P-glycoprotein consists of two homologous halves, each composed of a transmembrane domain and a nucleotide-binding domain. In order to understand how the domains interact in P-glycoprotein, we expressed each domain as a separate polypeptide and tested for associations using coimmunoprecipitation assays. We found that the interactions between the two halves of P-glycoprotein were mediated through associations between the two transmembrane domains as well as through the nucleotide-binding domains. In addition, the nucleotide-binding domain also associated with the transmembrane domain in each half of the molecule. By contrast, we could not detect any association either between the first nucleotide-binding domain and the second transmembrane domain, or between the second nucleotide-binding domain and the first transmembrane domain. We then tested whether individual domains associated with molecular chaperones, since biogenesis of P-glycoprotein appears to involve the chaperones calnexin and Hsc70. We found that calnexin associated only with the transmembrane domains, while Hsc70 associated only with the nucleotide-binding domains. These results suggest that noncovalent interaction between the domains of P-glycoprotein can contribute to structure and function of P-glycoprotein and that chaperones may participate in the folding of each domain.

P-glycoprotein, the product of the human MDR1 gene, is an energy-dependent transport protein which interacts with a wide variety of hydrophobic cytotoxic agents that do not have a common structure or intracellular target (see reviews by Roninson (1991) and Gottesman and Pastan (1993)). The protein is clinically important since it contributes to the phenomenon of multidrug resistance during chemotherapy of human cancers (see reviews by Endicott and Ling (1989), VanderBliek et al. (1986), 1988; Gros et al., 1991; Devine et al., 1992; Loo and Clarke, 1993a, 1993b, 1994a) and biochemical analyses (Bruggemann et al., 1989, 1992; Greenberger, 1993; Zhang et al., 1995a) indicate that residues within the TMD contribute to drug binding. The hydrophilic domains containing the consensus nucleotide binding folds (NBF) have been found to bind ATP (Azzaria et al., 1989; Baubichon-Cortay et al., 1994).

Functional studies suggest that different domains of P-glycoprotein must interact with each other for transport to occur. It has been demonstrated that ATPase activity is stimulated in the presence of drug substrates (Ambudkar et al., 1992; Sarkadi et al., 1992; Sharom et al., 1993; Al-Shawi and Senior, 1993; Shapiro and Ling, 1994). Coupling of ATPase activity to drug binding involves interactions between both homologous halves of the molecule since no drug-stimulated ATPase activity was observed when each half was expressed as a separate polypeptide (Loo and Clarke, 1994b).

The physical basis for interactions between domains is not known. Such interactions have been shown to be critical for function in bacterial ABC transporters, where individual domains are composed of separate polypeptides. In the histidine permease, for example, noncovalent interactions between all four domains were required to form a functional complex (Kerpola et al., 1991). In this study, we tested whether there are physical interactions between specific domains of P-glycoprotein by expressing each domain as a separate polypeptide and using coimmunoprecipitation assays. In addition, we tested whether chaperones may participate in the folding of the individual domains as there is evidence that they play a role in biosynthesis of the enzyme (Loo and Clarke, 1994c). We found that interactions between the homologous halves of the molecule are mediated by association between the TMDs as well as between NBFs. Specific associations were also observed between TMDs and NBFs. In addition, the molecular chaperone calnexin is involved in the folding of the TMDs, whereas Hsc70 participates in the folding of NBFs.

### EXPERIMENTAL PROCEDURES

**Generation of Constructs**—The cDNAs coding for either the NH2-terminal or COOH-terminal half-molecules of P-glycoprotein and containing the epitope for monoclonal antibody A52 were generated as described previously (Loo and Clarke, 1994b). To generate the NH2-terminal half-molecule of P-glycoprotein lacking the epitope for monoclonal antibody A52, the full-length MDR1-A52 cDNA in Bluescript (Stratagene), pBSGMDR1-A52, was linearized with HindIII (nt 2041) and the vector fragment ligated to generate pBSGMDR1COOH.

**NH2-terminal TMD (residues 1 to 379)**, which contains the first six transmembrane segments; TMD2, COOH-terminal TMD (residues 681-1025) which contains the last six transmembrane segments; MDR, multidrug resistance; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; NBF, nucleotide-binding domain; nt, nucleotide.
In order to express TMD1 (residues 1–379) containing the epitope for monoclonal antibody A52, pBSGMDR1-A52 was digested with BstBI (nt 1133) and HindIII (nt 3843), filled-in with Klenow and the vector fragment ligated with T4 DNA ligase.

The cDNA coding for TMD2 (residues 681–1025) and containing the epitope for monoclonal antibody A52 was created by digesting the cDNA coding for the COOH-terminal half-molecule of P-glycoprotein, pBSGMDR1A52NH2, with StuI (nt 3072) and EcoRV (nt 3849), and ligation of the vector fragment.

The cDNA coding for NBF1-A52 was obtained by ligating the cDNAs coding for residues 1–16 (EcoRI (nt 76) to Dral (nt 48)), residues 388–529 (SspI (nt 1159) to Apal (nt 1582)) and the epitope for monoclonal antibody A52 (Apal (nt 1582) to XhoI (nt 4061)). The Apal to XhoI fragments containing the A52 epitopes was isolated from pBSGMDR1A52ΔCOOH (Loo and Clarke, 1994b). The fragment coding for NBF1-A52 was subcloned into pMT21. In some experiments, NBF1 lacking the epitope for monoclonal antibody A52 was needed. The cDNA coding for NBF1 was obtained by ligating the cDNA coding for residues 1–16 with that coding for residues 388–682 (SspI (nt 1159) to XhoI (nt 4061)) isolated from pBSGMDR1ΔCOOH.

The cDNA coding for NBF2-A52 was constructed by ligating the fragment coding for residues 1025 to 1311 (StuI (nt 3072) to XhoI (nt 4061)) to pBSGMDR1A52ΔNH2, which had been linearized with HindIII, filled-in with Klenow fragment, and ligated with XhoI.

In some instances, NBF2 was fused to calbindin-D-28K. Fusion of NBF2 to calbindin (residues 19 to 261) (Parmentier et al., 1987) allowed us to differentiate NBF2 from TMD2 in SDS-PAGE.

The fragments coding for each domain of P-glycoprotein were subcloned into the EcorI and XhoI sites of the expression vector, pMT21, and the nucleotide sequence confirmed by sequencing (Sanger et al., 1977).

Cell Culture and Immunoprecipological Procedures—Forty hours after transfection of HEK 293 cells (10-cm diameter dish) with the desired cDNAs (Loo and Clarke, 1993a), the cells were washed with PBS and lysed with 1 mL of lysis buffer (10 mM Heps, pH 7.3, 150 mM NaCl, 1 mM CaCl2, 1% (w/v) digitonin, 10 mM iodoacetamide, 1% (v/v) aprotinin, 50 μg/ml 4-2-aminoethyl)-benzenesulfonyl fluoride and 5 μg/ml leupeptin). Insoluble material was removed by centrifugation at 26,000 × g for 15 min at 4 °C, and the lysates were incubated for 2 h at 4 °C with 15 μg of affinity-purified rabbit anti-calnexin antibody (Loo and Clarke, 1994c), 5 μg of affinity-purified rabbit anti-NBF1 (against residues 439–640) (Charuk et al., 1994), 2 μg of monoclonal antibody against calbindin-D-28K (Sigma), 10 μg of rat monoclonal antibody against Hsc70 (StressGen), or 2 μg with monoclonal antibody A52 as described previously (Loo and Clarke, 1994c). The immune complexes were collected by addition of protein A-Sepharose CL-4B (Pharmacia Biotech Inc.) and washed four times with buffer containing 10 mM Heps, pH 7.3, 150 mM NaCl, 1 mM CaCl2, and 0.2% (w/v) digitonin. The immunoprecipitates were analyzed by SDS-PAGE followed by immunoblot analysis and enhanced chemiluminescence (Pharmacia).

RESULTS

Expression and Glycosylation—Fig. 1 shows the four domains of P-glycoprotein and the composition of the constructs used to express each domain as a separate polypeptide. In most cases, the epitope for monoclonal antibody A52 was added at the COOH terminus of each construct to facilitate identification of the expressed mutant proteins, as well as for immunoprecipitation. We have previously found that the addition of an epitope tag to the full-length protein (Loo and Clarke, 1993a), or to either half-molecule forms of P-glycoprotein did not inhibit function (Loo and Clarke, 1994b).

Fig. 2 shows an immunoblot of whole cell extracts of HEK 293 cells expressing various domains of P-glycoprotein. Cells transfected with the cDNAs coding for the NH2-terminal (NBF1) and COOH-terminal (NBF2) nucleotide-binding domains, expressed polypeptides with apparent masses of 41 and 35 kDa, respectively, which are comparable to their predicted sizes. By contrast, both the NH2-terminal (TMD1) and the COOH-terminal (TMD2) transmembrane domains had apparent masses that were smaller than that predicted from their cDNA when analyzed by SDS-PAGE. TMD1 had an apparent mass of 39 kDa (unglycosylated, see below), compared to its predicted size of 44 kDa, while TMD2 had an apparent mass of 35 kDa, which is smaller that its predicted size of 40 kDa. The discrepancies in the apparent masses of the transmembrane domains is probably due to incomplete denaturation, since the samples were not heated after solubilization in SDS sample...
buffer. Heating of these samples, but not NBF1 or NBF2, caused aggregation of these polyptides such that they remained in the stacking gel.

To determine if the expressed domains were inserted into membranes of HEK 293 cells, membranes were prepared from cells transfected with the cDNA coding for each domain and then treated with sodium carbonate. Carbonate extraction of membranes removes all but integral membrane proteins (Fujiki et al., 1982). It was found that wild-type P-glycoprotein-A52 and the TMD1 and TMD2 polyptides were almost exclusively recovered in the pellet fractions, whereas NBF1 and NBF2 were present only in the supernatant fractions (data not shown). These results suggest that both TMD1 and TMD2 contain signals necessary for insertion into the membranes of the endoplasmic reticulum.

Human MDR1 contains three glycosylated sites in the first extracellular loop (Schinkel et al., 1993). Accordingly, we tested the sensitivity of the domain polyptides to endoglycosidase H digestion. Fig. 2 shows that the apparent molecular mass of TMD1 decreased from 46 kDa to approximately 39 kDa following endoglycosidase H treatment. By contrast, the TMD2, NBF1, or NBF2 were not sensitive to endoglycosidase H (Fig. 2) or N-glycanase F digestion (data not shown). These results suggest that only TMD1 was core-glycosylated and likely resides in the endoplasmic reticulum of transfected cells.

Associations between Domains—To test for associations between domains, co-immunoprecipitation assays were carried out on cell extracts prepared from cells which were cotransfected with cDNAs coding for various domains of P-glycoprotein. To confirm that this was a valid approach, we first assayed for association between the NH₂-terminal and COOH-terminal half-molecules of P-glycoprotein. In a previous study, it was found that drug-stimulatable ATPase activity was restored when both half-molecules were expressed together in the same cell (Loo and Clarke, 1994a), indicating that both halves can functionally interact. In this co-immunoprecipitation assay, a COOH-terminal half-molecule containing an epitope tag for monoclonal antibody A52 was used, while the NH₂-terminal half-molecule lacked this tag. Fig. 3A shows that the NH₂-terminal half-molecule could only be recovered by immunoprecipitation with monoclonal antibody A52 when coexpressed with the COOH-terminal half-molecule containing the A52 epitope tag. These results indicate that restoration of drug-stimulatable ATPase activity is due to physical association between the two half-molecules.

To identify the regions of interaction between the two halves of P-glycoprotein, we coexpressed the various domains (Fig. 1) of P-glycoprotein followed by immunoprecipitation with specific antibodies. Fig. 3B shows that TMD2 was recovered in the immune complex using an affinity-purified rabbit polyclonal antibody against NBF1, when coexpressed with the NH₂-terminal half-molecule. In the absence of the NH₂-terminal half-molecule, however, the antibody did not immunoprecipitate TMD2 (Fig. 3B). These results indicate that one possible site of interaction between the two half-molecules of P-glycoprotein is through the transmembrane domains.

To determine whether the cytoplasmic domains were also responsible for association of the two halves of P-glycoprotein, we coexpressed NBF1 containing an epitope for monoclonal antibody A52 together with NBF2 which had been fused to calbindin D-28K. Fig. 2 shows that TMD2 and NBF2 containing the A52 tags have approximately the same mass in SDS-PAGE. By fusing NBF2 to calbindin, the molecular mass of NBF2 is increased by approximately 25 kDa (see below), allowing it to be distinguished from TMD2 in SDS-PAGE.

Fig. 3C shows that NBF2-calbindin could be recovered with monoclonal antibody A52, only in the presence of NBF1. Similarly, when NBF1 was coexpressed with NBF2-calbindin and immunoprecipitated with monoclonal antibody against calbindin (Sigma), NBF1 could also be immunoprecipitated (Fig. 3E), indicating interaction between the two halves of P-glycoprotein.

Another potentially important association in P-glycoprotein is through association of the nucleotide-binding domains with the transmembrane domains. The observation that ATPase activity is stimulated by drug binding suggests a functional interaction between the cytoplasmic and transmembrane domains. Fig. 3D shows that, when NBF1 was coexpressed with either TMD1 or TMD2 and immunoprecipitated with antibody against NBF1, only TMD1 was immunoprecipitated. One possible explanation that TMD2 was not immunoprecipitated was that it was not expressed in the cells. We confirmed, however, that both NBF1 and TMD2 were indeed coexpressed by performing an immunoblot analysis on a sample of cells transfected with the cDNAs coding for both domains prior to immunoprecipitation studies (Fig. 4). These results suggest that NBF1 can associate with TMD1 but not with TMD2. Similarly, we attempted to identify associations of NBF2 with the transmembrane domains. Fig. 3E shows that there was association of NBF2 with TMD2 but not with TMD1.
amount of immunoreactive material of apparent mass between 50 and 100 kDa is due to reaction of the anti-mouse secondary antibody with the mouse monoconal antibody (anti-calbindin D) that was used during the immunoprecipitation. The immunoblot in Fig. 3E was exposed to film for a very short period of time compared to that in Fig. 4, to prevent masking of any signal by the immunoreactive material of 50–100 kDa. Therefore, the presence of a signal (Fig. 3E, lanes 2 and 4, respectively), suggests that these are significant associations. Extraction of cells with SDS (Fig. 4) or with digitonin (data not shown) resulted in the majority of TMD1 as well as the other domains being present in the soluble fraction.

These results show that there are physical interactions between the transmembrane and nucleotide-binding domains in each half of P-glycoprotein.

Association of Domains with Molecular Chaperones—We have previously demonstrated that the molecular chaperone calnexin associates with P-glycoprotein (Loo and Clarke, 1994c). Another class of chaperones which has been found to associate with ABC transporters are the Hsc/Hsp70 proteins. Yang et al. (1993) reported that this chaperone transiently associated with immature or mutant forms of the cystic fibrosis conductance regulator. In a previous attempt (Loo and Clarke, 1994c), we were unable to detect an association of Hsc/Hsp70 with P-glycoprotein expressed in NIH 3T3 cells, using a monoclonal antibody (Boehringer Mannheim) which cross-reacts with both constitutive and inducible forms of this protein. We now show that, by using a different monoclonal antibody that is specific for the constitutive form of Hsc70 (StressGen), an association between P-glycoprotein and Hsc70 could be demonstrated. As shown in Fig. 5, a monoclonal antibody to Hsc70 communoprecipitated only the immature form of P-glycoprotein (150 kDa). Similarly, the antibody also immunoprecipitated an improperly processed mutant (Pro709 → Gly) of P-glycoprotein (Fig. 5B). Immunoprecipitation of cell extracts with antibody to P-glycoprotein-A52 also resulted in the recovery of Hsc70 from cells transfected with cDNAs coding for wild-type or mutant Pro709 → Gly P-glycoprotein (Fig. 5C).

These results suggest that the molecular chaperone Hsc70 may also participate in the folding or quality control mechanism of P-glycoprotein biogenesis and that the various domains of P-glycoprotein may interact with specific chaperones during this process. Accordingly, communoprecipitation assays were carried out to test for associations of the molecular chaperones calnexin and Hsc70 with the half-molecules, TMD, and NBF domains of P-glycoprotein.

Fig. 6 shows that Hsc70 associates with both half-molecules of P-glycoprotein. This association is likely mediated through physical interaction with the nucleotide-binding domains since it was found that Hsc70 was present in the immune complexes from cells transfected with cDNAs coding for NBF1 or NBF2 and immunoprecipitated with monoclonal antibody A52, but not in the immune complexes from cells expressing TMD1 or TMD2. In addition, immunoprecipitation with anti-Hsc70 antibodies showed that NBF1 or NBF2 were recovered in the immune complexes but not TMD1 or TMD2 (Fig. 7). The SERCA1 Ca^{2+}-ATPase of sarcoplasmic reticulum was included as a control because it already contains the epitope for monoclonal antibody A52, and is localized to the endoplasmic reticulum in transfected mammalian cells (Maruyama et al., 1989). No Hsc70 was detected in the immune complexes from cells transfected with the DNA coding for SERCA1 Ca^{2+}-ATPase and immunoprecipitated with monoclonal antibody A52 (Fig. 6).

These results suggest that Hsc70 associations are specific. A similar approach was used to test for association of calnexin with each domain of P-glycoprotein. Fig. 8 shows that calnexin also associates with both half-molecules. The association with P-glycoprotein appears to be mediated through the transmembrane domains, since it was detected only in the immune complexes of the TMD1 and TMD2 but not in those of the nucleotide-binding domains. Similarly, when cell extracts of transfected cells were immunoprecipitated with anti-cal-
Potential problems in a coimmunoprecipitation approach to study protein interactions are nonspecific associations and aggregation after lysis of the cells. To overcome these problems, incubations in the presence of antibody were for short periods (2 h). In addition, controls containing only one domain were run in parallel (Fig. 3). In each case, nonspecific aggregation was not observed. Indeed, we found that the associations between domains decreased with longer incubation periods (18 h). The associations were specific since there were no associations between SERCA1 Ca$^{2+}$-ATPase and either NBF1, NBF2 or the NH$_2$-half molecule (data not shown). These results indicate that there are specific noncovalent associations between the various domains of P-glycoprotein.

An association between the two transmembrane domains was expected based on the results of photolabeling studies on P-glycoprotein. P-glycoprotein labeled with photoactive drug analogs followed by protease digestion revealed that the labeled fragments were from transmembrane domains from both homologous halves of P-glycoprotein (Bruggemann et al., 1989; Greenberger et al., 1991; Greenberger, 1993). About 50% of the label was found in each half of the molecule. In the presence of vinblastine, labeling of both halves was inhibited to a similar extent (Bruggemann et al., 1992). These results suggest that the labeling site is formed by both transmembrane domains.

An association between the nucleotide-binding domains, however, was rather surprising since there is no evidence of cooperativity in the ATPase activity of P-glycoprotein (Shapiro and Ling, 1994). Both nucleotide-binding domains appear to be capable of hydrolytic activity since low levels of ATPase activity could be detected when each half of P-glycoprotein was expressed as an individual polypeptides (Loo and Clarke, 1994b).

An association was also observed between the nucleotide-binding domains and the transmembrane domains. These interactions may be especially important for coupling of ATPase activity to drug binding, and could be mediated through the large cytoplasmic loops connecting the transmembrane segments. A number of mutations have been identified in these loops which alter the drug resistance profiles conferred by P-glycoprotein (Choi et al., 1988; Currier et al., 1992; Loo and Clarke, 1994a) and alter the pattern of coupling of drug binding to ATPase activity (Rao, 1995). Associations among residues within the four domains provide a mechanism for coupling of drug binding to ATPase activity, which is likely to be a key feature of drug transport.

Biosynthesis of the individual domains appears to be mediated through interactions of molecular chaperones. Association of calnexin with TMD1 is not surprising since this chaperone transiently associates with a wide variety of glycosylated proteins that are exported or targeted to the cell surface (reviewed by Bergeron et al., 1994). TMD2, however, was also found to associate with calnexin, although we found no evidence that this domain was glycosylated. Other nonglycosylated proteins have been identified which interact with calnexin, such as the nonglycosylated $\epsilon$-subunit of the T-cell receptor (Rajagopalan et al., 1994). In addition, calnexin remains bound to the class I major histocompatibility complex molecules after removal of oligosaccharides by endoglycosidase H. Initiation of binding by calnexin to the class I complex, however, was recently shown to require the presence of oligosaccharides (Zhang et al., 1995b). Apparently calnexin recognizes both carbohydrate and protein Determinants of selected proteins.

The molecular chaperone Hsc70 specifically associated with the nucleotide-binding domains. This class of chaperones has been implicated in stabilizing newly synthesized polypeptides, in mediating assembly of multimeric protein complexes, and in facilitating translocation of polypeptides across membranes (see reviews by Gething and Sambrook, 1992; McKay, 1993, and Becker and Craig, 1994). Although members of the Hsp70/Calnexin antibodies, followed by detection with monoclonal antibody A52 or vector alone (Control), only the transmembrane domains were recovered (Fig. 9). The association also appeared to be specific since no detectable calnexin was found in the immune complexes from cells transfected with the cDNA coding for SERCA1 Ca$^{2+}$-ATPase (Fig. 8). These results show that calnexin associates with transmembrane domains, whereas Hsc70 associates with the nucleotide binding domains.

**DISCUSSION**

Potential problems in a coimmunoprecipitation approach to study protein interactions are nonspecific associations and aggregation after lysis of the cells. To overcome these problems, incubations in the presence of antibody were for short periods (2 h). In addition, controls containing only one domain were run in parallel (Fig. 3). In each case, nonspecific aggregation was not observed. Indeed, we found that the associations between domains decreased with longer incubation periods (18 h). The associations were specific since there were no associations between NBF1 and TMD2 or between NBF2 and TMD1. Similarly, no associations were found between SERCA1 Ca$^{2+}$-ATPase and either NBF1, NBF2 or the NH$_2$-half molecule (data not shown). These results indicate that there are specific noncovalent associations between the various domains of P-glycoprotein.

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Hsc70 chaperone family are highly conserved, they show common and divergent specificities (Fourie et al., 1994). In general, they bind to a segment of the protein containing at least 7 residues that include large hydrophobic and basic amino acids with few or no acidic residues. The binding motif is best described as HyXHYXHYXHy, where Hy is a large hydrophobic or aromatic amino acid and X is any amino acid. There are several candidate regions in the nucleotide-binding domains of P-glycoprotein. For example, potential regions involved in interacting with Hsc70 include residues 397–403 (VFHVSYPS) in NBF1 and residues 1040–1046 (VFVNYPT) in the corresponding region of NBF2.

Association of each domain with at least one molecular chaperone suggests that folding of P-glycoprotein is carefully monitored for fidelity. This may account for retention in the endoplasmic reticulum of a large number of mutant P-glycoproteins in which only a single amino acid has been substituted (Loo and Clarke, 1993a, 1993b, 1994a, 1995). Elucidation of the sites of association between molecular chaperones and P-glycoprotein would help in understanding the role of the chaperones in the maturation of P-glycoprotein to form a functional transporter.

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