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Role of simvastatin and methyl-β-cyclodextin on inhibition of poliovirus infection

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Abstract

Cells exposed to simvastatin or to methyl-β-cyclodextrin show reduced poliovirus infection, without alteration in virus binding or on the kinetics of genome entry, suggesting that the steps which are altered are those post uncoating and genome entry. Reduction of infection by cyclodextrin is reversed by increasing MOI whereas that produced by simvastatin treatment is not, suggesting that the effects on infection are not due to a reduction in cholesterol. The differences in the characteristics of inhibition can be explained by the differential effects of the compounds. Cyclodextrin inhibits the store-operated calcium channels, suggesting that reduction in infection is through translational inhibition. Simvastatin produces vesicles from internal membranes which cannot sustain viral RNA synthesis, reducing infection through reduced transcription. The results indicate that the impact on viral infection by the cholesterol-modifying agents is due to the cellular changes produced rather than due to disruption of the cholesterol-rich domains.

Keywords: Poliovirus infection; SOCC; Cholesterol; Simvastatin; Cyclodextrin

Nonenveloped viruses bind to receptor(s) at the cell membrane and follow different pathways to internalize their genome [1]. Thus, the genome of simian virus 40 (member of the Poliomaviridae family) and echovirus 1 (from the Picornaviridae family) is internalized through caveolae [2,3], whereas canine parvovirus, adenovirus, and some picornavirus penetrate into the cell via clathrin-coated pits [4,5]. The strategy for entry of different members of a family of virus is also unique to each member; for example, the picornavirus human rhinovirus 2 and 14 are internalized by clathrin-mediated endocytosis but poliovirus is not [5,6]. There is also increasing evidence that specific membrane microdomains, defined by their low temperature insolubility in non-ionic detergents (rafts, DIMs) [7], play an important role in the entry of nonenveloped viruses into cells, and that manipulation of the components of these domains alters virus infection (reviewed in [1]). Disruption of rafts is generally accomplished either through metabolic depletion, by incubating cells in lipoprotein-depleted serum supplemented with statins [8], or through the use of agents that bind to and form water soluble complexes with cholesterol, such as methyl-β-cyclodextrin (MβCD) [9].

Pretreatment of HeLa cells with simvastatin leads to a substantial reduction in poliovirus (PV) infection as well as reduction in the translation of poliovirus proteins, without alteration in the binding of virus to the cell membrane or alteration of the kinetics of entry into cells [10]. It was shown recently that cholesterol extraction with MβCD also results in a reduction in poliovirus infection when MβCD is added to cells prior to poliovirus addition, and is less effective or even without effect if added at the time of the start of infection or later. Further, the effects of cyclodextrin were shown to be reversed by increasing the number of viral particles used to produce infection [11]. Since the

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characteristics of the inhibition of poliovirus infection by MβCD are different from those obtained in the presence of simvastatin, a series of experiments were conducted to understand the origin of the differences observed when using two different strategies to lower cholesterol. The data show that exposure of cells to simvastatin leads to vesiculation of internal compartments and, consequently, to a reduction in titer by a failure to form viral RNA replication complexes. The reduction in titer due to extraction of cholesterol with MβCD, on the other hand, can be explained by a cyclodextrin-induced inhibition of protein synthesis through inhibition of the store-operated calcium entry channels (SOCC). Thus, these results suggest that the reduction in poliovirus infection upon exposure of cells to cholesterol-reducing agents can be ascribed to cellular effects arising from the drugs used to modify the cholesterol concentration of the cell membrane, rather than due to disruption of rafts at the cell membrane.

Materials and methods

Reagents and antibodies. Actinomycin D and cell culture reagents were purchased from Gibco Laboratories (Grand Island, NY); simvastatin from Calbiochem (San Diego, CA); Alexa 555-conjugated cholera toxin subunit B (A555-CTB) from Molecular Probes (Carlsbad, CA), human poliovirus receptor antibody (clone D171) from NeoMarkers (Fremont, CA); mevalonate, neutral red and all other reagents from Sigma Chemicals (St. Louis, MO). Surindac sulfide was a gift from Dr. H. Aktas, Brigham and Women's Hospital, Boston, MA, and the antibody to CD55 a gift from Dr. Xuebin Qin, Brigham and Women's Hospital, Boston, MA.

Cells and viral infection. Suspension cultures of HeLa S3 cells (American Type Culture Collection, Manassas, VA) were maintained in Eagle's minimum essential medium supplemented with 10% fetal calf serum for a maximum of 8–10 passages. The cells were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum or on poly-L-lysin-coated coverslips. Stocks of wild-type Mahoney type 1 poliovirus were prepared at 37 °C from an original stock kindly provided by Dr. Marie Chow (Univ. Arkansas Medical School, Little Rock, AR). Infection of HeLa cells with poliovirus was carried out at the indicated multiplicity of infection (MOI) by first binding virus at 23 °C for 30 min, followed by two washes with virus-free media and incubation of infected cells at 37 °C. Poliovirus containing neutral red was prepared by infecting cells (10 MOI) in the presence of 4 μg/ml neutral red and maintaining the cultures in the dark [12]. Determination of viral titers and viral growth curves was performed using established procedures. All experiments described were repeated a minimum of three times per experimental condition.

Metabolic labeling of viral proteins. HeLa cells were infected at 37 °C for 3–5 h, washed three times with methionine/cysteine-free minimal essential medium, adding 100 μCi [^{35}S]methionine/cysteine mixture per milliliter in the last wash, and labeled for 45 min at 37 °C. The cells were then washed with ice-cold PBS, lysed, nuclei removed by centrifugation, and aliquots analyzed by SDS–PAGE on 12% polyacrylamide gels. The radioactivity in the individual bands was determined using a PhosporImager (Molecular Dynamics, Sunnyvale, CA).

Cholesterol depletion. Metabolic depletion of cholesterol was done according to the method described in [8], with minor modifications. Briefly, HeLa cells were grown for one or 2 days in depletion medium: DMEM supplemented with 5 μM simvastatin and 500 μM mevalonate containing either 5% or 2% fetal calf LPDS. Control cells were grown, in parallel, in DMEM supplemented with 5% FBS. As shown in Table 1, the cell growth and number was not significantly affected under these experimental conditions (even after 6 h in the presence of poliovirus) and neither was their viability, as determined by trypan blue exclusion. The cholesterol content of the cells was determined in lipid extracts of cell lysates [13] using the Amplex Red Cholesterol Assay (Molecular Probes, Carlsbad, CA).

Table 1
Cell survival and cholesterol content after simvastatin treatment

Condition	Number of cells (cells/plate) × 10^6		Cholesterol (pg/cell)
	0 h p.i.	6 h p.i.	
Control	3.2 ± 0.04		127 ± 25 (10)[a]
+PV		3.2 ± 0.01	
5% LPDS+Simv.	3.2 ± 0.07		61 ± 7 (10)
+PV		3.1 ± 0.08	
2% LPDS+Simv.	2.4 ± 0.08		34 ± 4 (7)
+PV		2.0 ± 0.05	

The values in the table are given as average and standard deviation.
[a] Numbers in parentheses indicate amount of determinations performed on independently prepared cells.

Extractions of cholesterol with MβCD were performed for 30 min at 37 °C, at an MβCD concentration of 10 mM in DMEM. After the extraction was completed, the cells were washed twice with PBS before adding poliovirus. Infection was done as described above.

Viral RNA synthesis. The synthesis of viral RNA was measured as described in [14]. Briefly, HeLa cells (3×10^6) grown in monolayer cultures were infected with poliovirus in the presence of 5 μg/ml Actinomycin D and 5 μCi/ml [^{3}H]uridine (Amersham Biosciences, Piscataway, NJ) and incubated at 37 °C. Cells were harvested at different times post infection (p.i.) by scraping into ice-cold phosphate buffer, TCA precipitated for 15 h at 4 °C and the precipitate adsorbed onto glass microfiber filters, air-dried, and the radioactivity counted by liquid scintillation.

Immunofluorescence and localization of the poliovirus receptor. HeLa cells were grown on poly-L-lysin-coated coverslips and either treated to lower their cholesterol or maintained in regular medium. At the end of the treatment period, the cells were washed and fixed for 20 min in PBS-containing 2% formaldehyde. To visualize the poliovirus receptor and GM1, cells were first incubated with the appropriate dilution of the poliovirus receptor antibody (D171), at 37 °C for 1 h, followed by incubation with the secondary antibody (Alexa 488-conjugated goat anti-mouse antibody, 1:500 dilution) (A488-D171) at 37 °C for 1 h. Cells were then incubated at 4 °C, for 20 min, with Alexa 555-conjugated cholera toxin subunit B (A555-CTB) to visualize GM1, washed and mounted with 50% glycerol. Inverting the order of addition of the fluorescent reagents produced the same results. Confocal images of the HeLa cell surface labeled with A555-CTB (red) and A488-D171 (green) were generated at the Nikon Imaging Center at Harvard Medical School on a Nikon E1000 Eclipse automated upright microscope, using a 60×, 1.4 NA objective lense. Images were collected using a Hamamatsu Orca II dual-mode cooled CCD camera and processed using the MetaMorph Imaging System software (Molecular Devices, Sunnyvale, CA). This work was conducted by Ms. Lara J. Petrak, at the Nikon Imaging Center.

Separation of Triton X-100 insoluble material. HeLa cells were treated as described above to lower their cholesterol. Untreated and treated cells were then washed with cold PBS and extracted on ice with 1% (wt/vol) Triton X-100, followed by homogenization for eight times on ice and centrifugation (4 °C) at 800*g* to remove nuclei. Lysates were brought to a concentration of 40% sucrose (final volume 1.6 ml) and overlaid with 1.6 ml 30% sucrose and 0.8 ml 5% sucrose in TNE. The samples were then spun in a Beckman rotor SW 60Ti for 18 h (4 °C) [15–17]. Fractions were collected starting at the top of the tubes and analyzed by Western blot of raft marker molecules CD55 and GM1.

Electron microscopy. For thin section electron microscopy, cells were fixed in 2.5% glutaraldehyde and 1% tannic acid in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h at room temperature, washed in 0.1 M cacodylate buffer, and then postfixed with 1% OsO4 in the same buffer. Samples were stained en bloc with 1% uranyl acetate, dehydrated in ethanol, and embedded in Epon 812 (Electron Microscopy Sciences, Fort

Washington, PA). Sections were stained with lead citrate and examined with a JEOL (Tokyo, Japan) 1200EX electron microscope in the laboratory of Dr. Daniel Goodenough, Harvard Medical School.

Measurements of internal Ca^{2+}. The changes in intracellular calcium concentration were measured as previously described [18,19]. Briefly, exponentially growing cells were detached, washed with PBS, and loaded with Fura-2AM (Molecular Probes, Eugene, OR) by incubation for 30 min at 37 °C. Cells were then transferred to a stirred, thermostated cuvette in a dual-wavelength spectrofluorometer system (Photon Technology International, Inc., Brunswick, NJ). Changes in internal calcium were then determined by the absorption shift of the dye by scanning the excitation spectrum between 340 and 380 nm, and monitoring emission at 510 nm. The results are displayed as the ratio of the emission at 340 to that obtained at 380.

Results

The first events in poliovirus infection involve binding to its receptor, a transmembrane protein of the IgG superfamily, followed by a conformational change and genome uncoating and entry (reviewed in [20]). Since the receptor is a transmembrane protein, it is possible that changes in the lipid composition of the plasma membrane could affect poliovirus infection, in particular if the receptor were located in the rafts, domains susceptible to changes in cholesterol content.

Simvastatin and methyl-β-cyclo dextrin disrupt the rafts: The poliovirus receptor localizes in detergent-insoluble membrane fractions (with the technical assistance of Ms. Lara J. Petrak).

The data in the literature indicate that metabolic depletion of cholesterol, as well as extraction of cholesterol with methyl-β-cyclodextrin, disrupts domains which are rich in cholesterol and insoluble in detergent at low temperature (DIM, rafts) [7,8,21]. However, the data shown in [11] indicate that this is not the case for HeLa cells exposed to MβCD. To determine if exposure of HeLa S3 cells to either simvastatin or to MβCD disrupts rafts, experiments were conducted to determine the effect of cholesterol depletion on markers for the regions enriched in cholesterol. HeLa cells were first treated either with simvastatin or with MβCD and subsequently exposed to Triton X-100 at low temperature. The cell-free supernatant was then analyzed for the effect of the ganglioside GM1, a marker for DIMs [15]. Fig. 1A shows that cholesterol depletion with simvastatin (5% LPDS) or with MβCD leads to a substantial migration of GM1 from the detergent-insoluble fractions (lanes 1–2) in untreated cells, to the detergent-soluble fractions (lanes 4–7) in low cholesterol membranes, indicating a disruption of the domains. Essentially the same results were obtained with another marker of rafts, CD55 [22] (not shown).

Further studies were undertaken to determine if the poliovirus receptor is located in the cholesterol-dependent domains, by staining HeLa cells with a mouse monoclonal anti-poliovirus receptor antibody and by labeling the ganglioside GM1 with the binding subunit of cholera toxin. The results indicate that the poliovirus receptor (PVR)

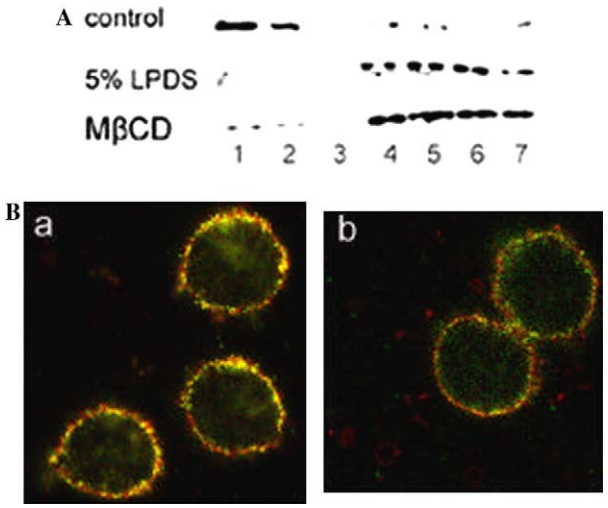

Fig. 1. The poliovirus receptor is located in rafts. (A) Effect of low cholesterol on raft integrity. HeLa cells were either untreated (control) or treated to lower cholesterol by incubation in simvastatin-containing, lipoprotein-deficient serum (5% LPDS) or incubation with methyl-β-cyclodextrin (MβCD) before lysis with Triton X-100 on ice. Lysates were subjected to sucrose density ultracentrifugation and fractions 1–7 collected from the top of the gradients and blotted for GM1. (B) Localization of the poliovirus receptor. Antibodies to the poliovirus receptor (green) and fluorescently labeled cholera toxin subunit B (red) (marker for GM1) were used to determine their colocalization at the plasma membrane (orange-yellow) by superimposing confocal images in (a) untreated cells and (b) simvastatin-treated cells.

resides in the cholesterol-sensitive domains as shown in Fig. 1B. The image shows yellow regions where the receptor colocalizes with the ganglioside GM1 (Fig. 1B-a). The right-hand panel (Fig. 1B-b) shows that there is reduced colocalization of PVR and GM1 in cells pretreated with simvastatin by the lower amount of yellow shown in the figure, corroborating the localization of PVR to the rafts.

Metabolic depletion of cholesterol reduces poliovirus infection

The localization of the poliovirus receptor in rafts suggests that disruption of these domains could affect PV infection. With this in mind, experiments were performed to test this hypothesis and to determine the general features of poliovirus infection in HeLa cells treated with simvastatin. As shown in Table 1 and Fig. 3, this procedure results in controlled depletion of cholesterol, without affecting cell viability within the time frame of the experiments.

The kinetics of virus growth in simvastatin-treated cells was found to be similar to that of untreated cells but with a clear reduction in the titer (Fig. 2A), even though the binding of poliovirus particles was unaltered by treatment with simvastatin (data not shown), as was also the case when cells were pretreated with MβCD [11]. Further shown in Fig. 2 is that titer is a function of the cholesterol remaining in the membrane (Fig. 2B) and that increasing MOI above three does not lead to recovery of the titer (Fig. 2C). This is in marked contrast with the results in [11], which indicate

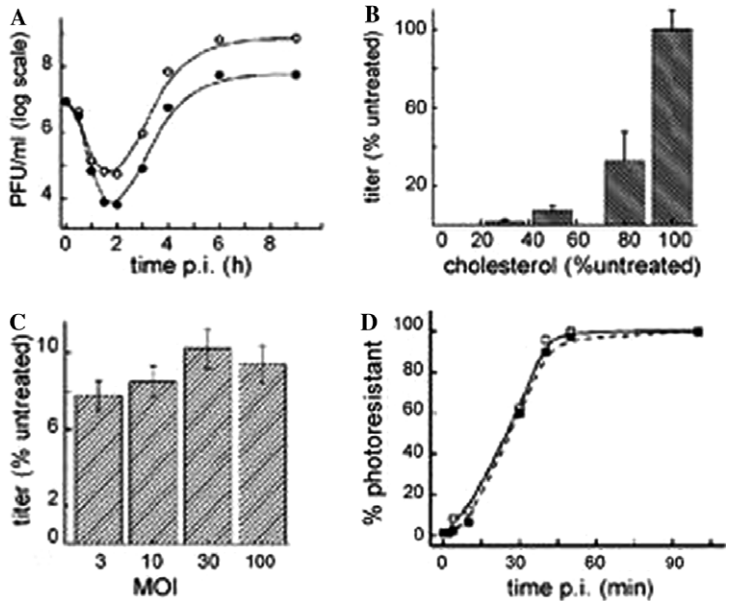

Fig. 2. Effect of reduced cholesterol on poliovirus growth curves and infection. (A) One-step growth curves of poliovirus infection of untreated HeLa cells (open circles) or HeLa cells treated to contain 50% cholesterol (closed circles) as described in Materials and methods. HeLa cell monolayers were infected at 3 MOI. Cells were harvested at the indicated times post infection (p.i.) and titers determined by plaque assays. Ratio of bound virus in treated cells to untreated cells (determined using ^{35}S-labeled virus) = 0.8 ± 01. (B) Dependence of titer on cholesterol concentration. Untreated HeLa cells or cells treated metabolically to lower cholesterol were infected with poliovirus and harvested at 6 h p.i. to determine PV titer. Data given are averages of at least four identical experiments (together with their standard deviation) relative to the average titer obtained in untreated cells. (C) Dependence of titer on poliovirus MOI HeLa cells containing ~50% cholesterol were infected at the indicated MOI. Titers were determined 6 h p.i. Data shown are the average (and SD) of at least three identical experiments plotted in the figure relative to the titer obtained in untreated cells. (D) Effect of low cholesterol on genome uncoating. HeLa cells were infected with virus labeled with neutral red and the infection started by addition of an agar overlay. At the indicated times after infection, the monolayers were exposed to white light for a period of 10 min and the plaques were developed in the dark for 48 h, stained and counted. Photoresistance is expressed as titer at each time point relative to the one obtained when plates were kept in the dark for 6 h. Open symbols: control cells; closed symbols: low cholesterol cells.

that infection can be recovered at high enough MOI in cells treated with MβCD. The next step in entry, uncoating of the viral genome, was studied in cells infected with virus grown in the presence of neutral red, which makes the virus light-sensitive during the initial stages of infection. The kinetics of light sensitivity correlate with the kinetics of RNA release, thus making it possible to study the viral uncoating [12]. HeLa cells were treated with simvastatin or with vehicle, infected with PV, and exposed to light at various times after the start of infection. Fig. 2D shows the titer (relative to that obtained in cells maintained in the dark) as a function of time post infection (p.i.) during which the cells were maintained in the dark. The data clearly show that the kinetics of uncoating of poliovirus in low cholesterol HeLa cells is statistically indistinguishable from the kinetics of virus entry in untreated cells, indicating that the inhibition of infection must arise post uncoating, either by impaired translation or impaired transcription of the viral genome.

Treatment with simvastatin or with methyl-β-cyclodextrin reduces the translation of poliovirus proteins

The effect of exposure of cells to cholesterol-lowering drugs on the viral translational capabilities was examined as described in Materials and methods, and the results shown in Fig. 3. Fig. 3A illustrates the reduction in the synthesis of viral proteins when cells are exposed to simvastatin. Lanes 1, 3, and 5 show the protein profile of cells containing 100%, 50%, and 30% cholesterol, respectively, and indicate that the protein profile of uninfected cells is similar at all three cholesterol concentrations. Lanes 2, 4, and 6 show the protein profile after infection with poliovirus. Comparison of lane 2 (control cells) with lanes 4 and 6 shows that when cells are exposed to simvastatin, the synthesis of poliovirus proteins is greatly reduced, even when the reduction in cholesterol is only 50% (lane 4), whereas the synthesis of cellular proteins is virtually unaffected.

The data in panels B and C show the results of increasing MOI on the viral protein synthesis of low cholesterol cells (lanes labeled "t"; lanes from cells with normal cholesterol: "c"). Panel B shows that treatment with MβCD does not affect the synthesis of cellular proteins (lanes 0), whereas there is a reduction in the viral protein synthesis when MβCD-treated cells are infected with poliovirus at 5 MOI (lanes "5", c and t). However, increasing the MOI to 100 restores the synthesis of viral proteins to a value almost identical to that of untreated cells (compare lanes "100", c and t). This result is consistent with the recovery of titer in MβCD-treated cells (cf legend), even though the DIMs are disrupted when cholesterol is extracted with MβCD (cf Fig. 1). In contrast, panel C, the gel electropho-

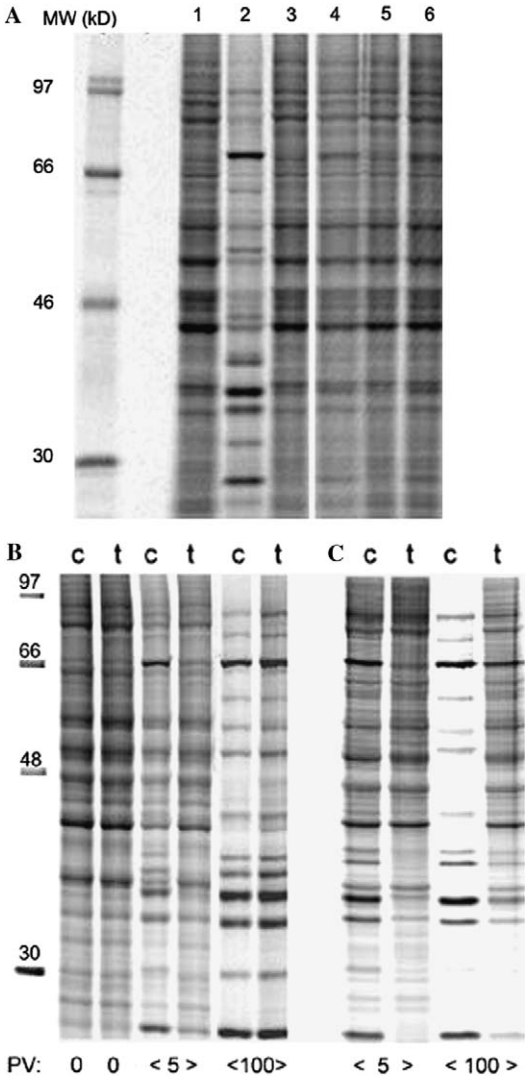

Fig. 3. Effect of low cholesterol on the translation of poliovirus proteins. (A) Effect of cholesterol content: HeLa cells were grown in 5% FBS (lanes 1 and 2) (control, 100% cholesterol) or in the presence of either 5% (lanes 3 and 4) or 2% (lanes 5 and 6) simvastatin-containing LPDS for 48 h and infected with poliovirus (5 MOI) for 6 h. ^{35}S-labeled amino acids were added during the last 45 min of infection and the cell extracts analyzed by SDS–PAGE (12%). Lane 1: control cells untreated and mock infected. Lane 2: control cells after infection with poliovirus. Lane 3: HeLa cells with ~50% cholesterol, mock infected and after poliovirus infection (lane 4). Lane 5: HeLa cells with ~30% cholesterol, mock infected and after PV infection (lane 6). Effect of MOI on translation: HeLa cells were depleted of cholesterol as indicated below, infected using two different MOIs and processed as detailed above. (B) Cell cholesterol was extracted using methyl-β-cyclodextrin (MβCD cells). The lanes are labeled at the top of the gel either "c" corresponding to lysates of untreated cells, or "t" for lysates of treated cells. At bottom of gel is indicated the MOI used for each of the lanes, ⟨0⟩ corresponds to mock infected cells, ⟨5⟩ cells infected at 5 MOI (titer:), and ⟨100⟩ at 100 MOI. Titer in untreated cells = 1.5×10^9 pfu/ml (5 MOI) and 2.8×10^9 pfu/ml (100 MOI). Titer in MβCD cells = 1.5×10^8 pfu/ml (5 MOI) and 1.3×10^9 pfu/ml (100 MOI). (C) Cell cholesterol was lowered by metabolic depletion (LPDS cells). Titer in untreated cells = 3.1×10^9 pfu/ml (5 MOI) and 5.2×10^9 pfu/ml (100 MOI). Titer in LPDS cells = 3.1×10^8 pfu/ml (5 MOI) and 4.5×10^8 pfu/ml (100 MOI). Total protein concentration ratio: low cholesterol cells/control = 1.02 (LPDS cells); 0.998 (MβCD cells).

resis of cells grown in the presence of simvastatin, shows again that viral protein translation is inhibited in low cholesterol cells (lanes 5, c and t) and that increasing the titer as high as 100 MOI does not increase the viral protein synthesis (lanes 100, c and t), consistent with the results shown in Fig. 2.

Effect of low simvastatin or methyl-β-cyclodextrin on transcription of the viral genome

To determine the steps affected during infection leading to the inhibition of viral protein synthesis in cells depleted of cholesterol, experiments were conducted to determine if transcription of the poliovirus genome is affected in low cholesterol cells. To this end, the synthesis of viral RNA was studied following infection with PV, in the presence of actinomycin D, to inhibit cellular RNA synthesis [14].

As shown in Fig. 4, the incorporation of ^{3}H-labeled uridine into newly synthesized RNA ([^{3}H]RNA) is approximately 70% lower in simvastatin-treated cells than the incorporation in untreated cells, at all time points. By contrast, the accumulation of [^{3}H]RNA in cells treated with MβCD is not significantly different than in control cells (cf Fig. 4). These results suggest that metabolic depletion of cholesterol leads to an impairment in the transcription of the viral genome, whereas extraction of cholesterol with MβCD does not.

Cellular effects of cholesterol depletion by simvastatin

The results described thus far strongly suggest that reduction of cholesterol using simvastatin or methyl-β-cyclodextrin produces different effects in cells, which in turn affects different steps in poliovirus infection. Previous work has shown that poliovirus infection leads to the generation

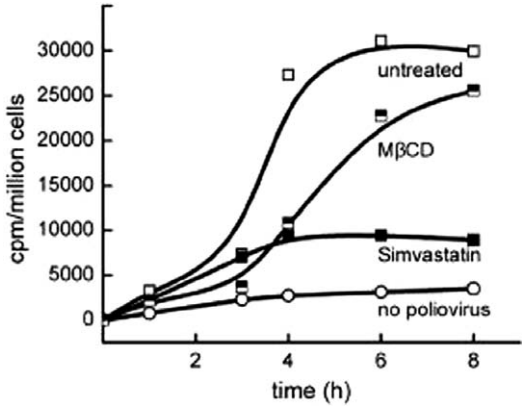

Fig. 4. Effect of low cholesterol on transcription of poliovirus RNA. HeLa cells were either untreated, grown in 5% fetal LPDS-containing simvastatin, or in the presence of 10 mM MβCD as described in Materials and methods and subsequently infected with poliovirus in the presence of actinomycin D and [^{3}H]uridine. At the indicated times after infection the cells were harvested and processed as described in Materials and methods. The points are average of results obtained in at least two separate experiments. No poliovirus refers to cells not exposed to either drugs or poliovirus.

of vesicles derived from intracellular membranes,and that these vesicles constitute the site for viral RNA replication ([23] and references therein). Further, if cells are first transfected with the viral proteins responsible for vesicle formation (2BC and 3A), subsequent exposure of these cells to poliovirus results in reduced susceptibility to PV infection [24,25]. If lowering cholesterol by treatment of HeLa cells with statins produced vesicles derived from internal membranes, as has been shown to occur in other cells [8], this could explain the reduced viral RNA transcription observed in the present study (cf Fig. 4).

The effects of treatment with simvastatin on the morphology of HeLa cells are shown in Fig. 5. Panels (A) and (B) correspond to control, untreated cells (100% cholesterol) before (A) and 6 h p.i. (B). The arrowheads point to the characteristic vesicles which form after infection [24]. Panels (C) and (D) are micrographs of uninfected, simvastatin-treated cells containing 50% (C) or 25% (D) cholesterol, respectively, showing the presence of vesicles of various sizes. Micrographs of cells treated with MβCD were indistinguishable from (A) (data not shown), indicating that this treatment does not alter cell morphology. The results shown in Fig. 5 indicate that the amount of intact internal membranes available as sites for binding of the poliovirus replicon is reduced in cells treated with simvastatin and this fact, together with previous data [24], provides circumstantial evidence that the impaired response to infection observed in cells treated with simvastatin is due to the formation of vesicles. Since the morphology of MβCD-treated cells is unaltered, the same explanation does not apply to these cells, consistent with the results shown in Fig. 4.

Cellular effects of cholesterol depletion by methyl-β-cyclodextrin

Work in several laboratories has established that exposure of cells to MβCD results in the inhibition of the store-operated calcium channel as well as inhibition of cell growth and cell cycle arrest in G1 [26–28]. Interestingly, an antiproliferative agent, clotrimazole (CLT), has also been shown to arrest cell cycle progression in G1 and to inhibit the store-operated Ca^{2+} entry channels [18,29–31]. Experiments were then conducted to determine if the inhibitory action of MβCD on poliovirus infection could be attributed to its inhibitory action on the SOCC, by comparing the results of poliovirus infection in the presence of methyl-β-cyclodextrin to those obtained in the presence of two other inhibitors of SOCC: clotrimazole and sulindac sulfide [31,32] and the results are shown in Fig. 6, indicating that both CLT and sulindac sulfide (SS) inhibit poliovirus infection in a time- and MOI-dependent manner, similar to the action of MβCD, even though neither CLT nor SS modify cellular cholesterol.

Fig. 6A shows that CLT and sulindac sulfate inhibit poliovirus infection if added before (−30 min) or at the

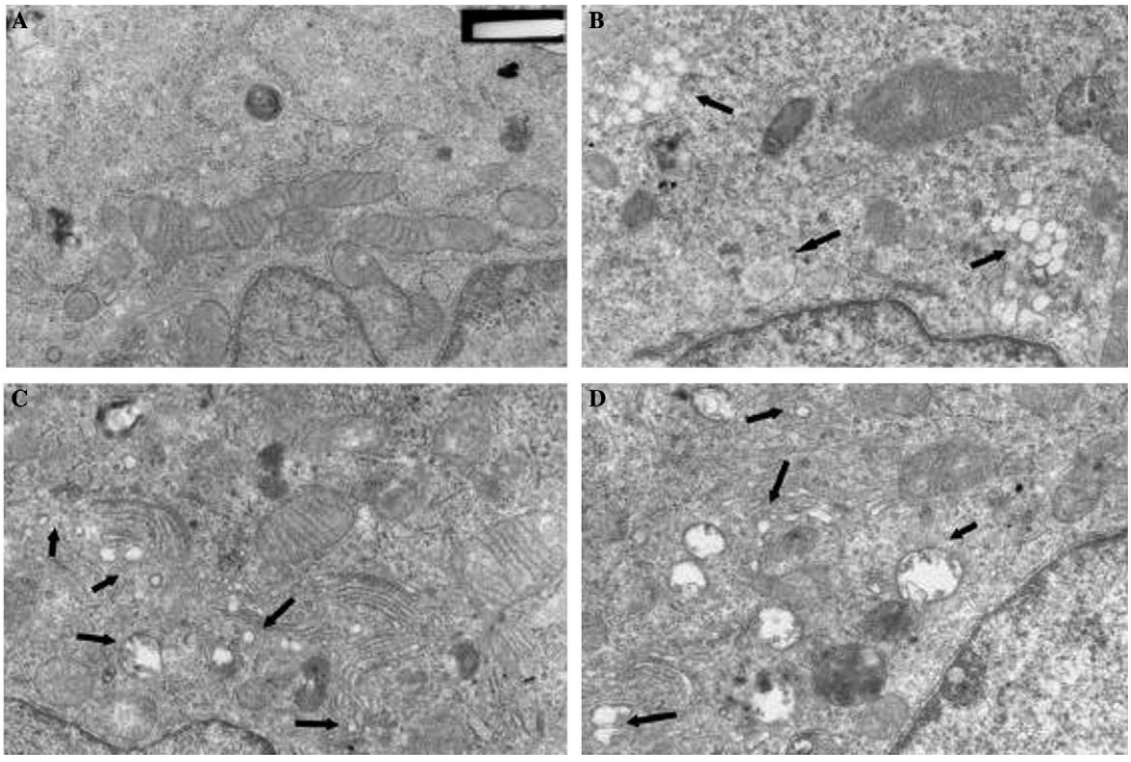

Fig. 5. Electron micrographs of low cholesterol HeLa cells. Control cells were processed before and after infection with poliovirus (10 MOI) as described in Materials and methods. LPDS cells were treated to contain 50% and 25% cholesterol. (A) Untreated cells, mock infected for 6 h; (B) infected with poliovirus for 6 h. Arrows point to vesicles of various size (arrows). (C) Uninfected cells grown in 5% LPDS to contain ~50% cholesterol. (D) Cells grown in 2% LPDS to contain ~25% cholesterol. Uninfected, low cholesterol cells show small vesicles similar to those in (B), as well as large rounded structures containing small vesicles (arrows in C and D). Other details in text. Bar 0.5 μm.

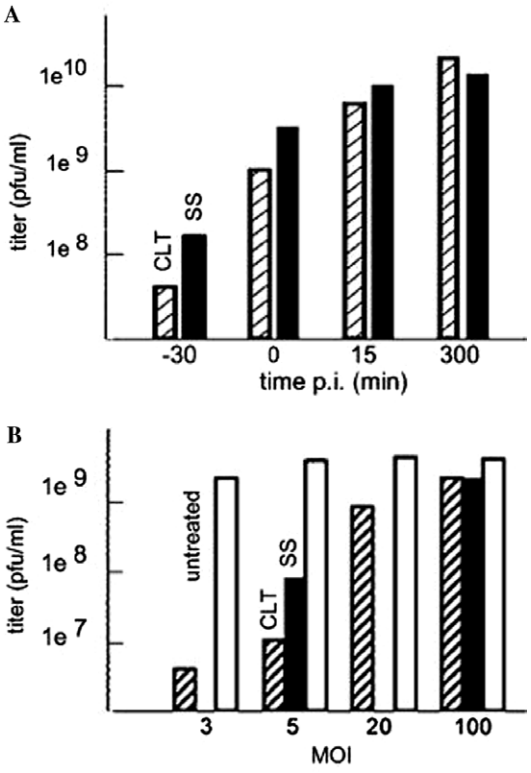

Fig. 6. Effect of inhibitors of store-operated Ca^{2+}-channels on poliovirus infection. (A) Inhibition of PV infection by CLT and SS depends on time of addition: CLT (20 μM) and SS (25 μM) were added to HeLa cells at the indicated times before or during infection with poliovirus (5 MOI). Last time point (300 min) corresponds to untreated cells. (B) Effect of MOI on titer of PV infected cells in the presence of CLT or SS. Cells were infected at the indicated MOI for 6 h, in the presence of 20 μM CLT, or of 25 μM SS, harvested, and the titer determined as indicated in Materials and methods.

time when infection starts (0), as was shown to occur when cells were exposed to MβCD [11]. Further shown in Fig. 6B is the fact that increasing the MOI when cells are exposed to CLT or to sulindac sulfate leads to an increase in titer, as is the case when cells are exposed to MβCD (cf Fig. 3 and [11]). The effects described were not found in cells treated with simvastatin, and they provide strong circumstantial evidence that methyl-β-cyclodextrin lowers poliovirus infectivity through the inhibition of the store-operated Ca^{2+} entry channels.

Discussion

The experiments described were undertaken to determine the mode of action of simvastatin and of methyl-β-cyclodextrin in their capacity to reduce infection by poliovirus, since these two agents, which have in common the ability to reduce plasma membrane cholesterol, affect PV infection differently [10,11] and thus the reduction of infectivity cannot be totally ascribed to the lower cholesterol. A first step in these experiments was to determine that treatment of HeLa S3 cells with simvastatin or with MβCD lowered cholesterol and produced disruption of the rafts, since Danthi and Chow did not find this to be the case for the cells they were working with [11]. The results illustrated in Fig. 1 indicate that cyclodextrin disrupts the rafts as judged by the migration of raft markers to detergent-sensitive domains at low temperature and further shows that the poliovirus receptor (PVR) resides in the cholesterol-rich domains. It is interesting to note that even though the receptor is part of the rafts, their disruption does not affect entry of poliovirus, as noted in Fig. 2 and as was found by Danthi and Chow [11]. This might be explained by the fact that in lipid bilayers containing the poliovirus receptor the complex formed by poliovirus and its receptor undergoes the temperature-dependent conformational change required for a successful infection, even in the absence of cholesterol [33]. The results obtained in cells exposed to either simvastatin or to MβCD would then be explained if the recruitment of receptors by poliovirus to form the temperature-dependent, infection permissive complexes were hampered by low cholesterol. However, if this were the case, increasing MOI would increase the probability of forming such complexes and thus bring titer to control values, independent of the methodology used to deplete cells of cholesterol. Since this is not the case, given that only those cells which have been treated with cyclodextrin respond to increasing MOI and recover titer, this strongly suggests that failure of receptor recruitment cannot be the explanation for the results obtained.

As shown in Fig. 3, cells exposed to the cholesterol-lowering drugs have a reduced translation of viral proteins which can be reversed by increasing the MOI when cells have been exposed to cyclodextrin (Fig. 3B), whereas this is not the case for cells treated with simvastatin (cf Fig. 3C) and the explanation in this case resides in the results in Fig. 4, which shows clearly that viral protein translation is inhibited due to the inhibition of viral RNA synthesis. By contrast, RNA synthesis is not much changed in cells treated with MβCD (cf Fig. 4).

The reversibility of the effects of MβCD on PV upon increasing MOI resembles the ability of high MOI to infect cells, even if they are non-permissive for PV infection [20], suggesting that the viral machinery is intact and that the treatment does not completely deplete cells of a component necessary for the life cycle of the virus, at least within the time frame of the experiments. In fact, it has been demonstrated that methyl-β-cyclodextrin does not only extract cholesterol from cell membranes but it also inhibits store-operated Ca^{2+} entry channels and cell growth, and produces cell cycle arrest in G1 [34,26,27,35]. Moreover, and as shown in Fig. 6, both clotrimazole and sulindac sulfate, compounds which inhibit the Ca^{2+}-entry channels and cell growth without changing cellular cholesterol [29,32], also inhibit poliovirus infection in a time- and MOI-sensitive manner, in much the same way as methyl-β-cyclodextrin does. These data taken together support the notion that MβCD (as well as clotrimazole and sulindac sulfide) decreases poliovirus infection as a consequence of its ability to inhibit the store-operated Ca^{2+} channels and, possibly

inhibiting the translation of a cellular protein necessary for effective poliovirus infection.

Exposure of cells to simvastatin produces vesiculation of internal membranes, as illustrated in Fig. 5. Interestingly, poliovirus infection produces vesiculation of internal membranes which are the site of RNA replication ([23,36] and references therein). Furthermore, formation of vesicles, for instance, by coexpression of 2BC and 3A prior to PV infection results in impaired infectivity [24,25,37], in much the same way as exposure of HeLa cells to simvastatin. It is also possible that the vesiculation induced in simvastatin-treated cells could result in a reduced level of infection by preventing the trafficking of cellular proteins required for viral replication, such as Sam68 [38].

In conclusion, the results presented show that there are differential effects when applying two different methods commonly used to modulate the cholesterol content of cells in order to investigate the role, if any, that rafts play in the life cycle of poliovirus. Moreover, the effects that these molecules have on the host are, in most likelihood, responsible for the reduction in poliovirus infection.

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References

[1] N. Chazal, D. Gerlier, Virus entry, assembly, budding and membrane rafts, Microbiol. Mol. Biol. Rev. 67 (2003) 226–237.
[2] H.A. Anderson, Y. Chen, L.C. Norkin, Bound simian virus 40 translocates to caveolin-enriched domains, and its entry is inhibited by drugs that selectively disrupt caveolae, Mol. Biol. Cell 7 (1996) 1826–1834.
[3] V. Marjomaski, V. Pietiainen, H. Matilainen, P. Upla, J. Ivaska, L. Nissinen, H. Reunanen, P. Huttunen, T. Hyypia, J. Heino, Internalization of echovirus 1 in caveolae, J. Virol. 76 (2002) 1856–1865.
[4] J.S. Parker, C.R. Parrish, Cellular uptake and infection by canine parvovirus involves rapid dynamin-regulated clathrin-mediated endocytosis, followed by slower intracellular trafficking, J. Virol. 74 (2000) 1919–1930.
[5] L. Snyers, H. Zwickl, D. Blaas, Human rhinovirus Type 2 is internalized by clathrin-mediated endocytosis, J. Virol. 77 (2003) 5360–5369.
[6] L. DeTulleo, T. Kirchhausen, The clathrin endocytic pathway in viral infection, EMBO J. 17 (1998) 4585–4593.
[7] K. Simons, W.L. Vaz, Model systems, lipid rafts and cell membranes, Annu. Rev. Biophys. Biomol. Struct. 33 (2004) 269–295.
[8] M. Hoa, S. Mukherjee, F.R. Maxfield, Cholesterol depletion induces large scale domain segregation in living cell membranes, Proc. Natl. Acad. Sci. USA 98 (2001) 13072–13077.
[9] A.E. Christian, M.P. Haynes, M.C. Phillips, G.H. Rothblat, Use of cyclodextrins for manipulating cellular cholesterol content, J. Lipid Res. 38 (1997) 2264–2272.
[10] A.V. Rodriguez, M.T. Tosteson, Cholesterol depletion reduces poliovirus infectivity and viral protein synthesis in HeLa cells, Biophys. J. 84 (2) (2003) 514a.
[11] P. Danthi, M. Chow, Cholesterol removal by methyl-beta-cyclodextrin inhibits poliovirus entry, J. Virol. 78 (2004) 33–41.
[12] D. Crowther, J.L. Melnick, The incorporation of neutral red and acridine orange into developing poliovirus particles making them photosensitive, Virology 14 (1962) 11–21.
[13] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Can. J. Biochem. 37 (1959) 911–917.
[14] S. Waggoner, P. Sarnow, Viral ribonucleoprotein complex formation and nucleolar cytoplasmic relocalization of nucleolin in poliovirus-infected cells, J. Virol. 72 (1998) 6699–6709.
[15] G. Brown, C.E. Jeffree, T. McDonald, H.W.M. Rixon, J.D. Aitken, R.J. Sugrue, Analysis of the interaction between respiratory syncytial virus and lipid-rafts in Hep2 cells during infection, Virologie 327 (2004) 175–185.
[16] P. Keller, K. Simons, Cholesterol is required for surface transport of influenza virus hemagglutinin, J. Cell Biol. 140 (1998) 1357–1367.
[17] A.D. Stuart, H.E. Eustace, T.A. McKee, T.D. Brown, A novel cell entry pathway for a DAF-using human enterovirus is dependent on lipid rafts, J. Virol. 76 (2002) 9307–9322.
[18] L.R. Benzaquen, C. Brugnara, H.R. Byers, S. Gatton-Celli, J.A. Halperin, Clotrimazole inhibits cell proliferation in vitro and in vivo, Nat. Med. 1 (1995) 534–540.
[19] N. Yamamoto, H. Watanabe, H. Kakizawa, M. Hirano, A. Kobayashi, R. Ohno, A study on thapsigargin-induced calcium ion and cation influx pathways in vascular endothelial cells, Biochim. Biophys. Acta 1266 (1995) 157–162.
[20] J.M. Hogle, Poliovirus cell entry: common structural themes in viral cell entry pathways, Ann. Rev. Microbiol. 56 (2002) 677–702.
[21] S. Mayor, S. Sabharanjak, F.R. Maxfield, Cholesterol-dependent retention of GPI-anchored proteins in endosomes, EMBO J. 17 (1998) 4626–4638.
[22] S. Bavari, C.M. Bosio, E. Wiegand, G. Ruthel, A.B. Will, T.W. Geisbert, M. Hevey, C. Schmaljohn, A. Schmaljohn, M.J. Aman, Lipid raft microdomains: a gateway for compartmentalized trafficking of Ebola and Marburg viruses, J. Exp. Med. 195 (2002) 593–602.
[23] D. Egger, N. Teterina, E. Ehrenfeld, K. Benz, Formation of the poliovirus replication complex requires coupled viral translation, vesicle production and viral RNA synthesis, J. Virol. 74 (2000) 6570–6580.
[24] M.W. Cho, N. Teterina, D. Egger, K. Bienz, E. Ehrenfeld, Membrane rearrangement and vesicle induction by recombinant poliovirus 2C and 2BC in human cells, Virology 202 (1994) 129–145.
[25] D.A. Suhy, J.T.H. Giddings, K. Kirkegaard, Remodeling the endoplasmic reticulum by poliovirus infection and by individual viral proteins: an autophagy-like origin for virus-induced vesicles, J. Virol. 74 (2000) 8953–8965.
[26] Young-Ae Choi, Byung Rho Chin, Dong Hoon Rhee, Han-Gon Choi, Hyeun-Wook Chang, Jung-Hye Kim, Suk-Hwan Baek, Methyl-beta-cyclodextrin inhibits cell growth and cell cycle arrest *via* a prostaglandin E2 independent pathway, Exp. Mol. Med. 36 (2004) 78–84.
[27] N. Kato, M. Nakamishi, N. Hirashima, Cholesterol depletion inhibits store-operated calcium currents and exocytotic membrane fusion in RBL-2H3 cells, Biochemistry 42 (2003) 11808–11814.
[28] T.P. Lockwich, X. Liu, B.B. Singh, J. Jadlowiec, S. Weiland, I.S. Ambudkar, Assembly of Trp1 in a signaling complex associated with caveolin-scaffolding lipid raft domains, J. Biol. Chem. 275 (2000).

[29] H. Aktas, R. Fluckiger, J.A. Acosta, J.M. Savage, S.S. Palakurthi, J.A. Halperin, Depletion of intracellular Ca^{2+} stores, phosphorylation of elF2alpha, and sustained inhibition of translation initiation mediate the anticancer effects of clotrimazole, Proc. Natl. Acad. Sci. USA 95 (1998) 8280–8285.

[30] H. Ouadid-Ahidouch, M. Rombaraki, P. Delcourt, A. Ahidouch, N. Joury, N. Prevarskaya, Functional and molecular identification of intermediate-conductance Ca-activated K channels in breast cancer cells: association with cell cycle progression, Am. J. Cell. Physiol. 287 (2004) C125–C134.

[31] C. Villalobos, R. Fonteriz, M.G. Lopez, A.G. Garcia, J. Garcia Sancho, Inhibition of voltage-gated Ca^{2+} entry into GH3 and chromaffin cells by imidazole antimycotics and other cytochrome P450 blockers, FASEB J. 6 (1992) 2742–2747.

[32] H. Weiss, A. Amberger, M. Widschwendter, R. Margreiter, D. Ofner, P. Dietl, Inhibition of store-operated calcium entry contributes to the anti-proliferative effect of non-steroidal anti-inflammatory drugs in human colon cancer cells, Int. J. Cancer 82 (2001) 882–887.

[33] M.T. Tosteson, H. Wang, A. Naumov, M. Chow, Poliovirus binding to its receptor in lipid bilayers results in particle-specific, temperature-sensitive channels, J. Gen. Virol. 85 (2004) 1581–1589.

[34] B. Bang, R. Gniandecki, B. Gajkowska, Disruption of lipid rafts causes apoptotic cell death in HaCaT keratinocytes, Ext. Dermatol. 14 (2005) 266–272.

[35] F. Tuluc, J. Meshki, S.P. Kunapuli, Membrane lipid microdomains differentially regulate intracellular signaling events in human neutrophils, Int. Immunopharmacol. 3 (2003) 1775–1790.

[36] V.R. Racaniello, Picornaviridae: the viruses and their replication, in: D.M. Knipe, P.M. Howley (Eds.), Fields Virology, vol. 1, Lippincott Williams and Wilkins, Philadelphia, PA, 2001, pp. 685–722.

[37] A. Schlegel, T.H.J. Giddings, M.S. Ladinsky, K. Kirkegaard, Cellular origin and ultrastructure of membranes induced during poliovirus infection, J. Virol. 70 (1996) 6576–6588.

[38] A.E. McBride, A. Schlegel, K. Kirkegaard, Human protein Sam68 relocalization and interaction with poliovirus RNA polymerase in infected cells, Proc. Natl. Acad. Sci. USA 93 (1996) 2296–2301.