A TOXIN-BASED PROBE REVEALS CYTOPLASMIC EXPOSURE OF GOLGI SPHINGOMYELIN

SUPPLEMENTAL DATA

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SUPPLEMENTAL METHODS

Expression and isolation of EqtII fused to GFP. The EqtII-GFP was constructed as a His_6 fusion protein, which contains an N-terminal hexa-histidine tag and the thrombin cleavage site. His_6-EqtII-GFP was expressed in an *E. coli* Rosseta (DE3) pLysS strain. 20 ml of overnight culture were used to inoculate 500 ml of M9-LB medium with 100 μg/ml of ampicilin and 20 μg/ml of cloramphenicol. Cells were cultured in an enriched M9-LB medium at 37 ºC to OD_{600}=0.6, then at 25ºC to OD_{600}= 0.8. Expression of EqtII-GFP was induced by addition of 0.25 mM isopropyl-β-D-thiogalactoside (IPTG) and a further shaking at 22ºC for 20 h. After 20 h cells were spun down and the pellets were frozen. Cells were thawed into buffer A (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0) with 10 mM imidazole, 20 mM β-mercaptoethanol, 1 mg/ ml lysozyme, 10 μg/ml DNase, 20 μg/ml RNase, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine (2 ml of lysis buffer per gram dry weight of bacteria). They were shaken for 45 min at 4 ºC with occasional vigorous shaking. Bacteria were sonicated on ice for 5 × 1 min with the amplitude 35% (Ultrasonic Processor VCX 750, Sonics&Materials, USA). Broken cells were centrifuged for 30 min at 4 °C at 15.500 rpm (Sigma 3K30 centrifuge). The supernatant was stored at 4 °C, and the pellet was washed with the same buffer (1 ml of buffer/g dry weight of bacteria). Shaking for 30 min on ice was followed by 3 × 1 min sonication and centrifugation at the same conditions. The supernatant was merged to the first one and pellet was washed again with the same buffer (1 ml of buffer/g dry weight of bacteria), shaken, sonicated and centrifuged at the same conditions. All three pellets were merged and applied to a 3-ml Ni-NTA column (Qiagen, Crawley, UK) equilibrated in buffer A with 10 mM imidazole and 20 mM β-mercaptoethanol. Following serial washing with 10 mM imidazole (3 × 5ml) and 20 mM imidazole in buffer A (4 × 5 ml), the His-tagged fusion protein was eluted with 3 ml 300 mM imidazole in buffer A. Protein was dialyzed against buffer A with 300 mM imidazole and pH 8.2. The fusion protein was further purified by FPLC on a 1 ml HiTrap™ Chelating HP column (Amersham Biosciences). Imidazole was removed by concentrating fractions containing partly purified fusion protein on Amicon ultra-4 (Ultracel-10k, Millipore) membrane and further purified on a Superdex HR75 column (Pharmacia) in a buffer A with 20 mM β-mercaptoethanol and pH 8.2. Fractions containing purified EqtII-GFP were pooled and concentrated again on Amicon ultra-4 membrane. The concentration of the protein was determined from the intensity of the band from SDS-PAGE gel and with bovine serum albumin (BSA) as the standard.

Hemolytic activity

Hemolysis of human or bovine red blood cells was measured at room temperature by using a microplate reader (MRX, Dynex, Germany). Blood was washed several times with 130 mM NaCl, 20 mM Tris-HCl, pH 7.4 (erythrocyte buffer) and proteins were diluted two-fold across the microtiter plate into a final volume of 100 μl of erythrocyte buffer. The same volume of erythrocyte suspension (A_{630}=0.5) was added to each well and hemolysis was monitored at 630 nm for 20 min at room temperature.

SUPPLEMENTAL FIGURES

Fig. S1

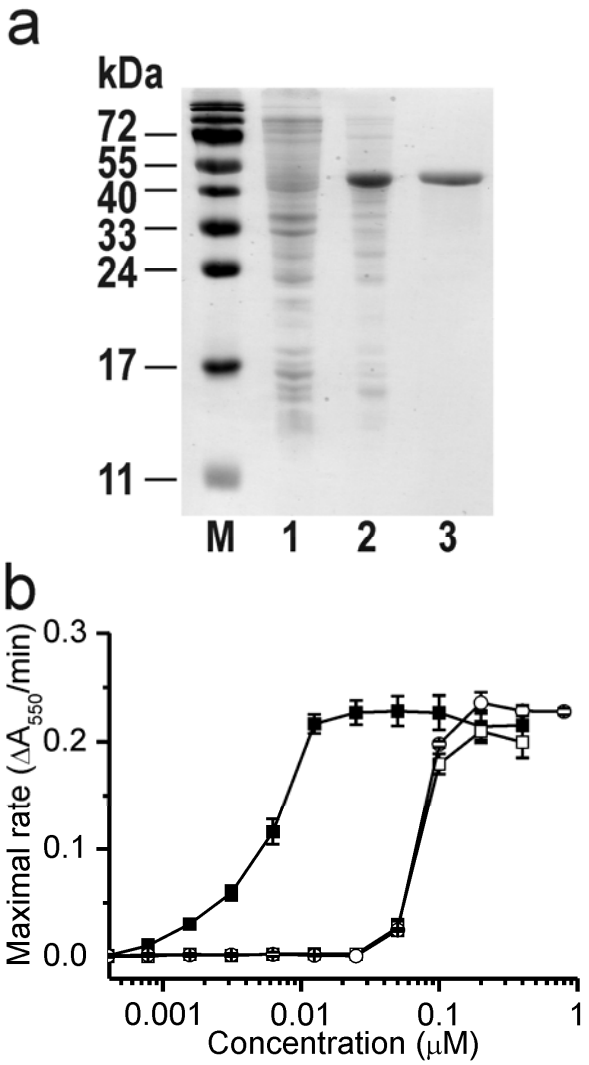

Fig. S1 Purification and hemolytical activity of recombinant EqtII-GFP.
(A) Purification of recombinant EqtII-GFP produced in *E. coli*. M, marker; 1, uninduced bacterial lysate; 2, induced bacterial lysate; 3, isolated EqtII-GFP. (B) Hemolytic activity of the native EqtII (solid squares); EqtII-GFP (open squares) and his-tagged EqtII (open circles). n=2-3; average ± S.D.

Fig. S2

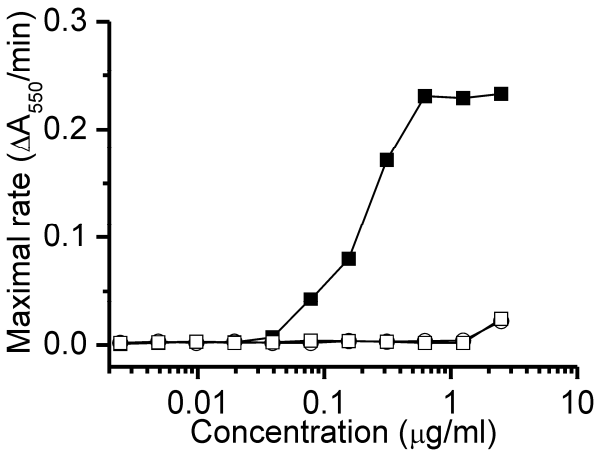

Fig. S2 Hemolytic activity of EqtII 8-69 mutant in different conditions.
Mutant of EqtII, in which residues 8 and 69 were replaced by cysteines, was preincubated in 20 mM Tris-HCl, 130 mM NaCl, pH 7.4 and with 10 mM dithiothreitol to obtain reduced form of the protein (solid squares), in 0.1 mM CuSO4, 0.3 mM 1,10-phenantroline to obtain oxidised form of the protein (open circles) or 13 mM reduced glutathione (open squares) for at least 30 min on ice. The residual activity was measured by a microplate reader as in Supp. Fig. 1B. The average of two independent experiments is presented

Fig. S3

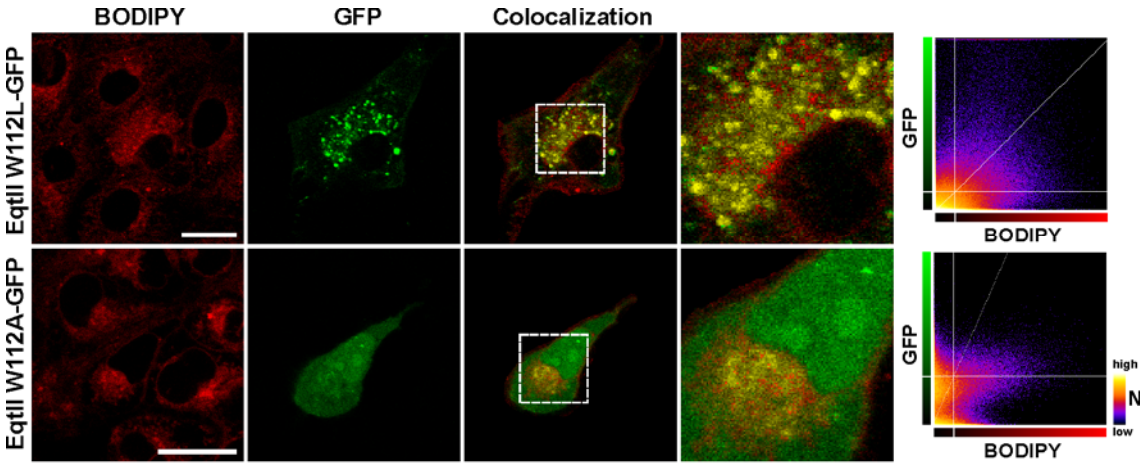

Fig. S3 Co-localization of EqtII W112L-GFP and EqtII W112A-GFP with BODIPY-TR ceramide. The experimental conditions are as in Figure 5. Mander's coefficients for the images presented here are 0.60 and 0.27 for EqtII W112L-GFP and Eqt W112A-GFP, respectively. Scale bar is 20 μm.