Studies on unusual cytoplasmic structures which contain rabies virus envelope proteins

Yajin Ni, Yasumasa Iwatani, Kinjiro Morimoto and Akihiko Kawai

Department of Molecular Microbiology, Faculty of Pharmaceutical Sciences, Kyoto University, Shimoadachi-cho, Sakyo-ku, Kyoto 606, Japan

We investigated unusual structures produced in BHK-21 cells infected with rabies virus (HEP-Flury strain). Sellers' staining of the cells revealed, in addition to Negri body-like structures (inclusion bodies), production of a fuchsin-stained cytoplasmic structure (FCPS) which encircled the nucleus. The frequency of the FCPS-forming cells increased as replication progressed. The FCPS was different from the inclusion body because the former contained the viral glycoprotein (G) and matrix protein (M2) antigens, while the latter contained nucleocapsid antigens. In the early phase of infection, we observed accumulation of viral envelope antigens in a cytoplasmic structure that was considered to be expanded rough endoplasmic reticulum (rER) because of its concomitant increase in BiP content. Time-course studies suggested that the envelope antigen-containing structure, which was not stained with basic fuchsin, translocated to the perinuclear region to form the FCPS. FCPS formation was dependent on incubation temperature and was decreased at 30 °C, while the development of virus-induced cytopathic effect (CPE) was delayed. When the incubation temperature was shifted up to 37 °C, FCPS formation was induced again and progression of CPE was accelerated in approximate proportion to the increasing number of FCPS-positive cells. From these studies, we conclude that viral G proteins gradually accumulate in the rER with M2 protein and the expanded rER converts eventually into the FCPS, which may be closely related to accelerated host cell death.

Introduction

Rabies virus is a typical neurotropic virus. Neurons in the brain are the major target of street rabies virus. The virus causes minor cytopathic changes in the brain, such as the formation of an inclusion body termed the Negri body in the cytoplasm of infected neurons (Matsumoto, 1970). On the other hand, brain-adapted or fixed laboratory strains of rabies virus cause severe cytopathic changes in the brain, where many degenerating neurons are observed, but Negri body formation in the neuron is usually rare, probably due to rapid progression of cell death (Miyamoto & Matsumoto, 1967). In neuronal tissues in culture, street rabies viruses and fixed laboratory strains cause cytological changes that correspond to those seen in the brain (Matsumoto & Kawai, 1969; Matsumoto et al., 1974), suggesting that the difference in cytopathic effects reflect primarily the different interactions of street and fixed strains with neuronal cells. Street virus does not replicate well in most non-neuronal cells in culture, while fixed laboratory strains replicate in some cell lines of non-neuronal origin, such as BHK-21 and CER cells. In these cells, the fixed strains produce cytoplasmic inclusion bodies of various sizes, but cytolysis progresses at a very slow rate. Accordingly, studies on rabies-induced CPE have so far not been performed extensively and the mechanism of CPE remains obscure.

To understand more about rabies virus-induced CPE, we have obtained BHK-adapted subclones of the HEP-Flury strain. Plaque-purified preparations of BHK-adapted HEP-Flury virus caused fast-progressing CPE in BHK-21 cell cultures only when the content of defective interfering (DI) particles in the virus stocks was reduced (e.g. by a one-cycle amplification of the plaque isolates in BHK-21 cell cultures; Kawai et al., 1975). When DI particles were added to the infected cultures, however, CPE was suppressed, and continued incubation of such cultures led to the establishment of persistent infection. Considering the different behaviour in vivo of street and fixed strains as well as our studies with BHK-adapted viruses, we assume that there are at least three viral factors which affect the progression of rabies virus-induced CPE. One is a regulatory
function which may be involved in regulation of CPE in the neurons and lost from the fixed strains. The second is a function which may be involved in regulation of CPE in the BHK-adapted fixed strain. The third is DI-mediated auto-interference.

Recently, we have observed in BHK-21 cell cultures infected with BHK-adapted HEP-Flury virus two types of cytoplasmic structure which were stained with a dye, basic fuchsin, contained in Sellers' staining solution (Sellers, 1927). One was the cytoplasmic inclusion body which contains accumulated viral nucleocapsids as demonstrated by immunofluorescence studies using antibodies against the viral nucleoprotein (N) and phosphoprotein (M1). The other is a fuchsin-stained cytoplasmic structure (FCPS), which seemed to be something other than a real inclusion body because of its location in the cell and an apparent relationship with enhanced progression of the CPE (A. Kawai, unpublished observation). The frequency of FCPS formation and CPE progression occurred almost in parallel. Accordingly, we further investigated the FCPS to elucidate the relationship between FCPS formation and progression of CPE. Immunofluorescence studies strongly suggested that the FCPS originates from expanded rough endoplasmic reticulum (rER), which contained accumulated viral envelope antigens, glycoprotein (G) and matrix protein (M2), as well as cellular BiP (a resident in the rER). In addition, FCPS formation was temperature-dependent and greatly suppressed at a lower temperature (30 °C), while a shift of incubation temperature to 37 °C induced FCPS formation. Time-course studies suggested that enlarged rER converted into FCPS.

Methods

- Rabies viruses. Most experiments were performed with a BHK-adapted subclone of the HEP-Flury strain (Kawai et al., 1975). For studying the reactivity with several monoclonal antibodies (MAbs) from the Wistar Institute (see below), a neurovirulent revertant of the HEP-Flury strain was used, because the original HEP-Flury strain did not react with MAb 194-2. The ERA strain was also investigated for comparison, for which we used the same virus stock as described previously (Sagara et al., 1995). Viruses were propagated and plaque-assayed using BHK-21 cell cultures as described previously (Kawai & Takeuchi, 1992). BHK-21 cells were propagated in Eagle's MEM supplemented with 5% calf serum and 10% Tryptose Phosphate Broth (Difco).

- Sellers' staining. Staining solution was prepared by mixing saturated solutions of basic fuchsin and methylene blue dissolved in methanol (Sellers, 1927). Infected cells sown on coverslips were dipped into Sellers' staining solution for several seconds, and then washed with running tap water. The specimen was placed on a glass slide and observed under a light microscope at a 400 × magnification.

- Antibodies. Polyclonal antibodies against the rabies virus glycoprotein (G), matrix protein (M2), nucleoprotein (N) and phosphoprotein (M1) were prepared by immunizing rabbits with the polypeptides extracted from the band of individual virion proteins separated in SDS–PAGE gels. We also used two types of G protein-specific MAbs (RG719 and #1-46-12). MAb RG719 is able to recognize denatured viral G protein, while MAb #1-46-12 reacts only with the native form of G protein (Ni et al., 1999). We also used several conformational epitope-specific MAbs which were a gift from the Wistar Institute (Philadelphia, PA, USA); MAbs 220-8, 101-1 and 1112-1 are specific for antigenic sites Ila, Iib and Iic, respectively, while MAbs 194-2 and 523-11 are specific for antigenic sites IIIa and IIIb, respectively (Lafon et al., 1983; Dietzschold et al., 1988).

Anti-BiP rabbit antiserum was prepared by immunizing rabbits with a BiP-mimicked synthetic (20-mer) oligopeptide together with complete Freund's adjuvant. The peptide was synthesized by mimicking the amino acid sequence of the C-terminal portion of the BiP molecule and conjugating with keyhole limpet haemocyanin before immunization.

- Immunofluorescence. BHK-21 cells sown on coverslips were infected with rabies virus. After incubation for an appropriate period as noted in the text, they were fixed with acetone for 2 min or 3% paraformaldehyde for 10 min at room temperature. Specimens were subjected to indirect fluorescent antibody (FA) staining: they were stained first with mouse monoclonal or rabbit polyclonal anti-G antibodies, and then with FITC- or rhodamine-conjugated second antibodies. In some experiments, Sellers' stained specimens were also subjected to FA staining, for which only the FITC-conjugated second antibody was used. FA-stained specimens were examined under an epifluorescence light microscope.

Results

Formation of the fuchsin-stainable perinuclear structure (FCPS)

When BHK-21 cells were infected with rabies virus (HEP-Flury strain) and stained with Sellers' staining solution 24–36 h after infection, we often noticed the formation of two types of unusual cytoplasmic structure that were stained with basic fuchsin. One was a ring structure which encircled the nucleus (referred to in this study as the fuchsin-stained structure that occupied a discrete broad area of the cytoplasm, as demonstrated in Fig. 1A; as indicated by arrowheads in Fig. 1A; as demonstrated in Fig. 3, this structure contains viral nucleocapsid antigens).

Relationship between the FCPS and G protein-enriched structures

The FCPS was next investigated by fluorescent antibody (FA) staining. FA staining with rabbit polyclonal anti-G antibody demonstrated three different distribution patterns of the antigen in infected cells (fixed with 3% paraformaldehyde followed by acetone permeabilization (Fig. 2A, B) or with acetone alone (Fig. 2H)). One is G antigen expressed on the cell surface (Fig. 2B). In many such cells we could also see one of two other distribution patterns which are characterized as G antigen accumulation in unusual cytoplasmic structures (referred to here as the G protein-enriched cytoplasmic structure; G-CPS). G-CPS can be classified into two types. One is a structure that occupied a discrete broad area of the cytoplasm,
Fig. 1. Sellers' staining of rabies virus-infected BHK-21 cells. BHK-21 cell cultures sown on cover-slips (1.0 x 10⁵ cells) were infected with rabies virus (HEP-Flury strain) at an m.o.i. of 3 p.f.u. per cell. After incubation for 36 h at 37 °C, the cover-slips were taken out and washed with PBS. The cells were fixed and stained by dipping into Sellers' staining solution for about 5 s and then washing in tap water. Some of the Sellers-stained specimens were further subjected to immunofluorescence study with murine anti-G MAb (RG719) and FITC-conjugated second antibody. After being mounted on a glass slide, the cover-slip was examined under a light microscope equipped with epifluorescence illumination apparatus. (A, B) Sellers' staining; (C) immunofluorescence. (B) and (C) were taken from the same microscope field. Arrows in (A) indicate the FCPS, and arrowheads indicate inclusion bodies. Bar in (A) indicates 20 μm.

located at some distance from the nucleus (indicated by arrowheads in Fig. 2A and more clearly seen in Fig. 2G; we refer to this as G-CPS type I). The other is a perinuclear ring structure (indicated by an arrow in Fig. 2A; we refer to this as G-CPS type II), which encircled the nucleus as demonstrated in a phase contrast image of the same cells (data not shown).

Based on its morphology, intracellular location and frequency, the FCPS visualized by Sellers' staining seemed to correspond to G-CPS type II. To examine this assumption, infected cells were doubly stained with Sellers' solution and then with anti-G antibody, followed by staining with a second antibody for FA staining. As shown in Fig. 1B and 1C, the morphological outline of the FCPS coincided exactly with that of G-CPS type II. On the other hand, Sellers' staining did not stain G-CPS type I (data not shown). These observations indicate that G-CPS type II is identical to the FCPS.

**Studies with anti-rabies G MAbs**

The G antigen-positive structures were then studied using anti-G MAbs. FA staining with a sequential epitope-specific anti-G MAb (RG719) demonstrated the presence of G-CPS types I and II in acetone-fixed infected cells (Fig. 2F, G). The MAb also detected G antigen on the cell surface when the cells were fixed with paraformaldehyde (Fig. 2E). In contrast, FA staining with a conformational epitope-specific anti-G MAb (#1-46-12) did not detect the G-CPS-associated G antigen in acetone-fixed infected cells (Fig. 2D). The MAb also detected the antigen exposed on the surface of paraformaldehyde-fixed (Fig. 2C) and acetone-fixed (Fig. 2D) cells (e.g. G antigen-positive microvilli-like structures were seen on the surface of the cells shown in Fig. 2C, D). We also checked this point by using other conformational epitope-specific MAbs (see Methods). MAbs 101-1, 1112-1 and 523-11 were unable to detect the G antigens in G-CPS types I and II, while MAbs 194-2 and 220-8 did (data not shown). These observations suggest that most G proteins in G-CPS types I and II are immature. The apparently selective staining with MAb RG719 of G-CPS types I and II in the acetone-fixed cells (Fig. 2F) may come from its easier accessibility or stronger affinity to the linear epitope on the immature G protein molecule than to the epitope on the mature protein exposed on the cell surface.
Fig. 2. For legend see Addis et al.
Double FA staining with MAb RG719 and anti-N antibody demonstrated different distributions of viral G antigen (Fig. 3A) and N antigen (Fig. 3B) in the cell. Most N antigen was found in the round or oval-shaped cytoplasmic inclusion bodies (Fig. 3B). Viral M1 antigen was also found in the inclusion bodies (data not shown), while the antigen of viral matrix (M2) protein (Fig. 3D) displayed a colocalized distribution with G antigen, especially in both types of G-CPS (Fig. 3C). These results strongly suggest that G-CPS types I and II are different from the inclusion bodies composed of viral nucleocapsids.

Since the G-CPS-associated G proteins were not recognized by some conformational epitope-specific MAbs, they seemed to be composed of immature forms or misfolded ones, and may have been retained in some cytoplasmic structure. To investigate their intracellular localization, we performed double FA staining by using MAb RG719 and an antibody against cellular BiP which is known to be located in the rER. As we expected, viral G antigen (Fig. 3E) and BiP antigen (Fig. 3F) displayed a colocalized distribution in G-CPS types I and II, indicating that such G antigens are accumulated in the enlarged rER.

**Effect of incubation temperature on G-CPS formation**

In our previous study on the temperature sensitivity of rabies virus replication (Kawai & Takeuchi, 1992), we noticed that the accumulation of viral G protein in the G-CPS was enhanced by elevation of incubation temperature. Consequently, we next examined whether G-CPS formation is temperature-dependent and is suppressed at lower temperatures. As shown in Fig. 4A, FA staining of cells incubated at 30 °C with MAb RG719 revealed normal distribution of G antigen on the cell surface, and no G-CPS formation was observed at 36 h. G-CPS type I, however, was formed at 30 °C but at a slow rate in the cultures kept for a longer period (60 h; Fig. 4F). This accumulation, however, was reversible and disappeared when the incubation temperature was elevated to 37 °C (Fig. 4B). The accumulation of G protein in the Golgi area might be caused by decreased efficiency of processing and/or transport of glycoprotein through the Golgi at the low temperature. In addition, it may not be harmful to the cell, since cell death was delayed (data not shown). In addition, as reported previously (Kawai & Matsumoto, 1977; Kawai & Takeuchi, 1992), progeny virus production was increased at lower temperatures.

Finally, we examined the effects of temperature shift-down on viral G protein (Fig. 5, Table 1). After 36 h of infection at 37 °C, type I was most prominent and type II was found only in a small fraction of the cells (Fig. 5A). After the temperature shift from 37 °C to 30 °C, the number of G-CPS type I-forming cells gradually decreased, and the number of G-CPS type II-positive and decaying (cell rounding) cells, as well as the ratio of G-CPS type II plus decaying cells to type I, was increased as the incubation period was prolonged (Fig. 5B, C). On the other hand, the frequency of G-CPS type II and decaying (ronding) cells was much increased in the cultures kept at 37 °C for 60 h (Fig. 5D). These results suggest that G-CPS formation is an irreversible process and that G-CPS type I is a precursor of type II.

**Possible relationship between the G-CPS/FCPS formation and cell death**

We have observed that the level of CPE increased, apparently in parallel, with the increasing frequency of FCPS as well as formation of G-CPS (types I and II) in the HEP virus-infected cultures (unpublished observations). In the present series of experiments, we observed that onset of CPE was greatly delayed when formation of FCPS and G-CPS (type I and II) was suppressed by lowering the incubation temperature (Fig. 4A, F). On the other hand, when the temperature was
Fig. 3. Double immunofluorescence studies to detect viral and cellular antigens colocalized with G antigen in rabies virus-infected cells. Rabies virus-infected cells were incubated for 36 h and fixed with acetone for 2 min at room temperature. They were doubly stained with murine anti-G MAb RG719 (A, C, E) and rabbit antibody against the viral N (B) or matrix protein (D) or cellular BIP protein (F). Then, they were stained with FITC conjugated (anti-mouse immunoglobulin) and rhodamine conjugated (anti-rabbit immunoglobulin) secondary antibodies. (A) and (B) show the same microscope field, stained with MAb RG719 and rabbit anti-N antibody, respectively. (C) and (D) were taken from the same cells stained with MAb RG719 and rabbit anti-M2 antibody, respectively. (E) and (F) show the same field stained with MAb RG719 and rabbit anti-BIP antibody, respectively. Arrowheads in (B) indicate inclusion bodies. Arrowheads in (E) and (F) indicate G-CPS type I, while arrows indicate type II.
shifted-up from 30 °C to 37 °C, we consistently observed the appearance of G-CPS types I and II several hours after shift-up; this was irreversible and always followed by cell death (Table 1). Accordingly, it seems likely that formation of G-CPS (types I and II) is a degenerative process, which is prominent in the case of HEP virus infection in culture. For comparison, in the cultures infected with the ERA strain of rabies virus, G-CPS formation was not frequent and G-CPS type II was rare even after 60–72 h of infection when the replication process had reached a plateau, and the progression of CPE was very slow (data not shown). The mechanisms of G-CPS formation as well as the origin of the difference in G-CPS formation between the two strains of rabies virus are under investigations.

Discussion

Sellers’ staining has long been used as a diagnostic method to visualize the Negri bodies formed in rabies virus-infected brain (Sellers, 1927). By using this method, we have observed, in rabies virus-infected BHK-21 cells, two kinds of virus-
Table 1. Effects of temperature shifts on G-CPS formation and CPE (cell rounding) in rabies virus-infected BHK-21 cell cultures

BHK-21 cells sown on cover-slips were infected with HEP virus (m.o.i. 5 p.f.u. per cell) and preincubated for 36 h at 30 °C (Expt. A) or 37 °C (Expt. B). The incubation temperature was shifted from 30 °C to 37 °C or 37 °C to 30 °C, and the frequency of G-CPS formation and cell rounding was determined by FA staining with MAb RG719 at each of the times indicated in the table (each value is the mean ± standard deviation of the values counted three times from ten microscope fields).

<table>
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<th>Cellular change</th>
<th>Time after temperature shift (h):</th>
<th>0</th>
<th>4</th>
<th>8</th>
<th>12</th>
<th>24</th>
<th>24 (Cont.)*</th>
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<tr>
<td><strong>Experiment A (Shift-up)</strong></td>
<td>G-CPS formation</td>
<td></td>
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<td></td>
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<tr>
<td>type I</td>
<td>&lt; 1</td>
<td>12 ± 2.6</td>
<td>26 ± 1.0</td>
<td>34 ± 2.6</td>
<td>43 ± 2.5</td>
<td>2 ± 1.5</td>
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<tr>
<td>type II</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>6 ± 1.0</td>
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<td>Cell rounding</td>
<td>&lt; 1</td>
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<td>&lt; 1</td>
<td>5 ± 1.0</td>
<td>12 ± 2.0</td>
<td>&lt; 1</td>
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<tr>
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<tr>
<td>type I</td>
<td>34 ± 2.6</td>
<td>ND</td>
<td>14 ± 1.5</td>
<td>ND</td>
<td>7 ± 1.0</td>
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<tr>
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<td>ND</td>
<td>36 ± 2.0</td>
<td>ND</td>
<td>21 ± 1.5</td>
<td>13 ± 1.5</td>
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<tr>
<td>Cell rounding</td>
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<td>71 ± 3.5</td>
<td>73 ± 1.0</td>
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* Control incubation for 24 h without the temperature-shift at 36 h, corresponding to 60 h incubation at 30 °C alone (Expt. A) or 37 °C alone (Expt. B).
ND, Not determined.

specific cytoplasmic structure which were stained with basic fuchsin contained in the staining solution; one is the Negri body-like structure composed of viral nucleocapsids (Hummeler et al., 1967, 1968); the other is the structure that we term FCPS. Our data strongly suggest that the FCPS is identical to G-CPS type II. G-CPS type I was not visualized by Sellers' staining, probably due to the lower density or absence of the fuchsin-stainable component(s). Both G-CPS types I and II contained BiP antigen, and type II seemed to originate from type I, which is the expanded rER where viral G proteins were accumulated together with another viral envelope protein (M2).

We observed that CPE progressed almost in parallel with the increasing number of FCPS-forming cells in HEP-virus infected cultures. Incubation of the cultures at 30 °C suppressed both FCPS/G-CPS type II formation and CPE. On the other hand, temperature-shift from 30 °C to 37 °C induced enhanced formation of G-CPS types I and II, followed by accelerated cell death, that began several hours after the appearance of G-CPS type II. Accordingly, it seems likely that formation of FCPS/G-CPS (types I and II) is a degenerative process resulting in damage to cellular integrity and may be closely related to cell death. In contrast, G-CPS formation was infrequent in ERA virus-infected cultures, and progression of CPE was very slow. At high temperature, 39.5–40.5 °C, however, ERA virus infection induced similar G protein-enriched cytoplasmic structures as seen in HEP virus-infected cells, which was also followed by enhanced cell death (unpublished observations). The ERA virus-induced enhanced cellular damage at higher temperature might account for the severe neuronal degeneration observed in brain infected with fixed rabies virus strains (Miyamoto & Matsumoto, 1967), since the temperature in the brain is much higher than 37 °C. In the case of street rabies virus infections, little cytopathic change is observed in the brain, except for Negri body formation. Accordingly, we suppose that the G protein-enriched structures are not produced in street rabies virus-infected brain, possibly due to regulated synthesis of envelope proteins, which would probably be essential for preserving the integrated neural network formation in order to accomplish virus transmission to the next victim animals.

The process of G-CPS/FCPS formation would progress in a step-wise manner as depicted in Fig. 6. In the early phase of infection, viral G proteins are processed normally and transported to the cell surface. In such cells, however, viral G proteins eventually begin to accumulate in the enlarged rER (formation of G-CPS type I), where viral M2 proteins are also found. Temperature-shift experiments suggested that G-CPS type I is a precursor of type II. There may be two possible mechanisms by which viral G proteins are accumulated in the rER. One is a relatively higher rate of viral glycoprotein synthesis at 37 °C than at 30 °C, which might result in
Fig. 5. Temperature shift-down experiment. Infected BHK-21 cells were prepared as described in Fig. 4, but preincubated at 37 °C for 36 h. The cultures were then transferred to 30 °C, and further incubated for 24 h. At various times after the shift, cells were fixed with acetone and FA-stained using MAb RG719. (A) 0 h after the shift; (B) 8 h; (C) 24 h. (D) Incubated at 37 °C for 60 h (no shift). Arrowheads indicate some decaying cells.
overproduction of the protein relative to the cellular capacity for post-translational processing, followed by retention of glycoproteins in the rER. This assumption is consistent with a recent report which described processing and/or intracellular transport of rabies virus G protein as being relatively slow (Whitt et al., 1991). Another possibility is that one of two envelope proteins of the virus is temperature-sensitive and causes the retention of viral glycoprotein in the rER (G-CPS type I formation) at 37 °C, whereas it works normally at lower temperatures contributing to increased progeny virus production as reported previously (Kawai & Takeuchi, 1992). Consistent with this assumption, we observed in our pulse-chase experiments that the viral matrix protein (M2) was quite unstable at 37 °C compared to 30 °C (unpublished observation). To examine whether the instability of M2 protein is responsible for causing the accumulation of viral glycoprotein in the rER at 37 °C, we are trying to isolate and investigate temperature-resistant mutants of the virus which do not form G-CPS at 37 °C.

We observed that rabies virus G protein tended to accumulate in the Golgi area at 30 °C. This accumulation, however, was not harmful to the cell and was reversed when incubation temperature was shifted up to 37 °C. On the other hand, G protein accumulation in G-CPS was irreversible, while at 37 °C G protein synthesis itself was not harmful to the cell, since permanent cell lines can be established which continued to produce the G protein (Morimoto et al., 1992). Extensive efforts have failed to result in isolation of cell lines constitutively producing the M2 protein (unpublished data). Persistent infection with rabies virus in culture can be characterized as a reduced rate of virus-induced cytolysis and virion formation (Kawai et al., 1975). Tuffereau et al. (1985) reported that the amount of M2 protein in persistent infection was greatly reduced, to less than 10% of that in acutely infected cells. Based on these facts, we propose that the viral matrix protein (M2) is cytotoxic, and its association with either G protein in the rER or with rER membrane may be harmful to the host cell, and may initiate G-CPS formation in infected cells. The matrix protein of another rhabdovirus, vesicular stomatitis virus, has also been reported to be cytotoxic (Blondel et al., 1990; Coulon et al., 1990; Melki et al., 1994; Ye et al., 1994).

**Note added during revision**

After submission of our manuscript, Gaudin et al. (1995) reported that viral G protein in the rER was conformationally immature and fusion-inactive, which supports our assumption that the G proteins accumulated in the G-CPS are immature. It also seems to be reasonable that the G proteins in the G-CPS displayed colocalized distribution with BiP, a resident in the rER.

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