MATURATION OF THE POLYMERASE POLYPROTEIN OF THE CORONAVIRUS MHV STRAIN JHM INVOLVES A CASCADE OF PROTEOLYTIC PROCESSING EVENTS

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1. ABSTRACT

The RNA polymerase gene of the murine coronavirus mouse hepatitis virus (MHV) encodes a polyprotein of greater than 750 kDa. The amino-terminal cleavage product of the MHV polymerase polyprotein, p28, has been shown to be cleaved from the polyprotein by the virus-encoded protease PCP-1. We aim to identify the MHV-JHM proteolytic products downstream of p28 and to determine which viral proteinase domains are responsible for generating each of them. To this end, we have generated antisera directed at specific MHV-JHM ORF1a regions and have used these antisera to identify six viral proteins, representing a large portion of ORF1a, from MHV-JHM-infected cells. These proteins include p28, p72, p65, p250, p210, and p27.

2. INTRODUCTION

Gene 1 at the 5' end of the genome of mouse hepatitis virus (MHV) encodes a large polyprotein precursor to the viral RNA polymerase complex. Gene 1 is 22 kilobases in length, contains two overlapping open reading frames (ORF1a and ORF1b), and is believed to encode a polyprotein of greater than 750 kDa (Lee *et al.*, 1991; Pachuk *et al.*, 1989). This polyprotein precursor is thought to be proteolytically processed by two viral papain-like cysteine proteinase domains, PCP-1 and PCP-2, and a poliovirus 3C-like pro-

teinase domain, 3C-pro, to generate the mature protein products associated with virus replication (Gorbalenya *et al.*, 1989). The release of the amino terminal cleavage product, p28, is mediated by PCP-1 (Hughes *et al.*, 1995; Dong and Baker, 1994; Baker *et al.*, 1993, 1989). This cleavage event is detectable both in vitro and in vivo. In addition, a 65 kDa protein encoded immediately downstream in MHV strain A59 is also believed to be released by PCP-1 activity (Bonilla *et al.*, 1997; Denison *et al.*, 1995).

Our goal is to identify the cleavage products generated downstream of p28 from cells infected with the neurotropic strain of MHV, strain JHM. We have recently published the in vivo identification of p72 and p65, two proteins immediately downstream of p28, and documented their precursor and product relationship (Gao *et al.*, 1996). Here we provide a preliminary report of the in vivo detection of three additional gene 1 products representing a large portion of the polyprotein encoded by ORF1a. These three proteins were detected by immunoprecipitation of viral proteins from infected cell lysates using polyclonal rabbit antisera generated against fusion proteins of GST with various regions of ORF1a.

3. MATERIALS AND METHODS

3.1. Infection of Cells and Preparation of Lysates

Monolayers of 80–100% confluent DBT cells were infected with MHV-JHM-x at a multiplicity of infection of 5–10 plaque-forming units/cell, and lysates prepared as described by Gao *et al.* (1996). Briefly, proteins were metabolically labeled between 6.0–6.5 hr post-infection with [³⁵S]methionine at a final concentration of 100 uCi/ml. Cells were then washed and incubated in MEM media containing a 10-fold excess of methionine for the indicated periods of time. Cells were lysed in a buffer containing 4% SDS, 3% DTT, 40% glycerol and 0.0625 M Tris, pH 6.8, and proteins solubilized by multiple passages through a tuberculin syringe.

3.2. Generation of Rabbit Antisera for Immunoprecipitation

A panel of antisera directed against GST-fusion proteins representing multiple domains within the MHV-JHM ORF1a were generated. Each fusion protein domain was RT-PCR amplified from total RNA of MHV-JHM-x infected DBT cells and directionally cloned into the bacterial expression vector pGEX-KG, kindly provided by Dr. Steven Broyles (Purdue University, West Lafayette, IN). Fusion proteins were induced and isolated following the method of Guan and Dixon (1991), and injected into rabbits intramuscularly and subcutaneously. Polyclonal immune sera were collected 7–10d following the final (3rd or 4th) injection.

3.3. Immunoprecipitation and SDS-PAGE

Virus-encoded proteins generated during infection of DBT cells were identified by immunoprecipitation from whole cell lysates prepared as described above. Lysates from approximately 1×10^6 cells were immunoprecipitated with 5–10 ul of immune serum in a total volume of 1 ml RIPA buffer, as previously described (Gao *et al.*, 1996). Proteins were electrophoresed on 5–15% gradient SDS-PAGE gels and analyzed by fluorography.

4. RESULTS AND DISCUSSION

To identify MHV-JHM ORF1a gene products, a series of antisera directed against ORF1a-GST fusion proteins (FPs) were generated. The fusion proteins were generated by cloning ORF1a RT-PCR products into the vector pGEX-KG (Figure 1A). Constructs were transformed into DH5 alpha cells, and FP production was induced by addition of IPTG to growth media. Bacterial cells were lysed by sonication, GST-FPs were purified by affinity chromatography and used for rabbit immunizations (Figure 1B).

Three polyclonal antisera were generated: anti-D3, anti-D5 and anti-D12. The D3-FP encompasses amino acids (aa's) 784–1037 (nt 2563–3326) of the polymerase polyprotein within the p72/p65 domains as well as the region downstream. The D5-FP encompasses aa's 1212–1365 (nt 3847–4393), including the catalytic His residue of the PCP-1 domain, and the D12-FP encompasses aa's 3340–3470 (nt 10231–10246), covering the catalytic His residue of the 3C-pro domain (Figure 2A).

Temporal processing of the MHV-JHM ORF1a polyprotein was analyzed in a pulse-chase experiment. Specific viral proteins p72, p65, p250, p210 and p27 were detected using two of the antisera described above (Figure 2B). P250 and p210 were also detected in experiments using the anti-D5 serum (data not shown). Based on the electrophoretic mobilities of the proteins and locations of the FPs used to generate the antisera (as well as previously published results identifying p72 and p65 using the anti-647 serum [Gao *et al.*, 1996]), the predicted locations of the protein products are depicted schematically in Figure 2A. Proteins p28, p72, p250 and p27 are detected immediately following pulse labeling of cells, whereas proteins p65 and p210 represent proteolytic products of the larger precursors. Two additional predicted protein products, p7 and p40, have not yet been detected from MHV-JHM-infected cells. This experiment also confirms our previously pub-

Figure 1. Production and purification of GST-fusion proteins. A) Schematic representation of the vector pGEX-KG provided by Dr. Steven Broyles (Purdue Univ., IN). RT-PCR products representing various domains within gene 1 were directionally cloned in frame with GST using *XbaI* and *HindIII*. B) Fusion proteins were isolated from sonicated bacterial lysates by affinity chromatography using glutathione-sepharose columns. Protein purity was assessed on Fast-Stained (Zion Research, Newton, MA) SDS-10% polyacrylamide gels, as shown here for the D3-FP. Lane 1, bacterial lysates; lane 2, column flow-through; lanes 3–5, column washes; lanes 6–9, D3-FP eluted by competition with glutathione. Molecular weight markers are labeled in kilodaltons on the left of the gel. Quantitation of proteins was achieved using the Bradford method (Bio-Rad, Richmond, CA) prior to injection into rabbits (200–600 ug per injection).

Figure 2. Synthesis and processing of MHV-JHM ORF1a polyproteins in vivo. A) The MHV-JHM ORF1a polyprotein precursor is processed into at least 8 protein products depicted here along with the antisera used to detect them. Putative protein products predicted based on the electrophoretic mobilities of other proteins are indicated by the (?). B) Temporal processing of the MHV-JHM ORF1a polyprotein. Pulse-chase immunoprecipitation analysis using a combination of the anti-D3 and anti-D12 antisera detects precursor-product relationships between p250-p210 and p72-p65, and an accumulation of p27 (3C-pro) during the chase. Molecular weight markers are labeled in kilodaltons on the left of the gel.

lished identification of p72 and p65 and their precursor-product relationship (Gao *et al.*, 1996). Furthermore, p27 (representing 3C-pro) is processed even at the earliest time point in the chase, and accumulates for the duration of the experiment. This implies that p27 is cleaved rapidly from the precursor as it becomes available, and unlike the HCV-229E 3C-like pro domain (Ziebuhr *et al.*, 1995), it is quite stable in vivo. This result is also consistent with the generation of a 27 kDa 3C-pro by MHV-A59 (Lu *et al.*, 1995), which has activity in vivo and in vitro (Seybert *et al.*, 1997; Lu *et al.*, 1996).

Further immunoprecipitation analyses of gene 1 proteolytic processing with additional antisera, including the indicated anti-D10 serum (Figure 2A), are needed to identify all of the viral products associated with viral replication, as are functional assays to determine the role of each subunit in the unique coronavirus discontinuous transcription mechanism. Such experiments are currently in progress.

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