Challenges in Tetraspanin Characterisation: Novel mAbs Against The Native Form of Tspan2

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Introduction

The plasma membrane in eukaryotic cells provides a key platform for cellular communications. Many elements cooperate this function, and transmembrane proteins provide a vital role in the association with the extracellular and intracellular signals. In mammals, the tetraspanins are a super-family of 33 transmembrane proteins that act as “membrane organisers”, interacting laterally with other membrane proteins to control biological activity within specialised microdomains (tetraspanin enriched microdomains or TEM). Tetraspanin proteins cross the membrane four times and span the cell surface to form two extracellular loops (EC1 and EC2), one small intracellular loop, and typically short cytoplasmic N- and C-termini. They are involved in many biological pathways in health and disease, but many members are poorly characterised at the protein level, in part due to the lack of specific antibodies that recognise the native proteins. Tspan2, a member of tetraspanin family, may play a role in the nervous system development through its signaling association in the early stages of oligodendrocytes terminal differentiation into myelin-forming glia. Previous studies have reported the involvement of Tspan2 in the cellular motility and invasiveness of the human lung cancer cells. (Hemler 2001; Otsubo et al. 2014)

Experimental Approach

In order to develop mAbs able to bind to the native Tspan2 molecule, the following distinct strategies have been pursued:

- Mouse myeloma (NS0) cell line transfected with the full-length native Tspan2GFP (pCMV6-AC-Tspan2-GFP) was used for immunisation. The use of whole mouse cells should direct the mouse immune response to native form of the protein that localised on the cell surface.
- A rapid initial hybridoma screen was devised using the recombinant version of the Tspan2 EC2 domain expressed as a GST fusion protein, for use in ELISA.

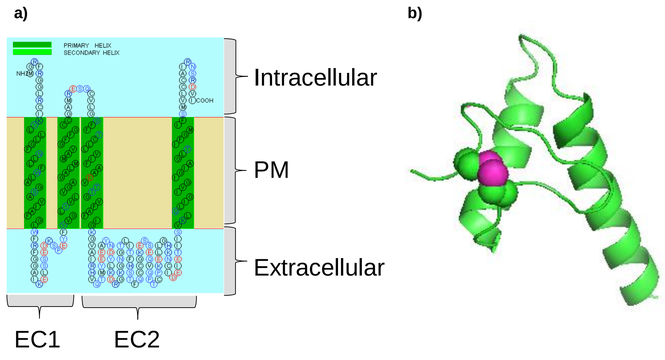

Figure 1. Prediction of Tspan2 secondary structure (a) and 3D structure of Tspan2EC2 (b).

Results

Tspan2EC2 was expressed using the pGEX-KG construct, and Rosetta Gami B(DE3) as an expression system. SDS-PAGE results show that recombinant Tspan2 has a molecular weight of approximately 37kDa with some free GST molecules present at 26kDa MW.

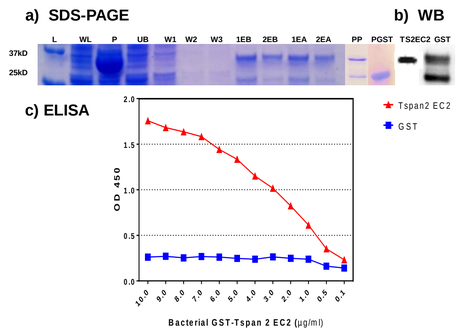

Figure 2. Tspan2EC2 recombinant protein analysis using SDS-PAGE (a). Samples represent different steps through the purification, from the whole lysate to pure Tspan2EC2. The Western blotting analysis (b). Samples were denatured in the presence of β-mercaptoethanol prior to loading. Titration of Tspan2EC2 (0.1-10 µg/ml) using the commercial rabbit anti-human Tspan2 polyclonal Abs (c).

Mouse Myeloma Cell Line (NS0) Transfection

NS0 cells were transfected with the full-length Tspan2GFP (pCMV6-AC-Tspan2-GFP, Origene), using the Lipofectamine 3000 transfection method. The transfection efficiency was tested by FACS (FACSCalibur, BD Biosciences), and the results showed that 50% of the NS0 cell are transfected and express GFP.

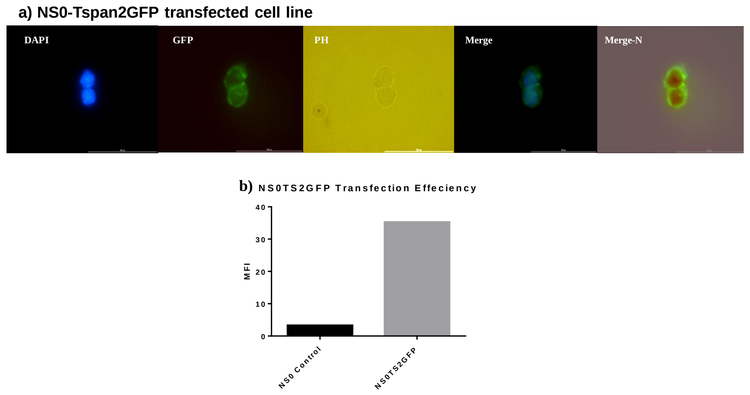

Figure 3. NS0-Tspan2GFP transfected cells. a) Fluorescence Microscopy images (100x objective), including DAPI and GFP channels, also the phase contrast (PH) and the merged images of DAPI+GFP (Merge); and with its negative background (Merge-N). b) illustrates the MFI of the NS0-Tspan2GFP transfected cells comparing with the negative control. *Bar* = 100µm.

Monoclonal Antibody Generation Against The Native Tspan2

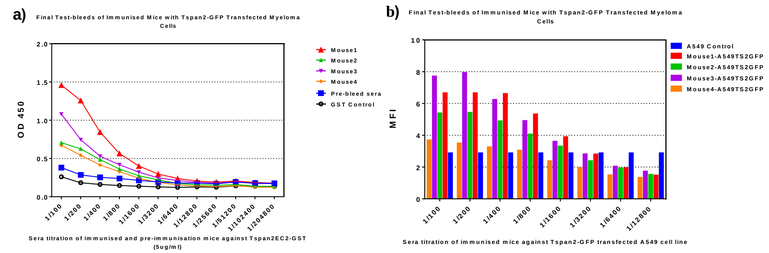

Figure 4. a) Final test-bleeds ELISA test for the mice 1-4. ELISA plates were coated with 5µg/ml recombinant Tspan2-EC2-GST. b) FACS results of mice sera titration against live A549 cells transfected with Tspan2-GFP.

The ELISA results of final test-bleeds indicate that the sera of mouse 1 and 3 showed the higher reactivity against the recombinant Tspan2EC2. Also, the FACS results demonstrated a high Ab titer of the binding with the native Tspan2 overexpressed on A549 cell line. The splenocytes of mouse 1, 3 and 4 have been fused with the mouse myeloma Sp2 cells to generate hybridomas. The hybridoma screen was carried out against the recombinant and the native Tspan2 in ELISA and FACS respectively. The positive and stable hybridomas for secreting anti-Tspan2 were further cloned to obtain a stable single cell colony.

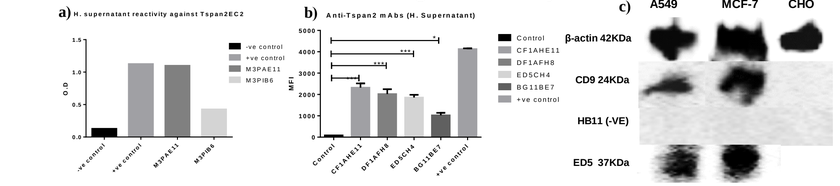

Figure 5. a) Shows the two hybridomas producing IgG mAbs against the Tspan2EC2, which were unstable. b) The MFI of four stable hybridoma supernatant (IgM mAbs) against native Tspan2 on live A549 cells. c) Immunoblotting of ED5 mAbs against A549, MCF-7 and CHO cell lysates. CD9 and ED5 mAbs bind only human tetraspanins, HB11 hybridoma supernatant used as a negative control. The data shown represent the mean ±SEM of three independent experiments (***, * p<0.05 in Dunnett's multiple comparisons test).

Conclusions

- Efficient expression of Tspan2 Large extracellular loop (EC2) using the bacterial system Rosetta Gami B (DE3).
- High transfection efficiency of native tetraspanins in mammalian cells, including NS0, CHO (data not shown), and A549 cell lines.
- The developed mAbs are of IgM isotype, and recognise the recombinant form of the protein in ELISA; the native molecule in WB and FACS assays.

Acknowledgement

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References

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