Proteins Specified by Sindbis Virus in HeLa Cells

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HeLa cells infected with Sindbis virus were found to contain five species of nonvirion proteins besides the structural proteins of the virus. Some of the nonvirion proteins were found to serve as precursors to the viral structural proteins in a pulse-chase experiment.

Several polypeptides other than the structural proteins have been detected in Sindbis (SB) virus-infected cells (4, 6, 7, 8, 11, 13, 14). A large viral protein called B1 protein (9.9×10^4 daltons) has been detected in baby hamster kidney (BHK) cells infected with SB virus (8). Infected chicken embryo (CE) cells incubated with a protease inhibitor accumulated a polypeptide possessing an electrophoretic mobility similar to the B1 protein found in infected BHK cells (4). Recently, we reported the presence of an even larger protein (13×10^4 daltons) functioning as a precursor to the structural proteins in aged CE cells infected with SB virus (11). A temperature-sensitive mutant of SB virus (*ts2*) accumulates a large polypeptide (11×10 to 13×10^4 daltons) in CE cells at the nonpermissive temperature (6, 13). The above protein contained tryptic peptides found in all of the three structural proteins (8). All the above observations led to the suggestion that proteins of SB virus are synthesized initially as a large polypeptide which is then cleaved to yield the individual proteins (4, 6, 7, 8, 11, 13, 14). The present report deals with experiments on the types of viral proteins specified by SB virus in HeLa cells.

The growth characteristics of SB virus in HeLa cells have recently been described (5). The exponential rise in virus synthesis begins after about 6 h after virus addition and continues until 12 h. Maximum yields of virus are obtained at about 36 h.

Preliminary experiments indicated that, even in the presence of dactinomycin, host cell protein synthesis was not substantially reduced during infection. Therefore, proteins from infected cells (labeled with ^3H -leucine) and uninfected cells (labeled with ^{14}C -leucine) were co-electrophoresed on polyacrylamide gels (PAG), and the virus-specific proteins were

distinguished from those of the host by the method of Zweernick and Joklik (18). This method has been used recently in the study of arbovirus-specific proteins in infected cells (17).

Experiments were done to determine the types and amount of virus-specific proteins synthesized in infected cells during several periods of the viral multiplication cycle. The results (not presented) indicated that at least five nonvirion (NV) proteins could be detected in the infected cells. There was no preferential labeling of any one of the viral proteins since the types and relative amounts of the polypeptides appearing in cells were similar regardless of the time after addition of virus to cultures. Additionally, we observed that NV polypeptides larger than the three structural proteins were the predominant species detected in infected cells during a brief pulse with ^3H -leucine. A pulse-chase experiment was performed to determine whether any of the polypeptides appearing in the infected cells served as precursors to the virus structural polypeptides. The results are illustrated in Fig. 1. Purified SB virions were found to contain three polypeptides (Fig. 1A) under the conditions of electrophoresis used here. These results are similar to those reported by Schlesinger et al. (9). The results presented in Fig. 1B-D represent the electrophoretic profiles of the proteins isolated from infected cells at 0, 30, and 60 min after the chase, respectively. At least eight virus-specific polypeptides can be distinguished in the profile presented in Fig. 1B. Three of these (E_1 , E_2 , and CP) may represent the structural proteins since their electrophoretic mobilities are similar to those obtained from the virions. Apparently, at least five NV proteins can be detected in the infected cells. We have designated them as NV-1, NV-2, NV-3, NV-4, and NV-5 in order of their decreasing electrophoretic mobilities. The

FIG. 1. Virus-specific proteins labeled during a pulse and subsequent chase. Six HeLa cell cultures were infected with SB virus and incubated at 37 C for 4 h in leucine-free growth medium containing 5 μ g of dactinomycin per ml. They were then exposed to ^{3} H-leucine (250 μ Ci/culture) for 2 min. Cultures receiving no virus served as controls and received ^{14} C-leucine (20 μ Ci/culture) for 2 min. At the end of the pulse the cultures were washed several times with cold phosphate-buffered saline, and then were incubated at 37 C with hypotonic medium containing 100 μ g of unlabeled leucine per ml (11). Hypotonic medium inhibits release of SB virus from infected cells (15) and ensures a minimal loss of labeled material during the chase interval. Two infected and two uninfected cultures were harvested at the start of the chase period. The same procedure was followed for cultures incubated for 30 and 60 min after the chase began. The isotopically labeled proteins from infected and uninfected cells were isolated by methods similar to those previously described (12). They were then dialyzed overnight against 0.0625 M Tris-hydrochloride, pH

predominant species of polypeptides containing the label after the pulse period are the NV-4 and NV-5 types. However, the results presented in Fig. 1C and D indicate that there is a progressive decrease in the amount of radioactivity in high molecular weight (HMW) proteins during the chase interval. Concomitantly, there is a progressive increase in the amount of radioactivity appearing in the three structural proteins. The distribution of the radioactivity associated with the various virus-specific polypeptides during the pulse and chase periods is summarized in Fig. 2. Based on the distribution of radioactivity in the three structural proteins during the chase period, the core protein, CP, appeared to be formed faster than the other two proteins. This is seen from the observation that there is very little difference in the amount of label appearing in CP between 30 and 60 min of chase. The present results suggest that the HMW polypeptides may serve as precursors to the structural proteins.

Attempts were made to determine the molecular weight of these viral polypeptides. The relative migration of polypeptides on SDS-PAG is directly proportional to the $\log_{10}$ of their molecular weights (10, 16). Therefore, the molecular weights of the above proteins were estimated by comparing their electrophoretic mobilities on PAG with those of known molecular weight. D-Amino acid oxidase, gamma globulin (H and L chains), and cytochrome c were used for this purpose. The estimated molecular weights of NV-1, CP, E₁, E₂, NV-2, NV-3, NV-4, and NV-5 are 23,500, 30,000, 45,000, 53,000, 65,000, 90,000, 110,000, and 130,000, respectively. The above values may be apparent, especially for the HMW proteins, since in the area of the origin of the gel accurate molecular weight determinations are difficult (2). Additionally, preliminary experiments indi-

6.8, containing 2% sodium dodecyl sulfate and 0.1% mercaptoethanol. Samples of 100 μ liters were made 5% in mercaptoethanol, 10% in sucrose, and 0.001% in bromophenol blue. They were then electrophoresed on the discontinuous-pH sodium dodecyl sulfate PAG electrophoresis system of Laemmli (3). Cylindrical separation gels were 10 cm long and contained 13% acrylamide. Cylindrical stacking gels were 1 cm long and contained 3% acrylamide. Samples were subjected to electrophoresis for 9 h at 3 mA per gel. The contributions of the host protein component to the infected cell profile in the electropherograms were eliminated by methods described in the text. A, SB virus structural proteins. Net SB virus-specific polypeptides at (B) 0 min, (C) 30 min, and (D) 60 min after chase.

FIG. 2. Time course of formation of viral-specific proteins. The amounts of radioactivity in each protein at the end of the pulse and chase periods were summed up from the profiles depicted in Fig. 1. After the 2-min labeling period, the net radioactivity (corrected for host proteins) in the profile 1B was 1.25×10^4 counts/min, whereas 30 and 60 min after chase they were 1.21×10^4 and 1.09×10^4 , respectively. The amount of radioactivity in each protein is expressed as the percentage of the net radioactivity present in the gel at the respective interval.

cate that some of the HMW proteins are glycoproteins since they can be labeled with radioactive glucosamine. Therefore, the estimates on the molecular weight of these proteins may not be real, since it is not known how glycosylation affects the electrophoretic mobilities of polypeptides (1).

Most of the viral polypeptides found in HeLa cells have counterparts in other host cells. On the basis of molecular weights, the NV-2 and NV-3 polypeptides may correspond to the PE₂ and B1 proteins detected in CE and BHK cells, respectively (7, 8). Recently, we showed that two HMW-polypeptides serving as precursors to the structural proteins are the predominant species appearing in infected aged CE cells during a brief pulse with ³H-leucine (11). Polypeptides NV-4 and NV-5 possess molecular

weights similar to the above polypeptides. The only exception appears to be the NV-1 protein described here. At present it is difficult to decide whether the NV-1 protein represents a true virus-specific polypeptide or a nonspecific degradation product of a larger polypeptide. However, a review of the electrophoretic profiles of SB virus-specific polypeptides published by others suggests the presence of a polypeptide with an electrophoretic mobility similar to that found for NV-1 (6, 11, 13, 14).

We have provided preliminary evidence to indicate that the NV-proteins found in HeLa cells may serve as precursors to the structural proteins. The results from the pulse-chase experiment support the above conclusion. The kinetics of appearance of radioactivity in the core protein, CP, was different from that of the other two structural proteins. This is exemplified by the observation that there was little increment in the amount of radioactivity appearing in the CP after 30 min of chase (Fig. 2). If it is assumed that all three structural proteins arise from a common precursor protein, then the present results would be consistent with the notion that cleavage of the capsid protein occurs soon after or during synthesis of the whole polypeptide chain. Alternatively, the results could be explained by assuming that the CP originates from a precursor other than that for the other two structural proteins. Confirmation of either of these two possibilities may be possible by analysis of the fingerprints of the various HMW-polypeptides detected in the HeLa cells. Towards this end we are purifying the viral proteins from the contaminating host proteins by immunoprecipitation with antiserum prepared against the virions.

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LITERATURE CITED

1. Bretscher, M. S. 1973. Membrane structure: some general principles. *Science* **181**:622-629.
2. Kiehn, E. D., and J. J. Holland. 1970. Synthesis and cleavage of enterovirus polypeptides in mammalian cells. *J. Virol.* **5**:358-367.
3. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.
4. Pfefferkorn, E. R., and M. K. Boyle. 1972. Selective inhibition of the synthesis of Sindbis virion proteins by an inhibitor of chymotrypsin. *J. Virol.* **9**:187-188.
5. Rosemond, H., and T. Sreevalsan. 1973. Viral RNAs associated with ribosomes in Sindbis virus-infected HeLa cells. *J. Virol.* **11**:399-415.
6. Scheele, C. M., and E. R. Pfefferkorn. 1970. Virus-specific proteins synthesized in cells infected with RNA⁺ temperature-sensitive mutants of Sindbis virus. *J. Virol.* **5**:329-337.

7. Schlesinger, S., and M. J. Schlesinger. 1972. Formation of Sindbis virus proteins: identification of a precursor for one of the envelope proteins. *J. Virol.* **10**:925-932.
8. Schlesinger, M. J., and S. Schlesinger. 1973. Large-molecular-weight precursors of Sindbis virus proteins. *J. Virol.* **11**:1013-1016.
9. Schlesinger, M. J., S. Schlesinger, and B. W. Burge. 1972. Identification of a second glycoprotein in Sindbis Virus. *Virology* **47**:539-541.
10. Shapiro, A. L., E. Vinuela, and J. V. Maizel, Jr. 1967. Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. *Biochem. Biophys. Res. Commun.* **28**:815-820.
11. Snyder, H. W., and T. Sreevalsan. 1973. Proteins specified by Sindbis virus in chick embryo fibroblast cells. *Biochem. Biophys. Res. Commun.* **54**:24-31.
12. Sreevalsan, T., and P. T. Allen. 1968. Replication of Western equine encephalomyelitis virus. II. Cytoplasmic structure involved in the synthesis and development of the virions. *J. Virol.* **2**:1038-1046.
13. Strauss, J. N., Jr., B. W. Burge, and J. E. Darnell. 1969. Sindbis virus infection of chick and hamster cells: synthesis of virus-specified proteins. *Virology* **37**:367-376.
14. Waite, M. R. F. 1973. Protein synthesis directed by an RNA⁻ temperature sensitive mutant of Sindbis virus. *J. Virol.* **11**:198-206.
15. Waite, M. R. F., and E. R. Pfefferkorn. 1970. Inhibition of Sindbis virus production by media of low ionic strength: intracellular events and requirements for reversal. *J. Virol.* **5**:60-71.
16. Weber, L., and M. Osborn. 1969. The reliability of molecular weight estimations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **224**:4406-4412.
17. Westaway, E. G. 1973. Proteins specified by group B togaviruses in mammalian cells during productive infections. *Virology* **51**:454-465.
18. Zweerink, H. J., and W. K. Joklik. 1970. Studies on the intracellular synthesis of reovirus-specified proteins. *Virology* **41**:501-518.