Characterization of Hepatitis C Virus E2 Glycoprotein Interaction with a Putative Cellular Receptor, CD81

MIKE FLINT, CATHARINE MAIDENS, LARRY D. LOOMIS-PRICE, CHRISTINE SHOTTON, JEAN DUBUISSON, PETER MONK, ADRIAN HIGGINBOTTOM, SHOSHANA LEVY, AND JANE A. MCKEATING

School of Animal & Microbial Sciences, University of Reading, Reading RG6 6AJ, and Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield S10 2HU, United Kingdom; Henry M. Jackson Foundation, Rockville, Maryland 20850; CNRS-UMR319, IBL/Institut Pasteur de Lille, 59021 Lille Cedex, France; and Department of Medicine, Division of Oncology, Stanford Medical School, Stanford, California 94305

Received 9 February 1999/Accepted 20 April 1999

Hepatitis C virus (HCV), the major cause of non-A, non-B viral hepatitis, is an enveloped virus classified in the Flaviviridae family, which also includes the flaviviruses (e.g., tick-borne encephalitis virus and dengue virus) and pestiviruses (e.g., bovine viral diarrhea virus and classical swine fever virus) (24). HCV replicates in the liver and induces a chronic infection, leading to cirrhosis and end-stage liver disease in approximately 20 to 30% of infected individuals. At present no prophylactic measures against HCV infection are available, and current therapies are unsatisfactory. Early events in Flaviviridae cell entry, replication and morphogenesis are not well understood (2, 10, 11, 14, 19, 25). One significant obstacle to progress in understanding HCV pathogenesis is the absence of suitable small-animal and cell culture models.

The structural proteins of HCV are believed to comprise the core protein and two predicted envelope glycoproteins (gps), E1 and E2. The majority of E1 and E2 gps expressed in vitro exist as high-molecular-weight disulfide-bridged aggregates (3–5, 7, 22). Hence, in order to study the biological activity of the HCV gps, it is critical to distinguish between gps undergoing productive folding and those following nonproductive pathway(s) resulting in misfolding and aggregation. The E2 gp extends from amino acids (aa) 384 to 746 (position within the polyprotein), and deletions removing the hydrophobic C-terminal region result in secretion of the ectodomain (17, 18, 29, 32). Comparison of E2661 with proteins truncated at position 688, 704, or 715 demonstrates that these deletions result in both reduced secretion and recognition by conformation-dependent monoclonal antibodies (MAbs). Secreted forms of E2661 gp were shown to contain minimal amounts of disulfide-bridged high-molecular-weight aggregates compared to the intracellular form(s) of the antigen (17a, 18). Antigenic characterization of secreted E2661 suggests that it folds in a way comparable to that observed in E1-E2 complexes and therefore makes an ideal soluble mimic of a viral ligand to study cellular receptor interactions.

Rosa and colleagues reported that a soluble truncated form of the HCV E2 gp bound to the surface of the T-cell line Molt-4 and that this interaction could be inhibited both by a MAb specific for the hypervariable region (HVR) and by HCV-infected chimpanzee sera (26). Recently, Pileri and colleagues (22) demonstrated that the cell surface-expressed molecule CD81 could interact with E2, suggesting that it may be the cellular receptor for HCV. CD81 is a member of the tetraspanin, or transmembrane 4, family, which traverses the membrane four times and has two extracellular (EC) loops of 28 and 80 aa, designated EC1 and EC2, respectively. Engagement of CD81 is reported to activate a variety of biologic responses including cell adhesion, morphology, proliferation, activation, and differentiation of T, B, and other cell types (16).

In this report, we define a number of potential contact sites between HCV E2 and the EC2 region of CD81, demonstrating that one or more of four amino acids within EC2 of CD81 are critical for this interaction. Various recombinant forms of the CD81 EC2 loop show differences in the ability to bind E2, suggesting that conformation of CD81 is important for E2 recognition. Regions of E2 involved in the CD81 interaction were analyzed, and our data suggest that the binding site is of a conformational nature involving aa 480 to 493 and 544 to 551 within the E2 glycoprotein. Finally, we demonstrate that ligation of CD81 by E2661 induced aggregation of lymphoid cells and inhibited B-cell proliferation, demonstrating that E2 interaction with CD81 can modulate cell function.

A truncated soluble form of the hepatitis C virus E2 glycoprotein, E2661, binds specifically to the surface of cells expressing human CD81 (hCD81) but not other members of the tetraspanin family (CD9, CD63, and CD151). No differences were noted between the level of E2661 binding to hCD81 expressed on the surface of rat RBL or KM3 cells compared to Daudi and Molt-4 cells, suggesting that additional human-cell-specific factors are not required for the primary interaction of E2 with the cell surface. E2 did not interact with African green monkey (AGM) CD81 on the surface of COS cells, which differs from the hCD81 sequence at four residues within the second extracellular region (EC2) (amino acids [aa] 163, 186, 188, and 196), suggesting that some or more of these residues defines the site of interaction with E2. Various recombinant forms of CD81 EC2 show differences in the ability to bind E2, suggesting that CD81 conformation is important for E2 recognition. Regions of E2 involved in the CD81 interaction were analyzed, and our data suggest that the binding site is of a conformational nature involving aa 480 to 493 and 544 to 551 within the E2 glycoprotein. Finally, we demonstrate that ligation of CD81 by E2661 induced aggregation of lymphoid cells and inhibited B-cell proliferation, demonstrating that E2 interaction with CD81 can modulate cell function.

* Corresponding author. Mailing address: School of Animal & Microbial Sciences, University of Reading, Reading RG6 6AJ, United Kingdom. Phone: (44) 1189 875 123, ext. 7892. Fax: (44) 1189 316 671. E-mail: j.a.mckeating@reading.ac.uk.
Materials and Methods

Antibodies. Directly labeled anti-human CD51 (bCD51), Biocytin (Amersham, Arlington Heights, III), anti-bCD9 (Coulter, Hialeah, FL), anti-bCD36, and CL-3 from clone 15A-2 were used according to the manufacturers’ protocols. Anti-bCD61 MAb 5A6 and 1D6 were described previously (16); bCD51 and 4T1-M1 were obtained from the Leukocyte Typing Workshop, and MAb 1.3.3.22 was purchased from Santa Cruz Biotechnology Inc., Santa Cruz, Calif. Anti-bCD51 was a gift from Leonie Ashman (Hansom Institute for Cancer Studies, Adelaide, South Australia, Australia).

MAbs to E2 were generated from rats after immunization with either baculovirus-expressed E2 (5A6) complexes or mammalian (human embryonic kidney [HEK]) cell-expressed E2 (5A6), and were epitope mapped using overlapping peptide-protein-E2 fusion proteins and overlapping peptides (29a). The E2-specific conformation-dependent MAbs (H53 and H60) were generated as previously reported (5). MAb 11/4b, specific for a linear epitope (SIRGKVQ) within the V3 region of HIV-1 gp120, was generated as previously described (5). MAb 6/53 was a gift from J. Garson (HepG2, Huh7, and PLC/PR5 cells were a gift from J. Garson [University College of London Medical School, London, England], Molt-4 and Daudi cells were obtained from the MRC ADP Repository. Rat basophilic leukemia (RBL-2H3) cells were routinely cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% (vol/vol) FCS. PLK3 cells were routinely subcultured in RPMI 1640 with 5% (vol/vol) FCS. COS cells or from stably transfected CHO cells. The fusion proteins were purified from the extracellular fluid by affinity chromatography on protein A-Sepharose (Pharmacia).

Expression of E2661. The open reading frame encoding E2661 was PCR-amplified from an existing cDNA clone sequence (pBTM116CMV-HC3; gift from C. M. Rice), using primers specific for aa 384 to 661. The primers were sense 5’-CCG GATT GTT GCT GTA AAG CAC TTC GCT GTC CGG GG-3’ and 5’-GTC CTT CAA GGA CAT GAC GAA TTA TTG CTT GAT G-3’ encoding the CD5 signal sequence and upstream of the Ig Fc region in plasmid CDM7B. The PCR product was digested with HindIII and BamHI and ligated into pGEX-2T (Pharmacia) which had been digested with HindIII and RsaI, resulting in a product of 4508 bp. The expression plasmid encoding E2661 was transferred into the yeast strain K480 (15A-2) to generate a stable transformant. The E2661-expressing yeast cells were cultivated to OD 1.0 at 57°C, with 25% glycerol (v/v). 2 M NaCl, 50 M Tris, 10 M MgSO4, 10 M MnCl2, 13 M K2HPO4, 3H-labeled 5-hydroxytryptamine (5-HT) from intracellular granules as described previously (31). Briefly, cells were incubated with [3H]5-HT (1 Ci/ml; Amersham) and washed twice with wash buffer (WB; 150 M NaCl, 5 M KCl, 10 M t-sorbitol, 1 M K2HPO4, 0.1 M HEPES pH 7.4, 1 M CaCl2, 0.1% [wt/vol] bovine serum albumin). After a 15-min preincubation at 37°C, this buffer was replaced and mock or E2661-containing concentrated supernatant diluted in WB buffer. Then, the yeast cells were washed twice with WB buffer, and 10 M anti-CD31 antibodies (5A6) or anti-epitope tag MAbs (11/4b) were added. After a further 10-min incubation at 37°C, the cells were washed twice more with WB buffer and 10 M anti-CD31 antibody-stimulated release assayed for background spontaneous release. Anti-CD31 MAb-stimulated secretion was typically 50% to 75% of the maximal secretory response to cross-linkage of surface-bound mE2 by optimal concentrations of antigen.

Results

Specificity of the HCV E2-CD1 interaction. E2661 has been reported to bind a range of cell lines at various levels; however, recognition is restricted to cells of human origin, suggesting that E2 interacts specifically with human CD51. Alternatively, the E2-CD1 interaction may be dependent on additional human cell-specific factors required for optimal CD1 binding.
tion and conformation. To investigate this further, RBL and KM3 (basophil and melanoma, respectively) rat cell lines, transfected to express hCD81, were tested for the ability to bind E2661. E2661 bound only to RBL and KM3 cells expressing hCD81; the level of binding correlated with hCD81 expression and was comparable to that found with various human cell types naturally expressing CD81 (Fig. 1; Table 1). It is worth noting that RBL cells naturally express rat CD81, and since no binding to the untransfected cells was seen, we can conclude that E2 does not bind rat CD81. In addition, E2661 was tested for its ability to interact with other members of the tetraspanin family. E2661 failed to interact with RBL cells expressing CD9, CD63, or CD151, confirming the specificity of the E2-CD81 interaction (data not shown).

Since it is known that simian COS cells (derived from African green monkeys [AGM]) express CD81 and that four amino acid changes, all within EC2, differentiate AGM CD81 from hCD81 sequences (16), we determined whether E2661 could interact with COS cells. COS were shown to express CD81 by their ability to bind MAbs JS81 (mean FI of 120.7) and JS64 (data not shown), two anti-CD81 MAbs demonstrating cross-species reactivity. In contrast, the human-specific anti-CD81 MAbs 5A6 and 1D6 failed to recognize CD81 on COS cells.

FIG. 1. Specificity of the E2-CD81 interaction. CD81 expression was monitored on parental (shaded graphs) and CD81-expressing (open graphs) KM3 (A) and RBL (B) cell lines with a pool of anti-CD81 MAbs (ID6, 4TM-1, and 1.3.3.22) and anti-mouse IgG-PE. Soluble E2661 was allowed to bind to KM3 (C) and RBL (D) parental cell lines (shaded graphs) and those stably expressing human CD81 (open graphs); cell-bound antigen was visualized with MAb 11/4b specific for the C-terminal tag, anti-rat IgG-PE, and FACS analysis.

FIG. 2. E2 does not bind COS cell-expressed CD81. KM3-CD81 and COS cells were monitored for CD81 expression with MAbs JS81 (A) and 5A6 (B). Soluble E2661 (open graphs) and mock antigen (shaded graphs) were monitored for binding to KM3-CD81 and COS cells (C) with MAb 11/4b. E2 and mock antigens were also tested for the ability to bind KM3 cells, resulting in a background median FI of 3.0.

# Table 1. CD81 dependency of E2 binding to various cell types

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Derivation</th>
<th>MAba</th>
<th>Anti-CD81</th>
<th>Irrelevant isotype control</th>
<th>E2661 gp in the presence ofb:</th>
<th>No addition</th>
<th>GST-EC2</th>
<th>Anti-CD81 5A6</th>
</tr>
</thead>
<tbody>
<tr>
<td>KM3</td>
<td>Rat melanoma</td>
<td>4.5</td>
<td>4.4</td>
<td>4.8</td>
<td>NT</td>
<td>NT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KM3-CD81</td>
<td>Rat melanoma</td>
<td>661.2</td>
<td>8.3</td>
<td>162.3</td>
<td>6.3</td>
<td>5.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HepG2</td>
<td>Human liver</td>
<td>12.4</td>
<td>13.7</td>
<td>12.9</td>
<td>11.6</td>
<td>13.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HuH7</td>
<td>Human liver</td>
<td>56.6</td>
<td>6.5</td>
<td>125.9</td>
<td>6.5</td>
<td>5.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLC/PR5</td>
<td>Human liver</td>
<td>63.2</td>
<td>5.9</td>
<td>110.5</td>
<td>7.3</td>
<td>4.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daudi</td>
<td>Human B cell</td>
<td>191.1</td>
<td>6.1</td>
<td>122.9</td>
<td>8.4</td>
<td>6.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Molt-4</td>
<td>Human T cell</td>
<td>250.3</td>
<td>2.4</td>
<td>664.4</td>
<td>5.9</td>
<td>6.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a CD81 expression was measured with MAb 5A6 (10 μg/ml), and background FI was measured with an irrelevant anti-gp120 isotype-matched control MAb.

*b E2661, at a concentration (1 μg/ml) shown not to saturate cell-expressed CD81 was incubated with GST-EC2 (50 μg/ml) for 1 h and subsequently tested for binding the various cell types. In addition, cells were incubated with 5A6 (10 μg/ml) for 1 h before being tested for the ability to bind E2661. Cell-bound E2 antigen was visualized with MAb 11/4b and anti-rat IgG-PE. NT, not tested.
E2661 gp failed to bind COS cells, suggesting that one or more of the changes at residues 163 (T/A), 186 (F/L), 188 (E/K), and 196 (D/E) in AGM CD81 are critical for interaction with E2. Since the only animal model currently available to study HCV replication is the chimpanzee, we determined whether E2661 gp could bind to chimpanzee cells and whether genetic polymorphisms exist between human, chimpanzee, and AGM CD81 sequences. E2661 gp was able to bind to immortalized chimpanzee B cells shown to express CD81 (data not shown). Consistent with this observation, no coding changes were seen between the chimpanzee and human sequences, although five silent polymorphisms, all of which were T/C changes, were observed (data not shown).

To elucidate the region of CD81 interacting with E2661, we tested the ability of a number of CD81 MAbs, all reactive with epitopes within the EC2 loop, to inhibit the E2661-CD81 interaction. MAbs 5A6, ID6, JS81, 4TM-1, and 1.3.3.22 were shown to saturate their ligand on RBL-CD81 cells by FACS and to inhibit E2661 interaction (Fig. 3). Although a residual amount of E2661 binding was observed in the presence of MAb 4TM-1, the levels were substantially (>10-fold) lower. In contrast, a control MAb, specific to major histocompatibility complex (MHC) class I, had no effect on E2661 binding to RBL-CD81 cells. Furthermore, we tested the ability of EC2-Fc fusion proteins inhibited E2661 binding to RBL-CD81 cells. To investigate this further, EC2-Fc and GST-EC2 proteins were analyzed by nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting. GST-EC2 existed as monomeric and dimeric forms, both of which were recognized by anti-CD81 MAbs, whereas only dimeric forms of EC2-Fc were detected (data not shown). E2661 gp was found to interact only with the monomeric form of GST-EC2, suggesting that this was the biologically active form (data not shown).

CD81 has been reported to associate in the plasma membrane with other molecules, such as CD19, CD21, and Leu-13, on B cells (1, 12, 33) and with CD4 and CD8 on T cells (13). We investigated whether E2661 would recognize CD81 expressed on cells of different lineages, where one might expect CD81 to exist in different oligomeric protein complexes. A number of cell lines were compared for CD81 expression and for the ability to bind a nonsaturating concentration of E2661 in the presence or absence of either the CD81-specific MAb 5A6 (10 µg/ml) or GST-EC2 (50 µg/ml). With a nonsaturating concentration of E2661, any interaction(s) with CD81 at the cell surface will be independent of CD81 expression levels, enabling one to directly compare cell types. Both hepatocyte lines, Huh7 and PLC/PR5, expressed low levels of CD81, whereas HepG2 cells did not express detectable levels of CD81 (Table 1). Such low-level CD81 expression is representative of the presence of sodium azide and tested for the ability to subsequently bind the CD81-specific MAbs. None of the MAbs bound RBL-CD81 cells in the presence of E2 gp, demonstrating reciprocal inhibition (data not shown).

Recombinant forms of hCD81 encoding both EC1 and EC2 or EC2 alone were expressed as N- or C-terminal fusion proteins with either human IgG Fc fragment or GST, respectively (16). EC1.EC2-Fc, EC2-Fc, and GST-EC2 were all able to bind CD81-specific MAbs (data not shown). These proteins were then tested for the ability to compete with cell-expressed CD81 for the binding of E2661. The GST-EC2 protein completely inhibited E2661 binding to RBL-CD81 (Fig. 4) and Molt-4 (data not shown) cells with a 50% inhibitory concentration of 12 µg/ml, demonstrating the critical role of the EC2 region in the E2-cell surface interaction. Neither of the Fc fusion proteins inhibited E2661 binding to RBL-CD81 cells. To investigate this further, EC2-Fc and GST-EC2 proteins were analyzed by nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting. GST-EC2 existed as monomeric and dimeric forms, both of which were recognized by anti-CD81 MAbs, whereas only dimeric forms of EC2-Fc were detected (data not shown). E2661 gp was found to interact only with the monomeric form of GST-EC2, suggesting that this was the biologically active form (data not shown).

CD81 has been reported to associate in the plasma membrane with other molecules, such as CD19, CD21, and Leu-13, on B cells (1, 12, 33) and with CD4 and CD8 on T cells (13). We investigated whether E2661 would recognize CD81 expressed on cells of different lineages, where one might expect CD81 to exist in different oligomeric protein complexes. A number of cell lines were compared for CD81 expression and for the ability to bind a nonsaturating concentration of E2661 in the presence or absence of either the CD81-specific MAb 5A6 (10 µg/ml) or GST-EC2 (50 µg/ml). With a nonsaturating concentration of E2661, any interaction(s) with CD81 at the cell surface will be independent of CD81 expression levels, enabling one to directly compare cell types. Both hepatocyte lines, Huh7 and PLC/PR5, expressed low levels of CD81, whereas HepG2 cells did not express detectable levels of CD81 (Table 1). Such low-level CD81 expression is representative of
Characterization of the E2 region(s) interacting with CD81.

We have generated a series of MAbs specific for linear and conformational epitopes within the E2 glycoprotein (summarized in Fig. 5). E2gp61 was incubated and captured on GNA lectin-coated EIA plates, and each of the MAbs was tested for the ability to bind. All of the MAbs were able to recognize GNA lectin-bound E2, albeit with different relative affinities, while MAb V3, specific for an epitope within HIV-1 gp120, did not react with E2gp61 (Fig. 6A). This panel of MAbs enabled us to study the regions of E2 involved in the CD81 interaction. Heat denaturation of E2gp61 (100°C) destroyed its ability to interact with CD81, suggesting that binding is dependent on conformation (data not shown). Initially, we wished to determine which epitopes of the E2gp61 were available for MAb recognition after interaction with cellular CD81. E2 was therefore allowed to bind to RBL-CD81 cells, and the MAbs were tested for the ability to recognize cell-bound antigen. As seen in Fig. 6B, a limited number of MAbs were able to recognize E2gp61-CD81 complexes. MAb V3 served as an internal control to determine background FL. As shown earlier (Fig. 1), MAb 11/4b, specific for the C-terminal tag, was able to bind E2gp61-CD81. Interestingly, MAbs H53 and H60, specific for conformational determinants, were also able to recognize E2gp61-CD81. However, only three of the linear MAbs tested, 7/59, 7/16b, and 6/1a, specific for epitopes including aa 384 to 391, 436 to 447, and 464 to 471, respectively, were able to recognize E2gp61-CD81 complexes (Fig. 6B). It is of interest that MAbs 6/16 and 6/82a bind the same epitope within the HVR as that recognized by MAB 7/59 but demonstrate different affinities for the E2gp61-CD81 complex. Furthermore, MAB 7/59 is an IgM, whereas both 6/16 and 6/82a are of the IgG1 isotype.

Masking of E2 epitopes in this assay could result from epitopes being directly involved in the CD81 interaction site. Alternatively, conformational changes may occur within E2gp61 as a result of interacting with CD81. To distinguish between these possibilities, we investigated whether preincubation of E2gp61 with the various MAbs could inhibit its subsequent interaction with CD81. E2gp61-MAb complex formation was demonstrated by capture of the complex by GNA lectin-coated EIA plates and visualization with an antispecies IgG-HRP conjugate (data not shown). E2gp61-MAb complexes were allowed to bind to RBL-CD81 cells and were visualized directly with an antispecies IgG-PE (Fig. 6C). The E2gp61-MAb complexes which bound to RBL-CD81 cells (11/4b, 7/59, 6/1a, H53, and H60) involved those MAbs previously shown to recognize E2gp61 when bound to CD81, with the exception of MAB 7/16b (Fig. 6C). The inability of the E2gp61-7/16b complex to bind CD81 may be due to steric blocking, suggesting that this region may not be directly involved in CD81 interaction. The ability of E2gp61-MAb complexes to bind cells, albeit with reduced signals, in the presence of MAbs 6/16, 6/82a, 3/11, 2/69a, 1/39, and H52 suggests that the epitopes recognized by these MAbs may not be directly involved in the CD81 interaction. However, binding of these MAbs may lead to occlusion of the CD81 binding site and hence a reduced interaction. E2gp61-MAb complexes involving MAbs 6/41a and 6/53 completely failed to bind CD81 cells (Fig. 6C). These data, together with the inability of MAbs 6/41a and 6/53 to bind to cell-associated E2 (Fig. 6B), suggest that their epitopes, aa 480 to 493 and 544 to 551, may be directly involved in the interaction with CD81.

Biological consequences of E2-CD81 interaction. MAbs to CD81 have been reported to induce cell aggregation and antiproliferative effects in CD81-expressing cell lines (reviewed in reference 16). We found that MAB 5A6 induced distinctive patterns of aggregation in both Daudi and Molt-4 cells; however antiproliferative effects were observed only in the Daudi B-cell line (data not shown). We therefore investigated whether the E2gp61 gp could induce similar effects in these cells. Daudi cells were incubated with E2gp61 in the presence or absence of MAbs H53 and 6/53, together or individually, a mock antigen preparation, and the CD81-specific MAB 5A6 at 37°C for 2 h. Cells were visualized by light microscopy, and changes in cell morphology were quantified by FSC/SSC FACs profiles (Fig. 7). MAB 5A6 rapidly induced aggregation to form cell chains, which led to increases in both FSC and SSC (Fig. 7B). Mock antigen had only a moderate effect on FSC and SSC, and no visible changes were apparent by light microscopy.
(Fig. 7C). In contrast, E2_{661} gp induced cell aggregation which was clearly visible by eye after 1 h and was similarly confirmed by changes in FSC and SSC (Fig. 7D). Incubation of the E2_{661} gp with MAb H53 prior to incubation with Daudi cells had no detectable effect on the aggregation patterns observed, whereas incubation with MAb 6/53 prevented aggregation (Fig. 7F). Treatment of cells with MAb H53 or 6/53 alone had no observable effects (data not shown). It is interesting that incubation of Daudi cells with E2_{661} gp induced a greater population of cells with increased SSC compared to that observed after MAb 5A6 treatment. The cell aggregation induced by both E2, and MAb 5A6 were dependent on ligand concentration(s), such that sequential dilutions of both agents reduced the level of aggregation observed to a negligible level (data not shown).

Antiproliferative effects of CD81 ligation were measured by incubation of the same ligands with Daudi cells at 37°C for 48 h. Viable cell counts were performed, and proliferation was determined. Over a 48-h period, the untreated cells proliferated 2.7-fold; MAb 5A6 reduced this proliferation by 45%. Mock antigen had no effect, whereas E2_{661} gp, independent of MAb H53, reduced proliferation by 36% (Fig. 8A). Neither 5A6 nor E2_{661} had any effect on the proliferation of KM3 or RBL cells expressing CD81 (data not shown). We have previously shown that antibody cross-linking of the tetraspan hCD63 on the surface of transfected RBL cells leads to cell activation and the concomitant release of intracellular granule contents (31). We therefore tested whether ligation of hCD81 by antibody and E2_{661} gp would have a similar effect. Addition of MAb 5A6 induced the release of 20% of the total cell-associated [3H]HT, whereas incubation with E2_{661} gp had no detectable effect. However, cross-linking of CD81-associated E2_{661} with MAb 11/4b stimulated a dose-dependent release of radioactivity (Fig. 8B), comparable to that induced by MAb 5A6.

**DISCUSSION**

Experiments presented here demonstrate that a truncated, soluble form of the HCV E2 glycoprotein, E2_{661}, binds specifically to the surface of cells expressing human CD81 but not other members of the tetraspan family (Fig. 1 and data not shown). No significant differences were noted between the level of E2_{661} binding to human CD81 expressed on the surface of rat RBL or KM3 cells compared to Daudi and Molt-4 cells, which naturally express CD81. Furthermore, recombinant GST-EC2 inhibited E2 binding to hCD81 at the surface of rat or human cells equivalently (Fig. 4 and data not shown). These data suggest that no additional human cell-specific factors are required for the primary interaction of E2 with the cell surface and, by inference, HCV attachment to the cell. The inability of E2_{661} to bind to untransfected RBL cells, which express high levels of endogenous rat CD81, demonstrate that E2 does not recognize rat CD81. Of more interest was the observation that E2_{661} failed to recognize AGM CD81 expressed on the surface of COS cells (Fig. 2). Since there are only four amino acid differences between human and AGM CD81, at residues 163, 186, 188, and 196 within the EC2 loop, these data suggest that one or more of these residues are critical for interacting with the E2 gp. Since the only animal model for studying HCV replication is the chimpanzee, it was important to demonstrate that E2_{661} gp binds chimpanzee cells expressing CD81 (data not shown). Consistent with this observation, no genetic polymorphisms, resulting in coding changes, were noted between the human and chimpanzee sequences.

The role of the EC2 region of CD81 in the association with E2 was further supported by the ability of both MAbs to this region, and a recombinant form of EC2, to inhibit E2_{661} binding to cells (Fig. 3 and 4). These observations are consistent with those reported by Pilieri and colleagues (22) demonstrating the ability of a recombinant EC2 protein to bind HCV virions. However, not all of the recombinant forms of the CD81 EC2 region were able to bind the E2_{661} gp, with only the GST-EC2 form demonstrating such activity (Fig. 3). In contrast, all of the recombinant proteins were able to bind the CD81-specific MAbs, which are specific for conformational epitopes dependent on disulfide bonding (data not shown). The dimerization of the Fc fusion proteins may affect EC2 conformation and hence its ability to interact with E2. Clearly, the requirements for E2 and MAb binding to CD81 are subtly different, such that E2 may behave more like the native CD81 ligand, which at present remains undefined.

CD81 exists on the cell surface as part of multimeric signaling complexes, the constituents of which vary between cell types (reviewed in reference 16). At present the expression levels of CD81 on primary hepatocytes, and the nature of any molecular interactions are unknown. We failed to detect CD81 expression on the hepatocyte cell line HepG2, but other hepatocyte and hepatoma cell lines expressed low levels of CD81 (data not shown). E2_{661} gp bound to B-cell (Daudi), T-cell (Molt-4), and hepatocyte (Huh7 and PLC/PR5) cell lines in a CD81-dependent manner, as determined by the ability of anti-CD81 MAbs and GST-EC2 to inhibit these interactions (Table 1). These data suggest that CD81 interaction(s) with cell surface proteins may not directly modulate E2 recognition; however, additional experiments on a wider range of cell types are required. We are presently establishing whether E2_{661} interaction with primary hepatocytes is CD81 dependent.

We were interested in defining the regions of E2_{661} that interact with CD81. It should be noted that heat-denatured E2_{661} gp failed to bind CD81 (data not shown), suggesting that the binding site is of a conformational nature. Initially, we used a panel of well-defined E2-specific MAbs to determine the...
accessibility of epitopes to antibody after CD81 complex formation (Fig. 6B). Surprisingly, the majority of MAbs were unable to recognize CD81-bound E2661. Epitopes recognized by MAbs 7/59, 7/16b, 6/41a, 6/53, and H60 are available for antibody binding after CD81 interaction, demonstrating that these regions are not involved in the interaction (Fig. 5 and 6).

It is of interest that MAbs 7/59, 7/16b, 6/41a, and 6/53 are able to recognize E2661 when bound to CD81 (Fig. 6B), its ability to inhibit E2661 cell attachment may be via a steric blocking effect. In contrast, MAbs 6/41a and 6/53 were unable to recognize E2661 when complexed with CD81 (Fig. 6B), and both inhibited E2661 attachment to cells, suggesting that amino acids within regions aa 480 to 493 and 544 to 551 are components of a discontinuous CD81-binding site (Fig. 5). It is worth noting that both peptide sequences PDRPRYCWHPY and PPLGNNWFG, recognized by MAbs 6/41a and 6/53, respectively, are conserved in E2 sequences from different subtypes, as would be expected for a protein region involved in receptor interaction(s).

Various investigators have reported that cross-linking of CD81 by MAbs leads to a number of different effects, including cell aggregation, antiproliferation, and calcium signalling (reviewed in reference 16). We therefore studied the biological consequences of the E2_{661}-CD81 interaction. E2_{661} was found to induce cell aggregation, independent of H53-mediated cross-linking, resulting in an FSC/SSC profile distinct from that seen after treatment of Daudi cells with MAb 5A6 (Fig. 7). Pretreatment of E2_{661} gp with GST-CD81 (10 μg/ml) inhibited the aggregation, confirming the CD81-dependent nature of the effect (data not shown). We found that the type of cell aggregation induced by MAb 5A6 in Molt-4 cells was distinctly different from that induced in Daudi cells, and the same effect was observed with the E2_{661} gp (data not shown). Given that E2_{661} induced aggregation in Daudi cells, we determined whether prolonged incubation induced any anti-proliferative effects. 5A6 and E2_{661} reduced cell proliferation by 45 and 36%, respectively, after a 48-h period (Fig. 8A). In contrast, binding of either ligand to CD81-expressing KB3 or RBL cells had no effect on aggregation or proliferation (data not shown).

The binding of E2_{661} gp failed to stimulate [\(^{3}H\)5-HT] secretion from CD81-transfected RBL cells although the anti-CD81 MAb, 5A6, was an effective stimulus (Fig. 8B). However, addition of E2_{661}, followed by MAb 11/4b, specific for the C-terminal epitope tag, induced 5-HT release, suggesting that although E2_{661} binding to CD81 does not stimulate secretion directly, the complex is retained at the cell surface and is available for antibody cross-linkage. RBL cell activation by antitetrabasin MAbs requires coligation with the high-affinity IgE receptor (11a); since the binding of E2_{661} does not appear to affect the ability of CD81 to interact with the IgE receptor, this provides evidence for the existence of separate binding sites on CD81 for membrane-associated and exogenous ligands. Since CD81 ligation can induce various effects in different cell types, it will be important to study the effects of the E2-CD81 interaction in primary cell types such as hepatocytes and B cells.

Activation of cells via the E2-CD81 interaction could prime cells for HCV replication and may affect expression of cell surface immunomodulatory molecules, possibly explaining the chronic nature of HCV infection. Clearly, it will be important to demonstrate whether CD81, either alone or with additional factors, functions as the HCV receptor in allowing virus-cell attachment and entry. Since CD81 is so widely expressed, it is

FIG. 7. Effect of E2_{661} on cell aggregation. Daudi cells were treated with no MAb (A), 5A6 (anti-CD81) at 1 μg/ml (B), mock antigen (C), E2_{661} antigen (5 μg/ml) (D), or E2_{661} antigen complexed with MAb H53 (E) or with MAb 6/53 (F). Cells were monitored for aggregation by visual light microscopic inspection and FACS analysis. Daudi cells treated with 5A6 and E2 showed signs of aggregation, forming long cell-cell chains. Results were quantified by analyzing FSC/SSC profiles of the cells by FACS analysis, where the percentage of cells with high FSC/SSC profiles is shown in the upper quadrant of each plot.
unlikely to be the sole factor determining HCV liver tropism. Since HCV cannot be propagated efficiently in vitro, answers to these questions will be difficult to obtain; however, information may derive from studies of pseudotypic viruses expressing chimeric HCV gp1 (9, 15). Alternatively, the E2-CD81 interaction may be important for the processing of viral proteins and intracellular virion formation. CD81 expression, in conjunction with other tetraspanins (CD37, CD53, CD63, and CD82), is enriched in the endocytic vacuoles involved in MHC class II processing and exosome formation (8). Furthermore, CD9, a related tetraspanin, has been shown to bind intracellular immature β1 integrin, which may lead to its retention in the endoplasmic reticulum as a means of controlling transport to the cell surface (27). A similar role for CD81 in HCV virion formation and export cannot be excluded by our data. Since E2 is believed to exist on the virus surface as a heterodimer with E1, it will be important to study the interaction of E1-E2 complexes with CD81 and to determine the biological consequences of CD81 engagement in cell types representative of those infected in vivo.

ACKNOWLEDGMENTS

J.A.M. thanks Peter Balfe and Jeff Almond for critical comments on the manuscript and Barbara Konig and Louise Wilson for technical expertise; J.D. thanks André Pillez for excellent technical assistance; and S.L. thanks Ching-Chi Kuo for technical expertise; J.D. thanks André Pillez for excellent technical assistance; and S.L. thanks Ching-Chi Kuo for technical assistance. J.A.M. thanks the Wellcome Trust and The Lister Institute (grants 90188 and 92177) for support. J.A.M. acknowledges the Wellcome Trust and The Lister Institute for funding this research. P.M. acknowledges the support of the Arthritis and Rheumatism Campaign (fellowship 1998-04410). S.L. was supported by grant CA34233 from the Public Health Service, National Institutes of Health. L.D.L.-P. acknowledges support from the U.S. Army Medical Research and Material Command (cooperative agreement DAMD17-93-V-3004).

REFERENCES


FIG. 8. Effect of E2 \(_{5A6}\) on Daudi cell proliferation and on RBL-CD81 granule release. (A) Daudi cells seeded at 10^5 cells/ml were either untreated or incubated with 5A6 at 1 µg/ml, E2661 antigen at 5 µg/ml alone or preincubated with 5A6 and E2661 antigen for 48 h at 37°C. Viable cell counts were performed after 48 h, and untreated cells were found to proliferate 2.7-fold. (B) RBL-CD81 cells were loaded overnight with [3H]5-HT and incubated with E2661 or mock antigen for 10 min. After washing, the cells were incubated with 11/4b (10 µg/ml) and [3H]5-HT release was measured. The results are shown as a percentage of the response to anti-ICD81 MAb 5A6 (10 µg/ml), and the mean values from two independent experiments are shown. The addition of E2661 antigen alone had no effect on [3H]5-HT release.
17a. McKeating, J. A. Unpublished data.
29a. Shotton, C. Unpublished data.