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Activation of Influenza A Viruses by Trypsin Treatment

HANS-DIETER KLENK, RUDOLF ROTT, MICHAELA ORLICH, AND JOCHEN BLÖDORN

Institut für Virologie, Justus Liebig-Universität, 6300 Giessen, Frankfurter Strasse 107, Germany

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A comparative analysis has been carried out on the infectivity of virus of several influenza A strains grown in different host systems. Strains A/swine/Shope/31 (HswlN1), A/PR/8/34 (HON1), A/FM/1 (H1N1), A/Singapore/1/57 (H2N2), A/equine/Miami/1/63 (Heq2Neq2), and A/chick/Germany/49 (Hav2Neq1) exhibit host-dependent differences in infectivity. Virions grown in embryonated eggs and cultures of chorioallantoic membrane cells are highly infectious, whereas virions grown in cultures of chick embryo cells have a low infectivity that significantly increases after treatment *in vitro* with trypsin. In contrast, fowl plague viruses do not show host-dependent variations in infectivity. Virions grown in all host systems tested are highly infectious, and the infectivity of virions grown in chick embryo cells cannot be enhanced by trypsin treatment.

The activation of virus particles appears to be based on the cleavage of hemagglutinin glycoprotein HA. This concept is supported by the following observations: (i) In virions of low infectivity only uncleaved glycoprotein HA can be detected. Virions of high infectivity exhibit complete or at least partial cleavage of the hemagglutinin. (ii) The activation of virions by trypsin treatment is always paralleled by cleavage of HA. (iii) Cleavage of HA is the only effect which can be detected after trypsin treatment. The neuraminidase is neither inactivated nor removed from the virion. (iv) Studies on recombinants of virus N and fowl plague virus (Rostock) show that host-dependent variation of infectivity and activation by trypsin, features specific for parent virus N, are found only with recombinant N(H)-FPV/Ro(N) but not with recombinant FPV/Ro(H)-N(N).

Efficient plaque formation and serial passages are possible only if highly infectious particles are formed in a given host system. Thus, all strains analyzed undergo, in the absence of trypsin, successive growth cycles in eggs and chorioallantoic membrane cells and form plaques in chorioallantoic membrane cells. In contrast, in chick embryo cells only viruses containing the fowl plague virus hemagglutinin produce plaques and replicate under multiple cycle conditions without the addition of trypsin.

The data show that cleavage of HA is not a precondition for virus assembly and hemagglutinating activity, but that it is necessary for infectivity. These findings are compatible with the hypothesis that, in addition to its role in adsorption, the hemagglutinin has another function in the infection process and cleavage is required for this function.

INTRODUCTION

The hemagglutinin of influenza virus consists of a 75,000–80,000-dalton glycoprotein that can exist either as a single polypeptide chain (HA) (Lazarowitz *et al.*, 1971, 1973a; Stanley *et al.*, 1973) or as a disulfide-bonded complex of two polypeptides with molecular weights of about 50,000 (HA_1) and 30,000 (HA_2) (Laver, 1971; Skehel and Schild, 1971; Klenk *et al.*, 1972a). Cell fractionation studies suggest that HA is synthesized on rough endoplasmic reticulum and migrates through smooth internal membranes to the plasma membrane where it is incorporated into mature viral envelopes (Compans, 1973; Stanley *et al.*, 1973; Klenk et al., 1974; Hay, 1974). HA_1 and HA_2 are derived from HA by proteolytic cleavage (Lazarowitz *et al.*, 1971; Klenk *et al.*, 1972b; Skehel,

1972), which may take place either on smooth internal membranes (Klenk *et al.*, 1974) or at the plasma membrane (Lazarowitz *et al.*, 1971; Hay, 1974). The proteolytic nature of the cleavage reaction has been established by the observations that incubation of virions *in vitro* with trypsin results in the conversion of HA to HA_1 and HA_2 (Lazarowitz *et al.*, 1973a) and that cleavage of HA can be blocked by a protease inhibitor (Klenk and Rott, 1973). The extent of cleavage has been shown to be host dependent and appeared to correlate with the cytopathic effect produced in the particular system studied (Lazarowitz *et al.*, 1973a; Stanley *et al.*, 1973). These findings suggested that the cleavage enzymes are host-specific proteases. In the MDBK cell-WSN strain system, plasminogen has been demonstrated to be involved in the cleavage process (Lazarowitz *et al.*, 1973b). Different virus strains grown in the same host cell have been found to contain either predominantly uncleaved or predominantly cleaved hemagglutinin glycoprotein, indicating that there are also strain-specific variations in the susceptibility to proteolytic enzymes (Klenk *et al.*, 1972a; Lazarowitz *et al.*, 1973a; Stanley *et al.*, 1973).

In the present study a series of influenza A strains has been grown in different host systems. Confirming the observations described above, we have found that cleavage of HA is both host dependent and strain dependent. The hemagglutinin glycoprotein of all strains investigated is present in the cleaved form, when the virus is grown in the embryonated egg or in cultivated chorioallantoic membrane cells. Most but not all strains exhibit uncleaved HA if the virus is grown in chick embryo cells. Evidence will be presented that cleavage of the hemagglutinin glycoprotein is necessary for the infectivity of influenza virions.

MATERIALS AND METHODS

Cells. Eleven-day-old embryonated eggs were used for the preparation of monolayer cultures of chick embryo (CE) cells (Zimmermann and Schäfer, 1960) and of chorioallantoic membrane (CAM) cells (Cursiefen and Becht, 1975).

Viruses. The following influenza A strains were employed: A/swine/Shope/31(Hsw1N1) which will be referred to as strain "Swine"; A/PR/8/34 (HON1), "PR8"; A/FM/1 (H1N1), "FM1"; A/Singapore/1/57 (H2N2), "Singapore"; A/equine/Miami/1/63 (Heq2Neq2) "Equine"; A/chick/Germany/49 (Hav2Neq1), "N"; A/FPV/Rostock (Hav1N1), "FPV/Ro"; and A/FPV/Dutch (Hav1Neq1), "FPV/Du". In addition, the recombinants A/FPV/Rostock (Hav1)-A/chick/Germany/49 (Neq1), "FPV/Ro(H)-N(N)," and A/chick/Germany/49 (Hav2)-A/FPV/Rostock (N1), "N(H)-FPV/Ro(N)," have been used. Recombinant FPV/Ro(H)-N(N) was obtained by double infection of chick embryo cells with anti-FPV/Ro neuraminidase serum present in the agar overlay medium, recombinant N(H)-FPV/Ro(N) was obtained by double infection of CAM cells with anti FPV/Ro(H)-N(N) serum present in the agar overlay.

Seed stocks were grown in the allantoic cavity of 11-day-old embryonated eggs and were stored as infected allantoic fluids at −80°.

Plaque assay in CE cells. Confluent monolayers on plastic petri dishes (5 cm in diameter) were rinsed with phosphate-buffered saline, pH 7.4, and infected with 0.2 ml of virus suspension diluted in the same buffer. After a 60-min adsorption at 37° the cultures were overlaid with 4 ml of medium 199 (Flow Laboratories) containing 0.08% bicarbonate and 0.7% Difco Bactoagar. Alternatively, reinforced Eagle's medium (Bablanian *et al.*, 1965) with 0.7% Difco Bactoagar was used. If trypsin was used in the overlay, it was added at a final concentration of 10 μg/ml. Cultures were kept for 3 days at 37° and then stained with 4 ml of phosphate-buffered saline, pH 7.4, containing 0.005% neutral red and 0.7% Difco Bactoagar. After 4 h of incubation at 37°, plaques were counted.

Plaque assay in CAM cells. Monolayers were washed twice with phosphate-buffered saline, pH 7.4, and infected as described above. The overlay consisted of medium 199 containing 0.09% bicarbonate, 2% fetal calf serum, and 0.7% Difco Bactoagar. After 3 days, plaques were visualized

by the addition of 2 ml of a trypan blue solution (0.05% in phosphate-buffered saline). After 2 h at 37° excess stain was removed and plaques were counted (Cursiefen and Becht, 1975).

Neuraminidase assay. The enzyme was incubated for 30 min at 37° in 0.5 ml of buffer with fetuin at a final concentration of 100 μg of *N*-acetylneuraminic acid (NANA)/ml (Drzeniek *et al.*, 1966). Free neuraminic acid was measured by the thiobarbituric acid method (Aminoff, 1966). One neuraminidase unit was defined as the amount of enzyme that releases 1 nmol of NANA in 1 min at 37°.

Hemagglutination titrations. Titrations were carried out according to the procedure of Davenport *et al.* (1960).

Virus growth. Confluent monolayers were inoculated at the appropriate multiplicities of infection. After 30-min adsorption the inoculum was removed. Infected CE cells were maintained in minimal medium (Eagle and Habel, 1956) with the twofold concentration of glucose (Scholtissek and Rott, 1961). Infected CAM cells were maintained in medium 199 with 2% fetal calf serum.

Radioactively labeled virus was grown in reinforced Eagle's medium (Bablanian *et al.*, 1965) with glucose and amino acids reduced to 33% of the usual concentration and containing [^{3}H]glucosamine, 2 μCi/ml, and ^{14}C-labeled amino acid mixture, 0.5 μCi/ml.

To obtain egg-grown virus, the allantoic cavity of 11-day-old embryonated eggs was inoculated with about 200 PFU. Fowl plague viruses were harvested after 24 hr, all other strains after 48 hr.

Virus purification. Virus was purified from cell culture medium and allantoic fluid by procedures described previously (Klenk *et al.*, 1972a).

Polyacrylamide-gel electrophoresis. Dissociation of virus samples with sodium dodecyl sulfate and mercaptoethanol, electrophoresis, and processing of radioactive gels have been carried out according to described procedures (Schwarz and Klenk, 1974). Unlabeled gels were stained with Coomassie brilliant blue and scanned in a Gilford linear transport.

Chemicals and isotopes. Reagents for polyacrylamide-gel electrophoresis, phenylmethyl sulfonylfluoride, *N*-tosyl-L-phenylalanyl chloromethane, *N*-tosyl-L-lysyl chloromethane hydrochloride, and trypsin, 2× crystallized, were obtained from Serva, Heidelberg, Germany. Protein [U-^{14}C]hydrolysate and D-[1-^{3}H]glucosamine hydrochloride (3 Ci/mmol) were purchased from Amersham Buchler, Braunschweig, Germany. Trypsin inhibitor from lima beans, type II L, was obtained from Sigma, St. Louis, Mo.

RESULTS

Requirement of Trypsin in the Overlay for Plaque Formation in Chick Embryo Cells

Several strains of influenza A virus, all grown in embryonated eggs, were tested for their ability to produce plaques in CE cells with or without trypsin (10 μg/ml) in the overlay medium (Table 1). Only the fowl plague viruses produced defined plaques equally well in the presence and absence of the enzyme; after 3 days such

TABLE 1

EFFECT OF TRYPSIN ON PLAQUE FORMATION IN CE CELLS

Virus	Hemagglutination activity of inoculum (HA units/ml)	Trypsin in overlay (10 μg/ml)	Plaque number (PFU/ml)	Plaque diameter (mm)
Swine	512	−	1.6×10^7	<0.5
		+	2.0×10^9	2–3
PR8	1024	−	3.4×10^7	<0.5
		+	2.9×10^9	1–4
FM1	1024	−	7.5×10^5	<0.5
		+	1.5×10^9	1–2
Singapore	1024	−	8.0×10^5	<0.5
		+	3.1×10^8	1–2
Equine	1024	−	2.2×10^6	<0.5
		+	1.8×10^9	3–4
N	1024	−	1.1×10^7	<0.5
		+	8.0×10^8	3
FPV/Ro	1024	−	1.2×10^{10}	2–3
		+	1.0×10^{10}	2–3
FPV/Du	1024	−	1.4×10^9	2–3
		+	1.7×10^9	2–3

plaques were fully developed, clear, and had a diameter of about 4 mm. All other strains tested exhibited very indistinct plaque formation if trypsin was absent. After 3 days, plaques appeared as white dots with a pale halo and an overall diameter of less than 0.5 mm. They were hardly visible and could be counted only with difficulty. However, when trypsin was added to the overlay, plaques were clearly visible with diameters ranging from 1.5 to 3 mm. In the presence of trypsin the number of plaques was at least 100 times higher, and plaquing efficiency increased almost to the level found with fowl plague virus (FPV).

Confirming and extending previous reports (Came *et al.*, 1968; Appleyard and Maber, 1974) our data show that only a few strains of influenza A viruses readily form plaques in chick embryo cells. However, trypsin significantly enhances the sensitivity of these cells as a plaque assay system for a wide spectrum of other strains.

Effect of Trypsin on Virus Replication in Chick Embryo Cells

Enhancement of plaque formation in chick embryo cells by trypsin might involve mechanisms that facilitate virus replication. We have studied, therefore, the effect of the enzyme on virus growth in these cells. The experiments have been performed with a strain not requiring trypsin (FPV/Ro) and two strains requiring trypsin for plaque formation (N and Swine). In each case cells were infected with egg-grown virus under single cycle and multiple cycle conditions with multiplicities of inoculation ranging from 30 to 0.003 PFU/cell. The growth curves of FPV/Ro and N are shown in Fig. 1. FPV/Ro grew to the same titer regardless of the multiplicity of infection. The peak of virus production was reached at about 12 hr under single cycle conditions and with the appropriate delay under multiple cycle conditions. Virus replication was analyzed in the presence and absence of trypsin in the culture medium. Growth curves were found to be identical under both conditions, indicating that trypsin had no effect on the replication of FPV/Ro. On the other hand, a requirement for the enzyme was observed in the case of virus N. When trypsin was added to the medium, the replication pattern was similar to that of FPV/Ro. With the length of the latent period depending on the multiplicity of inoculation, virus production rose to about the same titer under single cycle and multiple cycle conditions. In the absence of trypsin, however, this titer was obtained only under single cycle conditions. Under multiple cycle conditions, virus production progressively decreased as the multiplicity of inoculation was reduced. At a multiplicity of 0.003 PFU/cell virus production was no longer demonstrable. Furthermore, virus N can be serially passed in CE cells only if the medium contains trypsin. Virus was allowed to undergo four passages in the presence of and parallel passages in the

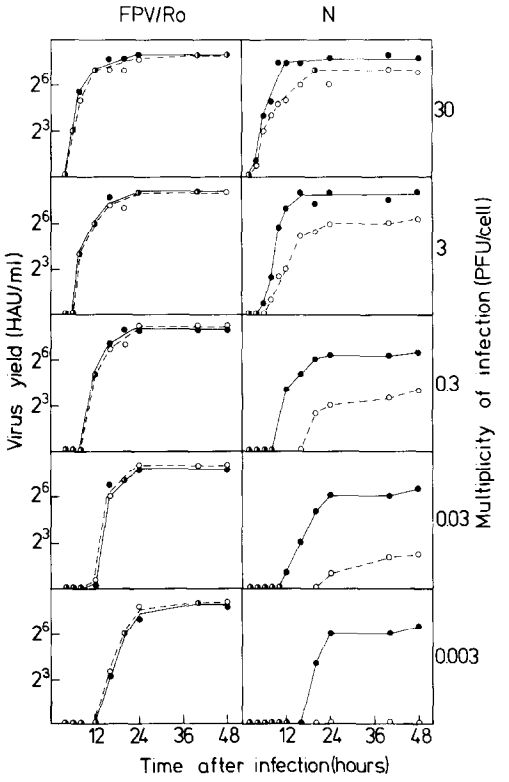

FIG. 1. Single and multiple cycle growth curves of strains FPV/Ro and N in CE cells. Virus replication was analyzed in the presence (●——●) and absence (○---○) of trypsin in the culture medium (5 μg/ml). Virus released into the medium was assayed. The inoculum was grown in embryonated eggs.

absence of the enzyme. To avoid the von Magnus phenomenon all passages were carried out at low multiplicities of infection, and virus was harvested 20 hr p.i. In the presence of trypsin, virus yields were only slightly reduced after the fourth passage. Without trypsin, however, already after the second passage virus could no longer be detected.

Similar experiments have been carried out with strain Swine, and it was found that this virus, too, could be grown in chick embryo cells under multiple cycle conditions only in the presence of trypsin. Thus, it appears that strains requiring trypsin for plaque formation, such as N and Swine, also require the enzyme to undergo successive growth cycles in chick embryo cells, whereas strains not requiring trypsin for plaque formation, i.e., the fowl plague viruses, readily replicate in the absence of the enzyme.

TABLE 2

EFFECT OF TRYPSIN TREATMENT ON INFECTIVITY OF INFLUENZA VIRUSES GROWN IN CE CELLS

Virus	Trypsin treatment[a]	Hemagglutination (HA units/ml)	Infectivity (PFU/ml)
Swine	–	64	2.0×10^6
	+	64	4.0×10^8
PR8	–	32	1.1×10^6
	+	32	2.9×10^7
FM1	–	32	2.3×10^6
	+	32	1.0×10^7
Singapore	–	32	1.3×10^5
	+	32	2.5×10^7
Equine	–	24	9.0×10^5
	+	24	1.0×10^8
N	–	128	6.0×10^5
	+	128	9.0×10^7
FPV/Ro	–	224	1.1×10^9
	+	224	1.1×10^9
FPV/Du	–	128	4.4×10^8
	+	128	4.0×10^8

[a] Trypsin treatment has been carried out as described in the text.

Enhancement of the Infectivity of the Virus Particle by Trypsin

As described above (Fig. 1), CE cells produce high yields of virus N under single cycle growth conditions without trypsin, but under multiple cycle conditions such production occurs only in the presence of the enzyme. This finding is compatible with the concept that CE cell-grown progeny virus differs from egg-grown input virus by a lower infectivity and that this difference could be overcome by trypsin. Thus, it appeared that the target of the trypsin action might be the virus particle.

To investigate this possibility directly, the effect of trypsin on CE cell-grown virus was analyzed. CE cells were infected with egg-grown stock under single cycle conditions. At 16 hr p.i. the culture medium was removed. Aliquots were incubated for 15 min at 37° with or without trypsin (10 μg/ml). Immediately after incubation, the samples were diluted with cold phosphate-buffered saline and assayed for hemagglutinating activity and infectivity. The results are shown in Table 2. Trypsin significantly enhances the infectivity of several strains. In the case of strains Swine, Singapore, and N, the increase is greater than 100-fold. It is not so marked but still higher than 10-fold in the case of PR8 and Equine. In contrast, the infectivity of the fowl plague viruses is not enhanced. It should be emphasized that there is no effect on the hemagglutinating activity with any of the strains tested.

From the data described so far, the following correlations can be made. Some virus strains form plaques and replicate under single cycle and multiple cycle conditions in CE cells equally well in the presence and absence of trypsin. CE cell-grown virus particles of these strains have a high infectivity that cannot be further enhanced by trypsin treatment. Other strains form plaques and replicate under multiple cycle conditions in CE cells only in the presence of trypsin. With these strains, trypsin significantly increases the infectivity of virions grown in CE cells.

We have also analyzed the effect of trypsin on the infectivity of virus N grown in two other host systems: the embryonated egg and CAM cells. Embryonated eggs are the common source for highly infectious influenza virus. CAM cells are another

host system in which all influenza strains studied replicate and form plaques in the absence of trypsin (Cursiefen and Becht, 1975). In contrast to CE cell-grown virus, the infectivity of virus N cannot be further increased if it is grown in eggs or in CAM cells (data not shown). This indicates that the infectivity depends on the host cell. The embryonated egg and CAM cells produce highly infectious virus, whereas CE cells produce virus of lower infectivity that can be altered by trypsin treatment to yield virus of high infectivity.

The plaque assays in these experiments were performed in CAM cells. As has been pointed out above, all virus strains investigated here produce distinct plaques in these cells without the addition of trypsin to the overlay. When chick embryo cells were used for the plaque assay, the difference between the infectivity of treated and untreated virus could not be demonstrated so clearly, presumably because the inoculum virus of the untreated control samples was activated by the enzyme added to the overlay.

The activation of the virus particle is also shown by the experiment described in Table 3. Strains Swine, PR8, and N, all grown in CE cells, were subjected to a second passage in these cells. Cell culture medium containing released virus of the first passage was incubated with and without trypsin and then used as inoculum for the second passage. The hemagglutination titers obtained in this passage clearly show that CE cell-grown virions of these strains had to be activated by trypsin in order to produce virus in the second passage. It should be mentioned that FPV did not show such a dependence on enzyme activation (data not shown).

Table 3 also shows that trypsin inhibitor from lima beans (5 μg/ml) blocks the activation if it is added to the virus simultaneously with the enzyme. If the inhibitor is added after trypsin treatment, activation is not blocked. This finding again demonstrates that trypsin acts on the virus particle, and it argues against the possibility that the enzyme present in the inoculum has any relevant effect on the host cell.

TABLE 3

EFFECT OF TRYPSIN INHIBITOR ON VIRUS ACTIVATION[a]

Virus strain	Virus yield (HA units/ml) after incubation of inoculum			
	Without trypsin[b]	With trypsin[c]	With trypsin and inhibitor[d]	With inhibitor after trypsin treatment[e]
Swine	2	64	2	64
PR8	2	32	2	32
N	2	64	2	64

[a] CE cells were infected with virus grown in the same host (m.o.i. = 50–100 PFU/ml). Virus was harvested 15 hr p.i. Before infection, the inoculum was incubated at 37°.

[b] Incubation, as described in footnote *a*, for 10 min without trypsin.

[c] Incubation, as described in footnote *a*, for 10 min with 5 μg of trypsin/ml.

[d] Simultaneous incubation for 10 min with 5 μg of trypsin/ml and 5 μg of trypsin inhibitor from lima beans/ml.

[e] Incubation, as described in footnote *a*, for 10 min with trypsin (5 μg/ml) and then for 10 min with trypsin inhibitor (5 μg/ml).

To investigate the problem further, whether an effect of trypsin on the host cell might enhance virus production, virus growth was analyzed in cells treated with trypsin before infection. Monolayers of CE cells were incubated for 30 min with trypsin (10 μg/ml) in medium lacking serum. The enzyme action was stopped by subsequent incubation with trypsin inhibitor (10 μg/ml for 10 min). After washing with phosphate-buffered saline, the monolayers were inoculated (m.o.i. = 0.3 PFU/ml) with strains Swine and N, both grown in CE cells. Infected cells were then incubated in the presence and absence of trypsin (10 μg/ml). Virus was produced only when trypsin was present during the replication period. Identical results were obtained from control cultures not preincubated with trypsin (data not shown). These results show that treatment of cells immediately before infection does not promote virus replication, again supporting the concept that an enzymatic action on the host

cell is not involved in the activation process.

Strain-Specific and Host-Dependent Variations of the Viral Protein Patterns and Their Relationship to the Infectivity of the Virion

In order to throw light on the molecular basis of the variations in infectivity described above, it was necessary to analyze the polypeptides of the virion. Detailed information on the influenza virus proteins has been presented in several recent reviews (Schulze, 1973; Klenk, 1974; White, 1974; Compans and Choppin, 1975) and shall only be briefly summarized here. The two principal nonglycosylated proteins are the nucleocapsid protein (NP) and the inner membrane protein (M). One or two high molecular weight proteins (P_1 and P_2) are found only in small amounts and might be associated with the nucleocapsid. The hemagglutinin is composed of glycoprotein HA or its cleavage products HA_1 and HA_2. Another glycoprotein (NA) forms the neuraminidase.

Figure 2 shows the polypeptides of FPV/Ro particles grown in CE cells. The hemagglutinin is present exclusively in the cleaved form. The HA_1 peak (MW 50,000) has an asymmetric shape suggesting heterogeneity. Two shoulders can be detected on its front slope, with molecular weights of 47,000 and 44,000. Previously one of these shoulders was tentatively attributed to the viral neuraminidase (Klenk *et al.*, 1972a). However, as will be shown below, the data obtained from recombinant virus N(H)-FPV/Ro(N) indicate that the glycoprotein of the FPV/Ro neuraminidase has a molecular weight of 58,000 and is located on the gel between proteins NP and HA_1. Complete cleavage of the hemagglutinin glycoprotein is also found in CE cell-grown virions of the Dutch strain of fowl plague virus (Table 4). The glycoprotein with a molecular weight of about 75,000 daltons found in these particles presumably corresponds to the viral neuraminidase as will be shown later.

Figure 2 also shows the polypeptide profile of virus N grown in CE cells. In contrast to the fowl plague viruses, the hemagglutinin is found exclusively in the uncleaved form. It should be mentioned that addition of calf serum or chicken serum to the culture medium did not cause cleavage of virus N hemagglutinin (data not shown). The virus N-CE cell system appears to be different in this respect from the WSN strain-MDBK cell system, where cleavage depends on the presence of serum. Lazarowitz and co-workers (1973b) reported that activators of serum plasminogen are released from MDBK cells and convert the zymogen to the active enzyme plasmin, which cleaves the HA polypeptide of the WSN strain.

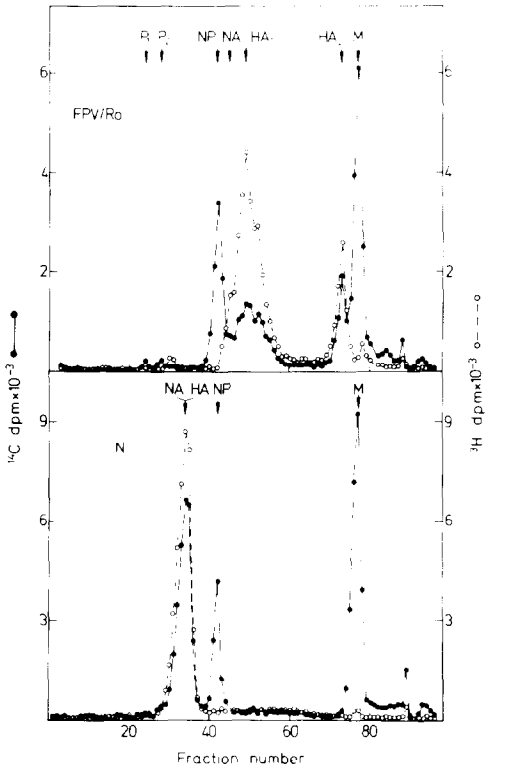

FIG. 2. The polypeptides of FPV/Ro virions (upper panel) and N virions (lower panel) both grown in CE cells. Purified virions, doubly labeled with [^{3}H]glucosamine and a mixture of ^{14}C-labeled amino acids, were subjected to polyacrylamide-gel electrophoresis. In this and all subsequent drawings of gel patterns the origin is on the left and the anode on the right.

Only uncleaved hemagglutinin glycoprotein was also found in CE cell-grown virions of strain Swine and strain PR8 (data not shown). Thus, there appears to be a

TABLE 4
GLYCOPROTEIN COMPOSITION OF INFLUENZA A VIRUSES

Virus strain	Host	Molecular weight[a]			
		HA	HA_1	HA_2	NA
Swine	CE cells	76,000	—	—	58,000
PR8	CE cells	72,000	—	—	62,000
N	CE cells	72,000	—	—	74,000
	CAM cells	72,000	48,000	32,000	74,000
	Chick embryo	—	46,000	31,000	76,000
FPV/Ro	CE cells	—	51,000	29,000	58,000
	Chick embryo	—	50,000	29,000	58,000
FPV/Du	CE cells	—	51,000	28,000	75,000
	Chick embryo	—	50,000	29,000	75,000

[a] Molecular weight determinations are based on electrophoretic mobilities using NP (MW 60,000) and M (MW 26,500) as internal standards (Klenk *et al.*, 1972a).

correlation between the cleavage of HA and the infectivity of virions grown in CE cells. In particles of strains PR8, Swine, and N which have a low infectivity, only uncleaved HA can be detected, whereas fowl plague virions which are highly infectious exhibit a high degree of cleavage.

It was now of interest to analyze the polypeptide patterns of virions grown in eggs where *all* strains are produced in a highly infectious form, as has been pointed out above. Such patterns are shown in Fig. 3. The profile of FPV/Ro is similar to that of CE cell-grown virus. The hemagglutinin glycoprotein is present only in the cleaved form. Complete cleavage is also found in egg-grown virions of the Dutch strain of fowl plague virus (data not shown).

The lower panel of Fig. 3 shows the polypeptide profile of egg-grown virus N. In contrast to virus derived from CE cells (compare with Fig. 2, lower panel), there is now extensive cleavage of the hemagglutinin. The polypeptide peak migrating in front of P_2 presumably represents the neuraminidase subunit rather than uncleaved HA. This conclusion is based on the following observations: (i) The single glycoprotein peak of virus N grown in CE cells (Fig. 2, lower panel) implies that the neuraminidase of this strain comigrates with glycoprotein HA. (ii) A glycoprotein with a molecular weight of about 75,000 is also found with FPV/Du (Table 4) and recombinant virus FPV/Ro(H)-N(N). Both strains contain the neuraminidase of virus N and

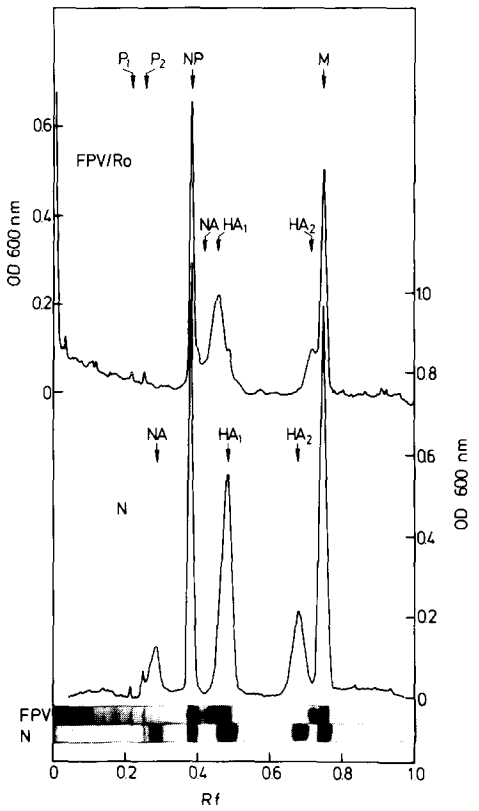

FIG. 3. The polypeptides of virions of strains FPV/Ro and N grown in the chick embryo. Photographs of polyacrylamide gels stained with Coomassie brilliant blue and the corresponding densitometer profiles are shown. There are several minor proteins of unknown identity on the gel derived from FPV/Ro virions. Among these, two bands have been consistently found: a band (MW 59,000) migrating in front of protein NP and another one (MW 46,000) migrating in front of glycoprotein HA_1.

the hemagglutinin of fowl plague virus which is found only in the cleaved form as demonstrated in the example of FPV/Ro (Figs. 2 and 3, upper panels).

Cleavage of HA can also be demonstrated if virus N is grown in CAM cells which, like the embryonated egg, produce virus of high infectivity. On the polyacrylamide gel shown in Fig. 4, HA_1 and HA_2 are clearly visible, although cleavage appears to be less extensive than in the egg as indicated by the comparatively high HA peak.

The data on the glycoprotein composition of the influenza strains analyzed in the present study are summarized in Table 4. They demonstrate that the cleaved form of the hemagglutinin glycoprotein cannot be detected in CE cell-grown virions of strains PR8, Swine, and N, whereas it is present in all virions grown in eggs and CAM cells and in fowl plague virions grown in CE cells. These results in combination with those on the biological activities of influenza virions indicate that particles of high infectivity contain the cleaved form of the hemagglutinin glycoprotein and that particles of low infectivity exhibit only the uncleaved form.

As described above, the infectivity of the inactive form of these viruses can be enhanced by trypsin. It was, therefore, of interest to see whether trypsin treatment converts the hemagglutinin from the uncleaved into the cleaved form. Aliquots of a preparation of purified virus N particles grown in CE cells were incubated in 50 μl of phosphate-buffered saline, pH 7.4, for 15 min at 37° with and without trypsin (10 μg/ml). Immediately after incubation, the samples were subjected to polyacrylamide-gel electrophoresis. Figure 5 demonstrates that trypsin treatment causes extensive cleavage of HA. A similar experiment has been performed on the Swine strain. CE cell-grown virions of this strain contain two glycproteins: the uncleaved hemagglutinin glycoprotein HA and the neuraminidase subunit NA (Table 4). After trypsin treatment a large part of HA is found in the form of the cleavage products HA_1 (MW 55,000) and HA_2 (MW 25,000). Thus, activation of virus particles by *in vitro* treatment with trypsin is accompanied by the cleavage of HA.

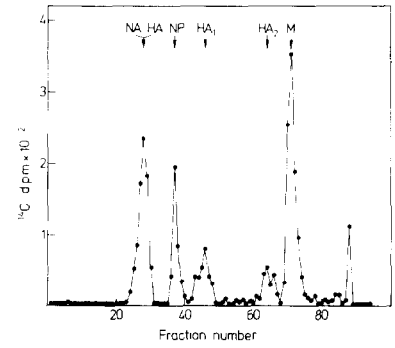

FIG. 4. Polypeptide profile of virus N grown in CAM cells. Purified virions labeled with a mixture of ^{14}C-labeled amino acids were analyzed by polyacrylamide-gel electrophoresis.

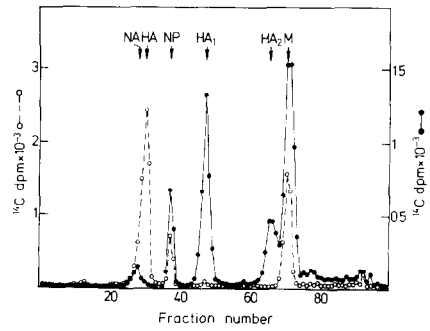

FIG. 5. Cleavage of glycoprotein HA of virus N by treatment with trypsin *in vitro*. A preparation of purified virus N grown in CE cells and labeled with a mixture of ^{14}C-labeled amino acids has been incubated with (●——●) and without (○- - -○) trypsin as described in the text. The trypsin-treated sample and the control which did not receive trypsin have been analyzed on separate polyacrylamide gels.

Effect of Trypsin Treatment on the Neuraminidase

In addition to the cleavage of HA, trypsin treatment might induce other alterations of the virus particle. Of particular importance would be an effect on the neuraminidase. By allowing elution of adsorbed virus particles, this enzyme conceivably might diminish the infectivity. Since trypsin has been reported to remove the neuraminidase from several influenza strains (Noll *et al.*, 1962), it was necessary to find out if such an effect occurred under the conditions of trypsin treatment used in

the present study and if this could account for virus activation. We have, therefore, analyzed neuraminidase activity of CE cell-grown virus N before and after trypsin treatment. Aliquots of a preparation of purified virus which was labeled with [^{3}H]amino acids were incubated for 15 min at 37° without and with trypsin at concentrations of 10 and 100 μg/ml, respectively. The samples were chilled and pelleted for 1 hr at 100,000 g. Aliquots were assayed for neuraminidase activity and subjected to polyacrylamide-gel electrophoresis. Trypsin treatment caused extensive cleavage of HA as already demonstrated (Fig. 5). Neuraminidase activity, however, was identical in treated and untreated samples.

A second type of experiment was designed to reveal whether neuraminidase was removed from N particles which were grown in the presence of trypsin. Infected CE cells were incubated with trypsin (5 μg/ml) and, in a simultaneous experiment, without the enzyme. At 16 h p.i. the medium was removed, dialyzed against phosphate-buffered saline and centrifuged for 1 h at 100,000 g. Aliquots of the supernatant fluid and the sediment resuspended in buffer were analyzed for hemagglutinin and neuraminidase activity. More than 90% of both activities was found in the pellet, and the neuraminidase activity was similar in particles grown in the presence and absence of trypsin.

These data show that trypsin has no effect on the neuraminidase activity, regardless of whether virus N is treated *in vitro* with trypsin or whether its production occurs in the presence of the enzyme. Thus, removal of neuraminidase can be excluded as a possible mechanism for the activation of virus N.

Trypsin Activation of Recombinants of FPV/Ro and Virus N

That the activation of virus N is due to trypsin action on the hemagglutinin and not on the neuraminidase can also be demonstrated by experiments employing the recombinants FPV/Ro(H)-N(N) and N(H)-FPV/Ro(N).

Table 5 shows plaque formation in CE cells. FPV/Ro(H)-N(N) produced defined plaques equally well in the presence and absence of trypsin. After 3 days they are fully developed with a diameter of about 4 mm. On the other hand, recombinant N(H)-FPV/Ro(N) produces satisfactory plaques only in the presence of trypsin. In the absence of the enzyme, plaques are ill defined and plaquing efficiency is low.

There is also a significant difference in infectivity between CE cell-grown virions of the two recombinants (Table 6). FPV/Ro(H)-N(N) virions have a high infectivity that cannot be further enhanced by trypsin. N(H)-FPV/Ro(N) virions, however, have a low infectivity that increases about 50-fold after trypsin treatment. On the other hand, if recombinant N(H)-FPV/Ro(N) is grown in eggs, it is highly infectious and cannot be further activated (data not shown).

The polypeptides of the recombinant viruses grown in CE cells have been analyzed by polyacrylamide-gel electrophoresis and are shown in Fig. 6. A comparison of the protein patterns of the recombinants

TABLE 5

EFFECT OF TRYPSIN ON PLAQUE FORMATION BY RECOMBINANTS FPV/Ro(H)-N(N) AND N(H)-FPV/Ro(N) IN CE CELLS

Recombinant	Hemagglutination activity of inoculum (HA units/ml)	Trypsin in overlay (10 μg/ml)	Plaque number (PFU/ml)	Plaque diameter (mm)
FPV/Ro(H)-N(N)	512	−	1.2×10^9	3–4
		+	1.3×10^9	3–4
N(H)-FPV/Ro(N)	1024	−	6.0×10^6	<0.5
		+	3.5×10^8	2

TABLE 6

EFFECT OF TRYPSIN TREATMENT ON INFECTIVITY OF RECOMBINANTS FPV/Ro(H)-N(N) AND N(H)-FPV/Ro(N) GROWN IN CE CELLS

Recombinant	Trypsin treatment[a]	Hemagglutination (HA units/ml)	Infectivity (PFU/ml)
FPV/Ro(H)-N(N)	–	128	1.5×10^8
	+	128	1.7×10^8
N(H)-FPV/Ro(N)	–	32	5.7×10^5
	+	32	2.4×10^7

[a] Trypsin treatment was carried out as described in Table 3.

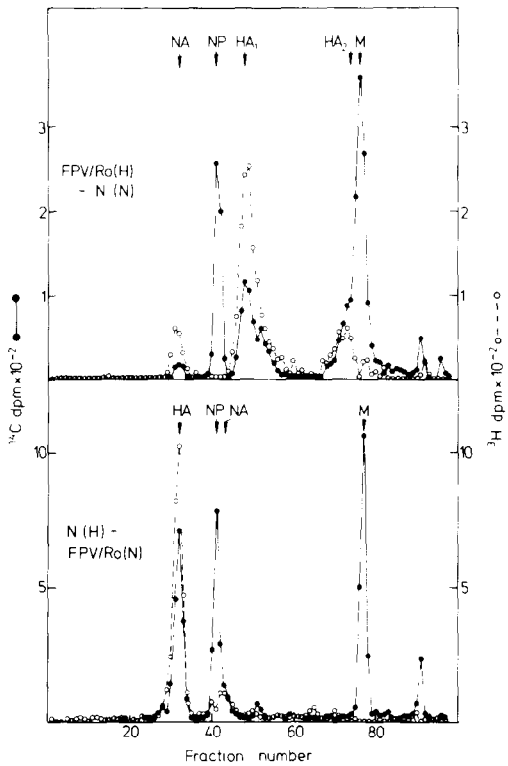

FIG. 6. The polypeptides of recombinants FPV/Ro(H)-N(N) (upper panel) and N(H)-FPV/Ro(N) (lower panel). Purified virions grown in CE cells and labeled with [^{3}H]glucosamine and a mixture of ^{14}C-labeled amino acids were analyzed.

with those of the parent viruses (Fig. 2) clearly demonstrates that FPV/Ro(H)-N(N) possesses the cleaved hemagglutinin glycoproteins HA_1 and HA_2 of FPV/Ro and the neuraminidase glycoprotein of virus N with a molecular weight of 75,000, whereas N(H)-FPV/Ro(N) contains the uncleaved hemagglutinin glycoprotein HA of virus N and the neuraminidase glycoprotein of FPV/Ro with a molecular weight of 58,000. *In vitro* treatment of recombinant N(H)-FPV/Ro(N) with trypsin results in complete cleavage of HA (data not shown). When this virus is grown in eggs, the hemagglutinin glycoprotein is present again exclusively in the cleaved form (data not shown).

Thus, only the recombinant possessing virus N *hemagglutinin* exhibits host-specific variation of infectivity and activation by trypsin, features typical for virus N. These features are not found with the recombinant possessing the *neuraminidase* of virus N. These findings are in agreement with the concept that the substrate for the trypsin action is the hemagglutinin and that its cleavage is an essential step in the activation mechanism.

Effect of Trypsin on Virus Adsorption

Cleavage of HA conceivably might be necessary for effective attachment of the virus to the cell surface, although this possibility does not appear very likely since nonactivated particles hemagglutinate as efficiently as activated ones (Table 2). To investigate this point further, the hemagglutinating activity of CE cell-grown virions of strain Swine was assayed with guinea pig and human erythrocytes in addition to chicken erythrocytes. Trypsin treatment did not alter the hemagglutinating capacity of the virus with any of these erythrocytes.

The ability of virions to adsorb to CE cells was also tested. A 1.5-ml culture medium of infected cells (HAT 128) was incubated for 15 min at 37° in phosphate-buffered saline with or without trypsin (10 μg/ml) and then mixed with 1 ml of packed CE cells. After 10-min adsorption at 0° the cells were pelleted at 200 $\times$ *g*. The supernatant fluid was found to be free of hemagglutinating activity, regardless of whether the virus was trypsin treated.

It could be argued that proteases on the cell surface might cleave HA, thus activating the hemagglutinin in the course of the hemagglutination reaction of the adsorp-

tion period. To eliminate this possibility the hemagglutinating activity of virus N derived from different hosts was assayed in the presence and absence of 0.1 m*M* phenylmethyl sulfonylfluoride, 0.1 m*M* *N*-tosyl-L-phenylalanyl chloromethane, and 0.1 m*M* *N*-tosyl-L-lysyl chloromethane hydrochloride. It was found that inhibition of proteolysis did not block hemagglutination (data not shown).

In order to demonstrate directly the possible cleavage of HA by cell surface proteases, the polypeptides of CE cell-grown virus N were analyzed after adsorption and elution from erythrocytes. A purified preparation of ^{14}C-amino acid- and [^{3}H]glucosamine-labeled virus (HAT 5120), 0.1 ml, was mixed with 0.2 ml of packed chicken erythrocytes. After 45 min at room temperature, 50 units of neuraminidase from *Vibrio cholerae* (Behringwerke, Marburg, Germany) in 1 ml of phosphate-buffered saline were added. After 3 h, the cells were pelleted by centrifugation at 1000 *g* for 10 min. The virus present in the supernatant fluid was pelleted by centrifugation at 100,000 *g* for 90 min. Polyacrylamide-gel electrophoresis revealed that the hemagglutinin glycoprotein was not cleaved after adsorption (data not shown).

These results demonstrate that the uncleaved hemagglutinin glycoprotein HA can participate in hemagglutination and adsorption to the cell surface as well as the complex of the cleavage products HA_1 and HA_2.

DISCUSSION

The influenza A viruses analyzed in the present study can be divided into two groups with respect to the infectivity of the virus particles. One group, comprising strains N, PR8, Swine, FM1, Singapore, and Equine, exhibits host-dependent differences in infectivity. Particles grown in eggs and in CAM cells are highly infectious. Particles grown in CE cells have a low infectivity that can be enhanced by trypsin treatment. The other group of influenza A strains represented by the fowl plague viruses does not show host-dependent variations in infectivity. Virions of these strains grown in all host systems tested are highly infectious, and CE cell-grown virus cannot be further activated by trypsin.

The activation of virus particles appears to be based on the cleavage of hemagglutinin glycoprotein HA. This concept is supported by the following observations: (i) In virions of low infectivity only uncleaved glycoprotein HA can be detected. Virions of high infectivity exhibit complete or at least partial cleavage of the hemagglutinin. (ii) The activation of virions by trypsin treatment is always paralleled by cleavage of HA. (iii) Cleavage of HA is the only effect that can be detected after trypsin treatment. The neuraminidase is neither inactivated nor removed. (iv) Host-dependent variation of infectivity and activation by trypsin, features specific for parent virus N, are found only with recombinant N(H)-FPV/Ro(N) but not with recombinant FPV/Ro(H)-N(N).

Efficient plaque formation and serial passages are possible only if highly infectious particles are formed in a given host system. Thus, all strains analyzed undergo, in the absence of trypsin, successive growth cycles in eggs and CAM cells and form plaques in CAM cells. In contrast, only viruses containing FPV hemagglutinin produce plaques and replicate in CE cells under multiple cycle conditions without trypsin, whereas all other strains require the enzyme. The trypsin requirement for plaque formation confirms a previous report by Appleyard and Maber (1974). Similar observations have been made by Came *et al.* (1968) who used pancreatin to facilitate plaque production. These authors were not able to detect an enhancement of infectivity after trypsin treatment of virus particles and suggested that the enzyme produced its effect by an action on the host cell rather than directly on the virion. This conclusion is in contrast to our observations. However, the activation experiments of Came *et al.* (1968) are not conclusive, since they were performed with egg-grown virus which, in the light of our observations, is synthesized in a highly infectious form and can, therefore, not be further activated by proteolytic enzymes.

It should be pointed out that inactive virus grown in CE cells shows some infec-

tivity, although at a significantly lower level than active virus. It is, therefore, not surprising that plaque formation in CE cells by strains producing inactive virus in this system is very inefficient but not totally absent. These findings suggest that, in a system which produces predominantly inactive virus, a small amount of infectious virus is always made due to cleavage of HA. This occurs, however, at a level too low to be detected on polyacrylamide gels. Alternatively, particles containing exclusively uncleaved HA might still be infectious at a very low level.

The observation that, depending on virus strain and host, influenza virions are formed which contain either uncleaved or cleaved HA glycoprotein indicates that cleavage is not necessary for virus assembly and release. Comparative analyses of hemagglutination and adsorption to host cells reveal that cleavage is not essential for these biological activities, either. The same conclusions have been drawn previously by Lazarowitz *et al.* (1973a) and Stanley *et al.* (1973) on the basis of similar observations. However, in contrast to the conclusion of these authors, our data indicate that cleavage is necessary for infectivity. The explanation for this discrepancy might be that these authors compared virus samples containing *predominantly* cleaved HA with samples containing *predominantly* uncleaved HA. Conceivably the small but electrophoretically clearly detectable amount of HA_1 and HA_2 present in the samples containing predominantly uncleaved HA might have been enough to allow a relatively high level of infectivity.

Influenza B viruses have also been reported to require trypsin for plaque formation in CE cells (Came *et al.*, 1968; Tobita and Kilbourne, 1974; Appleyard and Maber, 1974). Analysis of the proteins of an influenza B virus strain grown in hamster kidney cells revealed that cleavage of HA does not occur *in vivo* but can be accomplished *in vitro* by trypsin treatment (Choppin *et al.*, 1975). Cleavage is accompanied by an increase in infectivity (P. W. Choppin, personal communication). Thus, cleavage of HA as a precondition for infectivity might be a general phenomenon of influenza viruses.

Activation of virions by proteolytic enzymes is not confined to influenza virus. Recently Homma and Ohuchi (1973) and Scheid and Choppin (1974) reported that embryonated eggs produce infectious Sendai virus, whereas virions grown in a variety of other hosts are not infectious. Furthermore, they found that the uninfectious form can be activated by trypsin treatment and that activation is due to proteolytic cleavage of a large, biologically inactive precursor glycoprotein. The cleavage product is a smaller glycoprotein which is also found in egg-grown virus. It is involved in hemolysis, cell fusion, and initiation of infection. Thus, there is a striking similarity between this paramyxovirus and the influenza viruses with respect to host-dependent variation in infectivity and activation of an envelope glycoprotein by proteolytic cleavage. However, whereas there is good evidence that the activated paramyxovirus glycoprotein is involved in the fusion of the viral envelope with the cell membrane, thereby initiating infection, it is not yet understood why cleavage of glycoprotein HA is necessary for the infection process of influenza virus. The observation that cleavage of HA is a precondition for infectivity but not for hemagglutination is compatible with the concept that, in addition to its role in adsorption, the hemagglutinin has another function in the infection process, presumably at the stage of penetration, and that cleavage is required for this function.

The observations that cleavage of HA is a host-dependent phenomenon and that particles containing uncleaved HA have a low infectivity suggest that cellular proteases as activating enzymes play an important role in the host range and the spread of infection of an influenza virus.

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