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The E3 Protein of Bovine Coronavirus Is a Receptor-Destroying Enzyme with Acetylesterase Activity

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In addition to members of the *Orthomyxoviridae* and *Paramyxoviridae*, several coronaviruses have been shown to possess receptor-destroying activities. Purified bovine coronavirus (BCV) preparations have an esterase activity which inactivates *O*-acetylsialic acid-containing receptors on erythrocytes. Diisopropyl fluorophosphate (DFP) completely inhibits this receptor-destroying activity of BCV, suggesting that the viral enzyme is a serine esterase. Treatment of purified BCV with [^{3}H]DFP and subsequent sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the proteins revealed that the E3 protein was specifically phosphorylated. This finding suggests that the esterase/receptor-destroying activity of BCV is associated with the E3 protein. Furthermore, treatment of BCV with DFP dramatically reduced its infectivity in a plaque assay. It is assumed that the esterase activity of BCV is required in an early step of virus replication, possibly during virus entry or uncoating.

Members of at least three families of enveloped RNA viruses (i.e., *Orthomyxoviridae*, *Paramyxoviridae*, and *Coronaviridae*) bind to cell receptors containing sialic acid as receptor determinant (25, 27, 28, 34, 36). In addition, virus-associated receptor-destroying activities have been described for members of these virus families (9, 11, 20, 23, 26, 34). For parainfluenza and for influenza A and B viruses, the receptor-destroying enzyme is a neuraminidase, which removes sialic acids from cellular receptors. In parainfluenza viruses, the neuraminidase activity is located on the HN protein, which also possesses receptor binding/hemagglutinin activity (26). In influenza A and B viruses, the neuraminidase is a glycoprotein which is distinct from the hemagglutinin protein (for reviews, see references 1 and 17). In contrast, for influenza C viruses and for bovine coronavirus (BCV), the receptor-destroying enzyme is not a neuraminidase, but an acetylesterase, which removes acetyl groups from *O*-acetylated sialic acids (11, 34). The esterase activity of influenza C virus is associated with the HE protein, which also possesses receptor-binding and fusion activity (8, 10, 33). However, no direct evidence was available that a specific BCV protein was associated with the esterase activity of viral preparations (34).

In the present report, we show that the BCV esterase activity resides on the E3 glycoprotein, which is one of three known surface proteins of the virus (6, 14). Enzymatic activity is inhibited by diisopropyl fluorophosphate (DFP), indicating that the BCV receptor-destroying enzyme is a classical serine esterase, such as acetylcholinesterase (5). Furthermore, inhibition of the BCV acetylesterase by DFP inhibits viral replication, suggesting that the presence of an active viral esterase is essential for virus entry into host cells.

MATERIALS AND METHODS

Viruses and cells. The BCV seed virus was obtained from Duphar B.V. Weesp (Amsterdam, The Netherlands). BCV was grown in Madin-Darby bovine kidney (MDBK) cells and purified as described previously (34). Influenza A/WSN/33 virus was grown in Madin-Darby canine kidney (MDCK) cells as described previously (3). Erythrocytes derived from chickens (strain Rhode Island Red sex-linked chromosome X) were obtained from Pocono Rabbit Farm (Canadensis, Pa.).

Acetylesterase assay. Purified BCV preparations (5 μg) were incubated at room temperature in 1 ml of phosphate-buffered saline (PBS) containing 1 mM *p*-nitrophenylacetate. In order to prepare a 100 mM stock solution, *p*-nitrophenylacetate was dissolved in acetonitrile. The final acetonitrile concentration in the assay was 1%. Hydrolysis of the substrate was monitored at 400 nm with a chart recorder.

Enzyme inhibition assay. DFP, phenylmethylsulfonyl fluoride (PMSF), and *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) were dissolved in isopropanol in order to prepare 100× stock solutions. Purified BCV (5 μg) was preincubated with inhibitor for 10 min at room temperature in a volume of 50 μl. PBS was then added to a final volume of 990 μl. The reaction was started by the addition of 10 μl of substrate, and the activity was monitored at 400 nm.

Hemagglutination assay. A BCV suspension (22.5 μl containing 1,024 hemagglutination units) was mixed with 2.5 μl of 10 mM DFP (in 10% isopropanol), incubated at room temperature for 10 min, and used in the assay. Alternatively, DFP-treated BCV was purified over a 20 to 60% sucrose gradient. After the virus band was collected, the volume was adjusted to 500 μl with 20% sucrose. Controls were treated in the same way except that inhibitor was omitted. Hemagglutination assays were performed in V-shaped microtiter plates (Flow Laboratories, Inc., McLean, Va.) as described previously (22).

Plaque assays. BCV and influenza A/WSN/33 virus were incubated with 1 mM DFP for 10 min at room temperature, purified over a 20 to 60% sucrose gradient, and titrated on MDBK cells by standard procedures (24, 29).

Protein labeling with [^{3}H]DFP. Viral protein (140 μg) was labeled in PBS containing 0.1 mM [^{3}H]DFP (4.4 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.) for 30 min at room temperature. Labeled virus was purified from unin-

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corporated DFP on a 20 to 60% sucrose step gradient and 5-μg aliquots were analyzed on a sodium dodecyl sulfate-polyacrylamide gel (7% polyacrylamide) with or without beta-mercaptoethanol. After electrophoresis, gels were fixed, soaked in Amplify (Amersham Corp., Arlington Heights, Ill.), dried, and fluorographed for 2 to 6 days at −70°C. Lanes of the gel containing molecular weight standards (Bio-Rad Laboratories, Richmond, Calif.) were silver stained.

Protein labeling with [^{35}S]methionine. Confluent MDBK monolayers in 60-mm-diameter dishes were infected with BCV (multiplicity of infection of approximately 2) and incubated at 37°C with 3T3 medium (Dulbecco modified Eagle medium supplemented with 10% heat-inactivated fetal calf serum, 50 U of penicillin G per ml, and 50 μg of streptomycin sulfate per ml). At 24 h postinfection, monolayers were washed with PBS, and protein-labeling medium (Hanks balanced salt solution supplemented with 0.5% $NaHCO_3$, 0.004% [wt/vol] phenol red, 50 U of penicillin G per ml, 50 μg of streptomycin sulfate per ml, and 0.2% glucose) was added to the cells. After incubation at 37°C for 30 min, cells were washed with PBS and incubated at 37°C with labeling medium supplemented with 200 μCi of [^{35}S]methionine per ml (1,100 Ci/mmol; Dupont NEN). At 36 h postinfection, culture supernatants were collected, and labeled virus was purified on a 20 to 60% sucrose step gradient (see above).

RESULTS

Acetylesterase activity of BCV. In a previous article, we showed that an acetylesterase activity is associated with BCV. This enzyme releases acetate from bovine submaxillary mucin at a rate comparable with that of influenza C virus esterase (34). To allow a more detailed characterization of the BCV enzyme, the synthetic low-molecular-weight substrate *p*-nitrophenylacetate was selected. Enzymatic measurements using this substrate involve determination of the cleavage product *p*-nitrophenol at 400 nm. This procedure is much less cumbersome than the cascade assay used for measuring acetate that is released from bovine submaxillary mucin. A purified BCV preparation effectively hydrolyzed this *O*-acetylester (Fig. 1). For further characterization of the BCV enzyme, different inhibitors were tested. DFP, a serine esterase and protease inhibitor, completely inhibited the BCV esterase, when preincubated with the virus at a 1 mM concentration (Fig. 1). Serine protease inhibitors PMSF and TPCK partially inhibited the BCV esterase (Table 1). EDTA had no effect, indicating that divalent cations are most likely not required for enzymatic activity, and dithiothreitol actually enhanced the activity.

Specific labeling of the E3 protein of BCV by [^{3}H]DFP. Since DFP inhibits serine proteases and serine esterases by binding covalently to the serine on the active site (2, 5), BCV preparations were radioactively labeled with [^{3}H]DFP by incubation at room temperature for 30 min. Labeled virus was purified over a 20 to 60% sucrose step gradient, and viral proteins were analyzed on a sodium dodecyl sulfate-polyacrylamide (7% polyacrylamide) gel with and without beta-mercaptoethanol. After fluorography, a single labeled protein was detected, migrating with an apparent M_w of 62,000 (62K) under reducing conditions (Fig. 2, lane 2) and with an apparent M_w of approximately 125K under nonreducing conditions (Fig. 2, lane 3). In both instances, the [^{3}H]DFP-labeled protein migrated in the position of the [^{35}S]methionine-labeled E3 protein of BCV (Fig. 2, lanes 1 and 4). The

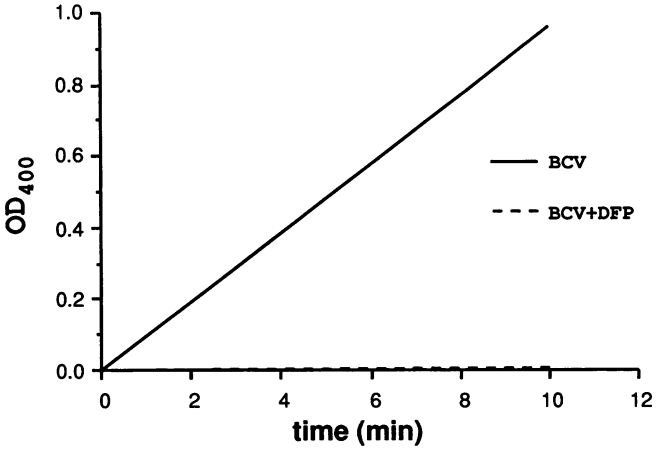

FIG. 1. Hydrolysis of *p*-nitrophenylacetate by BCV. Purified virus was incubated in a 1-ml cuvette containing 1 mM *p*-nitrophenylacetate in PBS–1% acetonitrile. For DFP treatment, 5 μg of virus was preincubated with 1 mM DFP for 10 min at room temperature (see Materials and Methods) and used in the assay. Hydrolysis of the chromogenic substrate was monitored at 400 nm using a chart recorder. OD_{400}, Optical density at 400 nm.

E3 protein of BCV is a homodimer composed of two 62K subunits, apparently connected by disulfide bridges, and it is one of the viral proteins recognized as making up the virion structure (7, 14, 15).

Hemagglutination of DFP-treated BCV. We then addressed the question of whether inactivation of the viral esterase affects the binding of BCV to erythrocyte receptors. BCV preparations were incubated with 1 mM DFP for 10 min at room temperature and purified over a 20 to 60% sucrose step gradient. DFP-treated and mock-treated BCV were collected, adjusted to a volume of 500 μl, and tested for receptor-binding activity by using a hemagglutination assay. No difference between treated and untreated BCV preparations was detected when hemagglutination assays were performed at 4°C, indicating that there was no requirement of an active esterase for binding to cell receptors (Fig. 3A). However, if the temperature was shifted up to 20°C and then to 37°C, untreated virus started to elute from erythrocytes as a result of the presence of the receptor-destroying activity. In contrast, the hemagglutination pattern of DFP-treated virus was stable under these conditions (Fig. 3B). The same result was obtained when BCV was incubated with 1 mM DFP and used directly in hemagglutination assays without prior purification over a sucrose gradient. Clearly, inhibition

TABLE 1. Effect of different inhibitors on BCV esterase[a]

Inhibitor	Concn (mM)	% Activity
DFP	1	<1
PMSF	1	91
TPCK	0.1	79
EDTA	10	98
DTT	10	140

[a] Purified BCV was preincubated with DFP (diisopropyl fluorophosphate), PMSF (phenylmethylsulfonyl fluoride), TPCK, (*N*-tosyl-L-phenylalanine chloromethyl ketone), EDTA (ethylenediaminetetraacetic acid), or DTT (dithiothreitol) at the indicated concentration for 10 min at room temperature in a volume of 50 μl. After 20-fold dilution with PBS, acetylesterase activity was determined by using *p*-nitrophenylacetate (1 mM) as the substrate (see Materials and Methods).

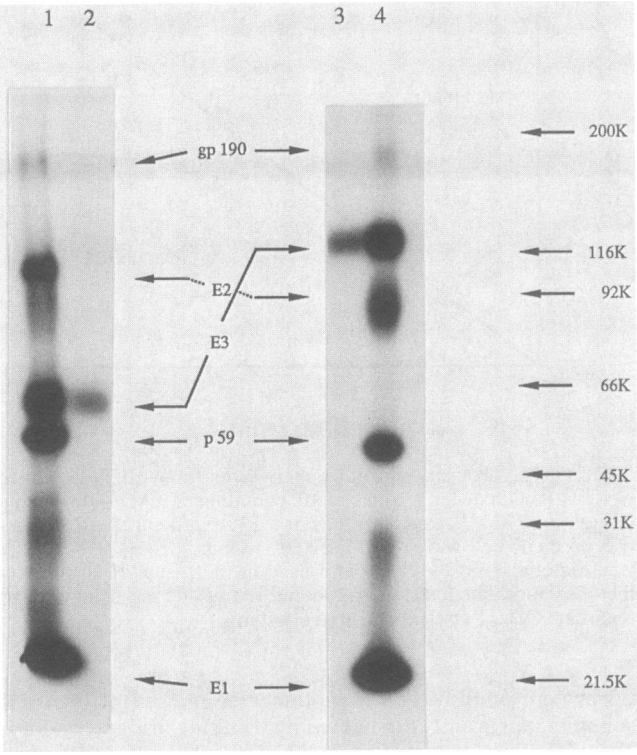

FIG. 2. Analysis of [^{3}H]DFP-labeled BCV proteins. [^{3}H]DFP-labeled and [^{35}S]methionine-labeled BCV was electrophoresed in a 7% polyacrylamide gel after solubilization in sample loading buffer with (lanes 1 and 2) or without (lanes 3 and 4) beta-mercaptoethanol. Lanes 2 and 3, [^{3}H]DFP-labeled BCV; lanes 1 and 4, [^{35}S]methionine-labeled BCV. Positions of viral proteins are indicated by arrows between lanes 2 and 3; positions of molecular weight markers (in thousands [K]) are shown on the right of both gels by arrows.

of the viral esterase activity stabilized binding of BCV to erythrocyte receptors.

Esterase activity required for BCV replication. In order to explore the biological significance of the BCV esterase during virus replication, infectivity titrations were performed with DFP-treated BCV. Gradient-purified DFP-treated and mock-treated BCV were tested for acetylesterase activity and hemagglutination titers and then were used

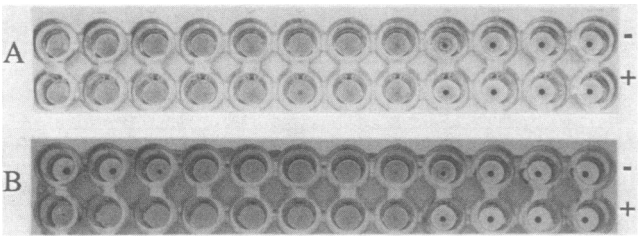

FIG. 3. Hemagglutination pattern of DFP-treated BCV. BCV (1,024 hemagglutination units) was incubated with 1 mM DFP for 10 min at room temperature. Hemagglutination was performed by using chicken erythrocytes and BCV preparations at serial 1:2 dilutions. (A) The microtiter plate was incubated at 4°C for 1 h and photographed. (B) The same plate was incubated overnight at room temperature, followed by incubation at 37°C for 30 min, and rephotographed. Symbols: +, BCV incubated with DFP; −, mock-treated BCV.

TABLE 2. Plaque formation of DFP-treated BCV preparations[a]

Expt no. and virus	HA titer[b]	% Esterase activity[c]	Titer (PFU/ml)
Expt 1			
BCV	2,048	100	2.6×10^7
BCV + DFP	2,048	<1	6.5×10^4
Expt 2			
BCV	128	100	2.5×10^6
BCV + DFP	128	<1	3.0×10^4
Expt 3			
BCV	8,192	100	5.4×10^7
BCV + DFP	8,192	<1	1.3×10^5
Expt 1			
A/WSN/33	8	NA	1.5×10^7
A/WSN/33 + DFP	8	NA	1.3×10^7
Expt 3			
A/WSN/33	128	NA	6.2×10^7
A/WSN/33 + DFP	128	NA	5.6×10^7

[a] BCV and influenza A/WSN/33 virus were incubated with and without 1 mM DFP and purified over a 20 to 60% sucrose gradient (experiment 1 and 2). Alternatively, DFP-treated virus was used without further purification (experiment 3).

[b] Reciprocal of the highest dilution of virus giving full hemagglutination after 60 min at 4°C.

[c] Measured with 1 mM *p*-nitrophenylacetate. NA, Not applicable.

in 10-fold serial dilutions for plaque assays. DFP-treated BCV had approximately 100- to 400-fold-lower infectivity titers than mock-treated BCV (Table 2). To exclude the possibility of unspecific effects caused by DFP, assays with influenza A/WSN/33 virus were done in parallel. This virus does not possess an esterase activity, and DFP treatment does not result in phosphorylation of a viral protein. No difference was found between the titer of DFP-treated and mock-treated influenza A/WSN/33 virus. These data strongly suggest that a functional BCV esterase is required for virus replication.

DISCUSSION

Previously we have shown that human coronavirus OC43 and BCV bind to sialic acid-containing cell receptors (34). Since the influenza C virus receptor-destroying enzyme, an *O*-acetylesterase (11), removed coronavirus receptors, it was concluded that OC43 and BCV recognize receptors similar to those of influenza C virus. In addition, a receptor-destroying or acetylesterase activity was found to be associated with BCV (34).

In order to determine whether the latter activity could be attributed to a viral protein, experiments were directed at analyzing the specificity and catalytic mechanism of the enzyme. Since the influenza C virus acetylesterase activity was conveniently measured with the low-molecular-weight substrate *p*-nitrophenylacetate (33), we also used this assay for monitoring the BCV enzyme. Of several inhibitors tested, only DFP, a serine protease and esterase inhibitor, completely inhibited the BCV esterase. Serine protease inhibitors PMSF and TPCK gave only partial inhibition, and no effect was detected by preincubation with EDTA, suggesting that divalent cations are not required for catalytic activity. Since the BCV esterase was quantitatively inhibited by DFP, it is suggested that the enzyme is a serine esterase. Similar findings were reported for other esterases (5, 19). By affinity labeling with the site-specific reagent DFP and analysis of the BCV protein on polyacrylamide gels, the E3 protein was identified as containing the viral esterase.

No difference in hemagglutination titers was observed between mock-treated and DFP-treated BCV in hemaggluti-

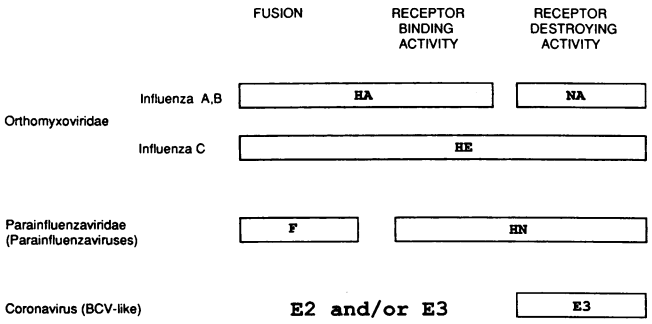

FIG. 4. Schematic representation of the functions of the surface glycoproteins of orthomyxoviruses, paramyxoviruses, and coronaviruses. Sendai virus and BCV are used as prototypes for paramyxoviruses and coronaviruses, respectively. HA, Hemagglutinin; NA, neuraminidase, HE, hemagglutinin-esterase; F, fusion protein; HN, hemagglutinin-neuraminidase; E2, peplomer protein; E3, coronavirus hemagglutinin-esterase. Boxes indicate proteins for which functions have been determined.

nation assays under standard conditions at 4°C. However, when the incubation temperature was raised, mock-treated BCV eluted from chicken erythrocytes, and only DFP-inactivated BCV retained its hemagglutination titer. These experiments suggest that inhibition of the receptor-destroying activity in BCV actually increases the stability of virus-receptor interactions. Similar results had been obtained earlier when the receptor-destroying activities of influenza viruses were inhibited (4, 11).

To investigate the role of the BCV esterase during virus replication, plaque assay experiments were performed. Following inactivation of the esterase with DFP, infectious virus was titrated in tissue culture. Compared with mock-treated BCV, virus with an inactivated esterase had an approximately 100- to 400-fold reduced titer. In contrast, the infectivity of DFP-treated influenza A virus was not diminished. Thus, data obtained from these experiments could indicate a direct involvement of the actylesterase in early phases of BCV replication. Since binding of BCV to sialic acid-containing receptors was not impaired by DFP inactivation of the esterase, it appears that the first step in viral replication, binding to cellular receptors, is independent of the presence or absence of a receptor-destroying activity. We thus speculate that an active esterase may be required for either endocytosis and/or uncoating of the virus with subsequent release of viral RNA into the cytoplasm.

There are now three RNA virus families that include viruses which have been found to possess receptor-destroying activities (Fig. 4). Neuraminidases are associated with influenza A and B viruses (1, 17) and with parainfluenza viruses (26). Esterases have been shown to be part of influenza C virus (8, 10, 11, 19, 33) and members of the coronavirus family, such as BCV (34). These receptor-destroying activities are either located in a separate protein (neuraminidases in influenza A and B viruses) or are part of a multifunctional protein as in the case of the HE protein of influenza C virus. Similarly, the receptor-binding activity may be localized in a separate protein (hemagglutinins of influenza A and B viruses) or it may be associated with a multifunctional protein (the HN and HE proteins of paramyxoviruses and influenza C virus). Finally, fusion activity may again be associated with a unique protein, as in the parainfluenza subgroup (27), or at the other extreme be part of the HE protein (10, 21), which possesses several functions.

Taking into account that BCV and OC43 are antigenically closely related to mouse hepatitis virus (12), yet appear to have an additional E3 surface glycoprotein, no biological function can be attributed to this protein by direct analogy. Since the receptor-binding and fusion activity in mouse hepatitis virus are located on the E2 protein (18, 31, 32, 35), it might be hypothesized that the E2 proteins of BCV and OC43 have homologous activities. But what, then, is the precise function of the E3 protein? Earlier studies revealed that the E3 protein of BCV is the viral hemagglutinin (15). Thus, is the E3 protein an additional receptor-binding protein or is it the only receptor-binding protein, with the E2 protein alone possessing fusion activity? The present study suggests that the E3 protein of BCV has an acetylesterase activity necessary for virus replication. Thus, the interesting possibility arises that this protein has receptor-binding as well as receptor-destroying activities. Although a great deal has already been learned about the molecular biology of coronaviruses (16, 30), identification of the precise roles of all proteins during coronavirus replication awaits further analysis. Additional experiments should also show whether all or only some coronaviruses possess receptor-binding/receptor-destroying activities and whether different strategies of attachment and uncoating prevail for different coronaviruses.

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