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Role of N-Linked Glycans of HCV Glycoprotein E1 in Folding of Structural Proteins and Formation of Viral Particles

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Abstract—Envelope proteins E1 and E2 of the hepatitis C virus (HCV) play a major role in the life cycle of a virus. These proteins are the main components of the virion and are involved in virus assembly. Envelope proteins are modified by N-linked glycosylation, which is supposed to play a role in their stability, in the assembly of the functional glycoprotein heterodimer, in protein folding, and in viral entry. The effects of N-linked glycosylation of HCV protein E1 on the assembly of structural proteins were studied using site-directed mutagenesis in a model system of Sf9 insect cells producing three viral structural proteins with the formation of virus-like particles due to the baculovirus expression system. The removal of individual N-glycosylation sites in HCV protein E1 did not affect the efficiency of its expression in insect Sf9 cells. The electrophoretic mobility of E1 increased with a decreasing number of N-glycosylation sites. The destruction of E1 glycosylation sites N1 or N5 influenced the assembly of the noncovalent E1E2 glycoprotein heterodimer, which is the prototype of the natural complex within the HCV virion. It was also shown that the lack of glycans at E1 sites N1 and N5 significantly reduced the efficiency of E1 expression in mammalian HEK293 T cells.

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Keywords: hepatitis C virus (HCV), HCV structural proteins, virus-like particles, N-glycosylation, site-directed mutagenesis, recombinant baculovirus, insect Sf9 cells, mammalian HEK293T cells

INTRODUCTION

In eukaryotic cells, many proteins are subject to posttranslational processing. One of the most common types of posttranslational protein modification is N-glycosylation, which involves the attachment of a weakly branched oligosaccharide chain composed of nine mannose (Man) and three glucose (Glc) residues to specific asparagine residues in Asn-X-Ser or Asn-X-Thr sequences (where X is any amino acid except for proline) [1]. Oligosaccharide attachment is coupled with protein folding; that is, the glycoprotein enters the calnexin–calreticulin cycle via specific interaction with lectin-like chaperons of the endoplasmic reticulum (ER), which guide its partial folding. The presence of N-oligosaccharides (N-glycans) can be involved in the stabilization of the protein structure and affect the specificity of protein–protein interactions [2–9], as well as plays a central role the folding of most membrane-bound and secreted glycoproteins. Glycosylation is a characteristic modification of surface proteins of enveloped viruses.

Hepatitis C virus (HCV) is the sole representative of the genus *Hepacivirus* of the family *Flaviviridae*. Its genome encodes a single precursor polyprotein of approximately 3000 amino acids, which is further processed in the ER to produce ten mature structural and nonstructural viral proteins [10–13]. The details of the HCV particle structure and its assembly remain poorly understood [12], and the processes of virion assembly and virus release from the cell are the least studied. It is reasonably supposed the virion properties depend on the glycosylation of HCV envelope proteins in the infected cell, their interactions, and the type of folding [14, 15].

HCV envelope glycoproteins E1 and E2 contain 5–6 and 9–10 potential glycosylation sites, respectively [11]. It should be noted that neither the exact number of E1 glycosylation sites, nor their actual involvement in protein modification in vivo has been determined so far.

Following glycosylation, HCV glycoproteins E1 and E2 may either dimerize, which produces functional noncovalent complexes, or form disulfide bonds that produce aggregates of incorrectly folded nonfunctional proteins. The formation of the complex is mediated by two cell chaperon proteins, calnexin and

Abbreviations: HCV, hepatitis C virus; VLP, virus-like particle; ER, endoplasmic reticulum; PFU, plaque-forming unit.

Primers used in the study

Primer	Nucleotide sequence, 5'–3'
16-Haml	CCA CTA CGA CAA TAC G
15-Hamr	GAG CAA GTC GAC GTG
26-N5tl	GGA CTG CCA ATG CTC AAT CTA TCC CG
17-N6tl	TGG TCA CTT ACA ACA GC
23-N5br	GAG CAT TGG CAG TCC TGC ACT GT
17-N6br	CTG TTG TAA GTG ACC AG
17-N7tl	GAA CTG GTC TAA GGT TC
17-N7br	AAC CTT AGA AGA CCA GTT CC
24r-Hpsr	AG CTG CAG TTA CCC GTC AAC GCC
29-EN1m	TTA TGA AGT GCG CCA AGT GTC CGG CAT AT
30-EN2m	AAC GAC TGC TCC CAA TCA AGC ATT GCG TAT
27-EN3m	GTT CAG GAG GGT CAA AGC TCC CGT TGC
24-EN4m	CGC GGC CAG GCA AGC CAG CGT CCC
19 Haml	CAC TAC GAC AAT ACG ACG T
18 Hamr	AGC AAG TCG ACG TGA CGT

calreticulin. Calnexin interaction with glycoproteins produces functional complexes, while calreticulin interacts with incorrectly folded glycoproteins that form aggregates [14–16]. Complexes of the first type serve to bind the virus to cell receptors and its entry into the cell; they affect its antigenic composition and probably play a certain role in the viral pathogenicity [17]. The aggregation of incorrectly folded glycoproteins can lead to the formation of defective virus particles, the ability of which to be released from or bind to host cells is probably suppressed [18–20].

We investigated the effects of N-glycans of the HCV glycoprotein E1 on the structural protein assembly and on the formation of viral particles using site-directed mutagenesis in a model system where virus-like particles (VLPs), which are morphologically similar to natural virions, are formed in insect cells transfected with baculovirus vectors that drive the expression of HCV structural proteins [15]. E1 mutants were generated by site-directed mutagenesis using standard double-stranded plasmid vectors.

EXPERIMENTAL

Bacterial cells and cell lines. The study involved *Escherichia coli* strains DH5*a* and DH10Bac (Gibco-BRL, United States), *Spodoptera frugiperda* cell line Sf9, and the human embryonic kidney cell line HEK293T.

Transformation of bacterial cells with plasmid DNA was performed as suggested by Amersham (United States). The isolation and purification of plasmids, endonuclease treatment, ligation, agarose gel electrophoresis, and other genetic engineering procedures were performed according to standard protocols [21].

Insect cells were cultured in Sf-900 II medium supplemented with 10% of fetal calf serum at 27°C using the previously developed techniques as described in [22]. The virus titer, recombinant virus amplification, Sf9 cell infection with the recombinant baculovirus, and virus expression were evaluated following the same instructions.

HEK293T human embryonic kidney cells were cultured at 37°C in a standard DMEM medium supplemented with 10% fetal calf serum, 4 mM L-glutamine, 1 mM sodium pyruvate, and 100 μg/mL streptomycin or 100 units/mL penicillin, in the atmosphere of 5% CO_2.

Recombinant constructs carrying cDNA of HCV structural proteins, recombinant bacmides, and recombinant bv-CE1E2 baculoviruses were constructed and analyzed as described previously [23].

Site-directed mutagenesis. A DNA fragment that corresponds to the cDNA sequence of HCV E1 was cloned into ClaI–PstI sites of a pKS(–) plasmid according to the standard protocol. A set of oligonucleotide primers was designed to obtain recombinant plasmids carrying E1 cDNA with point mutations at glycosylation sites (table). Each primer was 18–30 b long and contained a sequence encoding an N-glycosylation site (Asn-X-Thr/Ser; X ≠ Pro), with an Asn-encoding triplet substituted with a triplet encoding Gln.

Mutagenesis was performed using the method described by Drutsa et al. [24]. PCR was performed in a CycloTemp 107 thermal cycler (ResursPribor, Russia). PCR conditions were optimized empirically, so as to obtain a sufficient quantity of the target product in

the least possible number of reaction cycles. The presence of all planned nucleotide substitutions was confirmed by sequencing. The resulting mutant sequences were ligated into baculovirus vectors as described in [23]. The obtained plasmids were termed pFastBacHTb-CE1mutE2 and BacMam-CE1mutE2-GFP.

Total cell DNA was isolated from insect cells 72 h after infection with recombinant baculoviruses bv-CE1E2, bv-E1mut, bv-E1mutE2, bv-CE1mutE2 (5 PFU/cell) [23]. The presence of cDNA of structural HCV proteins in the total cell DNA was confirmed by PCR with primers to the pFastBacHT baculovirus vector. (forward, 5'-GTGGTTGGCTACGTATACTCC-3'; reverse, 5'-CCTCTACAAATGTGGTATGGC-3').

Microsome isolation. Seventy-two hours after infecting Sf9 cells with the bv-CE1E2 baculovirus recombinant (10 PFU/cell), 10^7 cells were collected from a monolayer culture and washed three times with phosphate buffered saline (PBS). Next, the cells were resuspended in 0.05 M Hepes–KOH buffer, pH 7.4, containing 1 mM EDTA, 1 mM DTT, 20% glycerin, and Protease Inhibitor Cocktail SetII, 1 : 200 (Calbiochem, United States) and destroyed in a Potter's homogenizer [30]. Intact cells and nuclei were removed by centrifugation as follows: 800 g at 4°C for 10 min, and microsomes were precipitated from supernatant as follows: 50000 g at 4°C for 60 min (Beckman Coulter Optima L-100XP centrifuge, rotor 50Ti). The pellet was resuspended in the same buffer without DTT and analyzed by Western blotting and electron microscopy.

HCV isolation and purification. A monolayer culture growing at 27°C was infected with the bv-CE1E2 baculovirus recombinant (20 PFU/cell). After 72 h, cells were collected (2×10^8), washed three times with PBS, resuspended in TNC lysis buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM PMSF, protease inhibitors (1 : 200) supplemented with 0.25% digitonin, and destroyed by ultrasonication. Cell debris was removed from homogenized lysates by low-speed centrifugation (1200 g, 15 min, 4°C). Next, HCV was concentrated in two steps, i.e., centrifugation through a 30% sucrose cushion (230000 g, 16 h, 4°C), and high-speed ultracentrifugation at 230000 g for 3 h, at 4°C. The HCV pellet was resuspended in TNC containing 1 mM PMSF and protease inhibitors (1 : 200). Purified HCV specimens were analyzed by electron microscopy and Western blotting [21, 25].

Anti-HCV antibodies. The study was performed using monoclonal mouse antibodies to HCV proteins E1 (Hep C E1 1879: sc-65459) and E2 (Hep C E2 BDI167: sc-57769) (Santa Cruz Biotechnology, United States), as well as monoclonal antibodies to calnexin (AF18) and calreticulin (FMC75) (Abcam, Great Britain). Polyclonal rabbit antibodies to structural protein C were kindly provided by Dr. M.G. Isagulyants (Ivanovskii Virology Institute, Moscow). Horseradish peroxidase-conjugated anti-mouse IgG antibodies (AB6706-1EA) (Sigma) were used as secondary antibodies.

Western blotting and immunoprecipitation. Sf9 cells were collected 72 h after infection with bv-CE1E2 (20 PFU/cell), washed three times with PBS (1.47 mM KH_2PO_4, 4.29 mM $Na_2HPO_4 \cdot 7H_2O$, 137 mM NaCl, 2.68 mM KCl), resuspended in TNC containing 0.25% digitonin, and destroyed by ultrasound. Cell debris was removed by centrifugation (15000 g, 15 min, 4°C). Cell lysate specimens containing 10 μg protein each were analyzed by PAGE in a 12% gel. Following electrophoresis, proteins were transferred onto a nitrocellulose membrane Hybond–ECL (Amersham Biosciences) by semi-dry electrotransfer. Membranes were washed in PBS containing 5% nonfat milk powder, incubated with primary antibodies to HCV structural proteins E1 and E2 (using 1 : 2000 dilution for E2 and 1 : 1500 dilution for E1), calnexin, and calreticulin (using 1 : 1000 and 1 : 2000 dilutions, respectively), followed by secondary antibodies (1 : 20000 dilution). Immune complexes were detected using chemiluminescent ECL and ECL Plus Western-blotting detection reagents and analysis systems, as recommended by Amersham Biosciences.

For the immunoprecipitation assay, bv-CE1E2-infected cells were collected 72 h after infection and lysed; cell debris and nuclei were removed. Structural proteins and their complexes were precipitated with monoclonal antibodies to HCV proteins E1 and E2, calnexin, and calreticulin (1 : 100 dilution, as recommended by the manufacturer). The precipitated proteins were separated by PAGE in 12% gel, transferred to a nitrocellulose membrane, and incubated with primary antibodies in dilutions indicated above, then treated with secondary antibodies.

Electron microscopy. An aliquot of purified HCV sample (~10 μL) was placed on a freshly ionized carbon collodion support film on a copper grid (150 mesh). After 3–5 min of adsorption, the excess solution was removed with filter paper, and 1.5% water solution of uranyl acetate was applied for 2 min for negative staining; specimens were air dried and studied using a JEM-100CX electron microscope (Jeol, Japan) with an accelerating voltage of 80 kV.

Glycosylation analysis by endoglycosidase H (Endo H) treatment. Cell lysate proteins were incubated with appropriate monoclonal antibodies at 4°C, and the resulting complexes were precipitated using protein-G-sepharose (BioVision, United States). One microliter of tenfold denaturing buffer (5% SDS, 0.4 M DTT) was added to 20 μg of protein precipitate, after which the mixture volume was brought up to 10 μL with water and the probe was boiled for 10 min. Next, the mixture volume was brought to 20 μL by adding 2 μL of tenfold reaction buffer G5 (50 mM Na cit-

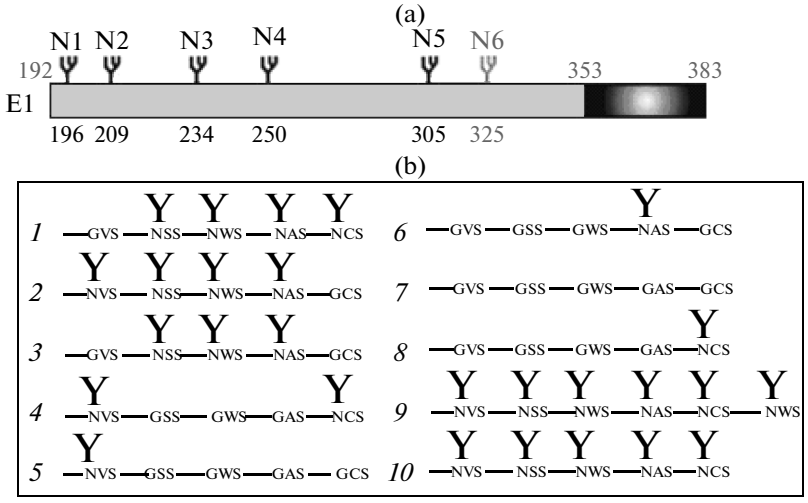

Fig. 1. N-glycosylation sites of HCV structural protein E1 and its mutant variants. (a) positions of glycosylation sites N1–N5 and the additional site N6 in the polypeptide chain of E1. (b) mutant variants of E1 with modified (disrupted) glycosylation sites: (*1*) N1; (*2*) N5; (*3*) N1 and N5; (*4*) N2, N3, and N4; (*5*) N2, N3, N4, and N5; (*6*) N1, N2, N3, and N5; (*7*) N1–N5; (*8*) N1, N2, N3, and N4, (*9*) with an additionally introduced glycosylation site N6; (*10*) wild-type variant of E1. Glycosylation sites are designated with Ys.

rate), 3 μL water, and 5 μL (5 units) of Endo H solution (P0702S BioLabs Inc., Great Britain). The probes were incubated for 15 h at 37°C and analyzed by PAGE in 12% gel.

Fluorescent microscopy and flow cytometry. HEK293T cells were analyzed using a Leica DMI 4000 microscope (United States) 72 h after transfection. Mammalian cells transfected with recombinant plasmids were harvested with trypsin 48 h after transfection, collected by centrifugation, washed twice with PBS, and analyzed in a Beckman Coulter EPICS flow cytometer (United States).

RESULTS AND DISCUSSION

In a previous work, we showed that insect cell cultures support the synthesis of structural HCV proteins, i.e., the core protein C and the envelope proteins E1 and E2 and posttranslational glycosylation of E1 and E2, as well as in vitro assembly and VLP formation [23]. In this study, we investigated the effects of E1 N-glycans on the formation of E1E2 heterodimers and VLP assembly in the model system of insect Sf9 cells transduced with pFastBacHT baculovirus vectors encoding a HCV polyprotein CE1E2 (genotype 1b, strain 274933RU [26]). For this purpose, site-directed mutagenesis in double-stranded plasmid vectors was used to obtain genetic constructs encoding ten different E1 variants with mutations at six glycosylation sites (Fig. 1). The resulting constructs were expressed in insect cells to analyze the efficiency of protein synthesis and the effects of the modification of the glycosylation site on the processing of structural HCV proteins, on gpE1 and gpE2 accumulation and glycosylation in the ER of insect cells, and on glycoprotein interaction with calnexin and calreticulin, as well as on the formation of E1E2 productive complexes and on VLP assembly. E1 expression in mammalian cells was studied using constructs that encode E1 with mutations at five glycosylation sites.

Effect of N-Glycans of HCV Glycoprotein E1 on Expression of Genes of Viral-Envelope Protein in Insect Cells

We found previously that insect cells efficiently support the posttranslational N-glycosylation of HCV envelope proteins, as shown by electrophoretic mobility of HCV proteins E1 and E2 produced in insect cells infected with the bv–E1E2 baculovirus in the presence or absence of tunicamycin, which blocks protein glycosylation [23]. In this study, the efficiency of the glycosylation of mutant E1 variants and the effects of N-glycans of E1 glycoprotein on the synthesis and processing of E1 and E2 were studied in insect cells transduced with baculovirus vectors that drive the expression of structural HCV proteins.

The expression of mutant E1 (E1mut) and wild-type E2 variants in insect cells and the efficiency of glycosylation of E1 variants with mutated glycosylation sites were analyzed by PAGE and immunoblotting with anti-E1 antibodies. The disruption of glycosylation sites in different combinations did not significantly affect the efficiency of E1 synthesis in insect cells, while electrophoretic mobility of mutant proteins increased with a decreasing number of glycosylation sites (Fig. 2a). The introduction of an additional glycosylation site (N6) into the E1 sequence did not

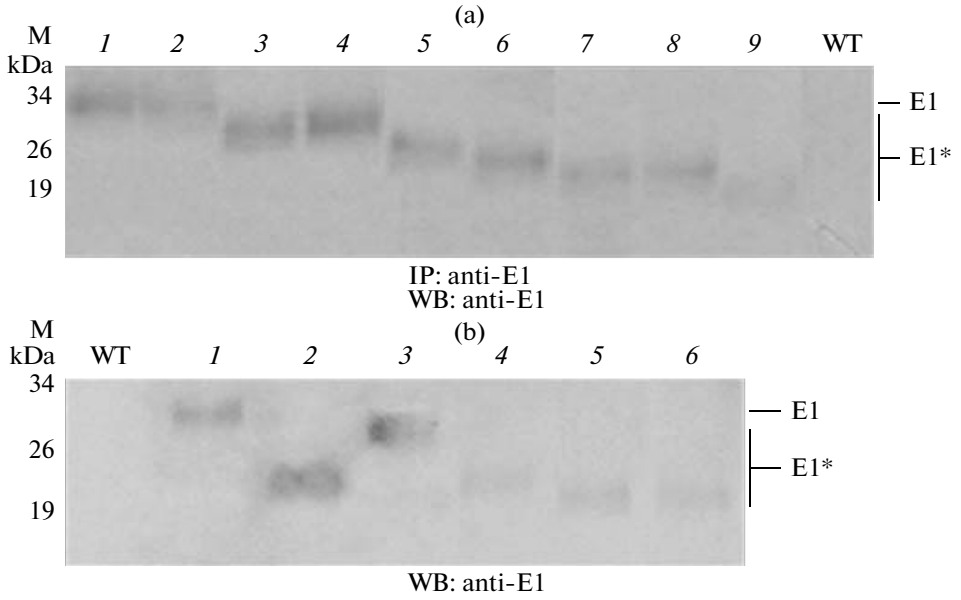

Fig. 2. Synthesis and glycosylation of mutant E1 variants together with the HCV protein E2 in Sf9 insect cells. (a) Western blotting with anti-E1 antibodies following PAGE in a 12% gel after preliminary immunoprecipitation. Cell lysates containing different E1 variants: (*1*) wild-type E1; (*2*) E1 with an additional glycosylation site N6; (*3*) mutation of the glycosylation site N1; (*4*) mutation of N5; (*5*. mutation of N1 and N5; (*6*) mutations of N2, N3, and N4; (*7*) mutations of N1, N2, N3, and N4; (*8*) mutations of N2, N3, N4, and N5; (*9*) mutations of all sites N1–N5. (b) Analysis of mutant E1 glycosylation together with the HCV protein E2 by Western blotting with anti-E1 antibodies. (*1*) wild-type E1; (*2*) wild-type E1 after endoglycosidase H treatment; (*3*) E1 with mutated N1 site; (*4*) E1 with mutated N1 site after endoglycosidase H treatment; (*5*) E1 with mutated N1 site from tunicamycin-treated cells; (*6*) E1 with all five glycosylation sites mutated. WT, wild-type baculovirus vector (negative control). Mutant proteins are designated as E1*. M, molecular weight marker, kDa. IP, immunoprecipitation, WB, western blotting (here and in Fig. 3).

affect the efficiency of E1 synthesis or its electrophoretic mobility. Experiments involving endoglycosidase H (EndoH) treatment of mutant E1 glycoproteins with subsequent Western blotting showed that mutant glycoproteins were glycosylated in insect cells. Mutant E1 variants were sensitive to endoglycosidase, although to a lesser extent than wild-type E1 (Fig. 2b).

Effect of N-Glycans of HCV Glycoprotein E1 on Formation of Productive E1E2 Complexes in Insect Cells

The effects of individual carbohydrate chains present at E1 glycosylation sites on the folding of both E1 and E2 can be analyzed by studying the formation of E1E2 heterodimers and their interaction with cellular chaperons calnexin and calreticulin. It is known that calnexin interacts with glycoproteins involved in the formation of functional complexes, whereas calreticulin interact with incorrectly folded glycoproteins that form aggregates [14–16]. Using antibodies to calnexin and calreticulin, we studied the effects of modifications of E1 glycosylation sites on the formation of functional and nonfunctional E1E2 heterodimers in insect cells. For this purpose, cell lysate proteins were first precipitated with anti-E2 antibodies, then separated by PAGE, transferred onto a nitrocellulose membrane, and detected with antibodies to calnexin (Fig. 3a) or calreticulin (Fig. 3b).

It was found that, in insect cells that express E1 mutant forms that lack any one of the four glycosylation sites (N2, N3, N4, or N6), the E1E2 complex was actually formed, although less efficiently than in those that express wild-type E1. At the same time, with an increasing number of disrupted E1 glycosylation sites, heterodimers interacted less with calnexin and more with calreticulin. Heterodimers that contain E1 with all glycosylation sites mutated, i.e., aggregates of incorrectly folded nonfunctional glycoprotein E1 and E2, interacted with calreticulin. The ability of incorrectly folded E1mut to interact with E2 also weakened, as shown by decreasing E2 quantities detected by coprecipitation with antibodies to calreticulin. Importantly, the formation of productive E1E2 complexes was impaired by the disruption of glycosylation sites N1 or N5. Apparently, the lack of carbohydrate chains at these particular positions prevents the formation of productive E1E2 complexes, and the carbohydrate chains attached at the sites N1 and N5 play the most important role in the correct glycoprotein folding. To sum up the results concerning the formation of an E1E2 complex in insect cells, we conclude that mutations of E1 N-glycosylation sites N2, N3, N4, and N6 have little effect on the assembly of the HCV envelope

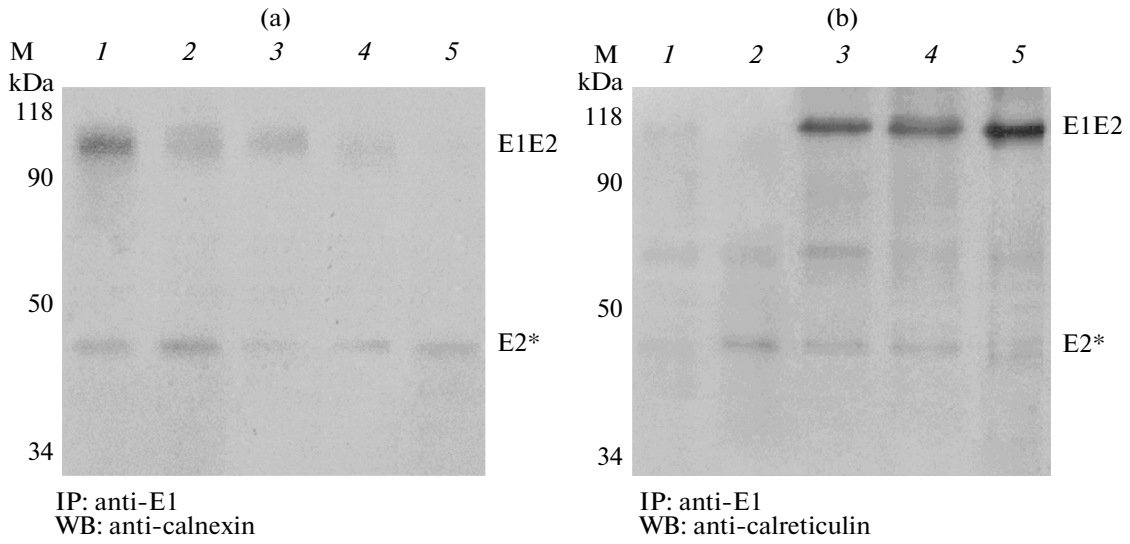

Fig. 3. Western blotting of E1E2 heterodimers produced in insect cells expressing mutant E1 variants with antibodies to calnexin (a) and calreticulin (b) after preliminary precipitation with anti-E2 antibodies. Cell lysates containing different E1 variants: (*1*) wild-type E1; (*2*) E1 with mutated N3 site; (*3*) E1 with mutated N5 site; (*4*) E1 with mutated N1 and N5 sites; (*5*) E1 with all five glycosylation sites mutated. M, molecular weight marker, kDa.

glycoproteins, while mutations of N1 and N5 interfere with this process.

Effect of N-Glycans of HCV Glycoprotein E1 on Formation of HCV Virus-Like Particles in Insect Cells

Previously, we showed that the expression of HCV proteins C, E1, and E2 in insect cells is accompanied by VLP formation [23]. In this study, we analyzed the effect of modification of E1 glycosylation sites on VLP formation in the same model system. For this purpose, microsomes were isolated from insect cells transduced with pFastBacCE1mutE2 vectors and analyzed by PAGE and Western blotting with antibodies to E2 (Fig. 4a) or by electron microscopy (Fig. 4b). The results suggest that mutations of E1 glycosylation sites did not prevent VLP formation in insect cells (Fig. 4); however, as follows from the experiments described above, if the glycosylation sites N1 or N5 were modified, VLPs would probably contain incorrectly folded glycoproteins.

Effect of N-Glycans of HCV Glycoprotein E1 on VLP Formation in Mammalian Cells

Based on the previously developed plasmid vector pFastBacMam1GFP [23], we constructed further vectors to express structural HCV proteins as a CE1E2 polyprotein in mammalian cells. Human HEK293T cells were transfected with pFastBacMam-CE1mutE2GFP vectors that encode wild-type proteins C and E2 and different E1 variants with mutations of glycosylation sites. The expression of mutant E1 forms (E1mut) and the efficiency of their glycosylation in human cells were analyzed by PAGE and subsequent immunoblotting (Fig. 5a).

As is demonstrated by Fig. 5, HEK293T cells synthesized mutant E1 variants, and the electrophoretic mobility of mutant proteins grew with a decreasing number of intact glycosylation sites. As was observed in insect Sf9 cells, the efficiency of E1 glycosylation in HEK293T cells depended on the presence of N-glycans at protein glycosylation sites. However, in contrast to insect cells, the efficiency of E1 synthesis in HEK293T cells also depended on the presence of N-glycans at potential glycosylation sites. We used fluorescent microscopy (data not shown) and flow cytometry (Fig. 5b) to find out whether the modification of E1 glycosylation sites affected the expression efficiency of HCV structural proteins by evaluating the level of CE1mutE2GFP polypeptide. It was shown that the disruption of E1 glycosylation sites N1 or N5 resulted in a considerable decrease in GFP fluorescence, which suggests the downregulation of CE1mutE2GFP synthesis compared to CE1E2GFP. On the other hand, the disruption of E1 glycosylation sites N2, N3, or N4 did not affect the level of CE1mutE2GFP expression.

Thus, we investigated whether the removal of N-glycans at particular positions of E1 affected its folding and the accuracy of the assembly of E1E2 complexes, which are functional envelope subunits of mature virus particles, and showed that the disruption of E1 glycosylation sites in different combinations did not have any significant effect on its expression in insect cells. On the other hand, the formation of func-

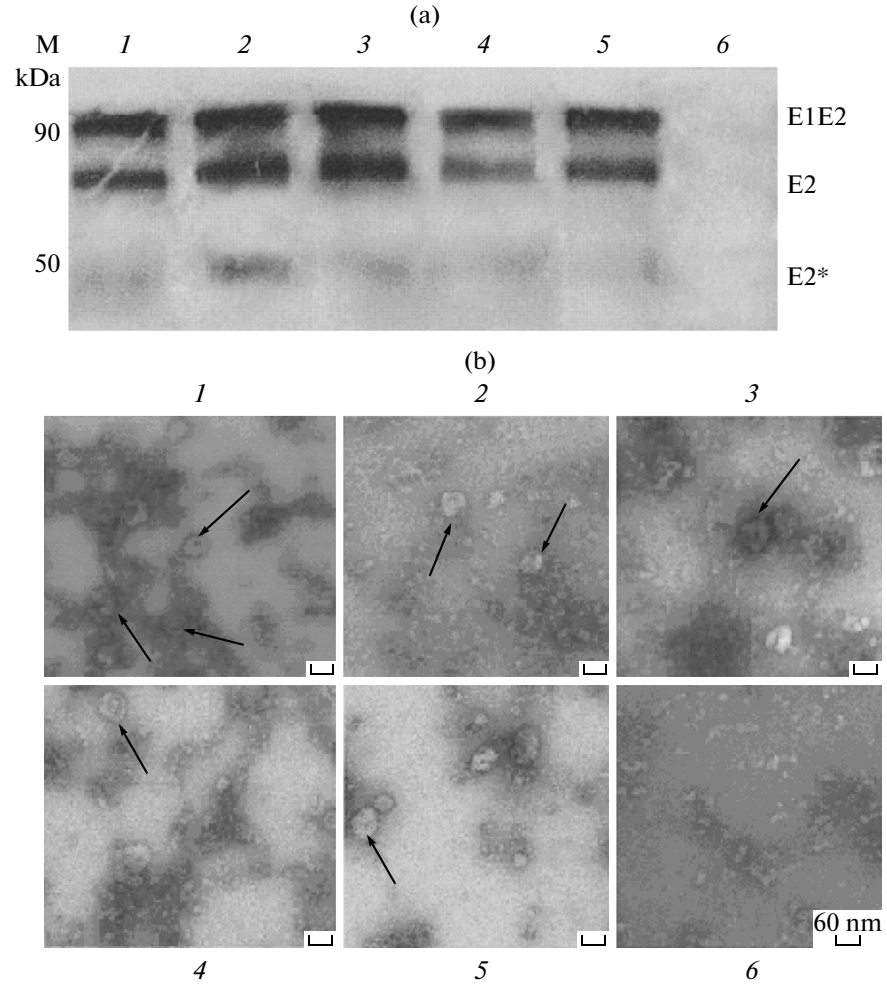

Fig. 4. VLPs produced in insect Sf9 cells expressing HCV structural proteins. (a) Western blotting of structural HCV proteins with anti-E2 monoclonal antibodies; VLPs were isolated from insect Sf9 cells infected with bv-CE1E2 baculoviruses: (*1*) wild-type bv-CE1E2; (*2*) bv-CE1E2 with mutated N1 site; (*3*) mutated N5 site; (*4*) mutated sites N2, N3, and N4; (*5*) all five glycosylation sites mutated; (*6*) bv-CAT/Gus. M is molecular weight marker, kDa. Partially deglycosylated protein is designated as E2*. (b) Electron microscopy of HCV VLPs in the microsomes isolated from Sf9 cells transfected with recombinant baculoviruses. VLPs are shown with arrows.

tional E1E2 heterodimers depends on the presence of N-glycans at E1 sites N1 and N5, and mutated glycoproteins are glycosylated less efficiently than wild-type E1. With decreasing number of intact glycosylation sites, E1mutE2 dimers interact more weakly with calnexin and more strongly with calreticulin. Glycoproteins with all mutated glycosylation sites only interact with calreticulin and formed nonfunctional aggregates.

The presence of structural HCV proteins in the ER and microsome fractions suggests that recombinant HCV proteins produced in insect cells, including mutated E1 variants, are incorporated in ER membranes, where their folding, E1mutE2 complex formation, and VLP formation take place. The presence of E1mut in VLPs produced in Sf9 cells indicates that E1 mutations do not affect VLP formation or yield in these cells. However, the lack of carbohydrate chains at glycosylation sites N1 and N5 results in incorrect protein folding, as well as, apparently, the accumulation of nonproductive E1E2 dimer aggregates in VLPs and, thus, the suppression of productive VPL assembly. The formation of incorrectly folded glycoprotein aggregates leads to the formation of defective virus particles that differ from wild-type virions and probably hampers the release of the virus from the cell and its ability to bind to host cells.

Our results indicate that mammalian cells support the production of mutant E1 glycoproteins included in VLPs. As in insect Sf9 cells, the lack of N-glycans at the sites N2, N3, or N4 does not affect the expression level of structural proteins of VLPs. However, the lack of N-glycans at the sites N1 or N5 resulted in a significant decrease in GFP fluorescence, which character-

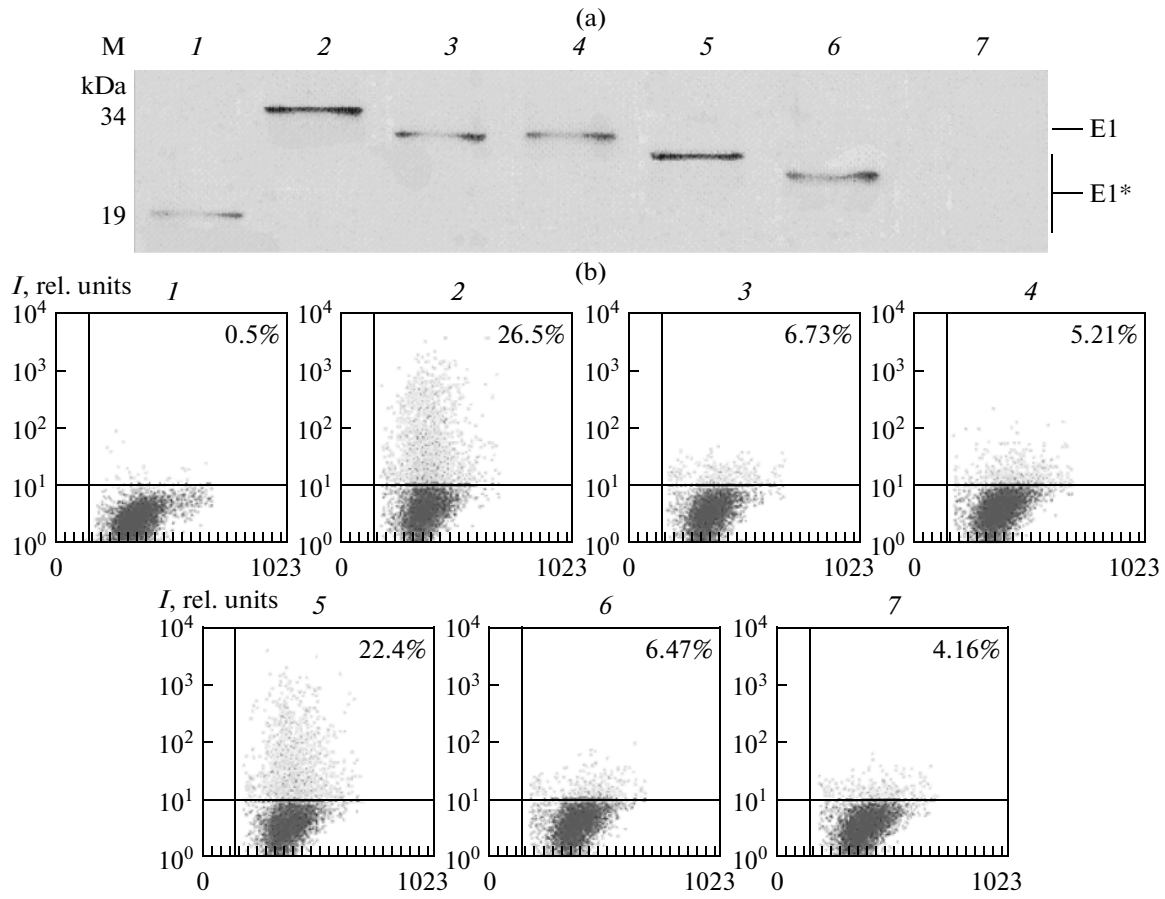

Fig. 5. Expression of mutant E1 proteins in HCV VLPs in HEK293T cells. (a) Western blotting with anti-E1 antibodies following PAGE in a denaturing 12% gel. Cell lysates containing different E1 variants: (*1*) E1 with all five glycosylation sites mutated; (*2*) wild-type E1; (*3*) E1 with mutated N1 site; (*4*) E1 with mutated N5 site; (*5*) E1 with mutated N1 and N5 site; (*6*) E1 with mutated sites N2, N3, and N4; (*7*) HEK293T cell lysate. Mutant proteins are designated as E1*. M, molecular weight marker, kDa. (b) Flow cytometry analysis of green GFP protein fluorescence in HEK293T cells transfected with recombinant pFastBacMam CE1E2GFP plasmid encoding E1 glycoprotein with different mutations. In diagrams, the *x* axis shows the relative linear cell size; *y* axis shows relative fluorescence intensity. HEK293T cells transfected with pFastBacMam CE1E2GFP plasmids encoding different E1 variants: (*1*) HEK293 cells; (*2*) wild-type E1; (*3*) E1 with mutated N1 site; (*4*) E1 with mutated N5 site; (*5*) E1 with mutated sites N2, N3, and N4; (*6*) E1 with mutated N1 and N5 sites; (*7*) E1 with all five glycosylation sites mutated.

izes the level of VLP protein synthesis. Therefore, in contrast to insect Sf9 cells, the efficiency of VLP protein expression in HEK293T cells depends significantly on the presence of N-glycans at the E1 glycosylation sites N1 and N5. These results agree with similar data on the role of N-glycans obtained in other modern expression systems [27, 28].

The further investigation of the effects of N-glycosylation of E1 and E2 envelope proteins on the formation of E1E2 complexes and VLP assembly in model systems of insect and mammalian cells should make it possible to specify the role of posttranslational modifications of structural HCV proteins in the virus life cycle and can provide a basis for the development of new approaches to antiviral therapy.

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