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Multidrug resistance-associated protein 1 as a major mediator of basal and apoptotic glutathione release

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ABSTRACT

The proteins responsible for reduced glutathione (GSH) export under both basal conditions and in cells undergoing apoptosis have not yet been identified, although recent studies implicate some members of the multidrug resistance-associated protein family (MRP/ABCC) in this process. To examine the role of MRP1 in GSH release, the present study measured basal and apoptotic GSH efflux in HEK293 cells stably transfected with human *MRP1*. *MRP1*-overexpressing cells had lower intracellular GSH levels and higher levels of GSH release, under both basal conditions and after apoptosis was induced with either Fas antibody or staurosporine. Despite the enhanced GSH efflux in *MRP1*-overexpressing cells, intracellular GSH levels were not further depleted when cells were treated with Fas antibody or staurosporine, suggesting an increase in GSH synthesis. *MRP1*-overexpressing cells were also less susceptible to apoptosis, suggesting that the stable intracellular GSH levels may have protected cells from death. Overall, these results demonstrate that basal and apoptotic GSH release are markedly enhanced in cells overexpressing MRP1, suggesting that MRP1 plays a key role in these processes. The enhanced GSH release, with a concurrent decrease of intracellular GSH, appears to be necessary for the progression of apoptosis.

1. Introduction

Glutathione (GSH) is involved in a number of biochemical processes and its levels in various cellular compartments are tightly controlled. The two primary mechanisms by which cells regulate intracellular GSH levels are by altering the rate of its biosynthesis and the rate of GSH export from cells. Interestingly, cells undergoing apoptosis release large quantities of GSH into the extracellular space by a transport-mediated process [1–6]. The significance of GSH release to the apoptotic process, and the transport mechanisms remain poorly defined. Because of the protective role of GSH, it has been hypothesized that GSH depletion increases reactive oxygen species which can act as second messengers to activate apoptotic pathways, or it may lead to a change in the redox state of proteins whose catalytic activity may be required for apoptosis to proceed [7,8].

The proteins responsible for GSH export remain largely unknown, although there is growing evidence that some of the multidrug resistance-associated proteins (MRP/ABCC) are involved in this process [4,9–19]. Among the MRPs, MRP1 is the best characterized [4,9,10]. MRP1 is expressed in all mammalian cells that have been examined, making it a likely candidate for the major GSH transporter, and several studies have shown that MRP1-overexpression is associated with lower levels of intracellular GSH and higher levels of extracellular GSH [11–15]. In addition, *Mrp1*−/− mice have higher GSH levels in tissues that normally express this protein, whereas GSH levels are unchanged in tissues that do not normally express Mrp1 [16,17]. The MRP proteins are also thought to be involved in apoptotic GSH release, but this is also not well defined. Other studies have associated increased cytotoxicity in cells overexpressing MRP1 to loss of intracellular GSH [18,19].

In addition to the MRPs, the rat organic anion transporting polypeptide 1 and 2 (Oatp1/Slc21a1 and Oatp2/Slc21a5) have also been shown to accept GSH as a substrate [20,21], and human OATP8 has been implicated in GSH transport [22–24]; however, recent studies failed to confirm the latter findings [4,25]. Based largely on the use of pharmacological inhibitors and indirect GSH detection methods, Franco and colleagues [23,24] concluded that cellular GSH export in Jurkat T cells is mediated by the OATP proteins. In contrast, Hammond et al. [4] used RNAi in the same cell model and a comparison with a different lymphocyte cell line to demonstrate that the MRP proteins rather than the OATP proteins are responsible for GSH export. The latter study demonstrated that decreasing *MRP1* expression in Jurkat cells leads to a decrease in both basal and apoptotic GSH release, suggesting that MRP1 is a major player in both these processes [4].

To further characterize the role of MRP1 in basal and apoptotic GSH export, the present study assessed GSH export in HEK293 cells stably overexpressing human *MRP1*. The results suggest that MRP1 is a major contributor to GSH export in this cell model.

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2. Materials and methods

2.1. Materials

Fas antibody clone CH-11 was purchased from MBL International Corporation (Woburn, MA). Fluorescent caspase 3 substrate Ac-DEVD-AMC was from Calbiochem (San Diego, CA), Annexin V-APC was from BD Pharmingen (San Jose, CA), calcein-AM was from Molecular Probes-Invitrogen (Carlsbad, CA) and [^{3}H]LTC$_4$ from PerkinElmer (Waltham, MA). All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Cell culture and transfection

HEK293 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Mediatech, Herndon, VA) and 5 mg/mL penicillin/streptomycin (Gibco, Grand Island, NY), and incubated at 37 °C in a 5% CO_2 atmosphere. Human *MRP1* cloned into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA) was kindly provided by Dr. Susan Cole, Queen's University, Ontario, Canada. HEK293 cells were transfected with *MRP1* using Fugene 6 transfection reagent (Roche, Notley, NJ). Stable cell lines were created by culturing transfectants in 800 μg/ml G418 (Invitrogen, Carlsbad, CA) for 3 weeks. Expression of MRP1 in the G418 resistant clones was determined by real time quantitative RT-PCR, and by immunoblot and functional analyses. For all experiments, 0.5×10^6 cells were plated per well of a 6-well plate. When confluent, cells were washed once with KH buffer (Krebs–Henseleit Buffer: 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 0.6 mM $MgSO_4$, 1.25 mM $CaCl_2$ and 10 mM Hepes/Tris; pH 7.5) and all experiments were performed in KH buffer containing 0.5 mM acivicin, an inhibitor of γ-glutamyl transpeptidase activity.

2.3. mRNA expression

Total RNA was isolated using the RNeasy Mini Kit and the RNase-Free DNase set (Qiagen, Valencia, CA). Oligonucleotide primers were designed using Primer Express 1.5 (Applied Biosystems, Foster City, CA) as previously described [4]. PCR analysis was performed using iScript One-Step RT-PCR kit with SYBR Green (Bio-Rad, Hercules, CA) and the Roto-Gene 3000 real time light cycler (Corbett Research, Phenix Corporation, Hayward, CA). Total RNA (10–100 ng) was used, and the data are presented relative to human β-actin.

2.4. MRP1 protein expression

Whole cell lysates were collected as previously described [26]. Briefly, cells were washed once in PBS and collected in 1X phosphate buffered saline solution (Invitrogen, Carlsbad, CA) containing 10 mM EDTA, 1 mM phenylmethanesulfonylfluoride (PMSF) and 1:100 dilution of mammalian protease inhibitor cocktail (Sigma, St. Louis, MO). After centrifugation at 10,000 ×*g* at 4 °C for 10 min, pellets were frozen overnight at −80 °C. Pellets were thawed on ice and lysed in 1× PBS solution containing 10 mM EDTA, 25 mM Tris–HCl, pH 7.4, 300 mM NaCl, 1 mM $CaCl_2$, 1% Triton X-100, 1 mM PMSF, and 1:100 dilution of mammalian protease inhibitor cocktail by passing through a 25-gauge needle. The lysate was centrifuged at 10,000 ×*g* for 5 min. The concentration of the supernatant (whole cell lysate) was determined using Bio Rad's DC protein assay. Protein (10 μg) was run on a 4–20% Tris–HCl Ready Gel (Bio-Rad, Hercules, CA) using a tank blotting system from Bio-Rad and detected using chemiluminescence (Perkin Elmer, Boston, MA). MRP1 primary antibody, MRPr1 (Axxora, San Diego, CA), was diluted in Tris-buffered saline Tween-20 (TBST) containing 1% low fat powered milk and 1% bovine serum albumin at a 1:200 dilution. The secondary antibody was horseradish peroxidase-conjugated goat anti-rat IgG (1:15000).

2.5. GSH release

Cells were preincubated for 20 min at 37 °C in KH buffer containing 0.5 mM acivicin to inhibit γ-glutamyl transpeptidase activity. The culture medium was collected to analyze for extracellular GSH at different times of incubation. Cells were lysed with 5% perchloric acid containing 1 mM EDTA, centrifuged at 18,000 ×*g* for 5 min and the resulting supernatant was analyzed for intracellular GSH using an enzymatic assay containing 5,5′-dithio-bis(2-nitrobenzoic acid) and glutathione reductase [27]. The pellet was dissolved in 1 M NaOH and used for protein analysis using the Lowry protein assay [28]. Results are expressed either as nmol GSH/mg protein or as the ratio of extracellular to intracellular GSH.

2.6. Calcein release activity

Cells were incubated with 1 μM calcein-AM at 4 °C for 1 h, washed with KH buffer, and resuspended in KH buffer plus 0.5 mM acivicin. Calcein in the extracellular buffer was analyzed by removing 200 μl of the medium at different times of incubation, and this was placed in a 96-well plate. For intracellular calcein, cells were lysed with KH buffer plus 2% Triton X-100, centrifuged for 5 min at 18,000 ×*g* and the supernatant added to the 96 well plate. Samples were analyzed on a SPECRTAmax Gemini XS spectrofluorometer (Molecular Devices Corporation, Sunnyvale, CA) at 37 °C, excitation 485; emission 530. Cells without calcein were measured to detect background fluorescence. Protein was analyzed using the DC protein assay (Bio-Rad, Hercules, CA). The calcein release data were expressed as average fluorescence/mg protein and then converted to percent of calcein released (supernatant) from total calcein made by cells (supernatant + cell lysate).

2.7. Membrane vesicle preparation

Plasma membrane vesicles were prepared from transfected HEK293 cells using sucrose gradients as previously described [29,30]. Briefly, cells were collected into a transport buffer solution containing 250 mM sucrose, 10 mM Hepes/Tris pH 7.5, 20 mM KCl, 0.20 mM $CaCl_2$, homogenized 20–25 times with a B pestle of a Dounce homogenizer on ice, and centrifuged at 800 ×*g*. The supernatant was layered onto sucrose gradients made up of 32% and 16% sucrose solutions, and centrifuged at 100,000 ×*g*. The discrete band formed at the 16–32% interface was collected and centrifuged further at 100,000 ×*g*. The resulting pellet was resuspended in the transport buffer described above and quantified using the DC protein assay (Bio-Rad, Hercules, CA).

2.8. Vesicle transport assay

ATP dependent transport of [^{3}H]LTC$_4$ was measured by rapid filtration through nitrocellulose filters (Millipore, Billerica, MA). Vesicles (8 μg) were incubated with 5 mM ATP or similar concentration of NaCl with 10 mM $MgCl_2$, 100 μg/ml creatine phosphokinase, 10 mM phosphocreatine, and 50 nM [^{3}H]LTC$_4$ (20 nCi) in transport buffer at 37 °C. Final incubation volume was 100 μl. After incubation, the reaction was stopped with 900 μl of an ice-cold buffer containing 300 mM sucrose, 10 mM Hepes/Tris pH 7.5, and 20 mM KCl, filtered through the nitrocellulose filters, and further washed with 4 ml of this wash buffer. After addition of 4 ml of OptiFluor (Perkin Elmer, Boston, MA), radioactivity associated with the filters was determined by liquid scintillation counting using a Beckman Coulter scintillation counter LS6500 (Fullerton, CA).

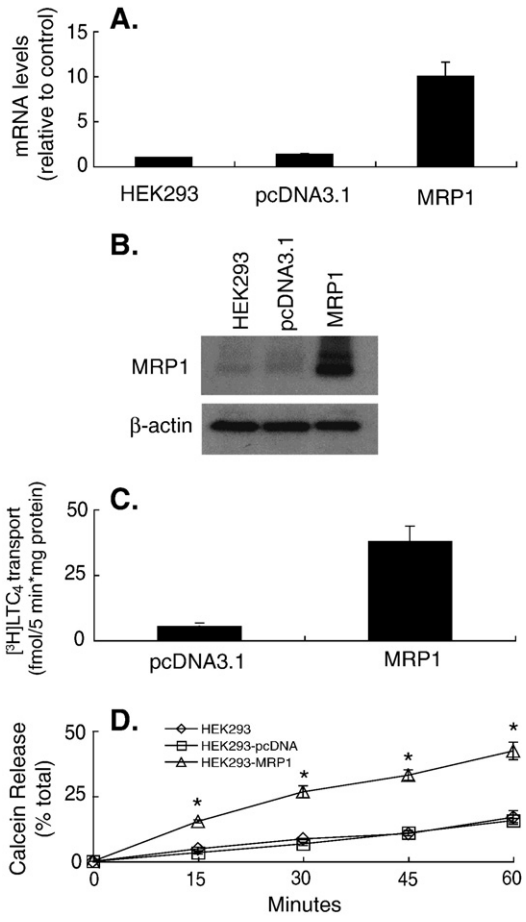

Fig. 1. *MRP1* mRNA and protein expression, and functional activity in transfected HEK293 cells. (A) Real time RT-PCR was performed on HEK293 cells transfected with human *MRP1* together with vector-transfected (pcDNA3.1) or parental cells (HEK293). *MRP1* levels were normalized to human β-actin and data are expressed as fold change relative to control (HEK293 cells). Values are means ± SE, $n=6$. (B) Western blot for human MRP1 protein expression (10 μg of protein was loaded for each sample). Blotting for human β-actin was done to ensure consistent protein loading. Blot is representative example of three experiments. (C) Membrane vesicles (8 μg protein) isolated from vector-transfected or *MRP1*-overexpressing HEK293 cells were incubated with 50 nM [^{3}H]LTC_4 with or without 5 mM ATP for 5 min. The ATP dependent uptake of [^{3}H]LTC_4 was determined. Data represent means ± SE, $n=3$. *Significantly different from vector-transfected cells, $P<0.05$. (D) Parental, vector-transfected, and cells transfected with *MRP1* were incubated for 1 h with 1 μM calcein-AM at 4 °C and percent of total calcein release was determined over 60 min. Values are means + SE, $n=3$. *Significantly different from control cells, $P<0.05$.

2.9. Plasma membrane integrity

Plasma membrane integrity was assessed by two methods: lactate dehydrogenase (LDH) release [31], and by propidium iodide exclusion as measured by flow cytometry. LDH release was expressed as a percentage of total LDH activity (lysed untreated cells), which was measured in cells lysed with 0.5% Triton X-100.

2.10. Phosphatidylserine externalization

At specified timepoints after treatment, cells were stained with Annexin V-APC and propidium iodide in KH buffer containing 2.5 mM $CaCl_2$. Cells were analyzed for propidium iodide exclusion and increases in Annexin V-APC staining using a Becton Dickinson FACSCalibur flow cytometer (Franklin Lakes, NJ) at the University of Rochester Flow Cytometry Core. Data were analyzed using Cell Quest software, gating out propidium iodide positive cells and including Annexin V positive cells for phosphatidylserine externalization.

2.11. Caspase 3-like activity

The caspase 3-like activity assay is based on one provided by BD PharMingen. Briefly, cells were placed in cell lysis buffer (10 mM Tris–HCl, 10 mM $NaH_2PO_4/NaHPO_4$ pH 7.5, 130 mM NaCl, 1% Triton X-100, and 10 mM sodium pyrophosphate) and frozen at −80°C until activity assay. Cell lysates were thawed and centrifuged at 18,000 ×*g* for 15 min at 4°C. The supernatants were then combined with 1× HEPES buffer (20 mM HEPES pH 7.5, 10% glycerol, and 2 mM DTT) and 30 μM Ac-DEVD-AMC. Caspase 3-like activity was measured as a change in fluorescence over 20 min using a SPECRTAmax Gemini XS spectrofluorometer (Molecular Devices Corporation, Sunnyvale, CA) at 37 °C.

2.12. DNA fragmentation

Cells were analyzed for a subG1 population which corresponds to cells with fragmented DNA using flow cytometry. At appropriate times, treated cells were trypsinized and centrifuged at 200 ×*g* for 7 min. The supernatant was removed and cells fixed in 70% ethanol and stored at 4 °C, until staining. For staining, cells were centrifuged at 200 ×*g* for 7 min, the supernatant removed and the pellet resuspended in 1 mg/ml RNase A in 1 X PBS. Cells were vortexed and incubated for 30 min at room temperature. After the incubation period, cells were centrifuged again at 200 ×*g* for 7 min and resuspended in 20 μg/ml PI solution. Flow cytometry was performed on a Becton Dickinson FACSCalibur flow cytometer (Franklin Lakes, NJ) at the University of Rochester Flow Cytometry Core.

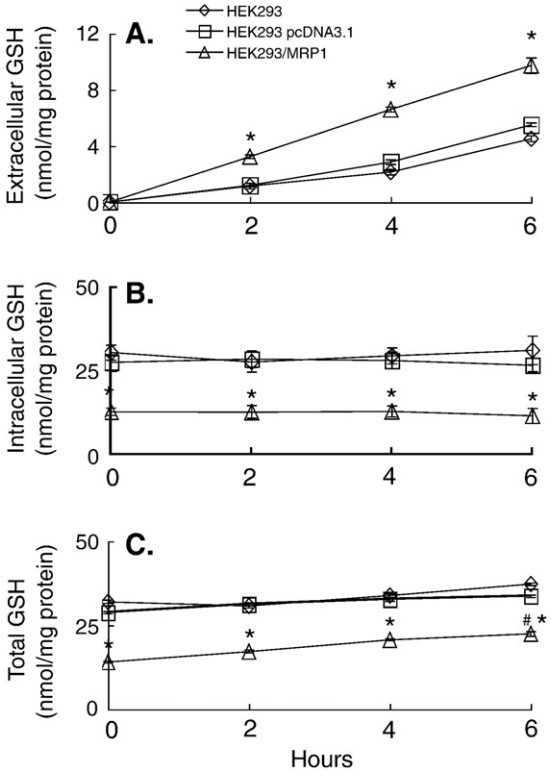

Fig. 2. HEK293 cells overexpressing MRP1 release significantly greater quantities of GSH when compared to parental or vector-transfected cells. Extracellular (A), intracellular (B), and total (i.e., extracellular plus intracellular) (C) GSH levels were measured in parental, vector-transfected or *MRP1*-overexpressing cells over 6 h. Values are means ± SE, $n=3$–6. *Significantly different from control cells, $P<0.05$. #Significantly different from time 0, $P<0.05$.

2.13. Statistical analysis

Statistical analysis was performed using Statview 5 (SAS Institute Inc., Cary, NC). Data were analyzed using one-way ANOVA and Fisher's PLSD posthoc analyses. In all cases p values of less than 0.05 were considered statistically significant.

3. Results

3.1. HEK293 cells overexpressing MRP1 demonstrate increased transport of LTC_4 and calcein

HEK293 cells and the empty vector transfected cells contained low levels of *MRP1* mRNA and protein (Fig. 1). Several *MRP1*-overexpressing clones were obtained and tested, including one in which *MRP1* expression was approximately 10-fold higher than in control cells (Fig. 1A). Protein expression, as determined by western blotting, corroborated the RNA data (Fig. 1B).

To test whether the expressed MRP1 protein was functional, transport activity of LTC_4 and calcein were determined. LTC_4 is a glutathione *S*-conjugate and a substrate for MRP1 [32–34], and previous studies have shown that calcein is a substrate for the MRP transporters [35,36]. Membrane vesicles from the *MRP1*-overexpressing cells exhibited increased [^{3}H]LTC_4 transport activity (Fig. 1C) indicating the presence of functional transporters. Of significance, calcein release in the *MRP1*-overexpressing cells was enhanced approximately 2 to 3 fold, indicating the presence of functional transporters at the plasma membrane (Fig. 1D). Total calcein (intracellular + extracellular) was constant throughout the experiment and was similar among the different transfectants (data not shown), indicating that the changes in calcein export observed were not due to insufficient loading of calcein-AM.

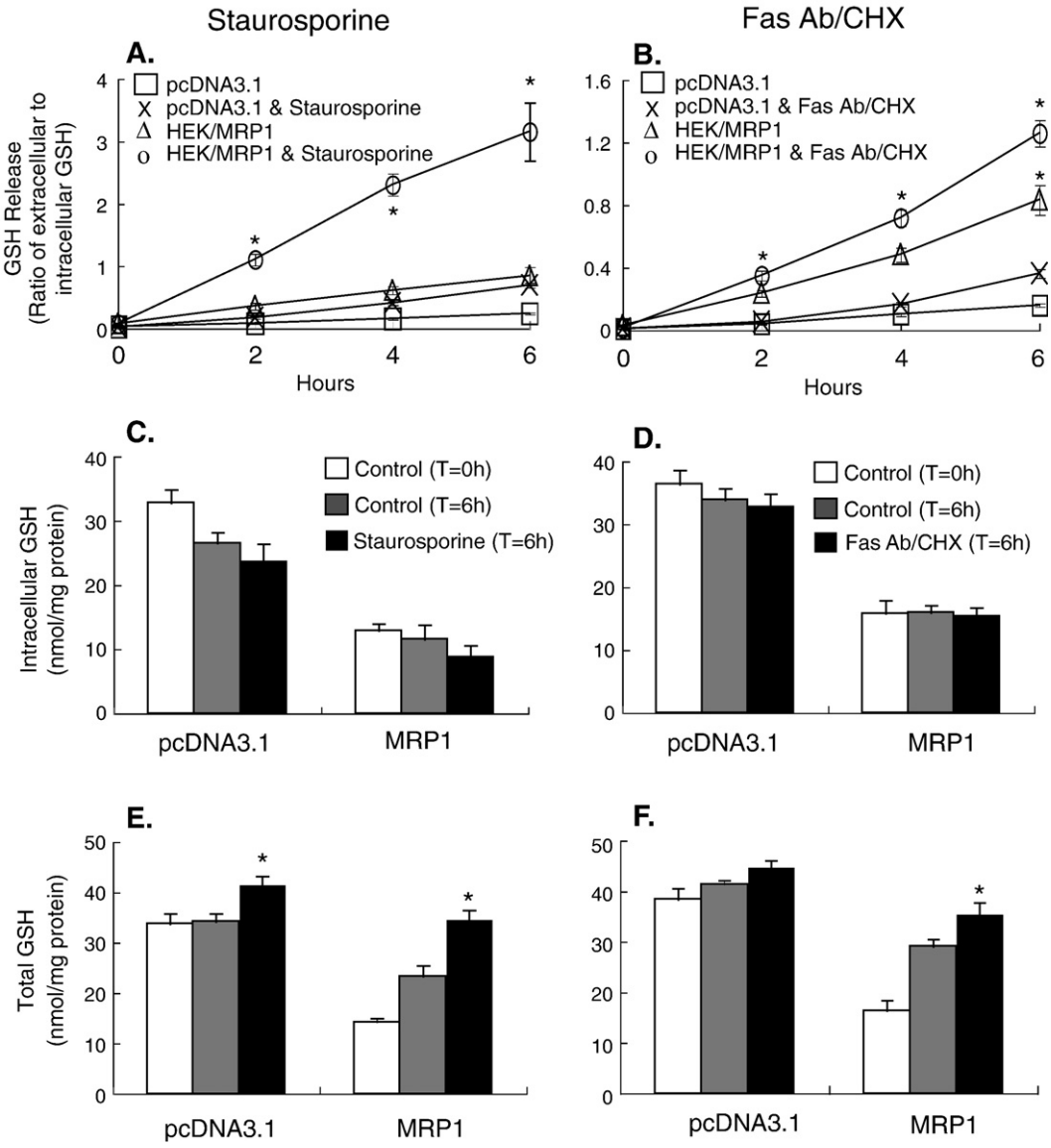

Fig. 3. Staurosporine or Fas Antibody/CHX significantly enhances GSH release from *MRP1*-overexpressing HEK293 cells. GSH release was measured in pcDNA3.1-, and *MRP1*-transfected cells (A, B). Cells were untreated (□, Δ) or treated with 10 μM staurosporine (×, ○) (A, C, E) or with 0.5 μg/ml Fas Antibody and 50 μg/ml CHX (×, ○) (B, D, F) for up to 6 h. Intracellular GSH (C, D) and total GSH (E, F) at time 0 (white bars) or at 6 h (grey or black bars), from either untreated cells (grey bars) or cells treated with 10 μM staurosporine (C, E) or 0.5 μg/ml Fas Antibody and 50 μg/ml CHX (D,F) (black bars). Values are means ± SE, n = 3–6. *Significantly different from control cells at 6 h, $P < 0.05$.

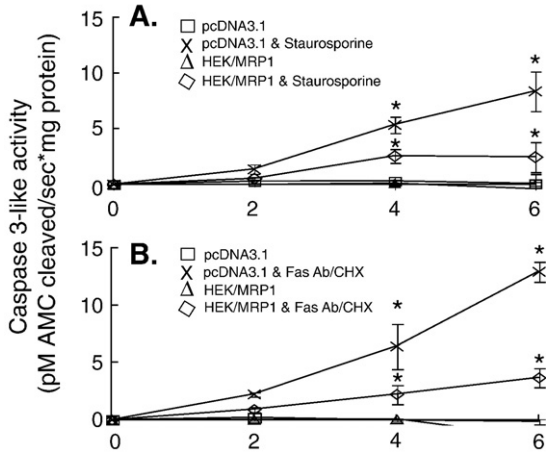

Fig. 4. Activation of caspase 3-like proteases in *MRP1*-overexpressing cells treated with staurosporine or Fas Antibody/CHX is significantly lower than in vector-transfected HEK293 cells. pcDNA3.1- and *MRP1*-transfected HEK293 cells were untreated (□, Δ) or treated with 10 μM staurosporine (×, ◊) (A), or treated with 0.5 μg/ml Fas Antibody and 50 μg/ml CHX (×, ◊) (B), for up to 6 h. Values are means ± SE, $n=3–5$. *Significantly different from control cells, $P<0.05$.

3.2. MRP1-overexpressing HEK293 cells have lower intracellular GSH levels and higher basal GSH export rates

The parental and empty vector-transfected cells released GSH relatively slowly over time, consistent with the low basal MRP1 expression in these cells, whereas *MRP1*-overexpressing cells released GSH at twice the rate of control cells (Fig. 2A). LDH release was low for all cells suggesting that GSH release was not due to a compromised plasma membrane (data not shown). HEK293-MRP1 cells also had less than half of the intracellular GSH found in the control cells (Fig. 2B). Despite the high rate of GSH efflux, the intracellular levels in the *MRP1*-overexpressing cells remained constant over the 6 h (Fig. 2B), suggesting that the GSH synthesis rate may be increased. Indeed, total GSH (i.e. extracellular plus intracellular) significantly increased over time in the HEK293-MRP1 cells, but remained relatively constant in the other cells (Fig. 2C).

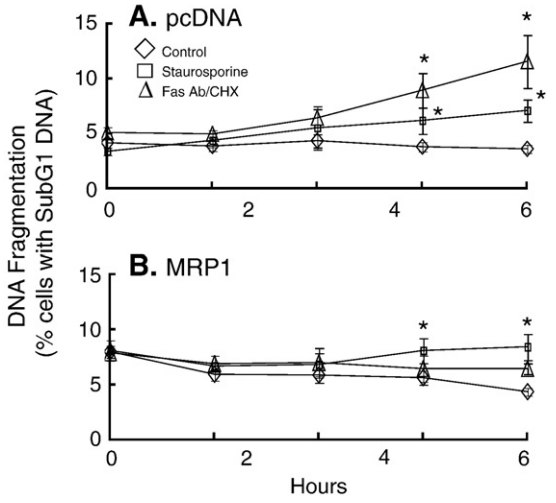

Fig. 5. Staurosporine and Fas antibody/CHX significantly increase the percentage of cells with subG1 DNA content in vector-transfected cells, and to a lesser extent in *MRP1*-transfected cells. pcDNA3.1 (A) and pcDNA3.1/MRP1-(B) transfected cells were untreated (◊) or treated with 10 μM staurosporine (□) or with 0.5 μg/ml Fas Antibody and 50 μg/ml CHX (Δ) for up to 12 h. Values are means ± SE, $n=4$. *Significantly different from untreated cells, $P<0.05$.

3.3. Staurosporine and Fas antibody/cycloheximide (CHX) enhance GSH release, and this is especially prominent in the MRP1-transfected cells

When treated with staurosporine or Fas antibody/cycloheximide (CHX), GSH release was increased in both the control and in the *MRP1*-overexpressing cells (Fig. 3A and B, respectively). At these doses of staurosporine and Fas antibody/CHX intracellular GSH levels were unaffected, although there was a downward trend (Fig. 3C and D). However, both apoptotic stimuli increased the total amount of GSH (i.e., intracellular plus extracellular GSH) in both vector- and *MRP1*-transfected cell lines, with the most striking increase observed in HEK293-MRP1 cells (Fig. 3E and F). As previously noted in Fig. 2, intracellular GSH levels in the HEK293-MRP1 cells were less than half of those of the vector-transfected cells without apoptotic stimuli (Fig. 3C and D). At 6 h after the apoptotic stimulus, total GSH (i.e. intracellular plus extracellular) in the *MRP1*-overexpressing cells was significantly higher, and both staurosporine and Fas antibody/CHX increased total GSH to such an extent that the levels were almost comparable to the GSH concentrations in the vector-transfected cells (Fig. 3E and F).

3.4. When treated with staurosporine or Fas antibody/CHX, caspase 3-like activity, DNA fragmentation, and phosphatidylserine externalization are elevated in vector-transfected cells, but to a lesser extent in the MRP1-overexpressing cells

To determine whether the enhanced GSH release observed in the HEK293-MRP1 cells was associated with altered susceptibility to apoptosis, different apoptotic endpoints were measured. Staurosporine and Fas antibody/CHX increased caspase 3-like activity in vector- and *MRP1*-transfected cells, but the level of caspase 3 activation in the HEK293-MRP1 cells was approximately half of that observed in the vector-transfected cells (Fig. 4). Another MRP1 clone with comparable levels of MRP1 mRNA and protein was also examined for GSH levels and caspase 3 activation giving similar results (data not shown). The reason for the decreased caspase activation despite the enhanced GSH release and the lower intracellular GSH levels in the HEK293-MRP1 cells is unclear, but may be due to an enhanced rate of GSH synthesis

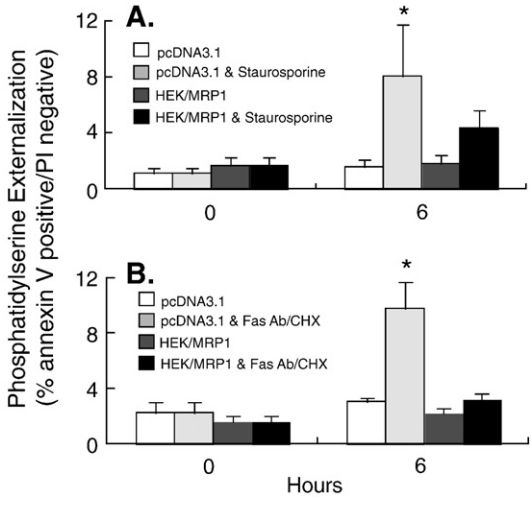

Fig. 6. Staurosporine-induced or Fas antibody/CHX-mediated increase in phosphatidylserine externalization is lower in *MRP1*-overexpressing cells. pcDNA3.1 transfected cells were untreated (white bars) or treated with apoptotic inducing agent (light grey bars). HEK293-MRP1 cells were untreated (dark grey bars) or treated with apoptosis inducing agent (black bars). Cells were analyzed for the percentage that were Annexin V positive and propidium iodide negative when treated with 10 μM staurosporine (A) or with 0.5 μg/ml Fas Antibody and 50 μg/ml CHX (B) for up to 6 h. Values are means ± SE, $n=3–5$. *Significantly different from control cells, $P<0.05$.

observed in the HEK293-MRP1 cells, as suggested by the increase in total GSH (Figs. 2 and 3).

DNA fragmentation, a late marker of apoptosis, was determined by measuring the percentage of cells with subG1 DNA content. In agreement with the caspase 3 activity data, the percentage of cells with subG1 DNA content increased in the vector-transfected cells treated with staurosporine or Fas antibody/CHX (Fig. 5A), but a smaller effect was noted in the *MRP1*-overexpressing cells (Fig. 5B). The percentage of cells with subG1 DNA at time zero was higher in the *MRP1*-overexpressing cells (Fig. 5B), but the significance of this is unknown. Because of this high percentage of *MRP1*-transfected cells with subG1 DNA at time zero, the difference in subG1 content between untreated and treated cells is less in the HEK293-MRP1 cells when compared to the vector-transfected cells (Fig. 5).

To determine the effect of MRP1 expression on phosphatidylserine externalization during apoptosis, HEK293-MRP1 and vector-transfected HEK293 cells were treated with staurosporine or Fas antibody/CHX, and phosphatidylserine on the extracellular membrane was assessed by labeling with fluorescently tagged Annexin V. Cells were also stained with propidium iodide to determine plasma membrane integrity. Staurosporine and Fas antibody/CHX significantly increased phosphatidylserine externalization in vector-transfected cells, but no significant increase in phosphatidylserine externalization was observed in the *MRP1*-overexpressing cells (Fig. 6). These data corroborate the results obtained from the caspase 3 activity and DNA fragmentation assays, and collectively suggest that the *MRP1*-overexpressing cells exhibit decreased sensitivity to apoptosis.

3.5. Vincristine stimulates GSH export in HEK293-MRP1 cells

Vincristine, a compound known to be co-transported with GSH via the MRP proteins [37,38], was used to further enhance GSH release in these cells. Vincristine significantly increased both basal and apoptotic GSH release in HEK293-MRP1 cells, but not in the vector-transfected cells (Fig. 7A). In addition, vincristine by itself or in combination with staurosporine or Fas antibody/CHX did not further increase caspase 3 activity in the vector-transfected cells (Fig. 7C). Total GSH (i.e., intracellular plus extracellular GSH) was unaffected (Fig. 7B). Thus, in agreement with the results presented above, the HEK293-MRP1 cells are relatively resistant to apoptosis.

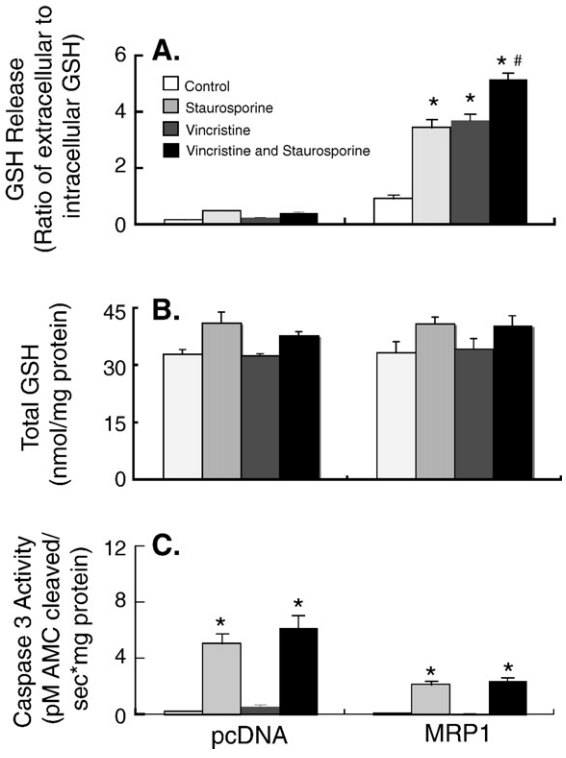

Fig. 7. Vincristine enhances basal and staurosporine-induced GSH release in *MRP1*-overexpressing HEK293 cells. pcDNA3.1-, and *MRP1*-transfected HEK293 cells were untreated (vehicle), treated with 10 μM staurosporine, 50 μM vincristine, or both staurosporine and vincristine for 6 h. GSH release (A), total GSH (B), and caspase 3-like activity (C) were measured. Values are means±SE, $n=3$. *Significantly different from control cells, $P<0.05$. #Significantly different from staurosporine-only treatment, $P<0.05$.

4. Discussion

The present study demonstrates that MRP1 is an effective mediator of both basal and apoptotic GSH export. Basal GSH efflux increased significantly in the *MRP1*-overexpressing cells, implicating MRP1 as an important mediator of basal GSH release, and extending previous observations in other cell lines [13,15,39]. Because of the enhanced basal GSH export in the HEK293-MRP1 cells, steady state intracellular GSH levels were significantly lower in these cells (Fig. 2A and B). Interestingly, when placed in KH buffer, intracellular GSH levels did not decrease over time in the HEK293-MRP1 cells despite the enhanced efflux, which is corroborated by the increase in total GSH over time, suggesting increased GSH synthesis. Because GSH synthesis is regulated via feedback inhibition [40], the high GSH export rate in the HEK293-MRP1 cells may stimulate GSH synthesis in an attempt to replenish and maintain intracellular levels. An upregulation of GSH synthesis was also previously noted in Fly-eco fibrosarcoma cells overexpressing *MRP1* [41].

Enhanced GSH release during apoptotic cell death has been previously observed [3,4,6], and recent work from our laboratory has implicated the MRP transporters, and in particular MRP1, in this process [4]. To further examine the role of MRP1 in apoptotic GSH release, HEK293-MRP1 cells were treated with staurosporine or Fas antibody/CHX to induce apoptosis. The results indicate that the MRP1 overexpressing cells had significantly higher levels of GSH release, supporting the role of MRP1 in GSH export during apoptosis.

Paradoxically, despite the high GSH export and low intracellular GSH levels in the MRP1-overexpressing cells, these cells were more resistant to apoptosis. It is now well established that enhanced GSH release, with a concurrent depletion of intracellular GSH levels, is important for the progression of apoptosis [2,4,42]. Stimulating GSH release in baby hamster kidney-21 (BHK) cells overexpressing *MRP1* with verapamil enhances phosphatidylserine externalization and caspase activation [19], whereas inhibiting GSH export reduces or slows down the appearance of apoptotic endpoints [2,4]. The present results demonstrate that HEK293-MRP1 cells had approximately 50% of the caspase 3 activity as the vector-transfected cells (Fig. 4). Further stimulation of GSH export with vincristine, a compound co-transported with GSH by the MRPs [37,38], did not increase caspase activation in HEK293-MRP1 cells (Fig. 7). In addition to low caspase activation, DNA fragmentation and phosphatidylserine externalization were significantly lower in the HEK293-MRP1 cells compared with vector-transfected cells (Figs. 5 and 6). Because effector caspases, such as caspase 3, are involved in the activation of downstream proteins in the apoptotic pathways, it is not surprising that other apoptotic endpoints were not altered. For instance, ICAD, an inhibitor of caspase activated DNase (CAD), is cleaved by caspase 3 thus releasing CAD which migrates to the nucleus and cleaves DNA [43,44]. The increase in DNA fragmentation is a later event compared to the activation of caspase 3 as observed in the vector-transfected cells (Fig. 5A) and is lower in the *MRP1*-overexpressing cells (Fig. 5B).

These data suggest that enhanced GSH release on its own is not sufficient to increase apoptosis in HEK293 cells overexpressing *MRP1*, but that a concurrent decrease in intracellular levels is also required. These findings provide insight into previous studies that reported that treatment of cells with compounds that stimulate GSH release is sufficient to induce cell death [18,19]. Intracellular GSH levels were not

depleted in the HEK293-MRP1 cells treated with staurosporine or Fas antibody/CHX despite elevated GSH release. This significant increase in total GSH over time in the MRP1-overexpressing cells (Figs. 3 and 7) suggests that there is an increase in GSH synthesis as the cells try to compensate for the loss of GSH. An increase in GSH synthesis has been observed in the highly metastatic B16 melanoma F10 (B16–F10) cell line when treated with agents that deplete GSH in an effort to induce cell death [45]. B16–F10 cells have high levels of intracellular GSH, and when stimulated to release GSH using verapamil or Bcl-2 antisense oligonucleotides they exhibit an increase in γ-glutamylcysteine synthetase (γ-GCS) activity and GSH levels [45]. One possible explanation for the increase in GSH synthesis is that the loss of GSH increases the amount of reactive oxygen species in the cells which have been suggested to induce transcription of both the heavy and light subunits of γ-GCS by the transcription factor Nrf2 [46,47]. Additionally, if cells use reactive oxygen species or changes in the redox state of proteins as signaling mechanisms in apoptotic pathways, an increase in the de novo synthesis of reduced GSH may prevent or attenuate these signals, slowing the progression of apoptosis and providing resistance to cell death.

Additionally, apoptotic endpoints measured in the vector-transfected HEK293 cells were not as high or did not occur as rapidly as previously observed in Jurkat cells when treated with Fas antibody or staurosporine [4]. Jurkat cells release a significantly greater quantity of GSH with a concurrent depletion of intracellular GSH when treated with either apoptotic agents [4]. Therefore, longer treatment of the vector-transfected HEK293 cells with the apoptotic agents may result in greater loss of intracellular GSH release and increased apoptosis.

Overall, the present results provide additional direct evidence that MRP1 is an effective mediator of GSH release under basal conditions, and during both death receptor-mediated and chemically-induced apoptosis. In addition, the results indicate that enhanced cellular GSH release with a concurrent decrease of intracellular GSH appears to be necessary for the progression of apoptosis.

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