Membrane fusion induced by influenza virus hemagglutinin requires protein bound fatty acids

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The low pH-dependent fusion of lipid membranes induced by two types of the fatty acylated influenza viral hemagglutinin has been studied by use of an energy transfer assay. When protein bound fatty acids were released from the hemagglutinin by hydroxylamine treatment viral fusion activity was inhibited. The extent of fusion inhibition correlates with the amount of fatty acids cleaved from the hemagglutinin. Virosomes prepared from fowl plague virus containing fatty acid free hemagglutinin showed a much lower fusion activity than control virosomes containing fatty acylated hemagglutinin. The hydroxylamine treatment applied has no detectable effects on the virus other than fatty acid release from its spike glycoproteins. These results support our previous hypothesis that protein bound fatty acids are involved in the induction of membrane fusion by the influenza hemagglutinin.

Acylprotein *Hemagglutinin* *Fusion* *Hydroxylamine*

1. INTRODUCTION

The fusion between lipid bilayers is a central step in many inter- and intracellular processes as well as during virus-cell interactions [1–3]. Enveloped viruses utilized as fusogens have revealed that their fusogenic membrane proteins are acylated with long chain fatty acids in a membrane near location [4]. To test for the functional significance of protein bound fatty acids in fusion we have initiated a study to compare the biological activities of viral acylproteins with or devoid of their fatty acid chains.

It is known that hydroxylamine releases ester-type linked fatty acyl chains [5,6] and we have recently reported that this cleavage of fatty acids from acylproteins reduces their hemolytic activity [7]. Since hemolysis alone is a questionable criteria for membrane fusion and difficult to quantitate, we made use of a resonance energy transfer assay [8] to monitor in a sensitive and quantifiable manner the fusion between viral or vesicular lipid bilayers with fluorescent target liposomes [9–12].

The results reported here indicate a correlation between the presence of protein bound fatty acids and the fusion activity of the hemagglutinins of different influenza viruses.

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In dedication to Professor R. Rott for his 60th birthday

2. MATERIALS AND METHODS

2.1. *Cells, virus, labeling procedures and polyacrylamide gel electrophoresis (PAGE)*

Chicken embryo fibroblasts (CEF) or Madin-Darby canine kidney cells (MDCK) were grown in Dulbecco's medium. At confluency chick embryo cells were infected with fowl plague virus (FPV, H7N1) and MDCK cells with virus N (H10N7), both influenza A viruses. For fatty acid labeling the growth medium was supplemented with 50 μCi/ml of [^{3}H]palmitic acid. Alternatively, FPV was grown in embryonated eggs and purified from the allantoic fluid by differential centrifuga-

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tion. For details of virus purification and PAGE see [7].

2.2. *Fusion assays through hemolysis and resonance energy transfer*

2.2.1. Hemolysis assay

Virus was adsorbed for 10 min at 4°C to a 1% suspension of chicken erythrocytes in 0.9% NaCl in a total volume of 1.2 ml before the pH was adjusted usually to pH 5.5 by adding 200 μl of a 1 M sodium acetate buffer. The mixtures were centrifuged after a 30 min incubation at 37°C for 5 min at 3000 rpm and the optical densities of the supernatants recorded at 540 nm. The efficiency and the maximal extent of hemolysis varied somewhat with the batch of erythrocytes utilized for each assay. The maximum value (A_{540nm} of 0.90–1.10) was taken as 100%.

2.2.2. Preparation of liposomes and virosomes

Large unilamellar vesicles (LUV) were prepared by reverse-phase evaporation (REV) in liposome buffer (100 mM NaCl, 10 mM Tris-HCl, 0.2 mM EDTA, 0.2 mM NaN_3), adjusted to pH 7.2 [13]. Viral or host cell lipids were used as a source for liposomal bilayer lipid. Phospholipid was determined as inorganic phosphate by the ammonium molybdate method [14]. The target liposomes contained 0.8 mol%, respectively, of *N*-(7-nitro-2,1,3-benzoxadiazol)dioleoylphosphatidylethanolamine (N-NBD-PE) and *N*-(lissamine-rhodamine-B-sulfonyl)dioleoylphosphatidylethanolamine (N-Rho-PE), all purchased from Avanti Biochemicals (Birmingham, USA). They were sized by extrusion with type SM 11307 Sartorius filters (Göttingen, FRG). Vesicles containing the viral glycoproteins (designated virosomes) were prepared from octyl-β-D-glucoside extracts from purified virus by the slow detergent dialysis procedure [15].

2.2.3. Fusion assay by resonance energy transfer

The resonance energy transfer (RET) assay was principally performed as described by Stegmann et al. [10] using a Perkin Elmer MPF 2A fluorospectrophotometer thermostatted at 37°C. Samples were incubated in a total volume of 2.7 ml liposome buffer containing the equivalent of 10 μmol phospholipid of labeled and 50 μmol of unlabeled liposomes. After adsorption (5 min) the pH was adjusted by adding 100 μl of a 1 M sodium acetate solution of the appropriate pH value and the increase of N-NBD-PE fluorescence was recorded at 530 nm. Incubations were terminated by adding Triton X-100 to a final concentration of 0.1% to measure maximal dequenching.

2.3. *Fatty acid cleavage from influenza hemagglutinin by hydroxylamine*

Deacylation of radiolabeled viral spike glycoprotein (HA) was as described before [7]. Incubation mixtures were diluted at least 10-fold for the hemolysis assay and 50-fold for the RET test. Fatty acid release was monitored by PAGE and fluorography. Radioactivity present in viral glycoproteins was quantitated by densitometric tracing of fluorograms.

2.4. *Hemagglutination assay*

A standard procedure was used throughout with 1% chicken erythrocytes in 0.9% NaCl. Usually 50 μl samples of either virus or virosomes were serially diluted and the hemagglutinin titres recorded 45 min after adding the blood cells.

3. RESULTS

3.1. *Fusion activity resides in the viral HA*

Before investigating the possible participation of protein bound fatty acids [20] in membrane fusion we verified first that fusion is induced exclusively by the viral acylprotein. Influenza virus induced fusion requires an acidic milieu and a hemagglutinin (HA) cleaved into a large (HA_1) and a small fragment (HA_2) [3,16,17]. Avian influenza virus N grown in MDCK cells is not fusogenic nor infectious because of its uncleaved HA. A short in vitro treatment with trypsin activates viral infectivity due to the upper mentioned cleavage of HA into HA_1 and the fatty acylated HA_2 [18,19]. Using this virus we can show an increase of N-NBD-PE fluorescence in our fusion assay only after trypsin activation (fig.1, upper scan). In contrast to virus N, FPV does contain the cleaved form of the HA spike glycoprotein, so that the fusogenic HA_2 is readily available. Its involvement in fusion under our conditions was verified by the complete proteolytic digestion of the HA spikes, which led to a drastic inhibition of fusion between 'naked' virus particles and target liposomes in the RET test

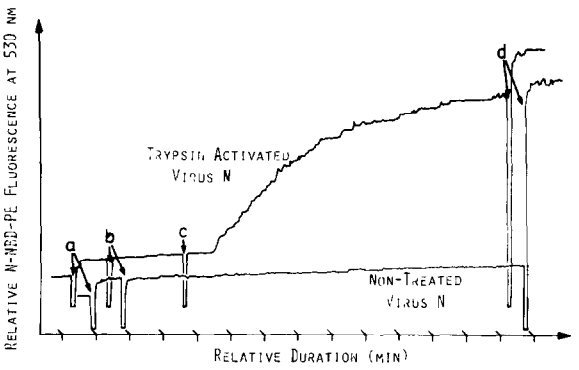

Fig.1. Fusion induced by trypsin activated virus N. Arrows at (a) mark the addition of virus N to the mixed target liposomes at neutral pH. At (b) the pH is adjusted to pH 5.5 by adding 1 M sodium acetate. For activation of virus N, trypsin (15 μg/ml final concentration) is added (c, upper scan only). The maximum value is reached by adding Triton X-100 (d). Fusion activity is not detectable without activation by trypsin (lower scan).

(not shown). Both virus species were only active in hemolysis and fusion at a pH below 5.6 (fig.1, see below).

3.2. *Effect of fatty acid release on the fusion activity of the influenza virus HA*

When FPV was incubated with increasing concentrations of hydroxylamine at 37°C prior to the RET fusion assay, a direct correlation between the doses of the drug and the inhibition of fusion and hemolytic activity was observed (fig.2a,b). Additionally, the degree of fusion inhibition depended on the duration of the respective incubation of virus particles with NH_2OH (fig.2a). Essentially the same results were obtained with trypsin activated virus N, which has an HA type (H10) different from FPV (H7) (not shown). As depicted in fig.3, the temperature used for the incubation with the drug is also crucial for the extent of inactivation. A 5 min treatment with 1 M hydroxylamine at 0°C reduced viral fusion activity only marginally while a 1 min, and even more so a 5 min, preincubation at 37°C inhibited the increase of fluorescence at 530 nm to 23% and almost 0% of the control value obtained with virus analysed immediately after adding hydroxylamine (100%). As with fusion activity the extent of fatty acid cleavage from the HA_2 of [^{3}H]palmitic acid labeled

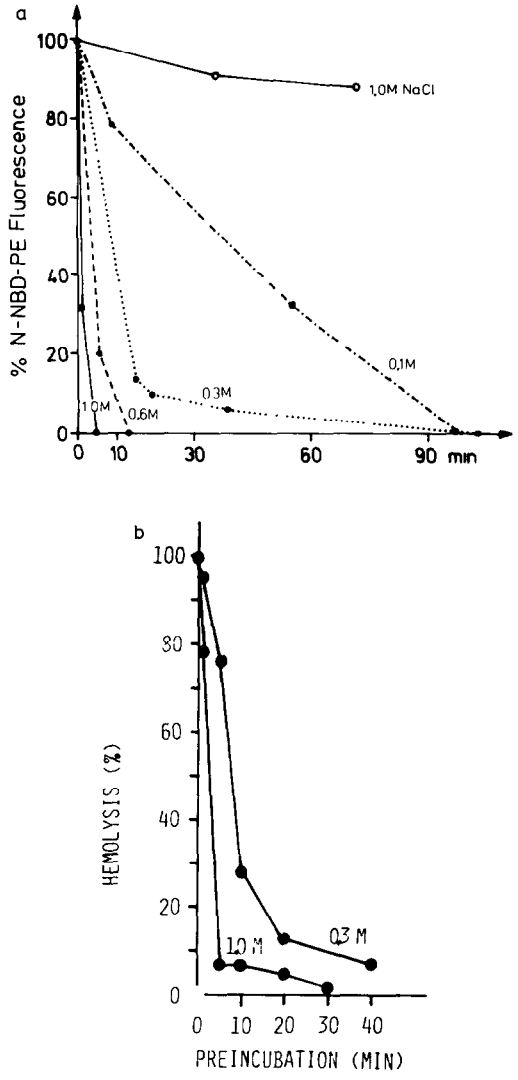

Fig.2. Effect of hydroxylamine on FPV induced fusion and hemolysis. (a) After preincubation of FPV (2^{16} HAU) with NH_2OH at 37°C fusion activity was quantitated by RET. NaCl-treated virus was tested as control. The control level of fusion (defined as 100% on the ordinate) represents 40% fluorescence increase at 530 nm as compared to the maximum value obtained after addition of Triton X-100. (b) FPV particles (equivalent to 1600 HAU) were preincubated with hydroxylamine (1.0 and 0.3 M) at 37°C for the indicated time periods and hemolysis measured.

FPV depended on the concentration of hydroxylamine used for deacylation. However the release of only a portion of the label is already quite effective in fusion inhibition (cf. fig.2 and table 1).

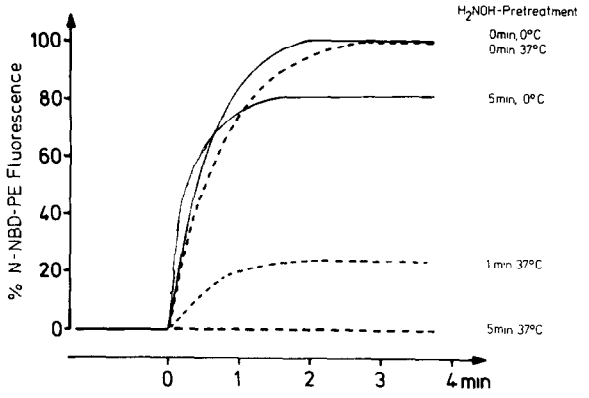

Fig.3. Fusion kinetics of FPV pretreated with 1 M NH_2OH (pH 6.5) at 0°C (——) and 37°C (– – –) for the indicated time periods as quantitated by RET. 0 min values (defined as 100%) refer to samples taken from the preincubation mixture immediately after adding hydroxylamine. As in fig.2a the control values are equivalent to 40% fluorescence as compared to the maximum values reached after addition of Triton X-100.

Although the reason for this feature is not known with certainty it may be due to a preferential release of the most fusogenic acyl species (e.g. oleic acid) present in the acylprotein [1,20].

Virosomes bearing acylated or fatty acid free glycoproteins on their surface were prepared by dialysis of octyl-β-D-glucoside extracts from NH_2OH- or NaCl-treated FPV. As shown in fig.4 control virosomes were more fusogenic (graph C) than the virosomes containing deacylated HA (graph D). It is noteworthy that a reduction of fusion activity from ~40% to 10–15% (relative to the 100% values reached with detergents) was observed with the control preparations (intact FPV treated with 1 M NaCl) as a result of overnight dialysis (unpublished). For this reason the inhibition of fusion by hydroxylamine in this experiment appears to be not as severe as shown for the non-dialysed virus (cf. fig.3).

Since a 30 min treatment with 1 M hydroxylamine at 37°C (pH 6.6) in addition to acyl cleavage from HA may potentially have other effects on the structural integrity of virus particles, their lipid composition, adsorption activity (hemagglutination) and immunoreactivity were tested. No major differences could be detected (not shown). To ensure that the HA_2 polypeptide itself is not damaged in any other way than just by release of the covalently bound acyl chains, this protein was isolated from polyacrylamide gels and subjected to proteolytic fingerprint and endgroup analysis. No difference in the HA fragment patterns could be detected and the latter analysis yielded the amino-terminal amino acid sequence Gly-Leu-Phe for both control and NH_2OH-treated HA_2 of FPV. These data indicate that inhibition of fusion by hydroxylamine is due to the release of fatty acids from the HA_2 polypeptide.

In contrast to the fusogenic polypeptide of other paramyxoviruses Sendai virus F-protein cannot be labeled with [^{3}H]palmitic acid ([19], Schmidt and Roßmann, unpublished). Therefore we compared its sensitivity to hydroxylamine with other enveloped viruses which do contain fusion active acylproteins. The results in table 2 demonstrate

Table 1

Release of fatty acids from HA_2 of [^{3}H]palmitic acid labeled FPV by hydroxylamine

	Percent radioactivity in hemagglutinin				
	0 min[a]	5 min	10 min	20 min	30 min
Control (1 M NaCl)	100	100	100	100	100
NH_2OH (0.3 M)	100	–	83	–	62
NH_2OH (0.6 M)	100	–	66	–	31
NH_2OH (1.0 M)	100	78	51	24	22

[a] Duration of incubation at 37°C prior to PAGE analysis followed by densitometry. The mean values of duplicate analyses are shown

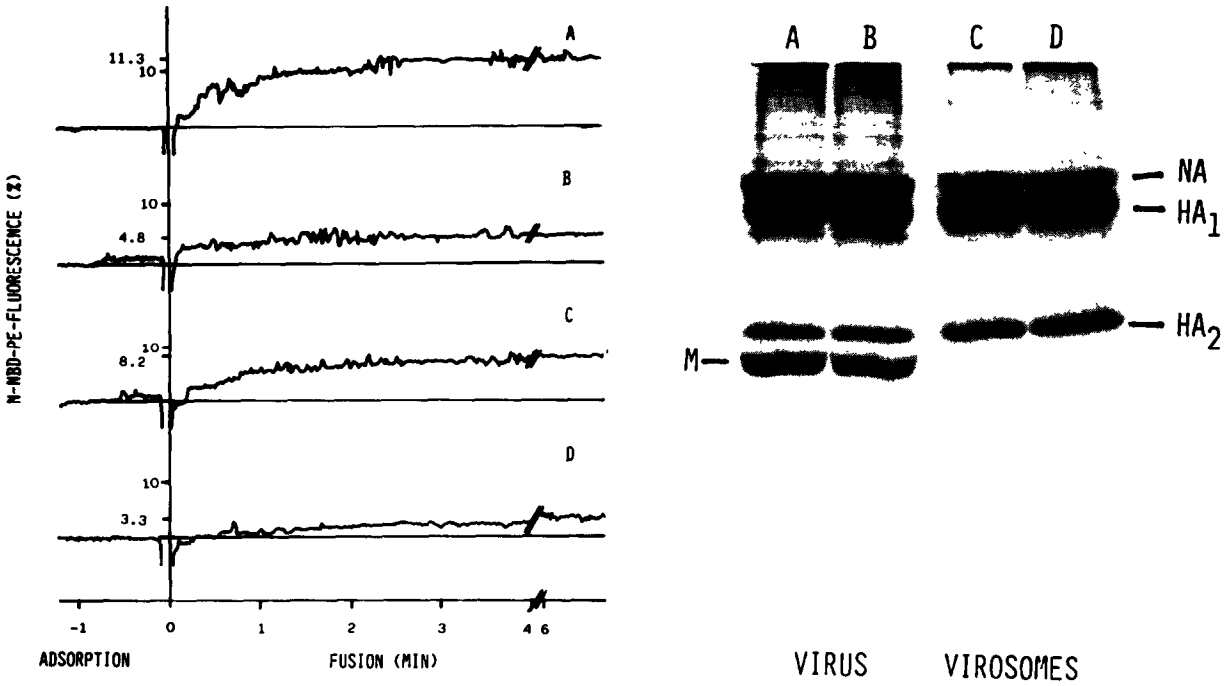

Fig.4. Kinetics of fusion induced by virosomes. Fusion was induced by FPV (equivalent to 2^{10} HA units) incubated for 200 min in PBS (A) or 1 M NH_2OH (B) at 37°C followed by dialysis or by virosomes with acylated (C) or fatty acid free HA_2 (D) derived from samples A and B (left panel). The Coomassie patterns of viral polypeptides after PAGE of the samples tested for fusion activity show no differences (right panel). The viral proteins are designated as follows: NP, nucleoprotein; NA, neuraminidase; $HA_{1/2}$, hemagglutinin; M, matrixprotein.

that fusion activity of all viruses with acylated spike proteins is inhibited by NH_2OH whereas the Sendai virus even shows a somewhat increased hemolytic activity.

Table 2

Effect of hydroxylamine on hemolysis

Virus	Acylproteins	pH[a]	Control	Hydroxyl-amine[b]
SFV	E1,E2	5.2	0.720	0.058
FPV	HA_2	5.5	0.619	0.041
FPV	HA_2	6.8	0.005	0.008
Virus N	HA_2	5.2	0.598	0.035
Virus N	HA_2	6.8	0.010	0.006
NDV	F-protein	6.8	1.242	0.805
Sendai virus	none	6.8	0.490	0.661

[a] pH during hemolysis test
[b] Treatment was for 30 min at 37°C with 1 M NH_2OH at pH 6.5

4. DISCUSSION

Hydroxylamine has been used as a reagent for virus inactivation long before its protein deacylating properties were discovered [5,21]. While its effect was then attributed to the modification of the viral genomic nucleic acids, NH_2OH has also been used for the cleavage of specific peptide bonds [22].

Despite this potential our control experiments reveal that neither the viral structural integrity nor the HA polypeptide itself were effected by the NH_2OH treatment applied here. Although as yet unknown NH_2OH-sensitive molecular requirements for fusion cannot be excluded completely, it is compelling to postulate that the inhibition of membrane fusion by hydroxylamine described in this report is due to the release of fatty acids from the hemagglutinin protein. This hypothesis is supported by the finding that fusion inhibition by hydroxylamine works also with other enveloped

viruses (e.g. SFV) which contain fatty acylated fusogenic spike proteins but not with Sendai virus, the F-protein of which is apparently not acylated. Thus, NH_2OH does not represent a general 'fusion-blocker' but seems to exert its effect only via the fatty acid cleavage from fusogenic acylproteins thereby implicating that fatty acids are not generally required for a protein to induce membrane fusion. However, once present in such proteins, the bound acyl chains may represent crucial triggers for this process.

Numerous studies have indicated an involvement of the hydrophobic amino-terminus of fusogenic viral polypeptides in membrane fusion [16–18,23]. Furthermore at least with the influenza HA it seems clear that intra- as well as intermolecular rearrangements occur prior to the merging of the two lipid bilayers involved [12,24]. The conclusions drawn from the results presented here are compatible with other hypotheses on the molecular mechanism of fusion proposed previously [3,12,24]. It is conceivable that protein linked fatty acids operate in concert with the above mentioned polypeptide regions to form an even bulkier hydrophobic 'drill' for both the close attraction of the viral lipid bilayer to the target membrane and its destabilisation to facilitate the fusion process.

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