Rapid Purification of Human P-glycoprotein Mutants Expressed Transiently in HEK 293 Cells by Nickel-Chelate Chromatography and Characterization of their Drug-stimulated ATPase Activities*

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P-glycoprotein containing 10 tandem histidine residues at the COOH end of the molecule was transiently expressed in HEK 293 cells and purified by nickel-chelate chromatography. The purified protein had an apparent mass of 170 kDa, and its verapamil-stimulated ATPase activity in the presence of phospholipid was 1.2 μmol/min/mg of P-glycoprotein. We then characterized P-glycoprotein mutants that exhibited altered drug-resistant phenotypes and analyzed the contribution of the two nucleotide binding folds to drug-stimulated ATPase activity. Mutation of residues in either nucleotide binding fold abolished drug-stimulated ATPase activity. The pattern of drug-stimulated ATPase activities of mutants, which conferred increased relative resistance to colchicine (G141V, G185V, G830V) or decreased relative resistance to all drugs (F978A), correlated with their drug-resistant phenotypes. By contrast, the ATPase activity of mutant F335A was significantly higher than that of wild-type enzyme when assayed in the presence of verapamil (3.4-fold), colchicine (9.1-fold), or vinblastine (3.7-fold), even though it conferred little resistance to vinblastine in transfected cells. These results suggest that both nucleotide-binding domains must be intact to couple drug binding to ATPase activity and that the drug-stimulated ATPase activity profile of a mutant does not always correlate with its drug-resistant phenotype.

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and subtracting the amount of protein obtained when the same number of vector-transfected cells was carried through the purification procedure. Yields of purified P-glycoprotein ranged from 6 to 12 μg.

Measurement of Mg^2+-ATPase Activity—Purified P-glycoprotein was diluted with an equal volume of 50 mM crude sheep brain phosphatidylethanolamine (Sigma), which had been previously washed with Tris-buffered saline to remove traces of phosphate and then sonicated. 100 ng of purified P-glycoprotein was incubated with the desired drug and ATPase activity initiated by addition of an equal volume of buffer containing 100 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM MgCl_2, and 10 mM ATP. The samples were incubated at 37°C for 40 min, and the amount of inorganic phosphate liberated was determined by the method of Chifflet et al. (1988).

RESULTS

Purification of P-glycoprotein—The cDNA coding for P-glycoprotein was modified to contain 10 histidine residues at the COOH end of the molecule. The presence of the tag did not affect function since transfection of mouse NIH 3T3 cells yielded drug-resistant colonies that had drug-resistant profiles that were similar to those of wild-type enzyme (data not shown).

To purify P-glycoprotein, its cDNA was transiently expressed in HEK 293 cells as they yield a relatively high level of P-glycoprotein. More than 80% of the P-glycoprotein could be extracted from the membranes with the detergent glycoprotein. More than 80% of the P-glycoprotein could be eluted with 80 mM imidazole wash; lanes 6, fractions eluted with 300 mM imidazole (4% of the eluted material and containing 500 ng of protein (lane 6B)). M, molecular weight markers.

Characterization of the ATPase Activities of Purified Wild-type P-glycoprotein and Mutants with Altered Drug-resistant Profiles—Fig. 2 shows that wild-type P-glycoprotein-(His)_10 in the presence of phosphatidylethanolamine had an ATPase activity of 0.11 μmol/min/mg of P-glycoprotein. In the absence of lipid, no ATPase activity was detected (<0.015 μmol/min/mg of P-glycoprotein). In the presence of verapamil and lipid, however, the ATPase activity increased about 11-fold (1.20 μmol/min/mg of P-glycoprotein). All ATPase activities were determined in the absence of EGTA and ouabain, as no Ca^{2+}-ATPase or Na^{+}-K^{+}-ATPase activity was detected (assayed ± 2 mM EGTA or ± 2 mM ouabain, respectively). Lower levels of stimulation were observed in the presence of 800 μM colchicine (3.1-fold) and vinblastine (5.8-fold). Maximal stimulation of ATPase activity in the presence of vinblastine occurred at 20–50 μM, and the activity decreased at higher concentrations of vinblastine.

We have previously identified residues in P-glycoprotein, which alter its ability to confer resistance to various cytotoxic drugs in transfected cells (Loo and Clarke, 1993a, 1993b, 1994a, 1994b). For example, mutants G141V or G830V conferred increased resistance to colchicine (about 3-fold) relative to that of wild-type enzyme while mutant F335A conferred decreased resistance to all drugs. In this study, we also included for comparison mutant G185V, which was recently shown by Rao (1995) to have increased verapamil- and colchicine-stimulated ATPase activities (2- and 3.3-fold, respectively). Therefore, to determine the effects of these drugs on the ATPase activities of these mutants, we purified and reconstituted each mutant P-glycoprotein into phospholipid and measured ATPase activity in the presence of various concentrations of vinblastine, colchicine, or verapamil. When analyzed by SDS-PAGE, the major protein in each purified preparation had an apparent mass of 170 kDa (corresponding to the fully glycosylated form of P-glycoprotein) and was present in similar amounts (Fig. 3). The maximal verapamil-stimulated ATPase activities of mutants G141V, G185V, and G830V were all slightly increased (1.4–1.7-fold) relative to that of wild-type enzyme (Fig. 2). The half-maximal stimulation of the ATPase activities of the glycine mutants was 9–16 μM, compared with 40 μM for the wild-type enzyme, suggesting that the mutants had increased affinity for verapamil. Vinblastine-stimulated ATPase activities of all three mutants, however, were similar to that of wild-type enzyme, whereas colchicine-stimulated ATPase activities were markedly increased (2.8–3.7-fold).
Mutant F978A, which confers little resistance to vinblastine, colchicine, doxorubicin, or actinomycin D in transfected cells, also showed little drug-stimulated ATPase activity, except at colchicine, doxorubicin, or actinomycin D in transfected cells, ated (150 kDa) forms of P-glycoprotein are indicated.

Procedures."Asamplecorrespondingto4%ofthepurifiedfractionwas subjected to SDS-PAGE, and the bands were visualized by Coomassie

Purification of P-glycoprotein Mutants

in HEK 293 cells and purified using nickel-chelate chromatography as described under "Experimental Procedures." A sample corresponding to 4% of the purified fraction was subjected to SDS-PAGE, and the bands were visualized by Coomassie Blue staining. The positions of the mature (170 kDa) and core-glycosylated (150 kDa) forms of P-glycoprotein are indicated.

DISCUSSION

Purification of P-glycoprotein using nickel-chelate chromatography following transient expression in HEK 293 cells has several advantages over purification from stable cell lines over-expressing P-glycoprotein (Urbatsch et al., 1994; Shapiro and Ling, 1994; Sharom et al., 1993) or following expression in insect Sf9 cells (Rao, 1995). The main advantage is that the expression, purification, and assay of ATPase activity for any mutant P-glycoprotein can be completed within 2 days, while the other methods often take months. A transient expression system also avoids the problems associated with potential recombination of the mutant P-glycoprotein cDNA with any endogenous P-glycoprotein genes. Although the level of expression of P-glycoprotein is relatively low in HEK 293 cells (0.1–0.3% by weight), the use of nickel-chelate chromatography provided a simple and efficient method to isolate highly purified P-glycoprotein in an active state. This procedure should be applicable to the study of the structure-function relationships of other eukaryotic membrane proteins.

In this study, we addressed two important questions concerning the structure and mechanism of P-glycoprotein: 1) are both ATP-binding sites required for drug stimulation of ATPase activity and 2) is there a correlation between the drug-resistant phenotype of a mutant and its pattern of drug-stimulated ATPase activity? Our results suggest that both nucleotide-binding sites must be intact for coupling of ATPase activity to drug binding. Mutation of the core (GK) amino acids in either homology A nucleotide-binding consensus sequence abolished basal as well as drug-stimulated ATPase activity. It is possible that both sites must be occupied simultaneously for coupling of drug binding to ATPase activity or that ATP binding occurs sequentially during the reaction cycle. There is, however, no evidence of cooperativity between the nucleotide-binding sites (Sharom et al., 1995).

Mutations that alter the drug-resistant profiles of P-glycoprotein also had profound effects on the pattern of drug-stimulated ATPase activities. For mutants G141V, G185V, G830V, and F978A, the pattern of drug-stimulated ATPase correlated with their relative drug-resistant profiles in transfected cells. In the glycine mutants, there was enhanced stimulation of ATPase activity by colchicine, whereas stimulation by vinblastine resembled that of wild-type enzyme. Similarly, in transfected cells, the relative resistance of these mutants to vinblastine was similar to that of wild-type enzyme, but the relative resistance to colchicine was elevated (about 3-fold). Mutant F978A conferred little resistance to all drug substrates in transfected cells, and the purified protein also showed ex-
tremely low levels of drug-stimulated ATPase activity. These results suggest that mutation of Phe-978 resulted in either decreased affinity (verapamil) and/or interference in coupling of drug binding to ATPase activity (vinblastine).

Purified mutant F335A P-glycoprotein, however, showed large increases in ATPase activity in the presence of all three drug substrates but conferred decreased relative resistance to vinblastine and only a small increase in resistance to colchicine in transfected cells (Loo and Clarke, 1993b). One explanation for this discrepancy is that mutation F335A alters the dissociation of vinblastine from P-glycoprotein such that the enzyme is slow in effluxing vinblastine. It is also possible that the mutation alters the conformation of the enzyme such that it is now in an “uncoupled” state. These possibilities could explain the fact that purified mutant F335A has a higher basal as well as drug-stimulated ATPase activity compared with wild-type enzyme.

The results of this study show that drug-stimulated ATPase activity of a mutant does not always correlate with its drug-resistant phenotype.

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REFERENCES

Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
Bradley, G., and Ling, V (1994) Cancer Metastasis Rev. 13, 223–233
Chiolfet, S., Torriglia, A., Chiesa, R., and Tolosa, S. (1988) Anal. Biochem. 168, 1–4
Endicott, J. A., and Ling, V. A. (1989) Annu. Rev. Biochem. 58, 137–171
Gottesman, M. M., and Pastan, I. (1995) Ann. Rev. Biochem. 62, 385–427
Janknecht, R., de Martynoff, G., Lou, J., Hipskind, R. A., Nordhem, A., and Stunneberg, H. G. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 8972–8976
Kajiji, S., Dreslin, J. A., Grizzuti, K., and Gros, P. (1994) Biochemistry 33, 5041–5048
Loo, T. W., and Clarke, D. M. (1993a) J. Biol. Chem. 268, 3143–3149
Loo, T. W., and Clarke, D. M. (1993b) J. Biol. Chem. 268, 31435–3149
Loo, T. W., and Clarke, D. M. (1993c) J. Biol. Chem. 268, 3145–3149
Loo, T. W., and Clarke, D. M. (1994a) Biochemistry 33, 31459–31463
Loo, T. W., and Clarke, D. M. (1994b) Biochemistry 33, 31469–31473
Loo, T. W., and Clarke, D. M. (1994c) Biochemistry 33, 31479–31483
Loo, T. W., and Clarke, D. M. (1995) Biochemistry 33, 3149–3153
Rao, U. S. (1995) J. Biol. Chem. 270, 6866–6870
Roninson, I. B. (ed) (1991) Molecular and Cellular Biology of Multidrug Resistance in Tumor Cells. Plenum Publishing Corp., New York
Sharom, F. J., Yu, X., and Doige, C. A. (1993) J. Biol. Chem. 268, 24197–24202
Sharom, F. J., Yu, X., Chu, J. W. K., and Doige, C. A. (1995) Biochem. J. 308, 381–390
Urbatsch, I. L., Al-Shawi, M., and Senior, A. E. (1994) Biochemistry 33, 7069–7076
Walker, J. E., Saraste, M., Runswick, M. J., and Gay, N. J. (1982) EMBO J. 1, 945–951