VIROLOGY

Conformational changes in the hepatitis A virus capsid in response to acidic conditions

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Low pH values encountered during uptake of viruses by receptor-mediated endocytosis have been shown to expose hydrophobic residues of many viruses and result in viral conformational changes leading to uncoating of the viral genome. An assay for hydrophobicity utilising the non-ionic detergent Triton X-114 was established, making use of metabolically-labelled hepatitis A virus (HAV). In this assay, hydrophilic proteins interact with the aqueous (buffer) phase, while hydrophobic proteins interact with the Triton (detergent) phase. HAV particles interact with the aqueous phase at neutral pH, whereas, under acidic conditions, HAV was found predominantly in the detergent phase. This indicates that the capsid of HAV undergoes conformational changes rendering the particle more hydrophobic under acidic conditions. A further two conformational changes were found in HAV on exposure to low pH, as detected by changes in buoyant density in CsCl gradients. These were maturation of provirions to virions and the formation of dense particles. These results may have implications for uncoating of the HAV RNA genome, and these conformational changes could represent intermediates in the viral uncoating process.

Introduction

Hepatitis A virus (HAV) is the sole member of the hepatovirus genus within the picornavirus family [1, 2]. Picornaviruses enter the host cell by the use of specific receptors, and the presence of a saturable HAV receptor on several cell lines has recently been demonstrated [3]. Early studies suggested that HAV entry into cells requires a cell-surface complex involving phospholipids, proteins and galactose [4], and recently two surface glycoproteins have been identified on African green monkey kidney cells as receptors for HAV [5, 6]. Attachment of HAV to susceptible cells is strongly dependent on the presence of calcium ions or acidic conditions [3, 7, 8]. Both calcium ions and low pH have been shown to increase the strength of the HAV–receptor interaction, resulting in decreased elution of bound particles from the cell surface [8].

After receptor binding, many viruses are thought to enter cells *via* coated pit regions on the cell surface. The mechanism of entry *via* coated pits and subsequent endocytosis seems to rely on the acidic pH in the vesicles because of two observations: (i) studies with lysosomotropic agents or weak bases, which raise the pH of endosomes to near neutrality, inhibit entry of many viruses; and (ii) viruses and toxins thought to enter cells *via* acidic vesicles require low pH to interact with membranes, including poliovirus (PV), the prototype of the picornavirus family [9].

The mode of entry of PV is thought to involve the formation of altered particles (A-particles) which have lost capsid protein VP4 and have a more hydrophobic exterior [9–11]. These particles are non-infectious and can be found eluting from cells after the viral attachment step [12–15]. A-particles are presumably capable of interaction with the endosomal membrane, facilitating release of viral RNA into the cytoplasm, by virtue of their hydrophobic nature.

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Preliminary experimental evidence available on the entry of HAV into susceptible cells suggests that HAV entry into cells occurs *via* receptor-mediated endocytosis, as HAV infection can be prevented by treatment of

cells with monensin, ammonium chloride, amantadine, dansylcadaverine and methylamine, agents that prevent endosomal acidification [16, 17]. In direct contrast with PV infection, the majority of HAV particles eluted from cells remain infectious and particles with altered sedimentation coefficients do not appear to be formed during receptor binding [8]. However, as the HAV VP4 molecule is thought to be extremely small [18] compared with that of most picornaviruses, its loss from virions would not necessarily be expected to lead to a detectable change in sedimentation coefficient. Therefore, it remained possible that hepatitis A virions do undergo conformational changes when exposed to the acidic conditions, but these cannot be detected by sedimentation on sucrose density gradients.

Alteration of HAV leading to uncoating of viral RNA is presumed to occur either within the environment of endosomes originating from clathrin-coated vesicles [16, 17], which are known to become acidic over time [19], or within vesicles derived from non-clathrin-coated regions of the cell surface, which can also become acidified [20]. Although HAV has been reported to be extremely stable under conditions of pH 1–11 at 20°C [21, 22] the effect of moderately low pH values such as those found within the endosomal pathway (pH 6.0–4.8), at 37°C on HAV particles, has never been examined. HAV particles may undergo subtle conformational changes on exposure to these conditions. Part of these conformational changes may cause HAV particles to become more hydrophobic, facilitate interaction with the endosomal membrane, and thus mediate viral uncoating.

This study set out to determine, by examining changes in hydrophobicity and buoyant density, whether HAV undergoes conformational changes in response to acidic conditions, such as those found within the endosomal pathways of cells [19, 20].

Materials and methods

Virus and cells

The strain of HAV used in these experiments is known as HM175A.2 and is a cytopathic variant which grows relatively rapidly in cell culture [23, 24]. Virus was propagated in BS-C-1 cells and purified by discontinuous glycerol/sucrose gradient ultracentrifugation as described by Bishop and co-workers [25].

Production of radiolabelled HAV

HAV was metabolically labelled with ^{35}S -methionine and ^{35}S -cysteine, as described elsewhere [26]. BS-C-1 cells were infected with HAV and incubated in culture medium for 12 h at 37°C. The medium was then removed and replaced with methionine- and cysteine-free medium and incubated for a further 3 h at 37°C, before the addition of medium containing (/ml) *c.*

150 μCi of $\text{L-}^{35}\text{S}$ -methionine and 30 μCi of $\text{L-}^{35}\text{S}$ -cysteine for incubation for a further 3 h at 37°C. After this time, label was removed and replaced with normal culture medium until the cells were harvested at 24–36 h after infection. Labelled virus particles were purified by linear sucrose density gradient ultracentrifugation.

Linear sucrose gradient ultracentrifugation

Radiolabelled HAV particles were purified by sucrose gradient ultracentrifugation [27]. Briefly, sucrose gradients of 10–30% w/v were prepared in Beckman SW41 tubes and allowed to equilibrate. Samples of cell lysate were layered on top and centrifuged for 2.5 h at 150 000 *g* at 4°C. Fractions were collected from the bottom of the tube and the first three or four fractions of 1 ml were pooled.

Hydrophobicity assay

A stock solution of Triton X-114 (Sigma) 4% w/v was prepared by dissolving 40 g of detergent in 1000 ml of 20m M sodium phosphate buffer (pH 7.5) containing 140 mM NaCl, by stirring overnight at 4°C. The resulting solution was then incubated at 37°C until two phases appeared, according to the method of Madhus and co-workers [9]. The upper phase, which consisted of buffer, was discarded and replaced by fresh buffer and this procedure was repeated three times. After the final condensation, the pre-treated Triton X-114 was made up to 200 ml in the same buffer.

Radiolabelled HAV was mixed with buffered saline of various pH values in siliconised microfuge tubes, before the addition of Triton X-114 at 4°C, by the method described by Skern *et al.* [28]. This solution became homogeneous with gentle mixing at temperatures of $\leq 18^\circ\text{C}$. Tubes were then placed at 37°C for 30 min to allow the phases to separate and centrifuged for 2 min at room temperature. The aqueous phase was removed and radioactivity in both phases was determined after precipitation with trichloroacetic acid.

Trichloroacetic acid precipitation

Samples of detergent or aqueous phase were spotted on to 25-mm disks of Whatman GF/A glass fibre paper and allowed to dry at 22°C. Disks were placed in a bath of ice-cold trichloroacetic acid (TCA) for 10 min, washed three times in cold TCA 5%, then washed once in cold absolute ethanol for 10 min. The disks were air-dried and covered with 3 ml of Insta-gel liquid scintillation cocktail (Packard Instrument, USA). After incubation at 22°C for 2–4 h, the amount of radioactivity was measured in a Packard liquid scintillation spectrophotometer.

Isopycnic CsCl gradient ultracentrifugation

CsCl was diluted to a buoyant density of 1.33 g/cm³. Gradients were pre-formed for 6 h at 18°C in a Beckman SW41 rotor at 150 000 *g*. A 1-ml volume of lysate (of varying pH) was layered on to the gradient and centrifuged for a further 18 h under the same conditions. Fractions of *c.* 0.6 ml were collected from the bottom of the gradient. The buoyant density of fractions was determined from their refractive index.

Detection of HAV RNA

Viral positive-stranded RNA was detected in CsCl gradient fractions by dot-blot hybridisation, as described by Anderson and Ross [18]. Briefly, HAV fractions were mixed with FSSC (6.1 M formaldehyde, 3 M NaCl, 0.3 M sodium citrate, pH 7.0), heated at 65°C for 15 min and applied to nitrocellulose membranes. Filters were then probed with ³²P-labelled negative-strand RNA probes, and exposed to pre-flashed film at -70°C. Densitometry was performed with the BioRad system and intensity values (adjusted for local background) were calculated as OD × mm². Values are relative and are comparable between gradients, but absolute values were not quantified. The accuracy in values of fractions 13 and 14 of the neutral pH gradient (Fig. 2b) was decreased due to the strong intensity of the signal which led to superimposition of the two signals (Fig. 2a). This analysis was kindly performed by Dr P. Gribbon at the University of Manchester.

Results

Effect of low pH on the hydrophobicity of HAV

As Triton X-114 is a mild detergent, native protein structure should be maintained during detergent treatment [29]. Bordier [30] demonstrated that hydrophilic proteins partition to the aqueous (top) phase and hydrophobic proteins are found in the detergent (bottom) phase. To determine whether HAV particles may become more hydrophobic and thereby facilitate interaction with the endosomal membrane, the ability of ³⁵S-labelled HAV to enter the detergent phase at pH 4.8, pH 5.5, or pH 7.6 was examined (Table 1; Fig. 1a). At neutral pH most of the radioactivity remained in the aqueous phase (92%), whereas at pH values of 5.5 or

4.8, increasing amounts of labelled virus were found in the Triton X-114 phase (85% and 91% respectively, compared with 8% at pH 7.6). These data indicate that HAV exposes hydrophobic domains at acidic pH.

This trend was confirmed in a further three experiments (Fig. 1b–d), where the effect of different pH ranges on the hydrophobicity of HAV particles were examined and compared with those of the initial experiment. In the first of these hydrophobicity assays (Fig. 1b) a fresh preparation of radiolabelled HAV was used, and this viral pool was exposed to pH values identical to those outlined in Table 1. Less virus was converted to the hydrophobic form at pH 5.5 and 4.8 than in the initial experiment. Presumably these differences were due to the use of a different viral pool and experimental variation.

The next purified viral pool was exposed to pH 7.0, 6.5 and 5.0 (Fig. 1c); *c.* 30% of the viral pool partitioned in the hydrophobic phase at pH 7.0, increasing to *c.* 40% at pH 6.5 and 65% at pH 5.0. These data further confirmed the trend that exposure of HAV viral particles to low pH resulted in an increase in hydrophobicity. A final preparation of radiolabelled viral particles was exposed to pH 7.0, 6.0 or 5.0 and partitioned in Triton X-114 before analysis (Fig. 1d); *c.* 30% of virus was found to be hydrophobic at pH 7.0, increasing to *c.* 60% at pH 6.0 and 65% at pH 5.0. These data indicate that HAV particles become more hydrophobic at low pH, presumably by the exposure of previously hidden hydrophobic domains.

Comparison of the numbers of hydrophobic particles at pH 7.6 and pH 7.0 (comparing the starting levels of hydrophobic virus in Figs. 1a and b with those of Figs. 1c and d) would further indicate some changes in hydrophobicity on lowering the pH from 7.6 to 7.0. This was confirmed when the hydrophobicity at pH 7.6 was directly compared with that at pH 7.0. Furthermore, the conversion of HAV to a hydrophobic particle occurred to a similar degree between pH 4.8 and pH 6.0 (Fig. 1a and d). In no instance was the transfer of HAV to the detergent phase complete, whether at pH 5.0 or pH 4.8, which are the most acidic pH values expected to be encountered in the endosomal pathway of cells [19, 31, 32].

Effect of low pH on HAV buoyant density

To determine whether low pH-induced conformational changes in the HAV capsid corresponded to conformational changes detectable by other means, isopycnic CsCl gradient ultracentrifugation was used to examine virus samples exposed to low (pH 4.8), neutral (pH 7.6), or high (pH 10.5) pH at 37°C for 1 h (Fig. 2); 19 fractions of *c.* 0.6 ml were collected from the bottom of centrifugation tubes. HAV RNA-containing particles in fractions were detected by dot-blot hybridisation

Table 1. Hydrophobicity of radiolabelled HAV particles, determined by partitioning into Triton X-114

pH value	Average cpm in		Percentage in phase	
	Aqueous phase*	Detergent phase*	Aqueous	Detergent
7.6	8274 SD 5	753 SD 57	92	8
5.5	1203 SD 22	6817 SD 171	15	85
4.8	778 SD 45	8036 SD 231	9	91

*Values are presented as average cpm and SD of duplicate samples.

Fig. 1. Percentage hydrophobicity percentage of counts in the detergent phase of HAV labelled with ^{35}S -methionine and ^{35}S -cysteine determined by partitioning into Triton X-114 after exposure to various pH values. These results demonstrated an increase in hydrophobicity of HAV when the pH became acidic. (a), data from Table 1; (b–d), data obtained from three separate experiments.

(Fig. 2a). The relative signal intensity was quantified by densitometry (Fig. 2b).

HAV virions (containing predominantly VP2 and little VP0) were expected to band at *c.* 1.34 g/cm^3 , whilst immature HAV provirions (containing predominantly VP0 and little VP2) were expected at *c.* 1.32 g/cm^3 [27, 33]. At neutral pH HAV RNA-containing particles were found mainly in fractions 13–15 (Fig. 2a and b), corresponding to a buoyant density of *c.* 1.32 g/cm^3 (Fig. 2c). This indicates that the viral preparation used contained a high proportion of immature HAV particles.

After exposure to acidic pH, HAV RNA-containing particles were predominantly found in fractions 2 and 3, with lower levels of RNA spread over fractions 10–

15, in contrast to the HAV particle profile at neutral pH (Fig. 2a and b). The more dense particles in fractions 2 and 3 had a buoyant density of $>1.4 \text{ g/cm}^3$, whilst the particles in fractions 8–12 had buoyant densities *c.* 1.34 g/cm^3 (Fig. 2c). This indicates that exposure to acidic conditions resulted in the conversion of immature provirions to virions and the formation of very dense ($>1.4 \text{ g/cm}^3$) HAV particles.

The HAV RNA-containing particle profile after exposure to alkaline conditions appeared intermediate to that observed on exposure of virus to acidic or neutral conditions (Fig. 2a and b). Whilst a small number of dense HAV RNA-containing particles were found in fractions 1 and 2, the majority were found in fractions 11–13 and had buoyant densities only slightly greater

Fig. 2. Effect of pH values 4.8, 7.6 and 10.5 on the buoyant density profile of HAV particles in CsCl gradients. Fractions of *c.* 0.6 ml were examined for (a) positive-stranded HAV RNA by dot-blot hybridisation and with (b) signal intensity measurement ($\square$ acidic, $\diamond$ neutral, $\circ$ alkaline) and (c) buoyant density determination from the refractive indices. (Note that much of fraction 1 of the acidic gradient had been pooled with fraction 2.)

than those found in fractions 13–15 at neutral pH. This indicates that some conversion of immature provirions to more mature particles, intermediate in nature to virions, may have been occurring. In addition, some RNA was found in fractions 17 and 18, corresponding to less dense regions of the gradient. The identity of these latter particles is unknown, but they may represent particles that are partially disrupted under the conditions used.

Similar HAV density profiles were detected on exposure of virus to acidic conditions. Dense HAV particles and a conversion of immature provirions to mature virions was seen after CsCl density gradients of particles exposed to pH values in the range 3.0–5.5. The neutral pH particle profile remained similar between pH values 7.0 and 8.5, thus differing somewhat from the data presented in Fig. 1, where conformational changes could be detected between pH 7.6 and 7.0. This difference could have a number of explanations and various hypotheses are being explored.

Discussion

HAV entering cells *via* the endosomal pathway would be exposed to conditions of acidic pH [16, 17, 20]. Therefore low pH might be expected to increase the hydrophobicity of viral particles, thus mediating interaction with the endosomal membrane and release of the viral RNA into the cytoplasm. Such a mechanism has been proposed for uncoating of PV, the type member of the picornavirus family [9]. Hydrophobic particles of PV, known as A-particles, are non-infectious and have lost capsid protein VP4, resulting in particles with altered sedimentation coefficients [34]. Altered PV particles are detected eluting from cultured cells early in infection, contrasting with HAV infection, where eluting particles retain infectivity and do not have any detectable changes in sedimentation coefficient [8]. This paper documents for the first time changes in HAV on exposure to acidic conditions, including a gain in hydrophobicity, providing a rationale for the HAV uncoating process.

The data presented here indicate that exposure of HAV to low pH causes conformational changes in the capsid, resulting in more hydrophobic particles, as detected by a hydrophobic partition assay. In no instance was the transfer of HAV particles from the aqueous to the detergent phase complete, indicating that not all particles were capable of undergoing the hydrophobic transition. This was despite the use of pH values of 4.8, representing the lowest pH values encountered in the endosomal/lysosomal pathway of cells [19, 31, 32]. Transfer of PV to the Triton X-114 detergent phase occurs half-maximally at pH 6.5 [9], whilst similar studies on the partition of human rhinovirus type 14 in Triton X-114 showed approximately half-maximal partition into the detergent phase at close to pH 3 [28]. However, the latter value is lower than that expected to be encountered during receptor-mediated endocytosis, and values this low were not examined.

HAV particles that do not undergo the hydrophobic transition and remain in the aqueous phase may be unable to uncoat. Alternatively, they may be empty viral capsids in the viral pool used for these studies.

Empty capsids, lacking viral RNA, may not be able to undergo the same conformational changes as infectious HAV particles. A further possibility is that low pH on its own is not capable of reproducing conditions encountered by viruses during cell infection, and that more particles would undergo the hydrophobic transition if the cellular environment could be reproduced more faithfully. Further research utilising the assay described here can be used to examine this in more detail.

The hydrophobic changes induced in PV during cell entry are enhanced by receptor binding as well as acidic conditions [9]. The contribution of HAV receptor binding to changes in hydrophobicity likewise could be examined by modifying the methods employed in the present study. Interestingly, HAV receptor binding has been shown to be enhanced under acidic conditions, confirming that some form of conformational change can occur under these conditions to enhance virus–receptor interactions [8]. Whether this relates to the virus or receptor, and how this relates to subsequent virus uncoating remains unclear.

When HAV particles were treated to acidic conditions, two conformational changes could be detected by alterations in viral buoyant densities after centrifugation over isopycnic CsCl gradients. Exposure of virus particles (original density of 3.2 g/cm³ at neutral pH) to low pH led to the formation of two further forms of HAV particles. The first type of particles had densities only slightly greater than those found at neutral pH (3.4 g/cm³ compared to 3.2 g/cm³) and formed a continuous spectrum with the original particles in the gradient. The density of these particles is consistent with those of mature HAV virions [27, 33], and is thought to occur *via* the autocatalytic cleavage of VP0 to VP2 which occurs under these conditions, converting immature provirions to mature virions [35]. Furthermore, there is direct preliminary evidence that acidic conditions lead to a more rapid autocatalytic cleavage of VP0 to VP2 than that occurring at neutral pH (Bishop and Anderson, unpublished observations). In the light of an increase in HAV hydrophobicity between pH 7.6 and 7.0 (Fig. 1) not correlating with the detection of particles with high buoyant density, the hydrophobic properties of purified preparations of HAV provirions and virions will be compared.

The second type of particle formed under acidic conditions has a dramatically altered density of *c.* 1.4 g/cm³ and was found at the bottom of CsCl gradients. Dense HAV particles have been detected previously in cell culture lysates [36, 37] and clinical samples [38–40], and are thought to result from a more open capsid configuration. Data on the infectivity of these three forms of HAV indicate that maturation of HAV provirions (1.32 g/cm³) to virions (1.34 g/cm³) results in an increase in infectivity

Fig. 3. Schematic representation of a working hypothesis of the entry and uncoating process of HAV in susceptible cells. Virions attach to cellular receptors (A) and are internalised by receptor-mediated endocytosis into endosomes (B). The endosomes are rapidly acidified due to the presence of an ATPase-dependent proton pump in the vesicle membrane. The first conformational change is caused by the autocatalytic cleavage of any VP0 molecules in viral capsids to VP2, creating mature virions. On exposure to low pH, virions are then converted to a more hydrophobic form (C), detectable by partitioning into Triton X-114. These particles are formed by the exposure of formerly hidden hydrophobic residues of viral capsid proteins and renders particles permeable to CsCl. This hydrophobic conformational change results in particles capable of being uncoated to release the viral RNA genome (D). Viral replication then ensues in the cytoplasm, leading to the production of intermediates in the viral morphogenic pathway (E), i.e., provirions and procapsids. Maturation of provirions to virions (F) finally completes the viral replication cycle, but this process is generally not completed and a full spectrum of immature viral particles is released from cells [25].

[27, 35]. However, dense HAV particles do not appear to be infectious (Bishop, unpublished observations).

Whilst the acidic conditions encountered during endocytosis may play a role in the entry of picornaviruses into cells, some more recent studies have questioned the role of vesicle acidification in the entry of PV into cells [41, 42]. Therefore, hypotheses involving the role of low ionic strength or divalent cations within endosomal vesicles have been put forward for PV uncoating [43]. The ability of these conditions to cause conformational changes in HAV can now be examined by modifications of the methods described here.

In conclusion, the results presented here identified three conformational changes in HAV in response to acidic conditions: (i) conversion of immature to mature virions, (ii) production of dense HAV particles and (iii) the generation of a more hydrophobic capsid structure. Preliminary evidence suggests that none of these changes is reversible (Bishop and Anderson, unpublished data). Clearly more work is needed to characterise these conformational alterations in more detail. A working model of how these changes might mediate viral uncoating is provided in Fig. 3.

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