

Membrane Permeability Induced by Hepatitis A Virus Proteins 2B and 2C and Proteolytic Processing of HAV 2BC

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The ability to rearrange membranes is a unique feature of nonstructural proteins 2B, 2C, and 2BC of some picornaviruses. To analyze in detail membrane binding of the respective proteins of hepatitis A virus (HAV), they were transiently expressed in the vaccinia/T7 system, and their effect on membrane permeability was studied using β -galactosidase as reporter. Although 2C had no effect, the significantly increased reporter activity observed in the extracellular space of 2B- and 2BC-expressing cells points to a specific effect of HAV proteins 2B and 2BC on membrane permeability. In biochemical fractionation studies, HAV 2C and 2BC showed properties of integral membrane proteins, whereas 2B was associated with membranes as a peripheral protein. Proteinase 3C-mediated cleavage of precursor 2BC *in vivo* was most efficient when the enzyme was coexpressed in its precursor forms P3 or 3ABC, which both include the membrane-anchoring domain 3A. 3ABC showed the same solubility pattern as 2BC, suggesting that colocalization of 2BC and 3ABC might be required for the efficient liberation of 2B and 2C and occurs on membranes that have been proposed as the site of viral RNA replication. © 1998

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INTRODUCTION

The wild-type hepatitis A virus (HAV) differs markedly from other picornaviruses in its slow and noncytolytic growth. Faster growing variants that have been isolated after several passages in cell culture carry mutations in the viral nonstructural proteins 2B and 2C in combination with mutations in other genomic regions, implying that these polypeptides might play important roles in HAV replication and adaptation to cell culture (Emerson *et al.*, 1993; Graff *et al.*, 1994). Until now, no distinct biochemical activity or functional role of either 2B or 2C or their common proteolytic precursor polypeptides 2BC could be shown, in part because none of these proteins were detected in HAV-infected cells.

All picornaviral structural and nonstructural proteins are formed by proteolytic cleavage of the polyprotein (P1-P2-P3), resulting in a large array of intermediate and mature products (Fig. 1). During the primary cleavage steps, the HAV precursor polypeptide of the structural proteins P1–2A is formed in addition to 2BC and P3. The latter two polypeptides give rise to the nonstructural proteins, all of which are supposed to be involved in picornaviral genome replication and to be part of the viral replication complex (Bienz *et al.*, 1983, 1987, 1990; Troxler *et al.*, 1992). The functions of the individual nonstructural

proteins from the P3 region have been studied in great detail for poliovirus and HAV. P3 of HAV constitutes the genome-linked replication primer 3B (VPg), its membrane anchor (3A), the only viral proteinase (3C^{pro}), and the RNA-dependent RNA polymerase (3D^{pol}). The orchestrated liberation of the mature viral polypeptides that is executed by the viral proteinase 3C^{pro} seems to be a prerequisite to control the various steps during the viral life cycle such as protein and RNA synthesis, as well as capsid assembly.

Multiple biochemical features have been ascribed to polio and HAV protein 2C, suggesting that this highly conserved polypeptide is involved in a variety of processes during viral replication. Biochemical and genetic analyses demonstrated RNA and membrane binding properties next to NTP binding and hydrolysis (Aldabe and Carrasco, 1995; Cho *et al.*, 1994; Echeverri and Dasgupta, 1995; Kusov *et al.*, 1998; Mirzayan and Wimmer, 1994; Rodriguez and Carrasco, 1993, 1995; Teterina *et al.*, 1997a). The biochemical properties, however, of picornaviral protein 2B and its role in virus replication are less well defined. When transiently expressed, enterovirus protein 2B enhances membrane permeability of mammalian and bacterial cells, blocks the protein secretory traffic (Doedens and Kirkegaard, 1995; Lama and Carrasco, 1992; van Kuppeveld *et al.*, 1997a, 1997b), and seems to localize to the outer surface of vesicles derived from the endoplasmic reticulum, which are the sites of viral ge-

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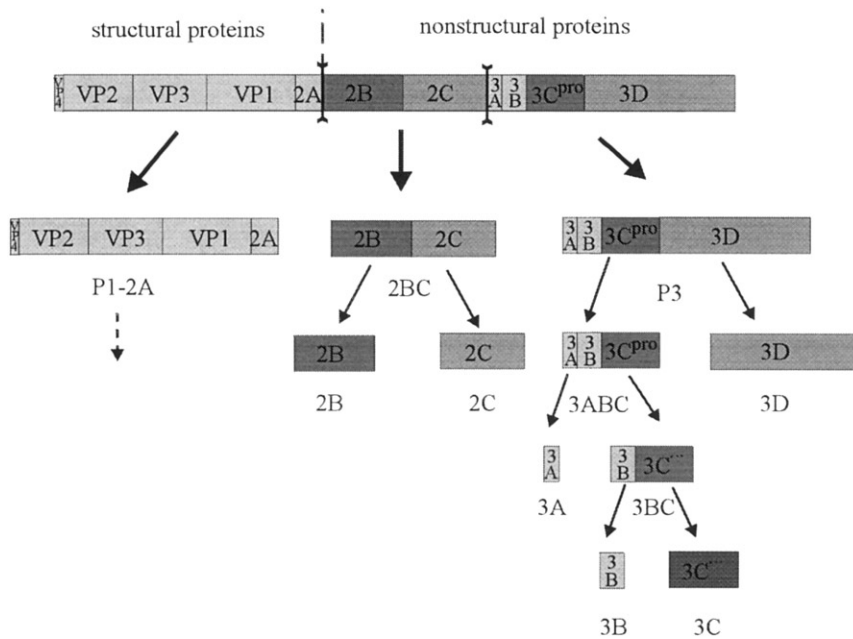


FIG. 1. Diagram of the HAV polyprotein and its 3C-mediated major processing leading to the liberation of the nonstructural proteins.

nome replication (Bienz *et al.*, 1987; Schlegel *et al.*, 1996; van Kuppeveld *et al.*, 1997a).

Evidence for the involvement of viral nonstructural proteins and vesicular membranes in picornaviral RNA replication was provided by immunocytochemistry of poliovirus-infected cells showing 3D, 3C, 2C, 2B, and viral RNA associated with vesicles (Bienz *et al.*, 1983, 1987, 1990; Schlegel *et al.*, 1996; Troxler *et al.*, 1992). Moreover, specific interaction of viral RNA with several viral proteins (some of which are highly hydrophobic) also support the notion that viral proteins might aid in the tight association of RNA replication with vesicle membranes (Banerjee *et al.*, 1997; Kusov *et al.*, 1998; Rodriguez and Carrasco, 1995; Xiang *et al.*, 1995). Possibly, as a prerequisite for the formation of the replication complex, vesicular membranes proliferate and are rearranged in poliovirus-infected cells (Aldabe and Carrasco, 1995; Bienz *et al.*, 1983; Schlegel *et al.*, 1996). A similar effect on membranes was observed when proteins 2C and 2BC of poliovirus and HAV were expressed in the absence of other viral proteins, pointing to their direct functional role in the formation of membrane vesicles (Aldabe and Carrasco, 1995; Cho *et al.*, 1994; Teterina *et al.*, 1997b). Neither its role in genome replication nor its biochemical features has been elucidated for HAV 2B, a protein significantly larger (28 kDa) than the respective polypeptide of other picornaviruses. To directly assess membrane association and localization, cells transiently expressing HAV proteins 2B, 2C, and 2BC were examined. Both 2BC and 2B increased the permeability of mammalian membranes that was demonstrated by the release of intracellular β -galactosidase. The efficiency of processing of 2BC by the viral proteinase 3C^{pro} and its precursors was

also studied *in vivo* to better understand the biogenesis and possible localization of HAV proteins 2B and 2C. Here, we present evidence that P3 and 3ABC, which is a stable intermediate product of P3 processing, catalyze proteolysis of 2BC more efficiently than 3C or other forms of the proteinase lacking the 3A domain.

RESULTS

Biochemical analysis of membrane association of HAV proteins 2B, 2C, and 2BC

HAV protein 2C was shown to bind to bacterial and mammalian membranes and to induce membrane rearrangement after expression in a recombinant system, in a manner similar to what was observed for HAV 2BC and the homologous poliovirus proteins (Cho *et al.*, 1994; Kusov *et al.*, 1998; Teterina *et al.*, 1997b). To investigate the mode of association to mammalian membranes in more detail, solubilization studies were performed using buffers that allow discrimination between peripheral and integral membrane proteins (Fujiki *et al.*, 1982; Tereshak, 1984). COS7 cells expressing 2B, 2C, and 2BC were fractionated and analyzed by immunoblot using anti-2B and anti-2C3A* (Fig. 2). Only minute amounts of the proteins were found in the PBS-soluble cytosolic fraction. The PBS-insoluble pellet was divided and treated under various conditions, including detergent (1% Triton X-100), 2 M urea, or high pH. Proteins 2B, 2C, and 2BC were completely soluble in PBS containing 1% Triton X-100, indicating that all polypeptides were associated with membranes. None of the proteins could be solubilized from the pellet by increasing the ionic strength (0.5 M NaCl, not shown),

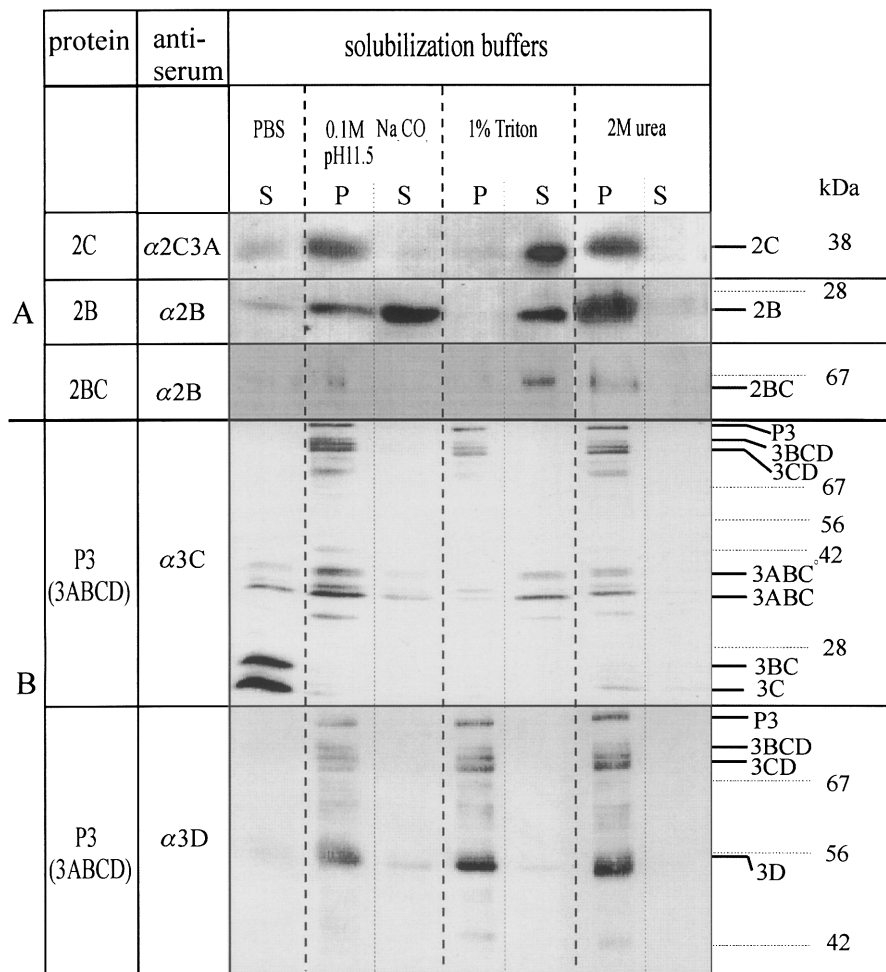


FIG. 2. Biochemical analysis of membrane association of HAV proteins 2B, 2C, 2BC (A), and P3, including its proteolytic products (B). COS7 cells transfected with cDNAs encoding the respective viral proteins and infected with vTF7-3 (Fuerst *et al.*, 1986; Probst *et al.*, 1997) were extracted with PBS by freeze-thawing. After centrifugation of the insoluble material, the pellet was further treated for 10 min with PBS supplemented with detergent or other components listed above the lanes. The soluble (S) and insoluble (P) fractions were separated by centrifugation (14,000 rpm) and analyzed by immunoblot using antisera as marked on the left. On the right, the position of HAV proteins and molecular weight standards (kDa) are indicated.

by chelating divalent cations (0.5 M EDTA, pH 8.5, not shown), or with mild chaotropic salt conditions (2 M urea), suggesting that none of the proteins were peripherally attached to the cytoplasmic side of membrane vesicles. However, under alkaline conditions (0.1 M Na₂CO₃, pH 11.5), ~90% of 2B was soluble, whereas 2C and 2BC were insoluble. At pH 11.5 (0.1 M Na₂CO₃), membranes are disrupted and converted to sheets without solubilizing them, resulting in a release of proteins trapped in membranous vesicles (Fujiki *et al.*, 1982). Under these conditions, integral membrane proteins sediment with the membrane sheets, and peripherally associated as well as soluble proteins remain in the supernatant. Following the definition of Fujiki *et al.* (1982), it can now be concluded from the solubility pattern that 2B might be localized peripherally to the luminal side of vesicles, whereas 2C and 2BC are integral membrane proteins.

Proteolytic processing of 2BC by 3C^{PRO} and its precursors

Intracellular protein targeting is generally determined during protein biogenesis. In the case of most secreted and membrane proteins, the signal peptide is responsible to direct the nascent protein chain to membranes. For proteins that arise by proteolytic processing of a precursor polypeptide, targeting follows other mechanisms (Rapoport *et al.*, 1996). To further our understanding on the targeted localization of 2B and 2C, the susceptibility of HAV 2BC to viral proteinase 3C^{PRO} and its precursors was assessed. Proteolytic cleavage of the picornaviral precursor protein, including that of HAV, has been studied in detail, and it has been shown that the amino acid sequence near the cleavage sites as well as secondary or tertiary structures might affect cleavage efficiency (Dougherty and Semler, 1993). In addition, polypeptides

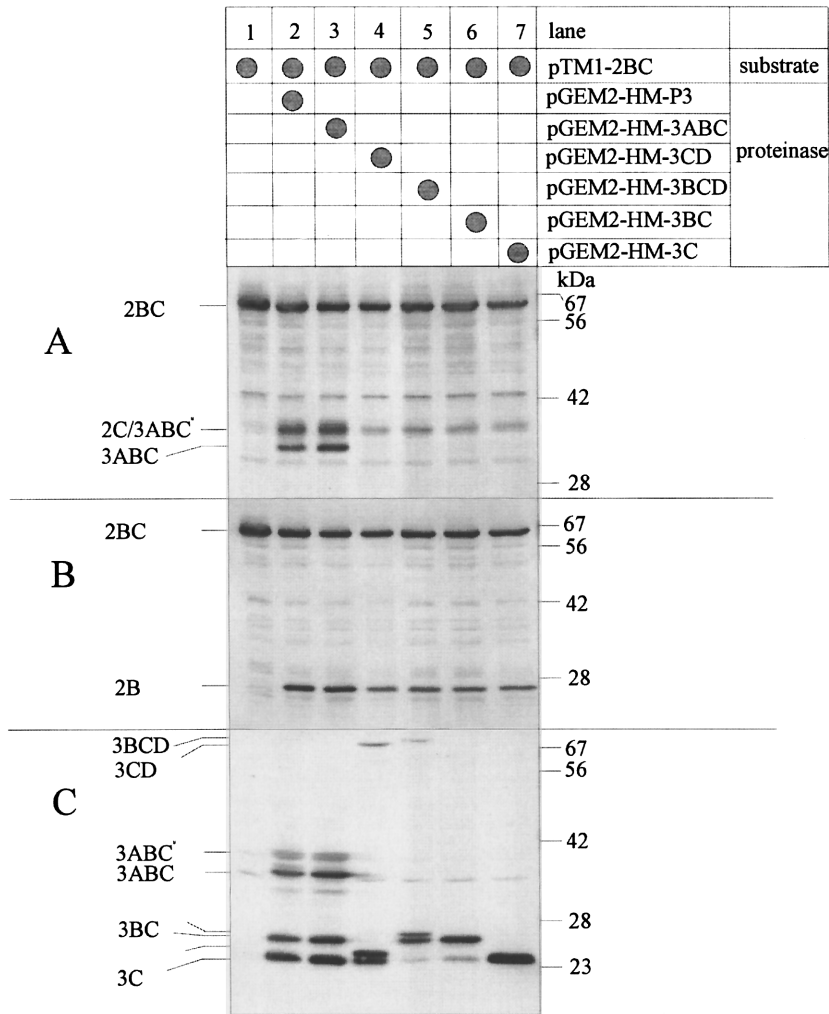


FIG. 3. Proteolytic cleavage of 2BC by 3C^{pro} and its proteolytically active precursors. cDNAs encoding HAV P3, 3ABC, 3BCD, 3CD, 3BC, and 3C were cotransfected with pTM1-2BC into COS7 cells, which were subsequently infected with vTF7-3. After 24 h of transient expression, the expression products were analyzed by immunoblot using anti-2C3A^{*} (A), anti-2B (B), and anti-3C (C). HAV proteins are marked on the left, and molecular weight standards are on the right.

flanking viral proteinases as well as other viral proteins have been shown to significantly improve precursor processing or the accessibility of the potential cleavage sites and to facilitate targeting of the mature products (Molla *et al.*, 1994; Santolini *et al.*, 1995). Because the only HAV proteinase 3C^{pro} is proteolytically active as mature protein and also in its precursor forms, it was interesting to test whether 3C-flanking domains might affect the substrate specificity and proteolytic activity of the enzyme. For this purpose, HAV 2BC was expressed as substrate for proteinase 3C^{pro} and its proteolytically active precursors (P3, 3ABC, 3BCD, 3BC, 3CD) in the vaccinia/T7 system (Fig. 3). Equal amounts of cell extracts were analyzed by immunoblot using antisera raised against a fusion protein containing 2C and parts of 3A (Fig. 3A), a 2B peptide (Fig. 3B), and 3C (Fig. 3C). As judged by the detection of 2B, all 3C-containing polypeptides were proteolytically active on 2BC (Fig. 3B). By

comparing the relative amounts of 2B, it was obvious that in cells coexpressing polypeptides P3 (lane 2) and 3ABC (lane 3), larger quantities of 2B were liberated from 2BC compared with those coexpressing 3CD (lane 4), 3BCD (lane 5), 3BC (lane 6), or 3C (lane 7). To test for correct processing of the nested set of 3C-containing proteins, the extracts were analyzed by immunoblot with anti-3C (Fig. 3C). As we have described elsewhere (Probst *et al.*, 1998), different amounts of the individual anti-3C reactive polypeptides were liberated from the various precursors. The variation in the total amounts of 3C-containing polypeptides most likely reflects the different transfer efficiency and immunogenicity of the individual 3C-containing polypeptides and not differences in the overall expression rate because the expression of each precursor is regulated by the exact same promoter (T7) and translation initiation sequence. It therefore can be concluded that the expression products derived from

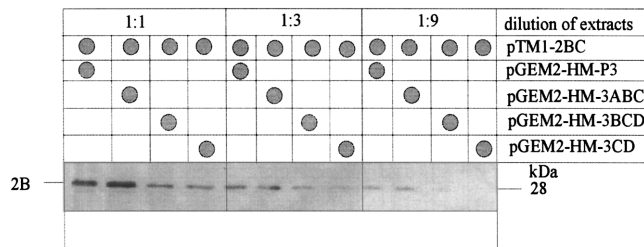


FIG. 4. Cleavage efficiency of different forms of the HAV proteinase on 2BC. Similar to Fig. 3, 2BC was coexpressed with P3, 3ABC, 3BCD, and 3CD in the vaccinia virus system. Cell extracts were obtained 24 h after transfection and analyzed in three different dilutions on the same gel. The amount of 2B was estimated by immunoblot using anti-2B.

pGEM-P3 and pGEM-3ABC specifically mediate more efficient cleavage of 2BC as compared to the others.

Among the anti-3C reactive polypeptides, 3ABC and 3ABC^o (probably a product of posttranslational modification of 3ABC) were also immunodetected by anti-2C3A* (Figs. 3A and 3C, lanes 2 and 3; Probst *et al.*, 1998). Because 2C comigrated with 3ABC^o, the 2BC cleavage efficiency could not be deduced from the amounts of 2C (Fig. 3A, lanes 2 and 3). To estimate the extent of 2BC cleavage mediated by some forms of the proteinase, different dilutions of the cell extracts were analyzed by immunoblot with anti-2B on the same gel. As shown in Fig. 4, at the 1:9 dilution, 2B was detectable only in extracts of cells coexpressing 2BC and P3 or 3ABC, whereas at the 1:3 dilution, 2B was visible in all extracts. This suggests that approximately three times more 2B is liberated by the 3A-containing proteinases P3 and 3ABC compared with 3BCD and 3CD. From these data, it can now be concluded that cleavage of 2BC *in vivo* is more efficiently catalyzed by 3A-containing forms of the proteinase compared with 3C and its precursors lacking 3A. This might imply that 3A can modulate the substrate specificity of 3C^{pro}, possibly by improving the accessibility to the cleavage site within 2BC.

To test the possibility that the improved substrate specificity of P3 and 3ABC for 2BC might be due to the joint localization of enzyme and substrate on membranes, the solubility of P3-derived products was determined similarly as described above for substrate 2BC and its products 2B and 2C (Fig. 2A). P3 was used as source of proteinase because both P3 and 2BC are the products primarily liberated from the polyprotein and thus are simultaneously present early in the virus life cycle (Fig. 1). The P3 processing products were identified by their electrophoretic mobility and immunoreactivity with anti-3C and anti-3D (Fig. 2B). Proteins 3BC, 3C, and small amounts of 3ABC were found in the PBS-soluble fraction. Some 3ABC was soluble at high pH, whereas the majority of 3ABC and 3ABC^o were solubilized with PBS containing 1% Triton X-100. P3, as well as 3BCD, and 3CD were insoluble in all buffers used (Fig. 2B, bottom), which is most likely due to their 3D content (Tesar *et al.*, 1994). Although we cannot exclude the possibility that P3

and 3ABC might have a higher specific activity on 2BC *in vitro* compared with 3C or 3BC, it is tempting to speculate from their similar solubility pattern that substrate 2BC and proteinase 3ABC might colocalize in or on membranes, which is possibly a prerequisite for efficient cleavage of 2BC.

Effects of HAV 2B, 2C, or 2BC on membrane permeability

Several reports have shown that enteroviral proteins 2B and 2BC enhance membrane permeability for low-molecular-weight compounds (Aldabe *et al.*, 1997; Doedens and Kirkegaard, 1995; van Kuppeveld *et al.*, 1997a). Based on their strong interaction with eukaryotic membranes (see above), the effect of HAV proteins 2B, 2C, and 2BC on the permeability of mammalian membranes was studied next. COS7 cells were cotransfected with equal amounts of 2B, 2C, 2BC, and lacZ-encoding cDNA, and intracellular and extracellular lacZ activity was determined 24 h later (Fig. 5). β -Galactosidase (lacZ) was chosen as reporter protein because of its large molecular mass, high solubility, stability, and easy enzymatic detection. The sum of intracellular and extracellular activities was compared among different coexpression assays (Fig. 5, top) and the relative amount of enzyme activity in the extracellular space was taken as a measure of membrane integrity (Fig. 5, bottom). To control for both, the possible effect of the relative expression rates and the influence of N-terminally flanking amino acid sequences, cDNA constructs (pTM, left panels of Fig. 5) containing the internal ribosomal entry site of the encephalomyocarditis virus (EMCV IRES), which allows for highly efficient internal initiation of translation, and a His-tag upstream of the HAV genes were compared with constructs lacking these elements (pET, right panels of Fig. 5). To ensure similar expression rates of both the reporter and the test gene, the pTM constructs were coexpressed with pEXT7-lacZ containing the EMCV IRES, whereas the pET constructs were coexpressed with pGEM1-lacZ. As a negative control and to normalize the amount of transfected DNA, the cDNA encoding HAV protein P1-2A was also coexpressed. As shown in Fig. 5 (top panels), none of the HAV P2 proteins had a pronounced effect on the overall expression rate of lacZ compared with the negative control, suggesting that these proteins are not cytotoxic. Probably, due to the competition for resources, β -galactosidase expression of pEXT7-lacZ in the presence of any of the IRES-driven HAV genes (pTMs) was markedly reduced (top left panel). No major effect on the expression rate of pGEM-lacZ was exerted by any of the pET constructs (top right panel). The high expression levels of the IRES-driven constructs (pTM and pEXT7) were confirmed by immunoblot analysis. By this test, we also verified the relative amounts of enzyme activity and excluded the possibility

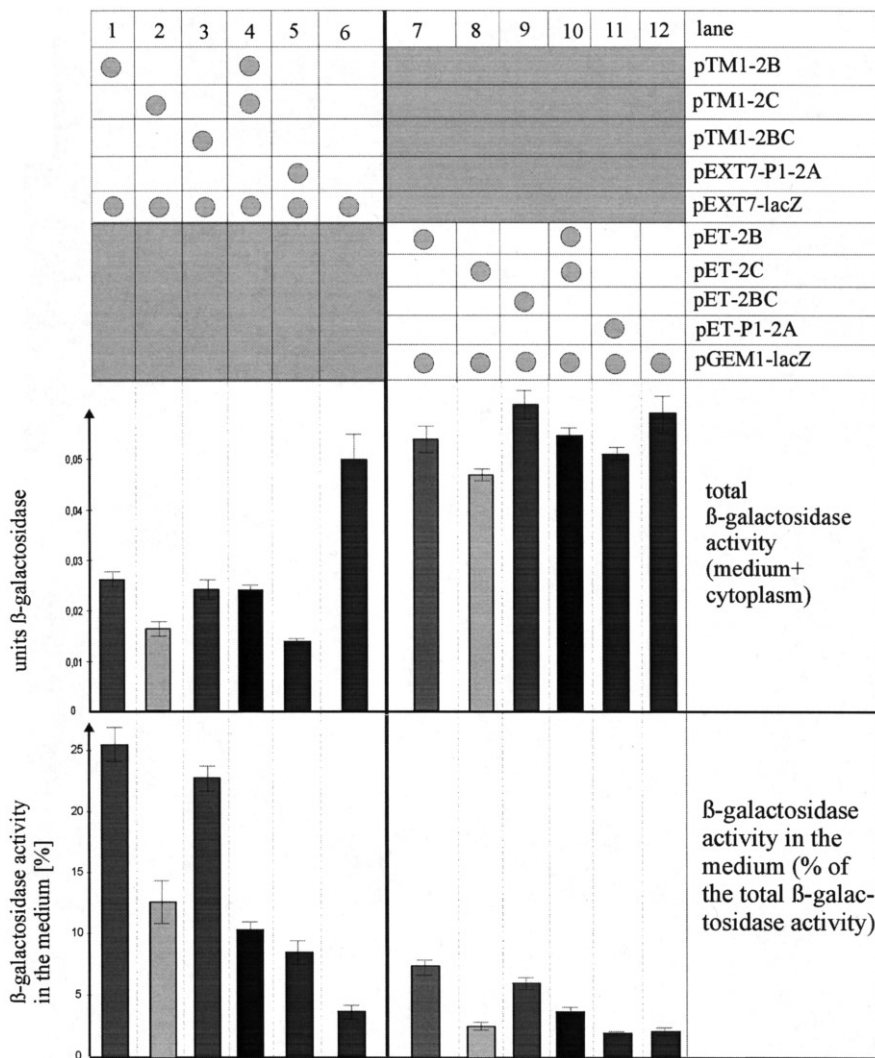


FIG. 5. Effect of 2B, 2C, and 2BC on cellular membrane integrity. Similar as described in Fig. 2, COS7 cells were cotransfected with equal amounts of cDNAs encoding either 2B, 2C, 2B + 2C, 2BC, or P1-2A together with lacZ and subsequently infected with vTF7-3. In the left panel, cDNA constructs contain the EMCV IRES and a His-tag upstream of the HAV genes; in the right panel, cDNAs containing neither of these elements were used for coexpression with a similar construct encoding lacZ. At 24 h postinfection, the medium was removed, and the cells were extracted by freeze-thawing. Using PNPG as substrate, the reporter enzyme activity was determined intracellularly and extracellularly and expressed in units β -galactosidase (1 U = 1 Δ OD_{405nm}/10 min). The lacZ activity in the medium was expressed as percentage of the total activity. Error bars indicate the confidence interval of measurements from duplicate plates and duplicate assays.

that any of the HAV P2 proteins might directly affect the reporter enzyme activity (data not shown). By comparing the medium of the different coexpression assays, a marked increase in lacZ activity was detected for 2B- and 2BC-expressing cells indicating that 2B and 2BC affect membrane permeability (Fig. 5, bottom, lanes 1, 3, 7, and 9). This effect was independent of the presence or absence of the IRES element or the His-tag because it was observed when either the pTM or pET constructs were expressed (compare left and right bottom panels of Fig. 5). The lacZ activity in the medium of cells expressing the pET constructs was lower than that of pTM-expressing cells, which is due to the fact that for normalization, the extracellular lacZ activity was taken as percentage of the total activity which was higher for the

pTM-expressing cells (see top panels). Although tightly associated with membranes, HAV protein 2C had no effect on the leakiness of mammalian membranes. In contrast, the permeabilizing effect of 2B seemed to be abrogated by 2C (compare lanes 1 with 4 or 7 with 10), which could possibly be a sign for the physical interaction of 2B and 2C. Taken together, these results indicate that the individual expression of HAV proteins 2B or 2BC specifically affects membrane integrity and causes an increase in membrane permeability.

DISCUSSION

Bacterial as well as mammalian expression systems have been used to assess biochemical features of indi-

vidual viral proteins and have allowed in part to relate the observed characteristics to their biological function or functions in the infected cell (Barco and Carrasco, 1998; Doedens and Kirkegaard, 1995; Teterina *et al.*, 1997b; van Kuppeveld *et al.*, 1997a). For HAV, recombinant systems are particularly useful to study proteins 2C and 2BC because these polypeptides as well as most other non-structural proteins of HAV have escaped detection in HAV-infected cells. Transient high-level expression with the aid of vaccinia virus vTF7-3 had shown that structural rearrangements of mammalian membranes were induced after expression of poliovirus proteins 2C and 2BC (Aldabe *et al.*, 1997; Cho *et al.*, 1994). Similar effects were observed with the homologous proteins of HAV (Teterina *et al.*, 1997b). However, in this report, reorganization of membranes was not correlated with biochemical features of the HAV proteins tested, nor was the effect of HAV 2B assessed. In initial experiments using various recombinant systems, we had tested membrane binding and found that none of the HAV P2 proteins are posttranslationally modified, despite of potential glycosylation sites (data not shown). Here, we provide biochemical evidence that none of the investigated HAV P2 proteins is cytosolic when expressed in mammalian cells. Based on their exclusive solubility with detergent, both proteins 2C and 2BC were characterized as integral membrane proteins. According to the definition of Fujiki *et al.* (1982) HAV protein 2B showed properties of a peripheral membrane protein and seemed to be located within membrane vesicles. This distinct feature of HAV 2B might be suggestive for function or functions that are temporally and spatially distinct from its precursor 2BC.

As shown by an increase in the extracellular levels of a reporter gene activity, both HAV proteins 2B and 2BC specifically altered membrane permeability of the expressing mammalian cell, whereas HAV 2C did not influence the release of intracellularly expressed β -galactosidase. As determined by the influx of hygromycin, similar effects on membrane traffic were obtained when poliovirus proteins 2B or 2BC were expressed in the absence of other viral proteins (Barco and Carrasco, 1998; Doedens and Kirkegaard, 1995). When the cytoplasmic calcium concentration was determined as a marker for vesiculation and membrane permeability, somewhat different effects of poliovirus proteins 2B, 2C, and 2BC were noticed. Only poliovirus protein 2BC augmented the level of free Ca^{2+} , whereas both 2B and 2C alone were ineffective (Aldabe *et al.*, 1997). Although not tested for 2BC, it was shown that coxsackievirus protein 2B seemed to be sufficient to modify membrane permeability and to increase the influx of extracellular Ca^{2+} and release of Ca^{2+} from endoplasmic reticulum stores (van Kuppeveld *et al.*, 1997a). Based on their localization to the viral replication complex, both proteins 2B and 2BC have been postulated to induce membrane vesicles, which might be a prerequisite for the formation of the

viral replication complex (Bienz *et al.*, 1987; Aldabe and Carrasco, 1995). To this point, none of the abovementioned observations on membrane rearrangement and permeability can be related to the replication of HAV *in vivo* because generally no gross effect on cell morphology (e.g., vesiculation, secretory pathway) and protein synthesis has been observed in HAV-infected cells (Gauss-Müller and Deinhardt, 1988). Possibly due to the very low amounts of HAV nonstructural proteins, membrane alterations are not detectable in infected cells.

In addition to the involvement of 2B and 2BC in the formation of the viral replication complex, the 2B- and 2BC-induced change of membrane permeability might be essential for the release of nonlytic picornaviruses. Induction of cell lysis and pore formation have been hypothesized to facilitate the release of cytolytic viruses (van Kuppeveld *et al.*, 1997a). It is intriguing to relate this observation on 2B of coxsackievirus to the distinct infectivity of recombinant HAV variants (Emerson *et al.*, 1993). Chimeric viruses produced from wild-type infectious viral genomes and carrying the 2B nucleotide sequence of a cell culture-adapted virus spread more rapidly to neighboring cells in culture that was determined by immunofluorescence. Although not directly examined for HAV, these data and our observation on membrane permeability can give support to the notion that HAV 2B and/or 2BC might be involved in viral particles release and that mutations in this genomic region might augment infectivity.

In the infectious cycle of picornaviruses, the functional requirements for individual viral proteins seem to be regulated by the proteolytic liberation of intermediate and mature polypeptides from the polyprotein. A fine tuning of processing can be achieved by both conformational and sequence-specific determinants of the substrates and/or by modification of the proteinase activity through additional components (e.g., viral or host proteins or membranes). In contrast to poliovirus, dipeptide pairs of different sequences can be cleaved by HAV 3C^{pro}, which was shown on synthetic peptides substrates, with the amino acid sequence at the 2B/2C and 2C/3A site being optimal (Jewell *et al.*, 1992). Requirements other than the primary sequence seem to be essential for the regulated cleavage at the different sites in the HAV polyprotein (Dougherty and Semler, 1993; Schultheiß *et al.*, 1994). As noted in a comparative study, rapid 3C-mediated cleavage of the poliovirus polyprotein occurs on membranes and is much less pronounced *in vitro* than *in vivo* (Lawson and Semler, 1992). The authors speculate that the *cis*-like situation that occurs *in vivo* makes proteolysis diffusion independent and more rapid. In other studies, it was shown that both polio and coxsackievirus proteins 2BC are poor substrates for 3C^{pro} *in vitro* (Molla *et al.*, 1994; van Kuppeveld *et al.*, 1996). Interestingly, the cleavage of poliovirus 2BC *in vitro* was improved when 3AB was added to the reaction mixture.

Here, we report a similar finding for cleavage of HAV 2BC. Analyzed *in vivo*, 2BC cleavage efficiency was significantly increased when P3 or 3ABC, instead of 3C^{pro}, was coexpressed. As both 3ABC and 2BC showed the same solubility pattern, it can be hypothesized that the membranous colocalization of 3ABC and 2BC might be a stringent requirement for the efficient cleavage of 2BC. Possibly, in HAV-infected cells where only minute amounts of these polypeptides are detectable (Gauss-Müller, unpublished observations), the necessary local concentration of both proteinase and substrate might only be ensured by their joint localization in or on membranes. In the vaccinia virus system, where the recombinant proteins are produced in large amounts, they possibly interact in a *cis*-like fashion, and the positive effect of 3A on the 2BC cleavage efficiency therefore might be underestimated. In addition, it is attractive to assume that membranous colocalization might be required for enzyme and substrate to acquire optimal conformation for productive cleavage. Taken together, it is tempting to speculate that the substrate specificity for efficient cleavage of 2BC can only be guaranteed by 3ABC, thus ensuring the regulated cleavage of 2BC and targeted liberation of its products.

MATERIALS AND METHODS

Construction of plasmids

To generate pET15b-2B, the 2B sense primer (5' CAT GCC ATG GGA CAT ATG GCC AAA ATT TCT CTT TT 3') and the 2B antisense primer (5' CCG GCT CAG CTC GAG TTA GGA TCC CTG AGT CCT TAA CTC CAT 3') were used for PCR amplification with pT7-HAV1 as template encoding the genome of the attenuated HAV strain HM175 (Harmon *et al.*, 1992). The PCR fragment was restricted with *Nde*I and *Esp*I and ligated into pET15b cut with the same enzymes. pET-2B was generated in the same way as pET15b-2B; however PCR-fragment and the vector were cut with *Nco*I-*Esp*I. pET15b-2BC was constructed similarly as pET15b-2B using the 2B sense primer and the 2C antisense primer (5'-GGC GAG CTC AAG CTT ACT GAG ACC ACA ACT CCA TGA A-3') for PCR amplification. pET-2BC was constructed by cutting pET15b-2BC with *Xba*I-*Nde*I, followed by the Klenow reaction and religation. To create pEXT7-2B, the 2B PCR fragment was restricted with *Nco*I and *Xho*I and ligated into pEXT7 cut with the same enzymes (Wilk *et al.*, 1992). Construction of pET-2C and pEXT7-lacZ was described before (Kusov *et al.*, 1998). To generate pTM1-2B, and pTM1-2C, pET15b-2B and pET15b-2C were cut with *Esp*I, incubated with Klenow polymerase, and then restricted with *Nco*I. Vector pTM1 was first restricted with *Xho*I, followed by the Klenow reaction and then with *Nco*I. For the construction of pTM1-2BC, pET15b-2BC was cut with *Hind*III, followed by the Klenow reaction and a second cut with *Nco*I. The 2BC fragment was ligated with pTM1

treated as described above. After ligation of the insert with the vector, cDNA constructs were created that encode the respective HAV gene products preceded by a 6 x His tag and the EMCV IRES. The sequences produced by PCR were verified by DNA sequencing. Construction of pEXT7-P1-2A and pGEM2-HM-P3, -3ABC, -3BCD, -3CD, -3BC, and -3C is described elsewhere (Probst *et al.*, 1998). For the construction of pGEM1-lacZ, pGEM1 was digested with *Hind*III and *Xmn*I. The resulting 1.8-kb fragment was ligated with a 4.9-kb fragment obtained after cutting pCH110 (Pharmacia) with the same restriction enzymes. To generate pET-P1-2A, the VP4 sense primer (5'-AAC GCT AGC AGA CAA GGT ATT TTC CAG ACT GTT-3') and the 2A antisense primer (AGA TGA TCA TTA TTG TGA AAA CAG TCC CTT CAT-3') were used for PCR amplification with pEXT7-HM/HM-P1-2A(E/S) as template (Probst *et al.*, 1996). The PCR fragment was restricted with *Nhe*I and *Bcl*I and inserted into pET11a cut with *Nhe*I and *Bam*HI.

Eukaryotic expression

In vitro transcription/translation was performed according to the instructions of the manufacturer (TNT, Promega) and as described previously (Schultheiß *et al.*, 1994). Transient expression in COS7 cells was as described using the vaccinia/T7 system (Fuerst *et al.*, 1986; Probst *et al.*, 1997).

Solubilization studies

COS7 cells were transfected with 1 μ g of cDNA of pET-2C, pET-2B, pET-2BC, or the pGEM1 constructs containing the HAV P3 region or parts of it. After infection with vaccinia virus vTF7-3 and incubation for 24 h, transfected COS cells were scraped off the plate, pelleted, suspended in 1.2 ml of PBS, and disrupted by three cycles of freeze-thawing. After centrifugation at 14,000 g for 10 min, the PBS-soluble supernatant was removed, and equal aliquots of the pellet were solubilized in the different buffers by shaking for 10 min at room temperature. The extracts were centrifuged as described above, and supernatants and pellets were analyzed by SDS-12% PAGE and immunoblotting using anti-2B directed against a synthetic peptide, anti-2C3A* (Jürgensen *et al.*, 1993), anti-3C (Schultheiß *et al.*, 1994), and anti-3D (Tesar *et al.*, 1994).

Studies on membrane permeability

COS7 cells (3×10^5) grown on 10-cm² wells were transfected with 0.5 μ g of HAV-encoding and 0.5 μ g of lacZ-encoding cDNA. After infection with vTF7-3, the medium (2 ml OptiMEM) and the cell layer were harvested in 200 μ l PBS-Tween, and the β -galactosidase activity of two independent transfection experiments was assayed in duplicate essentially as described previously (Sambrook *et al.*, 1989). LacZ activity was measured by

the increase of optical density at 405 nm using *para*-nitrophenyl- β -D-galactopyranosid as substrate. After calculation of the mean values (X) and the standard deviations (σ), the confidence interval (CI) was deduced by the following formula ($CI = X \pm 1.96 * \sigma/\sqrt{n}$), which represented a probability of error of 5%.

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