PROTEINS SPECIFIED BY SINDBIS VIRUS IN CHICK EMBRYO FIBROBLAST CELLS

H.W. Snyder and T. Sreevalsan

DEPARTMENT OF MICROBIOLOGY
GEORGETOWN UNIVERSITY, SCHOOL OF MEDICINE AND DENTISTRY
WASHINGTON, D.C. 20007

Received May 16, 1973

SUMMARY: Large amounts of high molecular weight polypeptides were detected in "aged" chick embryo fibroblast cells infected with Sindbis virus. These polypeptides were shown to be virus specific by several criteria. At least some of the above polypeptides were shown to be precursors to smaller viral structural proteins by a pulse-chase experiment.

INTRODUCTION: At present, the exact mode of formation of arboviral proteins in infected cells is far from clear. Virus specific polypeptides with molecular weights higher than those of the structural proteins have been detected in chick embryo fibroblast (CEF) cultures infected with wild-type Sindbis (SB) virus (1,2,3,4). One of these polypeptides has been shown to accumulate in infected cells incubated with an inhibitor of chymotrypsin (3). Also, Schlesinger and Schlesinger identified a precursor protein for one of the viral envelope proteins (5). Large virus specific polypeptides have been detected in CEF cultures infected with certain temperature sensitive (ts) mutants of SB virus, e.g. ts-11, ts-13 (1,4). Presumably these high moleuclar weight proteins are precursors to the smaller viral proteins. However, no direct evidence has been published to indicate that all the structural polypeptides originate in infected cells from precursor proteins. The present report deals with experiments which demonstrate that high molecular weight polypeptides can be accumulated in infected CEF under certain conditions. Evidence is presented suggesting that these polypeptides may serve as precursors for the viral structural proteins.

MATERIALS AND METHODS: CEF and Hela cell cultures were prepared by methods similar to those described earlier (6,7). CEF cultures were used at the end of one or seven days of incubation at 37°C. "Aged" cultures refer to those that were maintained at 37°C for seven days after seeding without addition of fresh medium. The details regarding the methods used for infecting cells with the virus, and the growth medium used, are published elsewhere (7). Both the HR and ts-2 strains of SB virus were used. The methods for obtaining stock suspensions of the labeled or unlabeled virus have been described previously (7).

Eagle's medium of low ionic strength (0.075M NaCl) was used instead of regular medium in experiments involving the pulse and chase of labeled proteins in infected cells. This hypotonic medium inhibits maturation of SB virus in CEF infected cells (8). Therefore, this procedure ensured a minimal loss of labeled virus from the infected cells during the chase interval.

Actinomycin D was obtained as a gift from Merck, Sharp, and Dohme, West Point, Pa. It was used at a concentration of 5 µg per milliliter of medium.

^{3}H-L-leucine (specific activity 115,000 mCi/mM) and ^{14}C-L-leucine (342 mCi/mM) were obtained from Amersham and Searles, Chicago, Ill.

Isotopically labeled proteins from infected and uninfected cells were isolated and electrophoretically analyzed on 7.5% polyacrylamide gels by methods similar to those described earlier (9).

RESULTS AND DISCUSSION: Group A arboviruses replicate poorly in CEF "aged" in vitro (10,11). Thus, cultures "aged" for seven days at 37°C yielded virus which was only 10% of that produced in cultures "aged" for one day. Incubation with actinomycin D or trypsinization relieved the viral inhibitory state of the "aged" cultures (10,11). Inefficient cleavage of the primary viral polypeptides (high molecular weight proteins) may be one of the reasons for the reduced yields of virus in "aged" cultures. If this is true, high molecular weight proteins should be the predominant species in "aged" cultures infected with SB virus. The electrophoretic patterns of labeled polypeptides appearing in one and seven day old

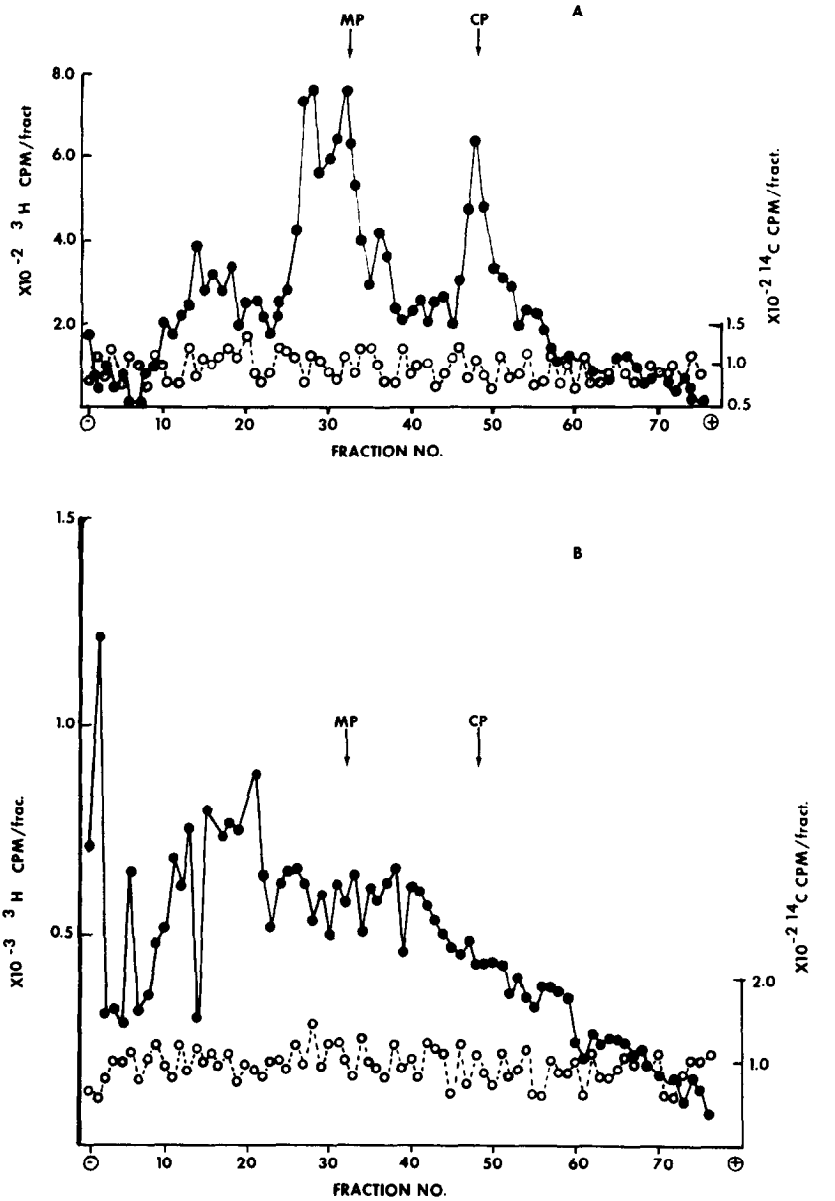

Figure 1. Electrophoretic patterns of proteins synthesized in one and seven day old CEF infected with SB virus. One or seven day old CEF cultures (8 x 10^6 cells/ culture) were infected with SB virus and incubated at 37°C for 4 hours in leucine-free growth medium. The cultures were then exposed to ^{3}H-leucine (50 µCi/culture) for 30 minutes. Cultures receiving no virus served as controls and were exposed to ^{14}C-leucine (10 µCi/culture) for 30 minutes. Cellular extracts were prepared from the infected and uninfected cells and processed for electrophoretic analysis. Mixtures of labeled proteins from infected and uninfected cells were co-electrophoresed at 4.5 milliamp/gel for 17.5 hours in 7.5 cm long polyacrylamide gels. (A) ^{14}C-labeled proteins from uninfected plus ^{3}H-labeled proteins from infected one day old cells. (B) ^{14}C-labeled proteins from uninfected plus ^{3}H-labeled proteins from infected seven day old cells. Arrows represent the positions at which the membrane (MP) and core (CP) proteins from SB virions migrate in the gels under similar conditions. ●———●, ^{3}H; o- - - - - o , ^{14}C.

CEF infected with SB virus were determined to test such a possibility and the results are presented in Figure 1. Distinct peaks of radioactivity corresponding to the two major structural polypeptides of the virus (membrane and core) are seen in the pattern from one day old cultures. However, no distinct peaks corresponding to the above proteins are seen in the pattern from seven day old cells. A majority of the labeled proteins appear to migrate in the gel with mobilities slower than those of the structural proteins. The possibility that they represent host proteins appears unlikely since labeled proteins (^{14}C-leucine) from uninfected cells migrated in the gel differently.

The following approach was used to establish that the high molecular weight proteins appearing in "aged" CEF were virus specific. The species of viral proteins synthesized should be similar regardless of the type of host cells used for infecting with SB virus. Conversely, the species of viral proteins synthesized in the same host should be different depending on the type of SB virus mutant used. Recently, a strain of Hela cells (Hela-R) has been shown to support the growth of SB virus (7). Therefore, we compared the types of viral proteins synthesized in Hela cells infected with the wild-type and the ts-2 mutant of SB virus. The results of such an analysis is shown in Figure 2. The predominant species of proteins synthesized in cells infected with wild-type virus were larger than the two structural proteins (Figure 2A). However, the major species of proteins appearing in ts-2 infected cells were polypeptides 1 and 2. The relative electrophoretic mobilities of these two proteins, as observed here, were similar to those reported by other workers (1,2,3). Additionally, it can be seen that the high molecular weight proteins observed in infected Hela cells possess electrophoretic mobilities different from those in uninfected cells. This, coupled with the observations on the types of proteins in Hela cells infected with the mutant virus, suggests that the high molecular weight proteins appearing in Hela cells are probably virus specific. The species of high molecular weight proteins observed in "aged" CEF cells were similar to those observed in Hela cells (compare Figures 1A and 2A) and therefore should also be specified by the virus.

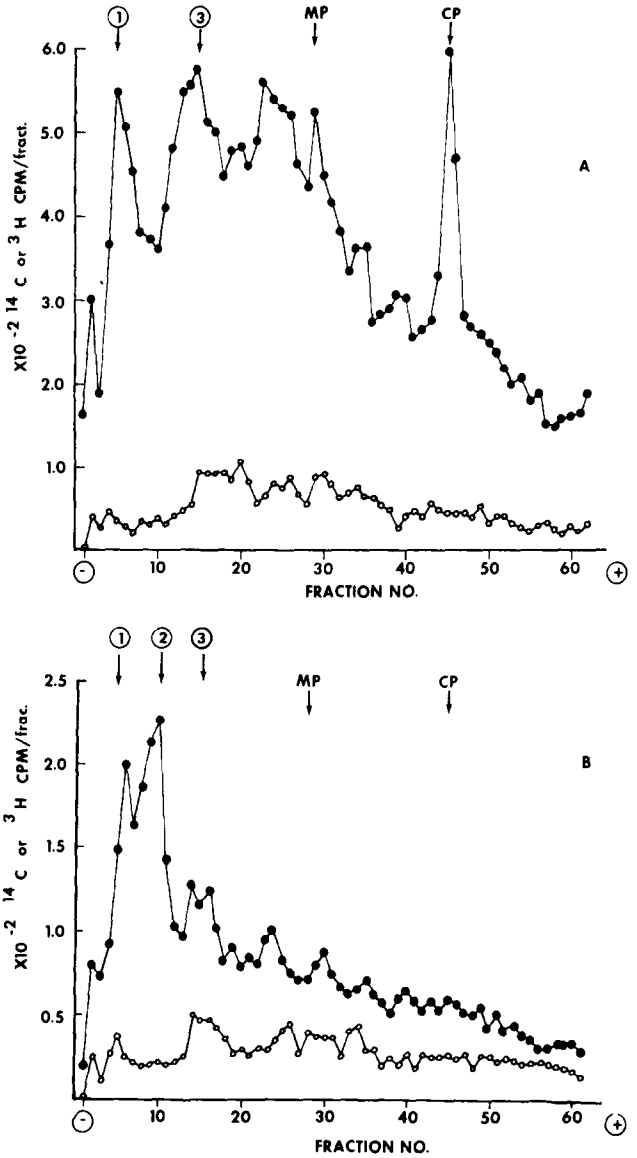

Figure 2. Electrophoretic patterns of proteins synthesized in Hela-R cells infected with SB virus and its mutant. Hela cell cultures (4×10^6 cells/culture) were infected with SB virus or the RNA^+ mutant ts-2. The cultures infected with SB virus were incubated at 37°C for 4 hours in leucine-free medium containing 5 µg actinomycin D/ml. and then exposed to ^{3}H-leucine (50 µCi/culture) for 30 minutes. The cultures infected with the ts-2 mutant were incubated at 27°C for 4 hours in leucine-fee medium containing actinomycin D. Then the cultures were rapidly warmed and incubated with fresh medium at 42°C for 1 hour. Tritiated leucine (50 µCi/culture) was added to the cultures and incorporation was allowed to occur for 1 hour at 42°C. Proteins from uninfected cells labeled with ^{14}C-leucine (10 uCi/culture) were prepared under conditions similar to those used for infected cells at 37°C or 42°C. Samples were prepared and analyzed by electrophoresis under conditions similar to those described in the legend to figure 1. (A) ^{14}C-leucine labeled proteins from uninfected plus ^{3}H-leucine labeled proteins from SB virus infected Hela cells at 37°C. (B) ^{14}C-leucine labeled proteins from uninfected plus ^{3}H-leucine labeled proteins from ts-2 infected Hela cells at 42°C. ●————●, 3H; o- - - - - - - -o ^{14}C.

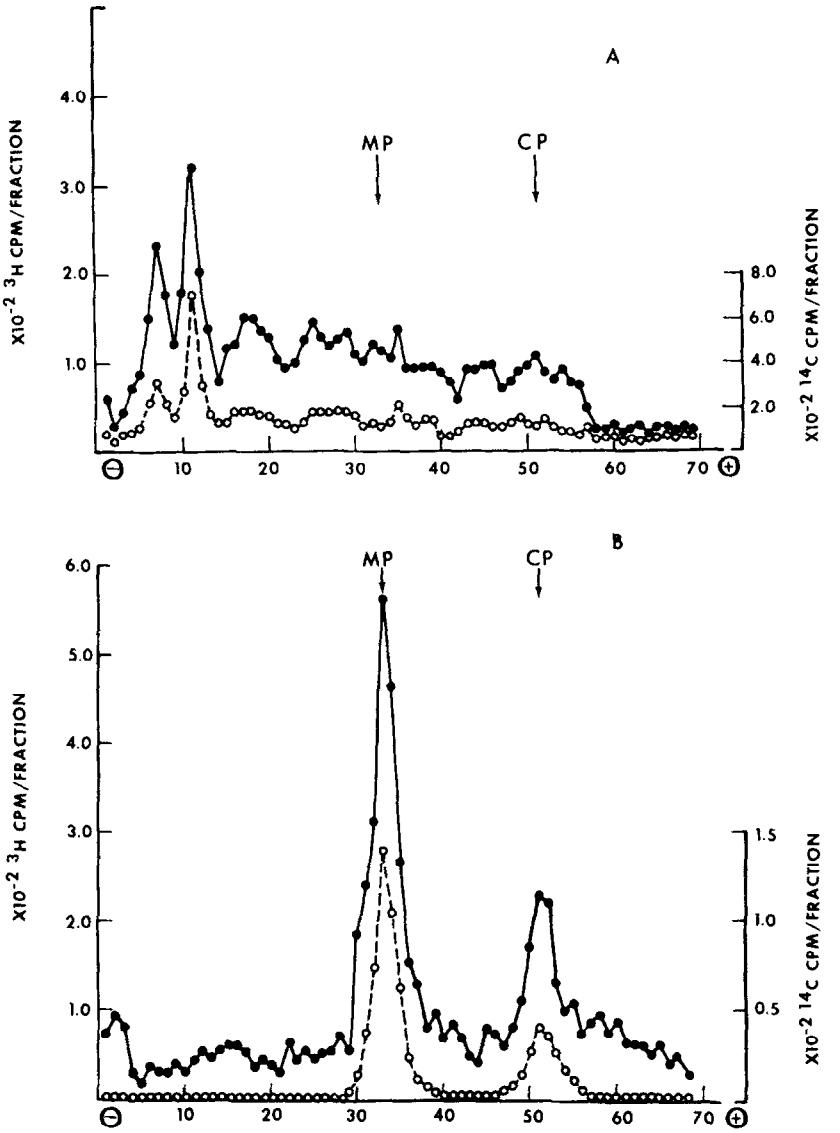

Figure 3. Virus specific proteins labeled during a pulse and subsequent chase. Four "aged" (seven days old) CEF cultures were infected with SB virus and incubated at 37°C for 4 hours in leucine-free growth medium containing 5 µg actinomycin D/ml. They were then exposed to ^{3}H-leucine (250 µCi/culture) for two minutes. At the end of the pulse the cultures were washed several times with cold phosphate buffered saline. The cultures were then incubated at 37°C with hypotonic medium containing 100 µg. unlabeled leucine/ml. Two cultures were harvested at the start and the rest at two hours after the chase began. The methods and conditions used for preparing labeled proteins from "aged" CEF cultures infected with the ts-2 mutant were the same as those described in the legend to figure 2. ^{14}C-labeled leucine (10 µCi/culture) was used for labeling the ts-2 infected cultures at 42°C. The proteins were prepared for electrophoresis and analyzed under conditions similar to those described in Figure 1. (A) ^{3}H-leucine lableld proteins from SB virus infected CEF cells 0 minutes after chase plus ^{14}C-leucine labeled proteins from ts-2 mutant infected CEF cells at 42°C. (B) ^{3}H-leucine labeled proteins from CEF cells at the end of two hours after the chase plus ^{14}C-leucine labeled proteins from SB virions. ●——●, ^{3}H; o- - - - -o ^{14}C.

It is possible that the high molecular weight proteins accumulating in "aged" CEF infected with SB virus may represent precursor proteins for the structural polypeptides. To test such a possibility the following experiment was done. A pulse-chase experiment with ^{3}H-leucine was performed in "aged" cells after infection with SB virus. In this experiment actinomycin D was used to release the "aged" CEF from the viral inhibitory state. The results of such an experiment are shown in Figure 3. The predominant species of polypeptides appearing during the pulse period (two minutes) consisted of proteins with electrophoretic mobilities similar to those of the two major polypeptides appearing in "aged" CEF infected with ts-2. The structural proteins (membrane and core) were poorly labeled under the above conditions. However, after a two hour chase with unlabeled leucine, about 85% of the label appearing in the high molecular weight region of the gel was confined in the region of the structural proteins. This suggests that the high molecular weight polypeptides detected during the pulse period may serve as precursors to the structural proteins.

The present results do not permit us to make any valid conclusions about Actinomycin D reversal of reduced SB virus production by "aged" CEF. Regardless of the exact nature of the actinomycin D effect, the results presented here represent direct experimental evidence suggesting that structural proteins of SB virus probably originate from high molecular weight polypeptides. The approximate molecular weights of the two major species of high molecular weight proteins observed in Figure 3 are 130,000 daltons and 120,000 daltons respectively. Proteins possessing similar molecular weights have been observed in cells infected with some RNA^{+} mutants, e.g. ts-2 (1,2,3). The present results show that the high molecular weight proteins detected in cells infected with the ts-2 mutant are identical in their electrophoretic mobilities to those found during a brief labeling period of cells infected with the wild-type. Additionally, we have shown here that the label present in such high molecular weight proteins appears in the structural proteins subsequent to a period of chase. Recent evidence indicates that a 26 to 28S form of viral RNA can function as a messenger in infected cells (7,12). If a messenger of that

size were to be translated monocistronically the approximate size of the theoretical protein would be 180,000 daltons. Based on this reasoning, the molecular weight of the largest protein observed here may be too small to represent the entire messenger. This anomaly may be due to the following reasons. Determination of the molecular weights of large proteins by electrophoresis on polyacrylamide gels may not be accurate since mobilities of proteins above 100,000 daltons could not be directly proportional to their molecular weights. Alternatively, there may exist a large segment in the 26 to 28S RNA which is not translated *in vivo*. The present results do not provide any evidence on how the smaller viral proteins originate from the high molecular weight precursors.

ACKNOWLEDGEMENTS: This work was supported by PHS research grant A1-09355-03 and training grant AI-298-07 from the National Institutes of Health.

REFERENCES:

1. Strauss, J.N. Jr., Burge, B.W. and Darnell, J.E., Virology, 37, 367, 1969.
2. Scheele, C.M, and Pfefferkorn, E.R., J. Virol., 5, 329, 1970.
3. Pfefferkorn, E.R. and Boyle, M.K., J. Virol., 9, 187, 1972.
4. Waite, M.R.F., J. Virol., 11, 198, 1973.
5. Schlesinger, S. and Schlesinger, M.J., J. Virol., 10, 925, 1972.
6. Sreevalsan, T., J. Virol, 6, 438, 1970.
7. Rosemond, H. and Sreevalsan, T., J. Virol., 11, 399, 1973.
8. Waite, M.R.F. and Pfefferkorn, E.R., J. Virol., 5, 60, 1970.
9. Sreevalsan, T. and Allen, P.T., J. Virol., 2, 1038, 1968.
10. Carver, D.H. and Marcus, P.I., Virology, 32, 247, 1967.
11. Lockart, R.Z., in "Medical and Applied Virology, Proceedings of the Second International Symposium," Ed. by Sanders, M. and Lennette, E.H., Warren H. Green, Inc., St. Louis, 45, 1966.
12. Kennedy, S.I.T., Biochem. Biophys. Res. Commun., 48, 1254, 1972.