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Review

Influenza A Virus Hemagglutinin–Neuraminidase–Receptor Balance: Preserving Virus Motility

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Influenza A viruses (IAVs) occasionally cross the species barrier and adapt to novel host species. This requires readjustment of the functional balance of the sialic acid receptor-binding hemagglutinin (HA) and the receptor-destroying neuraminidase (NA) to the sialoglycan-receptor repertoire of the new host. Novel techniques have revealed mechanistic details of this HA–NA–receptor balance, emphasizing a previously underappreciated crucial role for NA in driving the motility of receptor-associated IAV particles. Motility enables virion penetration of the sialylated mucus layer as well as attachment to, and uptake into, underlying epithelial cells. As IAVs are essentially irreversibly bound in the absence of NA activity, the fine-tuning of the HA–NA–receptor balance rather than the binding avidity of IAV particles *per se* is an important factor in determining host species tropism.

Highlights

Functional properties of the IAV HA and NA proteins need to be balanced to allow penetration of the heavily sialylated mucus layer, attachment to and endocytic uptake into underlying epithelial cells, and efficient spread of progeny virions.

The HA–NA–receptor balance is readjusted upon cross-species transmission as the sialoglycan repertoire differs between species. This adjustment is achieved via mutations in the receptor-binding site of HA, but may also be achieved by adaptation of the receptor-binding and -sialidase activity properties of NA.

IAVs are continuously in a receptor-bound state, which is highly dynamic resulting from multivalent, low-affinity HA–SIA interactions combined with NA activity and which allows virion movement on a receptor-containing surface.

Novel technological developments allow detailed kinetic analysis of the HA–NA–receptor balance.

Receptor Binding of Influenza A Virus

Influenza A viruses (IAVs) infect birds and mammals. Aquatic birds constitute the natural host reservoir of IAVs. Occasionally, these enveloped, negative-strand RNA viruses (Figure 1) cross the host species barrier and become established as viruses of humans or other mammals (e.g., porcine, equine, and canines). Their host tropism is determined by an interplay between different viral proteins and host factors that is crucial for efficient replication and transmission within a species. Host tropism switches require adaptation of IAV proteins to host factors of the novel host species. The IAV envelope carries trimeric hemagglutinin (HA) that binds to **sialic acid** (**SIA**) (see Glossary) receptors and its functional antagonist, the tetrameric receptor-destroying neuraminidase (NA) (Figures 1 and 2). Some 18 HA (H1–18) and 11 NA (N1–11) subtypes have been identified [1,2]. All combinations of H1–16 and N1–9 have been found in wild waterfowl IAVs [3]. Currently, H1N1 and H3N2 viruses are circulating in the human population. The question is not if, but when, a new animal IAV will manage to breach the host range barrier and cause the next influenza pandemic.

The receptor-binding properties of HA, a well-established host tropism determinant, need to change in order for an avian virus to evolve into a human virus. Avian viruses prefer binding to SIAs attached to cell-surface-associated glycan chains via an α2,3-linkage, while human viruses preferentially bind α2,6-linked SIAs [4,5]. A switch from α2,3 to α2,6 binding specificity requires several mutations in HA [6–8]. However, the situation is a little more complex. HA proteins do not bind with similar **affinity** to every sialoside containing appropriately linked SIAs. Internal sugars and their linkages also affect the HA-receptor binding specificity [9,10]. Also, the α2,6/α2,3 dichotomy is not absolute. Several H1N1 [11,12] and H3N2 [13] human viruses have been reported to also bind avian-type α2,3 receptors, whereas avian IAVs of several genotypes bind to α2,6 receptors [14–17], although none of the latter have (yet) breached the host range barrier. Once established as a human virus, the receptor binding properties further evolve with time, presumably in conjunction with antigenic drift [18] that predominantly occurs at positions surrounding the receptor-binding site [19,20]. As a consequence, successful human IAVs can acquire rather different HA binding properties [13,21,22]. Considering the functional antagonism of HA and NA, it seems likely that not the receptor-binding properties of HA alone determine host tropism, but that the activities of HA and NA need to be well balanced in relation to the host receptor repertoire for optimal viral fitness. Novel developments with respect to this HA–NA–receptor balance are the main focus of this review (for previous reviews on this topic see [23–27]).

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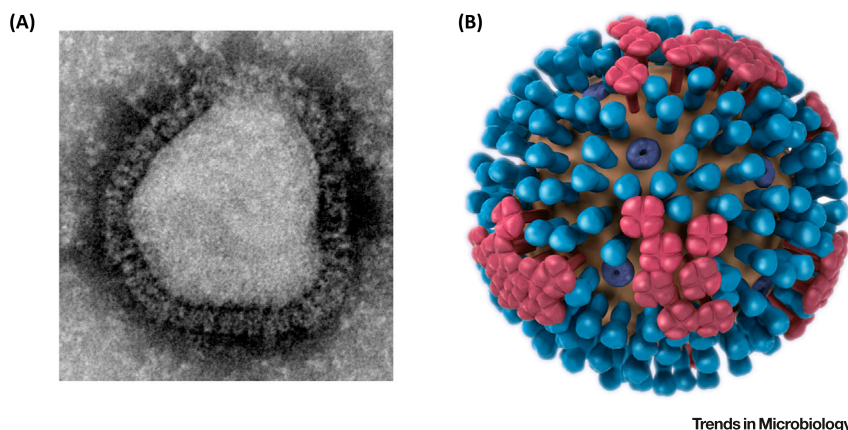

Figure 1. Influenza A Virus (IAV) Particle

(A) Electron micrograph of negatively-stained A/Netherlands/602/2009 (H1N1) virus particle (picture kindly provided by Jan van Lent, Laboratory of Virology, Wageningen University and Research). The virion has a diameter of approximately 100 nm.

(B) Graphic representation of a generic IAV particle (Centers for Disease Control and Prevention, National Center for Immunization and Respiratory Diseases). Hemagglutinin (HA) trimers are shown in blue, neuraminidase (NA) tetramers in red, and M2 ion channels in purple. The lipid envelope has a brownish color. Virions display more HA trimers than NA tetramers. NA tetramers may be evenly distributed on the virion surface or be present in a patch-like distribution [88,89,128].

HA–NA–Receptor Balance

We define the HA–NA balance as the balance between the activities of HA and NA on the full, highly diverse, spectrum of functional and decoy receptors present on cells and mucus. Multivalent IAV–receptor binding by low-affinity interactions of several HA trimers with sialosides [28,29] enables a dynamic binding mode. Individual HA–SIA interactions (K_D ∼1–20 mM) [30,31] are rapidly formed and broken without causing dissociation of the virus but providing access of NA to temporarily free SIAs. Sialidase activity by NA results in reduced SIA-receptor density which drives virus movement,

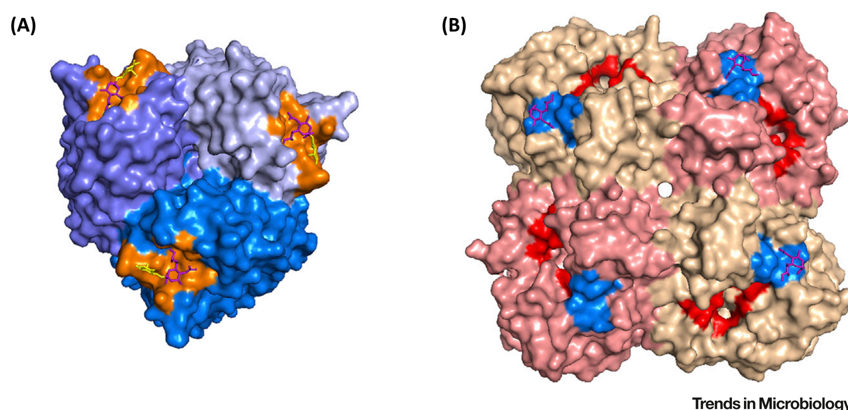

Figure 2. Structure of Hemagglutinin (HA) and Neuraminidase (NA)

(A) Top view of an HA trimer (H3; PDB 6BKM, [129]) in a surface representation. The receptor-binding site is colored orange. The sialic acid (SIA) and galactose residues in the receptor-binding sites are shown in a stick representation colored purple and yellow, respectively.

(B) Top view of an NA tetramer (N2; PDB 4H53, [130]) in a surface representation. The SIA contact residues in the catalytic site and 2nd SIA-binding site (2SBS) in each protomer are colored red and blue, respectively. SIA residues in the 2SBS are shown in a stick representation (purple).

Glossary

Affinity: affinity indicates how tightly a ligand binds to a protein and is commonly described by the dissociation constant K_D (the ligand concentration at which half of the protein-binding sites are occupied at equilibrium). We refer to affinity with respect to the interaction of a single HA protomer with a receptor.

Avidity: avidity refers to the accumulated strength of multiple individual binding interactions as they occur, for example, when a virion displaying multiple HA trimers interacts with a receptor-coated surface.

Biolayer interferometry (BLI): a label-free technology, based on an optical analytical technique, for measuring biomolecular interactions on a biosensor surface.

Clathrin-mediated endocytosis (CME): a process by which a cell internalizes extracellular components via inward budding of the plasma membrane. These inward buds are assembled with the help of clathrin molecules.

Glycan array: glycan arrays contain (synthetic) oligosaccharides immobilized on a solid support in a microarray format. They can be used for high-throughput analysis of protein–glycan interactions.

Macropinocytosis (MP): an endocytosis process that results in the uptake of liquid material by cells from their external environment via heterogeneously sized intracellular vesicles called macropinosomes.

Multivalent binding: refers to binding between two entities via multiple binding sites (e.g., virus particle and cell surface/mucin).

N-linked glycan: an oligosaccharide attached to an asparagine residue of a protein. N-glycosylation is a cotranslational process that occurs in the endoplasmic reticulum. N-glycans are modified during transport of proteins along the secretory pathway. The final structure of N-linked glycans is determined by the protein and the cell in which it is expressed and varies across species.

O-linked glycan: O-linked glycosylation is the attachment of a sugar molecule to serine or threonine residues in a protein. O-glycosylation is a post-translational modification.

as first demonstrated by microscopy [32], and ultimately causes dissociation [33,34]. Functionally balanced HA and NA activities are traditionally considered to be important for efficient release of newly assembled virus particles [35,36]. They are also essential, however, in enabling virus penetration of the heavily sialylated mucus layer overlaying epithelial cells (Figure 3), and in supporting virus interactions with host cell receptors that result in endocytic uptake (Figure 4) as is described in more detail below.

The HA–NA balance is likely tuned to the receptor repertoire IAV encounters in a specific host species. Cells expose a dense layer of sialylated glycans attached to proteins and lipids (Figure 3). Whether binding to specific sialoglycoproteins or -lipids is required for endocytic uptake and infection is not known. IAVs appear to preferentially bind to **N-linked glycans** on proteins [37], but these are not absolutely essential for host cell entry *in vitro* [38]. Functional receptors that are required for cell entry may be restricted to specific sialoglycan chains, as well as the proteins or lipids to which they are attached. For example, the voltage-dependent Ca^{2+} channel Cav1.2 was proposed as an IAV entry receptor [39]. IAV entry was inhibited by Ca^{2+} channel blockers; however, knockdown of Cav1.2 decreased the number of IAV-infected cells only twofold. Other specific membrane proteins of which the specific sialylation state affected IAV entry have been described, including fibronectin [40] and platelet-derived growth factor (PDGF)-β [41]. It can also not be excluded that cells present decoy receptors that will have a negative effect on endocytic uptake, although no such molecules have been described so

After addition of the initial sugar, other sugars can be attached. In general, O-glycans are shorter and less complex than N-glycans. Mucins contain many O-glycans.
Receptor tyrosine kinases (RTKs): transmembrane-containing tyrosine kinases that are cell-surface receptors for growth factors, cytokines, and hormones. Extracellular ligand binding will typically cause receptor di/oligomerization, which activates the receptor, thereby propagating a signal through the plasma membrane.
Sialic acid (SIA): a nine-carbon saccharide that generally occupies a terminal position of an oligosaccharide (referred to as sialoglycan). SIAs are generally attached to a penultimate galactose via an α2,3- or an α2,6-linkage.

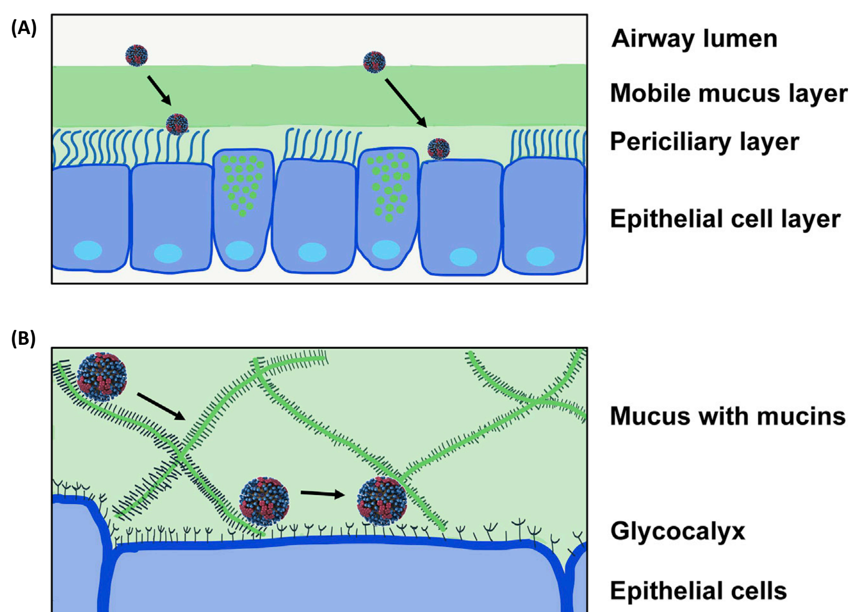

Figure 3. Influenza A Virus (IAV) Particles Penetrate the Mucus Layer of the Respiratory Tract
(A) IAV particles need to penetrate a gel-like mobile mucus layer as well as a brush-like periciliary layer to reach the underlying epithelial cells. The gel-like mobile mucus layer contains soluble mucins MUC5AC/5B, while the periciliary layer is occupied by membrane-spanning mucins and large mucopolysaccharides that are tethered to cilia, microvilli, and the epithelial cell surface [108]. Ciliated and nonciliated cells are shown, as well as mucus-producing goblet cells.
(B) Upon association of IAV particles with the mucus layer, they are continuously in a receptor-bound state. In the presence of mucus, inhibition of neuraminidase (NA) activity inhibits infection *in vitro* [43,94–96]. The dynamics of IAV–receptor interactions allow the particles to move through the mucus layer in an NA-dependent manner [60,97]. We speculate that virions are rolling over heavily sialylated, elongated mucin molecules until they reach the sialylated glycocalyx of the epithelial cells.

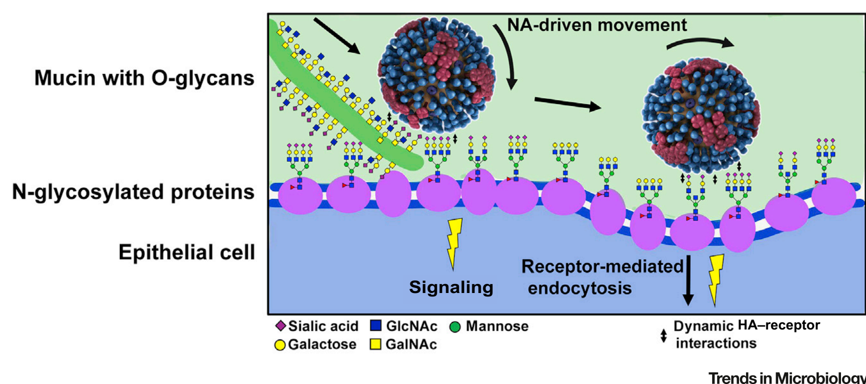

Figure 4. Dynamics of Influenza A Virus (IAV)–Receptor Interactions at the Mucus–Cell Interface
IAV particles are dynamically interacting with sialic acid (SIA) receptors present on O-glycans (present in high density on mucins) and on N-glycans (particularly present on the cell surface). Low-affinity hemagglutinin (HA)–SIA interactions [30,31] are rapidly formed and broken, thereby allowing neuraminidase (NA) access to these receptors. Upon local receptor destruction resulting from SIA cleavage, virions move to a higher receptor density-containing vicinity. This NA-dependent movement likely occurs in the mucus layer [60,97], but possibly also on the cell surface [32]. Binding of IAV particles to sialylated cell-surface receptors results in their (transient) clustering and activation, thereby inducing signaling events that trigger the uptake of IAV particles via receptor-mediated endocytosis [110–112].

far. The sialoside-rich mucus layer definitely acts as a decoy for IAV. The soluble mucins herein are elongated proteins (longer than 100 nm with an average radius of gyration of ~150 nm) [42], densely covered with short O-glycans forming a gel-like barrier to diverse pathogens (Figure 3; reviewed in [43]). Mucus also contains large numbers of potentially sialylated exosomes that may function as a decoy [44]. Thus, the sialoglycan repertoire of a host has a complex constitution and distribution of decoy and functional receptors. It varies between species [45–48], and therefore a species-specific HA–NA balance is potentially restricting crossing of the species barrier by IAV.

The restoration of a functional HA–NA–receptor balance by the selection of mutations in either HA or NA in response to an inhibitory decoy receptor was first shown 36 years ago [49]. Since then the reassortment of HA and NA genome segments from different viruses has frequently been shown to attenuate the resulting viruses. Acquisition of mutations in HA or NA after reassortment [50,51], or upon an otherwise disturbed balance [52–55], has been shown to restore fitness, and the requirement of a functional HA–NA–receptor balance is widely accepted now (reviewed in [23–27]). At the molecular level this HA–NA–receptor balance is, however, not well established. This is in part due to a biased focus on HA, but mainly because suitable assays for directly assessing the dynamics of virion–receptor interactions in the presence of NA activity are lacking. Studies using recombinant HA and NA proteins and monovalently displayed substrates are informative, but HA binding and NA sialidase activity inevitably occur at the multivalent configuration encountered at the virus–cell interface. Receptor-binding and sialidase-activity properties should therefore also be studied in the context of virus particles. Recently developed assays using **biolayer interferometry (BLI)** [33,56–58], or fluorescence imaging microscopy [32,59], allowed direct analysis of the dynamics of virus–receptor interactions. Initial interactions of virus particles with a receptor-coated surface are often short lived [59], presumably because they are driven by one or two HA–SIA binding events, each of which is rapidly formed and broken resulting from its very low affinity (K_D ~1–20 mM). However, the absence of NA activity also enabled virtually irreversible binding, as a result of multivalent HA–receptor interactions, to either a receptor-coated surface [33] or laterally mobile sialoglycolipid receptors embedded in a supported lipid bilayer [59]. Multivalent HA–receptor interactions, combined with NA activity, induced virus movement on a receptor-coated surface [32,33,60] until receptor density was decreased to an extent that allows virion elution [33]. Virus attached to sialoglycolipid receptors in a supported lipid biolayer were also shown to move by lateral diffusion of the attached

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sialoglycolipids in an NA-independent fashion [59]. Virion self-elution from a receptor-coated surface depended on the receptor-binding and -sialidase activity properties of HA and NA as well as on receptor density and identity [33,34]. The inferred speed of virus motility on a surface, and the self-elution rate, thus depend on the HA–NA–receptor balance governing the dynamics of virus–glycan interactions.

IAV cross-species transmission likely requires adjustment of the HA–NA–receptor balance as the sialoglycan repertoire differs between species [45–48]. Within the human host, changes in HA and NA activity resulting from antigenic drift may require functional coadaptation of HA and NA to restore the balance. This may be achieved by tuning the receptor (fine-)specificity and affinity of HA [61] and the activity of NA. Of note, all NA proteins preferentially cleave avian- over human-type receptors, although NA of human viruses cleaves human-type receptors relatively more efficiently [62,63]. One way to modulate NA activity is by mutation of the catalytic site which is, however, extremely conserved between avian and human viruses. Mutations in the active site are primarily observed upon development of resistance against NA inhibitors (NAIs) [64–67]. As an exception, recent human H3N2 viruses that depend on NA rather than HA for hemagglutination were recently shown to carry HA proteins with very weak receptor-binding properties and NA with presumed low activity due to a mutation in the catalytic site [68]. NA-dependent hemagglutination could also be observed upon passaging of H3N2 viruses on MDCK cells [69]. NA proteins with relatively low catalytic activity (low k_{cat}) were recently shown by BLI to contribute to the virion–receptor binding rate [33,57], which seems logical considering the relatively lower K_D of NA than of HA for binding to sialosides [70].

NA carries a second SIA-binding site (2SBS) adjacent to the catalytic site (Figure 2) which is highly conserved in most avian, but not in (pandemic) human viruses [34,63,71–73]. The 2SBS of different NA subtypes preferentially binds to avian-type receptors [34,63,74] and enhances cleavage of SIAs from multivalent receptor surfaces carrying these receptors. Binding via the 2SBS presumably enhances sialidase activity by bringing NA closer to its substrate, and is functionally reminiscent of the lectin-binding sites present in many glycosidases. The 2SBS may contribute to the virus–receptor binding rate in addition to HA [34,57] and the active site of NAs with low catalytic activity [33,57,75]. Altogether, this presents a range of options for fine-tuning the HA/NA/receptor balance that includes three receptor-binding sites (HA, and two on NA) and a cleavage site (NA), all organized in an oligomeric (trimeric HA, tetrameric NA) configuration (Figure 2). The loss of a functional 2SBS in the NA of human IAVs might have evolved as a functional prerequisite for restoring the functional balance in response to the new α2,6-linked human receptor repertoire that was encountered upon zoonotic transfer. Alternatively, the 2SBS may have lost its functional role in human IAVs and therefore have become apt to antigenic variation. Despite the general view that the primary change in adapting to a new host receptor repertoire takes place in HA, followed by further adaptations in NA, this is not the exclusive order of events. In the recently emerged H7N9 avian viruses mutation of the 2SBS, reducing binding to avian receptors, preceded the acquisition of the infamous Q226L mutation in HA which increased binding to human-type receptors [74]. Also in H9N2 avian viruses, mutation of the 2SBS is often found in company with the Q226L mutation in HA [76].

Additional mechanisms for tuning the HA–NA–receptor balance have been described. Changes in the length of the NA stalk domain usually (with some exceptions [77,78]) do not change the NA activity of a virus particle on soluble monomeric substrates or of the isolated NA protein. However, a shortened stalk affects virus replication *in vivo* and NA activity on multivalent substrates in the context of the viral particle *in vitro* [79,80], thereby, for instance, reducing virus self-elution from erythrocytes. Steric hindrance by HA was suggested to limit access of a retracted NA head domain to SIA receptors on the cell surface [81]. Several reports have demonstrated that altered stalk length differentially affects replication in different hosts, emphasizing the importance of the HA–NA–receptor balance on host specificity [82–84]. A shorter stalk is considered to be an adaptation of IAVs originating from the intestinal tract of waterfowl to the respiratory tract of chicken (reviewed in [85,86]). Also, virus particle morphology could have an impact on the HA–NA–receptor balance. In contrast to the mainly spherical morphology of laboratory-adapted IAVs harvested from *in vitro* cell cultures, clinical isolates often

contain filamentous IAVs (reviewed in [87]) displaying an asymmetric surface distribution of HA and NA [60,88–90] that may affect the HA–NA–receptor balance. Furthermore, mutations that affect intracellular transport and oligomerization of HA and NA [91,92] could affect their ratio and distribution.

Getting in

Transmission of respiratory IAVs to a new host occurs by the airborne route via respiratory droplets or aerosols as well as by direct contact with mucosal secretions [93]. The site of primary infection (intranasal, tracheal, lung) may affect pathogenicity but, at all entry sites, it is inevitable that virus particles are rapidly deposited on the mucosal surface and bind to mucins densely covered with sialylated **O-linked glycans** (Figure 4). Inhibition of NA activity in the presence of mucus inhibits infection *in vitro* [43,94–96] probably because, under these conditions, virus particles are not able to penetrate the mucus layer [97]. As outlined above, the HA–NA–receptor balance is critical for the interaction dynamics with an SIA receptor-coated surface. It is the NA activity that drives virus rolling/motility on such a receptor-coated substrate [32,33] which needs to be sufficiently cleared of receptors by NA activity before the virus can dissociate [33]. Considering the presence of the enormous amount of SIA receptors on mucins (reviewed in [98]) relative to the small number of IAV particles required for starting an infection [99–101], we consider that dissociation of virus particles from the highly organized mucus structure is not likely. Instead, we and others [32,33,60] propose that NA activity will locally decrease the receptor density, and the resulting SIA gradient will drive virus movement to areas of higher concentration. The first microscopic studies on IAV motility have identified NA activity-driven directional motility of spherical IAV particles on fetuin-coated glass slides [32]. Crawling as well as gliding motility (movement over short and longer distances, respectively) were observed. Variation in length of alternating phases of strong and weak attachment of the virus particles, dependent on the HA–NA–receptor balance, were proposed to give rise to the two mechanisms of motion [32]. Others have studied the motility of filamentous IAVs (up to 1 μm in length) on fetuin-coated surfaces and in mucus gels [60]. Filamentous IAVs appeared to move with higher directionality than the spherical particles studied in [32] when NA displayed a polarized distribution, restricted to one pole, on the viral filaments. The NA-rich pole functioned as an outboard engine, propelling the filament forwards. Directionality was lost when NA was randomly distributed. Remarkably, filamentous influenza C virus (ICV) particles, which carry their receptor-binding site and receptor-destroying activity in a single hemagglutinin–esterase–fusion (HEF) protein also displayed highly directional motion on a mucus-coated substrate [102] which was less obvious for spherical ICV particles. Thus, receptor cleavage in combination with rapid receptor exchange on individual receptor-binding sites may be a general driving force for the motion of viruses belonging to different classes on different substrates as, for instance, also proposed for β-coronaviruses [103] which harbor a hemagglutinin esterase that evolved from ICV HEF. Given the observed directionality of movement on sialylated substrates, we hypothesize that mucins are not merely IAV decoy receptors but actually support rapid directional transfer of virus particles through a viscous mucus layer. Movement of particles through mucus will obviously depend on the HA–NA–receptor balance. Too strong binding by HA in combination with an NA with low sialidase activity has been shown to slow down the speed of virus rolling *in vitro* [32–34], while particle morphology and HA–NA distribution affect directionality [60,102].

Upon crossing the viscous maze-like mucus layer, rich in soluble mucins, further penetration of a watery periciliary layer (PCL) is required in order to attach to sites on membranes of epithelial cells, resulting in endocytic entry. The PCL, from which soluble mucins are excluded, is formed by the cilia and microvilli that protrude from the membranes of ciliated cells, and the mucin-secreting goblet cells and serous cells, respectively [104]. These are the major cell types of the mammalian upper respiratory tract that face the mucus layer and of which at least ciliated and goblet cells can be infected by IAVs [105–107]. The narrow space (<200 nm) in between the cilia, filled with a glycocalyx composed of sialylated membrane-tethered mucins and other glycoconjugates, is impenetrable for beads >40 nm by diffusion [108]. Thus, entry of >100 nm diameter IAV particles by endocytosis, which is likely to occur only at the base of these protrusions, requires the type of active transport that could be provided by NA activity-driven rolling over sialylated substrates and will be dependent on the HA–NA–receptor balance. It cannot be excluded that IAV primarily enters cells at regions where cilia density is less, for instance at cell–cell borders, but also in this case it is crucial that the relatively small number of particles that start a new infection do not become trapped in between cilia.

Once in close contact with the surface of the cell body, the virus needs to bind (enigmatic) functional receptors that allow/induce endocytic uptake. IAV entry has been shown to proceed by **clathrin-mediated endocytosis (CME)** as well as **macropinocytosis (MP)** (reviewed in [109]). Both entry routes fully depend on sialylated cell-surface receptors [110]. The latter was shown to require signaling by **receptor tyrosine kinases (RTKs).** Several different RTKs are involved, and their effects may be redundant as well as additive [110,111]. CME of IAVs does not follow the ubiquitous route of surfing into preformed clathrin-coated pits but is characterized by the *de novo* formation of a clathrin coat at sites where virus particles have become immobilized [112]. Such a process requires yet undefined, transmembrane signaling as well as fixation at a spot by stable binding to (clustered) receptors. Many eukaryotic signaling receptors are heavily decorated with (sialylated) N-linked glycans. N-linked glycans have been shown to be important, but not absolutely required, for IAV entry. Especially in cells lacking sialylated N-glycans, decoy receptors were shown to interfere with entry [38]. We speculate that weak binding to O-glycans on mucins may not only be needed to move through the mucus layer but also to allow transfer of virions to higher affinity receptors at the cell surface, which may be N-linked glycans in particular. Multiantennary N-linked glycans containing multiple LacNAc repeats were, by structural modeling, suggested to associate simultaneously with two receptor-binding sites (RBSs) in single HA trimers of H3N2 viruses isolated since the 1990s. The HA proteins of these viruses display high **avidity** for such glycans and do not bind to short LacNAc antennae as analyzed by **glycan array** analysis [21].

Of note, binding receptors and signaling receptors at entry spots supporting CME or MP are not necessarily the same entities, as multivalent IAV particles can engage multiple different receptors simultaneously. Viruses bind to cell surfaces displaying heterologous sialoglycan receptors, enabling hetero-multivalent virion–cell interactions that will affect the HA–NA–receptor balance and the dynamics of virus–receptor interactions in ways that have not been explored yet. Cell–cell interaction studies have shown that short-lived interactions can lead to signaling, and it has been suggested that these weak interactions can be enabled by higher affinity interactions involving receptors that do not signal [113–115]. The same may hold true for weak interactions between virus and cell surface signaling receptors. Glycan arrays have so far mainly been employed for screening the binding of recombinant HA proteins. This approach focuses on identification of high-avidity binders whereas identification of low-affinity receptors necessarily relies on the highly **multivalent binding** mode of virions as used in BLI [33,57,58]. Indeed the occasional use of virus particles on glycan arrays has shown its potential to identify a broader spectrum of receptors than by recombinant proteins [11,13,116]. For instance, in contrast to the high specificity of recombinant HA of H7N9 for avian-type receptors [117], the corresponding H7N9 virus particles were shown to bind human- and avian-type receptors to similar levels [15]. Viruses could, in theory, move/roll in an HA–NA–receptor balance-dependent mode over the cell surface. However, this type of motility does not seem to be as crucial as that for penetrating the mucus layer as NA inhibitors have only a moderate effect on virus entry *in vitro* in a watery buffer of low protein content [38,96]. Possibly, under these conditions, IAV particles attach to abundantly present and highly mobile sialylated membrane protein receptors that surf with their cargo through the fluid lipid membrane over the cell surface. In the absence of NA activity, viruses do not roll [32,33], but, because of the high K_D of single HA–SIA interaction, we expect them to display NA activity-independent wobbling 'on the spot', which enables the virus particle to exchange bound receptors with closely adjoining receptors. Combined with surfing, such a mechanism may still allow the virus to recruit functional receptors required for entry.

Getting Out, and in Again

The crucial role for NA at the end of the infection cycle, when newly assembled particles at the cell surface are released from cells, has been abundantly documented. NA activity results in destruction of receptors that are otherwise bound by HA, both on the cell surface and on mucus. No release/aggregation of virus particles is observed when NA activity is blocked [35,118], showing the crucial function of NA in producing infectious progeny. NA sialidase activity is probably not required at the site of particle assembly *per se*, but expression of NA rather results in a cell surface devoid of sialosides, which also prevents superinfection [119]. Subsequent spreading of an infection differs from a primary infection in particle numbers (much higher [120]) and location (directly at the epithelial cell surface)

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and may have a different requirement for the HA–NA–receptor balance. Particles that are assembled are often spherical, but those of clinical isolates may also be filamentous (reviewed in [87,121]). Very long filamentous particles are particularly easily observed when they are cell-associated ([121] and references therein), but may be more labile than spherical particles when released from cells [122]. The filamentous particle phenotype is lost upon passaging *in vitro*, mostly resulting from mutations in M1, while this phenotype is maintained or may even be regained upon passaging *in vivo* [87,123], thereby suggesting an important, yet to be established, role for such particles *in vivo*. Filamentous particles may serve to efficiently spread from cell to cell when these cells are covered with a mucus layer. Alternatively, filamentous particles may be able to penetrate the mucus layer towards the luminal side and thereby be important for transmission. Of note, association of virus particles with mucus was shown to enhance particle stability in aerosols, and thereby transmissibility [124,125]. Likely, there is also an important role for the HA–NA–receptor balance in this respect, as, before infecting a new host, virus particles need to dissociate from these mucus molecules.

Concluding Remarks and Future Perspectives

Historically, identification of IAV receptors has mostly focused on the identification of receptors with high avidity for HA, despite the frequent demonstration of efficiently replicating IAVs that carry HAs with almost undetectable sialoglycan binding. At the same time, there has been a longstanding awareness of the requirement for an optimal HA–NA balance which, in simple terms, was defined as the need for matching HA affinities and NA activities in order to assure prevention of virions being trapped by decoy receptors but allowing binding to functional receptors on the cell surface. Only recently, the use of techniques that allow real-time detection of the dynamic interaction between IAV particles and receptor-coated surfaces, in the presence of NA activity, has permitted quantification of this balance. By multivalent interactions (avidity), HAs with low or relatively high binding affinity all support essentially irreversible virus binding to polyvalent receptor surfaces, as encountered in the mucus layer and on the cell surface, leading to the assumption that IAV particles are continuously in a receptor-bound state. It is the NA activity which, in conjunction with the K_D of monomeric HA–receptor interactions, enables virus motility on such surfaces and thereby assures efficient penetration of the mucus layer and migration over the cell surface to an as yet undefined spot that permits (signaling-induced) virus entry. Further development of microscopy and BLI-based techniques should help to answer numerous questions that address the basic principles of the HA–NA–receptor balance and its role in IAV–receptor interaction dynamics.

Clearly, this balance needs to be addressed in respect of the highly heterogeneous receptor repertoire that is encountered within a host. The use of recombinant glycoproteins in BLI is a step forward, and recent developments in binding cells to BLI sensors [126] have opened opportunities to directly examine virus–cell interactions by this technique. This should provide a quantitative description of species-specific IAV–host balance in order to understand which adaptations are required for crossing the species barrier. While not only the cell surface but also the mucus layer likely poses a selective barrier [43,127], their composition and interaction with IAV currently receives too little attention (see Outstanding Questions). Ultimately, studying the HA–NA–(decoy)receptor balance of viruses from different hosts, and the adjustment thereof by mutations in HA and NA, should have predictive value in linking naturally occurring amino acid substitutions to the potential of IAVs for altering their host range.

Outstanding Questions

The HA–NA–receptor balance of distinct viruses adapted to different (as well as to the same) host species remains to be determined. In view of the plethora of (decoy) receptors that viruses encounter *in vivo*, it is not yet known which (combinations of) receptors are best suited to perform these analyses. In order to directly compare the HA–NA–balance of different viruses, the same receptors need to be used.

Is the functional HA–NA–receptor balance required for efficient replication in, and transmission between, specific host species a very narrow balance, or is the required balance not so strict?

It is not known whether the functional HA–NA–receptor balances of different viruses adapted to different host species are distinct or (partly) overlapping. Does an overlapping HA–NA–receptor balance window provide an increased opportunity for cross-species transmission?

Do IAVs need to bind to specific functional receptors on host cells to initiate infection? How large is the repertoire of functional receptors, and what is their redundancy?

To what extent does mucus from different host species differ in sialoglycan make-up, and to what extent does the mucus layer function as a host range barrier? Does mucus function as a barrier at all for a well-adapted virus, or may it even enhance virus infection by helping viruses to reach the underlying epithelial cells?

To what extent should we consider that IAV particles are continuously in a receptor-bound state *in vivo* when we study the ability of antibodies or molecules to interfere with HA or NA functions *in vitro*?

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