Analyses of the Role of Structural Changes in the Regulation of Uncoating and Assembly of Alphavirus Cores

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In the late stages of alphavirus multiplication virus cores accumulate in the cytoplasm as stable structures, whereas they are unstable during the early stages of infection. Three types of explanation can be put forward to understand this phenomenon: (1) A cellular uncoating process is active early which is inactivated later, (2) the core structure differs between stable cores which accumulate and labile cores which are dissociated, (3) both mechanisms cooperate. A model based exclusively on the first principle involving core disassembly by cellular ribosomes has been proposed, but structural changes of the core may cooperate with the cellular uncoating process. We have therefore isolated cores of the Sindbis alphavirus from the cellular cytoplasm, from virus particles (vi-cores), and from low-pH-treated virus particles. Comparative analyses of the structure of these cores, using crosslinking and proteolytic digestion, and of the stability of the cores in the presence of ribosomes in vitro were performed. The structural comparisons indicate that the interactions between the molecular components of these cores are very similar and probably identical. In the presence of ribosomes vi-cores were slightly more stable than the other two types of cores. Evidence for a cellular uncoating mechanism is furnished by experiments which analyze the stability of cores in the presence of postmitochondrial cytoplasm or of 60 S ribosomal subunits derived from either uninfected or Sindbis virus-infected cells. The results obtained indicate that structural alterations of the core do not play a role in the regulation of disassembly and assembly of alphavirus cores. © 1996 Academic Press, Inc.

INTRODUCTION

Alphaviruses are enveloped plus-strand RNA viruses which form a genus in the Togavirus family (Murphy et al., 1995). The structure and replication of alphaviruses have been recently reviewed (Schlesinger and Schlesinger, 1996; Strauss and Strauss, 1994). The core of alphaviruses consists of the viral genome and 240 molecules of the viral core protein, which are probably organized into an icosahedral shell of triangulation number T = 4 (Choi et al., 1991; Paredes et al., 1992). The core accumulates in large amounts during viral replication in the cytoplasm of infected cells. Mature virus is formed by a budding process in which the cytoplasmic core (cy-core) is tightly enveloped by a modified cellular membrane containing the viral spike proteins to become a viral core (vi-core) (see Simons and Garoff, 1980, for a review). The interaction between spike proteins and the core is mediated by the cytoplasmic segment of the spike protein E2 and a complementary structure on the viral core (Lopez et al., 1994; Zhao et al., 1994). Since the E2 protein is present in the viral envelope in 240 copies this interaction probably occurs 240 times in the assembly of the mature virus (Paredes et al., 1993; Cheng et al., 1995) and furnishes the energy needed for the budding process (Suomalainen et al., 1992). Alphaviruses infect cells by an endosomal pathway (see Marsh and Hele-nius, 1989, for a review). At the pH of 5.5, present in this compartment, a reorganization of the spike proteins occurs which leads to fusion of the viral and the endosomal membrane followed by release of the core into the cytoplasm (Wahlberg et al., 1992; Bron et al., 1993; Justmann et al., 1993; Fuller et al., 1995). This core will be called postendosomal core (pe-core) in this article. During release the interactions between the E2 protein and the core have to be dissociated. This dissociation is most likely the consequence of reorganization of the viral glycoproteins in the endosome. These considerations show that the core is subjected to significant forces during establishment and dissociation of the spike–core interactions and lead to the question of whether structural differences exist between cy-cores, vi-cores, and pe-cores.

From the description given above it is not evident why alphavirus cores accumulate as stable structures late in infection, whereas they are unstable during the early stages of infection when they liberate the genome RNA for translation. Three different types of explanation can be put forward to understand the regulation of the stability of alphavirus cores: (1) The structure differs between stable cores which accumulate late in infection and labile cores which are dissociated early, (2) a cellular uncoating process is active in the early stages of infection which is inactivated during viral multiplication, (3) both types of mechanisms cooperate. A model based exclusively on the second principle has been proposed (Wengler, 1987).

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In this model alphavirus cores are unstable in the presence of cellular ribosomes, because a ribosomal binding site is exposed on the core surface, which leads to an interaction of cores with ribosomes followed by a transfer of core protein to ribosomes and a concomitant disassembly of cores. During virus multiplication newly synthesized viral core protein is bound to the ribosomes, saturates the ribosomes with core protein, and thereby inactivates the ability of the ribosomes to bind and disassemble the cores. On the other hand, structural changes of the core during the viral life cycle might occur and may cooperate with this cellular uncoating process. For example, the cy-core accumulating late in infection might have a structure which renders it rather stable against the disassembly by interaction with ribosomes and this conformation might be changed during the budding process. Similarly, the pe-core which is liberated into the cytoplasm in the early stages of infection might be especially labile against the cellular uncoating process.

The considerations presented above indicate that structural and/or functional differences might exist between cy-cores, vi-cores, and pe-cores. We have therefore attempted to isolate these cores specified by the Sindbis alphavirus and to compare their structural and functional properties. Since pe-cores do not accumulate after infection they cannot be isolated from infected cells. In order to overcome this difficulty virus particles were incubated at an endosomal pH in vitro, followed by isolation of the viral cores under appropriate conditions (see Results). The resulting cores are called artificial pe-cores and are used in the experiments. Comparative structural analyses involve chemical crosslinking of protein and proteolytic cleavage of cores. The stability of the cores in the presence of ribosomes in vitro was also analyzed. The results of these analyses are presented in this article.

MATERIALS AND METHODS

Materials and reagents

Trypsin (from bovine pancreas, treated with TPCK) was bought from Fluka. 3,3'-Dithio bis(sulfo succinimidylpropionate) (DTSSP) was obtained from Pierce. Nonidet-P40 (NP-40) detergent was purchased from Schwarz-Mann. Rabbit reticulocyte lysate (not treated with nuclease) was bought from Promega.

Isolation of cores

For isolation of cores, purified Sindbis virus was suspended in buffer containing 100 mM NaCl, 20 mM triethanolamine, pH 7.4 (TN buffer), NP-40 was added to 1% concentration, and the material was incubated at 20°C for 5 min followed by sucrose density gradient centrifugation (10-40% sucrose in TN), using the SW 60, the SW 41, or the SW 28 Beckman rotor. The optical density profile was determined in a flow through cuvette and fractions containing cores were pooled. For structural analyses either this core solution was used directly or cores were pelleted and resuspended in 1/50 volume of TN. Isolation of cores from the cytoplasm was performed as follows: BHK 21 cell monolayer cultures were harvested about 12 hr p.i. and suspended in TN buffer. Cores were isolated from the cells either by extraction with NP-40 (cy-cores (N)) or after mechanical lysis (cy-cores (G)). In the procedure involving NP-40, the cell suspension was adjusted to 1% NP-40, incubated on ice for 5 min, and subjected to 15 min centrifugation at 50,000 g. EDTA was added to the supernatant and cores were isolated by sucrose density gradient centrifugation as described above. In the procedure involving mechanical lysis, one volume of cell suspension was mixed with a half volume of glass beads (0.5 mm diameter) and the suspension was vortexed for 1 min. The homogenate was processed as described above for the NP-40 extract by centrifugation at 50,000 g, addition of EDTA to the supernatant, density gradient centrifugation, and isolation of cores from the gradient. Artificial postendosomal cores were isolated as follows: Virus was suspended in buffer containing 200 mM NaCl and 0.5 mM triethanolamine, pH 7.4, at a concentration of about 10 mg virus/ml. Two volumes of 100 mM MES buffer, pH 5.4, is then added to one volume of virus suspension and the solution is incubated at 37°C for 8 min. An increase in turbidity develops during the first 2 min. DTT and bromelain are then added to 5 mM and 0.5 mg/ml, respectively, and the material is incubated at 37°C for 20 min followed by inhibition of bromelain, using the inhibitor E 64 (100 μM final concentration) and neutralization by addition of 10 vol of buffer containing 250 mM Tris – HCl, pH 8.1. The virus is then recovered by a 20-min centrifugation at 400,000 g and resuspended in TN buffer. The efficiency of proteolytic cleavage of the viral spike proteins is analyzed at this stage by SDS-PAGE. Cores are then isolated by NP-40 treatment and gradient centrifugation as described above for viral cores. These cores are called artificial pe-cores. For the preparation of unlabeled cores for structural analyses virus or cells derived from 100 large (15-cm-diameter) petri dishes were processed, using the SW 28 rotor. For the preparation of [35S]methionine-labeled cores infected cells were labeled in three large petri dishes with 2.5 mCi of [35S]methionine between 2 and 8 hr p.i. Virus was isolated from the growth medium, harvested at 8 hr p.i., and the cells were lysed with glass beads. Cores were isolated as described above, except that the cores were localized according to the radioactivity profile of the gradients.

Crosslinking and protease treatments of cores

In a standard crosslinking reaction cores were incubated in the presence of 1 mM DTSSP at 0°C in TN buffer. The NaCl concentration was modified by addition of concentrated NaCl solution as indicated in the description.
of the experiments. Two types of core preparations were used: Either the sucrose density gradient fractions were used directly (low core concentration reaction) or cores pelleted from the gradient and resuspended in 1/50 vol of TN buffer (high core concentration reaction) were subjected to crosslinking. The reaction was blocked by addition of lysine to 10 mM. From the low core concentration reactions, 150-μl aliquots were removed, and cores were pelleted by centrifugation, resuspended in SDS–PAGE sample buffer without mercaptoethanol, and analyzed by SDS–PAGE. From the high core concentration reactions 3-μl aliquots were added directly to buffer without mercaptoethanol and analyzed by SDS–PAGE. Gels containing 7.5% acrylamide and 0.25% bisacrylamide were used for these analyses (Laemmli, 1970). In a standard protease reaction cores were incubated in the presence of 2 μg/ml of TPCK–trypsin at 0°C in TN buffer. The NaCl concentration was modified by addition of concentrated NaCl solution as indicated in the description of the experiments. All cleavages were performed under high core concentration conditions (about 2 mg/ml of protein). One-to-five-microliter aliquots were taken, added to 40 μl of SDS–PAGE sample buffer containing mercaptoethanol, and subjected to SDS–PAGE analyses (Laemmli, 1970) using gels containing 12.5% acrylamide and 0.4% bisacrylamide.

Analysis of core stability in vitro

Core stability was analyzed in vitro in rabbit reticulocyte lysate, in the cytoplasm of mock-infected and infected BHK cells, or in the presence of large ribosomal subunits isolated from uninfected or infected cells. In a typical experiment involving reticulocyte lysate 10 μl of [35S]methionine-labeled cores was added into 150 μl of S30 lysate at 0°C and concentrated NaCl was added as indicated in the description of the experiments. In accordance with the data given by the supplier (Promega) it was assumed that no endogenous NaCl was present in the lysates. Reactions were incubated at 0, 30, or 37°C and 50-μl aliquots were removed at various times, added into 200 μl of buffer containing 20 mM triethanolamine, pH 7.4, 2 mM EDTA, and kept at 0°C. For analysis of core disassembly the material was loaded onto a 10 to 40% sucrose density gradient in TN buffer and subjected to 1 hr centrifugation at 60,000 rpm in the SW 41 Beckman rotor. Ribosomal subunits were localized from the optical density profile of the gradient. The fate of the core protein was analyzed by determination of the radioactivity profile by liquid scintillation counting. Similar experiments were also performed using BHK cell cytoplasm. Postmitochondrial cytoplast was prepared from mock-infected and from SIN virus-infected cells at 14 hr p.i. Cells were suspended in TN buffer at 0°C, one-half volume of glass beads (0.5 mm diameter) was added, and the cells were homogenized by vortexing. The homogenate was decanted and mitochondria were removed by 20 min centrifugation at 50000 g. The supernatant, the postmitochondrial cytoplasm, is stored at −170°C prior to use. In a standard analysis of core stability 10 μl of labeled cores was added to 150 μl postmitochondrial cytoplasm at 0°C. The NaCl concentration was then adjusted by the addition of concentrated NaCl solution as indicated on the description of the experiments and the experimental analyses were performed as described above. Core stability was also analyzed in the presence of purified large ribosomal subunits. Subunits were isolated from the cytoplasm stored at −170°C (see above). Five hundred microliters of cytoplast was adjusted to 3 mM EDTA and subjected to 5 hr centrifugation at 41,000 rpm in the SW 41 Beckman rotor using a 10–40% w/w sucrose gradient in TN buffer. The 60 S ribosomal subunits were pooled from the gradients according to the optical density profile, dialyzed against TN buffer, stored on ice, and used during the next 4 days. In a typical analysis 150-μl subunits and 10 μl of labeled cores were mixed on ice, the salt concentration was adjusted as indicated in the description of the experiments, and the reactions were incubated at 30°C. The reaction products were analyzed by gradient centrifugation as described above for the reticulocyte lysate reactions.

RESULTS

Comparative analyses of the structure of vi-cores and cy-cores

In a first series of experiments the structure of vi-cores and cy-cores was compared. Vi-cores were prepared by removal of the viral membrane with NP-40. The cy-cores either were extracted from cells by NP-40 or were isolated from cells which were mechanically disrupted with glass beads; the resulting cytoplasmic cores are called cy-cores (N) and cy-cores (G), respectively. Core structures were compared by analyses of crosslinks generated by the homobifunctional amine reactive crosslinker DTSSP or of the proteolytic cleavages performed by trypsin. The authentic core protein has an apparent molecular weight of 33 kDa. A series of experiments involving these analyses under a variety of conditions of temperature, salt concentration, and concentrations of DTSSP or trypsin were performed. No significant differences between vi-cores and cy-cores were detected. The results of four analyses are presented in Fig. 1. Treatment with DTSSP at 0°C in TN buffer containing 100 mM NaCl and 20 mM triethanolamine, pH 7.4, crosslinks the core protein in the same manner in vi-cores and in cy-cores (Fig. 1A). If the salt concentration in this reaction is increased, high-molecular-weight polymers of core protein are generated much faster, but no difference can be detected between the vi-cores and the cy-cores (data not shown). The kinetics of proteolysis presented in Figs. 1B and 1C show that the cores are resistant against trypsin in the presence of 100 mM NaCl (Fig. 1B) and that this resistance is lost if the protease treatment is performed in
FIG. 1. Comparative structural analyses of viral and cytoplasmic SIN virus cores. The four parts of the figure show SDS-PAGE analyses of protein patterns stained with Coomassie blue, obtained from concomitant analyses of vi-cores, cy-cores (N), and cy-cores (G). In all analyses the reaction aliquots taken at the same time were analyzed on adjacent lanes of the gels in the order cy-cores (N), vi-cores, cy-cores (G). The authentic core protein has an apparent molecular weight of 33 kDa. An SDS-PAGE analysis of the protein patterns obtained after crosslinking cores by DTSSP (1 mM, 0°C) at low core concentrations in buffer containing 20 mM triethanolamine and 100 mM NaCl, pH 7.4, is shown in A (see Materials and Methods). Aliquots were taken from the reactions at the times indicated in the figure and subjected to SDS-PAGE on a gel containing 7.5% acrylamide. Marker proteins were separated on lane M. Analyses of the kinetics of proteolysis of the cores by trypsin are presented in B and C. Proteolysis was performed in the presence of 2 μg/ml of trypsin at 0°C, either in TN buffer (B) or in TN buffer containing 750 mM NaCl (C). Samples digested for 0, 2, 4, 8, 16, and 32 min were analyzed as indicated in the figure. An analysis of the proteolytic fragments generated from cores in the presence of 1.5 μg/ml of trypsin, using a single time of protease digestion (15 min) at 0°C at different NaCl concentrations, is presented in C. The NaCl concentrations are indicated in the figure. Gels containing 12.5% acrylamide were used in the analyses presented in B, C, and D; marker proteins were separated on lane M on these gels.

the presence of 750 mM NaCl (Fig. 1C). In the experiment presented in Fig. 1D, a single time of protease digestion was used and the concentration of salt was increased in a stepwise manner. It can be seen that the proteolytic cleavages which can be detected (Figs. 1C and 1D) occur in an identical manner in vi-cores and cy-cores.

Comparative analyses of the structure of vi-cores and artificial pe-cores

In contrast to the cytoplasmic cores which accumulate in large amounts late in infection, the pe-cores are transient structures. A procedure which would allow isolation of pe-cores from infected cells in amounts sufficient for performance of the structural analyses described above does not exist. Therefore, artificial pe-cores were prepared in vitro as follows (see Materials and Methods for details): Purified SIN virus was adjusted in vitro to the endosomal pH of 5.5 by addition of MES buffer, which did result in increased turbidity of the solution. If conformational alterations of spike proteins in the endosome lead to structural changes of the viral core, these changes should have occurred in this virus preparation. The isolation of cores from these virus particles is not trivial: Direct addition of NP-40 might lead to reversion of the structural alterations. Therefore, the spike proteins of virus particles were degraded at pH 5.5, using the nonspecific protease bromelain which is most active at acid pH. Bromelain is then inhibited and the turbid virus suspension is neutralized by dilution into neutral buffer. It is unlikely that after proteolytic degradation of the spikes neutralization can lead to a reversion of the virus struc-
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FIG. 2. Preparative density gradient centrifugation of vi-cores and artificial pe-cores. SIN virus was left either untreated (vi-cores) or was treated at low pH with bromelain (artificial pe-cores). Cores were isolated from these particles by treatment with NP-40 followed by preparative sucrose density gradient centrifugation (see Materials and Methods). The optical density profiles obtained in this centrifugation for material derived from control untreated virus particles and from acid and protease-treated viruses are shown in A and B, respectively. A 3-hr centrifugation at 30,000 rpm was performed in the SW 41 Beckman rotor. An aliquot of the samples which were loaded onto the gradients was subjected to analytical SDS-PAGE. The Coomassie blue-stained protein patterns obtained are shown in the inset in B: samples containing untreated virus, acid and protease-treated virus, and marker proteins are separated on lanes 1, 2, and 3, respectively.

ture to a neutral conformation. Virus particles are then pelleted by ultracentrifugation, untreated control virus is pelleted in the same experiment, both virus preparations are suspended in neutral buffer, and the cores are isolated by standard NP-40 treatment and gradient centrifugation. The optical density profiles of such gradients which allow localization of the cores are presented in Fig. 2. SDS-PAGE analyses of the preparations, taken prior to sucrose density gradient centrifugation, are presented as an inset in Fig. 2B. It can be seen that bromelain has degraded the spike protein efficiently. The cores derived from this virus preparation are therefore called artificial pe-cores. It can be seen that the sedimentation profiles of vi-cores and artificial pe-cores are qualitatively similar. The same series of comparative structural analyses which were performed with vi-cores and cy-cores were also done using vi-cores and artificial pe-cores. No significant differences were found between these cores in these experiments. The results of three analyses are presented in Fig. 3. The protein crosslinking patterns obtained by reaction with DTSSP in buffer containing either 100 or 200 mM NaCl are shown in Fig. 3A. The influence of increasing salt concentration on the susceptibility of the cores against trypsin is shown in Fig. 3B.

Comparative analyses of the stability of vi-cores, cy-cores, and artificial pe-cores in the presence of ribosomes

In earlier experiments the disassembly of alphavirus cores in the presence of ribosomes in rabbit reticulocyte lysates has been analyzed (Wengler et al., 1984, 1992). Viral cores were used exclusively in these experiments. Therefore, the possibility remains that in such experiments cy-cores are more stable than vi-cores or that a structural change which is not detected in the analyses reported above occurs during or after virus budding which leads to a labile structure of pe-cores.

The time course of disassembly of [35S]methionine-labeled cores in a rabbit reticulocyte lysate was therefore analyzed. A large series of experiments was performed in which either the stability of vi-cores and cy-cores or the stability of vi-cores and artificial pe-cores was analyzed concomitantly. Two parameters were changed in these analyses: the NaCl concentration in the reaction was adjusted to various concentrations between 50 and 300 mM and the reactions were performed at 0, 30, or 37°C. As an example of these experiments the time course of disassembly of artificial pe-cores and vi-cores at 30°C in the presence of 200 mM NaCl is shown in Fig. 4. It can be seen that both cores are dissociated with a concomitant transfer of core protein to the 60 S ribosomal subunit. The dissociation of the pe-core occurs slightly faster in this experiment than that of the vi-cores. This observation has been made in general in the stability analyses for both the pe-cores and the cy-cores. The most drastic effect was seen in the comparative analyses of vi-cores and cy-cores in reactions performed at 30°C containing 150 mM NaCl; cy-cores and vi-cores were completely dissociated after 12 and 20 min, respectively, in these reactions (data not shown). In contrast to the above considerations the cy-cores are not more stable against disassembly by ribosomes than the vi-cores, but are actually more labile. Therefore, it is unlikely that these differences in the core stability are involved in the regulation of core assembly and disassembly. This point will be examined further under Discussion.
vi-cores and cy-cores in these lysates was analyzed (Fig. 5). In accordance with the data shown in Fig. 4 it can be seen that if the cytoplasm derived from uninfected BHK cells is used, both cores are dissociated after 20 min incubation. These data show that the cy-cores are not inherently stable in the presence of ribosomes. On the other hand, it can be seen that both cores are stable in the cytoplasm derived from infected cells. These data indicate that during viral replication the dissociation of cores is regulated not by changes in the core structure but by alterations in a cellular uncoating mechanism.

It has been proposed that the ability of the cytoplasm to dissociate alphavirus cores involves interaction of 60 S ribosomal subunits with the viral core and that this interaction is inhibited by binding of newly synthesized viral core protein to the large ribosomal subunit during the later stages of viral replication (Wengler, 1987). The data presented in Fig. 5 then lead to the question of whether it is possible to reproduce the results obtained in these analyses by using 60 S ribosomal subunits derived from uninfected and infected cells instead of the complete cell lysate. The results obtained in such experiments are dependent on the temperature and salt concentration used in the reactions. The results obtained in an experiment in which the stability of vi-cores and cy-cores in the presence of ribosomal subunits in buffer containing 400 mM NaCl at 30 °C was analyzed are presented in Fig. 6. It can be seen that after 30 min incubation both the vi-cores and the cy-cores are completely dissociated in the presence of subunits derived from uninfected cells (Figs. 6A and 6C), whereas in the presence of subunits derived from infected cells at least half of both the vi-cores and the cy-cores are still apparently intact (Figs. 6B and 6D). These data show that both cores behave similarly in these analyses and that the 60 S ribosomal subunits derived from infected cells are much less active in core uncoating than the subunits derived from uninfected cells. These findings will be examined further under Discussion.

DISCUSSION

In order to discuss the implications of the above experiments it is useful to summarize our current understanding of the regulation of the stability of alphavirus cores. The model proposed is based solely on a cellular uncoating mechanism. Different lines of experimental evidence indicate that the disassembly is mediated by a transfer of core protein from the incoming core to the 60 S ribosomal subunit of the cellular ribosomes (see Wengler, 1987, for a review; Singh and Helenius, 1992). The site(s) on the ribosome involved in this reaction has not been characterized, but a sequence element of the core protein involved in the interaction has been identified by the following experiments (Wengler et al., 1992): Trypsin digestion of Sindbis virus core in the presence of about 700 mM NaCl does release the carboxy-terminal protease domain.
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FIG. 4. Analyses of the stability of vi-cores and artificial pe-cores in rabbit reticulocyte lysate. [35S]Methionine-labeled cores were isolated as described under Materials and Methods. 10 μl of these cores was added to 150 μl of rabbit reticulocyte lysate containing 200 mM NaCl at 0°. 50-μl aliquots were removed prior to incubation at 37 or after incubation at 37 for 4 or 16 min. Each aliquot was added into 200 μl of buffer containing 20 mM triethanolamine, 2 mM EDTA, pH 7.4, at 0° and the material was fractionated by 1 hr centrifugation at 60,000 rpm on a 10 to 40% sucrose density gradient using an SW 60 Beckman rotor. The optical density and radioactivity profiles were determined. The data obtained are shown in this figure. Reactions containing vi-cores incubated at 37 for 0, 4, or 16 min are analyzed on the gradients shown in A, B, and C, respectively. The corresponding reactions containing artificial pe-cores are shown in D, E, and F, respectively. The optical density profiles show two peaks which correspond to the large (60 S) and small (40 S) ribosomal subunits; the intact core sediments into the region comprising fractions four to six.

of the core protein as a soluble protein which has the residue M (106) as the amino-terminus (Strong and Harrison, 1990). This protein fragment does not bind to ribosomes. If endoproteinase Lys C is used in this reaction, a larger core protein fragment having the residue Q (94) as the amino-terminus is released. This fragment binds to ribosomes and inhibits the ability of cell lysates to disassemble viral cores in vitro. The core protein sequence K (99) PGKRQRMALKLEAD (113) which is highly conserved between different alphaviruses has therefore been proposed to represent the protein sequence which mediates binding of core protein to ribosomes. Trypsin cleavage of cores hydrolyzes the bond between R (105) and M (106) and thereby inactivates this site. Since this sequence is located in the central region of the core protein between the basic amino-terminal domain and the carboxy-terminal protease domain, this sequence has been named RBSc (central ribosome binding site) (Wengler et al., 1992). Newly synthesized alphavirus core protein remains associated to the 60 S subunit of cellular ribosomes during and after synthesis (Ulmanen et al., 1976) and cellular ribosomes contain bound viral core protein (Ulmanen et al., 1979). These data indicate that a modification of the 60 S ribosomal subunit by core protein binding during virus multiplication might inactivate the uncoating mechanism. In this model changes in the core structure play no role in the regulation of core stability. In all experiments mentioned above, cores derived from purified virus particles were used. Therefore, the possibility remains that structural alterations...
crosslinking indicate that the structural interactions between the molecular components of these cores are very similar and possibly identical. The data are in accordance with analyses of control and acid-treated virus by X-ray scattering (Stubbs et al., 1991). The analyses of the protease sensitivity of vi-cores, cy-cores, and artificial pe-cores not only show that the cores, which are protease resistant at 100 mM NaCl, are protease sensitive at 800 mM NaCl but that the transition to protease sensitivity with increasing salt concentration is indistinguishable of the core might occur during budding and/or in the endosome, which might cooperate with the above-mentioned processes in the regulation of the stability of alphavirus cores. To our knowledge a specific structural or functional comparison of the different alphavirus core structures has not been published.

The structural comparisons of Sindbis virus cy-cores, vi-cores, and artificial pe-cores by protease digestion and
between different cores (see Fig. 3B). Similar conclusions can also be drawn from the crosslinking experiments.

The structural experiments reported analyze the association of molecules within the core. Localized structural changes probably cannot be efficiently detected by these experiments. It might be argued that a localized change occurs in the RBS$_c$ site during the viral replication cycle. Cores accumulating in the cytoplasm might have an RBS$_c$ site inaccessible for ribosomes and this site would become exposed during or after viral budding. The structural analyses do not support such a hypothesis. As indicated above, the site of tryptic cleavage of the viral core protein is localized within the RBS$_c$ site. The analyses of the protease sensitivity of cores therefore show that the structure of this site, as measured by its accessibility to trypsin, is identical in cy-cores, vi-cores, and artificial pe-cores.

The stability of Sindbis cores in rabbit reticulocyte lysates was measured in experiments in which vi-cores were compared to cy-cores or artificial pe-cores. The two parameters varied in these experiments were the salt concentration in the reaction and the temperature. The kinetics of disassembly of the two cores were similar but consistently the vi-cores were slightly more stable than either the cy-core or the pe-core (see Fig. 4 for a typical experiment). These differences are small and they cannot explain the regulation of assembly and disassembly of cores during the viral life cycle. Cy-cores which are stable in the cellular cytoplasm in vivo are more labile in the reticulocyte lysate than the vi-cores, a finding that indicates that the differences observed in the lysate do not reflect biologically significant differences. A likely basis for the observed differences might be that the cores isolated from virus particles are constructed more regularly from the components than the cy-cores or the artificial pe-cores. Viral envelopment might select for regularly assembled cores or might correct small structural faults in the cores. This process could explain why the vi-cores consistently were slightly more stable than the other cores.

The data discussed so far provide indirect support for the proposed cellular uncoating mechanism. More direct evidence for this mechanism is furnished by two types of experiments reported above: (1) Vi-cores and cy-cores are unstable in the cytoplasm of uninfected BHK cells but are not disassembled in the cytoplasm derived from BHK cells 14 hr p.i. (Fig. 5). (2) The large ribosomal subunits derived from infected cells 14 hr p.i. are much less active in core disassembly than subunits from uninfected cells (Fig. 6). The presence of a small percentage of unmodified ribosomal subunits in the in vitro reactions obviously would lead to core disassembly. In the reactions involving cytoplasmic lysates large amounts of cores are present in the lysates derived from infected cells at 14 hr p.i. (data not shown). These cores represent a large reservoir of core protein and probably would deliver new core protein to those ribosomes which lose

their bound core protein during the in vitro incubation with the small amounts of labeled cores. If the purified large ribosomal subunits are used in these incubations, this mechanism of keeping the subunits saturated with core protein no longer exists. These processes would explain why the 60 S ribosomal subunits derived from the infected cells show some uncoating activity. These considerations might also be relevant for understanding that lysates derived from BHK cells 8 hr p.i. with the alphavirus Semliki Forest (SF) virus were active in uncoating labeled SF nucleocapsids (Singh and Helenius, 1992). It is possible that in vitro only under very favorable circumstances a condition of almost complete saturation of ribosomes with core protein can be sustained which is necessary to observe the inhibition of uncoating. The fact that in the experiments reported in this article lysates and ribosomes were isolated from rather late stages of infection might be relevant in this respect and differences might also exist between Semliki Forest virus and Sindbis virus-infected cell lysates in the possibility to establish such conditions in vitro. Furthermore, the synthesis of alphavirus-specific RNA and protein might occur in localized regions of the cytoplasm in the earlier stages of virus multiplication. The inhibition of the uncoating activity might then be localized to these regions and would not be detectable in total cell lysates. The in vitro disassembly reactions involving purified ribosomal subunits might represent a further step in an in vitro analysis of alphavirus uncoating but it will be of special importance to clarify in parallel experiments whether the phenomena detected in such reactions are also relevant in vivo.

Taken together the comparative structural analyses and the data obtained in the comparative analyses of the stability of cores in the presence of ribosomes indicate that structural alterations of the viral core probably do not play a role in the regulation of the stability of alphavirus cores. Obviously the experiments reported do not exclude the fact that local changes might occur at specific sites in the viral core, e.g., during the interaction of the E2 spike protein cytoplasmic domain with the viral core during budding at core protein residues participating in this reaction. The results obtained indicate that such changes remain localized and are not involved in the regulation of assembly and disassembly of the cores.

The regulation of disassembly and assembly of cores is a general problem in viral replication, which in most systems is not understood in molecular terms. Whether the mechanism proposed above for alphaviruses is used by other viruses as well is not known. The fact that the expression of viral coat protein in plant cells can induce resistance against infection by the corresponding or closely related viruses (see Beachy et al., 1990, for a review) indicates that these viruses may use similar mechanisms to regulate disassembly and assembly of viral ribonucleoproteins.
REFERENCES


