ANALYSIS OF THE FUNCTIONS OF CORONAVIRUS GLYCOPROTEINS BY DIFFERENTIAL INHIBITION OF SYNTHESIS WITH TUNICAMYCIN

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INTRODUCTION

The virion associated polypeptides of the A59 strain of mouse hepatitis virus (MHV) have been studied extensively (Sturman, 1977; Sturman and Holmes, 1977; Sturman et al., 1980) and are described by Dr. Sturman in this symposium. The nucleocapsid polypeptide N is phosphorylated and has a molecular weight of 50K. Two glycoproteins are associated with the viral envelope. The peplomers are composed of the glycoprotein E2(MW ≃ 180K) which may be proteolytically cleaved to yield two molecules which both migrate with an apparent molecular weight of 90K. The glycoprotein E1 (MW ≃ 23K) is deeply embedded in the viral membrane with the small glycosolated portion protruding. We have studied the intracellular synthesis of these structural polypeptides of A59 in the 17 clone 1 line (17 Cl 1) of spontaneously transformed BALB/c 3T3 cells. A59 acts as a moderate virus in 17 Cl 1 cells causing limited cell fusion. Virus particles are shed from intact cells following a 6 to 7 hour latent period at 37°, and the yield of infectious virus at 24 hours is 10^8 to 10^9 PFU/ml.

This report focuses on the intracellular synthesis of the virion-associated polypeptides of A59. We have studied the effects on A59 polypeptides of tunicamycin (TM) which prevents the formation of N-glycosidic linkages to polypeptides. Incubation with tunicamycin blocks the synthesis and incorporation into A59 virions of the peplomeric glycoprotein E2, but does not affect synthesis or glycosylation of the membrane glycoprotein E1. This observation suggests that unlike E2 and all other glycoproteins of enveloped viruses which have been studied to date, E1 may not be an N-linked glycoprotein. E1 may be the first O-linked viral glycoprotein. Formation of O-

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glycosidic linkages to cellular polypeptides such as mucin does not require transfer of oligosaccharides from dolichol linked-intermediates and is not inhibited by tunicamycin. Thus, mouse hepatitis virus apparently utilizes two different host cell mechanisms for glycosylation of the envelope glycoproteins E1 and E2. We have made use of the differential effects of tunicamycin on E1 and E2 to characterize the functions of these two viral glycoproteins.

SYNTHESIS AND PROCESSING OF THE STRUCTURAL POLYPEPTIDES OF A59 VIRUS

The normal intracellular synthesis of the structural polypeptides of A59 will be described first to provide a comparison with that observed in the presence of tunicamycin. These results differ in part from published reports on the intracellular synthesis of polypeptides of the A59, JHM and MHV3 strains of MHV (Bond et al., 1979; Siddell et al., 1980; Anderson, 1979).

Initial studies of virus infected cells were done using 1 hour labeling periods at different times after infection. By 4 hours after infection E1, E2 and N could be detected. Only the 180K form of E2 was observed in intracellular labeling experiments. The relative amounts of E1 and E2 synthesized at various times after infection were constant but the synthesis of N was not correlated to that of the glycoproteins. N appeared to be made in relatively large amounts early, whereas the maximal rate of synthesis of the glycoproteins occurred late in the infectious cycle.

In order to detect possible precursors to the structural polypeptides and to follow the fate of newly synthesized polypeptides within the infected cells, pulse chase studies were done at various times after infection. Figure 1 shows control and MHV-infected 17 Cl 1 cells labeled 6 hours after virus inoculation with a 15 minute pulse of ^{3}H leucine followed by chase periods of up to 6 hours in excess unlabeled leucine. At the end of the 15 minute pulse all three of the viral structural polypeptides were detected. E2 was found only in the 180K form and E1 appeared as a 20K form which was not glycosylated (data not shown). No change in molecular weight of E2 was observed. Thus the glycosylation of E2 may be a cotranslational event. In contrast, E1 was first synthesized as a 20K nonglycosylated protein and then chased into higher molecular weight forms up to 23K. The 23K form of E1 was glycosylated (data not shown). Thus glycosylation of E1 is a post translational event which occurs rather slowly. Pulse chase studies at later times after infection showed that a small amount of the nucleocapsid protein N chased into lower molecular weight forms which were not incorporated into virions. Another viral protein X (MW ≃ 17K) was detected about 2 hours after the pulse label. Since this polypeptide was not immunoprecipitable with anti-virion antibody, we do not yet know from which viral polypeptide it was generated.

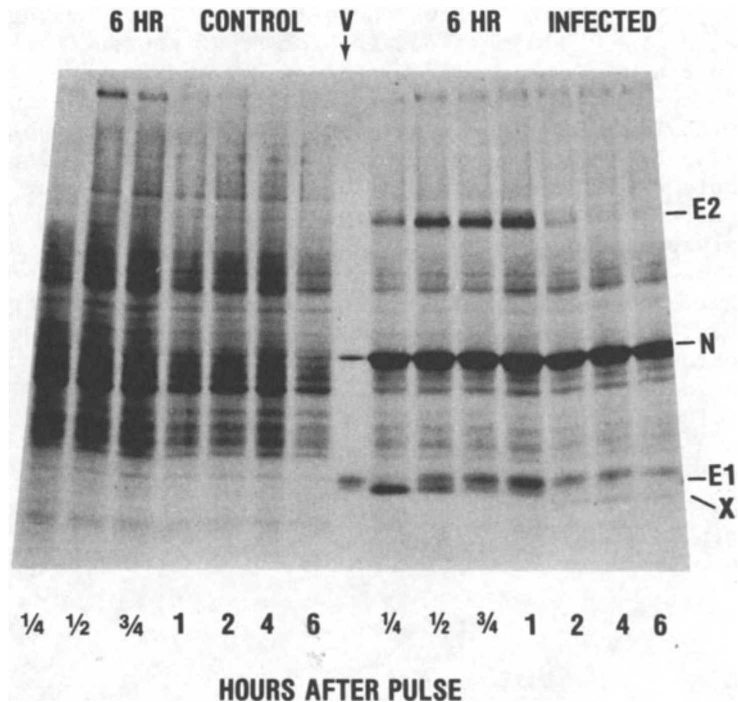

Fig. 1 Pulse chase study of control and A59 infected 17Cl 1 cells. Fluorograph of 5 to 20% polyacrylamide gradient slab gel with gradient purified A59 virion marker (V), and NP40 extracts of cells labeled for 15 min at 6 hrs after inoculation with 20µCi/ml ^{3}H leucine, and chased with excess cold leucine for the times indicated.

During the six hour chase period some labeled structural polypeptides were incorporated into virions which were released from the infected cells. The E2 synthesized 6 hours after infection was released from the cell in infectious virions within 2 hours after synthesis. Similarly the bulk of labeled E1 was released in virions within 2 hours. Both nonglycosylated and glycosylated forms of E1 were found in purified virions. No loss of the nucleocapsid protein N from the cells was detected. Apparently a large intracellular pool of N was synthesized early.

EFFECTS OF TUNICAMYCIN ON A59 GLYCOPROTEIN SYNTHESIS

When tunicamycin (TM) was added to cells 1 hour after virus inoculation and the cells were pulse labeled for 15 minutes with ^{3}H leucine at various times after infection, two surprising results were observed. First, glycosylation of E1 continued in the presence

of TM, and second, the incorporation of ^3H leucine into E2 was prevented by TM. Figure 2 shows the effects of TM on synthesis and processing of MHV polypeptides labeled at 10 hours after infection during a 2 hour chase period. At 10 hours the chase of E1 and E2 into virions was somewhat slower than at early times after infection. Cells with TM synthesized less E1 than untreated cells but the processing of the 20K form of E1 to 23K due to glycosylation was detectable within ½ hour after labeling. The glycosylation of E1 in the presence of tunicamycin clearly shows that E1 is unlike other viral glycoproteins which have been studied to date and suggests that E1 may be an O-linked glycoprotein. Studies by Nieman and Klenk (personal communication) and Sturman (personal communication) which have been presented at this meeting show that the oligosaccharide side chains of E1 are different from those of E2 and that the carbohydrate composition of E1 is similar to that of O-linked glycoproteins. The significance of this unusual mechanism for glycosylation will be discussed below.

The presence of tunicamycin did not alter the synthesis of the N polypeptide. However, in TM both control and infected cells showed three new cellular polypeptide species of molecular weights between 70 and 93K.

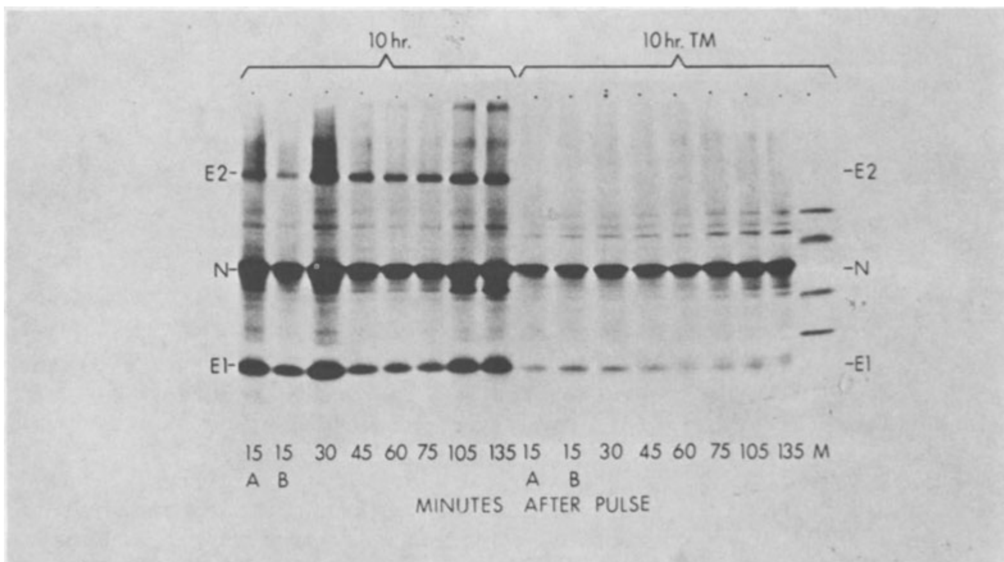

Fig 2. Effects of tunicamycin on A59 polypeptide synthesis. Fluorograph of 5 to 20% polyacrylamide gradient slab gel of NP40 extracts of A59 infected cells labeled for 15 min at 10 hrs after virus inoculation with 20µCi/ml ^3H leucine in the presence (TM) and absence of 0.5µg/ml of tunicamycin and then chased with excess cold leucine. A and B are replicate samples from identical plates and M is ^{14}C labeled molecular weight standards (30K, 46K, 69K and 92.5K).

In TM, synthesis of E2 was not detectable with the 15 minute pulse. Other viral glycoproteins such as the G protein of VSV are synthesized but not glycosylated in the presence of tunicamycin (Gibson et al., 1979). However, in TM, synthesis of the peplomeric glycoprotein of an oncorna virus was not detectable (Schwarz et al., 1976). It is possible that the E2 polypeptide was subject to rapid degradation if it was not glycosylated during translation. This degradation could occur either during or shortly after the

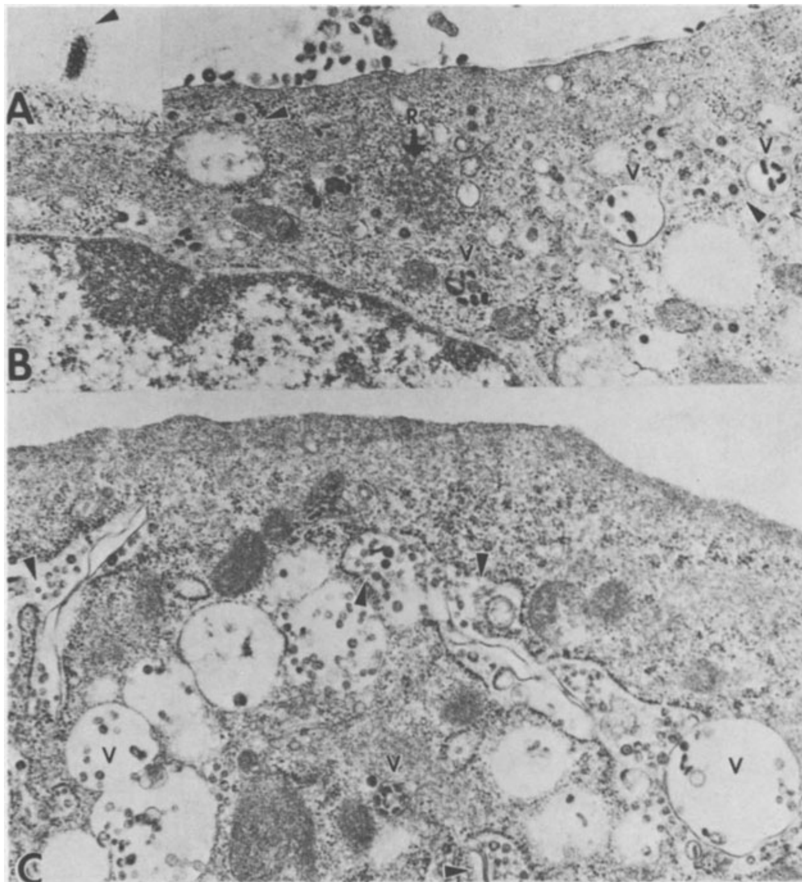

Fig 3. Effects of tunicamycin on virus maturation. 17 Cl 1 cells 24 hr after A59 infection (B) showed virions in RER (arrows), in smooth walled vesicles (V), and adsorbed to the plasma membrane and a reticular inclusion (R) in the cytoplasm. Insert (A) shows viral peplomers (arrow). TM treated cells (C) had virions in the RER, and smooth walled vesciles (V) but none on the plasma membrane. Arrows show long tubules in the RER. (A X60,000; B and C X23,000).

synthesis of the E2 polypeptide and could account for our failure to detect E2 in this pulse chase study (Klenk and Rott, 1980).

EFFECTS OF TUNICAMYCIN ON A59 VIRION FORMATION

The yield of infectious virus in the presence of tunicamycin was 0.1% of the yield in the absence of the drug at 24 hours. However transmission electron microscopic studies showed that virions are made in cells treated with TM (Fig. 3). A59 infected cells without TM (Fig. 3A and B) showed virions in the lumen of the rough endoplasmic reticulum (RER), in smooth walled vesicles (V) and adsorbed in large numbers to the plasma membrane by the tips of the peplomers. With TM (Fig 3C), A59 infected cells showed numerous virions in dilated cisternae of the RER and in smooth walled vesicles (V) but no virions adsorbed to the plasma membrane. In addition, numerous long tubules approximately 50nm in diameter were found in the lumen of the RER. Thus, virus particles were formed in TM and these virions migrated into smooth walled vesicles from which they could be discharged from the intact cells.

Virus particles released into the medium from intact TM treated cells were purified and concentrated by sucrose density gradient ultracentifugation and examined in negatively stained preparations (Fig. 4). Unlike virions purified from untreated infected cells which were covered with a thick layer of peplomers, virions released from TM treated cells had no surface peplomers. At this meeting, Dr. Sturman has shown that these virions from TM treated cells contained no E2, but showed normal ratios of E1 and N. The effects of tunicamycin on the replication of MHV are summarized in Table 1. The TM induced block in the synthesis of E2 does not prevent viral budding or the release of virions from infected cells. However, the released virions have no peplomers, contain no E2, are noninfectious, and cannot reabsorb to the surface of the infected cells.

FUNCTIONS OF THE GLYCOPROTEINS E1 and E2

We have made use of the differential inhibition by tunicamycin

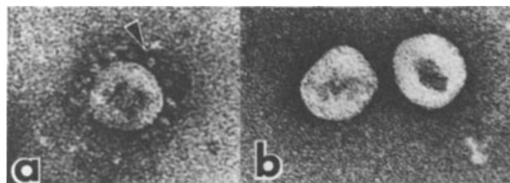

Fig 4. Effect of tunicamycin on virions. Virions purified from the medium over untreated cells (A) were covered with peplomers (arrow). No peplomers were seen on virions from TM treated cells (B) (X110,000).

Table 1. EFFECTS OF TUNICAMYCIN ON REPLICATION OF MHV

Yield of infectious virus decreased 1000X.

Synthesis of E2 blocked.

Synthesis and glycosylation of E1 not affected.

Virus budding continued.

Released virions contain N, E1 and RNA but no E2, are noninfectious and have no peplomers.

of the synthesis of the E1 and E2 glycoproteins of MHV and supporting data from other experiments to analyze the functions of the E1 and E2 glycoproteins. Our present concepts of the functions of E1 and E2 based on these studies are shown on Table 2.

That E2 is responsible for binding to susceptible cells is demonstrated by the inability of TM treated virions to bind to and infect cells and also by studies showing that binding of purified radiolabeled E2 to susceptible cells is prevented by pretreatment of the cells with concentrated MHV virions (data not shown).

The role of the peplomeric glycoprotein E2 in induction of cell fusion is suggested by the observation that tunicamycin, which prevents the synthesis of E2, also markedly reduces cell fusion in A59 infected L2 cells which are usually highly susceptible to fusion during replication of A59 (Fig. 5).

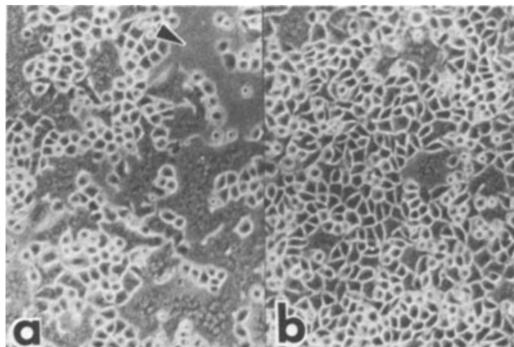

Fig 5. Inhibition of A59-induced cell fusion by tunicamycin. L2 cells 10 hours after inoculation were extensively fused (A). Arrow indicates area where syncytium has peeled off. Incubation of infected cells with 0.5μg/ml tunicamycin markedly reduced cell fusion (B).

Table 2. FUNCTIONS OF CORONAVIRUS GLYCOPROTEINS

E2, the peplomeric glycoprotein

1. Binding to susceptible cells.
2. Induction of cell fusion.
3. Induction of neutralizing antibody.

E1, the membrane glycoprotein

1. Determines location of viral budding.
2. Formation of viral envelope.

The role of E2 in cell fusion is also suggested by the observation that anti-E2 antibody but not anti-E1 antibody prevents fusion of MHV infected L2 cells (data not shown).

In neutralization tests anti-E2 antibody neutralized A59 virions more effectively than did anti-E1 antibody. These studies suggest that E2 is responsible for binding to receptors on susceptible cells and induction of cell fusion.

Immunofluorescent staining of MHV-infected cells with antibodies against purified E1 or purified E2 showed that these glycoproteins migrated differently within the infected cell (Doller and Holmes, 1980). Figure 6 shows MHV-infected 3T3 cells 8 hours after infection

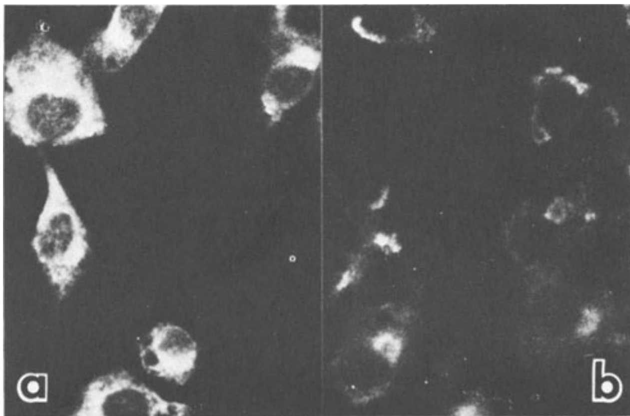

Fig 6. Localization of glycoprotein antigens. At 8 hrs after inoculation with A59, 17 Cl 1 cells were fixed with acetone and stained by the indirect fluorescent antibody technique with antibody to purified E2 (A) or antibody to purified E1 (B).

stained with anti-E2 or with anti-E1. Although the E2 antigen was distributed throughout the cytoplasm of the infected cells, the E1 antigen was restricted to tight clusters in the perinuclear area of the infected cells. This may correspond to the RER or Golgi apparatus of the infected cells. The localization of the E1 on these intracellular membranes may determine the site at which virions bud. It is possible that the restricted migration of E1 may be associated with the unusual glycosylation pattern of this glycoprotein. Perhaps E1 is glycosylated by the O-linked glycosylation pathway because it resembles cellular proteins which are localized in the RER or Golgi and which are glycosylated differently from the N-linked glycoproteins that rapidly disperse to the plasma membrane.

From the experiments showing formation of virions in the presence of tunicamycin it is clearly evident that E2 is not required for viral budding or for release of the virions from infected cells. Even in the TM-induced absence of E2 the formation of the viral envelope occurs in the intracytoplasmic membranes where E1 is localized and the nucleocapsid associates with the viral envelope normally. Thus, the E1 glycoprotein of this coronavirus appears to be functioning like the matrix or M proteins of other enveloped RNA viruses. However the matrix polypeptides of rhabdoviruses, paramyxoviruses, and orthomyxoviruses are not glycosylated.

Studies of E1 glycoprotein may serve as useful models for the synthesis, glycosylation and intracellular migration of cellular O-linked glycoproteins. The unique biochemical features of the E1 molecule may be responsible for important biological properties of coronaviruses such as budding from intracytoplasmic membranes and persistence of viral infection.

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