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Activation of multidrug efflux transporter activity at fertilization in sea urchin embryos (*Strongylocentrotus purpuratus*)

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Abstract

This study presents functional and molecular evidence for acquisition of multidrug transporter-mediated efflux activity as a consequence of fertilization in the sea urchin. Sea urchin eggs and embryos express low levels of efflux transporter genes with homology to the multidrug resistance associated protein (mrp) and permeability glycoprotein (p-gp) families of ABC transporters. The corresponding efflux activity is low in unfertilized eggs but is dramatically upregulated within 25 min of fertilization; the expression of this activity does not involve de novo gene expression and is insensitive to inhibitors of transcription and translation indicating activation of pre-existing transporter protein. Our study, using specific inhibitors of efflux transporters, indicates that the major activity is from one or more mrp-like transporters. The expression of activity at fertilization requires microfilaments, suggesting that the transporters are in vesicles and moved to the surface after fertilization. Pharmacological inhibition of mrp-mediated efflux activity with MK571 sensitizes embryos to the toxic compound vinblastine, confirming that one role for the efflux transport activity is embryo protection from xenobiotics. In addition, inhibition of mrp activity with MK571 alone retards mitosis indicating that mrp-like activity may also be required for early cell divisions.

Keywords: Multidrug efflux; Fertilization; Embryo; mrp; p-gp; Activation; psc833; mk571; Calcein-am; *Sp*ABCB1; *Sp*ABCC1; *Sp*ABCC2; Mitosis

Introduction

Fertilization results in physiological and structural modification of the egg. In sea urchins, the changes in egg activity result from a transient rise in calcium and permanent rise in intracellular pH; these initiate structural and enzymatic changes that ultimately lead to initiation of DNA synthesis and rapid cell divisions (Epel, 1990; Jaffe et al., 2001; Runft et al., 2002). The structural changes center on reorganization of embryonic surfaces, initially arising from cortical granule exocytosis and formation of the fertilization envelope. The exocytosis is followed by endocytosis and later sequential movements of vesicles to the cortex (Matese et al., 1997; Connor and Wessel, 1998; Smith et al., 2000). Some of these vesicles contain constituents of the extracellular matrix and their movement is sensitive to cytochalasin and latrunculin suggesting that the trafficking occurs on microfilaments (Ikebuchi et al., 2001; Wessel et al., 2002). It is also hypothesized that some of these vesicles contain amino acid transporters that are similarly inserted into the plasma membrane and then used to bring in amino acids from the surrounding seawater (Epel, 1972; Schneider, 1985; Wright and Manahan, 1989).

Here, we show that the fertilization response involves upregulation of multidrug efflux transport activity, as part of the re-organization of the cytoplasm and plasma membrane. This activity protects the embryo from exogenous toxicants and inhibition of the efflux activity affects cell division, suggesting that the transporter activity also has some role in passage through mitosis.

These multidrug (mdr) efflux transporters are members of the ATP Binding Cassette (ABC) super-family and actively

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export or sequester endogenous and exogenous compounds (Borst et al., 2000; Dean et al., 2001; Litman et al., 2001) including various xenobiotics (Leslie et al., 2001). The xenobiotic transporters include permeability glycoproteins (p-gp; Ambudkar et al., 1999), multidrug resistance associated proteins (mrp; Cole and Deeley, 1998) and White family/mitoxantrone (mxr) resistance proteins (Litman et al., 2000). In addition to providing protection by xenobiotic transport, these proteins regulate cellular and developmental physiology by efflux of endogenous signaling molecules. In *Dictyostelium*, the ABC transporter *RhT* regulates terminal stalk cell differentiation by efflux transport of DIF-1, an endogenously produced differentiation factor (Good and Kuspa, 2000). Efflux transporters are also involved in establishing the auxin gradients required for plant development (Friml et al., 2003; Gaedeke et al., 2001).

Most attention has focused on the protective role of these transporters in somatic cells but they are also important in embryo protection. For example both, embryos and adults of the marine echiuroid worm *Urechis caupo* express a p-gp transporter which protects them from naturally occurring microbial toxins (Hamdoun et al., 2002; Toomey and Epel, 1993; Toomey et al., 1996). Embryos of *Caenorhabditis elegans* express p-gp and mrp genes that are associated with tolerance to heavy metals (Broeks et al., 1996). Similarly mouse embryos have an efflux transport phenotype thought to be mediated by a p-gp transporter and which protects the embryo from xenotoxins (Elbling et al., 1993).

Previous work had suggested that early sea urchin embryos and larvae do not have p-gp-mediated toxicant defenses (Hamdoun et al., 2002; Toomey and Epel, 1993), although the adults can live in polluted environments. Here, we show that several mdr transporter-like genes are expressed in the embryo, including at least one with homology to p-gp and two with homology to mrps. The major efflux activity in the sea urchin egg is mrp-like and this activity is dramatically upregulated within 25 min of fertilization. This increase does not involve de novo transcription or translation, but rather the transporter proteins are present in eggs and their activity increases by a microfilament-dependent process, most likely involving movement of transporter vesicles to the egg surface and insertion. We show that this transport activity protects the embryo from toxic xenobiotic compounds such as vinblastine. Finally, inhibition of mrp-like transport activity slows progression through mitosis with a major effect on progression through anaphase. This suggests that mrp-mediated transport of some endogenous substance may also be required for early cell divisions.

Materials and methods

Animals

Purple sea urchins, *Strongylocentrotus purpuratus*, were kept in flow through sea water tanks (approximately 13°C) and fed kelp. Gametes were collected by intracoelomic injection with 0.5M KCl. Eggs were washed several times in 1.0 μm filtered seawater (FSW) prior to use in experiments. For individual embryo transport measurements, embryos were prepared by mixing eggs from 2 to 4 females, with diluted sperm from one male. Only batches of embryos with >90% successful development (assessed at the two-cell stage) were used for experiments. All experiments were conducted at 14°C.

Fluorophores and inhibitors

MK571 was obtained from Cayman Chemicals. PSC833 was a gift from Novartis (Basel, Switzerland). Reversin (R205) was initially obtained by gift of Dr. Balzas Sarkadi and later purchased from Sigma (St. Lois, MO). Actinonmycin-D, emetine and verapamil were also purchased from Sigma. Calcein-acetoxymethylester (c-am), cytochalasin-D and latrunculin-A were obtained from Calbiochem. Sytox-green was from Molecular Probes (Eugene, OR). DMSO was used as a solvent for all stocks. Chemical stocks were prepared at approximately 1000× the final desired concentration such that highest concentration of DMSO did not exceed 0.5% of the experimental volume; this solvent concentration has no effect on transport activity or activation of transport.

Degenerate PCR and cDNA library screening

Packed eggs or embryos were flash frozen in liquid nitrogen, homogenized in a liquid nitrogen cooled ceramic mortar, and then thawed into 10 volumes of homogenization buffer (Kalinowski et al., 2003) containing proteinase K (200 μg/ml). Homogenates were incubated for 45 min at 37°C before phenol-chloroform extraction and ethanol precipitation. RNA was further purified by 8 M lithium chloride precipitation (Sambrook and Russell, 2001). cDNA was synthesized with an oligo-dT 12–18 primer and Superscript Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Degenerate primers (Allikmets and Dean, 1998; Lee et al., 1998) were used to amplify conserved fragments of the corresponding transporter cDNAs by RT-PCR and clone a heterologous cDNA fragment containing a conserved 230-bp region of sea star (*Asterina miniata*) transporter cDNA; 5′TCGGGATCCAGGGAGAATATACTGTTTGGGGCACCATTGCAAGATGCAGAGTACCAGCGAGTGATCGAAGCCTGCGCGCTGGCCCCTGATCTGGACATGCTCCCAGCTGGAGATCTAACGGAGATTGGAGAAAAGGGAATTAACCTGAGTGGTGGTCAGAAGCAGAGGGTCAGTTTAGCTCGAGCCGTTTACAACAATGCCGACATCTATCTCCTTGACGACGAATTCCG3′. This fragment was random primed and used to screen cDNA macroarray filter libraries of *S. purpuratus* (Cameron et al., 2000; Clark et al., 1999) using standard filter hybridization techniques (Sambrook and Russell, 2001).

cDNA characterization

Clones corresponding to positive coordinates on the cDNA library screen were obtained from cDNA libraries maintained at Caltech as part of the Sea Urchin Genome Project (SUGP). Clones were grown on selective plates and individual colonies were selected for sequencing. cDNA inserts (pSport vector; Invitrogen) were analyzed for size on agarose gels and SP6 and T7 ends were sequenced (Davis Sequencing, Davis CA) to confirm the identity of each clone. All of the positive clones were completely sequenced using a transposon-based shotgun sequencing approach (GPS, New England Biolabs; Beverly MA) on an ABI 3100 sequencer using standard cycle sequencing protocols. Contiguous partial cDNA sequences were edited and assembled using Sequencher. Sequencing coverage was at least 2× for all sequences. The reading frame, homology and sequence analyses of the putative proteins encoded by these partial cDNAs were determined using tools at the Expasy Molecular Biology Server (http://us.expasy.org/) and at NCBI.

Real-time PCR

Real-time PCR was performed at the Lucy Whittier molecular core facility, UC Davis according to the following protocol. Fluorescent labeled TaqMan probe (5′ end, reporter dye FAM (6-carboxyflourescein), 3′ end, quencher dye TAMRA (6-carboxytetramethylrhodamine)) were designed using Primer Express software (Applied Biosystems, Foster City, CA). *Sp*ABCB1 primer sequences were forward 5′GGAGCTTGAGAAGCATTTCAGC3′ and reverse 5′AAGGAGGATGAAACCACACTCTG3′ with the probe sequence 5′TGATGCGAGTCTGGAAGCTCAACACC3′. *Sp*ABCC1 forward primer was 5′GATGGCGATGTAACGGACAGT3′ and reverse 5′TGATGGTCACCGAAAGATCGT3′ with the probe 5′CCGAGAGCGTGGGTAATTCACACGA3′. TaqMan PCR systems were evaluated according to Leutenegger et al. (1999).

For q-pcr experiments, three separate batches of sea urchin eggs and embryos were prepared for sample collection. Samples were taken immediately after termination of the experiments and lysed in 1× ABI lysis buffer (Applied Biosystems). Before RNA extraction, the lysates were transferred into 96 deep well plates and digested with Proteinase K (Invitrogen). The total RNA was extracted from the lysates using a 6700 automated nucleic acid (ANA) workstation (Applied Biosystems) according to manufacturer's instructions. Total RNA was eluted in 100 μl of elution buffer. Complementary DNA (cDNA) was synthesized using 100 units of SuperScript III (Invitrogen), 300 ng random hexadeoxyribonucleotide ($pd(N)_6$) primers (random hexamer primer) 10 U RNaseOut (RNase inhibitor), and 1 mM dNTPs in a final volume of 40 μl. Each PCR reaction contained 400 nM of each primer, 80 nM of the TaqMan probe and commercially available PCR mastermix (TaqMan Universal PCR Mastermix, Applied Biosystems) containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 2.5 mM deoxynucleotide triphosphates, 0.625 U AmpliTaq Gold DNA polymerase per reaction, 0.25 U AmpErase UNG per reaction and 5 μl of the diluted cDNA sample in a final volume of 25 μl. The samples were placed in 96 well plates and amplified in an automated fluorometer (ABI PRISM 7700 Sequence Detection System, Applied Biosystems). Amplification conditions were 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 60 s at 60°C.

Final relative quantitation was done using the comparative cycles to threshold (C_T) method (User Bulletin #2, Applied Biosystems) and is reported as relative transcription or the *n*-fold difference relative to eggs. The endogenous control for the sea urchin samples 18S rRNA was used to normalize the mrp TaqMan signals. The ΔC_T values were calibrated against the control ΔC_T values for each target gene. The relative linear amount of target molecules relative to the calibrator, was calculated by $2^{-\Delta\Delta CT}$.

Fluorescence microscopy

Accumulation of calcein was used to measure mrp transporter activity in eggs and embryos. Calcein-AM (c-am) is a non-fluorescent mrp and p-gp substrate. Eggs and embryos are incubated in 250 nM c-am and any c-am that enters the cell is rapidly hydrolyzed by esterases to form fluorescent, membrane-impermeant calcein, which is detected as intracellular fluorescence within minutes of extracellular c-am addition (Essodaigui et al., 1998). Thus, low transport activity results in high calcein accumulation and high fluorescence while increased transporter activity results in reduced intracellular accumulation of calcein and reduced fluorescence.

Visualization of DNA for cell division experiments was performed by fixing embryos overnight in 2% formaldehyde and 0.1% Tween 20. Fixative was washed out 2× in Tris-buffered saline with 0.1% Tween (TBST). Embryos were then resuspended in 10 nM Sytox green (Molecular Probes) in TBST. DNA was visualized with FITC wavelength filters (as described below).

Fluorescence imaging was performed using a fixed stage upright microscope (Olympus BX50-WI) with xenon illumination and a cooled (14°C) stage, or an inverted microscope (Zeiss Axiovert S-100; Germany) equipped with mercury illumination. Excitation was set to 488 ηm using a Lambda-10 automated filter wheel (Sutter Instruments, Novato, CA). Images were captured using a Photometrics Coolsnap FX cooled charge coupled device (CCD) camera (Olympus BX50-WI; Tokyo, Japan) or a Princeton Instruments cooled CCD camera (Zeiss S100). A 10× objective was used for all physiology experiments except the time-lapse recordings of eggs at fertilization which were collected using a 20× objective. Pseudo color micrographs and the visualization of DNA were performed with a 40× objective.

Data analysis and presentation

Fluorescence intensity of individual eggs and embryos was measured using image analysis macros provided with MetaMorph (Universal Imaging Corp., Downingtown, PA) software. All comparisons of changes in fluorescence intensity between treatments were made from data corrected for background (70 units). *P* values were computed from two sample *t* tests of means.

Results

Identification of transporter genes in the egg

We confirmed that several efflux transporter mRNAs are expressed in the sea urchin egg and we characterized partial cDNAs encoding large fragments of three unique mRNAs; *Sp*ABCB1 (1289AA; Genbank accession number CK995664), encoding a p-gp, and large fragments of two distinct mrp-like cDNAs, *Sp*ABCC1 (765AA; CK995665) and *Sp*ABCC2 (709AA; CK995667). BlastP comparison of the translated cDNAs on Genbank (NCBI) indicate highly significant homology ($E < 1 \times 10^{-50}$) to mammalian p-gp1, mrp2, mrp5, and sulfonylureas (ABCC8/9). Each partial mrp cDNA encoded about half of the expected protein with at least one ABC transporter ATPase domain and one ABC transporter type-1 integral transmembrane domain (Prosite). The p-gp cDNA was nearly full-length, as expected by homology to other p-gps. Multiple alignment of the protein sequences by ClustalW revealed that observed urchin sequences align with other vertebrate and invertebrate sequences consistent with their predicted BlastP identities (Fig. 1).

Basis of efflux transport assay

We adapted the methods of Homolya et al. (1996) and Essodaigui et al. (1998) which employ calcein-acetoxymethylester (c-am) to measure mdr transporter mediated transport activity (Essodaigui et al., 1998; Hollo et al., 1994, 1996, 1998; Homolya et al., 1996). Calcein-AM is a non-fluorescent compound that is a substrate of both mrp and p-gp. If the cell has low p-gp or mrp activity, or no activity, c-am accumulates in the cells and is rapidly hydrolyzed by non-specific esterases to form green, fluorescent calcein. Unlike c-am, free calcein is relatively membrane impermeant and therefore remains in the cell (Essodaigui, 1998). If a cell has high levels of p-gp or mrp transport activity, c-am is prevented from accumulating in the cell by efflux transport and fluorescent calcein accumulates to a lesser extent.

A specific test for multidrug transporter-mediated efflux activity is to measure intracellular calcein accumulation in the presence or absence of mdr inhibitors. If there is no or little transport activity, incubation in a transport inhibitor has little or no effect and there is no or little increased fluorescence in the cell. In contrast, if there is transport activity, incubation in an inhibitor results in increased calcein accumulation and fluorescence as compared to the fluorescence in the cell when no inhibitor is present.

Initiation of transport at fertilization

Fig. 2 compares the kinetics of calcein accumulation in the presence of 250 nM extracellular c-am in eggs and in 2–4 cell embryos. Calcein accumulates in unfertilized eggs in a linear fashion over time whereas in 2–4 cell embryos intracellular calcein fluorescence is barely detectable even after 2 h of incubation in c-am. The reduced calcein accumulation in embryos relative to eggs could be a consequence of reduction in calcein-AM permeability, a reduction in intracellular esterase activity, or result from increased expression of a calcein efflux transporter activity. Fig. 3 shows that embryos incubated in the presence of the mdr inhibitors cyclosporin-A, reversin-205, verapamil, PSC833, or MK571 accumulate increased levels of calcein. This indicates that the reduced accumulation in the embryos largely results from increased efflux transport, since the addition of the various mdr inhibitors results in calcein accumulation.

We found that differences in passive c-am permeability and esterase activity do not account for the changes in c-am accumulation at fertilization since the absolute fluorescence

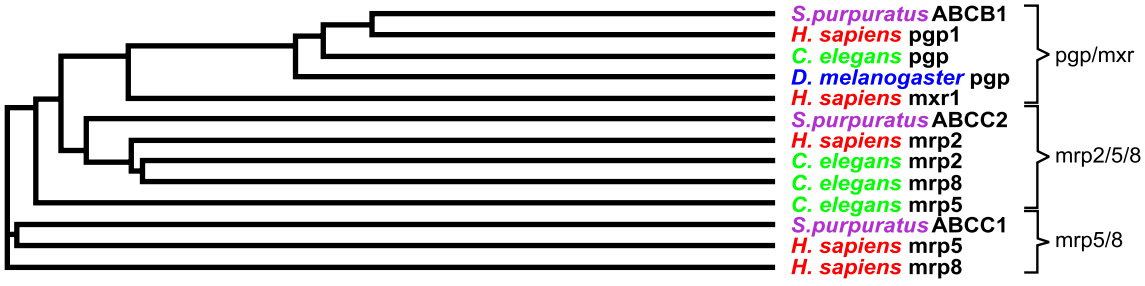

Fig. 1. Multiple sequence alignment (ClustalW) shows that the sea urchin multidrug transporter proteins and partial cDNAs segregate into three lineages relative to human and invertebrate mdr proteins. *Sp*ABCB1 is a p-gp that aligns more closely with p-gp and mxr (a functional half transporter) than any of the other proteins. The partial cDNAs *Sp*ABCC1 and *Sp*ABCC2 are both mrp-like and the former most similar to human mrp5 and 8, whereas the latter is closer to the other invertebrate (*C. elegans*) mrps and human mrp2.

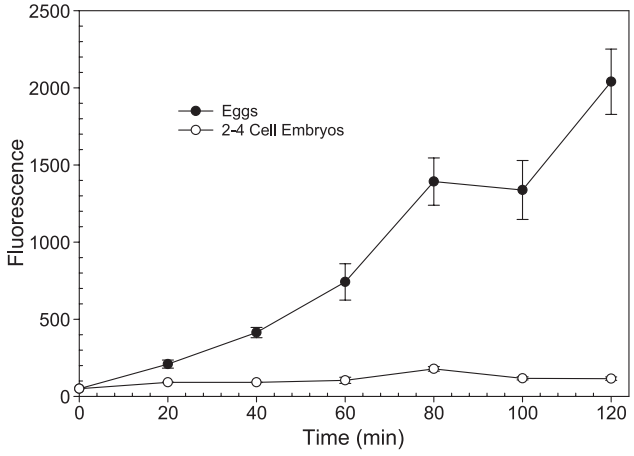

Fig. 2. Typical plot of calcein accumulation in unfertilized eggs and embryos (250 nM extracellular c-am). Calcein-am is excluded from embryos but in eggs it enters the cell, is hydrolyzed to fluorescent calcein, and accumulates in a linear fashion over time (points represent mean ± 1 SD of 20–100 individual embryos from four females).

levels of embryos treated with 10 μM MK571 or PSC833 were not significantly different (P = 0.34 and 0.4, respectively) from those of eggs treated with the same inhibitors (Figs. 4A, B). This indicates that the levels of c-am hydrolysis by esterases and passive permeability are equivalent in eggs and embryos; rather, fertilization results in a change in transport-inhibitor sensitive activity. Thus, the change in c-am accumulation that occurs post-fertilization is related to upregulation of transport activity. The results also demonstrate that there is a low level of MK571-sensitive transport in eggs prior to fertilization.

Fig. 5 shows continuous, time-lapse recording of calcein accumulation in seven sea urchin eggs at fertilization. In this experiment eggs were attached to a poly-lysine coated dish and recordings were started immediately after addition of c-am. Fluorescence was measured every 2 min and points represent the mean fluorescence intensity in seven embryos (±one standard deviation). As seen, fluorescence increases in a linear fashion prior to fertilization, indicating low efflux activity. Within 20–30 min after fertilization, a leveling of intracellular fluorescence accumulation occurs. This indicates that a distinct increase in efflux activity begins at 20–30 min after fertilization, and a corresponding reduction in the accumulation of intracellular calcein is observed. The addition of the mrp-specific inhibitor MK571 (10 μM) post-fertilization restores the unfertilized rate of calcein accumulation.

Mrp- and p-gp-specific embryo transport

The results in Figs. 3 and 4 show that both mrp and p-gp-like transport activities are upregulated in embryos but suggest that mrp is the major transporter since the increase in fluorescence is greater with the mrp-specific inhibitor MK571 than with the p-gp inhibitors cyclosporin-A, reversin 205, verapamil, and PSC833.

The results in Fig. 6 examine in greater detail the relative contribution of p-gp and mrp transporters to c-am efflux in embryos using the mrp- and p-gp-specific inhibitors, MK571 and PSC833. PSC833 is effective at lower concentrations but the effect saturates at about 3 μM and increased levels of drug do not result in any additional

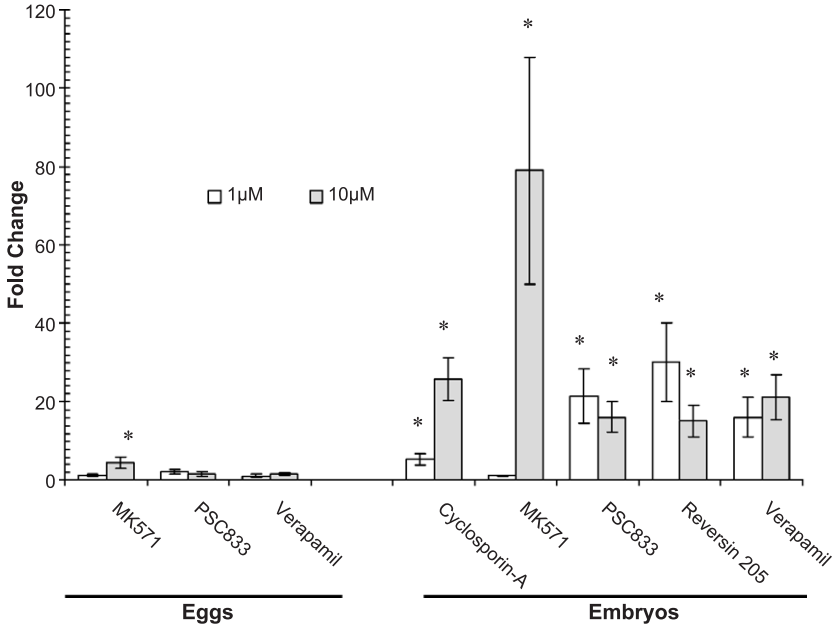

Fig. 3. Change in intracellular fluorescence in eggs and 2-cell embryos treated with mdr inhibitors for 90 min, as compared to untreated controls of the same developmental stage (bars represent means ± 1 SD of 3 batches of eggs or embryos). There is a small increase in fluorescence intensity in eggs treated with the mrp-specific inhibitor MK571 as compared to controls, but in embryos the fold change is much greater due to the significant decrease in calcein accumulation that occurs at fertilization. MK571, 10 μM, is more potent than any of the other p-gp-specific or general mdr inhibitors at inhibiting transport in the embryos.

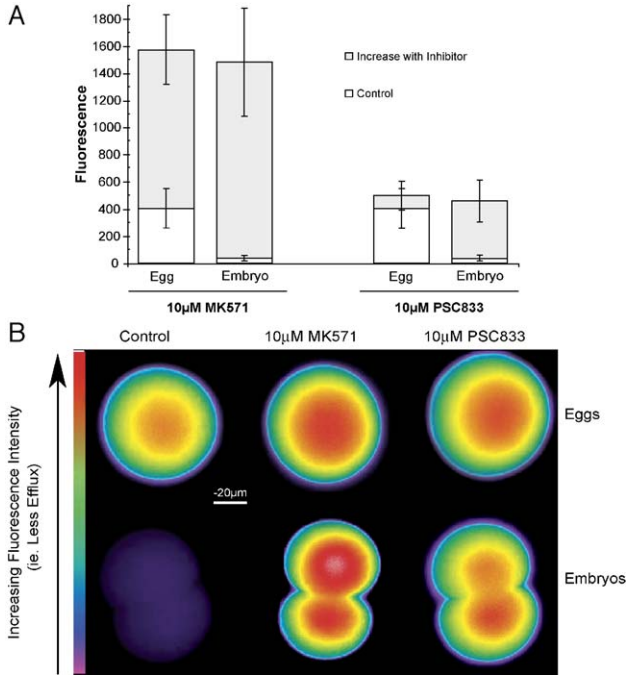

Fig. 4. (A) Absolute fluorescence levels in eggs and embryos before and after treatment with mrp- and p-gp-specific inhibitors (MK571 and PSC833; bars represent means ± 1 SD of 3 batches of eggs and embryos). The addition of inhibitors to embryos restores fluorescence to similar levels to eggs suggesting that "passive" permeability of eggs and embryos is similar but that MK571 and PSC833 sensitive transport increase at fertilization. (B) Representative pseudo-color micrographs of eggs embryos treated with mrp and p-gp inhibitors. Color bar (left) indicates relative fluorescence intensity (white is brightest fluorescence, violet dimmest).

accumulation. The effect of MK571 also saturates, but at a higher concentration. Importantly, the amount of intracellular fluorescence in saturating doses of MK571 is four to five times higher than when transport is maximally inhibited by PSC833, indicating a greater contribution of efflux transport activity from mrp transporters. These results indicate that both p-gp and mrp transport activity are present in these embryos, but that the p-gp contribution is minor compared to the mrp contribution.

Mechanism of transport initiation at fertilization

Translation and transcription

To determine the significance of transcription and translation in the upregulation of efflux transport, eggs were fertilized and appropriate inhibitors added after confirmation of fertilization envelope elevation (5 min post-fertilization (PF)). At 60 min PF the inhibitors were washed out and c-am was added. Embryos were incubated for 90 additional min and fluorescence intensities were measured at 150 min PF (2-cell stage).

Initiation of transport is not affected by inhibitors of transcription or translation, actinomycin and emetine, respectively (Fig. 7). As demonstrated previously (Kiefer et al., 1969; Wagenaar, 1983), embryos progress through first cleavage normally when treated with actinomycin but arrest prior to first cleavage with emetine treatment. The lack of effect of actinomycin and emetine on transport activity suggests that transcription and translation do not mediate post-fertilization changes of transporter activity. In addition, we directly measured the levels of transcripts of *Sp*ABCC1 and *Sp*ABCB1 in eggs and early embryos by real-time PCR (Table 1). These results showed an insignificant decrease in transcript abundance at fertilization followed by a later increase in transcript abundance 90–120 min PF. The increase in transcript abundance occurs later than the increase in transporter activity, confirming that the large induction in transporter activity occurs prior to de novo transcription of transporters. It is also important to note that the levels of these transcripts appear to be quite low (<1 copy/egg) as determined by comparison of C_T values in eggs to those from plasmid standards (data not shown).

Role of microfilaments in upregulation of transport

Earlier work has shown that transport systems for amino acids and nucleosides are also activated after fertilization and that this process is prevented by microfilament inhibitors (Epel, 1972; Schneider, 1985; Swezey et al., 1987). The stimulus-induced delivery of vesicles to the plasma membrane is typically a microfilament-mediated process requiring actin polymerization (Ikebuchi et al., 2001; Kato et al., 2004; Wessel et al., 2002). We examined the role of microfilaments in upregulation of transport using the actin polymerization inhibitors cytochalasin-D (CD) and latrunculin-A (Fenteany and Zhu, 2003; Schatten et al., 1986; Wessel et al., 2002) and found that both compounds prevented the upregulation of efflux transport activity since significantly more calcein accumulated in the embryos fertilized in these inhibitors than controls (Fig. 7). Because

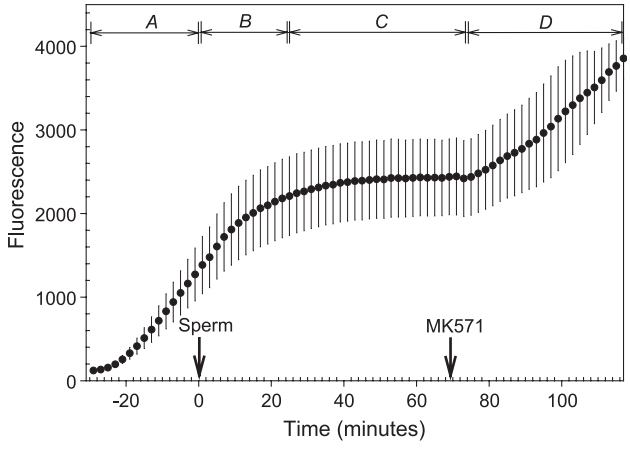

Fig. 5. Time-based measurement of calcein accumulation in eggs (n = 7) before, during and after fertilization. Calcein accumulates in a linear fashion in unfertilized eggs (A) but calcein accumulation stops within 25 min of fertilization (B) and remains at a steady state (C) until the mrp-specific inhibitor MK571(10 μM) is added (D). These results indicate that c-am transport is activated at fertilization.

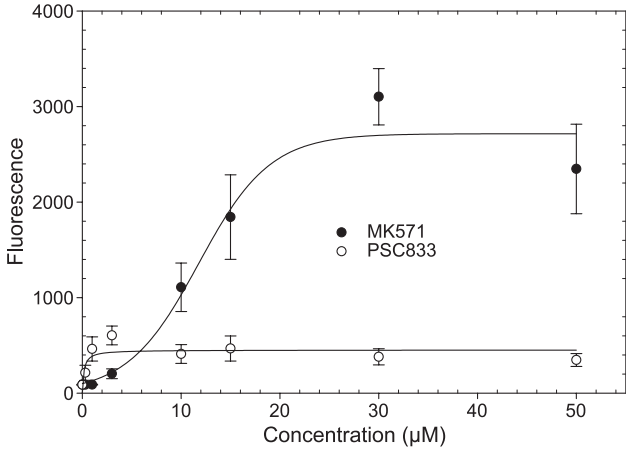

Fig. 6. Representative dose–response curves of the p-gp- and mrp-specific inhibitors (PSC833 and MK571, respectively) on calcein-am transport in 2 cell embryos. PSC833 inhibits transport at low doses, but MK571 is much more effective at high doses.

these inhibitors were added 5–10 min after fertilization and washed out prior to c-am addition this effect was not due to their competitive inhibition of transport activity.

Latrunculin caused a nearly 30-fold increase in calcein accumulation and its efficacy as an irreversible microfilament inhibitor was also evidenced by the fact that it blocked cell division, even though it was washed out (to prevent any possible interaction with the transporters as a competitive substrate for efflux) at 60 min post-fertilization. In contrast, with 2 μM cytochalasin-D, half of the embryos completed the first cell division consistent with reversible inhibition of actin polymerization in these embryos (Wessel et al., 2002). In cleavage-arrested, CD-treated embryos, there was a 7-fold increase in fluorescence relative to controls, indicating inhibition of the transporter. However, in CD-treated embryos that divided, fluorescence levels were similar to controls and we observed this effect even with 4 μM CD (not shown) suggesting recovery from CD treatment, rather than threshold dosage effects (Wessel et al., 2002). These results show that upregulation of transporter activity at fertilization is microfilament dependent.

Role of efflux transport in the embryo

Protection from xenobiotics

We tested the hypothesis that the major, mrp-mediated, transport activity protects embryos from xenotoxins by measuring the toxicity of the mrp substrate vinblastine to embryos during the first cell division in the presence or absence of 5 μM MK571. As seen in Fig. 8, MK571 increases sensitivity to vinblastine by at least one order of magnitude confirming that mrp activity is protective.

Progression through the cell cycle

We also observed that treatment of embryos with 10 μM or higher levels of MK571 alone, retards the first cell division. To analyze this in depth, we fixed embryos at various intervals following fertilization and chromosomal events during the cell cycle were visualized using the fluorescent DNA probe Sytox green. We found that embryos treated with 10 μM MK571 take a longer time to complete anaphase than do controls, though progression through earlier stages was not affected (Fig. 9A). Although the 10 μM MK571-treated embryos eventually complete cell division and continue to divide, many develop abnormally and have unevenly sized blastomeres (Fig. 9B) As opposed to the 10 μM concentration of MK571, concentrations of 15 μM or higher slowed progression through all stages of the cell cycle, and concentrations in excess of 20 μM stop cell division all together (not shown).

Discussion

This study shows a post-fertilization upregulation of efflux transporter activity that begins 25 min post-fertilization, coincident with the later responses to fertilization that include delivery of other stored maternal proteins to embryonic surfaces (Matese et al., 1997), pronuclear fusion, and DNA synthesis (Epel, 1990). The confluence of changes at this time may indicate a second phase of the post-fertilization events that is linked to resumption of the cell cycle (Jaffe et al., 2001).

This efflux activity is sensitive to inhibitors of mdr transporters, including specific inhibitors of p-gp and mrp, PSC833 and MK571, respectively (Gekeler et al., 1995; Liu et al., 2001; Mayer et al., 1997; Miller et al., 2002a), and is most effectively blocked by the mrp-specific inhibitor MK571. The data indicate that mrp-like activity is the major high-capacity transport activity in the sea urchin egg and embryo but the results also reveal a lower capacity, p-gp-like transport. The mrp activity appears to have several

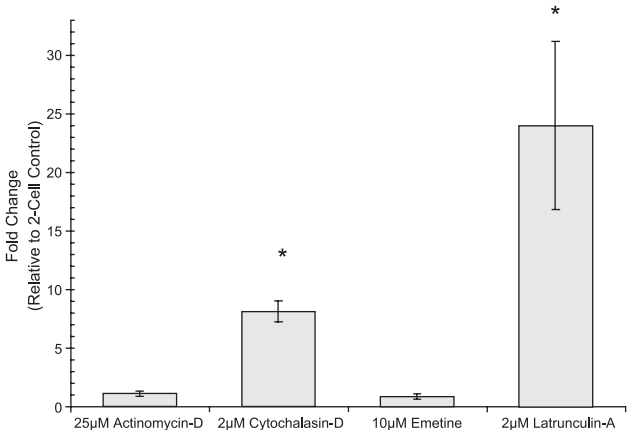

Fig. 7. Transcription and translation (actinomycin-D and emetine) inhibitors do not block the initiation of transport at fertilization, but cytoskeleton disrupting compounds cytochalasin-D and latrunculin-A do. This suggests that the initiation of transport occurs in some microfilament-dependent process such as movement of vesicles to the plasma membrane.

Table 1
Results of real-time q-pcr analysis of mrp (*Sp*ABCC1) and p-gp (*Sp*ABCB1) transporter mRNA abundance before and after fertilization are shown

Time PF (min)	C_T 18s rRNA	C_T *Sp*ABCC1	C_T *Sp*ABCB1	Fold change *Sp*ABCC1 (relative to egg)	Fold change *Sp*ABCB1 (relative to egg)
0	14.3 (±0.4)	34.4 (±1.1)	29.1 (±0.8)	–	–
30	15.8 (±1.2)	37.4 (±1.6)	31.4 (±2.8)	−5 (±3.4)	−2 (±3.2)
60	16.6 (±0.4)	37.2 (±0.9)	31.7 (±1.5)	−0.37 (±1.9)	−0.25 (±0.4)
90	15.6 (±1.5)	35.9 (±0.4)	30.7 (±2.6)	−0.9 (±1.8)	−0.2 (±0.9)
120	15.0 (±0.5)	34.5 (±2.0)	28.9 (±0.8)	4 (±3.2)	2.4 (±1.5)

The cycles to threshold (C_T) values for each transcript measured are shown. The high C_T values for mrp and p-gp suggest that these are low abundance transcripts. A standard curve relative to a recombinant standard suggests they are both at <1 copy/egg (not shown). The fold change in mrp and p-gp is expressed relative to the value of the internal standard (ribosomal RNA) for each sample. Given the low abundance of these transcripts, the changes in transcript abundance are not likely to be significant. Values represent the means (±one standard deviation) for three batches of eggs and embryos.

roles. Inhibition of activity enhances toxicity of vinblastine, indicating that this efflux transport can protect against xenobiotics, as seen in other organisms. Inhibition of the transporter also retards progression through the cell cycle, with major effects on anaphase. This effect on mitosis and the timing of upregulation of activity, point to a role for efflux transport in promoting entry or progression through the first cell division.

Our molecular data demonstrate that several mdr transporter mRNAs are present in the egg including *Sp*ABCB1, *Sp*ABCC1, and *Sp*ABCC2, and that they encode gene products that could play roles in the observed xenobiotic efflux. *Sp*ABCB1 encodes an efflux transporter similar to p-gp, an extensively studied xenobiotic transporter that transports c-am and various xenobiotics in mammalian cells (Ambudkar et al., 1999). *Sp*ABCC1 encodes a protein with homology to mammalian mrp5 which is an efflux transporter with broad substrate specificity and the capacity to transport characteristic mrp substrates including cyclic nucleotides, anti-viral drugs and anti-cancer drugs (Kruh and Belinsky, 2003). *Sp*ABCC1 was highly represented in the sea urchin egg cDNA library and an analysis of the translated partial cDNA shows that the protein it encodes shares 65% amino acid identity to murine mrp5 in the conserved ABC transporter ATPase domain and an overall identity of about 22% to the same murine protein. In plants mrp5 homologs establish auxin gradients that regulate root development (Friml et al., 2003; Gaedeke et al., 2001). SpABCC2 encodes a protein with homology to mammalian sulfonylurea receptors which regulate K+ conductance in insulin-sensitive cells and with transport properties similar to those of mrp5 (Bryan and Aguilar-Bryan, 1997; Kruh and Belinsky, 2003).

In addition to molecular approaches, different fluorophores are used to identify the transporters responsible for particular transport phenotypes (see Litman et al., 2001 for review). For example, while c-am is extruded by both p-gp and mrp proteins, rhodamine is only transported effectively by cells that express p-gps. Consistent with this observation, *Urechis* embryos and larvae express p-gp, and are capable of high-capacity rhodamine transport, while sea urchin eggs, embryos and larvae are not (Hamdoun et al., 2002; Toomey and Epel, 1993). It is probable that these same studies did not identify drug-sensitive p-gp transport in sea urchin eggs and embryos because rhodamine was used at concentrations (1 μM) that saturate the low-capacity p-gp-like transport we identified here.

Similarly, our previous studies did not detect p-gp protein (Hamdoun et al., 2002; Toomey and Epel, 1993) on western blots of sea urchin egg proteins using the monoclonal antibody C219 which reacts strongly with the conserved the epitope VQEALD (Georges et al., 1990). The corresponding epitope in sea urchin p-gp has a single amino acid substitution of the glutamate with aspartate, which could account for the lack of immunoreactivity of C219 with sea urchin p-gp. Given the molecular evidence and PSC833 sensitivity, it is clear that sea urchin embryos have some p-gp-mediated efflux activity.

Analysis by real-time-PCR (RT-PCR) shows that *Sp*ABCC1 and *Sp*ABCB1 are rare transcripts and that their level does not significantly increase until 90–120 min post-fertilization. In contrast, transport activity increases within 25 min of fertilization and the increase in efflux activity occurs in the presence of protein and RNA synthesis inhibitors. These data then all indicate that the

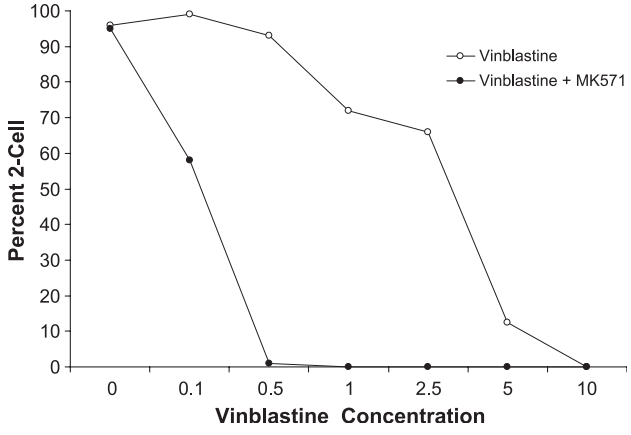

Fig. 8. Competitive inhibition of mrp activity with 5 μM MK571 sensitizes the embryo to vinblastine measured as failure to enter and complete first cell division. Vinblastine was added 45 min PF (after efflux transport is initiated) either in the presence or absence of MK571 (n = 100 embryos per point). At 145 min PF embryos were fixed and scored for completion of first cell division.

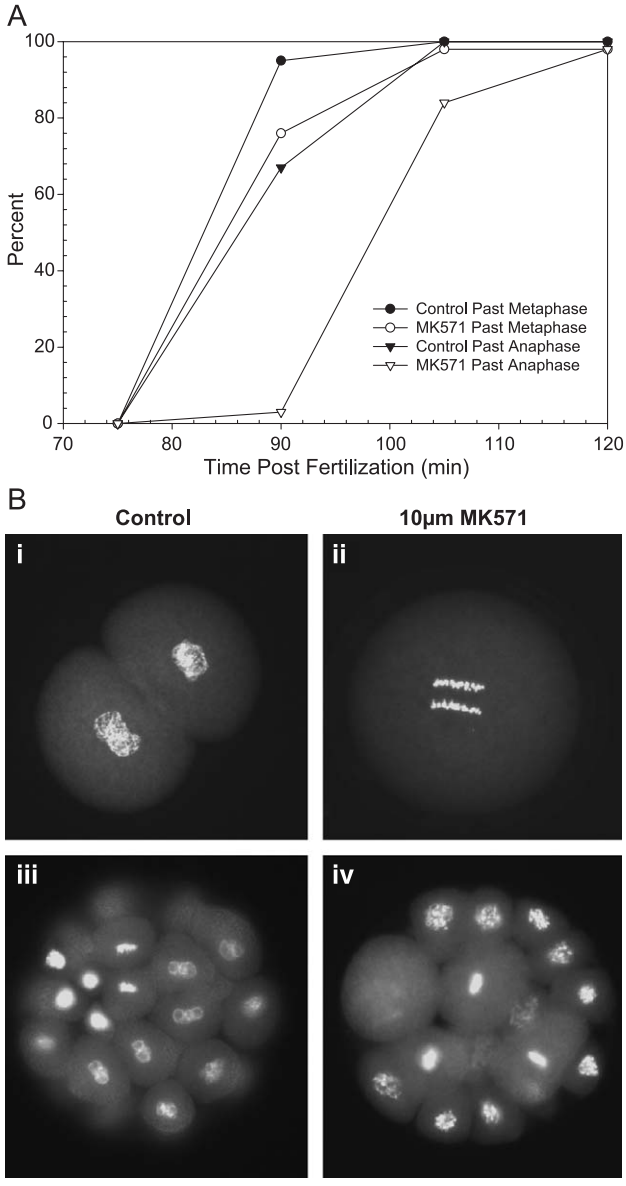

Fig. 9. (A) Graph shows the progression of control and 10 μM MK571-treated (5 min PF) embryos through first cell division (n = 50–100 embryos per point). Progression through metaphase is similar in controls and MK571-treated embryos. In contrast at 90 min post fertilization >60% of control embryos are past anaphase, while virtually none of the MK571-treated embryos have completed this stage. These MK571-treated embryos later complete anaphase demonstrating MK571-induced developmental delay rather than cell death. (B) Micrograph. (i) Control embryos enter prophase of the second cell division by 120 min following fertilization. In contrast, cell division is retarded in MK571-treated embryos and anaphase is especially prolonged (ii). Embryos treated with 10 μM MK571 eventually complete first cell division but some of them develop abnormalities, including uneven blastomeres, as seen in the 32-cell embryos shown (iii and iv).

upregulation of mdr efflux activity at fertilization is mediated post-translationally.

The upregulation of mrp following fertilization may share a common mechanism with the previously described increase in activity of amino acid transporters after fertilization. Activation of mrp and amino acid transport activity are both insensitive to pharmacological inhibition of transcription or translation, sensitive to microfilament polymerization inhibitors and activity becomes apparent after cortical exocytosis (Epel, 1972; Schneider, 1985). This similar behavior among transporters suggests that they are translocated to the plasma membrane in vesicles after fertilization. Indeed, this may be part of a cascade of vesicle movement events after fertilization which also includes movement and exocytotic deposition of vesicle contents (Matese et al., 1997) and, in at least some cases, this movement is similarly carried out on microfilaments (Kato et al., 2004).

This phenomenon is analogous to numerous other types of signal-induced changes in cellular transport physiology that are mediated by delivery of transporters to the plasma membrane (see Bryant et al., 2002 for review), including the well-studied example of insulin-induced translocation of the glucose transporter GLUT4 to the membrane (Watson and Pessin, 2001). The microfilament-mediated increase in mrp activity in the sea urchin embryo at fertilization may also be accompanied by other post-translational events such as protein kinase-mediated upregulation of pre-existent and/or newly inserted membrane transporter activity (Miller et al., 2002b).

One role of these transporters at fertilization is protection, as is demonstrated by the sensitization of embryos to vinblastine by competitive inhibition of mrp activity with MK571. This role is similar to the protective functions ascribed to these transporters in somatic cells and consistent with their expression in the adult blood–brain barrier (Miller et al., 2002a), blood–testis barrier (Melaine et al., 2002), and in intestinal epithelia (Miller et al., 2002b). Similarly, this is also consistent with a role for these transporters in protection of embryos and fits with their expression both in embryos (Broeks et al., 1996; Elbling et al., 1993; Toomey and Epel, 1993) and the placenta (Leazer and Lassen, 2003; Pascolo et al., 2003).

An unexpected finding was that embryos treated with MK571 alone exhibit a mitotic delay seen as a retardation of progression through anaphase at 10 μM MK571 and overall retardation at higher levels of the inhibitor. One possibility is that the effect is a non-specific inhibition of one of the enzymes involved in mitotic progression, such as polo or aurora kinase. A second possibility is that the transporter is pumping out some generally toxic substance produced by metabolic activity and when this is not removed cell activity is retarded with anaphase being particularly sensitive. A reasonable candidate in this regard is 4-hydroxynonenal which is a toxic lipid peroxidation product associated with free radical production and which is a known mrp substrate (Renes et al., 2000). A third possibility is that there is some regulatory substance that needs to be removed in order for the cell to enter anaphase. Regulatory proteins are released by the kinetochore and most emphasis has focused on inductive roles of these agents; perhaps, there is also a negative

agent that must be effluxed from the cell for anaphase to commence.

Possible candidates for such a negative agent in sea urchins are leukotrienes, which are derived from arachidonic acid oxidation by specific lipoxygenases and which are mrp substrates (see Funk, 2001 for review). Indeed, the active moiety of MK571 is a leukotriene analog and MK571 can also act as a leukotriene receptor antagonist, but the K_d for the receptor antagonism is in the 10 nM range (Lynch et al., 1999), whereas transport inhibition occurs in the 10-μM range which is also where the effect on mitosis is observed. Leukotrienes have signal transduction roles and most work has focused on them as paracrine effectors (Funk, 2001).

If leukotrienes are involved in regulating mitosis in the one cell stage of the sea urchin embryo they most likely act intracellularly, perhaps as some part of a checkpoint, and efflux from the cell then allows progression through the cell cycle. Silver has noted changes in leukotrienes during the cell cycle in sand dollar eggs and posited an effect on nuclear envelope breakdown (Silver, 2001). Perry and Epel (1985) and Hawkins and Brash (1997) have described lipoxygenase activities in sea urchin eggs which similarly point to active arachidonic acid metabolism in these cells. However, a specific 5-lipoxygenase has not been identified.

This study points to dual roles for mrp transporters in development. One is the well-known effect on toxicant efflux. The other is a possible role in pumping out endogenous compounds as part of cell regulation. Such roles have recently been described for establishing auxin gradients in *Arabidopsis* (Friml et al., 2003) and differentiation in *Dictyostelium* (Good and Kuspa, 2000).

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