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Research paper

Signal dependent transport of a membrane cargo from early endosomes to recycling endosomes

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ABSTRACT

Many membrane cargoes undergo endocytosis and intracellular recycling to the plasma membrane via the early endosomes and the recycling endosomes. However whether specific sorting signals are required for transport from early endosomes to recycling endosomes is not known and the current view is that transport to the recycling endosomes is by a passive default process. Here we show that the cytoplasmic tail of the neonatal Fc receptor (FcRn) contains discrete signals for endocytosis and for sorting to the recycling endosomes. The FcRn cytoplasmic tail has previously been shown to contain the unusual WISL motif for AP2/clathrin-mediated endocytosis. By analysing FcRn mutants and CD8/FcRn chimeric molecules, we have identified an extended WISL sequence (GLPAPWISL) which promotes sorting from the early endosomes to the recycling endosomes. The insertion of GLPAPWISL into the cytoplasmic tail of CD8 resulted in efficient endocytosis and trafficking to the recycling endosomes, with only low levels detected in the late endosomes. Replacement of the highly conserved GLAPAP sequence within the GLPAPWISL motif with alanine residues resulted in endocytosis of the CD8/FcRn chimera to the early endosomes which was then trafficked predominantly to the late endosomes rather than the recycling endosomes. These studies demonstrate that signals within the cytoplasmic domains of membrane cargo can mediate active transport from early to recycling endosomes.

1. Introduction

Specific transport mechanisms are required to direct molecules to their defined location and to maintain the identity and function of individual compartments (van Vliet et al., 2003). Sorting of cargo is mediated by short sequences of amino acid residues, called sorting signals or motifs (Bonifacino and Traub, 2003). Sorting signals are recognized and captured by components of coat complexes that promote transport vesicle/carrier formation (Bonifacino and Traub, 2003; Cai et al., 2007). Most endosomal-lysosomal sorting signals characterized to date are contained within the cytoplasmic tail of transmembrane proteins and the major classes of endocytosis signals are the tyrosine-based and di-leucine-based motifs (Bonifacino and Traub, 2003).

Upon internalization from the plasma membrane, cargos are transported to the early endosomes. Here, cargo can be targeted for degradation by transport to late endosomes/lysosomes. Alternatively, proteins can undergo endocytic recycling either directly to the plasma membrane, or indirectly via the recycling endosome (Hsu et al., 2012; Hsu and Prekeris, 2010) or the TGN (Lieu and Gleeson, 2011). Endocytic recycling of membrane cargo back to the plasma membrane is important for a variety of cellular processes including signalling, nutrient uptake, cell–cell adhesion and cell development (van Ijzendoorn, 2006). Endosomal sorting is orchestrated by the generation of membrane tubules emanating from early endosomes which pinch off to become tubular carriers (Cullen, 2008). Membrane tubule formation is mediated by sorting nexin (SNX) Bar proteins which induce curvature of membranes (Peter et al., 2004; van Weering et al., 2012). In addition, SNX proteins can also recognise cargo for inclusion into tubular carriers (van Weering and Cullen, 2014). For example, SNX27 interacts with NPxY/NxxY sorting motifs for fast retrieval of cargo back to the cell surface (Balana et al., 2011; Ghai et al., 2013). Specific sorting motifs have also been identified for cargo which are transported in a retromer-dependent manner from the early endosomes to the TGN

Abbreviations: FcRn, neonatal Fc receptor; TfR, transferrin receptor; TGN, trans-Golgi network; M6P-R, mannose-6-phosphatemannose-6-phosphate receptor; GFP, green fluorescent protein.

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(Seaman, 2007). However, it remains unclear whether specific sorting signals are required for cargo sorting from the early endosomes to recycling endosomes. Recycling endosomes are a subpopulation of endosomes that are typically located deeper in the cell and centred around the microtubule-organizing centre (Perret et al., 2005; van Ijzendoorn, 2006).

A commonly held view is that cargo may be delivered to the recycling endosomes by a default pathway. The suggestion of a default pathway was initially based on studies which failed to identify sorting signals of the transferrin receptor (TfR) and the observation that lipids were endocytosed and recycled with similar kinetics as the transferrin receptor (Mayor et al., 1993). The proposal that endosomal sorting of membrane cargo to the recycling endosome represents a default pathway mediated by tubules which are continuously formed from the early endosomes (van Weering and Cullen, 2014) has dominated the protein recycling field (Hsu et al., 2012; Maxfield and McGraw, 2004; van Weering and Cullen, 2014).

More recently a L-F/R-F motif in the cytoplasmic domain of TfR has been identified to function as a sorting motif for the recycling of TfR to the cell surface (Dai et al., 2004; Li et al., 2007). An adaptor known as ACAP1 (ARF GAP with coiled-coil ankyrin repeat and pH domain-containing protein 1) was shown to bind to two distinct phenylalanine-based sequences (LF, RF) in the cytoplasmic tail of TfR and promote the recycling of TfR from recycling endosomes to the cell surface (Dai et al., 2004). This study showed that sorting motifs are relevant in the slow recycling pathway. Nonetheless, whether active sorting is required to deliver TfR, or any other receptor, to the recycling endosomes from early endosomes remains unclear.

The neonatal Fc receptor (FcRn) is a recycling receptor that functions in the neonate and the adult (Roopenian and Akilesh, 2007). FcRn is a membrane-bound heterodimer consisting of a MHC class I-like heavy chain (~40 kDa) and a non-covalently associated light chain, β2 microglobulin (β2m) (Burmeister et al., 1994). There is considerable interest in this receptor given its role in rescuing endocytosed IgG and albumin from lysosomal degradation by capturing these ligands in acidic intracellular compartments, and recycling them back to the cell surface where they dissociate at neutral pH and are released, thereby extending their lifetime within the circulation (Martins et al., 2016). FcRn contains conserved WXXϕ and acidic di-leucine (DXXXLL) motifs in the cytoplasmic tail (Tesar and Bjorkman, 2010) across a number of species, including human, rat, bovine and murine (Tesar and Bjorkman, 2010). The μ subunit of AP-2 has been shown to interact with the WXXϕ motif (Wernick et al., 2005) to promote endocytosis. Substitution of tryptophan to alanine decreased FcRn endocytosis (Wu and Simister, 2001). The "di-leucine" motif in the cytoplasmic domain of FcRn has also been linked to efficient and rapid internalization of FcRn (Stefaner et al., 1999; Wu and Simister, 2001). How FcRn is diverted from early endosomes to recycling endosomes to evade the late endosomal-lysosomal pathway and to be sorted into the recycling pathway remains unknown. Here we have analysed the sorting motifs in the cytoplasmic tail of FcRn that are responsible for sorting into the endocytic-recycling pathway and have identified a signal which promotes active transport from the early endosomes to the recycling endosomes.

2. Materials and methods

2.1. Cell culture and transient transfections

HeLa cells were maintained as a semi-confluent monolayer in Dulbecco's Modified Eagle's media (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS), 2 mM glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin (C-DMEM). For transient transfections, HeLa cells were seeded as monolayers in 12-well plates with coverslips. Cells were transfected with plasmid DNA (0.1-1.0 μg/well) using Fugene 6 or Fugene-HD (Promega, USA) according to manufacturer's protocol. Transfected cells were cultured for 24 h.

2.2. Plasmids and antibodies

GFP-Rab11 is an N-terminal fusion construct with GFP, as described (Zhang et al., 2004). Rabbit polyclonal anti-human EEA1 (cat.2411) was purchased from Cell Signalling (USA). Rabbit anti-LAMP1 (cat. Ab24170) and rabbit anti-GRASP65 (cat. Ab30315) were purchased from Abcam (UK) and mouse monoclonal anti-human CD8α (RPA-T8, cat 14-0088) from eBioscience (USA). Goat anti-rabbit IgG Alexa Fluor 488, goat anti-rabbit IgG Alexa Fluor 568, goat anti-rabbit IgG Alexa Fluor 647, goat anti-mouse IgG Alexa Fluor 448, goat anti-mouse IgG Alexa Fluor 568, goat anti-mouse IgG Alexa Fluor 647, goat anti-rat IgG Alexa Fluor 568 were purchased from Molecular Probes (Invitrogen, USA).

2.3. FcRn constructs

2.3.1. pEGFP-C1-CD8/FcRn

The luminal and transmembrane domains of CD8a were amplified from pDC315-CD8/CI-M6PR construct using the primers 5′-AATGCTAGCCCACCATGGCCTTACCAGTGACC-3′ and 5′- ACCAAGCTTGTTGCAGTAAAGGGTGAT-3′, which contain *Nhe*I and *Hind*III restriction sites. The sequence encoding the cytoplasmic domain of human FcRn was amplified from the Hu-FcRn-pMAT construct using the primers 5′-ATTAAGCTTCGGAGAATGAGAAGCGGAC-3′ and 5′-ACCGGTACCTCATCAGGCGGTGGCGGGGATC-3′, which contained *Hind*III and *Kpn*I restriction sites. The resulting PCR products were digested with *Hind*III and then a ligation performed to generate the CD8-FcRn DNA fragment which was purified, and digested with *Nhe*I and *Kpn*I and cloned into pEGFP-C1, previously digested with *Nhe*I and *Kpn*I to remove EGFP tag. The EGFP tag was replaced with the CD8/FcRn fragment. The sequence of pEGFP-C1-CD8/FcRn was confirmed by DNA sequencing.

2.3.2. pEGFP-C1-CD8/FcRn-18

CD8/FcRn-18 was generated by removing the C-terminal 18 amino acids from the cytoplasmic tail of FcRn. The corresponding DNA was amplified from the pEGFP-C1-CD8/FcRn construct using 5′-AATGCTAGCCCACCATGGCCTTACCAGTGACC-3′ and 5′-GATGGTACCTCATCAAGGTGTAGGCAGCAGCACGCC-3′. The resulting product was digested with *Nhe*I and *Kpn*I and cloned into pEGFP-C1 (previously digested with *Nhe*I and *Kpn*I) and the construct confirmed by DNA sequencing.

2.3.3. pEGFP-C1-CD8/FcRn-28

CD8/FcRn-28 was generated by removing the last 28 amino acids from the C-terminus of the cytoplasmic domain of FcRn. The corresponding DNA was amplified from the pEGFP-C1-CD8/FcRn construct using 5′-AATGCTAGCCCACCATGGCCTTACCAGTGACC-3′ and 5′- GATGGTACCTCATCATCCCCGCAGGCTGATCCAG −3′. The resulting product was digested with *Nhe*I and *Kpn*I and cloned into pEGFP-C1(previously digested with *Nhe*I and *Kpn*I) and the construct confirmed by DNA sequencing.

2.3.4. pEGFP-C1-CD8$_{WISL}$

The CD8$_{WISL}$ construct was generated by inserting the residues "WISL" after the first 10 amino acids of the cytoplasmic tail of CD8 (equivalent to the position of WISL in wild type cytoplasmic tail of FcRn), by the overlapping PCR method. DNA