Isolation and characterization of single-chain Fv genes encoding antibodies specific for *Drosophila Poxn* protein

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Abstract The usefulness of intrabodies as specific inhibitors of gene function has been extensively demonstrated in cell culture assays. However, very few experiments have been conducted with intrabodies expressed in whole organisms. To evaluate the intrabody technology in *Drosophila*, we focused on *poxn* protein, since its effects can be easily studied. We purified the recombinant *poxn* protein. We next isolated three single-chain variable fragments (scFv) which specifically recognize *poxn* protein. Two scFvs, designated α-Poxn2 and α-Poxn4, react with both denatured and native Poxn with half maximal inhibition values of 100 nM and 40 nM, respectively. The α-Poxn5 scFv also recognizes denatured Poxn but either does not recognize native Poxn or its half maximal inhibition value for native Poxn is high.

Key words: *Poxn*; Intrabody; Single-chain variable fragment; *Drosophila*

1. Introduction

Because of the explosive growth in the cloning and sequencing of genes, a major task for the coming years will be to unravel the function of these genes. A key element in the functional analysis of a gene is the capability to inactivate this gene. It is therefore of crucial importance to develop gene inactivation techniques which are broad range and susceptible to precise experimental control. In addition, development of techniques for selective gene inactivation would facilitate the development of new anti-gene therapies, as well as novel strains of plants, animals and other industrially important organisms that no longer possess a particular undesired trait.

Intracellularly expressed antibodies are referred to as intrabodies. Numerous studies with cell cultures have demonstrated the feasibility of intrabody-mediated gene modulation (reviewed in [1–3]).

The results obtained with cell cultures cannot always be extrapolated to intact multicellular organisms [4]. For instance, studies on cultured cells do not address the question of non-specific toxic effects in whole organisms because many functions are likely to go undetected in culture assays. This limitation may apply to a large number of genes, since genetic evidence from *Drosophila* indicates that most organism-lethal mutations are not cell-lethal [5]. Thus, it would be desirable to assess the validity of the intrabody approach in whole organisms.

A few studies have already demonstrated the feasibility of intrabody-mediated gene modulation in plants (for examples see [6,7]). Further experiments, using different targets in different tissues and organs and eventually in a wide range of different organisms, should allow to assess the general utility of intrabodies.

Drosophila's short reproductive cycle, its large body of experimental data and mutants, and its well-advanced genetics and molecular biology [8,9] make it particularly well suited for the study of intrabodies in different tissues and organs in the context of a whole organism. As a first step towards the evaluation of intrabody technology in *Drosophila*, we have focused on the isolation of genes encoding antibodies specific for Poxn.

The *poxn* protein, encoded by the *paired box neuro* (*poxn*) gene, specifies poly-innervated, as opposed to mono-innervated, external sense organs in *Drosophila* [10,11]. *Poxn* is responsible for all the differences between mono- and poly-innervated external sense organs, including the differences in their axonal pathways and target recognitions in the central nervous system [12,13].

In this report we describe the purification of the *poxn* protein, and the isolation and characterization of both monoclonal antibodies and scFvs with specificities for Poxn. To our knowledge, this is the first study that reports the cloning and characterization of genes encoding antibodies specific for a *Drosophila* gene product. In the accompanying paper, we evaluate the intrabody technology in *Drosophila* using these scFvs.

2. Materials and methods

2.1. Expression and purification of poxn protein

Escherichia coli strain BL21(DE3) [14] was transformed with plasmid pAR3040 [15] harboring the 1.01 kb *Bam*HI cDNA fragment spanning most of the *poxn* coding region fused to the T7 promoter

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Abbreviations: ABTS, 2′,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) diammonium; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; BSA, bovine serum albumin; cDNA, complementary DNA; CDR, complementarity determining region; EDTA, ethylene diamine tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; globH5, globular domain of histone H5; HRP, horseradish peroxidase; IPTG, isopropylthio-β-D-galactoside; mAb, monoclonal antibody; NBT, nitrotetrazolium chloride blue; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; scFv, single chain variable fragment; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; V_H, heavy chain variable domain; V_L, light chain variable domain

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[10,11]. Poxn was overexpressed as described [16] except that the culture was supplied with 150 μg/ml rifampicin 30 min after induction with 1 mM IPTG. The resulting inclusion bodies were recovered and Poxn was refolded in vitro according to the previously described protocols [17,18]. Alternatively, addition of rifampicin was omitted from the above-mentioned protocol and cells were harvested by centrifugation, washed with PBS and resuspended in 1/50 volume of PBS containing 1 μg/ml pepstatin A, 1 mM EDTA and 1 mM PMSF. Sonication was carried out on ice until the cell suspension became clear. The lysate was centrifuged at 14 000 rpm for 30 min at 4°C. The supernatant containing soluble Poxn was recovered, diluted two times with buffer A (50 mM NaCl, 50 mM sodium phosphate, pH 6.7) and passed through a 0.2 μm filter. 24 ml of diluted sample was applied at a flow rate of 2 ml/min on a MonoS 10/10 column (Pharmacia Biotech) pre-equilibrated with buffer A. The column was then washed with 50 ml of buffer A. Bound proteins were eluted with 80 ml of a linear gradient of 50 mM to 1 M NaCl in 50 mM sodium phosphate, pH 6.7 at the same flow rate as above. Fractions of 8 ml were collected. Poxn was mainly present in fractions 12, 13 and 14 as revealed by SDS-PAGE. The concentration and purity of Poxn were higher in fraction 12 than in fractions 13 and 14. Fractions 12 from several rounds of chromatography were pooled, concentrated 3–4 times by ultrafiltration using Amicon (cut-off 10 kDa) and then passed through a 0.2 μm filter. 5 ml of this sample was chromatographed over a Superdex 200 gel filtration column in PBS at a flow rate of 1 ml/min. Fractions were collected every 4 min and analyzed by SDS-PAGE. Fractions 6 and 7 which contained Poxn were pooled and stored at −70°C until further use.

2.2. Immunization of mice

Adult NMRI and NZB mice (10 of each strain) were each immunized subcutaneously with 15 μg of Poxn emulsified with an equal volume of Freund's complete adjuvant. After 3 weeks, primed mice were again immunized as above but with Freund's incomplete adjuvant. Seven days later, the serum antibody titers were analyzed by ELISA and immunolabelling of *Drosophila* embryos [19]. Four weeks after the second immunization, the antigen-responding mice received an intraperitoneal injection of 10 μg Poxn in PBS. Five days later, the spleens of mice which gave high titers of α-Poxn antibodies were used for the isolation of B-lymphocytes.

2.3. Isolation of mAbs

Spleen cells from immune NZB mice were fused with the mouse myeloma cell line NSO as described [20]. The hybridoma cells were screened by ELISA and immunolabelling of *Drosophila* embryos [19]. The determination of mAbs isotypes was carried out using the InnoLia mouse mAb isotyping kit (Innogenetics).

2.4. DNA manipulations

DNA manipulations were carried out following standard procedures [21]. PCR screening of the recombinant clones and PCR-*Mva*I and -*Sau*3AI fingerprinting were as described [22]. Nucleotide sequences were determined by the dideoxynucleotide chain termination method [23].

2.5. Library construction

mRNA was prepared from the pooled splenocytes of positive mice using the QuickPrep Micro mRNA purification kit (Pharmacia Biotech). The resulting mRNA was then used for first-strand cDNA synthesis using the Ready-To-Go-T-Primed first-strand kit (Pharmacia Biotech). V_H and V_L genes were amplified separately from the cDNA with 35 cycles of PCR using an equimolar mixture of the primers VHBACK-1 (5′-GAGGTCCAGCTCGAGCAGTCTGG-3′), VHBACK-2 (5′-AGGTCCAGCTGCTCGAGTCTGG-3′), VHFOR (5′-CTATTAACTAGTGACAGTGACCAGAGTCCCTTGGCCCC-3′), VLBACK (5′-GTGCCAGATGTGAGCTCGTGATGACCCAGTCTCCA-3′) and VLFOR (5′-GCAGCATTCTAGAGTTTGATTTCCAGCTTGGTCCC-3′). Each PCR reaction was performed in a 100 μl volume containing 1–2 μl of the first-strand cDNA synthesis mixture, 20 nmol of each dNTP, 20 pmol of each primer, 2.5 units of Taq polymerase and PCR buffer (Boehringer Mannheim). Each PCR cycle consisted of 1 min denaturation at 94°C, 2 min annealing at either 51°C with primer VHBACK-2 or 58°C with the other primers, and 2 min extension at 72°C. The V_H and V_L repertoires were separately digested with restriction enzymes *Spe*I and *Sac*I, respectively, to produce DNA ends free from tailing nucleotides. The digested V_H and V_L repertoires were purified from 1% agarose gel using GeneClean II kit (Bio 101). The linker DNA [24] was amplified from a recombinant pHEN1 containing a scFv gene with 35 cycles of PCR (94°C for 1 min, 65°C for 1.5 min and 72°C for 1 min) using primers LINKFOR (5′-TGGAGACTGGGTCATCACGAGCTCCGATCCGCC-3′) and LINKBACK (5′-GGGACTCTGGTCACTGTCACTAGTGG-3′). The amplified linker was purified from the gel using MERMAID kit (Bio 101). The gel-purified V_H and V_L repertoires in combination with linker were then assembled to generate the scFv repertoire as described [25] except that the first seven cycles were each carried out at 94°C for 1.5 min, 51°C for 1.5 min and 72°C for 2 min without primers. Primers VHBACK-1-*Sfi*I (5′-CTCGCAACTGCGGCCCAGCCGGCCATGGCCGAGGTCCAGCTCGAGCAG-3′), VHBACK-2-*Sfi*I (5′-CTCGCAACTGCGGCCCAGCCGGCCATGGCCGAGGTCCAGCTGCTCG-AG-3′) and VLFOR-*Not*I (5′-AACAGTTTCTGCGGCCGCAGCATTCTAGAGTTT-3′) were then added and the reaction mixture was amplified for 25 cycles (94°C for 1.5 min, 68°C for 1.5 min and 72°C for 2 min). The assembled products were digested with *Sfi*I and *Not*I, gel-purified using GeneClean II kit (Bio 101) and ligated into pHEN1 vector [26]. The ligated DNA was then electroporated into *E. coli* XL2Blue-MRF′ (Stratagene). After electroporation, cells were grown, plated and stored as a library stock [22].

2.6. Rescue of phagemid library and panning

Rescue of phagemid particles was as described [22] except that M13K07 helper phage was used and 45 min after addition of helper phage, IPTG was added to final concentration of 1 mM. Rescued phages were blocked with 1% BSA (w/v) containing the MonoS 10/10 fractions of an induced culture of *E. coli* BL21(DE3) harboring non-recombinant pAR3040 vector. The fractions used correspond to the ones where Poxn, in lysate from bacteria containing the recombinant vector, elutes. Blocked phages were panned against Poxn as follows. Wells of 96-well ELISA plates (Nunc-Immuno plate, MaxiSorp) were coated with 60 μl of 50 μg/ml Poxn in PBS per well at room temperature for 4–6 h and blocked with PBS-1% BSA (w/v). In addition, uncoated wells were blocked as above to serve as negative control. Phages were incubated in Poxn-coated and negative wells, unbound phages removed and the bound phagemid particles eluted and propagated as in [22] except that PBS, 0.02% Tween 20 was replaced by PBS, 0.05% Tween 20.

2.7. Phage ELISA assays

Rescue of phagemid from individual clones for monoclonal phage ELISA was carried out as in Section 2.6. Wells of 96-well microtiter plates were coated with 60 μl of either 5 μg/ml Poxn, 500 μg/ml lysozyme or 1 mg/ml globH5 [27] in PBS. The remaining protein binding sites were blocked as for panning. The monoclonal phage ELISA was performed using 50 μl of culture supernatant per well and the detection module of recombinant phage antibody system (Pharmacia Biotech) according to the manufacturer's recommendations. Polyclonal phage ELISA (5×10^9 phage particles per well) was as outlined for monoclonal phage ELISA.

2.8. Expression of scFv proteins

ScFv proteins were expressed in *E. coli* strain TG1. Induction of protein synthesis and isolation of scFv fragments from the periplasmic space have been described [28].

2.9. Indirect ELISA using soluble scFv fragments

The coating and blocking of the wells were as described in Section 2.7. 50 μl of periplasmic extracts was added to each well and incubated for 2 h. The wells were washed five times with PBS, 0.05% Tween 20. Anti-c-myc-tag antibody 9E10 was added to each well and allowed to react for 2 h. The wells were washed as above and then incubated with anti-mouse IgG-HRP conjugate (Sigma) for 1 h. After five washes, binding was detected using ABTS/H_2O_2 as substrate. All steps were carried out at room temperature.

2.10. ELISA competition test for specificity and half maximal inhibition measurements

The ELISA competition test and half maximal inhibition measurements were performed as in [29,30].

2.11. SDS-PAGE and Western blotting

SDS-PAGE and Western blot analysis of the proteins separated by SDS-PAGE were essentially as in [31,32], respectively.

3. Results

3.1. Expression and purification of the poxn protein

To ensure high levels of expression, Poxn was initially expressed in the presence of rifampicin. Rifampicin has no effect on T7 polymerase but inhibits the transcription by *E. coli* RNA polymerase, thus increasing the expression of genes driven by the T7 promoter [16]. Expression of Poxn in the presence of rifampicin yielded high levels of Poxn which accumulated as inclusion bodies. The fusion protein appeared in SDS-polyacrylamide gel with a molecular mass of 47 000 Da (Fig. 1, lane 2). The inclusion bodies were recovered and used for purification and refolding of Poxn (Fig. 1, lane 3). However, the use of this procedure was hampered by the low recovery of Poxn as a soluble fraction after refolding, and by a poor reproducibility. Since the aggregation problem may be circumvented by lowering the expression levels [33], the rifampicin was omitted from the expression protocol. This resulted in lower levels of Poxn but, interestingly, the expressed protein was found almost exclusively in the soluble fraction. The soluble Poxn was first partially purified from the *E. coli* cell lysate supernatant by cation-exchange chromatography through a MonoS 10/10 column. The Poxn in fraction 12 was about 60–70% pure as estimated by Coomassie brilliant blue staining of proteins resolved by SDS-PAGE (Fig. 1, lane 4). Fractions 12 from several rounds of chromatography were used for further purification of Poxn by gel filtration on Superdex 200. Poxn was more than 90% pure after gel filtration (Fig. 1, lane 5). The protein bands with lower electrophoretic mobilities than Poxn, present in the sample recovered after gel filtration, are likely aggregates of Poxn (Fig. 1, lane 5). This conclusion is based on the fact that these bands are also present in the in vitro refolded sample (Fig. 1, lane 3); and since Poxn was excised from the gel prior to refolding, it cannot be contaminated with proteins with migration patterns so different from that of Poxn. However, these proteins did not react with mAbs specific for Poxn (Fig. 2A). This might be due to the inaccessibility of these antibodies to their corresponding epitopes in aggregates because of the possible in-

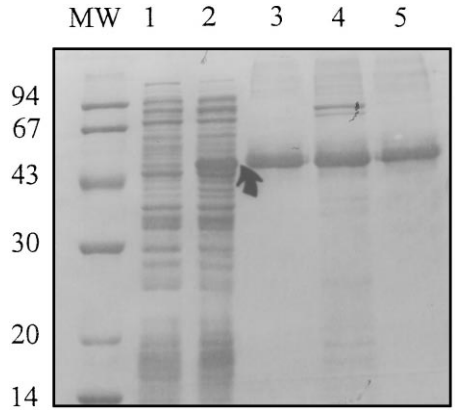

Fig. 1. SDS-PAGE analysis of protein samples at different stages of purification of Poxn. Various preparations were electrophoresed in 12.5% SDS-polyacrylamide gel and stained with Coomassie brilliant R-250. Lane MW, molecular weight protein markers, sizes in kDa are indicated on the left of the figure; lane 1, total lysate of IPTG induced *E. coli* BL21 (DE3) cells containing non-recombinant pAR3040 vector; lane 2, total lysate of IPTG induced *E. coli* BL21 (DE3) cells harboring recombinant pAR3040 vector containing the 1.01 kb *Bam*HI cDNA fragment of *poxn*, the arrow indicates the position of the overexpressed Poxn; lane 3, Poxn excised from SDS-polyacrylamide gel, electroeluted and refolded in vitro; lane 4, fraction 12 from MonoS 10/10 column (concentrated 4 times); lane 5, pool of fractions 6 and 7 from Superdex 200 gel filtration column (concentrated 3 times).

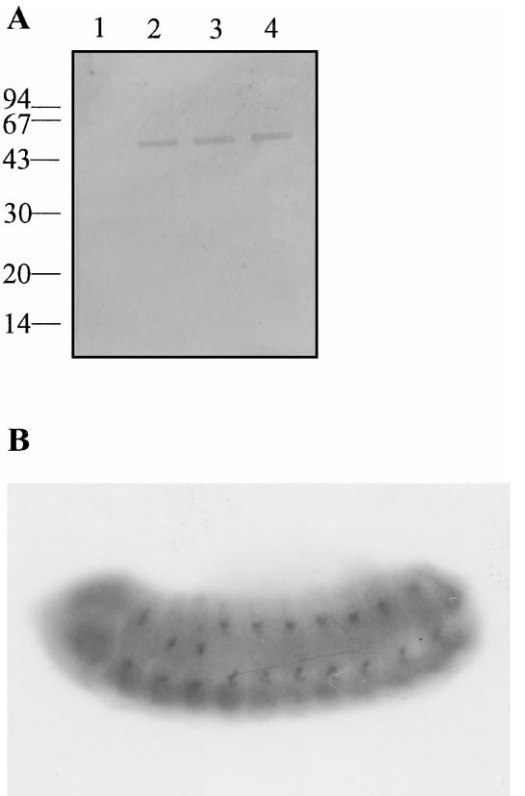

Fig. 2. Analysis of the purified Poxn and anti-Poxn mAbs. A: Western blot. Protein samples were transferred to nitrocellulose membrane, reacted first with mAb17B7B10 and then with anti-mouse IgG-alkaline phosphatase conjugate. Bound antibodies were detected using NBT/BCIP as substrate. The protein samples are as follows: lanes 1, 2, 3 and 4, respectively, the same as lanes 1, 2, 4 and 5 in Fig. 1. B: Immunolabelling of *Drosophila* embryos [19] with mAb 17B7B10. The immunolabelling pattern precisely parallels the spatial and temporal patterns of expression of *poxn* described in [10,11]. Similar results were obtained with mAb 17B7A12.

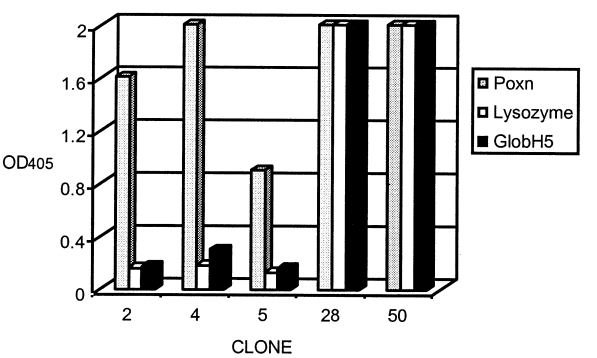

Fig. 3. Monoclonal phage ELISA to assay the specificity of the binding of five clones isolated by panning against Poxn. Clones 2, 4 and 5 represent α-Poxn2, α-Poxn4 and α-Poxn5 scFvs, respectively. Clones 28 and 50 each contain a truncated V_H.

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EVQLEQSGAELVRPGASVRLSCKASGYTFT DYEMH WVKQTPVHGLEWIG GIDPETDSTAYNQKFNH KATLTADKSSSTAYMELRSLTFEDSAVYYCT
EVQLEQSGPELVKPGASVKISCKASGYASS NSWMN WVKQRPGKGLEWIG WISPGDGNTYYNGKFKG KATLTADKSSSTAYMQLSSLTSEDSAVYFCA
EVQLEQSEAELVRPGASVKLSCTASGFNIK DDYMH WVKQRPEQGLEWIG WIDPEDGETKYAPKFQD KATITADTSSNTAYLQLTSLTSEDTAVYYCT

R SPIYYYSSSSAMDY WGQGTLVTVTS / GGGGSGGGGSGGGGS / ELVMTQSPSSLSASLGERISLTC RASQDIYGSLN----- WFQQKPDGTIK
D GFAY---------- WGQGTLVTVTS / GGGGSGGGGSGGGGS / ELVMTQSPLSLSVTIGQPASISC KSSQSLLHSDGKTYLN WLQQRPGQSPK
T LGSSLPY------- WGHGTLVTVTS / GGGGSGGGGSGGGGS / ELVMTQSPLSLSVTIGQPASISC KSSQSLVYSDGKTYLN WLQQRPGQSPK

LLIY GTSSLDS GVPKRFSGSRSGSDYFLTISSLESEDFADYYC LQYASSPFT FGSGTKLEIKL        α-poxn2
RLMY QVSKLDP GIPDRFSGSGSETDFTLKISRVEAEDLGVYYC MQGTHYPLT FGDGTKLEIKL        α-poxn4
RLIY QVSKLDP GIPDRFSGSGSETDFTLKISRVEPEDLGVYYC LQGTNYPLT FGTGTKLEIKL        α-poxn5
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Fig. 4. Amino acid sequence of the anti-Poxn scFvs. The linker sequence is shown in bold and between slashes. The polarity of scFvs is as follows: V_H-linker-V_L. CDRs are underlined. Dashes have been introduced to align the CDRs.

volvement of these epitopes in aggregation. Although less likely, we cannot formally exclude the possibility that these aggregates are composed of proteins other than Poxn.

3.2. *Generation of monoclonal antibodies specific for the poxn protein*

To confirm that the purified protein is Poxn, the purified protein was used as immunogen for the production of mAbs, and the mAbs generated against the purified protein were tested by immunolabelling of *Drosophila* embryos for their specificities for Poxn. The purified protein did not elicit antibodies in NMRI mice. In contrast, 8 out of 10 NZB mice, immunized with purified protein, produced α-Poxn antibodies. However, the mice with the highest titers of α-Poxn antibodies died soon after the second immunization. Hybridomas were produced using the immune NZB mice. Two mAbs, 17B7A12 and 17B7B10 (IgG1 class), were isolated. Western blots with either mAb reveals a single reactive band corresponding to the bulk of protein obtained by cation-exchange chromatography and gel filtration (Fig. 2A). Immunolabelling of *Drosophila* embryos shows that these mAbs label specifically those *Drosophila* cells that express Poxn (Fig. 2B). These experiments confirm that the overexpressed and purified protein with apparent molecular mass of 47 000 Da is indeed Poxn.

3.3. *Isolation of α-Poxn scFv antibodies*

A scFv library was constructed from the V_H and V_L repertoires of NZB mice immunized with Poxn. The library contained 2×10^7 individual colonies. PCR screening of 100 randomly chosen colonies demonstrated that a full-length scFv insert was present in 85% of the colonies, whereas about 10% of the colonies contained a short (probably rearranged) insert. The insert diversity of recombinant colonies containing a full-length scFv insert was assessed by PCR-*Mva*I fingerprinting and shown to be about 85% (data not shown).The library was subjected to four rounds of panning against Poxn, and the specific enrichment values (calculated as the number of phages eluted from Poxn-coated wells divided by the number of phages eluted from BSA-coated wells) for scFv phages recognizing Poxn were 14, 14, 21 and 115, respectively, after the first, second, third and fourth rounds of panning. Polyclonal mixtures of phagemid particles, produced by repropagation of the phages eluted after each round of phage selection, were also tested by ELISA for enrichment of scFv phages specific for Poxn. A sharp increase of signal was noticed after the fourth round of panning. The results of polyclonal phage ELISA were consistent with enrichment values outlined above.

Following the fourth round of panning, 120 clones obtained by infection with the eluted phages were individually tested by ELISA. Out of 120 clones, 68 produced phages that bound to Poxn. PCR and restriction analyses of these 68 clones demonstrated that two clones, designated clone 28 and clone 50, have an insert of about 300 bp while other clones have an insert with the size expected for the scFv fragment. The insert

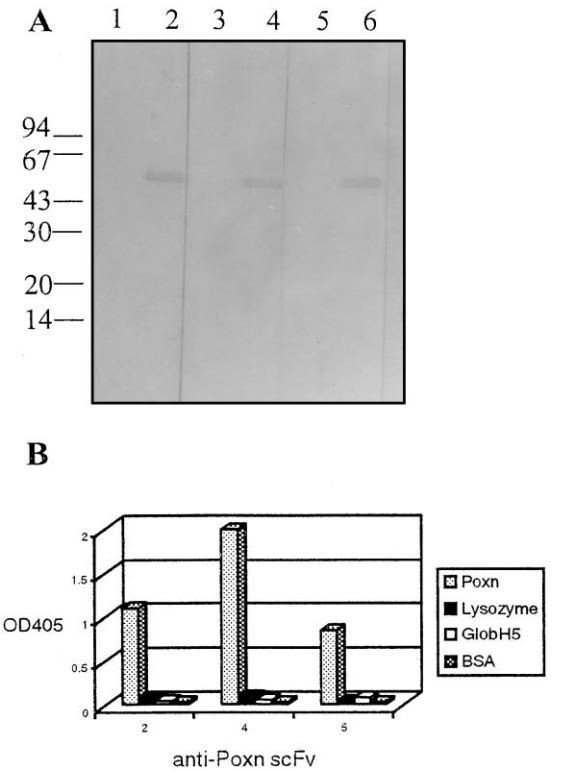

Fig. 5. Specificity of soluble scFvs. A: Western blot analysis of α-Poxn scFvs. Proteins were electrophoresed in 12.5% SDS-polyacrylamide gel, transferred to nitrocellulose membrane and reacted first with scFvs, then with anti-c-myc tag 9E10 antibody and finally with anti-mouse IgG-alkaline phosphatase conjugate. The bound antibodies were detected by NBT/BCIP as substrate. The protein samples transferred to membrane are as follows: lanes 1, 3 and 5, total lysate of induced *E. coli* BL21(DE3) cells containing non-recombinant pAR3040 vector; lanes 2, 4 and 6, total lysate of induced *E. coli* BL21(DE3) cells harboring recombinant pAR3040 vector containing the 1.01 kb *Bam*HI cDNA fragment of *poxn*. The scFvs used are as follows: lanes 1 and 2, α-Poxn2 scFv; lanes 3 and 4, α-Poxn4 scFv; lanes 5 and 6, α-Poxn5 scFv. B: Indirect ELISA assay. Binding was determined by ELISA to a variety of immobilized proteins: Poxn (5 μg/ml), lysozyme (500 μg/ml), GlobH5 (1 mg/ml) and BSA (10 mg/ml). Experiments were carried out with periplasmic extracts of transformed *E. coli* TG1.

diversity of the 66 putative positive clones with expected insert size was assessed by PCR-*Mva*I and -*Sau*3AI fingerprinting. Based on *Mva*I restriction enzyme digestion patterns, three different groups were identified and all clones belonging to one group had the same *Sau*3AI restriction enzyme digestion pattern (data not shown). Therefore, one clone from each group was chosen for further analysis. These clones were designated α-Poxn2, α-Poxn4 and α-Poxn5.

3.4. Specificity of the reactive phages

To determine the specificity of the selected clones, monoclonal phage ELISA was carried out using Poxn and two irrelevant antigens as controls, lysozyme and globH5. Clones 28 and 50 gave similar absorbance values for different proteins indicative of the polyspecificity or sticky behavior of these clones. Except for clones 28 and 50, absorbance values obtained with Poxn-coated wells were much higher than those obtained with wells coated with lysozyme or globH5 (Fig. 3). Taking into account that in this assay, concentrations of lysozyme and globH5 are, respectively, 100 and 200 times higher than that of Poxn, these results confirm that α-Poxn2, α-Poxn4 and α-Poxn5 scFvs are highly specific.

3.5. Sequence analysis of the reactive clones

Fig. 4 shows the amino acid sequence of α-Poxn2, α-Poxn4 and α-Poxn5 scFvs as deduced from the nucleotide sequence. Each scFv consists of unique V_H and V_L domains. Clones 28 and 50 carry only a part of a V_H sequence but V_L is missing (data not shown), consistent with our previous observation that only an insert of 300 bp was identified by restriction analysis of these two clones.

3.6. Specificity of the soluble α-Poxn scFv antibodies

α-Poxn2, α-Poxn4 and α-Poxn5 scFvs were expressed as soluble antibody fragments. After IPTG induction, a protein band with an apparent molecular mass of about 29 000 Da was detected in Western blot with the anti-c-myc 9E10 antibody (data not shown). Western blotting experiments with periplasmic extracts demonstrated that these scFvs specifically recognize Poxn (Fig. 5A). The same extracts were also screened by ELISA for binding to a panel of different proteins and the scFv fragments were found to be highly specific (Fig. 5B).

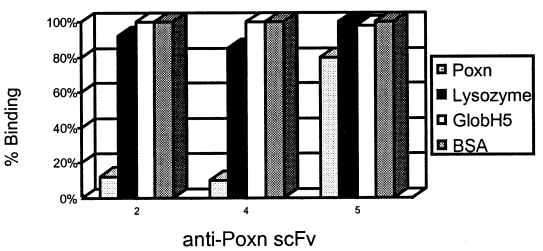

Fig. 6. ELISA competition test. Wells were coated with 1 μM Poxn and the binding of soluble scFvs to immobilized Poxn was competed with 1 μM Poxn, 1 μM lysozyme, 4 μM GlobH5 or 150 μM BSA. The percentage of binding is expressed as follows: [absorbance (A_{405}) in the presence of competitor divided by absorbance (A_{405}) in the absence of competitor]×100. The scFv samples used were periplasmic extracts of transformed *E. coli* TG1.

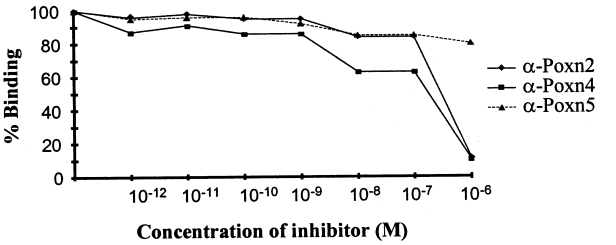

Fig. 7. Inhibition ELISA to determine half maximal inhibition values of anti-Poxn scFv fragments. Wells were coated with 1 μM Poxn and the binding of soluble scFvs to immobilized Poxn was competed with different amounts of Poxn. The percentage of binding is expressed as in legend for Fig. 6.

3.7. ELISA competition test and half maximal inhibition measurements

As illustrated in Fig. 6, the binding of α-Poxn2 and α-Poxn4 scFvs to Poxn coated on solid phase was efficiently inhibited by preincubating the antibody with 1 μM solution of native Poxn. This concentration of Poxn did not inhibit the binding of α-Poxn5 scFv. Therefore, at least, α-Poxn2 and α-Poxn4 scFvs recognize native Poxn. Irrelevant antigens such as lysozyme, globH5 and BSA, even if used at much higher concentrations than Poxn, failed to compete with Poxn for binding to these scFvs confirming that these antibody fragments are indeed specific for Poxn (Fig. 6). To determine the half maximal inhibition values of the scFvs for Poxn, experiments were performed at constant but non-saturating concentrations of the scFvs as determined in the titration ELISA test. These experiments showed that α-Poxn2 and α-Poxn4 scFvs react with native Poxn with half maximal inhibition values of 100 nM and 40 nM, respectively (Fig. 7). The analysis of the binding curves suggests that half maximal inhibition value for α-Poxn5 scFv, if any, is higher than 1 μM (Fig. 7). Alternatively, α-Poxn5 scFv may react only with denatured Poxn.

4. Discussion

Intrabody technology is one of the most novel and promising developments in the rapidly evolving area of gene modulation technology. Numerous studies on cultured cells have provided secure foundation for the use of intrabodies as reproducible, simple, broad-based and specific reagents for the modulation of gene function (reviewed in [1–3]). To provide a basis for the evaluation of intrabody technology in *Drosophila*, we have purified the *Drosophila poxn* protein, and isolated scFv genes that encode antibodies specific for Poxn.

The apparent molecular mass of the Poxn (47 000 Da) expressed in *E. coli* is higher than the expected molecular mass of 38 144 Da calculated from the amino acid composition of Poxn. The fact that staining pattern of *Drosophila* embryos with mAbs that specifically recognize the 47 000 Da protein precisely parallels the spatial and temporal patterns of the expression of *poxn* during embryonic development proves that the purified protein is Poxn. As described for *Drosophila fushi tarazu* and *krüppel* proteins [34,35], the high proline content (9.2%) of recombinant Poxn may be responsible for its anomalous migration.

The fact that Poxn did not elicit any detectable antibodies in NMRI mice suggests that the self-tolerance profile of mouse may include Poxn. We thus switched to NZB mice

which make autoantibodies more easily than other strains and are therefore recommended for immunization with well-conserved antigens [31]. Apparently, high titers of α-Poxn antibodies were lethal for these mice, in line with the idea that these antibodies may indeed cross-react with mouse antigen(s).

Since scFvs with good expression levels and high specificities are readily selected from very large and diverse antibody libraries, a scFv library was constructed using spleen cells of the immunized NZB mice. From this library, we first isolated five clones giving strong ELISA signals on Poxn. However, two of these clones, clones 28 and 50, showed strong signals with irrelevant antigens as well. Since these two clones contain only a part of a V_H, their reactivity with different antigens might result from the stickiness of the truncated mouse V_H. Three scFvs show a high specificity of binding to Poxn. The specific recognition of Poxn by these scFvs in Western blot suggests that these scFvs recognize denatured Poxn. Likewise, ELISA competition test reveals that at least two of these scFvs, α-Poxn2 and α-Poxn4, recognize native Poxn as well. Thus, the epitopes recognized by α-Poxn2 and α-Poxn4 scFvs are probably linear epitopes that are also exposed in native Poxn. Binding of α-Poxn5 scFv to Poxn coated on solid phase was not inhibited by incubation of antibody with 1 μM Poxn, suggesting that this antibody reacts with an antigenic determinant which is hidden in native Poxn and becomes exposed upon coating the protein on a solid phase. Alternatively, in addition to the recognition of denatured Poxn, α-Poxn5 scFv may react with native Poxn but with high half maximal inhibition value. We could not test the latter possibility, because Poxn aggregates at concentrations above 1 μM.

The monoclonal antibodies isolated in this study can be used for further analysis of the role of *poxn* in the development of the fly nervous system, as has also been documented by using these mAbs to isolate and characterize *poxn* mutations that affect the adult chemosensory organs [36].

In addition, using the α-Poxn scFvs, we demonstrate the feasibility of intrabody-mediated gene modulation in *Drosophila* (see accompanying paper [37]).

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