

Comparison of Anti-Shadoo Antibodies – Where is the endogenous Shadoo protein?

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Abstract—Shadoo protein (Sho) was described in 2003 as the newest member of Prion protein superfamily [1]. Sho has similar structural motifs like prion protein (PrP) that is known for its central role in transmissible spongiform encephalopathies. Although a great number of functions have been proposed, the exact physiological function of PrP is not known yet. Investigation of the function and localization of Sho may help us to understand the function of the Prion protein superfamily.

Analyzing the subcellular localization of YFP-tagged forms of Sho, we detected the protein in the plasma membrane and in the nucleus of various cell lines. To reveal the localization of the endogenous protein we generated antibodies against Shadoo as well as employed commercially available anti-Shadoo antibodies: i) EG62 anti-mouse Shadoo antibody generated by Eurogentec Ltd.; ii) S-12 anti-human Shadoo antibody by Santa Cruz Biotechnology Inc.; iii) R-12 anti-mouse Shadoo antibody by Santa Cruz Biotechnology Inc.; iv) SPRN antibody against human Shadoo by Abgent Inc. We carried out immunocytochemistry on non-transfected HeLa, Zpl 2-1, Zw 3-5, GT1-1, GT1-7 and SHSY5Y cells as well as on YFP-Sho, Sho-YFP, and YFP-GPI transfected HeLa cells. Their specificity (in antibody-peptide competition assay) and co-localization (with the YFP signal) were assessed.

Keywords—Shadoo, prion protein, immunocytochemistry, antibody-peptide competition assay, antibody.

I. INTRODUCTION

SHADOO of the prion protein shares similar motifs and functional analogies with the prion protein. The exact function of the prion protein is not known yet, although it would help to discern the mechanism of pathogenesis of transmissible spongiform encephalopathies, in which the prion protein has a central role. [2] Studying other members of the prion protein superfamily could help in understanding the function of these proteins.

In earlier work, we found that Shadoo might have dual localization as YFP-Sho was observed in the plasma membrane and in the nucleus of several cell lines. Moreover, a nuclear localization signal was identified in Shadoo that induce its nuclear and nucleolar accumulation. Shadoo appears to have a passive nuclear entry; however its nuclear accumulation requires ongoing active processes [3]. To discern the localization of the endogenous Shadoo an immunocytochemistry study is conducted here. These

experiments were facilitated by generating five different antibodies in rabbit and hen and purchasing all three antibodies against Shadoo that was commercially available at the time.

II. MATERIALS AND METHODS

A. Plasmid Construction

To generate YFP-Sho, the ER-targeting signal sequence of mouse Shadoo was cloned into pEYFP-C1 (Clontech) between NheI and AgeI restriction sites, and the mouse Shadoo core sequence and the GPI-signal sequence were cloned into EcoRI and BamHI restriction sites. Used primers: AAGGGCTAGCCACCATGAACTGGACTGCTGCCACG; GGGGGGACCGGTAAGGCGCTACAGCTGTCA; GGCGCGGAATTCTAAGGGCGGCCGCGGAGGC; CGCGCCGGATCCTCATCCACGATCAGGAAGATG).

B. Cell Culture

Cells (HeLa, SH-SY5Y, Zpl2-1, Zw3-5, GT1-1, GT1-7) were grown at 37°C in a humidified atmosphere of 5% CO₂ in high-glucose Dulbecco's Modified Eagle medium (DMEM, Lonza) supplemented with 10% heat inactivated fetal bovine serum (Lonza) and containing 100 units/ml penicillin and 100 µg/ml streptomycin (Lonza).

C. Transfection

Cells were cultured on Labtek II 8 well chambers (Labtek), seeded a day before the transfection at a density of 2x10⁴ cells/well. The next day, at around 50% confluence, cells were transiently transfected with plasmid constructs using Turbofect (Fermentas), briefly as follows: DMEM was changed on the cells to fresh one. Typically 500 ng plasmid and 0,5µl Turbofect was mixed in 50µl serum free DMEM and the mixture was incubated for 40 minutes at RT prior adding to cells. Transfection medium was changed to fresh supplemented DMEM after 3 hours of incubation.

D. Used Antibodies

We have generated five different antibodies in hen and rabbit, however only one of them seemed to show some specificity against Shadoo. This antibody was generated against a C-terminal peptide sequence of mouse Shadoo by Eurogentec Ltd. (EG62). Furthermore we bought all commercially available antibodies, such as S-12 α-Sho antibody (sc-136910) against human Shadoo and R-12 α-Sho antibody (sc-136909) against mouse Shadoo from Santa Cruz Biotechnology Inc., and SPRN antibody (C-term) (AG)

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(AP4754b) against human Shadoo from Abgent Inc. The blocking peptide's sequence was available in the case of EG and AG antibodies. We could determine the sequence of the blocking peptide (sold by Santa Cruz) of R-12 antibody by mass spectrometry. Comparison of the known blocking peptide sequences are summarized on Figure 1.

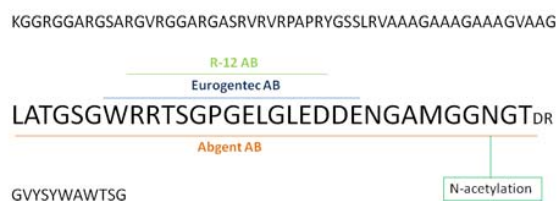


Fig. 1 Targeted Shadoo sequence of the analyzed anti-Shadoo antibodies

E. Immunocytochemistry

Cells were fixed using 4% paraformaldehyde in PBS for 10 minutes. Blocking and permeabilizing mixture contains Triton-X 100 and BSA. Primary antibodies were used in 1:100 dilution, secondary antibody was used in 1:400 dilution (DyLight 649 anti-rabbit). Nuclei were stained by Hoechst 33342 (5 μ M) (Invitrogen). Antibody-peptide competition assay: 50-5000 fold excess of peptide was incubated with primary antibodies for 1 hour at room temperature before immunocytochemistry staining.

F. Confocal Microscopy

Confocal laser scanning microscopy was performed using an Olympus FV1000 confocal laser scanning microscope (Olympus Life Science Europe GmbH, Hamburg, Germany). Picture capturing and analysis were performed using Fluoroview software (Olympus).

III. RESULTS

A. Localization Pattern by Different Anti-Shadoo Antibodies

To visualize the endogenous Shadoo we performed immunocytochemistry staining on different cell lines (human: HeLa, SH-SY5Y, mouse: GT1-1, GT1-7, Zpl2-1, Zw3-5). The EG and S-12 antibodies have a profound nuclear staining on all investigated cell lines (Fig. 2). By contrast the pattern of the R-12 and AG antibody staining varies among the investigated cell lines. R-12 antibody shows nuclear staining on HeLa cells and some kind of fibril-like staining on Zpl2-1, Zw3-5, GT1-7 and SH-SY5Y. R-12 antibody did not show structure specific staining on GT1-1 cells. AG antibody which was raised against a human Shadoo fragment, shows nuclear staining on the human cell lines examined (Fig. 2). However its staining on the mouse cell lines was not clear. For example very faint nuclear staining could be observed on Zpl2-1 cells in G0 phase, however spindle-like structures are stained in dividing cells (Fig. 2).

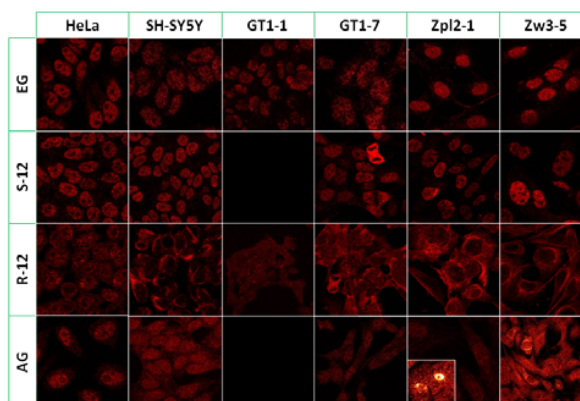


Fig. 2 Cell panel of the analyzed anti-Shadoo antibodies

B. Antibody Staining on YFP-Sho transfected HeLa Cells

In order to check the specificity of the above mentioned antibodies we investigated the co-localization of the antibody staining with YFP fluorescence on YFP-Sho transfected HeLa cells. EG and R-12 antibodies gave plasma membrane and ER/Golgi-like vesicles staining on YFP-Sho transfected HeLa cells. Although these antibodies showed nuclear staining on HeLa cells, the intensity of this staining does not seem to increase when YFP-Sho was detected in the nucleus. S-12 antibody staining did not change in YFP-Sho transfected HeLa cells, this antibody showed similar nuclear staining as on untransfected HeLa cells. The staining did not co-localize the plasma membrane and ER/Golgi-like signals either. AG antibody gave plasma membrane, ER/Golgi-like vesicles and increased nuclear staining in HeLa cells transfected by YFP-Sho (Fig. 3). Data not shown in the case of Sho-YFP and YFP-GPI.

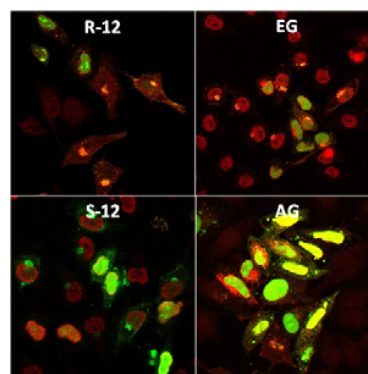


Fig. 3 Merged images of YFP-Sho transfected HeLa cells by different antibodies (green = YFP; red = antibody)

C. Antibody-Peptide Competition Assay

Testing whether the immunocytochemistry staining are specific (i.e. whether the antibody binds by its paratope) antibody-peptide competition assays were carried out on HeLa cells. In order to assess if these antibodies are independent (i.e. they recognize different epitopes) all of the antibodies were also pre-incubated with the blocking peptides related to the other antibodies.

The EG peptide was able to compete the binding of its own antibody and of the S-12 anti-Sho antibody. The R-12 peptide was able to decrease the intensity of the S-12 antibody staining. By contrast its own antibody required a vast amount of peptide (5000 fold excess). The S-12 peptide was able to block only the binding of its own antibody. The AG peptide was tested only against its own antibody and required 500 fold excess for successful competition. The S-12 blocking peptide was able to decrease the intensity only of the S-12 antibody staining. As three of the blocking peptides as well unrelated control peptides were able to compete the S-12 antibody binding, we could not prove the specificity of S-12 antibody in these experiments (Table I).

TABLE I
BLOCKING EFFICIENCY OF THE DIFFERENT ANTI-SHADOO PEPTIDES

	EG peptide	S-12 peptide	R-12 peptide	AG peptide
EG	200x	-	-	
S-12	200x	50x	200x	
R-12	-	-	5000x	
AG	-	-	-	500x

IV. CONCLUSION

Taken together the results from the experiments on transfected cells and from the antibody-peptide competition assay, they do not support the claim that the S-12 antibody is an anti-Shadoo antibody, since the staining does not co-localize with YFP-Sho. R-12 and EG antibodies recognized the transfected Shadoo fusion protein in the ER/Golgi-like vesicles and in the plasma membrane. However in the case of R-12 antibody the antibody-peptide competition assay was unsuccessful indicating that the nuclear staining of R12 is unspecific. The AG antibody seems to be specific to Shadoo as it showed the best co-localization with the YFP-Sho signal in transfected HeLa cells. The epitope of EG and AG antibodies are independent from each other and both of them showed nuclear staining; however the cross-reactions seen on Western blot (data not shown) prevent us from drawing a definite conclusion about Shadoo's nuclear localization (Table II).

TABLE II
COMPARISON OF DIFFERENT ANTI-SHADOO ANTIBODIES

	Cell panel	YFP-Sho co-localization	Competition
EG	nuclear	plasma membrane, ER/Golgi-like	EG peptide ✓
R-12	HeLa: nuclear others: fibril-like	plasma membrane, ER/Golgi-like	X
S-12	nuclear	X	S-12, R-12, EG peptide ✓
AG	human: nuclear others:	plasma membrane, ER/Golgi-like, nuclear	AG peptide ✓

ABBREVIATIONS

Sho: Shadoo, PrP: prion protein, ER: endoplasmic reticulum

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REFERENCES

- [1] M. Premzl, Shadoo, a new protein highly conserved from fish to mammals with similarity to prion protein, *Gene*, 2003, 314:89-102
- [2] B. Caughey, Protofibrils, pores, fibrils, and neurodegeneration: separating the responsible protein aggregates from the innocent bystanders, *Annu Rev Neurosci*, 2003, 26:267-298
- [3] E. Tóth, The highly-conserved, N-terminal (RXXX)₈ motif of mouse Shadoo mediates nuclear accumulation, *BBA Mol Cell Res*, submitted for publication