Influenza virus budding from the tips of cellular microvilli in differentiated human airway epithelial cells

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The epithelium of conducting airways represents the main target for influenza virus in mammals. However, the peculiarities of virus interactions with differentiated airway epithelial cells remain largely unknown. Here, influenza virus budding was studied in differentiated cultures of human tracheobronchial epithelial cells using transmission electron microscopy. Budding of spherical and filamentous virions was observed on the apical surfaces of cells with no association with cilia and secretory granules. Quantitative analysis of the distribution of viral buds on the cell surface indicated that the tips of the microvilli represented a prominent site of influenza virus budding in the human airway epithelium. As the microvilli of differentiated cells are involved in many fundamental cell functions, these data will prompt further studies on the biological significance of microvilli-associated budding for virus replication, transmission and pathogenicity.

INTRODUCTION

Influenza virus assembly and budding takes place at the apical surface of infected cells (reviewed by Nayak et al., 2009). Many previous electron microscopic (EM) studies on virus infection in non-differentiated cell lines have revealed a relatively uniform distribution of budding virus particles on the cell surface (Herrero-Uribe et al., 1983; Itoh et al., 2009; Klenk et al., 1970; Mora et al., 2002). By contrast, some EM studies on virus infection in both differentiated and non-differentiated cells have found abundant (Blaskovic et al., 1972; Morgan et al., 1961) or even preferential (Bruce et al., 2012) budding of virions from microvilli-like surface projections. Although these reports suggested that cellular microvilli might represent an important budding site, convincing quantitative data confirming these morphological observations are still absent.

In mammals, influenza viruses replicate primarily in the epithelium of conducting airways, which consists of several distinct cell types with different functions and represents a complex system controlling appropriate mucus production and coordinated ciliary activity (Fahy & Dickey, 2010; Tomashesfki & Farver, 2008). Budding from the planar surface, cilia and the bases and tips of microvilli has been observed in limited EM studies of infected airway epithelial tissues of animals (Blaskovic et al., 1972; Castleman et al., 2010; O’Neill et al., 1984), but the predominant sites of budding in different cell types are currently unclear.

To study the features of influenza virus budding under conditions mimicking natural influenza infection in humans and to understand whether virus budding cooperates with some specialized surface structures or takes place throughout the whole apical plasma membrane domain, we used cultures of differentiated human tracheobronchial epithelial (HTBE) cells. These cultures are pseudostratified and polarized; they contain basal, ciliated and mucus-secreting cells, and represent a good model for the morphology and functions of human airway epithelium in vivo (Gray et al., 1996; Matrosovich et al., 2004). Our quantitative analysis of the distribution of influenza virus buds on ultrathin EM sections of infected HTBE cultures demonstrated preferential virus budding from the tips of cellular microvilli.

RESULTS AND DISCUSSION

HTBE cultures were infected with the H1N1 2009 pandemic virus isolate A/Hamburg/5/2009. We fixed infected cultures at 24 h post-inoculation to ensure infection of all susceptible cell types and to avoid significant cytopathic effects occurring at later time points. The influenza virus-infected HTBE cells were examined using ultrathin sections of cells embedded in epoxy resin. To focus on virus budding, we analysed virions at the pre-scission stages, as judged by the lack of complete separation of the viral and plasma membranes (Fig. 1a–c). Isolated virions and virions loosely attached to the membrane (Fig. 1d) were excluded from the analysis. For each budding virus particle, the host-cell type was defined as either ciliated (containing cilia and/or basal bodies, Fig. 2), secretory (containing secretory granules, Fig. 3) or undefined. The latter cell type included poorly differentiated cells, which lacked cilia, basal bodies and secretory granules but had an apical domain (Fig. 4). Some
cells were represented on sections by fragments that were too small to judge cellular differentiation status unambiguously. These cells were also listed as undefined. Noticeably, the morphology of virus-infected HTBE cells did not undergo significant cytopathological changes at 24 h post-inoculation (Fig. 5a, compare with Figs 2–4). At the same time, budding virions coated with viral surface glycoproteins were clearly distinguishable from pleomorphic structures present on the apical surface of HTBE cells (compare Fig. 5b with Fig. 1b, or Fig. 5c with Figs 2c and 3a, or Fig. 5c with Fig. 2a and 4c).

Our analysis of 300 sections from two replicate infected cultures identified 140 budding virions representing random sampling (Table 1). The majority of buds (61) were found on the surface of undefined cells, followed by secretory cells (53) and ciliated cells (26). This pattern correlated with the relative abundance of these cell types in HTBE cultures.

Remarkably, two distinctive sites of virus budding were found in all cell types, namely the planar surface of the plasma membrane and the cellular microvilli (Fig. 1a–c, Fig. 2b, c, Figs 3, 4 and 6). No budding virions were associated with secretory granules (Fig. 3a, c). Our finding that the cilium membrane is not involved in influenza virus budding is in agreement with a previous study in chicken embryo tracheal organ cultures (Blaskovic et al., 1972). However, it is not consistent with an in vivo study of canine influenza virus (Castleman et al., 2010). Thus, further studies are needed to clarify whether budding from cilia is host or virus-strain specific.

The majority of ultrathin sections analysed contained cells with abundant microvilli (Fig. 6a, c). However, there were also cell sections that either completely lacked microvilli or had a single short microvillus (Fig. 6b). Because cells with a flat surface are not typical of normal HTBE in vivo (Carson et al., 1985), we analysed such sections separately. For each electron micrograph, we calculated the number of microvilli per 1000 nm of apical surface length. Ninety-one of 140 budding virions (65%) were found in cells that had more than two microvilli per 1000 nm (Fig. 6a and Table 1, cells designated M). The majority (62–68%) of the viral buds in these cells were associated with microvilli. The remaining 49 budding virions were found in cells with fewer than two microvilli per 1000 nm (Fig. 6b and Table 1, cells designated L). Budding from microvilli was less
pronounced in these cells than in M cells but was still quite significant (22–57 % of total virus buds in L cells were located on microvilli; Table 1).

Among 74 buds with a microvillar location, 45 buds (61 %) were associated with the tip of the microvilli (Figs 1c, 2b, c; 3a and 4b and Table 1). The profile of the tip of the microvilli was usually at least tenfold smaller than the profile of the microvillar lateral membrane on the ultrathin sections (Figs 2a, b, 3a, 4b, c and 6a). Thus, the observed distribution of the buds could not be explained by their random distribution over the whole surface of the microvilli. These results indicated that virus assembly and budding from microvilli occurred predominantly at their tips.

Influenza viruses are pleomorphic and contain both spherical and filamentous particles (Bruce et al., 2012; Calder et al., 2010; Choppin et al., 1960; Itoh et al., 2009). In the ultrathin sections studied here, virus buds were found at different stages of viral bud formation (Fig. 6d–f; see also Figs 2b, c, 3 and 4b, c). The viral buds formed had either a spherical or an elongated form (Fig. 6e, f; see also Figs 2c and 4a). The production of very long filamentous particles (up to 1750 μm in length) was detected rarely and only from secretory cells (Fig. 6g–i). Among a total of 108 budding particles analysed for their morphology (Table 2), 34 buds (31 %) were elongated by a factor of 1.5 or more along one axis and were counted as filamentous. Spherical filamentous buds were found on all cell types, with filamentous buds being associated more often with microvilli than with the planar membrane (Table 2).

Our study demonstrated that different types of HTBE cells are involved in productive influenza virus infection. Quantitative analysis showed that, in addition to the planar plasma membrane, cellular microvilli represent a prominent

Fig. 3. EM analysis of influenza virus budding in secretory cells. (a) Viral buds located on microvilli. (b, c) Viral buds on the planar area of the plasma membrane. Arrows indicate viral buds and arrowheads indicate exocytosis of secretory granules. In each panel, the left images show an overview and the right images show a higher magnification of the boxed area. Bars, 1000 nm (a, left image), 500 nm (b, left image), 250 nm (c, left image), 100 nm (a–c, right images).

Fig. 4. EM analysis of influenza virus budding in undefined cells. (a) Viral buds located on the planar area of the plasma membrane. (b, c) Viral buds located on the tip and side of microvilli, respectively. Arrows indicate viral buds, arrowheads indicate cross-sectioned microvilli and a lipid vesicle is indicated by an asterisk. In each panel, the left images show an overview and the right images show a higher magnification of the boxed area. Bars, 1000 nm (a, left image); 500 nm (b, c, left images); 100 nm (a–c, right images).
site of influenza virus budding in human airway epithelium. Microvilli are characteristic cell-surface structures of differentiated cells involved in many fundamental cell functions, such as regulation of substrate transport and energy metabolism, chemoreception, signalling and shedding of the cellular membrane from their tips in the form of vesicles (reviewed by Lange, 2011). The protein and lipid composition of microvilli, including the composition of cholesterol-based lipid rafts, differs significantly from that of the planar plasma membrane (Röper et al., 2000). These

**Fig. 5.** EM analysis of non-infected differentiated HTBE cultures. (a) Apical surface of ciliated and secretory cells. White double arrows indicate tight junctions and black arrowheads indicate secretory granules. The boxed areas in (a) are shown at a higher magnification in (b)–(d). (b) Multiple threads of glycocalyx at the surface of the microvilli are indicated by arrows. (c) Branched microvilli are indicated by arrowheads. (d) Cross-sectioned cilia and a lipid vesicle (arrow). Bars, 1000 nm (a); 100 nm (b–d).

**Fig. 6.** EM analysis of differentiated HTBE cultures infected with influenza virus, showing examples of the different types of cell surface and diverse viral buds. (a, b) Apical surface of cells with high (a) and low (b) numbers of microvilli. These two types of cell surface were designated M and L, respectively, in Tables 1 and 2. The grey bar with arrowheads indicates 1000 nm. (c) Higher magnification of the boxed area in (a). Arrows indicate viral particles and arrowheads indicate microvilli. (d) Initial stage of viral bud formation. (e, f) Formation of a vesicular viral bud and an elongated viral bud, respectively. (g–i) Budding of long filamentous viral particles from the surface of a secretory cell. The boxed area in (h) is shown at a higher magnification in (i). Arrows indicate filamentous viral particles and arrowheads indicate secretory granules filled with electron-translucent material at the periphery and electron-dense material in the centre. Bars, 250 nm (a–c, g); 100 nm (d–f, i); 500 nm (h).

**Table 1.** Number of influenza virus buds at the planar cell membrane and on microvilli of different cell types identified on ultrathin sections of infected HTBE cultures

L indicates cells with fewer than two microvilli per 1000 nm of the apical surface profile (for example, see Fig. 6b), whilst M indicates cells with two or more microvilli per 1000 nm of the apical surface profile (for example, see Fig. 6a). Numbers in parentheses show the percentage of buds on the side and tip of the microvilli, or just on the tip, as indicated, with respect to the total number of buds in that type of cell.

<table>
<thead>
<tr>
<th>Budding site</th>
<th>Secretory cells</th>
<th>Ciliated cells</th>
<th>Undefined cells</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L</td>
<td>M</td>
<td>L</td>
<td>M</td>
</tr>
<tr>
<td><strong>Planar membrane</strong></td>
<td>13</td>
<td>13</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td><strong>Microvilli</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Side + tip</td>
<td>6 (32)</td>
<td>21 (62)</td>
<td>4 (57)</td>
<td>13 (68)</td>
</tr>
<tr>
<td>Side</td>
<td>2</td>
<td>11</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Tip</td>
<td>4 (67)</td>
<td>10 (48)</td>
<td>4 (100)</td>
<td>11 (85)</td>
</tr>
</tbody>
</table>
structural and functional distinctions of microvilli may have considerable implications with regard to the mechanisms of influenza virus assembly and budding, as well as the lipid and protein composition of the virus particles. Thus, future studies are warranted to specify these mechanisms, characterize distinctions in the properties of virus particles assembled at the planar cell membrane and at the microvilli, and assess potential effects of microvilli-associated budding on virus replication, transmission and pathogenicity.

**METHODS**

**Viruses.** Swine-origin pandemic influenza virus A/Hamburg/5/2009 (H1N1pdm) was isolated from clinical material in Madin–Darby canine kidney (MDCK) cells, expanded by one additional passage in MDCK cells and stored in aliquots at −80 °C.

**Cells and virus infection.** HTBE cultures were grown on Transwell membrane supports under air–liquid interface conditions and infected with A/Hamburg/5/2009 at a m.o.i. of 0.1 as described previously (Matrosovich et al., 2004, 2007). Virus-inoculated cultures were incubated under air–liquid interface conditions at 33 °C for 24 h and fixed directly on membrane supports with 4% paraformaldehyde for 16 h at 4 °C.

**Electron microscopy.** Transwell membranes with fixed cells were cut into small segments, post-fixed with 1% osmium tetroxide, dehydrated in a graded ethanol series and embedded in a mixture of Epon and Araldite. Ultrathin cross-sections were cut and contrast stained with uranyl acetate and lead citrate, and electron micrographs were acquired using a Zeiss 109 transmission electron microscope at 80 kV as described previously (Kolesnikova et al., 2000, 2007).

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**REFERENCES**


