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Role of the Glutathione Redox Cycle in Acquired and de Novo Multidrug Resistance

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Drug resistance represents a major obstacle to successful cancer chemotherapy. However, the specific biochemical mechanisms responsible for clinical drug resistance are unknown. In these studies resistance to the antitumor agent adriamycin was found to involve two mechanisms, one that decreased drug accumulation by the P170 mechanism and another that altered the glutathione redox cycle, an important pathway in the detoxification of reactive oxygen. This dual mechanism of drug resistance was demonstrated in cell lines that had acquired the multidrug-resistant phenotype and in human colorectal cancer cells with de novo resistance. These studies support a model of acquired and de novo multidrug resistance that includes alterations in both drug accumulation and the glutathione redox cycle.

MULTIDRUG RESISTANCE (MDR) describes a problematic form of resistance in which tumor cells that become refractory to treatment with one drug develop cross resistance to a variety of agents that include antitumor antibiotics such as the anthracyclines, vinca alkaloids, and epidophyllotoxins (*1*). A characteristic feature of MDR cells is a decrease in drug accumulation that is associated with the increased production of a membrane glycoprotein termed P170 (*2*). This protein binds a drug and facilitates efflux by an energy-dependent process (*3*). The gene coding for P170 (*mdr*-1) has been cloned (*4*) and was expressed at levels that are proportional to the degree of resistance in several multidrug-resistant cell lines (*5*). P170 is also expressed in human tumor tissues including tumors of the colon, adrenal, ovary, and breast (*6*). Although the increased expression of P170 is an important component of acquired MDR, it is unlikely to be the sole mechanism of resistance for the broad spectrum of compounds that encompass MDR (*7*). In primary or de novo forms of resistance, for example, a clear relation between the expression of P170 and tumor response to chemotherapy has not been established (*6, 7*).

Evidence of a role for "non–P170-mediated" mechanisms of resistance in MDR cells was demonstrated in the following studies, in which we used the Ca^{2+} channel antagonist verapamil (Ver) (Table 1 and Fig. 1). Verapamil was previously shown to bind to the active site of P170, thereby inhibiting drug efflux (*8*). However, we observed that Ver treatment did not confer full sensitivity to the anthracycline adriamycin (Adr) in MDR human breast carcinoma (MCF/Adr) and murine leukemia (P388/Adr) cell lines. These cells display the MDR phenotype of cross-resistance, decreased drug accumulation, and overexpression of the *mdr*-1 gene (*9, 10*). The MCF/Adr and P388/Adr cells were, respectively, 94 and 37 times as resistant to Adr as drug-sensitive cell lines (Table 1). Treatment with Ver (10 μg/ml) partially restored Adr sensitivity, decreasing the 50% inhibitory concentration (IC_{50}) value for Adr by about seven times in each cell line. This dose of Ver produced a sixfold increase in the steady-state concentration of [^{3}H]-daunomycin (Fig. 1), whereas MCF/Adr cells accumulated 1/30 as much [^{3}H]daunomycin as the parent cell line. The maximum effect achieved with Ver (50 μg/ml) resulted in a ninefold increase in drug uptake and in a corresponding decrease in the IC_{50} value to one-ninth that of Adr. These studies demonstrate that altered drug accumulation alone cannot account for Adr resistance in MCF/Adr and P388/Adr cell lines.

Studies of Batist *et al.* (*11*) showed that a 45-fold increase in glutathione *S*-transferase (GST) activity occurs in MCF/Adr cells. The GSTs are a multigene family of isoenzymes that catalyze the conjugation of glutathione (GSH) to a variety of electrophilic compounds as the first step in a detoxification pathway leading to mercapturic acid formation (*12*). However, MCF/Adr cells express a specific anionic isoenzyme of GST that presumably possessed high levels of intrinsic peroxidase activity (*11*). Peroxidase activity mediated by GST, otherwise known as non–selenium-dependent glutathione peroxidase (GSH-Px) (*12*), was believed to account for the decreased formation of hydroxyl radicals observed in MCF/Adr cells exposed to Adr (*13, 14*). The generation of oxyradicals produced by the redox cycling of the quinone moiety of Adr is believed to contribute to the antitumor activity of this and related compounds (*15*). Therefore, it is

Table 1. Enhancement of Adr cytotoxicity by BSO and Ver. The IC_{50} values were calculated as the concentration of Adr that inhibits day 4 [^{3}H]thymidine incorporation by 50% in human breast (MCF and MCF/Adr) and colon cancer cells (MIP-101 and DLD-1), and inhibits growth at 72 hours by 50% in P388 cells. Log phase monolayer cultures of human breast or colon cells, and suspensions of P388 cells were incubated with Adr for 3 hours. Cells were washed free of drugs and allowed to grow in RPMI 1640 medium supplemented with 10% fetal bovine serum. After 72 hours, P388 cell growth was assessed by counting (Coulter Electronics), and MCF and colon cell growth was measured by incorporation of [^{3}H]thymidine (6.7 Ci/m*M*; New England Nuclear). [^{3}H]Thymidine was incubated with cells for 24 hours and incorporation into trichloroacetic acid–precipitable material was determined (*25*). BSO treatment was 200 μ*M* for MCF and colon cells and 10 μ*M* for P388 cells, and was administered for 24 hours before the Adr. This treatment decreased GSH concentrations in the various cell lines by 75 to 90% without affecting cell doubling times. DOP is defined as the degree of potentiation calculated by IC_{50} control/IC_{50} treated. Ver was added simultaneously with Adr at doses of 10 μg/ml in MCF and colon cells, and 5 μg/ml in P388. Values are the means of four to six experiments, with SE less than 10% of the reported values.

Source	IC_{50} values for Adr (μ*M*)						
	Control	BSO	(DOP)	Ver	(DOP)	BSO/Ver	(DOP)
Cell lines							
MCF	0.16	0.13	(1)	0.08	(2)	0.08	(2)
MCF/Adr	15.0	3.40	(4)	2.30	(7)	0.15	(100)
P388	0.01	0.01	(1)	0.03	(0.3)	0.02	(0.5)
P388/Adr	0.37	0.18	(2)	0.05	(8)	0.01	(37)
Colon lines							
DLD-1	0.20	0.05	(4)	0.10	(2)	0.02	(10)
MIP-101	2.00	0.70	(3)	0.20	(10)	0.08	(25)

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possible that elevated levels of antioxidant enzymes (for example, GSH-Px) may confer resistance to Adr. However, the role of GST activity and its associated peroxidase activity in Adr resistance has not been established.

We conducted experiments with MCF/Adr cells to determine the effect of inhibiting GSH-dependent peroxidase activity on Adr resistance. Specific inhibitors of GSH-Px activity are not available; however, GSH-Px requires GSH and the "GSH redox cycle" to sustain activity (Fig. 2) and, therefore, can be inhibited by depleting cells of GSH (*16*). This depletion was achieved by treating cells with buthionine sulfoximine (BSO), a selective inhibitor of GSH synthesis (*17*). Exposure to BSO (0.2 m*M* for 24 hours) resulted in a 90% decrease in cellular GSH content without toxicity. That GSH-Px activity was inhibited by this treatment was indicated by the decrease in the stimulation of the hexose monophosphate shunt (HMPS). Treatment of MCF/Adr cells with Adr or with the oxidant *tert*-butyl hydroperoxide (tBH) resulted in a marked decrease in reduced GSH, reflecting its consumption by GSH-Px, and a corresponding stimulation of the HMPS (Table 2). The HMPS activity was increased in order to supply reducing equivalents [that is, nicotinamide adenine dinucleotide phosphate (NADPH)] for the reduction of oxidized glutathione (GSSG), and its activation is a useful index of GSH redox cycling catalyzed by GSH-Px (Fig. 2) (*18*). Treatment with BSO markedly decreased the stimulation of the HMPS caused by tBH, but only partially inhibited the Adr-induced activation of the HMPS (Table 2). This partial inhibition was due to the fact that NADPH is required for the single electron reduction of Adr, to form semiquinone-free radicals, in addition to its role in maintaining the GSH redox state (*15*). In contrast, utilization of NADPH by tBH was due solely to the consumption of NADPH by GSH-reductase. These findings on the inhibitory effect of BSO on GSH redox cycling was confirmed in studies in which GSH-reductase activity was inactivated by treating cells with the 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) (Table 2). The inhibitory effect of BCNU treatment was due to binding of the active site of the reductase by an isocyanate derivative of the parent nitrosourea (*19*). Treatment with BCNU blocked the recycling of GSSG to GSH, resulting in an even further decrease in the ratio of GSH/GSSG after tBH or Adr administration (Table 2). Inhibiting GSH-reductase activity also had a sparing effect on NADPH, as reflected by a decrease in the tBH-induced stimulation of the HMPS. The present studies establish the pharmacologic conditions in which BSO can modify GSH-Px activity in situ and are consistent with a report by Durse *et al.* (*14*) showing that Adr stimulated a twofold increase in the formation of hydroxyl radicals in BSO-treated MCF/Adr cells.

BSO treatment alone was less effective than Ver at reversing Adr resistance in MCF/Adr cells (Table 1). Depletion of cellular GSH to levels that inhibited GSH-Px activity decreased by four times the IC_{50} value for Adr. Thus, neither BSO nor Ver alone could restore the nearly 100-fold resistance to Adr in this MDR cell line. However, the combined effect of BSO and Ver completely restored Adr sensitivity in MDR cells, while producing only a twofold increase in sensitivity in the parent MCF-7 cell line. Moreover, BSO treatment had no effect on drug accumulation either alone or in combination with Ver (Fig. 1), showing that BSO was acting by a mechanism separate from that of Ver.

The data with MCF/Adr cells indicate that GSH-dependent peroxidase activity and P170 have a role in Adr resistance. In subsequent studies we used BSO and Ver to determine the role of these mechanisms in other drug-resistant cell lines (Table 1). The multidrug resistant cell line P388/Adr, like MCF/Adr, was selected for resistance to Adr and displays the "classic MDR phenotype" (*9*). BSO and Ver decreased the IC_{50} value for Adr to one-half and one-eighth as much, respectively, and in combination completely restored Adr sensitivity. These treatments had little or no effect on the parent P388 cell line. More interesting was the finding that both BSO and Ver increased Adr sensitivity in human colon cell lines derived from patients that had not received prior chemotherapy. The MIP-101 and DLD-1 are poorly differentiated colon adenocarcinoma

Table 2. Effect of BSO and BCNU on the activation of the GSH redox cycle in multidrug-resistant MCF/Adr. GSH and GSSG were determined by the high-performance liquid chromatography (HPLC) method of Fariss and Reed (*26*). HMPS was determined in separate monolayer cultures of 2×10^6 log phase cells that were incubated for 30 min in 2 ml of Hanks balanced salt solution containing 1 m*M* glucose and [$^{14}C_1$]glucose (final specific activity, 1 μCi/2 μmol; New England Nuclear), in septum-stoppered T25 flasks (Corning). $^{14}CO_2$ was trapped on filter disks soaked in 2*N* NaOH (*18*). All values are the means ± SEM of four experiments. N.D., not determined.

Treatment	GSH (nmol/mg protein)	GSSG (nmol/mg protein)	GSH/GSSG	HMPS activity (% control)
Control	39 ± 3.9	3.1 ± 0.05	12.6	100 ± 12
BCNU* (25 μ*M*)	41 ± 3.4	2.9 ± 0.06	14.2	94 ± 23
BSO† (0.2 m*M*)	N.D.	N.D.	N.D.	109 ± 17
Adr‡ (1 m*M*)	27 ± 3.5	1.6 ± 0.5	16.7	530 ± 34
Adr + BCNU	29 ± 2.3	4.4 ± 1.0	6.6	N.D.
Adr + BSO	N.D.	N.D.	N.D.	307 ± 41
tBH (0.25 m*M*)	24 ± 0.4	4.3 ± 0.2	5.6	976 ± 42
tBH + BCNU	14 ± 1.2	8.1 ± 0.6	1.7	305 ± 69
tBH + BSO	N.D.	N.D.	N.D.	278 ± 39

*After 30 min of incubation with BCNU (25 μ*M*) (National Cancer Institute), cells were washed free of drugs with phosphate-buffered saline and were incubated in complete medium for 2 hours before the addition of tBH or Adr. This allowed for the recovery of GSH levels, whereas GSH reductase activity was irreversibly inhibited by more than 95% (*21*). This treatment was not found to be toxic by trypan blue exclusion. †Cells were treated with BSO as described in the legend to Table 1. ‡Adr and tBH were added in 100 μl of Hanks balanced salt solution and cells were treated for 30 min prior to determination of GSH, GSSG, or HMPS activity.

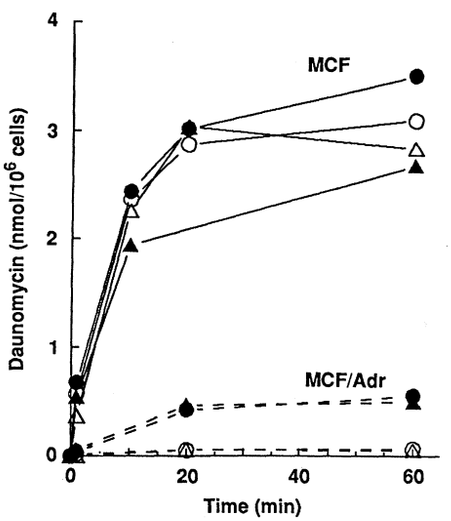

Fig. 1. Effect of Ver and BSO on the net accumulation of [^{3}H]daunomycin into MCF and MCF/Adr cells. Cell suspensions (2×10^6 cells per milliliter) were incubated with 10 μ*M* daunomycin (specific activity 1 mCi/10 μ*M*) in RPMI 1640 medium supplemented with 10% fetal bovine serum and 25 m*M* Hepes. Ver (10 μg/ml) was added immediately before daunomycin. BSO (0.2 m*M*) was administered continuously for 24 hours prior to and during drug uptake. Incubations were terminated by layering 0.5 ml of cells over silicone oil and centrifugating at 12,000*g* for 1 min. Cell pellets were dissolved in 1*N* NaOH for 1 hour at 80°C, neutralized with glacial acetic acid, and counted by liquid scintillation spectrometry. Solid lines represent drug-sensitive MCF cells and dashed lines represent drug-resistant MCF/Adr cells. ○, control; ●, Ver; △, BSO; and ▲, BSO/Ver. Results are the means of duplicate determinations.

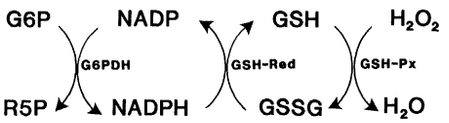

Fig. 2. The glutathione redox cycle. G6P, glucose-6-phosphate; R5P, ribulose-5-phosphate; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); GSH, glutathione (reduced form); GSSG, glutathione (oxidized form); G6PDH, glucose-6-phosphate dehydrogenase; GSH-Red, glutathione reductase; and GSH-Px, glutathione peroxidase.

cell lines. However, the MIP-101 cell line was established from a liver metastasis and was also more anaplastic than the tumor used to establish the DLD-1 cell line (*20*). The MIP-101 cells were ten times as resistant to Adr compared to DLD-1 cells and were also more responsive to Ver (Table 1). The Ver-mediated sensitization of MIP-101 cells was accompanied by a sixfold increase in the steady-state concentration of [^{3}H]daunomycin (*21*). The DLD-1 cells accumulated three times more daunomycin than MIP-101 cells; however, Ver increased drug accumulation by only 1.5 times in these more drug-sensitive colorectal carcinoma cells (*21*). These observations suggest that tumor progression in colon cancer may be correlated with the expression of a de novo MDR phenotype. The effect of BSO in these colon cells was comparable to the effect on MDR cells, the IC_{50} value for Adr being decreased to one-third to one-fourth as much. The combined effect of BSO and Ver treatments were additive and increased Adr sensitivity by 10- and 25-fold, respectively, in DLD-1 and MIP-101 cells.

These cell lines differed with respect to the specific GSH-dependent mechanism of Adr resistance. The Adr resistance was correlated with elevated GST activity only in MCF/Adr cells (Table 3), whereas P388/Adr and MIP-101 cells had one-third to one-fourth as much GST activity as their corresponding drug-sensitive cells lines. Moreover, GST-associated peroxidase activity (that is, non–selenium-dependent GSH-Px) did not correlate with Adr resistance because virtually all of the increased GSH-peroxidase activity present in the Adr-resistant cells was the selenium-dependent form (Table 3). This was established by the substrate affinity of the cellular peroxidase for hydrogen peroxide (H_2O_2) (Table 3). The GST-associated form of GSH-Px can reduce a variety of organic peroxides [for example, cumene hydroperoxide (CuOOH)] but shows little activity toward hydrogen peroxide, whereas selenium-dependent GSH-Px metabolizes both hydrogen and organic peroxides (Table 3) (*22, 23*). The Adr resistance correlated with a two- to fourfold increase both in the concentration of GSH and in selenium-dependent GSH-Px activity in P388/Adr and MIP-101 cells. However, in MCF/Adr cells, Adr resistance was correlated with a sixtyfold increase in selenium-dependent GSH-Px activity and a 75% decrease in GSH. These results demonstrate the importance of selenium-dependent GSH-Px and of the associated GSH redox capacity as biochemical mechanisms that contribute to Adr resistance.

The increased GSH redox capacity observed in cell lines with acquired MDR was probably the result of an adaptive response to reactive oxygen species generated by the metabolic activation and subsequent redox cycling of the selecting agent, Adr. This is consistent with a mechanism of Adr cytotoxicity involving the generation of reactive oxygen (*15*). An increase in the cellular GSH redox capacity may be a general phenomenon in MDR because many of the drugs that develop cross-resistance to Adr are also known to redox cycle and thereby generate reactive oxygen (*24*). The etiological basis for the expression of the MDR phenotype in de novo resistant colon cancer is not understood. However, Cowan and colleagues (*10*) have shown that similar biochemical changes occur in MDR human breast carcinoma cells and in hyperplastic rat liver nodules induced by carcinogen treatment. Thus, an MDR phenotype may be expressed in certain forms of carcinogen-induced neoplasias.

The present studies suggest that we may need to broaden our definition of the MDR phenotype to include alterations in the GSH redox cycle in addition to decreased drug accumulation and increased production of P170. These studies also show that a similar dual mechanism of resistance may occur in previously untreated human colorectal cancer, a tumor that is inherently refractory to chemotherapy. A better understanding of the mechanisms of clinical resistance may require further elucidation of tumor-reactive oxygen detoxification pathways.

Table 3. GSH and GSH-dependent enzyme activities in multidrug and de novo resistant cancer cells. Total GSH-Px activity (non–selenium- and selenium-dependent GSH-Px) was determined using CuOOH (1.25 m*M*) as a substrate by the method of Paglia and Valentine (*23*). Selenium-dependent GSH-Px activity was determined by the same enzymatic method with the exception that H_2O_2 (0.25 m*M*) was used as the substrate as described by Shrene *et al.* (*22*). GST activity was determined with 1-chloro-3,4-dinitrobenzene as described by Habig *et al.* (*27*). GSH was measured by the cycling reduction assay of Tietze (*28*). Values are the means ± SD of triplicate determinations.

Source	GSH-Px*		GST*	GSH†
	CuOOH	H_2O_2		
Cell lines				
MCF	0.5 ± 0.3	0.4 ± 0.1	2 ± 0.1	9.5 ± 0.3
MCF/Adr	27 ± 5.0	24 ± 4.0	78 ± 5.4	2.3 ± 0.2
P388	1.2 ± 0.3	1.0 ± 0.2	9 ± 0.9	2.2 ± 0.2
P388/Adr	4.0 ± 0.7	3.1 ± 0.5	3 ± 0.5	4.3 ± 0.3
Colon lines				
DLD-1	4.5 ± 0.7	3.7 ± 0.05	87 ± 6.3	10 ± 1.0
MIP-101	16 ± 4.5	13 ± 3.4	21 ± 3.1	29 ± 1.2

*Nanomoles per minute per 10^6 cells. †Nanomoles per 10^6 cells.

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Conversion of a PI-Anchored Protein to an Integral Membrane Protein by a Single Amino Acid Mutation

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Qa-2, a cell-surface glycoprotein anchored by phosphatidylinositol (PI), is structurally related to the class I transplantation antigens H-2 K, D, and L, which are integral membrane glycoproteins. The predicted transmembrane segment of Qa-2 differs from those of H-2 K, D, and L by the presence of an aspartate in place of a valine at position 295. A single base change that replaced this aspartate with valine resulted in cell-surface Qa-2 molecules that were insensitive to hydrolysis by a PI-specific phospholipase C and more resistant to papain cleavage, properties shared by H-2D. Cells expressing Asp → Val mutant Qa-2 proteins were still able to attach a PI anchor to endogenous proteins such as Thy-1 and J11D. It therefore appears that this single amino acid change converts Qa-2 from a PI-linked form into an integral membrane protein.

UNTIL RECENTLY IT WAS BELIEVED that all membrane-bound proteins are integrated into the lipid bilayer by a membrane-spanning segment of hydrophobic amino acids interacting noncovalently with the nonpolar tails of membrane lipids. In the past few years, however, it has become clear that a number of proteins are anchored instead to one leaflet of the lipid bilayer by covalent linkage to phosphatidylinositol (PI) (*1*). Although the signals mediating this novel posttranslational modification are not well understood, alignment of carboxyl-terminal regions of the proteins, predicted from their respective cDNA or genomic DNA sequences, has revealed common features among polypeptides with PI anchors. The common features are a truncated or weakly hydrophobic membrane-spanning segment and little or no cytoplasmic tail (*1*).

Qa-2, a cell-surface glycoprotein expressed on subpopulations of hematopoietic cells (*2*), was shown to be anchored by PI (*3–5*), since it could be released from the cell membrane by treatment with a PI-specific phospholipase C (PI-PLC). Qa-2 is otherwise structurally similar to the H-2 class I transplantation antigens K, D, and L, which are integral membrane glycoproteins with strongly hydrophobic transmembrane segments and cytoplasmic tails of about 30 amino acids (*6, 7*). Exon exchange between transplantation antigens and the *Q7* gene, which encodes Qa-2 (*4, 8, 9*), showed that the signals for PI modification of Qa-2 are located in the carboxyl-terminal region of the molecule (*4, 5*). By exchanging segments of the *Q7* and *H-2* genes of the C57BL/10 mouse (H-2^b haplotype), we mapped the signals for PI modification of Qa-2 to a region of 80 amino acids that differs from H-2D^b most significantly by the presence of a charged aspartate residue (Asp^{295}) in place of valine in the transmembrane segment and by a cytoplasmic tail of only three basic amino acids (*5*). The transmembrane segment of all transplantation antigens that have been sequenced has a hydrophobic or neutral amino acid at this position (*7*), and the unusual features of *Q7* led Steinmetz *et al.* (*10*) to propose that *Q7* might be a pseudogene. To distinguish which of these features is responsible for the modification of Qa-2 by PI, we replaced the Asp^{295} with Val and treated transfected thymoma cells with PI-PLC to test whether the mutant Qa-2 is linked to PI.

The transmembrane Asp^{295} codon of a Q7^b cDNA was converted to Val by site-directed mutagenesis (Fig. 1). A segment of DNA containing the mutated codon was inserted in place of a corresponding segment from the wild-type Q7^b cDNA expression vector to ensure that no other mutations were introduced aberrantly. R1.1 thymoma cells were transfected with the mutant Q7^b cDNA vector as described (*8*), and G418-resistant clones were screened for expression of cell-surface Qa-2. Two strongly positive clones (Q7V3R and Q7V6R) were independently isolated and used for further study.

```
H-2D^b   284 ...MVIVAVLGVLGAMAIIGAVVAFVM... 307
                |  ||  ||| ||||||||||
Q7^b     287 ...MATIAVVVDLGAVAIIGAVVAFVM... 310
                      ↑
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Fig. 1. Alignment of the transmembrane peptide segments of H-2D^b and Q7^b. The arrow refers to the Q7^b Asp^{295} that was mutated to Val by antisense oligonucleotide 5′-TCCAAGGA CAACCAC-3′, which differs from the wild-type sequence (*8*) by a single base (underlined). A 672-bp Kpn I–Dra III fragment (positions 415 to 1087) (*8*) was removed from the Q7^b cDNA expression vector and site-directed mutagenesis was performed by using a procedure for gapped-plasmid heteroduplexes (*17*). Colonies were screened with the ^{32}P-labeled oligonucleotide, and mutant DNAs were purified after retransformation of plasmids from positive clones. The Kpn I–Dra III segment containing the mutated codon was sequenced by a modified chain-termination method used for supercoiled plasmids (*18*). This segment was excised from the mutated plasmid and inserted in place of a corresponding segment from the wild-type Q7^b cDNA expression vector. Mutant DNAs were electroporated into R1.1 cells, and stable transfectants were selected in growth medium containing G418 (1 mg/ml) (*8*).

The PI-PLC sensitivity of Qa-2 molecules on the surface of clones Q7V3R and Q7V6R was compared to that of clone Q7c26R, an R1.1 cell line transfected with a wild-type Q7^b cDNA construct (*8*), which expresses the PI-anchored form of Qa-2 (*5*). As expected, the Q7c26R cells showed a marked reduction in staining (about tenfold) after PI-PLC treatment (Fig. 2A). In contrast, Asp^{295} → Val mutant Qa-2 molecules on Q7V3R and Q7V6R were completely resistant to PI-PLC hydrolysis in that virtually identical profiles were obtained from mock-treated and PI-PLC–treated groups (Fig. 2A). It therefore appears that the Asp^{295} → Val mutant Qa-2 is no longer modified by PI but, like H-2D^b, integrates into the lipid bilayer.

To be certain that the Q7V3R and Q7V6R cells retained the ability to covalently modify proteins with PI, two other proteins endogenous to R1.1 cells and known to be PI-anchored, Thy-1 (*11*) and J11D (*12*), were analyzed for their sensitivity to PI-PLC. There is little difference in the PI-PLC sensitivity of Thy-1 and J11D among the Q7c26R, Q7V3R, and Q7V6R cell lines (Fig. 2, B and C). Not all of these molecules are removed by PI-PLC treatment, although this unexplained phenome-

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