successful application of these fluorescent timers to monitor intracellular trafficking of lysosome-associated membrane protein type 2A (LAMP-2A). The success of this approach was strongly reliant on the authors' experimental design. Subach *et al.* first identified key residues involved in chromophore formation and maturation of the mCherry protein by carefully examining its X-ray crystal structure. In addition, they studied reports regarding the effect of amino acid substitutions in the structures of DsRed variants on the maturation of their respective chromophores. The knowledge thus gained allowed the authors to chose specific amino acids to be included in their mutagenesis studies. They then subjected the chosen residues to multiple saturated mutagenesis cycles. This strategy permitted the construction of a bacterial library, which they then screened for clones with varying maturation rates. This pool yielded three different fluorescent proteins with distinctive blue-to-red conversion times, which were isolated and characterized with regard to their fluorescence excitation and emission properties at different pHs and temperatures and in different cell types.

Following these studies, the authors used the new fluorescent timers to investigate the intracellular trafficking of LAMP-2A and elucidate the mechanism by which LAMPs reach the lysosomes. To accomplish this, several types of mammalian cells were transfected with fusion proteins consisting of LAMP-2A attached to a fluorescent timer. Because of the very slow maturation times of the fluorescent proteins, their emission was observed for more than 60 h, long enough for the fusion proteins to migrate from the Golgi apparatus to the endosomes. By calculating the ratios of the red and blue forms, and fitting the obtained data to a kinetic model, the ages of the fusion proteins could be determined. Correlating the ages of LAMP-2A in the various compartments within the cell allowed the researchers to determine the trafficking path of LAMP-2A through the cell. The mechanism by which LAMP-2A reaches the endosomes had been a topic of ongoing discussion for quite some time: some investigators believed that the protein follows a direct path to reach the endosomes, whereas others had suggested that it meanders through an indirect route to arrive to its final destination. The new data presented by Verkhusha *et al.* demonstrate that the trafficking of LAMP-2A from the Golgi apparatus to the endosomes follows an indirect pathway, as suggested by Storrie *et al.*[5] (Golgi → plasma membrane → early and recycling endosomes → late endosomal-lysosomal components). Therefore, the newly prepared fluorescent timers not only have shown their usefulness as general *in vivo* imaging reagents, but also have provided fundamental information regarding mechanisms of traffic and transport within the cells.

Just as occurred in the evolution of the green fluorescent protein, from a single protein to protein families with a multitude of emission colors, we believe that research on these fluorescent timers will continue to advance until an array of such proteins with varied maturation times and colors is available. The current work of Verkhusha *et al.* demonstrates the advantage of preparing and using fluorescent timer proteins for dynamic studies involving long time frames. Indeed, this report is the first *in vivo* analysis of the spatial and temporal characteristics of a lysosomal membrane protein. Armed with these newly developed tools, scientists will be able to observe not only the location of a particular protein but also its transport mechanism. Although these new proteins may be invaluable for slow dynamic studies, they are not appropriate for imaging fast cellular processes. Thus, the quest for fluorescent timers with fast color-conversion times continues. We are living in an era in which molecular biology is continuously evolving and providing us with new methods that, along with rational design, permits the creation of a myriad of new proteins with unprecedented characteristics. Fluorescent timers are certainly unique, and they form a new class of proteins exceptionally suited for *in vivo* imaging and for enhancing the understanding of cellular events.

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'Sialidase sensitivity' of rotaviruses revisited

Kalyan Banda, Gagandeep Kang & Ajit Varki

Rotaviruses have been designated as 'sialidase sensitive' or 'sialidase insensitive', based on how their entry into cells is affected by treating cells with sialidases. A new study uses multiple methods, including saturation transfer difference NMR spectroscopy, to elucidate interesting interactions involving terminal and internal sialic acid moieties, concluding that 'sialidase insensitive' does not mean 'sialic acid independent'.

Rotaviruses are non-enveloped, double-stranded, segmented RNA viruses that are the leading cause of severe diarrhea in infants and children worldwide[1]. They primarily infect mature cells of the small intestine, causing diarrhea in part by diminishing the cells' absorptive capacity. Rotavirus attachment to host cells involves complex interactions between two rotavirus outer-layer viral proteins, VP7 and VP4 (the spike protein), and several possible receptors whose roles remain largely unresolved. Infectivity is enhanced by trypsin cleavage of VP4 into a C-terminal fragment, VP5*, and an N-terminal domain, VP8*, both of which remained associated with the triple-layered virion. Previous studies had suggested that cell-surface sialic acids[2] play a role in mediating viral entry[3], and the differing effects of sialidase pretreatment on infection led to the designation of a class of 'sialidase insensitive/sialic acid-independent' rotaviruses[3,4]. Now, a new study by Haselhorst *et al.*[5] instead shows that sialidase-insensitive viruses bind to internal sialic acids that are resistant to sialidase action.

In the search for rotavirus attachment sites, several studies suggested that sialylated glycosphingolipids (gangliosides) (**Fig. 1**) are the initial receptors and showed differences in human and animal rotavirus hemagglutination of erythrocytes, a heavily sialylated cell type[3]. Treatment of target

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cells with sialidases decreased the infectivity of hemagglutinating viruses; and this could be specifically traced to VP8* and to the region between amino acids 93 and 208 in particular. In contrast, infectivity with hemagglutination-negative rotaviruses was resistant to sialidase, suggesting sialic acid independence. However, other studies showed that triple-layered particles of both sialidase-sensitive and sialidase-insensitive strains could bind to certain gangliosides[6] including the sialic acid–containing GM3, casting doubt on the appropriate definition of the sialidase classifications.

Indeed, the presumption that sialidase insensitivity equates to sialic acid independence did not account for the fact that many sialidases cannot cleave the nonterminal ('internal') sialic residues of gangliosides such as GM1 (**Fig. 1**). The paper by Haselhorst *et al.* now provides the first direct evidence for the binding of an internal sialic acid by VP8* of a sialidase-insensitive strain. The authors used NMR spectroscopy and infectivity assays on sialidase-sensitive and sialidase-insensitive strains (porcine strain CRW8 and human strain Wa, respectively) to demonstrate interactions of VP8* with terminal and internal sialic acid moieties. Saturation transfer difference (STD) NMR spectroscopy showed that CRW8 VP8* bound the terminal α2-3-linked *N*-acetylneuraminic acid (Neu5Ac) sialic acid residue of the glycan chains of ganglioside GD1a (aceramido-GD1a), but not of aceramido-GM1, which lacks this terminal Neu5Ac residue. In contrast, Wa VP8* did not bind the GD1a glycan, but bound aceramido-GM1, which has an internal α2-3-linked Neu5Ac residue (**Fig. 1**). In keeping with this, removal of terminal α2-3-linked-Neu5Ac actually gave *increased* binding by Wa, which was decreased by incubation with the α-methylglycoside of Neu5Ac. Notably, the sialidase used is from *Vibrio cholerae*, a bacterium whose toxin binds to GM1 but not GD1a (**Fig. 1**). This differential receptor utilization was corroborated by decreased infectivity of each strain following preincubation with the homologous but not the heterologous VP8*, a decrease upon incubation with corresponding antisera and a dose-dependent blockade of Wa by cholera toxin.

Many further complexities in the binding preferences of various strains need to be considered. For example, further investigations with GM3, untested in the current study, should shed more light on rotavirus recognition. The single oxygen atom difference between Neu5Ac and *N*-glycolylneuraminic acid (Neu5Gc) has also been previously shown to influence rotavirus recognition, further suggesting a complex interaction surface and demonstrating additional selectivity between viruses[6]. Structural comparison of VP8* cores of the sialidase-sensitive strain RRV and the sialidase-insensitive human virus DS-1 also showed that although the sialic acid–binding cleft is wider in the human strains, the adjacent extended binding region is highly conserved, and is postulated to be capable of interacting with glycan linkages proximal to the sialic acid moiety[7]. This may allow VP8* to bind internal sialic acids linked to distal sugar residues in glycolipids. It is likely that these and other fine differences in the receptor specificity of sialidase-insensitive strains explain many varying results in the literature. Regardless, it now seems likely that most, if not all, rotaviruses require sialic acids for host cell recognition, with variations in types, linkages and presentation of sialic acid moieties.

How might the different rotavirus specificities play out *in vivo*? Interestingly, age-related changes in ganglioside expression occur in early infancy, as in pigs and rodents, where Neu5Gc-GM3 is predominant for the first 3–4 weeks after birth[8]. Also, during evolution, humans acquired a mutation that abrogated synthesis of the CMP-Neu5Gc sialylation donor from the CMP-Neu5Ac precursor[2]. The subsequent altered interplay with pre-existing rotaviruses might explain the unusual sialidase resistance of most human rotavirus strains, and possibly the predilection of bovine-human 'reassortant' (combinatorial) strains for human neonatal infections, as described from the Indian subcontinent[9]. In this regard, it is also notable that dietary Neu5Gc (which is present in cow's milk but not in human milk) can be metabolically incorporated into the gut epithelium, generating receptors for a Neu5Gc-preferring toxin[10].

Overall, it is clear that much more needs to be learned about the rotavirus sialic acid interactions, an area of study that may eventually lead to better approaches toward understanding and treating this potentially deadly disease.

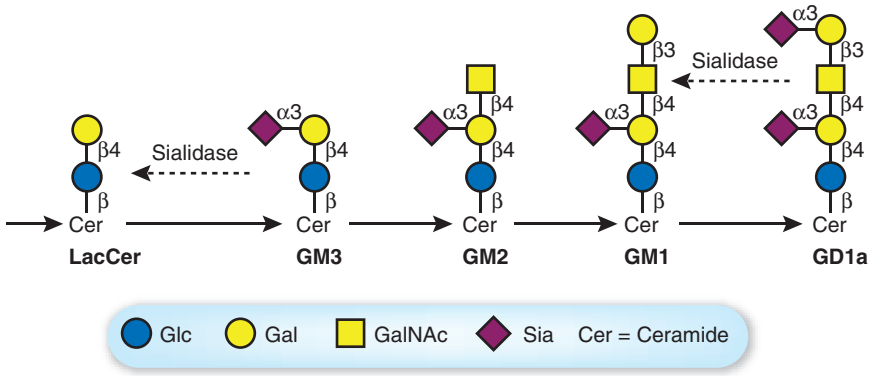

Figure 1 Ganglioside biosynthetic pathways and sialidase action. Gangliosides are polar lipids, with a core ceramide and attached neutral glycan chains consisting of monosaccharides such as glucose (Glc), galactose (Gal) and *N*-acetylgalactosamine (GalNAc), with a variable number of terminal or subterminal sialic acid (Sia) residues. Linkages of the neutral monosaccharides are from the C-1 position, and from C-2 for sialic acids. Solid arrows show pathways for biosynthesis of these types of gangliosides. Sialidase enzymes, for example, from *V. cholerae* remove terminal sialic acids (see dotted arrows) but typically cannot remove subterminal ('internal') sialic acids. The study by Haselhorst *et al.*[5] shows that a sialidase-insensitive rotavirus binds to the internal sialic acid of GM1 ganglioside.

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