A New Determinant of Endoplasmic Reticulum Localization Is Contained in the Juxtamembrane Region of the Ectodomain of Hepatitis C Virus Glycoprotein E1*

Received for publication, December 28, 1999, and in revised form, April 11, 2000 Published, JBC Papers in Press, April 26, 2000, DOI 10.1074/jbc.M910400199

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Hepatitis C virus glycoproteins E1 and E2 do not reach the plasma membrane of the cell but accumulate intracellularly, mostly in the endoplasmic reticulum. Previous studies based on transient expression assays have shown that the transmembrane domains of both glycoproteins are sufficient to localize reporter proteins in the endoplasmic reticulum and that other localization signals may be contained in the ectodomain of E1 protein. To identify such signals we generated chimeric proteins between E1 and two reporter proteins, the human CD8 glycoprotein and the human alkaline phosphatase, and analyzed their subcellular localization in stable as well as transient transfectants. Our results showed that (i) an independent localization determinant for the endoplasmic reticulum is present in the juxtamembrane region of the ectodomain of E1 protein and (ii) the localization dictated by this determinant is either due to direct retention or to a recycling mechanism from the intermediate compartment/cis-Golgi complex region, which is clearly different from those previously described for other retrieval signals. These results show for the first time in mammalian cells that the localization in the endoplasmic reticulum of transmembrane protein can be determined by specific targeting signals acting in the lumen of the compartment.

HCV is a major agent of chronic hepatitis and liver diseases in humans throughout the world. It is classified in the Flaviviridae family because of its similarity in genomic organization to flavivirus and pestivirus. The genome, a single positive-strand RNA, is translated into a polyprotein of about 3,000 amino acid residues, which is processed by host and viral proteases to generate at least 10 polypeptides (reviewed in Ref. 1). Little is known on HCV replication and assembly because the virus does not infect laboratory animals and tissue-cultured cells. Only recently Lohmann et al. (2) succeeded in designing a self-replicating subgenomic viral RNA, and to date biogenesis of HCV proteins has been studied only by cell-free transcription/translation and transfection assays. These experiments show that co-translational cleavages in the N-terminal region of HCV nascent chain release the two putative envelope viral glycoproteins, E1 and E2 (3). E1 and E2 are intrinsic membrane proteins with an N-terminal ectodomain that is heavily N-glycosylated and a C-terminal hydrophobic TMD, whose precise length has not been established. When expressed in tissue culture cells, E1 and E2 interact with each other and with resident chaperones forming two types of complexes: heterogeneous aggregates containing E1 and E2, possibly associated by intermolecular disulfide bonds, as well as calreticulin and calnexin and a non-covalent heterodimer presumably representing the correct folding state that precedes the formation of the viral envelope (4–6). Independently of their oligomeric status, E1 and E2 localize predominantly in the ER and are not transported to the cell surface (4, 6–8). This localization suggests that HCV budding occurs entirely on intracellular membranes, thus helping HCV to minimize the host immune surveillance and determine persistent infection.

Mutational analysis in transfected cells allowed the identification of two independent ER retention signals in the TMDs of E1 and E2 glycoproteins; replacement of the TMD of human CD4 protein with the C-terminal hydrophobic region of E1 or E2 resulted in the ER localization of the chimeric protein (9–11). However, removal of the E1 TMD or its substitution with the TMD and the cytosolic domain of CD8 still resulted in localization in the ER (9, 12). Conversely, E2 deleted of the TMD or with a different TMD was secreted or located on the plasma membrane, respectively (10, 12). Thus, it is likely that other determinant(s) for ER localization is contained in the luminal portion of E1 protein. On this line, E1 deleted of the TMD and of the juxtamembrane portion of the ectodomain was secreted (12). However, the native, correctly folded configuration of E1 and E2 glycoproteins is still unknown; thus, evaluating whether ER localization of recombinant forms of these proteins is determined by a specific signal or by misfolding is a constant problem.

To overcome this problem, we decided to search for localization signals in E1 glycoprotein by fusing part of it to the human glycoprotein CD8 or to the human alkaline phosphatase (AP) (13). CD8 is a class I transmembrane protein that is quickly
transported to the cell surface and has been extensively used as a reporter protein to study localization signals (14–17). Maturation of its O-linked oligosaccharides has been thoroughly characterized (18, 19), and it has been established that sugar modifications mark transport in different compartments of the secretory pathway. Finally, in contrast to HCV E1, folding of CD8 can be monitored by using conformation-sensitive antibodies and by the formation of disulfide-linked homodimers. Human placental AP is a glycoprotein anchored to the cell surface via a phosphatidylinositol-glycan moiety (20, 21). Several laboratories have shown that the catalytic domain of this enzyme retains its activity when fused to different types of TMDs (22, 23). Thus, the use of this protein as a reporter offers the possibility of very easily testing the folding state of the chimeric proteins by measuring its enzymatic activity. We have constructed several chimeric proteins between these two reporter proteins and the C-terminal region of E1 protein and expressed them either stably in rat thyroid cells (FRT) or transiently in human hepatoma cells (HuH-7). Analysis of the intracellular localization of these chimeras indicated the presence of a new localization determinant in the viral glycoprotein.

**EXPERIMENTAL PROCEDURES**

Materials—All culture reagents were supplied by Sigma-Aldrich. Solid chemicals and liquid reagents were obtained from Merck, Farmitalia Carlo Erba (Milano, Italy), Serva Feinbiochemics (Heidelberg, Germany), BDH. All radiochemicals were obtained from NEN Life Science Products. Protein A-Sepharose CL-4B and the ECL kit were from Amersham Pharmacia Biotech. Endoglicosidase H and neuraminidase enzymes and all enzymes used in molecular cloning were obtained from Roche Molecular Biochemicals, New England BioLabs (distributed by Microgels, Napoli, Italy), or Promega Italia Srl (Milano, Italy). Oligonucleotides were synthesized by Primm Srl (Milano, Italy). The following antibodies were used: mouse mAb OKT8 (anti-CD8 protein), mouse mAb N1 (anti-CD8 protein), rabbit polyclonal anti-CD8 and rabbit polyclonal anti-SSα (16); mouse mAb G10-1 (anti-CD8 protein) (24); rabbit polyclonal anti-calnexin (17); rabbit polyclonal anti-calcitulin (Stress-Gen, Canada); rabbit polyclonal anti-alkaline phosphatase (Rockland, Gilbertsville, PA); mouse mAb anti-pleural alkaline phosphatase (Chemicon International, Inc., Temecula, CA); peroxidase-conjugated anti-mouse and anti-rabbit IgG (Sigma-Aldrich); Texas Red-conjugated anti-mouse IgG and fluorescein-conjugated anti-rabbit IgG (Jackson ImmunoResearch Lab, West Grove, PA).

Construction of Chimeric Plasmids and cDNA Fragments—cDNA fragments were cloned in the desired expression vector by standard DNA protocols or by PCR amplification of the area of interest using synthetic oligonucleotides with the appropriate restriction sites. PCR amplification was performed according to the procedure of Barnes (25) to minimize the possibility of unwanted second-site mutations. Site-directed mutagenesis was carried out by inserting the mutations in the PCR primers. The expression vector (plasmid CD8) was a plasmid containing the human CD8a cDNA downstream of the promoter and the enhancer from the Friend murine leukemia virus (26). This plasmid was mutated in order to contain a SpI site and a ClaI site between amino acids 160 and 161 of CD8 protein, where all HCV or SV fragments were cloned. Plasmid CD8-E199–142 contains HCV sequence from nucleotide 1200 to nucleotide 1568 of the HCV BK strain (27) corresponding to residues 99–112 of E1 protein. All the other HCV sequences were amplified from plasmid CD8-E199–129. Plasmid CD8-E199–155 contains HCV fragment encoding residues 155–192 of E1 protein, followed by a stop codon. HCV cDNA fragments contained in the plasmids CD8-E199–154, CD8-E199–145, and CD8-E199–142 encode, respectively, residues 99–154, 99–148, and 99–142 of the E1 protein. Plasmid CD8-SV5-SV differs from CD8-E199–142 that the HCV fragment was amplified with primers substituted with HCV nucleotides ATGATGATG, coding for methionines 131–133, into AGCAGCAGC, coding for three serines.

Plasmids expressing the AP chimeric proteins were prepared from the pEGFP-C1 plasmid (Clontech) by substituting the CD8 ectodomain sequence with AP sequence in two steps. First, plasmids CD8, CD8-SV, CD8-E199–142 and CD8-E199–155 were digested with BglII (unique site in the promoter region) and SpII to remove only the CD8 ectodomain. Then they were ligated with a fragment BglII/SpII amplified by PCR from promoter region in plasmid CD8 and contained a mutagenized unique site for HindIII. The ligated plasmids were again digested with HindIII and ligated to the soluble AP cDNA fragment HindIII/SpII1 derived by PCR from the plasmid pBC12/RSV/SEAP in which the stop codon in position 1529–31 of cDNA sequence was removed (13). All plasmids were verified by DNA sequencing.

**Cell Culture and Transfection—**Parental FRT cells were cultured as described previously (19) and transiently transfected with Lipofectin reagent (Life Technologies, Inc.) according to manufacturer’s instructions. Stable transformants were obtained by co-transfecting cells with recombinant plasmids and the plasmid PSV2neo, selected in the presence of G418 (Life Technologies, Inc.) and screened by indirect immunofluorescence. HuH-7 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and transfected by using FuGENE6 transfection reagent (Roche Molecular Biochemicals) according to the manufacturer’s instructions. 36 h after transfection, cells were analyzed by indirect immunofluorescence and Western blotting.

**Indirect Immunofluorescence—**Cells grown on glass coverslips were fixed with 4% formaldehyde for 20 min at room temperature and made permeable with 0.1% Triton X-100 in phosphate-buffered saline. Cells were labeled with the appropriate antibodies and with fluorescein and Texas Red-conjugated secondary antibodies. To separately stain the cell surface and the intracellular membranes, after fixation cells were incubated with a specific polyclonal antibody, then permeabilized and incubated with a specific monoclonal antibody. Coverslips were mounted in Mowiol and viewed by epifluorescence on a Zeiss axiostar photomicroscope with a 100× planar objective.

**Metabolic Labeling, Preparation of Cells Extracts, Immunoprecipitation, SDS-PAGE, and Western Blotting—**Stably transfected cells were allowed to grow to subconfluence and then were manipulated for [35S]cysteine, [3H]GlcN, or [3H]Man labeling, as performed previously (19). Cells were lysed with 20 mM Tris-HCl, pH 7.4, 1% SDS, 20 mM EDTA, 100 mM sodium chloride; total extracts were passed through a syringe needle, and aliquots were then used for immunoprecipitation, SDS-PAGE, and Western blotting as detailed previously (28, 29). For immunoprecipitation with the conformation-sensitive antibodies, OKT8 and G10-1, cells were lysed with 20 mM Tris-HCl, pH 7.4, 1% Triton X-100, 20 mM N-ethylmaleimide. To calculate the half-life times, the amounts of protein were determined by scanning densitometry of the Coomassie blue-stained gel developed after autoradiography. Mean values of four experiments were considered.

**Endoglicosidase H (Endo H) and Neuraminidase Digestions—**Treatment with Endo H and neuraminidase were performed as indicated in the manufacturer’s instructions.

**Analysis of [3H]GlcN- and [3H]Man-labeled Glycans—**All analysis were performed on CD8-derived proteins immunoprecipitated after 16 h of labeling with [3H]GlcN or [3H]Man and subjected to SDS-PAGE. Areas of polycrylamide gels corresponding to bands visualized by fluorography were excised and digested overnight with Pronase at 60°C. The glycopeptides isolated by Bio-Gel P-4 filtration (400 mesh) column (1 × 75 cm) were analyzed for the radioactivity amino-sugar composition as described previously (19). N-Glycans generated by Endo H treatment were isolated by Bio-Gel P-4 filtration and analyzed by HPLC as described (30).

**Assay for Alkaline Phosphatase Activity—**Transfected cells were lysed with 20 mM Tris-HCl, pH 9.8, 100 mM NaCl, 1 mM MgCl2, Triton X-100 0.5%. Aliquots of cell lysates were assayed for alkaline phosphatase activity as described previously (31). The increase in light absorption at 405 nm was measured after 20 min of incubation with the specific substrate p-nitrophenyl phosphate. The amounts of alkaline phosphatase chimeras were determined as arbitrary units by scanning densitometry of the Western blot film used by the NIH Image program. The specific activities were calculated dividing the absorbance units for the protein amount measured.

**RESULTS**

**The C-terminal Region of Glycoprotein E1 Determines Localization of the Reporter Protein CD8 in the Endoplasmic Reticulum—**It was previously reported that E1 with the C-terminal 72 residues deleted was efficiently secreted (12); thus we focused on this portion of the protein and fused it to the C terminus of the CD8 ectodomain (Fig. 1A). For cloning conven-

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Endo H digests N-glycans of high mannose type, and resistance to Endo H digestion indicates that a glycoprotein has moved from the ER to the medial- and trans-Golgi, where high mannose chains are converted to complex type chains. Neuraminidase removes sialic acid residues from both N- and O-glycans. These residues are added in the trans-/trans-Golgi network; thus, sensitivity to neuraminidase indicates passage of a glycoprotein through these compartments. In agreement with previous studies (19), wild type CD8 was almost completely or completely converted into a mature, neuraminidase-sensitive form after 1 or 4 h of chase (Fig. 3). As expected, CD8 was always resistant to Endo H digestion. CD8-E199–192 was mostly present as a 33-kDa band that did not show any modification in its electrophoretic mobility over time. At all time points analyzed, this protein was completely sensitive to Endo H and resistant to neuraminidase digestion, indicating the absence of complex type N-glycans and sialic acid residues, respectively. These modifications suggested an incomplete maturation and a premedial-Golgi location of CD8-E199–192 at steady state.

To define more precisely the intracellular distribution of CD8-E199–192, we characterized its carbohydrate chains by gel filtration and HPLC. Fig. 4 shows that the [3H]GlcN-labeled Pronase glycopeptides from CD8-E199–192 contained GlcNAc but not GalNAc, indicating the presence of N-glycans and the absence of O-glycans (Fig. 4, panel a). In addition, the gel filtration of [3H]Man-labeled glycopeptides revealed that they were entirely Endo H-sensitive (Fig. 4, panel b), and HPLC analysis of oligosaccharides generated by Endo H showed the presence of two major species Man$_6$GlcNAc and Man$_7$GlcNAc (Fig. 4, panel c). These results suggested that the N-glycans were modified only by the ER glucosidases I and II and partially by the ER mannosidase (33). Taken together, these data indicate that CD8-E199–192 does not receive Golgi complex modifications because it is modified at the N-glycans only by ER resident enzymes and does not carry O-glycans.

Two ER Localization Determinants Are Contained in the C-terminal Region of E1 Glycoprotein—Next we analyzed the subcellular localization of a chimeric protein containing the CD8 ectodomain fused to the hydrophobic tail of E1 (Fig. 1A, CD8-E1$_{155–192}$). Immunofluorescence analysis of stable transfectants expressing CD8-E1$_{155–192}$ showed also that this chimeric protein was not expressed on the cell surface but was localized intracellularly (Fig. 5, a–b), in close association with the ER membrane as indicated by co-localization with the ER marker protein calnexin (Fig. 5, a–b). The level of expression of CD8-E1$_{155–192}$ in the stably transfected FRT cells was low, as reported for the chimeric form of CD4 containing the TMD of E2 (10). For this reason, we could not perform a biochemical analysis to study the glycan processing and the maturation of the protein. A possible explanation for this finding is that the TMD of E1 protein has a toxic effect on the cells. Interestingly, rapid cell lysis, probably due to permeability changes of the inner membrane, has been recently documented in prokaryotic cells expressing this region (34). These results were entirely consistent with the previously described identification of an ER retention signal in the TMD domain of E1 (9).

To assess the presence of additional ER localization signal(s) in the luminal portion of E1 protein, we prepared three additional constructs containing amino acids 99–154, 99–148, and 99–142 of E1 inserted between the ectodomain and the TMD of CD8 (see Fig. 1A). By immunofluorescence analysis of transiently transfected cells, we observed that chimeric proteins encoded by these three constructs were also localized intracellularly and could not be detected on the cell surface (data not shown). Thus, we focused on the construct containing the shortest fragment (Fig. 1A, CD8-E1$_{199–142}$) and analyzed its intracel-
lular localization in stably transfected cells in comparison to an ER marker protein (Fig. 6). The immunofluorescence results showed that CD8-E199–142 displayed the same subcellular localization observed for CD8-E199–192 and CD8-E1155–192, thus indicating that the 44 lumenal amino acids of E1, independently of the transmembrane domain, determine localization of CD8 protein in the ER.

Then we analyzed glycosylation of CD8-E199–142. Pulse-chase experiments revealed that CD8-E199–142 was present mostly as a 34-kDa band, completely sensitive to Endo H and resistant to neuraminidase, which did not change electrophoretic mobility during the chase (Fig. 7A); a faster migrating band, which displayed a similar sensitivity to glycosidases, appeared in variable amounts in different experiments. This molecular form was not recognized by an antibody against the CD8 tail (data not shown) and could be due to an endoproteolytic cleavage(s) of unknown nature, resulting in the removal of the C-terminal tail. The analysis by gel filtration of the [3H]GlcN-labeled glycan moiety confirmed the data obtained with CD8-E199–192 (Fig. 7B). Also CD8-E199–142 did not contain GalNac but only N-glycans of complex type, indicating the absence of Golgi-enzyme modifications.

**ER Localization of CD8-E199–142 Protein Is Not Due to Misfolding**—Protein misfolding in the lumen of the ER prevents export toward the Golgi complex (35). To rule out the possibility that CD8-E199–142 was retained in the ER by quality control mechanisms recognizing misfolded proteins, we performed different experiments. First, we analyzed the folding status of this chimeric protein by using two different conformation-sensitive antibodies, OKT8 and G10-1, and by assessing the formation of disulfide-linked homodimers (18). Since both antibodies recognized human CD8 in immunofluorescence assays (16, 24), we tested whether they were also able to immunoprecipitate CD8-E199–142. Indeed, CD8-E199–142 was immunoprecipitated by the two conformation-sensitive antibodies as efficiently as the wild type CD8 (Fig. 8A). In addition, comparison of the electrophoretic mobility of the chimeric protein in reducing and non-reducing conditions revealed that, similarly to wild type CD8, CD8-E199–142 molecules were mostly associated in disulfide-linked homodimers (Fig. 8B) and did not form disulfide-linked aggregates (data not shown). Next we tested if CD8-E199–142 was bound to ER chaperones. The ER chaperones calnexin and calreticulin are involved in the quality control of several N-glycoproteins, including HCV E1 and E2 (36, 37). Both chaperones associate with glucosylated N-glycans bearing proteins transiently (if productive folding intermediates) or stably (if misfolded or incompletely folded), causing their ER retention. Thus we performed co-immunoprecipitation experiments of pulse-chase-labeled proteins. As already observed by Tatu and Helenius (38), several high molecular weight proteins were co-immunoprecipitated from cell lysates and mock-treated (-) or treated with Endo H (H) or neuraminidase (N). Samples were analyzed by SDS-PAGE, followed by fluorography. Numbers on the right and on the left indicate the positions on the same gel of a marker protein. Only the relevant portion of the gel is shown. Note that the stability of CD8 protein over time was not observed in other pulse-chase experiments.
The intracellular distribution of CD8-E199–142 and CD8-SV did not vary, but a much higher transfection efficiency was observed, in place of the HCV E1 insert, 44 amino acids derived from the juxtamembrane portion of the SV E1 glycoprotein (Fig. 1A, CD8-SV). SV E1 glycoprotein has the same membrane topology of HCV E1 and CD8 proteins and is quickly transported to the plasma membrane (28). Therefore, we expected CD8-SV to be transported to the cell surface unless misfolded because of the SV E1 insertion. As shown in Fig. 6, unlike CD8-E199–142, CD8-SV was clearly expressed on the cell surface, although less efficiently than wild type CD8. This result was confirmed by the finding that newly synthesized CD8-SV was converted to a mature, terminally glycosylated form (see below).

Taken together, these results strongly support that ER localization of CD8-E199–142 was not due to misfolding and quality control problems. However, although very unlikely, ER localization could be the result of an extremely fast degradation rate of a properly folded protein, so as to prevent its export. Thus we performed pulse/chase-labeling and immunoprecipitation experiments to determine the half-lives of CD8, CD8-E199–142, and CD8-SV. By quantitative analysis of the autoradiogram (data not shown) we calculated a half-life of 8 h for CD8 and CD8-SV and 5 h for CD8-E199–142. Albeit slightly shorter than that of CD8, the half-life of CD8-E199–142 was more than 10-fold longer than the half-time of surface expression of wild type CD8 (18), indicating that ER localization was not the consequence of a faster degradation. Conversely, it is conceivable that the accelerated turnover of CD8-E199–142 is the result of its localization in the ER.

Terminal Glycosylation and Transport to the Plasma Membrane of Mutagenized CD8-E199–142—To further verify the specificity of the localization determinant present within residues 99–142 of HCV E1, we substituted different residues by site-directed mutagenesis and analyzed the intracellular localization of each mutant in transiently transfected HuH-7 cells. The intracellular distribution of CD8-E199–142 and CD8-SV did not vary, but a much higher transfection efficiency was obtained in HuH-7 with respect to FRT cells. Replacement with serine at three consecutive methionine residues, positions 131–133, affected subcellular localization (Fig. 1, A and B, CD8-E199–142M). As shown in Fig. 9, CD8-E199–142M protein was clearly expressed on the surface of HuH-7 cells as the control CD8-SV protein, whereas CD8-E199–142 protein was completely intracellular. Next, we performed a biochemical analysis of the chimeric proteins expressed in HuH-7 cells. To obtain results more directly comparable with the immunofluorescence data, we looked at the total population of the chimeric proteins by Western blotting. This approach showed that also in HuH-7 cells, CD8-E199–142 protein migrated as a single band of about 34 kDa, sensitive to Endo H and resistant to neuraminidase. Conversely, CD8-E199–142M protein was present in two forms of 34 and 37 kDa (Fig. 10, see also Fig. 7). The 34-kDa form responded to the glycosidases digestions as the 34-kDa form of CD8-E199–142, whereas the 37-kDa form was sensitive to

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Retention time (min)

Fractions

Fractions

CD8-E199–142 protein has only high mannose N-linked oligosaccharides. CD8-E199–142 protein was immunoprecipitated from stably expressing FRT cells labeled for 16 h with [3H]GlcN or [3H]Man and visualized by SDS-PAGE, followed by fluorography. The radioactive band was excised from the gel, digested with Pronase, and subjected to Bio-Gel P-4 filtration as indicated under “Experimental Procedures.” a, elution pattern of the glycopeptides labeled with [3H]GlcN. Arrow 1 indicates the void volume, where O-linked oligosaccharides of wild type CD8 and N-glycans of complex type are eluted. Arrow 2 indicates the elution volume of high mannose glycopeptides from Tamm-Horsfall glycoprotein. The histogram on top of the panel shows the percent radioactivity recovered as GlcN and GalN in the pooled fractions 19–26 according to the procedure previously described (19). b, analysis of [3H]Man-labeled oligomannosides from CD8-E199–142. Bio-Gel P-4 filtration of 19–26 pooled fractions of panel a before (solid line) and after (dashed line) Endo H treatment. Arrows 1 and 2 are as in the panel a, and arrow 3 shows the elution volume of high mannose oligosaccharides from Tamm-Horsfall glycoprotein (released by Endo H). c, HPLC analysis of the oligomannosides of the peak fraction marked by arrow 3 in panel b. Arrows on the top indicate the retention times of marker oligosaccharides. Arrow 9, ManoGlcNAc; arrow 8, ManoGlcNAc; arrow 7, ManoGlcNAc; arrow 6, ManoGlcNAc; arrow 5, ManoGlcNAc; arrow 4, ManoGlcNAc; arrow 3, ManoGlcNAc; arrow 2, ManoGlcNAc; arrow 1, ManoGlcNAc; arrow 0, GlcNAc.
neuraminidase and largely resistant to Endo H (Fig. 10). As expected, CD8-SV also showed two bands of 30 and 33 kDa. The 33-kDa form was sensitive to neuraminidase, whereas none was sensitive to Endo H since no N-glycosylation sites are present in the SV E1 fragment (Fig. 10). The most likely interpretation of these results is that the newly synthesised forms of CD8-E1$_{99-142}$M (34 kDa) and CD8-SV (30 kDa) proteins are exported to the Golgi complex, where they are converted to the slower migrating, terminally glycosylated forms and eventually transported to the plasma membrane. These findings were confirmed by pulse-chase labeling experiments (data not shown). In conclusion, since it is extremely unlikely that the mutagenesis of three residues could cause refolding of an otherwise unfolded protein, these results provide further evidence that the ER localization was not due to misfolding of the 44 residues sequence and demonstrate that at least one of three methionines residues in positions 131–133 of HCV E1 is essential for the function of the localization determinant.

The 44 Lumenal Amino Acids of E1 Determine Intracellular Localization of Another Reporter Protein—To investigate whether the E1 determinant functioned as a localization signal in another context, we constructed additional chimeric proteins containing the catalytic domain of AP (13) instead of the ectodomain of CD8 (Fig. 11). The chimeric protein comprising the extracellular domain of AP fused to the TMD and cytoplasmic tail of CD8 (AP8, Fig. 12) was expressed on the plasma membrane of transiently transfected HuH-7 cells. The chimeric protein containing the SV E1 44 amino acids fragment between the AP and CD8 domains was also localized on the cell surface (AP8-SV, Fig. 12). On the contrary, the chimeric protein AP8-E1$_{99-142}$ containing the HCV E1 insert was localized exclusively in intracellular membranes (Fig. 12). However, also in this case, mutagenesis of the three methionines resulted in transport to the cell surface (AP8-E1$_{99-142}$M, Fig. 12). All chimeric forms of AP were detected by Western blot analysis (data not shown), and most importantly, all were enzymatically active; the specific activity ranged from 14.7 for AP8-SV to 8 for AP8-SV and AP8-E1$_{99-142}$M and 6.3 for AP8-E1$_{99-142}$ (see “Experimental Procedures” for details). Thus, the insertion of 44 residues (independently from their sequence) between the AP catalytic domain and CD8 moiety resulted only in a partial decrease of enzymatic activity, with no obvious correlation with the intracellular localization of the chimeric constructs. In conclusion, all together these data represent further evidence in favor of the role of the E1 lumenal region as a specific determinant for ER localization.
DISCUSSION

To understand the molecular mechanism that prevents the export to the cell surface of the HCV E1 glycoprotein, we have investigated the intracellular localization of chimeric proteins containing different domains of E1 and demonstrated that, in addition to the already described determinant present in the TMD region, a different localization determinant is present in the juxtamembrane region of the ectodomain of E1 protein. Both determinants independently are able to localize the reporter protein mostly in the ER. The ER localization dictated by the lumenal determinant is either due to direct retention or to a recycling mechanism that is clearly different from the ones previously described for proteins bearing KDEL, KKXX, or TMD retrieval signals.

Several lines of evidence support the conclusion that the HCV E1 sequence contained in CD8-E199–142 represents a novel type of ER localization determinant. The immunofluorescence results, which show a clear ER pattern of labeling, and the characterization of the oligosaccharide chains, which indicates complete absence of Golgi processing, unequivocally demonstrate that CD8-E199–142 is localized almost exclusively in the ER. The proper folding of the chimeric proteins, suggested by the reactivity to conformation-specific mAbs, by the correct dimerization and by the absence of association with ER chaperones, indicates that localization in the ER is not due to the quality control mechanisms operating in this compartment. The finding that the same HCV E1 region determines ER localization of another reporter, AP, without affecting its enzymatic activity strongly supports the result obtained with CD8. Finally, the different subcellular localization of the control constructs CD8-SV and AP8-SV, together with the loss of ER localization after a limited mutagenesis of the HCV E1 determinant, reinforces the notion of a specific signal.

The new localization determinant of HCV E1 protein has two important features. (i) It is present in the ectodomain of an intrinsic membrane protein, and thus, it has to perform its function in the lumen of the secretory apparatus, and (ii) it apparently operates independently of the quality control mechanisms that prevent export from the ER of misfolded proteins. Both findings were unexpected, given the evidence available in the literature. The KKXX and RRXX motifs are currently the best characterized examples of localization signals of transmembrane proteins. Both are exposed on the cytosolic side of
the membrane (15, 40), and it has been documented that the KKXX motif operates by directly binding to COP-I cytosolic proteins (41). In addition, there are several documented examples of ER localization mediated by TMDs. In some cases TMDs are both necessary and sufficient to induce ER localization and function by a recycling mechanism involving the Golgi complex receptor Rer1p (42–43). In other cases, TMDs cooperate with cytosolic or lumenal domains to specify ER localization. For example, the TMD and the lumenal pentapeptide EGHRG of the asialoglycoprotein receptor H2a form a complex determinant for ER localization by virtue of the quality control mechanism (44, 45). To our knowledge, the only transmembrane protein with an ER localization motif in the lumenal domain is the yeast Sec20 protein (46, 47). This protein is a class II transmembrane protein and contains the HDEL sequence at its C terminus. This motif (KDEL in higher eukaryotes) is the prototype for ER localization signals of “soluble” proteins present in the ER lumen, like BiP, protein disulfide isomerase (PDI), and many others (48) and determines a retrieval process mediated by a receptor located in the Golgi complex (49, 50). The function of the HDEL motif in the case of the Sec20 protein remains to be established. Anyhow, the general rule is that ER localization determinants of transmembrane proteins are located either in the cytosolic or in the TMD regions but not in the lumenal ectodomain. Therefore, a challenging task will be to understand the mechanism of action of the localization determinant present in the lumenal domain of HCV E1 protein.

According to our data, HCV E1 protein has two localization determinants, which apparently operate in an independent fashion. Other membrane proteins have been shown to contain multiple signals for localization. The targeting of ERGIC-53 protein to the early secretory pathway is a result of retention and retrieval mechanisms modulated by several co-operating signals (51); rabbit cytochrome P450 C1 and 2C2 contain signals for ER localization in the transmembrane and the C-terminal cytosolic domains (52); rubella virus E1 glycoprotein has a complex ER localization signal that requires both the transmembrane and the cytosolic regions of the protein (53). Interestingly, it has been proposed that rubella E1 protein would control virus assembly by its slow folding and transport rate to the Golgi complex, the site of virus budding (53). HCV E1 protein might play a similar role in virus assembly because it folds slowly, representing the rate-limiting step in the formation of E1-E2 complexes (8), and most likely has a major role.
in controlling the intracellular localization of the envelope proteins. Current opinion is that ER residency of proteins that have successfully undergone the quality control process is achieved by two, different but potentially overlapping, mechanisms (reviewed in Ref. 54): dynamic recycling, in which proteins are continuously retrieved from post-ER compartments, and direct retention, in which proteins are excluded from transport vesicles because they are probably bound to large heterodimeric complexes with other resident proteins. The last mechanism has been frequently hypothesized, but little direct evidence is available in the literature (55, 56); indirectly, it relies mostly on the large mass of data showing retention in the ER of newly synthesized proteins during the quality control process (35). Conversely, dynamic recycling from the Golgi complex has been demonstrated in many instances; the KDEL receptor, which is normally located in the Golgi complex, moves to the ER when a vast excess of KDEL-bearing proteins is transiently expressed by the cell. Modifications of the oligosaccharides chains due to enzymes localized in the Golgi complex have been shown in recombinant reporter proteins localized in the ER (48). Extending the rationale of this approach, the absence or the presence of Golgi-type modifications in the oligosaccharides chains of ER resident proteins has been used as a direct criteria to discriminate between retention and retrieval mechanisms. For example, direct retention has been claimed for the TMD-dependent ER localization of HCV glycoproteins and of chimeric CD4 proteins because of the absence of mannose residue-trimming of the N-linked glycans (9, 10, 57). However, the Golgi mannosidase responsible for the trimming from Man$_9$-GlcNAc$_2$ to Man$_6$-GlcNAc$_2$ has not been precisely located among cis- and medial-Golgi complex; thus, a recycling from a very early Golgi location cannot be excluded. In the present work, we could examine not only the trimming of high mannose glycans but also the presence of $O$-glycosylation of CD8 protein. It is widely accepted that the addition of GalNAc residues, i.e. the start of $O$-glycosylation, is the most cis-Golgi complex enzymatic marker available (58, 59). We found no GalNAc in the chimeric reporter CD8-E$_{99-142}$; nonetheless, we believe that these results may only suggest, not demonstrate, a mechanism of direct retention in the ER. Several considerations must be taken into account. First, a quick retrieval from the Golgi complex back to the ER and/or inaccessibility of the protein to the cis-Golgi enzymes could yield the observed results; second, a recycling from the intermediate compartment could not be detected by any assays given the absence of specific enzymatic modifications localized in this compartment; third, we have recently observed by confocal and immunoelectron microscopy that a portion of HCV E1 and E2 glycoproteins is present in the intermediate compartment/cis-Golgi region of stably expressing CV1 cells. Therefore, we think that the available results allow only to (i) exclude a dynamic recycling such as the one operating by both KDEL and KKXX motifs, which certainly results in a passage of the protein through the Golgi complex, or (ii) hypothesize either a direct retention or a dynamic recycling from the intermediate compartment. Overall, the last possibility goes along better with all the new data that underline the role of the cross-talk between ER, intermediate compartment, and Golgi complex in the function of the early segment of the secretory pathway (60).

Acknowledgments—We thank Dr. J. A. Ledbetter for the generous gift of antibody and Bruno Mugnoz for excellent technical assistance.

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