Intracellular location and translocation of silent and active poliovirus replication complexes

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Replication of poliovirus (PV) genomic RNA in HeLa cells has previously been found to start at distinct sites at the nuclear periphery. In the present study, the earliest steps in the virus replication cycle, i.e. the appearance and intracellular translocation of viral protein and negative-strand RNA prior to positive-strand RNA synthesis, were followed. During translation, positive-strand RNA and newly synthesized viral protein presented as a dispersed endoplasmic reticulum (ER)-like pattern. Concomitant with translation, individual PV vesicle clusters emerged at the ER and formed nascent replication complexes, which contained newly synthesized negative-strand RNA. The complexes rapidly moved centripetally, in a microtubule-dependent way, to the perinuclear area to engage in positive-strand viral RNA synthesis. Replication complexes made transcriptionally silent with guanidine/HCl followed the anterograde membrane pathway to the Golgi complex within the microtubule-organizing centre (MTOC), whereas replication complexes active in positive-strand RNA synthesis were retained at the nuclear periphery. If the silent replication complexes that had accumulated at the MTOC were released from the guanidine block, transcription was not readily resumed. Rather, positive-strand RNA was redistributed back to the ER to start, after a lag phase, translation, followed by negative- and positive-strand RNA synthesis in replication complexes migrating to the nuclear periphery. As some of the findings appear to be in contrast to events reported in cell-free guanidine-synchronized translation/transcription systems, implications for the comparison of in vitro systems with the living cell are discussed.

INTRODUCTION

During replication in their host cells, all positive-strand RNA viruses studied so far provoke membrane modifications that result in the formation of virus replication complexes. This process creates distinct, new compartments in the cell where replication of the viral genome takes place. Different viruses utilize different ways to assemble their replication complexes. For example, hepatitis C virus (HCV) inserts its RNA-dependent RNA polymerases into membranes in the absence of other viral proteins (Ivashkina et al., 2002; Schmidt-Mende et al., 2001), although ultimately forming a multiprotein complex (Egger et al., 2002; Gosert et al., 2002; Mottola et al., 2002; reviewed by Moradpour et al., 2003). Coronavirus and plant alphaviruses use viral membrane-bound proteins as organizer proteins of the replication complex (Brockway et al., 2003; Chen & Ahlquist, 2000; Chen et al., 2003; Prod’homme et al., 2003) and the picornaviral polymerase associates in a protein precursor stage with nascent replication complexes that form concomitantly with translation in cis (Collis et al., 1992; Egger et al., 2000; Jurgens & Flanagan, 2003).

The replication complex of poliovirus (PV), the prototype member of the family Picornaviridae, has been extensively studied. It contains all viral structural and non-structural proteins (Bienz et al., 1992; Egger et al., 1996, 2000; Girard et al., 1986; Takegami et al., 1983) and RNA replication occurs on the cytosolic surface of membraneous vesicles that aggregate into a functional rosette-like structure (Bienz et al., 1992; Choe & Kirkegaard, 2004; Egger et al., 1996).

The different virus replication steps have to follow each other in a coordinated way. Despite extensive investigations on the individual steps, there are still many open questions and discrepancies (reviewed by Agol et al., 1999), for example, concerning the influence of certain elements on RNA replication, such as the internal ribosome entry site (Borman et al., 1994; Murray et al., 2004) and the cis-acting replication element (Goodfellow et al., 2003; Morasco et al., 2003; Murray & Barton, 2003; Paul et al., 2000). Such discrepancies may in part be explained by the use of different experimental approaches, i.e. cell extract-based translation/transcription or chemically defined systems, or the use of living cells. In vitro systems can allow elegant analysis of independent replication steps. In vivo, however, most of the replication steps are linked and associated with, if not dependent on, cellular structures, such as the initiation of
positive-strand RNA synthesis, which was found to be dependent on specialized vesicular membranes in the replication complex (Egger et al., 1996).

PV-induced vesicles are considered to be derived from components of the cellular anterograde membrane traffic system (Rust et al., 2001; Suhy et al., 2000). This pathway establishes a membrane flow, along microtubules (MTs), from the endoplasmic reticulum (ER) to the microtubule-organizing centre (MTOC) harbouring the Golgi complex (Rios & Bornens, 2003; Scales et al., 1997; Thyberg & Moskalewski, 1999), with which the vesicles ultimately fuse (Klumperman, 2000; Scales et al., 1997). However, PV vesicles, although derived from the anterograde membrane pathway, do not appear to fuse with the Golgi complex (Bolten et al., 1998; Rust et al., 2001).

In PV-infected neurons, migration of intact virions, enclosed in endosomes, over long distances along axons was found to be necessary to establish infection in a cell (Mueller et al., 2002; Ohka et al., 1998). In HeLa cells, intracellular translocation of components of the PV replication machinery was recently inferred from the detection of perinuclear start sites for RNA replication, which were quite distant from the peripheral decapsidation site of the viral genome (Egger & Bienz, 2002). Since virions were reported to be decapsidated rapidly in HeLa cells (Huang & Bienz, 2002). Since virions were reported to be decapsidated rapidly in HeLa cells (Huang et al., 2000), genomic viral RNA rather than virions might be transported to the putative replication start sites. Such a process would be comparable to the transport described for cellular RNAs, which are translocated to a translation site at which their translation product is needed (Derrigo et al., 2000; Huang et al., 2003).

The present paper was aimed at elucidation of the translocation of viral molecules and the location of virus-specific processes within the cytoplasm of HeLa cells at early stages after PV infection. Rather than using PV constructs with specific replication defects, we used inhibitors of transcription, viral transcription or intracellular transport to manipulate defined steps in PV replication. We found that, in parallel with viral protein synthesis at the ER, PV vesicles and negative-strand RNA appeared, thus forming nascent replication complexes. The complexes rapidly moved, in a MT-dependent way, to the perinuclear area where they were retained. Inhibition of translocation did not affect virus replication, suggesting flexibility in the intracellular location of viral RNA replication. Replication complexes rendered transcriptionally inactive by guanidine/HCl were retained at the nuclear periphery, but moved on to the MTOC. After release of the guanidine block, no immediate restoration of function of the replication complex components at the MTOC could be observed. Rather, positive-strand RNA redistributed to the ER and restarted the virus replication cycle by initiating translation, followed by negative- and positive-strand RNA synthesis. This differed from results reported from cell-free translation/transcription systems, where pre-initiation complexes, formed during a translation period with guanidine-inhibited RNA synthesis, started transcription within minutes after guanidine release (Barton & Flanagan, 1997).

Functional assays using in vitro systems (Barton et al., 1995; Molla et al., 1991; Paul et al., 1998) are increasingly being used to complement in vivo methods. Although individual PV replication steps are generally faithfully reflected by in vitro systems, our findings suggest that quantitative as well as qualitative differences between the living cell and in vitro assays may exist.

**METHODS**

**Cells, virus infection and inhibitors.** HeLa cells were grown as monolayer cultures and infected with PV Mahoney type 1 at an m.o.i. of 15 at 36 °C. Infections were left to proceed in Eagle’s MEM containing 5% fetal calf serum. Quantification of PV progeny by plaque titration was done using standard procedures in confluent HeLa cell monolayers. For mixed infections with PV type 1 and type 2, cells were infected sequentially as described in Egger & Bienz (2002).

Cycloheximide (CHI; 200 μM) and puromycin (200 μM; Calbiochem) were used as inhibitors of translation. Guanidine/HCl (Calguiri & Tamm, 1968), a reversible inhibitor of PV transcription, was added to cells at a concentration of 2 mM. The guanidine block was reversed by washing cells three times with medium. To disrupt MTs, cells were kept for 10 min at 0 °C (Ward et al., 2001). Nocodazole (5 μM; Sigma) was added to prevent repolymerization of MTs upon rewarming to 36 °C. The disruption of MTs was confirmed by immunofluorescence (IF).

**IF and fluorescent in situ hybridization (FISH).** For IF or FISH, cells were grown on glass cover slips and fixed as described previously (Bolten et al., 1998). The protocol for strand-specific detection of RNA, including input RNA by FISH, has been described in detail (Bolten et al., 1998; Egger & Bienz, 2002). Briefly, for the detection of positive-strand PV RNA, an FITC-labelled RNA probe of negative polarity spanning nt 4440–7440 (numbers correspond to genomic PV RNA) was used. For the detection of negative-strand PV RNA, an FITC-labelled probe of positive polarity spanning nt 1–6867 was hybridized to the cells after thermal denaturation of the specimens. Strand specificity of the riboprobes was ascertained on Northern blots of RNA from infected cells and purified virions.

For visualization of mixed replication complexes containing sequences from PV types 1 and 2, two FITC-labelled, PV type 1-specific riboprobes (nt 3083–3185 and nt 2687–2784) and two Alexa 546-labelled, PV type 2-specific probes (nt 3085–3187 and nt 2675–2784) were mixed and hybridized to cells infected with PV types 1 and 2 as described previously (Egger & Bienz, 2002).

For indirect IF, the following antibodies directed against PV or cellular proteins were used: monoclonal antibody (mAb) anti-PV 2B, 1D3.B1 (Egger et al., 1996), rabbit polyclonal anti-giantin Ab (Covance) and anti-β-tubulin Ab (Chemicon). The secondary Abs used were goat anti-mouse Ab coupled to Alexa 488 or TexasRed and goat anti-rabbit Ab coupled to TexasRed (all from Molecular Probes).

**Light (LM), confocal laser-scanning (CLSM) and electron (EM) microscopy.** IF and FISH preparations were viewed under a Nikon Eclipse 800 microscope with appropriate filters and photographed on Kodak P3200 Tmax b/w film. Positives were printed in greyscale or appropriately false-coloured from scanned films using Silverfast and Adobe Photoshop software. Image acquisition at the CLS microscope and bleeding controls from one detection channel into the other were done as previously described (Rust et al., 2001).
To obtain EM sections of PV-infected cells oriented in the same way as in LM preparations, cells were grown on a tissue culture grade plastic support (Thermanox; Nunc) fitted into a 3.5 cm Petri dish. Cells were fixed at different times post-infection (p.i.) with 2.5% glutaraldehyde and 2% OsO4. The dehydrated cells were embedded in Epon-812 resin on the support in the following way: the support was placed on a thin layer of fluid resin with the cells facing the resin and then several resin-filled standard EM gelatin capsules were inverted and placed on to the other side of the support. After polymerization, the support was cut around the individual capsules. This resulted in capsules with one end fitting into the microtome chuck and carrying on the other end cells embedded in a thin layer of Epon and facing the microtome knife. The cells could thus be sectioned in the same plane as that observed by LM, which resulted in serial sections from the top of the cell down to the support, comparable to a through-focus series by LM.

**Northern blot hybridization.** RNA was phenol/chloroform extracted from PV-infected cells at the indicated times p.i., ethanol precipitated, loaded on to a denaturing 1% agarose gel and blotted. For the detection of positive- or negative-strand viral RNA, hybridization was done with the two strand-specific riboprobes described above for FISH, which were DIG-labelled instead of FITC-tagged. Since positive-strand RNA is not yet present in a large excess over negative-strand RNA at early stages of infection, a two-cycle RNase protection assay for negative-strand RNA detection was not required. Hybridized probes were visualized with alkaline phosphatase-coupled anti-DIG Fab fragment (Roche) and ECL (CDP-Star; Roche) and quantified with a Beckman densitometer.

**Western blotting.** Infected cells were lysed, the nuclei removed by centrifugation and the supernatant electrophoresed on a 10% polyacrylamide gel before blotting on to a nitrocellulose membrane. Viral proteins containing the 2C moiety were detected with mAb anti-2C 11/2.B1.1 (Egger et al., 1996) and a secondary anti-mouse Ab conjugated to peroxidase (Dako), visualized with ECL (Advance Western Blotting Detection kit; Amersham). The 2C-containing proteins were quantified with a Beckman densitometer.

**RESULTS**

**Intracellular location of early PV replication steps**

To elucidate the origin and formation of the previously reported perinuclear start sites for PV RNA replication (Egger & Bienz, 2002), we determined the intracellular location of the initial virus replication steps preceding positive-strand RNA synthesis, i.e. translation and negative-strand RNA synthesis.

Input positive-strand PV RNA could be found by strand-specific FISH to be dispersed in the cytoplasm of the newly infected cell at 40 min p.i. (data not shown; and Egger & Bienz, 2002). The first immunofluorescent signal of 2B, a non-structural and thus newly made viral protein, was found as tiny dots spread out in the cytoplasm at around 1.5 h p.i. (data not shown). In parallel with the onset of the bulk of viral protein synthesis (see Fig. 6a), by 2.5 h.p.i. FISH and IF signals had evolved into a typical, feathery ER-like pattern (Fig. 1a and b). This pattern was interpreted to reflect viral translation at the ER. At a slightly later stage, immunofluorescent preparations demonstrated the appearance of small granules intermixed with the featherly translation pattern (Fig. 1c).

To identify these structures and to facilitate comparison of the intracellular location and distribution of PV-related structures in EM and LM pictures, a procedure was developed that allowed us to section cells for EM in the same plane as that observed by LM, i.e. parallel to the surface...
of the culture vessel (see Methods). By EM, individual vesicles appeared concomitantly with the onset of viral translation (Fig. 2a). Slightly later, as soon as the immunofluorescent translation pattern emerged, EM showed clusters of a few vesicles spread out in the cytoplasm and always associated with the ER (Fig. 2b).

Negative-strand RNA was visualized by FISH as small granules dispersed in the cytoplasm and in a perinuclear location (Fig. 1d). Its earliest detection was towards the end of the period showing the viral translation pattern. Transcription of input positive-strand RNA into negative-strand RNA can occur only after cessation of translation (Barton et al., 1999; Gamarnik & Andino, 1998; Parsley et al., 1997). The simultaneous occurrence of translation and transcription can be explained by a slightly asynchronous replication of the several viral genomes present in the same cell. In parallel with the disappearance of the translation pattern, negative-strand RNA signals rapidly migrated to and accumulated in a perinuclear area, simultaneously decreasing in number (Fig. 1e).

Concomitantly, the newly made PV vesicles visualized by IF of viral protein 2B also accumulated in the perinuclear area (Fig. 1f), similar to the positive-strand RNA-specific FISH signal, which condensed and underwent a comparable migration process (Fig. 1g). Compatible with the LM data, EM preparations of parallel cultures, sectioned as outlined above, showed vesicle clusters surrounding the nucleus (Fig. 2c).

Our observations indicated a translocation of input viral RNA from the cell surface to a perinuclear area in several steps. First, the RNA invades the cytoplasm reaching the ER where translation ensues. Consequently, PV vesicles arise and translation eventually gives way to negative-strand RNA synthesis. The complexes of newly formed vesicles, viral protein, input positive-strand and newly made negative-strand RNA rapidly move towards the nucleus. After analysing large numbers of cells, we concluded that this migration took place during or shortly after negative-strand RNA synthesis and thus represented nascent replication complexes.
Nocodazole inhibits migration of nascent replication complexes but not virus replication

To evaluate the underlying cellular transport mechanism for the migration of nascent replication complexes, cells were depleted of MTs by chilling and nocodazole treatment. Around 2–5 h p.i., such cells showed the usual translation pattern in immunofluorescent and FISH preparations (data not shown; cf. Fig. 1a and b), indicating that transport of the genomic positive-strand RNA to the ER was not MT dependent. However, migration of the emerging replication complexes to the perinuclear area was blocked in the absence of MTs and the complexes remained dispersed at 3–5 h p.i. (Fig. 1h and i). In line with the LM data, EM pictures of cells not treated with nocodazole showed vesicle clusters located around the nucleus (Fig. 2c), whereas in nocodazole-treated cells, the vesicle clusters were dispersed throughout the cytoplasm (Fig. 2d). Comparison of Fig. 2(e) and (f) showed that ultrastructural details of individual vesicle clusters in cells with or without nocodazole treatment were identical. The structural features characterizing PV vesicles have been described previously (Bienz et al., 1987, 1994; Rust et al., 2001).

To test the possible influence of migration on virus replication, virus progeny of nocodazole-treated and untreated cells were titrated by plaque assay. Fig. 3(a) shows that, in cation, virus progeny of nocodazole-treated and untreated cells processed for CLSM. Fig. 3(b) shows that nocodazole treatment decreased the proportion of mixed replication complexes formed in cells doubly infected with two virus strains (Egger & Bienz, 2002). Thus, coalescence was tested to the same level whether the MTs were intact or not.

Thus, MT-dependent translocation of nascent replication complexes from the ER to the perinuclear area did not seem to be a prerequisite for the functionality of replication complexes, indicating that onset of positive-strand RNA synthesis may take place at, but does not depend on, perinuclear replication start sites.

Migration causes nascent replication complexes to coalesce

The accumulation of nascent replication complexes in a perinuclear region was found to coincide with a reduction in number (Fig. 1e). Coalescence of complexes during migration would increase the number of mixed replication complexes formed in cells doubly infected with two virus strains (Egger & Bienz, 2002). Thus, coalescence was tested after inhibition of ER-to-nucleus migration by nocodazole. Replication complexes containing one or both species of PV RNA were quantified in PV types 1 and 2 doubly infected cells processed for CLSM. Fig. 3(b) shows that nocodazole treatment decreased the proportion of mixed replication complexes to around 50 %, whereas in untreated cells the percentage was up to 80 % in line with previously published data (Egger & Bienz, 2002).

These findings indicated that migration of nascent replication complexes towards the nucleus favours their coalescence. Although not necessary for virus replication, this process may still be evolutionarily advantageous for the virus by enhancing virus recombination.

Newly synthesized viral proteins are necessary for translocation of viral products

To determine the viral product(s) triggering the migration of nascent virus replication complexes towards the nucleus, we analysed cells with CHI- or puromycin-inhibited translation or with ongoing translation and guanidine-blocked viral transcription.

If CHI was added at the time of infection, translation was completely inhibited (Fig. 4a) and input viral RNA molecules were found in discrete dots dispersed in the cytoplasm (Fig. 4b). Guanidine added at the time of infection, while blocking RNA replication, allowed viral translation to proceed as visualized by the appearance of the non-structural protein 2B (Fig. 4c). The newly made viral protein (Fig. 4c) associated with the non-replicating input positive-strand viral RNA migrated towards the nucleus (Fig. 4d). Surprisingly, the destination of the migrating virus molecules was altered under these conditions and resulted in a juxtanuclear spot rather than a perinuclear ring.

To evaluate the influence of ongoing translation on the ER-to-nucleus migration, translation was inhibited by CHI or puromycin approximately at the peak of viral protein synthesis (2–5 h p.i.), i.e. when some newly made vesicles and proteins were already present. One hour after addition of the inhibitors, viral proteins visualized by IF using Ab against protein 2B were found at the perinuclear area and in a juxtanuclear spot [Fig. 5a(i)] and 1 h later, all of the protein was accumulated at the juxtanuclear spot [Fig. 5a(ii)]. Labelling with Ab against the Golgi marker giantin identified this juxtanuclear spot as the Golgi-containing MTOC [Fig. 5a(i) and (ii)]. Thus, PV protein 2B and vesicles
ultimately migrated to and accumulated in the MTOC. The Golgi remained structurally intact during the blocked infection [Fig. 5a(i) and (ii)].

Most of the viral RNA in such cells remained at the site of translation, i.e. the ER [Fig. 5a(iii)]. This could be explained by cleavage and release of peptides from the polysomes,
whereas the viral mRNA was retained at those sites. RNA retention was slightly less pronounced after puromycin treatment [Fig. 5a(iii)] compared with CHI treatment (not shown), which is known to stabilize (‘freeze’) polysomes (Barton et al., 1999; Seiser & Nicchitta, 2000).

The findings indicated that complexes composed of newly synthesized viral proteins and vesicles but devoid of RNA have a tropism for the MTOC region and that the complexes reach this region by migrating via the perinuclear area.

Inhibition of viral RNA replication extends migration of nascent replication complexes to the MTOC

The described migration of non-functional replication complexes to the MTOC could mean that the perinuclear accumulation of functional complexes could be a result of retention at the nucleus and that the transcriptional activity of a virus replication complex could be pivotal for its intracellular location. Therefore, we tested whether the presence of viral RNA or continuing RNA transcription was necessary for retention by blocking ongoing viral RNA synthesis with guanidine.

The intracellular location of viral protein and RNA in relation to the Golgi complex and the MTOC was visualized by CLSM at 4 h p.i. in cells with functional replication complexes or in cells treated with guanidine at 2 h p.i., i.e. at the earliest stages of RNA synthesis (see time course of RNA replication in Fig. 6a). Functional replication complexes did not show an affinity for the Golgi apparatus or the MTOC as visualized in double-labeling immunofluorescent preparations with Ab against the cis-Golgi marker giantin and the viral protein 2B, irrespective of whether MTs and the Golgi were intact [Fig. 5b(i)] or disrupted by nocodazole treatment [Fig. 5b(ii)]. Inhibition of viral transcription at 2 h p.i. by guanidine resulted in a redistribution of the viral protein 2B towards the Golgi complex resulting in a redistribution of the viral protein 2B towards the Golgi complex within the MTOC region was observed. This redistribution was also found for the non-capsid proteins 3A, 2C and VPg (not shown) and for positive-strand RNA, which co-localized with viral protein 2B in the MTOC [Fig. 5b(iv)]. There was no extensive association between inactive replication complexes and Golgi components as shown by only a moderate co-localization of the two markers [Fig. 5b(iii), shown in yellow].

The experiments showed that membraneous complexes composed of vesicles, viral protein and RNA continue their migration from the nucleus to the MTOC only if not involved in viral RNA synthesis. Thus, the MTOC tropism of PV vesicles appears to be counteracted by ongoing RNA synthesis, resulting in retention of active replication complexes at the nucleus.

Resumption of virus replication after release from a guanidine block

We tested whether the viral molecules in the presumably ready-to-go replication complexes in the MTOC region would resume replicative activity after release from a guanidine block. PV-infected cells were guanidine treated at 2 h p.i., washed at 4 h p.i. and then tested for viral protein and negative- and positive-strand RNA synthesis in parallel with IF and FISH analysis to locate viral components.

Unexpectedly, there was no immediate formation of active replication complexes upon release from the guanidine block, but rather a lag phase before the reappearance of viral synthetic activities (Fig. 6a). Translation was resumed after about 30 min and negative- and positive-strand RNA synthesis after 1 h following release from the guanidine block. During the lag period, viral protein and positive-strand RNA migrated away from the Golgi into the cytoplasm (Fig. 6b), transforming after 1 h into typical translation patterns [Fig. 6b, (i)–(ii) and (vii)–(viii)]. Thirty minutes later, accumulation of negative- and positive-strand RNA containing replication complexes [Fig. 6b(iii), (vi) and (ix)] in the perinuclear region was observed.

Our findings indicated that after releasing guanidine-blocked viral transcription, the silent replication complexes apparently were not able to resume transcription. Since pre-initiation complexes formed in cell-free translation/transcription systems following guanidine treatment engage in negative- and positive-strand RNA synthesis immediately after reversal of a guanidine block (Barton & Flanagan, 1997), our data suggest that difference(s) exist in function(s) of structures involved in viral RNA replication in cell-free systems and the living cell.

DISCUSSION

Previous observations on the relocation of viral molecules and virus-induced structures in PV-infected cells (Egger & Bienz, 2002) prompted us to investigate further the role and mechanisms of migration of viral components to specific intracellular sites or compartments during the early stages of a PV infection.

In HeLa cells, decapsidation of PV RNA is thought to proceed at the cell periphery (Danthi & Chow, 2004). It is controversial whether this takes place at the cell membrane or, after uptake of the virions, from endocytotic vesicles (DeTulleo & Kirchhausen, 1998; Kronenberger et al., 1997; see also Ohka et al., 2004). The notion that decapsidation of PV proceeds within 20 min of adsorption of the virus to the cell (Huang et al., 2000) implies that the FISH signal at 40 min p.i. represented predominantly freshly decapsidated RNA. The migration of viral input RNA in HeLa cells is in contrast to neuronal cells, where endocytosed PV particles migrate along MTs in axons over long distances to the cytoplasm (Mueller et al., 2002; Ohka et al., 1998), comparable with adeno- and herpesviruses, which are transported by MTs across the cell to reach the site of replication in the nucleus (Sodeik, 2000).

Translation, the first step in PV replication, has been
suggested to take place at the ER (Egger et al., 2000; Roumiantzef et al., 1971), which delivers PV translation products to membranes (Echeverri & Dasgupta, 1995; Towner et al., 1996). As reported previously for HCV translation (Brass et al., 2002), PV translation at the ER can also be considered signal recognition particle-independent and most likely follows the concept put forward recently (Potter & Nicchitta, 2000). Accordingly, initiation of translation occurs at ER-bound ribosomes and remains at the ER if the nascent polypeptide contains domains for membrane association, such as PV proteins 2B, 2C, 3A and precursors. Only if the translation product is a cytosolic protein would the polysome be released from the ER, which is not the case for the PV polyprotein.

After approximately 1·5 h p.i., the FISH signal detecting viral positive-strand RNA adopted an ER-like pattern and a matching pattern was obtained by IF using Ab against the non-structural PV protein 2B. This is in line with viral translation proceeding in a membrane-bound fashion at the ER. Both IF and FISH ER patterns, dominant for almost an hour, gradually transformed into randomly dispersed granules, representing small clusters of vesicles as shown by EM. Thus, vesicles are formed immediately after the appearance of viral proteins. The close spatial and temporal association of membrane-bound translation and vesicle formation automatically ensures membrane specificity of the process of vesicle formation and obviates targeting signals of PV proteins for particular membranes.

The site at which negative-strand RNA can be detected at the earliest time point in the virus replication cycle was considered as the site of its synthesis. In our experiments using FISH, negative-strand RNA was detected towards the end of the predominant translation pattern in small dots similar to those formed by the protein 2B-containing vesicular clusters. Since negative-strand RNA was previously found to be vesicle associated (Bolten et al., 1998), we regarded the emerging vesicular clusters as the site of synthesis of this RNA species.

The post-translational transport of vesicular clusters from the dispersed translation sites to the perinuclear area was sensitive to nocodazole. This treatment impairs MTs and, consequently, causes fragmentation of the Golgi apparatus (Thyberg & Moskalewski, 1999). It is noteworthy that dispersed replication complexes and dispersed Golgi remnants (Thyberg & Moskalewski, 1999). It is noteworthy that dispersed replication complexes and dispersed Golgi remnants were not, or only rarely, associated with each other. Therefore, we concluded that the observed inhibition of migration of replication complexes by nocodazole was a direct result of the depolymerized MTs and not of a disintegrated Golgi carrying replication complexes along.

By using a combination of cytoskeleton inhibitors, it has been shown that cytoskeletal elements are dispensable for PV replication (Doedens et al., 1994). This is in contrast to HCV, where a functional cytoskeleton was found to be required for RNA synthesis (Bost et al., 2003). This finding might point to an important difference in the assembly and maintenance of the replication complexes of the two viruses. Our experiments confirmed that functional MT and thus translocation of replication complexes to the nuclear periphery are not mandatory for PV replication. The biological significance of the migration process is open. However, our experiments with cells infected with two different strains of PV suggested that migration could perhaps enhance encounter of viral genomes early in infection and thus promote recombination (Egger & Bienz, 2002). This might be sufficient to select for conservation of the propensity of the replication complexes to migrate towards the nucleus.

Our experiments with the translation inhibitor CHI indicated that translation was necessary for the MT-mediated, centripetal migration of vesicle clusters. However, viral translation cannot be uncoupled from formation of PV vesicles and thus one could interpret our results to mean that it is the newly formed PV vesicles that are prone to engage in migration.

After addition of translation or transcription inhibitors, the goal of migration changed according to the functional state of the replication complex. The migration of active replication complexes was halted at the nuclear periphery, whereas vesicle clusters moved on to the MTOC if transcriptionally inactivated by guanidine or CHI. The observed action of CHI on transcription can be explained by our finding that CHI dissociates RNA from vesicle–protein complexes. That the functionality of the replication complexes rather than a chemical effect of an inhibitor determined the final goal of migration was suggested by the finding that two inhibitors with different modes of action led to the same altered location.

Vesicular clusters, whether transcriptionally active or silent, use the MT system for their centripetal migration. Silent clusters follow the entire MT trail towards the Golgi area in the MTOC, comparable to the vesicles of the anterograde membrane pathway (Klumperman, 2000; Scales et al., 1997) from which the PV vesicles originate (Rust et al., 2001; Suhy et al., 2000). That functional replication complexes are

Fig. 6. Kinetics and location of viral synthetic activities in PV-infected cells in the absence of guanidine (−gua) or after guanidine release (gua 2–4 h) where guanidine was added to the cells at 2 h p.i. and removed at 4 h p.i. (arrows). (a) For quantification, cultures were processed at the indicated times for Western blots and developed with anti-2C Ab (i) or for negative-strand (ii) or positive-strand (iii) RNA-specific Northern blots and hybridized with DIG-labelled RNA probes. All blots were quantified by densitometry. (b) After guanidine removal, the location of PV protein 2B (i–iii), negative-strand RNA (iv–vi) or positive-strand RNA (vii–ix) was determined by IF and strand-specific FISH at 0·5 (i, iv, vii), 1·0 (ii, v, viii) and 1·5 (iii, vi, ix) h after release from the block. Bar, 20 μm.
blocked in migration at the nucleus, similar to antero-grade transport vesicles made migration-incompetent in uninfected cells (Scales et al., 1997), could be due to either a loss of MT association or by mechanical hindrance of their transport due to size and rigidity of active replication complexes. Either mechanism could be overcome by guanidine treatment, which can loosen the tight configuration of the replication complex (Bienz et al., 1990) and presumably affect the presence of viral or cellular components on the surface of the complex, thus influencing its behaviour towards MTs. Whether these effects result from inhibition of the guanidine-sensitive ATPase function of protein 2C (Pfister & Wimmer, 1999) is unknown.

In investigating the reversibility of the guanidine block, we found differences between the in vitro translation/transcription system and the living cell. In a cell-free system, guanidine allows extended translation with the accumulation of so-called pre-initiation complexes (Barton & Flanagan, 1997). These complexes are blocked at the level of initiation of negative-strand RNA synthesis. After guanidine removal, negative-strand and subsequently positive-strand RNA synthesis starts within a few minutes. Re-addition of guanidine again specifically blocks initiation of negative-strand RNA synthesis (Barton & Flanagan, 1997).

In vivo, removal of the drug caused transcription to be resumed only after a considerable lag phase, a relocation of viral RNA from the MTOC to the ER and a period of translation at the ER. This was unexpected, since the inactive replication complexes contained the necessary components for viral RNA replication, i.e. vesicles, viral protein and RNA (see Fig. 5b), although a small amount of RNA might have been degraded during the guanidine block (see Fig. 6a).

The observed lack of a direct resumption of transcription in vivo suggested a process that rendered replication complexes irreversibly replication incompetent, thus necessitating their de novo synthesis. It is unlikely that this resulted from irreversible damage of protein 2C by guanidine, since in vitro the action of guanidine is easily reversed. Although the cause for the presumable irreversibility of the guanidine block of pre-existing replication complexes in vivo is not known, we speculated that it might be due to additional effects of guanidine if PV replication takes place in and interacts with the intricate membraneous network in the living cell.

Several other important differences between in vitro systems and the infected cell have already been described. In vitro systems were found to exhibit an optimal but unbalanced replication (Verlinden et al., 2002). Translation can be extended over hours, leading to concentrations of viral protein not observed in the living cell. RNA replication has been described to be associated with membrane fragments that are morphologically different from canonical PV vesicles (Fogg et al., 2003).

In the living cell, the incoming virus needs to establish its infection in an environment that necessitates replication steps of high specificity. The virus has to recruit resources used by the cell and also to keep its comparatively low amounts of input and progeny molecules close together, and hence compartmentalize its replication in a replication complex. Importantly, guanidine— as well as brefeldin A-resistant mutants (Crotty et al., 2004; Pincus & Wimmer, 1986) do not escape from the activity of the respective inhibitors by using alternative membrane structures as replication complexes. Rather, adaptive mutations seem to lead to altered protein–protein interactions (Crotty et al., 2004) that still allow induction and utilization of membrane structures that are, as judged by ultrastructural analysis, identical to the replication complexes observed after wt PV infection (unpublished data). Conceivably, the tight interconnection of structure and function in vivo precludes flexibility for the virus in the use of replication-relevant structures. Thus, the complexity of the interaction of the virus with the cellular organization should not be underestimated.

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