Influenza A virus (IAV) enters host cells after attachment of its hemagglutinin (HA) to surface-exposed sialic acid. Sialylated N-linked glycans have been reported to be essential for IAV entry [Chu VC, Whittaker GR (2004) Proc Natl Acad Sci USA 102:18153–18158], thereby implicating the requirement for proteinaceous receptors in IAV entry. Here we show, using different N-acetylgalactosaminyl transferase 1 (GnT1)-deficient cells, that N-linked sialylated glycans can mediate, but are not required for, entry of IAV. Entry into GnT1-deficient cells was fully dependent on sialic acid. Although macropinocytic entry appeared to be affected by the absence of sialylated N-glycans, dynamin-dependent entry was not affected at all. However, binding of HA to GnT1-deficient cells and subsequent entry of IAV were reduced by the presence of serum, which could be reversed by back-transfection of a GnT1-encoding plasmid. The inhibitory effect of serum was significantly increased by inhibition of the viral receptor-destroying enzyme neuraminidase (NA). Our results indicate that decoy receptors on soluble serum factors compete with cell surface receptors for binding to HA in the absence of sialylated N-glycans at the cell surface. This competition is particularly disturbed by the additional presence of NA inhibitors, resulting in strongly reduced IAV entry. Our results indicate that the balance between HA and NA is important not only for virion release, but also for entry into cells.

N-linked glycosylation | hemagglutinin-neuraminidase balance | CHO lect1 cells | CHO 15B cells

Virus entry pathways are defined largely by the interactions between virus particles and their receptors at the cell surface (1). Influenza A virus (IAV) is an enveloped, negative-strand RNA virus that causes respiratory and/or intestinal infections in a variety of animal hosts, including birds and mammals. The IAV envelope contains two glycoproteins: the hemagglutinin (HA) protein, which is responsible for virus cell attachment and fusion, and the neuraminidase (NA) protein, which is the receptor-destroying enzyme essential for release of the virus from the host cell after budding. IAV attaches to host cells by binding of HA to sialic acids (SIA) present on the host cell surface. Attachment is followed by entry via endocytic routes delivering the virus to the acidic environment of the late endosome that triggers HA-mediated fusion. The interactions of HA with SIA likely are important determinants not only of attachment, but also of virus uptake, intracellular trafficking, and fusion.

Cell surface glycan composition is complex and varies between host and cell type. Sialylated glycans are attached to membrane phospholipids or to membrane proteins via asparagine (N-linked glycans) or serine/threonine (O-linked glycans) residues. How and to what extent binding of IAV to specific sialoglycans determines subsequent endocytosis remains unclear. N-linked glycans have been shown to be required for IAV entry, even though O-linked glycans and/or glycolipids permit efficient sialic acid-dependent binding of IAV to cells devoid of sialylated N-linked glycans (2). Thus, it appears that beyond the well-established requirement for binding to either α2–6 SIALs of human IAVs or α2–3 SIALs of avian IAVs, a specific subset of glycoprotein receptors is required for productive entry.

The IAV entry process is complicated by the virus’s ability to enter cells via more than one endocytic pathway. Clathrin-mediated endocytosis (CME) has been identified as an entry route for IAV, but other endocytic routes may be used as well (3, 4). Recently, macrophagocytosis was identified as an alternative IAV entry route (3). A requirement for specific receptors in these entry pathways was inferred from the finding that CME of IAV specifically requires the endocytic adaptor epsin 1 (5). Furthermore, the activation of tyrosine kinase receptors like EGFR is known to be required for efficient IAV entry (6), whereas entry via macropinocytosis, but not CME, is reduced by tyrosine kinase receptor inhibitors (3). The binding of IAV to specific receptors may influence selection of a specific endocytic entry pathway, and also may play a role in determining host and cell specificity.

In the present study, we investigated the entry of IAV into N-acetylgalactosaminyl transferase 1 (GnT1)-deficient cell lines. Such cell lines produce N-linked glycoproteins with immature glycans that cannot be sialylated, although SIALs are present on O-linked glycoproteins and glycolipids (7–9). Our results demonstrate, in contrast to conclusions drawn by Chu and Whittaker (2), that CME of IAV is not affected by GnT1 deficiency. However, N-linked glycans are required for efficient IAV entry in the presence of serum, and furthermore, depending on the glycosylation state of the host cell surface, NA activity is crucial for entry in a serum-rich environment.

Results

Chu and Whittaker (2) showed that GnT1-deficient CHO lec1 cells cannot be infected by IAV (strain A/WSN/33) and influenza B viruses. They found that the virus did bind, but was arrested at the plasma membrane, indicating that IAV internalization somehow requires N-linked glycoproteins. The recent description of two alternative, condition-dependent entry pathways for IAV (3) prompted us to reinvestigate IAV entry in relation to glycoprotein maturation. Fig. 1A shows an endpoint titration of IAV (strain WSN) on three GnT1-deficient cell lines. Compared with the parent CHO Pro5 cells, a 4-log drop in titer was observed on the CHO lec1 cells, in agreement with Chu and Whittaker’s findings (2). In contrast, GnT1-deficient CHO 15B cells and HEK293S cells yielded titers similar to those obtained using their parent cell lines (CHO kl cells and HEK293T cells, respectively).

We specifically addressed the requirement of N-linked glycosylation in IAV entry. A luciferase-expressing WSN virus (10) was used in a quantitative IAV entry assay (3). Entry was


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1To whom correspondence should be addressed. E-mail: c.a.m.dehaan@uu.nl.

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performed for 2 h in PBS, after which the medium was replaced by full growth medium containing bafilomycin A1 (BafA1) to prevent any further entry. Whereas at a low multiplicity of infection (MOI), the entry levels in GnT1-deficient HEK293S cells and CHO 15B cells were somewhat reduced compared with their parent cells, efficient entry was observed at higher MOI (Fig. 1D and F). In contrast, and in agreement with the results of Chu and Whittaker (2), entry in CHO lec1 cells was almost completely abolished (Fig. 1E). Replication of vesicular stomatitis virus (VSV), which enters by CME (11), was not affected in CHO lec1 or CHO 15B cells (Fig. 1B and C). Based on these findings, we conclude that N-linked glycans are not required for IAV entry.

We next compared the CHO lec1 and CHO 15B cells. The GnT1-negative status of these cells was analyzed by determining the glycosylation state of heterologously expressed HA containing several N-linked glycans. HA transiently expressed in GnT1-deficient CHO lec1 and CHO 15B cells comigrated on SDS/PAGE, moving faster than HA derived from the parent cells, consistent with their expected lower molecular mass because of their incomplete N-glycosylation (Fig. 2A). EndoH glycosidase cleaves high-mannose oligosaccharides, but not complex N-linked oligosaccharides. Indeed, HA expressed in CHO lec1 and CHO 15B cells, but not that expressed in parent cells, was sensitive to EndoH digestion (Fig. 2A). Digestion with PNGaseF glycosidase, which removes all N-linked glycans, resulted in the faster and similar migration of HA derived from all four cell lines. We conclude that the GnT1-deficient CHO lec1 and CHO 15B cell lines express N-linked high-mannose oligosaccharides, whereas their parent cells express complex N-linked glycans.

We also examined the binding efficiency of HA to the four CHO cell lines. For this, cells were grown and fixed in 96-well plates and incubated with Strep-tagged recombinant trimeric HA (strain WSN) that was precomplexed with antibodies labeled with HRP for detection. Binding efficiency of recombinant HA to CHO lec1 cells (Fig. 2C) and CHO 15B cells (Fig. 2B) was similar but clearly reduced compared with binding to their parent CHO k1 and CHO Pro5 cells (by approximately twofold to eightfold, depending on the HA concentration). In all four cell lines, binding was completely dependent on SIAs, as demonstrated by the complete abolishment of binding after pretreatment of cells with *Vibrio cholera* NA (VCNA). These findings indicate that HA binds with similar efficiency to the N-linked sialoside-deficient CHO 15B and CHO lec1 cells. Thus, a quantitative difference in binding efficiency does not explain the observed difference in IAV infectability of these two cell lines.

We recently demonstrated that serum induces an efficient dynamin-independent macropinocytosis-like IAV entry pathway that acts in addition to the CME pathway (3). Consequently, here we examined the effect of serum on IAV entry in the GnT1-deficient CHO cell lines. (A) TCID_{50} determination after 72 h of IAV (strain WSN) incubation on the indicated WT (gray bars) or GnT1-deficient cells (black bars), demonstrating efficient infection of CHO 15B and 293S GnT1-deficient cells. (D–F) Entry of increasing amounts of a luciferase-expressing IAV into WT (gray bars) or GnT1-deficient cells (black bars) as determined by luciferase expression. The x-axis indicates the amount of virus stock in inoculum; with 5 μL, ~90% of CHO k1 or CHO Pro5 cells become infected. The y-axis represents relative light units. (B and C) Control infections by luciferase-expressing VSV.

**Fig. 2.** Analysis of N-linked glycosylation in WT and GnT1-deficient CHO cell lines. (A) Western blot of HA produced in the indicated cell lines and treated with EndoH or PNGaseF. HA produced in GnT1-deficient cells (Lec1 and 15B) is completely sensitive to EndoH digestion, confirming the absence of GnT1 activity in these cells. (B and C) Binding of purified soluble trimeric recombinant HA (strain WSN) to the indicated cell lines in 96-well plates. The x-axis represents HA concentration (in micrograms). NA indicates pretreatment of cells with NA. Binding was quantified by HRP-linked secondary antibodies.
deficient CHO cell lines. We found significantly reduced entry in
the presence of serum in CHO 15B cells (Fig. 3B) compared with
WT CHO k1 and CHO Pro5 cells (Fig. 3A and C). No entry was
observed in CHO lec1 cells regardless of the absence or presence of
serum (Fig. 3D). Removal of sialic acids by pretreating cells
with VCNA demonstrated that IAV entry is sialic acid-de-
dependent under all conditions (Figs. 3E and F).

In nature, IAV must find its functional receptors on specific
target cells amid a plethora of decoy receptors on soluble pro-
teins, the extracellular matrix, and the surfaces of nonpermissive
cells to which it may bind. Thus, we examined the effect of serum
on the binding of HA to the various CHO cells. Clearly, the
addition of 10% serum to the binding buffer diminished HA
binding to CHO 15B and CHO lec1 cells to a very low level, but
had little effect on HA binding to the parent CHO k1 and CHO
pro5 cell lines (Fig. 4 A–D). Serum is known to contain large
amounts of sialylated proteins. Sialylated surface receptors
and serum proteins likely compete for binding to HA. An absence
of complex N-linked glycans might make CHO 15B and CHO lec1
cells very ineffective competitors for binding. Similar results were
obtained on assessment of binding of virus particles rather than
recombinant HA protein (Fig. S1).

We next examined the role of the viral NA in coping with
potential decoy receptors on soluble serum proteins. We studied
entry into CHO k1 and CHO 15B cells in the presence of the
viral NA inhibitors zanamivir (Fig. 4E) and oseltamivir carbox-
ylate (Fig. 4F), the activated form of the prodrug oseltamivir. NA
inhibitors were present only during the entry phase, in the
presence or absence of 10% serum. After 2 h, the different entry
media were replaced by full growth medium containing BafA1.
The two NA inhibitors did not inhibit entry in either cell line in
PBS; entry of IAV was even increased in the WT cells, likely due
to inhibition of cleavage of functional cell surface receptors,
in agreement with previous observations (12). However, very strong
inhibition of entry into CHO 15B cells by both inhibitors was
observed in the presence of serum. In contrast, entry into CHO
k1 was inhibited by only approximately twofold in the presence of
serum. No inhibition was observed after the addition of oseltamivir
carboxylate after entry (Fig. 4H), indicating that NA
inhibition prevents IAV infection at a stage before or during
entry. The inactive prodrug oseltamivir also did not inhibit entry
(Fig. 4G). We conclude that NA activity is required for IAV
entry in the presence of serum. Compared with WT cells, entry
into GnT1-deficient cells is much more dependent on NA
activity. This correlates well with the poor binding of HA to these
cells in the presence of serum.

Although the foregoing results explain the deleterious effect
of serum on IAV entry in GnT1-deficient cells, they do not explain
the differences in entry between CHO lec1 cells (only very
limited entry under any conditions) and CHO 15B cells (efficient
entry in PBS and reduced entry in the presence of serum). Both
GnT1-deficient cell lines have been selected after chemical
mutagenesis (7, 8) and thus may harbor an unknown number of
mutations in other genes. On the other hand, the parent cells
might already harbor the underlying defect of the lec1 cells.
Consequently, we examined the effect of enzymatic removal of

![Fig. 3.](image)

**Fig. 3.** Serum inhibits IAV entry into GnT1-deficient cell lines. (A–D) Entry of luciferase-expressing IAV (x-axis, virus stock (microliters); y-axis, relative light units) into the WT CHO cell lines k1 (A) and Pro5 (C) and their derivative
GnT1-deficient cell lines 15B (B) and lec1 (D) was studied in the absence
(black bars) or the presence (gray bars) of 10% FCS. (E and F) Entry into cells
pretreated with NA (VCNA) demonstrates that entry in all cells was strictly
dependent on sialic acid.
N-linked glycans from the parent CHO Pro5 and CHO k1 cell lines by pretreatment with PNGaseF. Whereas in PBS, no significant effect of PNGaseF treatment was observed in either CHO Pro5 or CHO k1 cells (Fig. 5A), a significant (approximately threefold) reduction of entry was observed in the presence of serum in both cell lines. Thus, PNGaseF-treated CHO k1 and CHO Pro5 cells display similar IAV entry characteristics as the GnT1-deficient CHO 15B cells.

We next examined whether complementation with GnT1 could restore IAV entry into CHO lec1 and CHO 15B cells. IAV entry into CHO 15B cells transfected with GnT1 was even higher than that observed in the parent CHO k1 cell line (Fig. 5B); however, GnT1-transfected CHO lec1 cells still did not support an entry level comparable to that of the parent CHO Pro5 cells (Fig. 5C). Entry was restored to only ~26% in PBS and ~64% in serum. We conclude that sialylated N-linked glycans are required for efficient entry in the presence of serum but are dispensable for entry in PBS. Our results also indicate that the CHO lec1 cells harbor an additional defect that affects entry of IAV in the absence of sialylated N-linked sugars.

In our recent study of IAV entry in presence of serum, we discovered the coexistence of a macropinocytosis-like IAV entry route (sensitive to EIPA) and a dynamin-dependent entry route (sensitive to dynasore, the sole available IAV entry route in PBS) (3). Only the combined presence of EIPA and dynasore could block IAV entry into HeLa cells, A549 cells, and CHO k1 cells in the presence of serum, indicating a high degree of redundancy for these routes. In the present study, confirming and extending our previous results, we show that in PBS, IAV entry into the parent CHO cells (Fig. 5 D and E) and GnT1-deficient CHO cells (Fig. 5 F and G) was fully sensitive to dynasore, whereas in serum, only the combination of dynasore and EIPA resulted in strong inhibition. The simultaneous addition of dynasore and EIPA specifically inhibits IAV entry; addition at 2 h post-infection had no effect, and cell viability was unaffected (Fig. S2). To assess whether complex N-linked glycans are required for a specific IAV entry route, we calculated the fold inhibition of entry by dynasore in the presence of serum (Fig. 5F, Inset). Even though the absolute level of entry into CHO 15B cells was reduced by ~10-fold in the presence of serum (Fig. 5F), a difference in the inhibition of entry by dynasore in the presence of serum was observed between parent CHO k1 cells and GnT1-deficient CHO 15B cells (2.1-fold vs. 5.5-fold) (Fig. S2). As expected, the expression of GnT1 in CHO 15B cells (Fig. 5H) reduced the inhibition by dynasore in the presence of serum (by 2.6-fold). However, entry levels in serum were lower in GnT1-transfected CHO lec1 cells that in the parent CHO Pro5 cells and were only partly resistant to dynasore (6.7-fold inhibition vs. 2.6-fold inhibition) (Fig. 5I). We conclude that in GnT1-deficient cells, dynasore-sensitive entry (presumably CME) is the sole available entry pathway in the absence of serum. In the presence of serum, entry levels are reduced compared with the parent cells, and the residual entry levels in CHO 15B cells are more sensitive to dynasore, suggesting that N-linked glycans are required for the optimal induction of a macropinocytosis-like IAV entry pathway.

**Discussion**

In a series of elegant experiments, Chu and Whittaker (2) demonstrated that GnT1-deficient CHO lec1 cells were not very susceptible to IAV infection, even though binding was not dramatically affected by the absence of mature N-linked glycans. In the present study, using binding and infection assays fundamentally different from those used by Chu and Whittaker, we reached the same conclusion. However, the results of our experiments with another GnT1-deficient CHO cell line, CHO 15B, and with GnT1-deficient HEK 293S cells, which are highly susceptible to IAV infection, cast these results in a very different light.

Clearly, sialylated N-glycans are not required for IAV entry per se. Efficient entry of IAV was readily observed in GnT1-deficient CHO 15B and HEK 293S cells. Moreover, entry was not affected by enzymatic removal of N-linked glycans from WT cells, at least in the absence of serum. In contrast to CHO 15B cells, in CHO lec1 cells IAV entry was strongly affected regardless of the presence or absence of serum, and could not be fully restored by transfection with GnT1-encoding plasmids. We conclude that the CHO lec1 cells must carry an additional genetic defect. This defect does not involve the absence of sialylated cell surface receptors on the CHO lec 1 cells, given that these cells contain sialylated glycosphingolipids (2) and contain similar O-linked glycans as their WT counterparts (13). Indeed, HA binding was similar in both GnT1-deficient CHO cell types.

In principle, IAV can bind to any cell surface-exposed sialo-glycan fitting into the binding site of a specific HA, after which entry can follow. These sialoglycans can be glycolipids, N-linked glycoproteins, or O-linked glycoproteins. The premise underlying Chu and Whittaker's investigation of CHO lec1 cells is that despite efficient IAV binding to the cell surface (presumably to

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**Fig. 5.** (A) Pretreatment of WT CHO Pro5 cells (black bars) and CHO k1 cells (gray bars) with PNGaseF reduces entry of luciferase-expressing IAV in the presence of serum. Entry levels are plotted relative to the levels observed in nontreated cells; approximately 10% of WT CHO cells became infected at the concentration of virus used. (B and C) Transfection of GnT1-deficient 15B cells (B) and lec1 cells (C) with a GnT1 expression plasmid 24 h before inoculation fully or partially restored IAV entry into GnT1-deficient 15B and lec1 cells, respectively. (D–I) Analysis of pathway-specific entry of IAV into WT (D and E), GnT1-deficient (F and G), or GnT1-transfected (H and I) CHO cells in the presence or absence of serum. Inhibitors were 80 μM dynasore (DY), 80 μM EIPA (EI), and both DY and EI. (F, Inset) Fold inhibition of entry by 80 μM dynasore in the presence of 10% FCS in k1 cells (D), 15B cells (F), and GnT1-transfected 15B cells (H). *P < 0.01.
O-linked glycans and gangliosides), the specific signaling required to initiate viral entry is abrogated, leaving the virus trapped on the surface (2). This hypothesis implies that IAV receptor specificity extends beyond the basic interaction of HA with a sialoglycan. In addition, the de novo formation of clathrin-coated pits at the site of IAV attachment and the specific requirement of the endocytotic adaptor protein epsin 1 for CME suggests the involvement of specific signals. However, the observation that dynamin-dependent entry of IAV into CHO 15B cells was not reduced under serum-free conditions indicates that CME of IAV is not dependent on IAV binding to complex N-linked glycans for any putative signaling event.

Recently, IAV entry was shown to involve the activation of tyrosine kinase growth factor receptor signaling on virus binding (6). The macrophagocytosis-like entry route induced in the presence of serum was found to be specifically sensitive to inhibitors of tyrosine kinase growth factor receptors (3). Our present results suggest that this route, which is dynasore-resistant (3), is affected in CHO 15B cells. In the presence of serum, IAV entry was more sensitive to dynasore in CHO 15B cells than in the parent CHO k1 cells. This greater sensitivity in CHO 15B cells was no longer observed after transfection of Gnt1-encoding plasmids. We speculate that these findings might be attributed to the absence of sialylated N-glycans on the growth factor receptors present on the CHO 15B cells and the resulting diminished binding of IAV to these receptors.

Although sialylated N-glycans are not required for IAV entry per se, serum negatively affects IAV entry in the absence of (but not in the presence of) cell-surface-exposed mature N-linked glycans. In the presence of serum, binding of IAV HA to CHO 15B cells was reduced to very low levels, whereas entry of IAV was highly dependent on NA activity. In contrast, binding of HA to the parent CHO k1 cells was not affected, and IAV entry was only moderately affected by NA inhibitors. These results indicate that soluble serum factors might function as decoy receptors. This function becomes much more apparent in the absence of sialylated N-glycans at the cell surface and in the presence of NA inhibitors. The neutralization of inhibitory decoy receptors present on soluble molecules in mucus or serum by the viral NA has long been proposed as an important function for this protein (14, 15), although to date little direct evidence to support this notion has been provided (reviewed in refs. 16 and 17).

Our data suggest that the balance between receptor binding by HA and cleavage by NA of competing decoy and functional entry receptors is an important determinant of viral infectivity in vivo. This balance might be disturbed by the presence of decoy receptors that cannot be cleaved by NA, as has been demonstrated by, for instance, the inhibition of human H3N2 virus by horse serum. Horse serum proteins are enriched in 4-O-acetylated sialic acids, which are well bound by the virus’s NA but hydrolyzed poorly by its NA. The virus can successfully escape from this inhibition by increasing the avidity of its NA against 4-O-acetylated decoy receptors or decreasing the binding of HA to these receptors, both of which restore the HA–NA balance (18–20). Our results suggest that changes in the avidity of viral binding to host cells (due to, e.g., changes in the cells’ repertoires of glycosylation enzymes) might be another important factor affecting the intricate balance between HA and NA. This becomes especially apparent in the presence of decoy receptors under serum-rich conditions. Rather than studying entry by preabsorption of virus to cells at 4 °C in protein-free media, as is common practice, including serum during entry experiments is appropriate, because it more closely mimics the natural (i.e., soluble protein-rich) in vivo conditions.

**Experimental Procedures**

**Cells, Viruses, and Infection Assays.** Madin–Darby canine kidney (MDCK-II), HEK293T, and HEK293S Gnt1− cells were maintained as described previously (3, 21). CHO Pro5, CHO lec1, CHO k1, and CHO 15B cells (all from American Type Culture Collection) were maintained at 37 °C in N-MEM (Gibco/Invitrogen) containing 10% FCS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. Influenza A/WSN/33 (H1N1) was grown in MDCK cells as described previously (3). Infection with VSV–FL, a firefly luciferase encoding VSV virus, was described previously (22). Virus was stored at –80 °C, and virus titers were determined by measuring the TCID50 on MDCK-II cells. The IAV–WSN swine pseudovirus (WSN–Ren) harbors an HA segment in which the HA coding region is replaced by Renilla luciferase (10). WSN–Ren is produced in an MDCK cell line that stably expresses the HA of WSN (10). Infections with WSN or WSN–Ren were performed as described previously (3) in the presence or absence of 10% FCS at a specific MOI. Entry assays were performed by replacing WSN–Ren and entry medium after 2 h with full growth medium containing 10 nM BafA1 (3). Luciferase activity was determined after 16 h. When indicated, cells were treated before infection with PNGase F (New England BioLabs) or VCNA (Roche) as described previously (3, 21). Cell viability (WST assay; Roche) was not affected.

**Chemicals.** Stocks of BafA1, dynasore, and EIPA (all from Sigma-Aldrich) were dissolved in DMSO. Zanamivir was obtained from GlaxoSmithKline. Oseltamivir carboxylate and oseltamivir were a gift from Roche Pharmaceuticals.

**HA Expression and Binding.** Soluble trimeric recombinant HA (strain WSN) was produced, purified, and tested for binding efficiency to the four CHO cell lines as described previously (21). In brief, cells (10^6 per well) were fixed in 96-well plates. HA was precomplexed with HRP-linked anti–Strept-tag mouse anti-body and with HRP-linked anti-mouse IgG (4:2:1 molar ratio) before incubation at the indicated concentrations.

Glycosylation patterns of HAs expressed in the four CHO cell lines were examined by SDS/PAGE, followed by Western blot analysis (21). When indicated, HA was incubated with EndoH glycosidase or PNGase F (New England BioLabs) before electrophoresis in accordance with the manufacturer’s instructions.

**Gnt1 Transfections.** Transient transfection of CHO 15B and CHO lec1 cells with a Gnt1 (rat) expression plasmid (Mammalian Gene Collection; Thermofisher Scientific) was performed using JetPrime transfection reagent (Westburg) in accordance with the manufacturer’s instructions. Transfection efficiency was determined to be ~65% for both cell lines (FC5, 100 U/mL of penicillin, and 100 μg/mL of streptomycin) by microscopic examination of cells transfected in parallel with a GFP expression plasmid. Cells were used for entry assays at 24 h after transfection.

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Examination of Virus Binding by Confocal Immunofluorescence Microscopy. CHO k1 and CHO 15B cells were grown to near-confluency on glass coverslips in 24-well plates. Cells were washed twice with PBS containing 1 mM CaCl$_2$ and 0.5 mM MgCl$_2$ (PBS/Ca/Mg) and incubated with influenza A virus (IAV; strain WSN) at a multiplicity of infection of 2 (as determined by TCID$_{50}$) for 2 h at 20 °C. Endocytosis does not occur at this temperature. Incubations with IAV were performed in PBS/Ca/Mg or PBS/Ca/Mg containing 10% (vol/vol) FCS. Cells were washed twice with PBS/Ca/Mg and then fixed and permeabilized with 100% methanol. After blocking for 15 min with 10% normal goat serum, the cells were incubated for 1 h with a monoclonal antibody directed against the IAV nucleoprotein (HB-65; kindly provided by Ben Peeters, Central Veterinary Institute, Lelystad, Netherlands). After washing, the cells were incubated with a 1:400 dilution of Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes) secondary antibody for 1 h. Nuclei were subsequently stained with DAPI, and after three washing steps, the coverslips were mounted in FluorSave (Calbiochem). Immunofluorescence staining was analyzed using a confocal laser-scanning microscope (Leica SPE-II).

Determination of Effects of Dynasore and 5-(N-ethyl-N-isopropyl)amiloride on Postentry Events and Cell Viability. CHO k1 cells were grown in 96-well plates and incubated for 3 h in the presence of 80 μM dynasore (Sigma-Aldrich), 80 μM 5-(N-ethyl-N-isopropyl)amiloride (EIPA; Sigma-Aldrich), or 80 μM dynasore plus 80 μM EIPA. Cell viability was determined by a Wst-1 cell viability assay (Roche) in accordance with the manufacturer’s instructions. Both inhibitors also were examined at 80 μM concentrations for postentry effects on IAV infection. The entry assay was performed as described in Experimental Procedures. To determine the post-entry effects of both inhibitors, CHO k1 and CHO 15B cells were inoculated with IAV WSN-Ren for 2 h in PBS containing 10% FCS in the absence of inhibitors. The inhibitors were subsequently added to the BafA1-containing culture medium, and were replaced after 3 h by culture medium containing only BafA1. For comparison, effects on entry were examined as well. Luciferase activity was determined as described in Experimental Procedures.

![Fig. S1. Effect of serum on IAV (strain WSN) binding to CHO k1 and CHO 15B cells. Virus was bound to cells at 20 °C for 2 h in the presence or absence of 10% serum as indicated. Nuclei were stained with DAPI (blue), and virus was stained using an anti-nucleoprotein monoclonal antibody (green).](image-url)
Fig. S2. The effect of dynasore (DY) and EIPA (EI) on postentry events and cell viability. Dynasore (DY) and EIPA (EI) were used at 80 μM. (A–D) Luciferase activity plotted relative to the controls with no inhibitors added (−). Inhibitors were present during entry of IAV (A and B) or postentry (C and D) using CHO k1 cells (A and C) or CHO 15B cells (B and D). (E) Cell viability of CHO k1 cells after incubation with the indicated inhibitors plotted relative to the control (−).