Chikungunya virus emergence is constrained in Asia by lineage-specific adaptive landscapes

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Adaptation of RNA viruses to a new host or vector species often results in emergence of new viral lineages. However, lineage-specific restrictions on the adaptive processes remain largely unexplored. Recently, a Chikungunya virus (CHIKV) lineage of African origin emerged to cause major epidemics of severe, persistent, debilitating arthralgia in Africa and Asia. Surprisingly, this new lineage is actively replacing endemic strains in Southeast Asia that have been circulating there for 60 y. This replacement process is associated with adaptation of the invasive CHIKV strains to an atypical vector, the *Aedes albopictus* mosquito that is ubiquitously distributed in the region. Here we demonstrate that lineage-specific epistatic interactions between substitutions at amino acid positions 226 and 98 of the E1 envelope glycoprotein, the latter of which likely resulted from a founder effect, have for 60 y restricted the ability of endemic Asian CHIKV strains to adapt to this new vector. This adaptive constraint appears to be allowing invasion of the unoccupied vector niche by *Ae. albopictus*-adapted African strains. These results underscore how different adaptive landscapes occupied by closely related viral genotypes can profoundly affect the outcome of viral evolution and disease emergence.

The ability of RNA viruses to emerge and cause human disease often reflects their ability to exploit new ecologic contacts and rapidly adapt to new amplification hosts or vectors. These adaptations can lead to the expansion of viral ecological niches and often facilitate introductions into new geographic ranges. Numerous examples have demonstrated that this emergence process often relies on the acquisition of only one or a few adaptive mutations that allow RNA viruses to overcome host-specific barriers (1–5). These mutations can occur readily because of the high error frequency of viral RNA-dependent RNA polymerases, followed by efficient positive Darwinian selection of mutant strains in the extremely large viral populations within infected hosts (6). However, much less attention has been paid to understanding the limitations on these processes that may restrict viral adaptation, and how potential genetic constraints influence virus evolution in nature. Improved understanding of these adaptive limitations is critical for understanding and predicting future emergence events and for designing intervention strategies (7).

Chikungunya virus (CHIKV: *Togaviridae*: *Alphavirus*) is an enveloped, single-stranded, positive-sense RNA virus transmitted by mosquito vectors. CHIKV is endemic to both Africa and Asia, although transmission cycles differ considerably on these continents. CHIKV is primarily maintained in Africa via a zoonotic sylvatic cycle that relies on nonhuman primates as reservoir hosts and arboreal, primatophilic *Aedes (Stegomyia)* spp. mosquitoes (e.g., *Aedes furcifer* and *Aedes africanus*) (8). In contrast, in Asia humans serve as the primary hosts of CHIKV, with *Aedes aegypti* traditionally serving as the primary vector in most urban epidemics (9). Phylogenetic studies and historical analyses have indicated that major epidemics in India and Southeast Asia resulted from the introduction of CHIKV from Africa beginning as early as the 18th century (10) and continuing into the 21st century (9). Although there is no evidence that CHIKV persisted in Asia following a well-documented outbreak in 1788 (10), an introduction that occurred around 1950 or earlier resulted in an endemic Asian lineage of CHIKV that still persists there (Fig. S1).

Since 2004, CHIKV has exploded onto the global scene as a major emerging pathogen in a series of devastating outbreaks that have infected up to 6.5 million people (11) and have been associated with several thousand human deaths worldwide (12–14). Phylogenetic studies demonstrated that these outbreaks were associated with at least three independent CHIKV lineages that emerged almost simultaneously in different parts of the world (15–20). The major CHIK outbreaks were caused by virus strains of the Indian Ocean lineage (IOL), which evolved from the East-Central-South-African (ECSA) enzootic genotype. This lineage first emerged in Kenya in 2004 and subsequently spread to several Indian Ocean islands, India, and Southeast Asia (Fig. S1). A CHIKV strain of the IOL, presumably transported from India by a viremic traveler, was also responsible for the small 2007 CHIKV outbreak in Italy (21). The second series of outbreaks, caused by phylogenetically distinct CHIKV strains also belonging to the ECSA genotype, began in 2006 in Cameroon and spread to Gabon in 2007 (19). The third CHIKV lineage was responsible for a 2006 outbreak in Malaysia and belonged to the original endemic Asian genotype (17).

An intriguing feature of 2006 to 2009 CHIK epidemics in Southeast Asia was that most virus strains isolated there did not belong to the endemic Asian genotype that has been circulating there for six decades (22); instead, they belonged to the newly introduced IOL (16, 18). Sequences generated from recent Southeast Asian outbreaks suggest that the IOL strains are actively replacing endemic Asian strains there. The best example was documented in Malaysia: the initial 2006 outbreak there was caused by an endemic Asian strain. However, the subsequent 2008 epidemic of greater magnitude was caused by the newly introduced IOL (17, 23) (Fig. S1*B*). This finding suggested that this shift in viral genotypes could be the major factor influencing the reemergence of CHIKV in Southeast Asia.

Among several factors associated with the recent emergence and spread of CHIKV, a prominent role has been attributed to the adaptation of the emerging IOL strains to the mosquito, *Aedes albopictus*, previously considered to be only a secondary vector. Unlike past epidemics mediated by *Ae. aegypti*, *Ae. albopictus* has served as the primary CHIKV vector during the majority of recent outbreaks, including those on several islands of the western Indian Ocean, parts of India, Singapore, Malaysia, Thailand, Sri Lanka, Gabon, and Italy (18, 24–28). Phylogenetic and epidemiologic studies indicate that CHIK outbreaks in regions highly infested with *Ae. albopictus* were always associated with an alanine-to-valine substitution in the E1 envelope glycoprotein (E1-A226V), which was selected convergently by

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different CHIKV lineages (15, 18, 20, 23, 25, 27). Laboratory studies, including reverse genetics, confirmed the role of this mutation in increased infectivity and transmissibility by *Ae. albopictus* (2, 29). Surprisingly, despite its dramatic effect on transmissibility, this mutation has not been detected in previous CHIKV strains of the ECSA or in endemic Asian lineages.

Although *Ae. albopictus* is native to Southeast Asia, there is no evidence that this species has played a major role in transmission of endemic Asian CHIKV strains. Conversely, *Ae. aegypti* had been consistently incriminated as the epidemic vector before the introduction of the IOL strains in 2007. However, the recent *Ae. albopictus*-transmitted Southeast Asian epidemics indicate that *Ae. albopictus* can be highly efficient vector, posing the question of why the E1-A226V mutation had not been observed previously nor been selected during six decades of transmission there. Stated another way, given an approximately 60-y head start in adapting to *Ae. albopictus*, why has the Southeast Asian genotype been losing its competition with the IOL since 2007? A possible explanation is suggested by previous studies indicating that other genome regions can modulate the effect of the E1-A226V mutation on CHIKV fitness for *Ae. albopictus* (30). We therefore hypothesized that Southeast Asian lineage-dependent epistatic genome interactions limit the penetrance of the E1-A226V adaptation to *Ae. albopictus*, which facilitated the invasion and establishment of *Ae. albopictus*-adapted IOL strains in that region.

Results

To study restrictions on the adaptability of endemic Southeast Asian CHIKV strains for *Ae. albopictus*, we first analyzed the effect of the E1-A226V adaptive mutation on the fitness of endemic Asian strain RSU1 (Fig. S1*A*), isolated in Indonesia in 1985 (hereafter ID85). We tested *Ae. albopictus* colonies originating from Galveston, Texas and Thailand. Viruses expressing E1-226A (ID85-GFP-226A) or E1-226V (ID85-GFP-226V) residues were generated from green fluorescent protein (eGFP)-expressing infectious cDNA clones, and their relative infectivities for *Ae. albopictus* were determined by oral exposure to artificial blood meals containing serial 10-fold CHIKV dilutions. The eGFP expression was used to facilitate detection of infection using fluorescence microscopy. Previously, it was shown that the adaptive effect of the E1-A226V mutation is primarily manifested by increased *Ae. albopictus* infectivity (2, 29, 30), and that CHIKV-expressing eGFP exhibits similar infectious characteristics in mosquitoes as wild-type (non-eGFP) viruses (31–33). Oral infectious dose 50% values (OID_{50}) of ID85-GFP-226A and ID85-GFP-226V viruses were almost identical ($P > 0.1$) for both mosquito colonies (Table 1). In contrast, as reported earlier, the infectivity of LR-GFP-226V virus expressing the E1-226V residue in the background of the LR2006 OPY1 (hereafter LR) CHIKV strain belonging to the IOL (Fig. S1*A*) was nearly 100-times higher compared with that of the identical virus with an E1-226A residue ($P < 0.01$). Similarly, introduction of the E1-A226V substitution into the genetic background of the SL-CK1 strain (hereafter SL07), also belonging to the IOL (Fig. S1*A*), led to nearly 100-fold increase in infectivity for *Ae. albopictus* ($P < 0.01$) (Table 1).

Table 1. Effect of the E1-A226V mutation on infectivity of Asian and IOL strains of CHIKV for *Ae. albopictus* mosquitoes (Galveston and Thailand colonies)

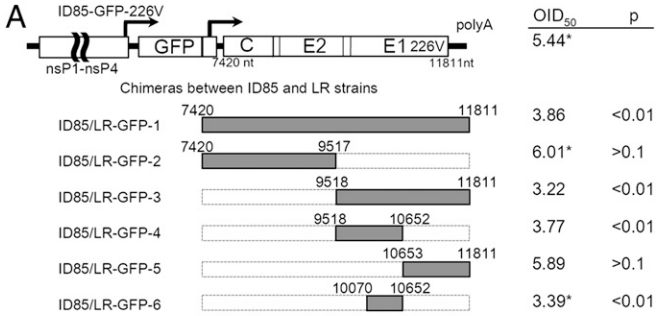
		Galveston		Thailand	
Virus	E1-226	OID50	*P*	OID50	*P*
ID85-GFP-226A	A	5.74*	$P > 0.1$	5.54	$P > 0.1$
ID85-GFP-226V	**V**	5.44*		5.11	
ML06-GFP-226A	A	4.81	$P > 0.1$	4.65	$P > 0.1$
ML06-GFP-226V	**V**	4.40		4.30	
LR-GFP-226A	**A**	4.83	$P < 0.01$	5.22	$P < 0.01$
LR-GFP-226V	V	3.02		3.17	
SL07-GFP-226A	A	5.27	$P < 0.01$	5.37	$P < 0.01$
SL07-GFP-226V	**V**	3.21		3.55	

The E1-226 mutated residues for each CHIKV background are in bold and underlined. OID_{50} values are expressed as Log_{10} pfu/mL. *P* values are listed for the comparison of OID_{50} values between viruses with E1-226V and E1-226A residues in same genetic background. Asterisks stand for OID_{50} values calculated as an average of two independent experiments.

These results suggested that endemic Asian CHIKV strains might be insensitive to the *Ae. albopictus*-adaptive effects of the E1-A226V substitution. However, to rule out the possibility that the ID85 strain is atypical of endemic Asian strains or the possibility that, since 1985, Asian strains have accumulated compensatory mutations that modulate the effect of E1-A226V, we repeated these analyses using the last endemic Asian strain MY002IMR/06/BP (hereafter ML06) reported in 2006 from Malaysia. Because no virus isolates from this outbreak were available to us, the genome of the ML06 strain was synthesized based on its GenBank sequence (EU703759.1). A cDNA clone was subsequently used to generate mutants of interest. As for the ID85 strain, oral infectivity of ML06 viruses expressing E1-226A (ML06-GFP-226A) or E1-226V (ML06-GFP-226V) in the ML06 background were almost identical ($P > 0.1$) when tested in both Galveston and Thailand colonies of *Ae. albopictus* (Table 1), indicating that insensitivity to the adaptive effect of the E1-A226V mutation is a common feature of endemic Asian CHIKV strains.

To elucidate the genetic determinants that restrict penetrance of the E1-A226V substitution on fitness of Asian CHIKV strains for *Ae. albopictus*, a series of chimeric viruses was constructed based on Asian strain ID85 and IOL strain LR, all of which contained the E1-226V residue. A comparison of their oral infectivity for the Galveston strain of *Ae. albopictus* revealed that a single genomic region spanning nucleotides 10,070 to 10,652 and encoding amino acids 27 to 220 of the E1 glycoprotein was responsible for the decreased infectivity of the ID-85 strain (Fig. 1*A*). In this region, the ID85 and LR strains differ by six amino acids. However, only positions E1-98 and E1-211 were consistently associated with sensitivity of various CHIKV strains to the *Ae. albopictus*-adaptive effect of the E1-A226V mutation (Fig. S2). Residues E1-98T and E1-211E are present in all sequenced, endemic Asian CHIKV, and absent in all other CHIKV strains, including all IOL isolates sequenced to date (Fig. S1*A*; see GenBank). Therefore, we hypothesized that one or both of these amino acids affect penetrance of the E1-A226V substitution. To test this, the E1-98A and E1-211K residues from the LR strain were introduced individually into the ID85-GFP-226V clone and infectivity was evaluated in Galveston *Ae. albopictus*. The single E1-T98A substitution generated a nearly 100-fold increase ($P < 0.01$) in infectivity of the ID85-GFP-226V virus, up to levels characteristic of IOL viruses, such as LR-GFP-226V and SL07-GFP-226V (Fig. 1*B*). The single E1-E211K mutation was not associated with a significant increase in CHIKV infectivity for *Ae. albopictus* compared with the ID85-GFP-226V virus ($P > 0.1$) (Fig. 2*B*). Moreover, double expression of the E1-98A and E1-211K residues in the ID85 background with the E1-A226V substitution did not result in any increase in CHIKV infectivity for *Ae. albopictus* compared with the ID85 strain with E1-T98A and E1-A226V substitutions (Fig. S3*A*), indicating that a single epistatic interaction between positions E1-226 and E1-98 influences CHIKV fitness in *Ae. albopictus*.

To confirm the critical role of the E1-T98A substitution in penetrance of the E1-A226V substitution, we introduced this mutation into the wild-type ID85-GFP-226A genome with E1-226A. Infectivity of the resultant virus (ID85-GFP-226A-98A) for Galveston *Ae. albopictus* was not significantly different from that of ID85-GFP-226A (Fig. 1*B*), indicating that the E1-98A and E1-226V residues must be expressed simultaneously to enhance infectivity of the ID85 strain for *Ae. albopictus*. To demonstrate that the epistatic interactions between E1-98A and E1-226V are



B

CHIKV background (mosquito colony)	E1-226	E1-98	E1-211	OID_{50}	p
ID85-GFP (G)	V	A	E	3.48	p<0.01
ID85-GFP (G)	V	T	K	4.81	p>0.1
ID85-GFP (G)	A	A	E	5.33	p>0.1
ML06-GFP (G)	V	A	E	2.35	p<0.01
ML06-GFP (G)	A	A	E	4.70	p>0.1
LR-GFP (G)	V	T	K	5.00	p<0.01
ID85-GFP (T)	V	A	E	3.39	p<0.01
ML06-GFP (T)	V	A	E	2.36	p<0.01

Fig. 1. Genetic determinants restricting the effect of the E1-A226V mutation on infectivity of Asian CHIKV strains for *Ae. albopictus*. (*A*) Schematic representation and infectivity (OID_{50}) of the chimeric viruses constructed based on genomes of ID85 (white) and LR (gray) CHIKV strains for *Ae. albopictus* (Galveston). The *P* value for each chimeric virus is derived from a comparision of OID_{50} values between it and ID85-GFP-226V. (*B*) Infectivity of CHIKV expressing residues of interest at positions E1-226, E1-98, and E1-211 in the backbone of ID85, ML06, and LR strains for Galveston (G) and Thailand (T) *Ae. albopictus*. The *P* value for each virus is derived from a comparison of OID_{50} values between it and the virus with the same genetic background that has valine at position E1-226, and strain-authentic residues at positions E1-98 and E1-211. The mutated residues for each background are in bold and underlined. OID_{50} values are expressed as Log_{10} pfu/mL. Asterisks stand for OID_{50}-values that were calculated as an average of two independent experiments.

not restricted to the ID85 strain, the E1-98A residue was also expressed in the background of endemic Asian strain ML06. As expected, infectivity of ML06-GFP-226V-98A for Galveston *Ae. albopictus* increased about 100-fold compared with the same CHIKV with only the single E1-A226V substitution ($P < 0.01$), whereas expression of the E1-98A residue in the presence of E1-226A did not lead to a significant change in infectivity ($P > 0.1$) (Fig. 1*B*). In addition, introduction of the Asian-genotype E1-98T residue into the IOL background with the E1-226V residue reduced *Ae. albopictus* infectivity by approximately 100-fold ($P < 0.01$) compared with the wild-type IOL strain (LR-GFP-226V) (Fig. 1*B*). These data indicate that the E1-98T residue, which is common to all sequenced strains of the endemic Asian genotype, limits the adaptive effect of the E1-A226V mutation regardless of the CHIKV backbone.

Geographic strains of *Ae. albopictus* can differ in their susceptibility to infection with CHIKV (34). Therefore, to demonstrate that the effect of the E1-98T substitution on penetrance of the E1-A226V adaptive mutation is not restricted to the Galveston strain of *Ae. albopictus*, viruses expressing combinations of E1-A226V and E1-T98A mutations in the background of the ID85 and ML06 strains were also tested using the Thailand colony. As with Galveston mosquitoes, the OID_{50} values of the double mutants (E1-T98A and E1-A226V) were almost 100-fold lower for Thailand mosquitoes compared with viruses that expressed only the E1-226V residue ($P < 0.01$) (Fig. 1*B*).

In addition to initial oral infection that is dependent upon infection of midgut epithelial cells, transmission of arboviruses by mosquitoes requires dissemination into the hemocoel followed by spread and replication in other organs, including the salivary glands. To investigate if the E1-98T residue that restricts CHIKV infectivity for *Ae. albopictus* is also associated with a decreased dissemination needed for transmission, we used a competition assay similar to that described previously (2). A synonymous mutation A6454C was introduced into the nsP4 gene of the ID85 and ML06 strains to create a recognition sequence for the ApaI restrictase. *Ae. albopictus* (Thailand) were fed blood meals containing equal amounts of viruses expressing the E1-98T or E1-98A residues in the backgrounds of either the ID85 or ML06 CHIKV strains containing the E1-226V residue; one of the viruses in the competition mixture was labeled by the ApaI marker. Ten days postinfection (dpi), the presence of disseminated viral infection in individual mosquitoes was analyzed by the detection of cytopathic effects after inoculation of Vero cells with mosquito heads and legs homogenates, followed by RT-PCR of viral RNA from the culture supernatants. ApaI digestion of the resultant amplicons followed by gel electrophoresis was used to differentiate which of the two competitors developed disseminated infections (Fig. 2). The presence of the E1-98T residue in the backbone of the ID85 and ML06 strains expressing the E1-A226V mutation was associated with significantly lower rates of dissemination ($P < 0.01$, Fisher's exact test) compared with the same viruses with the E1-T98A substitution, regardless of the titers of viruses in the blood meal (Fig. 2 *A* and *B*). These data further support our observations that the E1-98T residue in endemic Asian CHIKV strains limits the adaptive effect of the E1-A226V mutation in *Ae. albopictus* mosquitoes. Interestingly, a similar difference in dissemination efficiency (7- to 10-fold) was observed in competition experiments between LR-226V-ApaI (2) (IOL genotype) and ID85-226V-98T (Asian genotype), indicating that, even after acquiring the E1-A226V substitution, Asian strains would be less fit for *Ae. albopictus* transmission compared with viruses of the IOL (Fig. 2*C*). The E1-E211K mutation had no effect on dissemination of the ID85 strain with E1-226V and E1-T98A residues (Fig. S3*B*), supporting the conclusion that only one epistatic interaction between amino acids at E1-226 and E1-98 influences CHIKV fitness in *Ae. albopictus*.

To directly demonstrate that an epistatic effect of the E1-98T residue of endemic Asian CHIKV strains restricts positive selection of the *Ae. albopictus*-adaptive E1-A226V substitution, we performed an experiment where the ML06 CHIKV strain expressing the single substitution (E1-A226V), or double mutations (E1-A226V, E1-T98A), was serially passaged alternately in mosquitoes and Vero cells in the presence of a 100- or 1,000-fold excess of wild-type ML06 with the E1-226A and E1-98T residues (Fig. 3). After two consecutive passages in *Ae. albopictus* of mixtures containing only the E1-98T residue, only wild-type virus was recovered from mosquitoes with disseminated infections (Fig. 3*A*). In contrast, even a 1,000-fold excess of wild-type virus in the starting blood meal did not prevent the selection in disseminated viral populations of CHIKV with simultaneous expression of the E1-A226V and E1-T98A substitutions (68.4% frequency of wins, $n = 19$) (Fig. 3*B*). These data indicate that, for endemic Asian strains of CHIKV, the simultaneous acquisition of two independent E1 mutations would be required for efficient adaptation to *Ae. albopictus*. In contrast, for IOL strains, the same fitness advantage and selection efficiency could be gained by the acquisition of a single amino acid substitution. Therefore, in regions of Southeast Asia where *Ae. albopictus* is abundant, we expect IOL CHIKV strains to have a selective advantage over endemic Asian strains despite the decades-long presence of only the latter. The observation that selection of the two mutations required for adaptation is inefficient may explain the ongoing shift in CHIKV genotype frequency in Asia.

CHIKV strains of the Asian genotype have been circulating in Southeast Asia, where *Ae. albopictus* is highly abundant, for at least 60 y. Considering the high evolutionary rates of CHIKV [estimated as 4.3×10^4 subs/nt per year for Asian strains (22)], it

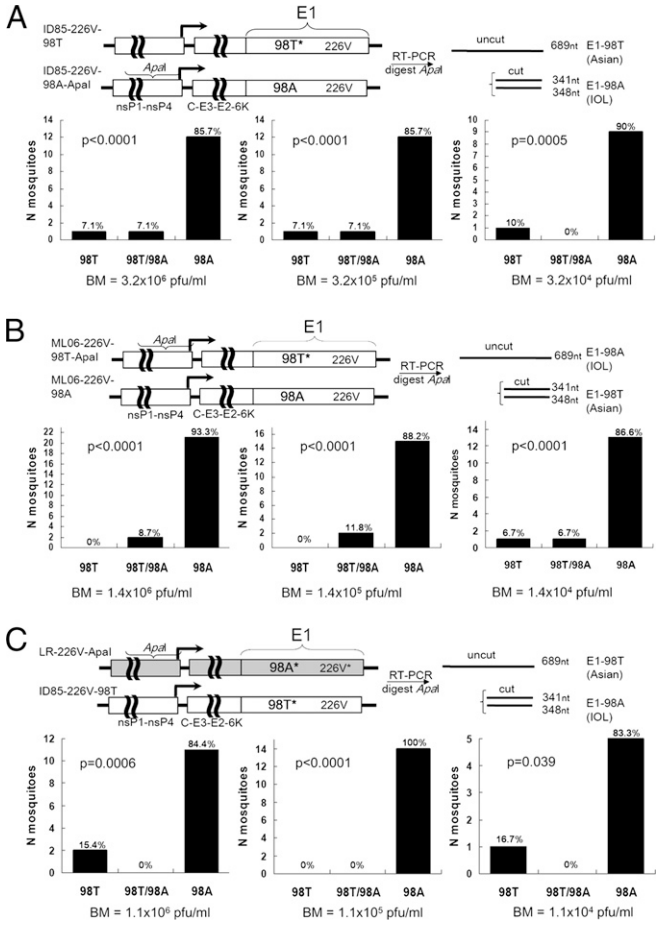


Fig. 2. Effect of the E1-98T residue on dissemination of CHIKV strains in *Ae. albopictus*. Above each figure is a schematic representation of the viruses used in the competition assay. Asterisks indicate authentic residues for the respective CHIKV background. Thailand *Ae. albopictus* mosquitoes were presented with blood meals containing the indicated amount of a 1:1 mixture (based on plaque-forming units) of viruses expressing E1-98T and E1-98A residues in the backbone of ID85 (*A*) or ML06 (*B*) strains and processed at 10 dpi. Graphs show numbers and proportions of mosquitoes containing viruses expressing only threonine (98T), alanine (98A), or containing both viruses (98T/98A) in mosquitoes heads and legs (representing disseminated infections). (*C*) Competition between viruses of IOL (LR-226V-ApaI) and Asian strain ID85 with E1-A226V mutation. 98T indicates mosquitoes with disseminated infection of only the Asian strain; 98A indicates only the IOL strain; 98T/98A indicates mosquitoes that contained both viruses. Differences in dissemination efficiencies between 98T and 98A viruses were compared with a one-tailed Fisher's exact test.

is surprising that this virus could not accrue two point mutations (E1-A226V, E1-T98A) to increase transmissibility by *Ae. albopictus*. A possible explanation is that past CHIKV transmission in Asia was primarily dependent on *Ae. aegypti*, which represented a stronger selective force than *Ae. albopictus*. If mutations that provide a fitness advantage in *Ae. albopictus* have a deleterious effect in *Ae. aegypti*, they may have been negatively selected. To test this hypothesis, we analyzed the effect of individual mutations E1-A226V and E1-T98A on CHIKV fitness in *Ae. aegypti*. The oral infectivity for *Ae. aegypti* (Thailand) of viruses expressing either E1-A226V or E1-T98A in the background of Asian strains ID85 and ML06 was almost identical to that of the parental viruses ID85-GFP-226A and ML06-GFP-226A ($P > 0.1$) (Fig. S4*A*). Similarly, no significant differences were observed in the relative dissemination efficiency in *Ae. aegypti* of viruses expressing E1-A226V and E1-T98A in the background of the ID85 strain, compared with the wild-type virus containing residues E1-226A and E1-98T (Fig. 4 *B* and *C*). These data indicate that CHIKV adaptation to *Ae. albopictus* via the E1-A226V and E1-T98A mutations does not compromise fitness for *Ae. aegypti*.

Another possible explanation for the failure of CHIKV to acquire the two *Ae. albopictus*-adaptive mutations in Southeast Asia is that they compromise the infection or viremia induction in human amplifying hosts. However, earlier studies demonstrated that the E1-A226V mutation does not affect CHIKV replication in infant mice (2), models for human infection (35). In competition experiments, we also could not detect any difference in replication of CHIKV with either E1-98T or E1-98A residues in 2- to 3-d-old mice (Fig. S5). These findings indicate that mutations associated with CHIKV adaptation to *Ae. albopictus* probably do not affect viral fitness in the vertebrate host. Overall, our data support the hypothesis that the lack of selection for one of the two required mutations (E1-T98A) and the low probability of the simultaneous acquisition of two independent mutations is largely responsible for the inability of Asian CHIKV strains to adapt to *Ae. albopictus* mosquitoes over the course of 60 y.

Discussion

In this study we demonstrated that Asian CHIKV strains are impaired in their ability to adapt to *Ae. albopictus* mosquitoes via acquisition of the E1-A226V substitution, and that a single residue (E1-98T) is a key determinant that restricts this adaptation. These data further explain epidemiological observations that, despite its abundance, *Ae. albopictus* was never incriminated as a primary CHIKV vector in Asia before 2007. The inability to adapt to *Ae. albopictus* apparently prevented the Asian endemic CHIKV strains from efficient utilization of the substantial human *Ae. albopictus* transmission cycle niche available in Southeast Asia, thus facilitating the establishment of the *Ae. albopictus*-adapted IOL CHIKV strains; this explains the ongoing shift in CHIKV genotypes in Southeast Asia, which may eventually lead to complete extinction of the endemic Asian genotype.

It is intriguing that, despite the high mutation frequency and rapid replication of RNA viruses (36), adaptation of Asian CHIKV strains to *Ae. albopictus* has not occurred during the previous 60 y of circulation since their introduction into Asia. This phenomenon could be explained in terms of the classic population biology theory of adaptive landscapes. It postulates that in nature, viral populations exist at the isolated peaks of high fitness on adaptive landscapes, and that their movement to a higher adaptive peak is restricted by adaptive valleys of lower fitness, or possibly even by plateaus (Fig. 4) (37, 38). Although we did not find evidence indicating that mutations that increase CHIKV fitness in *Ae. albopictus* cause deleterious effects in either *Ae. aegypti* or in vertebrate hosts (as assessed in mice), we cannot rule out the possibility of additional epistatic mutations with subtle effects on fitness that cannot be measured using our methods. However, we believe that the observed epistatic interaction between residues E1-226 and E1-98 in Asian strains alone has probably prevented CHIKV evolution to the higher adaptive peak characterized by efficient virus transmission by *Ae. albopictus*. This epistatic interaction would dramatically increase the length of the fitness plateau that Asian strains must traverse to reach the *Ae. albopictus*-adaptive peak (Fig. 4*B*). Because the presence of an alanine at E1-98, characteristic of IOL strains, does not inhibit the role of mutations at E1-226, there is no comparable fitness plateau to traverse by IOL strains, allowing more rapid transition to the E1-226V fitness peak (Fig. 4*A*). Thus, our findings represent some of the strongest evidence supporting the role of the complex adaptive landscapes in the arbovirus emergence.

Interestingly, recent CHIKV outbreaks demonstrate that, even when no epistatic interactions limit the adaptive effects of the E1-A226V mutation, its selection still takes about 1 y (~15–23

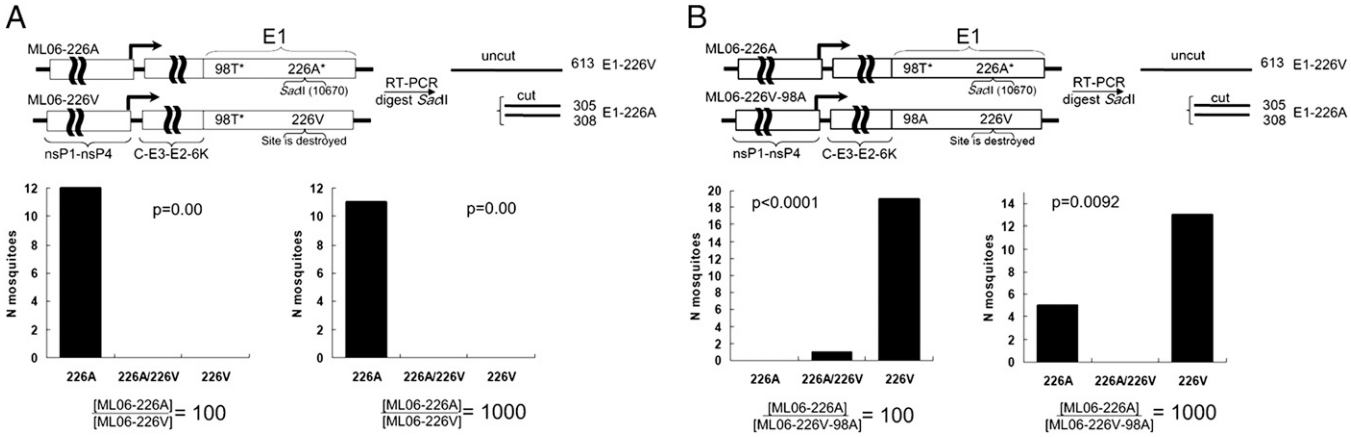


Fig. 3. Effect of the E1-98T residue on positive selection of the E1-A226V mutation in CHIKV populations transmitted by *Ae. albopictus* (Thailand). CHIKV expressing the single E1-A226V mutation (*A*) or two mutations (E1-A226V, E1-T98A) (*B*) in the background of ML06 strain were passaged twice in *Ae. albopictus* in the presence of a 100- or 1,000-fold excess of wild-type ML06 virus with alanine at E1-226. Asterisks indicate authentic residues for the ML06 strain. At the bottom is the ratio of the viruses presented to *Ae. albopictus* in the starting blood meals. Graphs show number of mosquitoes after the second passage that contain only wild-type (226A), only mutant (226V), or both (226A/226V) strains in heads and legs. The differences in proportions of mosquitoes containing either 226A or 226V viruses were tested for significance with a one-tailed Fisher's exact test.

transmission cycles, assuming 15–20 d per cycle) for IOL CHIKV populations to fix the E1-A226V mutation in *Ae. albopictus*-infested regions (15, 27). This finding indicates that selection of adaptive mutations in CHIKV, and possibly other arboviruses, is a relatively inefficient process. This inefficiency could be explained by genetic bottlenecks that the virus encounters at various stages of mosquito infection (39) (Fig. 2 and Fig. S3*B*), which may reduce the effect of positive selection that only operates efficiently on large populations (40). This effect of genetic drift would have an even stronger effect on natural selection if the adaptive mutations were affected by epistatic interactions, indicating that the latter could play a major role in determining evolution of the particular viral lineages in certain environments. Moreover, arboviral evolution can be constrained by host alternation (41). Thus, the ecological complexity of CHIKV in Asia, which allows virus transmission by *Ae. albopictus* and *Ae. aegypti*, may have a stronger constraining effect on adaptation of Asian CHIKV lineage to *Ae. albopictus* compared with IOL strains, especially considering that *Ae. albopictus*-adaptive mutations do not affect CHIKV fitness in *Ae. aegypti* (Fig. S4).

Another important question concerning the maintenance of CHIKV in Asia is why introduction of the *Ae. albopictus*-adapted African strains did not occur earlier, considering the continuous exchange of travelers between these two regions. Previously, we demonstrated that the majority of CHIKV strains of the ECSA genotype are restricted in their ability to adapt to *Ae. albopictus* because of the presence of another epistatic residue: isoleucine at position E2-211 (30). This residue, similarly to E1-98T, restricts the adaptive effect of the E1-A226V mutation in *Ae. albopictus*. However, IOL CHIKV strains have threonine at E2-211, which has no effect on CHIKV sensitivity to the E1-A226V mutation. Therefore, it is likely that the same processes that have prevented adaptation of the Asian strains to *Ae. albopictus* limit the potential for strains of the ECSA genotype to be introduced and established in the Asia.

The E1-98T residue is found in all endemic Asian strains sequenced to date, yet in no other CHIKV lineages. This finding, combined with results indicating that the E1-T98A residue by itself has no detectable effect on CHIKV fitness for infection of either mosquito vectors or for mice, suggests that the E1-98T residue may have become established in the endemic Asian lineage via a founder effect. We cannot rule out the possibility that the threonine residue is adaptive for humans or for another Asian vertebrate amplifying host not yet identified. However, it seems likely that the establishment of the threonine residue and its limitation on the ability of CHIKV to emerge via adaptation to *Ae. albopictus* was a stochastic event.

The precise molecular mechanisms underlying the roles of mutations at positions E1-226 and E1-98 on CHIKV fitness in *Ae. albopictus* have yet to be elucidated. In alphaviruses, the E1 envelope glycoprotein is primarily responsible for fusion of viral and cellular membranes within the endosomes (42). A detailed analysis of various mutations at position E1-226 demonstrated that an increased fitness of the CHIKV in *Ae. albopictus* is associated with an increase in interactions between methyl or methylene groups of the aliphatic amino acids at E1-226 (such as V, I, L, and M) with methylene groups of proline at the position E1-86 (33). The E1-86P is located at the base of the fusion loop, which becomes inserted into the target endosomal membrane during virus entry (Fig. S6). The insertion of the fusion loop is a highly regulated process that is triggered by low pH and the presence of specific lipids in the target endosomal membrane (43). The E1-98 residue, which regulates CHIKV sensitivity to the E1-A226V mutation, is located in the opposite side of the fusion loop base in proximity to position E1-86, suggesting a possible interaction between these residues. It is likely that mutations at E1-98 and E1-226 (via position E1-86) directly modulate flexibility of the fusion loop, depending on the specific environment of endosomal compartments, and thus regulate the

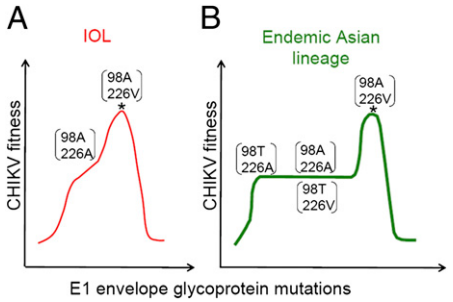


Fig. 4. CHIKV strains of the IOL (*A*) and endemic Asian lineage (*B*) exhibit different fitness landscapes with regard to amino acid substitutions in the E1 glycoprotein. Asterisks indicate *Ae. albopictus*-adaptive peaks.

CHIKV fusion dynamics with *Ae. albopictus* membranes during virus entry.

In summary, our results demonstrate that complex epistatic interactions can constrain the ability of a mosquito-borne alphavirus to adapt to a new vector, and thus can restrict epidemic emeregence. The adaptive landscape differs significantly between major CHIKV lineages, and an adaptive plateau leading from the endemic Asian lineage peak to a higher fitness for transmission by *Ae. albopictus* apparently prevented the adaptation of this lineage to this vector for at least 60 y. This failure of the endemic Asian genotype to traverse this fitness plateau now appears to be allowing it to be displaced by the newly emerged IOL CHIKV genotype, which adapted to *Ae. albopictus* via a single mutation owing to the lack of a comparable epistatic barrier. These findings underscore how different adaptive landscapes occupied by closely related viral genotypes can profoundly affect the outcome of viral evolution and disease emergence. They also emphasize the need to understand the molecular basis of viral proteins, their structure, and interactions with host cells that affect the ability of arboviruses to be transmitted by mosquitoes.

Materials and Methods

Details are available in *SI Materials and Methods*. Briefly, full-length infectious clones with and without eGFP for the LR strain were described previously (2, 31). Infectious clones of ID85, ML06, and SL07 strains were constructed using conventional PCR-based cloning methods (44). Detailed information for all plasmids is available from the authors upon request. Infectious viruses were generated by electroporation of the infectious clone-derived RNA into BHK-21 cells. Viral titers were determined by titration on Vero cells, as previously described (3). See Tables S1 and S2 for recovery of the viruses after electroporation of in vitro transcribed RNA and GenBank CHIKV strain information, respectively.

Mosquitoes were handled as described previously (32). The Galveston colony of *Ae. albopictus* was established in 2003. Thailand colonies of *Ae. albopictus* and *Ae. aegypti* were established from mosquito eggs collected in 2009 in Bangkok. The OID_{50} experiments were performed using eGFP-expressing viruses, as described previously (2, 30). To measure CHIKV fitness using competition assays, the pair of viruses that differed by mutations of interest in the E1 gene were competed, with one of the viruses containing the synonymous mutation 6454A→C, which creates a site for ApaI restrictase. A 1:1 ratio of the viruses was used to prepare infectious blood meals, which were orally presented to *Ae. albopictus* (Thailand colony) or *Ae. aegypti*. At 10 dpi, mosquitoes were processed as described in *SI Materials and Methods* (Fig. S7). Differences in dissemination efficiencies were tested for significance with a one-tailed Fisher's exact test.

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