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Role of clathrin-mediated endocytosis during vesicular stomatitis virus entry into host cells

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Abstract

Vesicular stomatitis virus (VSV) is well established to enter cells by pH-dependent endocytosis, but mechanistic aspects of its internalization have remained unclear. Here, we examined the functional role of clathrin in VSV entry by expression of a dominant-negative mutant of Eps15 (GFP-Eps15Δ95/295), a protein essential for clathrin-mediated endocytosis. Whereas expression of GFP alone had no effect on VSV infection, expression of GFP-Eps15Δ95/295 severely limited infection. As independent ways to examine clathrin function, we also examined cells that had been treated with chlorpromazine and utilized small interfering RNA (siRNA) techniques. Inhibition of clathrin-mediated endocytosis by chlorpromazine treatment, as well as clathrin knock-down using siRNA duplexes directed against the clathrin heavy chain, also prevented VSV infection. In combination with previous morphological approaches, these experiments establish clathrin as an essential component needed for endocytosis of VSV.

Keywords: Clathrin; Endocytosis; Virus entry; RNAi

Introduction

Vesicular stomatitis virus (VSV) is a member of the Rhabdoviridae that has been well established to enter cells via endocytosis (Marsh and Helenius, 1989). The virus binds ubiquitously to cells via a phosphatidyl serine receptor (Schlegel et al., 1983) and fuses with endosomal membranes in a pH-dependent manner (Blumenthal et al., 1987; Gaudin, 2000). This endocytic entry of VSV is similar to other members of the Rhabdoviridae, such as rabies virus (Lewis et al., 1998). The basic mechanism of pH-dependent endocytosis for VSV has been known for many years and is well documented (Heine and Schnaitman, 1971; Matlin et al., 1982; Schlegel et al., 1981; Superti et al., 1987). However, many of the molecular details of VSV entry remain to be established.

The best-characterized pathway of endocytosis relies on the formation of clathrin-coated pits and vesicles (Brodsky et al., 2001), but there are other non-clathrin pathways that exist in the cell (Johannes and Lamaze, 2002). In principle, any of these pathways can be utilized by viruses to infect host cells (Russell and Marsh, 2001; Sieczkarski and Whittaker, 2002a). The most comprehensive studies of VSV entry have relied heavily on a morphological study by electron microscopy. In some of these studies, the majority of incoming viruses were shown to be present in pits and vesicles with electron-dense coats; however, non-coated vesicles were also observed (Matlin et al., 1982; Superti et al., 1987). In contrast, other investigators, also using electron microscopy, showed a preponderance of viruses in large non-coated vesicles, which were possibly macropinosomes (Cernescu et al., 1990). Unlike other viruses (e.g., influenza virus and Semliki Forest virus) for which entry has been studied morphologically, VSV is relatively

Abbreviations: VSV, vesicular stomatitis virus; GFP, green fluorescent protein; EGF, epidermal growth factor; SFV, Semliki Forest virus; CCV, clathrin-coated vesicle; siRNA, small interfering RNA.

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inefficient in its binding and entry (Matlin et al., 1982)—raising the possibility that morphological approaches may not be the most reliable way to examine entry into host cells for VSV.

In recent years, molecular approaches have been increasingly employed to study virus entry. In these type of experiments, dominant-negative forms of cellular proteins are overexpressed and can give quantitative analysis of functional virus entry (Sieczkarski and Whittaker, 2002a). Because of the possible problems with the morphological study of VSV entry by electron microscopy, and because VSV has a ubiquitous receptor lacking conventional signals for receptor-mediated endocytosis, we addressed in this paper the functional role of clathrin during VSV entry. By overexpression of a dominant-negative form of Eps15 and by use of chlorpromazine, both of which efficiently block clathrin-mediated endocytosis, we show that the functional pathway of VSV entry is via clathrin-coated pits.

Results

Disruption of clathrin-mediated endocytosis using dominant-negative mutants of Eps15

Our first experimental approach to determine the functional role of clathrin in VSV entry utilized a dominant-negative form of the cellular Eps15 protein. Eps15 was originally identified as a substrate for receptor-activated epidermal growth factor (EGF) tyrosine kinase and has since been found to be ubiquitously and constitutively associated with the AP-2 adaptor protein, and plays an important role in clathrin-mediated endocytosis (Benmerah et al., 1999). It has been described previously that the overexpression of a protein lacking the second and third of the three EH domains of Eps15 (Eps15Δ95/295) results in a dominant-negative protein, which inhibits clathrin-mediated endocytosis—effectively blocking uptake of cellular ligands such as transferrin and EGF (Benmerah et al., 1999), without affecting non-clathrin pathways (Nichols et al., 2001). Blockage of Eps15 function also prevents entry and infection of Semliki Forest virus, Sindbis virus and adenovirus, but not influenza virus or rotavirus (Carbone et al., 1997; Meier et al., 2002; Sanchez-San Martin et al., 2004; Sieczkarski and Whittaker, 2002b).

To confirm that we had a functional block in clathrin-mediated endocytosis, we first performed control experiments with transferrin. We expressed a green fluorescent protein-tagged version of Eps15Δ95/295 (GFP-Eps15Δ95/295) in both HeLa cells (Fig. 1) and BHK cells transiently expressing the human transferrin receptor (not shown). Cells were starved for 30 min in serum-free medium and Alexa 568-labeled transferrin was added for 20 min at 37 °C to allow internalization and cells were acid washed to remove any uninternalized ligand. Expression of GFP-Eps15Δ95/295 resulted in a pronounced inhibition of transferrin internalization (Fig. 1d), whereas in control cells expressing GFP alone transferrin was internalized normally (Fig. 1b). These data confirm that expression of GFP-Eps15Δ95/295 resulted in an effective and specific block in clathrin-mediated internalization.

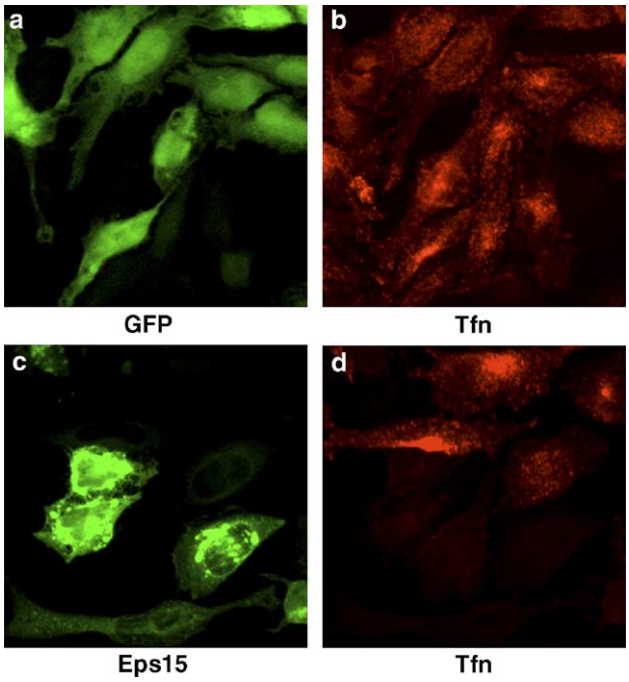

Fig. 1. GFP-Eps15Δ95/295 effectively inhibits transferrin internalization. HeLa cells were transiently transfected with GFP or with GFP-Eps15Δ95/295, and then Alexa 568-labeled transferrin was bound and internalized, followed by an acid wash to remove un-internalized transferrin. Fluorescence microscopy of GFP or GFP-Eps15Δ95/295 (Eps15) is shown along with Alexa 568-labeled transferrin (Tfn).

We next examined VSV infection of HeLa cells expressing GFP-Eps15Δ95/295, as well as GFP alone as a control (Fig. 2). We used a relatively low multiplicity of infection (<1 pfu/cell) to ensure that our results reflected the infection of single virus particles in each cell (DeTulleo and Kirchausen, 1998). Expression of GFP alone had no effect on the ability of cells to become infected (Figs. 2Aa–c), whereas in cells expressing GFP-Eps15Δ95/295, we saw an almost complete block in virus infection (Figs. 2Ad–f). Quantitation of our microscopy data showed a dramatic drop in virus infection; from around 30% of cells being both transfected and infected to around 5%. These data clearly show an effect of GFP-Eps15Δ95/295 expression and suggest that VSV requires clathrin-mediated endocytosis for productive infection.

As HeLa cells are not typically used for productive VSV infection, and to determine if the effects of GFP-Eps15Δ95/295 were cell type specific, we also used BHK cells as an experimental system. As with HeLa cells, BHK cells showed a dramatic reduction in VSV infection when expressing GFP-Eps15Δ95/295 (Figs. 3a–c), which was not apparent in control cells expressing GFP only (not