Supporting Information

Conserved Walker A Cysteines 431 and 1074 in Human P-glycoprotein Are Accessible to Thiol-Specific Agents in the Apo and ADP-Vanadate Trapped Conformations

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Table S1

Table S1: Cell surface expression and transport function of C431/C1074 and N611C/T1256C P-gp mutants in HeLa cells.

Mutation(s)	Cell surface expression	Transport Function (%)			
		Cal-AM	**Rh123**	**NBD-CsA**	**Dauno**
C431/A1074C	90-100	95-100	90-100	90-100	95-100
N611C/T1256C	40-50	90-100	90-100	90-100	90-100

Supplementary Table Legend

The Bacmam baculovirus transduced HeLa cells were evaluated for cell surface P-gp expression using MRK-16 antibody and for transport function by following the accumulation of fluorescent substrates including rhodamine 123 (Rh123) (1.3 μM), NBD-cyclosporine A (NBD-CsA) (0.5 μM), daunorubicin (dauno) (0.5 μM) and calcein-AM (cal-AM) (0.5 μM) in the absence and presence of 1 μM tariquidar. The cells were washed and analyzed by flow cytometry. The level of protein expression and transport activity in HeLa cells expressing the mutant P-gps was compared to that of the cysless-WT (taken as 100%). The values indicate the range of cell surface expression and steady-state accumulation from at least three independent experiments.

Supplementary Figure Legends

Figure S1: Disulfide cross-linking of cysless WT and single cysteine mutant P-gps with crosslinker M17M. The DTT-free crude membranes of High-Five insect cells expressing cysless WT, C431 and C1074 were treated with and without M17M (200 μM) for 15 min at 4 ^{o}C. The reactions were stopped by the addition of 5 X SDS-PAGE sample buffer without reducing agent (βME) and samples were subjected to immunoblot analysis on SDS/PAGE (7% gel). The amount of protein loaded for cysless WT, C431 and C1074 are 0.1 μg, 0.25 μg and 1 μg, respectively. An arrow shows the position of the un-crosslinked P-gp. Similar results were obtained in two independent experiments.

Figure S2: Treatment of N611C/T1256C in the apo state with crosslinker M17M. The DTT-free crude membranes of High-Five insect cells expressing N611C/T1256C were treated with 200μM of the homobifunctional disulfide cross-linker M17M for 15 min at 4 ^{o}C. The reactions were stopped by the addition of SDS sample buffer containing no reducing agent (βME) and samples were subjected to immunoblot analysis on SDS/PAGE (7% gel). Arrows show the position of uncrosslinked P-gp in both apo and ADP-V_i trapped conformations.

Figure S3: Inhibition of P-gp ATP hydrolysis by C431/C1074C in the presence of vanadate (0.3 mM). P-gp ATPase activity remained inhibited by vanadate in the closed conformation. In this experiment, 10 μg of protein in the ADP-V_i trapped conformation was reassessed for V_i-sensitive P-gp mediated ATPase hydrolysis as described in Experimental Procedures. Figure is representative of two experiments with C431/C1074.

Figure S4: Concentration-dependent effect of MTS-verapamil on cysless WT, single (C431 and C1074) and double (C431/C1074) mutant P-gp-mediated ATP hydrolysis. Inhibition of cysless WT (●), single C431 (■) and C1074 (▲) and double C431/C1074 (▼) mutant P-gp ATPase activity by MTS-verapamil. Vanadate-sensitive P-gp mediated ATPase hydrolysis was measured as described in Experimental Procedures in the presence of increasing concentrations of MTS-verapamil (0 – 10 μM). Data represent the mean values [Error bars = SD (n = 3)].

Figure S5: Effect of MTS-verapamil and MTS-rhodamine on single C431 and C1074 and double C431/C1074 P-gp mediated ATP hydrolysis under different assay conditions. Vanadate-sensitive P-gp mediated ATPase hydrolysis was measured as described in Experimental Procedures in the presence of MTS-verapamil (10 μM) (A) and MTS-rhodamine (10 μM) (B). Vanadate-sensitive P-gp mediated ATPase hydrolysis was carried out in the absence of DTT in the ATPase buffer (solid bars), with the addition of 4 mM DTT (bars with diagonal lines) and with the pre-incubation of 10 mM ATP and 20mM $MgCl_2$ (bars with dots). Data represent the mean values of two experiments.

Figure S6: Effect of fluorescein maleimide on cysless WT, single (N611C and T1256C) and double (N611C/T1256C) mutant P-gp mediated ATP hydrolysis. (A) Inhibition of cysless WT (●), single N611C (▲) and T1256C (▼) and double N611C/T1256C (♦) mutant P-gp ATPase activity by FM. Vanadate-sensitive P-gp mediated ATPase hydrolysis was measured as described in Experimental Procedures in the presence of increasing concentrations of FM (0 – 25 μM). (B) Inhibition of single

(N611C and T1256C) and double (N611C/T1256C) mutant P-gps ATPase activity when ATP is added prior to FM. Vanadate-sensitive P-gp mediated ATPase hydrolysis was measured as described in Experimental Procedures in the presence of increasing concentrations of FM (0 – 25 μM). Data represent the mean values (Error bars showing ± SD are shown for n = 3). The inhibition curves were generated by non-linear regression (one phase decay) using GraphPad Prism 5.

Figure S7: Disulfide cross-linking of C431/C1074 mutant P-gp with crosslinker M17M in the presence of drug substrates. The DTT-free crude membranes of High-Five insect cells expressing C431/C1074 were treated with cyclosporine A, QZ 59 SSS, actinomycin D at indicated concentrations for 5 mins at room temperature prior the addition of M17M (200μM) for 15 min at 4^{o}C. The reactions were stopped by the addition of 5 X SDS-PAGE sample buffer without reducing agent (βME) and samples were subjected to immunoblot analysis on SDS/PAGE (7% gel). Arrows show the positions of the crosslinked (X-linked) and un-crosslinked P-gp in the presence of various drug substrates as given above the immunoblot.

Figure S1

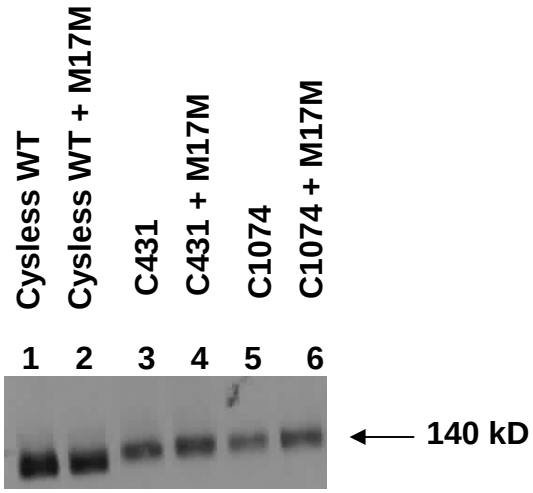


Figure S2

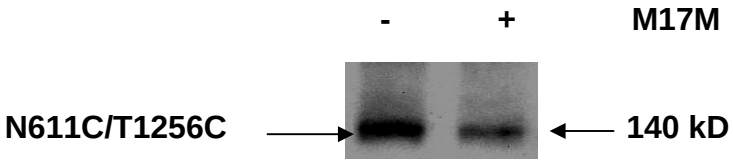


Figure S3

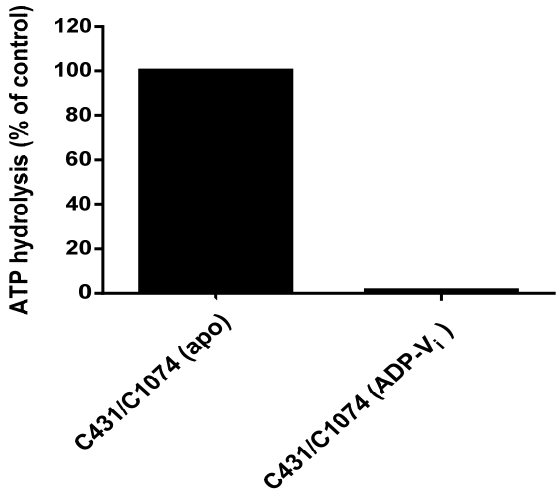


Figure S4

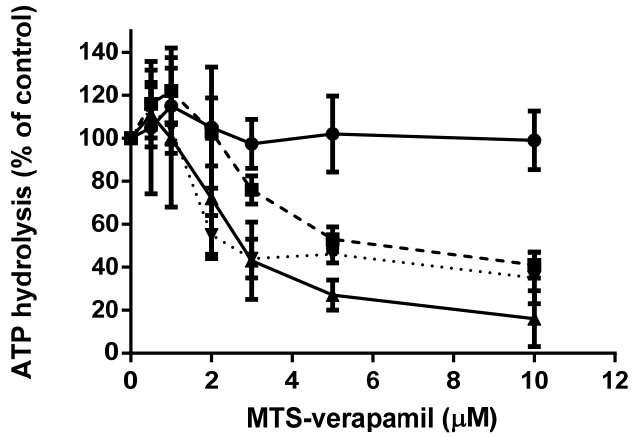
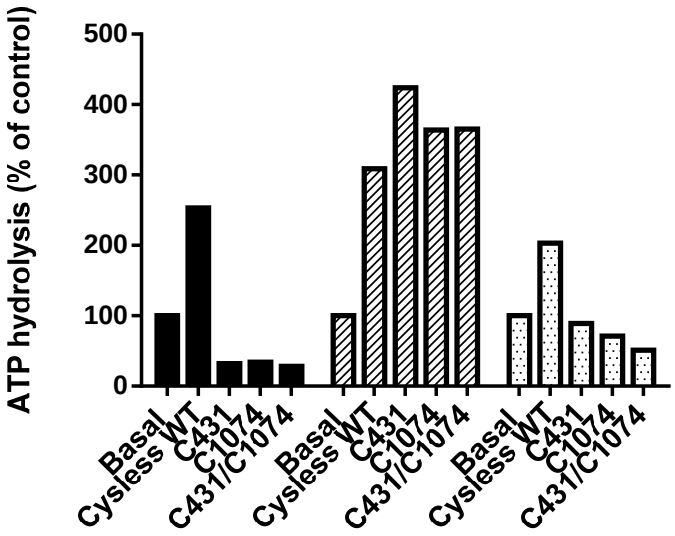


Figure S5

A



B

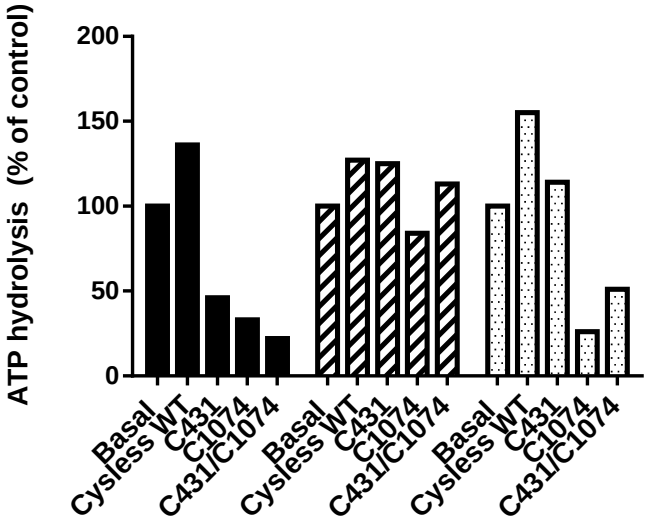
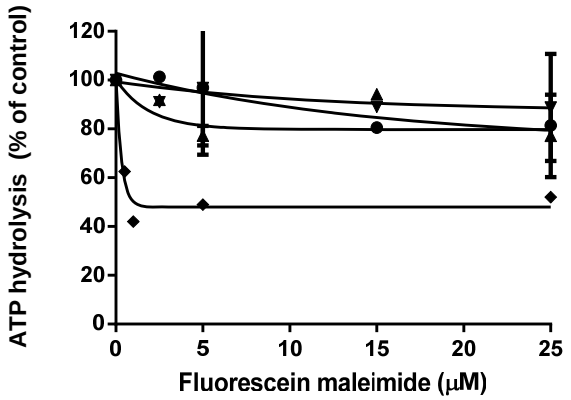


Figure S6

A


ATP hydrolysis (% of control)
120
100
80
60
40
20
0
0
5
10
15
20
25
Fluorescein maleimide (μM)

B

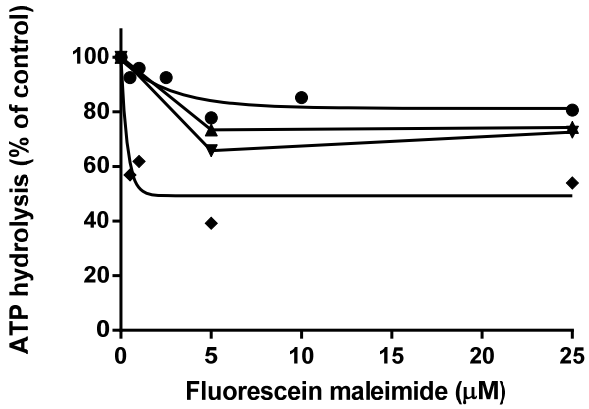
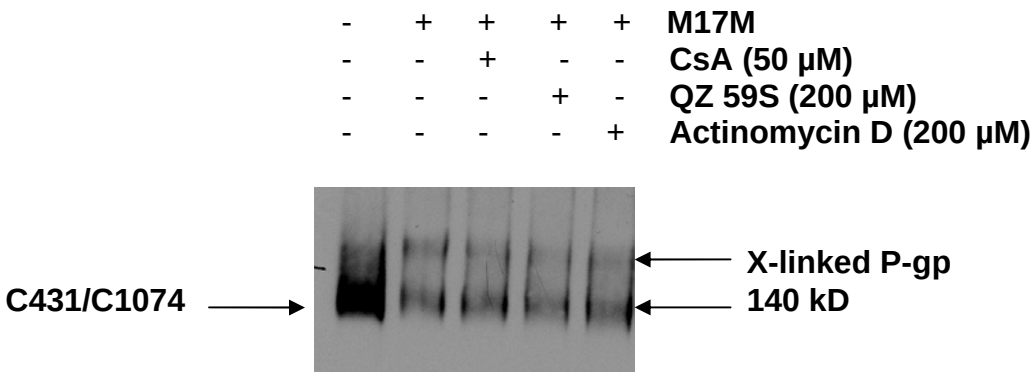

ATP hydrolysis (% of control)
100
80
60
40
20
0
0
5
10
15
20
25
Fluorescein maleimide (μM)

Figure S7


- + + + + M17M
- - + - - CsA (50 μM)
- - - + - QZ 59S (200 μM)
- - - - + Actinomycin D (200 μM)
C431/C1074
X-linked P-gp
140 kD