

Michelob_x is the missing inhibitor of apoptosis protein antagonist in mosquito genomes

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Apoptosis is implicated in the life cycle of the malaria parasite in mosquitoes. The genome project for the primary malaria vector *Anopheles gambiae* showed a significant expansion of the inhibitor of apoptosis protein (IAP) and caspase gene families in comparison with *Drosophila*. However, because of extensive sequence divergence, no orthologue was identified for the reaper/grim-like IAP antagonist genes that have a pivotal role in cell death regulation in *Drosophila*. Using a customized searching strategy, we identified *michelob_x(mx)*, a gene not predicted by the genome project, as the missing IAP antagonist in the *An. gambiae* and other mosquito genomes. Mx has a highly conserved amino-terminal IAP-binding motif. Expression of Mx induces rapid cell death in insect cell lines and is a potent tissue ablator *in vivo*. Its proapoptotic activity is totally dependent on the IAP-binding motif. Like reaper in *Drosophila*, *mx* is transcriptionally induced by ultraviolet irradiation to mediate cell death.

Keywords: reaper; grim; apoptosis; *Anopheles gambiae*; *Aedes aegypti*

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INTRODUCTION

Controlled cell death by apoptosis has an important role in insect host defence against parasites and other pathogens. In mosquitoes, apoptosis of midgut epithelial cells is observed after cell invasion by the *Plasmodium* ookinete, an essential step in the transmission life cycle of the malaria parasite (Abraham & Jacobs-Lorena, 2004). The destruction of the cell during apoptosis is initiated by the activation of caspases, which is controlled by several inter-related pathways (Danial & Korsmeyer, 2004). Mainly through

studies on *Drosophila melanogaster*, we know that inhibitor of apoptosis proteins (IAPs) and IAP antagonists have a principal role in regulating cell death in insects. IAPs inhibit the activity of caspase. This inhibition is removed by the IAP antagonist after its binding to IAP (Palaga & Osborne, 2002).

In *D. melanogaster*, deletion mutants that involve the three IAP antagonists reaper, hid and grim, essentially all programmed cell death is blocked and the cell death response to cytotoxic stimuli is strongly impeded (White *et al*, 1994). Reaper, Hid, Grim (RHG) proteins share little overall sequence similarity except for a short, 7-amino-acid IAP-binding motif (IBM) at their amino-terminus. IBM binds to the surface groove formed in the baculoviral IAP repeats (BIR) domains of IAP, and thus releases caspases from inhibition by IAP (Wu *et al*, 2001). The functional mechanism of IBM is highly conserved. The binding of the IBM from human IAP antagonist Smac to Xiap is structurally similar, if not identical, to the binding of insect IBM to Diap1 (Wu *et al*, 2001).

Although most IAPs and caspases are ubiquitously expressed, Reaper/Grim-like IAP antagonists have restricted expression. During normal development, they are expressed in cells destined to die. These proapoptotic genes are also transcriptionally activated to mediate cell death in response to cytotoxic stimuli such as ionizing irradiation. As the IBM is at their extreme N terminus, the nascent proteins have their IBM exposed because of the removal of methionine in eukaryotic cells. This is in contrast with the other class of IAP antagonists, such as Smac and caspase 9, that require post-translational cleavage to expose their IBM. Although both classes of IAP antagonists exist in *Drosophila*, the Reaper/Grim-like IAP antagonists have not been identified outside the *Drosophila* genus, with the exception of a Reaper orthologue in the blowfly (Chen *et al*, 2004). This is apparently because of the rapid divergence of these proteins, as well as the small size of the signature IBM. The absence of reaper/grim-like genes in mosquitoes left a significant lacuna in our comprehension of cell death regulation in these disease-transmitting insects. Using a customized search strategy, we identified potential reaper/grim-like genes in mosquito genomes. Structural and functional comparison of Mx in mosquitoes versus Reaper/Grim in fruitflies showed interesting insights into the function and evolution of this family of proteins.

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RESULTS AND DISCUSSION

The *Anopheles gambiae* genome project identified 12 caspases and 7 IAPs, representing a significant increase compared with *Drosophila*, which has 7 caspases and 4 IAPs (Christophides *et al*, 2002). Four of the mosquito IAPs seem to be orthologues of Diap1, the *Drosophila* IAP that binds to RHG proteins and has a central role in regulating caspase activation and cell death (Palaga & Osborne, 2002). The significant increase of caspases and death-regulating IAP genes may reflect the functional requirement of fine-tuning cell death in response to parasites and viruses commonly encountered as a consequence of blood feeding on infected vertebrates. However, the key regulators of this pathway, the IAP antagonists such as Reaper and Grim in *Drosophila*, were not identified because of 'rapid sequence diversification' (Christophides *et al*, 2002). To circumvent this problem, we first identified orthologues of RHG proteins in distantly related *Drosophila* species such as *Drosophila virilis* and *Drosophila mojavensis*. Using the orthologue sequences, we were able to build a hidden Markov model (HMM)-based profile for the IBM motif (Bailey & Elkan, 1994). A motif search program (Zhou *et al*, 1999) was then customized to search for potential open reading frames (ORFs) in the mosquito genome that have an IBM, immediately following the methionine. Several putative matches were identified among genomic or expressed sequence tags (ESTs) of *An. gambiae* and were found to be conserved in *Aedes* mosquitoes as well. One of them, *michelob_x(mx)*, was chosen for functional characterization because of the presence of ESTs in *Aedes aegypti* complementary DNA libraries made from animals fed with virus-contaminated blood.

Using the sequence information for primer design, genomic fragments of around 900 base pairs that contained *mx* were amplified from the genomic DNA of *Aedes albopictus* and *An. gambiae*. Although both the amplified fragments contained an intron, transfecting either fragment into *Drosophila* S2 cells was sufficient to induce rapid cell death, which could be blocked by *diap1*. The intron-less *Ae. albopictus mx* cDNA was then obtained through reverse transcription-PCR (RT-PCR), using RNA extracted from C6/36 cells treated with 254 nm ultraviolet (UV) irradiation, and used for functional characterization of *Mx*. In addition, *mx* cDNA clones for *Ae. aegypti* were identified by searching The Institute for Genomic Research (TIGR) *Ae. aegypti* Gene Index (<http://www.tigr.org>). The expression of *mx* in *An. gambiae* was also verified by identifying a corresponding EST (BM653729) from the A.Gam.ad.cDNA1 library as well as RT-PCR testing of RNA samples extracted from adult animals.

Examination of the sequences indicated that *Mx* proteins from the three mosquito genomes have an invariable N-terminal IBM that is almost identical to that of the *Drosophila* Grim protein (Fig 1). As expected, sequence alignment outside the IBM region showed little overall similarity. However, two structural features beyond local amino-acid identity are worth mentioning. First, there is a polyglutamine stretch of 10–20 residues immediately following the IBM (Fig 1). This feature was also observed for Grim, and is conserved among *Mx* proteins from different mosquito species. The length of this polyglutamine region varies in different species and its function remains unclear. Second, the carboxy-terminal region of *Mx* is rich in arginines and lysines, a feature that is shared by Reaper but not Grim.

Our results suggest that it is inappropriate to designate *Mx* as the orthologue of Reaper or Grim because it lacks a Grim helix 3 (GH3) domain. That is, sequence alignment as well as motif analysis using MEME (Multiple EM for Motif Elicitation) failed to identify a discernible GH3 motif in the *mx* sequence. The GH3 domain, shared by *Drosophila* IAP antagonists Grim, Reaper and Sickie, can induce cell death in the absence of the IBM motif. The proapoptotic activity of GH3 is mediated either through induction of IAP degradation (Olson *et al*, 2003) or by the release of cytochrome *c* from mitochondria (Claveria *et al*, 2002). It has been found that the region connecting IBM and GH3 in Reaper has similarity with a family of nonstructural viral proteins and can inhibit cellular protein translation (Colon-Ramos *et al*, 2003). This region does not seem to be present in *Mx*.

When *mx* was expressed in *Drosophila* S2 cells or in the mosquito C6/36 cells (*Ae. albopictus*), it induced cell death within 20 h of transfection, similar to what has been observed for *Drosophila* IAP antagonists. However, unlike Reaper and Grim, which retain proapoptotic ability even when their N-terminal IBM is removed, the proapoptotic activity of *Mx* is totally dependent on its N-terminal IAP-binding motif (Fig 2A). When this motif, composed of amino acids 2–8, was removed, the killing ability of *Mx* was abolished (Fig 2A,B). This is not due to protein stability, as there were much higher levels of mutant *Mx*(–IBM) protein than wild-type *Mx* (Fig 2B). As the remaining proapoptotic activity of Reaper and Grim, both without IBM, is mainly mediated by their GH3 domains, the fact that *Mx* minus IBM has no proapoptotic activity verifies the functional absence of a GH3 motif.

The only *Drosophila* IAP antagonist that does not have a functional GH3 motif is head involution defective (*Hid*). Like *Mx*, *Hid* minus IBM has no cell-killing function (Vucic *et al*, 1998). However, we do not believe that *Mx* is the orthologue of *Hid*, for two reasons. First, the IBM of *Mx* (AIAF) is the signature for Reaper/Grim-like IAP antagonists (Zhou, 2005). Second, our search identified another potential IAP antagonist (*Michelob_y*) in the mosquito genomes (L.Z., unpublished data) that has sequence features more reminiscent of *Hid* and Sickie, although functional analyses have yet to be performed.

To test the proapoptotic activity of *Mx in vivo*, we generated a transgenic fly strain carrying P{UAS-*mx*}. The P52Gal4 insertion directs Gal4 expression in a set of discrete cells, including neurons and glia in the central nervous system midline as well as some ventral epidermal cells (Melnattur *et al*, 2002). This Gal4 strain has been used successfully in characterizing functional interaction as well as the functional difference of various *Drosophila* IAP antagonists (Zhou *et al*, 1997; Wing *et al*, 1998). When the P{UAS-*mx*} strain was crossed with the P52Gal4:UAS-*lacZ* strain, the progenies carrying both p{UAS-*mx*} and P52Gal4:UAS-*lacZ* were embryonic lethal. Anti- β -galactosidase (β -Gal) staining showed that many epithelial cells, as well as the ventral unpaired median neurons expressing P52Gal4, were eliminated by *mx* (Fig 2D–G). Expression of *reaper* or *hid* alone in this context induces little cell death, and only when both are expressed together can significant tissue ablation be achieved (Zhou *et al*, 1997). In this regard, *mx* is similar to *grim*, in that expression by itself is able to cause tissue ablation *in vivo* (Wing *et al*, 1998). It should be noted that the ability of individual IAP antagonists to induce cell death differs markedly, depending on tissue types and developmental

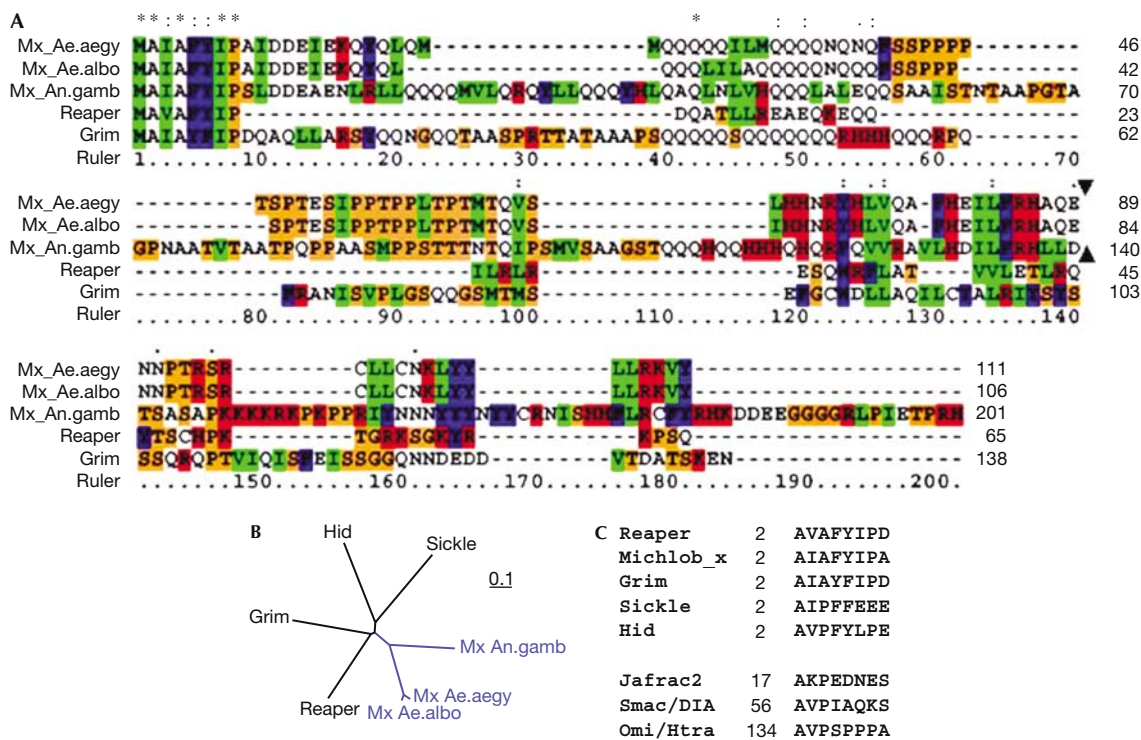


Fig 1 | Comparison of Mx sequences with Reaper and Grim. (A) Alignment of Mx sequences from *Anopheles gambiae* (An.gamb), *Aedes albopictus* (Ae.albo) and *Aedes aegypti* (Ae.aegy), and Grim (NP_524137) and Reaper (NP_524138) sequences from the fruitfly. The relative position of the single intron (indicated with solid triangle) in *mx* genes is conserved between *Anopheles* and *Aedes*. There are no introns in *grim* or *reaper*. The colour scheme reflects percentage identity among the five aligned sequences (red, 100%; blue, > 75%; and green, > 50%). (B) Protein distance tree showing that Mx is slightly closer to Reaper and Grim than to Hid and Sickle. The scale bar reflects the rate of nucleotide change per position. (C) Comparison of the inhibitor of apoptosis protein (IAP)-binding motifs in fly (Reaper, Grim, Hid (NP_524136), Sickle (NP_524139) and Jafrac2 (Q9V3Q4)), mosquito (Michlob_x) and human (Smac/IDIA (NP_063940), Omi/Htra (O43464)) IAP antagonists. Numbers indicate the position of the first Ala in the host proteins.

stages. For instance, expression of *hid* alone in the eye disc during the third-instar larval stage was able to induce significant cell death (Grether *et al*, 1995).

As in *Drosophila* IAP antagonists, proapoptotic activity of Mx is dependent on the cellular level of IAPs (Fig 3A). Cell death induced by Mx is blocked when the DNA ratio for *mx* and *diap1* is 1:2. By contrast, a ratio of 1:5 failed to block completely the induction of cell death by Grim or Reaper, presumably owing to the fact that GH3 can induce cell death through IAP degradation (Colon-Ramos *et al*, 2003) or through IAP-independent mechanisms (Holley *et al*, 2002; Yoo *et al*, 2002). In this sense, Mx functions similar to the GH3-less Reaper(107), which has two helix-disturbing substitutions in the GH3 domain. Cell death induced by Mx is also efficiently blocked by expressing p35, a caspase inhibitor from a baculovirus.

A direct interaction between Mx and IAP is verified by co-immunoprecipitation assay. Although Mx interacts directly with Diap1 when both are expressed in mammalian cells, there is no interaction when the IBM is removed (Fig 3B), indicating that the interaction between Mx and Diap1 is mediated through the IBM. Expression of Mx in mammalian cells (H1299 and MCF7) induces cell death within 24 h of transfection. Collectively, this evidence indicates that Mx induces cell death by removing the inhibitory

effect of IAP on caspase activation, and probably acts solely through this mechanism. The absence of a GH3 domain in Mx may indicate that the joining of both GH3 and IBM in *Drosophila* IAP antagonists occurred after the separation of *Drosophila* and mosquito lineages about 250 million years ago (Rohdendorf, 1974). This is a likely scenario given that a protein that has only a GH3 domain but does not have an IBM was recently identified in *Drosophila*, which has functional interaction with the GH3-less IAP antagonist Hid (L. Zhou, in preparation). Identifying orthologues of Reaper/Mx from other insects or vertebrates should provide further insight into this hypothesis. Alternatively, it is possible that GH3 was lost during the evolution of Mx in mosquitoes, after mosquito/*Drosophila* divergence.

An important characteristic of *reaper*-like IAP antagonist genes is that they are regulated at the transcriptional level. During development, *reaper*, *grim* and *sickle* are expressed specifically in cells just before their genetically programmed death. Expression of *reaper*, *sickle* and *hid* is also induced after cytotoxic stimuli such as X-ray or UV treatment. To test whether *mx* is also transcriptionally regulated, we treated the mosquito C6/36 cell line with 10 mJ/cm² UV and monitored the expression of *mx* by quantitative PCR. A significant increase in *mx* was observed 30 min after the treatment, and after 1 h the level of *mx* in irradiated cells was more

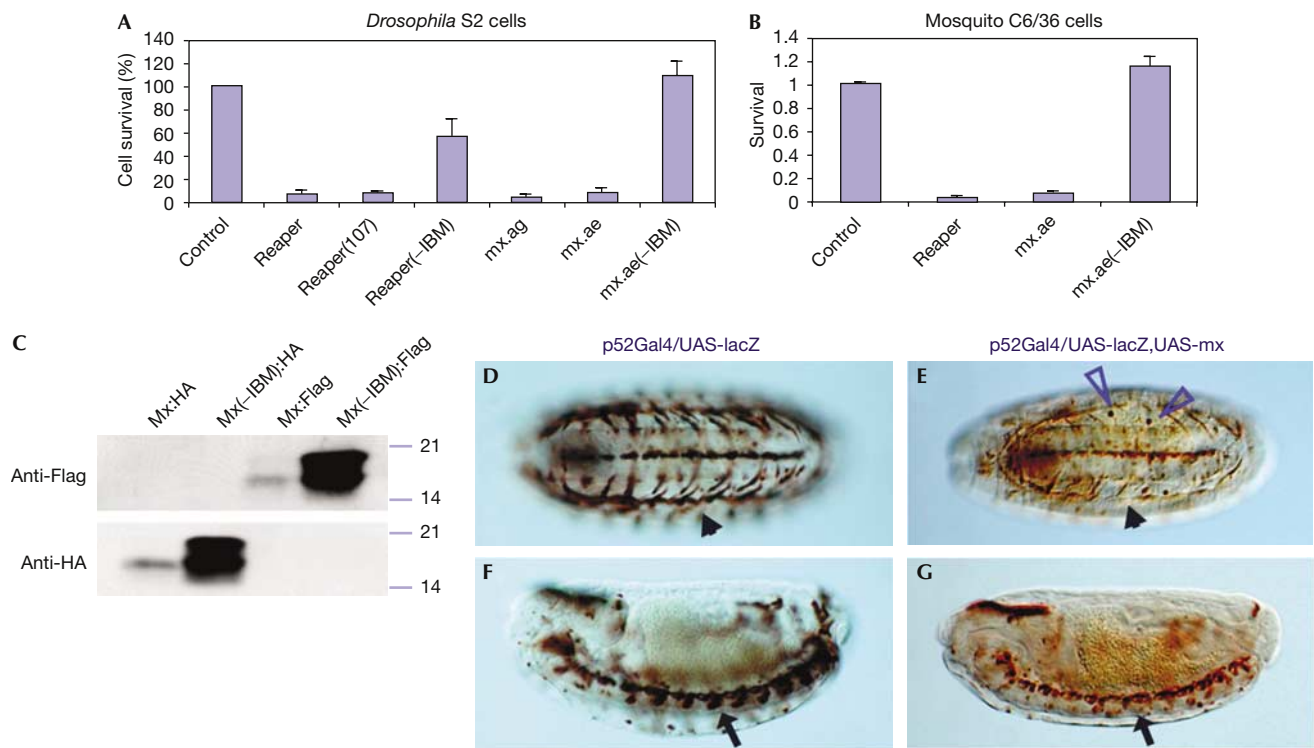


Fig 2 | The IAP (inhibitor of apoptosis protein)-binding motif (IBM) is required for the proapoptotic activity of Mx. (A) Expression of Mx in S2 cells induces cell apoptosis similar to Reaper, or Reaper(107), which has two helix-disturbing substitutions in its GH3 (Grim helix 3) domain (Leu35Pro and Ala36Pro). Although Reaper (-IBM) retained partial proapoptotic activity, removal of the IBM (amino acids 2–8) from Mx totally abolished its proapoptotic activity. Error bars represent the standard error of 4–5 measurements. *mx.ag*, *mx* cloned from *Anopheles gambiae*; *mx.ae*, *mx* cloned from *Aedes albopictus*; *mx* from *Aedes aegypti* behaved the same as the two presented here (data not shown). (B) Expression of *mx* induced cell death in mosquito C6/36 cells, which is dependent on the IBM domain. Mx from *Ae. albopictus* was used. (C) Western blot analysis of cell lysates from transfected H1299 cells. At 48 h after transfection, there is significant accumulation of mutant protein Mx(-IBM) in cells transfected with pRK5-Mx(-IBM):HA (haemagglutinin) or pRK5-Mx(-IBM):Flag. In contrast, there is little accumulation of the wild-type protein in cells transfected with pRK5-Mx:HA or pRK5-Mx:Flag. Equal amounts of whole-cell lysates were loaded on all lanes. (D–G) Expression of *mx* in transgenic fly strain induces cell/tissue ablation. P52Gal4 is an enhancer trap insertion in the GP150 gene (Melnattur *et al*, 2002). It guides the expression of UAS-lacZ in ventral musculature (arrowhead in (D), ventral view, stage 15) as well as in the central nervous system midline glia and neurons (arrow in (F), sagittal view, stage 15). Expression of *mx* (E,G) caused the elimination of muscle cells; the arrowhead in (E) (ventral view, stage 15) points to the empty space that should be occupied by the β -galactosidase (β -Gal)-expressing muscle cells (compare with (D)). Corresponding with the disappearance of β -Gal-positive muscle cells is the emergence of round β -Gal-positive apoptotic inclusion bodies (blue triangles in E) inside migrating microphages. Similarly, expression of *mx* also eliminated midline neurons (arrow in G, compare with F).

than fourfold that in control cells (Fig 4). This induction of *mx* by UV was followed by massive apoptosis that initiated about 2 h after treatment. Introducing double-stranded RNA (dsRNA) for *mx* through transient transfection before UV irradiation significantly suppressed UV-induced cell death. This suppression of cell death is significant at a low dosage (1 mJ/cm² UV). This is not surprising, as studies in *Drosophila* have shown that several cell-death regulatory genes are induced by irradiation to mediate cell killing. The relatively low transfection efficiency (5–10%) prevented us from measuring the extent to which *mx* expression is suppressed by RNA interference. However, even if dsRNA caused a complete block of *mx* expression, cell death can still proceed if the level of other proapoptotic genes is high enough. This may explain why, at higher dosages (>5 mJ/cm² UV), dsRNA for *mx* is not able to rescue the cells (Fig 4B). This evidence suggests that, similar to what we know about cell-death regulation in *Drosophila*, cell

death in mosquitoes is also controlled by several regulatory genes. Identifying the other components of the cell-death regulatory network will be necessary for a comprehensive understanding of cell-death regulation in mosquitoes.

In summary, using a customized motif search strategy, we have successfully identified and characterized the first IAP antagonist, *mx*, in mosquito genomes. Although the Mx IBM region is nearly identical to that of the *Drosophila* Grim protein, it lacks a GH3 domain, indicating evolutionary diversification among the mosquito and *Drosophila* lineages. Furthermore, removal of the *mx* IBM completely abolishes its apoptotic activity. Like *reaper*, *mx* is also transcriptionally regulated to induce cell death. At present, a role for Mx in regulating cell death during virus and parasite infection in mosquitoes is unknown. A further analysis of the functional role of *mx* should provide more insight into pathogen–host interaction and cell-death regulation in mosquitoes. It is

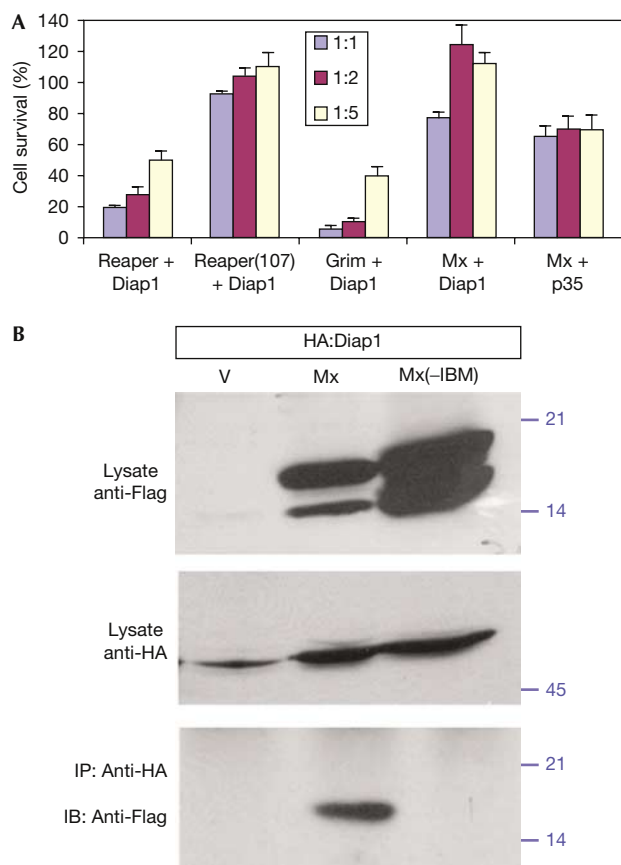


Fig 3 | Mx-induced cell death is blocked by Diap1 and P35. (A) Unlike Reaper- or Grim-induced cell death, which cannot be totally blocked by Diap1, cell death induced by Mx is readily blocked by Diap1, the principal death-inhibiting IAP (inhibitor of apoptosis protein) from the fruitfly, and by p35, a viral caspase inhibitor. In this regard, it is similar to Reaper(107), which has two helix-disrupting substitutions in the GH3 domain. The colour of the bars reflects the ratio of the two testing constructs in a total of 0.9 μ g per sample. Error bars represent the standard error of repeated measurements ($n=3$). (B) Direct interaction between Diap1 and Mx is detected by co-immunoprecipitation (IP). This interaction is abolished when the IBM (amino acids 2–8) is removed from Mx. NCI-H1299 cells were transfected with 1 μ g of pRK5-HA (haemagglutinin):Diap1 along with 3 μ g of pRK5-Mx:Flag, or pRK5-Mx(IBM):Flag, or empty vector. At 48 h after transfection, cells were lysed. Equal amounts of whole-cell lysates were immunoprecipitated with an anti-HA antibody (12CA5). The immunoprecipitate was resolved by SDS-polyacrylamide gel electrophoresis and transferred to the membrane, followed by immunoblotting (IB) with anti-Flag.

worth noting that because *mx* was not predicted by the *An. gambiae* genome project, there is no probe set for this gene in the popular 'Plasmodium/Anopheles Genome Array' manufactured by Affymetrix. Gene expression profiling using this DNA array would have totally missed information on important cell-death regulatory genes such as *mx*. This serves as a reminder that the efficacy of genomic analysis can only be as good as our understanding of the genome.

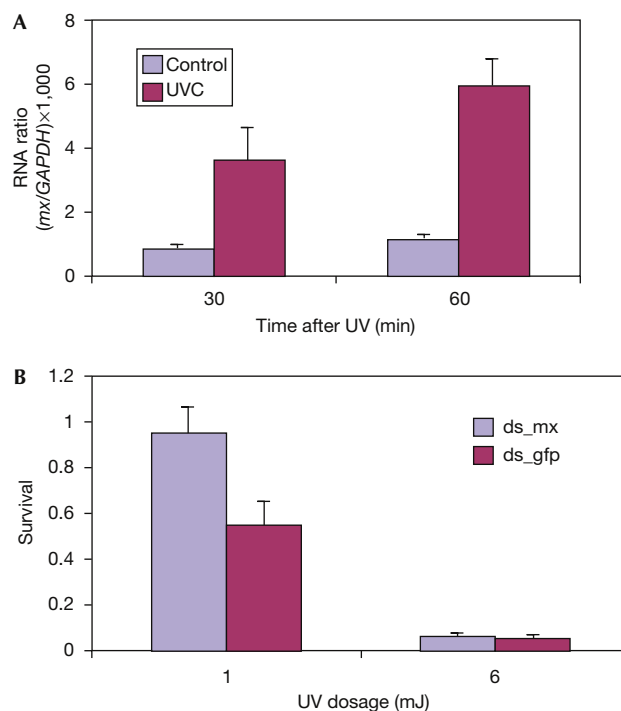


Fig 4 | *mx* is transcriptionally upregulated by ultraviolet light to mediate cell death. (A) *mx* RNA is rapidly induced after ultraviolet (UV) treatment of C6/36 mosquito cells. Units are relative to the messenger RNA level of the housekeeping gene *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase). Error bars represent standard errors ($n=3$). (B) Transfection of double-stranded *mx* RNA (*ds_mx*) suppresses UV-induced cell death in C6/36 cells. Double-stranded green fluorescent protein (GFP) RNA (*ds_gfp*) was used as a control. Transfection of dsRNA and DNA was followed by 2 h recovery before UV treatment. Error bars represent standard errors ($n=4$ for 1 mJ and $n=3$ for 6 mJ).

Speculation

It is possible that *mx*, like *reaper* in *Drosophila*, has a pivotal role in regulating cell death during development and in response to environmental stress and infection.

METHODS

Data-mining strategy. Models and scoring matrices for the IBM motifs were built with the MEME program (Bailey & Elkan, 1994), and tested empirically by searching the Swiss-Pro annotated protein data set (see Supplementary information III online). Genomic sequences for *Anopheles* and *Aedes* were obtained from Ensembl and TIGR, respectively. A motif search program implemented in C was customized to search for the IBM motifs in the genomic as well as EST sequences from mosquito genomes (Zhou et al, 1999).

Cloning *mx*. Genomic DNA flanking the predicted ORF of *mx* was first amplified from *Ae. albopictus* and *An. gambiae* genomic DNA and tested for cell-killing ability in S2 cells. An intron-less cDNA was then obtained by RT-PCR using RNA extracted from UV-irradiated C6/36 mosquito cells. Other *mx* cDNA clones were isolated from the *Ae. aegypti* cDNA libraries.

RNA interference assay in C6/36 cells. Transcription, annealing and purification of dsRNA were carried out using the MEGAscript RNAi kit from Ambion (Austin, TX, USA). dsRNAs were transfected to C6/36 before UV irradiation.

(For a more detailed description of methods, as well as additional sections on *in vivo* and *in vitro* cell death assay, UV treatment of C6/36 cells, co-immunoprecipitation, and western blot analysis, please refer to supplementary information I online.)

Supplementary information is available at *EMBO reports* online (<http://www.emboareports.org>).

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