

# A Molecular Mechanism of the Fusion Reaction between HVJ (Sendai virus) Envelope and Erythrocyte Membrane

Kimiko ASANO

**ABSTRACT:** A Molecular mechanism of the fusion reaction between HVJ envelope and erythrocyte membrane was studied. N-terminal domain of the F<sub>1</sub> subunit of F(fusion) protein was assigned to the fusogenic domain from results of several experiments including limited digestion with protease, surface labeling, and photo-crosslinking. This domain is exposed on the protein surface despite its hydrophobicity, and closely opposed to cell membranes when the virion bound to the target surface.

Comparison of amino acid sequences of the fusogenic domains found in F (or F-like) protein of paramyxoviruses with other hydrophobic sequences, such as signal peptides and transmembrane sequences, revealed that only fusogenic domains maintain very high homology of amino acid sequences. A hypothesis that some membrane components might bind to the domain and cause membrane fusion was thus proposed. Experimental tests of this hypothesis revealed a temperature-dependent binding of cholesterol to F protein. Evidence supporting the notion that the fusogenic domain is the binding site of cholesterol was obtained. Furthermore, peptides with inhibitory activity against the fusion reaction and infection were shown to bind to the fusogenic domain of F protein and to hamper cholesterol binding. Thus, the binding of cholesterol in the target membrane with the N-terminal portion of the F<sub>1</sub> subunit seems to be an important step in the fusion reaction.

**Key words:** HVJ, Membrane fusion, Limited proteolysis, Hydrophobic domain, Cholesterol binding, Virus inhibitory peptides

## INTRODUCTION

The fusion of biological membranes seems to be an important and fundamental event among the cellular functions. At the subcellular level, membrane fusion such as pinocytosis,

phagocytosis, and phagosome-lysosome fusion, as well as exocytotic processes is widely observed in most cells<sup>1)</sup>. Fusion between plasma membranes is known to occur in myoblast formation, fertilization, and pathological syncytium formation.

Although the mechanism of these membranes fusion reactions, at the cellular and subcellular levels may not be completely the

---

Division of General Education, College of Medical Technology, Kyoto University.  
Received July. 8, 1988

same, there will be a common mechanism in the fusion reaction, since the basic structure of biological membranes is lipid bilayers, and the principle to construct this structure is hydrophobic interaction, which exhibits no specificity among membrane components. Thus, close contact of two membranes and some perturbation of the bilayer structure may lead to a continuation of two membranes, *i.e.*, membrane fusion. But close apposition of two biological membranes does not always result in fusion of these membranes; therefore, there must also be mechanisms which suppress the occurrence of membrane fusion between biological membranes. And ways to break such suppression might be provided with the fusogenic agents.

The HVJ-induced cell fusion reaction discovered by Okada<sup>2)</sup> has served as a good experimental model for studies of membrane fusion, because in this system rapid and extensive membranes fusion can be attained under easily controllable conditions. Human erythrocytes are extensively used for the study of membrane fusion reactions. The fusion reaction of human erythrocytes induced by HVJ can be divided into several stages as depicted in Fig. 1.

Stage I of the reaction is cell agglutination. This stages is dependent on the pre-

sence of active HN (hemagglutinin and neuraminidase) protein on the surface of the virus particles, and the presence of virus receptors on the plasma membrane of the cell. Both glycolipids and glycoproteins which contain terminal N-acetylneuraminic acid are known to serve as binding sites for the HN spikes.

Stage II is viral envelope-cell membrane fusion. In this step, the viral contents (an RNA-protein complex called nucleocapsids) are released into the cells (Fig. 2). Concomitant with this step, hemolysis was induced except when a virus preparation harvested at a very early phase of infection of embryonated eggs is used. In the case of virus particles thus prepared, it has been known that freeze-thawing of such virus preparations activates hemolytic activity without affecting cell fusion-inducing activity<sup>4)</sup>. Thus, this phenomenon may depend on the leakiness of the virus envelope. This stage is highly dependent on the reaction temperature. At 20°C and lower, almost no reaction can proceed, whereas an increase in the reaction temperature above this level up to about 40°C enhances the reaction extensively. The presence of active F protein on the viral envelope is absolutely necessary for this step.

Stage III is cell-cell fusion of plasma membranes of adjacent cells.

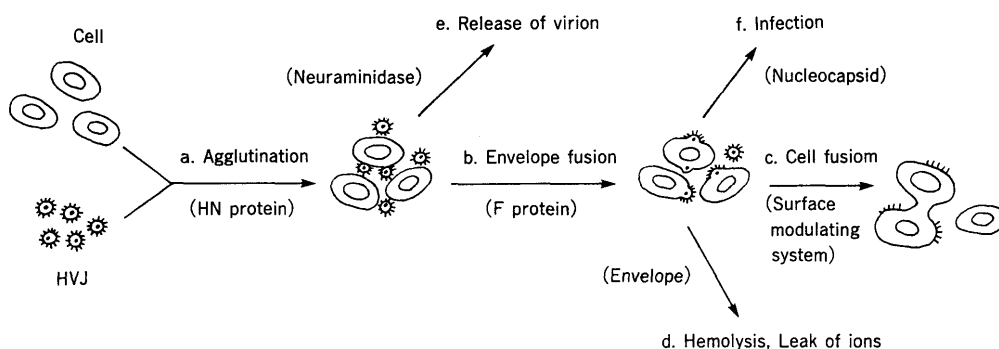


Fig. 1<sup>3)</sup>. Schematic representation of HVJ-induced cell fusion reaction

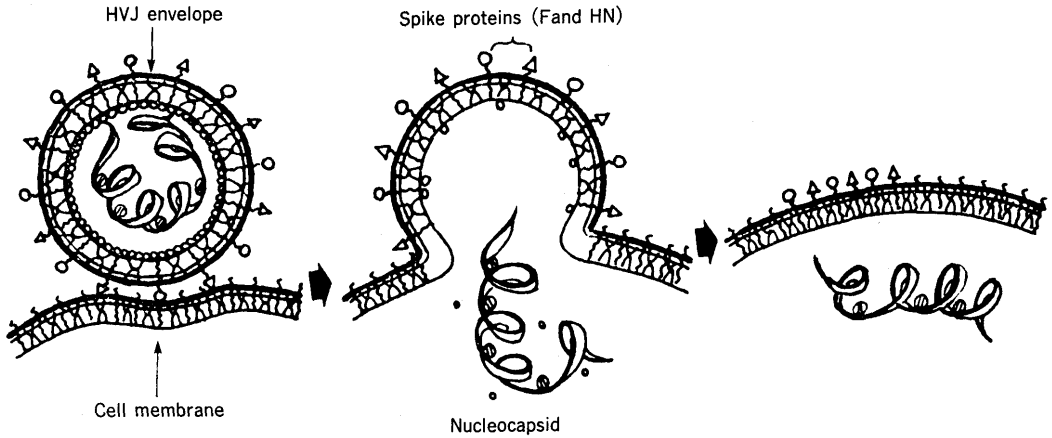


Fig. 2<sup>3)</sup> Schematic illustration of virus envelope-cell membrane fusion.

In this review, we will describe a possible molecular mechanism of the membrane fusion with the special reference to the fusion reaction between HVJ envelope and erythrocytes membrane.

1. *Structural Requirements for Hemolytic Activity of F Protein of HVJ*

The importance of F protein in the fusogenic activities of HVJ was first reported by Homma and his colleagues<sup>5)</sup>. During studies on host-dependent modification of HVJ, they found that virus preparations grown in L cells

are different from those grown in embryonated eggs. Although L cell-grown virus preparations exhibit hemagglutinating and neuraminidase activities and can infect embryonated eggs, neither fusogenic activities nor infectivity for cell cultures could be detected.

Comparing the chemical composition of egg-grown HVJ with L cell-grown HVJ, they found that two glycoprotein bands, F<sub>1</sub> and F<sub>2</sub>, were missing from the L cell-grown preparation, and that instead a new glycoprotein band (designated as F<sub>0</sub>) which molecular weight almost corresponded to the sum of F<sub>1</sub> and F<sub>2</sub>

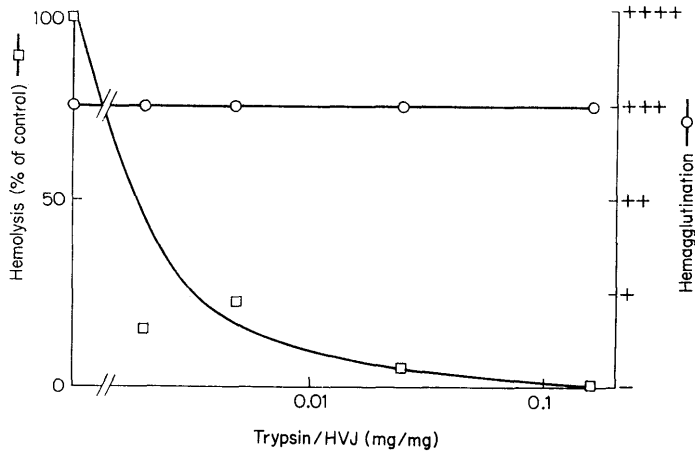


Fig. 3<sup>9)</sup>. Selective inactivation of hemolytic activity of HVJ by tryptic digestion

was present in L cell-grown virus preparations. They further found that limited proteolysis of L cell-grown samples with trypsin resulted in the activation of fusogenic abilities in parallel with the splitting of  $F_0$  to  $F_1$  and  $F_2$ .

Since no enzymatic activity was detected in F protein and since a rather high density on the surface of proteoliposomes was required for induction of hemolysis and cell fusion by reconstituted viral envelopes<sup>6)</sup>, we are tempted to speculate that F protein by itself interacts directly with the target cell membrane and induces structural perturbations which lead to membrane fusion.

This section describes effects of limited digestion of HVJ by different proteases on biological activities of the virus particles. From these results it can be concluded that the N-terminal hydrophobic region<sup>7,8)</sup> of  $F_1$  is exposed to the surrounding medium, and splitting off of the segment by either chymotrypsin or thermolysin digestion results in the complete cessation of the hemolytic activity of the virus particle.

When egg-grown HVJ was digested by trypsin, hemolytic activity was greatly reduced whereas hemagglutination was unimpaired<sup>9)</sup> (Fig. 3). Examination of virus particles treated with trypsin by SDS (sodium dodecylsulfate) gel electrophoresis under reducing conditions revealed that the  $F_1$  band disappeared after the digestion, and new bands designated as  $F_{1a}$  and  $F_{1b}$  appeared. No appreciable change can be detected in the HN and  $F_2$  bands. That trypsin digestion splits  $F_1$  into  $F_{1a}$  and  $F_{1b}$  was supported by the fact that the digested samples have F protein band when electrophoresed without splitting the disulfide bonds; therefore,  $F_{1a}$  and  $F_{1b}$  (and also  $F_2$ ) are still connected with interpeptide bond(s), and breaking the disulfide bonds with sulf-

**Table 1<sup>9)</sup>.** Properties of Fragments of F Protein.

Protein or Fragment	$M_r$	N-terminus
F ( $F_1$ -S-S- $F_2$ )	66,000	Phe
$F_1$	51,000	Phe
$F_2$	15,000	blocked
$F_{1a}$	32,000	Phe
$F_{1b}$	19,000	Ser > Ala

hydryl compounds yields three glycopeptides ( $F_{1a}$ ,  $F_{1b}$  and  $F_2$ ) from the digested F protein molecules.

Tight binding of these fragments of F protein to the viral envelope even after splitting the interchain disulfide bonds was shown as follows: trypsin-digested virus particles were incubated with 40 mM dithiothreitol and 8 M urea at 37°C for 1 h. After washing the samples two times with the same medium, the treated virus particles were subjected to SDS gel electrophoresis. No fragment was removed by such treatment, suggesting penetration of each individual fragment to the viral membrane, although the possibility of tight non-covalent binding by some membrane non-penetrating fragments to membrane-penetrating segments of F Protein cannot be entirely excluded.

To learn the origin of  $F_{1a}$  and  $F_{1b}$  fragments, F protein was purified from trypsin-digested virus particles, then subjected to SDS gel electrophoresis, and the bands corresponding to  $F_{1a}$  and  $F_{1b}$  were extracted, then the N-terminal amino acids were determined by the dansylation technique. Since  $F_{1a}$  retained the original N-terminus of  $F_1$ , as shown in Table 1,  $F_{1a}$  seemed to be derived from the N-terminal segment of  $F_1$ . Therefore,  $F_{1b}$ , which has either Ser or Ala as its N-terminus was assumed to be the C-terminal segment of  $F_1$ . From the apparent molecular weights of these two fragments ( $F_{1a}$ , 32,000 daltons; and

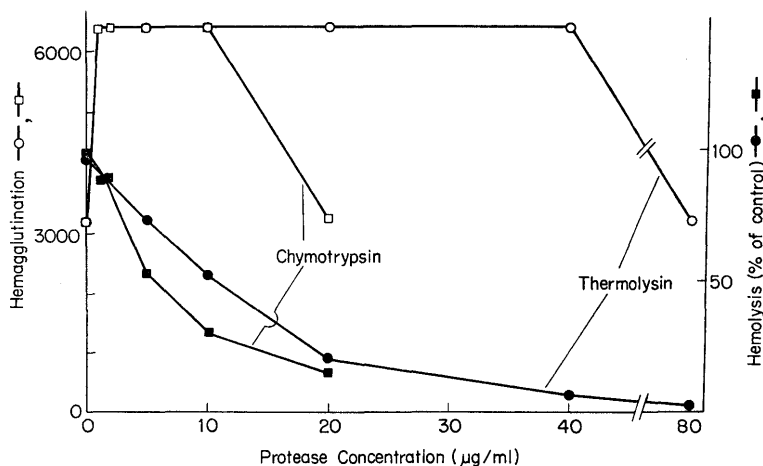


Fig. 4<sup>9)</sup>. Selective inactivation of hemolytic activity of HVJ by chymotrypsin or thermolysin

F<sub>1b</sub>, 19,000 daltons), there is no apparent loss of small fragments from F<sub>1</sub> upon tryptic digestion.

When egg-grown HVJ was digested with graded amounts of chymotrypsin or thermolysin, hemolytic activity was greatly impaired at low concentration of the protease. Hemagglutination was not reduced at these concentrations although digestion by higher concentrations of these proteases resulted in diminished hemagglutination activity of the treated virus particles (Fig. 4). Cell fusion inducing activity was also lost in parallel with hemolytic activity. Unlike tryptic digestion, no appreciable decrease in F<sub>1</sub>, estimated by staining with Coomassie brilliant blue, was detected by either chymotryptic digestion or thermolysin digestion. Even at very high concentrations of chymotrypsin, which split off almost all the HN protein, the F<sub>1</sub> and F<sub>2</sub> bands were not appreciably decreased.

Moreover, hybrid envelopes prepared with trypsin-inactivated virion (HN, active; F, inactive) and chymotrypsin-digested sample (HN, inactive; F, unknown) were active for hemagglutination but inactive for hemolysis<sup>10)</sup>.

Since hybrid envelopes similarly prepared with trypsin-inactivated sample and dithiothreitol-treated virion (HN, inactive; F, active) exhibited both hemagglutinating and hemolytic activity, it can be concluded that the F protein of chymotrypsin and thermolysin-digested samples had lost its biological activity without appreciably changing its content and molecular weight.

As the next step, we purified F protein from untreated and protease-treated virus particles. Close examination of the SDS gel electrophoretic behavior of these F protein preparations revealed that the molecular weight of F<sub>1</sub> has decreased by about 3,500 daltons after chymotryptic digestion, and by about 2,500 daltons after thermolysin digestion.

To confirm these results, we used the dansylation technique to determine the identities of the N-termini of the modified F<sub>1</sub>'s. Direct dansylation of the virus particles, followed by SDS gel electrophoresis and identification of the dansylated amino acids of each band were performed. The splitting of the N-terminal segment of F<sub>1</sub> by chymotrypsin and thermolysin, was evidently shown by newly

detected additional amino acid residues of Ala and Ile, respectively. Based on these results, it is tempting to speculate that the  $\text{NH}_2$ -terminal hydrophobic segment removed from the  $\text{F}_1$  subunit by chymotrypsin or thermolysin digestion is required for fusogenic activity of the protein, and also that the segment is exposed to the surface of the protein, and therefore, preferentially splits off with the proteases. Thus, the loss of an appreciable length of the N-terminal segment, by treatment with either chymotrypsin or thermolysin, may be correlated with the loss of biological activities of F protein.

Because the hydrophobic  $\text{NH}_2$ -terminal segment is still intact after inactivation by trypsin digestion, some explanations for this result were needed. Therefore, the surface iodination technique was applied to this system. F protein prepared from surface iodinated followed by trypsin digested virion did not lose the iodine label as expected, and the iodine label is almost equally distributed between  $\text{F}_{1a}$  and  $\text{F}_{1b}$ . Trypsin splitting of iodinated, then chymotrypsin-digested virus particles similarly produced  $\text{F}_{1a}$ - and  $\text{F}_{1b}$ -like fragments, but the iodine label in the  $\text{F}_{1a}$ -like fragments was very scant in this doubly-digested sample compared with that in the chymotrypsin-untreated sam-

ples.

On the other hand, iodination of virion after trypsin digestion resulted in very low iodination of the  $\text{F}_{1a}$  fragment, although the iodination level of  $\text{F}_{1b}$  and the other surface components did not decrease. This could be explained easily if we assume that higher-order structures of F protein are changed after trypsin digestion, and therefore, the hydrophobic  $\text{NH}_2$ -terminal segment is buried in the hydrophobic interior of the protein. The above assumption was supported also by circular dichroism measurement in the far ultraviolet region of F, trypsin digested F and thermolysin digested F proteins. Similar changes of the spectra in the secondary structure were also found in the case of proteolytic activation of  $\text{F}_0$  to  $\text{F}_1$  and  $\text{F}_2^{1D}$ . Fig. 5 illustrates the structure of several derivatives of F protein described above.

## 2. Fusogenic Sequences of Enveloped Virus

Hydrophobic nature of the N-terminal portion of  $\text{F}_1$  subunit of F protein is known to be a common feature with all fusogenic paramyxoviruses so far studied<sup>7,8,12</sup>. Furthermore, this segment of F protein of HVJ seems to be exposed on the protein surface despite the hydrophobic nature of this portion as described

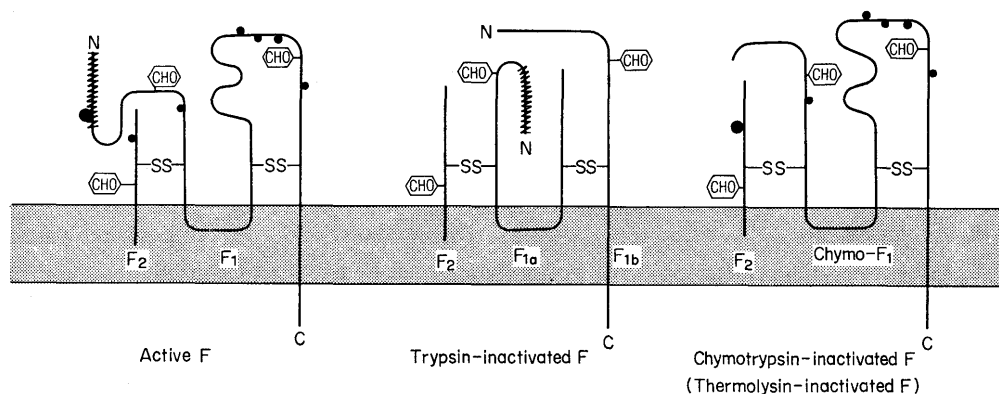


Fig. 5<sup>3</sup>. Model of several derivatives of F protein

Table 2<sup>13,14</sup>. Comparison of Hydrophobic Sequences of Viral Glycoproteins.\*

strain	signal	fusogenic	transmembrane
<b>I. Paramyxoviridae</b>			
<b>1. paramyxoviruses</b>			
HVJ (Z)	MTAVIQRSQCISTSLVVLTTLVSCQIPRD	FFGAVIGTIALGVATSAQI TAGIALA	TVITHVVMVLLVVHIVHIVLVYRLRR
SV5	MGTHIQGLVVSCLLAGASLD	FAGVVIGLAAALGVATAAQV TAAVALV	VLSHAIICLSGLGLIHLLSVVVWKLK
NDV-MIYA	MASRSSTRIPAPLMLTWIALALGCVE	FIGAIHGSVALGVATAAQI TAASALIQA	RLTNTSALITYLVLTVISLVCGLSLVLACYLMHK
NDV-AVIC	MGPRSSTRIPPLMLTIRIALALSCVH	FIGAIHGSVALGVATAAQI TAASALIQA	KLTSTSAITVIALTAISLVCGLSLVLACYLMVK
human P13	MPTSILLIITMIMA SFCQID	FFGGVIGTIALGVATSAQI TAAVALV	HQSSTTHIIVLIMHILFHNVTHIAVKVYR
Mumps		FAGIAIGIAALGVATAAQV TAAV	
<b>2. morbilliviruses</b>			
Measles	MSIMGLKVNVS AIFMAVLLTLQTPTGQIH	FAGVVLAGAALGVATAAQITAGIAL	KGLSSTSIVYILIAVCLGGLIGIPALICCCGR
canine DV		FAGVVLAGAALGVATAAQITAGIAL	
<b>3. pneumovirus</b>			
Res. Syn. V	MELLILKANAITTILTAVTFCFAS	FLG FLLGVGSAI ASGVAVS	KSTTNIMITTHIIVHIVILLSLIAVGLLLKAR
<b>II. Influenza viruses</b>			
A/PR/8/34	MKANLLVLLCALAAADA	GLFGAIAAGFIEGGWTGMIDGW YGYHH	ILAIYSTVASSLVLLVSLGATSFWMC
A/JAP/305/57	MAIHYLILLFTAVRG	GLFGAIAAGFIEGGWQGMVDGWYGYHH	ILAIYATVAGSLSLAIMMAGISFWMC
A/AICHI/2/68	MKTIALSYIFCLALAG	GLFGAIAAGFIENGWEGMIDGW YGFRH	ILWISFAISCFLLCVVLL GFIMWAC
A/FPV/ROSTOCK/34	MNTQILVFALVAVIPTNA	GLFGAIAAGFIENGWELVDGW YGFRH	ILWFSFGASCFLLLAIAV GLVFICV
B/LEE/40	MKA-IVLL-MVVTNSA	GFFGAIAAGFIEGGWEGMIAGW HGYTS	ILLYYSTAAASSLAVTLMTAIFIVYMV
C/Cal/78	MFFSLLMLGLTEA	IFGIDDLIIGLLFVAIVEAGGYL LGS	FYWWGSSGLAITAANLMAALVISGIA
<b>III. Vesicular Stomatitis Viruses</b>			
NEW JERSEY	MLSYLIFALAVSPILGK	KDGVSNPFGFPQSCGYGTVD <sup>AE</sup> A	SSLMGVLAHVIGFVILMFLIK
INDIANA	MKCLLYLAPLPIGVNCK	KQGIWLNPGFPQSCGYATVD <sup>AE</sup> A	SSIASFFFHIGLHGLFLVLR
<b>IV. TOGA viruses</b>			
SINDBIS		DYTCKVFGGVYYPF WGAQCFC <sup>DSE</sup> N	LFQGASSLLIIGLMIFACSMMLTST
SFV		DYQCKVYVTGVYPFMWGGA <sup>YCFDSE</sup> N	ISGGLGA FAIGAILVLVVTCIGL
<b>V. RETRO viruses</b>			
MMTV (MOUSE, B)	KTSQTPQNSLTLFLALLSVLGGPPPVGT	FVAAILGISALIAHTSFAVATTALVK	WTQYFIFIGVGLLLVIVL MIFPIVFQ CLAK
MoMLV (MOUCE, C)	KNKVNP <sup>R</sup> GPLIPLILLMLRGVST	EPVSLTLALLLGGGLTMGGIAAGIGTGTALMATQAFQQL	SPWFTTLISTIMGPLIVLL MILLFGP CLNR
AKV (MOUCE, C)	KNQVNPWGPLIVLLILGGVNP	EPVSLTLALLLGGGLTMGGIAAGVGTGTALVATQQFQQL	SPWFTTLISTIMGPLIHL LILLFGP CLNR
FMLV (MOUSE, C)	RDLLIPLILFLSLK <sup>GARS</sup>	EPVSLTLALLLGGGLTMGGIAAGVGTGTALVATQQFQQL	SPWFTTLISTIMGPLIHL LILLFGP CLNR
ATLV (HUMAN, C)	MGKFLATLILFFQFCPLIFGD	AVPVAVVWLVSALAMGAGVAGGITGSM <sup>SLASGK</sup>	ALQTGITLVALLLVILAG PCILR

\* Charged residues were underlined.

in the preceding section. These properties were taken as the evidence for the direct interaction of the segments with the target cell membranes, and for the participation in the fusion reaction. Therefore, these were sometimes called fusogenic domain.

It was pointed out previously, however, that the homology of such putative fusogenic segments between F proteins of the paramyxoviruses is very high, compared with other hydrophobic stretches of the same F proteins, such as signal peptides and transmembrane sequences as shown in Table 2. In case of influenza viruses, the highest (about 79%) homology was observed for fusogenic sequences of A-type viruses, whereas transmembrane sequences showed only a marginal homology and signal peptide was devoid of homology. A similar tendency was also observed within rhabdo viruses, toga viruses and retro viruses.

Murine leukemia viruses showed a rather high homology for their transmembrane sequences, but the homology is still higher for fusogenic sequences. This might mean that these viruses have common ancestor and they are differentiated into respective strains not so long ago on the genetic (or evolutionary) time scale, compared with the other viruses. Toga virus E1 glycoproteins have about 60 residue long putative signal sequences which are quite different from the other signal peptides, but rather have some similarity with the leader peptides of mitochondrial proteins, therefore, they are not cited in the Table 2. Interestingly, ATLV of human origin showed some homology with Friend murine leukemia virus. The very high homology in fusogenic sequences compared with other hydrophobic sequences might suggest that this hydrophobic sequence may be required not only for the simple hydrophobic interaction with the

lipid bilayers but also for the specific interaction with other membrane component(s)<sup>159</sup>.

### 3. Mechanism of Membrane Fusion Reaction

Direct contact of F<sub>1</sub> subunit with the target cell membrane was shown by using photoaffinity labeling experiments. For the study of the possibility, lipid soluble photoaffinity labels were incorporated into the plasma membrane, and after contact with the virus particles at ice-cold temperature or 37°C, photolysis was performed and cross-linking of these lipidic labels to HN and F protein were studied. Among the several labels tested, the results with the smallest label employed, *i.e.* <sup>125</sup>I-TID [3-trifluoromethyl-3-(m(<sup>125</sup>I)iodophenyl)diazirine], will be described. This label was incorporated into resealed ghosts of human erythrocytes, washed and then mixed with HVJ. Photolysis was performed either at ice-cold condition or after incubation at 37°C for 10 min. Then samples were solubilized with 1% Triton X-100, centrifuged and the resultant supernatants were applied to SDS gel electrophoresis either directly or after precipitation with anti-F or anti-HN antibodies. HN protein was cross-linked with the photoaffinity label at both temperatures. Similarly, labeling of F protein was detected either at ice-cold temperature or 37°C by the reagent. These results suggest that F protein are in close contact with the target cell membrane even under ice-cold conditions at which no fusion reaction was known to proceed.

Now, the prerequisites seem to be fulfilled to consider a possibility that the fusogenic sequence of F<sub>1</sub> may have a specific affinity to some membrane lipids. As a candidate of such compounds, cholesterol seems to be most promising, since it was reported that cholesterol is required for HVJ-induced fusion reaction of



proteoliposomes containing glycoporin. Low pH-induced fusion of liposomes with Semliki forest virus was also reported to require cholesterol or other sterols with 3- $\beta$ -hydroxyl group<sup>12,16</sup>.

Furthermore, HVJ-induced lysis of the liposomes under an influence of intact F protein was reported to be enhanced by the presence of cholesterol in the liposomes<sup>17</sup> although effect of lipid composition of the liposomes on fusion efficiency is much more complicated, and the cholesterol effect is not always observed<sup>16</sup>. Thus, binding of cholesterol with the fusogenic sequence of F protein was suspected<sup>13,15</sup>.

At the same time, it was hypothesized that the inhibition of the virus-cell membrane fusion with the analogues of N-terminal portion of the F<sub>1</sub> subunit might be due to the binding of these analogues with this fusogenic sequence. At first, the binding of cholesterol with purified F protein and the action of

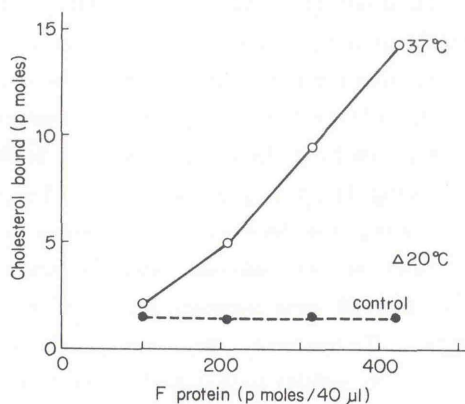


Fig. 6<sup>15</sup>. Binding of cholesterol to F protein. control: cholesterol was added after the incubation with anti-F

inhibitory peptides on virions were substantiated by us<sup>13,15</sup>, and then evidences for the binding of these compounds with the fusogenic sequence were obtained<sup>20</sup>.

4. *Binding of Cholesterol with Fusogenic Hydrophobic Sequences of F Protein.*

Complex Formation of N-terminus of F<sub>1</sub> with Cholesterol

N-terminus: N-PhePheGlyAlaValIleGlyThrIleAlaLeuGlyValAlaThrAlaThrAlaAlaGlnIleThr-

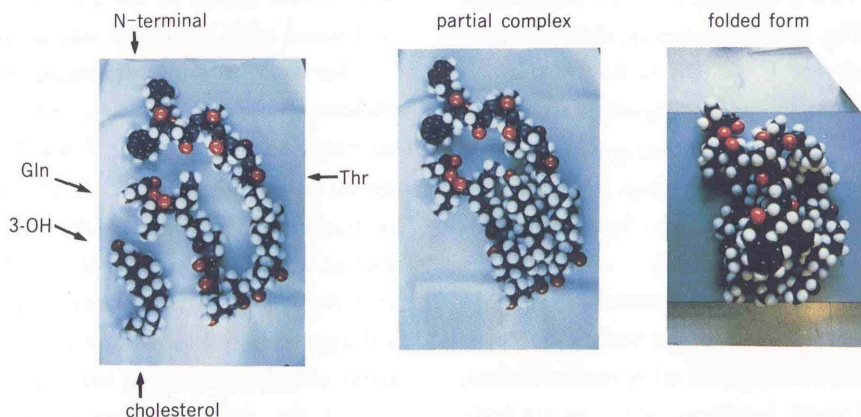


Fig. 7<sup>13</sup>. Molecular models of complexes of fusogenic domain of HVJ with cholesterol. Black: carbon, White: hydrogen, Blue: nitrogen, Red: oxygen, Yellow: sulfur or phosphorus.

In order to obtain experimental proofs, specific binding of  $^3\text{H}$ -cholesterol to purified F protein was measured after stopping the reaction by adding the anti-F antibody and exhaustive washing the complex with a buffer containing Triton X-100. As shown in Fig. 6, no binding was detected if the mixture was incubated at  $20^\circ\text{C}$ , whereas extensive binding was observed after incubation at  $37^\circ\text{C}$  for 2 hours. Temperature dependency observed here is very similar to that of the overall envelope fusion reaction<sup>19</sup>. Although saturation of F protein with cholesterol is difficult to attain with several technical problems, subquantitative binding of cholesterol was attained. This binding was stable even after washing with serum albumin-containing buffer.

To know what part of the F protein can interact with cholesterol, Stuart-type molecular models of N-terminal domain of  $\text{F}_1$  and its cholesterol complex were constructed. By postulating hydrogen bond formation between  $3\text{-}\beta\text{-hydroxyl}$  of cholesterol and amide group of  $^{18}\text{Gln}$  of HVJ sequence, and hydrophobic interaction between the rest of the cholesterol structure and the fusogenic amino acids sequences, such a complex could be constructed easily<sup>13</sup> (Fig. 7). Consequently, the cholesterol binding site of F protein could be this N-terminal domain, and reorganization of the domain to its cholesterol binding form could be a temperature dependent step. Cholesterol-peptide complex can be also be formed with SFV (Semliki Forest Virus).

Rate of binding of cholesterol to F protein can not 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

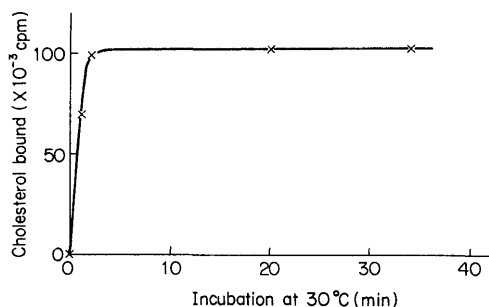


Fig. 8<sup>20</sup>. Effect of incubation time on binding of cholesterol of F-protein.

cholesterol was developed utilizing charge modified nylon membrane filters. By this method, binding of cholesterol with F protein could be stopped with relatively short duration of incubation. As shown in Fig. 8, binding reached nearly saturated level only after 2 min 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<sup>19,21</sup>.

##### 5. *Binding Specificity of the Sterols with F Protein*

To manifest the binding specificity of different sterols with F protein, effect of dilution with several sterols on the binding of labelled cholesterol with F protein was studied.

For competition experiments, radioactive cholesterol concentration was set at partially saturated concentration of  $7.8 \mu\text{M}$ , and cold sterols were added to reach a saturation concentration of  $38.8 \mu\text{M}$ . As shown in Table 3 Part(a), addition of cold cholesterol to increase cholesterol concentration from  $7.8 \mu\text{M}$  to  $38.8 \mu\text{M}$  apparently decreased the amount of radioactive cholesterol binding, indicating that dilution of the radioactive species occurred, although calculated amount of total bound cholesterol did not decrease. When same amount of  $5\alpha\text{-cholestane-3-one}$  or ergosterol were added,

**Table 3<sup>20</sup>** Effect of Cold Sterol Addition on Binding of Cholesterol with F Protein

Part (a)				
Cold sterol added to reaction medium	Total sterol concentration ( $\mu\text{M}$ )	Bound cholesterol		
		cpm	cpm (%)	amounts (%)
cholesterol	7.8	39,126	(100)	(100)
+cholesterol	38.8	8,136	21	105
+5 $\alpha$ -cholestane-3-one	38.8	8,489	22	—
+ergosterol	38.8	9,791	25	—

Part (b)			
Exp. No.	Washing medium	Bound cholesterol	
		cpm	(%)
I	Triton X-100 only	33,786	(100)
	+cholesterol	18,825	56
	+5 $\alpha$ -cholestane-3-one	13,918	41
	+5 $\alpha$ -cholestane (A/B-trans)	13,071	39
	+phosphatidylethanolamine	28,156	83
II	Triton X-100 only	104,905	(100)
	+cholesterol	67,215	64
	+tetrahymanol	53,153	51
	+digitonin	75,381	72
	+5 $\beta$ -choestane (A/B-cis)	96,927	92
	+squalene	88,029	84

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 (1  $\mu\text{g}/\text{ml}$ ). As shown in Table 3 Part(b), addition of cholesterol replaced the

radioactive cholesterol on F protein. Similar replacement was observed with 5 $\alpha$ -cholestane-3-one, 5 $\alpha$ -cholestane (A/B trans) and tetrahymanol, and with digitonin to a lesser extent, whereas 5 $\beta$ -choestane (A/B cis), squalene and phosphatidylethanolamine were almost inactive in the replacement.

#### 6. Cholesterol Binding Site of F Protein

To check the previous postulation that cholesterol binds to the fusogenic domain of F protein (which locate in N-terminal segment of the F<sub>1</sub> subunit) rather specifically, we examined whether cholesterol specifically bind with non-fusogenic 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<sup>6</sup>. This

**Table 4<sup>20</sup>** Binding of Cholesterol to F, Thermolysin-truncated F and HN Proteins

Glycoproteins	Bound cholesterol	
	cpm	(%)
Intact F	10,550	(100)
Thermolysin-digested F	4,829	46
Thermolysin-digested F + Z-D-Phe-Phe-Gly	2,253	21
Intact HN	0	0

protein showed no cholesterol binding activity (Table 4).

When F protein was digested with thermolysin to remove a 2,500 dalton segment from the N-terminus of F<sub>1</sub> subunit, it simultaneously lost its fusogenic activity<sup>9)</sup>, and this modification greatly diminished the cholesterol binding ability. Remaining binding activity may be due to the incomplete digestion of F<sub>1</sub> subunit which is usually observed in this type of experiment, 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 are comparison of circular dichroism spectra of F and thermo-F proteins. The difference of these spectra was as little as expected from the fact that roughly 26 residues were splitted off from the N-terminal of F<sub>1</sub> by thermolysin digestion and remaining structure may keep the native conformation.

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<sub>1</sub> subunit of F protein<sup>22)</sup>, only one (or a few nearby) residue is sensitive to trypsin digestion of intact F protein<sup>9)</sup>. Therefore, limited digestion by trypsin can be used for detection of small conformational changes of F protein. From the experimental results, degree of digestion of F and thermo-F proteins which was depicted by appearance of F<sub>1a</sub> and F<sub>1b</sub> fragments was almost the same under the condition where undigested F<sub>1</sub> or thermo-F<sub>1</sub> was still remaining. Thus, induction of gross conformational change by truncation

of N-terminal portion of F<sub>1</sub> with thermolysin is unlikely. Therefore, an experimental support for the specific binding of cholesterol with the N-terminal hydrophobic portion of F<sub>1</sub> is now offered.

Moreover, it was reported that hydrophobicity of the fusogenic domain of F protein is much lower than transmembrane sequences of F protein and other transmembrane protein<sup>23)</sup>. Our calculation according to Kyte & Doolittle<sup>24)</sup>, or Hoop & Woods<sup>25)</sup> also confirmed this conclusion. This result, as well as the fact that 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 hypothesis that this fusogenic sequence of F<sub>1</sub> subunit specifically interacts with cholesterol seems to be reasonable.

Cholesterol concentration required for binding was rather low (half-maximal binding was observed at 4-5  $\mu$ M) under artificial Triton X-100 containing conditions. Since concentration of cholesterol in plasma membranes is very high (about 20% w/w), 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 is substantiated.

In case of sterol binding with F protein, requirement of 3-hydroxylgroup or long hydrophobic tails was not observed as above, unlike binding of sterols with several other membrane-penetrating proteins, such as streptolysin O<sup>26)</sup>, or perfrinolysin<sup>27)</sup>. But this binding is specific in a sense that the binding ability of sterol-related compounds depends on the presence of cholesterol-uncleus or three-

Complex Formation of N-terminus of F<sub>1</sub> of HVJ with Inhibitory Peptides

N-terminus: N-Phe-Phe-Gly-(Ala-Val-.....)

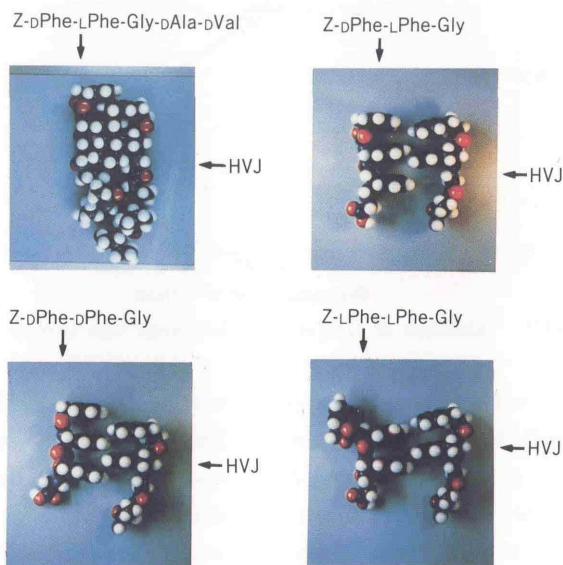


Fig. 9<sup>13)</sup>. Complex formation of inhibitory peptides of virus infection with amino terminal segment of F<sub>1</sub> of HVJ

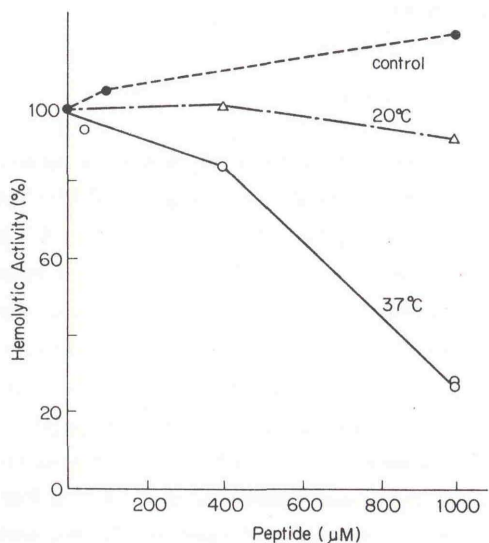


Fig. 10<sup>15)</sup>. Inhibition of hemolytic activity of HVJ by preincubation with Z-D-Phe-Phe-Gly. control: minuspincubation with the peptide

dimensionally similar A/B ring *trans* structure, *i.e.* the A/B ring *cis* compound and squalene without ring structure showed no binding affinity or even so it was greatly reduced. Thus, the binding specificity of F protein for sterols is different from any other cases 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<sup>28)</sup> is entirely different from that of HVJ F protein.

7. Inhibition of HVJ-induced Hemolysis with Peptide Derivatives

Richardson *et al.*<sup>28,29)</sup> reported previously that some peptide derivatives analogous to the N-terminal portion of the F<sub>1</sub> subunit, such as

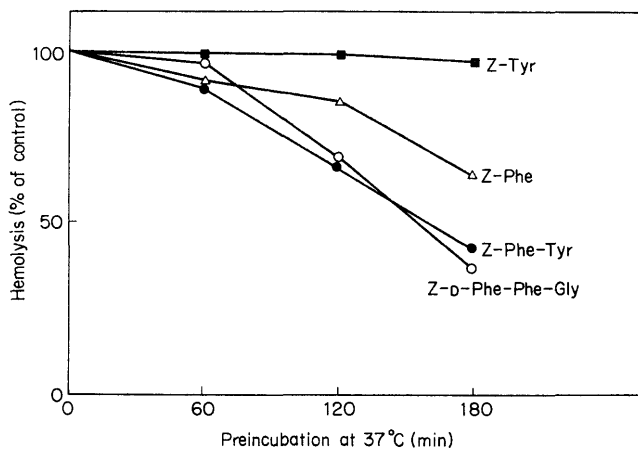


Fig. 11<sup>20)</sup>. Inhibition of HVJ-induced hemolysis with peptide derivatives

Z-D-Phe-phe-Gly (Z: carbobenzyoxy; amino acyl residues were usually L-configuration, but in case of D-configuration they were preceded with D) show an inhibitory effect on paramyxovirus infection. From the similarity of the structure of these inhibitors with amino terminus of F protein, they postulated the presence of a cellular receptor for amino terminus of F<sub>1</sub> and competition of binding to this receptor works as a mechanism of inhibition. However, this hypothesis can not easily explained following results that carbobenzyoxy (Z) group attached to amino terminus is required for efficient inhibition and peptides containing D-amino acids are more inhibitory than those containing only L-isomers.

Thus, a similar approach described in the previous section was taken for studies on the mechanism of inhibition of the fusion reaction with a peptidic inhibitor of paramyxoviruses. We made molecular models of inhibitory peptides, and found out that those with a high inhibitory activity can make a seemingly stable complexes with amino terminal segment of F<sub>1</sub> (Fig. 9). But in case of less active Z-Phe-Phe-Gly and azidobenzoyl-D-Phe-Phe-Gly complex formation seems to be inefficient. To test our

new hypothesis, HVJ was incubated with Z-D-Phe-Phe-Gly at different temperatures, pelleted and resuspended in fresh incubation buffer without an inhibitor and the hemolytic activity was determined. As depicted in Fig. 10, hemolysis (due to envelope fusion) was almost completely inhibited at 1 mM only when incubation was done at 37°C. Incubation at lower temperatures did not result in any appreciable inhibition.

To study the interaction site between the inhibitory peptide and viral component, we searched for effective peptide(s) among available peptide derivatives which may be labeled with iodine. As shown in Fig. 11, Z-Phe-Tyr was found to be inhibitory, whereas, Z-Tyr did not show inhibitory effect which may serve as controls. Since prolonged incubation was necessary to obtain the appreciable inhibition with Z-D-Phe-Phe-Gly, effect of incubation with Z-Phe Tyr was compared with other peptides. Time course of the Z-Phe-Tyr inhibition enhancement was similar to that of Z-D-Phe-Phe-Gly, whereas prolonged incubation with Z-Tyr did not result in detectable inhibition.

Dose response curve of Z-Phe-Tyr was similar to that of Z-D-Phe-Phe-Gly. Further-

more, Z-Phe-Tyr or Z-D-Phe-Phe-Gly at 1 mM, exhibited no effect (neither inhibitory nor stimulatory) on hemagglutination reaction of HVJ under the same condition for detection of hemolysis inhibition. Thus, Z-Phe-Tyr was selected as a candidate for the iodination studies. Preliminary study showed that a monoiodinated derivative, Z-Phe-MITyr, was inhibitory to the hemolytic reaction.

#### 8. *Binding of Iodinated Derivatives of Z-Phe-Tyr with HVJ virions and Purified F protein*

Radioiodinated peptides (Z-Phe-MITyr and Z-Phe-DITyr) were prepared, and were incubated at 30°C for 2 h with intact virions or purified F protein, and the bound peptide was quantified by a radioactivity measurement. From the results the peptide was shown to bind to both virion and F protein. 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 (data not shown). Assuming that the affinity of Z-Phe-Tyr is similar to those of Z-Phe-MITyr and Z-Phe-DITyr, amount of bound peptides to the target can be calculated at each concentration. The resultant concentration-dependency of the binding which 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,

**Table 5<sup>20</sup>.** Binding Specificity of Z-Phe-MITyr with HVJ Glycoproteins

Glycoprotein	amount (μg)	Bound Peptide (cpm)
F protein	24	792
HN protein	28	106

proper controls are necessary. As such control, binding of Z-Phe-MITyr to HN protein which has a hydrophobic transmembrane domain was measured. As shown in Table 5, HN protein showed only a marginal binding with Z-Phe-MITyr.

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 F<sub>1</sub> subunit being the binding site of inhibitory peptides from the molecular modeling was not yet clear. Thus, binding of the radioactive peptides with modified F protein which lacks the N-terminal portion of F<sub>1</sub> subunit was measured. As summarized in Table 6, binding of

**Table 6<sup>20</sup>.** Effect of Protease Treatments on Z-Phe-MITyr and Z-Phe-DITyr<sup>a</sup> Binding Ability of HVJ Virions and Purified F Protein

Virion	Bound Peptides	
	Z-Phe-MITyr cpm	Z-Phe-DITyr cpm
Untreated	4,689	6,266
Chymotrypsin-truncated	2,523	3,742
Thermolysin-truncated	1,973	3,123
Protein (pmol)	Bound Peptides/Protein	
	Z-Phe-MITyr	Z-Phe-DITyr (mol/mol)
F 164	0.24	0.13
F 329	0.23	0.24
Thermo-F 71	0.05	0

<sup>a</sup>: diiodotyrosine

the peptides with chymotrypsin- or thermolysin-truncated virions were substantially decreased. Furthermore, interaction of the peptides with purified thermolysin-truncated F protein drastically decreased, although interaction of anti-F antibody with the modified F protein did not appreciably change (data not shown).

#### 9. *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 domain of the F<sub>1</sub> subunit, binding of these two compounds to F protein may (or may not) be affected by each other. Accordingly, effect of a preincubation with inhibitory peptides on cholesterol binding was studied. As shown in Table 7, cholesterol binding was appreciably inhibited by pretreatment with inhibitory peptides, whereas effect of non-inhibitory Z-Gly-Phe was negligible.

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 ring<sup>13,15</sup>. Afterwards, 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

have appeared<sup>30,31</sup>. Although our previous models which employed parallel packing of the aromatic rings that may also be favorable for the carbonyl oxygen-aromatic interaction<sup>32</sup>, it was equally possible to construct the "herringbone" packing network of five aromatic rings which are clustered at the N-terminal of F<sub>1</sub> subunit and at the inhibitory peptides. Enhancement of the inhibition by introduction of carbobenzyoxy 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 2,500 daltons of F<sub>1</sub> 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, although no such drastic dissociation with Triton X-100 was observed with complex of non-aromatic ring containing cholesterol and F protein. Moreover, it is interesting in this respect that non-inhibitory Z-Gly-Phe, Z-Glu-Phe and Z-Glu-Tyr have more space in-between the aromatic rings than that of inhibitory peptides.

#### *FINAL REMARKS*

Taken these results into consideration, it can be said that a direct participation of the N-terminal fusogenic sequence of F<sub>1</sub> and a direct interaction of this domain with membrane cholesterol in the membrane fusion reaction between viral membrane and other cell membranes are further substantiated.

Some comments may 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 reaction in a sense that this reaction requires intactness of two viral pro-

**Table 7**<sup>20</sup>. Effect of Preincubation with Peptide Inhibitors on Cholesterol Binding of F-Protein

Addition	Concentration	Inhibition (%)
DMSO <sup>a</sup> control	—mM	(0)
Z-D-Phe-Gly	1.6	42
Z-Phe-Tyr	1.7	23
Z-Phe	2.7	23
Z-Gly-Phe	2.4	4

<sup>a</sup>: dimethylsulfoxide as solvent



teins, F and HN. Thus, we think that the notion described above, *i.e.* specific binding of a membrane protein (F protein in this case) with cholesterol of target membrane may be important for fusion reaction, can not 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 was provided. It is also known that some types of liposomes can fuse together quite efficiently under appropriate conditions without 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.

#### ACKNOWLEDGEMENTS

I would like to thank Prof. Yoshio Okada and Prof. Takashi Uchida of Institute for Molecular and Cellular Biology, Osaka University, for their kind help in the preparation of HVJ and valuable discussions. I am also indebted to Prof. Akira Asano of Institutes for Protein Research, Osaka University, for his helpful collaboration and discussion.

#### REFERENCES

- 1) Poste, G.: Mechanisms of virus-induced cell fusion. *Int. Rev. Cytol.* 33: 157-252, 1972.
- 2) Okada, Y.: The fusion of Ehrlich's tumour cells caused by HJV virus *in vitro*. *Biken J.* 1: 103-110, 1958.
- 3) Asano, A. & Asano, K.: Mechanism of HVJ-induced cell fusion. In *Structure, Dynamics and Biogenesis of Biomembranes*, ed. Sato, R. & Ohnishi, S., p. 57-78, 1982.
- 4) Homma, M., Shimizu, K., Shimizu, Y. K. & Ishida, N.: On the study of Sendai hemolysis. Complete Sendai virus lacking in hemolytic activity. *Virology* 71: 41-47, 1976.
- 5) Homma, M. & Ohuchi, M.: Trypsin action on the growth of Sendai virus in tissue culture. III. Structural difference of Sendai virus grown in egg and tissue culture cells. *J. Virol.* 12: 1457-1465, 1973.
- 6) Ozawa, M. & Asano, A.: The preparation of cell fusion inducing proteoliposomes from purified glycoproteins of HVJ (Sendai virus) and chemically defined lipids. *J. Biol. Chem.* 256: 5954-5956, 1981.
- 7) Gething, M. J., White, J. M. & Waterfield, M. D.: Purification of the fusion protein of Sendai virus: analysis of the NH<sub>2</sub>-terminal sequence generated during precursor activation. *Proc. Nat. Acad. Sci. USA.* 75: 2737-2740, 1978.
- 8) Richardson, C. D., Sheid, A. & Choppin, P.: Specific inhibition of paramyxovirus replication by oligopeptides with amino acid sequences similar to those at the N-termini of the F<sub>1</sub> or HA<sub>2</sub> viral polypeptides. *Virology* 105: 205-222, 1980.
- 9) Asano, K., Murachi, T. & Asano, A.: Structural requirement for hemolytic activity of F-glycoprotein of HVJ (Sendai virus) studied by proteolytic dissection. *J. Biochem.* 93: 733-741, 1983.
- 10) Ozawa, M., Asano, A. & Okada, Y.: Biological activities of glycoproteins of HVJ (Sendai virus) studied by reconstitution of hybrid envelope and by concanavalin A-mediated binding: a new function of HANA protein and structural requirement for F protein in hemolysis. *Virology* 99: 197-202, 1979.
- 11) Hsu, M.-C., Scheid, A. & Choppin, P.: Activation of the Sendai virus protein (F) involves a conformational change with exposure of a new hydrophobic region. *J. Biol. Chem.* 256: 3557-3563, 1981.
- 12) White, J., Kielian, M. & Helenius, A.: Membrane fusion proteins of enveloped animal viruses. *Quarterly Reviews of Biophysics* 16: 151-195, 1983.

- 13) Asano, A. & Asano, K.: Molecular mechanism of virus entry to target cells. *Tumour Research* 19: 1-20, 1984.
- 14) 浅野 朗, 浅野仁子: ウイルス糖タンパク質の構造と膜融合と AIDS, *細胞工学* 6(10): 781-787, 1987.
- 15) Asano, K. & Asano, A.: Why is a specific amino acid sequence of F glycoprotein required for the membrane fusion reaction between envelope of HVJ (Sendai virus) and target cell membranes? *Biochem. Int.* 10: 115-122, 1985.
- 16) White, J. & Helenius, A.: pH-dependent fusion between the Semliki Forest virus membranes and liposomes. *Proc. Nat. Acad. Sci. USA.* 77: 3273-3277, 1980.
- 17) Kundrot, C. E., Spangler, E. A., Kendall, D. A., Macdonald, R. C. & Macdonald, R. I.: Sendai virus-mediated lysis of liposomes requires cholesterol. *Proc. Nat. Acad. Sci. USA.* 80: 1608-1612, 1983.
- 18) Klappe, K., Wilschut, J., Nir, S. & Hoekstra, D.: Parameters affecting fusion between Sendai virus and liposomes, role of viral protein, liposome composition and pH. *Biochemistry* 25: 8252-8260, 1986.
- 19) Maeda, T., Asano, A., Ohki, K. & Ohnishi, S.: A spin-label study on fusion of red blood cells induced by Hemagglutinating Virus of Japan. *Biochemistry* 14: 3736-3741, 1975.
- 20) Asano, K. & Asano, A.: Binding of cholesterol and inhibitory peptide derivatives with the fusogenic sequence of F-glycoprotein of HVJ (Sendai virus): Possible implication in the fusion reaction. *Biochemistry* 27: 1321-1329, 1988.
- 21) Sekiguchi, K. & Asano, A.: Participation of spectrin in Sendai virus-induced fusion of human erythrocyte ghosts. *Proc. Nat. Acad. Sci. USA.* 75: 1740-1744, 1978.
- 22) Miura, N., Ohtsuka, E., Yamaberi, N., Ikehara, N., Uchida, T. & Okada, Y.: Molecular cloning of a full-length cDNA encoding the hemagglutinin-neuraminidase (HN) glycoprotein of Sendai virus (HVJ). *FEBS Lett.* 188: 112-116, 1985.
- 23) Davis, N. G. & Hsu, M.-C.: The fusion related hydrophobic domain of Sendai F protein can be moved through the cytoplasmic membrane of *Escherichia coli*. *Proc. Nat. Acad. Sci. USA.* 83: 5091-5095, 1986.
- 24) Kyte, J. & Doolittle, R. F.: A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* 157: 105-132, 1982.
- 25) Hoop, T. P. & Woods, K. R.: Prediction of protein antigenic determinants from amino acid sequences. *Proc. Nat. Acad. Sci. USA.* 78: 3824-3828, 1981.
- 26) Johnson, M. K., Geoffroy, C. & Alouf, J. E.: Binding of cholesterol by sulfhydryl-activated cytolysins. *Infect. Immunol.* 27: 97-101, 1980.
- 27) Hase, J., Mitsui, K. & Shonaka, E.: Clostridium perfringens exotoxins. IV Inhibition of the  $\theta$ -toxin induced hemolysis by steroids and related compounds. *Jpn. J. Exp. Med.* 46: 45-50, 1976.
- 28) Rice, C. M. & Strauss, J. H.: Nucleotide sequence of the 26 mRNA of Sindbis virus and deduced sequence of the encoded virus structure proteins. *Proc. Nat. Acad. Sci. USA.* 78: 2063-2066, 1981.
- 29) Richardson, C. D. & Choppin, P. W.: Oligopeptides that specifically inhibit membrane fusion by paramyxoviruses: Studies on the site of action. *Virology* 131: 518-532, 1983.
- 30) Burley, S. K. & Petsko, G. A.: Aromatic-interaction: A mechanism of protein structure stabilization. *Science (Washington, D. C.)* 229: 2328, 1985.
- 31) Singh, J. & Thornton, J. M.: The interaction between phenylalanine rings in proteins. *FEBS Lett.* 191: 1-6, 1985.
- 32) Thomas, K. A., Smith, G. M., Thomas, T. B. & Feldman, R. J.: Electronic distribution within protein phenylalanine aromatic rings are reflected by the three-dimensional oxygen atom environments. *Proc. Nat. Acad. Sci. USA.* 79: 4843-4847, 1982.