The Minimum Functional Unit of Human P-glycoprotein Appears to be a Monomer*

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Several studies have demonstrated the presence of oligomers of P-glycoprotein in multidrug-resistant cells. The minimum functional unit of P-glycoprotein, however, is not known. In order to determine whether the functional unit is an oligomer, we tested for associations between P-glycoproteins containing either a histidine tag or the epitope tag for monoclonal antibody A52 at the COOH-terminal end of the molecule. Both tagged molecules were active and had indistinguishable drug-resistance profiles. The tagged P-glycoproteins were expressed contemporaneously in HEK 293 cells, purified by nickel-chelate chromatography followed by immunoblot analysis. We found that P-glycoprotein-A52 did not copurify with functionally active P-glycoprotein-(His)10 even when the former was overexpressed relative to the histidine-tagged protein. Similar results were obtained with phosphorylation-deficient mutants of P-glycoprotein. By contrast, we could purify and reconstitute drug-stimulated ATPase activity when the half-molecules NH2-terminal half-(His)6/COOH-terminal half-A52 or NH2-terminal half-A52/COOH-terminal half-(His)10 were coexpressed in HEK 293 cells. These results suggest that nickel-chelate chromatography may be a suitable method for studying protein-protein interactions in membrane proteins and that the minimal functional unit of P-glycoprotein is likely to be a monomer.

P-glycoprotein is an ATP-dependent pump that transports a wide range of cytotoxic compounds out of the cell (1, 2). It may be one of several mechanisms responsible for multidrug resistance during chemotherapy. P-glycoprotein is expressed in a wide variety of tissues and cells. Its physiological role is unknown but it may act to protect the organism from endogenous and exogenous cytotoxic compounds. This is supported by studies on "knock-out" mice that were homozygous for a disruption of the mdr1a gene (3–5).

Cloning and sequencing of human MDR1 (6) showed that P-glycoprotein contains 1280 amino acids organized into two tandem repeats of 610 amino acids joined by a linker region of 60 amino acids. Each repeat consists of an NH2-terminal hydrophobic domain containing six potential transmembrane-spanning helices followed by a hydrophilic domain containing a nucleotide-binding site. The arrangement is supported by the results of topology studies on the full-length molecule (7, 8).

The mechanism by which P-glycoprotein is able to couple ATP hydrolysis to the efflux of a broad range of lipophilic compounds is unknown. Although each half-molecule of P-glycoprotein possesses basal ATPase activity, drug-stimulated ATPase activity was only observed when both halves were expressed contemporaneously (9). Both nucleotide-binding sites are also essential for function since mutations in either site (10, 11) or chemical modification of either site (12, 13) inactivates any coupling of ATPase activity to drug binding.

P-glycoprotein has been detected to be present in the membrane in the form of monomers, dimers, and higher oligomers (14–17). In the membranes of multidrug-resistant cells, the majority of P-glycoprotein exists in the form of dimers and higher oligomers (17). It is not known, however, whether P-glycoprotein can function as a monomer. Knowledge of the minimal functional unit of P-glycoprotein is important for understanding the mechanism of P-glycoprotein function. Drug-binding site(s) formed by a monomer would likely have significantly different implications than that formed by oligomers.

Our approach to identifying the minimum functional unit was to coexpress P-glycoproteins containing either a histidine tag or the epitope for monoclonal antibody A52 at the COOH-terminal end of the molecule and test for associations using nickel-chelate chromatography. Our results suggest that the minimal functional unit is a monomer.

EXPERIMENTAL PROCEDURES

Generation of Constructs—Full-length MDR1 cDNA and the cDNAs coding for the NH2- and COOH-terminal half-molecules, modified to encode the epitope for monoclonal antibody A52 at the COOH-terminal ends of the proteins, were inserted into the mammalian expression vector pMT21 as described previously (9, 18). Oligonucleotide-directed mutagenesis was carried out as described previously (18). For purification purposes, a full-length MDR1 cDNA and the cDNAs coding for the half-molecules were modified to encode for 10 histidine residues at the COOH ends of the proteins. The sequence at the COOH terminus of the full-length and COOH-terminal half-molecule of P-glycoprotein that would normally end as TKRQ now became TKRA(His)6LDPRQ. The sequence at the COOH terminus of the NH2-terminal half-molecule was RKL(A(His)6)LDPRQ.

Purification of P-glycoprotein Mutants and Measurement of Mg2+-ATPase Activity—For purification of P-glycoprotein mutants, HEK 293 cells transfected with the cDNA coding for the histidine- and/or A52-tagged P-glycoproteins were solubilized with 1% (w/v) n-dodecyl-β-D-maltoside and the mutant P-glycoproteins purified by nickel-chelate chromatography. Drug-stimulated ATPase activity was determined as described previously (11).

Immunological Procedures—The purified samples were subjected to SDS-PAGE, electrophoresed onto nitrocellulose, and developed with monoclonal antibody A52 (19), or with a rabbit polyclonal antibody against P-glycoprotein followed by enhanced chemiluminescence (Amersham) as described previously (20). To test for association between A52- and histidine-tagged P-glycoproteins, a coimmunoprecipitation approach was used. Both cDNAs were coexpressed in HEK 293 cells. The cells were solubilized with Tris-buffered saline (10 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing...
1% (w/v) CHAPS, and the insoluble material was removed by centrifugation at 16,000 *g* for 15 min. The supernatant was transferred to a fresh tube, and 20 *μ*g of monoclonal antibody A52 was added. After incubation at 4 °C for 2 h, the immune complexes were collected by using protein A-Sepharose CL-4B, and the beads were washed 4 times with Tris-buffered saline containing 0.1% (w/v) CHAPS. The immune complexes were then incubated in solubilization buffer (50 mM NaPO4, pH 8.0, 500 mM NaCl, 50 mM imidazole, 1% (w/v) n-dodecyl-β-D-maltoside). The beads were removed by centrifugation, and the supernatants were subjected to nickel-chelate chromatography as described above.

### RESULTS

**Coexpression and Interaction of Tagged Half-molecule Forms of P-glycoprotein—**Our approach to determining the minimum functional unit of P-glycoprotein was to coexpress and purify by nickel-chelate chromatography a histidine-tagged P-glycoprotein and a P-glycoprotein containing a monoclonal antibody A52 epitope tag at the COOH-terminal end of the protein and then assay for the presence of oligomers by immunoblot analysis with monoclonal antibody A52. Addition of a monoclonal antibody A52 epitope tag (18) or a histidine tag (11) at the COOH-terminal end of human P-glycoprotein had no detectable adverse effect on P-glycoprotein function. In addition, the presence of a histidine tag provides a simple and rapid method of purifying P-glycoprotein by nickel-chelate chromatography (11).

To test the feasibility of such an approach, we tested for interactions between half-molecules of P-glycoprotein containing either a histidine tag or the epitope for monoclonal antibody A52 at the COOH terminus. In a previous study (9), we showed that it was possible to express each half of P-glycoprotein as a separate polypeptide. Restoration of drug-stimulated ATPase activity was observed only when both half-molecules were expressed contemporaneously in insect cells. These results suggested that the two half-molecules could associate in vivo to form a functional complex. Since both of these half-molecules contained the epitope tag for monoclonal antibody A52 at their COOH terminus, it appeared that addition of residues to the terminus of either polypeptide did not interfere with their association or function. Accordingly, we constructed cDNAs coding for the NH2- and COOH-terminal half-molecules of P-glycoprotein containing 10 tandem histidine residues at their COOH terminus. The cDNAs for the histidine-tagged half-molecules were coexpressed transiently in HEK 293 cells with half-molecules containing the epitope tag for monoclonal antibody A52. The transfected cells were solubilized with n-dodecyl-β-D-maltoside detergent, centrifuged to remove insoluble material, and the supernatants were subjected to nickel-chelate chromatography. In previous studies (11) we first prepared membranes from transfected cells before solubilization with detergent. To minimize any potential loss of interaction between the two half-molecules and loss of material during preparation of membranes, the expressed polypeptides were solubilized directly from transfected cells. This modification of the procedure does not interfere with subsequent purification and measurement of the drug-stimulated ATPase activity of the full-length P-glycoprotein. Following nickel-chelate chromatography, the isolated proteins were reconstituted with phosphatidylethanolamine, and drug-stimulated ATPase activity was measured. Phosphatidylethanolamine was used for reconstitution as it forms aggregates of membrane sheets rather than sealed vesicles (21). A large excess of lipid (lipid:protein; 200:1) was necessary for maximal ATPase activity. Fig. 1 shows that stimulation of ATPase activity in the presence of saturating levels of verapamil (1 mM) was observed in the samples isolated from cells transfected with the full-length P-glycoprotein-(His)10 cDNA (11.4-fold), cotransfected with the NH2-terminal half-molecule-(His)10 and COOH-terminal half-molecule-A52 cDNAs (9.0-fold) or cotransfected with COOH-terminal half-molecule-(His)10 and NH2-terminal half-molecule-A52 cDNAs (7.9-fold). No verapamil-stimulated ATPase activity was observed, however, in those samples isolated from cells transfected with only the cDNAs for the NH2- or COOH-terminal half-molecules.

Immunoblot analysis (Fig. 2A) shows that the histidine-tagged NH2-terminal half-molecule could be purified by nickel-chelate chromatography. Comparison of equivalent loading of the flow-through and eluted fractions following purification shows that more than 50% of the histidine-tagged half-molecule could be recovered by nickel-chelate chromatography (Fig. 2A). To confirm that drug-stimulated ATPase activity was due to co-purification of both half-molecules, the purified proteins were subjected to immunoblot analysis with monoclonal antibody A52. As shown in Fig. 2B, about 50% of the A52-tagged COOH-terminal half-molecule could be recovered by nickel-chelate chromatography when it was coexpressed with the histidine-tagged NH2-terminal half-molecule. Similarly, about half of the A52-tagged NH2-terminal half-molecule was recovered when coexpressed with the histidine-tagged COOH-terminal half-molecule. The A52-tagged NH2-terminal half-molecule was not recovered when expressed alone or when coexpressed with the histidine-tagged COOH-terminal half-molecule. These results show that the two differently tagged half-molecule forms of P-glycoprotein could physically associate to form a functional complex, even in the presence of the relatively high ionic strength buffer (0.5 M NaCl and 0.3 M imidazole) used during purification. Therefore, the approach of using polypeptides with different tags and purification by nickel-chelate chromatography to study protein:protein interactions appeared feasible. We then tested for interactions between full-length P-glycoprotein-(His)10 and P-glycoprotein-A52.

**Coexpression and Isolation of Full-length P-glycoproteins Containing Different Tags—**The cDNAs coding for P-glycoprotein-A52 and P-glycoprotein-(His)10 were transfected into HEK 293 cells either individually or together at ratios of P-glycopro-

1 The abbreviation used is: CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
Coexpression of Tagged P-glycoproteins Lacking Phosphorylation Sites—It was possible that negligible amounts of P-glycoprotein-A52 copurified with P-glycoprotein-(His)_{10} because only low levels of dimers or oligomers were present in our expression system. It has been observed that monomeric forms of P-glycoprotein are much more extensively phosphorylated than oligomeric species (17). These authors postulated that a dephosphorylation event may precede oligomer formation. Accordingly, we constructed a phosphorylation-deficient mutant by mutating the five serine residues (Ser-661, -667, -671, -675, and -683) that are within the consensus sites for phosphorylation by protein kinase C to alanine. In agreement with the results of Germann et al. (22), we also found that the mutant was not phosphorylated following metabolic labeling of the transfected cells with {sup}{32}P{sub}orthophosphate (data not shown). The phosphorylation-deficient mutant was also modified to contain either a ten-histidine tag or the epitope for monoclonal antibody A52 at the COOH terminus of the molecule. The phosphorylation-deficient P-glycoprotein-(His)_{10} mutant was active and had a drug-stimulated ATPase activity of 1.08 μmol/min/mg of P-glycoprotein in the presence of 1 mM verapamil. This was similar to the specific activity of wild-type P-glyco-

protein-(His)_{10} (1.20 μmol/min/mg of P-glycoprotein). These phosphorylation-deficient mutants of P-glycoprotein were coexpressed, purified by nickel-chelate chromatography, and the eluted fractions were subjected to immunoblot analysis with monoclonal antibody A52 (data not shown).

Coimmunoprecipitation Assay—It was possible that our inability to detect an association between A52- and histidine-tagged P-glycoproteins was that they only formed homodimers between two histidine-tagged proteins or two A52-tagged proteins. To rule out this possibility, we tested for the presence of P-glycoprotein-A52/P-glycoprotein-(His)_{10} heterodimers in vivo by combining immunoprecipitation with nickel-chelate chromatography. The rationale for this approach was that if P-glycoprotein-A52/P-glycoprotein-(His)_{10} dimers were present, then histidine-tagged P-glycoprotein should coimmunoprecipitate with P-glycoprotein-A52 in the presence of monoclonal antibody A52. The presence of a relatively high concentration of n-dodecyl-β-d-maltoside (1%) and NaCl (0.5 M) in the solubilization buffer used for subsequent nickel-chelate chromatography should result in the disruption of any association between P-glycoprotein-A52 and P-glycoprotein-(His)_{10}. P-glycoprotein-(His)_{10} can then be recovered by nickel-chelate chromatography. As shown in Fig. 4, this was indeed the case. P-glycoprotein-(His)_{10} was recovered after immunoprecipitation with monoclonal antibody A52 followed by nickel-chelate chromatography only if it was coexpressed with the A52-tagged P-glycoprotein (Fig. 4B). P-glycoprotein-A52 did not bind to the nickel column and was detected only in the flow-through fractions (Fig. 4A). Similarly, there was no nonspecific immunoprecipitation of P-glycoprotein-(His)_{10} by monoclonal antibody A52. Therefore, it appears that the histidine- and A52-tagged P-glycoproteins can associate in vivo. This association was not disrupted by the presence of CHAPS and the low salt concentration (0.15 M NaCl) used during immunoprecipitation. In contrast, the presence of n-dodecyl-β-d-maltoside (1% (w/v)) and the high salt concentration (0.5 M NaCl) during nickel-chelate chromatography disrupted any association between P-glycoprotein-A52 and P-glycoprotein-(His)_{10}. These results suggest that P-glycoprotein recovered by nickel-chelate chromatography was monomeric.

Size Exclusion Chromatography—To further test whether purified P-glycoprotein-(His)_{10} was monomeric, it was subjected to size exclusion chromatography on a Sepharyl S-300 (Pharmacia) column, that has a useful range of M_{r} 1 × 10^{4} to 1.5 × 10^{6} for globular proteins. Chromatography was carried
out in the same buffer used for nickel-chelate chromatography (10 mM Tris-HCl, pH 7.5, 500 mM NaCl, 70 mM imidazole, pH 7.0, 0.1% (w/v) n-dodecyl-β-D-maltoside). The fractions were subjected to immunoblot analysis and compared to the elution positions of standard proteins. Fig. 5A shows that the majority (>80%) of freshly purified P-glycoprotein-(His)$_{10}$ eluted close to the catalase marker protein that has a molecular mass of 232 kDa and a Stokes radius of 52 Å. A small amount (10–20%) of P-glycoprotein-(His)$_{10}$ eluted in the void volume and may represent aggregated material. The amount of aggregated material increased substantially if the purified protein was frozen and thawed repeatedly or stored for several hours at 4 °C (Fig. 5B). Under these conditions, almost all of the purified protein eluted in the void volume. On SDS-PAGE, P-glycoprotein has an apparent mass of 170 kDa, while its apparent molecular mass by gel exclusion chromatography is approximately 230 to 250 kDa. These results suggest that purified P-glycoprotein probably associates with one micelle of n-dodecyl-β-D-maltoside (aggregation number 98, micellar weight 50,000) which would result in a protein-detergent complex with an mass of about 220 kDa. A dimer would have an estimated mass of at least 440 kDa. Therefore, the observation that purified P-glycoprotein-(His)$_{10}$ elutes close to catalase is consistent with its being in the monomeric form. This is in contrast to the Band 3 protein from erythrocytes. This polytopic membrane protein (molecular mass of 95 kDa) eluted between thryoglobulin (667 kDa) and ferritin (440 kDa) protein markers during gel exclusion chromatography in the presence of C$_{12}$E$_{8}$ detergent (23, 24). Its large size was consistent with its being a dimer-detergent complex, and it was calculated that each monomer associated with one micelle of detergent.

**DISCUSSION**

Contemporaneous expression of proteins containing different tags is a powerful method for studying potential interactions between proteins. It is possible to study their interactions in vivo by using immunoprecipitation assays (20). The major disadvantages of immunoprecipitation assays are the potential for aggregation of the polypeptides and the low recovery of material for functional assays. In this study, we have used nickel-chelate chromatography to study protein-protein interactions. This method allows for the rapid isolation of interacting subunits and in sufficient amounts for measurement of activity. The two halves of P-glycoprotein were coexpressed and purified as a functional complex by nickel-chelate chromatography. The presence of a relatively high concentration of detergent (1%) and salt (0.5 M NaCl and 0.3 M imidazole) did not appear to disrupt the interaction between the two halves. By contrast, these conditions resulted in the disruption of any association between the A52- and histidine-tagged full-length P-glycoproteins (Fig. 4). Therefore, noncovalent interactions between the monomers of an oligomer are likely to be weaker than the noncovalent interactions between the two halves of P-glycoprotein, since it was possible to co-purify the two half-molecule forms of P-glycoprotein.

Most membrane transport proteins are oligomeric; composed of dimers or higher oligomers (25, 26). The presence of at least a dimer of P-glycoprotein has been shown by radiation inactivation analysis of membranes from drug-resistant cells (14), chemical cross-linking experiments (15), analysis of binding of monoclonal antibody MRK-16 to a discontinuous epitope (16), and by sucrose gradient sedimentation of detergent-solubilized enzyme (17). In this study, however, we were unable to detect association between P-glycoprotein-A52 and P-glycoprotein-(His)$_{10}$ when using only nickel-chelate chromatography, even when there was a relative excess of P-glycoprotein-A52. One possible explanation for this observation was that dimer formation occurred preferentially between two A52-tagged P-glycoproteins or between two histidine-tagged P-glycoprotein molecules. We were, however, able to demonstrate that P-glycoprotein-A52 and P-glycoprotein-(His)$_{10}$ can associate in vivo by first immunoprecipitating the heterodimers and then subjecting the immunoprecipitates to nickel-chelate chromatography. The results suggest that functional P-glycoprotein isolated by nickel-chelate chromatography is a monomer. In support of this conclusion is the finding that purified P-glycoprotein migrates with an apparent mass of about 230 kDa when subjected to gel exclusion chromatography.

The role of P-glycoprotein oligomers in the cell is not known. One possibility is that they are involved with trafficking between different compartments within the cell. Poruchynsky and Ling (17) reported that oligomerization of P-glycoprotein occurs early in biosynthesis in the endoplasmic reticulum. Perhaps oligomerization of P-glycoprotein is required before the
enzyme can leave this compartment. Mutants of P-glycoprotein that are retained in the endoplasmic reticulum may remain associated with molecular chaperones (27) because they are unable to form oligomers. Once in the plasma membrane however, endocytosis and subsequent degradation of P-glycoprotein may be modulated by monomer/dimer interactions as postulated by Poruchynsky and Ling (17). Further studies are needed to test these postulates.

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REFERENCES

1. Gottesman, M. M., and Pastan, I. (1993) Annu. Rev. Biochem. 62, 385–427
2. Endicott, J. A., and Ling, V. A. (1989) Annu. Rev. Biochem. 58, 137–171
3. Schinkel, A. H., Smit, J. J. M., van Tellingen, O., Beijnen, J. H., Wagenaar, E., van Deemter, L., Moi, C. A. A. M., van der Valk, M. A., Robanus-Maandag, E. C., te Riele, H. P. J., Berne, A. J. M., and Borst, P. (1994) Cell 77, 491–502
4. Schinkel, A. H., Moi, C. A. A. M., Wagenaar, E., van Deemter, L., Smit, J. J. M., and Borst, P. (1995) Eur. J. Cancer 31A, 1295–1298
5. Schuetz, E. G., Schinkel, A. H., Relling, M. Y., and Schuetz, J. D. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4001–4005
6. Chen, C., Chin, J. E., Ueda, K., Clark, D. P., Pastan, I., Gottesman, M. M., and Robinson, I. B. (1986) Cell 47, 381–389
7. Loo, T. W., and Clarke, D. M. (1995) J. Biol. Chem. 270, 843–848
8. Kast, C., Canfield, V., Levenson, R., and Gros, P. (1996) J. Biol. Chem. 271, 9240–9248
9. Loo, T. W., and Clarke, D. M. (1994) J. Biol. Chem. 269, 7750–7755
10. Azzaria, M., Schurr, E., and Gros, P. (1989) Mol. Cell. Biol. 9, 5289–5297
11. Loo, T. W., and Clarke, D. M. (1995) J. Biol. Chem. 270, 21449–21452
12. Loo, T. W., and Clarke, D. M. (1995) J. Biol. Chem. 270, 22957–22961
13. Urbatsch, I. L., Sankaran, B., Bhagat, S., and Senier, A. E. (1995) J. Biol. Chem. 270, 26956–26961
14. Boscoiboinik, D., Debanne, M. T., Stafford, A. R., Jung, C. Y., Gupta, R. S., and Epand, R. M. (1996) Biochim. Biophys. Acta 1307, 225–228
15. Naito, M., and Tsuruo, T. (1992) Biochem. Biophys. Res. Commun. 185, 284–290
16. Georges, E., Tsuruo, T., and Ling, V. C. (1993) J. Biol. Chem. 268, 1792–1796
17. Poruchynsky, M. S., and Ling, V. C. (1994) Biochemistry 33, 4163–4174
18. Loo, T. W., and Clarke, D. M. (1995) J. Biol. Chem. 268, 3143–3149
19. Zuberzycka-Gaarn, E., MacDonald, G., Phillips, L., Jorgensen, A. O., and MacLennan, D. H. (1984) J. Bioenerg. Biomembr. 16, 441–446
20. Loo, T. W., and Clarke, D. M. (1995) J. Biol. Chem. 270, 21839–21844
21. Shapiro, A. B., and Ling, V. C. (1994) J. Biol. Chem. 269, 3745–3754
22. Germann, U. A., Chambers, T. C., Ambudkar, S. V., Licht, T., Cardarelli, C. O., Pastan, I., and Gottesman, M. M. (1996) J. Biol. Chem. 271, 1708–1716
23. Casey, J. R., and Reithmeier, R. A. F. (1993) Biochemistry 32, 1172–1179
24. Sekler, I., Kopito, R., and Casey, J. R. (1995) J. Biol. Chem. 270, 21028–21034
25. Klingenberg, M. (1981) Nature 290, 449–454
26. Reithmeier, R. A. F. (1994) Curr. Opin. Cell Biol. 6, 583–594
27. Loo, T. W., and Clarke, D. M. (1994) J. Biol. Chem. 269, 28683–28689