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The S2 Subunit of the Spike Glycoprotein of Bovine Coronavirus Mediates Membrane Fusion in Insect Cells

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The hemagglutinin/esterase (HE), spike precursor (S) and the S1 and S2 subunits of the spike precursor protein of bovine coronavirus were expressed in *Spodoptera frugiperda* (Sf9) cells, and the cell-fusing activity of each recombinant glycoprotein was examined. Extensive syncytia formation was observed in cells infected with the S2 recombinant but not with the HE or S1 recombinant baculoviruses. Fusion of Sf9 cells expressing the intact S protein precursor was evident after trypsin treatment. These results demonstrate that proteolytic cleavage of the S spike precursor is required for fusion induction and that the fusion is mediated by the S2 subunit. These observations may reflect the biological role of the S2 subunit in fusion–penetration during bovine coronavirus infection.

The envelopes which surround many viruses are derived from host cell membranes and contain multiple copies of viral glycoproteins which form the surface projections of the virions required for host cell binding and penetration. Two mechanisms for virus penetration of the target cell have been proposed (*1*). In the case of paramyxoviruses, for example, after initial binding of the cell surface, the viral envelope fuses with the plasma membrane directly, releasing the nucleocapsids into the interior of the cell (*2*). In contrast, alphaviruses, rhabdoviruses, and orthomyxoviruses are internalized by endocytosis and fusion of the envelope with endosomal membranes is activated in an acidic, intracellular compartment (*3*).

Bovine coronavirus (BCV) contains two major membrane glycoproteins; the hemagglutinin/esterase (HE) and the spike (S) protein (*4, 5*). The HE glycoprotein is a disulfide-linked 140K homodimer of a 62K monomer, and exhibits hemagglutinating and sialic acid-containing receptor-destroying activities (*6–8*). The 180K S glycoprotein is cleaved into S1 and S2 subunits at amino acids 768–769 and forms the large club-shaped projections characteristic of coronaviruses (*9, 10*). The S2 subunit is associated with the viral envelope and forms the stalk of the surface projection while the S1 subunit is believed to form the bulbous head of the projection. Studies using a series of monoclonal antibodies have demonstrated that BCV-neutralizing epitopes are located on the HE glycoprotein (*11*) and the S1 subunit of the S glycoprotein (*11, 12*). The biological significance of the S2 subunit of BCV has not been determined yet.

Although cell fusion has been observed in cultures infected with many coronaviruses, there is no direct evidence as to which viral components are involved (*13, 14*). One approach to elucidate the function of individual viral components is to express the genes for the individual protein and examine their biological activities in the absence of other viral components. Recently, the *Autographa californica* nuclear polyhedrosis baculovirus has been developed as a helper-independent expression vector. The recombinant proteins expressed by baculovirus appear to retain the biological activities, such as immunogenicity, enzymatic activity, and other physicochemical properties (*15, 16*). Several viral fusion proteins expressed in insect cells have been also demonstrated to be biologically active (*17, 18*). Thus, in order to identify the viral membrane glycoprotein which induces cell fusion by BCV, we expressed the HE, the S, and the S1 and S2 subunits of the S glycoprotein using recombinant baculoviruses, and examined the cell-fusing activity of each recombinant polypeptide.

The construction of recombinant plasmids of the HE (*8*), S (*9*), and S1 (*12*) genes of BCV have been described in detail elsewhere. Briefly, in order to express the S1 subunit, the S gene was digested with *Tth*III, which cleaved the S gene within the sequences encoding the proteolytic cleavage site of the S precursor glycoprotein (nucleotides 2294–2295). The 5′ 2294-nucleotide fragment was fused to a universal translational terminator and inserted into the *Bam*HI site of baculovirus transfer vector pAcYM1 (*19*). Because the S2 subunit is derived by proteolytic cleavage of the S precursor polypeptide, the cDNA sequences encoding the S2 subunit lack a translation initiation codon and membrane translocation sequence. Therefore, the cDNA sequence encoding the S2 subunit was fused to the amino-terminal signal sequence of the BCV HE gly-

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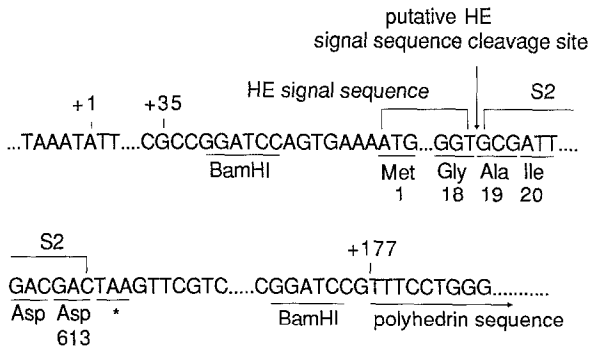

FIG. 1. Nucleotide and deduced amino acid sequences of the junctions of pAcDS2. Numbers indicate nucleotide positions (top) encoding the polyhedrin protein of baculovirus (A of initiating ATG as +1) and amino acid positions (bottom) of the recombinant S2 polypeptide. Note that the G at +3 position was changed to T in pVL941.

coprotein. Plasmid pCVE3 (*20*), which contains the cloned HE gene of BCV in the *Bam*HI site of pTZ18R (Pharmacia), was digested with *Sty*I, blunt-ended by S1 nuclease treatment and digested with *Sal*I. A 2.0-kb *Sca*I–*Sal*I fragment containing the cDNA sequences encoding the S2 subunit was obtained from the plasmid pCVE2, which contains the BCV S gene cloned in the *Bam*HI site of pTZ18R and was ligated into the linearized pCVE3. This recombinant plasmid was subsequently modified by site-directed mutagenesis, yielding plasmid pAcDS2, which contained the complete BCV HE signal sequence (*21*) fused to the S2 subunit through the alanine residue located at the amino terminus of the mature S2 subunit (*10*) (Fig. 1). The cloned genes were introduced into the genome of *A. californica* nuclear polyhedrosis virus (AcMNPV) by homologous recombination, and recombinant viruses were isolated by plaque hybridization and subsequent purification (*22*). Recombinant baculoviruses containing the HE, S, S1, and S2 genes were designated BVLHE (*8*), vAcS (*9*), vAcS1 (*12*), and vAcS2, respectively.

Synthesis of BCV-specific polypeptides in *Spodoptera frugiperda* (Sf9) cells infected with the recombinant viruses was examined at 24 hr postinfection. Rabbit antisera to BCV precipitated a 57K polypeptide when analyzed under reducing conditions in the cells infected with BVLHE (Fig. 2, lane 2). The 57K polypeptide represents the monomer of the HE glycoprotein. Cells infected with vAcS produced a 170K polypeptide. This indicates that the S polypeptide was produced but proteolytic cleavage was not occurring to a significant extent in Sf9 cells (Fig. 2, lane 3). A 95K and an 80K polypeptide of the S1 and S2 subunits were immunoprecipitated from cells infected with vAcS1 and vAcS2, respectively (Fig. 2, lanes 4, 5).

In order to determine if any of the recombinant polypeptides were capable of inducing cell fusion, Sf9 cells were infected with the recombinant baculoviruses at an m.o.i. of 5–10 PFU per cell and incubated at 28° in TNM-FH media containing 10% fetal bovine serum (*22*). At 36 hr postinfection, the medium was replaced with TNM-FH in which the pH varied between 5.0–6.5. In order to examine syncytia formation at alkaline pH and because TNM-FH becomes turbid at alkaline pH, replicates of the infected cells were overlayed with 1.5% agarose in PBS which had been adjusted to a pH range of 5.0–8.0. Syncytia formation was monitored by phase-contrast microscopy. Fusion was not detected in cultures infected with wild-type AcMNPV, BVLHE, or vAcS1 recombinant baculoviruses over the pH range examined (Figs. 3A, 3B, 3D). In contrast, fusion of vAcS-infected cells was apparent within 2 hr after a pH shift to 5.3 (Fig. 3C). Extensive syncytia formation was observed in vAcS2-infected cells (Fig. 3E) and continued to increase over 8 hr of observation until giant syncytia composed of approximately 100–200 cells were observed (Fig. 3F). When polyclonal BCV antibodies were included in the media, the fusion by the S and S2 polypeptides was partially inhibited (Table 1). These observations demonstrate that the S2 subunit of the spike glycoprotein of BCV can induce cell fusion in the absence of other viral components.

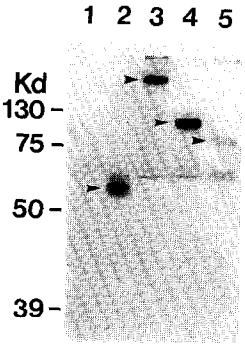

FIG. 2. Synthesis of BCV-specific recombinant polypeptides in insect cells. Sf9 cells were infected with recombinant baculoviruses at an m.o.i. of 5–10 PFU/cell and incubated at 28° in TNM-FH supplemented with 10% fetal bovine serum (GIBCO). At 24 hr postinfection, the media was replaced with methionine-free Grace's medium and the cells were starved for 1 hr. Cells were radiolabeled for 2 hr by adding [^{35}S]methionine (800 Ci/mmole, Amersham) to 120 μCi/ml. Cell lysates were prepared with RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 150 m*M* NaCl, 50 m*M* Tris–HCl, pH 7.4, 10 m*M* EDTA, 0.1% SDS) and immunoprecipitated with BCV-specific rabbit antisera using a suspension of 10 mg protein A–Sepharose (Pharmacia). The immune complexes were dissociated with 10% SDS, 25% glycerol, 10 m*M* β-mercaptoethanol, 10 m*M* Tris–HCl, pH 6.8, 0.02% bromphenol blue) and electrophoresed on a 7.5% discontinuous SDS–polyacrylamide gel followed by autoradiography. Lane 1, AcMNPV; lane 2, BVLHE; lane 3, vAcS; lane 4, vAcS1; lane 5, vAcS2.

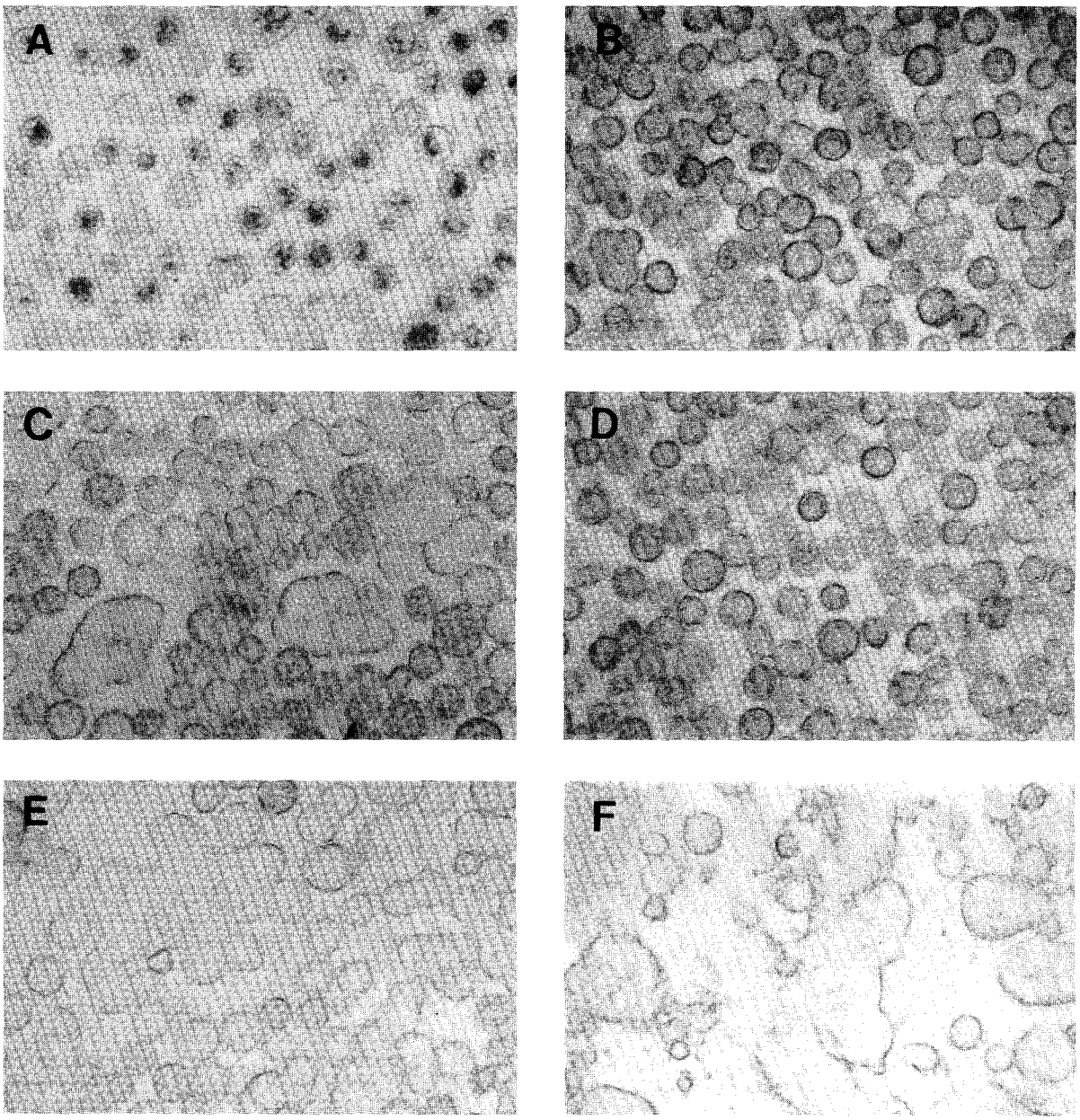

FIG. 3. Cell fusion induced by vAcS2 recombinant baculovirus. At 36 hr postinfection, the media were replaced with the TNM-FH, pH 5.3. Cell fusion was monitored after 4 hr with an inverted phase-contrast microscope (Zeiss Model IM35; magnification, 200 ×). A, AcMNPV; B, BVLHE; C, vAcS; D, vAcS1; E, vAcS2; F, vAcS2 (after 8 hr).

The low level of fusion observed in vAcS-infected cells (Fig. 3C) was thought to be due to a low level of cleavage of the S polypeptide. Partial cleavage of the recombinant S polypeptide of BCV in Sf9 cells has been demonstrated by a pulse-chase experiment (*12*). In order to further determine the effect of proteolytic cleavage on the cell fusing activity of the recombinant S polypeptide precursor, vAcS-infected cells were treated with 20 μg/ml of trypsin for 10 min in PBS, pH 8.0, prior to a shift to pH 5.3. As shown in Table 1, trypsin treatment increased the extent of cell fusion in vAcS-infected cells to a level similar to that seen in vAcS2-infected cells. This result demonstrates that cleavage of the S precursor is required for induction of cell fusion as previously reported in coronavirus-infected cells (*23, 24*).

We have demonstrated that a polypeptide composed of signal sequence of the BCV HE glycoprotein followed by the S2 subunit of the BCV S glycoprotein induces cell–cell fusion in Sf9 cells. Although it is not clear whether the HE signal sequence is cleaved in insect cells, the alanine residue at position 19 derived

TABLE 1

FUSION ACTIVITIES MEDIATED BY RECOMBINANT BACULOVIRUSES IN SF9 CELLS[a]

Treatment	AcNPV	BVLHE (HE)	vAcS (S)	vAcS1 (S1)	vAcS2 (S2)
TNM-FH	–	–	++	–	++++
Antibody[b]	–	–	+	–	++
Trypsin[c]	–	nd[d]	++++	nd	nd

[a] Cell fusion was induced at 36 hr p.i. by replacing the media with TNM-FH, pH 5.3, and fusion was monitored after 4 hr of pH-shift. The degree of fusion was presented as + (approx 20%), ++ (40%), ++++ (over 80%).

[b] Final dilution of 1/100 of anti-BCV rabbit antisera was added to the media at the time of pH-shift.

[c] At 36 hr p.i., cells were treated with trypsin for 10 min in PBS, pH 8.0 (final concentration of 20 μg/ml, TPCK, Sigma), and shifted to pH 5.3 in TNM-FH containing 10% fetal bovine serum.

[d] Not done.

from the N-terminus of the S2 subunit provides a favorable context for cleavage of the HE signal sequence (*25*). The HE signal peptide of 18 amino acids does not seem to contribute to the fusogenicity of the recombinant S2 polypeptide since the HE polypeptide expressed with BVLHE did not induce fusion (Fig.3B). We have concluded that the domain responsible for the cell fusion is located on the S2 subunit. The location of fusion domain on the S2 subunit of BCV is consistent with previous suggestions that the crucial fusogenic domain in MHV resides in the C-terminal half (S2 portion) of the peplomer protein (*26, 27*).

Fusion of insect cells infected with vAcS was dramatically increased by trypsin treatment (Table 1). Therefore, it is clear that proteolytic cleavage is required to induce the fusion activity of both the recombinant S polypeptide in insect cells and the authentic S polypeptide produced in BCV-infected cells (*14*). It has been observed that fusogenic polypeptides which require proteolytic activation often contain a hydrophobic domain located at the N-terminus which is exposed as a result of proteolytic cleavage of the precursor (*2*). In other instances, internal fusogenic domains that are masked at physiological pH are uncovered and activated by a conformational change which occurs in acidic compartments within the infected cell (*28*). A hydropathy plot of the BCV S2 subunit indicates that there are seven hydrophobic domains in addition to the putative membrane anchor domain. The N-terminus of the S2 subunit of BCV appears to be largely hydrophilic. The first hydrophobic domain (VTIDCSAFVCGDYAAC) is located 54 amino acids downstream from the cleavage site of the S precursor. A similar situation has been observed for Semliki Forest virus where the fusogenic domain of the E1 glycoprotein is located at amino acids 80–96 from the N-terminus (*29*). The significance of the internal hydrophobic domains of the BCV S2 subunit with respect to fusogenicity remains to be determined.

Recently, Sturman *et al.* (*30, 31*) have demonstrated that the both S1 and S2 subunit glycoproteins of mouse hepatitis coronavirus (MHV A-59) undergo conformational changes at pH 8.0 and 37°, resulting in the release of the S1 from virions. Furthermore, based upon the observation of MHV-induced cell fusion under mildly alkaline conditions, both of the subunits have been suggested to correlate with the coronavirus infectivity and cell fusion. However, we have not detected fusion with the S1 subunit (Fig. 3D). In Semliki Forest virus, although both E1 and E2 glycoproteins undergo conformational changes at fusion pH, only E1 is necessary and sufficient to mediate membrane fusion (*32*). Similarly, a conformational change of the BCV S1 may be important for exposing the fusogenic domain of the S2 subunit by releasing the S1 rather than being directly involved in fusogenicity.

Acidic conditions required for fusion in insect cells by the recombinant S2 subunit seem to indicate the involvement of an acidic compartment in initiation of BCV infection. Earlier evidence also suggested that MHV infection involved acidification of endosomal vesicles (*33*). However, fusion of mammalian cells infected by BCV or other coronavirus has been observed at mildly alkaline conditions ranging from pH 7.5 to 8.0 (*14, 30*), suggesting that coronavirus may enter the target cells by direct fusion of the viral envelope with the plasma membrane. In addition, we have previously observed dramatic differences in glycosylation of the BCV spike glycoproteins between insect cells and mammalian cells (*12*). The glycosylation differences may result in the conformational changes and the altered optimum pH for fusogenicity of the recombinant S2 subunit. Thus, expression of the S2 subunit polypeptide in mammalian cells should help to determine if post-translational processing of the S2 subunit has a direct bearing on the ability to mediate cell fusion at alkaline pH.

Previous reports have indicated that the S2 subunit of the S polypeptides of other coronaviruses contain neutralizing epitopes as well as determinants of virulence and tissue tropism (*26, 34–36*). Deregt *et al.* (*5*) have produced a panel of monoclonal antibodies specific for the S polypeptide of bovine coronavirus, all of which bind the S1 subunit (*12*). As fusion of the viral envelope with cellular membranes is a critical requirement for virus infection, we are preparing antisera directed against specific regions of the S2 subunit in order to identify the amino acids mediating membrane

fusion and to determine if the fusogenic domain of the S2 polypeptide constitutes an important immunological determinant.

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