Immunoaffinity-Purification and Identification of Teleost Thy-1

Sören-Oliver Deininger[1], Michael Przybylski[2], Alexander Reuter[3] and Claudia A. O. Stuermer[3]
[1]Bruker Daltonik GmbH, Leipzig, Germany, [2]Department of Chemistry, University of Konstanz, Konstanz, Germany, [3]Department of Biology, Neurobiology, University of Konstanz, Konstanz, Germany

Introduction

Goldfishes are able, in contrast to higher vertebrates, to regenerate injuries in their central nervous system(CNS). Regeneration in the CNS of goldfishes has been widely studied. The visual system of the goldfish has been of particular interest, since after transection of the optic nerve pathfinding errors of the regenerating axons in the retina can be easily observed. Further, it is possible to study the effect of drugs or proteins on the axonal pathfinding by injetiong them into the eye.

Monoclonal antibodies have been created by immunizing mice with liposomes generated from regenerating optic nerves. The resulting clones were screened on retina wholemounts [1].

The protein that is recognized by mAb M802 is unique, because this protein is not present on embryonal developing axons. It only appears on developing axons in the retina after the fish has reached a size of approx. 1.2 cm and disappears some time after the axons have reached their destination in the optic tectum.

After transection of the optic nerve, however, all axons (including the early axons, that have never expressed the M802-Antigen), start to express the M802-Antigen. It has therefore been suspected, that the M802-Antigen has a specific role in the regeneration of the Goldfish CNS [1].

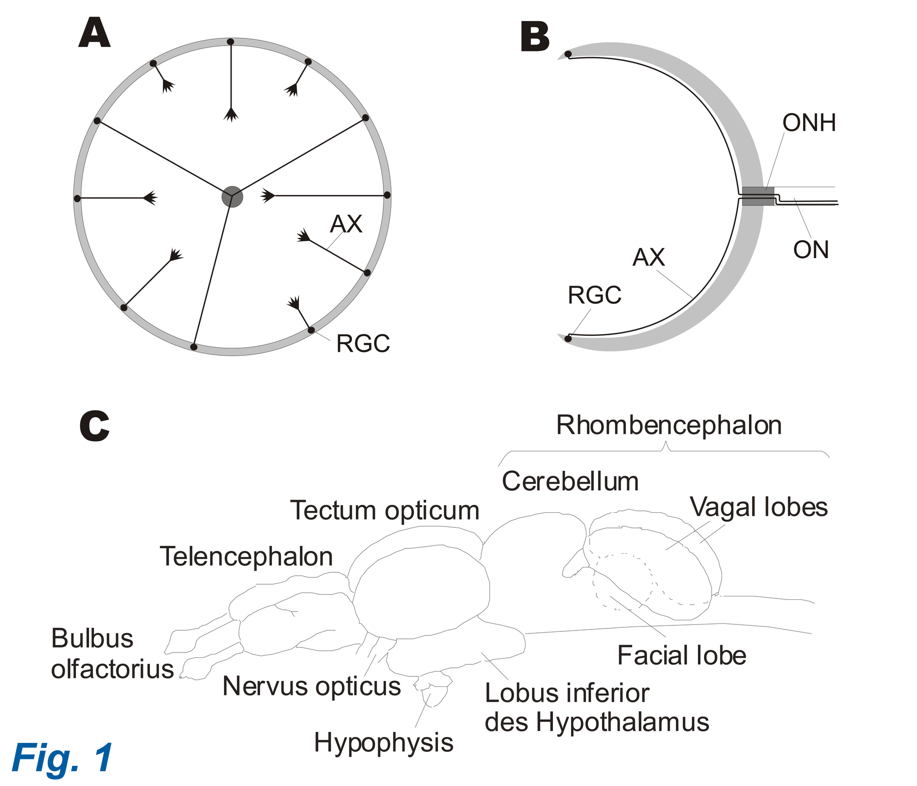


Fig. 1
A) and B) Schematic view of the pathfinding of axons in the retina from top (A) and in cross-section (B) Retinal ganglion cells (RGC) send the axons (AX) radial to the optic nerve head (ONH) and further into the optic nerve
C) The goldfish brain

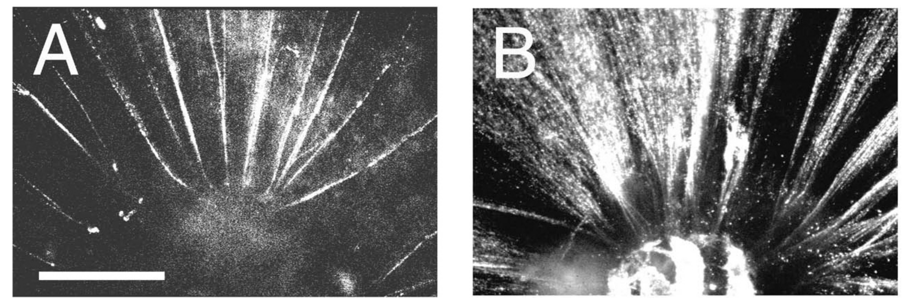


Fig. 2 Unfixed retinal explants of adult goldfish retina immunostained with mAb M802
A) young axons are stained
B) after transection of the optic nerve, all axons regenerate and are stained

Besides in young adult or regenerating axons, the M802 antigen can also be found in low concentrations in the vagal lobes of the goldfish brain and in the fibroblast-like goldfish cell line CAR

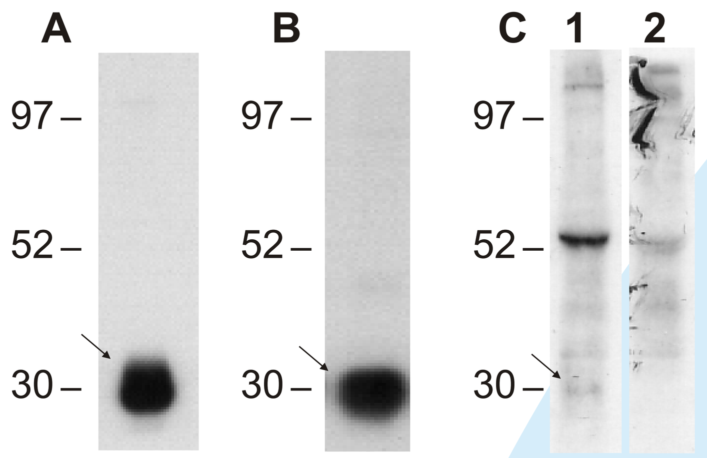


Fig. 3 Immunoblot analysis with mAb M802
A) Membranes from regenerating axons
B)CAR-cell lysate
C) 1: Membranes from vagal lobes
2:Control with secondary antibody only

The CAR cells grow very slow and optic nerve transection of goldfishes is a laborous animal experiment, therefore the optimization of the immunoaffinity-purification was initially done with vagallobe-membrane-lysate in TBS, 1% Triton X-100(TX-100). Mab 802 was coupled to NHS-activated sepharose with 2µg antibody per 10µl sepharose. The purification was done with 10µl mAb-Sepharose in a microcolumn

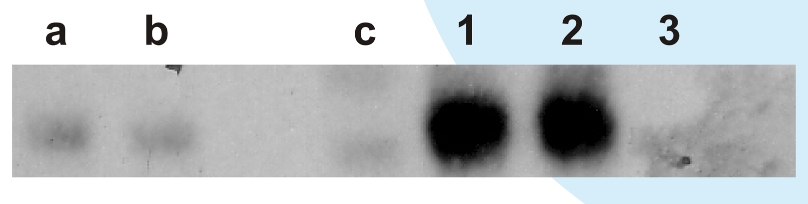


Fig. 4 Immunoblot of affinity-purification of vagallobe membrane lysate a)Lysate before purification b)Lysate after purification (10 µl) c)mAb-Sepharose after elution. 1)-3) elution fractions (0.25µl)

With 1% TX-100 in the lysis buffer, the M802 antigen could be significantly enriched in the elution fractions from the affinity columns, however the concentration of the antigen after the affinity-column did not appear to be decreased (Fig. 4)

The lysis buffer was changed to contain 1% TX-100, 0.5 % deoxycholate, 0.1% SDS (RIPA-Buffer). With this lysis buffer, it was possible to achieve complete binding of the antigen to the column (Fig. 5). This may indicate, that the protein is contained in lipid rafts which are too large to enter the sepharose beads, and which are not dissolved in TX-100 but in RIPA-buffer.

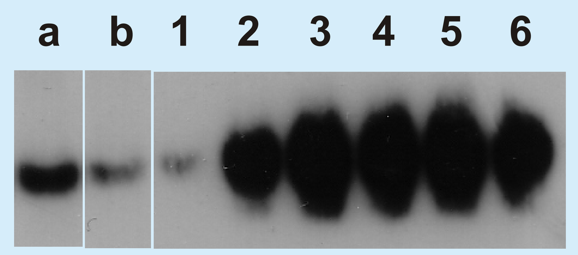


Fig. 5 Immunoaffinity-purification of vagallobe membrane lysate in RIPA-buffer a)Lysate before purification(10 µl) b)Lysate after purification(10 µl) 1)-6) elution fractions,(0.25 µl)

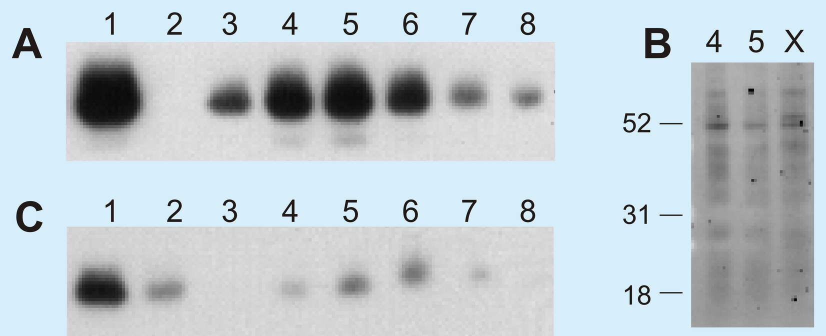


Fig. 6
A) Immunoblot of affinity-purification of CAR-cell lysate 1) Lysate before affinity column(10µl) 2) Lysate after affinity column (10µl) 3)-8) elution fractions (0.25µl) B) SDS-Page of fractions 4 and 5; X: control with nonspecific antibody C)Immunoblot of affinity-purification of CAR-cell lysate after extensive washing on column 1) Lysate before affinity column(10µl) 2) Lysate after affinity column (10µl) 3)-8) elution fractions (0.25µl)

The affinity purification of CAR-cell lysate showed complete binding of the antigen (Fig 6A). The SDS-PAGE analysis of the eluates showed nonspecific background, but no antigen band could be observed (Fig 6B). To reduce the nonspecifically bound proteins in the eluate, the volume to wash the antibody column was increased from 10x to 50x the column volume. During this extensive washing the M802-antigen was washed off the affinity column (Fig. 6C), indicating a low affinity

Because a significant amount of antigen was washed off the column during the affinity purification, a batch-protocol was used, in which the binding and washing was done with mAb-sepharose in microreaction tubes, buffer exchanges were done by spinning down the sepharose and pipetting off the supernatant. To yield low volumes of the eluate, the beads were transfered to a column for elution

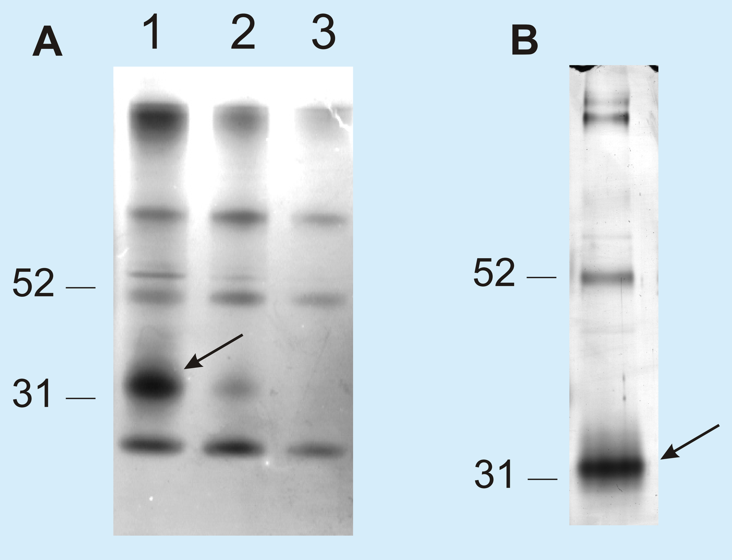
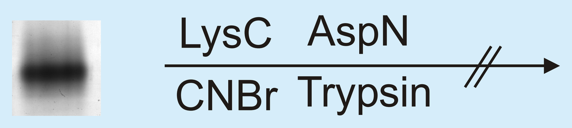


Fig. 7 SDS-PAGE analysis of eluates after affinity purification A) from vagallobe membrane lysate 1)-3) consecutive elution fractions B) from CAR-cell lysate, antigen containing fractions were pooled

After affinity-purification and elution, the M802-antigen could be obtained as band in SDS-PAGE. From 20 ml of CAR-cell lysate with 80 µl of mAb Sepharose with 10 µg mAb per µl sepharose, 1.2µg of M802-antigen were obtained.

In Gel Digestion of M802 antigen



The M802 antigen was essentially resistant to proteolysis. Neither digestion with Trypsin, Lys-C, Asp-N or CNBr yielded sufficient peptides to obtain sequence information and identification of the unknown antigen.

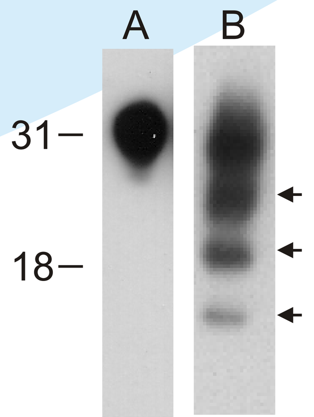


Fig. 8 Immunoblot of M802 antigen A) before and B) after enzymatic deglycosylation with PNGase-F in solution. After deglycosylation three additional bands appear, thus the M802-antigen contains at least three N-linked glycan chains, which contribute 50% to the apparent molecular weight

The high amount of glycosylation of the M802-antigen leads to a high resistance to proteolysis. The enzymatic deglycosylation led to a small fraction of deglycoylated protein, but most of the protein remained intact (Fig. 8). To overcome this limitation, a in-gel-deglycosylation was performed

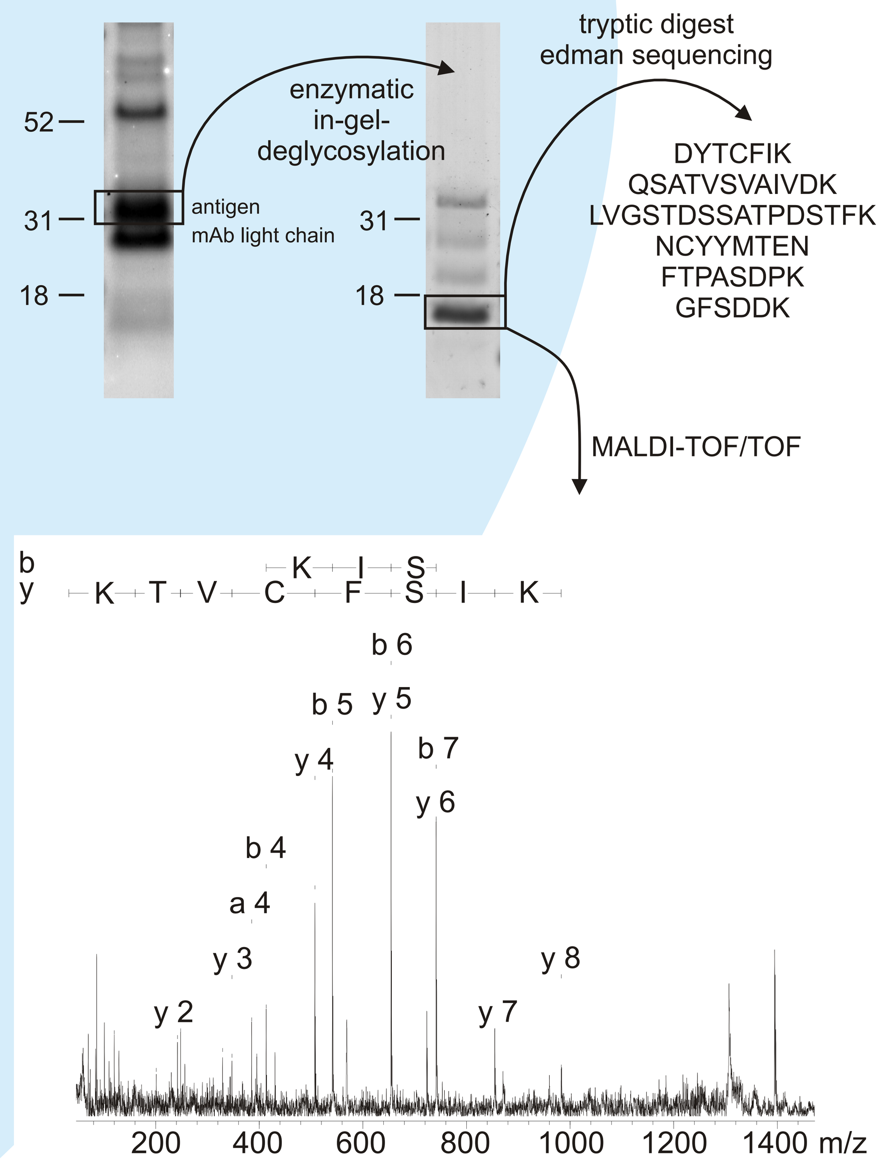


Fig 9 In-gel-deglycosylation of M802 antigen, subsequent reelectrophoresis, Edman-sequencing [2] and MALDI-TOF/TOF de-novo-sequencing. The mass spectrum was recorded on a Ultraflex-TOF/TOF, de-novo-sequencing was done with the RapiDeNovo-Software (Bruker Daltonics)

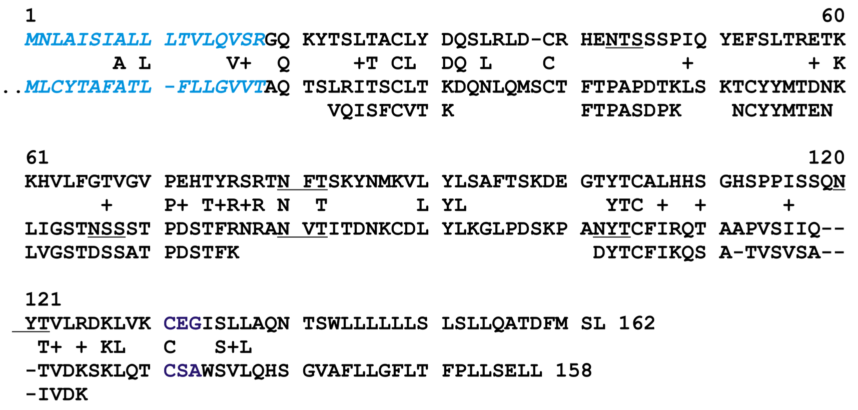


Fig 10 Alignment of human (Hs) and zebrafish (Dr) Thy-1 precursor protein and the sequences obtained from M802 antigen (Ca). Potential glycosylation sites are underlined

A BLAST-search with the obtained sequences resulted in several zebrafish EST-Tags homologue to mammlian Thy-1. With this information it was possible to clone the zebrafish Thy-1 sequence (Fig 9)[2]

Conclusion

Enzymatic in-gel-deglycosylation is a possibility to obtain sequence information from highly glycosylated proteins that are otherwise resistant to proteolysis.

The M802 antigen could be identified as teleost Thy-1. Although long known in mammals, its function is not yet understood. There are significant differences between mammal and teleost Thy-1. The former is a high abundant protein in brain, is expressed after neurons have reached their destination and remains on the surface. The latter is low abundant in brain, and appears only on young axons. The identification of teleost Thy-1 may therefore lead to a more detailed understanding of the function of this protein

Methods

Immunoaffinity purification of M802 antigen
CAR-Cells and Membranes from Vagallobes and Retina were lysed in TBS, 1% Triton X-100 (TX-100), 0.5% Deoxycholate, 0.1% SDS, 1 mg/ml BSA.
mAB M802 was coupled to NHS-activated Sepharose 4B Phast Flow Beads (Amersham Pharmacia Biotec) at a concentration of 10µg antibody/µl beads. The Lysate was incubated with mouse IgG Beads (Sigma, 10µl beads / 200µl lysis buffer) 4° C for 4 h. The supernatant was recovered, M802 antibody beads were added and incubated overnight at 4°C. The supernatant was removed and beads were subsequently washed with lysis buffer, TBS buffer + 1% TX-100, TBS buffer with 900 mM NaCl + 1% TX-100, TBS buffer + 1% TX-100 and preelution buffer (TBS + 0.2% dodecylmaltoside). Beads were transferred to a column and eluted dropwise with elution buffer (0.2% dodecylmaltoside in 0.1% TFA). Antigen containing fractions were identified by dot-blotting and probing with mAB M802.
In Gel Deglycosylation. M802 antigen was lyophilized and redissolved in SDS sample buffer for alkylation (0.4 M Tris, 1% SDS, 10 mM EDTA, Orange G, 26 mM DTT, pH 8.6) and incubated at 96 °C for 5 min. After cooling down, 1/10 vol of a 20% (w/v) iodoacetamide solution was added and incubated at room temperature for 30 min. The sample was run on a NuPAGE 4-12% gradient gel (Invitrogen) and stained with colloidal Coomassie blue. The M802 band was excised, destained, washed with water, shrunk by addition of 70% acetonitrile and dried. 50 µl (2.5 Units) of PNGaseF solution (Sigma) was dialyzed against PBS with 20 mM EDTA and 1% n-octylglycoside. The gel pieces were rehydrated with the PNGaseF solution and incubated overnight at 37° C. After washing with water, the slices were dehydrated by addition of acetonitrile, dried, rehydrated with Novex NuPAGE Sample Buffer (Invitrogen) and homogenized in an Eppendorf tube. The slurry was transferred to a NuPAGE 4-12% Gel and reelectrophoresed.

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