide (0.03%). Tissues were counterstained with hematoxylin, dehydrated, and mounted in Permount.

**RESULTS AND DISCUSSION**

**Epitope Mapping.** Overlapping hexapeptides covering the entire 211-amino acid fragment from the C-terminal cytoplasmic domain of hamster Pgpl [shown previously to contain all three mAb-binding sites (27)] were synthesized on polypropylene pins (Fig. 1). Each successive pin contained the last five residues of the preceding one and the following amino acid in the sequence. A library of ~250 peptides, including all 206 hexapeptides to span this segment of the protein, was screened by ELISA with each mAb to determine its binding sequence. mAb C219 reacted more strongly with the two peptides 198 and 199 than with any other peptides tested. mAb C494 reacted with four successive peptides (peptides 17–20), giving a stronger signal than mAb C219. In contrast, mAb C32 binding to synthetic hexapeptides was substantially weaker. The strongest binding of mAb C32 occurred over two peaks, peptides 119, 120 and 125–129. As detailed below, the weakness of these signals is attributed to the small size of the hexapeptide relative to the size of the mAb C32 epitope.

The results in Fig. 2 represent the relative intensities of the ELISA signals obtained for the binding of the three mAbs to the generated synthetic peptides. The movement of the 6-amino acid window defines the boundaries of the continuous epitopes and identifies some amino acids critical for binding. For example, mAb C219 bound strongly to the amino acid sequence [in single-letter code (28)] QVEALD with Val-506 and Asp-511 representing two critical amino acids required for antigen recognition. mAb C494 bound to four hexapeptides covering the amino acid sequence KP-NLLEGNY with Thr-323 and Glu-325 as the critical residues. Differences in the number of hexapeptides involved in antibody binding when using this assay are likely related to the size of the epitope recognized by a given antibody and the number of residues (distance) between the critical amino acids in a given epitope. mAb C32-binding domain covers a stretch of 13 amino acids (GDNRSRVYSQDEIER), with four critical amino acids associated with its binding—Asp-427 and Val-431 from the first domain and Glu-436 and Glu-438 from the second. The weak signals seen for mAb C32 in Fig. 1 were due to the small size of the synthetic hexapeptides used to probe for its epitope. A comparable ELISA signal to that seen with mAb C494 was obtained with mAb C32 when the complete sequence of 13 amino acids (GDNRSRVYSQDEIER) was synthesized on these pins (Inset of Fig. 1, mAb C32 graph). Extension of the peptide sequences for the mAb C219 and C494 epitopes, however, did not result in any increased signal.

The amino acid sequences of the antigenic peptides from Fig. 1 were located on the full structure of Pgpl, as shown in Fig. 2. mAb C219 binds to an amino acid sequence 6 residues away from the consensus sequence of the B site of the proposed ATP-binding domain. A homologous amino acid sequence for mAb C219 is also found in the N-terminal half of Pgpl. The epitope recognized by mAb C494 is on the other side of the ATP-binding domain to that of mAb C219. mAb C32 binds to a region positioned between the A and B sites of the postulated ATP-binding domain. However, the N-terminal half of Pgpl does not contain a homologous sequence for the mAb C494 or C32 epitope.

**Identification of mAbs As Gene-Specific Probes.** Fig. 3 lists the relative signals from the ELISA performed by using analogous peptide sequences from the different Pgpl isoforms. The binding of mAb C219 to the peptide sequence VQAALD in the N-terminal half of rodent Pgp isoforms was stronger than that seen for the epitope sequence VQALD in the C-terminal half, due to a single amino acid substitution (alanine substituted for Glu-508). The substitution of valine
for Glu-508 in the N-terminal half of human class I Pgp isoform (VQVALD) reduced its binding to the antibody (Fig. 3). Hamster Pgp-specific cDNA clone, encoding the ATP-binding domain in the N-terminal half of Pgp, was shown to contain the mAb C219 epitope. A protein fragment with the expected molecular mass was immunoprecipitated from an in vitro expression system using only the mAb C219. These results further corroborate those seen using the synthetic peptides (J. Endicott, E.G., and V.L., unpublished observation).

Results from the ELISA for binding of peptide analogs of the mAb C494 epitope indicated that substitution of a single critical amino acid (Thr-323) by tryptophan in class II hamster (pgp2) or lysine in class III hamster (pgp3) and human (mdrl) causes complete loss of antibody recognition. These findings confirm that mAb C494 is a specific immunological probe for the expression of human (mdrl) and hamster (pgp1) Pgp class I isoform (Fig. 3). Because in vitro transfection studies have shown that human class I gene (mdr1) can confer MDR in a variety of cells (29), positive staining with mAb C494 may be predicted in cells expressing the MDR phenotype in human tissues. Analogs for only part of the mAb C32-binding domain (SQDEIERE) were synthesized for comparison between the different isoforms of Pgp because the remainder of the same antibody binding domain is conserved among the different members of Pgp gene families. The signals in Fig. 3 show the effect of a single critical amino acid substitution on binding of mAb C32 to this peptide (e.g., antibody is not bound when valine is substituted for Glu-438). Therefore, mAb C32 should bind most strongly to hamster

Fig. 2. Epitope location of the three mAbs on Pgp. Schematic representation of the tandemly repeated Pgp molecule (hamster pgpl) shows 12 putative transmembrane domains (numbers 1–6 and 1′–6′). The two proposed A and B ATP-binding domains are indicated by the hatched squares. The sites and the amino acid sequences (epitopes) recognized by the three mAbs are indicated by arrows and within the boxes for mAb C219, C494, and C32; the peptide numbers correspond to the hexapeptides of Fig. 1. OD signals from the ELISA are indicated as a relative value with five pluses representing the highest OD with that mAb.

Fig. 3. Comparison of antibody binding to peptide analogs of their binding sequences. The different peptide analogs for mAbs C219, C494, and C32 have been tested for binding to their respective antibodies. OD signals from ELISA were converted to plus signs, as in Fig. 2. Species origin and gene member of the Pgp gene family (as found in the literature) are in parentheses. Amino acids (single-letter code) in boldface represent different amino acids in analog than in antibody-binding sequences.

Table 1. Epitope distribution of Pgp isoforms

<table>
<thead>
<tr>
<th>Pgp isoform class*</th>
<th>mAb C219</th>
<th>mAb C32</th>
<th>mAb C494</th>
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</thead>
<tbody>
<tr>
<td>Hamster</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I (pgpl)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>II (pgp2)</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>III (pgp3)</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Human</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I (mdr1)</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>III (mdrl)</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II (mdr1)</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>III (mdrl)</td>
<td>+</td>
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*Ng et al. (19).
class I and II products and much more weakly to the other Pgp isoforms. In agreement with the above conclusion, previous results have shown that hamster Pgp binds more strongly to mAb C32 than that of mouse and human using a Western (immunologic) blot technique (30).

**Immunohistochemical Staining.** Serial sections of each tissue were incubated with each of the three mAbs, either with or without a peptide containing the respective epitope sequence. Specific staining by mAb against Pgp was defined as staining that could be completely abolished by competition with 100-fold molar excess of the epitope-containing peptide. This allowed positive identification of epitope-specific staining and contrasted with conventional immunohistochemical staining in which an “irrelevant” antibody is used as negative control.

Staining with all three antibodies, in which there was no competition among them, was frequently observed. Notably, this included strong membrane staining of epithelial cells of seminal vesicles, distinct staining of cells of colonic crypts, and moderate intracytoplasmic staining of hepatocytes. Such staining is probably due to nonspecific interactions with the antibody through regions other than those containing the paratope domain. Thus, use of peptides in a competitive binding assay can clearly result in enhanced specificity and sensitivity of the immunohistochemical procedure.

The epitope-mapping studies predict that three distinct patterns of reactivity with the mAb panel would result from the expression of each of the three hamster Pgp isoforms (Table 1).

Staining of intestinal tissue sections with mAb C494 alone (Fig. 4A) and in the presence of a peptide containing the mAb C494 epitope (Fig. 4B) revealed a strong signal for the luminal surface of colonic epithelial cells, which was abolished when free peptide was added. A similar staining was seen with

![Fig. 4. Epitope-specific staining of Pgp isoforms. Ascending colon stained with mAb C494 (A) or mAb C494 in the presence of C494 peptide (KPNTLEGNVK) (B). Adrenal gland stained with mAb C32 (C) or mAb C32 in the presence of C32 peptide (GDNSRVSQDIEERAC) (D). Skeletal muscle from chest wall stained with mAb C219 (E) or mAb C219 in the presence of C219 peptide (VVQELDKAREGRTC) (F). (A and B, X70; C and D, X90; E and F, X55.)](image-url)
mAbs C219 and C32 (data not shown). This staining pattern is consistent with the predominant expression of the class I Pgp isorm (see Table 1). The immunohistochemical staining shows that the class I isorm has a polarized distribution in the colonic epithelial cell membrane that is consistent with a membrane transport function.

Fig. 4 C and D shows the staining of adrenal tissue sections with mAb C32 without and with its epitope, respectively. The plasma membrane of adrenal cortical cells stained with mAbs C32 and C219 but not with mAb C494 (data not shown), indicating the predominant expression of the class II Pgp isorm (see Table 1). Previous reports of Pgp expression in the adrenal gland suggest that Pgp may be involved in the transport of corticosteroids. The detection of Pgp in the steroid-producing zona fasciculata and zona reticularis in this study is consistent with the above hypothesis. However, cortical cells in the zona glomerulosa, also known to produce steroids, do not express detectable levels of Pgp, and this suggests that Pgp is not a general mechanism for secreting steroids, but that it may be a marker of differentiation of specialized cortical cells. Because the immunohistochemical technique described here is semiquantitative, it was not possible to determine whether tissues exhibiting the class I pattern of staining also expressed much lower levels of the other two isorms. Similarly, tissues exhibiting the class II staining pattern may also express the class III isorm at low levels (Table 1).

Hamster skeletal muscles incubated with mAb C219 (Fig. 4F) showed staining of approximately 5% of muscle fibers. However, the staining was completely abolished in the presence of peptide containing the mAb C219 epitope (Fig. 4F). Staining of skeletal muscle with mAbs C32 or C494 did not reveal epitope-specific staining (data not shown). This staining pattern is specific for class III isorm expression alone (Table 1). The expression of the class III isorm was previously seen in Northern blot analysis (mdr2 positive) of mouse muscle tissue (22). However, whether the expression of the class III isorm was present in only a subset of muscle fibers, as shown here, could not be determined in that study. The restricted expression within muscle tissue suggests the presence of some specialized muscle fibers not previously recognized. The distribution of this isorm within muscle fibers seen under high magnification follows a fairly regular, coarse, transverse pattern, in addition to patchy areas of plasma membrane staining (data not shown). Thus, the class III Pgp isorm may be part of an energy-dependent mechanism for the transport of metabolites, possibly hormones, across the complex membrane system of these specialized skeletal muscle fibers. Ultrastructural studies are ultimately required to definitively localize Pgp to subcellular structures in skeletal muscle fibers.

In summary, previous immunohistochemical studies of Pgp expression in normal tissues and tumor samples have provided insight into the cellular localization of this membrane glycoprotein (12–18); however, distinguishing what classes of Pgp isorms are expressed at the protein level has not been possible. Results of the epitope-mapping studies described here show that the three classes of mAbs, represented by C219, C32, and C494, may be used in combination to determine the pattern of expression of the Pgp isorms in normal and tumor tissues. This technique may also have applications in studies for investigating the correlation of Pgp isorm expression with patient response to cancer chemotherapy.

We thank Roman Zastawny for preparing the figures and helpful discussion. We are grateful to Dr. Carlos Cordon-Cardo for valuable advice and Andes Mah for his technical assistance concerning immunohistochemistry procedures. We also thank our colleagues for their critical reading of the manuscript. This work was supported by the National Cancer Institute of Canada, and by Public Health Service Grant CA37130 from the National Institutes of Health.