Trypsin is associated with the rotavirus capsid and is activated by solubilization of outer capsid proteins

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The rotavirus capsid is made up of three concentric protein layers. The outer layer, consisting of VP7 and VP4, is lost during virus entry into the host cell. Rotavirus field isolates can be adapted to high-titre growth in tissue culture by treatment with trypsin and by supplementing the culture medium with trypsin, which cleaves VP4 into two fragments, VP8* and VP5*. It is known that protease inhibitors reduce the replication of rotavirus in vitro and in vivo and also diminish disease symptoms in a mouse model. To clarify the molecular basis of these observations, a series of assays were conducted on purified rotavirus particles grown in the presence of trypsin. Results of HPLC and mass spectrometry followed by N-terminal sequencing showed that viral particles contain molecules of trypsin. When associated with triple-layer particles (TLPs), trypsin is inactive and not accessible to protease inhibitors, such as aprotinin. When the outer layer is solubilized by calcium-chelating agents, VP5*, VP8* and VP7 are released and the associated trypsin is activated, allowing cleavage of the viral capsid proteins, as well as other exogenous proteins. It is shown that addition of trypsin inhibitors significantly reduces synthesis of viral mRNA and viral proteins in cells and has a major inhibitory effect if present when virus enters the cell. These data indicate that incorporation of trypsin into rotavirus particles may enhance its infectivity.

INTRODUCTION

Rotaviruses, forming a genus of the family Reoviridae, are a leading cause of severe gastroenteritis in young children worldwide (Parashar et al., 2003). Rotavirus entry into cells may take place either by endocytosis followed by membrane vesicle solubilization (Chemello et al., 2002) or by direct penetration of the cell membrane (Kaljot et al., 1988). This process is associated with solubilization of the outer capsid proteins, most likely due to the low calcium concentration in the cytoplasm (Ruiz et al., 2000).

Rotaviruses particles have a complex architecture of three concentric capsid layers (Prasad et al., 2001). The innermost capsid layer, composed of VP2, is surrounded by the intermediate capsid layer composed of VP6. The outermost layer is composed of the glycoprotein VP7 and the spike protein VP4. VP7 is a calcium-binding glycoprotein that interacts with integrins during the final virus adsorption step (Lopez & Arias, 2004).

Trypsin treatment of viral particles modifies the structure of VP4 and is correlated with an enhancement of infectivity by a mechanism that is not yet clearly established (Crawford et al., 2001). Conversely, protease inhibitors can prevent the development of rotavirus-induced diarrhoea and reduce virus replication in cell culture (Katyal et al., 2001; Vonderfecht et al., 1988). The two trypsin cleavage products of VP4 (VP8* and VP5*) remain associated with the virion (Dormitzer et al., 2004). VP5* contains a putative fusion domain and VP8* mediates initial cell attachment (Lopez & Arias, 2004).

In this study, we show that trypsin molecules are tightly associated with the virion. Trypsin activity is inhibited when associated with virions, but is activated upon outer capsid layer solubilization. Inhibition of virus-associated trypsin by addition of protease inhibitors to cell culture medium reduces primary transcription of the genome, and thus virus replication.

METHODS

Cells and virus. The RF strain of rotavirus (G6, P[1]) was propagated in monkey kidney MA104 cells in Eagle’s minimum essential medium (EMEM) supplemented (trypsinized; TR) or not (non-trypsinized; NTR) with 0.5 μg trypsin ml−1 (type IX; Sigma-Aldrich) and also with 600 μg penicillin and 100 μg streptomycin

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ml⁻¹ (Biovalley). Infections were carried out at an m.o.i. of 0.5 p.f.u. per cell in the presence (0.5 µg ml⁻¹) or absence of trypsin. Viral infectivity was titrated by plaque assay (Poncet & Cohen, 1989) or by immunofluorescence (IF) as described previously (Ciarcia et al., 1994).

**Purification of rotavirus triple- (TLPs) and double- (DLPs) layer particles.** Rotavirus-infected MA104 cells were incubated for 4 days at 37 °C and frozen when a complete cytopathic effect (CEP) was reached. The thawed tissue-culture suspension containing the virus and cell debris was subjected to ultracentrifugation for 60 min in a 45Ti rotor at 90,000 g at 4 °C. The supernatant was discarded and the pellet treated with Vertrel XF as described by Mendez et al. (2000). The aqueous phase was then mixed with CaCl₂, adjusted to obtain a refractive index of 1.369 and centrifuged for 16 h at 80,000 g at 4 °C. The upper (TLPs) and lower (DLPs) bands were collected separately and each centrifuged in CsCl again as described above. Particles were kept suspended in CaCl₂ at -4 °C and salt was removed by a spin column (Sephadex G25; Pharmacia).

**Detection of trypsin and trypsin activity.** Trypsin activity was analysed by zymogram assay or SDS-PAGE. A combination of 1 µg BSA, 1 µg trypsin inhibitor (TIU) and 1 µg aprotinin [4-4 tripeptide amide (TIA)] was added to 4 µg desalted TLP or DLP in 20 mM Tris/HCl, pH 7.4, 50 mM NaCl and 100 µM CaCl₂, and incubated for 15 min at 37 °C. EGTA was added before incubation but after addition of BSA or aprotinin. Samples, in Laemmli sample buffer without reducing (2% SDS, 10% glycerol, 0.025% bromophenol blue) or reducing (plus 700 µM 2-mercaptoethanol) conditions, were incubated at room temperature for 5 min and analysed by PAGE. PAGE was performed using the NuPage system (Invitrogen), in Tris–MES (pH 7) running buffer as recommended by the manufacturer. Following electrophoresis, gels were stained with 1% Coomassie blue R-250. Gels were destained (Silver Stained Plus kit; Bio-Rad). Zymogram analysis was performed in Novex 4–16% gels (Invitrogen) with then silver-stained (Silver Stained Plus kit; Bio-Rad). Sequencing and mass spectrometry.

Peptides present in fractions collected from RPLC analysis were characterized by N-terminal sequencing using Edman chemistry, with a Perkin-Elmer Procise 494 HT protein sequencer. Proteins were identified by searching amino acid sequence databases using the MS-Search program (http://prospector.ucsf.edu/).

**Rotavirus infection and inhibition assays.** For viral infection, 5 x 10⁵ MA104 cells were plated in 6-well plates, incubated for 72 h and then washed three times with EMEM and incubated for 4 h with EMEM. Cells were infected with purified rotavirus TR-TLPs (RF bovine strain) at an m.o.i. of 0.5 p.f.u. per cell. Virus adsorption was performed for 1 h at 15 or 37 °C, supplemented or not with 624 TIU aprotinin 1⁻¹ (Sigma-Aldrich). The inoculum was then removed and cells were washed three times with EMEM and incubated for 5 or 18 h with EMEM at 37 °C with 5% CO₂ in the presence of various concentrations of aprotinin (1-625–2496 TIU 1⁻¹).

For preparation of cell lysates, cells were harvested by treatment with trypsin at 5 or 18 h post-infection (p.i.) and washed twice with EMEM by centrifugation at 200 g for 5 min at room temperature. Cell pellets were subjected to three freeze–thaw cycles, resuspended in 1 ml EMEM and clarified by centrifugation at 16,000 g for 5 min. Infectious virus titres were determined by indirect IF assay using MA104 cell monolayers grown in 96-well microtitre plates (Ciarcia et al., 1994).

For detection of rotavirus antigens, cell lysates were prepared at 5 h p.i. using the protocol described above. ELISA was used as described previously (Schwartz-Cornil et al., 2002). To determine the toxicity of protease inhibitors, infected and mock-infected cell monolayers were incubated with 0.1-1% Trypan blue prepared in PBS for 30 min at 37 °C and live cells were counted.

**Preparation of total mRNA and real-time PCR assays.** MA104 cells (1.5 x 10⁵ cells per well) were plated in 24-well plates for 72 h. Cells were infected with RF rotavirus at an m.o.i. of 0.5 p.f.u. per cell for 1 h at 15 or 37 °C. Cells were then washed three times with EMEM and incubated in EMEM containing different amounts of aprotinin (624–624 TIU 1⁻¹) for 3 h at 37 °C. The total RNA in the pellets was extracted using TRizol reagent (Invitrogen). One-tenth volume of the total cellular RNA was heat-denatured at 65 °C for 5 min and used as template for reverse transcription (RT). RT reactions were performed using 50 U Superscript II reverse transcriptase (Invitrogen) at 42 °C for 50 min in the presence of 20 mM Tris/HCl (pH 8.4), 50 mM KCl, 5 mM magnesium acetate, 10 mM DTT, 40 U RNaseOut (Invitrogen) and 12-5 mg random hexanucleotide primers µl⁻¹ (Invitrogen). A 1:125 dilution of the RT reaction was used for quantitative RT-PCR (Q-RT-PCR) analysis.

Primers were generated for Q-RT-PCR amplification of rotavirus RF segment 6 [sense, 5'-GCTTTAAAAGCAAGTCTTCAAC-3' (positions 2–24); antisense, 5'-GTTAATTACCAATCTTCAG-3' (positions 166–188)] and the human cellular housekeeping gene gamma-carboxyglutamate-3-phosphate dehydrogenase (GAPDH) [sense, 5'-GGGCGCCTGGTACACAGGGCTGC-3' (positions 118–141); antisense, 5'-GAGGCCCAGACCTCTCAGTG-3' (positions 383–407)]. Each primer pair was used at a concentration of 150 nM in a 25 µl reaction mixture. Real-time PCR was performed using the Stratagene MX3000p apparatus and the Brilliant SYBR Green Q-PCR Master Mix (Stratagen). The method and the quantification procedure have been extensively described elsewhere (Overbergh et al., 1999). The conditions used for Q-PCR consisted of denaturation for 15 s at 95 °C, annealing for 18 s at 55 °C and amplification for 15 s at 72 °C for 40 cycles, with an initial step of denaturation at 95 °C for 10 min. Following each cycle, accumulation of PCR products was detected by monitoring the increase of fluorescence of the dsDNA-binding SYBER Green reporter dye. Each PCR amplification was performed in duplicate, in optical 96-well reaction plates with optical caps (Stratagen). Analysis and quantification were performed using the Stratagene MX3000p 2.00 software. A range from 1:25 to 1:250 000 dilutions of total cDNA was used to define standard curves for each primer pair and to quantify primer efficiency, which reflects the capacity to amplify small amounts of target. Efficiency of primers for rotavirus gene 6 and the GAPDH gene was measured to be 102-3 and 100-8%, respectively. The amount of gene 6-specific mRNA was related to that of GAPDH gene-specific mRNA in each sample. Standard curves were used to estimate the relative amounts of DNA. Following the PCRs, a melting curve was established in the range of 60 to 95 °C, to identify amplified products by their melting temperature (T_m). No amplification was observed in the absence of template.
RESULTS

Association of trypsin with purified TLPs

Rotavirus virions can be generated in the presence (TR) or absence (NTR) of porcine trypsin. The protein composition of highly purified bovine rotavirus RF virions generated under these different conditions was analysed by HPLC followed by MALDI-TOF analysis. Individually purified TR-TLPs and TR-DLPs from the same virus preparations were incubated overnight with formic acid, subjected to centrifugation and the supernatants processed for HPLC. As shown in Fig. 1, a series of peaks was observed with TR-TLPs but not with TR-DLPs, indicating that the material released by formic acid treatment originated from the outer shell of the TR-TLPs. The dominant peak present in the TR-TLP sample was analysed by MALDI-TOF mass spectrometry and was found to contain a single species having a molecular mass of 23 461 Da, corresponding to that of mature porcine trypsin (23 400 Da). The amino acid sequence of the material present in this peak, IVGGYTXAAN, was determined using Edman sequencing, and corresponds to the N terminus of mature porcine trypsin. We estimated that 10–100 molecules of trypsin were associated with each TR-TLP, based on the number of molecules detected by the Edman reaction, the starting concentration of TR-TLPs and the assumption that all trypsin molecules present on the TR-TLPs. The major peak of TR-TLPs profile are indicated.

Fig. 1. HPLC analysis of TLP and DLP. Rotavirus RF TR-DLPs and TR-TLPs (200 µg each) were incubated overnight in 5% formic acid and then subjected to centrifugation and the supernatants were analysed by HPLC. The resulting chromatograms are shown. The molecular mass (determined by mass spectrometry) and the N-terminal sequence of the species present in the major peak of TR-TLPs profile are indicated.

Trypsin is associated with the rotavirus capsid

thrombin was not detected (data not shown). These results indicate that trypsin molecules are associated with purified rotavirus TR-TLPs.

Trypsin associated with purified TLPs is activated upon solubilization of outer capsid proteins

To determine whether the trypsin associated with TR-TLPs was enzymically active, TR-TLPs were incubated in the presence or absence of BSA at 37 °C for 15 min and then analysed by SDS-PAGE under reducing conditions to estimate the hydrolysis of BSA substrate. When EGTA, a calcium-specific chelating agent, was added to TR-TLPs prior to incubation to solubilize the outer protein shell of the rotaviral capsid (Cohen et al., 1979; Estes et al., 1979), neither VP7 nor VP* were visible on the gel, contrary to the results obtained in the absence of EGTA treatment (Fig. 2a, lanes 2 and 3). Protein hydrolysis was not observed with TR-DLPs in the presence or absence of EGTA (Fig. 2a, lanes 4 and 5). Under these conditions, BSA was not cleaved when incubated with TR-TLPs or TR-DLPs (Fig. 2a, lanes 6 and 8). When EGTA was added to TR-TLPs prior to the addition of BSA, the band corresponding to whole BSA was not visible and neither were the viral proteins VP7 and VP* (Fig. 2a, lane 7). Again, protein hydrolysis was not observed with TR-DLPs (Fig. 2a, lane 9).

Following silver staining of the same gel (Fig. 2b), protein hydrolysis products were revealed in EGTA-treated TR-TLPs (Fig. 2b, lane 3) but not in TR-DLPs, even when they were treated with EGTA (Fig. 2b, lanes 4 and 5). This observation suggested that these additional bands indeed consisted of degradation products of VP5* and VP7. When TR-TLPs were treated with EGTA and then incubated with BSA, additional bands were also observed (Fig. 2b, lanes 3 and 7). These bands were absent when TR-TLPs were incubated with BSA alone (Fig. 2b, lane 6) or when TR-DLPs were incubated with BSA regardless of prior treatment with EGTA (Fig. 2b, lanes 8 and 9). These observations suggest that the additional bands in lane 7 corresponded to VP5*, VP7 and BSA degradation products. It should also be noted that a low molecular mass band was present in all TR-TLP samples (Fig. 2b, lanes 2, 3, 6 and 7), but not in the TR-DLP samples (Fig. 2b, lanes 4, 5, 8 and 9). This band could correspond to the TR-TLP-associated trypsin molecules described above.

These data show clearly that the trypsin molecules are neither free nor active in mature TR-TLPs, as would be expected if the purified virion preparations were contaminated with trypsin from the cell culture medium, but are closely associated with the virus outer layer. Trypsin can be activated only when the TR-TLP outer layer is solubilized, resulting in the degradation of outer capsid proteins VP7 and VP5* and of exogenously added substrates, such as BSA.
Trypsin is not accessible to protease inhibitors in TLPs

To clarify the location of the trypsin within TR-TLPs, we attempted to inhibit its proteolytic activity in vitro by addition of aprotinin, a powerful serine protease inhibitor. After electrophoresis under non-reducing conditions, proteins with protease activity can be visualized on a zymogram as white bands. Using this methodology, trypsin molecules can be observed as a white band migrating with the appropriate molecular mass (Fig. 3a, lane 1) and a minor band, which is most likely due to the non-reducing conditions of the electrophoresis. Proteins without protease activity can be visualized after Coomassie blue staining of the zymogram (Fig. 3a, lane M). Inhibition of trypsin activity by addition of aprotinin results in the disappearance of the white bands correlated with the protease activity of trypsin and appearance of a low molecular mass band corresponding to aprotinin (Fig. 3a, lanes 2 and 3). Addition of EGTA inhibited neither trypsin (Fig. 3a, lane 4) nor aprotinin (Fig. 3a, lane 5). Thus, treatment at room temperature with 2% SDS in the presence of EGTA and electrophoresis under non-reducing conditions did not alter the activity of trypsin or aprotinin.

When TR-TLPs were heated to 80°C in the presence of 2% SDS, viral proteins VP2, VP6, VP7 and VP5 were visible following electrophoresis and Coomassie blue staining (Fig. 3a, lane 6). This observation is consistent with the total disruption of TR-TLPs under these conditions. Trypsin molecules freed from the TR-TLPs appeared as a white band with the same molecular mass as purified trypsin (Fig. 3a,
lanes 1 and 6). Thus, treatment at 80 °C in 2 % SDS totally disrupted TR-TLPs and inactivated trypsin, which can be renatured and reactivated in the gel following non-reducing electrophoresis by incubation of the gel in renaturation buffer to remove SDS.

When TR-TLPs treated for 5 min at room temperature in 2 % SDS were subjected to zymogram analysis, trypsin activity could be seen as a white smear in addition to the sharp white band described above (Fig. 3a, lane 7). This smear may be interpreted as a progressive release of the trypsin associated with the viral outer proteins, due to incubation with 2 % SDS, or migration into the gel. Addition of aprotinin to TR-TLPs prior to incubation in 2 % SDS did not significantly reduce the trypsin activity (Fig. 3a, lanes 7 and 8). The presence of a smear suggests that the TR-TLPs were not totally disrupted after incubation at room temperature, and that the capsid proteins either did not enter the gel or migrated as complexes of various molecular masses. This interpretation was confirmed by ethidium bromide staining to visualize the genomic dsRNA present in the gel. The majority of nucleic acids, probably associated with viral proteins, was present at the top of the gel, whereas after treatment at 80 °C all the individual dsRNA segments could be visualized (Fig. 3b, lane 6 and 7–10).

When TR-TLPs were treated at room temperature with EGTA in 2 % SDS prior to zymogram analysis, trypsin activity could also be seen as a sharp white band, but none of the viral proteins were visible (Fig. 3a, lane 9). When aprotinin was added prior to EGTA treatment, the bands corresponding to VP7 and VP5* were again visible (Fig. 3a, lane 10). Thus, aprotinin can inhibit trypsin only when present during solubilization of the outer capsid proteins by EGTA.

The smearing of the protease activity confirmed the tight binding of trypsin to TR-TLPs, since their association resists 2 % SDS at room temperature but not at 80 °C, and indicated that the trypsin is not accessible to aprotinin before solubilization of the outer protein layer.

Effect of aprotinin on rotavirus multiplication

To determine the functional role of trypsin molecules associated with infectious virus, we examined whether protease inhibitors have an effect on virus replication. After adsorption of purified TR-TLPs onto cells, aprotinin was added to the cell culture medium. The effect of the addition of this protease inhibitor was evaluated by determining the amount of rotavirus antigen present in infected cells 5 h p.i. by ELISA. The dose–response curve of aprotinin on antigen production is shown in Fig. 4. Aprotinin significantly reduced the production of viral antigen and addition of 624 TIU l⁻¹ resulted in an eightfold reduction of the viral titre recovered at 18 h p.i. (data not shown). Even at the highest dose used, aprotinin did not have a toxic effect on MA104 cells, as estimated by trypan blue exclusion assay (data not shown). This last observation is in accordance with previous studies showing the very low cytotoxicity of this serine protease inhibitor (Shah et al., 2004). These findings show that addition of serine protease inhibitors to cell culture medium reduces rotavirus antigen production in a dose-dependent manner.

Sequential addition of aprotinin during infection by RF strain

The role of virion-associated trypsin at specific steps during the viral life cycle was investigated by infecting MA104 cells with pure TR-TLPs, in the presence or absence of aprotinin, and measuring viral protein synthesis by ELISA. Maximum inhibition of viral protein synthesis was reached when aprotinin was present during both adsorption at 15 °C and incubation for 5 h at 37 °C (Fig. 5, bar 5). Presence of aprotinin during adsorption alone was not sufficient to obtain maximal inhibition, suggesting that the binding of viral particles to cells is not the step in the viral life cycle affected by the presence of aprotinin (Fig. 5a, bar 0). Maximal inhibition efficiency was reached when aprotinin was present at the time when cells were transferred from 15 to 37 °C following viral adsorption (Fig. 5a, bar 1), as well as when present only at 37 °C after the adsorption step (Fig. 5a, bar 6). The inhibition efficiency was equivalent regardless of when aprotinin was removed from the cell culture medium following transfer to 37 °C (Fig. 5a, bars 1–5). Inhibition was not observed when aprotinin was added to cell culture medium immediately after TR-TLP adsorption at 37 °C (Fig. 5b), suggesting that, under these conditions, all TR-TLPs have entered the cells and were not accessible to aprotinin.

These results indicate that aprotinin has a major inhibitory effect when present during recovery of endosomal activity in
cells. Thus, trypsin associated with TR-TLPs most likely plays a role during the first steps of viral entry.

**Early viral transcription is reduced in the presence of aprotinin**

To address which early step of the viral life cycle is affected by aprotinin, transcription of viral mRNAs was examined. MA104 cells were infected with purified TR-TLPs and incubated in the presence of different concentrations of aprotinin. Total mRNA was then extracted, denatured at 65°C and then the random-primed RT reaction was performed. To estimate viral transcription, the amount of rotavirus gene 6 mRNA present in the experimental samples was determined by Q-RT-PCR and normalized using the amount of cellular GAPDH mRNA present in each sample.

To evaluate the amount of viral dsRNA which could be detected under the conditions of denaturation used, we performed a control experiment by extracting total RNA following viral adsorption at 15°C (0 h p.i.). This control experiment showed that the viral dsRNA remaining associated with the cells were not amplified by Q-RT-PCR when RNA was heated to 65°C prior to RT (data not shown). Thus, the signal detected under our experimental conditions corresponds to the gene 6 mRNA present in the cell cytoplasm and not the gene 6 dsRNA. It is a measure of the viral mRNAs synthesized in the cell and, hence, an evaluation of the quantity of virus which has passed the cellular membrane.

After infection of cells followed by incubation with various amounts of aprotinin, total RNA was extracted at 3 h p.i. and viral mRNAs were quantified. As the concentration of aprotinin added to the medium increased, viral transcription decreased (Fig. 6). Because trypsin was not added during infection, this result may indicate that inhibition of virion-associated trypsin interferes with viral penetration and thus viral transcription.

**DISCUSSION**

We have shown that porcine trypsin molecules are associated with the outer protein layer of rotavirus virions propagated in cell culture medium containing porcine trypsin. Our data support the hypothesis that trypsin is specifically incorporated into or onto rotavirus virions during virus replication. The TLPs used were highly purified by two rounds of isopycnic ultracentrifugation. TLPs and DLPs were purified simultaneously from the same virus preparation and by the same protocol, but trypsin was present only in TR-TLPs, not in TR-DLPs. Trypsin...
associated with virions was inactive, and its activity was recovered only when the outer capsid was solubilized. If trypsin was present in the purified virion preparations as a contaminant, it would have been active even before solubilization of the outer capsid, and unbound trypsin would also have been inhibited by aprotinin before, as well as after, solubilization of the outer capsid. We have also detected trypsin in other rotavirus strains (bovine UK, simian RRV and SA11 4F, porcine OSU and human WA) by zymogram analysis (data not shown), indicating that this association is not specific to the bovine RF strain. Moreover, antibodies specific for porcine trypsin were present in three different sera generated from animals immunized with purified TR-TLPs, suggesting that immunization with TR-TLPs generates anti-trypsin antibodies (data not shown).

The small number of trypsin molecules present in each TR-TLP and the fact that trypsin migrates in SDS-PAGE at approximately the same position as VP8* might explain why trypsin present on TR-TLPs has not been observed previously.

**How and when does trypsin gain access to the virus?**

The porcine origin of the trypsin associated with TR-TLPs was established unambiguously by protein sequencing. We have shown that trypsin is not active when the viral outer capsid is intact. It is possible that, following virus assembly, extracellular trypsin binds to VP4 and/or VP7, which could block the enzymic activity of the protease in a manner similar to trypsin inhibitors like serpins (Ye & Goldsmith, 2001). Another possibility is that trypsin enters cells due to altered cell permeability (Obert et al., 2000) or during virus entry, as was shown for the toxin z-sarcin (Liprandi et al., 1997), and is then incorporated into the virus particle during virus maturation. A similar hypothesis has already been presented to explain the differences in VP4 conformation between TR-TLPs and NTR-TLPs seen by electron cryomicroscopy (Crawford et al., 2001).

**Virus-associated trypsin: nature versus nurture?**

In this study we used the bovine rotavirus RF strain adapted to cell culture, but trypsin was also found in other cell cultured-adapted rotavirus strains. Rotavirus infectivity in cell culture is enhanced by trypsin treatment, and cleavage of VP4 by trypsin occurs in vivo in the lumen of the intestine prior to infection of enterocytes in animal models (Ludert et al., 1996). It is possible that viruses capable of incorporating trypsin molecules have a selective advantage during adaptation to cell culture.

Our results raise the question of the possible function of virus-associated trypsin in the viral life cycle of rotavirus. During rotavirus infection, viruses could acquire trypsin while passing through the gastrointestinal tract, an environment very rich in proteases. Again, rotaviruses capable of acquiring trypsin would have a selective advantage by being able to infect intestinal cells efficiently. Further studies need to be performed to verify whether trypsin can be detected in TLPs prepared from rotavirus-infected animals, in other enteric viruses or in other viruses requiring trypsin for optimal growth (e.g. influenza viruses; Klenk et al., 1975).

**A role for the virus-associated trypsin in rotavirus entry**

The results presented here indicate that addition of aprotinin to the cell culture medium inhibits viral multiplication, as seen by the decrease in RNA and viral protein synthesis in the presence of aprotinin. The protease inhibitor did not interfere with virus attachment, since the presence of aprotinin during adsorption alone was not sufficient to obtain maximal inhibition. These observations are in accordance with previous results showing that trypsin treatment of rotavirus does not attenuate viral attachment to cell membranes (Kaljot et al., 1988). The inhibition by aprotinin was more efficient when the protease inhibitor was present during recovery of endosomal activity during the transition from 15 to 37°C. This suggests that, to be efficient, aprotinin has to enter the cell simultaneously with TR-TLPs. Q-RT-PCR results indicated that early viral transcription was reduced in the presence of aprotinin following adsorption, suggesting that aprotinin, and hence trypsin, acts after virus binding, but prior to initiation of viral transcription. These results are in agreement with previous observations showing that (i) infection with trypsin-treated virus leads to greater levels of RNA synthesis early in infection, (ii) trypsin converts non-infectious virions into infectious virions by allowing them to be decapsidated into the cell (Clark et al., 1981), (iii) NTR-TLPs enter the cells more slowly than TR-TLPs and produce less virus (Crawford et al., 2001; Kaljot et al., 1988) and (iv) NTR-TLPs never produce virus titres as high as those produced by TR-TLPs, even when NTR-TLPs are treated.
exogenously with a high concentration of trypsin (Crawford et al., 2001).

Although we cannot exclude an effect of trypsin or aprotinin on the cell itself, our results showing activation of trypsin upon solubilization of the viral outer capsid agree with previous experiments that have suggested a role for outer capsid proteins in membrane solubilization. TR-TLPs are able to solubilize pig jejunum brush border membrane vesicles charged with carboxyfluorescein (CF) after addition of EGTA or heating at 60°C (Ruiz et al., 1994). Membrane solubilization was not observed with NTR-TLPs, unless they were treated first with EGTA and then with trypsin. Similar results were observed with intact cells by measuring incorporation of ethidium bromide (Ruiz et al., 1997). These results were explained by the effect of VP5*, which is able to solubilize membranes (Denisova et al., 1999), but membrane permeabilization can also be obtained in the absence of VP4. Trypsin treatment of VP7, solubilized from virus-like particles composed of VP2, VP6 and VP7 (VLP 2/6/7), is required to induce permeabilization of membrane vesicles detected by measurement of the release of CF (Charpilienne et al., 1997). Conversely, it has been shown that addition of a monoclonal antibody specific for VP7 neutralizes rotavirus infection by impairing virus outer capsid solubilization (Ludert et al., 2002). Altogether, these results indicate that VP7 solubilization is required for rotavirus infection and that solubilized and trypsinized VP7 is capable of permeabilizing cellular membranes in the absence of VP4.

We propose that, during rotavirus infection, trypsin molecules are bound to the outer capsid layer in an inactive form, and are activated with the solubilization of VP7 and VP4 due to a drop in calcium concentration in the endosomal vesicle (Chemello et al., 2002) (Fig. 7). Activated trypsin may then cleave VP7 and VP4 into fragments capable of disrupting cellular membranes. However, virions could possibly enter the cell by an endosome-independent mechanism (Lopez & Arias, 2004). We propose that, following rotavirus attachment, and a local drop in calcium concentration near the plasma membrane which partially solubilizes the viral outer capsid, VP7 or VP4 cleaved by trypsin could then permeabilize the plasma membrane. This step could be inhibited in the presence of aprotinin, which may enter cells along with the virus and gain access to trypsin during outer capsid solubilization. Entry of macromolecules present in the medium during rotavirus infection has already been described (Liprandi et al., 1997). This would lead to DLPs gaining access to the cytoplasm to begin actively transcribing viral mRNA to complete the next step in the viral life cycle.

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