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Nipah virus entry into the host cell does not occur by fusion at the plasma membrane but by the endocytic mechanism of macropinocytosis

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The Henipaviruses Nipah virus (NiV) and Hendra virus (HeV) are zoonotic biosafety level 4 (BSL-4) paramyxoviruses which have emerged respectively in Asia and Australia over the past ten to fifteen years. Despite NiV infection causing a fatal encephalitis in man with up to 92% mortality, and outbreaks occurring in Asia on an annual basis, no anti-viral treatments or prophylaxis are currently available against this dangerous pathogen. Furthermore, the future development of NiV or HeV vaccines is unlikely due to commercial considerations.

There are evidences that NiV enters the host cell by an endocytic route, namely receptor-mediated macropinocytosis. The identification of an endocytic pathway as the method of entry used by NIV is important as it should open the door for the development of a much-needed low-cost antiviral treatment. Hitherto, the Henipaviruses were believed to enter the host cell like other paramyxoviruses – by fusion of the virion envelope with the host cell's plasma membrane at neutral pH. Paramyxovirus entry is thought to be mediated by the concerted action of the viral glycoproteins, the attachment protein (NiV-G in the case of NiV) and the viral fusion protein (NiV-F) following attachment to the receptor. Although the formation of giant multinucleated cells called syncytia (cell-cell fusion involving the plasma membrane) is a hall-mark of NiV infection, our results suggest that NiV entry occurs via fusion with an intracellular membrane following endocytosis rather than directly with the plasma membrane.

The cellular receptor of NiV is ephrinB2 whose cellular function is to act as a ligand for members of the EphB class of receptor tyrosine kinases such as EphB4. Ephrins and Ephs are plasma membrane-bound proteins that play an important role in development. For example, in the case of ephrinB2 and EphB4 they are expressed respectively on venous cells and arterial cells and play an important role in vasculogenesis. Essentially, when an EphB4-expressing arterial cell encounters another during development of the vascular system the result is adhesion but contact between an EphB4-expressing arterial cell and an ephrinB2-expressing venous cell results in bi-directional *trans*-endocytosis of receptor-ligand complexes and subsequent cellular repulsion. Our results, generated by a combination of mutagenesis, fluorescent tagging and confocal microscopy suggest that the NiV attachment glycoprotein NiV-G mimics EphB4 thereby allowing NiV to enter by a similar mechanism.

After previously mapping the ephrinB2 binding site on the NiV-G globular head we intended to map the NiV-G binding site on ephrinB2 using receptor downregulation as an assay for mutagenesis experiments. In the absence of an effective antibody specific for human ephrinB2 we tagged the receptor with GFP. This strategy has allowed us to show not only that ephrinB2 is internalized upon interaction with NiV-G but also that the two proteins are internalized together into large (more than 0.5µm diameter) intracellular vesicles. The size of these vesicles and the finding that ephrinB2 expression provokes filopodia formation led us to hypothesize that the mechanism involved was macropinocytosis. Macropinocytosis is an active endocytic pathway which is constitutive in dendritic cells and macrophages that serves to take up fluid and exogenous antigens from the extracellular milieu but can also be induced transiently in many cell types by interaction with specific ligands such as epidermal growth factor (EGF). When EGF binds to its receptor EGFR a rapid internalization of both proteins is induced and the resulting intracellular vesicle is sorted to the lysosomes by a kinase-dependent pathway. Importantly, macropinocytosis has been shown to occur in regions of the plasma membrane where ruffling - the formation of lamellipodia and filopodia - is taking place.

Our results suggest that the cytoplasmic

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domain of ephrinB2 is required for entry but is dispensable for post-entry virus spread via syncytia formation. Intracellular signaling from the cytoplasmic domain appears to be required as NiV entry is abrogated by the mutation of a single tyrosine residue. Furthermore, we find that NiV entry is Rac1- and Cdc42-dependent. These small GTPases are molecular switches which control the actin cytoskeleton and hence the dynamics of filopodia formation. From our results we hypothesize that when NiV-G (or EphB4) interacts with ephrinB2 a single tyrosine in the cytoplasmic domain becomes phosphorylated triggering a signalling cascade involving Rac1 and Cdc42 which results in the retraction of filopodia necessary for macropinocytosis to occur. Importantly, we find that NiV entry is abrogated by EIPA, an analog of amiloride, which is a specific inhibitor of macropinocytosis. Moreover, the lysosomotropic drug chloroquine (CQ) which acts by raising the pH of subcellular compartments such as late endosomes and lysosomes abrogates NiV entry *in vitro*. Interestingly, the protease cathepsin L which cleaves and activates NiV-F localizes in these cellular compartments and requires a low pH for activity. We hypothesize that by raising the pH, CQ entraps NiV within the intracellular vesicle thus terminating entry. We intend to test both CQ and EIPA *in vivo* using our animal model the hamster and are confident that a novel low-cost antiviral treatment can now be developed not only for NiV but also HeV.

(A paper describing these results has been submitted to PLoS Pathogens and is currently in revision).