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Further Studies on the Mechanism of Rabies Virus Neutralization by a Viral Glycoprotein-Specific Monoclonal Antibody, #1-46-12

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Abstract: **We previously reported that a conformational epitope-specific monoclonal antibody (mAb; #1-46-12) neutralized the rabies virus by binding only a small number (less than 20) of the antibody molecules per virion, while a linear epitope-specific mAb (#7-1-9) required more than 250 IgG molecules for the neutralization. We also isolated both the epitope-negative (R-31) and -positive (R-61) escape mutants that resisted mAb #1-46-12. Co-infection studies with wild type (wt) and R-61 mutant have shown that although the infectivity of R-61 mutant was not affected by the binding of about 300 IgG molecules per virion, incorporation of a small number of wt G protein into the R-61 virion resulted in dramatic loss of the resistance. In this study, we further investigated properties of the mutant G proteins. The R-61 G protein lost reactivity to the mAb when solubilized, even keeping a trimer form, suggesting that membrane-anchorage is essential for the maintenance of its epitope-positive conformation. On the other hand, incorporation of wt G proteins into the R-31 virions did not affect their resistance to the mAb very much. Although we have not so far found the presumed conformational changes induced by the mAb-binding, we think that these results are not inconsistent with our previously proposed novel model (referred to as a domino effect model) for the virus neutralization by mAb #1-46-12 other than a classical spike-blocking model, which implicates successive spreading of the postulated antibody-induced conformational changes of G protein to the neighboring spikes until abolishing the host cell-binding ability of the virion.**

Key words: **Virus neutralization, Monoclonal antibody, Rabies virus, Escape mutants, Neutralization mechanism, Glycoprotein mutant, Neutralizing monoclonal antibody**

The rabies virion is a bullet-shaped enveloped particle, which is surrounded by about 600 spikes composed of homo-trimers of a single type of viral G protein. The G protein is a typical type-I transmembrane glycoprotein, and has been extensively studied for many years because of its important roles in the viral life-cycle (receptor recognition and binding, envelope fusion with the endosomal membrane in the early phase of infection, and envelope formation with the matrix protein, leading to the budding of progeny virions) and the roles in the neuropathogenesis of a fatal disease in humans and other mammals as well as in the induction of protective immunity (7, 13).

As for the host defense systems against pathogenic microbes, microbe-specific antibody production is one of the most important events in animals, and a major role of such antibodies is the impairment of the target microbes. For inducing the protective immunity against rabies virus infection in humans and domestic animals, inactivated vaccine is used. The G protein is a major target of the antiviral antibody production in the body, and such antibodies are believed to be involved in certain antiviral activities against the infection, including the virus neutralization by their direct binding to the

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Abbreviations: CBB, Coomassie brilliant blue; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid; FA, fluorescent antibody; FFI, focus-formation inhibition; FITC, fluorescein isothiocyanate; IgG, immunoglobulin-gamma; G, glycoprotein; mAb, monoclonal antibody; MEM, minimum essential medium; MOI, multiplicity of infection; pAb, polyclonal antibody; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PFU, plaque-forming unit; SDS, sodium dodecyl sulfate; SH, sulfhydryl; wt, wild type.

virions, although such activities have not yet been established as the virus neutralization with the help of complement system (e.g., antibody-dependent complement-mediated virolysis) nor the killing of infected cells (i.e., antibody-dependent, cell-mediated or complement-mediated cytotoxicity) (see a review by Lafon (10)).

To date, coupled administration of rabies vaccine and the anti-rabies hyper-immune serum derived from domestic animals has been applied to the post-exposure prophylaxis for the victims bitten by rabid dog or wild animals. It has also been suggested recently that a mixture of some anti-G monoclonal antibodies (mAbs) of known nature would be more effective for the post-exposure treatment than the antiserum of animal origin (4, 12). For this purpose, however, extensive studies are still necessary to learn more about the effects of anti-G mAbs on the viral infectivity and host cell viability, including studies on effects of anti-G mAbs on the G protein functions and conformations as well as studies on the epitope structures and mapping on the G protein.

Mechanisms of the virus neutralization by specific antibodies have long been studied. In many virus cases, it has been shown that the number of virion-bound IgG molecules that is required for neutralization of about 63% (i.e., $1-1/e$) infectivity was correlated with the surface area of the virion, giving a formula, $n=0.0033\times A$, where "n" and "A" denote the number of IgG molecules per virion required for 63% neutralization and the mean value of the virion surface area, respectively (1). In the case of rabies virus, this formula seemed apparently to fit well as reported by Flamand et al. (2), showing the requirement of about 225 IgG molecules per virion for the neutralization.

Our recent studies on the rabies virus neutralization by anti-G mAbs suggested, however, that virus neutralizing mechanisms of anti-G mAbs differed from each other probably depending on the antibody types and properties of the epitopes (e.g., epitope structures and location on the G protein molecule; 5). A linear epitope-specific anti-G mAb, #7-1-9, showed the lowest virus-neutralizing activity, and required binding of 255 IgG molecules per virion for the neutralization, being consistent with a report by Flamand et al. (2). On the other hand, with one of conformational epitope-specific anti-G mAbs (#1-46-12) of strong virus-neutralizing activity, we could show that the number of bound antibodies to cover at most 3-4% of the spikes on the rabies virion (i.e., less than 20 IgG molecules per virion) was enough for the neutralization of infectivity. Based on these observations, we proposed previously a domino effect model of conformational change of the virion spikes, which is initiated by binding of only a small number of antibodies to the spikes, and is spread to the neighboring spikes until the host cell-binding ability of the whole virion is lost, although precise mechanisms of the virus neutralization remain to be elucidated.

In this study, we tried to understand possible mechanisms of virus neutralization by antiviral antibody. For this purpose, we used two types of escape mutants of rabies virus that resisted the neutralization by mAb #1-46-12; one (i.e., mutant R-31) lacked the 1-46-12 epitope, and the other (i.e., mutant R-61) preserved it (6). Interestingly, the R-61 virus was not neutralized even by the binding of more than 250 IgG molecules of mAb #1-46-12, although the binding kinetics of this mAb to R-61 was almost the same as that of the wt virus. We performed co-infection studies with these escape mutants, comparing the properties of the progeny viruses from the co-infected cultures with wt virus and one of the two escape mutants under various infection conditions. The data obtained in this study are not inconsistent with our previously purposed novel hypothesis (domino effect model; 5) proposed previously for the virus neutralization by binding of a small number of mAb #1-46-12 to the rabies virion (5).

Materials and Methods

Viruses and cells. Rabies viruses were the BHK-21-adapted HEP-Flury strain (wild type; wt) and the two types of escape mutants derived from the HEP-Flury strain [clone R-31: 1-46-12 epitope-negative (group I); clone R-61: the epitope-positive (group II)], which escaped from the neutralization by mAb #1-46-12 (6). Preparation of these mutants was described in our previous study (6). All virus clones were plaque-purified and propagated in BHK-21 cells, and were titrated by plaque formation assay using BHK-21 cells as described previously (8). BHK-21 cells were propagated in Eagle's minimum essential medium (MEM) supplemented with 5% calf serum and 10% Tryptose phosphate broth (Difco).

Virus infections for production of phenotype-mixed virions. BHK-21 cells were co-infected with the wt virus and an escape mutant (R-31 or R-61) at an MOI of 3 PFU/cell under various mixing conditions of the two viruses. At 48 hr of infection, the culture medium was harvested to recover the progeny viruses, and subjected to the examination of their sensitivity to neutralization by mAb #1-46-12 by focus inhibition assay (see below).

Antibodies. Monoclonal antibodies (anti-G mAbs) and the rabbit polyclonal antibodies (pAbs) against the rabies virus G and N proteins were the same as those used in our previous study (6). In this study, culture

fluids of the hybridoma cells were mostly used as the source of mAbs.

Virus neutralization assay. Sensitivity of the progeny viruses of co-infections to anti-G mAbs was examined by a modified method of the focus formation inhibition (FFI) assay described by Louie et al. (11). In brief, virus suspensions were diluted to give a concentration of 1×10^5 PFU/ml, and mixed with the same volume of serial 2-fold dilutions of mAb #1-46-12, whose original concentration was about 10 μg IgG/ml. After incubation for 1 hr at 37 C, the mixtures were inoculated to monolayer cultures of BHK-21 cells on glass cover slips. At 24 hr post infection (p.i.), cells were fixed with acetone and stained first with rabbit pAb against the rabies virus nucleoprotein (N), and then with the FITC-conjugated anti-rabbit immunoglobulin (Ig) goat antibody (Cappel). Fluorescent antibody (FA)-stained specimens were observed under an epifluorescent light microscope to count numbers of fluorescent foci in 20 microscopic fields for each specimen. The efficiency of the neutralization was calculated by dividing the focus numbers of the test sample with that of the untreated control.

Detection of virion bound IgG. One milliliter of culture fluid of the rabies virus-infected BHK-21 cell cultures, which contained about 1 μg of the progeny virus, was mixed with the same volume of adequately diluted mAb preparation. After incubation for 1 hr at 37 C, the antibody-bound virions were recovered by ultracentrifugation at 35,000 r.p.m. for 1.5 hr through a 20% sucrose cushion. After wiping off the residual droplets of the supernatant on the inside wall of the centrifuge tube with Kimwipes, each virion pellet was dissolved in a small quantity of SDS-PAGE sample buffer (9). The samples were then subjected to SDS-PAGE, in which a known concentration of mouse immunoglobulin sample was co-electrophoresed as a reference for estimation of the amount of bound mAb as described previously (5). The gel was processed for chemiluminescent immunoblotting assay using nitrocellulose membrane filter (type BA85; Schleicher & Schuell) and peroxidase-conjugated rabbit anti-mouse IgG antibody (Cappel) as well as a luminescence assay kit (Western Blotting Luminol Reagent, SC-2048; Santa Cruz Biotechnology). Chemiluminescence emitted by the luminol oxidation was exposed to X-ray film (Kodak X-Omat AR film). Density of the heavy chain band of each sample was compared with that of the reference using the NIH Image soft.

Protease treatment of rabies virions. One milliliter of the culture fluid of rabies virus-infected BHK-21 cell cultures, which contained about 1 μg of the virus, was mixed with mAb #1-46-12 at a concentration of 0.1 μg IgG/ml or PBS (control). After 1 hr incubation at 37 C, virions were recovered by ultracentrifugation at 35,000 r.p.m. for 1.5 hr through a 20% sucrose cushion. The pellet was resuspended in a citrate-phosphate buffer of optimal pH for the following protease treatments. Virion sample was divided into several aliquots, one of which was used for determining the residual infectivity by focus inhibition assay as described above. Others were used for protease digestions by mixing either with trypsin (Sigma-Aldrich Co.), chymotrypsin (Sigma-Aldrich Co.), proteinase K (Wako Pure Chemical Industries, Ltd.), elastase (Sigma-Aldrich Co.), papain (Wako Pure Chemical Industries, Ltd.), Bromelain (Sigma-Aldrich Co.), pepsin (Wako Pure Chemical Industries, Ltd.), Protease from *Streptomyces griseum* (Sigma-Aldrich Co.), or pronase E (Sigma-Aldrich Co.) at a concentration of 1 μg/ml in the presence or absence of 0.01% Triton X-100. After incubation at 37 C for 2 hr, 1/4 volume of 4× SDS-PAGE sample buffer was added to each sample, which was then boiled immediately to inactivate the proteases. The samples were applied to 10% SDS-PAGE, followed by staining with Coomassie brilliant blue (CBB).

Results

Properties of the Progeny Viruses Recovered from the Co-infected Cultures with the Wt and R-31 Viruses

To learn more about the properties of the escape mutants as well as to elucidate the possible mechanism of rabies virus neutralization by anti-G mAb #1-46-12, we compared properties of the G protein between the wild type (wt; HEP-Flury strain) and two groups of the mutants. For this purpose, we first examined antibody-sensitivity of the phenotype-mixed progeny viruses recovered from the co-infected cultures with wt and its mutant viruses. In our previous study (5), we have noted that most of the progeny viruses recovered from the co-infected cultures with the wt virus and the epitope-positive escape mutant (group II; R-61) at a ratio of 1:1 were as highly sensitive to mAb #1-46-12 as the wt virus, although the R-61 virus itself was completely resistant to the mAb.

To compare the previous co-infection studies on the R-61 mutant, we first performed co-infection experiments with the wt virus and an epitope-negative escape mutant (group I; R-31), and investigated the effect of incorporation of wt G proteins into the R-31 virion. BHK-21 cells were co-infected with the wt virus and R-31 at an MOI of 3 PFU/cell under various mixing conditions of the two viruses (under this infection condition, every cell was shown to be infected with the viruses by an immunofluorescence test done at 24 hr

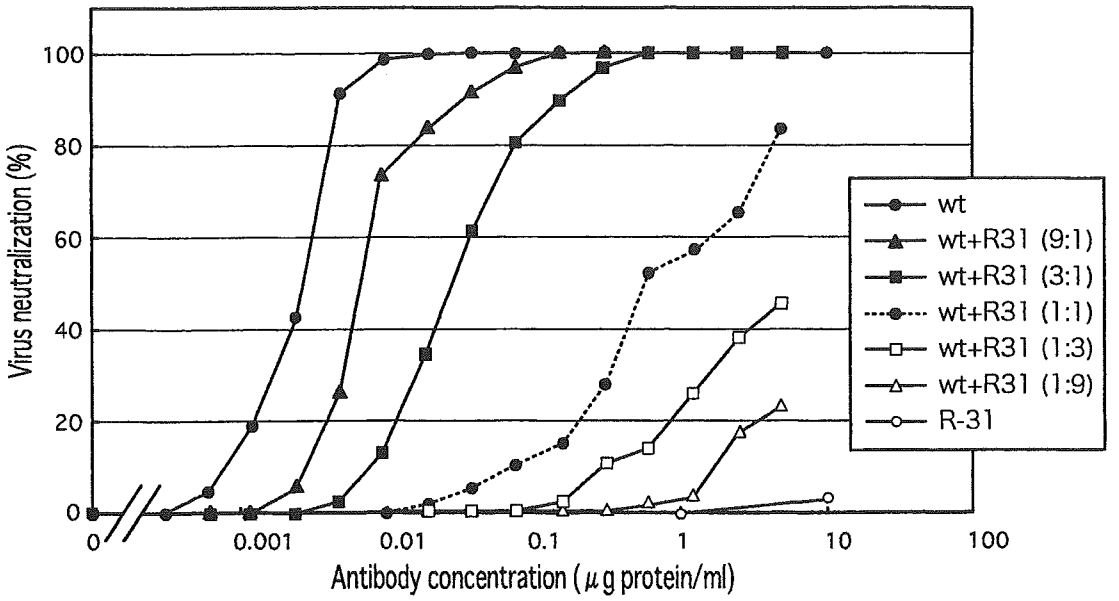

Fig. 1. Neutralization efficiency of the phenotype-mixed rabies virions by anti-G mAb #1-46-12. The wt virus of HEP-Flury strain and an escape mutant (R-31) were mixed at various ratios and inoculated to BHK-21 cell cultures (MOI=3 PFU/cell). After 48 hr of incubation at 37 C, progeny virions were harvested and examined for their sensitivity to mAb #1-46-12 at various antibody concentrations. Symbols: —●—, wt alone; —▲—, wt+R-31 at a ratio of 9:1; —■—, wt+R-31 at a ratio of 3:1; ···●···, wt+R-31 at a ratio of 1:1; —□—, wt+R-31 at a ratio of 1:3; —△—, wt+R-31 at a ratio of 1:9; —○—, R-31 alone.

after the infection using the anti-rabies nucleoprotein antibody; data not shown). At 48 hr p.i., the culture medium was harvested to examine the sensitivity of the progeny virions to neutralization by mAb #1-46-12. Unlike the case with the R-61 mutant, progeny viruses recovered from the co-infected cultures with wt and R-31 viruses at a ratio of 1:1 were mostly resistant to the mAb under the neutralization condition that was enough for 100% inactivation of the wt virus (Fig. 1). In addition, we could see a positive correlation between the mixing ratio of R-31 to wt virus and the grade of resistance of the progeny virions to neutralization by mAb #1-46-12. In other words, incorporation of a certain amount of R-31 G protein into the wt virion seemed to endow the virions with the resistance to the mAb. This point was further investigated as follows.

Evidence for the Phenotypic Mixing of Wt and R-31 G Proteins in the Progeny Virions

As described previously (5), based on the slower migration rate of R-61 mutant G protein in SDS-PAGE gel than that of the wt G protein owing to its additional carbohydrate side chain, we could confirm that, under the co-infection condition with wt and R-61 viruses at a ratio of 1:1, both the wt and R-61 G proteins were incorporated roughly equally into the population of progeny virions. This observation suggested that the spikes of each virion recovered from the co-infected cultures were mostly composed of mixtures of the wt and R-61 types (i.e., phenotype-mixed), and that incorporation of a small amount of wt G protein into the R-61 virion was enough for increasing the sensitivity to the neutralization by mAb #1-46-12.

The same assay technique was not applicable to analyses of the phenotype-mixed progeny virions recovered from the co-infected cultures with wt and R-31 viruses, because both R-31 and wt G proteins migrated at a roughly similar rate in the SDS-PAGE gel. Accordingly, we tried to estimate the relative content of the wt and R-31 G proteins in the progeny virions by comparing the amounts of mAbs that were bound to the progeny virions.

When co-infection was done at a 1:1 mixing ratio of wt and mutant viruses, the amount of IgG bound to the progeny virions was roughly half that of the wt virions under the test condition of the antibody concentration at 0.1 μg IgG/ml (Fig. 2A). Even at a higher concentration of 1 μg IgG/ml, the amount of IgG bound to the progeny virions was still much smaller than that to the wt virions incubated with the mAb at 0.1 μg IgG/ml. FA staining of co-infected culture with wt and R-31 viruses demonstrated that almost all cells were FA-stained with anti-G mAb #1-46-12, indicating that almost all cells allowed the wt virus to replicate in the cultures (data not shown). As for the neutralization sensitivity to mAb #1-46-12, 80% of the progeny viruses recovered

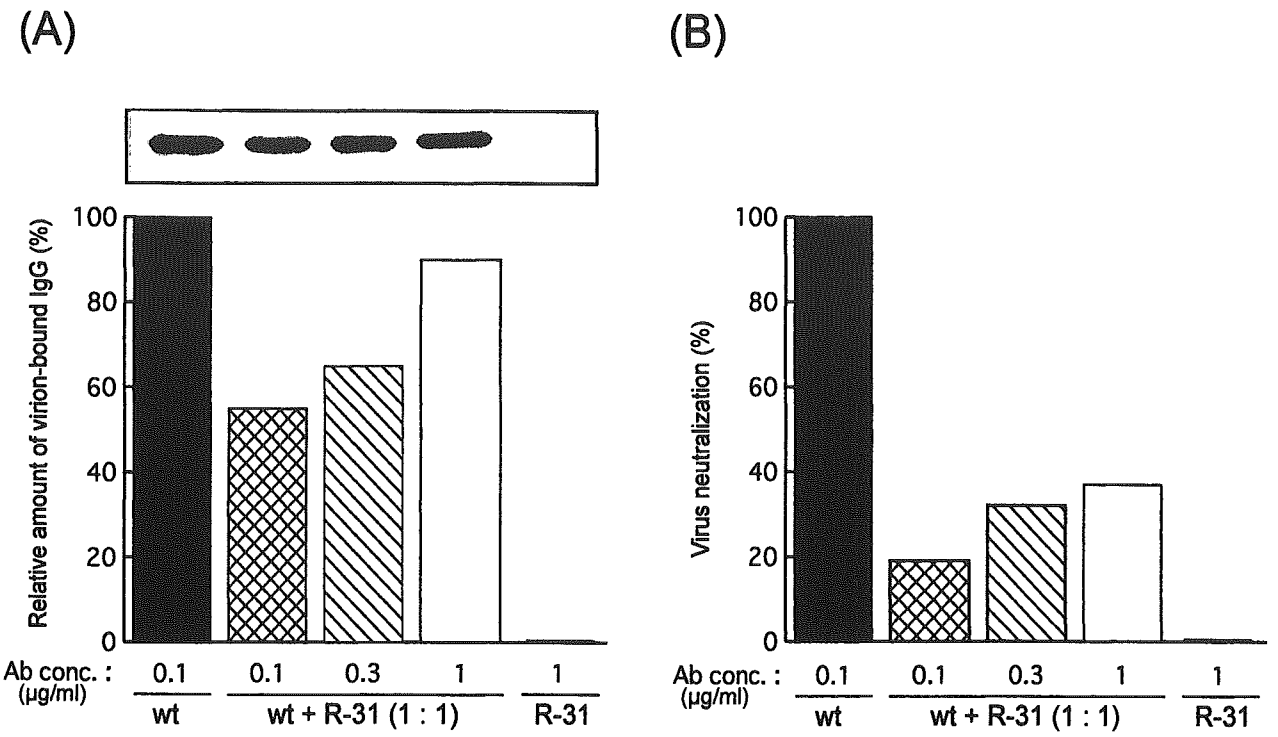

Fig. 2. Relationship between the phenotype mixing of progeny virions and their sensitivity to neutralization by mAb #1-46-12. The wt virus of HEP-Flury strain and R-31 mutant were mixed at a ratio of 1:1 and inoculated to BHK-21 cell cultures (MOI=3 PFU/cell). After 48 hr of incubation, progeny virions in the culture fluids were mixed with the same volume of mAb #1-46-12 solutions at various concentrations and incubated for 1 hr at 37 C. Then, the virions were subjected to estimation of the relative content of virion-bound antibodies (A) and determination of residual infectivity (B). (A) The virion-bound IgG of each sample was estimated by chemiluminescent immunoblotting assay as described in "Materials and Methods." Density of the heavy chain band of each sample was compared with that of the reference using the NIH Image software, and the relative amounts of the virion-bound mAbs under various antibody concentrations were calculated by dividing the values for the test samples by that of control (wt alone), and plotted as bars. (B) Neutralization efficiency was examined by FFI assay, and shown in % of untreated control for each neutralization condition. Bars: closed bar, wt virus incubated with 0.1 μg IgG/ml of mAb #1-46-12; cross-hatched, hatched and open bars, phenotype-mixed viruses incubated with 0.1, 0.3 and 1 μg IgG/ml of mAb #1-46-12, respectively.

from the co-infected cultures were resistant to the mAb at a concentration of 0.1 μg IgG/ml, while almost 100% of the wt viruses were neutralized by the mAb at this concentration (Fig. 2B). Even at a concentration as high as 1 μg IgG/ml, more than 60% of the progeny viruses from the co-infected cultures still showed resistance to the neutralization.

From these results, it was mathematically suggested that spike G proteins of at most 20% of the virions recovered from the co-infected culture were composed of the wt G protein alone, being sensitive to neutralization by mAb #1-46-12, while the spikes of more than 30% of the virions were composed of both wt and R-31 G proteins (i.e., phenotype-mixed) probably at varying ratios. The latter virions, however, were resistant to the neutralization; in other words, it was suggested that the incorporation of certain amounts of wt G proteins into the R-31 virions did not affect the resistant nature of the virions to mAb #1-46-12. In addition, normal functions of the R-31 G protein required for the early phase of infection might not be affected by the mAb binding to the neighboring wt G proteins coexisting on the same virion.

Anchorage-Dependency of the R-61 Mutant G Protein

Preservation of a full range of conformational flexibility of the G protein would be necessary to achieve its biological functions required for the early phase of virus infection as well as for the virion formation by budding process. But the two groups of mutant G proteins displayed different behavior in association with the wt G protein coexisting on the same phenotype-mixed virion. Accordingly, we next compared properties of the wt and mutant G proteins in a solubilized form with NP-40 or CHAPS (the latter preserves the trimer form of the spike, while the former disrupts it)

and checked whether or not the mutation affected the conformational epitopes recognized by other anti-G mAbs.

As shown in Table 1, FA staining and immunoprecipitation studies showed that both types of the escape mutants preserved the antigenicity to all of our stocks of anti-G mAbs, except for mAb #1-46-12. In this study, however, the R-61 mutant G protein was not precipitated by mAb #1-46-12 when solubilized with NP-40, although this mutation did not affect the antigenicity detected by the FA staining. Similar results were also obtained with the NP-40-solubilized R-61 G protein from the purified virion, having lost the reactivity with the mAb #1-46-12 in the immunoprecipitation assay (data not shown).

Since NP-40 disrupted the trimer form of G protein spikes, we next performed similar immunoprecipitation studies using the other detergent (e.g., CHAPS) that preserves the trimer form of G protein. As shown in Table 2, however, the CHAPS-solubilized R-61 G protein from the infected cells was not recognized by mAb #1-46-12, while its reactivity to other anti-G mAbs was not affected (data not shown). Similar results were also obtained with the CHAPS-solubilized R-61 G proteins from the purified virions (data not shown). The results suggest strongly that the non-reactivity with mAb #1-46-12 of the solubilized R-61 G protein is not caused by loss of the trimer form, but rather due to removal of the lipid bilayer. On the other hand, R-31 G proteins lacked the 1-46-12 epitope simply due to a single amino acid substitution in the epitope region (6), and were not recognized by mAb #1-46-12 under any reaction conditions we tested (Table 2).

Table 1. Comparison of the reactivity of the wild-type (wt) and mutant viruses against the anti-G mAbs on the cell surface and in the NP-40-extracted infected cell lysates

mAb	wt		R-31		R-61	
	FA[a]	IP[b]	FA	IP	FA	IP
#1-46-12	+	+	−	−	+	−
#1-76-11	+	+	+	+	+	+
#1-56-13	+	+	+	+	+	+
#4-7-15	+	+	+	+	+	+
#4-61-31	+	+	+	+	+	+
#5-5-45	+	+	+	+	+	+
#4-11-42	+	+	+	+	+	+
#4-45-33	+	+	+	+	+	+
#6-35-23	+	+	+	+	+	+
#1-30-44	+	+	+	+	+	+
#4-50-42	+	+	+	+	+	+
#7-1-9	+	+	+	+	+	+

[a] Fluorescent antibody staining of G antigen on the cell surface.

[b] Immunoprecipitation of G antigen in the NP-40-extracted infected cell lysates.

Search for Possible Alteration in the G Protein Conformation Induced by Binding of a Small Numbers of Anti-G mAb #1-46-12

To think more about the flexibility of the rabies virus G protein from different viewpoints, we next tried to detect certain presumed conformational changes induced after the mAb binding to the virion. We previously reported that binding of a small number of anti-G mAb (#1-46-12) to less than 5% of the wt virion spikes was enough for the neutralization of its infectivity, which was shown to result in a loss of the receptor-binding ability of the virion (5). From this study, it was strongly suggested that binding a small number of mAb #1-46-12 to cover only a small portion (less than 5%) of the G protein spikes of the virion might have induced certain conformational changes in all spikes of the whole virion to destroy their receptor-recognizing and/or binding ability. In addition, it has been reported that the rabies virus G protein takes three different conformations depending on the environmental pH conditions, which could be differentiated by altered reactivity to certain anti-G mAbs and/or by increased sensitivity to some proteases (3). Accordingly, it was of interest to examine whether the G protein undergoes similar conformational changes after binding of the neutralizing mAb #1-46-12.

To check this possibility, we examined the reactivity of wt virions with the acid-sensitive epitope-specific anti-G mAb (#1-30-44) as well as with other anti-G

Table 2. Comparison of the G protein between the wild-type virus and the escape mutants that survived the neutralization by mAb #1-46-12

Detection method	Treatment	Form and location of G protein	Virus		
			wt	R-31	R-61
FA[a]	Formalin-fixed	trimer; cell surface	+	−	+
IP[b]	−	trimer; virion	+	−	+
	NP-40-extracted	monomer; cell lysates	+	−	−
	CHAPS-extracted	trimer; cell lysates	+	−	−

[a] Fluorescent antibody staining with mAb #1-46-12.

[b] Immunoprecipitation with mAb #1-46-12.

mAbs after the neutralization by binding of a small number of anti-G mAb #1-46-12. The virions were first incubated with a low concentration (0.1 μg IgG/ml) of mAb #1-46-12, which resulted in covering about 5% of the virion spikes on average but was enough for 100% neutralization of the infectivity, and then incubated with other anti-G mAb at a higher concentration (i.e., 1 μg IgG/ml), which was followed by recovering the antibody-bound virions by ultracentrifugation through a 20% sucrose cushion. Then, recovered virions were subjected to SDS-PAGE and western blotting with rabbit anti-mouse IgG antibody for detecting the virion-bound mAbs. A small aliquot of each mAb-bound virion sample was subjected to the focus formation assay to confirm the neutralization of viral infectivity. As shown in Fig. 3, although the amount of mAb #1-46-12 was enough for the wt virus neutralization (data not shown), binding of a small amount of mAb #1-46-12 to the virion did not affect additional binding of any other anti-G mAb tested, including the acid-sensitive conformational epitope-specific mAb #1-30-44. From these results, it was suggested that, even if the presumed conformational change(s) was induced by binding of the mAb #1-46-12, such change(s) would not be big, would not affect the conformational epitopes recognized by mAb #1-30-44 and other anti-G mAbs we tested, and would not be the same as the acid-induced change.

We finally tried to find possible changes of the virion G proteins that could be recognized by increased sensitivity to proteases after the binding of mAb #1-46-12. Virions were first incubated with a low concentration (0.1 μg IgG/ml) of mAb #1-46-12, and then recovered by ultracentrifugation through a 20% sucrose barrier. The virion pellet was resuspended for digestion with either of several kinds of proteases listed in Table 3, followed by SDS-PAGE analysis. A small aliquot of the mAb-treated virions was also checked to confirm the neutralization (data not shown). The results are summarized in Fig. 4 and Table 3, showing that no obvious difference was detected in any case of the protease digestion between the mAb-treated and untreated virions. Internal virion proteins (N, P, and M proteins) were affected by either of these proteases only in the presence of Triton X-100. This result indicates that the virion envelope was kept intact, not allowing the proteases to invade the virion, even after the antibody binding or during the protease digestion.

Discussion

In this study, we performed several experiments to understand the possible mechanism of rabies virus neutralization by mAb #1-46-12, including the co-infection studies with wt virus and the two types of escape mutants (R-31 and R-61) that survived the neutralization by mAb #1-46-12. We found that neutralization sensi-

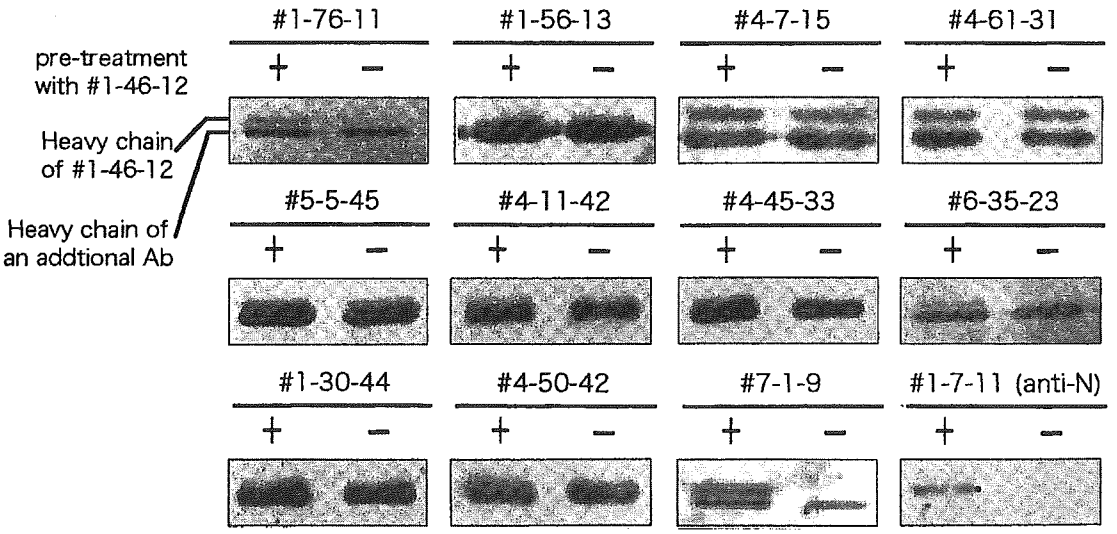

Fig. 3. No effect of mAb #1-46-12 binding on the G protein conformation recognized by other conformational epitope-specific anti-G mAbs. Rabies virions (about 1 μg protein) were mixed with mAb #1-46-12 at a concentration of 0.1 μg IgG/ml, and incubated for 1 hr at 37 C, followed by addition of the other mAb into the reaction mixture at a concentration of 1 μg IgG/ml. After further incubation for 1 hr at 37 C, the virion-bound antibodies were recovered by ultracentrifugation at 35,000 r.p.m. for 1.5 hr through a 20% sucrose cushion, and subjected to SDS-PAGE and immunoblotting with rabbit peroxidase-conjugated anti-mouse IgG antibody. Only the heavy chain band of mAbs tested (thick band) and mAb #1-46-12 (thinner band) were shown here. The antibody #1-7-11 is an anti-N mAb, being used as a negative control of this experiment, and did not bind to the virion even after the binding of small amount of mAb #1-46-12 (indicated by asterisk).

Table 3. Investigation of the sensitivity to proteases of the rabies virion before and after the binding of neutralizing mAb #1-46-12[a)]

Protease	Sensitivity to protease			
	Native virion	Neutralized virion		
	G	G	Internal proteins (N, P, M)	
			TX (−)	TX (+)
Serine proteases				
Trypsin	−	−	−	+
Chymotrypsin	−	−	−	+
Proteinase K	+	+	−	+
Elastase	−	−	−	+
SH proteases				
Papain	+	+	−	+
Bromelain	−	−	−	+
Acidic endoprotease				
Pepsin	+	+	−	+
Others				
Protease of *Streptomyces griseum* (Actinase E+Pronase E)	+	+	−	+
Pronase E	+	+	−	+

[a)] Experiments were performed as described in Fig. 4. Data for the trypsin digestion were transcribed from the results shown in Fig. 4 depicted as (+) and (−) for sensitive and insensitive to trypsin, respectively. Results for other proteases were also transcribed similarly into (+) or (−) as done for the trypsin digestion. TX: Triton X-100.

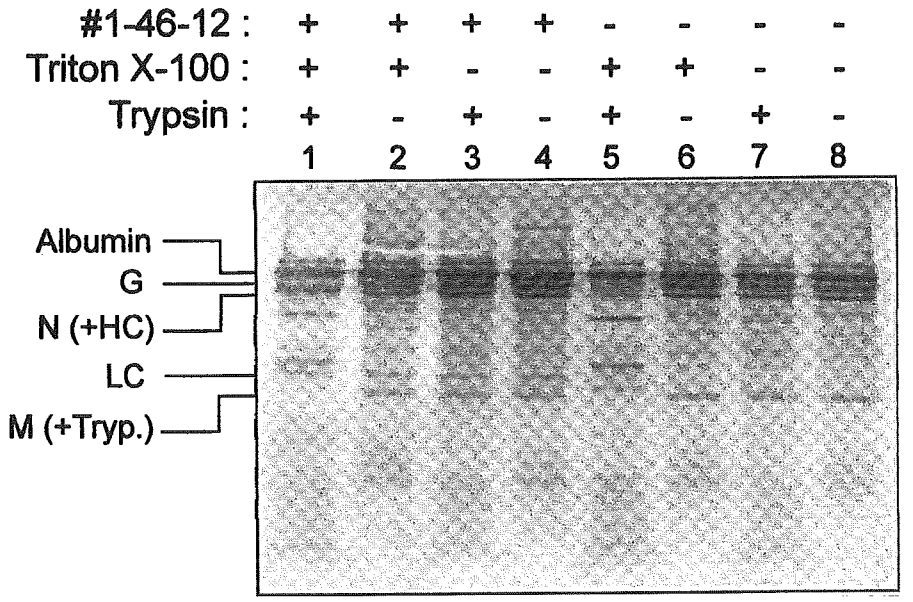

Fig. 4. Investigations of protease-sensitivity of the wt rabies virions after the neutralization by mAb #1-46-12. Purified virions (1 μg protein) of the wt HEP-Flury strain were incubated without or with mAb #1-46-12 at a concentration of 0.1 μg IgG/ml. After incubation for 1 hr at 37 C, the virions were recovered by ultracentrifugation at 35,000 r.p.m. for 1.5 hr, and suspended in appropriate buffer for digestion with proteases, including trypsin and other proteases listed in Table 3. After addition of trypsin or other protease at a final concentration of 1 μg/ml and/or Triton X-100 to the mAb-bound virion suspensions, the reaction mixtures were incubated for 2 hr at 37 C for protease digestion, and then mixed with the SDS-PAGE sample buffer, followed by SDS-PAGE and CBB staining. Only a representative result of the trypsin digestion is shown here.

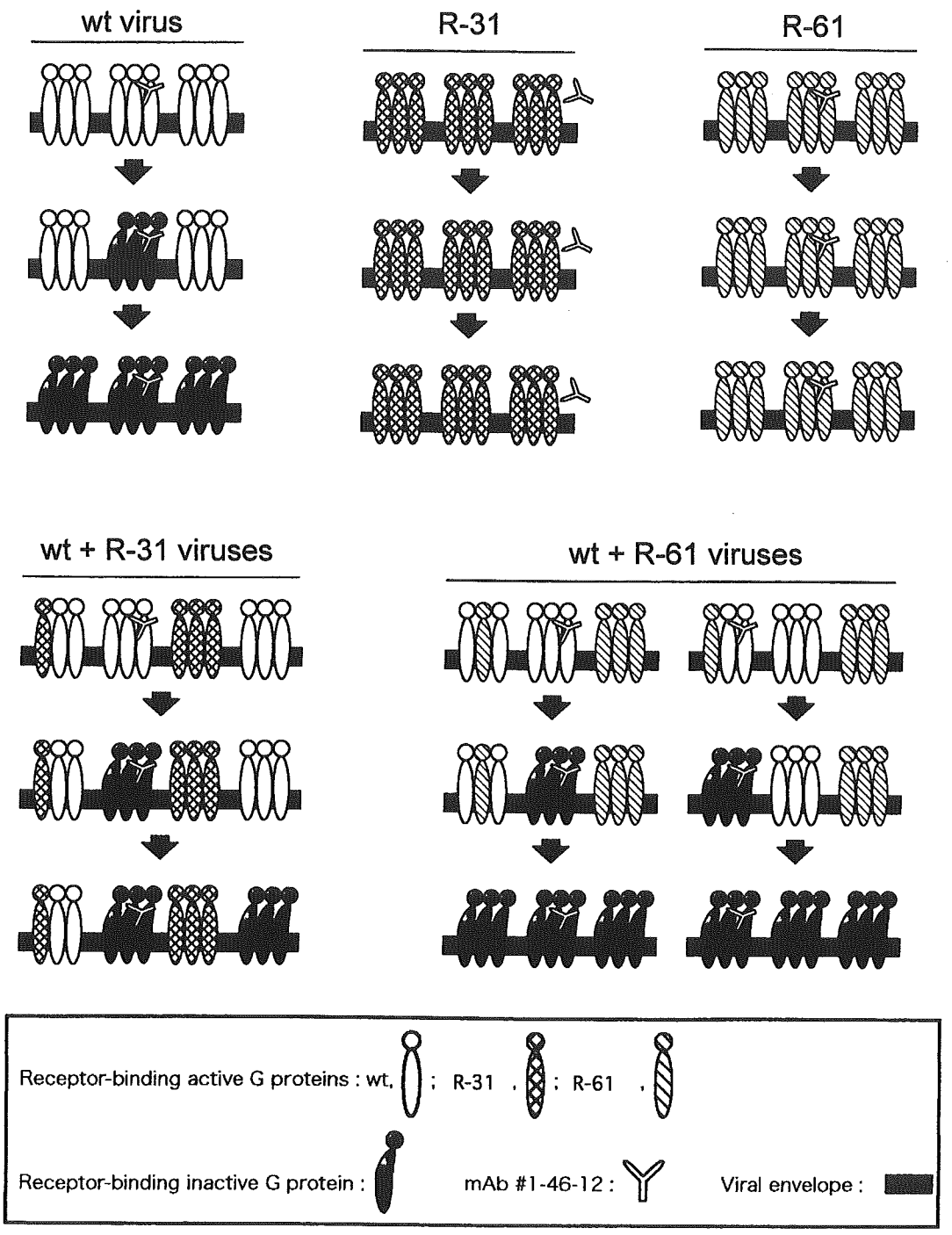

Fig. 5. A hypothetic domino effect model for the possible mechanism of rabies virus neutralization by mAb #1-46-12 and for the escape mutations to this antibody (see the text). The group I (R-31) is the 1-46-12 epitope-negative mutant, while the group II (R-61) is the epitope-positive mutant (5, 6).

tivity of phenotype-mixed progeny virions recovered from the co-infected cultures were different depending on the combination and ratio of wt and escape mutant viruses in the inocula. The epitope-negative mutant (R-31) G protein lacked the 1-46-12 epitope due to an amino acid substitution in the epitope region (6). In the case of mixed infections with the wt and the R-31 viruses, the progeny virions displayed stronger resistance to mAb #1-46-12 than the wt virus (Fig. 1). In this case, it is also suggested that incorporation of a certain number of R-31 G protein molecules into the progeny virion is enough for the virus to be resistant to the antibody. This observation also suggests that coexistence of wt and R-31 G protein spikes on the same viri-

on greatly reduced the sensitivity to mAb #1-46-12.

In contrast, progeny virions that were recovered from the co-infected cultures with the wt virus and an epitope-positive escape mutant (R-61) showed strong sensitivity to the antibody even under a co-infection conditions at a ratio of 1:1 (5). This observation means that incorporation of only a small number of wt G protein into the R-61 virion is enough to endow the virion with high sensitivity to neutralization by mAb #1-46-12. In addition to different neutralization sensitivities of these phenotype-mixed virions, we observed that the virions which were neutralized by the binding of a small number of mAb #1-46-12 had lost their receptor-binding ability (5), probably due to conformational change of the whole virion G proteins.

However, in the case of the R-61 virus, binding of 300 IgG molecules per R-61 virion resulted in little inactivation of the viral infectivity, and might not have induced such conformational change(s). Additional *N*-glycosylation at Asn-37 might be responsible for acquisition of this resistance, since this potential glycosylation site of rabies virus G protein is usually not glycosylated except for the case of R-61 virus (6, 14). *N*-Glycosylation at Asn-37 did not affect the antigenicity of the G protein to mAb #1-46-12, and seems to be involved in the structural stability of the G protein even after the mAb binding.

As shown in Table 2, however, the mutant R-61 G proteins lost their antigenicity (or reactivity) due to conformational changes after removal of the lipid bilayer, probably not due to disruption of the trimer form, when they were solubilized with NP-40 or CHAPS, implying that the R-61 G protein required the membrane anchorage for keeping the original epitope-positive conformation. Additional glycosylation at Asn-37 and the membrane anchor might contribute to the structural stability of the protein to preserve the epitope-positive conformation, and it behaves like an intact molecule even after the antibody binding.

It has been known that the pH-induced conformational change of G protein confers on the protein more increased sensitivity to certain proteases or induces loss of its reactivity to some mAbs (3). We tried to find evidence for such postulated structural change(s) induced by mAb binding. After many tests, however, we were not able to find such changes so far. At least, we can say that such a conformational change of G protein induced by binding of mAb #1-46-12 might be different from that which is induced after being exposed to acidic conditions, because the G protein preserved the acid-sensitive 1-30-44 epitope even after the binding of mAb #1-46-12 (Fig. 3).

Together with these observations, we hypothesize again a domino effect model described in our previous study (5) as a possible mechanism of virus neutralization by mAb #1-46-12 as well as for explaining the two different ways of acquiring the resistant nature of the virus shown for mutants R-31 and R-61 as illustrated in Fig. 5. Binding of a small number of mAb #1-46-12 to the wild type virion might cause the mAb-induced certain conformational change(s) of G protein, which would spread to the neighboring antibody-free spikes like a "domino effect," resulting in losing the original receptor-binding conformation of the spikes. In the case of co-infection with R-61 mutant, if a small number of wt G proteins are incorporated into the R-61 virion, R-61 G proteins behave like the wt G protein to change its conformation due to the "domino effect" after the mAb binding to the wt G protein. Coexistence of wt G proteins with the R-31 G protein on the same virion did not affect the normal functions of R-31 G protein that are required for the early phase of virus infection even after the binding of mAb #1-46-12 to the wt G proteins.

Although further studies are necessary, this kind of antibody will be an ideal drug for post-exposure treatment of rabies virus infection because of its excellence at recognizing a highly conserved epitope of the virus and the requirement of less than 20 molecules of bound antibody for the neutralization, and may provide a key for designing and developing effective drugs that will attack the epitope site of the viral G protein to cause its conformational change(s).

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Further Studies on the Soluble Form (Gs) of Rabies Virus Glycoprotein (G): Molecular Structure of Gs Protein and Possible Mechanism of the Shedding

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***Abstract*: In this study, we investigated the antigenic structures and maturation of some C-terminal-deficient derivatives of rabies virus glycoprotein (G). The Gs protein, a soluble form of G protein shed from infected cells, displayed antigenicity to most of our conformational epitope-specific anti-G mAbs, but took the 1-30-44 epitope-deficient conformation (termed G_C form). (The 1-30-44 epitope was acid-sensitive and dependent on two separate regions, the Lys-202-containing and Asn-336-containing regions; Kankanamge et al., Microbiol. Immunol., 47: 507–519). Intact G proteins took the 1-30-44 epitope-positive form (referred to as G_B form) on the cell surface, but not inside the cell. A deletion mutant G(1-429) (termed GΔTC), lacking the transmembrane (TM) and cytoplasmic domains, was shown to be accumulated in the rough endoplasmic reticulum (rER) with BiP and did not seem to be shed. Another C-terminal-deficient mutant G(1-462) (termed CT1) was deprived of the whole cytoplasmic domain except for a basic amino acid left at the C-terminus, but was transported to the cell surface, where it showed pH-dependent cell fusion activity and almost full antigenicity to most of the anti-G mAbs with the exception of very weak antigenicity to mAb #1-30-44. No Gs protein could be detected in the CT1-producing cultures. Based on these results, we think that the cytoplasmic domain was not necessary for the G protein to be transported to the cell surface, but was necessary to keep its 1-30-44 epitope-positive G_B conformation. Gs proteins might have lost the C-terminal regions during the maturation process after being exported from the rER.**

***Key words*: Rabies virus, Glycoprotein, Gs protein, Shedding, Monoclonal antibody, Conformational change, Non-native conformation**

The rabies virus genome encodes a single type I glycoprotein (G). G proteins are synthesized in the rough endoplasmic reticulum (rER), assembled into a non-covalent trimer form (6, 27), and transported to the Golgi apparatus, where they are further processed, and exposed to the cell surface with a mature conformation (3, 5, 8). A portion (about 10%; 18) of G proteins are released from the infected cells in a soluble form (Gs) into the culture medium (Gs shedding). The Gs protein is a C-terminal-deficient protein, lacking the greater part of the TM and whole cytoplasmic domains, and also shown to be not very immunogenic in animals (2). At present, however, the molecular structure and shedding mechanism of Gs protein as well as its role(s) in the infection process *in vivo* yet remain to be elucidated.

We have recently characterized our conformational epitope-specific anti-G monoclonal antibodies (mAbs; 9–11), which might be usable for investigating the molecular structures of Gs protein as well as for elucidating possible reason(s) why the Gs protein is not very

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Abbreviations: Arg, arginine; Asn, asparagine; CBB, Coomassie brilliant blue; cDNA, complementary deoxyribonucleic acid; DOC, deoxycholate; EDTA, ethylenediaminetetraacetic acid; FA, fluorescent antibody; FITC, fluorescent isothiocyanate; G, glycoprotein; G_B, B-form of viral G protein; G_C, C-form of viral G protein; Gs, soluble form of viral G protein; IP, immunoprecipitation; Lys, lysine; mAb, monoclonal antibody; MEM, minimum essential medium; NP-40, Nonidet-P 40; pAb, polyclonal antibody; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; rER, rough endoplasmic reticulum; RIPA, radioimmunoprecipitation assay; RVV-T7, T7 RNA polymerase gene-containing recombinant vaccinia virus; SDS, sodium dodecyl sulfate; TM, transmembrane; VSV, vesicular stomatitis virus.

immunogenic in animals. For instance, one (#1-30-44) of those mAbs recognizes the acid-sensitive epitope of mature G protein, and two amino acids in separate regions, Lys-202 and Asn-336, have been suggested to be involved in the epitope formation (10). Using this mAb, rabies virus G protein was shown to take either of two conformations under neutral pH conditions, the 1-30-44 epitope-positive (referred to as G_B) and the negative (referred to as G_C) ones (11).

Using these anti-G mAbs, we tried in this study to elucidate more precisely the molecular structures of rabies virus Gs protein and the possible mechanisms of Gs protein shedding. Results showed that the Gs protein displayed antigenicity to most of our conformational epitope-specific anti-G mAbs, except for mAb #1-30-44 (hence the Gs protein was assumed not to take the G_B, but the G_C conformation that was termed for the G protein which lacked conformational 1-30-44 epitope under neutral pH; 11). Further studies were also performed to learn more about the shedding mechanism, for which two C-terminal deficient mutants (GΔTC and GΔC) were prepared. The G(1-429) mutant (termed GΔTC), which lacked the whole TM and cytoplasmic domains, was not detected in culture fluids of cDNA-transfected cells, but was found to be accumulated in the rER and Golgi area. Another C-terminal-deficient mutant G(1-462) [termed GΔC (=CT1)], which preserved the TM, but lacked 43 amino acids from the C-terminus, was efficiently transported to the cell surface, where it showed low pH-dependent cell fusion activity and antigenicity to a panel of our conformational epitope-specific anti-G mAbs, except for very weak antigenicity to the 1-30-44 epitope-specific mAb. The results obtained so far suggest that the cytoplasmic domain is not essential for correct folding and maturation of G protein, but seems to be necessary to keep the 1-30-44 epitope-positive mature form. The Gs protein did not seem to take G_B conformation, but the G_C or G_C-like conformation. Intracellular G protein was 1-46-12 epitope-negative, but the Gs protein shed into the culture fluid was 1-46-12 epitope-positive, and no Gs protein could be detected in the infected cells. Accordingly, we would like to assume that Gs protein is produced by removal of the TM and cytoplasmic domains from a full-sized G protein by proteolytic cleavage during the maturation process, probably at or near the final step of the maturation process.

Materials and Methods

Virus and cell cultures. Rabies viruses used in this study were the BHK-adapted HEP-Flury strain (12). Virus stocks were prepared by plaque-purification and amplification in BHK-21 cell cultures, and were plaque assayed using BHK-21 cells as described previously (14). BHK-21 cells were propagated in Eagle's MEM supplemented with 5% calf serum and 10% Tryptose phosphate broth (Difco).

cDNA-transfections. The G cDNA used in this study was the same one prepared for the HEP-Flury strain of rabies virus as described previously (19, 20). The G cDNA was inserted into a T7 promoter-regulated expression vector pCDM8 as described previously (21). From this G cDNA, we prepared two kinds of C-terminal-deleted mutants, G(1-429) (referred to as GΔTC) and G(1-462) (referred to as GΔC or CT1). GΔTC was prepared by removing a short upstream sequence (residues 430–439) from the TM, the whole TM and the cytoplasmic domains, while CT1 was prepared by deleting the cytoplasmic domain except for one amino acid (Arg-462) left at the C-terminal (Fig. 3). These deletion mutants were inserted into pCDM8 vector (inserted downstream of the T7 promoter to be expressed by transcription with T7 RNA polymerase; reconstructed expression vectors were referred to as pCDM8-G, pCDM8-GΔTC and pCDM8-CT1). G gene expression was performed by transfecting plasmid DNAs to BHK-21 cells according to a protocol of the calcium phosphate method (1) with the help of recombinant vaccinia virus (RVV-T7) which provided T7 RNA polymerase as described previously (10).

Antibodies. Rabbit polyclonal antibodies against the whole G protein of rabies virus HEP strain (anti-G pAb) and the C-terminal-specific antibodies (prepared against its C-terminal-mimicked synthetic oligopeptide; anti-G_{CT} pAb) were the same as described previously (18).

All anti-G monoclonal antibodies (mAbs) used in this study were prepared and some were characterized in our previous studies (9–11, 22). Most studies were performed by using mAbs #1-30-44 (specific for a low pH-sensitive conformational epitope of G protein; 10), #1-46-12 (specific for a highly conserved conformational epitope; 9), and #7-1-9 (linear epitope-specific; 22). In this study, culture fluids from the hybridoma cultures were mostly used as the mAb source. Antibody concentration of each mAb stock was estimated by semi-quantitative immunoblot assay using a mouse immunoglobulin sample of known concentration and peroxidase-conjugated anti-mouse goat antibody (Cappel) as described previously (9).

Indirect fluorescent antibody (FA) staining. Cell cultures were performed on glass cover slips. At appropriate time of incubation, cells were fixed with 10% formalin in PBS(−) for 1 hr at neutral pH (pH 7.4), after which specimens were treated with 0.1% Triton X-100

(in the case of detecting both the intracellular and cell surface antigens) or mock-treated (in the case of detecting the cell surface antigens alone). They were stained with rabbit anti-G pAb or mouse anti-G mAb, followed by staining with goat FITC-conjugated second antibody (Cappel).

Radioactive labeling. Culture medium of the rabies virus infected and cDNA-transfected BHK-21 cells was replaced with methionine-free medium at an appropriate time as described in the text. After incubation for 30 min, the culture medium was replaced again by fresh methionine-free medium, to which L-[^{35}S]methionine (final concentration =10 μCi/ml) was added for metabolic radiolabeling of the cells for 6 hr. Then, the radiolabeled cells were washed with PBS(−) three times, followed by lysing in 100–200 μl of RIPA II buffer [1% NP-40, 1% sodium deoxycholate (DOC), 150 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, 1 mM pefabloc SC (Merck) and 25 μg/ml leupeptin; pH 7.4] for immunoprecipitation with anti-G mouse mAbs or rabbit antibody. To obtain the particulate-free fraction of the culture fluids of the virus infected cultures, the fluids were placed on a 20% sucrose cushion and ultracentrifuged at 40,000 r.p.m. for 90 min at 4 C.

Immunoprecipitation and autoradiography. Radiolabeled cell lysates (10–20 μl) were mixed with 1–2 μg of anti-G mAbs or 1–2 μl of rabbit anti-G pAb and placed on ice for 2 hr, followed by recovery with Pansorbin (commercial product of insoluble protein A of *Staphylococcus aureus*; Calbiochem; 10–20 μl) for 2 hr at 4 C as described previously (15). In the case of precipitation with mouse mAb, precipitates were recovered with the help of rabbit second antibody against mouse immunoglobin. Precipitates were dissolved in 50 μl of sample lysis buffer for SDS-PAGE (16), and applied to 10–12% SDS-PAGE. After the electrophoresis, the gels were stained with Coomassie brilliant blue R-250 (CBB), then dried onto 3MM filter paper (Whatman), and exposed to an imaging plate for autoradiographic analysis in a Bio-Imaging Analyzer BAS2000 (Fuji Photo Film Co., Ltd.).

SDS-PAGE. Assay samples were dissolved in sample lysis buffer [125 mM Tris-HCl, 4.6% sodium dodecyl sulfate (SDS), 10% 2-mercaptoethanol, 0.005% bromophenol blue and 20% glycerol; pH 6.8]. They were applied to 10% polyacrylamide gels prepared with Laemmli's discontinuous buffer system (16). After the electrophoresis, protein bands in the gels were subjected to immunoblotting (see below), or stained with CBB and dried on filter paper. Estimation of molecular weight of the proteins concerned was performed by comparing the mobility of coelectrophoresed SDS-PAGE Molecular Weight Standards (Bio-Rad; for immunoprecipitation).

Semi-quantitative chemiluminescent immunoblot assay. Cells were lysed in SDS-PAGE sample buffer and applied to 10% SDS-PAGE gel. After electrophoresis, samples in the gel were electrically blotted onto nitrocellulose membrane filter (type BA85; Scleicher & Schuel) using a semi-dry type blotting apparatus. The blotted membrane filter was subjected to the usual blocking procedures with skim milk, and then incubated with murine mAbs or rabbit anti-G pAb, followed by incubation with horseradish peroxidase-conjugated second antibody (goat). Luminescent light was emitted by luminol oxidation using a commercial chemiluminescence assay kit (Santa Cruz Biotechnology), and exposed to X-ray film (Kodak X-Omat AR film). For estimation of apparent molecular weight of the proteins concerned, Rainbow marker and Dr. Western were coelectrophoresed. Density of the developed band was measured using a scanner and application software (NIH Image 1.6.2).

Estimation of the amounts of mAbs bound to the cell surface. Mouse mAb solution (10 μg/ml) was poured onto the formalin-fixed cell monolayers, and incubated for 1 hr at room temperature. After the cells were washed three times with PBS(−), the cell-bound antibodies were recovered by dissolving them in SDS-PAGE sample buffer and were applied to semi-quantitative chemiluminescent immunoblot assays with peroxidase-conjugated anti-mouse Ig antiserum (goat), for which a mouse antibody stock of known concentration was used as a standard as described previously (11).

Results

Properties of the Gs Protein Produced in the Virus Infected Culture

First, we checked the Gs protein shedding into the rabies virus-infected BHK-21 cell cultures by the immunoprecipitation method using two kinds of anti-G rabbit polyclonal antibodies (pAb) which are directed either to whole G protein or to its C-terminal region (termed anti-G and anti-G_{CT} pAbs). As shown in Fig. 1, anti-G pAb detected the 62 and 56-kDa polypeptides in the culture fluids from the virus infected cultures (lane 4), while the latter was not recognized by anti-G_{CT} pAb (lane 1) as reported previously (18). After removal of the particulate fraction from the culture medium by ultracentrifugation, only the 56-kDa band was left in the medium (Fig. 1; lane 3), and was not precipitated by anti-G_{CT} pAb (lane 2). These results indicate that, in addition to virion-associated G proteins, a soluble form (Gs) of G protein was shed into the infected culture fluids.

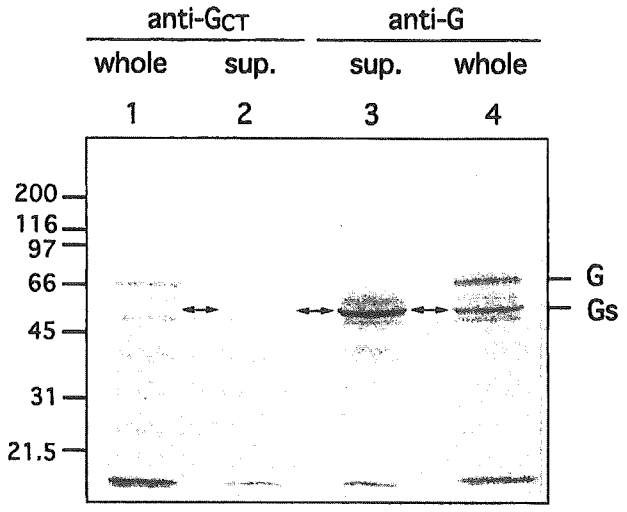

Fig. 1. Demonstration of a C-terminal-deficient G protein derivative in the rabies virus-infected BHK-21 cell culture. Rabies virus (HEP-Flury strain) infected BHK-21 cell monolayer cultures were incubated in the presence of L-[^{35}S]methionine (10 μCi/ml) for 6 hr from 40 hr after the infection. Culture fluids were recovered and clarified by high speed centrifugation (8,000 r.p.m. for 5 min). The supernatant was recovered and split into two equal parts, one of which was then subjected to immunoprecipitation with rabbit antibody against the whole G protein (anti-G) or its C-terminal region (anti-G_{CT}). The other half was first subjected to ultracentrifugation (35,000 r.p.m. for 1.5 hr) to remove the particulate fraction, and the supernatant was processed for immunoprecipitation as done for the whole culture fluid. Lanes 1 and 4: whole culture fluid; lanes 2 and 3: the same culture fluid from which the particulate fraction was removed. [Antibodies] Lanes 1 and 2: anti-G_{CT} pAb; lanes 3 and 4: anti-G pAb. Arrows indicate the position of Gs protein.

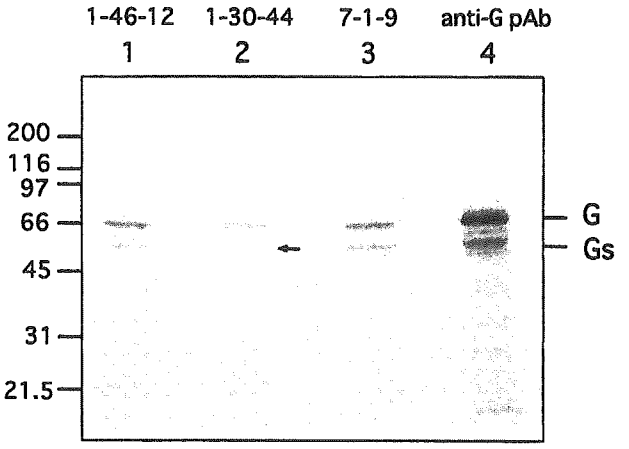

Fig. 2. Antigenic profile of Gs protein displayed by its reactivity to anti-G mAbs. Culture fluids were recovered from the radiolabeled rabies virus-infected cultures, and subjected to immunoprecipitation with anti-G pAb or either of anti-G mAbs (see "Materials and Methods"). Lanes 1 to 3: mAbs #1-46-12, #1-30-44, #7-1-9, respectively; lane 4: anti-G pAb. No Gs protein was detected on lane 2 (indicated by an arrow).

Table 1. Antigenic difference between the full-sized G protein of rabies virus and its C-terminal-truncated soluble form (Gs)

Anti-G mAbs	Epitope structure	G	Gs
#1-30-44	Conformational	+	−
#1-46-12	Conformational	+	+
#1-76-11	Conformational	+	+
#4-11-42	Conformational	+	+
#4-45-33	Conformational	+	+
#4-50-36	Conformational	+	+
#4-56-33	Conformational	+	+
#4-61-31	Conformational	+	+
#5-5-45	Conformational	+	+
#6-35-23	Conformational	+	+
#7-1-9	Linear	+	+

Antigenicity to anti-G mAbs was examined at neutral pH (7.4) by immunoprecipitation assay in the same way as described in Fig. 2.

The Gs protein in culture fluids was further examined with anti-G mAbs. As shown in Fig. 2 and Table 1, Gs proteins were recognized by most of our conformational epitope-specific anti-G mAbs except for one mAb, #1-30-44 (Fig. 2, lane 2), the mAb that recognized an acid-sensitive conformational epitope that is formed by two separate regions (Lys-202-containing and Asn-336-containing regions) of G protein (10). These observations indicate that the Gs protein took a 1-30-44-epitope-negative conformation (referred to as the Gc form), while the 1-30-44 epitope-positive conformation (referred to as the G_B form) was detected in the authentic G protein (Table 1; 11).

Studies on the C-Terminal-Deleted Mutant GΔTC

To investigate more precisely the conditions for the antigenic maturation of Gs protein, we prepared a deletion mutant of the G cDNA encoding a C-terminal-truncated G(1-429) (termed GΔTC protein), which lacked a short sequence of the upstream region (amino acid residues 430 to 439) and the whole transmembrane (residues 440 to 461) and cytoplasmic (residues 462 to 505) domains (Fig. 3). The GΔTC, however, could not be detected in the cDNA-transfected culture fluid (Fig. 4, lane 6). Longer exposure for detecting the weak chemiluminescence did not result in detecting the GΔTC band from the culture fluids (data not shown). Immunofluorescence with anti-G mAb (#7-1-9, a linear epitope-specific mAb; 22) did not detect any antigen on the cell surface when formalin-fixed cells were examined without treatment with Triton X-100 (data not shown), but did detect the antigen in the cell after the Triton-treatment as shown in Fig. 5A. Double immunofluorescence studies with anti-G mAb (#7-1-9; Fig. 5A) and rabbit anti-BiP antibody (Fig. 5B) suggested that

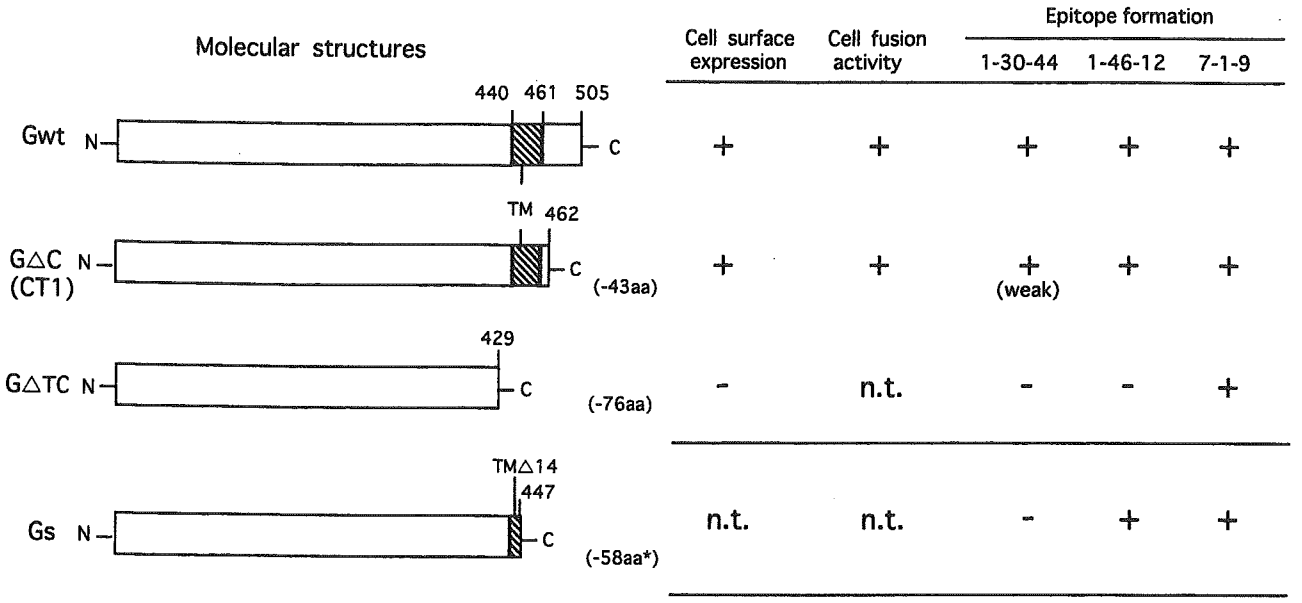

Fig. 3. Schematic illustration of the C-terminal-deficient G protein derivatives and a summary of characterizations. Amino acid residues 462 to 505 compose the original cytoplasmic domain. TM: transmembrane domain (amino acid residues 440 to 461); n.t.: not tested; *: cited from Dietzschold et al. (2).

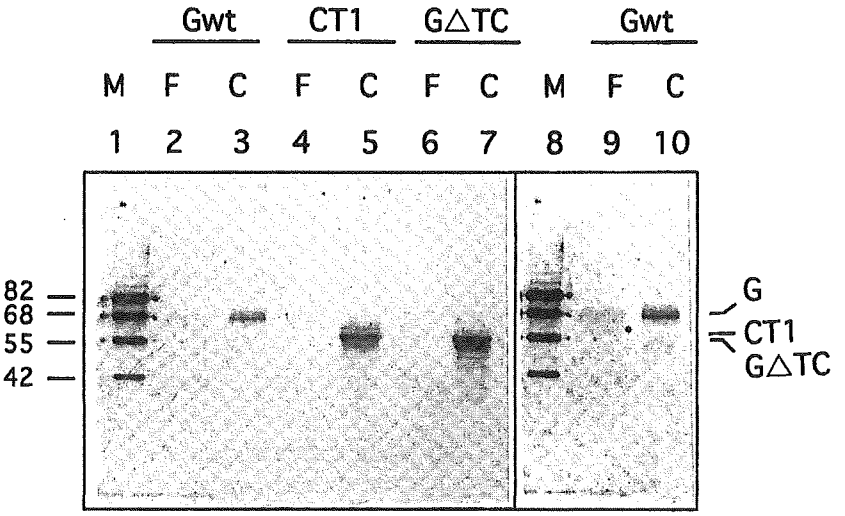

Fig. 4. Little or no shedding of soluble G proteins in the G protein derivative-producing cultures. BHK-21 cells were transfected with the G cDNA encoding wild type G protein, or the CT1 or GΔTC mutant protein with RVV-T7 (see "Materials and Methods"). After 12 hr incubation, culture fluids and cells were recovered separately. Culture fluids were processed for concentration of the G protein derivatives, if present, by precipitation with 10% TCA, followed by washing with 0.1 N HCl, then with cold acetone, and dried. Precipitates were dissolved in SDS-PAGE sample buffer and subjected to SDS-PAGE and immunoblotting with anti-G pAb. Chemiluminescence was detected under two different exposure periods. As for the longer period, only the data for the wild type are shown (lanes 9 and 10), but the data for other samples were omitted. Lanes 1 and 8: marker ("Dr. Western"; M); lanes 2, 4 and 6: culture fluids (F) from the wild type G, CT1 and GΔTC-producing cultures, respectively; lanes 3, 5 and 7: cell lysates (C) of the wild type G, CT1 and GΔTC-producing cells, respectively. Lanes 9 and 10: the same immunoblot of the sample on lanes 2 and 3, but exposed for the longer period. The asterisk depicted on lane 9 indicates the position of Gs protein.

GΔTC did not seem to be exported, but was accumulated in the cytoplasm, probably in the rough endoplasmic reticulum (rER) since it was shown to be colocalized with Bip in the cell (Fig. 5B). Fluorescent antibody (FA) staining also showed that GΔTC retained the immature or misfolded form as implicated by absence of its antigenicity to conformational epitope-specific anti-G mAbs (a representative result is shown in Fig. 6B). In

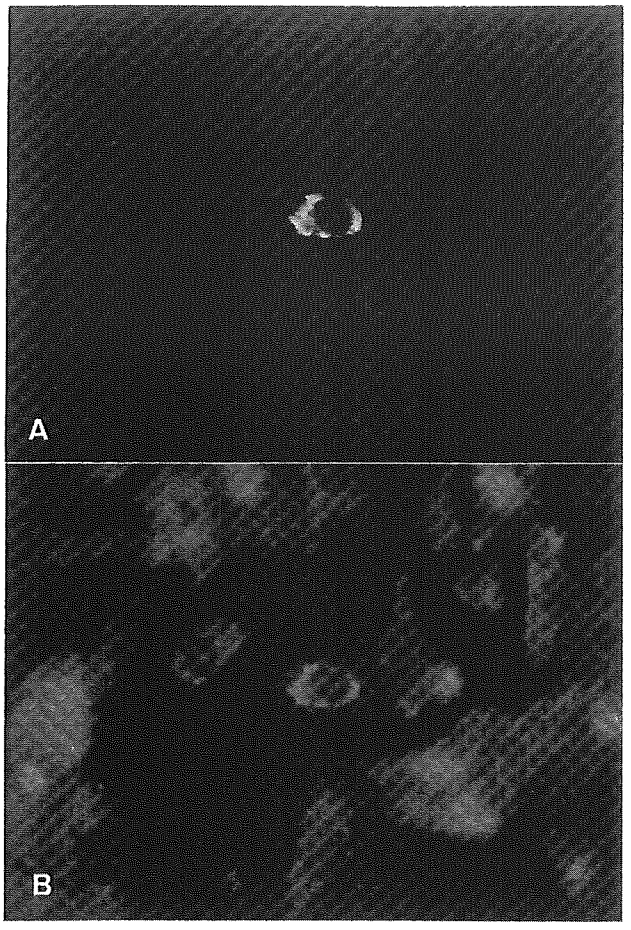

Fig. 5. Double immunofluorescence study of the GΔTC mutant. The GΔTC cDNA was transfected to BHK-21 cells with the help of RVV-T7. After 16 hr incubation, the cells were fixed with 10% formalin (30 min at room temperature), and doubly stained with mouse anti-G mAb #7-1-9 (A) and rabbit anti-BiP pAb (B), followed by a second staining with goat anti-mouse Ig antibody (FITC) and the anti-rabbit Ig antibody (rhodamine), respectively. A single G antigen-positive cell is seen in Panel A, and the antigen is colocalized in the cytoplasm with the BiP antigen shown in Panel B.

contrast, wild type G proteins were transported to the cell surface, which contained the antigenically and functionally mature form as previously demonstrated by FA staining using mAbs #1-46-12 and #1-30-44 and by their pH-dependent cell fusion activity (data not shown; 11, 23). Wild type G proteins inside the cell, however, were negative for the FA staining with conformational epitope-specific anti-G mAbs, including mAb #1-30-44 (data not shown). From these results, we conclude that the GΔTC mutant took the immature or misfolded form probably being intimately associated with BiP and accumulated in the rER (in other words, removal of the TM and cytoplasmic domains from the G protein may impair the maturation of its ectomain conformation, resulting in its accumulation in the rER).

Studies with a Cytoplasmic Domain-Deficient Mutant (CT1)

We next examined another C-terminal-deficient mutant G(1-462) (termed CT1), which lacked 43 amino acids from the C-terminus, with one basic amino acid (Arg-462) of the cytoplasmic domain left at the C-terminus (Fig. 3). Expression of CT1 mutant was performed similarly in BHK-21 cells using pCDM8 vector and RVV-T7. As shown in Figs. 7A and 7C, the CT1 was efficiently transported to the cell surface as wild type G protein, where it showed antigenicity to almost all of the conformational epitope-specific anti-G mAbs (representative data with mAbs #1-46-12 and #7-1-9 are shown in Fig. 7A and 7C), except for very weak antigenicity to mAb #1-30-44 (Fig. 7B). The CT1 protein on the cell surface was competent for cell fusion activity that was seen when the cells were exposed to the acidic conditions (data not shown; 11). These observations indicate that only a small fraction of the C-terminal-deficient CT1 proteins were taking the functionally mature G_B conformation on the cell surface.

We further checked the weak antigenicity of CT1 against mAb #1-30-44 by comparing the amounts of mAbs #1-30-44 and #1-46-12 bound to the cell surface of the cDNA-transfected cells. As shown in Fig. 8, the amount of each mAb bound to the cell surface was rough in proportion to the intensity of the fluorescence in the FA-stained specimens shown in Fig. 7. The molecular ratio of the amounts of mAb #1-30-44 to #1-46-12 bound to the cell surface of the wild type G protein-producing cells was about 1:4 as reported previously (11). The ratio of the amount of mAb #1-30-44 to that of mAb #1-46-12 bound to the CT1-producing cells was much smaller (about 1:6) when compared with that of wild type G protein-producing cells (Fig. 8B), suggesting that only a very small portion of CT1 proteins took the G_B conformation on the cell surface.

Immunoblotting studies demonstrated that no Gs protein could be detected in the CT1-producing cultures (Fig. 4, lane 4; more precisely, the amount of such protein, if any, would be at the undetectable level when compared with that from the wild type G cDNA-transfected cultures shown on lanes 2 and 9 in Fig. 4). Although the Gs protein could be detected in the wild type G cDNA-transfected cultures, the amount was very small (Fig. 4, lane 9) (a slightly dense band seen at the position for the full-sized G protein on lane 9 might be the G protein that has come from the G protein-producing but dying cells). In the lysates of G cDNA-transfected cells, however, no Gs-like protein could be detected (Fig. 4, lanes 3 and 10). Even when exposure time was prolonged for detecting the chemiluminescence, no band was detected from the CT1-producing

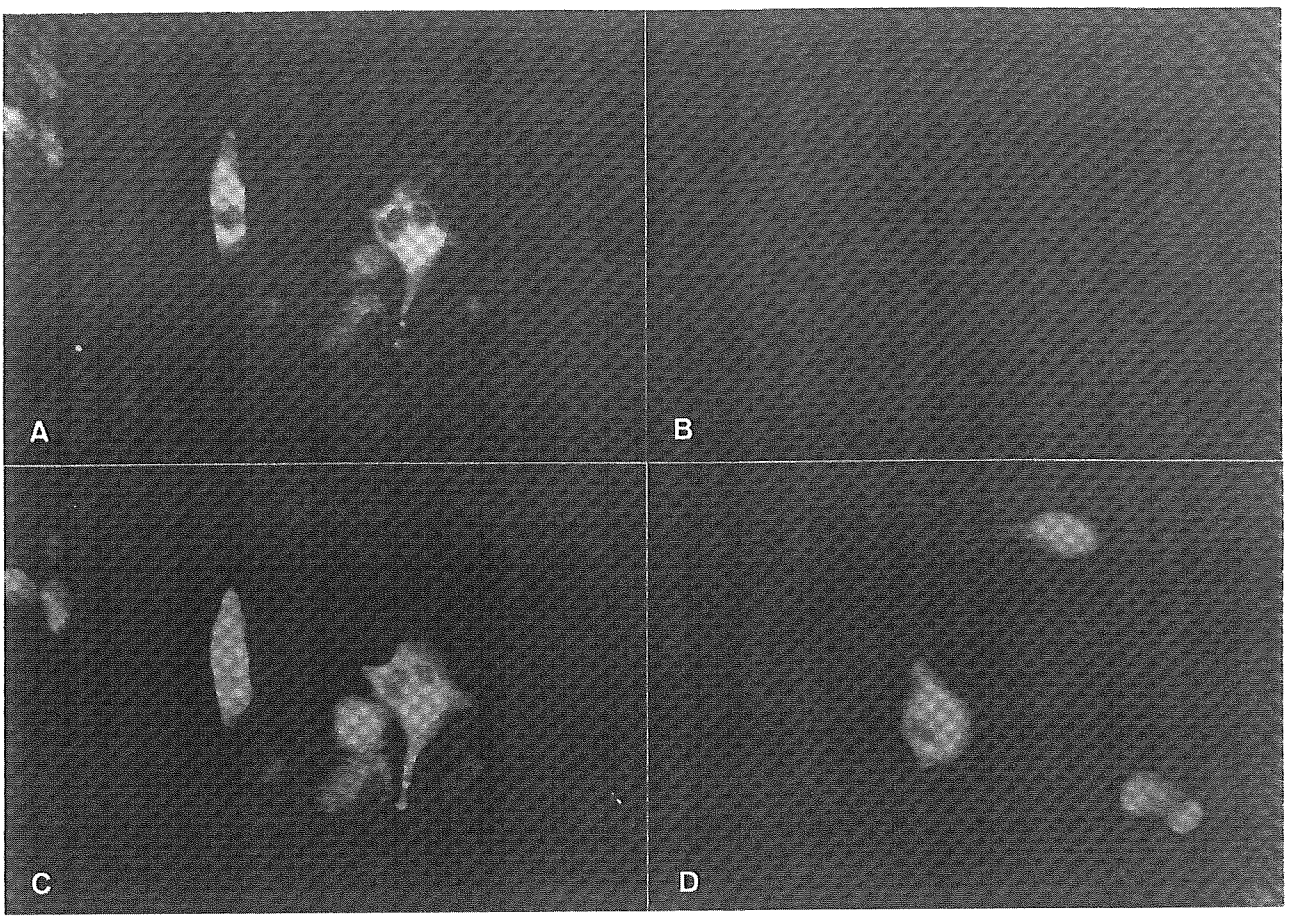

Fig. 6. Double immunofluorescence study of GΔTC mutant with anti-G mAbs. The GΔTC cDNA was transfected to BHK-21 cells as described in Fig. 5. After 16 hr incubation, the cells were formalin-fixed (30 min at room temperature) and doubly FA-stained with anti-G mAb (FITC) and anti-G pAb (rhodamine) after permeabilization with 0.2% Triton X-100. A and C (photographs of the same microscopic field): stained with anti-G mAb #7-1-9 (FITC) and rabbit anti-G pAb (rhodamine), respectively; B and D (photographs of the same microscopic field): stained with anti-G mAb #1-30-44 (FITC) and rabbit pAb (rhodamine), respectively.

nor from the GΔTC-producing cultures (data not shown).

Discussion

In this study, we investigated the antigenicity of some C-terminal-deficient forms of rabies virus G protein using conformational epitope-specific anti-G mAbs. Based on the reactivity with these mAbs, the Gs protein in the virus-infected culture fluids was assumed to take the G_C or G_C-like form, the G_C form which has been defined for the 1-30-44 epitope-negative conformation of G protein under the neutral pH (the epitope-positive conformation is termed the G_B-form; 11). Ectodomain of the G_C form of G protein and Gs protein displayed almost the same antigenic profile in terms of their reactivity to a panel of our conformational epitope-specific anti-G mAbs including #1-30-44 antibody (Table 1).

Concerning our 14 neutralizing anti-G mAbs (11), only the 1-30-44 epitope was absent from the Gs protein (Table 1). The mAb #1-30-44 might be very important in terms of its ability to recognize not only the difference between the G_B and G_C forms of the authentic G protein which are taken under neutral pH conditions (11), but also the difference between the authentic G and soluble Gs proteins (Fig. 2). The latter difference might possibly be responsible for the immunogenic defect reported by Dietzschold et al. (2); that is, the Gs protein is not very immunogenic compared with the authentic G protein, and induced much lower titers of virus-neutralizing antibody than authentic G protein in animals. From these considerations, we assume that preservation of the 1-30-44 epitope-positive conformation might be essential for the rabies virus antigens to show strong immunogenicity in animals, and that mAb #1-30-44 might be a good tool for checking the quality of rabies vaccine and storage conditions.

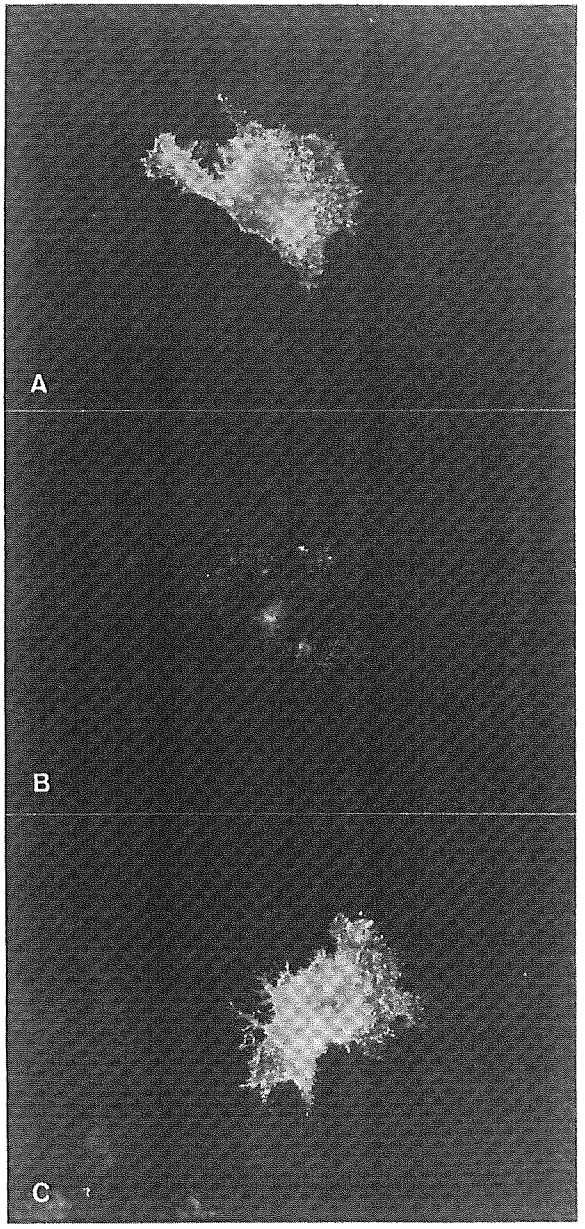

Fig. 7. Immunofluorescence study of the CT1 mutant. The CT1 cDNA was transfected to BHK-21 cells with RVV-T7. After 16 hr incubation, the cells were formalin-fixed, and FA-stained with either of anti-G mAb #7-1-9 (A), #1-30-44 (B) and #1-46-12 (C).

As to the 1-30-44 epitope, we previously reported that at least two amino acids located in two separate regions (i.e., Lys-202 and Asn-336) are involved in the epitope formation (10, 11). The Lys-202 is included in the neurotoxin-like region (17), while Asn-336 is located close to Arg-333, substitution of which affects the neurotropic nature of rabies virus *in vivo* (24, 26). The 1-30-44 epitope was acid-sensitive, but was also destroyed spontaneously by conformational changes of G protein occurring under neutral pH conditions (11). Based on their reactivity with mAb #1-30-44, we reported recently that about two thirds of the HEP-Flury virus G proteins took the G_C conformation on the cell surface (11). We further think that the 1-30-44 epitope-negative G_C form is not the same as the A-form of G protein (denoting the acid-induced fusion-active form; 5, 7), because the G_C form is detected even under neutral pH conditions, and is not fusion-active (11). Trimer formation of G protein is not required for forming the 1-30-44 epitope, because treatment of G proteins/virion spikes with Triton X-100 is known to solublize them into a monomer form, while we previously observed that Triton-solubilized G proteins preserved the reactivity to mAb #1-30-44 (11). Accordingly, 1-30-44 epitope formation of the G and G-related proteins was independent of trimer formation, but simply dependent on the conformation of each moleule.

Although the soluble form of rabies virus G protein (Gs) has been intensively studied for more than 20 years, there is no convincing explanation of the mechanism of Gs protein shedding. In our previous study (13), we observed that spikeless virions were produced when the pH of rabies virus-infected culture medium was reduced to less than 6.9–7.0, and that spikeless virions contained a counterpart fragment of Gs protein that might have been derived from the root (TM and cytoplasmic domains) of the protein. The ratio of G_B/G_C was decreased in relation to decreasing pH of the culture medium (our unpublished data). Based on these considerations, we speculate a possible mechanism for Gs protein shedding as follows: certain proteolytic enzyme(s) would be involved in Gs protein production, and the G_B form is resistant to such protease involved in Gs protein shedding, while the G_C form is sensitive to the protease. The Gs protein is produced by a proteolytic cleavage from the full-sized G protein, probably in the G_C form, which might be from the G proteins exposed on the cell surface or near the final step of its maturation in the trans-Golgi network. These possibilities are now under investigation.

Based on our present data for the G protein mutant (CT1), we think that the CT1 protein was quite efficiently transported to and expressed on the cell surface of the transfected cells, but only a small portion of CT1 proteins displayed the 1-30-44 epitope-positive G_B-conformation and most of the CT1 proteins were the 1-30-44 epitope-negative non-G_B form (Figs. 7B and 8), probably taking the G_C-like (or possibly G_D) conformation (G_D form is like the G_C form which lacks the 1-30-44 epitope, but is different from the G_C form in its inability to recover the G_B form after being solubilized from the cell membrane; 11). From these studies, we assume that the cytoplasmic domain of rabies virus G protein might not be essential for antigenic and func-

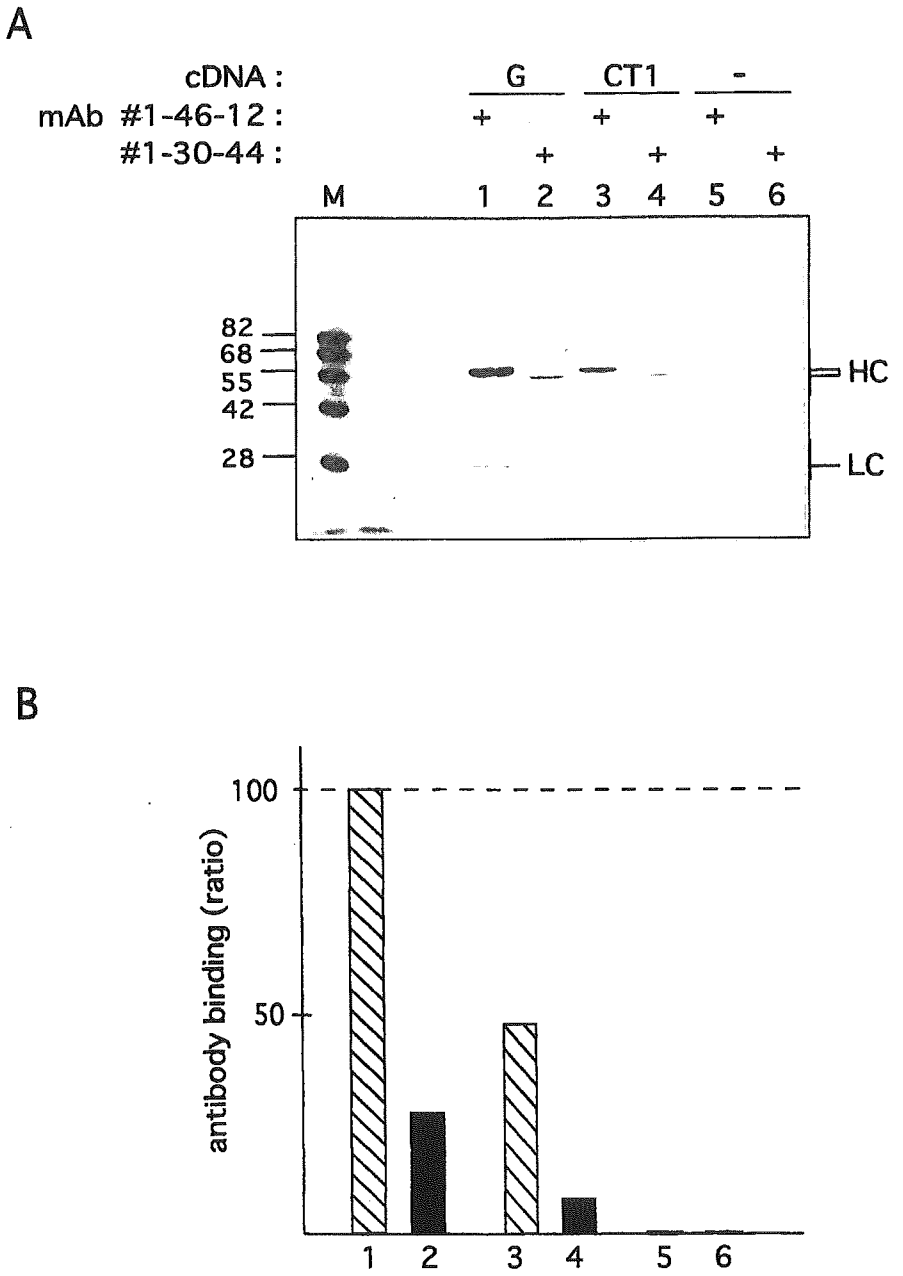

Fig. 8. Antibody binding assays of the G and CT1-producing cells. (A) G cDNA-transfection was performed to produce the wild type G or the CT1 mutant. After incubation for 16 hr, the cells were formalin-fixed (30 min at room temperature), and then incubated with 10 μg of anti-G mAb (#1-46-12 or #1-30-44) for 2 hr at room temperature. After washing the cells three times with PBS(−), the cell-bound mAbs were recovered by dissolving in SDS-PAGE sample buffer and applied to 10% SDS-PAGE, and then processed for the chemiluminescent immunoblotting with peroxidase-conjugated anti-mouse Ig. Lanes 1 and 2: wild type G-producing cells; lanes 3 and 4: CT1 mutant-producing cells; lanes 5 and 6: control cells (empty vector-transfected). Lanes 1, 3 and 5: mAb #1-46-12; lanes 2, 4 and 6: mAb #1-30-44. (B) After developing the immunoglobulin band on X-ray film, the density of the heavy chain (HC) band was measured using a software NIH-Image and depicted as a bar for each band (density of the heavy chain band on lane 1 was set as 100%).

tional maturation of ectodomain, but might be required at least for keeping the G_B conformation on the cell surface and probably on the virion also. In other words, truncation of the cytoplasmic domain may affect the stability of G_B conformation, and most non-G_B form of CT1 protein might further be fixed in the G_C-like or G_D form by intramolecular sulfhydryl bonding.

As noted above, the CT1-producing cells expressed large amounts of CT1 proteins on the cell surface, but most of them were shown to be in the non-G_B form,