RELATEDNESS OF VIRION AND INTRACELLULAR PROTEINS OF THE MURINE CORONAVIRUSES JHM AND A59

Clifford W. Bond, Kevin Anderson, Susan Goss, and
Lisa Sardinia

Department of Microbiology
Montana State University
Bozeman, MT. 59717

INTRODUCTION

Coronaviruses are a group of RNA viruses with positive polarity that cause a wide variety of diseases in many species including man. The structural proteins of coronaviruses have been studied extensively (reviewed in Robb and Bond, 1979). However, little attention has been focused on the intracellular proteins (Bond et al. 1979, Anderson et al. 1979, Siddel et al. 1980).

We have described 9 intracellular and viral-specific proteins of the murine coronaviruses JHM and A59 (Bond et al. 1979). Four of these 9 viral-specific proteins were identified as structural proteins.

In this communication, we compare the virion and intracellular viral-specific proteins of JHM and A59 viruses and describe post-translational events observed in murine coronaviral-specific protein synthesis.

MATERIALS AND METHODS

Cells

The source and growth of the murine cell lines 17CL-1 and DBT have been described previously (Sturman and Takemoto, 1972; Hirano et al. 1974; Bond et al. 1979).

Virus

The source and growth of the A59 and JHM virus stocks have been described previously (Bond et al. 1979). Cells were infected in suspension, and virus was adsorbed at 37° for 30 min at a cell density of 10^7 cells/ml. Adsorbed cells were centrifuged, resuspended in prewarmed (37°) Dulbecco's Modified Eagle's Medium with 2% fetal bovine serum (DME2) at a density of 2×10^6 cells/ml, plated into plastic culture dishes at a density of 3×10^5 cells/cm², and reincubated at 37°.

Preparation of Intracellular Proteins

Cells were infected by JHM or A59 virus at a multiplicity of infection (MOI) of 0.3 plaque forming units (PFU)/cell, and incubated at 37°. At the labeling time indicated in the experiment, the cell layers were washed twice with methionine-free DME2, and pulse-labeled with ³⁵S-methionine (100 µCi/ml) in methionine-free DME2 for the time period indicated. At the end of the pulse-period, the cell layers were washed twice with serum-free complete medium (DME0), and chased with DME2 or lysed in situ. Cells were lysed in situ at 0° with buffer B10 [10 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 0.5% (vol/vol) NP40, 0.1% (wt/vol) sodium dodecyl sulfate (SDS), 1% (vol/vol) Aprotinin, 50 µg/ml ribonuclease, 50 µg/ml deoxyribonuclease] and the cytoplasmic lysates were stored at -20°.

Immunoprecipitation

Cytoplasmic lysates were immunoprecipitated as follows. A cytoplasmic lysate representing 1.25×10^6 cells (50 µl) was diluted 10 fold with buffer B11 [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.02% (wt/vol) sodium azide, 0.05% (vol/vol) NP40, 1% (vol/vol) Aprotinin, 0.1% (wt/vol) bovine serum albumin]. Twenty µl of antisera (either mouse-anti-JHM virus or mouse-anti-A59 virus) were added to the suspension, incubated for one hr at 0°, and the immune complexes were precipitated with 50 µl of 10% (vol/vol) fixed Staphylococcus aureus (Cowan) by incubation for one hr at 0°. Anti-viral antisera were prepared as previously described (Robb and Bond, 1979), and fixed S. aureus (Cowan) were prepared as described by Kessler (1975). The pellets were washed 3 times with buffer B11, and resuspended in 20 µl of 20 mM DTT, 1% (wt/vol) SDS. The proteins were eluted and reduced by incubation at room temperature for 15 min followed by heating at 60° for 5 min. The S. aureus (Cowan) were removed by centrifugation and the supernatant fraction was alkylated with N-ethylmaleimide at 0° for one hr as described by Crawford and O'Farrell (1979).

Preparation of Virion Proteins

Cells were infected by JHM virus or A59 virus at an MOI of 0.1 PFU/cell as described above and incubated at 33°. At 10 hrs PI, the infected cells were washed twice with methionine/5 DME2, and labeled with ^{35}S -methionine (50 $\mu\text{Ci}/\text{ml}$) in methionine/5 DME2. The cell culture fluid was harvested at 70% cell lysis (24-30 hrs PI) and centrifuged at 2000 x g for 20 min. The supernatant fraction was layered onto a step gradient consisting of layers of 10%, 17%, and 40% (wt/wt) potassium tartrate in PNE buffer [30 mM PIPES (pH 6.5), 100 mM NaCl, 1 mM EDTA], and centrifuged at 153,400 x g for 90 min at 4°. The fraction containing infectious virus was diluted with PNE buffer, layered onto a linear gradient consisting of 10 to 40% (wt/wt) potassium tartrate in PNE buffer, and centrifuged to equilibrium at 208,800 x g for 5 hrs at 4°. Fractions were pooled on the basis of radioactivity, and Aprotinin was added to 1% (vol/vol). The suspension was dialyzed against 0.01 M NH_4HCO_3 , lyophilized, and the resulting powder was resuspended in buffer B10. The suspension was adjusted to 20 mM DTT, 1% (wt/vol) SDS, and heated to 45° for 5 min. The proteins were alkylated with N-ethylmaleimide for one hr at 0° as described by Crawford and O'Farrell (1979).

Polyacrylamide Slab Gel Electrophoresis

Reduced and alkylated proteins were electrophoresed on 6%, 8%, or 10% polyacrylamide slab gels as described by Laemmli and Favre (1973) with the following exception. The resolving gel was supplemented with 0.5% (wt/vol) linear polyacrylamide. Volumes representing approximately equivalent cell numbers were loaded in each lane of the slab gel.

RESULTS

Cells (17CL-1) were infected with A59 virus or mock infected and labeled with ^{35}S -methionine for 30 min, 60 min, 120 min or 180 min beginning at 8 hrs PI (Figure 1). This time point was chosen because it was a time of maximum protein synthesis, and was during the time of logarithmic production of infectious virus at 37°. The density of the major viral-specific protein bands (150K, 60K, 57K, 54K, and 23K) was a function of the length of the labeling time. The 22K protein was not detectable until the length of the labeling period was 120 min. The 22K protein was readily detectable in a 180 min labeling period. An identical experiment was done with JHM virus, and similar data was obtained (data not shown). These data suggest that the 22K protein may be the result of posttranslational modification of a precursor protein. To test this hypothesis, the following experiment was done.

Cells (17CL-1) infected with A59 virus or mock infected were pulse-labeled with ^{35}S -methionine for 30 min, and chased for 0 min, 20 min, 40 min, 60 min, 80 min, and 100 min. Immunoprecipitates were electrophoresed on either 10% (Figure 2) or 6% (Figure 3) polyacrylamide slab gels. The results clearly indicate that the 23K protein band lost density, and the 22K protein band gained density during the chase (Figure 2). The density of the 150K band decreased during the chase (Figure 3). However, there was no concomitant increase of a corresponding protein during the chase. There was no significant evidence of posttranslational cleavage of precursor proteins in any other region of the gels. An identical experiment was done with JHM virus and similar data were obtained. Identical experiments were done using DBT cells infected by JHM virus or A59 virus, and similar results were obtained.

Fig. 1. The spectrum of viral-specific proteins synthesized by 17CL-1 cells infected by A59 virus or mock infected as a function of the length of the labeling period. Cells were infected with A59 virus (lanes 1, 3, 5, 7) or mock infected (lanes 2, 4, 6, 8), labeled with ^{35}S -methionine for 30 min (lanes 1, 2), 60 min (lanes 3, 4), 120 min (lanes 5, 6) or 180 min (lanes 7, 8), and immunoprecipitated. The immunoprecipitates were reduced and alkylated, and electrophoresed on an 8% polyacrylamide slab gel as described in Materials and Methods.

Structural proteins of purified JHM and A59 virions were compared with the intracellular proteins described in the previous experiments. The results are shown in Figure 4. The 63K intracellular protein of JHM virus and the 60K protein of A59 virus comigrate with the respective structural proteins of purified virions. The 150K and 22K proteins are not evident in the virion lanes of Figure 4. However, both were faintly evident in the original autoradiograms suggesting that these proteins are precursors to the slower migrating species. The 23K intracellular protein of JHM virus migrated slightly differently in comparison with the structural proteins of JHM virions. The 23K intracellular protein of A59 virus migrated slightly faster than the structural proteins of A59 virions. These data suggest that the 23K protein is not directly assembled into the virion without modification.

Fig. 2. Pulse-chase of viral-specific proteins synthesized by 17CL-1 cells infected by A59 virus or mock-infected. Cells infected by A59 virus (lanes 2-7) or mock infected (lanes 1, 8) were pulse-labeled with ^{35}S -methionine for 30 min, chased for 0 min (lanes 1, 2), 20 min (lane 3), 40 min (lane 4), 60 min (lane 5), 80 min (lane 6) or 100 min (lanes 7, 8), and immunoprecipitated. The immunoprecipitates were reduced and alkylated, and electrophoresed on a 10% polyacrylamide slab gel as described in Materials and Methods.

DISCUSSION

The data presented above demonstrate that the 23K intracellular protein of JHM and A59 viruses is processed to a 22K protein which is assembled into the virion. The 23K intracellular protein does not appear to be assembled directly into the virions of JHM and A59 without processing to the 22K species because it migrates differently than the structural proteins of these viruses. The 61K and 56K intracellular proteins of JHM virus, and the 57K and 54K intracellular proteins of A59 virus are not represented in the respective virions. Anderson et al. (1979) suggested that these proteins are the result of posttranslational modification of the 63K protein (JHM) or the 60K protein (A59). The data shown in Figures 2 and 3 do not support this contention.

The 150K intracellular protein bands of JHM and A59 viruses decrease in density during the chase (Figure 3). We have previously shown that this protein is glycosylated by glucosamine and mannose (Bond et al. 1979). This protein is incorporated into the virion

Fig. 3. Pulse-chase of viral-specific proteins synthesized by 17CL-1 cells infected by A59 virus or mock infected. The reduced and alkylated immunoprecipitates described in the legend to Figure 2 were electrophoresed in the corresponding lanes of a 6% polyacrylamide slab gel as described in Materials and Methods.

Fig. 4. Comparison of the intracellular and virion proteins of JHM virus and A59 virus. Cells were infected by JHM virus (lanes 3, 4), A59 virus (lanes 8, 9) or mock infected (lanes 1, 2, 6, 7), pulse-labeled with ^{35}S -methionine for 30 min, chased for 0 min (lanes 1, 3, 6, 8) or chased for 100 min (lanes 2, 4, 7, 9), and immunoprecipitated. Virion proteins of JHM (lane 5), and A59 (lane 10) were prepared as described in Materials and Methods. The immunoprecipitates and virion proteins were reduced and alkylated, and electrophoresed on an 8% polyacrylamide slab gel as described in Materials and Methods.

directly (Figure 4), and is probably then glycosylated to the slower migrating species found in the virion.

The pulse-chase experiments were done using DBT cells as well as 17CL-1 cells. Similar results were obtained. These results indicate that the posttranslational modifications are viral-specific, and not cellular artifacts. Aprotinin, a protease inhibitor, was utilized throughout the processing steps to eliminate protease activity that would result in cleavage artifacts.

Further work including tryptic peptide mapping of the structural and intracellular viral-specific proteins is in progress to further define the relatedness of the intracellular and virion proteins of the murine coronaviruses JHM and A59.

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