Supporting Online Material for

Molecular Basis for the Nerve Dependence of Limb Regeneration in an Adult Vertebrate

Anoop Kumar, James W. Godwin, Phillip B. Gates, A. Acely Garza-Garcia, Jeremy P. Brockes*

*To whom correspondence should be addressed. E-mail: j.brockes@ucl.ac.uk

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This PDF file includes:

Materials and Methods
Figs. S1 to S5
Table S1
References
Supporting Online Material

Materials and Methods

Yeast two hybrid screen
The yeast two hybrid screen was performed using the duplex-A yeast system (Origene Technologies, Rockville). The Prod 1 sequence coding for the 68 amino acid protein without signal sequences was cloned into pEG202-NLS to give a protein with an N-terminal LexA sequence fused to Prod 1, which bound LexA operators but did not autoactivate reporter genes. We screened 2 million clones from a normal newt forelimb cDNA library constructed in pJG4-5 as well as 1.5 million from a newt blastema library. 127 clones which were both leu positive and X-Gal positive on galactose plates, and leu negative and X-Gal negative on glucose plates were sequenced. The majority of these clones were false positives identified by other investigators (see http://www.fccc.edu/research/labs/golemis/InteractinTrapInWork.html). Four of the clones were identified as orthologs of the Xenopus protein XAG2, and named newt Anterior Gradient (nAG). One other secreted protein was identified as positive, a member of the resistin family. The largest nAG clone, missing 36 amino acids at the N terminus was found to interact with Prod 1 and not with two control constructs, pRHFM and pBAIT, supplied by Origene. A full length clone was obtained by extension from normal limb cDNA and cloned into the expression vector pCINeo (Promega). The sequence of nAG is deposited in GenBank, accession number EF667357.

Retrival of sequences, multiple alignment and phylogenetic analysis
Protein sequences related to nAG were retrieved from the non-redundant NCBI database, the Ambystoma EST Database (1), and the Axolotl EST Database (2) using blastp or tblastn (3). The sequences from the Ensembl (4) family ENSF00000005359 were also incorporated in the analysis.

An initial multiple sequence alignment of all sequences was constructed using MAFFT 5.8 E-INS-I (5). Redundant (>99% identity), fragmentary, and non-metazoan sequences were then identified and removed. A preliminary Neighbour-Joining phylogenetic analysis, as implemented in MAFFT, unambiguously highlighted (bootstrap value of 100) a subset of the sequences that were closely related to the endoplasmic reticulum protein ERp19 (6). Most of the sequences of this ERp19 family were removed to reduce the computational cost of subsequent analyses.
A new multiple sequence alignment was computed for the 47 sequences selected for inclusion in the final analysis. Bayesian inference as implemented in the program MrBayes 3.1.2 (7) was used to compute the probable phylogenetic relationship between the sequences. The program was run with default parameters using a mixture of models for amino acid substitution. Calculations were run for 1,000,000 generations, with tree sampling every 100 generations. At the completion of the run, the average standard deviation of split frequencies (a measure to determine if stationarity has been reached) had an acceptable value of ~0.04. The first 25% of trees were discarded, and the remaining trees were used to compute the clade credibility (posterior probability, in the case of Bayesian analysis) values.

**Protein interaction assays**
The 68 amino acid Prod 1 sequence was cloned into pET15-b (Novagene) and grown in BL21DE3 Origami (Novagene). After induction with IPTG, the Prod 1 protein with N-terminal his tag was purified by Ni chromatography. N-terminal his tag and C terminal myc tag versions of nAG (160 amino acids) and CTGF (373 amino acids) were also prepared. The purified proteins were incubated, generally at 0.5 µg/ml, in binding buffer (PBS pH 7.4, 50 mM dithiothreitol, 0.1% Tween 20, 0.25 mg/ml BSA) for 3 hr at 4 ºC. We added 20 µl anti mouse IgG magnospheres (Dynal) prebound to monoclonal mouse anti-myc (Sigma) and rotated for 2 hr at 4 ºC. The beads were isolated with a magnet and washed 4 times with PBS with 0.1% Tween 20, and eluted by boiling in SDS sample buffer with 50 mM dithiothreitol. Aliquots were run on a 12% SDS gel (Invitrogen) and electroblotted onto nitrocellulose. The membranes were first probed with mouse anti-myc and donkey anti-mouse IgG labelled with IR700 (Lycor), then with rabbit anti Prod 1 (reagent 683 from Morais da silva et al) and goat anti-rabbit IgG-IR 800 (Lycor). The membranes were scanned with the Odyssey Imaging Scanner (Lycor) and are presented as anti Myc or anti Prod 1 (Fig 1C). Although the immunoreactivity of the CTGF band is lower than that of the nAG band, the protein amounts were equal.

For assay of the interaction of proteins expressed in mammalian cells, Cos 7 cells were grown to confluence and transfected, using Lipofectamine (Invitrogen), with a plasmid expressing nAG with a C terminal myc tag. After 48 hr the medium was changed to OptiMEM (Gibco) without
serum. After 48 hr further culture, the medium was harvested, and incubated for 30 min at room temperature with live mouse PS cells (8) transfected 4 days earlier with a plasmid encoding Prod 1 with a N terminal flag tag. After washing in Hepses buffered DMEM with 1% BSA (HB buffer), the cells were reacted with monoclonal anti-myc, or anti-flag (to determine expression on the surface of Prod 1) for 1 hr at 4 ºC, followed by phosphatase labelled rabbit anti-mouse IgG for 30 min at 4 ºC. Cells were washed in HB with levamisole (Vectastain) to inhibit non-specific phosphatase and developed in the standard phosphatase substrate BCIP/NBT.

Animal methods

Adult newts (Notophthalmus viridescens) were obtained from Charles D. Sullivan and Co. (Tennessee). After surgery, animals were maintained at a temperature of 24 ºC. Animal husbandry, amputation and denervation methods were as described elsewhere (9, 10). For electroporation of adult limbs, newts were anesthetised in 0.1% tricaine, and the amputated limb was passed through a hole in a rubber jacket so as to insulate the rest of the body from the current flow, and held with the electrodes in a recessed chamber under amphibian saline. We used customised platinum disc electrodes modelled on those described (11). The stump was injected under the wound epithelium with 1µl of DNA solution (5mg/ml) through a glass needle with a 0.2µm diameter tip, using a Picospritzer III (Intracel, Herts, UK) with 5 msec pulse length. The DNA was electroporated with 5 pulses (frequency 200 PPS, duration 100 msec, voltage 100 V/cm) using a SD9 electroporation device (ETL, Cortland, New York, USA). The animals were allowed to recover in sterile water with 0.5% sulfamerazine for 12-24 hrs. The image of RFP expression (Fig 4A) was obtained with a stereofluorescence (Leica) microscope where the brightfield image has been merged with the fluorescent one.

Antibodies and immunohistochemistry

Rabbits were immunised (Eurogentec, Belgium) with KLH conjugated to peptides for amino acids 31-45 (reagent 223) or 132-146 (224) of the nAG sequence and the sera were affinity purified by adsorption and elution from the appropriate immobilised peptide (performed by Eurogentec). The antibodies were characterised by immunoblotting extracts of bacteria expressing nAG, or extracts and conditioned medium of Cos 7 cells transfected with the nAG plasmid.
For analysis of expression of nAG and other antigens in sections of newt limbs, limbs were collected and fixed in 4% ice-cold paraformaldehyde for 16-18 hrs at 4 ºC, washed 2× in PBS and embedded in Tissue Tek-II. Longitudinal or cross sections of the limbs were serially sectioned in a cryostat (Leica) at 12 µm. The sections were collected in Superfrost slides and stored at -30 ºC until use.

For nAG staining, the sections were air dried and rehydrated in PBS, then immersed in PBS containing 0.3% Triton X-100 for 15min. The slides were rinsed again in PBS and blocked in PBS with 10% goat serum (10%GS-PBS). Peptide antibodies 223 and 224 were diluted 1:100 in GS-PBS and spun at 20800g for 15min to remove aggregates. Parallel control antibodies were concentration-matched affinity purified rabbit polyclonal antibodies, either anti-adeno associated virus, (Eurogentec) or anti mammalian P110α (a gift from Prof. Peter Shepherd); these were routinely compared with omission of the first layer antibodies. The slides were incubated overnight at 4 ºC, washed 2× in PBS, and incubated for one hr with goat anti-rabbit IgG coupled to Alexa 488 (Molecular Probes-Invitrogen) diluted at 1:1000 in 10% GS-PBS. The sections were washed again (2×) in PBS and mounted in fluorescent mounting medium (Dakocytomation).

For acetylated tubulin staining, the sections were reacted with antibody (Clone 6-11-B-1, Sigma) at a dilution of 1:1000. The secondary antibody was goat anti-mouse IgG coupled to Alexa 488 (Molecular Probes-Invitrogen) at 1:1000 dilution, or class specific (IgG2b, Southern Biotechnology) at 1:500 when co-staining with nAG antibodies.

For HNK (Leu7) staining, antibodies (IgM; refs, 9, 12) were used at a dilution of 1:100. The sections were incubated overnight with the primary antibodies and processed as described previously. Texas red conjugated goat anti-mouse IgM (1:500) was used as the second layer. Muscle-specific myosin heavy chain antibodies (13) were used at 1:500 dilution. The secondary antibodies were goat anti mouse IgG coupled to Alexa 488 as above.
For colocalisation studies the respective primary antibodies were incubated together at 4 ºC overnight, washed extensively and reacted with the second layers sequentially. The nuclei were counterstained with Hoechst 33258 (2 µg/ml).

The sections were observed under a Zeiss Axiskop2 microscope and images were acquired with a Hamamatsu Orca camera using Openlab (Improvision) software. Wherever comparative analysis between control and denervated samples were performed, all images were acquired with identical camera settings and illumination control. Post processing (contrast enhancement) was equally applied to all matched experimental and control samples using Openlab software routines.

The triple-stained samples were observed under a Leica spectral scanning confocal microscope with 63x oil objective and images were acquired as sequential image stacks from each channel and were subsequently merged to obtain composite projections.

**Assays on cultured limb blastemal cells**

Newt limb blastemas (generally 30-50) were harvested at the early to mid bud stage and the wound epidermis was removed. In some cases the blastemas were denervated 5 days earlier. The blastemal mounds were dissociated in serum free Amphibian Modified Eagles Medium (AMEM) and transferred to collagen-coated microwells. A detailed protocol will be published elsewhere. The cells were incubated with AMEM + 1% Fetal Bovine Serum for 72 hr before addition of conditioned medium.

Conditioned medium (serum free DMEM) was harvested from Cos 7 cells transfected with nAG plasmid or red fluorescent protein (RFP) plasmid as control. After removal of debris by centrifugation, the medium was adjusted to urodele osmolarity by addition of distilled water, and concentrated 100× in Centricon concentrators (cutoff molecular weight 5kD) according to the manufacturers instructions. The experimental and control concentrated media samples were added directly to the microwells with BrdU at 10 µM and left for 96 hrs. After fixation in 1% PFA, the cells were stained with mouse anti BrdU and Alexa Fluor anti mouse IgG, and counterstained with Hoechst. Microwells were scanned automatically under a Zeiss Axiovert 200
inverted microscope using Axiovision software with Mosaic-X module and a motorised stage. A full 6x6 field dual channel scan was performed under the 10x objective with Hoechst and FITC filter channels. The resulting mosaic image was stitched and exported as a TIFF image for each channel. For counting the cells, the TIFF images were imported into Image-Pro Plus 4.5 (Media Cybernetics) and cell quantitation was performed with the count/size function. The area filter was set to a constant value for all scans to exclude debris. Data for 8 independent assays with experimental and control conditioned media is given in Table S1.
Table S1. Summary of S phase re-entry assays performed on limb blastemal cells stimulated with either experimental (nAG) or control (RFP) media.

<table>
<thead>
<tr>
<th>Conditioned media</th>
<th>Total number of cells</th>
<th>Percent (%)</th>
<th>Fold increase</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Hoechst</td>
<td>BrdU-positive</td>
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</tr>
<tr>
<td>RFP 1</td>
<td>1036</td>
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</tr>
<tr>
<td>nAG 1</td>
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<tr>
<td>RFP 2</td>
<td>1054</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>nAG 2</td>
<td>1270</td>
<td>127</td>
<td>10</td>
</tr>
<tr>
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<td>5</td>
</tr>
<tr>
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<td>325</td>
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</tr>
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<td>21</td>
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<tr>
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<tr>
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<td>RFP 7 (denervated)</td>
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<tr>
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<tr>
<td>nAG 8 (denervated)</td>
<td>595</td>
<td>11</td>
<td>1.85</td>
</tr>
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</table>

Assays 7 and 8 were performed on cells taken from blastemas denervated 5 days earlier.
Figure S1 Comparison of staining of nerve and glands with antibodies 223 and 224 against non-overlapping sequences of nAG. Sections of a regenerating newt limb at day 11 pa were stained in parallel with 223 (A, B) or 224 (C, D) or no first layer antibody (E). A, C; staining of distal nerve sheath (NS), B, D; staining of gland in wound epidermis (arrowed), E; note absence of reaction. Scale bar, 100µm.
Figure S2  Expression of nAG in glands of a normal newt limb. Section of a limb showing A, weak staining of glands (arrowed) with antibody 223, and B, staining with control antibodies to adeno associated virus. The arrow identifies a gland which is not stained. M, muscle; B, bone. Scale bar, 100µm.
**Figure S3** Expression of nAG in the nerve sheath (NS) in relation to regenerating axons in an amputated newt limb. Longitudinal section of distal peripheral nerve at day 10 pa, showing expression of A, nAG; B, HNK1; C, Hoechst labelled nuclei and D, merged A and B to show association of nAG expression with regenerating nerve. Scale bar, 200 µm.
**Figure S4** Expression of nAG in a multicellular gland in the wound epidermis. Section of a gland (arrowed) in the blastema at 12 days pa, showing A, differential interference contrast image; B, nAG staining; C, Hoechst labelled nuclei. WE, wound epidermis, scale bar, 100 µm.
Figure S5  Immunoblots of 4 independent preparations of concentrated nAG or RFP conditioned media for assays on cultured cells. Conditioned media of transfected Cos 7 cells was made and concentrated as described above, run on 12% SDS gels and immunoblotted with antibody 223, followed by anti rabbit IgG labelled with IR800 (Lycor). The markers (red) are labelled with IR700 (Lycor). Lanes 3,5,7 & 9 are nAG media, 2,4,6 and 8 are RFP control. Note the presence of the 18kD band in 3,5 and 7 and the lower level in 9; the 3 strongly positive samples were used in cell assays. Lane 10 is a sample of the myc-tagged nAG which runs at 25kD.

References