Binding of Cholesterol and Inhibitory Peptide Derivatives with the Fusogenic Hydrophobic Sequence of F-Glycoprotein of HVJ (Sendai Virus): Possible Implication in the Fusion Reaction†

Kimiko Asano
College of Medical Technology, Kyoto University, Kyoto 606, Japan
Akira Asano*
Department of Biochemistry, Cancer Research Institute, Sapporo Medical College, Sapporo 060, Japan
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ABSTRACT: Specificity of the binding of sterols and related compounds with purified F-protein (fusion protein) of the HVJ (Sendai virus) was studied by binding competition with [^{3}H]cholesterol. Requirement for cholesterol or the A/B ring trans structure and nonrequirement for the 3-hydroxyl group were found in this binding. Binding of ^{125}I-labeled Z-Phe-Tyr, an inhibitory peptide of viral membrane–cell membrane fusion, was studied by using purified proteins and virions. F-Protein and virions showed a specific binding with the peptide, whereas the result was negative with hemagglutinin and neuraminidase protein. Thermolysin-truncated F-protein (an F-protein derivative deprived of a 2.5-kDa fragment from the N-terminal of the F_1 subunit and without fusogenic activity) exhibited a considerably diminished binding ability both to cholesterol and to inhibitory peptides. Therefore, the N-terminal hydrophobic sequence that was previously assigned as fusogenic seems to be the binding site of these molecules. In support of this, the binding of cholesterol with F-protein was inhibited by Z-Phe-Tyr and other fusion inhibitory peptides, whereas it was not affected with non-fusion-inhibitory Z-Gly-Phe. These results are discussed in relation to the notion that the binding of the N-terminal portion of the F_1 subunit of F-protein with cholesterol in the target cell membranes facilitates the fusion reaction.

Paramyxoviruses, such as HVJ[1] (Sendai virus), are known to induce the cell fusion reaction (Okada, 1969). The fusion reaction also occurs very efficiently between a viral membrane of the paramyxoviruses and a target cell membrane under physiological conditions (Apostlov & Almeida, 1972; Shimizu et al., 1976; Maeda et al., 1975). A viral glycoprotein named F is thought to show a direct function to induce this fusion reaction (Homma & Ohuchi, 1973; Scheid & Choppin, 1977).

The hydrophobic nature of the N-terminal portion of the F_1 subunit of F-protein (fusion protein) is known to be a common feature with all fusogenic paramyxoviruses so far studied (Gething et al., 1978; Richardson et al., 1980, 1986; White et al., 1983; Paterson et al., 1984; Varsanyi et al., 1985, 1987; Sever et al., 1985; Spriggs et al., 1986). Furthermore, this segment of the F-protein of HVJ seems to be exposed on the protein surface despite the hydrophobic nature of this portion (Asano & Asano, 1982; Asano et al., 1983). These properties were taken as evidence for direct interaction of the segments with the target cell membranes and for participation in the fusion reaction. Therefore, these were sometimes called fusogenic segments.

It was pointed out previously, however, that the homology of such putative fusogenic segments among F-proteins of the paramyxoviruses was very high compared with other hydrophobic stretches of the same F-proteins, such as signal peptides and transmembrane sequences (Asano & Asano, 1984, 1985). Therefore, this hydrophobic sequence may be required not only for the simple hydrophobic interaction with the lipid bilayers but also for the specific interaction with (an)other membrane component(s) (Asano & Asano, 1985).

Furthermore, HVJ-induced lysis of the liposomes under the influence of intact F-protein was reported to be enhanced by the presence of cholesterol in the liposomes (Kundrot et al., 1983; Hsu et al., 1983), although the effects of lipid composition of the liposomes on fusion efficiency are much more complicated, and the cholesterol effect is not always observed (Klappe et al., 1986). Thus, binding of cholesterol with the fusogenic sequence of F-protein was suspected (Asano & Asano, 1984, 1985).

At the same time, it was hypothesized that the inhibition of the virus–cell membrane fusion with the analogues of the N-terminal portion of the F_1 subunit might be due to the binding of these analogues with this fusogenic sequence. Although the binding of cholesterol with purified F-protein and the action of inhibitory peptides on virions were substantiated previously (Asano & Asano, 1984, 1985), evidence for the binding of these compounds with the fusogenic sequence was missing. In this paper, we will present some evidence that cholesterol and inhibitory peptides bind to the segment of F-protein that is required for the fusogenic activity.

Experimental Procedures

Materials. Purification of the HVJ, Z strain, grown in embryonated hen eggs was performed as described previously (Maeda et al., 1975). Purification of the F-protein was per-

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[1] Abbrevations: Z, carbobenzoxy; MIT, monoiodotyrosine; DIT, diiodotyrosine; SDS, sodium dodecyl sulfate; DMSO, dimethyl sulfoxide; HVJ, hemagglutinating virus of Japan (Sendai virus); Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; TBS, Tricine-buffered saline consisting of 40 mM Tricine–KOH buffer (pH 7.6), 0.14 M NaCl, and 5.4 mM KCl; thermo-F, thermolysin-truncated F-protein; DPO, 2,5-diphenyloxazole; Tris-HCl, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; HN, hemagglutinin and neuraminidase; PMSF, phenylmethanesulfonyl fluoride.

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formed at 4 °C as follows: about 100–150 mg of protein of the HVJ virions was solubilized with 100 mL of TBS (40 mM Tricine–KOH buffer, pH 7.6, containing 140 mM NaCl and 5.4 mM KCl) supplemented with 2% Triton X-100 and 1 M NaCl, and centrifuged at 100000*g* for 1 h. The resultant supernatant was dialyzed overnight against 1 L of the medium consisting of 10 mM Tris-HCl buffer (pH 7.6), 0.15 M NaCl, and 0.1% Triton X-100. After centrifugation of the dialyzed sample at 10000*g* for 15 min, the supernatant was applied on a DEAE-Sepharose CL-6B column (1 × 30 cm) that was equilibrated with the same medium used for dialysis. The applied column was washed with the equilibrium medium and then eluted with a linear gradient of 0.15–0.5 M NaCl in 10 mM Tris-HCl buffer (pH 7.6) or with 0.5 M NaCl in the same buffer. HN-protein was purified as described previously (Ozawa et al., 1979a). Antibodies against them were raised in rabbits by a conventional method and were partially purified as described previously (Mimura & Asano, 1982).

Iodination of Z-Phe-Tyr was performed either by the peroxidase method or by the chloramine T method. The peroxidase method was performed as follows: Z-Phe-Tyr (4.5 μmol), potassium iodide (48.2 μmol), and 2 mCi of carrier-free $Na^{125}I$ were dissolved in 2 mL of TBS, and 0.5 mg of lactoperoxidase (Sigma Chemical Co.) in 0.5 mL of TBS and 0.5 mL of 0.03% H_2O_2 was added to the solution. Reaction was performed at 30 °C for 5 min. Then the reaction mixture was lyophilized, extracted with acetone, and chromatographed on a silica gel plate (Kieselgel 60 F, Merck) by using a solvent consisting of 95 mL of chloroform, 5 mL of methanol, and 1 mL of acetic acid. Iodination with chloramine T was similarly done, except that the reaction was performed in water containing 10 mg of chloramine T at 4 °C. Bands corresponding to Z-Phe-MIT and Z-Phe-DIT were cut and extracted with acetone, and identification of these compounds as to whether it was monoiodinated or diiodinated was done by an ultraviolet absorption spectrum measurement and thin-layer chromatography after the carboxypeptidase Y digestion.

Z-Phe-Tyr and other peptide derivatives were purchased from Peptide Institute, Inc. (Osaka, Japan). Tetrahymanol was kindly provided by Prof. Y. Nozawa of Gifu University, Medical School. [1α,2α(n)-^{3}H]Cholesterol (44 Ci/mmol) was obtained from Amersham. The type C glass fiber filter was a product of Whatman.

Sterol Binding. Binding of cholesterol with F-protein was performed similarly as described before (Asano & Asano, 1985). For preparation of the cholesterol solution, [^{3}H]-cholesterol was dried in a test tube, the appropriate amount of cold cholesterol was added, and the mixture was dissolved with a propylene glycol–dioxane (2:1) mixture (usually 1.5–1.8 μCi of hot and 0.4 nmol of cold cholesterol/2 μL). Two microliters of this solution was added to 50 μL of the reaction medium, which consisted of 10 mM Tris-HCl buffer, pH 7.6, 0.1% Triton X-100, and 0.15 M NaCl and also contained intact or protease-digested viral proteins. After incubation at 30 or 37 °C for 10–30 min, 10–20 μL (0.4–0.5 mg of protein) of anti-F antibody (or anti-HN antibody when HN-protein was used) was added, and incubation was continued for 2 h at 20 °C. For control experiments, cholesterol was added after the incubation with the antibody (2 h, at 20 °C), mixed well, and then filtered immediately. Control values are subtracted from the experimental values to calculate the amount of bound cholesterol. Cholesterol-containing precipitates were filtered by using a glass-fiber filter and washed 10 times with 1 mL each of a medium consisting of 10 mM Tris-HCl buffer, pH 7.6, 1.0% Triton X-100, and 0.15 M NaCl. Radioactivity was measured with Beckman liquid scintillation spectrometer LS100.

Rates of binding of cholesterol to F-protein were determined by a modification of the usual method described above to avoid prolonged incubation with the antibody. Thus, collection of the precipitates on glass-fiber filters was omitted, and instead reaction was stopped by the addition of 10 volumes of the chilled washing buffer; F-protein and the complex were absorbed on charge-modified nylon membrane filters (ZetaProbe blotting membrane; Bio-Rad, Richmond, CA). The membrane was supplied as rectangular sheets, but disks were made from them, and they were prewashed with cold cholesterol (1 μg/mL) in 1% Triton X-100. The duration of incubation with cholesterol was changed as indicated in the figure legend. Washing of filters and radioactivity measurement were performed as described above.

Hemolysis Measurement. The hemolytic activity of the virions was determined as described in a previous paper (Asano et al., 1983) by using the human erythrocytes as target cells. For inhibition studies, preincubation of the virions with peptide derivatives at 30 °C for 2 h was performed as described previously (Asano & Asano, 1985).

Binding of Peptide Derivatives to Purified F-Protein and Virions. Binding of the radioactive peptide derivatives to F-protein was carried out under conditions similar to those employed for the hemolytic inhibition as follows. Peptide derivatives including radioiodinated peptides were dissolved in 0.1 N ammonia water. The reaction medium consisted of 10 mM Tris-HCl buffer (pH 7.6), 0.15 M NaCl, 0.1% Triton X-100, 15–25 μg of F-protein, and an indicated amount of peptide derivatives. After incubation at 30 °C for 2 h, F-protein was precipitated by incubation with anti-F antibody for another 2 h. The resultant precipitate was collected on the glass-fiber filters and washed 5 times with 1 mL of Tris–saline medium (10 mM Tris-HCl, pH 7.6, and 0.15 M NaCl) each. For control experiments, F-protein was incubated with the antibody for 2 h, and then the radioactive peptide employed for the respective experiments was added and filtered immediately. Control values were subtracted from the experimental values to calculate the bound peptides. Binding of the radioactive peptide derivatives to the virions was performed similarly, except that Triton X-100 was omitted from the reaction medium and anti-F antibody precipitation was not employed. Radioactivity was measured with either a Beckman liquid scintillation spectrometer (LS 100) or a Packard-Prias γ-counter.

Protease Digestion of Virions or Purified F-Protein. Proteolysis of the virions was performed in TBS including 1 mg/mL of the viral protein and 50 μg/mL of either thermolysin (Boehringer) or chymotrypsin (Sigma, Type I). $CaCl_2$ (5 mM) was added when thermolysin was used. After digestion at 37 °C for 2 h, the reaction was terminated by adding 20 μL of PMSF (100 mM in 2-propanol) and then centrifuged with an Eppendorf centrifuge and suspended in 200 μL of TBS.

Digestion of the purified F-protein (140 μg) with thermolysin (8 μg) or chymotrypsin (8 μg) was performed in 1 mL of 10 mM Tris-HCl buffer (pH 7.6) containing 0.1% Triton X-100 and 0.15 M NaCl (5 mM $CaCl_2$ was added when thermolysin was used). Other conditions for incubation and termination of the reaction were similar to those described for virions. Digested preparations were rechromatographed on a DEAE-Sepharose column that was used for the isolation of pure F-protein. The peak appearing at the same position was collected, and its purity was checked by SDS gel electrophoresis (Figure 1).

Table I: Binding of Cholesterol to Purified F-Protein[a]

cholesterol added		radioactivity in		bound cholesterol		
[^{3}H]cholesterol (μCi)	total (μM)	exptl[c] (cpm)	control (cpm)	(pmol)	F-protein used (pmol)	chol/F[b] (%)
0.5	7.7	26 150	2300	19.13	259	7.4
1.5	7.8	13 674	1770	3.22	61.6	5.2
1.5	7.8	45 059	1133	11.88	87.6	13.6
1.5	7.8	35 179	1393	9.14	123	7.4
1.5	7.8	16 808	1669	4.1	30.8	11.3
1.5	7.8	13 208	1133	3.3	30.8	10.7
1.5	7.8	14 880	685	3.8	66.5	5.7
1.5	7.8	13 375	475	3.5	66.5	5.3

[a] Binding was performed as described under Experimental Procedures except that indicated amounts of F-protein and [^{3}H]cholesterol (plus 0.39 nmol of cold cholesterol) were used. Controls were zero time controls as described under Experimental Procedures. Amounts of F-protein were expressed as picomoles of a 61 665-kDa subunit. Each F-protein of the upper section is from four different preparations, and those of the lower section are a pair of F-proteins from two different preparations. [b] Cholesterol/F-protein ratio; 1:1 binding was taken as 100%. [c] Experimental samples.

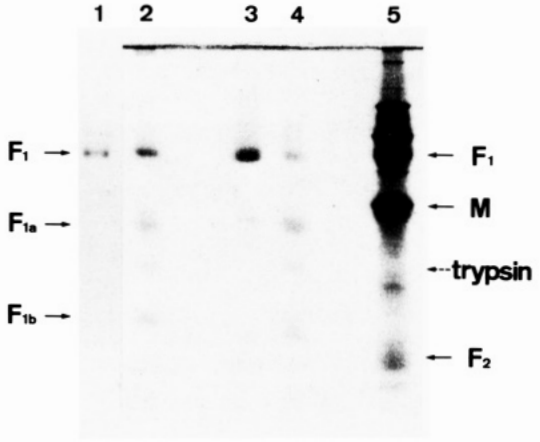

FIGURE 1: SDS gel electrophoresis of F-protein, thermolysin-truncated F-protein, and their trypsin-digested samples. (Lane 1) Purified F-protein (1.5 μg); (lane 2) trypsin-digested F-protein (2.4 μg F-protein); (lane 3) purified thermolysin-truncated F-protein (2.5 μg protein); (lane 4) trypsin-digested thermo-F protein (2.5 μg protein); (lane 5) HVJ virion (12 μg protein). 12.5% gel.

Trypsin digestion of purified F-protein and thermolysin-truncated F-protein was performed simultaneously in 10 mM Tris-HCl buffer (pH 7.6) containing 0.15 M NaCl and 0.1% Triton X-100 at an F-protein concentration of 10 μg/86 μL for 60 min at 37 °C with 1 μg of trypsin. The reaction was terminated by the addition of 2 μL of PMSF (100 mM).

Other Methods. Protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. SDS gel electrophoresis was performed by the method of Laemmli (1970).

Circular dichroism (CD) spectra were measured in a buffer mixture of 5 mM Tris-HCl buffer (pH 8.0) and 5 mM potassium phosphate buffer (pH 8.0) containing 0.075 M NaCl and 0.05% Triton X-100 at 25 °C by using a 1-mm light path cuvette on a Jasco J-500 spectropolarimeter equipped with a data processor for CD. The CD data were expressed as mean residue ellipticity, $[\theta]_{MRW}$. Curve-fitting analysis of the CD spectra was performed basically as described by Chang et al. (1978) by use of a computer program kindly provided by Dr. K. Yutani of Institute for Protein Research, Osaka University. Prediction of secondary structures from amino acid sequences was performed as described by Chou and Fasman (1978).

RESULTS

Cholesterol Binding to Purified F-Protein. As reported previously, cholesterol binds to pure F-protein in a temperature-dependent manner. For a further confirmation that the bound radioactivity is not from radioactive impurity or some

FIGURE 2: Identification of F-protein bound cholesterol by thin-layer chromatography. Binding of cholesterol with F-protein was carried out as described under Experimental Procedures except that the total volume was increased to 200 μL and about 12 μCi of cholesterol and 19 μg of purified F-protein were used. After incubation for the binding, F-protein was precipitated by the addition of 80 μL (2 mg of protein) of anti-F-protein antibody (20 °C, 2 h). Resultant precipitates were filtered with glass-fiber filters (type C), washed with a medium containing 1% Triton X-100 (1 mL each, 10 times), washed with water 2 times, and then dried in a vacuum desiccator. Bound radioactive material was extracted from the glass-fiber filter with a chloroform–methanol (1:1) mixture, concentrated, and spotted on a silica gel plate with 10 μg of cold cholesterol (lane 2). As a control, 0.15 μCi of [^{3}H]cholesterol was spotted on lane 1. The silica gel plate was chromatographed with chloroform as the solvent. Radioactive spots were detected by fluorography using DPO as a fluor. Dashed line shows the solvent front, and an arrowhead indicates the origin where samples were spotted.

cholesterol metabolite but elicited by cholesterol itself, the bound radioactive material was extracted with a chloroform–methanol (1:1) mixture and chromatographed on a silica gel plate. As shown in Figure 2, the extracted material showed an R_f value identical with that of authentic [^{3}H]cholesterol and also cold cholesterol (data not shown). Table I shows the ratio of bound cholesterol to F-protein (mol/mol of subunit; F-protein seems to be an oligomer[2] of 61 665-kDa subunit)

[2] The oligomeric structure of F-protein of HVJ has not been extensively studied yet. But from a chemical cross-linking study, some evidence for oligomeric nature of the molecule (dimer or higher) was reported (Markwell & Fox, 1980). A similar conclusion can also be drawn from an electron microscopy study in which a molecular dimension of F-protein was found to be 21 × (3–7) nm (Shimizu et al., 1974).

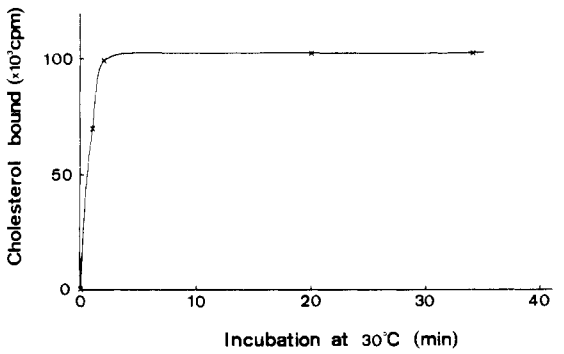

FIGURE 3: Effect of incubation time on binding of cholesterol to F-protein. Reaction conditions were the same as the other experiments except that 10.2 μg of purified F-protein was used. Reaction was stopped by the addition of 10 volumes of the chilled washing medium. Filtration with a charge-modified nylon membrane, washing, and counting were performed as described under Experimental Procedures. Control counts in which radioactive cholesterol was added after dilution with the cold medium and added just before the dilution were 17.9 and 19.2 × 10^3 cpm, respectively. Average of these controls was subtracted from the total counts.

(Miura et al., 1985) measured with different preparations of F-protein. Depending on the experimental conditions and/or F-protein preparation, about 5–14% of the F-protein subunit was found to bind cholesterol under all conditions employed. When the same F-protein preparation and antibody were used, reproducibility was much higher than that among experiments using different preparations (Table I, lower section).

This stoichiometry may represent a lower limit of binding ability, since a rather high concentration of the detergent, Triton X-100, must be employed in the reaction mixture and wash medium to obtain reproducible results and low nonspecific binding; thus, some bound cholesterol may be dissociated during this procedure. In fact, increasing the wash cycle with 1% Triton X-100 to 13 times resulted in an appreciable (about 25%) decrease in the cholesterol binding. Other possibilities which may result in low stoichiometry are that cold cholesterol contained in the viral membranes might have been bound to F-protein during the solubilization and purification procedures in which Triton X-100 was used or that one cholesterol molecule binds to one molecule but not to one subunit of F-protein. Partial denaturation of F-protein is also a possibility.

The rate of binding of cholesterol to F-protein cannot be measured by the antibody-precipitation method used for the other measurements, since prolonged incubation with the antibody was required for precipitation of F-protein. Therefore, a different method of collection and washing of F-protein and its complex with cholesterol was developed utilizing charge-modified nylon membrane filters. By this method, binding of cholesterol with F-protein could be stopped with a relatively short period of incubation. As shown in Figure 3, binding reached a nearly saturated level only after 2 min of incubation. Thus, the rate of binding of cholesterol with F-protein is about 5–10 times faster than the rate of envelope fusion reported before (Maeda et al., 1975; Sekiguchi & Asano, 1973).

Binding Specificity of the Sterols with F-Protein. To manifest the binding specificity of different sterols with F-protein, the effect of dilution with several sterols on the binding of labeled cholesterol with F-protein was studied. To select appropriate concentrations of cholesterol for study of such competition experiments, the effect of cholesterol concentrations on the amounts of cholesterol bound to F-protein was studied. As shown in Table IIA, binding of cholesterol was

Table II: Effect of Cold Sterol Addition on Binding of Cholesterol with F-Glycoprotein

Part A[a]

cold cholesterol added (μM)	total cholesterol (μM)	bound cholesterol: counts (cpm)	bound cholesterol: amt (pmol)	bound cholesterol: % of max
none	0.23	13110	0.45	1.15
1.55	1.78	29329	7.91	20.3
7.75	7.98	23905	28.9	74.1
25.87	26.1	9874	39.00	(100)

Part B[b]

cold sterol added to reaction medium	total sterol concn (μM)	bound cholesterol: counts	bound cholesterol: counts (%)	bound cholesterol: amt (%)
cholesterol (7.8 μM)	7.8	39126	(100)	(100)
+cholesterol	38.8	8136	21	105
+5α-cholestan-3-one	38.8	8489	22	
+ergosterol	38.8	9791	25	

Part C[c]

expt	washing medium	bound cholesterol: counts	bound cholesterol: amt (%)
I	Triton X-100 only	33786	(100)
	+cholesterol	18825	56
	+5α-cholestan-3-one	13918	41
	+5α-cholestane (A/B trans)	13071	39
	+phosphatidylethanolamine	28156	83
II	Triton X-100 only	104905	(100)
	+cholesterol	67215	64
	+tetrahymanol	53153	51
	+digitonin	75381	72
	+5β-cholestane (A/B cis)	96927	92
	+squalene	88029	84

[a] Binding was performed as described under Experimental Procedures except that 16 μg of F-protein, 0.5 μCi of [^{3}H]cholesterol, and indicated amounts of cold cholesterol were added in a final volume of 52 μL. After this period, 20 μL of anti-F antibody (25 mg of IgG fraction/mL of TBS) was added to the reaction mixture and then incubated for 2 h at 20 °C. Resultant precipitates were collected on glass-fiber-filter disks and washed. [b] Reaction conditions were similar to those described in (A) except that 5.4 μg of F-protein, 1.5 μCi of [^{3}H]cholesterol, and an indicated amount of cold cholesterol or its analogues were used. [c] Reaction conditions were similar to those described in (B) except that about a 3-fold concentration of [^{3}H]cholesterol was used in experiment II. Cold analogues were not included in the reaction medium; instead, these analogues were added to the washing medium at a concentration of 1 μg/mL.

dependent on its concentration and approached saturation around 26 μM under the experimental conditions employed (such as in the presence of 0.1% Triton X-100).

Therefore, for competition experiments, radioactive cholesterol was set at a nearly but not completely saturated concentration of 7.8 μM, and cold sterols were added to reach a saturation concentration of 38.8 μM. As shown in Table IIB, addition of cold cholesterol to increase cholesterol concentration from 7.8 to 38.8 μM apparently decreased the amount of radioactive cholesterol binding, indicating that dilution of the radioactive species occurred, although the calculated amount of total bound cholesterol did not decrease. When the same amount of 5α-cholestan-3-one or ergosterol was added, the binding of radioactive cholesterol also decreased. These results indicate that these two sterols compete with cholesterol at the binding site on F-protein.

Competition of the binding with cholesterol was also studied by adding several sterols and other lipidic compounds to the washing medium. As shown in Table IIC, addition of cholesterol replaced the radioactive cholesterol on F-protein. Similar replacement was observed with 5α-cholestan-3-one, 5α-cholestane (A/B trans), and tetrahymanol and with digitonin to a lesser extent, whereas 5β-cholestane (A/B cis),

Table III: Binding of Cholesterol to F-, Thermolysin-Truncated F-, and HN-Proteins[a]

glycoprotein	bound cholesterol counts	%
intact F	10550	(100)
thermolysin-digested F	4829	46
thermolysin-digested F + Z-D-Phe-Phe-Gly	2253	21
intact HN	0	0

[a] Conditions for binding and sample washing were similar to those described under Experimental Procedures except that 0.42 nmol of cholesterol (including 1.5 μCi of [^{3}H]cholesterol), 5 μg of F-protein, 4.6 μg of thermolysin-truncated F-protein, or 5.8 μg of HN protein was used. F-Protein and HN-protein were precipitated with anti-F antibody or with anti-HN antibody, 0.5 mg in TBS, respectively.

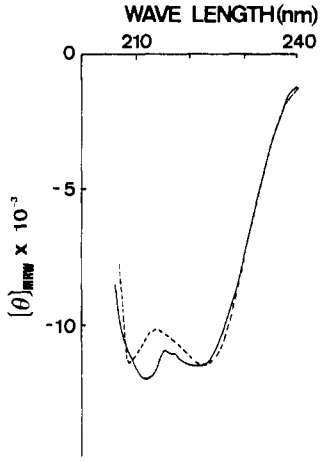

FIGURE 4: CD spectra of F- and thermolysin-truncated F-proteins. Data of multiple scans were accumulated by a data processor, averages were plotted, and mean residue ellipticity was calculated from the data. Concentrations of F- and thermo-F-proteins were 67 and 62 μg/mL, respectively.

squalene, and phosphatidylethanolamine were almost inactive in replacement.

Cholesterol Binding Site of F-Protein. To check the previous postulation that cholesterol binds to the fusogenic sequence of F-protein (which locates in the N-terminal segment of the F_1 subunit) rather specifically, we examined whether cholesterol specifically binds with nonfusogenic proteins. HN-Protein of the HVJ is known to have hemagglutinating and neuraminidase activities, but it is not fusogenic by itself, although intactness of this protein is required for the fusion reaction (Ozawa et al., 1979a,b; Ozawa & Asano, 1981). This protein showed no cholesterol binding activity (Table III).

When F-protein was digested with thermolysin to remove a 2500-Da segment from the N-terminal of the F_1 subunit, it simultaneously lost its fusogenic activity (Asano & Asano, 1983), and this modification greatly diminished the cholesterol binding ability (Table III). Remaining binding activity may be due to the incomplete digestion of the F_1 subunit, which is usually observed in this type of experiment (Asano et al., 1983), since this remaining binding activity was inhibited by an inhibitory peptide (see below).

To check the other possibility that loss of cholesterol binding of thermolysin-truncated F-protein (thermo-F) is due to gross conformational change of the molecule, we used two different methods. One of them is comparison of circular dichroism spectra of F- and thermo-F proteins. As shown in Figure 4, the mean residue ellipticities of these proteins were quite similar. By curve-fitting analysis, slight decreases of α-helix (from 42.7 to 36.9%) and β-sheet (12.0 to 5.7%) were found without appreciable change of β-turn structure. Secondary

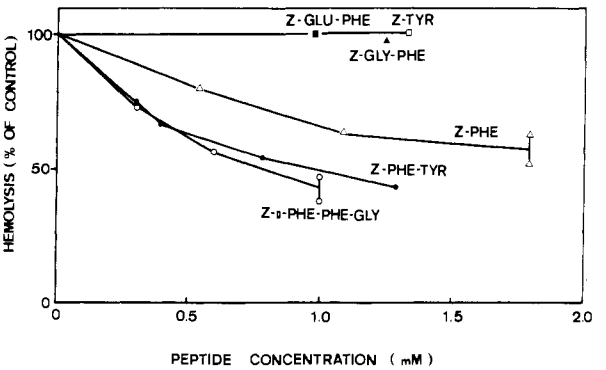

FIGURE 5: Inhibition of HVJ-induced hemolysis with peptide derivatives. Preincubation with peptides and the hemolytic activity measurement were performed as described under Experimental Procedures. (○) Z-D-Phe-Phe-Gly; (●) Z-Phe-Tyr; (△) Z-Phe; (▲) Z-Gly-Phe; (■) Z-Glu-Phe; (□) Z-Tyr.

structure predicted by the Chou and Fasman (1978) method was β-sheet for the first 23 residues from the N-terminal of F_1 (the last 11 of these residues have some possibility of α-helix conformation also) and α-helix for the adjacent 22 residues. Since roughly 26 residues may be split off from the N-terminal of F_1 by thermolysin digestion (Asano et al., 1983), a partial decrease in α-helix and β-sheet structures by thermolysin digestion can be explained at least partly by a split off of the N-terminal segment.

Furthermore, as a very sensitive method for detection of protein conformational change, we used limited digestion by trypsin. Although 20 Lys and 25 Arg residues are present in the F_1 subunit of F-protein (Miura et al., 1985A), only one (or a few nearby) residue is sensitive to trypsin digestion in intact F-protein (Asano et al., 1983). Therefore, limited digestion by trypsin can be used for detection of small conformational changes of F-protein. As was shown in Figure 1, the degree of digestion of F- and thermo-F proteins (lanes 2 and 4) shown by the appearance of F_{1a} and F_{1b} fragments was almost the same under the condition that undigested F_1 or thermo-F_1 was still remaining. Thus, induction of gross conformational change by truncation of the N-terminal portion of F_1 with thermolysin is unlikely.

Inhibition of HVJ-Induced Hemolysis with Peptide Derivatives. Richardson et al. (1980, 1983) reported previously some peptide derivatives analogous to the N-terminal portion of the F_1 subunit, such as Z-D-Phe-Phe-Gly, show an inhibitory effect on paramyxovirus infection. Recently, we found that preincubation of HVJ virions with this peptide resulted in the inhibition of virus-induced hemolysis, which is a result of membrane fusion reaction between the viral envelope and erythrocyte membrane (Shimizu et al., 1976; Maeda et al., 1975; Sekiguchi & Asano, 1978).

To study the interaction site between the inhibitory peptide and viral component, we searched for an effective peptide(s) among availabe peptide derivatives that may be labeled with iodine. As shown in Figure 5, Z-Phe-Tyr was found to be inhibitory, whereas Z-Glu-Phe, Z-Gly-Phe, and Z-Tyr did not show an inhibitory effect and may serve as controls. Since prolonged incubation was necessary to obtain the appreciable inhibition with Z-D-Phe-Phe-Gly, the effect of incubation with Z-Phe-Tyr was compared with other peptides. The time course of the inhibition enhancement was similar in these two peptides, whereas prolonged incubation with Z-Tyr did not result in detectable inhibition (data not shown).

The dose–response curve of Z-Phe-Tyr was similar to that of Z-D-Phe-Phe-Gly (Figure 3). Furthermore, Z-Phe-Tyr or Z-D-Phe-Phe-Gly at 1 mM exhibited no effect (neither inhi-

Table IV: Apparent Inhibition of the Binding of Z-Phe-MIT with Cold Z-Phe-Tyr[a]

	peptide concn		radioactive Z-Phe-MIT bound	
expt	Z-Phe-Tyr (mM)	total (mM)	F-protein (cpm)	virion (cpm)
I	0	0.09	461	5114
	0.25	0.34	193	5796
	0.5	0.59	126	3872
II	0	0.138	2950	
	0.5	0.638	776	
	1.0	1.138	453	

[a] Binding with purified F-protein was performed as described under Experimental Procedures except that either 4.51 nmol of radioactive Z-Phe-MIT (171 000 cpm) in experiment I or 13.75 nmol (550 000 cpm) in experiment II, the indicated amount of cold Z-Phe-Tyr, and either 20 μg of F-protein or 50 μg protein of HVJ virions were used in experiment I and 145 μg of F-protein were used in experiment II. When virions were used, Triton X-100 was omitted from the medium. Total volume was increased to 100 μL in experiment II. After the binding with F-protein, either 20 μL of anti-F antibody (0.5 mg in TBS) in experiment I or 40 μL of the antibody in experiment II was added to each tube, and further incubation was done for 2 h at the same temperature. Other conditions used were as described under Experimental Procedures. Radioactivity bound in zero time control samples were 84 cpm for F-protein and 217 cpm for HVJ virions in experiment I and 180 cpm for experiment II, respectively.

Table V: Binding Specificity of Z-Phe-MIT with HVJ Glycoproteins[a]

			radioactivity in		
glycoprotein	amt (μg)	amt (nmol)	exptl[b] (cpm)	control (cpm)	bound peptide (cpm)
F-protein	24	0.39	908	116	792
HN-protein	28	0.44	235	129	106

[a] Binding measurement was performed as described under Experimental Procedures except that the indicated amounts of purified F- or HN-proteins and 90 μM (188 000 cpm) of Z-Phe-MIT were added. Molecular mass of the subunits of F- and HN-proteins was based on nucleic acid sequence studies (Miura et al., 1985a,b). [b] Experimental samples.

bitory nor stimulatory) on the hemagglutination reaction of HVJ under the same conditions for detection of hemolysis inhibition (data not shown). Thus, Z-Phe-Tyr was selected as a candidate for the iodination studies. Preliminary study showed that a monoiodinated derivative, Z-Phe-MIT, was inhibitory to the hemolytic reaction (data not shown).

Binding of Iodinated Derivatives of Z-Phe-Tyr with HVJ Virions and Purified F-Protein. Radioiodinated peptides (Z-Phe-MIT and Z-Phe-DIT) were prepared and incubated at 30 °C for 2 h with intact virions or purified F-protein, and the bound peptide was quantified by a radioactivity measurement. As shown in Table IV, peptide was bound to both samples. To check the specificity of this binding, dilution of the radioactive peptide with cold Z-Phe-Tyr was studied. As a result, dilution was clearly observed with the purified F-protein. On the assumption that the affinity of Z-Phe-Tyr is similar to those of Z-Phe-MIT and Z-Phe-DIT, the amount of peptide bound to the target can be calculated at each concentration (data not shown). The resultant concentration dependency of the binding shows a saturation at 1 mM was compatible with that of the hemolysis inhibition.

Since the hydrophobic nature of inhibitory peptide derivatives is evident, some interaction of these peptides with lipid bilayers or hydrophobic transmembrane domain must be considered. Some anomaly and low competition observed in dilution experiments using virions may indicate the presence of such interaction. Thus, for detection of the specific binding of the peptides with F-protein, proper controls are necessary.

Table VI: Effect of Protease Treatments on Z-Phe-MIT and Z-Phe-DIT Binding Ability of HVJ Virions[a] and Purified F-Protein[b]

	bound peptides			
virion	Z-Phe-MIT (cpm)	Z-Phe-MIT (%)	Z-Phe-DIT (cpm)	Z-Phe-DIT (%)
untreated	4689	(100)	6266	(100)
chymotrypsin truncated	2523	54	3742	60
thermolysin truncated	1973	42	3123	50

		bound peptides			
		Z-Phe-MIT		Z-Phe-DIT	
protein	amt (pmol)	amt (pmol)	peptide/protein (mol/mol)	amt (pmol)	peptide/protein (mol/mol)
F-protein	164	39.1	0.24	20.9	0.13
F-protein	329	77.0	0.23	77.8	0.24
thermo-F[c]	71	3.9	0.05	0	0

[a] Binding was performed as described under Experimental Procedures except that 50 μg of protein of HVJ virions was used. Either 66 000 cpm of Z-Phe-MIT (3.0 nmol) or 57 000 cpm of Z-Phe-DIT (1.3 nmol) was added. Radioactive counts in control samples were 479 cpm for Z-Phe-MIT and 636 cpm for Z-Phe-DIT. [b] Experiments were performed as described for the upper section except that the indicated amounts of purified protein were used. Controls for F-protein were 187 cpm for Z-Phe-MIT and 289 cpm for Z-Phe-DIT. [c] Thermolysin-truncated F-protein.

Table VII: Effect of Preincubation with Peptide Inhibitors on Cholesterol Binding of F-Protein[a]

addition	concn (mM)	inhibition (%)
DMSO control		(0)
Z-D-Phe-Phe-Gly	1.6	42
Z-Phe-Tyr	1.7	23
Z-Phe	2.7	23
Z-Gly-Phe	2.4	4

[a] Conditions for preincubation and binding reactions were similar to those described under Experimental Procedures except that 4 μg of F-protein was used, and cholesterol was added to the reaction mixtures after the preincubation with peptide inhibitors was completed. After the binding reaction with cholesterol for 30 min, anti-F antibody (0.5 mg in TBS) was added and incubated for 2 h at 20 °C. Resultant precipitates were collected on glass-fiber filters and were washed as described under Experimental Procedures.

As such control, binding of Z-Phe-MIT to HN-protein that has a hydrophobic transmembrane domain was measured. As shown in Table V, HN-protein showed only a marginal binding with Z-Phe-MIT.

Although binding of the peptides with F-protein was substantiated by these studies, evaluation of the previous hypothesis that the N-terminal fusogenic sequence of the F_1 subunit is the binding site of inhibitory peptides from the molecular modeling was not yet clear. Thus, binding of the radioactive peptides with modified F-protein that lacks the N-terminal portion of the F_1 subunit was measured. As summarized in Table VI, binding of the peptides with chymotrypsin- or thermolysin-truncated virions was substantially decreased. Furthermore, interaction of the peptides with purified thermolysin-truncated F-protein was drastically decreased (Table VI), although interaction of anti-F antibody with the modified F-protein was not appreciably changed (data not shown).

Inhibition of Cholesterol Binding to F-Protein with Peptide Inhibitors. Since the binding sites of cholesterol and inhibitory peptides on F-protein were both assigned as the fusogenic N-terminal sequence of the F_1 subunit, binding of these two compounds to F-protein may (or may not) be affected by on another. Accordingly, the effect of a preincubation with in-

hibitory peptides on cholesterol binding was studied. As shown in Table VII, cholesterol binding was appreciably inhibited by pretreatment with inhibitory peptides, whereas the effect of noninhibitory Z-Gly-Phe was negligible.

Discussion

Fusion of HVJ membrane with other cell membranes proceeds in several steps. The first step is the binding of virions to a cell surface, which is mediated by the interaction of HN-protein of the virion with sialic acid containing components of the target cell surface. This step is usually measured as a virion-induced cell agglutination that occurs at 4 °C. We have previously shown by using a lipid-soluble photoaffinity label, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine, inserted in the plasma membrane that F-protein of the attached virion exists very close to lipid bilayers of target cells at this step of the reaction (Asano & Asano, 1984). As the temperature increases, the membrane fusion proceeds rapidly. Although the requirement of active F-protein for the fusion reaction is well established (Homma & Ouchi, 1973; Scheid & Choppin, 1975), the mechanism by which F-protein perturbs the membrane structure and how it induces the membrane fusion reaction are not well understood. However, the hypothesis that F-protein somehow interacts directly with the lipid bilayers of the plasma membrane and thus results in membrane fusion is well accepted.

F-Protein contains a stretch of hydrophobic aminoacyl residues located at the N-terminal of the F_1 subunit, and it was thought to be the membrane interaction site from its hydrophobic nature (Gething et al., 1978; Hsu et al., 1981). On the basis of the susceptibility of this sequence to a limited digestion with aminopeptidase M, thermolysin, or chymotrypsin and to an iodination reaction, we have previously shown that this portion is exposed on the surface of the protein despite its hydrophobicity (Asano et al., 1983; Asano & Asano, 1982). Thus, the postulation above seems likely.

Specific interaction of this segment with cholesterol (or some other membrane component) was suspected mainly from the fact that the homology of amino acid sequences among fusogenic paramyxoviruses are very high compared with other hydrophobic sequences of F-protein, i.e., the hydrophobic core of signal peptides or transmembrane sequences (Asano & Asano, 1984). This hypothesis may be meaningful because a high cholesterol content of the plasma membrane is well-known.

In a previous paper, we reported that cholesterol was rather specifically bound to the F-protein, judging from the result that bound cholesterol was still sustained after 10 washings with a medium containing 1% Triton X-100 (Asano & Asano, 1985). Washing of these samples 10 times with cold sterol-containing medium or washing them 3 times more with the same medium after regular washing appreciably decreased the binding as described under Results. Thus, this binding seems to be reversible even in a complex form with anti-F antibody. Furthermore, specificity of the binding was confirmed by the facts that very low binding of cholesterol was seen with control samples in which filtration was performed immediately after the cholesterol addition and with the other viral membrane protein, HN.

To study whether this cholesterol binding is specific to the fusogenic sequence or not, we utilized the truncation of F-protein with thermolysin (or chymotrypsin). Limited digestion of HVJ virions (or purified F-protein) with thermolysin or chymotrypsin results in production of nonfusogenic form(s) of F-protein (Asano & Asano, 1983). This is due to a splitting off of the N-terminal portion of the F_1 subunit. Evidence supporting this conclusion is as follows: (1) The molecular mass of F_1 was decreased detectably (about 2500 Da by thermolysin digestion and 3500 Da by chymotrypsin). (2) The N-terminal amino acid of truncated F_1 was mostly different from the original terminal Phe (Ile in the case of thermolysin and Ala in case of chymotrypsin). (3) Elution profiles of the truncated F-protein from DEAE-Sepharose CL-6B were almost identical with the intact one. In the case of HVJ, a 26-residue stretch of hydrophobic amino acids exists at the N-terminal of the F_1 subunit, and the molecular mass of this segment is calculated to be 2414 Da. Thus, thermolysin or chymotrypsin digestion seems to split off most, if not all, of the fusogenic hydrophobic sequences.

Therefore, we compared the cholesterol binding ability of thermolysin-truncated F-protein with that of the intact one. As described above, the thermolysin-truncated protein showed considerably decreased binding of cholesterol. Residual cholesterol binding ability of thermo-F-protein may be due to inability to prepare completely truncated F-protein (Asano et al., 1983; and data not shown). Although the degree of inactivation is not paralleled with truncation, this can be explained by several postulations, such as stronger resistance of active form(s) to the proteolysis or a possibility that only one intact chain in the oligomeric molecule is required for activity. Further studies may be required for complete understanding of this result. A possibility that inactivation by thermolysin truncation was due to gross conformational change of the protein induced by the proteolysis can be denied by the results of CD measurements and limited trypsin digestion that were described under Results. Therefore, an experimental support for the specific binding of cholesterol with the N-terminal hydrophobic portion of F_1 is now offered.

Furthermore, the hydrophobicity of the fusogenic sequence of F-protein is much lower than that of the transmembrane sequences of F-protein and other transmembrane proteins [Davis & Hsu, 1986; and our calculation according to the method of Kyte and Doolittle (1982) or Hoop and Woods (1981)]. This result, as well as the fact that the hydrophobic transmembrane sequence of HN-protein does not bind with cholesterol under our experimental condition, indicates that simple hydrophobic interaction may not be so important in the interaction of the fusogenic sequence with cholesterol. Accordingly, a hypohtesis that this fusogenic sequence of the F_1 subunit specifically interacts with cholesterol seems to be reasonable.

Unlike binding of inhibitory peptides to F-protein that requires prolonged incubation, binding of cholesterol to this protein was very rapid, being completed within a few minutes, at least under the experimental conditions employed. Furthermore, the cholesterol concentration required for binding was rather low (half-maximal binding was observed at 4–5 μM) under artificial Triton X-100 containing conditions. Since the concentration of cholesterol in plasma membranes is very high (about 20% w/w) (see textbooks of biochemistry or cell biology such as *Molecular Biology of the Cell*), its concentration in target membranes may not be a limiting factor for the binding reaction. Thus, participation of cholesterol binding of F-protein in the fusion reaction with plasma membranes has to be considered seriously.

Binding of sterols with several other membrane-penetrating proteins is already known. For example, binding of cholesterol to a cytolytic protein toxin, streptolysin O, was reported (Johnson et al., 1980). Possible binding of cholesterol to another bacterial toxin, perfrinolysin, was also described (Hase et al., 1976). These proteins are reported to bind with sterols

possessing a 3β-hydroxyl group, and 3-keto and some other derivatives also showed a weak binding affinity. Moreover, fusion of the Semliki Forest virus with liposomes under mild acidic conditions was reported to require the presence of cholesterol or other sterols having a 3β-hydroxyl group in liposomes (White et al., 1983). Since both cis and trans compounds were equally effective, configuration at the A/B ring junction of the molecules seems to be unimportant for the Semiliki Forest virus induced fusion reaction.

In the case of sterol binding with F-protein, the requirement of a 3-hydroxyl group or long hydrophobic tails was not observed as above. But this binding is specific in the sense that the binding ability of sterol-related compounds depends on the presence of cholesterol nucleus or a three-dimensionally similar A/B trans ring structure; i.e., the A/B ring cis compound and squalene without ring structure showed no binding affinity or the binding affinity was greatly reduced. Thus, the binding specificity of F-protein for sterols is different from that of any other case such as streptolysin O or Semliki Forest virus. This is in accordance with the fact that the primary sequence of the putative fusogenic sequence of Semliki Forest virus E protein (Rice & Strauss, 1981) is entirely different from that of HVJ F-protein.

Inhibition of the paramyxovirus infection such as measles virus and canine distemper virus with peptide derivatives including Z-D-Phe-Phe-Gly, which has some similarity with the N-terminal segment of the F_1 subunit, was reported previously (Richardson et al., 1980, 1983). Although the reason was unknown, the presence of the carbobenzoxy group at the N-terminal was required for an efficient inhibition. Since a few hours of incubation was normally employed for the infection inhibition studies, we tried a long-term incubation of virions with the inhibitory peptides and found that a prolonged incubation was essential to inhibit the membrane fusion reaction determined by a hemolysis measurement. No inhibition was seen in the hemagglutination reaction upon preincubation with inhibitory peptide derivatives under similar conditions. As we used virions in previous studies (Asano & Asano, 1985), the binding site of these inhibitors on virions could not be determined at that time.

Therefore, we tried a direct measurement of the binding of inhibitory peptides with viral components. Binding of the labeled peptide(s) with whole virions or protease-truncated virions revealed a partial specificity of the binding; however, the presence of nonspecific interaction (probably due to the hydrophobic nature of the peptide derivatives) was evident. Thus, the binding of inhibitory peptides with purified F-protein was studied.

The apparent affinity of the iodinated Z-Phe-Tyr was found to be in good agreement with the concentrations required for a hemolysis inhibition. Substoichiometric binding of the peptides with F-protein (less than 25% of the F_1 subunit was occupied with the peptides) may be due to rather a low affinity of the peptides, since concentrations required for half-maximal inhibition of hemolysis were higher than 10^{-4} M. Direct binding of these inhibitory peptides with the N-terminal hydrophobic portion of the F_1 subunit was supported by experiments that used thermolysin- or chymotrypsin-truncated F-proteins.

As discussed earlier (Asano & Asano, 1985), the lack of binding of similar peptides, such as Z-D-Phe-Phe-(^{125}I)Tyr, with paramyxovirus virions reported previously (Richardson & Choppin, 1983) may be due to the short period of incubation (15 min at 30 °C). The reason why longer incubation time at temperatures above 20 °C is required for the interaction of F-protein with inhibitory peptides is not fully understood at present. The necessity of a local unfolding or folding of the protein structure for the binding may be an explanation for these temperature dependencies.

We have postulated previously from the consideration with the molecular models that the N-terminal Phe-Phe sequence interacts with the inhibitory peptides with three aromatic rings (Asano & Asano, 1984, 1985). Later, papers reporting a notion that several forms of aromatic–aromatic interaction, in which "herringbone" packing of the benzene rings predominates, is a common feature in the stabilization of protein structures appeared (Burley & Petsko, 1985; Singh & Thornton, 1985). Although our previous models that employed parallel packing of the aromatic rings may also be favorable for the carbonyl oxygen–aromatic interaction (Thomas et al., 1982), it was equally possible to construct the herringbone packing network of five aromatic rings that are clustered at the N-terminal of the F_1 subunit and at the inhibitory peptides (data not shown). Enhancement of the inhibition by introduction of a carbobenzoxy group to the N-terminal can be easily explained by this aromatic interaction. Our finding that these peptides bind to the N-terminal portion of about 2500 Da of the F_1 subunit support this notion.

Furthermore, washing of the peptide–F-protein complexes with an aromatic detergent (Triton X-100, 1%) released the bound peptides almost completely (data not shown), although no such drastic dissocation with Triton X-100 was observed with a complex of a nonaromatic ring containing cholesterol and F-protein. Moreover, it is interesting in this respect that noninhibitory Z-Gly-Phe, Z-Glu-Phe, and Z-Glu-Tyr have more space in between the aromatic rings than do the inhibitory peptides.

Taking these results into consideration, it can be said that a direct participation of the N-terminal fusogenic sequence of F_1 and a direct interaction of this segment with membrane cholesterol in the membrane fusion reaction between viral membrane and other cell membranes are further substantiated.

Some comments should be added to avoid any misunderstanding. Among all membrane fusion phenomena, membrane fusion between Sendai virus envelope and target cell membranes seems to be one of the most strictly regulated fusion reactions in the sense that it requires intactness of two viral proteins, F and HN, for this reaction. Thus, we think that the notion described above, i.e., specific binding of a membrane protein (F-protein in this case) with cholesterol of a target membrane may be important for fusion reaction, cannot always be applied to other membrane fusion phenomena. For example, it is well-known that Sendai virions fuse with artificial lipid bilayer membranes of quite different compositions under different (sometimes denaturing) conditions if appropriate binding or collision frequency with virus particles is provided. It is also known that some types of liposomes can fuse together quite efficiently under appropriate conditions without the addition of proteins. Induction mechanisms of the membrane fusion reactions could be different in these cases, although the final event, i.e., membrane fusion due to hydrophobic interaction, may be the same.

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Registry No. Z-Phe, 1161-13-3; Z-Phe-Tyr, 2537-91-9; Z-Phe-Phe-Gly, 57092-54-3; cholesterol, 57-88-5; 5α-cholestan-3-one, 566-88-1; ergosterol, 57-87-4; digitonin, 11024-24-1; 5α-cholestane, 481-21-0; tetrahymanol, 2130-17-8.

REFERENCES

Apostolov, K., & Almeida, J. D. (1972) *J. Gen. Virol. 15*, 227–234.

Asano, A., & Asano, K. (1982) *Structure, Dynamics and Biogenesis of Biomembranes* (Sato, R., & Ohnishi, S., Eds.) pp 57–78, Plenum, New York.

Asano, A., & Asano, K. (1984) *Tumor Res. 19*, 1–20.

Asano, K., & Asano, A. (1985) *Biochem. Int. 10*, 115–122.

Asano, K., Murachi, T., & Asano, A. (1983) *J. Biochem. (Tokyo) 93*, 733–741.

Burley, S. K., & Petsko, G. A. (1985) *Science (Washington, D.C.) 229*, 23–28.

Chang, C. T., Wu, C. S. C., & Yang, J. T. (1978) *Anal. Biochem. 91*, 13–31.

Chou, P. Y., & Fasman, G. D. (1978) *Annu. Rev. Biochem. 47*, 251–276.

Davis, N. G., & Hsu, M.-C. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 5091–5095.

Gething, M. J., White, J. M., & Waterfield, M. D. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2737–2740.

Hase, J., Mitsui, K., & Shonaka, E. (1976) *Jpn. J. Exp. Med. 46*, 45–50.

Homma, M., & Ohuchi, M. (1973) *J. Virol. 12*, 1457–1465.

Hoop, T. P., & Woods, K. R. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3824–3828.

Hsu, M.-C., & Choppin, P. W. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7732–7736.

Hsu, M.-C., Scheid, A., & Choppin, P. W. (1981) *J. Biol. Chem. 256*, 3557–3563.

Hsu, M.-C., Scheid, A., & Choppin, P. W. (1983) *Virology 126*, 361–369.

Johnson, M. K., Geoffroy, C., & Alouf, J. E. (1980) *Infect. Immunol. 27*, 97–101.

Klappe, K., Wilschut, J., Nir, S., & Hoekstra, D. (1986) *Biochemistry 25*, 8252–8260.

Kundrot, C. E., Spangler, E. A., Kendall, D. A., MacDonald, R. C., & MacDonald, R. I. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1608–1612.

Kyte, J., & Doolittle, R. F. (1982) *J. Mol. Biol. 157*, 105–132.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Maeda, T., Asano, A., Ohki, A., Okada, Y., & Ohnishi, S. (1975) *Biochemistry 14*, 3736–3741.

Markwell, M. A. K., & Fox, F. (1980) *J. Virol. 33*, 152–166.

Mimura, N., & Asano, A. (1982) *J. Cell Biol. 93*, 899–909.

Miura, N., Ohtsuka, E., Yamaberi, N., Ikehara, M., Uchida, T., & Okada, Y. (1985a) *Gene 38*, 271–274.

Miura, N., Nakatani, Y., Ishiura, M., Uchida, T., & Okada, Y. (1985b) *FEBS Lett. 188*, 112–116.

Molecular Biology of the Cell (1983) (Alberts, B., Bray, D., Lewis, J., Roff, M., Roberts, K., & Watson, J. D., Eds.) Garland, New York.

Okada, Y. (1969) *Curr. Top. Microbiol. Immunol. 48*, 102–128.

Ozawa, M., & Asano, A. (1981) *J. Biol. Chem. 256*, 5954–5956.

Ozawa, M., Asano, A., & Okada, Y. (1979a) *J. Biochem. (Toyko) 86*, 1361–1369.

Ozawa, M., Asano, A., & Okada, Y. (1979b) *Virology 99*, 197–202.

Paterson, R. G., Harris, T. J. R., & Lamb, R. A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 6706–6710.

Rice, C. M., & Strauss, J. H. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2062–2066.

Richardson, C. D., & Choppin, P. W. (1983) *Virology 131*, 518–532.

Richardson, C. D., Scheid, A., & Choppin, P. W. (1980) *Virology 105*, 205–222.

Richardson, C. Hull, D., Greer, P., Hasel, K., Berkovich, A., Englund, G., Bellini, W., Rima, B., & Lazzarini, R. (1986) *Virology 155*, 508–523.

Scheid, A., & Choppin, P. W. (1977) *Virology 88*, 54–66.

Sekiguchi, K., & Asano, A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1740–1744.

Sever, A. C., Smith, J. A., Waxham, M. N., Wolinksy, J. S., & Goodman, A. M. (1985) *Viology 144*, 373–383.

Shimizu, K., Shimizu, T. K., Kohama, T., & Ishida, N. (1974) *Virology 62*, 90–101.

Shimizu, Y. K., Shimizu, K., Ishida, N., & Homma, M. (1976) *Virology 71*, 48–60.

Singh, J., & Thornton, J. M. (1985) *FEBS Lett. 191*, 1–6.

Spriggs, M. K., Olmsted, R. A., Venkatesan, S., Coligan, J. E., & Collins, R. L. (1986) *Virology 152*, 241–251.

Thomas, K. A., Smith, G. M., Thomas, T. B., & Feldmann, R. J. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4843–4847.

Varsanyi, T. M., Jornvall, H., & Norrby, E. (1985) *Virology 147*, 110–117.

Varsanyi, T. M., Jornvall, H., Orvell, C., & Norrby, E. (1987) *Virology 157*, 241–244.

White, J., Kielian, M., & Helenius, A. (1983) *Q. Rev. Biophys. 16*, 151–195.