P-glycoprotein inhibits caspase-8 activation but not formation of the death inducing signal complex (disc) following Fas ligation

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Abstract

Previous studies by our laboratory have shown that the drug transporter protein P-glycoprotein, P-gp, can specifically inhibit Fas-induced caspase-3 activation and apoptosis. Importantly, inhibition of both caspase-3 activation and cell death could be reversed by pharmacological and antibody inhibitors of P-gp function. However, the molecular mechanisms underpinning P-gp-mediated resistance to Fas-induced cell death and caspase activation remained unknown. We therefore sought to identify the point(s) within the death receptor pathway at which P-gp exerted its inhibitory effect and to determine whether the ATPase activity of P-gp was required. Structure-function analysis determined that ATP hydrolysis was necessary for P-gp to confer resistance to Fas-induced caspase activation and cell death. Importantly, although both FADD and caspase-8 were recruited to the Death Inducing Signal Complex (DISC) in wild-type P-gp expressing cells following Fas ligation, subsequent activation of caspase-8 at the DISC was inhibited. The ability of P-gp to inhibit caspase-8 activation was also ATP dependent. These studies demonstrate that P-gp inhibits Fas-induced caspase-8 activation but not formation of the DISC and that this activity of P-gp is dependent on ATP hydrolysis.
Cell Death and Differentiation (2002) **9**, 1266–1272. doi:10.1038/sj.cdd.4401081

Abbreviations: P-gp, P-glycoprotein; DISC, death inducing signaling complex; MDR, multidrug resistance; TNF, tumor necrosis factor; GFP, green fluorescence protein; FLIP, Flice-like inhibitory protein

Introduction

P-glycoprotein, (P-gp), is a large (170 kDa) ATP-dependent transporter protein that can confer multidrug resistance (MDR) by actively effluxing drugs from target cells.[1] Expressed on apical membranes of vital organs and at blood–tissue barriers, P-gp has been shown to protect these tissues from damage induced by xenotoxins.[1] P-gp is also expressed on hematopoietic cells, however a physiological role in these cells has yet to be identified. Although P-gp has been demonstrated to efflux a wide range of structurally and functionally diverse compounds, no model for the mechanism(s) by which P-gp effluxes cytotoxic drugs can account for its broad specificity.[1,2] The ATP binding sites within the molecule are well defined and previous studies have determined that both sites are required for transporter activity.[3–5]

In addition to providing resistance to cytotoxic drugs, P-gp[+ve] multidrug resistant cells are often cross-resistant to a number of cell death stimuli, such as radiation,[1,6,7] although the mechanism(s) of resistance to these stimuli are not well characterized. We and others have examined the effect of P-gp on apoptosis induced by the tumor necrosis factor (TNF) family of death receptors (Fas, TNFR).[8–11] We have previously shown that P-gp can specifically inhibit Fas and TNF-induced caspase-3 activation and cell death. Importantly, inhibition of both caspase-activation and cell death were reversed by P-gp antagonists. However, the molecular mechanisms underpinning this novel function of P-gp remained unknown. The order of molecular events during Fas-induced cell death has been intensely studied over the past decade and several steps prior to caspase activation have been identified. We sought to determine the most proximal step(s) during Fas-induced cell death that was affected by P-gp expression. In addition, we examined whether the efflux/ATPase function of P-gp was necessary to confer resistance to Fas-induced cell death.

Overexpression of P-gp using common transfection methods has proven difficult. Therefore, most studies undertaken to examine the role of P-gp in apoptosis have utilized cell lines that have been selected using high doses of chemotherapeutic drugs to induce expression of P-gp. While much useful information has been gathered from these experiments, drug selection can induce a myriad of genetic changes that may directly or indirectly affect sensitivity to cell death stimuli. To counteract this potential problem, we expressed P-gp on the surface of CEM T-leukemia cells by retroviral gene transduction in the complete absence of drug selection. Since we were interested in examining the role of P-gp efflux function in the regulation of Fas-induced apoptosis, we also generated P-gp[+ve] CEM cells carrying a double mutation in the Walker A repeat regions of P-gp which are essential for ATPase activity and drug efflux function.[4] The results herein describe the effects of wild-type and mutant P-gp on Fas-mediated cell death and define the most proximal point in the Fas-signaling pathway that is affected by P-gp.

Results

Expression of wild-type and mutant P-gp by retroviral transduction

Expression of cell surface wild-type and mutant P-gp and intracellular GFP in retrovirus transduced CEM cells were assessed by flow cytometry (Figure 1A). P-gp was equivalently expressed on the surface of cells infected with wild-type P-gp (CEM^{GFP}-P-gp^{WT}) and mutant P-gp (CEM^{GFP}–P-gp^{MUT}), but not on P-gp^{-ve} cells infected with GFP alone (CEM^{GFP}) (Figure 1A). All cell lines expressed high levels of intracellular GFP. Expression of wild-type or mutant P-gp had no substantial effect on the relative levels of cell surface Fas compared to CEM^{GFP} cells (Figure 1A).

^{123}Rh accumulation studies were performed to examine the efflux activity of CEM^{GFP}–P-gp^{WT} and CEM^{GFP}–P-gp^{MUT}. Following incubation of these cells with ^{123}Rh, CEM^{GFP}-P-gp^{WT} cells showed a markedly reduced ^{123}Rh accumulation compared with that seen with CEM^{GFP} or CEM^{GFP}–P-gp^{MUT} cells (Figure 1B). Pre-treatment of CEM^{GFP}–P-gp^{WT} cells with the pharmacological P-gp inhibitor verapamil, inhibited ^{123}Rh efflux, as observed by the increase in fluorescence to the levels seen in CEM^{GFP} or CEM^{GFP}–P-gp^{MUT} cells (Figure 1B). Thus, wild-type P-gp expressed on the surface of CEM cells was capable of effluxing P-gp substrates, while mutant-P-gp had no efflux activity.

Expression of wild-type, but not mutant P-gp confers resistance to Fas-induced cell death

We next assessed the sensitivity of the CEM cells to Fas-mediated apoptosis. CEM^{GFP}–P-gp^{WT}, CEM^{GFP}-P-gp^{MUT}, and CEM^{GFP} cells were labeled with ^{51}Cr and then cultured with APO-1-1 anti-Fas antibody and cross-linked with protein A
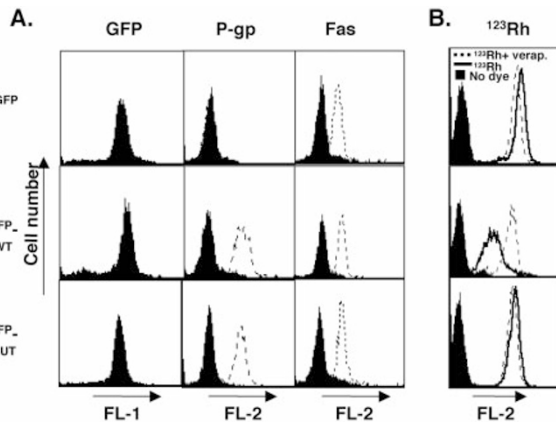

Figure 1 The expression of wild-type and mutant P-gp by retroviral transduction. (**A**) Expression of GFP (left hand column-shaded histogram) and P-gp (middle column-dashed line) and Fas (right hand column-dashed line) after infection and sorting. CEM cells expressing either wild-type P-gp (CEM^{GFP}–P-gp^{WT}), mutant P-gp (CEM^{GFP}–P-gp^{MUT}), or GFP alone (CEM^{GFP}), were stained and analyzed by flow cytometry using mAbs specific for human P-gp (MRK16) or Fas (CH-11). Fluorescence staining in each case is compared with cells stained with isotype control mAbs (shaded histograms middle and right hand columns). (**B**) Cells were incubated without (shaded histogram) or with ^{123}Rh (solid line) for 30 min and dye uptake was assed by flow cytometry. In some cases cells were pre-treated with 10 μM verapamil (dashed line) for 30 min and then cultured with ^{123}Rh

for 0–6 h. CEM^{GFP}–P-gp^{MUT}, and CEM^{GFP} cells displayed significant cell death after 6 h incubation with APO-1-1 (Figure 2A). By contrast and consistent with our previous studies using drug-selected P-gp expressing cells,[9] cell death induced by Fas antibodies in CEM^{GFP}–P-gp^{WT} cells was significantly attenuated over both a dose and time range of APO-1-1 (Figure 2A, B). Taken together, these results suggested that P-gp ATPase and/or efflux function was necessary to confer resistance to Fas-induced cell death. These data were confirmed using the CH-11 IgM anti-Fas monoclonal antibody to cross link the Fas receptor (Figure 2C). Consistent with the data shown in Figure 2A, B, the CEM^{GFP}–P-gp^{WT} cells were significantly less sensitive to Fas-induced apoptosis compared to the CEM^{GFP}–P-gp^{MUT}, and CEM^{GFP} cells. To determine whether inhibition of Fas-mediated cell death could be reversed using inhibitors of P-gp function, CEM^{GFP}–P-gp^{WT}, and CEM^{GFP} cells were pre-treated with either verapamil, or the P-gp-specific antagonistic antibody, UIC-2 and then cultured with CH-11 anti-Fas antibody. Both verapamil and UIC-2 reversed P-gp-mediated inhibition of Fas-induced apoptosis (Figure 2D), demonstrating that inhibition of Fas-killing was specifically mediated by P-gp. These results were consistent with those from our previous studies using CEM cells that were induced to overexpress P-gp following selection in the chemotherapeutic drug doxorubicin.[9,10]

Wild-type P-gp inhibits Fas-induced caspase activation

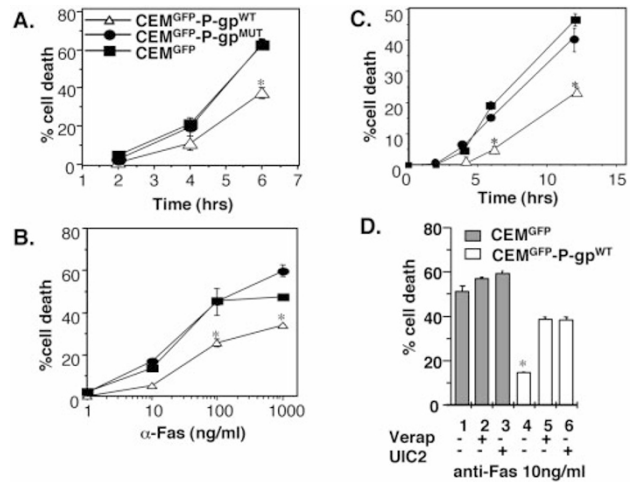Following ligation of the Fas receptor at the cell surface, FADD and caspase-8 are recruited, the DISC is formed and

Figure 2 Wild-type, but not mutant P-gp confers resistance to Fas-mediated cell death. CEM cells expressing either wild-type P-gp (CEM^{GFP}–P-gp^{WT}), mutant P-gp (CEM^{GFP}–P-gp^{MUT}), or GFP alone (CEM^{GFP}), were labeled with ^{51}Cr and then treated for (**A**) 0–6 h. with 100 ng/ml APO-1-1 anti-Fas antibody and protein A; (**B**) for 6 h with 0–1000 ng/ml APO-1-1 anti-Fas antibody and protein A; (**C**) 0–12 h. with 10 ng/ml CH-11 anti-Fas antibody. (**D**) CEM^{GFP}–P-gp^{WT} and CEM^{GFP} cells were labeled with ^{51}Cr, pretreated with verapamil (10 μM) or monoclonal antibody UIC-2 (5 μg/ml) 30 min and then cultured for 20 h with 10 ng/ml CH-11 anti-Fas antibody. For all experiments, data were calculated as the mean$\pm$S.E. of triplicate samples and are representative of at least three separate experiments and statistical differences ($P<0.001$ as determined by unpaired t-test) are denoted by asterisk

caspase-8 is auto-activated.[12] Since only wild-type P-gp inhibited Fas-mediated cell death, we were interested in determining the effects of wild-type and mutant P-gp on caspase activation. Lysates from CEM^{GFP}–P-gp^{WT}, CEM^{GFP}–P-gp^{MUT}, and CEM^{GFP} cells treated with 0–100 ng/ml APO-1-1 anti-Fas antibody and protein A for 4 h were assessed for cleavage of caspase-8, caspase-3, and the caspase-3 substrate, PARP. As seen in Figure 3A, cleavage of caspase-8 to the active p18 protein was significantly
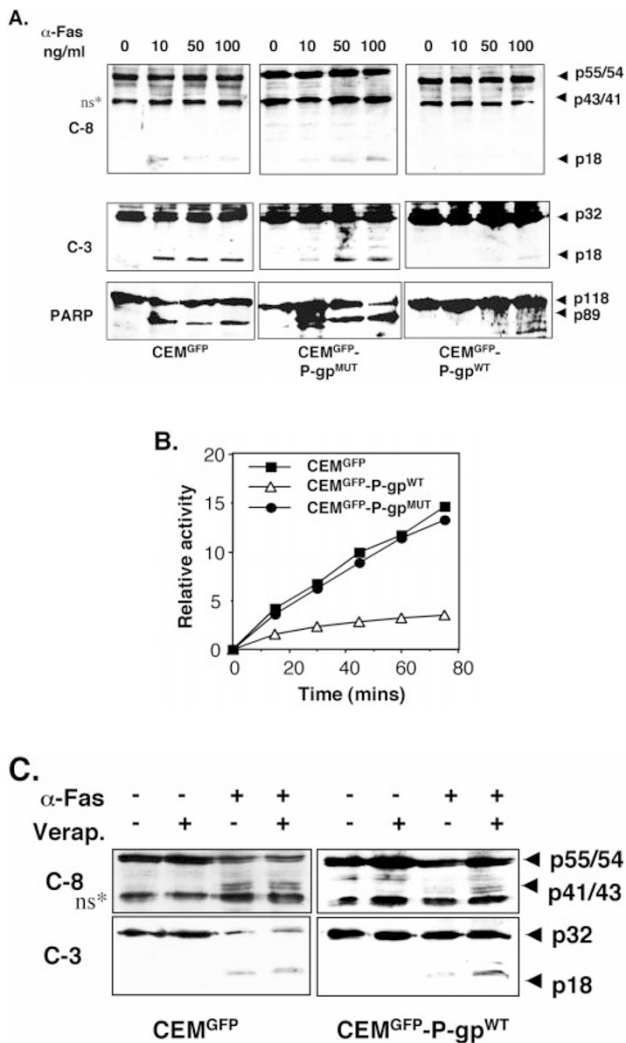

Figure 3 Wild-type P-gp inhibits Fas-induced caspase-3 activation. (**A**) CEM cells expressing either wild-type P-gp (CEM^{GFP}–P-gp^{WT}), (CEM^{GFP}–P-gp^{MUT} or GFP alone (CEM^{GFP}), were treated for 4 h. with 0–100 ng/ml APO-1-1 anti-Fas antibody and protein A. Whole cell lysates were examined for cleavage of PARP, Caspase-8 (C-8) and Caspase-3 (C-3). Arrows indicate the cleavage products. ns* indicates non-specific bands. (**B**) Cytosolic lysates from cells treated with 50 ng/ml APO-1-1 anti-Fas antibody and protein A for 4 h were incubated with the caspase-3 substrate Ac-DEVD-pNA and substrate cleavage measured by change in optical density (O.D.). Relative fold activity was determined by dividing O.D. readings of samples treated with anti-Fas antibody by O.D. of control untreated samples. These results are representative of at least three different experiments. (**C**) To inhibit P-gp function, CEM^{GFP}–P-gp^{WT} and CEM^{GFP} cells were pre-treated with 10 μM verapamil for 30 min and then cultured with 50 ng/ml APO-1-1 anti-Fas antibody and protein A for 6 h. In these experiments, the 43/41 kDa proteins were the most clearly detected caspase-8 cleavage product

reduced in CEM^{GFP}–P-gp^{WT} cells compared with CEM^{GFP}-P-gp^{MUT}, and CEM^{GFP} cells. In addition, both caspase-3 and PARP were processed similarly in CEM^{GFP}–P-gp^{MUT}, and CEM^{GFP} cells while processing of these molecules in CEM^{GFP}–P-gp^{WT} cells was significantly attenuated (Figure 3A). These results indicated that only wild-type P-gp inhibited Fas-mediated caspase activation. These results were confirmed by DEVDase activity assays for caspase-3 activity (Figure 3B). Ligation of the Fas receptor resulted in increased caspase-3 activity in CEM^{GFP}–P-gp^{MUT} and CEM^{GFP} cells, however, very little caspase-3 activity was observed in lysates from Fas-treated CEM^{GFP}–P-gp^{WT} cells.

To determine whether inhibition of caspase activation in CEM^{GFP}–P-gp^{WT} cells could be reversed by blocking P-gp efflux function, cells were pre-treated with verapamil then cultured with APO-1-1 and protein A (Figure 3C). Both caspase-3 and caspase-8 cleavage were enhanced by addition of verapamil to CEM^{GFP}–P-gp^{WT} cells, with caspase-8 cleavage increasing from 7 to 39% and caspase-3 cleavage from 8 to 21% (Figure 3C). Verapamil had no effect on the cleavage of caspase-8 or caspase-3 in CEM^{GFP} cells. Taken together these experiments clearly demonstrated that inhibition of caspase cleavage was specifically due to functional P-gp expression since inhibition by either mutation or pharmacological inhibitors restored Fas-induced caspase-activation.

Expression of wild-type or mutant P-gp does not alter expression of FLIP or FADD

Overexpression of intracellular FLIPs (Flice-like inhibitory proteins) can inhibit Fas-mediated cell death by competing with caspase-8 for binding to FADD and the Fas receptor.[13] Resistance to Fas-mediated apoptosis has also been associated with deletion of the adapter molecule FADD.[14] Therefore, it remained possible that the protein levels of the adapter molecule FADD or FLIP were altered in CEM^{GFP}–P-gp^{WT} cells and thereby responsible for the inhibition of caspase activation. To examine these possibilities, Western blot analysis for FADD and FLIP expression was performed on whole cell lysates from the retrovirus transduced cells (Figure 4). As shown in Figure 4, all cells displayed equivalent levels of both FADD and FLIP regardless of P-gp expression. Hence, inhibition of Fas-mediated apoptosis induced by P-gp is not due to altered levels of FLIP or FADD in CEM^{GFP}–P-gp^{WT} cells.

Wild-type, but not mutant P-gp inhibits caspase-8 auto-activation and cleavage at the DISC

Since the expression of wild-type P-gp inhibited processing of caspase-8, the most proximal caspase in the Fas-mediated pathway, it was possible that this occurred at the level of DISC formation. To determine the point at which P-gp was inhibiting Fas-mediated apoptosis, DISC formation was examined in CEM^{GFP}–P-gpWT, CEM^{GFP}–P-gp^{MUT}, and CEM^{GFP} cells. Cells were treated for or 0–20 min with APO-1-1 anti-Fas antibody, the DISC was immunoprecipitated and Western blots for caspase-8 and FADD were performed (Figure 5). The p55/54 pro-forms of caspase-8 were associated with the Fas-
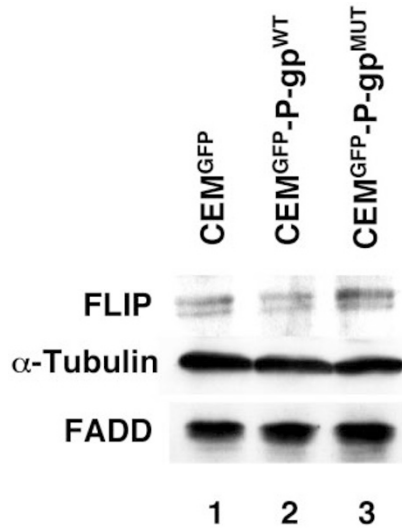
Figure 4 Expression of wild-type or mutant P-gp does not alter expression of FLIP or FADD. Whole cell lysates from CEM cells expressing either wild-type P-gp (CEM^{GFP}–P-gp^{WT}), mutant P-gp (CEM^{GFP}–P-gp^{MUT}), or GFP alone (CEM^{GFP}) were examined for expression of endogenous FADD, FLIP and α-tubulin
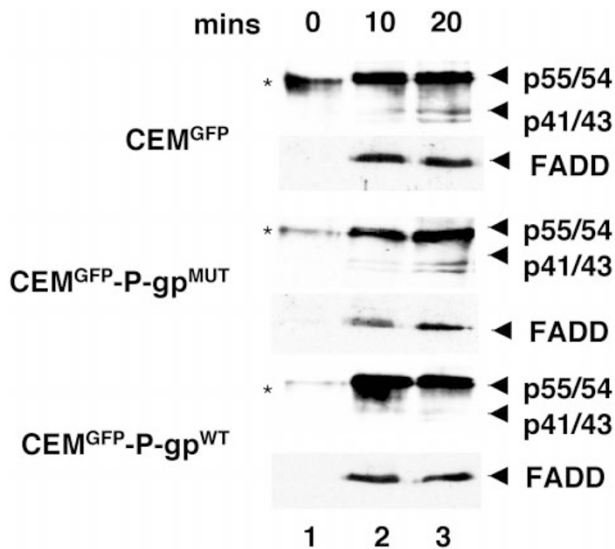

Figure 5 Wild-type, but not mutant P-gp inhibits caspase-8 autoactivation and cleavage at the DISC. CEM cells expressing either wild-type P-gp (CEM^{GFP}–P-gp^{WT}), mutant P-gp (CEM^{GFP}–P-gp^{MUT}), or GFP alone (CEM^{GFP}) were treated for 0–20 min with 1 g/ml APO-1-1 anti-Fas antibody and were immunoprecipitated with protein A sepharose beads. Cells were lysed and IP lysates were then assessed for the presence of caspase-8 and FADD by Western blot analysis using anti-caspase-8 and anti-FADD mAbs. Arrows indicate the p55/54 pro-forms of caspase-8 and processed p43/41 caspase-8 still bound to the Fas receptor. Note the IgG heavy chain denoted by * runs as a band just below the p55 proforms of caspase-8 and can be seen in the 0 min time point

receptor regardless of P-gp expression (Figure 5). However, processing of caspase-8 to p43/41 products was significantly inhibited in CEM^{GFP}-P-gp^{WT} cells. Densitometry analysis revealed that processed caspase-8 in cells treated for

20 min with anti-Fas antibody represented 34 and 46% of total bound protein in CEM^{GFP}–P-gp^{MUT} cells and CEM^{GFP} cells, respectively, compared to just 11% in CEM^{GFP}–P-gp^{WT} cells (Figure 5). Furthermore, equivalent amounts of FADD were associated with the DISC in all cell lines tested, demonstrating that the differences in caspase-8 activation could not be due to deficient FADD recruitment in CEM^{GFP}-P-gp^{WT} cells (Figure 5). Similar results were observed in drug-selected P-gp^{+ve} cells (data not shown). Thus, wild-type but not ATPase-defective P-gp inhibits caspase-8 activation following DISC formation but does not affect recruitment of FADD or pro-caspase-8.

Discussion

The results presented herein demonstrated that wild-type P-gp can inhibit Fas-mediated apoptosis and mutation of specific amino acids necessary for ATPase activity abolished this activity of P-gp. Importantly, these studies revealed that P-gp does not interfere with the formation of the DISC since equivalent amounts of pro-caspase-8 were associated with the Fas-receptor. Rather, we demonstrated that P-gp inhibited the processing and activation of caspase-8. We used a retrovirus system to express P-gp in the complete absence of drug selection; hence our results were not confounded by unknown genetic alterations introduced by drug selection, although data from this study confirmed our earlier studies in drug-selected cells describing the inhibition of Fas-mediated apoptosis by P-gp.[9]

The fact that mutations in the ATP binding regions abolished P-gp-mediated Fas resistance suggests that ATP binding and/or hydrolysis are required for its caspase-inhibitory effects. However, verapamil inhibited efflux function and Fas-resistance (Figure 1B, 2D), yet is known to increase ATPase activity.[1] It is therefore unlikely that P-gp-mediated resistance to Fas is due to depletion or alteration of intracellular ATP levels. The ability of P-gp to inhibit Fas-induced apoptosis may hinge on the specific conformation of P-gp within the plasma membrane. Recent studies have demonstrated that P-gp undergoes changes in conformation during the ATP catalytic cycle, which is initiated upon nucleotide binding.[15] Since mutations within the Walker A repeats abolish ATP binding, mutant P-gp may not be capable of assuming conformational states required to protect against Fas killing. This notion is supported by evidence that mAb UIC2, which reversed P-gp mediated resistance to Fas, traps P-gp in a transient conformation to inhibit drug efflux function.[16]

To date, viral and cellular FLIPs, cow pox virus CrmA, adenovirus protein E3-14.7 kDa, activation of Protein Kinase C (PKC) and depletion of glutathione have been implicated in resistance to the Fas-mediated cell death pathway by inhibiting activation of caspase-8 through various mechanisms.[13,17–21] Although the Fas-death receptor pathway is perhaps the best-characterized cell death pathway, all of the components of the Fas-DISC may not be entirely known. Indeed, recent reports have identified other unknown proteins that can bind to and possibly regulate caspase-8.[22] Therefore, although it presently remains unclear how P-gp is inhibiting caspase-8 activa-

tion, it may be regulating an as yet uncharacterized component of the DISC. Clearly the manner by which P-gp regulates cell death and caspase-activation requires further investigation, however, at least two possibilities can be considered given the most recent developments in the model of Fas-induced cell death.

A potential mechanism by which P-gp could regulate Fas-mediated cell death is suggested by recent work implicating both Fas and P-gp in association with components of the actin cytoskeleton.[23,24] Luciani et al. recently demonstrated that P-gp associates with actin through ezrin, radixin and moesin (ERM) proteins in CEM cells.[24] This interaction correlated with uropod formation and multi-drug resistance, however, the effects of pharmacological and antibody inhibitors on P-gp-ERM association and uropod formation were not investigated. A separate study demonstrated that Fas associated with ezrin in uropods of CEM cells and activated CD4+ T-cells.[23] Disassociation of Fas and ezrin caused resistance to Fas-mediated apoptosis, although the precise events in the signaling pathway that were inhibited (ie, DISC formation, caspase-8 activation) were not explored. Hence, an interaction between P-gp and ezrin may affect a necessary interaction between Fas and ezrin, thereby inducing resistance to Fas-induced apoptosis.

Several groups have shown that Fas forms clusters or caps upon receptor triggering in a ceramide-dependent fashion,[25] and these clusters are internalized into lipid rafts.[26] These findings are supported by recent observations by Hueber et al. that Fas is constitutively partitioned into sphingolipid and cholesterol rich membrane rafts.[27] P-gp can regulate both cholesterol trafficking[28] and the transport of sphingolipids.[29,30] Therefore, it remains possible that P-gp may inhibit Fas-mediated apoptosis by disrupting formation of sphingolipid and cholesterol rich membrane rafts, inhibiting signaling events downstream of receptor aggregation and DISC formation.

The physiological relevance of P-gp mediated regulation of Fas-induced cell death remains to be resolved. However, that functional P-gp inhibits Fas-mediated apoptosis in T-cells suggests P-gp may play a physiological role in immune cell functions by controlling cell death and survival. In support of this hypothesis, Gollapudi and co-workers recently reported that treatment with anti-P-gp antibodies induced apoptosis in activated primary human blood mononuclear cells (MNCs).[31] Cell death was dependent on activation of the Fas receptor pathway, as Fas-Fc, an inhibitor of Fas receptor-Fas ligand interactions, and caspase inhibitors inhibited MNC apoptosis.

In addition to inhibiting cell death, a role for P-gp during cellular differentiation has also been proposed since P-gp expression is down-regulated during differentiation of pluripotent stem cells along the myeloid lineage[32] and overexpression of P-gp in murine stem cells induces a myeloproliferative syndrome.[33] Interestingly, Fas and TNFR have been demonstrated to negatively regulate self-renewal of multipotent long-term repopulating hematopoietic stem cells.[34] Taken together, these findings suggest that P-gp may influence self-renewal decisions in repopulating stem cells by preventing Fas-induced apoptosis.

We and others have demonstrated that P-gp can inhibit Fas-induced apoptosis and caspase-3 activation.[9–11,35] The studies presented herein are the first to demonstrate that P-gp can inhibit the activation of caspase-8 while not affecting DISC formation and provide an explanation as to why expression of P-gp is associated with loss of Fas-induced caspase-3 activation and apoptosis. Furthermore, we illustrate for the first time that the ATP binding sites within P-gp are necessary to confer resistance to Fas-induced caspase activation and cell death. These findings provide novel molecular detail as to how P-gp can inhibit death-receptor mediated apoptosis.

Materials and Methods

Cell culture and retroviral gene transduction

All cells were grown in RPMI medium 1640 supplemented with 10% (vol/vol) fetal calf serum, 2 mM glutamine, 100 units/ml of penicillin, and 100 mg/ml of streptomycin (GIBCO, Grand Island, NY). Wild-type and mutant MDR1 coding regions for P-gp were cloned into the retrovirus vector plasmid MSCV, a bicistronic vector expressing green fluorescent protein (GFP). The 4.2 kB EcoR1–EcoR1 fragment from huMDR1 clone huEWT (kindly provided by Paul Roepe, Memorial Sloan Kettering Cancer Center, New York) was cloned into the EcoR1 sites of the MSCV vector (provided by Steve Jane, Bone Marrow Institute, Royal Melbourne Hospital, Melbourne, Victoria). The mutant P-gp used in the following studies contained double lysine to alanine substitutions at amino acids 233 (K433M) and 1076 (K1076M) within the Walker A motifs, which disrupted nucleotide binding, and ATPase and drug-efflux activity.[4,16] Mutant constructs containing double lysine to alanine substitutions at 433(K433M) and 1076(K1076M) amino acids were generated by excising 4.1 kB Eag1–Eag1 fragment from clone pUCFVXMΔH3–Sal(ΔAUG)-MDR/Neo (provided by Igor Roninson, Chicago University, Chicago, IL) and cloned into MSCV vector. To generate high titre virus supernatant, plasmids were co-transfected with an amphotropic 'helper' packaging plasmid pEQ-Pam3(−E) into 293T cells by calcium phosphate precipitation. After 48 h transfected cells were analyzed by FACs for GFP expression. Supernatants from cells expressing high levels of GFP were stored in aliquots at −70°C. CCRF-CEM (CEM) were cultured with viral supernatant and 4 μg/ml polybrene for 3 days, changing media and adding new supernatant and polybrene every 8 h. Transduced cells were sorted using a Becton Dickinson FACs for GFP and P-gp expression using monoclonal antibody (mAb) MRK-16 against P-gp. The highest 10% of cells expressing GFP and P-gp were then single cell cloned (plated at less than one cell per well in 96 well plate and expanded). The expression of cell surface P-gp and Fas was assessed by flow cytometry using the MRK 16 (Kamiya) anti-P-gp and CH-11 (Upstate Biotechnology) anti-Fas monoclonal antibodies respectively.

Cell death assays

Three million cells were labeled with ^{51}Cr as previously described.[9] Cells (2×10^5 ml) were either treated with the following anti-Fas mAbs: clone CH-11 (10 ng/ml); or clone APO-1-1 (0–1000 ng/ml) (Kamiya) cross-linked with Protein A (1/10 000 of mAb concentration) (Boeringher Mannheim). In some cases, cells were pre-treated with P-gp inhibitors, verapamil (10 μM) or mAb UIC-2 (5 μg/ml). Supernatants were harvested after 2–20 h and measured for ^{51}Cr

radioactivity. % specific lysis=[(experimental release – spontaneous release)/(maximum release – spontaneous release)] × 100. Data are calculated as the mean ± S.E. of triplicate samples and are representative of at least three separate experiments.

Western blotting

Cells (5×10^5), either treated with APO-1-1 or CH-11 anti-Fas antibodies as above or untreated, were washed twice in PBS, and lysed on ice for 30 min in NP40 lysis buffer (0.5% NP-40, 5 mM $MgCl_2$, 25 mM KCl, 10 mM TrisHCl pH 8.0) containing Complete-mini protease inhibitor cocktail tablets (Roche Molecular Biochemistry, Germany). Cell debris was removed by centrifugation at 13 000 r.p.m. for 1 min, and the supernatant retained. Loading of equal amounts of protein was ensured by estimating the protein concentration by Bradford assay. Proteins were separated by SDS – PAGE (10 – 15% gels), and transferred onto Immobilon-P nitrocellulose transfer membrane (Millipore Corporation, Bedford, MA, USA), in a Trans-Blot SD semi-dry transfer cell (BioRad, USA). Blots were probed with the following primary antibodies: anti-human caspase-3 mAb (Transduction Laboratories, Lexington, KY, USA); anti-human FLICE mAb (gift from Marcus Peter, University of Heidelberg, Germany); anti-human FADD mAb (Transduction Laboratories); anti-human FLIP rabbit polyclonal (Upstate Biotechnology, Lake Placid, NY, USA); anti-human PARP polyclonal antibody (Boehringer Manneheim); anti-human -tubulin mAb (Sigma, St. Louis, MS, USA). HRP-Rabbit anti-Mouse or Swine anti-Rabbit (Dako, Glostrup, Denmark) secondary antibodies were added and bound proteins were and visualized by Enhanced Chemiluminescence (ECL) Western Blotting Detection reagents (Amersham Pharmacia Biotech, Buckinghamshire, UK). Per cent cleavage of caspase-3 and caspase-8 were determined by densitometry using BioRad Quantity One Software.

DEVDase assays

Cells (1×10^6) were treated for 4 h in the presence of 50 ng/ml APO-1-1 anti-Fas antibody and protein A. Cells were washed twice in ice-cold PBS and lysed in Nonidet P-40 lysis buffer without protease inhibitors and DEVDase assays were performed as previously described.[35]

Immunoprecipitation of the death inducing signal complex (DISC)

The amount of DISC-associated caspase-8 and FADD was determined as follows: P-gp^{+ve} and P-gp^{-ve} CEM cells were treated with 1 μg/ml anti-APO-1-1 antibody and Protein A (0.2 ng/ml) or Protein A alone for 0 – 20 mins at 37°C. Cells were washed twice with ice-cold PBS and lysed in lysis buffer [30 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride and small peptide inhibitors, 1% Triton X-100 and 10% glycerol]. Untreated controls were then supplemented with anti-APO-1-1. The Fas-DISC was then precipitated for 4 h at 4°C with protein A-Sepharose (Pharmacia, Freiburg, Germany). After precipitation, beads were washed five times with 20 volumes of lysis buffer. Western blots for caspase-8 and FADD were performed as described above.

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