Effects of Manipulating Apoptosis on Sindbis Virus Infection of *Aedes aegypti* Mosquitoes

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Improved control of vector-borne diseases requires an understanding of the molecular factors that determine vector competence. Apoptosis has been shown to play a role in defense against viruses in insects and mammals. Although some observations suggest a correlation between apoptosis and resistance to arboviruses in mosquitoes, there is no direct evidence tying apoptosis to arbovirus vector competence. To determine whether apoptosis can influence arbovirus replication in mosquitoes, we manipulated apoptosis in *Aedes aegypti* mosquitoes by silencing the expression of genes that either positively or negatively regulate apoptosis. Silencing of the *A. aegypti* anti-apoptotic gene *iap1* (*Aeiap1*) caused apoptosis in midgut epithelium, alterations in midgut morphology, and 60 to 70% mosquito mortality. Mortality induced by *Aeiap1* silencing was rescued by cosilencing the initiator caspase gene *Aedronc*, indicating that the mortality was due to apoptosis. When mosquitoes which had been injected with *Aeiap1* double-stranded RNA (dsRNA) were orally infected with Sindbis virus (SINV), increased midgut infection and virus dissemination to other organs were observed. This increase in virus infection may have been due to the effects of widespread apoptosis on infection barriers or innate immunity. In contrast, silencing the expression of *Aedronc*, which would be expected to inhibit apoptosis, reduced SINV midgut infection and virus dissemination. Thus, our data suggest that some level of caspase activity and/or apoptosis may be necessary for efficient virus replication and dissemination in mosquitoes. This is the first study to directly test the roles of apoptosis and caspases in determining mosquito vector competence for arboviruses.

Each year over 500 million people are infected with mosquito-borne diseases such as malaria, dengue fever, and yellow fever, which cause significant morbidity and mortality worldwide (39, 40). Moreover, traditional mosquito control measures are in danger of becoming less effective due to increasing levels of insecticide resistance (17, 27). These issues highlight the necessity for more sophisticated strategies for mosquito vector control in the prevention of mosquito-borne diseases. One potential approach to vector control is to interrupt vector competence for pathogens by genetic manipulation of mosquito vectors, but this approach requires more detailed knowledge of the molecular interactions between vectors and pathogens.

Sindbis virus (SINV) is the type species of the genus *Alphavirus* in the virus family *Togaviridae* and is associated with occasional outbreaks of human disease in Africa, Europe, and Asia. While SINV was originally isolated from *Culex* mosquitoes (32), a large number of mosquito species are able to act as vectors for SINV in nature, including species from the genera *Culex*, *Culiseta*, *Ochlerotatus*, *Aedes*, and others (13). SINV is an important tool for studying the interactions between arboviruses and mosquitoes, both because the molecular biology of SINV replication has been studied extensively and because SINV can be transmitted in the laboratory by *Aedes aegypti*, which is one of the main vectors responsible for transmission of dengue and yellow fever viruses. Thus, even though *A. aegypti* is not considered an important natural vector for SINV, the *A. aegypti*-SINV pair has been studied extensively as a model to understand arbovirus-mosquito interactions.

We are only beginning to learn about antiviral defense mechanisms in mosquitoes at the cellular level (reviewed in reference 9). The best-studied mechanism identified to date is RNA interference (RNAi). During replication of the viral RNA genome, double-stranded RNA replication intermediates are formed and

recognized by the RNAi machinery, stimulating degradation of the viral RNA. Studies have found that the replication of several arboviruses, including dengue fever virus (10, 29), o'nyong-nyong virus (15), and SINV (4, 5), is repressed by mosquitoes through activation of the RNAi machinery. In addition to RNAi, the Toll pathway may be involved in the antiviral defense. Components of the Toll pathway are upregulated in *A. aegypti* after a SINV blood meal (30), and reducing or activating the Toll signaling pathway affects dengue virus replication in *A. aegypti* (41). After SINV infection, transcript levels of JNK pathway genes and several serpin genes are altered (30). Also, after o'nyong-nyong virus infection of *Anopheles gambiae*, the gene encoding the heat shock protein cognate 70B was upregulated, and silencing this gene resulted in a higher level of virus replication (31).

Apoptosis has been shown to act as an antiviral defense in certain mammalian and insect systems, although most examples involve DNA viruses rather than RNA viruses (6, 18). Many arboviruses, which are almost all RNA viruses, induce apoptosis as a natural consequence of infection in vertebrate cells but tend to cause nonlytic, persistent infections in mosquito cells (14). However, there are several intriguing observations indicating that

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apoptosis can occur during arbovirus infection in mosquitoes. Cytopathology, sometimes with features of apoptosis, has been observed in the midgut and/or salivary gland after infection with arboviruses (2, 20, 37, 38). In one case, apoptosis in the midgut was correlated with resistance to infection by West Nile virus (33), while in another case, apoptosis occurring in salivary glands late after infection correlated with reduced virus transmission (11, 12). Furthermore, engineering infectious SINV cDNA clones to express proapoptotic genes converted SINV infection of cultured mosquito cells from persistent to lytic, thereby reducing long-term production of virus associated with persistent infection (35, 36). The midgut and salivary gland are known to be potential barriers for arboviruses (1), but the mechanisms involved in penetrating these tissues are not understood. Any physical changes in these barriers, such as increased cell death associated with virus infection, could potentially have either positive or negative effects on virus replication and dissemination. However, no causative data exist that directly link apoptosis to effects on virus vector competence in mosquitoes.

The molecular pathways that regulate apoptosis in insects have been studied most thoroughly in *Drosophila* (for a review, see reference 44), but recent studies indicate that the process is regulated similarly in *A. aegypti* (3, 16, 36). The core apoptosis pathway in *A. aegypti* consists of the initiator caspase Dronc (AeDronc) and its adaptor protein AeArk, the effector caspases CASPS7 and CASPS8, the inhibitor-of-apoptosis protein AeIAP1, and the IAP antagonist proteins AeMichelob_x and IMP. Based on these recent studies (3, 16, 36) and extrapolating from what is known in *Drosophila*, a model has emerged where in unstimulated cells, AeDronc is activated constitutively by AeArk, but levels of active AeDronc are normally kept in check by AeIAP1, which can inhibit AeDronc as well as CASPS7 and CASPS8. Following an apoptotic stimulus, AeIAP1 levels are depleted due to its interaction with AeMichelob_x and IMP. Active AeDronc then accumulates and activates CASPS7 and CASPS8, leading to death of the cell.

Consistent with this model, silencing of *Aeiap1* by double-stranded RNA (dsRNA) induces rapid and dramatic apoptosis in the *A. aegypti* cell line Aag2, while silencing of *Aedronc* in Aag2 cells suppresses apoptosis triggered by a variety of stimuli (16). It has also been shown that topical application of *Aeiap1* dsRNA causes high mortality in adult *A. aegypti* mosquitoes (26) and that environmental stress leads to increased *Aeiap1* transcript levels in *A. aegypti* (25). These results support the idea that AeIAP1 may play an important role in apoptosis *in vivo*. In this study, we directly tested the effects of inducing or inhibiting apoptosis on virus replication and dissemination across midgut and salivary gland barriers. We found that silencing of *Aeiap1* in adult female mosquitoes directly induced high mortality, similar to what was previously reported. Furthermore, we show for the first time that silencing *Aeiap1* leads to apoptosis *in vivo* and alteration of midgut morphology. When mosquitoes with silenced *Aeiap1* or *Aedronc* expression were infected with SINV, we found that silencing of *Aeiap1* caused increased SINV infection and dissemination, while silencing of *Aedronc* caused decreased SINV infection, providing the first direct evidence that apoptosis can affect vector competence in mosquitoes.

MATERIALS AND METHODS

Insect rearing. *A. aegypti* Rexville D mosquitoes, obtained from Carol Blair (Colorado State University), were reared at 27°C and 80% humidity

under a 12-h light/12-h dark regimen. Adults were maintained on 10% sucrose solution, sugar, raisins, and fresh water. Naive adult females were collected at 1 day posteclosion for use in RNAi experiments.

Gene silencing by RNAi. The full-length coding sequences of *Aeiap1*, *Aedronc*, or the chloramphenicol acetyltransferase gene (*cat*; used as a negative control) were PCR amplified with T7 polymerase promoter sites incorporated onto both ends. The PCR products were used as templates to synthesize double-stranded RNA (dsRNA) using an AmpliScribe T7 high-yield transcription kit (Epicentre Biotechnologies). The dsRNA was concentrated to 5 µg/µl in diethyl pyrocarbonate (DEPC)-treated water, and 69 nl of the dsRNA was injected into 1-day-posteclosion females using a Nanoject II injector (Drummond Scientific). When two dsRNAs were coinjected, the two dsRNAs were mixed together at a total concentration of 5 µg/µl and delivered by a single injection of 138 nl (two pulses of 69 nl each). Three days after dsRNA injection, live mosquitoes were collected for gene expression analysis or for viral blood feeding.

SINV preparation and infection by blood meal. Stocks of SINV strain 5'dsMRE16ic-eGFP (8) were prepared by transfection of *in vitro*-transcribed, capped viral RNA into BHK cells, and titers were determined as previously described (35). Blood feeding was conducted in a closed hood within a certified Arthropod Containment Level 2 facility. Tissue culture fluid containing 5'dsMRE16ic-eGFP ($10^{6.5}$ PFU/ml) was mixed 1:1 with defibrinated sheep blood (Colorado Serum Company), warmed to 37°C, and placed in a Hemotek 5W1 membrane feeding system (Discovery Workshops), where female mosquitoes were allowed to probe and feed through a stretched sheet of Parafilm for 45 min. Fully engorged mosquitoes were collected and maintained with food and water. At 7 days after the blood meal, midguts and salivary glands were dissected, and samples were examined by fluorescence microscopy in a blind manner. To determine infection prevalence, the percentage of mosquitoes that exhibited enhanced green fluorescent protein (eGFP) expression in the midgut, foregut, and hindgut was recorded. To assess the level of virus infection, a procedure modified from that of Myles et al. (21) was used, in which infection scores were calculated by estimating the percentage of surface area of each midgut, foregut, and hindgut that was eGFP positive and multiplying this number by a factor of 1, 2, or 3 according to whether the intensity of eGFP fluorescence was low, medium, or high, respectively. For example, a midgut that was 30% infected and displayed medium fluorescence intensity was recorded as having an infection score of 30×2 , or 60. To minimize bias, samples were analyzed independently by two different individuals in a blind manner, and the results were found to be similar. To assess virus dissemination, the percentage of mosquitoes exhibiting eGFP expression in salivary glands or eyes was recorded.

Real time reverse transcription-PCR (RT-PCR). RNA was isolated from homogenized tissues of pools of 10 individual mosquitoes using Trizol (Invitrogen) and treated with Turbo DNA-free DNase (Ambion). Equal amounts of RNA (1 to 3 µg depending on the transcript being analyzed) were used to synthesize cDNA using reverse transcriptase (Promega) and oligo(dT) primer. The resulting cDNA was analyzed for expression of *Aeiap1*, *Aedronc*, and *actin6* with previously described primers (3). Expression was initially analyzed by agarose gel electrophoresis to verify the correct size for the amplicons. Real-time PCR was performed using the Bio-Rad iCycler optical module. Cycle thresholds (C_T) were determined using iQ SYBR green Supermix according to the manufacturer's instructions (Bio-Rad). Relative expression levels were calculated using the formula $2^{-\Delta C_T}$, where $\Delta C_T = C_T(\text{Aeiap1 or Aedronc}) - C_T(\text{actin})$ (3).

Caspase assay. To detect caspase activity, mosquito tissues from 10 pooled individuals per treatment were homogenized in lysis buffer (20 mM HEPES KOH [pH 7.5], 50 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol [DTT], 250 mM sucrose) with complete mini-EDTA-free protease inhibitor (Roche Applied Science). Protein concentration was determined by absorbance at 280 nm, and 50 µg of protein was mixed in 100 µl reaction buffer (100 mM HEPES [pH 7.4] containing 2 mM DTT, 0.1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfon-

ate [CHAPS], 1% sucrose) with 200 μ M Ac-DEVD-AFC (MP Biomedical), an effector caspase substrate, and incubated for 15 min at 37°C. Fluorescence (excitation, 405 nm; emission, 535 nm) was monitored as previously described (35).

Transmission electron microscopy (TEM). Dissected mosquito midguts were fixed in 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2 to 7.4) for 16 h at room temperature with constant rotation. Samples were washed (3 times for 5 min each time) in 0.1 M sodium cacodylate buffer at room temperature and then postfixed in 2% osmium tetroxide in 0.1 M sodium cacodylate buffer at room temperature with constant rotation. Samples were dehydrated in an ascending acetone series (50 to 100%), infiltrated with Embed 812/ Araldite resin at room temperature with constant rotation, and then embedded in flat molds. The resin was cured in a drying oven at 60°C for 24 h. Silver to gold sections were cut on a Leica Ultracut-S ultramicrotome and placed on 200-mesh copper grids. Images were collected with a CM 100 (FEI Company) transmission electron microscope.

Light microscopy. Purple to green, semithick (200 to 250 nm) sections of embedded TEM samples were heat fixed onto glass microscope slides. Sections were then stained with epoxy tissue stain (Electron Microscopy Sciences) containing toluidine blue and basic fuchsin and viewed on a Zeiss Axioplan2 upright microscope.

RESULTS

Silencing *Aeiap1* causes mortality in *Aedes aegypti* mosquitoes, which can be rescued by silencing *Aedronc*. Silencing *Aeiap1* in *A. aegypti* Aag2 cells causes spontaneous apoptosis, while silencing of *Aedronc* protects Aag2 cells from apoptosis induced by UV or cytotoxic drugs (16). A recent study reported that topically applied *Aeiap1* dsRNA causes mortality of adult female *A. aegypti* (26), but the mechanism of death was not investigated. We hypothesized that the mortality observed in mosquitoes following *Aeiap1* RNAi was due to widespread apoptosis in the mosquito tissues.

To test this hypothesis, we utilized adult *A. aegypti* females of the Rexville D strain, which has been used extensively in studies with SINV (22–24). Adult female Rexville D mosquitoes were injected with 345 ng dsRNA corresponding to either *Aeiap1*, *Aedronc*, or a negative-control sequence not present in the *A. aegypti* genome (the bacterial *cat* gene) and monitored for survival. *Aeiap1* dsRNA caused rapid and significant mosquito mortality, with approximately 45% survival after only 1 day and 30 to 40% survival at 14 days after injection with *Aeiap1* dsRNA (Fig. 1). When the amount of injected *Aeiap1* dsRNA was increased from 345 to 2,100 ng, there was no significant increase in mortality (data not shown).

Interestingly, there was a statistically significant difference (log rank test, $P < 0.0001$) in mortality between mosquitoes injected with *Aedronc* dsRNA and control *cat* dsRNA but not between *Aedronc* dsRNA and phosphate-buffered saline (PBS) injection (Fig. 1A). These data suggest that introduction of any dsRNA, even nonspecific dsRNA, into the hemocoel may induce a stress response, and silencing *Aedronc* helps protect against mortality caused by this stress.

To further examine the cause of the increased mortality associated with injection of dsRNA, we tested whether silencing *Aedronc* would rescue the mortality induced by silencing of *Aeiap1*. When Rexville D mosquitoes were injected with *Aeiap1* dsRNA together with *Aedronc* dsRNA, mortality was significantly reduced compared to that induced by *Aeiap1* dsRNA plus PBS (Fig. 1B). The ability of silencing *Aedronc* to protect against the mortality induced by silencing *Aeiap1* strongly suggests that apoptosis is responsible for the increased mor-

FIG 1 Survival of mosquitoes after injection of dsRNA. Rexville D mosquitoes were intrathoracically injected with the indicated dsRNAs or the same volume of PBS. Mock-injected mosquitoes were manipulated similarly to the other groups but were not injected. Each treatment was performed on 100 mosquitoes, and survival was recorded over 14 days. The survival curves were compared by the log rank test: ****, $P < 0.0001$; **, $P < 0.005$; *, $P < 0.05$.

tality due to silencing *Aeiap1* and also indicates that silencing *Aedronc* is able to inhibit apoptosis *in vivo*, similar to results obtained using Aag2 cells (16).

To verify whether the genes targeted by dsRNA were being silenced, we harvested surviving mosquitoes at 3 days after dsRNA injection and examined *Aeiap1* and *Aedronc* transcript levels. Semiquantitative RT-PCR indicated that even in the mosquitoes which had survived the treatment, the mRNA levels of *Aeiap1* in both midgut and carcass (defined as the rest of the mosquito without the midgut) were decreased compared to levels obtained after *cat* dsRNA or PBS treatments (Fig. 2A). Attempts to quantify gene knockdown by real-time RT-PCR (Fig. 2B) were complicated by variation between mosquitoes (data not shown), and presumably also by the fact that gene knockdown occurred in only a subset of cells in the injected mosquitoes. However, the resulting data indicated that the transcript levels of *Aeiap1* and *Aedronc* were signif-

FIG 2 Analysis of gene silencing. (A) Mosquitoes injected with *Aeiap1* or control *cat* dsRNA or PBS were dissected at 3 days postinjection, and total RNA from midguts or carcasses (defined as the rest of the mosquito, minus the midgut) derived from pools of 10 mosquitoes was analyzed by RT-PCR using primers for *Aeiap1* and *actin*. (B) Real-time RT-PCR was performed on RNA at 3 days postinjection from pooled midguts of mock-injected mosquitoes or mosquitoes injected with the indicated dsRNAs or PBS. Transcript levels of *Aeiap1* and *Aedronc* were analyzed in midguts by real-time RT-PCR relative to *actin*, as described in Materials and Methods. Results are means $\pm$ standard errors from two independent experiments.

icantly decreased in the midgut at 3 days after injection of their corresponding dsRNAs, compared to levels in mosquitoes injected with PBS or *cat* dsRNA (Fig. 2B). Because of the trauma associated with injection, these control injected samples are the most relevant controls. Mosquitoes injected with PBS or control dsRNA tended to have higher *Aeiap1* transcript levels and lower *Aedronc* transcript levels than mock-injected individuals (Fig. 2B), although this was not statistically significant in most cases.

Silencing *Aeiap1* induces widespread apoptosis in midgut epithelium. To directly determine whether silencing *Aeiap1* induced apoptosis in mosquitoes, we first investigated whether caspase activity was induced by injection of *Aeiap1* dsRNA, since silencing *Aeiap1* in Aag2 cells induces caspase activation and apoptosis (16). Using the effector caspase substrate Ac-DEVD-AFC, we found that midgut tissue from *Aeiap1* dsRNA-injected mosquitoes had a significantly higher level of caspase activity than the other treatments (mock injection, PBS, *cat* dsRNA, or *Aedronc* dsRNA) at 1, 2, and 3 days after injection (Fig. 3A). In the rest of the carcass, caspase activity was also increased by *Aeiap1* RNAi at 1 and 2 days after injection (Fig. 3B), but the increase in caspase activity was not as pronounced as in the midgut.

Examination of midguts from *A. aegypti* injected with *Aeiap1* dsRNA revealed that the midguts often displayed altered gross morphology and were more fragile than midguts from control mosquitoes, including ones injected with *cat* or *Aedronc* dsRNA (data not shown). To study this more closely, we examined the morphological features of the midguts by microscopy of tissue sections. In mock-injected and *cat* dsRNA-injected mosquitoes, the midgut epithelium had a normal appearance, with organized columnar epithelial cells and an intact brush border layer of microvilli (Fig. 4A and B). However, in *Aeiap1* dsRNA-injected mosquitoes, the midgut epithelium was highly disorganized and microvilli were much shorter and exhibited gaps (Fig. 4A and B).

Examination of the nuclei of midgut epithelial cells by transmission electron microscopy (TEM) also revealed the presence of condensed chromatin, a hallmark of apoptosis, in the nuclei of midgut epithelial cells from mosquitoes injected with *Aeiap1* dsRNA (Fig. 5). These results confirm that widespread apoptosis occurred in midgut epithelium after mosquitoes were injected with *Aeiap1* dsRNA.

Inducing or inhibiting apoptosis influences SINV infection and dissemination. In order to be transmitted by mosquitoes, viruses have to overcome several barriers in the mosquito, including the midgut infection and escape barriers and salivary gland transmission and escape barriers (1). Although SINV has not been shown to cause apoptosis during infection of *A. aegypti*, apoptosis has been observed in other arbovirus-mosquito combinations. Therefore, either increasing or decreasing apoptosis might be expected to influence the ability of SINV to successfully breach these barriers, either by affecting the amount of virus amplification or by affecting the physical integrity of the barriers. To test the effects of inducing or inhibiting apoptosis on SINV infection and dissemination, we injected Rexville D mosquitoes with *cat*, *Aeiap1*, or *Aedronc* dsRNAs, and 3 days later the surviving injected mosquitoes were given a blood meal containing 5' dsMRE16ic-eGFP, a strain of SINV that is efficient at midgut infection and dissemination in *A. aegypti* and expresses enhanced green fluorescent protein (eGFP) (8). At 7 days after the blood meal, the infection prevalence (percentage of infected mosquitoes) and infection patterns were examined by observing eGFP expression. No significant differences were observed between dsRNA treatments in the ability of 5' dsMRE16ic-eGFP to establish infection in the midgut, foregut, and hindgut (Fig. 6A to C, left). However, by scoring the infected midguts individually, we found that midguts from *Aeiap1* dsRNA-treated mosquitoes had significantly higher infection scores (calculated by multiplying the estimated percentage of sur-

FIG 3 Caspase activity in midgut and remaining carcass following injection of dsRNA. Rexville D mosquitoes were injected with dsRNA (*Aeiap1*, *Aedronc*, or *cat*) or PBS or mock injected. Midguts were dissected at 1, 2, or 3 days postinjection, and pooled tissues from 10 individuals were homogenized in lysis buffer. Caspase activity was determined in midguts (A) and remaining carcass (B) using the effector caspase substrate Ac-DEVD-AFC. Data are means $\pm$ standard errors of the means from three independent experiments, and statistical significance (****, $P < 0.001$; ***, $P = 0.001$; **, $P < 0.01$; *, $P < 0.05$) was calculated with Student's *t* test for comparison with the data from *cat* dsRNA treatment at the same time point.

face area of each midgut that was eGFP positive by the intensity of eGFP fluorescence, as described in Materials and Methods) than those from *cat* dsRNA-treated mosquitoes, while midguts from *Aedronc* dsRNA-treated mosquitoes had significantly lower infection scores than those from *cat* dsRNA-treated mosquitoes (Fig. 6A, right). Similar results were also observed in foregut and hindgut (Fig. 6B and C, right). These results suggested that inducing caspase activation and apoptosis (by silencing *Aeiap1*) caused increased SINV replication in the gut, while inhibiting caspase activation and apoptosis (by silencing *Aedronc*) caused decreased SINV replication.

To investigate the effects of inducing or inhibiting apoptosis on SINV midgut escape and ability to establish disseminated infection, we examined the occurrence of infection in salivary glands and eyes. We found that a significantly higher percentage of *Aeiap1* dsRNA-treated mosquitoes exhibited eGFP expression in salivary glands and eyes than *cat* dsRNA-treated mosquitoes (Fig. 7). This result may be due to the damage sustained by the midgut due to widespread apoptosis following *Aeiap1* RNAi. More interestingly, however, mosquitoes injected with *Aedronc* dsRNA had a significantly lower rate of disseminated infection in the salivary glands than *cat* dsRNA-injected mosquitoes. The percentage of mosquitoes with infection in the eyes was also lower in *Aedronc* dsRNA-treated than in *cat* dsRNA-treated mosquitoes, but the difference was not statistically different (Fig. 7). These results indicate that inhibiting apoptosis by silencing *Aedronc* reduced viral dissemination from the midgut.

DISCUSSION

There have been past reports of apoptosis occurring in certain combinations of arboviruses and mosquitoes (2, 11, 12, 20, 33, 37, 38). Whether or not a specific arbovirus induces apoptosis in a particular mosquito probably depends on a multitude of factors, including the genetic backgrounds of both the virus and the vector. Attempts to determine whether various strains of SINV induce apoptosis in *A. aegypti* are in progress. However, regardless of whether apoptosis occurs in this particular virus-vector combination, our aim in this study was to directly test the effects of manipulating apoptosis on infection of a mosquito vector by an arbovirus. To this end, we modulated the apoptotic pathway *in vivo* by silencing the apoptosis inhibitor *Aeiap1* or the initiator caspase *Aedronc* and then asked whether manipulating apoptosis affected the ability of SINV to infect *A. aegypti*. Our data indicate that inducing apoptosis caused increased midgut infection and virus dissemination, while inhibiting apoptosis had the opposite effect.

This reciprocal effect of apoptosis on virus infection is the opposite of what one would expect if apoptosis has an antiviral function. How, then, do we explain these results? First, these observations were based on systemic induction or inhibition of apoptosis prior to infection, which differs from apoptosis being stimulated in isolated cells by virus replication. The positive effect that inducing widespread apoptosis had on SINV infection could be due to degradation of structural barriers or inhibition of innate immunity. Indeed, our observations indicate that the midguts of mos-

FIG 4 Midgut morphology following injection with dsRNA. Rexville D mosquitoes were mock injected or injected with dsRNA (*cat* or *Aeiap1*), and midguts were dissected at 3 days postinjection. (A) Midgut tissue was fixed, embedded, sectioned, and stained with epoxy tissue stain. Arrows indicate midgut microvilli, which are intact in mock- and *cat*-injected samples but not in *Aeiap1*-injected samples. The midgut lumen is also indicated (L). (B) TEM images of midguts injected with *Aeiap1* dsRNA or mock injected, showing microvilli (arrows).

quitoes injected with *Aeiap1* dsRNA were drastically altered in morphology, which might allow virus to physically escape the midgut more easily. The major gross pathology we observed following injection of *Aeiap1* dsRNA was in the midgut. This may have been due to the midgut being more amenable than other organs to RNAi following intrahemocoelic injection (for example, taking up dsRNA more efficiently) or to midgut cells being more sensitive to apoptosis following silencing of *Aeiap1*. It is possible that apoptosis also occurs in other organs that we did not examine in detail, but at least at the gross level of organ morphology, the main effects we observed were in the midgut. It should also be noted that these observations were made in mosquitoes that survived at least 3 days after injection of *Aeiap1* dsRNA; more extensive pathology may have occurred in other organs in mosquitoes that did not survive at least 3 days. In addition to physical effects on the integrity of the midgut epithelium, other physiological changes were probably also induced in midguts after *Aeiap1* RNAi, such as changes in the peritrophic membrane, changes in expression of innate immunity genes, or alteration of midgut pH. All of these factors could contribute to the mosquitoes becoming more susceptible to infection after treatment with *Aeiap1* dsRNA. If *Aeiap1* dsRNA is to be used as an insecticide for mosquitoes, as has been proposed (26), possible effects on mosquito innate immunity should be considered, since our results indicate that such treatment could make surviving mosquitoes more susceptible to virus infection.

FIG 5 Midgut epithelial cells from mosquitoes with silenced *Aeiap1* exhibit apoptotic nuclei. Midguts from mosquitoes injected with *Aeiap1* dsRNA at 2 days postinjection (top) or mock injected (bottom) were examined by TEM. Examples of two types of cells are shown, one that exhibits condensed and marginalized chromatin (arrows in top panels), a hallmark of apoptosis, and one with normal euchromatin and heterochromatin (bottom). Nuclei are outlined.

However, although the positive effects of *Aeiap1* silencing on SINV infection could be due to side effects associated with inducing widespread apoptosis, the ability of *Aedronc* RNAi to reduce disseminated SINV infection hints at a more interesting mechanism. Although we did not attempt to directly determine whether RNAi of *Aedronc* actually inhibited apoptosis *in vivo*, it is likely that it does so, since silencing *Aedronc* reduced the mortality associated with silencing *Aeiap1*, and because silencing *Aedronc* is highly effective at inhibiting apoptosis in Aag2 cells in response to several types of stimuli, including UV radiation, cytotoxic drugs, and *Aeiap1* RNAi (16). In addition, *Dronc* is required for almost all apoptosis in *Drosophila* (7, 34, 43).

One possible explanation for the inhibitory effect of silencing *Aedronc* on SINV is that a low degree of apoptosis or caspase activation is stimulated by SINV and that this aids in replication and/or dissemination from the midgut in *A. aegypti*. For example, it has been shown that caspase activity is required for escape of baculovirus from the midgut of its lepidopteran host, due to the involvement of caspases in remodeling of basal laminae (19). Thus, if SINV stimulates cellular caspases to degrade basal laminae, and if this aids in midgut escape, the effect of *Aedronc* RNAi may be due to decreased caspase activation.

Another intriguing possibility is that inducing or inhibiting apoptosis affects the RNAi response *in vivo*. An earlier study showed that systemic spread of RNAi is required for inhibiting virus replication and lethal SINV infection in *Drosophila*, and although the mechanism of release of dsRNA from infected cells is unknown, the authors of that study speculated that it may require

FIG 6 Virus infection in the midgut (A), foregut (B), and hindgut (C) of mosquitoes injected with dsRNAs. Rexville D mosquitoes were injected with dsRNA (*cat*, *Aedronc*, or *Aeiap1*), and 3 days later the surviving mosquitoes were given a blood meal containing SINV strain 5' dsMRE16ic-eGFP. At 7 days postinfection, the midgut, foregut, and hindgut were analyzed for the presence of eGFP, indicating infection prevalence (left), or for infection scores (determined as described in Materials and Methods), indicating the extent of infection (right). For infection prevalence, the means of two independent trials are shown and were compared by Student's *t* test; for infection scores, combined individual scores were compared by Student's *t* test (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$). Sample sizes were 32, 27, and 15 for *cat*, *Aedronc*, and *Aeiap1*, respectively.

cell death (28). Therefore, manipulating apoptosis could affect systemic RNAi spread. However, in this scenario, decreased cell death by RNAi of *Aedronc* would be expected to decrease the systemic RNAi response and allow higher virus replication, which is opposite to what we observed. Perhaps more interestingly, a recent report indicated that apoptosis has a suppressive effect on RNAi in *Drosophila*, in both dying cells and adjacent cells (42). Thus, induction of apoptosis by injection of *Aeiap1* dsRNA could suppress RNAi in adjacent cells and allow SINV to replicate to higher levels. In order to explain the reduced infection observed when *Aedronc* is silenced, one would have to postulate that some

level of apoptosis occurs naturally during SINV infection and that preventing this low level of apoptosis helps boost the RNAi response, thereby downregulating virus replication.

In summary, silencing *Aeiap1* in adult female *A. aegypti* mosquitoes caused apoptosis and alterations in midgut morphology, increased mosquito mortality, and enhanced susceptibility to SINV infection. Meanwhile, silencing *Aedronc* protected mosquitoes against mortality induced by *Aeiap1* silencing or injection of nonspecific dsRNA and reduced SINV infection and dissemination. Given the importance of RNAi in controlling SINV infection in *A. aegypti* (4, 5), and given the possible effects of apoptosis on

FIG 7 Virus dissemination in mosquitoes injected with dsRNAs. Mosquitoes were injected with the indicated dsRNA, and 3 days later, the surviving mosquitoes were given a blood meal containing SINV strain 5'dsMRE16ic-eGFP. At 7 days postinfection, disseminated infection was assessed by recording the percentage of mosquitoes expressing eGFP in salivary glands (A) and eyes (B). The means of two independent trials are shown and were compared by Student's *t* test (**, $P < 0.01$; *, $P < 0.05$). Sample sizes were 40, 37, and 26 for *cat*, *Aedronc*, and *Aeiap1*, respectively.

RNAi (42) and the effects of manipulating apoptosis on SINV infection that we have observed, interplay between these two arms of the immune response could be involved in explaining our results. This study represents the first attempt to directly test the effect of inducing or inhibiting apoptosis on arbovirus vector competence. Further work in this area promises to reveal additional important information about the interactions between the mosquito innate immune system and arboviruses. In particular, the use of alphavirus transducing systems to manipulate apoptosis only in infected cells and the study of specific vector-virus pairs that occur more commonly in nature will help to further define the role of apoptosis in determining vector competence.

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