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Grant Title: A novel structure of GPI anchored proteins and its physiological role

Abstract

Objectives: Glycosylphosphatidylinositol (GPI) is a glycolipid that anchors 150 or more proteins to the mammalian cell surface. GPI precursor has three mannoses and all of them are modified by ethanolamine-phosphate (EthN-P). It has been believed that EthN-P linked to the third mannose is always used as a bridge to the protein whereas one linked to the second mannose is removed after GPI is attached to proteins. The EthN-P seemingly transiently linked to the second mannose is important because mutations in PIGG, which catalyzes this modification, cause inherited GPI deficiency characterized by neuronal dysfunction. However, a role of this EthN-P has been unclear because GPI-anchored proteins (GPI-APs) such as CD59 and DAF are normally transported and expressed with the normal structure at normal level in mammalian *PIGG*-knockout (KO) cells. We tried to elucidate the mechanism of neuronal abnormalities in the PIGG deficiencies.

Methods: We established the HEK293 cells highly expressing HFGF-CD59 (CD59 fused with epitope tags and GST for easy purification) and knocked out *PIGB* in these cells. HFGF-CD59 proteins were solubilized by PI-PLC-treatment from the wild type and the *PIGB*-KO cells, affinity-purified from the supernatants and subjected to SDS-PAGE and in-gel trypsin digestion. The C-terminal peptide linked to GPI glycan was determined by LC-ESI-mass spectrometry (MS). Similarly, HA-NT5E and HA-NTNG2, candidate GPI-APs with the novel structure, expressed on the Expi293F cells, were purified and analyzed.

Results: We found that *PIGB*-KO cells lacking the third mannose and *PIGO*-KO cells lacking EthN-P linked to the third mannose expressed low levels of GPI-APs and that those residual GPI-APs were lost by further knockout of *PIGG*, indicating that the EthN-P on Man2 can be used to bridge CD59 and DAF in a low efficiency. In the MS sample from the *PIGB*-KO cells, precursor ions corresponding to the C-terminal 11 amino acid peptide linked to GPI having two Man and a HexNAc side chain were found. MS/MS analysis showed not only the GPI diagnostic fragments but also the fragments that are diagnostic of peptide-attachment to Man2- linked EthN-P. These fragment ions were also found in approximately 10% of precursor ions which have three Man in wild type cells. These results indicate that approximately 10% of CD59 protein is expressed with this novel structure in wild type cells. We next hypothesized that some GPI-APs are preferentially linked in this way and the expression of these proteins might be lower in *PIGG*-KO cells than in wild type cells. Through the quantitative proteomics analysis and analysis by flow cytometry, NTNG2 and NT5E (CD73) were picked up and HA tagged NT5E and NTNG2 were affinity purified to demonstrate attachment of these proteins to Man2-linked EthN-P by LC-MS/MS. MS/MS analysis of precursor ions indicated not only the GPI diagnostic fragments, but also the fragments which are specific to the novel structure. These specific fragment ions were present in all precursor ions from NTNG2 and NT5E. These results indicated that NT5E and NTNG2 expressed in wild type HEK293 cells has the preference to PIGG dependent structure. Therefore, reduced levels of these specific GPI-APs may result in the clinical symptoms associated with *PIGG*-IGD.