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Structural Proteins of Chikungunya Virus

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Polyacrylamide gel analysis of the structural proteins of African and Asian strains of Chikungunya virus, an alphavirus, showed that both strains contain three structural proteins: glycosylated E1 and E2, embedded in the viral envelope, and a nonglycosylated nucleocapsid protein. In pulse-chase experiments the precursor protein PE2 was chased into glycoprotein E2, which migrated slightly faster than did glycoprotein E1. The third Chikungunya glycoprotein, E3, was not associated with mature virions but was released into culture fluids. With glycoproteins E1 and E2, separated by glass wool column chromatography, it was shown that hemagglutinating activity is associated with glycoprotein E1.

Chikungunya (CHIK) virus belongs to the *Alphavirus* genus (4). First observed in epidemic form in Africa (21), it causes a disease clinically similar to Dengue fever. The virus was also isolated from humans in Thailand in the course of an epidemic of hemorrhagic fever in 1958 (11). Serologically, CHIK virus belongs to the Semliki Forest (SF) subgroup of alphaviruses (4). Serological grouping of alphaviruses is based upon their cross-reactivity in hemagglutination inhibition or neutralization tests (6). The envelope glycoproteins of alphaviruses are the distinguishing factors in these biological tests; therefore, a basic understanding of these proteins as antigens is required in alphavirus research.

In this communication we report the isolation and analysis of the structural proteins of CHIK virus from both African and Asian strains.

The prototype African strain of CHIK virus was obtained from the Yale Arbovirus Research Unit, Yale University School of Medicine, New Haven, Conn. One Asian strain (BaH 306) was isolated from acute-phase serum drawn from a child with acute hemorrhagic fever in Bangkok, Thailand, and identified as CHIK virus by T. Ogata and A. Oya in our laboratory in 1958 (unpublished data.). The other Asian strain was kindly supplied by Donald S. Burke, U.S. Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand. This strain (D77-012) was isolated from acute-phase plasma drawn from a 5-year-old Thai child hospitalized with undifferentiated fever. The virus was isolated by intrathoracic inoculation of the plasma into *Aedes aegypti* mosquitoes and detected in sonicated thorax-abdomen suspensions by plaquing on LLC-MK2 cells (D. S. Burke, personal communication). Seed viruses were prepared by passaging the viruses once in suckling mouse brain tissues and once in Vero cell cultures. Infected culture fluid was centrifuged at 9,500 × *g* for 15 min, and the supernatant was distributed into small vials and stored at −80°C until use. Infected cultures were incubated at 34°C, because virus yields of both Asian strains are reduced at 37°C, although the African strain did not show temperature sensitivity at 37°C (unpublished data). Preparation of virions and labeling of viral proteins were essentially the same as described previously for western equine encephalitis (WEE) virus (14). Viral proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described for WEE virus (13, 23).

The purified virions (BaH 306) banding on sucrose-TNE (0.05 M Tris-hydrochloride, 0.1 M NaCl, 1 mM EDTA [pH 7.4]) buffer density gradients were examined in an electron microscope (Fig. 1a). The virions showed quite uniform enveloped spherical particles ca. 70 nm in diameter which are common to all alphaviruses (17, 24). A similar electron micrograph was obtained when a specimen of the African strain banding at the same density was examined (data not shown). SDS-PAGE analysis of these purified virions revealed the presence of a broad band corresponding to envelope glycoproteins as previously reported (19) and a capsid (C) protein similar to those of other alphaviruses (Fig. 1b). As shown in Fig. 1b, envelope glycoproteins E1 and E2 of CHIK virus migrate very close to one another on the gel, and therefore, the E1 and E2 bands are not clearly identified.

Pulse and chase experiments were performed to reveal the two bands of CHIK virus glycoproteins and to determine which protein migrates faster on the gel. At 8 h postinfection, infected Vero cells were labeled with [^{3}H]leucine for 15 min, the isotope-containing medium was removed, and the cultures were chased with cold leucine for various periods of time as shown in Fig. 2. After a 90-min chase, the 65K precursor protein PE2 had disappeared almost completely and a new protein band migrating below the E1 protein appeared. This indicates that the E2 protein, cleaved from PE2, migrates faster than the E1 protein (1).

Therefore, the envelope of CHIK virus contains two glycoproteins, E1 and E2, which nearly comigrate on the gel. Recently, Konishi and Hotta (18) also reported two envelope polypeptides from CHIK virus, but they did not determine which protein was E1 or which was E2. The structural proteins of CHIK virus migrate on SDS-PAGE in the order E1, E2, and C, a pattern different from that of SF virus or Venezuelan equine encephalitis virus polypeptides (7, 10).

To demonstrate that both E1 and E2 are glycosylated, we compared proteins synthesized in the presence and absence of tunicamycin with SDS-PAGE. This drug prevents glycosylation of the polypeptide chains of alphavirus glycoproteins and the cleavage of PE2 to E2; the final products are unglycosylated forms of PE2 and E1 with higher electrophoretic mobilities (9, 13, 23). As shown in Fig. 3, the unglycosylated forms of PE2 and E1 migrated more rapidly than those of untreated controls.

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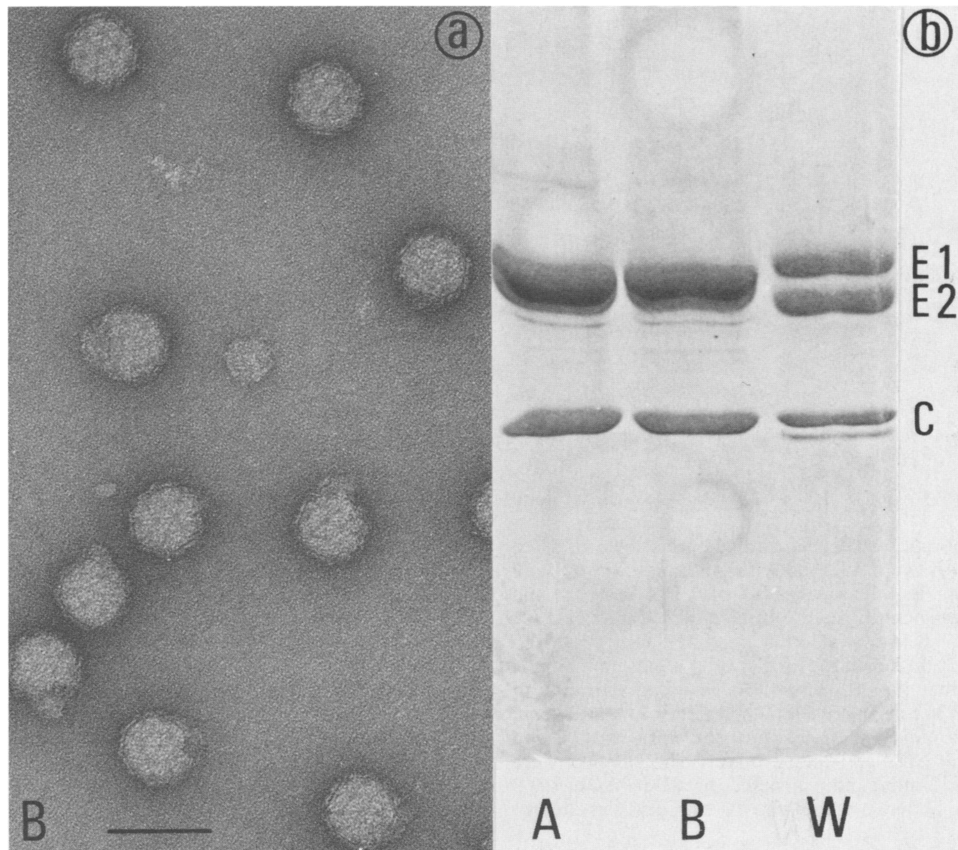

FIG. 1. (a) Electron micrograph of purified CHIK virions. CHIK virions from strain BaH 306 (B) were purified and negatively stained with an aqueous 2% solution of sodium phosphotungstate at pH 7.2 for 30 s. The specimens were examined with a Hitachi H-500 electron microscope at 75 kV. Bar, 100 nm. (b) SDS-PAGE pattern of purified CHIK virions. Viral proteins from the purified virions were electrophoresed on a 7.5% SDS-polyacrylamide gel, and the gel was stained with Coomassie brilliant blue. African strain (A) and Asian strain BaH 306 (B) of CHIK virus and WEE virus (W) were used.

To detect E3 of CHIK virus, the virus was labeled with [^{35}S]methionine and purified as described previously (23), and then purified virions were analyzed on a gradient SDS-polyacrylamide gel. As shown in Fig. 4, no band smaller than the capsid protein was visible. However, when virus-infected cultures were labeled with [^{35}S]methionine and then culture fluids were analyzed on the gel, a band with a molecular weight of ca. 11,000 appeared in addition to structural proteins E1, E2, and C (Fig. 4). This protein, E3, was labeled with [^{3}H]mannose (data not shown). These results indicate that, as with Sindbis (SIN) and WEE viruses, E3 of CHIK virus is not associated with virions but is released into culture fluids.

We were also interested in separating the CHIK virus glycoproteins to study the E1-E2 interaction, since it has been suggested that E1-E2 association in alphaviruses differs from virus to virus (8). To examine this, we tried to isolate and separate the two glycoproteins without loss of biological activity. For SIN and WEE viruses it has been possible to separate E1 and E2 in the presence of nonionic detergents by using a glass wool column under appropriate ionic conditions, by isoelectric focusing, or simply by solubilizing E1 specifically from a virus pellet in low-salt conditions (3, 5, 8, 12, 20). It has been reported that glass wool column chromatography provides a reproducible separation of E1 and E2 of WEE virus (25). In several preliminary experiments, we found that the association between E1 and E2 of CHIK virus was tighter than that of WEE and SIN viruses (2, 25) and that it was impossible to separate E1 from E2 under the same conditions that were used for WEE virus glycoproteins. Therefore, the conditions for the solubilization of CHIK virus envelope proteins were modified as stated in the legend to Fig. 5; both high pH and high ionic strength were used to dissociate E1 and E2.

Figure 5A shows a typical elution pattern of E1 and E2 of CHIK virus from a glass wool column. There are two major peaks and one minor peak of radioactivity. An analysis of pools of fractions by SDS-PAGE is shown in Fig. 5B. When the concentration of NaCl in the linear gradient reached 0.5 M, pure E1 was preferentially eluted in the first major peak; there were some fractions containing a reassociated E1-E2 complex, which eluted ahead of the main peak. A second peak was obtained at a NaCl concentration of ca. 0.9 M. This peak contained E2-rich fractions, but pure E2 was not obtained, as shown in Fig. 5B. The third (minor) peak, obtained by chasing with SDS, also contained both E1 and E2.

Hemagglutinating activity was associated with only the fractions from the first peak, and we could not find this activity in fractions from the second peak. These results reveal that hemagglutinating activity is associated with E1 and that E2 may not be involved in this activity, as indicated

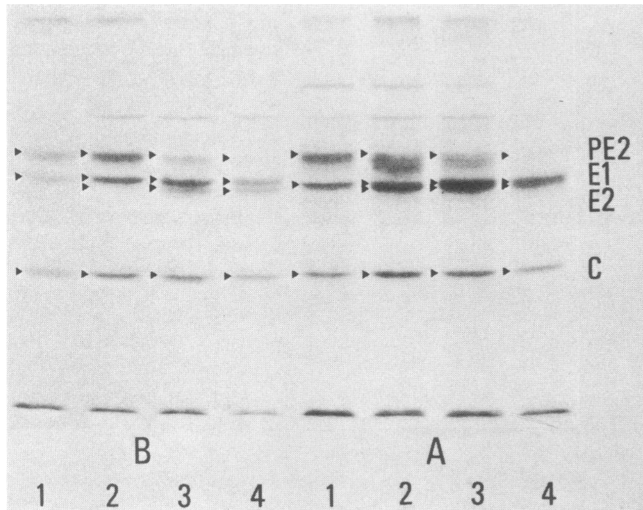

FIG. 2. Electrophoretic profiles of viral proteins from infected cells. Vero monolayers were infected with African strain (A) and Asian strain BaH 306 (B) at a multiplicity of 5 PFU per cell and incubated at 34°C. Some cultures were labeled with [^{3}H]leucine (20 μCi/ml) for 15 min at 8 h postinfection (lanes 1). Other infected cultures were washed and incubated further after a pulse in minimal essential medium containing 30 times the normal concentration of unlabeled leucine for 30 min (lanes 2), 60 min (lanes 3), and 90 min (lanes 4). After each pulse or chase, the cells were washed and dissolved in 100 μl of SDS gel sample buffer. The samples were heated at 100°C for 2 min and subjected to SDS-PAGE (10% polyacrylamide gel). After electrophoresis the gel was fluorographed.

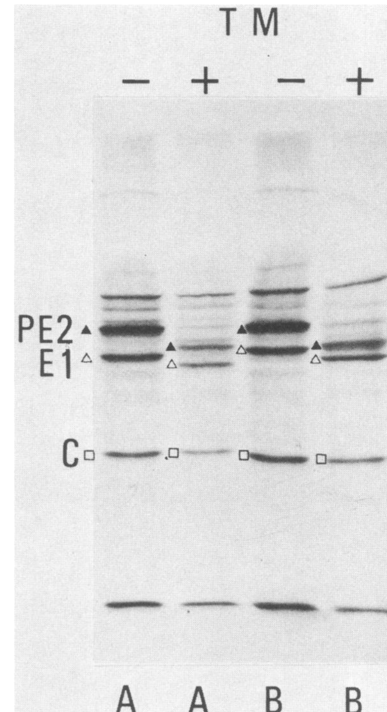

FIG. 3. Viral proteins synthesized in Vero cells treated with TM. Vero cells were infected with African strain (A) or Asian strain BaH 306 (B) at 34°C. Cells were labeled with [^{3}H]leucine (20 μCi/ml) for 30 min at 8 h postinfection. TM (0.5 μg/ml) was added to one set of cultures at 1 h postinfection. Viral proteins were analyzed by SDS-PAGE (10% polyacrylamide gel) as described in the legend to Fig. 2. Symbols: +, TM-treated cells; −, untreated cells.

by the ratios of E1 to E2 in fractions from the second peak.

CHIK, SF, and O'nyong nyong viruses are antigenically related viruses belonging to the SF virus complex (4). Glycoproteins of CHIK virus are not as well characterized as those of SF virus, which is one of most well-known alphaviruses on the molecular level. Therefore, we isolated and examined the two glycoproteins of CHIK virus and compared their properties with those of SF virus glycoproteins (8, 22). Since CHIK virus displays some variability in antigenic specificity (4), we used three CHIK viruses from different sources, one African and two Asian strains. We noted some biological differences between African and Asian strains: for example, the two Asian strains make plaques (2.56 mm in diameter) larger than those made by the African strain (1.54 mm) in Vero cells and show temperature sensitivity at 37°C, although in SDS-PAGE, these three strains do not differ significantly.

We can conclude from our studies that (i) E1 of CHIK virus has hemagglutinating activity, as is the case for SF virus (8), (ii) E3 of CHIK virus is not associated with virions but is released into culture fluids, in contrast to E3 of SF virus (10), and (iii) the E1-E2 association of CHIK virus is tighter than those of SIN and WEE viruses. This association force of CHIK virus glycoproteins is very similar to that of SF virus glycoproteins (8; unpublished data). In glass wool column chromatography, the solubilized E1 of WEE virus was eluted as a homogeneous protein with 0.2 M NaCl (25), whereas the dissociation of E1 from E2 of CHIK virus required a higher salt concentration (0.5 M). In CHIK virus, after the dissociation of the E1-E2 complex, some E1 may reassociate with E2 before it is bound to glass wool. This

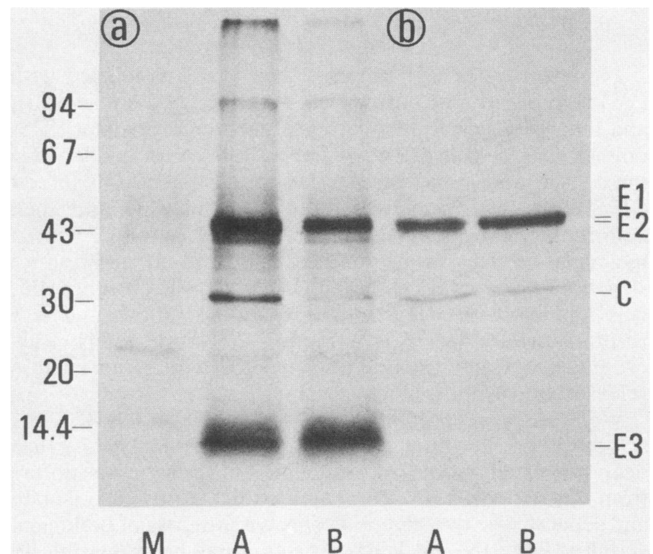

FIG. 4. Electrophoretic profiles of CHIK virus E3. Vero cells were infected with African strain (A) or Asian strain BaH 306 (B) or mock infected (M) and labeled with [^{35}S]methionine. The culture fluids were analyzed by SDS-PAGE (a). CHIK virions labeled with [^{35}S]methionine were purified and analyzed on a 7.5 to 20% gradient gel (b). The numbers on the left refer to molecular weight markers (in thousands).

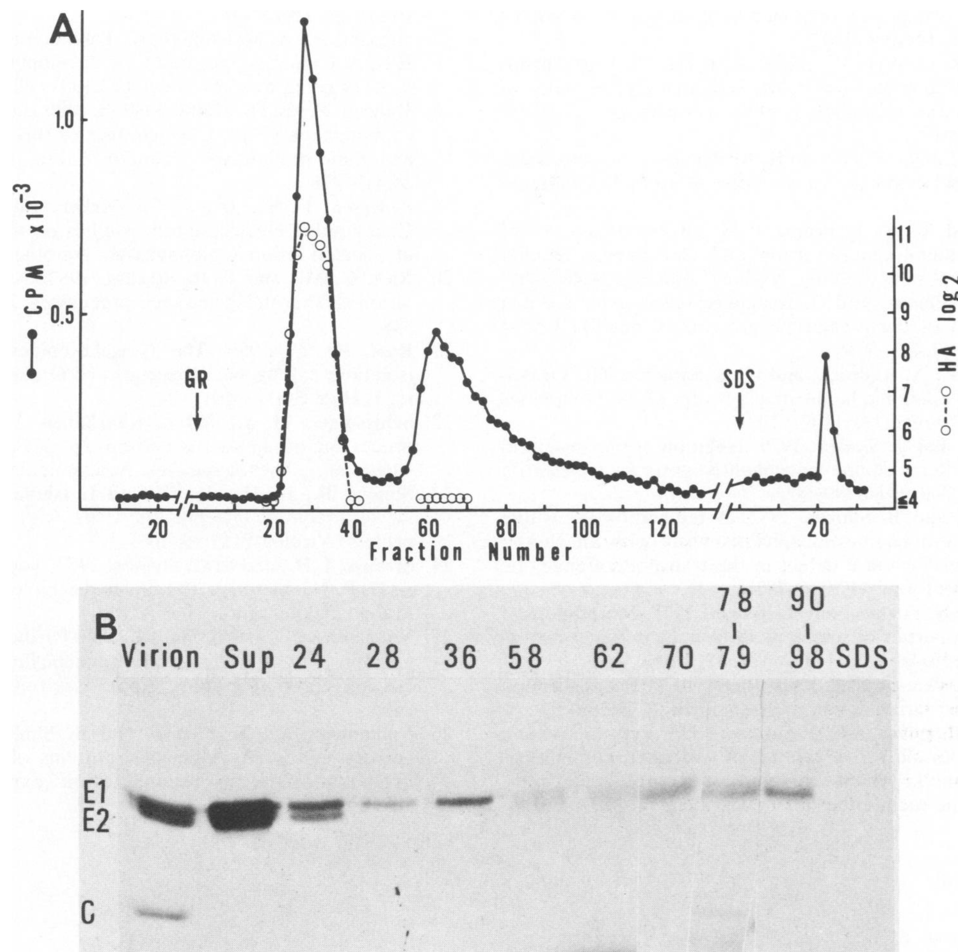

FIG. 5. (A) Chromatography of CHIK virus E1 and E2 on a glass wool column (1.5 by 33 cm). Purified [^{35}S]methionine-labeled virus (strain BaH 306) was disrupted by Nonidet P-40 (1% final concentration) in 50 mM Tris-hydrochloride–0.4 M NaCl (pH 8.0) and centrifuged at 240,000 × g for 60 min. The supernatant was dialyzed against starting buffer (25 mM succinate, 0.3 M NaCl [pH 6.0]). The dialyzed sample (1.45 ml, 86,000 cpm) was applied to the column, which was washed with starting buffer and then eluted with a gradient solution consisting of 100 ml of starting buffer and 100 ml of elution buffer (50 mM Tris-hydrochloride/1.5 M NaCl [pH 9.5]). The gradient buffer change is indicated by an arrow (GR). Finally, the column was chased with SDS buffer (50 mM Tris-hydrochloride, 1% SDS [pH 9.0]). The SDS buffer change is indicated by an arrow (SDS). The column was operated at a flow rate of ca. 1 ml/7.2 min, and 2-ml fractions were collected. Symbols: ●, counts per minute per 0.5 ml; ○, hemagglutination (HA) units per 0.5 ml. (B) Pooled fractions from glass wool column chromatography analyzed by SDS-PAGE. Collected samples were dialyzed against TNE buffer before electrophoresis. Some fractions were put into cellulose tubing (Visking Co.) and embedded in Ficoll-400 (Pharmacia Fine Chemicals, Inc.) for concentration before application to the gel. Numbers on the top of each lane refer to fraction numbers shown in (A). Sup, Supernatant.

may be a reason why it is difficult to obtain pure E2 from CHIK virus.

The isolation of the spike glycoproteins without denaturation is important for the study of the different biological properties of the glycoproteins, such as the hemagglutinating activity of E1 (5, 12, 25), and also for in vitro reconstitutions of viral membranes (15, 16, 26). In addition to these, protein sequence information obtained directly or deduced from subgenomic 26S RNA sequence will be most useful in the evaluation of the relationships among the members of the SF virus complex and the mechanism of evolution within the *Alphavirus* genus.

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