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Loss on Serial Passage of Rhesus Monkey Kidney Cells of Proteolytic Activity Required for Sendai Virus Activation

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Primary and secondary cultures of rhesus monkey kidney cells supported multiple-cycle replication of Sendai virus, but later passages lost this ability, and this was reflected in decreased plaque formation. Multiple-cycle replication also did not occur in LLC-MK_2 cells, a continuous line of RMK cells. Failure of replication in serially passed cells was correlated with a decrease in proteolytic cleavage of a viral surface glycoprotein (F_0), and the ability of cells to support multiple-cycle replication and plaque formation could be restored by the addition of trypsin (0.3 μg/ml) to the overlay medium. The use of wild-type virus, which requires trypsin, and protease activation mutants that require chymotrypsin or elastase for activation has provided evidence that the activating protease supplied by primary or secondary cells has trypsin-like activity. Inactive virus, with uncleaved F_0 glycoprotein, adsorbed to primary or secondary cells but did not infect them, even though such cells possess the enzyme that is capable of cleaving the F_0 glycoprotein of virus synthesized in these cells. The inability of these cells to activate adsorbed virus indicates that the activating protease that they possess is inaccessible to adsorbed virus, although it can act on the F_0 glycoprotein during virus maturation in these cells. These data provide a biochemical explanation for the failure of later passages of a cell strain or a continuous cell line to support the replication of a paramyxovirus.

Infectivity of Sendai virus and virus-induced cell fusion and hemolysis are dependent on the proteolytic cleavage of a virion glycoprotein, F_0, which yields two disulfide-linked polypeptide chains, F_1 and F_2 (12, 22, 24). Some cells, such as MDBK (22) and L cells (12), yield inactive virions that possess the F_0 glycoprotein precursor. When F_0 on such inactive virus is cleaved with trypsin in vitro, fully active, infective virus is produced that is similar in biological activities to the virus produced in the chorioallantoic sac of the chicken embryo (12, 22) or in secondary calf kidney cells (29). These and other studies have indicated that the host range and tissue tropism of paramyxoviruses is dependent on the availability of the appropriate activating cellular protease.

Plaque formation, which requires multiple-cycle replication, occurs in several primary and secondary cell cultures (8, 13, 25, 26). When MDBK cells are inoculated with infective virus, a single cycle occurs because the virus released is noninfective. However, plaque formation does occur in MDBK (23) or LLC-MK_2 cells (27) if trypsin is present in the overlay because multiple-cycle replication can occur due to cleavage of the F_0 glycoprotein by the exogenous protease.

In the past, primary cultures of certain cells, e.g., monkey kidney, were commonly employed for the propagation of viruses, but the ability to support replication of the same viruses was lost or significantly reduced when the cells were serially passed (2, 21) or when continuous lines were established (17). We report here that serially passed rhesus monkey kidney (RMK) cells, which support multiple-cycle replication and plaque formation by Sendai virus in primary and secondary cultures, lose this ability in later passages due to a loss of a trypsin-like proteolytic activity.

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MATERIALS AND METHODS

Cells. Primary cultures of RMK cells were prepared from monkey kidneys (7) or purchased as confluent monolayers from Flow Laboratories, Rockville, Md. Cells were passed by removal from the substrate with trypsin (0.25%) (1:300, ICN Pharmaceuticals, Cleveland, Ohio) and ethylenediaminetetraacetic acid (0.05%), diluted 1:5, and grown in plastic flasks or petri dishes (Falcon Plastics, Baltimore, Md.) in reinforced Eagle medium (REM) (1) with 10% calf serum. Monolayer cultures of a variant of the MDBK line of bovine kidney cells (6) and of the LLC-MK_2 line of

rhesus monkey kidney cells were grown in REM supplemented with 10% fetal calf serum or 5% calf serum, respectively.

Virus. Seed stocks of wild-type (WT) Sendai virus were grown in the allantoic sac of 11-day-old embryonated chicken eggs inoculated with ~10^6 egg infective doses. Protease activation (pa) mutants of Sendai virus were grown in the allantoic sac of 11-day-old embryonated chicken eggs inoculated with ~10^4 plaque-forming units (23). With mutants pa-c1, -c2, and -e2, which require activating protease for multiple-cycle replication, 10 μg of elastase in 0.1 ml of phosphate-buffered saline was injected into the allantoic sac at the time of infection. pa-e1 and pa-e3 to -e8 were grown without added protease (23).

Growth, isotopic labeling, and purification of virus. Virus was grown in confluent monolayers as described previously (23). For labeling of primary cultures, [^{3}H]leucine (44 μCi/ml) was added to two monolayers in 120-cm^2 bottles after the adsorption period; for secondary or subsequent passages, 5 μCi/ml was added to 20 10-cm petri dishes. After 24 to 48 h, the medium was harvested, and debris was removed at 6,000 × *g* for 20 min. Virus was pelleted at 17,000 × *g* for 2 h, resuspended in phosphate-buffered saline by Dounce homogenization, layered onto a 15 to 40% potassium tartrate gradient, and centrifuged in a Spinco SW41 rotor for 1 h at 30,000 rpm. The virus band was collected, dialyzed against phosphate-buffered saline, and pelleted in an SW50.1 rotor for 1 h at 30,000 rpm.

Hemagglutination. Hemagglutination titrations were performed as described previously (23).

Plaque assays. Plaque assays were performed in confluent monolayers of RMK, MDBK, or LLC-MK$_2$ cells in 60-mm petri dishes as described previously (23). Replicate, prediluted aliquots of seed stocks were used to minimize dilution errors. After a 2-h adsorption period at 37°C, the monolayers were washed with warm phosphate-buffered saline before addition of the agar overlay. As indicated below, in some assays trypsin or elastase (0.3 μg/ml) was present in the overlay. Monolayers were stained with neutral red 3 to 4 days postinfection (5), plaques were counted at 4 to 6 days, and the average diameter of 15 to 20 random plaques was determined.

Polyacrylamide electrophoresis. Samples were made 1% in sodium dodecyl sulfate and 1% in 2-mercaptoethanol, and 10% polyacrylamide slab gels were run in a discontinuous buffer system as described previously (15).

Fluorography and radioactivity measurements. For detection of [^{3}H]leucine on the slab gels, fluorography (3) was done as described previously (14).

Chemicals and isotopes. Components for polyacrylamide gels were obtained from Ames Co., Miles Laboratories, Elkhart, Ind.; acetylated trypsin, α-chymotrypsin, and elastase were obtained from Sigma Chemical Co., St. Louis, Mo.; trypsin inhibitor from soybean was obtained from Miles Laboratories, Kankakee, Ill.; and [^{3}H]leucine was obtained from New England Nuclear Corp., Boston, Mass.

RESULTS

Efficiency of plaque formation in serially passed RMK cells. Replicate dilutions of infective egg-grown Sendai virus were titrated on serial passages of RMK cells with or without *N*-acetyl trypsin (0.3 μg/ml) in the overlay. The cells were passed at a dilution of 1:5 and could usually be passed five to six times before senescence. All experiments were done on cells that could be carried for at least one more viable passage. As shown in Table 1, without trypsin the number and size of plaques formed decreased markedly in the third and fourth cell passages, whereas in the presence of trypsin, plaque formation was restored to the level of the first passage. Thus, later cell passages sustain multiple-cycle infection with reduced efficiency. Figure 1 illustrates the plaque morphology in secondary cultures of RMK cells. Without trypsin, 90 plaques were formed with an average diameter of 1.3 mm; with trypsin, the number increased to 128, and the diameter increased to 2.4 mm. Although plaque size and number did not decrease significantly until the third passage in the experiment shown in Table 1, Fig. 1 shows a reduction on the second passage, illustrating some variation in the exact passage number in which the decrease in multiple-cycle replication occurs. The somewhat lower plaque titer in primary than secondary cells in Table 1 probably reflects the fact that the primary cultures are a heterogeneous mixture of cell types from the kidney (9), whereas second-passage cells are a more homogeneous population.

Multiple-cycle replication in a continuous line of RMK cells. The decline in efficiency of plaque formation with cell passage was further examined using LLC-MK$_2$ cells, a continuous line of RMK cells that have undergone innumerable passages. As shown in Fig. 2, Sendai virus could undergo multiple-cycle replication only when trypsin was added to the medium. To yield a detectable hemagglutination in a single

TABLE 1. *Effect of trypsin on plaque formation by Sendai virus in serial passages of RMK cells*

Cell passage	Plaques[a]			
	Trypsin (0.3 μg/ml)		No trypsin	
	No.	Diam (mm)	No.	Diam (mm)
1	54[b]	NM[c]	50	NM
2	85	2.1[d]	79	2.0
3	57	1.8	21	1.3
4	53	1.7	15	0.4

[a] The virus inoculum was grown in the chorioallantoic sac of the chicken embryo and, at the dilution used, produced ~70 plaques in MDBK cells in the presence of trypsin. Trypsin was present in the agar overlay during the assay.

[b] Average count on three monolayers.

[c] NM, Not measured, plaques ~2 mm.

[d] Average of 15 to 20 plaques.

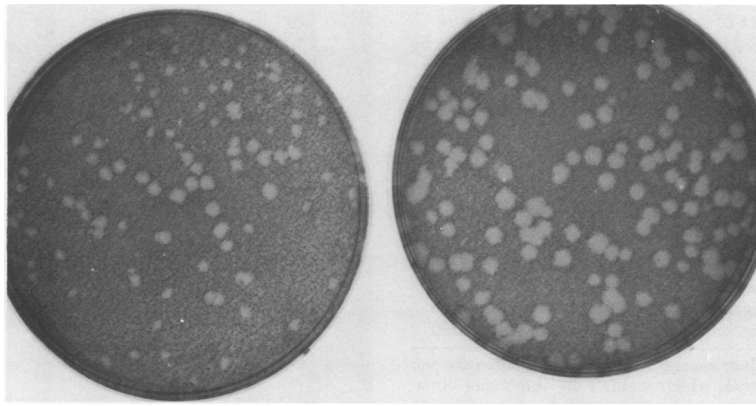

FIG. 1. *Enhancement by trypsin of plaque formation by egg-grown Sendai virions in secondary cultures of RMK cells. Confluent monolayers were inoculated with equal concentrations of virus. Left, no trypsin; right, N-acetyl trypsin (0.3 μg/ml) present in the agar overlay.*

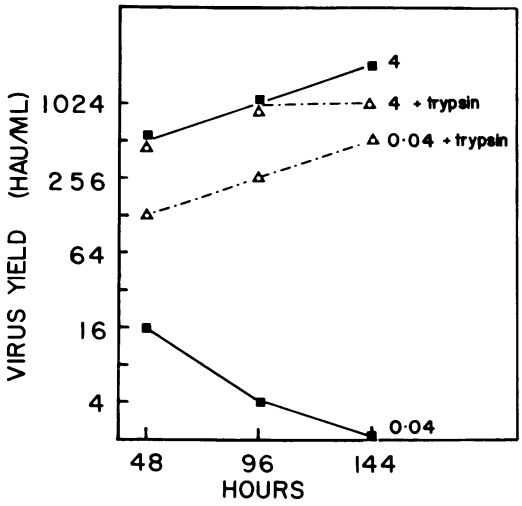

FIG. 2. *Effect of trypsin on the replication of Sendai virus in LLC-MK₂ cells inoculated at different multiplicities. Confluent monolayers were inoculated with egg-grown virus at a multiplicity of 4 or 0.04, and after a 2-h adsorption period, were washed with phosphate-buffered saline, and 2.5 ml of reinforced Eagle medium was added with (△- - -△) or without (■——■) N-acetyl trypsin (0.1 μg/ml). Virus yield was assayed by hemagglutination titrations at times indicated.*

cycle, at least 0.5% of the cells must be infected by the input virus; at a lower initial infection rate, the presence of trypsin is required. A similar pattern has been observed in Sendai virus infection of MDBK cells, which also yields noninfective virus in the absence of trypsin (23). As with MDBK cells, trypsin is also required for plaque formation in LLC-MK₂ cells. Thus, LLC-MK₂ cells do not provide the enzyme required for virus activation and appear to represent the final expression of the reduction in efficiency of activation shown above with the serial passages of RMK cells.

Activation of trypsin-sensitive pa mutants by secondary RMK cells. To investigate the specificity of the proteolytic activity present in the early passages of RMK cells, a set of pa mutants of Sendai virus was used (23). Whereas WT virus can be activated and forms plaques with trypsin, the pa mutant pa-c1 can be activated with chymotrypsin or elastase, but not trypsin; mutants pa-e1 and pa-e3 through -e8 can be activated by trypsin or elastase; and pa-e2 can be activated with elastase only. These pa mutants were used as probes to determine the activating protease associated with early-passaged RMK cells. WT and pa mutants were plaqued on secondary RMK, LLC-MK₂, and MDBK cells with or without the addition of specific proteases. Because WT virus will not grow in the presence of elastase or chymotrypsin, it was plaqued in the presence of trypsin (0.3 μg/ml). The pa mutants were plaqued in the presence of elastase, because each of them is activated by this enzyme, and pa-c1 and -e2 are not activated by trypsin. As shown in Table 2, with the appropriate activating enzyme, plaque formation of each virus in secondary RMK, LLC-MK₂, and MDBK (data not shown) cells was efficient. In the absence of the appropriate protease, LLC-MK₂ cells could not support plaque formation with any of the viruses; thus these cells did not provide any of the specific proteases required for virus activation. However, early-passage RMK cells could support plaque formation with high efficiency of those viruses that are activated by trypsin, WT, and pa mutants e1 and e5 through e8 (data not shown), but not the mutants that require proteases other than trypsin, pa-c1, and -e2. Thus, the activating protease that was present in early-passage cells,

TABLE 2. *Effect of specific proteases on plaque formation in LLC-MK$_2$ and secondary RMK cells by pa mutants of Sendai virus*

Virus and protease	Plaques[a]			
	Secondary RMK cells		LLC-MK$_2$ cells	
	No protease	Protease	No protease	Protease
WT, trypsin (0.3 μg/ml)	65	74	0	77
pa-c1, elastase (0.3 μg/ml)	18[b]	186	0	196
pa-e1, elastase (0.3 μg/ml)	115	140	0	149
pa-e2, elastase (0.3 μg/ml)	23[b]	165	0	190

[a] The virus inoculum was grown in the chorioallantoic sac of the chicken embryo, which yields fully infectious virus. Replicate monolayers were inoculated with the same dilution of WT and pa mutants. Trypsin or elastase (0.3 μg/ml) was present in the agar overlay with WT and pa mutants, respectively.

[b] Plaques were faint and small (<0.5 mm in diameter). Normal plaques, seen on the other plates, were distinct and ≤1.5 mm in diameter.

but lost on serial passage, has trypsin-like activity.

Decreased cleavage of F_0 in later passages of RMK cells. Because the ability of late-passage RMK cells to plaque Sendai virus is decreased and virus activation is dependent upon the cleavage of the F_0 glycoprotein, it therefore seemed likely that the late-passage cells did not efficiently cleave the F_0 glycoprotein to F_1 and F_2. Figure 3 shows an electropherogram of virus from first-, fourth-, and fifth-passage RMK cells. There were different amounts of radioactivity in each lane; however, comparison of the relative amounts of the F_0 and F_1 glycoproteins indicates a decrease in the extent of cleavage of F_0 with increasing passage number. To quantitate this, the F_0 and F_1 bands of each lane were cut out, solubilized, and counted. Table 3 shows the percent cleavage of F_0 in the various cell types. Decreased cleavage of F_0 was correlated with the decreased ability of later-passage RMK cells to support plaque formation. MDBK cells, in which egg-grown Sendai virus cannot plaque without exogenous trypsin, cleaved the F_0 protein by only 16%.

Inability of early-passage RMK cells to activate MDBK-grown Sendai virus. Because early-passage RMK cells are capable of producing virus with cleaved F_0 and, therefore, of sustaining multiple-cycle replication, we investigated whether these cells could activate adsorbed, inactive virions with uncleaved F_0 glycoprotein. Primary and secondary RMK cells were inoculated with noninfective MDBK-cell-grown virions. The HN glycoprotein of such virions possesses full hemagglutination and neuraminidase activities, and thus adsorption, which occurs through this glycoprotein, is normal (22). However, when RMK cells were inoculated with 53 plaque-forming units of inactive virus (as assayed on MDBK cells in the presence

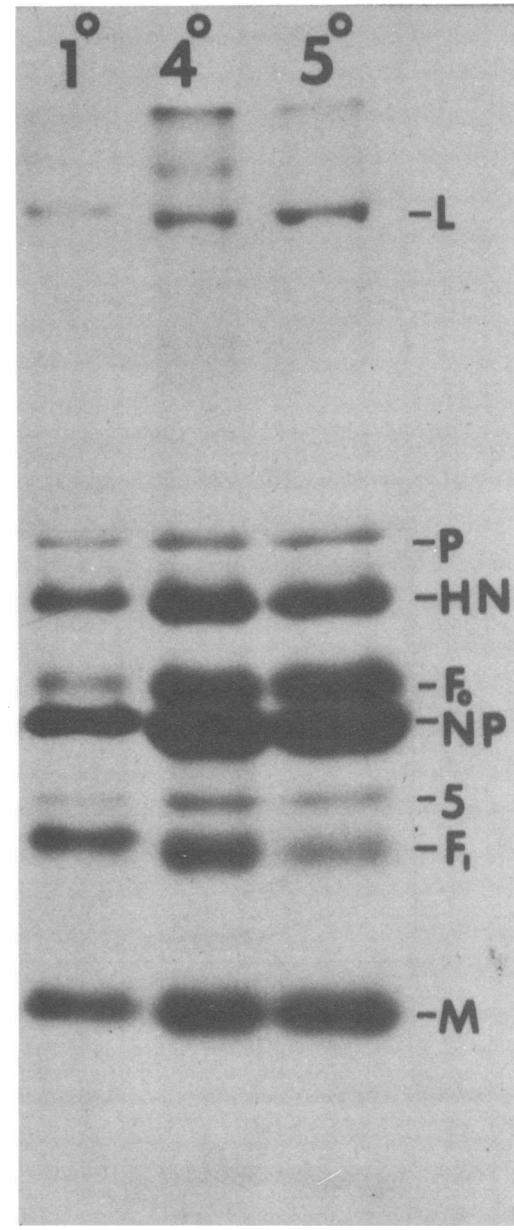

FIG. 3. *Polyacrylamide gel electrophoresis of the polypeptides of Sendai virions grown in primary, fourth-, and fifth-passage RMK cells. Confluent monolayers were inoculated with egg-grown virus at a multiplicity of 30 plaque-forming units per cell, and virus was grown in the presence of [^{3}H]leucine as described in the text. Virions were purified, disrupted with sodium dodecyl sulfate and 2-mercaptoethanol, and subjected to electrophoresis on a 10% gel followed by fluorography.*

TABLE 3. *Quantitation of the cleavage of the Sendai virus F_0 polypeptide in various cell cultures*

Cells	Polypeptide (cpm)[a]		% Cleavage[b]
	F_0	F_1	
Primary RMK	565	831	60
Fourth-passage monkey kidney	2,751	1,220	31
Fifth-passage monkey kidney	3,279	991	23
MDBK	1,646	319	16

[a] Radioactivity in polypeptides was determined by cutting the appropriate bands from the gels shown in Fig. 3 and solubilizing them as described in the text. Background determinations were made from non-polypeptide-containing areas from the same lanes and subtracted from the values for F_0 and F_1.

[b] % Cleavage = $[F_1/(F_0 + F_1)] \times 100$. No correction was made for the small amount of label incorporated into F_2 (24) that runs with the dye front and has not been determined directly.

of trypsin), no plaques were formed, but when trypsin (0.3 μg/ml) was present in the overlay, 63 plaques were detected. Thus, the early passages of RMK cells were unable to activate adsorbed virus, even though these cells possess the requisite proteolytic activity to achieve the cleavage of F_0 and activate virus produced in these cells. A similar result was obtained when early-passage RMK cells were inoculated with inactive MDBK-grown Sendai virions and maintained in liquid medium; hemagglutination titers were not detected unless trypsin was added to the growth medium.

DISCUSSION

These studies have shown that primary and secondary cells support multiple-cycle replication of Sendai virus, but lose this ability in later passages due to a decline in proteolytic activity that activates virus by cleavage of the F_0 glycoprotein. The restoration of multiple-cycle replication in later cell passages by addition of trypsin to the medium and experiments with pa mutants that require specific proteases have suggested that the protease is trypsin-like in activity, and is thus similar to that found in the allantoic sac of the chicken embryo (22, 23).

The location of the activating protease in RMK or chorioallantoic membrane cells has not yet been established; however, several lines of evidence have suggested that it may be associated with the plasma membrane. Activating protease has not been found in the culture medium or allantoic fluid (22), and F_0, but not its cleavage products, has been found in cytoplasmic extracts of infected RMK cells (14), yet mature virions produced by chorioallantoic membrane or RMK cells contain the cleaved glycoprotein. Experiments with another paramyxovirus, simian virus 5, are also compatible with cleavage of F_0 at the plasma membrane, although this has not yet been established (20). However, cleavage may not occur only on the plasma membrane in all paramyxovirus cell systems; Nagai et al. (19) suggested that cleavage of the F_0 glycoprotein of Newcastle disease virus occurs in association with intracellular membranes in BHK21-F cells. The direct demonstration of the location of the enzyme and its characterization await further experiments. It should be noted that cleavage of the hemagglutinin protein of influenza virus, which enhances infectivity, has been demonstrated at the plasma membrane (15, 16).

It is of interest with regard to the location of the protease that primary and secondary RMK cells, which produce infectious virus with cleaved F glycoprotein, cannot activate adsorbed inactive virus, although such virus can be activated by added trypsin. Thus, the enzyme, although clearly present in the cell and probably associated with the plasma membrane where cleavage occurs during maturation, is not accessible to adsorbed virus. Shibuta (25) found that cynomologous monkey kidney cells supported plaque formation by virus grown in chicken embryos or secondary cynomologous monkey kidney cells, but not by virus grown in a variety of cell lines or chicken embryo fibroblasts, a finding that can now be explained in terms of cleavage by the former cells.

The inability of cells that possess the activating protease and produce infectious virus to activate adsorbed inactive virus may be of significance in disease. In an infected host, if cells of one tissue were capable of activating inactive virions produced by other cells, failure of cleavage would be less significant because virus could be activated after adsorption to other cells. On the other hand, the observed inability of cells to activate adsorbed inactive virus, even though they possess the necessary protease, would tend to limit the spread of infection in a host. It is of interest that virulence of Newcastle disease virus strains for chickens correlates with cleavage of viral glycoproteins by host enzymes (18).

The present results indicate that a decline in proteolytic activation of Sendai virus is another property of serially passed RMK cells, which usually have a limited life span in vitro. After five or more passages, these cells reach senescence and can no longer be serially passed. Hayflick (10) and others have studied senescence in diploid WI-38 human fetal lung fibroblasts, which can usually be passed 40 to 50 times before senescence. One finding was an increase in lysosomal enzyme activity (for review see reference 11). Bosmann et al. (4) and Sun et al.

(28) also found increases in six acid hydrolases on passage of WI-38 cells. Bosmann found that a cathepsin-like enzyme remained essentially constant on cell passage; however, a neutral proteolytic activity declined to an undetectable level when the cells reached a senescent phase. The decline in virus-activating protease in RMK cells resembles this and is in contrast to the increase seen in other proteolytic activities in aging cultured cells (11).

The loss on passage of the ability to cleave the F_0 glycoprotein has provided a biochemical explanation for failure of later passages or a continuous cell line to support replication of a paramyxovirus. Failure of serially passed cells to support replication of viruses that grow in primary cells has repeatedly been observed in the past, and with enteroviruses another explanation was found. McLaren et al. (17) showed that primary RMK or human amnion cells could be infected by Coxsackie A9 virus, whereas continuous lines of these cells were resistant due to the loss of virus receptors. In another study (2) human amnion cells became resistant to Coxsackie A23 virus by the fourth passage. The mechanisms involved in the loss on serial passage of enterovirus receptors or of the proteolytic activity required for cleavage of paramyxovirus glycoproteins are unknown. If is of interest that both events involve changes in the plasma membrane of the cell. It is also not clear whether the loss is due to the selection on passage of cells with a lower level of this protease, or whether it is part of a general decrease in proteases and perhaps other enzymes. However, these results have emphasized the role of host enzymes in determining the outcome of infection, not only with respect to cell type but also the metabolic state of the cell.

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