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NOTES

Virus-Directed Post-Translational Cleavage in Sindbis Virus-Infected Cells

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The viral polypeptides synthesized in cells coinfected with group C and group D or E Sindbis virus mutants were studied. Cleavage of the ts2 protein occurs in cells coinfected with ts2 and ts20. Since the ts2 protein fails to chase in cells infected with ts2 alone, the activity effecting this cleavage must be, at least in part, virus specified.

Virions of the alphavirus Sindbis are composed of at least three species of protein (20). The core protein, C, has a molecular weight of approximately 30,000 (24) and associates with the virion 42S RNA (2) to form an icosahedral nucleocapsid (4). The nucleocapsid buds through modified regions of the plasma membrane of the infected cell (1, 3, 16), becoming surrounded by an envelope (1, 4) composed of a lipid bilayer (12) from which the two glycoproteins, E1 and E2, have been shown to project (10, 20, 24). Each of the glycoproteins has an apparent molecular weight of approximately 50,000 (20, 24). Semliki forest virus, another alphavirus, has been shown to possess an additional glycoprotein, E3 (11).

E2 and E3 have been shown to be cleaved from a nonstructural precursor polypeptide, PE2, as a terminal event in the maturation of the virion (13, 21). Mutants of complementation groups D (12a) and E (13) are defective in this cleavage. Tryptic peptide maps (19) and pulse-chase experiments (6) suggest that E1 and PE2 are cleaved from another nonstructural glycoprotein, B, which accumulates in Sindbis-infected BHK cells (23). B and C are postulated to be formed by the initial cleavage (19) of the primary translation product (18) of the Sindbis 26S mRNA (17). A polypeptide of the appropriate size accumulates at the expense of envelope and core proteins in cells infected with several Sindbis mutants of complementation group C, including ts2 (18). This polypeptide has since been referred to as the ts2 protein. Recent support for such a cleavage sequence (14) has come from the elegant experiments of J. C. S. Clegg (9), who found only one site on the Semliki forest virus mRNA for initiation of protein synthesis in vivo.

Several lines of evidence, however, have suggested that the postulated cleavage sequence may be an oversimplification of the intracellular events leading to the formation of the virion structural proteins. First, as previously reported (18), the high-molecular-weight polypeptide produced at nonpermissive temperature in cells infected with group C mutants cannot be chased into cell-associated structural or nonstructural proteins upon shift to permissive temperature (Fig. 1). Also, proteins synthesized in vitro from wild-type mRNA do not include polypeptides with molecular weights comparable to that of the ts2 protein. Instead, small polypeptides containing C fingerprints (7) or discrete C and B proteins (22) are made. Large-molecular-weight polypeptides are, however, made in vitro from ts2 mRNA (8, 22). These observations suggest the possibility that multiple initiation and termination sites exist on the mRNA and that the ts2 protein may be a nonfunctional polypeptide produced by the failure of termination sequences at the 3′ end of the capsid portion of the message (8). A major observation that is inconsistent with this model is the complementation between mutants of group C and those of other RNA$^+$ complementation groups (5). Therefore, the polypeptide profiles of cells coinfected with complementing mutants seemed likely to provide insight into the mechanism of Sindbis virus protein synthesis.

Chicken embryo fibroblast cultures infected with ts2, ts23 (group D), ts20 (group E), or mixtures of ts2 with ts23 or ts20 were labeled with [^{35}S]methionine at nonpermissive temperature (41°C) for 1 h, digested, and subjected to polyacrylamide gel electrophoresis. Figure 2 shows that the high-molecular-weight polypep-

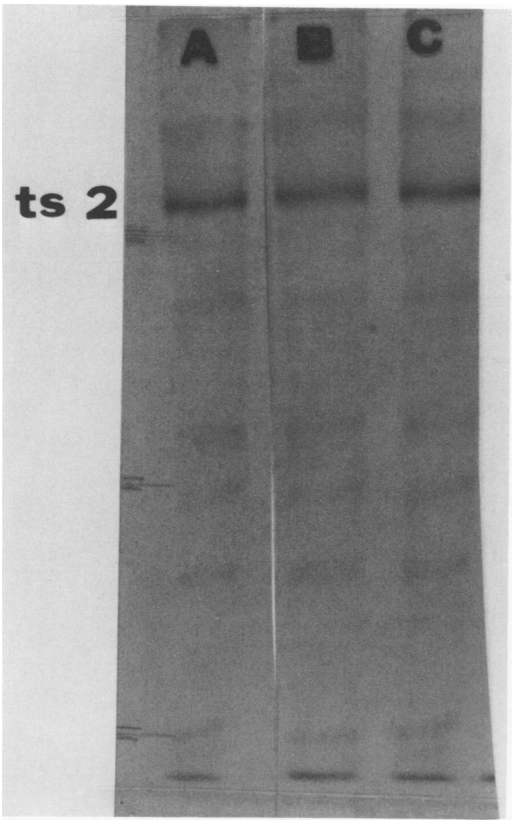

FIG. 1. *Polypeptides produced in ts2-infected cells. SPAFAS (Norwich, Conn.) chicken embryo fibroblast secondary cultures were infected with ts2 at a multiplicity of 10. Cells were incubated at 29°C in Eagle minimal essential medium lacking methionine, to which 3% dialyzed fetal calf serum (Microbiological Associates, Inc., Bethesda, Md.) and 5 μg of actinomycin D (Sigma Chemical Co., St. Louis, Mo.) per ml were added. After 8 h, medium prewarmed to 45°C was added, and the cultures were shifted to 41°C. Two hours later, [^{35}S]methionine was added to a final concentration of 50 μCi/ml, and the cultures were incubated for 7 min at 41°C. The pulse (lane A) was harvested and processed immediately for gel analysis. The cultures to be chased were washed three times with medium prewarmed to 45°C, to which had been added 100×-concentrated methionine and 100 μg of cycloheximide per ml. One set of cultures was incubated at 29°C (lane B); the second set was incubated at 41°C (lane C) for 45 min before the cultures were harvested. To harvest the cultures, the medium was removed, and the plates were floated on an ice-water slurry, washed three times with ice-cold phosphate-buffered saline supplemented with 100×-concentrated methionine, and digested by addition to the monolayers of 4% sodium dodecyl sulfate, 20% glycerol, and 10% β-mercaptoethanol in 0.125 M Tris, pH 6.8. After heating to 100°C for 2 min, the samples were subjected to slab polyacrylamide gel electrophoresis in the discontinuous system described by Laemmli (15). Approximately equal amounts of radioactivity were applied to each lane.*

tide produced in ts2-infected cells (lanes A and G) also appears in cells coinfected with ts2 and ts20 (lane B) or ts2 and ts23 (lane F). It is absent from cells infected with ts20 (lane C), ts23 (lane E), or wild type (lane D). When cells were incubated at 41°C throughout infection or at 29°C for 8 h and then shifted to 41°C prior to labeling, identical results were obtained. The presence of the ts2 protein in the doubly in-

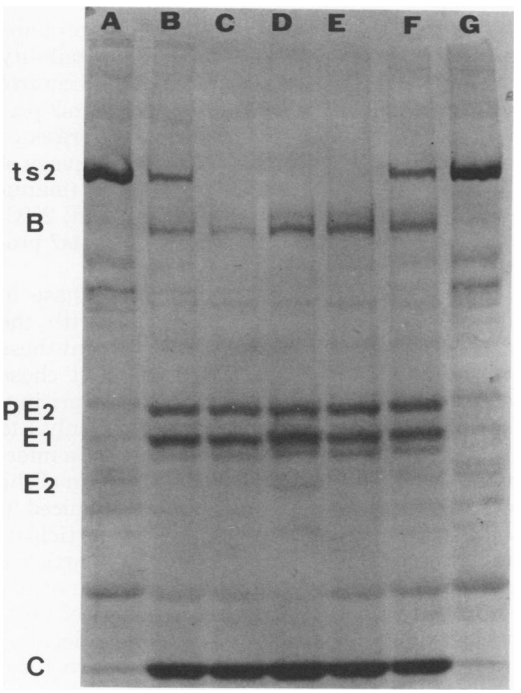

FIG. 2. *Polypeptides produced in singly and doubly infected cells. SPAFAS (Norwich, Conn.) chicken embryo fibroblast secondary cultures were infected with ts2, ts20, ts23, or mixtures of ts2 with ts20 or ts23 at a multiplicity of 10 PFU of each mutant per cell. Cells were incubated at 29°C in Eagle minimal essential medium lacking methionine, to which 3% dialyzed fetal calf serum (Microbiological Associates) and 5 μg of actinomycin D (Sigma) per ml were added. After 8 h, medium prewarmed to 45°C was added, and the cultures were shifted to 41°C. Two hours later, [^{35}S]methionine was added to a final concentration of 5 μCi/ml, and the cultures were incubated for 1 h at 41°C. The medium was removed, and the cells were harvested and processed for gel analysis as described in Fig. 1. Approximately equal amounts of radioactivity were applied to each lane. Lanes A and G, ts2; lane B, ts2 plus ts20; lane C, ts20; lane D, wild type; lane E, ts23; lane F, ts2 plus ts23.*

fected cells indicates that if the ts2 protein is cleaved in these cells, its cleavage occurs more slowly than in wild-type-infected cells.

To demonstrate that cleavage of the ts2 protein occurs in doubly infected cells, cultures coinfected with ts2 and ts20 were pulse-labeled at 41°C for 7 min and then chased at 41 or 29°C for 45 min. Figure 3 shows that the ts2 protein present in the pulse (lane A) chases both at permissive (lane B) and nonpermissive (lane D) temperatures. The high background of radioactivity in the lower-molecular-weight proteins appearing in the pulse precludes the possibility of demonstrating cleavage products. To control against nonspecific degradation of the ts2 protein, other cultures were chased in the presence of insulin, which interferes with the cleavage of Sindbis virus nonstructural proteins (manuscript in preparation). Lanes C (insulin, 29°C) and E (insulin, 41°C) indicate that the ts2 protein is not degraded.

The capacity of the ts2 protein to chase in doubly infected cells is compatible with the ability of group C mutants to complement those of groups D and E. The very efficient chase observed in these experiments is surprising, however, since double infections yield only 1 to 10% of the progeny produced in wild-type infections (5). Therefore, only a small fraction of the cleavage products of the ts2 protein produced in coinfected cells appears capable of participating in the production of infectious particles. Nevertheless, the results presented here firmly support the hypothesis that a series of posttranslational cleavages of a high-molecular-weight precursor yields the virion structural proteins.

Because the ts2 protein from pulse-labeled, coinfected cultures chases efficiently, the reason for its accumulation in coinfected cultures during a steady-state label is unclear. Under steady-state conditions, however, nearly all of the ts2 protein can be found in the 12,000 × *g* pellet of infected cell homogenates (data not shown). Perhaps its association with membrane is unfavorable for cleavage.

The results presented suggest two possible lesions for ts2. First, an amino acid substitution could change the conformation of the high-molecular-weight precursor such that its cleavage could not occur. In the coinfected cells chased at nonpermissive temperature, then, cleavage would proceed due to intracistronic complementation, the complementing mutant producing a protein complexing with the mutant protein, altering its conformation such that it would be cleaved.

The second possible lesion would involve a mutation unrelated to the cleavage site of the ts2 protein, probably inactivating a virus-specified protease. In this case, the ts2 protein would be cleaved in coinfected cells at nonpermissive temperature by a diffusible protease activity provided by the complementing virus. Such an activity has been suggested previously (8, 22).

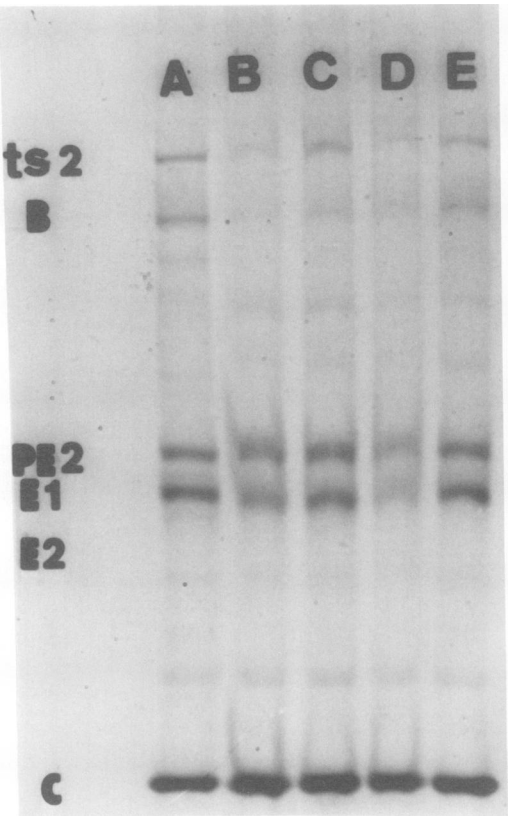

FIG. 3. *Pulse-chase polypeptide profiles of ts2-ts20 double-infected cells. Cells were infected with a mixture of ts2 and ts20 and incubated as described for Fig. 1. After 2 h at 41°C, cultures were pulse-labeled with 50 μCi of [^{35}S]methionine per ml for 7 min. The pulse (lane A) was harvested, and cultures to be chased were washed three times with medium prewarmed to 45°C, to which had been added 100×-concentrated methionine and 100 μg of cycloheximide per ml. One untreated culture (lane B) and one insulin-treated culture (lane C) were shifted to 29°C, and an untreated culture (lane D) and insulin-treated culture (lane E) were chased at 41°C.*

Neither of these possible lesions, however, accounts for the failure of the ts2 protein to cleave during single infection upon shift to the permissive temperature. Protein produced after the shift would be expected both to complex the ts2 protein produced at nonpermissive temperature, thereby modifying its conformation such

that it could be cleaved, and to cleave it by direct proteolytic activity. The real lesion is more likely to be a combination of the two. It is clear, though, that the complementing virus directs the synthesis of a diffusible factor responsible for the cleavage of ts2 protein in coinfected cells. The protein involved in cleavage may prove to be the capsid protein.

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