Identification of epitopes associated with different biological activities on the glycoprotein of vesicular stomatitis virus by use of monoclonal antibodies

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Summary. Thirteen monoclonal antibodies (MAbs) to the glycoprotein (G) of vesicular stomatitis virus (VSV) serotype Indiana were prepared and examined for their effects on various biological activities of VSV, including in vitro infection, hemagglutination, adsorption to cells, and mediation of cell fusion. Competitive binding assays with these MAbs revealed the presence of at least seven distinct antigenic determinants (epitopes) on the G protein. In some cases, overlappings among epitopes to various degrees were observed as partial inhibition or binding enhancement. The MAbs to all the epitopes but one (epitopes 1–6) reacted with the denatured G protein in a Western immunoblot analysis. Four of the epitopes (epitopes 2, 4, 5, and 7) were involved in neutralization and two (epitopes 1 and 2) in hemagglutination inhibition. None of the MAbs inhibited the adsorption of radiolabeled VSV to BHK-21 cells; the MAbs to epitope 2 slightly enhanced the virus adsorption. All neutralization epitopes except epitope 2 (epitopes 4, 5, and 7) were associated with inhibition of VSV-mediated cell fusion. These results show a direct spatial relationship between the epitopes recognized by the MAbs and functional sites on G protein and further insights into the structure and function of G protein.

Introduction

Many enveloped viruses including vesicular stomatitis virus (VSV), family *Rhabdoviridae*, genus *Vesiculovirus*, transfer their nucleocapsids to the cytoplasm of host cells by the adsorption and receptor-mediated endocytosis, followed by fusion with the endosomal membrane [20, 21]. The glycoprotein (G) of VSV

is the sole protein anchored in the viral envelope and plays a critical role in this early stage of virus infection. Many biological properties of G protein are associated with the virus entry [37], which include adsorption to host cells [3, 6], hemagglutination (HA) [9, 23], and mediation of in vitro cell-cell fusion [7, 38]. The cell-cell fusion occurs only at low pH, which mimics the acidic environment of the endosomal lumen [7, 38]. As expected from its central role in infection, the G protein also gives rise to and reacts with neutralizing antibodies [12]. In recent years, much effort has been made to reveal the structure-function relationships of the G protein, especially regarding its role in fusion [2, 10, 27, 29, 39, 40], but the underlying molecular mechanisms are still poorly understood.

One approach to the structure-function relationship of surface glycoproteins of viruses is to analyze for the sites and effects of the monoclonal antibody (MAb) binding [4, 13, 31, 33]. Production of MAbs against G proteins of two major serotypes of VSV (Indiana and New Jersey) has been reported by two research groups [5, 14, 15, 36]. These MAbs have mainly been used to map neutralization and non-neutralization epitopes on G protein and to analyze the mutation leading to antigenic variations of G protein [8, 11, 16–18, 35]. The effects of the MAbs specifically reacting with G protein on biological functions other than neutralization have not been reported.

In the present study, we prepared thirteen MAbs specific for seven distinct epitopes on G protein of VSV-Indiana and examined for their effects on various biological activities of VSV including in vitro infection, HA, adsorption to the cells, and mediation of cell-cell fusion. Our findings defined the spatial relationship between the epitopes recognized by the MAbs and the functions of G protein.

Materials and methods

Virus

The San Juan strain of VSV-Indiana originally provided by Dr. R. R. Wagner, University of Virginia, was obtained from Dr. K. Yamamoto, National Institute of Health, Tokyo. The virus stock was prepared by infecting BHK-21 cells (Japanese Cancer Research Resources Bank) at a multiplicity of 0.1 PFU/cell. Virus harvested at 22 h postinfection was concentrated by ultrafiltration and ultracentrifugation [22, 25], and purified by sucrose density gradient centrifugation [22]. This preparation containing 1.1 mg/ml of viral protein $(2.3 \times 10^{11}\,\text{PFU/ml})$ was stored at $-80\,°C$. The protein content of the preparation was determined with BCA protein assay reagent (Pierce Chemical Co., Rockford, IL, U.S.A.) with bovine serum albumin (BSA) as a standard. Virus infectivity was determined by plaquing on monolayer cultures of BHK-21 cells [41].

Isolation of the G protein and immunization

The G protein was extracted from the purified virus with 30 mM octyl-β-D-glucopyranoside (Sigma Chemical Co., St. Louis, MO, U.S.A.) as described by Petri and Wagner [30]. After removal of the nucleocapsids by ultracentrifugation at 150,000 × **g**, the supernatant containing G protein was dialyzed against 10 mM HEPES (pH 7.4) containing 0.15 M NaCl.

G protein thus obtained was free from any other virus protein in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) stained with Coomassie brilliant blue.

For immunization, female BALB/c mice were subcutaneously injected twice each with 25 µg of the purified G protein emulsified in the same quantity of Freund's complete and Freund's incomplete adjuvants, respectively. Then, additional two intraperitoneal injections with 40 µg of G protein were given. Three days before fusion for hybridoma production, the final 40 µg of G protein was injected intravenously.

Generation of monoclonal antibody-producing hybridomas

The spleen cells of the immunized mice and SP 2/O-Ag 14, BALB/c mouse non-secretory plasmacytoma cells, were fused with polyethylene glycol according to Oi and Herzenberg [28] or by the novel VSV-mediated cell fusion method described previously [25, 26]. Media preparation and HAT selection of hybridomas were described previously [25]. In 2 weeks, the hybridomas were screened for production of anti-G protein antibody by enzyme-linked immunosorbent assay (ELISA) with purified G protein as the antigen (see below). The positive cultures were cloned several times by the limiting dilution method. The isotypes of the specific antibodies were determined with a mouse monoclonal antibody isotyping kit (Amersham International, Buckinghamshire, England).

Preparation and purification of monoclonal antibodies

Thirteen hybridomas were established and each was over-grown in about 1 l of a serum-free medium (Iscove's modified Dulbecco's medium, Sigma) containing 1 mg/ml of BSA, 1 mM sodium pyruvate (Gibco, Grand Island, NY, U.S.A.), 8 µg/ml of bovine insulin (Sigma), 5 µg/ml of iron-saturated human transferrin (Miles Scientific, Naperville, IL, U.S.A.), 50 µM 2-mercaptoethanol, 20 µM ethanolamine, 2.5 µg/ml of linoleic acid, 2.5 µg/ml of oleic acid, 2.5 µg/ml of palmitic acid, and 10 µg/ml of gentamicin. MAb in the culture supernatant was precipitated with ammonium sulfate at 40% saturation and the precipitate was further purified by high performance liquid chromatography on hydroxyapatite beads [42] or by affinity chromatography on protein G-Sepharose (Pharmacia, Uppsala, Sweden). The eluate was concentrated to 4 ml by membrane ultrafiltration (30,000 mol. wt. cut-off Centriprep; Amicon, Danvers, MA, U.S.A.), and dialyzed against phosphate-buffered saline (PBS). The antibody concentration was determined from absorbance at 280 nm with an extinction coefficient of 1.4 per mg of protein. For use as negative controls in various assays, two MAbs prepared at National Institute of Health were purified by the same procedure. One of them was IgG1 specific for the core antigen of feline immunodeficiency virus (unpubl.) and the other was IgG2a specific for sheep red blood cells (not cross-reactive with goose erythrocytes) [25].

ELISA

Production of anti-G protein antibody in hybridoma culture supernatants was examined by ELISA. Wells of microtiter plates (Costar 3690; Costar, Cambridge, MA, U.S.A.) were coated with the purified G protein (1 µg/ml) in 50 mM sodium carbonate buffer (pH 9.6) for 2 h at room temperature. The wells were washed with PBS containing 0.05% (v/v) Tween 20 (PBS-Tween) and blocked overnight at 4 °C with 0.5% (w/v) gelatin in PBS. After washing, each culture supernatant was added to the wells and the plates were incubated for 1 h at room temperature. The antibody bound was detected by incubation for 1 h at room temperature with alkaline phosphatase-conjugated goat anti-mouse IgG + M (Tago 6553; Tago Inc., Burlingame, CA, U.S.A.) diluted 5,000-fold in PBS-Tween. The enzyme reaction was started by adding 1 mg/ml of p-nitrophenylphosphate (Wako Pure Cemical Ind., Osaka, Japan) in 1% (v/v) diethanolamine (pH 9.8) containing 0.5 mM MgCl$_2$. The

absorbance at 410 nm was measured with an EIA autoreader (Sanko Junyaku Co., Tokyo, Japan). As a positive control, a 2,000-fold dilution of mouse immune serum was used. This control usually showed an absorbance of approximately 1.0 after incubation for 20 min at room temperature. The well with absorbance higher than 0.2 was regarded as positive.

Purified MAbs were titrated by the same ELISA to compare the relative reactivities, in which the wells of the plates were coated with VSV virions (2 μg/ml) instead of G protein. The relative reactivity was defined as the concentration of MAb needed to attain 50% of the absorbance value of the positive control.

Western blotting

Purified VSV was separated by SDS-PAGE in 10% polyacrylamide gel under reduced conditions. The proteins separated were then blotted onto immobilone membrane (Millipore Corp., Bedford, MA, U.S.A.) according to Towbin et al. [34]. The blots were allowed to react with culture supernatants of established hybridomas, and specific bands were visualized with alkaline phosphatase-conjugated goat anti-mouse IgG + M (Tago 6553) and the BCIP/NBT phosphatase substrate system (Kirkegaard & Perry Lab. Inc., Gaithersburg, MD, U.S.A.).

Biotinylation of MAbs and competitive binding assay

A 500-μg portion of each purified MAb was mixed with 100 μg of biotinyl N-hydroxy-succinimide ester (NHS-LC-Biotin, Pierce) in 0.5 ml of 0.1 M sodium bicarbonate (pH 8.4). After incubation for 4 h at room temperature, the mixtures were dialyzed extensively against PBS at 4 °C; BSA was then added to a final concentration of 5 mg/ml.

For competitive binding assay, the wells of plates were coated with purified VSV (2 μg/ml) and blocked with 5% (w/v) unfatted bovine milk in PBS. Serial 10-fold dilutions of unlabeled competitor MAb (2×10^{-1} to 2×10^{-5} mg/ml) were added to the wells (40 μl/well) and the plates were incubated for 3 h at room temperature. Subsequently, 40 μl of biotinylated MAb was mixed with competitor MAb. The concentrations of biotinylated MAbs were 1 μg/ml for 2B9 and 3B1, 0.5 μg/ml for V20 B12, and 0.25 μg/ml for the other MAbs. These concentrations were about half-maximal in their titration curve and were within the range where the binding was linear. After incubation overnight at 4 °C, biotinylated MAb bound was detected with a 5,000-fold dilution of alkaline phosphatase-conjugated streptavidin (Bethesda Research Lab., Gaithersburg, MD, U.S.A.). Dilution was made in PBS-Tween containing 5% (w/v) unfatted bovine milk. The enzyme reaction and absorbance determination were carried out as described above. All assays were performed in duplicate and the results were expressed as the percentage of binding calculated with the formula:

$$\text{Binding}(\%) = \frac{\text{average of absorbance in the presence of competitor}}{\text{average of absorbance in the absence of competitor}} \times 100$$

Neutralization assay

For the neutralization assay, the stock of VSV was diluted to a final concentration of approximately 1,000 PFU/ml with bicarbonate-free Eagle's minimum essential medium (MEM, Nissui Pharmachemical Co., Tokyo, Japan) containing 0.2 mg/ml of BSA and 20 mM HEPES (pH 7.2). The virus was mixed with an equal volume of each of serial twofold dilutions of each purified MAb (from 250 μg/ml) in the same medium. The mixtures were incubated for 1 h at 37 °C and then plated in duplicate on monolayers of BHK-21 cells in 6-well culture plates for plaque assay (100 μl/well). The neutralization antibody titer

was defined as the reciprocal of the highest dilution reducing more than 50% of the plaques of the control without MAb.

Hemagglutination inhibition assay

Hemagglutination inhibition (HI) was assayed with 4 or 8 hemagglutinating units of VSV in V-bottom microtiter plates as described by Halonen et al. [9], except that goose erythrocytes were used after the treatment with trypsin (Sigma) at 100 µg/ml for 30 min at 37 °C. This pretreatment of the erythrocytes enhanced HA, thus increasing the sensitivity of HI [19; unpubl. data]. The reciprocal of the highest dilution of purified MAb causing complete inhibition of hemagglutination was taken as the HI titer.

Virus adsorption assay

For adsorption assays, ^{35}S-labeled VSV was prepared by the addition of 20 µ Ci/ml of L-[^{35}S]methionine (Amersham) to the infection medium as described by Bailey et al. [1]. The radiolabeled virions were concentrated and purified as described for the unlabeled virus. The final preparation was free of contaminating labeled materials as judged by SDS-PAGE and autoradiography. The final preparation contained 3.4×10^{10} PFU/ml (1.8 mg/ml viral protein) with a specific activity of 1.5×10^4 cpm/µg.

The radiolabeled virus absorbed to cells was quantified essentially as described by Matlin et al. [21]. BHK-21 cells grown to confluency in 12-well culture plates (about 10^6 cells/well) were washed twice with the binding medium, bicarbonate-free MEM buffered with 20 mM HEPES (pH 7.2) containing 2 mg/ml of BSA, and cooled for 5 min on ice. The radiolabeled purified virus (33,500 cpm) was mixed with each purified MAb at various concentrations in the binding medium. The mixtures were incubated for 1 h at 37 °C, chilled, and then plated in duplicate on the BHK-21 cell (100 µl/well). After incubation for 1 h on ice, unbound virus was removed. The cells were washed four times with the binding medium. The cells bound with the virus were solubilized in 0.4 ml of Solubable (NEN Research Product, Boston, MA, U.S.A.), and its radioactivity was measured with a liquid scintillation counter. The average radioactivities bound to the cells in the presence of MAb were expressed as the percentage of the radioactivity bound in the absence of MAb. Nonspecific interaction of the virus with the cells and the surface of the plates was minimized by adding BSA to the binding medium. The addition of BSA reduced the nonspecific binding of labeled VSV to the surface of the plates from 9.3% to less than 1.1% of the input radioactivity. The amount of virus used was within the range where the radioactivity bound to cells increased proportionally with the amount of the input virus.

Inhibition of VSV-mediated cell fusion

The effect of MAbs on VSV-mediated cell fusion was assayed by inhibition of polykaryon formation of BHK-21 cells [24, 38]. The cells in 24-well culture plates (about 1.5×10^5 cells/well) for 18–24 h were washed twice with the ice-cold binding medium (bicarbonate-free MEM buffered with 20 mM HEPES, pH 7.2, containing 2 mg/ml of BSA). The purified virus (25 µg) in 200 µl of the cold binding medium was applied onto the cells. After incubation for 1 h on ice to allow viral adsorption, free virus was removed, and the cells were treated on ice for 45 min with 25 µg/ml of each MAb in 200 µl of the binding medium. After removing the MAb solutions, 0.5 ml of prewarmed (37 °C) acidic medium, bicarbonate-free MEM buffered with 10 mM MES (pH 5.5), was added for triggering fusion and the plates were incubated for 2 min at 37 °C. The medium was replaced with 0.5 ml of the prewarmed binding medium and the cells were incubated for an additional hour at 37 °C. After fixation with 20% formalin in PBS and staining with hematoxylin, inhibition of polykaryon for-

mation was examined under a phase-contrast microscopy. The amount of the virus used in this assay was enough to induce fusion in about 80% of the cells.

Results

Production and characterization of anti-G protein MAbs

Two fusion experiments yielded 13 stable hybridomas secreting MAbs specifically reacting with G protein of VSV. Their characteristics are listed in Table 1. Their relative reactivities varied over a 40-fold range, but were still within a relatively high range compared with those of other MAbs to different antigens determined by us. All the MAbs except for P2F3 reacted with G protein in Western blotting analysis.

Epitope assignments of MAbs by competitive binding assay

Competitive binding ELISA was carried out among these MAbs to classify the epitopes of G protein recognized by them. Typical results of the competitive binding assay are shown in Fig. 1, in which the binding of biotinylated 1A7 MAb to G protein was challenged by several unlabeled MAbs. We observed four types of competition. In addition to homologous MAb (1A7), P2F9 completely inhibited the binding. 5C6 partially inhibited the binding. 3F4, P2E11 or P2F3

**Table 1.** Characteristics of anti-G protein monoclonal antibodies

Designation	Isotype[a]	Relative reactivity[b] in ELISA (ng/ml)	Western[c] blotting	Epitope[d]
1A7	IgG1	128	+	1
P2F9	IgG1	14	+	1
V11A2	IgG1	32	+	2
V17E8	IgG1	64	+	2
2B9	IgG2b	391	+	3a
3B1	IgG2b	391	+	3a
V20B12	IgG1	592	+	3b
3F4	IgG1	98	+	4
5E11	IgG1	43	+	4
5C6	IgG1	28	+	5
P2E11	IgG1	195	+	6
V12B3	IgG1	43	+	6
P2F3	IgG2a	49	−	7

[a] All MAbs had κ light chain
[b] Relative reactivity was defined in ELISA as the MAb concentration needed to attain an absorbance of 0.6 when a positive control (immunized mouse serum diluted 2,000-fold) had an absorbance value of 1.2
[c] Reactivity to G protein in Western blotting analysis
[d] Epitopes (antigenic determinants) were identified by competitive binding assay among MAbs as described in Table 2
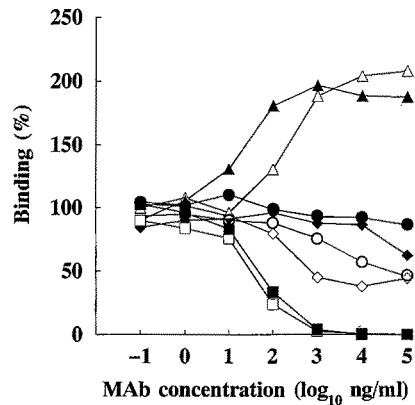
Fig. 1. Typical results of the competitive binding assay with biotinylated 1A7 MAb. Each competitor antibody at various concentrations was added to VSV-coated wells of ELISA microtiter plates followed by the addition of biotinylated 1A7 MAb. The binding percentages were estimated from the absorbance at 410 nm in the presence of competitor compared with that in the absence of competitor. Competitors: ■ 1A7 (homologous); □ P2F9; ▲ V11 A2; △ 2B9; ◆ 3F4; ◇ 5C6; ● P2E11; ○ P2F3

did not show any marked effect on the binding. On the other hand, V11 A2 and 2B9 obviously enhanced the binding. Similar four types of competition were observed with other combinations of biotinylated and unlabeled MAbs (Table 2). The 13 MAbs were assigned to seven distinct epitopes on G protein based on the complete inhibition. When an unlabeled MAb inhibited the binding of a biotinylated MAb at 100 μg/ml (at least 100-fold excess of biotinylated MAbs) to less than 10% and when this inhibition was observed in pair-wise assays, both MAbs were considered to share the same (or a closely adjacent) epitope. These seven epitopes were designated as Ep 1 to Ep 7. The MAbs to the same epitope showed similar patterns of partial inhibition or enhancement of binding against MAbs to different epitopes (Table 2). Only MAbs to Ep 3 were subgrouped into two based on the pattern; MAbs to Ep 3a had no effect on the binding of MAbs to Ep 2, whereas MAb to Ep 3b enhanced the binding of MAbs to Ep 2.

Effects of MAbs on the infectivity and hemagglutinating activity of VSV

The 13 MAbs were assayed for the neutralizing activity by the plaque reduction test (Table 3). The MAbs assigned to four (Ep 2, Ep 4, Ep 5 and Ep 7) of the seven epitopes had neutralizing activities. The neutralization titers of the MAbs to Ep 5 and Ep 7 were about 10-times higher than the others.

Inhibition of hemagglutination by the MAbs was also examined (Table 3). The MAbs assigned to two epitopes (Ep 1 and Ep 2) had higher HI activity than the others. Goose erythrocytes used in this experiment were pretreated with trypsin to enhance the sensitivity of HI. Untreated erythrocytes gave similar results, although higher MAb concentrations were required (data not shown).

Table 2. Topographical analysis of epitopes on G protein of VSV-Indiana by competitive binding assay[a]

Unlabeled competitor MAb	Biotinylated MAb													Epitope[b]
	1A7	P2F9	V11A2	V17E8	2B9	3B1	V20B12	3F4	5E11	5C6	P2E11	V12B3	P2F3	
1A7	++	++	e	e	e	e	e	–	–	–	–	–	–	1
P2F9	++	++	e	e	e	e	e	–	–	–	–	–	–	1
V11 A2	e	e	++	++	e	e	e	++	+	–	–	–	–	2
V17 E8	e	e	++	++	e	e	e	+	+	–	–	–	–	2
2B9	e	e	–	–	++	++	++	–	–	–	–	–	–	3 a
3B1	e	e	–	–	++	++	++	–	–	–	–	–	–	3 a
V20 B12	e	e	e	e	++	++	++	–	–	–	–	–	–	3 b
3F4	–	–	–	–	–	–	–	++	++	–	–	–	++	4
5E11	–	–	–	–	–	–	–	++	++	–	–	–	++	4
5C6	+	+	–	–	+	+	+	–	–	++	–	–	++	5
P2 E11	–	–	–	–	–	–	–	–	–	–	++	++	++	6
V12 B3	–	–	–	–	–	–	–	–	–	–	++	+	++	6
P2F3	–	–	–	–	+	+	+	–	–	+	–	+	++	7

[a] + + Inhibition of binding to ≤ 10% at a competitor concentration of 100 µg/ml; + partial inhibition of binding to ≤ 50% at a competitor concentration of ≤ 10 µg/ml, but ≥ 10% at 100 µg/ml; – no competition; e enhancement of binding to ≥ 140% at a competitor concentration of ≤ 100 µg/ml

[b] MAbs were classified in the same epitope when the unlabeled MAb decreased the binding of the biotin-labeled MAb to less than 10% of control (+ +) in pair-wise competitive binding assay. MAbs to epitope 3 were divided into two subgroups based on the difference in the competition to MAbs to epitope 2

Table 3. Neutralization and hemagglutination inhibition activities of anti-G protein MAbs

Epitope	MAb	Neutralization[a]	HI[b]	
			4 HAU	8 HAU
1	1A7	< 1	128	32
	P2F9	< 1	256	64
2	V11A2	128	128	16
	V17E8	64	32	8
3 a	2B9	< 1	8	< 1
	3B1	< 1	8	< 1
3 b	V20B12	< 1	8	2
4	3F4	64	2	1
	5E11	128	1	< 1
5	5C6	2048	4	4
6	P2E11	< 1	4	< 1
	V12B3	< 1	8	2
7	P2F3	1024	16	4

[a] Titers for neutralization represent the reciprocal of the highest twofold dilution of purified MAb (125 µg/ml initial) causing more than 50% reduction in the plaque number
[b] Titers for HI represent the reciprocal of the highest twofold dilution of purified MAb (250 µg/ml initial) inhibiting HA caused by 4 or 8 HAU of VSV. Control IgG1 and IgG2a MAbs and the serum-free medium for growing hybridomas showed no HI activity (< 1)

Effects of MAbs on virus adsorption to cells

We examined MAbs for the influence on the adsorption of radiolabeled VSV to BHK-21 cells. MAbs to Ep 1 and Ep 3 had very little, if any, effects on the virus adsorption (Fig. 2 a and c) similar to the control MAbs (Fig. 2 h). MAbs to Ep 4, Ep 5, Ep 6 and Ep 7 slightly reduced the virus binding (Fig. 2 d, e, f and g). Even at the highest concentration (125 µg/ml), they exerted partial inhibition (65–75% of the binding of control). On the other hand, MAbs to Ep 2 slightly enhanced the VSV adsorption only at certain concentrations (Fig. 2 b). In another experiment with a different preparation of radiolabeled VSV with a higher specific activity, similar results were obtained: no MAb completely inhibited the virus binding and MAbs to Ep 2 enhanced the virus binding (data not shown).

Effects of MAbs on VSV-mediated cell-cell fusion

We examined MAbs for the effects on VSV-mediated polykaryon formation of BHK-21 cells to test whether the MAbs inhibit the fusion induced by VSV. Extensive cell fusion was induced by acid treatment of the virus-bound cells (Fig. 3 o) but not by the same treatment of unbound cells (Fig. 3 p). MAbs reacting with Ep 4 (Fig. 3 g and h), Ep 5 (Fig. 3 i), and Ep 7 (Fig. 3 l) completely inhibited polykaryocyte formation. The other MAbs (Fig. 3 a–f, j, and k), as
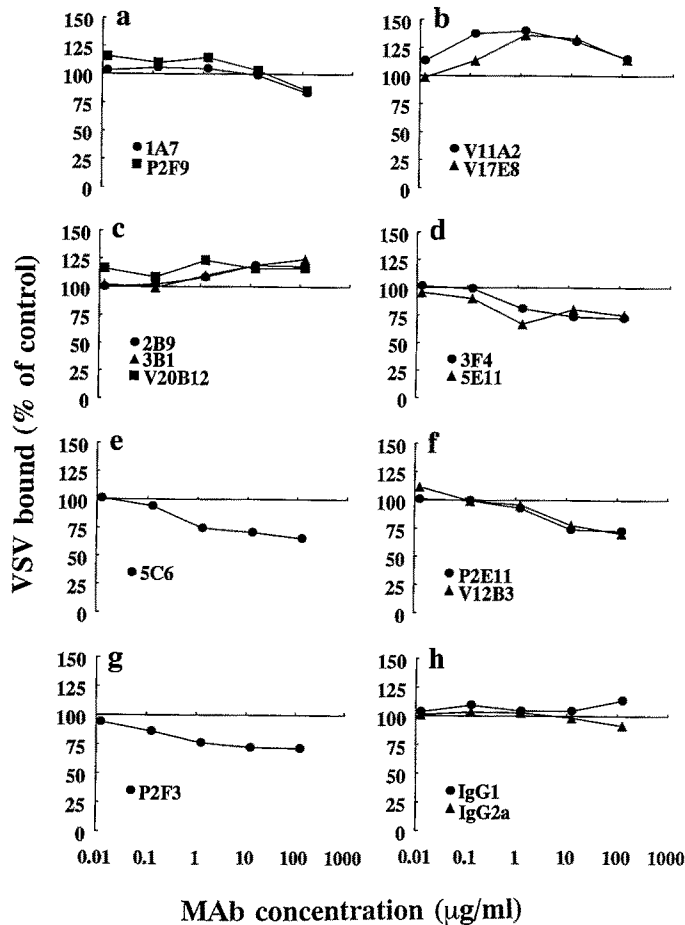
Fig. 2. Effects of anti-G protein MAbs on adsorption of VSV to BHK-21 cells. Purified radiolabeled virions (33,500 cpm of VSV corresponding to 2.2 μg) were incubated with various concentrations of the purified MAbs for 1 h at 37 °C, chilled, and then plated on BHK-21 cells in 12-well plates. After exposure of the cells to the virus-MAb mixture for 1 h on ice, the cells were washed, and the cell-bound radioactivity was measured. The average counts of the duplicate wells are expressed in percentage of those in the absence of MAb. Approximately 22% of the input radioactivity remained cell-bound in the MAb-free control wells. Results with **a** Ep 1, **b** Ep 2, **c** Ep 3, **d** Ep 4, **e** Ep 5, **f** Ep 6, and **g** Ep 7 are shown. **h** Results with monoclonal mouse Ig G1 and Ig G2a used as controls

well as control Ig G1 and Ig G2 a (Fig. 3 m and n), did not inhibit the polykaryocyte formation at all.

Discussion

In this paper, we prepared thirteen MAbs specifically recognizing seven distinct epitopes on G protein of VSV-Indiana and identified the epitopes involved in neutralization, HA, viral adsorption, and mediation of cell fusion by measuring the effects of each MAb on these biological functions. The results are sum-
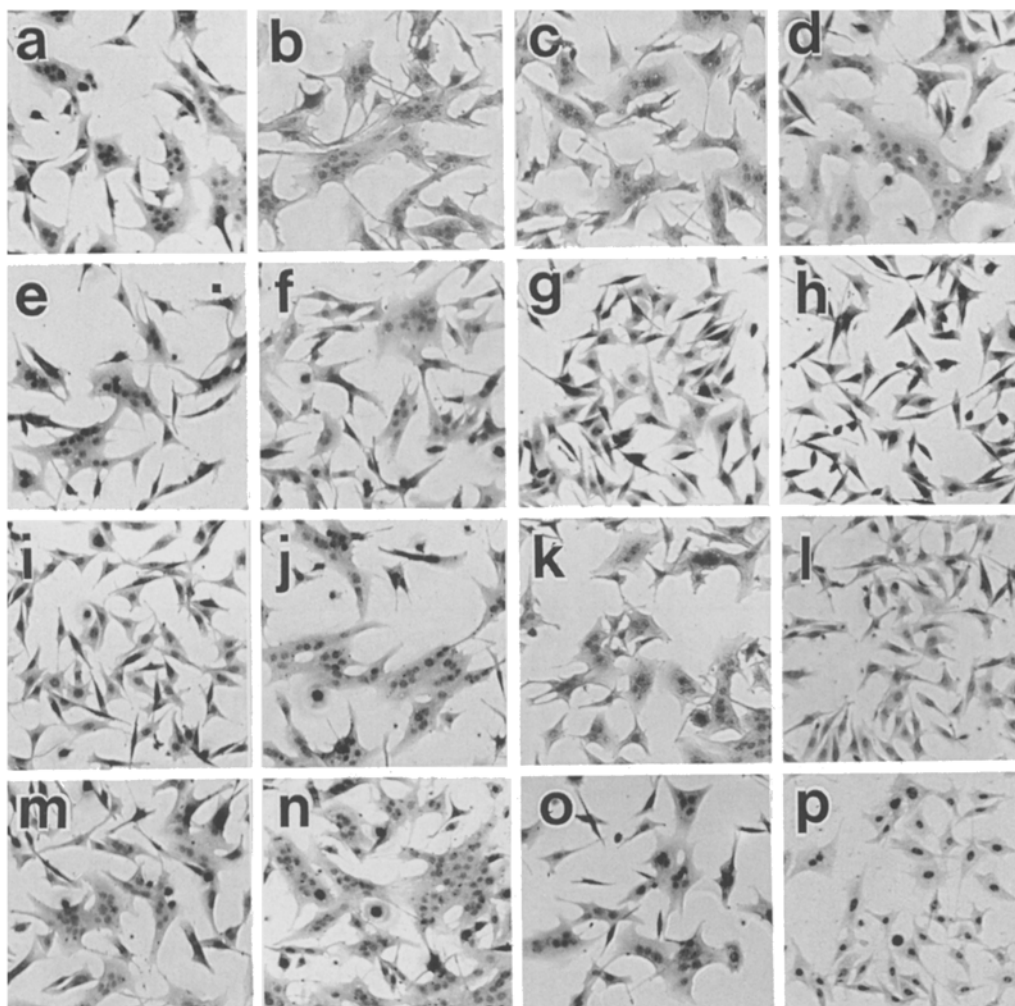
Fig. 3. Inhibition of VSV-mediated BHK-21 cell fusion with anti-G protein MAbs. Sub-confluent monolayers of BHK-21 cells were absorbed with purified VSV at 4 °C, and the inoculum was replaced by medium containing each MAb. After incubation on ice for 45 min, the fusion was triggered by a brief exposure of prewarmed pH 5.5 medium and postincubated in pH 7.2 medium at 37 °C for 90 min before fixation and staining. Photographs were then taken with a camera connected to a phase-contrast microscope (original magnification: × 100). **a** 1A7 (Ep 1); **b** P2F9 (Ep 1); **c** V11A2 (Ep 2); **d** V17E8 (Ep 2); **e** 2B9 (Ep 3 a); **f** V20B12 (Ep 3 b); **g** 3F4 (Ep 4); **h** 5E11 (Ep 4); **i** 5C6 (Ep 5); **j** P2E11 (Ep 6); **k** V12B3 (Ep 6); **l** P2F3 (Ep 7); **m** Ig G1 (control); **n** Ig G2a (control); **o** MAb free; **p** mock. Inhibition of fusion was observed in **g, h, i,** and **l**

marized in Table 4. Of the seven epitopes defined by MAbs, only Ep 3 and Ep 6 were not related to any function. Ep 1 was related to HA, but not to any other biological function. The other epitopes (Ep 2, Ep 4, Ep 5, and Ep 7) were involved in neutralization. Ep 2 was related to HA and viral adsorption efficiency, whereas the other neutralization epitopes (Ep 4, Ep 5, and Ep 7) were related

Table 4. Association of each epitope on G protein with different biological activities

Epitope	MAb	Neutrali-zation[a]	Hemagglu-tination inhibition[b]	Inhibition of virus adsorption[c]	Inhibition of cell fusion[d]
1	1A7	−	+	−	−
	P2F9	−	+	−	−
2	V11A2	+	+	e	−
	V17E8	+	+	e	−
3a	2B9	−	−	−	−
	3B1	−	−	−	NT[e]
3b	V20B12	−	−	−	−
4	3F4	+	−	−	+
	5E11	+	−	−	+
5	5C6	+ +	−	−	+
6	P2E11	−	−	−	−
	V12B3	−	−	−	−
7	P2F3	+ +	−	−	+

[a] + +, +, and − Neutralization titers of ⩾ 1,000, ⩾ 50 and > 1, respectively for 50% reduction of plaques, shown in the third column of Table 3

[b] + and − HI titers against 4 HAU of ⩾ 32 and ⩽ 16, respectively, given in the 4 th column of Table 3

[c] − No inhibition or slight inhibition of adsorption (65–125%); e enhancement of adsorption (⩾ 135%) in the virus binding assay shown in Fig. 2

[d] + Inhibition of fusion; − no inhibition

[e] Not tested

to the cell fusion activity. We reported here for the first time identification of the G protein epitopes associated with HA and fusion activities.

In the competitive binding assay, the mutual complete competition of paired MAbs revealed the presence of at least seven distinct epitopes on G protein of VSV-Indiana. In addition, some topographical relationships among some of these epitopes were suggested by partial inhibition or enhancement of the binding of MAbs (Table 2). In particular, association among Ep 1, Ep 2, and Ep 3 would be quite possible since the mutual binding enhancement of the respective MAbs was observed. Such enhancement is probably due to an advantageous allosteric alteration of G protein after binding with the first MAb, thereby resulting in increased binding of the second MAb. Similar competitive binding assays with anti-G protein MAbs were reported by Volk et al. [36] and Le-Francois and Lyles [14, 15], who demonstrated 11 and 10 epitopes on G protein of VSV-Indiana, respectively. The enhancement of binding was found also by LeFrancois and Lyles [14, 15].

Of the seven epitopes identified on G protein, all but one (Ep 1–6) reacted with respective MAbs even in Western blot analysis. These are presumably linear epitopes not dependent on the secondary structure. The MAbs assigned

to four epitopes (Ep 2, Ep 4, Ep 5, and Ep 7) had VSV-neutralizing activity, although of varying efficiency. Previous reports also demonstrated the same number of neutralizing epitopes on G protein of VSV-Indiana [14, 36].

MAbs to Ep 1 and Ep 2 showed HI activity. The proximity between these HI epitopes were suggested by the competitive binding assay and it is likely that these epitopes concurrently form the functional domain for the HA activity. These HI epitopes were not always neutralization epitopes and were different from the fusion-inhibition epitopes. This indicates that the sites involved in HA activity are different from those involved on the other functions. In general, viral HA is equivalent to the viral attachment to cells. However, the HI MAbs did not inhibit the VSV binding to BHK-21 cells. The little correlation between these two activities is likely ascribed to the difference of the target cells and/or conditions in these assays. The result of another experiment showed that the HI MAbs markedly inhibit the binding of radiolabeled VSV to goose erythrocytes, in which the binding is measured under the same condition as the HI assay (data not shown).

Attempts to identify the cell-binding domain of G protein were unsuccessful in this study. In the binding assay, no MAb completely inhibited the VSV adsorption (Fig. 2). MAbs to Ep 4, Ep 5, Ep 6, and Ep 7 at high concentrations slightly inhibited VSV adsorption, but such low inhibitory effects were not related to the efficient neutralization or HI. The lack of the complete inhibition suggests that all neutralizing MAbs prepared in this study block the virus infection at a step subsequent to adsorption. On the other hand, a certain concentration of MAbs to Ep 2, one of the neutralization epitopes, rather enhanced VSV adsorption. Although it is difficult to explain the biological significance of this enhancing effect, a similar enhancement of the VSV adsorption by immune serum was reported by Schlegel and Wade [32]. They suggested that the VSV-antibody complex binds to a different or an additional cell binding site, thus altering the adsorption efficiency. The same explanation may be given to the enhancing effect of MAbs to Ep 2.

In the fusion inhibition assay, MAbs reacting with three epitopes (Ep 4, Ep 5, and Ep 7) inhibited the VSV-mediated cell fusion. These epitopes are probably located on or close to the fusogenic domain of G protein. Although hydrophobic domains involved in fusion have been identified in several viral fusion proteins [10, 27], such a domain has not been identified in G protein of VSV [27]. A most recent finding that introduction of a glycosylation site into residue 117 of G protein resulted in fusion-defective mutant suggests that the residues 118 to 136 are involved in the fusion activity [39]. Some of our fusion-inhibiting MAbs may recognize these residues and the location of the fusion-inhibition epitopes will be required. We found the presence of multiple epitopes related to the fusion activity. This suggests that these different regions of G protein contribute to the fusion activity in partnership and might explain the lack of highly hydrophobic fusion sequence in the G protein of VSV. All fusion-inhibiting MAbs had the neutralizing activity. In the VSV infection

process, after endocytosis of the bound virion, the fusion of viral envelope with the endosomal membrane is necessary for the entry of the nucleocapsids into the cytoplasm [20, 21]. Fusion-inhibiting MAbs neutralize VSV probably by blocking the fusion stage of the infection process.

To analyze further the structure-function relationship of G protein, our studies are currently aimed at locating these epitopes on the G protein by testing the reactivity of the MAbs to G protein fragments expressed in *Escherichia coli*.

Acknowledgements

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