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Thermolysin Activation Mutants with Changes in the Fusogenic Region of an Influenza Virus Hemagglutinin

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Influenza virus A/seal/Mass/1/80 (H7N7) mutants were obtained; the hemagglutinins (HAs) of the mutants were not activated by trypsin, as in the wild-type virus, but by thermolysin. The mutants grew efficiently under multiple replication cycle conditions and formed plaques in chicken embryo cells only when thermolysin was added to the culture medium. They exhibited hemolytic activity and induced protective immunity in chickens after an asymptomatic course of infection. Nucleotide sequencing of the HA gene and direct amino acid sequencing showed that insertion of a single leucine into the fusion peptide of the HA2 chain close to the cleavage site and a shift of the cleavage site toward the C terminus by one amino acid were responsible for the changes in the biological properties of the thermolysin activation mutants. Revertants could be obtained when trypsin or trypsin-like endoproteases were present in the virus-producing system.

Penetration of influenza virus into the host cell occurs by fusion of the viral envelope with cellular membranes, mediated by the viral spike glycoprotein hemagglutinin (HA). Posttranslational cleavage of an inactive precursor molecule, HA0, into the two disulfide-linked polypeptides, HA1 and HA2, by appropriate endoproteases is essential for the formation of the fusion-active HA and, consequently, of infectious virus particles (7). The sequence of the N terminus of HA2 produced by the cleavage process is hydrophobic and highly conserved among influenza viruses and becomes exposed and relocated at low pH (2, 18). All evidence presented supports the concept that the structure at the C terminus of HA1 is the key factor that determines HA cleavability and activation. Mammalian influenza viruses and the apathogenic avian influenza viruses, which cause local infections, have a single arginine at the cleavage site. Trypsin or trypsin-like proteases, such as plasmin (8, 14), the blood clotting factor X in the allantoic fluid of chicken eggs (5), tryptase Clara associated with Clara cells of the bronchiolar epithelia (6), and inflammatory (14) and bacterial proteases (14, 16), have been identified as enzymes that recognize monobasic cleavage sites of HA. Ubiquitous enzymes, such as the subtilisin-related furin, proved to activate the HAs of the highly pathogenic avian influenza viruses, which cause a generalized infection. This type of enzyme usually recognizes the multibasic cleavage site R-X-(K/R)-R motif (15, 17). The cleavability of HA can be modulated by mutations at or at some distance from the cleavage site (11, 12). In the present report we describe a new type of influenza virus mutant in which HA had lost the ability to be activated by trypsin-like proteases but could be activated by thermolysin. The thermolysin activation mutants provide further evidence that not only the structure at the C terminus of HA1 but also the sequence of the N terminus of HA2 is critical for HA activation.

Egg-grown influenza virus A/seal/Mass/1/80 (H7N7) (wild-type [wt] virus), which contains a single arginine at the HA cleavage site and is produced in infectious form only in a restricted number of cell types (9, 10), was serially passaged at a multiplicity of infection (MOI) of about 10^{-2} PFU per cell in nonpermissive chicken embryo cells (CEC) in the absence of trypsin but in the presence of 10 μg of thermolysin per ml in the culture medium. Medium taken after incubation of infected cells for 72 h at 37°C was used for further passages. After five passages, virus particles were isolated which formed clearly visible plaques in CEC when thermolysin was present in the agar overlay. Three plaque mutants (Th1 to Th3), purified by five plaque-to-plaque passages, were used for further investigations.

When CEC were infected at a high MOI (~10 PFU per cell) with mutants Th1 to Th3 in the absence of exogenous proteases, virus yield, as determined by hemagglutination, was similar to that of the wt virus. Progeny viruses, however, were not infectious. They could be activated by in vitro treatment with 5 μg of thermolysin per ml.

In contrast to the wt virus, Th1 to Th3 are not produced in CEC in the presence of trypsin under multiple replication cycle conditions (MOI of $\sim 10^{-3}$ PFU per cell). As expected, the mutants grew efficiently, however, in the presence of at least 0.5 μg of thermolysin per ml in the culture medium (Fig. 1). The patterns of activation by trypsin and thermolysin were identical to those found by the plaque assays. The thermolysin-activated mutants lysed chicken erythrocytes in a manner similar to that of the trypsin-activated wt virus. However, the pH optimum for hemolysis was 5.0 compared with 5.4 for the wt virus, suggesting a conformational change of the fusion peptide (2, 4). Th mutants, activated in vitro by thermolysin, produced negligible clinical signs and lung lesions over a 14-day incubation period after intratracheal inoculation into chickens. Nevertheless, Th mutants induced the production of HA-inhibiting antibodies and protective immunity in the chicken against challenge with 10^5 PFU of the highly pathogenic seal virus variant SC35 (9), indicating that at least a single growth cycle had occurred. A minimal mass of viral antigen must have been produced, since inoculation of an equal amount of inactivated virus did not result in protection. Cleavage of HA of the Th mutants is shown by polyacrylamide gel electrophoresis (Fig. 2). When [^{35}S]methionine-labeled purified virus preparations of wt virus and the Th mutants obtained from the medium of infected CEC were compared, it became evident that the HA0 polypeptides of the Th mutants are cleaved after treatment with thermolysin and trypsin, although their HAs were not activated by the latter protease, in

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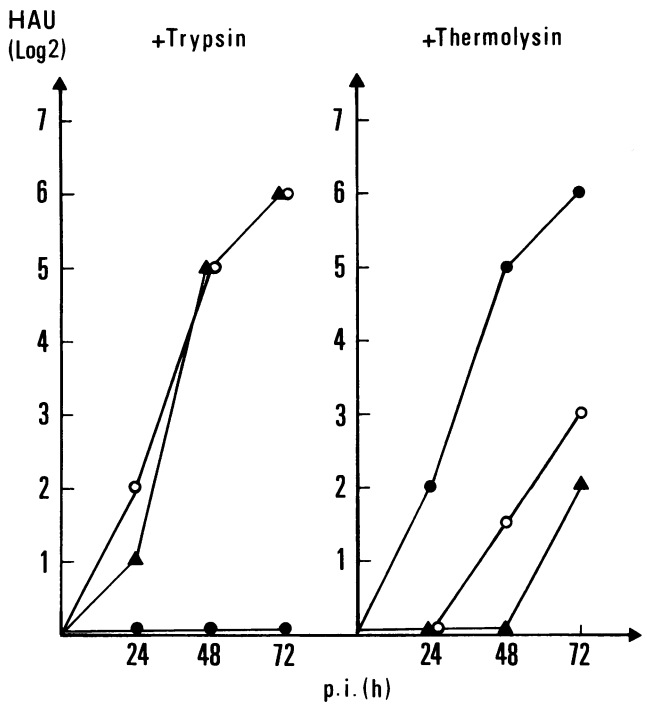

FIG. 1. Growth of thermolysin activation mutant Th1 (●), the revertant Th1T (○), and wt seal influenza virus (▲) in CEC in the presence of 1 μg of thermolysin or trypsin per ml. Cells were infected with an MOI of 10^{-3} PFU per cell. After incubation for 72 h at 37°C, HA titers in the medium were determined at the time points indicated. p.i., postinfection; HAU, HA units.

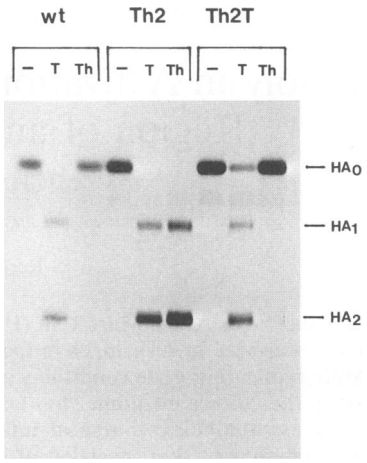

FIG. 2. Proteolytic cleavage of the HA of thermolysin activation mutant Th2, the revertant Th2T, and the wt virus. CEC were labeled with 100 μCi of [^{35}S]methionine 5 to 20 h after infection with 10 PFU of the virus per cell. Virus obtained from the medium and purified by centrifugation was treated with 5 μg of thermolysin (Th) per ml or 5 μg of trypsin (T) per ml for 15 min at 37°C or not treated (–). HA was immunoprecipitated by standard procedures using an HA (H7)-specific monoclonal antibody and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions (1).

contrast to HA0 of wt virus. No differences were found in the migration of the HA polypeptides of the different virus preparations synthesized in CEC under appropriate virus growth conditions.

The Th mutants were found to be genetically stable when passaged at both high and low MOIs in CEC in the presence of 1 μg of thermolysin per ml in the medium. However, after 2 to 3 passages at an initial MOI of 10 PFU per cell of the Th mutants in CEC in the presence of trypsin, revertants were obtained; the HAs of these revertants were proteolytically cleaved and activated by trypsin but not by thermolysin, similar to the wt virus (Fig. 2). Under multiple and single growth cycle conditions, infectious revertants, similar to the wt virus, were produced only when trypsin was added to the culture medium. Plaque formation was also found to occur only in the presence of trypsin. When 11-day-old chicken embryos were infected with 10^3 PFU of thermolysin-activated Th mutants, infectious virus was detected only when thermolysin was applied. On the other hand, when infected with 10^7 PFU, the HA of the progeny virus obtained after a single passage was cleavable by thermolysin as well as by trypsin. Since the trypsin-like blood clotting factor X is present in the allantoic fluid of the chicken embryo (5), the conditions of the two systems responsible for obtaining revertants are similar: trypsin-like endoproteases must be present in the environment.

Nucleotide sequence analyses by the dideoxynucleotide chain termination method (9, 13) of the HA genes of the Th mutants and the deduced amino acid sequences revealed no amino acid exchange at HA1. With all three Th mutants, however, an insertion of Leu between Phe at position 3 and Gly-4 at HA2 of wt virus was found (Fig. 3). Additional amino acid replacements of Phe-3 to Leu or to Ile occurred for Th2 and Th3, respectively. Direct amino acid sequencing (3) of HA2 of the Th2 mutant revealed Leu-Leu-Leu-Gly-Ala-Ile-Ala at the N terminus and the loss of Gly-1, which is present directly adjacent to the cleavage site of all influenza viruses analyzed so far (18). Remarkably, the revertants showed similar amino acid substitutions at the fusion peptide of HA2, but in these cases the insertion of Leu was adjusted by the removal of Phe-3 (Th1T) or Leu-2 (Th2T), respectively.

The results obtained from this study point again to the high variability of influenza virus. The fact that thermolysin activation mutants could be isolated after a few passages suggests that they have been selected from a genetically heterogeneous virus population by the selection pressure of the enzyme. Thermolysin is not produced in cells of the respiratory tract, explaining why the virus was nonpathogenic for chickens after intratracheal application of the virus. Nevertheless, a single cycle of replication must have occurred, because the in vitro thermolysin-activated virus used for infection induced solid protective immunity.

The primary specificity of thermolysin is for the amino groups of hydrophobic amino acid residues. Therefore, one might have expected that the cleavage site by this protease would be the peptide bond between Gly-1 and Leu-2 of HA2, which was indeed the case. In light of previous studies, which showed that thermolysin cleaves the HA polypeptides of H3 and H10 subtype viruses at the same site, resulting in an inactive HA (3), it was of special interest that the HAs of the Th mutants described here were transformed into an active state. They exhibited hemolytic activity, although with a significant decrease in the pH threshold, and the virus particles became infectious through the cleavage reaction. This apparent discrepancy can be explained by the finding that the fusion peptide of the Th mutants has acquired an insertion of a single apolar amino acid, leucine, close to the cleavage site. Since no other mutation was found in the HA of the Th1 mutant, it is obvious that this insert altered the N-terminal sequence of

Virus	activated by	Sequences											
		← HA1			HA2 →								
wt	Trypsin	...CCA P	AAG K	ACC T	AGA R ↓	GGA G	CTT L	TTT F	---	GGA G	GCA A	ATT I	GCT... A
Th1	Thermolysin	*	*	*	AGG R	*	↓ *	*	CTT L	*	*	*	*
Th1T	Trypsin	*	*	*	AGG R ↓	*	*	---	CTT L	*	*	*	*
Th2	Thermolysin	*	*	*	AGG R	*	↓ *	TTG L	TTA L	*	*	*	*
Th2T	Trypsin	*	*	*	AGG R ↓	*	---	CTG L	TTA L	*	*	*	*
Th3	Thermolysin	*	*	*	AGG R	*	↓ *	ATT I	CTT L	*	*	*	*

FIG. 3. Nucleotide sequences of the HA gene and the predicted amino acid sequence around the cleavage site (arrows) of wt, the thermolysin activation mutants (Th1 to Th3), and the revertants (Th1T and Th2T) of the seal influenza virus. The single-letter amino acid code was used for the predicted protein sequences. An asterisk denotes the same nucleotide or amino acid as that found in wt virus; - - - indicates that the nucleotide and amino acid are missing.

HA2 in such a way that it retained the fusion activity, although the N-terminal Gly was lost. This result would imply that not an absolute conserved sequence but the length of the hydrophobic amino acid sequence at this region is required for the fusion activity of HA and thereby for the infectivity of the virus. This conclusion was confirmed by the revertants obtained, which were no longer activated by thermolysin, but by trypsin, like the wt virus. They contained Leu at the same position at which the insert was introduced into the Th mutants, but on the other side they have lost a Leu or Phe at position 2 or 3, respectively, so that the number of the apolar amino acids remained the same as that of the wt virus, including the N-terminal Gly of HA2.

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