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Glycoprotein E1 of Hog Cholera Virus Expressed in Insect Cells Protects Swine from Hog Cholera

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The processing and protective capacity of E1, an envelope glycoprotein of hog cholera virus (HCV), were investigated after expression of different versions of the protein in insect cells by using a baculovirus vector. Recombinant virus BacE1[+] expressed E1, including its C-terminal transmembrane region (TMR), and generated a protein which was similar in size (51 to 54 kDa) to the size of E1 expressed in swine kidney cells infected with HCV. The protein was not secreted from the insect cells, and like wild-type E1, it remained sensitive to endo-β-*N*-acetyl-D-glucosaminidase H (endo H). This indicates that E1 with a TMR accumulates in the endoplasmic reticulum or *cis*-Golgi region of the cell. In contrast, recombinant virus BacE1[−], which expressed E1 without a C-terminal TMR, generated a protein that was secreted from the cells. The fraction of this protein that was found to be cell associated had a slightly lower molecular mass (49 to 52 kDa) than wild-type E1 and remained endo H sensitive. The high-mannose units of the secreted protein were trimmed during transport through the exocytotic pathway to endo H-resistant glycans, resulting in a protein with a lower molecular mass (46 to 48 kDa). Secreted E1 accumulated in the medium to about 30 μg/10^6 cells. This amount was about 3-fold higher than that of cell-associated E1 in BacE1[−] and 10-fold higher than that of cell-associated E1 in BacE1[+]-infected Sf21 cells. Intramuscular vaccination of pigs with immunoaffinity-purified E1 in a double water-oil emulsion elicited high titers of neutralizing antibodies between 2 and 4 weeks after vaccination at the lowest dose tested (20 μg). The vaccinated pigs were completely protected against intranasal challenge with 100 50% lethal doses of HCV strain Brescia, indicating that E1 expressed in insect cells is an excellent candidate for development of a new, safe, and effective HCV subunit vaccine.

Hog cholera virus (HCV) is a member of the *Pestivirus* genus of the *Flaviviridae* (4). The virion is a small spherically shaped particle which consists of a hexagonally shaped core (22), containing the viral positive-stranded RNA genome, which is surrounded by an envelope containing three viral glycoproteins, E1 (gp51 to gp54), E2 (gp44 to gp48), and E3 (gp31) (35, 47).

HCV causes a highly contagious and often fatal disease in pigs which is characterized by fever and hemorrhages and can run an acute or chronic course. Outbreaks of the disease occur intermittently in several European countries and can cause large economic losses. Vaccination of pigs with a live attenuated HCV vaccine strain, the C strain, protects pigs from hog cholera and elicits a strong antibody response against envelope protein E1 (33). In addition, strongly neutralizing monoclonal antibodies (MAbs) directed against E1 have been prepared (48).

Recently, cDNA sequences encoding different versions of E1 of HCV strain Brescia (25) have been inserted into the gX locus of pseudorabies virus (PRV) (37). The PRV recombinants contain the sequence of E1 without (M203) and with (M204 and M205) a C-terminal transmembrane region (TMR). E1 expressed in SK6 cells infected with M203 is secreted into the medium. In contrast, E1 expressed in cells infected with M204 and M205 accumulates in the cell. Pigs inoculated with M204 and M205 develop high levels of neutralizing antibodies against HCV and are protected against challenge with a lethal dose of HCV, whereas pigs inoculated with M203 develop low levels of neutralizing antibodies and are partly protected upon challenge (37).

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Because the use of recombinant vaccines in the field is still a matter of controversy, the development of a safe vaccine such as a nonreplicating E1 subunit vaccine would be advantageous. However, the advantage of a vaccine vector like PRV for the expression of E1 is its capacity for antigen amplification during viral replication in the vaccinated animal. In contrast, subunit vaccines are capable of eliciting a protective immune response only when a large amount of antigen is applied. Many examples have shown that the baculovirus-insect cell system supports high-level expression of heterologous proteins (13, 19, 21). Moreover, except for the modification and trimming of the N-linked core oligosaccharides, most aspects of the processing of proteins, such as phosphorylation, acylation, proteolytic cleavage, and cellular targeting, appear to proceed similarly in insect and mammalian cells (13, 19, 21). The baculovirus system has therefore become very popular and has been proven to be very successful in vaccine development (18, 29, 39). The studies described in the present paper were primarily undertaken to investigate whether E1 of HCV, synthesized in insect cells, could elicit a protective immune response in pigs against hog cholera. To this end, sequences encoding E1 with and without a TMR were fused to the signal sequence of gX of PRV (28) and inserted into the p10 locus of a baculovirus vector. The effect of the TMR on the processing, localization, and production of E1 in insect cells as well as the protective capacity of E1 will be described.

MATERIALS AND METHODS

Cells and viruses. *Autographa californica* nuclear polyhedrosis virus (AcNPV) and recombinant viruses were propagated in the *Spodoptera frugiperda* cell line Sf21 (38). Sf21 cells were grown as monolayers in either TC100 medium (6)

supplemented with 10% fetal bovine serum and antibiotics or SF900 serum-free medium (GIBCO-BRL) plus antibiotics. For cotransfection, Sf21 cells were grown in TNM-FH medium (9) supplemented with 10% fetal bovine serum plus antibiotics.

Swine kidney cell line SK6 (11) was used to propagate HCV strain Brescia and PRV recombinant viruses M203 and M204. SK6 cells were grown in Eagle basal medium plus 5% fetal bovine serum and antibiotics.

Cloning of the gX signal sequence and the E1-encoding fragments. The gX signal sequence was constructed by annealing a 3′ stretch of seven complementary nucleotides of a 51-mer oligonucleotide, 5′-pGTTACATCGATAGATCT CAACAATGAAGTGGGCAACGTGGATTCTCGCCCT-3′, and a 49-mer oligonucleotide, 5′-pAGATTGGATCCGGC CACGACGGTGCGGACCACGAGGAGCCCGAGGGC GA-3′, and by filling in the 5′ single strands in a polymerase chain reaction with *Taq* polymerase (Perkin Elmer). A flanking *Bgl*II site included in the sequence of the 51-mer and a flanking *Bam*HI site included in the sequence of the 49-mer were digested, and the *Bam*HI-*Bgl*II fragment was isolated from an agarose gel by electroelution. The fragment was ligated into the calf intestinal alkaline phosphatase-treated *Bam*HI site of the pAcAS3 vector (40), and ligation mixtures were used to transform *Escherichia coli* DH5α cells by electroporation with a Gene-pulser (Bio-Rad) to give pAcAS3gX with a unique *Bam*HI site. Plasmid DNA was recovered by standard methods and was purified by CsCl centrifugation (30).

DNA fragments encoding E1 with and without TMR (E1+TMR and E1−TMR, respectively) were obtained by polymerase chain reaction with a cDNA clone of HCV strain Brescia, VP6 (25), as template in a 37-cycle amplification with *Taq* polymerase (Perkin Elmer). Noncoding, flanking *Bam*HI sites were included in the sequence of the forward and reverse primers used to amplify the E1 fragments. The crude polymerase chain reaction products were *Bam*HI digested, and the E1 fragments were isolated from an agarose gel by electroelution and ligated into the calf intestinal alkaline phosphatase-treated *Bam*HI site of the pAcAS3gX vector (see above) to give pAcAS3gX.E1 with and without TMR (pAcAS3gX.E1+TMR and pAcAS3gX.E1−TMR, respectively). As a result of an error which introduced a mismatch in the reverse polymerase chain reaction primer, the *Bam*HI site at the 3′ end of the E1−TMR fragment was absent. Unexpectedly, cloning of this uncleavable *Bam*HI terminus in the pAcAS3gX *Bam*HI-calf intestinal alkaline phosphatase vector resulted in a pAcAS3gX.E1−TMR transfer vector with an insert which was correct in sequence (see below) and orientation but lacked the 3′ terminal *Bam*HI site. Therefore, this transfer vector was still suitable to construct the E1−TMR recombinant baculovirus.

Transfection and selection of baculovirus recombinants expressing E1. Confluent monolayers of Sf21 cells (2×10^6) grown in T25 flasks were cotransfected with 1 μg of wild-type AcNPV DNA isolated from extracellular budded virus particles, and 2 μg of either pAcAS3gX.E1+TMR or pAcAS3gX.E1−TMR by the calcium phosphate precipitation technique described by Summers and Smith (31). Cells were grown in TNM-FH medium for 4 days, after which the medium was collected. Recombinant viruses expressing β-galactosidase were isolated from the medium in a plaque assay including 2.5 μg of Bluo-Gal (GIBCO-BRL) per ml in a TC100 agar overlay. Blue plaques were picked and were used to infect confluent monolayers of Sf21 cells grown in M24 wells. After 4 days, the cells were fixed and tested for expression of E1 by immunostaining with horseradish peroxidase (HRPO)-conjugated MAb 3 as described by Wensvoort et al. (48). AcNPV recombinant viruses expressing E1 were plaque purified three times, and virus stocks were prepared and stored at 4°C.

DNA and RNA analysis. Viral and cellular DNAs were isolated from Sf21 cells infected with wild-type and recombinant AcNPV viruses as described by Summers and Smith (31). Southern blots of restriction enzyme-digested viral and cellular DNAs were hybridized (30) with a nick-translated probe of HCV cDNA clone VP6 (25) and showed that the DNA sequences encoding E1 were correctly inserted in the p10 locus of baculovirus.

The nucleotide sequence of the junction between E1 and the simian virus 40 (SV40) terminator in the DNA of AcNPV recombinant viruses was determined by the dideoxy chain termination method with T7 DNA polymerase (Pharmacia) and an E1-specific oligonucleotide primer, p428 (5′-pAAC TGTCAAGGTGCAT-3′; nucleotides 3170 to 3186 in the cDNA sequence of HCV strain Brescia [25]). Circularized 5.2-kb (E1+TMR) or 5.1-kb (E1−TMR) viral fragments, prepared by agarose gel fractionation of *Xho*I-digested DNA isolated from the nuclei of Sf21 cells infected with recombinant AcNPV viruses, were used as templates. The sequence at the 3′ junction between the E1−TMR fragment and the vector showed that the E1 and vector sequences were fused by ligation of a vector having a degraded *Bam*HI site (GATCC of the vector is missing) and a blunt E1 fragment. The vector sequence downstream of this junction was intact. The sequence around the 3′ junction between E1+TMR and the vector indicated that no cloning artifacts were introduced at this fusion site.

Cytoplasmic RNAs isolated from Sf21 cells infected with wild-type and recombinant AcNPV viruses (3) were analyzed in a neutral agarose gel (23). A Northern (RNA) blot was made on a Hybond-N membrane (Amersham) and hybridized with the VP6 probe as described above. To account for variation in gene doses, the probe was washed off and the blot was reprobed with a nick-translated fragment encoding a part of the polyhedrin gene. The fragment was isolated from a plasmid containing the AcNPV *Eco*RI-i fragment (kindly provided by J. Vlak).

Radiolabeling and analysis of proteins. Confluent monolayers of Sf21 cells (3×10^6) grown in T25 tissue culture flasks were infected with wild-type or recombinant AcNPV virus at a multiplicity of infection of 5 to 10 $TCID_{50}$ (50% tissue culture infective doses) per cell for 1.5 h at room temperature. The virus was removed, the monolayer was washed once with SF900 medium, and the cells were grown in SF900 medium for 40 h. The medium was removed, and the cells were incubated 1 h in methionine-free Grace medium (6). The cells were labeled with 40 μCi of [^{35}S]methionine (Amersham) per ml in methionine-free Grace medium for 6 h. The medium was harvested and clarified by centrifugation for 10 min at $600 \times g$. The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in 1 ml of PBS-TDS (1% [vol/vol] Triton X-100, 0.5% [wt/vol] sodium deoxycholate, 0.1% [wt/vol] sodium dodecyl sulfate [SDS] in PBS). Lysates were sonified and clarified by centrifugation for 10 min at $80,000 \times g$. Lysates and medium were divided in aliquots of 200 μl, and the aliquots were stored at −20°C. SK6 cells infected with PRV recombinants M203 and M204 were labeled with 100 μCi of [^{35}S]methionine per ml as described by van Zijl et al. (37). Medium and lysates were prepared as described above. Incorporation of [^{35}S]methionine was measured by trichloroacetic acid precipitation.

Aliquots of the medium and lysates corresponding to 3,000 incorporated counts per s were immunoprecipitated as follows. Samples were incubated for 16 h at 4°C with 2 μl of a mixture of the E1-specific MAbs 3, 6, and 8 (1:1:1) (37). Immunocomplexes were bound to 1 mg of protein A Sepharose by incubation for 2 h at 4°C under light shaking. The protein A beads were washed four times by centrifugation for 1 min at 1,000 × *g*, and then the beads were suspended in ice-cold PBS-TDS. Precipitated proteins were dissolved by boiling the beads for 3 min in sample buffer (60 mM Tris-Cl [pH 6.8], 2% [wt/vol] SDS, 10% [vol/vol] glycerol, 5% [vol/vol] 2-mercaptoethanol, 0.01% [wt/vol] bromophenol blue) and were analyzed by SDS-10% polyacrylamide gel electrophoresis (PAGE) (37). After electrophoresis, the gels were fixed in 10% (vol/vol) methanol–7% (vol/vol) acetic acid, dried under vacuum, and exposed to Hyper-MP films (Amersham).

Pulse-chase labeling of proteins was performed as described above, except that the cells were pulse-labeled for 15 min with 100 μCi of [^{35}S]methionine per ml and chased for the indicated period with SF900 medium. Lysates and media were prepared and analyzed as described above.

Endo H and PNGase F digestion. Immunocomplexes bound to the protein A beads were incubated in 20 μl of 100 mM sodium acetate (pH 5.5)–1% (vol/vol) Nonidet P-40 (NP-40)–1 mM phenylmethylsulfonyl fluoride and digested with 20 mU of endo H (endo-β-*N*-acetyl-D-glucosaminidase H; Boehringer-Mannheim) at 37°C for 16 h. Then, 20 mU of fresh endo H was added, and the mixture was incubated for another 2 h at 37°C. After addition of sample buffer, the mixture was boiled for 3 min and analyzed as described above.

Digestion with PNGase F (glycopeptidase F; Boehringer-Mannheim) was performed in the same way as digestion with endo H, except that the protein A beads were incubated in PBS containing 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, and 800 mU of enzyme.

ACA for detection of E1. E1 expressed by the recombinant viruses was detected in an E1-specific antigen capture assay (ACA) as described by Wensvoort et al. (46). Briefly, four different MAbs directed against four distinct antigenic domains of E1 (44) are used in this ACA. MAb 3 is used as capture antibody. Bound E1 is detected with HRPO-conjugated MAb 6, MAb 8, or MAb 13 and tetramethylbenzidine (Sigma) as substrate. Optical density is measured at 450 nm, and the titer of an E1 sample is calculated as the sample dilution with an optical density of half the maximum value of a control sample.

Time course of E1 production. Confluent monolayers of Sf21 cells grown in T25 flasks were infected with BacE1[−] or BacE1[+] at a multiplicity of infection of 5 to 10 $TCID_{50}$ per cell for 1.5 h at room temperature. The virus was removed, and the cells were washed twice with SF900 serum-free medium and supplied with 4 ml of fresh SF900 medium. After 0, 16, 24, 40, 48, 64, 72, and 98 h, the cells were suspended in the medium and centrifuged for 10 min at 600 × *g*. Cell pellets and media were separated, and media were clarified by centrifugation for 10 min at 1,400 × *g*. Virus titers in the media were determined by end-point dilution at the same time points after infection (31). The cells were washed twice with 2 ml of SF900 medium and suspended in 1 ml of SF900 medium, and the suspensions were freeze-thawed twice. To 0.5 ml of the freeze-thawed cell lysate, an equal amount of PBS containing 2% (vol/vol) NP-40 was added, and the mixture was incubated for 1 h at room temperature. Untreated and NP-40-treated cell lysates and media were analyzed for E1 in the ACA.

Immunoaffinity purification of recombinant E1. MAb 3 (16 mg), purified from ascites fluid by saturated $(NH_4)_2SO_4$ precipitation, was coupled to 0.6 g of CNBr-activated Sepharose-4B (Pharmacia) according to the manufacturer's instructions. Confluent monolayers of Sf21 cells grown in six T150 flasks (±1.2 × 10^8 cells) were infected with BacE1[−] at a multiplicity of infection of 5 to 10 and grown for 98 h in SF900 medium. The medium (±170 ml) was harvested and mixed with the MAb 3-coupled beads, and the mixture was incubated for 16 h at 4°C under light shaking. The beads were packed in a column and washed with PBS. Bound E1 was eluted with 0.1 M glycine-HCl (pH 2.5). The eluate was immediately neutralized with 25 μl of 3M Tris-Cl (pH 10.0) per ml. Samples were tested for E1 in the ACA as described above. The purity of E1 in the eluted fractions was determined by SDS-PAGE. Fractions containing 90% pure E1 were pooled, and the amount of protein in this pooled fraction was determined by a Lowry assay.

Immunization and challenge exposure of pigs. Groups of two specific-pathogen-free, 10- to 12-week-old pigs were inoculated intramuscularly with a double water-oil emulsion (1, 7) of immunoaffinity-purified E1 on day 0. Pigs 1, 2, 3, and 4 were inoculated with 20 μg of E1, and pigs 6, 7, 8, and 9 were inoculated with 100 μg of E1. After 28 days, pigs 3 and 4 were vaccinated again with 20 μg and pigs 8 and 9 were vaccinated with 100 μg of immunoaffinity-purified E1 (see above). Pigs 5 and 10 (control pigs) were inoculated on day 0 with a double water-oil emulsion of SF900 medium from Sf21 cells infected with wild-type AcNPV and vaccinated again with the same inoculum on day 28. Serum samples were taken on days 0, 14, 28, and 42. Pigs of all groups were challenged intranasally on day 42 with 100 50% lethal doses of HCV strain Brescia 456610 (33), a challenge dose that, in unprotected pigs, leads to acute disease characterized by high fever and thrombocytopenia starting at days 3 to 5 and to death at days 7 to 11. Heparinized (EDTA) blood samples were taken on days 38, 40 (4 and 2 days before challenge, respectively), 42, 45, 47, 49, 52, 54, and 56. Thrombocytes in EDTA blood samples were counted, and HCV titers in freeze-thawed leucocyte fractions were determined by end-point dilution titration on SK6 cells. All animals were observed daily for signs of disease, and body temperatures were measured. Titrations were read by immunostaining with MAb-HRPO conjugates (48). Neutralizing antibody titers against HCV strain Brescia in serum samples were determined in a neutralizing peroxidase-linked antibody (NPLA) assay (32). NPLA titers are expressed as the reciprocal of the serum dilution that neutralized 100 $TCID_{50}$ of strain Brescia in 50% of the replicate cultures.

RESULTS

Construction, selection, and characterization of recombinant viruses expressing E1. Transfer vectors pAcAS3gX.E1+TMR and pAcAS3gX.E1−TMR were constructed as depicted in Fig. 1.

Sf21 cells were cotransfected with pAcAS3gX.E1+TMR and pAcAS3gX.E1−TMR and wild-type AcNPV DNA isolated from extracellular virus particles. Polyhedrin-positive plaques expressing β-galactosidase were isolated and analyzed for expression of E1 by immunostaining with MAb 3. One plaque-purified E1−TMR virus (BacE1[−]) and one plaque-purified E1+TMR virus (BacE1[+]) were used to prepare virus stocks with titers of ±10^8 PFU/ml.

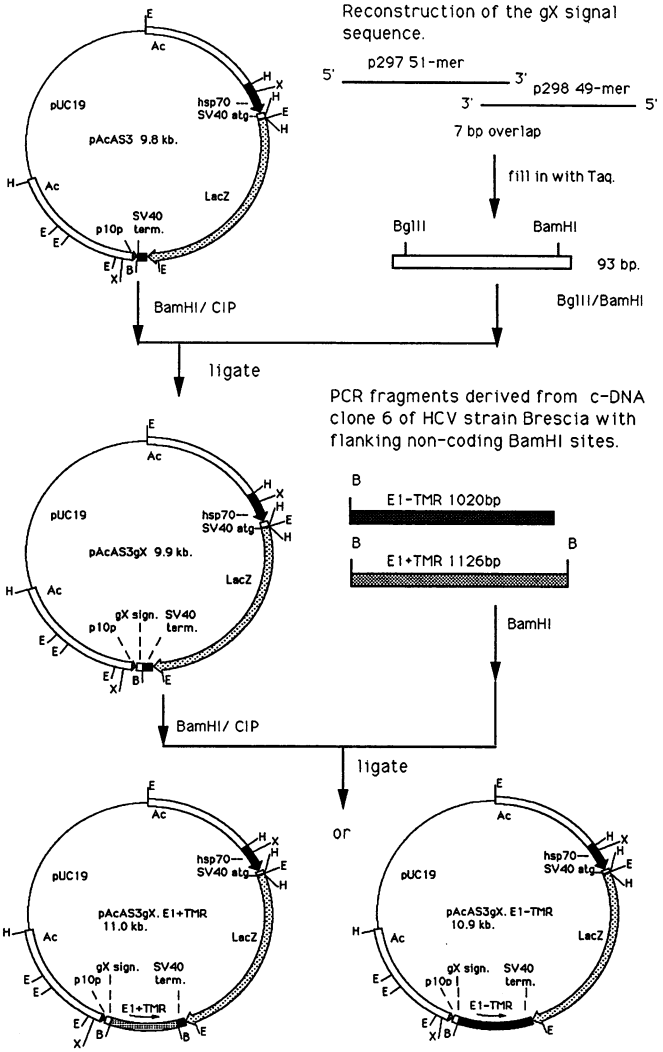

FIG. 1. Scheme of the construction of the transfer vectors pAcAS3gX.E1−TMR and pAcAS3gX.E1+TMR. Arrows show the directions of transcription of the hsp70 (LacZ) and p10 promoters. Ac, AcNPV DNA; p10, p10 promoter; hsp70, *Drosophila melanogaster* hsp70 promoter; SV40 term, SV40 transcription termination sequence; SV40 atg, SV40 initiation sequence; LacZ, *E. coli lacZ* gene; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; X, *Xho*I. PCR, polymerase chain reaction; CIP, calf intestinal alkaline phosphatase.

The E1 expression products observed in BacE1[+]- and BacE1[−]-infected cells were further characterized by radioimmunoprecipitation with a mixture of three E1-specific MAbs (Fig. 2). E1 precipitated from the lysate of Sf21 cells infected with BacE1[+] migrated as a doublet with a molecular mass of 51 to 54 kDa (lane 6). The protein was similar in size to wild-type E1 (lane 1). No E1 was precipitated from the medium of Sf21 cells infected with BacE1[+] (lane 7). E1 precipitated from the lysate of Sf21 cells infected with BacE1[−] also appeared as a doublet and, as expected, was slightly smaller (49 to 52 kDa) than wild-type E1 (lane 4). A doublet of 46 to 48 kDa was precipitated from the medium of cells infected with BacE1[−] (lane 5). The higher mobility of this protein indicated that the processing of this product was different from that of the cell-associated protein.

Localization of E1. To compare the processing of glycans

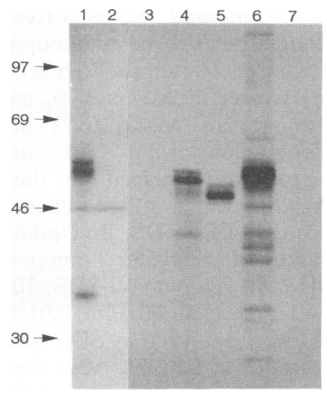

FIG. 2. Radioimmunoprecipitation assay with E1-specific MAbs 3, 6, and 8 (1:1:1 mixture) of media and lysates of Sf21 cells infected with BacE1[−], BacE1[+], or AcNPV. Cells were labeled at 44 h after infection with 40 μCi of [^{35}S]methionine per ml for 6 h. Immunoprecipitates were analyzed by SDS-10% PAGE and visualized by autoradiography. Wild-type E1, immunoprecipitated from the lysate of [^{35}S]cysteine-labeled SK6 cells infected with HCV strain Brescia (37), was run in parallel. Lanes: 1, wild-type E1; 2, AcNPV cell lysate; 3, AcNPV medium; 4, BacE1[−] cell lysate; 5, BacE1[−] medium; 6, BacE1[+] cell lysate; 7, BacE1[+] medium. Molecular weight calibration (10^3) is indicated to the left of the autoradiograph.

of E1 in insect and mammalian cells, we infected these cells with baculovirus and PRV recombinants expressing E1−TMR and E1+TMR, respectively. The construction of the PRV recombinants (M203 and M204) has been described previously (37). The cell-associated and secreted proteins were immunoprecipitated and treated with endo H or PNGase F (Fig. 3). Cell-associated E1 expressed by M204 and BacE1[+] had molecular masses similar to that of wild-type E1 (51 to 54 kDa), and both were sensitive to PNGase F and endo H. Both enzymes reduced the 51- to 54-kDa doublet to a single band of 44 kDa, confirming our

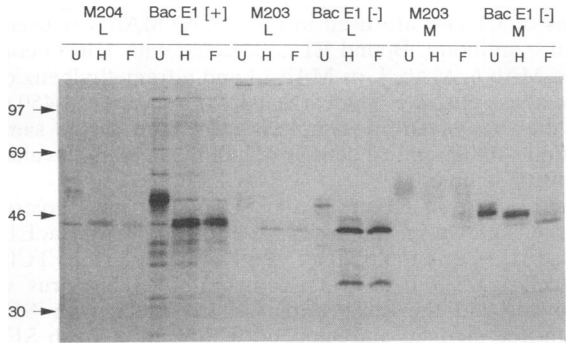

FIG. 3. PNGase F and endo H digestion of E1 products expressed by BacE1[−], BacE1[+], PRV E1[−] (M203), and PRV E1[+] (M204). Sf21 cells were infected with BacE1[−] or BacE1[+] and at 44 h after infection were labeled with 40 μCi of [^{35}S]methionine per ml for 6 h. SK6 cells were infected with M203 or M204 and labeled at 6 h after infection with 100 μCi of [^{35}S]methionine per ml for 16 h. Immunoprecipitates of lysates (L) and media (M) were divided in three fractions. One part was digested with PNGase F (F), one part was digested with endo H (H), and one part was left untreated (U). Samples were analyzed by SDS-10% PAGE and visualized by autoradiography. Molecular weight calibration (10^3) is indicated to the left of the autoradiograph.

previous observation that the 51- to 54-kDa doublet originates from heterogeneity in N-linked glycosylation of the E1 polypeptide (47). Cell-associated E1 products expressed by M203 and BacE1[−] were similar in size (49 to 52 kDa) and, as expected, smaller than E1 of M204 and BacE1[+]. The former proteins were also sensitive to digestion with PNGase F and endo H, resulting in the appearance of a single band of 42 kDa. A doublet of 38 to 41 kDa precipitated from the lysate of cells infected with BacE1[−] was also reduced in size to a single band of 32 kDa by endo H and PNGase F. This doublet was only present in the lysate of BacE1[−]-infected cells and was probably the result of cleavage by an insect cell-specific cellular protease. The sensitivity to endo H of cell-associated E1 with and without TMR, expressed in insect and mammalian cells, indicates that the N-linked glycans are of the high-mannose type and suggests that these E1 products are retained in the ER or *cis*-Golgi compartment of the cell. E1 secreted from M203- and BacE1[−]-infected cells was highly resistant to endo H. In mammalian cells, endo H resistance is due to processing of the N-linked high mannoses to a complex glycan in the *trans*-Golgi region of the cell (20). In insect cells, about 50% of the high-mannose glycans are processed in the Golgi compartments to the smaller endo H-resistant glycan, $GlcNac_2$-Man-Man_2, whereas the remainder has a variable number of mannose residues (10, 14). This explains the higher mobility of secreted E1 compared with cell-associated E1 of Sf21 cells infected with BacE1[−] (Fig. 2, lanes 4 and 5) and the partial deglycosylation by endo H of the major fraction of secreted E1, resulting in a product with a mobility slightly higher than that of untreated E1 (Fig. 3, BacE1[−] M, lanes U and H). A minor fraction of secreted E1 is even completely digested by endo H to a band of 42 kDa.

The 46- to 48-kDa E1 secreted from cells infected with BacE1[−] or the 55- to 65-kDa E1 secreted from cells infected with M203 were partly digested by PNGase F to a single band of 42 kDa. This band and the 42-kDa band observed after endo H digestion of E1 secreted from insect cells (see above) were similar in size to that obtained after PNGase F and endo H treatment of cell-associated E1 derived from M203 and BacE1[−]. It probably represents the unglycosylated backbone of E1−TMR.

Expression and secretion of E1. The levels of expression of E1 in Sf21 cells and in the medium were determined at different time points after infection (Fig. 4). Titers of extracellular virus were determined at the same time points after infection. No significant growth differences between BacE1[−] and BacE1[+] were found (results not shown). Although the amount of E1 detected in infected cells reached a plateau at 40 h after infection, the level of E1 in the medium accumulated to a maximum at 64 h after infection (compare Fig. 4A and B) and was about three times higher than the amount of cell-associated E1−TMR. Practically no E1 was detected in the medium of cells infected with BacE1[+]. Treatment of the lysate of these cells with NP-40 resulted in a 30-fold increase of the titer of E1, whereas the titer of E1 of the lysate of cells infected with BacE1[−] increased no more than 2-fold. Treatment of the media with NP-40 had no effect on the titer of E1.

The maximum amount of E1−TMR secreted in the medium was calculated from the results of the ACA by using the 90% pure E1 fraction (purified by immunoaffinity chromatography; see Materials and Methods) as standard and was about 20 μg/ml or 30 μg/10^6 cells.

Although no difference in virus growth was observed, the production of E1 in cells infected with BacE1[−] was about

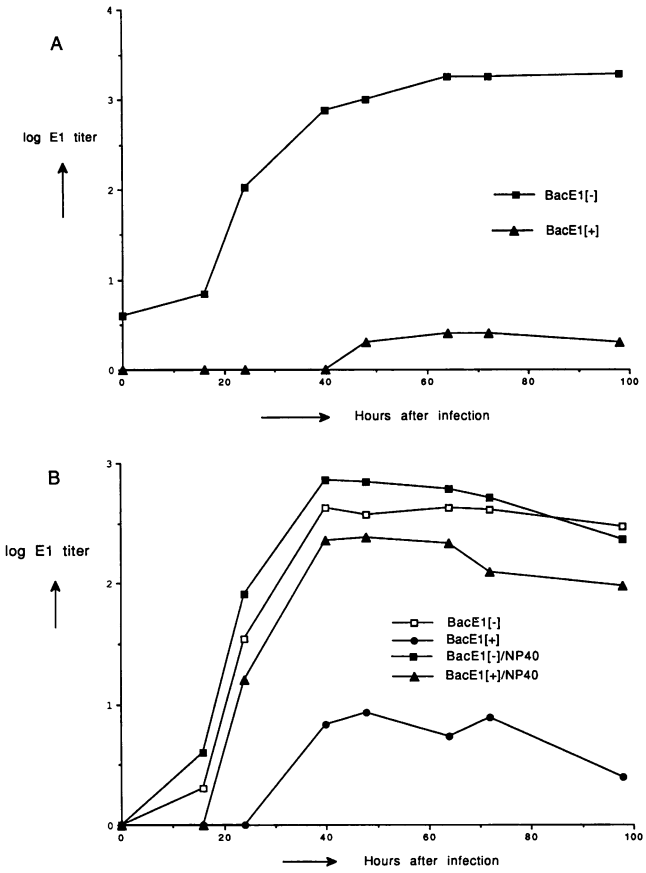

FIG. 4. Time course of E1 production in Sf21 cells infected with BacE1[−] and BacE1[+]. ACA (enzyme-linked immunosorbent assay) titers of E1 in the media (A) and in the cells (B) before and after NP-40 treatment at 16, 24, 40, 48, 64, 72, and 98 h after infection are shown. Titers are expressed as $\log_{10}$ values.

10 times higher (Fig. 4A and B) than in cells infected with BacE1[+]. Analysis of the cytoplasmic RNA of Sf21 cells infected with BacE1[−] and BacE1[+] on Northern blots showed that there was only a slightly higher level of E1-specific RNA in cells infected with BacE1[−] than in cells infected with BacE1[+] (results not shown). However, this difference was not large enough to account for the 10-fold-lower production level of E1 in cells infected with BacE1[+] than in cells infected with BacE1[−]. Because no degraded E1 products were precipitated from lysates of [^{35}S]methionine-labeled Sf21 cells infected with BacE1[+], the E1+TMR protein is not instable. Probably, accumulation of large amounts of E1+TMR in the membranes of the ER inhibits protein syntheses, resulting in a much lower production level of E1+TMR than of E1−TMR.

To study the kinetics of secretion of E1, cells infected with BacE1[−] were pulse-labeled for 15 min at 40 h after infection and chased for different time intervals (Fig. 5). E1 was first detected in the medium after a chase period of 40 min. However, after a chase period of 240 min, 40% of the labeled E1 was still associated with the cells. A part of these counts was made up by the C-terminally processed form of E1 of 38 to 41 kDa. This form of E1 was not transported through the Golgi stack at all, and remained cell associated.

Vaccination and challenge of pigs. To perform the first

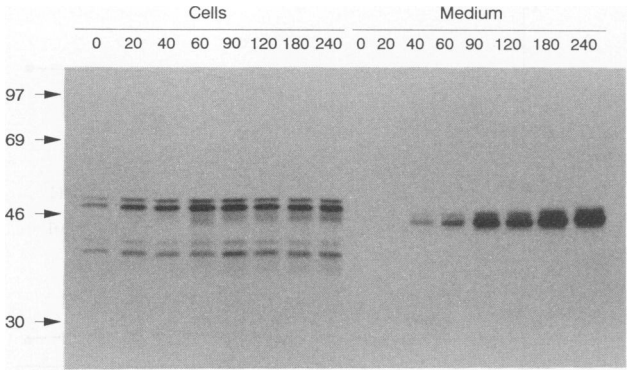

FIG. 5. Kinetics of secretion of E1 without TMR expressed in Sf21 cells infected with BacE1[−]. Sf21 cells were infected with BacE1[−] and pulse-labeled at 40 h after infection with 100 μCi of [^{35}S]methionine per ml for 15 min and chased with complete medium for the times indicated above each lane. Immunoprecipitated proteins from cell lysates and media were analyzed by SDS-10% PAGE and visualized by autoradiography. Molecular weight calibration (10^3) is indicated to the left of the autoradiograph.

vaccination experiment under optimal conditions, we prepared a pure and concentrated E1 fraction. Therefore, we fractionated E1 from the SF900 serum-free medium of cells infected with BacE1[−] by immunoaffinity chromatography with MAb 3 coupled to CNBr-Sepharose. Pigs were inoculated and challenged according to the regimen indicated in Table 1. None of the pigs showed fever after inoculation with the vaccine preparation. Within 4 weeks, pigs inoculated with 20 μg of E1 developed high NPLA titers (>3,000). A booster response was observed in pigs between weeks 4 and 6, after the second vaccination. However, between weeks 4 and 6, NPLA titers also significantly increased in most of the pigs which were vaccinated only once. Moreover, NPLA titers at 6 weeks in pigs which were vaccinated once did not correlate with the doses of E1 used. In fact, pigs which were vaccinated with 20 μg of E1 raised NPLA titers that were as high as or higher than those of pigs vaccinated with 100 μg of E1.

Irrespective of their NPLA titers, however, all vaccinated animals were completely protected against a lethal challenge with HCV strain Brescia. No animals showed any signs of disease, thrombocyte counts stayed within the normal range (175×10^3 to 587×10^3/μl), and no virus was detected in leucocyte fractions taken up to day 14 postchallenge. In contrast, the two control animals developed fever (>40°C) from days 4 and 5 on, showed a rapid decline of thrombocyte counts, became recumbent at day 6, and were killed when moribund at day 8 postchallenge. At day 5, one control animal was viremic (titer, $10^{2.1}$ $TCID_{50}$ per ml), and at day 7, both animals were viremic (titers, $10^{4.4}$ and $10^{2.8}$ $TCID_{50}$ per ml).

DISCUSSION

Many examples of the accurate recognition of the processing and targeting signals of heterologous proteins by insect cells have been described elsewhere (13, 19, 21). In the present study, we show that a gX–E1+TMR fusion protein is also properly processed and targeted in insect cells. Both in mammalian and in insect cells, recombinant E1+TMR appeared to be cell associated and sensitive to endo H and PNGase F, indicating that in the absence of HCV infection E1+TMR remained localized to the membranes of the ER or *cis*-Golgi region. In HCV-infected cells, E1 is also located intracellularly and sensitive to endo H (24). Therefore, we conclude that E1 is localized to the membranes of the ER or *cis*-Golgi region irrespective of the signal sequence used (gX or E1). Furthermore, the fact that E1−TMR is secreted from both insect and mammalian cells and the fact that E1+TMR is sensitive to endo H indicate that the TMR of E1 not only serves to anchor the protein into the cellular membranes but also functions as a signal which targets E1 to the ER or the *cis*-Golgi region.

The efficiency of secretion of heterologous proteins from insect cells is unpredictable and largely dependent on the protein under investigation and possibly also on the temporal expression of the baculovirus promoter used (8, 13, 26). Secretion of gX–E1−TMR proceeded efficiently, resulting in a threefold-higher level of secreted E1 than intracellular E1. It remains to be determined which factors influence secretion of E1 from insect cells. In some cases, it was shown that the use of specific signal sequences may positively influence

TABLE 1. Immunization of pigs[a]

Dose (μg)	Pig no.	Neutralizing antibody titer on the following days[b]:				Results of HCV challenge[c]		
		0	14	28	42	Disease	Viremia	Death
20	1	<6.25	150	4,800	>6,400	−	−	−
20	2	<6.25	25	3,200	4,800	−	−	−
20 (×2)	3	<6.25	19	1,600	>6,400	−	−	−
20 (×2)	4	<6.25	75	2,400	>6,400	−	−	−
100	6	<6.25	25	2,400	2,400	−	−	−
100	7	<6.25	19	2,400	4,800	−	−	−
100 (×2)	8	<6.25	150	1,600	>6,400	−	−	−
100 (×2)	9	<6.25	19	200	>6,400	−	−	−
None	5	<6.25	<6.25	<6.25	<6.25	+	+	+
None	10	<6.25	<6.25	<6.25	<6.25	+	+	+

[a] Two groups of four specific-pathogen-free pigs were inoculated intramuscularly on day 0 with doses of 20 and 100 μg of immunopurified E1, respectively. On day 28, two of the four pigs of each group were vaccinated again with a dose of E1 identical to that used on day 0. On day 42, the pigs were challenged intranasally with 100 50% lethal doses of HCV strain Brescia 456610 (33). All animals were observed daily for signs of disease, and body temperatures were measured.

[b] The neutralizing antibody titer is expressed as the reciprocal of the serum dilution neutralizing 100 $TCID_{50}$ of HCV strain Brescia in 50% of the replicate cultures (32).

[c] Fever, anorexia, thrombocytopenia, and paralysis were regarded as signs of disease. Thrombocytopenia and viremia after HCV challenge were recorded by taking heparinized blood samples on days 38, 40, 42, 45, 47, 49, 52, 54, and 56, counting the thrombocytes, and determining HCV titers in the leucocyte fractions. The two control pigs were killed when moribund.

the level of secretion of heterologous proteins from insect cells (2, 34). However, in other cases, such as that of urokinase-type plasminogen activator, the native signal peptide of the protein was cleaved efficiently and allowed for efficient secretion (90%) of the protein (12). The effect of the signal sequence of gX on secretion of E1 is unknown but will be studied by evaluating secretion of E1−TMR that has its own signal sequence (25, 41). Secreted E1 probably accumulates in the lumen of the ER and is directed to a site from which Golgi vesicle formation starts (27). A minor fraction of E1−TMR, however, remained cell associated. This suggests that besides the TMR there may be other retention signals on E1 or that E1 interacts with permanent residents of the ER or *cis*-Golgi region of insect cells. The aberrant C-terminal cleavage of a part of the cell-associated E1−TMR by an insect cell-specific protease is probably also a result of the retention of E1−TMR at a site in the ER or *cis*-Golgi region where this protease is located. Because the resulting 38- to 41-kDa protein was not secreted, no transit Golgi vesicle formation seems to take place at this particular site.

Transport to the cell surface and secretion of E1−TMR took no more than 55 min in insect cells and proceeded at a rate similar to that in mammalian cells (24). Secretion of E1 from insect cells was fast compared with secretion of several other glycoproteins. Transport of human immunodeficiency virus gp160 lacking a C-terminal TMR took 4 h because of sorting of the protein into a degradative pathway (43). Probably because of inefficient oligomerization, secretion of a transmissible gastroenteritis virus spike protein without a TMR was observed only after 24 h (5). For the same reason, the processing of influenza virus hemagglutinin in insect cells was found to be retarded (15). Abundant previous work suggests that secretory and membrane glycoproteins are secreted properly only if they are correctly folded and oligomerized in the ER; if not, they are not moved (17, 27). In line with this, the rate-limiting step during secretion was found to be transport from the ER to the Golgi region. In HCV-infected cells, E1 forms homo- and heterodimers (35, 42, 47). We did not study homodimerization of E1 in insect cells and do not know its effect on the rate of secretion of E1 without TMR. However, the fast secretion of E1 without TMR suggests that it is correctly processed, folded, and possibly also dimerized in insect cells. Irrespective of this, the secreted form of E1−TMR (Fig. 5; migrating just below the cell-associated E1−TMR protein) was not detected in the cell lysate before it was detected in the medium, suggesting that the rate-limiting step during secretion of E1 from insect cells is also transport from the ER to the Golgi region.

The proper folding of E1+TMR and of both the secreted and cell-associated forms of E1−TMR was also inferred from the reaction pattern of these proteins in the ACA with four MAbs (MAbs 3, 6, 8, and 13), which each recognize a discontinuous epitope (36) in one of the four distinct antigenic domains of E1 of strain Brescia (results not shown) (44). This indicated that E1 expressed in insect cells is antigenically indistinguishable from wild-type E1.

However, the most convincing evidence for the antigenic integrity of E1 was obtained from the vaccination experiments with pigs. Secreted E1, purified and concentrated from serum-free medium of BacE1[−]-infected Sf21 cells by immunoaffinity chromatography, induced a protective immune response in pigs at the lowest dose (20 μg) tested. Pigs with C strain-induced antibody titers (measured by the NPLA) of ≥50 are protected from hog cholera and do not transmit the virus to nonprotected contact pigs after challenge with virulent HCV (33). Animals inoculated once with 20 μg of E1 developed NPLA titers of 3,000 and higher, indicating that a much lower dose of E1 might be sufficient to induce a protective immune response. Moreover, the rise of antibody titers against E1 proceeded much faster in pigs vaccinated with insect cell-produced E1 than in pigs infected with low-virulent-field strains of HCV or in pigs vaccinated with the C strain (46) or with PRV M204 (45). Experiments in pigs to test lower doses of E1 directly prepared from serum-free medium are in progress. Furthermore, the length of protection induced by an E1 subunit vaccine has to be determined. The outcome of these experiments will determine the efficacy of an E1-based vaccine and the feasibility of its commercialization. Since animals infected with pestiviruses raise antibodies against at least two viral glycoproteins, namely E1 and E2 (16), such a vaccine would also allow for serological differentiation between vaccinated and infected animals.

In conclusion, the data described in the present report indicate that E1 is highly promising as a new, safe, effective, and differentiable HCV subunit vaccine. Such a vaccine would allow for a controlled eradication of hog cholera.

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