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# Functional evaluation of tryptophans in glycolipid binding and membrane interaction by HET-C2, a fungal glycolipid transfer protein

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ABSTRACT

HET-C2 is a fungal glycolipid transfer protein (GLTP) that uses an evolutionarily-modified GLTP-fold to achieve more focused transfer specificity for simple neutral glycosphingolipids than mammalian GLTPs. Only one of HET-C2's two Trp residues is topologically identical to the three Trp residues of mammalian GLTP. Here, we provide the first assessment of the functional roles of HET-C2 Trp residues in glycolipid binding and membrane interaction. Point mutants HET-C2$^{W208F}$, HET-C2$^{W208A}$ and HET-C2$^{F149Y}$ all retained > 90% activity and 80–90% intrinsic Trp fluorescence intensity; whereas HET-C2$^{F149A}$ transfer activity decreased to ~55% but displayed ~120% intrinsic Trp emission intensity. Thus, neither W208 nor F149 is absolutely essential for activity and most Trp emission intensity (~85–90%) originates from Trp109. This conclusion was supported by HET-C2$^{W109Y/F149Y}$ which displayed ~8% intrinsic Trp intensity and was nearly inactive. Incubation of the HET-C2 mutants with 1-palmitoyl-2-oleoyl-phosphatidylcholine vesicles containing different monoglycosylceramides or presented by lipid ethanol-injection decreased Trp fluorescence intensity and blue-shifted the Trp $\lambda_{max}$ by differing amounts compared to wtHET-C2. With HET-C2 mutants for Trp208, the emission intensity decreases (~30–40%) and $\lambda_{max}$ blue-shifts (~12 nm) were more dramatic than for wtHET-C2 or F149 mutants and closely resembled human GLTP. When Trp109 was mutated, the glycolipid induced changes in HET-C2 emission intensity and $\lambda_{max}$ blue-shift were nearly nonexistent. Our findings indicate that the HET-C2 Trp $\lambda_{max}$ blue-shift is diagnostic for glycolipid binding; whereas the emission intensity decrease reflects higher environmental polarity encountered upon nonspecific interaction with phosphocholine headgroups comprising the membrane interface and specific interaction with the hydrated glycolipid sugar.

In filamentous fungi such as *Podospora anserina*, cell-cell recognition associated with heterokaryon fusion and vegetative incompatibility is regulated by *het* genes [1]; [2]. The heterokaryon compatibility gene, *het-c2*, encodes HET-C2, a protein with similar conformational architecture to human glycolipid transfer protein, i.e. GLTP-fold [3]; [4]; [5]; [6]; [7]; [8]. HET-C2 uses its all α-helical, two-layer 'sandwich' topology to bind and transfer single glycosphingolipid (GSL) molecules between membranes in vitro [7]; [8]; [9]; [10]. To acquire and deliver glycolipids, HET-C2 must interact transiently and reversibly with membranes. Thus, HET-C2 possesses the defining features of peripheral amphitropic membrane proteins, which have affinity for both aqueous and nonpolar environments but require neither post-translational modifications nor anchor proteins for reversible interaction with membranes [3]; [5]; [8].

Currently, there is much interest in defining the specific and non-specific ways that membrane lipid composition can target amphitropic proteins, such as HET-C2 and human GLTP, to select sites in cells. One approach to track protein interaction with membranes relies on the environmentally responsive fluorescence of tryptophan (Trp) to avoid disturbances introduced by extrinsic labels. Human GLTP contains

*Abbreviations:* GLTP, glycolipid transfer protein; GSL, glycosphingolipid; MonoGlycCer, monoglycosylceramide; GalCer, galactosylceramide; GlcCer, glucosylceramide; POPC, 1-palmitoyl- 2-oleoyl-*sn*-glycero-3-phosphocholine; SUVs, small unilamellar vesicles

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three Trp residues [11]. A complicating feature of human GLTP Trp fluorescence is the dramatically different contribution of each Trp to the total emission, with Trp96, Trp142, and Trp85 accounting for 70–75%, 15–20%, and 5–10% of the signal [12]. The situation is made more complex by the so-called 'signature' Trp fluorescence response triggered in GLTP upon interaction with membranes containing glycolipid [11]; [12]; [13]; [14]. The resulting drop in fluorescence intensity (~40%) and 12–13 nm blue shift in the emission wavelength maximum ($\lambda_{max}$) correlates with glycolipid binding via stacking of the initial ceramide-linked sugar headgroup over Trp96. This positioning of Trp96 in the GSL headgroup recognition center was initially observed by X-ray diffraction [3]; [4]; [5]; [6]. Point mutation to either Phe or Ala supported the importance of Trp96 for proper function of the glycolipid headgroup recognition center [3]. Double mutation of Trp to Phe (homo) in various combinations verified the importance of Trp96 but provided limited insights into the functionality of other Trp residues [13]. A hetero double mutation strategy involving replacement of Trp with Phe and Tyr enabled adequate protein viability to dissect the various functional roles played by each of GLTP's three Trps including the participation of Trp142 in the initial membrane docking event [12]. The importance of Trp142 to GLTP functionality was made dramatically clear by the severely impaired membrane partitioning and loss of glycolipid transfer induced by Trp142-to-Ala point mutation [15]. Yet, mapping of the complete GLTP-fold membrane interaction site remains defined mostly by modeling [5]; [8]; [12]; [16]; [17]; [18]; [19].

The HET-C2 GLTP-fold contains only two Trp residues. Trp208 forms the C-terminus in the HET-C2 GLTP-fold and resides on the protein surface as determined by X-ray diffraction (1.9 Å) [8]. The location and accessibility of Trp208 differ from GLTP Trp85 and Trp142, the latter which participates in the initial events of membrane docking [12]; [15]; [20]. In contrast, Trp109 is structurally homologous with Trp96 in human GLTP/glycolipid complexes suggesting a stacking function that helps orient the ceramide-linked sugar for hydrogen bonding with conserved Asp, Asn, Lys, and His in the glycolipid headgroup recognition center [8]. In the present study, we provide the first evaluation of Trp functionality in the fungal GLTP-fold using point mutation approaches. Our study provides evidence for: i) Trp109 playing a key role in the binding of glycolipid as well as enhancing HET-C2 partitioning to the POPC membranes; ii) Phe149, which replaces Trp in some other GLTP orthologs, regulating membrane interaction needed for efficient and rapid transfer of simple uncharged GSLs; iii) W208 playing a minimal role regulating the transfer activity of monoglycosylceramides (MonoGlycCer) and membrane partitioning of HET-C2.

## 1. Experimental procedures

### 1.1. Expression and purification of wild type HET-C2 and mutants of HET-C2

The *P. anserina* ORF encoding HET-C2 (NCBI GenBank # U05236) was subcloned into pET-30 Xa/LIC (Novagen) by Ligation Independent Cloning [8]. HET-C2 mutants (W208F, W208A, F149Y, F149A, H101A, W109Y and W109Y-F149Y) were produced by QuikChange mutagenesis (Stratagene, La Jolla, CA) and confirmed by sequencing. Mutant and wild-type constructs (pET-30 Xa/LIC; Novagen) were transformed into BL21 cells, grown in Luria-Bertani medium at 37 °C, induced with 0.1 mM IPTG, and then grown 16–20 h at 15 °C. Soluble protein from lysate was isolated by Ni-NTA affinity chromatography. Final purification was accomplished by FPLC SEC using a HiLoad 16/60 Superdex-75 prep grade column (Amersham). Protein purity was verified by SDS-PAGE.

### 1.2. Glycolipid transfer of HET-C2

Radiolabeled glycolipid transfer between vesicles was measured at 37 °C by incubating with HET-C2 (0.2–0.5 μg) with donor vesicles [1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) + 10 mol% dipalmitoyl phosphatidic acid] containing [$^3$H]-GalCer (2 mol%), a trace of nontransferable [$^{14}$C]-tripalmitin and ten-fold excess of POPC acceptor vesicles. After recovery of the acceptor vesicles by passage of the mixture over DEAE-Sepharose minicolumns, glycolipid transfer was quantified by liquid scintillation counting [21].

### 1.3. Preparation of vesicles

Lipid mixtures, dissolved in dichloromethane, were dried under a gentle stream of nitrogen in a glass test tube. Final traces of solvent were removed by vacuum desiccation for > 3 h. The dried lipid film was hydrated by vortexing for 5 min with 10 mM phosphate buffered saline (pH 7.4). Small unilamellar vesicles (SUVs) were prepared by intermittent probe sonication of the lipid suspension for about 30–45 min at room temperature. Residual multilamellar vesicles and titanium probe particles were removed by centrifugation at 100,000*g* for 90 min. Analysis by size exclusion chromatography confirmed average diameters of ~25–30 nm for SUVs [22].

### 1.4. Fluorescence measurements

Trp fluorescence was measured at 25 °C from 310 to 420 nm with a SPEX FluoroMax steady state fluorimeter (Horiba Scientific) using excitation and emission band passes of 5 nm while exciting at 295 nm. Protein concentration was kept at $A_{295} < 0.1$ to avoid inner filter effects [14]. For membrane interaction studies, the Trp emission signals of wtHET-C2 and mutants (1 μM) were measured before and after addition of increasing amounts of POPC vesicles lacking or containing glycolipid (20 mol%). HET-C2 binding of glycolipid also was assessed by titration-microinjection of glycolipids (or other lipids) dissolved in ethanol [14]. Measurements were performed under constant stirring adding small aliquots (1 μl) of GSL, dissolved in ethanol (0.1 mM), to protein (1 μM; 2.5 ml).

#### 1.4.1. Binding/partitioning coefficient analyses

Because the Trp emission peak undergoes a dramatic $\lambda_{max}$ blue-shift (355 to ~348 nm) upon glycolipid binding, intensities at 353 nm were used to evaluate binding isotherms to avoid problems discussed by [23]. The fraction of binding sites (α) occupied by glycolipids was calculated by Eq. (1):

$$\alpha = (F - F_o)/F_{max} \quad (1)$$

where $F_o$ and $F$ are the Trp emission intensities of GLTP in the absence and presence of glycolipid, respectively, and $F_{max}$ is the emission intensity of the fully liganded GLTP, i.e. at excess glycolipid [24]; [25]. $F_{max}$ was determined by plotting $1/(F - F_o)$ vs. $1/L$ and extrapolating $1/L = 0$, where $L$ equals the total glycolipid concentration. $\Delta F_m$ (maximum fluorescence change when the protein is completely saturated with glycolipid) was determined by plotting $1/L$ (glycolipid concentration) and $1/\Delta F$ (decrease in fluorescence intensity). The bound glycolipid concentration was calculated using the relationship:

$$[\text{Bound Lipid}] = -\text{protein concentration} \times \Delta F/\Delta F_m \quad (2)$$

The free lipid concentration was calculated as:

$$[\text{Free lipid}] = [\text{Total lipid}] - [\text{Bound lipid}] \quad (3)$$

$K_d$ values shown in Table 3 were determined by nonlinear least-squares (NLLSQ) fitting of bound lipid vs. free lipid. NLLSQ and regression analyses and data simulations were performed using OriginPro 7.0 software (MicroCal, Inc., Northampton, MA) and Prism 5 (GraphPad Software, Inc. La Jolla, CA) to avoid biases associated with linear transformations, i.e. Scatchard analysis. Our previous mass spectroscopy analyses of HET-C2:glycolipid complexes indicate binding of one glycolipid per protein [8].