Characterization of Bovine Viral Diarrhoea-Mucosal Disease Virus-specific Proteins in Bovine Cells

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SUMMARY

The presence of virus-specific polypeptides in bovine viral diarrhoea-mucosal disease (BVD) virus-infected bovine cells was studied by radiolabelling in the presence of a hypertonic initiation block (HIB) and by analysis by SDS–PAGE. These experiments were complemented by radioimmunoprecipitations with anti-BVD hyperimmune serum of infected cells labelled under isotonic conditions. A total of 12 polypeptides (Mr 165, 135, 118, 80, 75, 62, 56 to 58, 48, 37, 32, 35 and 19, all × 10^3) were identified in infected cells. Time course analysis of the induction of the viral polypeptides indicated that they could be detected as early as 4 h post-infection and their synthesis reached a plateau between 12 and 20 h post-infection. The most abundant polypeptides were the ones that could be detected earliest. HIB was found to be an excellent adjunct to existing techniques in the identification of viral polypeptides. Seven of these polypeptides had not been reported previously. This is the first report of the direct detection of BVD virus-induced polypeptides in infected cells without the aid of immunoprecipitation. The sum of the molecular masses of these polypeptides is greater than the coding capacity of the genome; therefore precursor–product relationships must exist between these polypeptides.

INTRODUCTION

Bovine viral diarrhoea-mucosal disease (BVD) virus infection is among the most common viral infections of cattle. On the basis of physical, chemical and biological properties, the agent has been classified in the family Togaviridae, genus Pestivirus (Westaway et al., 1985a). Recently, members of the genus Flavivirus were shown to have a genome structure and a replication strategy fundamentally different from those of the alphaviruses and as a result the family Flaviviridae was established (Rice et al., 1985; Westaway et al., 1985b). It is not known at the present time if BVD virus belongs in one of these two families because very little is known about its replication strategy and genome structure.

Previous reports on the virus-induced proteins of BVD virus are conflicting (Matthaeus, 1979, 1980; Purchio et al., 1984). These reports rely exclusively on the specificity of antibodies for the identification of the viral polypeptides. Using a direct method of identification of virus-specific polypeptides in infected cells and the comparative results of radioimmunoprecipitation (RIP), we report here the identification of BVD virus-induced proteins in bovine cell cultures infected with a cytopathic isolate of BVD virus. In the companion paper (Donis & Dubovi, 1987) we report on the presence of oligosaccharide moieties in some of the polypeptides reported here.

METHODS

Cell cultures and virus. Foetal bovine testicle (FBT) cells prepared by standard trypsin dispersion methods were cultured in Eagle's minimum essential medium with Earle's salts (MEME) supplemented with 10% lamb serum (Gibco). Cell cultures used in these experiments were from the third to fifth passage and determined to be free of non-cytopathic BVD virus and mycoplasma. The Singer cytopathic isolate of BVD virus was obtained from
National Veterinary Service Laboratories, Ames, Iowa, U.S.A. The virus was plaque-purified twice and a stock was prepared in FBT cells and stored frozen at −70 °C. This stock had an infectivity titre of 10^8.3 median tissue culture infective doses per ml (TCID₃₀/ml).

**Isotopic labelling and preparation of cell extracts.** FBT cells cultured in 12-well plates (Flow) for at least 48 h before use were infected with BVD virus at an input multiplicity of 100 to 300 TCID₃₀/cell or mock-infected with medium only. Infections were initiated by rinsing the cell sheet with phosphate-buffered saline (PBS) (137.5 mM-NaCl, 2.5 mM-KCl, 8.5 mM-Na₂HPO₄·1·5 mM-KH₂PO₄, pH 7.2), adding the virus inoculum and adsorbing it to the cells for 1 h at 37 °C. At the end of this period, the inoculum was removed, the monolayer rinsed with PBS and overlaid with MEME with 5% lamb serum. When used, actinomycin D (Sigma) was added to the culture medium at a final concentration of 500 ng/ml at 4 h post-infection (p.i.) (except when indicated otherwise) and kept during all subsequent steps until cell harvesting. At various times after infection, monolayers were rinsed with PBS once, with precursor-free medium once and then overlaid with the latter medium. Precursor-free medium refers to either methionine-, cysteine- or leucine-free MEME. After a starvation period of 1 h, the culture medium was made hypertonic to block protein synthesis initiation by the addition of 150 mM excess NaCl (unless otherwise indicated in figure legends) 20 min before the labelling started and was maintained thereafter. This procedure is referred to as hypertonic initiation block (HIB) (Nuss & Koch, 1976; Nuss et al., 1975). In the absence of HIB, the labelling started directly after the starvation period. Proteins were labelled by the addition of either L-[³⁵S]methionine (radioactive concentration 12 mCi/ml, specific activity 1330 Ci/mmol) to a final concentration of 200 µCi/ml, L-[³⁵S]cysteine (radioactive concentration 18.16 mCi/ml, specific activity 1400 Ci/mmol) to a final concentration of 200 µCi/ml, or L-[4,5-³H]leucine (radioactive concentration 1 mCi/ml, specific activity 58 Ci/mmol) to a final concentration of 100 µCi/ml. Cultures were incubated for different time periods at various conditions after infection as indicated in each figure legend. When labelling was completed, the medium was aspirated and the monolayers were rinsed with PBS. The cells were scraped from the wells into PBS using a rubber policeman and were pelleted by centrifugation for 45 s in a microcentrifuge at 2000 g. A cytoplasmic extract from the cells of one well (approximately 1·5 × 10⁶ cells) was prepared by adding 50 µl of TNE buffer (20 mM-Tris-HCl pH 8·0, 100 mM-NaCl, 5 mM-EDTA) containing 1% Triton X-100 and protease inhibitors (1 mM-1,10-phenanthroline, 50 µM-phenylmethylsulphonyl fluoride (PMSF), 50 µM-sodium metabisulphite, 20 µg/ml leupeptin, 50 µg/ml crude ovomucoid trypsin inhibitor, 50 µg/ml crude soybean trypsin inhibitor, 50 µg/ml Aprotinin (Sigma)). Following 10 strokes with a loose fitting Teflon pestle (Kontes, Vineland, N.J., U.S.A.), the nuclei were pelleted by centrifugation at 20000 g for 2 min. The supernatant fluid (cytoplasmic extract) was assayed and stored at −20 °C until used for RIP or for direct analysis by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS–PAGE).

**Radioimmunoprecipitation.** Cell extracts centrifuged at 10000 g for 5 min to eliminate insoluble matter were adjusted to a final concentration of 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS in TNE buffer containing 100 µM-PMSF. Addition of 10 µl of anti-BVD hyperimmune serum to samples of a cell extract containing approximately 2 × 10⁶ d.p.m. was followed by mixing and overnight incubation at 4 °C. The immune complexes were isolated by adding 100 µl of immunosorbant per reaction. The immunosorbs was a 10% suspension of Pansorbin (Protein A-containing fixed Staphylococcus aureus, strain Cowan I, Calbiochem-Behring) washed three times with TNE containing 0.5% Triton X-100 and 0.1% SDS, and then incubated for 1 h at 37 °C with a solution of 1% gelatin in TNE and washed once again before use as indicated above. Following incubation at 22 °C for 1 h on a rocking platform, the immune complexes bound to the immunosorbs were collected by centrifugation at 10000 g for 5 min and the supernatant fluids were stored frozen at −20 °C until needed. The acid-insoluble radioactivity present in these samples was assayed by radioimmunoprecipitation in the presence of sodium dodecyl sulphate (SDS–PAGE).

**Preparation of antiserum.** A male bovine calf, 2 months of age, was inoculated twice, 3 weeks apart, with a killed BVD vaccine (Triangle 1, Fort Dodge Laboratories, Fort Dodge, Iowa, U.S.A.). One month later, it was inoculated intravenously with 10⁶ TCID₃₀/ml of the Singer isolate of BVD virus. Serum was collected 4 weeks later. This antiserum had a virus neutralization titre of 25000 when tested against 100 TCID₃₀ of the homologous virus.
**RESULTS**

*bVirus proteins*

**Analysis of lysates labelled under HIB**

BVD virus infection does not induce an efficient shut off of host cell protein synthesis until the later stages of infection when cytolytic changes are evident. As a result, initial attempts to visualize viral polypeptides in radiolabelled infected cell lysates were unsuccessful due to the high levels of incorporation of labelled amino acid precursors into cellular proteins. To circumvent this problem, we attempted to block cellular protein synthesis selectively using transcription and translation inhibitors such as actinomycin D, α-amanitin and hypertonicity. Actinomycin D is commonly used to allow direct detection of viral polypeptides in cells infected with RNA viruses. Addition of actinomycin D to FBT cells shortly after infection produced a significant degree of cell toxicity, even at concentrations as low as 500 ng/ml, resulting in significant loss of cells from the monolayer by 12 h p.i. Furthermore, treatment of FBT cells with actinomycin D at this low concentration resulted in a reduction in incorporation of radiolabelled amino acid precursor of only 70% by 12 h p.i. This level of incorporation was still unsatisfactory for visualization of viral polypeptides. A hypertonic extracellular environment established by the addition of NaCl shortly before the radiolabelling step was found to be an effective means of detecting virus-induced polypeptides since initiation of translation of cellular messages is more sensitive to extracellular hypertonicity than that of viral messages (Nuss *et al.*, 1975). This procedure, known as HIB has been used in other virus systems to facilitate the identification of virus-induced polypeptides (Nuss *et al.*, 1975; Tsurumi *et al.*, 1983; Lamb *et al.*, 1978). When 150 mM-NaCl in excess of the normal NaCl content of MEME was added to FBT cells, incorporation of radiolabelled amino acids in treated cultures was reduced to only 2 to 3% of control values. The use of a combination of both actinomycin D (400 ng/ml) and low level HIB (75 mM-NaCl) was also found to be useful under our experimental conditions.

When extracts from cells labelled with L-[4,5-3H]leucine under HIB were analysed by SDS-PAGE (Fig. 1), we identified the presence of the following virus-induced polypeptides: 118K, 80K, 62K, 58K to 56K, 48K, 37K and 19K. The 118K was often very faint and not readily detectable under these labelling conditions. The other viral polypeptides could be easily identified since there were only a few polypeptides in the mock-infected cell extract lane. With the leucine label the 80K, 37K and 19K appeared as the most highly labelled polypeptides. Viral infection appeared to inhibit the incorporation of L-[4,5-3H]leucine into cellular proteins under HIB conditions further, since most of the cell-encoded polypeptides present in the mock-infected cells were absent in infected cells. Analysis of cell extracts labelled with L-[35S]methionine under HIB (Fig. 2) indicated the presence of viral polypeptides with Mr (× 10⁻³) of 165, 118, 80, 56 to 58, 48 and 37. The 165K polypeptide was well resolved in gradient SDS–PAGE gels but the 56K to 58K and 62K comigrated as a broad band under these conditions. With the methionine label, the 80K polypeptide was efficiently labelled but the 37K polypeptide labelled less well. Labelling cells with L-[35S]methionine under a mild HIB and in the presence of actinomycin D resulted in the confirmation of previously identified viral polypeptides such as 165K, 118K, 80K, 62K, 56K to 58K, 48K, 37K and 19K (Fig. 3, lanes 1 and 2). Two previously undetected species were evident, 135K and a faint species migrating with an apparent size of 25K. Using the conditions just described, the incorporation of [35S]cysteine into infected cells (Fig. 3, lanes 3 and 4) yielded results comparable to those obtained with L-[35S]methionine except that one extra polypeptide with molecular mass of 32K was identified while the 37K polypeptide was absent. The significance of this is currently being further investigated, since the 32K polypeptide was not evident in [3H]leucine- or [35S]methionine-labelled infected cells. To confirm the presence of the 25K and 19K polypeptides, we labelled infected and mock-infected cells with L-[35S]methionine and L-[35S]cysteine (Fig. 4). The two polypeptides of interest, 25K and 19K were clearly visualized above the background incorporation into cellular polypeptides. Alpha-amanitin was also used to shut off transcription by RNA polymerase β and thereby reduce the background of cellular protein synthesis. The results of these experiments were comparable to those obtained using actinomycin D and HIB (data not shown).
Fig. 1. BVD virus-induced polypeptides in infected FBT cells. Extracts from mock-infected (lane 1) and infected (lane 2) cells were labelled with L-[4,5-3H]leucine from 10 to 11 h p.i. in the presence of HIB and analysed in a 10% SDS–polyacrylamide gel.

Fig. 2. High molecular weight viral polypeptides present in BVD virus-infected cells. Cell extracts from mock-infected (lane 1) and infected (lane 2) cells were labelled with [35S]methionine for 40 min at 13 h p.i. in the presence of HIB, and separated in a 5 to 15% acrylamide gradient SDS–polyacrylamide gel.

**Immunoprecipitation analysis**

Analysis by RIP assays of lysates from infected cells labelled with L-[35S]methionine in isotonic medium followed by SDS–PAGE yielded results in close agreement with those obtained by HIB. The presence of an additional, less abundant species of 75K was observed in
Fig. 3. BVD virus-induced polypeptides in infected FBT cells. FBT cells infected (lanes 2 and 4) and mock-infected with BVD virus (lanes 1 and 3) were labelled for 1 h from 8 to 9 h p.i. in the presence of 0.5 μg/ml of actinomycin D and 75 mM excess NaCl with L-[^35S]methionine (lanes 1 and 2) or L[^35S]cysteine (lanes 3 and 4). The harvested cells were lysed and separation was carried out by SDS-PAGE.

Fig. 4. Low molecular weight polypeptides induced in BVD virus-infected cells. Infected (lane 2) and mock-infected (lane 1) FBT cells were labelled for 2 h (8 to 10 h p.i.) in the presence of 0.4 μg/ml of actinomycin D and 75 mM excess NaCl with [^35S]methionine and[^35S]cysteine. Cell extracts were separated by SDS-PAGE.

radioimmunoprecipitated samples (Fig. 5) while the 135K, 32K, 25K and 19K polypeptides were absent. The comparison of the profile of a typical RIP with that of cells labelled in hypertonic medium (compare Fig. 5 with Fig. 1, 2) indicated that there were differences in the relative proportions of individual viral polypeptides depending on the method used to identify them. Either the RIP failed to bind viral polypeptides quantitatively or HIB had a differential effect on the translational efficiency of different polypeptides or a combination of both.
Fig. 5. Radioimmunoprecipitation of BVD virus-infected FBT cells with hyperimmune serum. Lysates from FBT cells infected with BVD virus (lanes 2 and 4) or mock-infected (lanes 1 and 3) were labelled for 1.5 h with [35S]methionine at 15 h p.i. without inhibitors of protein synthesis. Extracts were radioimmunoprecipitated with anti-BVD hyperimmune serum and separated by 10% SDS-PAGE. Lanes 3 and 4 are a longer exposure of lanes 1 and 2 to show the less abundant proteins.

Although the 37K polypeptide was fairly abundant in lysates labelled under HIB (Fig. 1, 2), it was poorly immunoprecipitated by the hyperimmune serum used (Fig. 5, 6). The 56K to 58K pair appeared to be two distinct entities as they were well separated in some experiments (see Fig. 1, Fig. 5, lane 2) but in others, they migrated as a broad band (Fig. 2, 6). We believe that this is the consequence of heterogeneity in their oligosaccharide moieties (Donis & Dubovi, 1987). The 75K polypeptide was presumably immunoprecipitated with high efficiency since it was undetectable in infected cells labelled in hypertonic medium but was visible by RIP. Alternatively we could hypothesize that the translation of this polypeptide is more sensitive to HIB; a similar phenomenon has been described for vesicular stomatitis virus (Nuss & Koch, 1976). The 118K was apparently bound with high efficiency by the antibodies used since it was more prominent in radioimmunoprecipitated samples than in HIB-labelled extracts. Factors such as the relative amount of antibodies of a given specificity and their relative avidities influence the outcome of a RIP assay. The absence of the 25K and 19K polypeptides in radioimmunoprecipitated lysates was probably the result of the poor immune response that these polypeptides elicited in the hyperimmunized animal. Typically 1-5 to 3% of the total radioactive protein present in the infected cell lysate was immunoprecipitated with the hyperimmune serum. If a sequential RIP was performed with the same lysate, no viral polypeptides were immunoprecipitated in the second assay. RIP of mock-infected cell lysates with hyperimmune serum, or of infected cell lysates with normal serum, bound only 0.3 to 0.5% of the input radioactivity.

**Time course of synthesis of viral polypeptides**

Virus-induced polypeptides could be detected as early as 4 h p.i. by the RIP assay and reached a maximum rate of synthesis between 12 and 20 h p.i. (Fig. 6). After this time the rate of synthesis reached a plateau and remained at similar levels until cell functions were significantly altered. Cytopathology, in the form of cytoplasmic vacuoles, could be detected as early as 14 h p.i. and became extensive with massive detachment of cells from the dish by 24 h p.i.
DISCUSSION

There is general agreement between the results of direct analysis of cell extracts labelled under HIB and the analysis of cell lysates labelled under isotonic conditions and subjected to immunoprecipitation with hyperimmune serum; polypeptides found in infected cells labelled in hypertonic medium that were also detected in RIP-analysed extracts were 165K, 118K, 80K, 56K to 58K, 48K and 37K. The 135K, 25K and 19K polypeptides found in [3H]leucine-HIB- or [35S]methionine-[35S]cysteine-labelled cell extracts were only seen in RIP-analysed [35S]methionine cell extracts after prolonged exposure of autoradiograms (data not shown). We speculate that this is the result of poor recognition of these polypeptides by the antiserum. The 32K polypeptide was not detected in [35S]methionine-labelled cell extracts immunoprecipitated with hyperimmune serum. The 37K polypeptide was immunoprecipitated with low efficiency by the antiserum used in these experiments but could usually be visualized with standard exposures. The majority of sera collected from naturally BVD virus-infected animals also do not precipitate this polypeptide. The maximal immune response against this polypeptide is found in late convalescent serum (R. O. Donis & E. J. Dubovi, unpublished results). Four polypeptides (135K, 32K, 25K and 19K) were detected in infected cells by shut off of host cell macromolecular synthesis only and their viral specificity remains to be confirmed by an independent method. The use of monospecific polyclonal antiserum prepared against the purified proteins, monoclonal antibodies or molecular cloning techniques are among the possible alternatives to establish unequivocally their viral origin.
Matthaeus (1980) reported the existence of one unglycosylated, 85K, and two glycosylated, 57K and 44K, polypeptides in crude cell extract preparations. Our results are in agreement except for small differences in the molecular weights which could be in part the result of studying different virus isolates.

The inability to detect other polypeptides in the extracts was probably the result of the ammonium sulphate precipitation step used to remove high mol. wt. proteins. Our results confirm but also significantly extend the findings of Purchio et al. (1984) who detected the polypeptides 115K, 80K and 55K by RIP using a different cytopathic isolate of BVD. Polypeptides of M, 45K and 38K were described by these authors as minor polypeptides. We show here that both of them are present in large quantities in infected cells labelled with L-[^3H]leucine under HIB conditions. In addition, Purchio et al. (1984) make no mention of the 165K, 135K, 75K, 62K, 25K and 19K polypeptides. The difference in our results is probably the result of the use of different isotopes and antisera in our experiments. This is an example of one of the limitations of RIP analysis, only viral proteins that induce a significant antibody response in the animal can be identified by that method. Immunodominance of certain viral proteins in the animal host chosen for the preparation of hyperimmune serum is one of the potential sources of bias, resulting in their preferential recognition over less immunogenic polypeptides. Clearly, the use of major and minor to describe proteins detected only by RIP is inadvisable. The second problem inherent to RIP assays is the possible coimmunoprecipitation of cellular proteins tightly bound to viral proteins or of two viral polypeptides bound together, a problem that can be partially resolved by immunoblotting. We were able to identify 118K, 80K and 56K to 58K in immunoblots using hyperimmune bovine serum and 125I-labelled affinity-purified rabbit anti-bovine IgG. The polypeptides not detected in this system are presumably those inducing antibodies to conformational epitopes not recognized after denaturation (R. O. Donis & E. J. Dubovi, unpublished results). In contrast, RIP analysis showed the presence of a 75K polypeptide which was not detected in HIB-labelled cell extracts. Evidence to support its viral origin is derived from the analysis of different field isolates of viruses in which the 75K polypeptide displays considerable size variability and from radiolabelling with carbohydrate (R. O. Donis & E. J. Dubovi, unpublished results; Donis & Dubovi, 1987). If these were cellular polypeptides coimmunoprecipitated as a result of a strong bond with a viral polypeptide, we would expect them to have a consistent size in the same cells infected with different isolates of BVD virus.

We report here the identification of seven previously unreported polypeptides in infected cells of M, ($\times 10^{-3}$) 165, 135, 75, 62, 32, 25 and 19. In addition, we confirm by independent means the viral origin of the polypeptides with M, ($\times 10^{-3}$) 118, 80, 56 to 58, 48 and 38 previously identified by immunological methods only. The quantification of bound and unbound radiolabelled polypeptides in RIP reactions demonstrates the relatively low rates of viral protein synthesis in infected cells. Only 2 to 3% of the total incorporated radioactivity is virus-specific (as recognized by the antibodies present in the antiserum). Taking into consideration that these experiments were carried out in contact-inhibited quiescent cells, we can speculate that the viral protein represents an even smaller proportion of the total cellular protein. This is in agreement with several observations, such as the need to concentrate cell lysates, inability to detect viral proteins in infected cell lysates analysed by SDS–PAGE stained with Coomassie Brilliant Blue, and the difficulties encountered in the preparation of purified viral proteins to be used in enzyme immunoassays. The time course of synthesis of viral polypeptides shows that polypeptides can be detected by 4 h p.i., that is, towards the end of the first third of an infectious cycle of approximately 12 h at 37 °C (R. O. Donis, unpublished observation). The rate of synthesis reached a maximum at a time when the first cycle of growth was close to completion. The polypeptides that appeared to be the most abundant in infected cells seemed to be the ones that were detectable in the early stages of infection. We believe precursor–product relationships probably exist between some of these polypeptides since the sum of their molecular masses is greater than the coding capacity of the virus. The reported size for the BVD virus genome of 12.5 kilobases (Renard et al., 1985) would be translated into a maximum of 500K of protein (unglycosylated), assuming non-overlapping reading frames, whereas the sum of the masses of polypeptides detected by us is 853K. The precursor–product relationships could involve
proteolytic processing and/or oligosaccharide addition and trimming as well as fatty acid acylation all of which could alter the real and apparent Mr of the polypeptide backbones. Evidence derived from the tryptic peptide maps of the 118K and 80K polypeptides strongly suggests that the former is a precursor of the latter (Purchio et al. 1984). The results of preliminary pulse and chase experiments were not rewarding, as only the two largest polypeptides (165K and 135K) transferred label to other polypeptides, but the recipients could not be identified unequivocally. In this respect BVD virus is similar to the members of Flaviviridae, since no clear precursor–product relationships could be established for the polypeptides induced in infected cells by these viruses on the basis of pulse–chase experiments. Presumably, this is the result of cotranslational proteolytic processing. Conversely, BVD virus differs in this aspect from the alphaviruses in which proteolysis of the precursor polypeptides could be easily established by label transfer. The function of each of these polypeptides in the replication cycle of the virus is not clear at this time, except for the 56K to 58K polypeptide which is present in the virion envelope (R. O. Donis & E. J. Dubovi, unpublished observations).

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