Influenza virus is a major human pathogen that causes annual epidemics and occasional pandemics. Moreover, the virus causes outbreaks in poultry and other animals, such as pigs, requiring costly and laborious countermeasures. Therefore, influenza virus has a substantial impact on health and the global economy. Here, we review entry of this important pathogen into target cells, an essential process by which viral genomes are delivered from extracellular virions to sites of transcription/replication in the cell nucleus. We summarize current knowledge on the interaction of influenza viruses with their receptor, sialic acid, and highlight the ongoing search for additional receptors. We describe receptor-mediated endocytosis and the recently discovered macropinocytosis as alternative virus uptake pathways, and illustrate the subsequent endosomal trafficking of the virus with advanced live microscopy techniques. Release of virus from the endosome and import of the viral ribonucleoproteins into the host cell nucleus are also outlined. Although a focus has been on viral protein function during entry, recent studies have revealed exciting information on cellular factors required for influenza virus entry. We highlight these, and discuss established entry inhibitors targeting viral and host factors, as well as the latest prospects for designing novel ‘anti-entry’ compounds. New entry inhibitors are of particular importance for current efforts to develop the next generation of anti-influenza drugs – entry is the first essential step of virus replication and is an ideal target to block infection efficiently.

**Introduction**

Influenza A virus (IAV), the causative agent of influenza, is a large burden to the economy and public health worldwide. With waterfowl as the primary reservoir, the virus is able to infect a wide variety of birds and mammals, including humans. Due to this trait, zoonotic spillovers occur occasionally and can lead to pandemics with severe consequences for the human population. The swine-origin H1N1 virus from the 2009 pandemic and the H5N1 and H7N9 avian influenza viruses are recent examples of animal viruses that acquired the potential to infect and cause disease in humans. A detailed understanding of the viral life cycle is required to assess or predict the impact of circulating as well as newly emerging viruses but also to develop anti-influenza drugs. The entry process of the virus represents a favourable target for drug development, as inhibition of this first step of virus infection should result in an efficient block of virus propagation. One possibility is to target viral proteins essential for entry, e.g. the receptor-binding protein haemagglutinin (HA). An alternative approach is to target cellular proteins required for entry. Whilst in the latter case toxicity could represent an obstacle, this strategy would offer the advantage that resistance is less likely to occur. In addition, many viruses use similar entry routes and so it is conceivable that broad-spectrum antivirals could be developed.

IAV belongs to the family Orthomyxoviridae and possesses a segmented, negative-sense RNA genome. Unlike most RNA viruses, IAV replicates in the nucleus. Therefore, the virus has to overcome several barriers on its way to the site of replication and, simultaneously, avoid being recognized by the innate immune system. IAV entry is a dynamic process that requires the completion of six individual steps: attachment to target cells (I), internalization into cellular compartments (II), endosomal trafficking to the perinuclear region (III), fusion of viral and endosomal membranes (IV), uncoating (V) and import of the viral genome into the nucleus (VI) (Fig. 1). Here, we summarize how the virus manages to successfully enter target cells and to transport its genetic material to the nucleus. Furthermore, we discuss which host factors are required by the virus to complete these processes and which inhibitors are available to block individual steps of the IAV entry pathway.

**IAV attachment to host cells**

**Sialic acid is the receptor for IAV**

The initial step of the viral entry process is the attachment of IAV to the host cell. The primary receptor for IAV is...
*N*-acetylneuraminic acid (also called sialic acid), and this receptor is recognized and bound by the viral membrane protein, HA (Palese & Shaw, 2007). Sialic acid is the distal residue in oligosaccharide chains of *N*- and *O*-linked glycoproteins and lipids. Often, sialic acid is attached to the underlying galactose by α-2,3 or α-2,6 linkages. This linkage and the resulting structural consequences influence how IAV can bind to its receptor.

HA is a multifunctional IAV protein mediating virus attachment and fusion. There are 18 different HA subtypes known, of which 16 circulate in waterfowl and two (H17 and H18) have been isolated from bats (Tong et al., 2012, 2013). Of note, H17 and H18 do not bind to sialic acid; the receptor for these viruses is not yet known (Sun et al., 2013; Tong et al., 2013; Zhu et al., 2013b). HA is expressed as a trimer on the virion surface. The stalk region of HA connects HA to the virion envelope by a short hydrophobic sequence (Skehel & Wiley, 2000). This region is heavily glycosylated on conserved epitopes, which appear to be required for the stability and structure of the molecule (Roberts et al., 1993). The globular head is also glycosylated, but the glycosylation pattern and type can be highly variable in different HA subtypes. The receptor-binding pocket (RBP) is located on the distal end of the HA trimer at the globular head (Fig. 2a) and is highly conserved among different HA subtypes. Mutations in residues of the RBP and those in close proximity can drastically alter the receptor specificity of HA (Connor et al., 1994; Liu et al., 2009; Xu et al., 2010). Sialic acid has been shown to occupy the whole RBP and to be the major point of contact between the virus and the cell (Weis et al., 1988). The interaction between sialic acid and HA is believed to be of low affinity. To increase the overall strength of the interaction, multiple HA molecules are used to bind to several glycoproteins, resulting in high-avidity binding to the cell surface (Sauter et al., 1989).

The structure and conformation of HA determines the receptor specificity of IAV. It is well established that avian strains prefer sialic acid receptors with an α-2,3 linkage, whilst human IAV strains generally possess a high receptor specificity for α-2,6-linked sialic acid (Weis et al., 1988; Gamblin et al., 2004; Stevens et al., 2006b). In addition, studies using glycan arrays have shown that modifications on the underlying sugar chains are also recognized by HA and influence the binding of HA to sialic acid (Stevens et al., 2006a). More recent data suggest that the linkage of sialic acid is not the only determinant of receptor-binding specificity but that the topological structure of sialic acid...
contributes to the specificity and affinity of HA binding to sialic acid. It has been shown that human IAV strains preferentially bind to long, umbrella-shaped sialic acid molecules with α-2,6 linkage, whilst avian strains generally bind to short sialic acid molecules that adopt a cone-like structure (Chandrasekaran et al., 2008). There are also reports that alternative glycosylations can be recognized by certain IAV strains, e.g. it has been shown that N-glycolylneuraminic acid linked to galactose by an α-2,3 linkage can serve as a receptor for IAV in the duck intestine (Ito et al., 2000). Recent structural studies on receptor binding of H5 and H7 viruses have further developed our understanding of differential receptor specificity: for an H5N1 virus that had been selected to transmit in the ferret model, it was shown that binding of α-2,6 sialic acid occurred in a similar orientation as in pandemic human viruses (Xiong et al., 2013a). In contrast, the orientation of sialic acid was different when the avian H5 was analysed in combination with α-2,6 sialic acid. HA from an H7N9 virus that has emerged recently in China was able to bind α-2,6 sialic acid efficiently but in a different orientation compared with human pandemic viruses (Steinhauer, 2013; Xiong et al., 2013b). Moreover, these recent studies also suggest that not only might efficient binding to α-2,6 sialic acid be required for human receptor specificity but also a reduction in binding efficiency to α-2,3 sialic acid. For the impact of receptor specificity on tropism, host range and pathogenicity of IAV, see Matrosovich et al. (2009), Imai & Kawaoka (2012) and Wilks et al. (2012).

Often, virus entry is a multi-step process in which abundant, low-affinity receptors are utilized for initial contact of viral particles with the cell. Subsequently, binding of high-affinity receptors results in complete attachment and may trigger uptake of a particle. Whilst it is generally accepted that sialic acid is the main receptor for IAV, there is still debate as to whether IAV requires additional host factors for successful attachment and entry into target cells. It has been observed that IAV binding to sialylated receptors does not always result in internalization of the virus into the host cell (Carroll & Paulson, 1985). Furthermore, some desialylated cells retain the ability to bind IAV (Stray et al., 2000; Thompson et al., 2006), indicating that sialic acid might not be the sole receptor required for IAV attachment. Annexin V and 6-sulfo sialyl Lewis X receptors have been proposed as potential additional receptors for IAV attachment (Table 1) (Huang et al., 1996; Gambaryan et al., 2008). Furthermore, IAV is able to attach to but not infect cells with a defect in complex N-linked glycosylation, suggesting the requirement of an additional factor other than sialic acid for efficient virus infection (Chu & Whittaker, 2004). These results were later refined, as it was found that proteins containing N-linked glycans are of importance for virus entry via macropinocytosis, whilst clathrin-mediated endocytosis (both discussed below) was not affected by the absence of N-linked glycosylation and that entry of IAV was completely dependent on the presence of sialic acid (de Vries et al., 2012). For dendritic cells and macrophages, there are studies indicating that C-type lectin receptors such as macrophage mannose receptor (MMR),

![Fig. 2. Structure of HA. (a) Structure of the HA of IAV A/SouthCarolina/1918 based on the results of Gamblin et al. (2004) [Protein Data Bank (PDB) accession no. 1RUZ]. The trimeric complex of HA is shown with one monomer highlighted in colour. HA1 is depicted in red, HA2 in blue and the RBP in green. (b) The pre- and post-fusion conformations of HA are shown (Bullough et al. 1994; PDB accession no. 1HTM). For the post-fusion conformation, only the structure of the part represented in blue could be resolved. HA1 was not included in the structure and was modelled according to Palese and Shaw (2007).](http://vir.sgmjournals.org)
globular head (Yoshida distinguished: antibodies binding conserved epitopes in the globular head (Yoshida et al., 2009; Whittle et al., 2011; Ekiert et al., 2012) and antibodies recognizing conserved sites in the stem of HA (reviewed by Ekiert & Wilson, 2012). The latter type of antibody does not inhibit attachment but blocks fusion and is therefore discussed in the section on viral fusion. Antibodies that recognize conserved epitopes in the globular head can block attachment of different influenza virus subtypes and hold promise for the development of antiviral drugs.

Besides mAbs, soluble sialic acid analogues that block the RBP of HA have been suggested as potent inhibitors of IAV infection. Both soluble α-2,3- and α-2,6-linked sialic acid can be found in mucus as well as in exosomes released from airway epithelial cells (Baum & Paulson, 1990; Kesimer et al., 2008; Upham et al., 2010; Londrigan et al., 2011). However, it remains to be determined whether these receptors alone are sufficient or whether additional co-receptors are required for viral uptake.

### Inhibitors of virus attachment

Inhibition of virus attachment might be an attractive strategy for inhibiting IAV infection at the earliest step. Several approaches to block the interaction between HA and sialic acid have been proposed (Table 2). Inhibitors can work by either binding to the HA globular head to prevent interactions with the receptor or by acting on the receptor sialic acid. mAbs that bind HA are the most prominent example of inhibitors acting on the virus. Numerous mAbs have been generated and found to inhibit virus replication in cell-culture and animal models (reviewed by Martinez et al., 2009). Importantly, most mAbs that target the globular head of HA bind and neutralize only the HA they were generated against and a few closely related HAs. However, in recent years broadly neutralizing mAbs that bind conserved epitopes in HA have been developed, and these antibodies show promise as inhibitors of many different influenza virus strains (Corti & Lanzavecchia, 2013). Two types of antibody can be distinguished: antibodies binding conserved epitopes in the globular head (Yoshida et al., 2009; Whittle et al., 2011; Ekiert et al., 2012) and antibodies recognizing conserved sites in the stem of HA (reviewed by Ekiert & Wilson, 2012). The latter type of antibody does not inhibit attachment but blocks fusion and is therefore discussed in the section on viral fusion. Antibodies that recognize conserved epitopes in the globular head can block attachment of different influenza virus subtypes and hold promise for the development of antiviral drugs.

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### Table 1. Host factors involved in IAV entry

<table>
<thead>
<tr>
<th>Host factor</th>
<th>Entry step</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sialic acid</td>
<td>Attachment</td>
<td>Palese and Shaw (2007)</td>
</tr>
<tr>
<td>C-type lectins</td>
<td>Attachment</td>
<td>Reviewed by Londrigan et al. (2011)</td>
</tr>
<tr>
<td>Annexin V</td>
<td>Attachment</td>
<td>Huang et al. (1996)</td>
</tr>
<tr>
<td>6-Sulfo sialyl Lewis X</td>
<td>Attachment</td>
<td>Gambaryan et al. (2008)</td>
</tr>
<tr>
<td>Dynamin</td>
<td>Internalization</td>
<td>Roy et al. (2000)</td>
</tr>
<tr>
<td>Actin</td>
<td>Internalization</td>
<td>Gottlieb et al. (1993)</td>
</tr>
<tr>
<td>Clathrin</td>
<td>Internalization</td>
<td>Matlin et al. (1991)</td>
</tr>
<tr>
<td>Epsin-1</td>
<td>Internalization</td>
<td>Chen &amp; Zhuang (2008)</td>
</tr>
<tr>
<td>EGFR</td>
<td>Internalization</td>
<td>Eierhoff et al. (2010)</td>
</tr>
<tr>
<td>c-Met kinase</td>
<td>Internalization</td>
<td>Eierhoff et al. (2010)</td>
</tr>
<tr>
<td>PLC-γ1</td>
<td>Internalization</td>
<td>Zhu et al. (2013a)</td>
</tr>
<tr>
<td>Rab5</td>
<td>Endosomal trafficking</td>
<td>Sieczkarski et al. (2003)</td>
</tr>
<tr>
<td>Rab7</td>
<td>Endosomal trafficking</td>
<td>Sieczkarski et al. (2003)</td>
</tr>
<tr>
<td>PKC βII</td>
<td>Endosomal trafficking</td>
<td>Sieczkarski et al. (2003)</td>
</tr>
<tr>
<td>Cullin 3</td>
<td>Endosomal trafficking</td>
<td>Huotari et al. (2012)</td>
</tr>
<tr>
<td>HDAC8</td>
<td>Endosomal trafficking</td>
<td>Yamauchi et al. (2011)</td>
</tr>
<tr>
<td>vATPase</td>
<td>Endosomal acidification</td>
<td>Guinea et al. (1995)</td>
</tr>
<tr>
<td>CD81</td>
<td>Fusion</td>
<td>He et al. (2013)</td>
</tr>
<tr>
<td>ITCH</td>
<td>Uncoating</td>
<td>Su et al. (2013)</td>
</tr>
<tr>
<td>Karyopherin (z1; z3; z5)</td>
<td>Import</td>
<td>Wang et al. (1997)</td>
</tr>
<tr>
<td>Ran</td>
<td>Import</td>
<td>O’Neill et al. (1995)</td>
</tr>
<tr>
<td>p10</td>
<td>Import</td>
<td>O’Neill et al. (1995)</td>
</tr>
<tr>
<td>CSE1L</td>
<td>Import</td>
<td>Konig et al. (2011)</td>
</tr>
</tbody>
</table>
The conjugation of the sialidase to amphiregulin containing an epidermal growth factor-like domain is required for the effective targeting of epithelial cells. DAS181 possesses antiviral activity against a broad variety of IAVs and influenza B viruses in cell culture (Nicholls et al., 2013).

**Table 2. Inhibitors of IAV entry**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Entry step</th>
<th>Potential as drug</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>mAbs (HA-RBP)</td>
<td>Attachment</td>
<td>Yes</td>
<td>Reviewed by Clementi et al. (2012)</td>
</tr>
<tr>
<td>SA mimics</td>
<td>Attachment</td>
<td>Yes</td>
<td>Reviewed by Vanderlinden &amp; Naesens (2013)</td>
</tr>
<tr>
<td>SA binders</td>
<td>Attachment</td>
<td>Yes</td>
<td>Reviewed by Vanderlinden &amp; Naesens (2013)</td>
</tr>
<tr>
<td>Sialidases e.g. DAS181</td>
<td>Attachment</td>
<td>Yes/phase II trial</td>
<td>Reviewed by Nicholls et al. (2013)</td>
</tr>
<tr>
<td>Dynasore</td>
<td>Internalization</td>
<td>Laboratory use</td>
<td>de Vries et al. (2011)</td>
</tr>
<tr>
<td>EIPA</td>
<td>Internalization</td>
<td>Laboratory use</td>
<td>de Vries et al. (2011)</td>
</tr>
<tr>
<td>Receptor tyrosine kinase inhibitors</td>
<td>Internalization</td>
<td>Yes</td>
<td>Eierhoff et al. (2010); de Vries et al. (2012)</td>
</tr>
<tr>
<td>LJ001</td>
<td>Internalization</td>
<td>Yes</td>
<td>Wolf et al. (2010)</td>
</tr>
<tr>
<td>Bafilomycin A1</td>
<td>Endosomal acidification</td>
<td>Laboratory use</td>
<td>Guinea et al. (1995)</td>
</tr>
<tr>
<td>mAbs (HA stalk)</td>
<td>Fusion</td>
<td>Yes</td>
<td>Reviewed by Corti &amp; Lanzavecchia (2013)</td>
</tr>
<tr>
<td>Small-molecule inhibitors (HA stalk)</td>
<td>Fusion</td>
<td>Yes</td>
<td>Reviewed by Vanderlinden &amp; Naesens (2013)</td>
</tr>
<tr>
<td>Amantadine</td>
<td>Uncoating</td>
<td>Approved</td>
<td>Davies et al. (1964)</td>
</tr>
<tr>
<td>Rimantadine</td>
<td>Uncoating</td>
<td>Approved</td>
<td>Rabinovich et al. (1969)</td>
</tr>
<tr>
<td>Benzyl-substituted amantadine derivatives</td>
<td>Uncoating</td>
<td>Yes</td>
<td>Wang et al. (2013)</td>
</tr>
<tr>
<td>Importazole</td>
<td>Import</td>
<td>Laboratory use</td>
<td>Chou et al. (2013)</td>
</tr>
</tbody>
</table>

**Entry routes used for IAV internalization**

**Internalization of IAV**

Upon attachment to the host cell, IAV is taken up into the cell. Imaging studies revealed early on that the virus enters the cell by receptor-mediated endocytosis (Patterson et al., 1979; Matlin et al., 1981; Yoshimura et al., 1982). It was shown that cold-bound virus was – upon raising the temperature to 37 °C – not fusing at the plasma membrane. Virus uptake occurred within minutes after the temperature rise as the half-life time of attached viral particles to become resistant to treatment with sialidases was between 10 and 15 min (Matlin et al., 1981). IAV was internalized mainly into clathrin-coated but also into uncoated vesicles. This already suggested that IAV is able to utilize multiple endocytosis routes, not only clathrin-mediated endocytosis. Later, it was demonstrated that the virus can still infect cells defective in clathrin- and caveolin-dependent pathways (Sieczkarski & Whittaker, 2002). In line with these data, imaging studies with single viral particles showed that IAV can utilize clathrin- and non-clathrin entry routes in parallel (Rust et al., 2004). Recent studies identified macropinocytosis as an alternative pathway exploited by IAV (de Vries et al., 2011). Macropinocytosis refers to the uptake of large-sized cargo through the actin-dependent formation of large endocytic vesicles called macropinosomes. IAV enters the cell by clathrin-mediated endocytosis in the absence of serum, and this pathway can be blocked efficiently using the dynamin inhibitor dynasore. However, if serum is present during infection, IAV is taken up into cells by dynamin-dependent and -independent entry routes. A complete block of internalization was only achieved when cells were treated with dynasore in combination with ethylisopropylamiloride (EIPA) (de Vries et al., 2011). EIPA is an inhibitor of Na⁺/H⁺ exchangers, which was shown to block macropinocytosis by preventing elevation of the cytosolic pH, which in turn affects activation of GTPases required for actin remodelling (Koivusalo et al., 2010). Further studies showed that the choice of entry route is likely to be cell-type dependent (De Conto et al., 2011) and that filamentous IAV preferentially uses macropinocytosis for internalization (Rossman et al., 2012). The latter can explain earlier observations on the entry process of filamentous IAV: it had been demonstrated that acidification of endosomes was required but dynamin seemed to be dispensable (Sieczkarski & Whittaker, 2005).

To date, it is not clear whether binding of HA to sialylated glycans is sufficient to initiate internalization of viral particles. Several studies indicate that additional receptors may be required to orchestrate virus uptake (Table 1). It was demonstrated that the formation of clathrin-coated pits occurs at faster pace at virus-attached spots than in other areas at the cell surface (Rust et al., 2004). These data indicate that IAV specifically triggers its uptake via clathrin-mediated endocytosis and is therefore likely to interact with additional cell-surface receptors to activate downstream signalling processes required for internalization. The adaptor protein Epsin-1 localizes to attachment sites of IAV and this coincides with the formation of clathrin-coated pits at that site. In addition, knockdown of Epsin-1 inhibits clathrin-mediated endocytosis of IAV but not of other ligands such as transferrin (Chen & Zhuang, 2008). Therefore, Epsin-1 is an adaptor recruited specifically for clathrin-mediated IAV entry, indicating that IAV
triggers certain pathways that differ from classical clathrin-mediated endocytosis events.

There is growing evidence that receptor tyrosine kinases (RTKs) may play an important role in the uptake of IAV particles. It has been shown that IAV attachment activates epidermal growth factor receptor (EGFR) and that activated EGFR promotes virus uptake into target cells. Similar results have been obtained for the c-Met kinase, so the authors speculate that IAV attachment to the cell surface results in lipid raft formation, which serves as a signalling platform to trigger RTK activation leading to virus internalization (Eierhoff et al., 2010). Recently, it has been demonstrated that activation of phosphoinositide-specific phospholipase C (PLC), which acts downstream of EGFR, is required for entry of H1N1 viruses (Zhu et al., 2013a). Interestingly, in this study, both H1N1 and H3N2 viruses were able to activate EGFR following attachment but only H1N1 viruses also activated PLC. These results indicate that different IAV subtypes are capable of specifically activating distinct signalling pathways to facilitate entry. Supporting evidence for the involvement of RTKs in IAV entry comes from inhibitor studies showing that many RTK inhibitors decrease IAV internalization by macropinocytosis (de Vries et al., 2011). Indeed, N-linked glycans, present on membrane receptors, have been shown to be important for IAV entry in the presence of serum (de Vries et al., 2012).

Nevertheless, the authors clearly demonstrated that IAV entry was dependent on sialic acid under all experimental conditions. In summary, there is evidence for activation of common RTK cascades involving protein kinase C (PKC), MEK/ERK and phosphatidylinositol-3-OH kinase (PI3K)/AKT by IAV infection, but it remains to be determined how these pathways contribute to virus entry into host cells.

**Inhibitors of internalization**

Compounds that inhibit virus endocytosis would be of great clinical use, as a large array of viruses enter cells by endocytosis. However, most inhibitors used in tissue-culture experiments such as dynasore or EIPA are cytotoxic in higher concentrations and with prolonged exposure and are therefore not suitable for clinical use. In addition, RTK inhibitors are problematic as most currently available compounds lack specificity and target a variety of RTKs. An interesting compound is Lj1001, which affects membrane fluidity and curvature through O2-mediated lipid oxidation. Therefore, its antiviral activity is restricted to enveloped viruses (Wolf et al., 2010; Vigant et al., 2013).

**Endosomal trafficking of IAV**

**From early to late endosomes**

The endosomal system is well described as a cellular sorting system for incoming extracellular material and intracellular vesicles (reviewed by Mellman, 1996). Influenza viruses are taken up by endocytosis or macropinocytosis and exploit the transport system via distinct endosomal stages and simultaneously occurring changes in pH to release their viral ribonucleoprotein complexes (vRNPs) into the cytoplasm. Upon internalization by either uptake pathway, the virus first localizes to early endosomes and then reaches late endosomes. Endosomal trafficking is known to be a non-linear pathway with a multitude of different branches leading to degradation of extracellular compounds and membrane recycling (Steinman et al., 1983). Here, we focus on how IAV exploits the endosomal pathway.

Before IAV reaches its fusion site, it has to pass different stages of the endocytic machinery, which is assembled and constantly renewed around the internalized virus particles (Rust et al., 2004; Cocucci et al., 2012). A difficulty in detecting these stages of viral trafficking is the short time span that viruses remain in the endosomal compartment. Penetration of vRNPs into the cytoplasm can be detected several minutes after virus binding to the cell, and vRNPs reach the nucleus within the first hour (Martin & Helenius, 1991). With the aim of visualizing viruses along the endosomal trafficking pathway, synchronized infection was established as an important tool that allows monitoring early infection events (Matlin et al., 1981). It has been demonstrated that endosomal trafficking of the virus involves actin- and microtubule-dependent processes (Nielsen et al., 1999; Sun & Whittaker, 2007; De Conto et al., 2012). Using single virus trajectories from imaging fluorescently labelled virions, viral transport was dissected into three different stages (Lakadamyali et al., 2003). First, the virus is transported in the cell periphery and this process has been demonstrated to be actin-dependent. This is followed by the second stage, which is marked by rapid dynein-directed movement. Finally, movement of virions along microtubules to the perinuclear region can be defined as stage three. This transport pattern correlates to a large extent with well-established endosomal routes: early endosomes (EEs) containing cargo are transported away from the cell surface via actin-dependent transport. EEs are then transported via the motorproteins kinesin-1 and dynein along microtubules towards the nucleus. Simultaneously, EEs constantly exchange vesicles with the trans-Golgi network, thereby undergoing a maturation process (reviewed by Huotari & Helenius, 2011). Rab5 and additional proteins such as early endosomal autoantigen 1 (EEA1) and PI3K are major regulators of this maturation process and are used as marker proteins to stain EEs (Fig. 3) (Bucci et al., 1992; Mu et al., 1995; Simonsen et al., 1998; Christoforidis et al., 1999a; Fujioka et al., 2011). Late endosomes (LEs) are formed from EEs during their microtubule-dependent transport into the perinuclear region by acquiring intraluminal vesicles during vesicle exchange with lysosomes or other late endosomes (Luzzio et al., 2007; Huotari & Helenius, 2011). LEs contain integral membrane proteins such as lysosomal membrane protein 1 (LAMP1) and their pH drops from 6.8–5.9 in EEs to 6.0–4.8 in LEs (Maxfield & Yamashiro, 1987). The progression from EEs to LEs is indicated by the so-called ‘Rab switch’ from Rab5 for EEs to Rab7 for LEs (Rink et al., 2005). Rab proteins are cellular GTPases that are recruited to vesicle membranes and play a key role in regulating endosomal trafficking. Different Rab proteins are required...
for different steps in vesicular transport, although some follow their endosomal compartments throughout maturation of endosomes (Zerial & McBride, 2001).

With respect to IAV infection, both Rab5 and Rab7 have been shown to be required (Sieczkarski & Whittaker, 2003). Moreover, PKCβII has been linked to IAV trafficking in endosomes. Infection of cells pre-treated with inhibitors against PKCβII leads to the accumulation of viral particles in LEs, without fusion taking place (Sieczkarski et al., 2003).

Other important players in LE trafficking are histone deacetylases. Depletion of histone deacetylase 8 (HDAC8) results in dysregulation of microtubule organization, centrosome function and maturation of LEs to lysosomes and subsequently in a decrease in viral replication (Yamauchi et al., 2011). Furthermore, Cullin 3, which is a scaffolding protein for an E3 ubiquitin ligase complex, has been shown to be required for IAV entry at the level of LEs (Huotari et al., 2012). Importantly, depletion of Cullin 3 also inhibits trafficking of other cargos such as epidermal growth

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**Fig. 3.** Super-resolution microscopy of IAV in endosomes. A549 lung epithelial cells were infected with IAV (A/WSN/33, m.o.i. 25) for 30–180 min. Virus was added to the cells in an initial cold binding step to synchronize the infection process. Cells were fixed and stained for the nucleus (DAPI staining) (blue) and nucleoprotein (NP) (green) (a), nucleus, NP and EEA1 (red) (b, i) or NP and EEA1 (b, ii–iv). Images were acquired by standard immunofluorescence microscopy [confocal laser scanning microscopy (CLSM), a and b, i] or super-resolution microscopy [stimulated emission depletion (STED), b, ii–iv]. In (b, iii, iv), rendered (IMARIS) images of viral particles within endosomes are shown. In (b, iv), the transparency of the endosomal staining was increased to allow visibility of viral particles inside the respective endosomes. p.i., Post-infection.
factor (EGF), indicating that this pathway is required for transport of a variety of cargos and is not used solely by IAV.

**Fusion of IAV**

**Fusion between viral and endosomal membranes**

Preceding the nuclear transport of IAV, fusion of viral and endosomal membranes is required to release vRNPs into the cytoplasm. This process is driven by a low-pH environment and the class I fusion protein of IAV, the HA. LEs possess an acidic environment and thus facilitate the induction of influenza virus HA-dependent fusion at pH 5.0 (Maeda & Ohnishi, 1980; Daniels et al., 1985; White & Wilson, 1987). Interestingly, HAs of different subtypes display varying pH optima for fusion, and HAs of human isolates require lower pHs than avian isolates of the same subtype (Galloway et al., 2013). During the acidification process of endosomes, proton pumps, which deliver protons into the endosomal lumen and thereby ensure stepwise acidification, exhibit a crucial function (Galloway et al., 1983; Pérez & Carrasco, 1994). These so-called v-ATPases consist of two complexes, one membrane-associated V0 complex and a soluble cytosolic V1 complex, which hydrolyses ATP as the driving force of acidification (reviewed by Forgac, 2007). Once IAV is in the acidic environment of the LE, HA undergoes conformational changes that expose the fusion peptide and position it towards the endosomal membrane (Fig. 2b) (Carr & Kim, 1993; Bullough et al., 1994; Chen et al., 1999). It was shown that intermediate stages dependent on pH and membrane proximity exist (Korte et al., 1999; Leikina et al., 2002). Following the final conformational changes, the fusion peptide is inserted into the target membrane, which brings the viral and endosomal membranes into close proximity (Tsurudome et al., 1992; Weber et al., 1994; Durrer et al., 1996). Of note, whilst the crystal structures of pre- and post-fusion HA have been solved, the structures of the intermediate stages are not known so far and can only be modelled based on the pre- and post-fusion structures. For the fusion process, it was shown that several HA trimers promote membrane fusion by simultaneous conformational changes and release of folding energy (Markovic et al., 2001). To proceed with fusion between viral and endosomal membranes, HA trimers tilt at the fusion site and the outer leaflets of the membranes interact with each other in a hemi-fusion stage (Tatulian et al., 1995; Chernomordik et al., 1998). Finally, both membranes fuse and a so-called fusion pore is established (Spruce et al., 1989; Melikyan et al., 1993a, b). Through this fusion pore, vRNPs can be released into the cytoplasm.

Limited information is available regarding cellular factors required for fusion of IAV. As discussed above, the vATPase complex is essential for acidification of the endosome, a prerequisite for fusion. Only very recently, the tetraspanin CD81 has been identified as another cellular player in the fusion process (He et al., 2013). Approximately 50% of internalized IAV localizes to CD81- and Rab5-positive endosomes and fusion has been observed to occur in these vesicles. In the absence of CD81, fusion is reduced by 50%. It is currently unknown how CD81 contributes to fusion, but CD81 seems to mark a productive entry route for IAV.

**Inhibitors of fusion**

Inhibition of viral fusion can be achieved by inhibition of acidification in endosomes (Table 2). One of the most potent inhibitors for this purpose is bafilomycin A1 (BafA1). Bafilomycins belong to a family of macrolide antibiotics that have been shown to inhibit vacuolar-type proton pumps involved in viral entry (Bowman et al., 1988; Ochiai et al., 1995). Similar effects have been shown for diphyllyn and saliphenylhalamide, other vacuolar-type ATPase inhibitors (Huss & Wieczorek, 2009; König et al., 2010; Müller et al., 2011). Small molecules that bind to the stem region of HA and thereby hinder the conformational changes required for fusion represent another class of fusion inhibitors. The first compound of this group to be discovered was tertiary butylhydroquinone, which has been crystallized in complex with HA (Bodian et al., 1993; Russell et al., 2008). Later, several compounds that act in a similar way were described: BMY-27709, CL-385319, RO5464466, stachylin and 4c (Luo et al., 1997; Plotch et al., 1999; Yoshimoto et al., 1999; Vanderlinden et al., 2010; Zhu et al., 2011). Unfortunately, resistance mutations in HA can develop rapidly within a few passages of the virus and confer resistance to this type of compound. Arbidol has also been identified as an inhibitor of membrane fusion. Mutations rendering viruses resistant to arbidol have been mapped to HA and seem to impact on the acid stability of HA (Leneva et al., 2009). Of note, arbidol is approved as an anti-influenza drug in Russia. Alternatively, the stem region of HA can be targeted by broadly neutralizing antibodies as mentioned above (Corti & Lanzavecchia, 2013). Such antibodies bind to a region of HA that is conserved even between different subtypes, and this enables the antibodies to inhibit many different strains of IAV (Okuno et al., 1993; Throsby et al., 2008; Ekiert et al., 2009; Sui et al., 2009; Corti et al., 2011). Whilst they do not prevent binding to the host cell, they interfere with the conformational changes required for fusion. These antibodies represent promising drug candidates but also could help in the design of a vaccine that provides protection against a broad range of IAV strains.

**Uncoating**

**Release of vRNPs into the cytoplasm**

Upon fusion of viral and endosomal membranes, IAV uncoating is completed with the release of the vRNP into the cytosol. This process requires coordinated action of the viral proteins M2 and M1. In the intact virus particle, M1 forms a structured layer underneath the viral membrane and can be visualized by electron microscopy (Ruigrok et al., 2000; Calder et al., 2010; Fontana et al., 2012; Fontana & Steven, 2013). It is assumed that M1 plays a
crucial role in the architecture of the virion by linking the viral membrane containing the glycoproteins with the RNP in the virus core. Whilst the expression of viral glycoproteins can result in the production of virus-like particles, even in the absence of M1, this matrix protein is required for production of infectious virions (reviewed by Rossman & Lamb, 2011). This is supported by the observation that M1 determines the shape of the virion: exchange of M1 is sufficient to change the morphology of virions from spherical to filamentous (Roberts et al., 1998; Bourmakina & García-Sastre, 2003; Elleman & Barclay, 2004). It is currently unclear how M1 interacts with the viral membrane and/or glycoproteins; specific binding domains have not yet been mapped (Zhang & Lamb, 1996; Schmitt & Lamb, 2005). For the interaction of M1 with the vRNPs, it has been demonstrated that the middle domain of M1 is responsible for binding to nucleoprotein (NP) on the RNP (Noton et al., 2007).

During the uncoating process, the interaction of M1 with the viral membrane as well as the interaction of M1 with the vRNPs has to be released in order to allow complete uncoating and subsequent transport of the RNP into the nucleus. This requires the activity of the viral protein M2. M2 was identified in 1981 as the second protein encoded by segment 7 of IAV (Lamb & Choppin, 1981). It was found to form tetramers that are present in virions (Zebedee & Lamb, 1988; Holsinger & Lamb, 1991; Sugrue & Hay, 1991) and was described to possess ion-channel activity selective for monovalent ions (Pinto et al., 1992; Chizhmakov et al., 1996). Interestingly, the ion-channel activity of M2 is regulated by pH: at lower pH, the ion-channel activity increases, and histidine 37 of M2 is crucial for this regulation (Pinto et al., 1992). The transmembrane pore of the channel is lined by a series of amino acids that all lie on the same side of an α-helix; four of these helices from the four monomers form the channel (Grambas et al., 1992; Pinto et al., 1992; Wang et al., 1993; Stouffer et al., 2008). During the entry process of IAV, the ion-channel activity of M2 is required for uncoating; upon acidification of the endosome, M2 mediates proton influx from the endosome into the virion, resulting in a decrease in the pH within the virus particle (Wharton et al., 1994). This M2-mediated change in pH is required for the detachment of M1 from the vRNPs, resulting in the release of the vRNPs into the cytoplasm (Zhirlinov, 1990). Furthermore, it was observed that M1 separates from the vRNPs before they are imported into the nucleus (Bukrinskaya et al., 1982; Martin & Helenius, 1991). Interestingly, this initial RNP nuclear import can be blocked by expression of M1, but brief low-pH treatment can in turn relieve the block mediated by M1 (Bui et al., 1996). These observations have led to the current model in which the pH drop in the virion within the endosome causes conformational changes in M1, and subsequently the interaction between the RNPs and M1 is weakened or lost. The changes in M1 conformation have been visualized by electron microscopy: the helical structure of the M1 layer in the virion is lost in acid-treated virions (Calder et al., 2010; Fontana et al., 2012). Before the loss of the M1, structure rearrangements in the M1 layer could be detected (Fontana & Steven, 2013). It is currently unclear how the conformational change occurs, but it has been suggested that the linker region between the N- and C-terminal domain of M1 is important: in vitro the linker peptide changed its conformation upon a pH drop but only in the presence of zinc ions, which have been detected in influenza virions (Elster et al., 1994; Okada et al., 2003). Not much is known about the involvement of cellular factors in this process yet. Very recently, the E3 ubiquitin ligase Itch was reported to be required for efficient uncoating (Su et al., 2013). The authors demonstrated that Itch gets phosphorylated and recruited to endosomes upon IAV infection where it ubiquitinates M1 and thereby facilitates release of the vRNPs.

**Inhibitors of uncoating**

Amantadine is the best-known example for an inhibitor of M2. Its antiviral activity was first described in 1964 (Davies et al., 1964). Later it was found that it targets the M2 protein of IAV and thereby exerts its antiviral function (Skehel et al., 1978; Hay et al., 1985). Rimantadine is structurally similar and also blocks M2; together, this drug class is called adamantanes. Unfortunately, resistance to the adamantanes can be achieved by just a single amino acid change in M2 and this has no or very little impact on viral fitness (Hay et al., 1986; Hayden et al., 1991; Sweet et al., 1991). Moreover, resistance to the adamantanes has become widespread since the beginning of the 21st century and therefore current guidelines do not recommend the use of adamantanes (Bright et al., 2005). A recent study reported on the development of novel M2 inhibitors that can block adamantane-sensitive as well as -resistant strains (Wang et al., 2013). It is therefore conceivable that novel M2-inhibiting drugs might become available for clinical use in the future (Table 2).

**Nuclear import of vRNPs**

**Import of vRNPs into the nucleus**

After completion of the uncoating process, the RNP are transported into the nucleus. Early studies observed that NP accumulates in the nucleus whilst M1 is distributed between the cytoplasm and nucleus (Martin & Helenius, 1991). Given the size of the vRNPs, it was hypothesized that an active, energy-dependent process would mediate their import. Of note, RNP microinjected into the cytoplasm of cells were also capable of entering the nucleus (Kemler et al., 1994). In 1995, O’Neill and co-workers demonstrated that the viral RNA was not able to enter the nucleus; addition of NP was required. Moreover, they showed that, at 0 °C, NP docks to the nuclear envelope in the presence of karyopherins and is imported into the nucleus upon addition of the cellular import factors Ran and p10 proteins and a temperature shift to 20 °C (O’Neill et al., 1995). This clearly showed that viral RNPs are imported via the cellular karyopherin import pathway. All
protein components of the RNP, the three polymerase subunits and NP, possess nuclear localization signals (NLSs). Nevertheless, import of the RNP only depends on the NLS in NP (O’Neill et al., 1995; Cros & Palese, 2003). First, an unconventional NLS in the N terminus was described in NP (Wang et al., 1997). Later, a second bipartite NLS was identified between aa 198 and 216 (Weber et al., 1998), as well as a third one around aa 320–400 (Bullido et al., 2000). For import of the RNPs, the unconventional NLS seems to be the most important (Cros & Palese, 2003). On the cellular side, karyopherins z1, x3 and x5 have been identified as the main importins for RNPs (Table 1) (O’Neill et al., 1995; Wang et al., 1997; Melen et al., 2003). In addition, CSE1L, a cellular factor required for cycling of karyopherins between the nucleus and cytoplasm, has been shown to be required for import of RNPs early in infection (König et al., 2010). Later in infection, when the individual polymerase proteins get imported, the NLSs on the polymerase subunits become important and other karyopherins are involved. This topic has been reviewed by Hutchinson & Fodor (2012). Upon import into the nucleus, the karyopherins bind to RanGTP, which results in release of cargo, and this marks the end of the viral entry process.

**Outlook**

Entry of IAV into target cells is the very first step of the viral life cycle and as such is crucial for the establishment of infection. The receptor specificity of the viral HA determines the tropism of the virus, thereby contributing to the outcome of disease, and potentially to virus spread between susceptible hosts. In recent years, our understanding of the differential receptor specificity between avian and mammalian influenza viruses has greatly improved, and exciting structural insights have been obtained. However, more work is still required to fully understand and predict the receptor specificity of all HA subtypes. For entry into target cells, the virus relies on and exploits existing cellular pathways of transporting cargo, and thus the entry process is a complex interplay between virus and host cell. Advances in live cell microscopy are of great value in tracking virions during entry in real time and in monitoring the interaction of the virus with cellular factors and compartments. Novel findings indicate that virus uptake and trafficking may not be equal to that of other cargo transported into the cell. Instead, IAV specifically recruits factors facilitating entry and activates signalling molecules such as RTKs within minutes after infection. Future studies will shed light on how these host factors contribute to virus entry on a molecular level. The increasing insight into these processes can be exploited to develop means of inhibiting the virus early in infection. Novel treatment options may be directed specifically against IAV or may be of broad antiviral efficacy if targeting entry routes used by several viruses. In the near future, we may obtain a detailed insight into the cell biology of IAV entry and profit from newly developed antivirals targeting host factors rather than viral proteins, thereby minimizing the occurrence of resistance as observed with the M2 and NA inhibitors.

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