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Intracellular Cleavage of Human Influenza A Virus Hemagglutinin and Its Inhibition

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Abstract—Replication of human influenza A viruses and proteolytic cleavage of the viral glycoprotein HA0 → HA1/2 were studied in passaged cultures of epithelial cells of the mucosal membrane of human large intestine (CACO-2 line), dog kidney cells (MDCK), and monkey kidney cells (CV-1). Cleavage of the viral glycoprotein HA0, synthesis of activated virions, multicycle virus infection, and effective production of viral foci under an agarose overlayer were found in CACO-2 cells. By pulse–chase labeling of viral glycoproteins, testing the sensitivity to endoglycosidase-H of the viral glycoproteins HA0 and HA1/2 synthesized, and inhibiting the HA0 proteolysis with brefeldin A, the HA0 → HA1/2 proteolysis was established to occur in the late stages of intracellular transport in the *trans*-Golgi and plasma membrane areas of the cells. Proteolysis of the viral glycoprotein HA0 in CACO-2 cells was suppressed by aprotinin, a natural inhibitor of serine proteinases. Unlike MDCK and CV-1 cells resistant to apoptosis induced by influenza virus, CACO-2 cells retained their viability for 2-3 days after infection with human influenza A virus.

Key words: influenza virus, replication, hemagglutinin cleavage, aprotinin

Nine structural proteins (PB1, PB2, PA, NP with molecular weight of 56 kD, M1 (27 kD), M2, NEP, NA, HA) and two nonstructural proteins NS1 and PB1-F2 are found in the structure of influenza A virus. Hemagglutinin (HA) of influenza virus is located on the surface of the viral particle and is responsible for the penetration of the virus into a target cell. This function requires the HA0 molecule (75 kD) to be proteolytically cleaved to two subunits, HA1 (55 kD) and HA2 (20 kD), that activates hemagglutinin and makes the virus infectious for target cells [1, 2]. It should be emphasized that host proteinases are responsible for the cleavage of the influenza virus hemagglutinin, and this process underlies the pathogenesis of viral infection [3].

Abbreviations: PB1, PB2, PA) proteins of polymerase complex; NP) main nucleocapsid protein; M1) virion matrix protein; M2) ionic channel protein; NEP) nuclear export protein; NA) neuraminidase; HA) hemagglutinin; NS1) nonstructural protein 1; PB1-F2) virus mitochondrial protein; HA0) uncleaved hemagglutinin; HA1, HA2) large and small subunits of hemagglutinin, respectively; CACO-2) cell line of human large intestine; CV-1) line of monkey kidney cells; MDCK) line of dog kidney cells; DMEM) Dulbecco's modified Eagle's medium.

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The HA molecule is cleaved in a strictly determined proteolytic site [4]. By primary structure of the proteolytic site of HA, all influenza viruses are subdivided into two large groups. The first group includes two viral subtypes H5 and H7 with several Arg residues in the proteolytic site of HA (the multi-arginine site). Such hemagglutinin is cleaved intracellularly in the *trans*-Golgi apparatus under the influence of subtilisin family proteinases [5]. Viruses with the multi-arginine HA leave the infected cells in a fully infectious state with HA1/2 and effectively infect adjacent target cells. The second group consists of viruses whose HA is cleaved in a site containing a single Arg residue (the mono-arginine site). This group includes all viruses of human influenza (H1-H3 subtypes) and the majority of avian and animal viruses (subtypes H1-H15 except H5 and H7). HA0 of these viruses is cleaved, as a rule, outside the cell (or on the plasma membrane of the cell) by extracellular trypsin-like proteinases [6]. Viruses of this group leave the infected cells in a non-active state with HA0 and need an additional activation in the intercellular medium to become infectious for new cells [7].

This rule of the extracellular proteolysis of mono-arginine hemagglutinin of human influenza A viruses is likely to be not very strict. In a published work [8] the

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intracellular cleavage HA0 → HA1 + HA2 of human influenza virus A/WSN/33 (H1N1) was found in a culture of bovine cells (the line MDBK). The proteolysis occurred at the stage of the penetration of the virus into the target cell, and the authors suggested that a proteinase should exist in endosomes of the MDBK cells capable of activating the influenza protein HA0 with the mono-arginine proteolytic site.

The present work performed on the culture of passaged cells of human large intestine carcinoma (the CACO-2 line) extends these observations and casts some doubt on the dogma about the obligatory extracellular proteolysis of hemagglutinin of human influenza epidemic viruses. The intracellular cleavage of the viral protein HA0 with the mono-arginine site and activation of human influenza A viruses are shown for the first time. HA0 is shown to be sensitive to proteolysis by aprotinin, which is an inhibitor of serine trypsin-like proteinases. It is also shown that CACO-2 culture of human cells has an increased resistance to apoptosis induced by influenza infection and maintains for several days the reproduction of human influenza A virus.

MATERIALS AND METHODS

Viruses and cells. Human influenza viruses A/Aichi/2/68 (H3N2) and A/PR/8/34 (H1N1) were propagated in 9-day-old chicken embryos as described in [9]. Passaged cultures of human large intestine carcinoma cells, dog kidney cells, and monkey kidney cells (CACO-2, MDCK, CV-1 lines, respectively) were grown on polystyrene dishes in the Dulbecco's modified Eagle's medium (DMEM) in the presence of 10% fetal calf serum (Gibco BRL, Germany). After infection with the viruses, the CACO-2 cells were incubated in the serum-free medium.

Pulse–chase labeling of cellular polypeptides. Proteins of the infected cells were labeled with a mixture of [^{35}S]cysteine and [^{35}S]methionine (Promix, Amersham, England) dissolved in the DMEM without these amino acids (Sigma, USA) at the concentration of 70 μCi/ml within 45 min (the pulse). After the pulse labeling, the medium was removed, and the cells were washed and incubated in DMEM containing a tenfold excess of unlabeled cysteine and methionine (the chase). The labeled cells were subjected to electrophoresis in polyacrylamide gel.

Immune precipitation of labeled viral proteins. The pulse–chase labeled infected CACO-2 cells (~10^6) were homogenized in 400 μl of phosphate buffer (PB: 10 mM Na_2HPO_4/NaH_2PO_4, pH 7.2, 2.7 mM KCl, 137 mM NaCl) supplemented with 0.1% BSA, 0.005% SDS, 0.025% Triton X-100, 0.2% Tween 20, 400 μM benzamidine (Sigma), 200 trypsin-inhibiting units (TIU) of aprotinin (AWD, Germany) per 1 ml, then destroyed for 2 min with a Branson-450 ultrasonic disintegrator (Branson, USA), and incubated with rabbit antibodies to the whole virus for 1.5 h at 5°C. Then the suspension was supplemented with protein A conjugate with Sepharose (Pharmacia-Biotech, England) to the concentration of 4% and incubated for 1 h at 4°C. The resulting complexes of polypeptide–antibody–protein A-Sepharose were washed four times with PB at 2000*g* for 8 min and then dissolved in a dissociating buffer containing 2% SDS and 10 mM dithiothreitol at 96°C for 5 min. The dissociated proteins were analyzed in polyacrylamide gel with subsequent detection of labeled polypeptides by radioautography on Kodak Biomax film (USA).

Identification of the glycosylation profile of the HA protein, cell treatment with brefeldin A. The glycosylation profile of the HA protein was determined by sensitivity of the carbohydrate component to glycosidase F and endoglycosidase H (BioLabs, Germany) under denaturing conditions according to the firm's protocol. After the treatment of cell homogenates with glycosidase, the polypeptide composition was analyzed by western-blot-electrophoresis with antiviral antibodies. Brefeldin A (Sigma) was added to the culture medium from the stock solution prepared in ethanol (5 mg/ml) to the final concentration of 10 μg/ml immediately after the pulse labeling of cell proteins with a subsequent incubation in this medium during the chase. Then the labeled proteins were analyzed in polyacrylamide gel with subsequent radioautography of the gels.

Titration of virus with immune complexes in cell cultures. A monolayer of CACO-2 or MDCK cells in 24-well plates was infected for 1 h with variously dissolved viruses at 37°C, and after the infection the cell monolayer in the well was covered with 0.6 ml of 1% agarose in DMEM supplemented with 0.1% BSA, penicillin (25 U/ml), and streptomycin (25 μg/ml), or additionally trypsin (0.1 μg/ml). At different times of incubation (in the time interval from 10 to 48 h), the cells were fixed for 20 min with 2% *p*-formaldehyde in PB and permeabilized with 0.2% nonionic detergent NP-40 for 15 min. Then the monolayer was incubated for 1 h at 20°C with monoclonal antibodies to the influenza protein NP (A3 clone; collection of the Center for Disease Control, Atlanta, USA) and then with the anti-species peroxidase conjugate. Immune complexes were stained with a water-insoluble tetramethylbenzidine (TMB) substrate True Blue (KPL, USA).

Electrophoresis in polyacrylamide gel and identification of proteins by western-blotting. Electrophoresis of the polypeptides was performed in polyacrylamide gel with SDS in a mini-apparatus (7 × 8 cm) as described in [10]. Before the electrophoresis, the specimens to be analyzed were dissolved in buffer containing 1% SDS, 10 mM dithiothreitol (DTT), and 10% glycerol. After the electrophoresis, the polypeptides were by a semi-dry method transferred onto a Protrane membrane (Schleicher and

Schuell, Germany) [10]. The membrane was incubated with antiviral antibodies dissolved in PB supplemented with 0.5% BSA for 2 h at 20°C, and immune complexes were identified with an anti-species peroxidase conjugate (Dako, Denmark) by enhanced chemiluminescence (ECL) with an ECL-supersubstrate (Pierce, USA).

Analysis of apoptotic fragmentation of DNA. Low-molecular-weight DNA fragments of cell chromatin were isolated by salt extraction in the presence of SDS [11]. The cells (~10^6) were suspended in PB, precipitated at 1500*g* for 15 min, the cell precipitates were suspended in 80 μl of PB, and then successively mixed with 300 μl of the buffer containing 20 mM Tris-HCl (pH 7.6), 10 mM EDTA, 0.6% SDS, and 100 μl of 5 M NaCl. The resulting suspension was incubated for 12 h at 4°C to produce SDS-precipitates of unfragmented DNA. Then the suspension was centrifuged at 14,000*g* for 20 min. The supernatant fluid was successively treated with RNase A (1 mg/ml) (Quiagen, Germany) and proteinase K (0.2 mg/ml) (Sigma) for 45 min at 37°C, and DNA was precipitated with three volumes of 96% ethanol. The precipitated DNA was dissolved in buffer TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.8) and analyzed by electrophoresis in 1.6% agarose gels containing ethidium bromide (1 μg/ml).

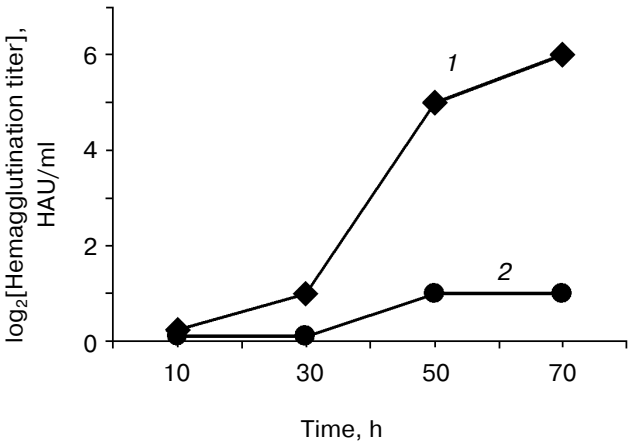

Fig. 1. Multicycle reproduction of influenza A virus in CACO-2 culture. Cell cultures CACO-2 (*1*) and MDCK (*2*) were infected with influenza virus A/Aichi/68 at the multiplicity of ~0.001 plaque-forming units per cell and then incubated in DMEM without serum. At fixed time intervals (the abscissa axis) the virus titer was determined in the culture fluid by its ability to agglutinate chicken erythrocytes. Along the ordinate axis is $\log_2$ of hemagglutination titer. The hemagglutination titer determined in hemagglutination units (HAU) is the inverse value of the maximum dilution of the virus causing hemagglutination of 0.5% suspension of chicken erythrocytes.

RESULTS

In the first part of the work, multicycle infection with different human influenza A viruses was studied in CACO-2 culture. The cell monolayer was infected at the multiplicity of ~0.001 plaque-forming units per cell, and accumulation of the virus in the culture medium was determined. In CACO-2 effective replication occurred, and a significant amount of the virus up to the titer of 2^5-2^6 hemagglutination units per 1 ml was accumulated 48-72 h after the infection, whereas in MDCK cells unable to cleave the viral HA0 no multicycle reproduction of the virus occurred (Fig. 1). Therefore, it was suggested that the CACO-2 cell could cleave the viral HA0 and activate the virus, thus providing for the multicycle reproduction in the culture. To support this hypothesis, the ability of influenza viruses to produce foci in CACO-2 and MDCK cultures under the agarose overlayer with trypsin and without it was also studied. In CACO-2 cells, viral foci were effectively produced in the absence of trypsin in the agarose overlayer, whereas in MDCK culture trypsin was required to produce foci (Fig. 2). Similar results were obtained with A/PR/8/34 virus (not shown). These findings confirmed that in CACO-2 cells human influenza viruses were activated by a cell proteinase and infected the adjacent cells by production of foci of the infected cells.

In the next experiments, we studied the intracellular cleavage HA0 → HA1/2 analyzing the profile of newly synthesized proteins by pulse–chase with S^{35}-labeled amino acids. Only uncleaved HA0 was found in the CACO-2 cells infected with A/Aichi/68 virus after the labeling for 40 min (Fig. 3). But after the chase for 60 min cleaved HA1/2 was detected, and its intracellular content after the chase for 2.5 h was 30-40% of the HA0 content. Similar data were also obtained for A/PR/34 virus (not shown). Thus, these observations suggested that the proteolysis of hemagglutinin of human influenza A viruses occurred inside the cells.

Then sensitivity of the HA0 → HA1/2 proteolysis was tested to various inhibitors of serine proteinases: to aprotinin isolated from bovine lungs [12], an inhibitor of the plasmin system ε-aminocaproic acid (ε-ACA) [13]; α1-antitrypsin (α1-AT) and α2-macroglobulin (α2-MAG) from blood serum [14]; and trypsin inhibitor from chicken eggs [15]. Figure 3 shows a noticeable cleavage of HA0 with production of HA1 and HA2 in the control cells without inhibitors. A similar protein spectrum suggesting the lack of inhibition of the HA0 → HA1/2 proteolysis was found in the cells in the presence of ε-ACA, α1-AT, α2-MAG, and the egg inhibitor (not shown). On the contrary, a noticeable inhibition was found in the infected CACO-2 cells in the presence of aprotinin, and this suggested that the proteinase responsible for proteolysis of HA0 is a serine proteinase and seems to be vulnerable to exogenous inhibitors of the aprotinin group.

Then the effect of aprotinin, which inhibits the intracellular proteolysis of HA0, was studied on appearance of the HA0 glycoprotein in the extracellular virus.

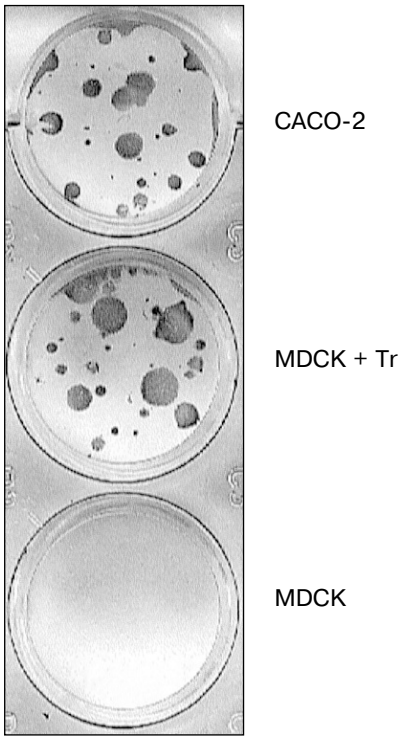

Fig. 2. Formation of influenza virus foci in CACO-2 and MDCK cultures. CACO-2 and MDCK cell cultures were infected with A/Aichi/68 virus with multiplicity of ~50 plaque-forming units per well. The cell monolayer was covered with an overlayer of agarose with trypsin (+Tr) and without it and incubated for 30 h. The monolayer was fixed with *p*-formaldehyde, and viral foci were identified with antibodies to the viral protein NP and by *in situ* staining with a water-insoluble dye True Blue.

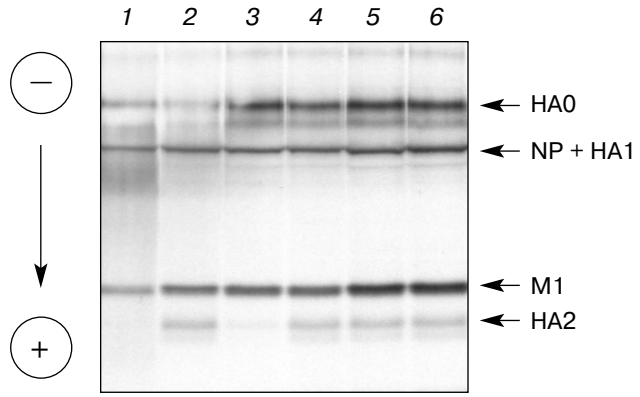

Fig. 3. Synthesis of viral A proteins in CACO-2 cells and the effect of proteinase inhibitors. The monolayer of CACO-2 cells was infected with A/Aichi/68 virus at the multiplicity of ~5 plaque-forming units per cell, 6.5 h after the infection the cell proteins were labeled for 35 min (the "pulse" (*1*)), and then the labeled cells were incubated for 2.5 h in the medium with excess unlabeled methionine and cysteine (*2*) which was also supplemented with aprotinin (150 trypsin-inhibiting units per 1 ml) (*3*), 50 mM ε-aminocaproic acid (ε-ACA) (*4*), 0.3 μM α1-antitrypsin (α1-AT) (*5*), or 0.05 μM α2-macroglobulin (α2-MAG) (*6*). Viral polypeptides were precipitated with rabbit antibodies to the whole virus prepared with protein A–Sepharose. The precipitated polypeptides were analyzed by electrophoresis in polyacrylamide gel with subsequent radioautography.

The protein composition was studied of the influenza virus synthesized in CACO-2 cells in the presence of aprotinin. Figure 4 shows (lanes *3* and *4*) that the virus synthesized in the CACO-2 contained a significant part of the hemagglutinin cleaved to HA1/2 similarly to the virus synthesized in chicken embryos (lane *1*). Judged by scanning the gels, about 80% of HA0 molecules in the virus were cleaved. On the contrary, in MDCK cells a virus was synthesized which mainly contained HA0 (lane *2*). Thus, in the virus synthesized in CACO-2 cells the cleaved hemagglutinin was predominant, and such an activated virus caused a multicycle virus reproduction, whereas no cleavage occurred in MDCK and the multicycle virus reproduction did not develop. On addition of aprotinin into the culture fluid, CACO-2 cells produced a virus with prevalence of uncleaved HA0 (Fig. 4, lane *3*). These findings fully confirm the conclusion about the synthesis of activated virus by CACO-2 cells and the inhibitory effect of aprotinin on the HA0 → HA1/2 proteolysis.

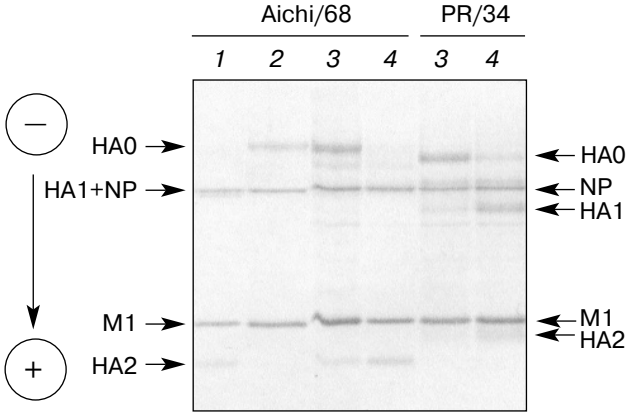

Fig. 4. Protein composition of the virus synthesized in CACO-2 cells in the presence of aprotinin. MDCK (*2*) and CACO-2 (*3*, *4*) cells were infected with A/Aichi/68 and A/PR/8/34 viruses at the multiplicity of one plaque-forming unit per cell and incubated in DMEM (*2*, *4*) or in the medium supplemented with aprotinin (200 trypsin-inhibiting units per 1 ml) (*3*). Twenty-six hours after the infection, a virus was isolated from the culture fluid, and the viral proteins were analyzed by electrophoresis in polyacrylamide gel and stained with Coomassie Blue R-350; *1*) preparation of Aichi/68 virus grown in chicken embryos.

To determine in what cell compartment the HA0 proteolysis occurs, the resistance of the carbohydrate component of glycoproteins HA0, HA1, and HA2 to endoglycosidase H (endo-H) was studied. The glycoproteins are known to acquire a complex-like carbohydrate component during the synthesis in the middle area of the Golgi apparatus and become resistant to endo-H [16]. Glycosidase F, which produces HA0f, HA1f, and HA2f, was used in these experiments to control the complete removal of carbohydrates from the glycoproteins. On treatment with endo-H of the pulse–chase-labeled cell homogenates, only HA0 (and mainly only after the pulse labeling) was cleaved by endo-H with production of HA0h and HA0h*, whereas after the chase HA0 was already resistant to endo-H and the above-mentioned products were not found. This suggested that the uncleaved HA0 was displaced into the middle area of the Golgi apparatus. The product HA0h* in CACO-2 cells seemed to be a fraction of HA0 molecules which have some potential unglycosylated sites and, as a result, have lower molecular weight. Second, proteolysis products HA1 and HA2 resulting after the chase (Fig. 5a, lane *1*) were resistant to endo-H and did not change their electrophoretic mobility in gel after the treatment with endo-H, which suggested their generation in the *trans*-Golgi and plasma membrane area when they already had the mature carbohydrate complex-like component (Fig. 5a, lane *2*). The totality of data suggested that HA0 was cleaved during the late secretory stage after the middle Golgi area—in the *trans*-Golgi and plasma membrane of the cell.

We also studied the effect on the HA0 proteolysis of brefeldin A, which is known to damage the *cis*- and middle areas of the Golgi apparatus and disturb the transport of mature glycoproteins from the Golgi into the plasma membrane [17]. In the infected cells treated with brefeldin A, only uncleaved glycoprotein HA0 was found, and the proteolysis products HA1/2 were absent after the 2.5-h chase, whereas in the cells without brefeldin HA1/2 were clearly visible (Fig. 5b). Moreover, damage to the Golgi apparatus on treatment with brefeldin A seemed to result in insufficient glycosylation of HA0 and production of HA0g (Fig. 5b, lane *4*). These findings supported the conclusion that to provide for the HA0 → HA1 + HA2 proteolysis HA0 should be transported in the late stages of the intracellular route into the *trans*-Golgi area and/or plasma membrane.

In the last part of the work, involvement of the apoptosis system in the death of influenza-infected CACO-2 cells was studied. Levels of the influenza-induced apoptosis were compared in the lines of CV-1 monkey and MDCK dog cells where the typical apoptosis develops 16-20 h after the infection with virus [10] and in the human cell line CACO-2. The cells were infected with A/Aichi/68 and A/PR/34 viruses at the multiplicity of ~2 plaque-forming units per cell, and the cell death was determined with a light microscope by apoptotic fragmentation of chromosomal DNA and proteolysis of the viral protein NP (56 kD) → aNP (53 kD) due to cell caspases [10]. The CV-1 and MDCK cells died and unfastened from the glass 18-22 h after the infection, whereas the CACO-2 cells displayed only a weak cytopathic effect after 48 h, and their death could be recorded only by 60-70 h after the infection (not shown). Moreover, in the CV-1 (Fig. 6a) and MDCK (not shown) cells the apoptotic fragmentation of DNA was noticeable with produc-

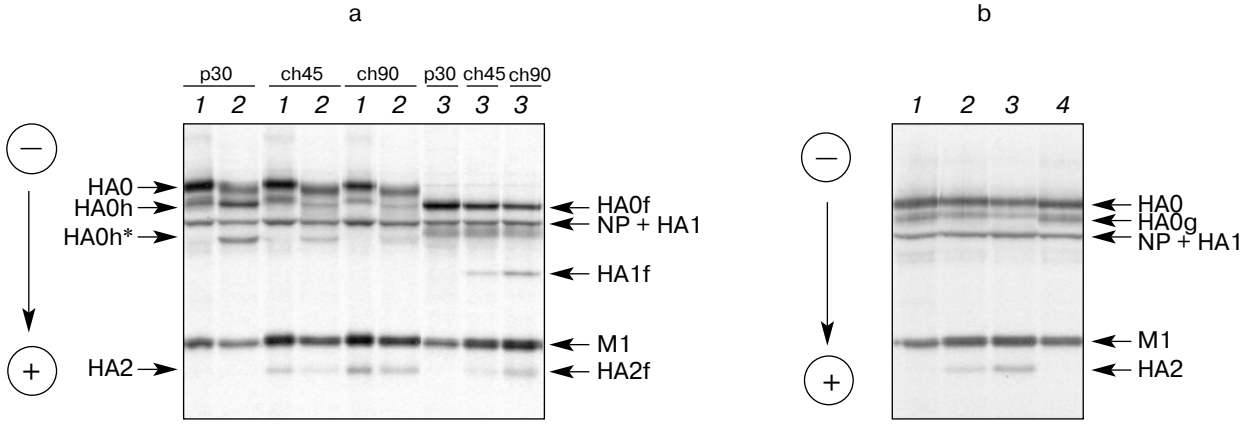

Fig. 5. Analysis of sensitivity to endoglycosidase H of the synthesized glycoproteins of influenza virus (a) and the effect of brefeldin A on their synthesis in CACO-2 (b). Culture of CACO-2 was infected with A/Aichi/68 virus and 6.5 h after the infection was labeled as described in the legend to Fig. 3. The cell homogenates untreated (*1*) and treated with endo-H (*2*) and glycosidase F (*3*) were analyzed in polyacrylamide gel with subsequent radioautography. In experiments (b) the cells after the pulse (*1*) were "chased" either in usual medium for 60 and 150 min (*2*, *3*) or in medium containing brefeldin A (10 μg/ml) for 150 min (*4*).

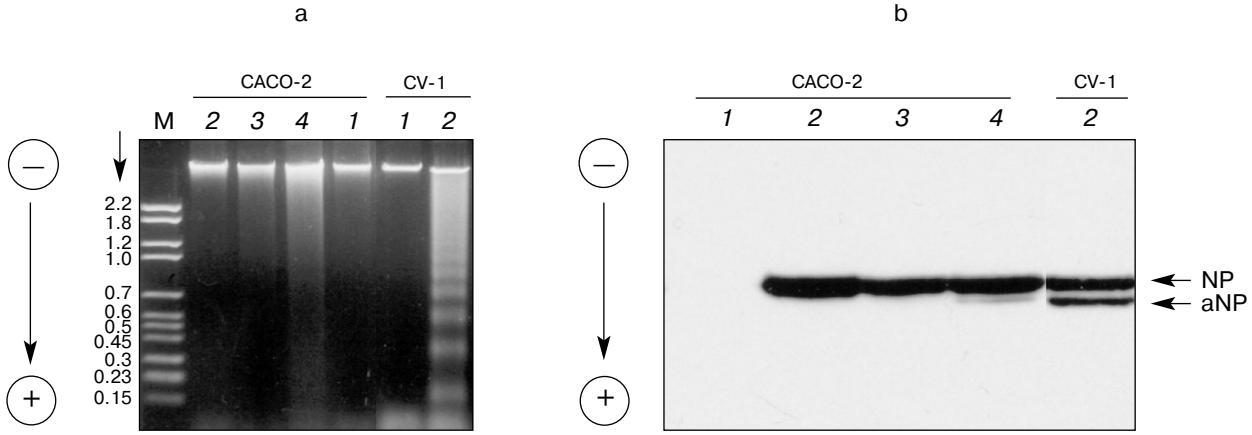

Fig. 6. Determination of apoptosis markers in CACO-2 cells infected with human influenza A virus. CACO-2 and CV-1 cells were infected with influenza virus A/Aichi/68 at the multiplicity of 4 plaque-forming units per cell and then incubated in DMEM without serum. Twenty-two (lane *2*), 46 (*3*), and 72 h (*4*) after the infection fragmentation of the chromatin DNA was determined by electrophoresis in 1% agarose gel (a); DNA from uninfected cells (lane *1*). On the left, marker DNA fragment size in kb. The accumulation of apoptotic viral protein aNP in the cells infected with A/Aichi/68 virus 22 (*2*), 36 (*3*), and 52 h (*4*) after the infection was determined by polyacrylamide gel-western-blotting with anti-NP monoclonal antibodies and peroxidase conjugate by enhanced chemiluminescence (b); uninfected cells (*1*).

tion of typical low-molecular-weight apoptotic fragments of 1.0-0.15 kb. In the CV-1 and MDCK cells a significant amount of the apoptotic form of the viral protein aNP was detected (Fig. 6b) that suggested the development of apoptosis and noticeable activation of caspases in these cells [10]. On the contrary, no apoptotic fragmentation of DNA was detected in the CACO-2 cells up to 72 h after the infection (Fig. 6a) and only minor amounts of the protein aNP were found (Fig. 6b). These observations suggested that the cell culture CACO was resistant to apoptosis caused by influenza virus, and its death seemed to develop through necrosis and be significantly slower than in the case of influenza apoptosis.

DISCUSSION

Findings of the present work show that human influenza A viruses possessing a surface glycoprotein HA with a single Arg residue in the proteolytic site can be cleaved HA0 → HA1/2 inside CACO-2 cells. CACO-2 is the line of epithelial cells of the mucosal membrane of human large intestine in which some cells can acquire signs the differentiation of the monolayer has been completed [18]. Our findings suggest a possibility of the intracellular proteolysis of HA0 with the mono-arginine proteolytic site in the epithelial cells of mucosal membranes. It is not excluded that a similar intracellular cleavage of HA0 of human influenza A viruses can also occur inside human respiratory epithelium cells. Our data question the generally accepted dogma about the extracellular proteolysis of such hemagglutinins of human epidemic influenza A viruses.

The cleavage of viral glycoprotein HA and the associated activation of the virus in the CACO-2 culture promoted the effective multicycle replication of the virus in this culture. This feature allows us not to add trypsin to the cells to provide for the effective reproduction of the virus and makes the CACO-2 culture of human cells very useful for preparation of clinical isolates of influenza viruses from patients, for experiments with production of virus variants by methods of "inverse genetics", for titration of viruses by plaques or immune foci, etc.

Our results have shown that the cleavage of the mono-arginine HA0 → HA1/2 can occur in the cells on the late stages of transport of glycoproteins in the *trans*-Golgi and/or plasma membrane region. In this connection it is interesting that H5 and H7 hemagglutinin types with the polyarginine proteolytic site are cleaved in the majority of cells by the ubiquitous serine proteinase of the subtilisin family which circulates between the *trans*-Golgi and plasma membrane [6]. Thus, it seems that in several types of epithelial cells, such as CACO-2, the HA0 → HA1/2 proteolysis of viruses with mono- and poly-arginine hemagglutinins can occur similarly in the late stages of the intracellular transport from the *trans*-Golgi into the plasma membrane. Note that we have also found the intracellular HA0 → HA1/2 cleavage and activation of human epidemic influenza viruses in epithelial cells of the mucosal membrane of human nasopharynx adenoids, and this activation was also sensitive to aprotinin (unpublished data). These observations suggest that epithelial cells of mucosal membranes contain a proteinase which can intracellularly activate the HA protein of human influenza viruses and, similarly to subtilisin proteinases,

be located and circulate between the *trans*-Golgi and plasma membrane. However, unlike subtilisins, this proteinase has to be specific for mono-arginine sites.

We suggested earlier that influenza could be treated by inhibition of proteolysis of the viral protein HA0 and suppression of the virus activation in the infected body [19]. Aprotinin, which is a registered drug and permitted for use in humans seems to be the most promising anti-influenza inhibitor [20]. The findings of the present work support these suggestions and show a noticeable inhibition by aprotinin of the intracellular HA0 $\rightarrow$ HA1/2 proteolysis of human influenza viruses in CACO-2 culture of epithelial cells of human mucosal membrane, and this is in agreement with data on the curative effect of aprotinin in humans and animals infected with influenza [20].

Influenza viruses are known to cause cell death by apoptosis in the late stages of the infection [21, 22]. In the present work, we have shown the resistance of CACO-2 cells to apoptosis induced by human influenza A viruses. Even 72 h after the infection with influenza virus along with obvious death and degeneration of the cell monolayer, these cells did not display such features of apoptosis as fragmentation of the chromosomal DNA, activation of caspases, and accumulation of the apoptotic protein aNP. Moreover, the infected CACO-2 cells were highly permeable and susceptible for staining of the nuclear DNA with propidium iodide (data not presented) that suggests the necrotic death of CACO-2 cells in influenza infection. The culture of epithelial cells of swine pulmonary tissue (SJPL line) was earlier described which similarly to CACO-2 was resistant to apoptosis when infected with influenza viruses and displayed features of necrotic death at the late stages of the infection [23]. The resistance mechanisms of CACO-2 and SJPL cells to apoptosis and also the mechanism of necrotic death of cells infected with influenza virus remain unclear and will be a subject of further studies.

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