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Modulating Poly(A)-specific ribonuclease (PARN) activity in vitro by RNA binding proteins

Master’s degree project
Modulating Poly(A)-specific ribonuclease (PARN) activity \textit{in vitro} by RNA binding proteins

mRNA degradation is an important step in the regulation of gene expression. The first step in mRNA decay is removal of the poly(A)-tail in the 3’-end, carried out by the poly(A)-specific ribonuclease (PARN). However, the mRNA exists as a complex with RNA binding proteins when deadenylation is about to start. The aim of this study was to investigate how the RNA-binding proteins poly(A) binding protein (PABP) and p100 affect the activity of PARN. Recombinant PABP, p100 and PARN was expressed and purified. The RNA-binding capacity of PABP and p100 was investigated \textit{in vitro}. PABP was found to inhibit the effect of PARN \textit{in vitro} when low mRNA concentration (relative to PABP) was used. However, at high mRNA concentration, PABP showed both stimulatory and inhibitory effects on PARN depending on the specific concentrations provided. Therefore, the amount of PABP in the cytoplasm might be a way for the cell to control mRNA degradation.

Keywords
mRNA degradation, deadenylation, PARN, RNA binding proteins
Sammanfattning


Generna för de RNA-bindande proteinerna poly(A)-bindande protein (PABP) och p100 klonades i bakterier, som sedan användes för att producera proteinerna. PABP och p100 renades sedan fram. Den RNA-bindande förmågan hos PABP och p100 studerades genom att de fick binda ett budbärar-RNA. Det visade sig att PABP band bra till RNA. Tyvärr band ej p100 RNA. Därför nöjde jag mig med att undersöka hur PARN påverkades av PABP. Jag fann att mycket PABP motverkade aktiviteten hos PARN. Jag fann också att enzymet fungerade bättre om en mindre mängd PABP användes.

Konzentrationen av RNA-bindande proteiner i våra celler är därför ett möjligt sätt att reglera mängden tillverkat protein från våra gener.

Modulating Poly(A)-specific ribonuclease (PARN) activity in vitro by RNA binding proteins

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INTRODUCTION

In eucaryotic cells, gene expression is regulated at different levels. The nuclear transcription is most frequently regulated. However, before the transport to the cytoplasm, several modifications of the mRNA occur, e.g. capping, splicing and polyadenylation. Capping is addition of a nucleotide structure at the 5’ end. The cap consists of two methylated guanosine residues connected by a 5’–5’-triphosphate bridge. Splicing removes the non-coding introns present in pre-mRNA and at the 3’ end, a poly(A) tail is synthesized, consisting of around 200 adenosine residues. These modifications, as well as the final cytoplasmic translation, can also act as targets for regulation.

The concentration or the steady state level of mRNA in the cytoplasm reflects the amount of mRNA available for translation by the ribosomes. The steady state level is a result of transcription and degradation. mRNA degradation is coupled to decapping and deadenylation. Thus, the rates of these two events will determine the stability or half-life of mRNA. In yeast, two major degradation pathways are known, and in both the first step to occur is deadenylation (1), (fig 1). In the major pathway, decapping occurs after deadenylation, which precedes the degradation of the mRNA by the 5’→3’ Xrn1p exonuclease. In the alternative pathway the cap is not removed after deadenylation, instead the mRNA is degraded in the 3’→5’ direction by the exosome complex. The composition of the exosome is unknown. However, several nuclease activities are associated with it.

Poly(A) specific nucleases from different organisms have been found (Reviewed in 2), but the exact functions and interactions with other factors are far from understood. Three poly(A) nuclease activities are known in eucaryotes: poly(A) nuclease (PAN) (3,4,5), poly(A)-specific ribonuclease (PARN) (6,7,8) and the activity associated with the caf1/ccr4 complex (9). PARN was identified in HeLa cell extracts (10). It requires a 3’-located hydroxyl group and releases free 5’ AMP as product (11). Only poly(A) is efficiently degraded and the mRNA body is unaffected by the nuclease. PARN has been cloned and expressed as a recombinant protein in Escherichia coli followed by purification. The size of the full-length protein is 74 kDa (8). In calf thymus extracts, a 54 kDa fragment of PARN has been identified, purified and characterized (6). PARN exists as a trimer.
and degrades poly(A) in a highly processive mode. Three different studies (12, 13, and 14) show that PARN degrades capped substrates \textit{in vitro} more efficiently than uncapped mRNA:s. A model has been proposed (12), where the cap-binding site of the enzyme is different from its active site (fig 2).

![Fig 2: A model of PARN in action. Courtesy of Helena Nordvarg.](image)

In the cytoplasm, mRNA:s usually do not exist as naked molecules. Instead they are in complex with RNA binding proteins (1). It is such an mRNA/protein complex PARN has to encounter as substrate, when it is about to start the deadenylating process. It is not completely understood how RNA binding proteins affect the PARN activity. The poly(A) is covered with poly(A) binding protein (PABP) (fig 1), one of the most abundant RNA binding proteins in the cytoplasm. One PABP covers about 27 nucleotides (15). The structure of PABP has recently been solved (16). The interactions with poly(A) occur through conserved residues in the RNA recognition motifs (RRM:s) (fig 3). In yeast, PAN is dependent on PABP (4, 5) and mutations in PABP led to extended poly(A) tails (3). In contrast, the association of poly(A) binding proteins with human or \textit{Xenopus} mRNA:s appears to protect the transcript from deadenylation (17, 18). It has also been shown \textit{in vitro} that the activity of PARN from calf thymus is inhibited when PABP is bound to the mRNA (7).

![Fig 3: Structure of two RRM:s in PABP binding to a stretch of eight adenosines (From Deo et. al 1999).](image)

A protein of size 100 kDa (p100) copurified with the nuclease activity when PARN from calf thymus extracts was purified in Virtanen’s lab. It was believed that the p100 was the poly(A) specific nuclease since it copurified with the nuclease activity. However, this was later shown to be incorrect, since the activity was associated with the 54 kDa fragment of PARN (6). The native p100 is a poly(A) binding protein (Martinez and Virtanen, personal communication). The gene encoding p100 has been identified and the corresponding cDNA has been cloned in the lab. Due to a sequencing mistake in the original paper (19) describing the p100 gene, the AUG start codon was incorrectly identified. This was later corrected and the real AUG was found to be located 75 nucleotides upstream. Due to this sequencing mistake two versions of p100 were cloned and expressed. p100 refers to the full-length initiating at the correct AUG, while p100s (for “short”) refers to a shorter version obtained by initiation at the 2:nd and incorrectly determined start codon (fig 4).
Fig 4: Multidomain composition of p100. Domains 1-4 (green rectangles) are SNase homologues. Domain 5 consists of a tudor domain (red oval) and a truncated SNase-fold. p100s is an N-terminal deletion of p100.

p100 was initially identified as a human EBNA-2 co-activator (100kD) called “p100 co-activator”. p100 activates transcription by forming a link between EBNA 2 (Epstein-Barr virus nuclear protein 2) and the human transcription factor TFII E (19). The co-activator also binds ssDNA. Two different bioinformatical studies (20, 21) suggested that p100 consists of five domains (fig 4). The first four are repeats, each of which is a staphylococcal nuclease (SNase) homologue. However, the catalytic amino acids present in nuclease are missing in p100, but the domains could still serve to bind DNA without catalytic activity. SNase homologues consist of two subdomains of which the larger belongs to the oligonucleotide/oligosaccharide-binding (OB)-fold superfamily. The fifth domain is special in the way that it only contains the smaller subdomain of the nuclease structure. The OB-fold is replaced by a structure found in multiple copies in the tudor protein from Drosophila melanogaster. Therefore this special domain is called a ‘tudor domain’ and may function in RNA-binding or in binding to other proteins (reviewed in 22).

The function of p100 is not yet known. It has been implicated to function in several steps of gene regulation, for example as a transcriptional co-activator as described above. Further, it is stated that p100 functions in a signal transduction pathway as a link between the serine/threonine kinase Pim-1 and the DNA-binding domain of the transcription factor c-Myb (23). p100 has also been found in endoplasmic reticulum and lipid droplets of milk secreting cells (24) suggesting that it has a function additional to its role as a co-activator. Finally we have the reason to believe (Virtanen, personal communication) that it is associated with mRNA degradation.

Here we wanted to study how RNA binding proteins (PABP and p100) affect the activity of PARN. We described a procedure to produce p100 in E. coli. We investigated the RNA binding properties of PABP and p100. Finally, we studied how PABP affected PARN activity.

MATERIALS AND METHODS

Preparation of competent cells

One ml of an overnight culture of the E. coli strain BL21(DE3) was added to 100 ml LB (Luria-Bertani broth). Cells were incubated at 37°C with shaking, until OD<sub>600</sub> reached 0.5. The flask was chilled on ice for 20 min and cells collected by centrifugation at 1200 x g for 5 min at 4°C. Cells were resuspended in 10 ml of ice-cold TSS solution (85% LB-medium, 10% PEG (wt/v), 5% DMSO (vol/vol), 50 mM MgCl₂ (pH 6.5)) and aliquoted before frozen in -70°C.

Transformation

The genes encoding the p100 and p100s proteins had been cloned into the pET-32 expression vector (Novagen) and the PABP-gene had been cloned into the pET-19b expression vector (Novagen) (Martinez, personal communication).
Competent cells (BL21(DE3)) were thawed on ice 1 h. 40 µl bacteria were added to 1.5 µl plasmid solution and the mixture was kept on ice for an additional 20-30 min before incubation at 42°C 40-45s. After incubation on ice for 2 min, 360 µl room-tempered LB medium was added and the bacteria were grown at 37°C for 1 h. The cultures were spread on dried plates containing ampicillin (50 µg/ml) and grown overnight at 37°C.

Expression of recombinant proteins in E. coli

To express p100, 50 ml over night culture was inoculated into 750 ml fresh LB media at room temperature. Carbenicillin (50 µg/ml) was used as selection. The culture was grown overnight at room temperature. Expression was induced at OD600 of 1.4 by the addition of 1mM isopropyl-β-D-thiogalactopyranoside (IPTG). The time of induction was 2 h at room temperature. The cells were collected by centrifugation at 5000 rpm for 20 min at 4°C.

Expression conditions for PABP was: LB medium containing carbenicillin (50 µg/ml final concentration), 1mM IPTG at OD 600 of 1.1, 2 h induction at 37°C before harvesting. PARN (full size 74 kDa) was expressed in TB containing 50 µg/ml carbenicillin (final concentration), induced with 1 mM IPTG (final concentration) at OD600 ranging from 1 to 1.5 and grown in 37°C 1 h 30 min before harvesting.

Expression of recombinant proteins in insect cells

p100 was expressed in insect cells using baculovirus as expression vector (Bac-To-Bac baculovirus expression systems (Life technologies)). The p100 gene had previously been cloned into the plasmid pFASTBAC HTb (Life technologies) and transformed into the E. coli strain DH10 BAC HTb (Life technologies) to produce bacmids (thankfully provided by Y. Ren). Sf9 cells were grown for two weeks in TC 100 medium (Life technologies) at 27°C using plastic culture flasks. Transfection with bacmids was made in a 6-well microtiter plate. The virus-containing supernatant was collected 48h later and stored in the dark at +4°C. The cells were lysed and protein expression was investigated by SDS-PAGE gel analysis. The supernatant was used to infect a large cell culture. After 72h, the cells were washed with 0.9% NaCl and collected by centrifugation at 4000 rpm for 10 min at 4°C. They were resuspended in 5 ml Extraction/Wash Buffer (20 mM Tris-HCl pH 7, 0.5 M KCl, 0.5% NP-40, 10% Glycerol, 2.5 mM Imidazole, 5 mM β-Mercaptoethanol) and stored at -70°C.

Extraction procedure

The harvested E. coli cells were resuspended in 20 ml Extraction/Wash buffer. The harvested insect cells were already frozen in extraction/wash buffer (see above). Samples from both E.coli and insect cells were sonicated 3x15s with a pause on ice between each burst. The cell extracts were centrifuged at 10 000 rpm for 20 min at 4°C and the supernatant saved for purification of p100, PABP and PARN respectively.

Purification

Talon His-bind metal affinity chromatography

Cell free extracts were passed through 0.45 µm filters (Whatman) before loaded on 2 ml gravity flow columns (Bio-Rad) packed with 0.5 ml Talon Metal Affinity Resin (Clonetech Laboratories). The column was washed with 15 ml extraction/wash buffer followed by 10 ml wash buffer (20 mM Tris-HCl pH 7, 0.5 M KCl, 10% Glycerol, 2.5 mM imidazole in the purification of PARN or 10 mM imidazole in the purification of p100 and PABP). The
protein was eluted by addition of 2 ml elution buffer (20 mM Tris pH 7, 0.5 M KCl, 10% Glycerol, 52 mM imidazole in the purification of PARN or 150 mM imidazole in the purification of p100 and PABP). Fractions of 0.5 ml were collected. Samples from input, flow-through and each fraction were analysed by SDS-PAGE.

Poly(A) sepharose chromatography

The identified fractions containing p100 or PABP from the Talon purification step was diluted 20-fold in Buffer D (10% Glycerol, 20 mM HEPES, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol (DTT), 100 mM KCl). pH 7 was used for PABP and pH 5 for p100. The dilution was loaded on a gravity flow column (Bio-Rad) packed with 0.5 ml poly(A) on sepharose (Pharmacia Biotech), equilibrated with 8 ml Buffer D. The column was further washed with 7 ml Buffer D before elution with 2 ml Buffer D (containing 1.0 M KCl) collected in 0.5 ml fractions. Samples from input, flow-through and each fraction were analyzed by SDS-PAGE.

7-methyl-GTP sepharose chromatography

The Talon purified fractions containing PARN were pooled and dialysed over night in Buffer D (containing 50 mM KCl at pH 7). The column matrix, containing immobilized 7-Me-GTP on sepharose (Pharmacia Biotech), was equilibrated with Buffer D (containing 50 mM KCl, pH 7) before the dialysed sample was loaded. The column was washed with 10 ml Buffer D (containing 200 mM KCl) and the protein eluted by increasing the KCl conc. to 2 M. 0.5 ml fractions were collected and analyzed by SDS-PAGE after TCA precipitation (see below).

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS gels were electrophorated in transfer buffer (192 mM glycine, 25 mM Tris-HCl, 20% (v/v) MeOH) to Immobilon membranes (Millipore) over night at 30 V at 4°C using a Mini trans-blot transfer cell (BioRad). The membranes were incubated in BLOTTO (5% non-fat dry milk (Semper) in Phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 8.9 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.3)) for 1 hour at room temperature. The primary anti-p100 antibody (diluted in BLOTTO 1:500) was added and the membranes shaken 2 hours at room temperature. The membranes were washed three times (1, 20 and 20 min) with BLOTTO. Secondary antibody (anti rabbit with horseradish peroxidase), diluted in BLOTTO 1:1000, was added and incubated with the membrane for 30 min at
room temperature. The membranes were washed in PBS with 0.5% Tween 20 for 5 min followed by four changes for 30 min. ECL reagents (Pharmacia) were mixed and immediately added to the membrane. Exposure to photographic film for 25 s to 2 min was performed.

**Determination of protein concentrations**

p100, p100s and PABP concentrations were determined using the Bio-Rad protein assay kit (no. 500-0006) and bovine gamma globuline (concentration 1.46 µg/µl) as a reference. The concentration of PARN was estimated by comparing the corresponding band with markers of known concentration on the SDS-PAGE gel.

**Preparation of RNA substrates**

RNA substrates, L3 (A30) capped at the 5’ end with m7GpppG (Pharmacia Biotech) or non-capped, were synthesized in vivo transcription using T3 RNA polymerases (Promega no. P208C) and plasmid pT3-L3 (A30) (10) digested with NsiI, as DNA template. RNA substrates were labeled either in their bodies or in their homopolymeric tails by inclusion of radioactively labeled mononucleotides during in vitro transcription (10,11). The specific radioactivities of the included radioactive mononucleotides were 40 Ci/mmol in the transcription mixture for body labeling (α-32P-UTP) or 5 Ci/mmol in the transcription mixture for tail labeling (α-32P-ATP). Transcribed RNA was purified according to Moore and Sharp (28).

**Determination of RNA concentrations**

The concentration of the RNA substrates were determined by the use of the relationship: (cpm of free nucleotide/molar amount of free nucleotide)=(cpm of RNA-sample/molar amount of RNA-sample). The counts were performed on the same day and the calculated concentration was corrected for the amount of the specific nucleotide in the RNA-sample.

**RNA-binding assay**

Proteins for RNA-binding assays were microdialyzed 45 min on a Nitrocellulose filter (0.025µm (Millipore) floating on the surface of Buffer D (10% Glycerol, 20 mM HEPES pH 7, 1.5 mM MgCl2 , 0.2 mM EDTA, 0.5 mM DTT, 100 mM KCl,) at +4°C. Conditions for in vitro RNA-binding were: 1.5 mM MgCl2, 2.5% (w/v) PVA (Sigma P-8136, Mw 10 000), 100 mM KCl, 0.2 units RNA guard (RNase inhibitor), 0.3-0.5 nM RNA substrate (32P-UTP labeled), 20 mM HEPES-KOH (pH 7), 0.1 mM EDTA, 0.5 mM DTT, 10% glycerol and protein (0.04-40 ng/µl)(0.5-500 nM). Reaction volume was 24 µl and incubations were performed at 30°C for 10 min. The RNA-binding capacity was investigated by loading 10 µl of the reaction mixture on a native gel containing 3% polyacrylamide and 0.5% agarose at 4°C. The gel was fixed in 10% acetic acid and dried 1 h before exposed over night to a PhosphorImager screen (Molecular Dynamics). The screen was scanned and the result was analyzed using the Image-Quant software (Molecular Dynamics).

**In vitro deadenylation**

The in vitro deadenylation reactions were performed in the same conditions as the RNA-binding assay (see above). Tail labeled RNA substrate was used. The total reaction volume was 12 µl. The final RNA-concentration was 0.8 nM-20 nM as indicated. PABP ranged from 5 to 20 nM as indicated and PARN was present at a final concentration of 0.3 nM. 1 µl of PABP was added and the reaction mixture was pre-incubated for 10 min at 30°C. 1 µl of PARN, dialysed against BufferD pH 7 before assayed, was added. After incubation for 1 to 12 min at 30°C (as indicated), the deadenylation was stopped.
by applying 1 µl of the mixture onto a Thin layer chromatography (TLC)-plate. The TLC plate was run in 0.4 M KH₂PO₄ buffer pH 3.5 to separate the free ATP:s from the substrate. The plate was dried before exposed over night to a PhosphorImager screen (Molecular Dynamics). The screen was scanned and the result was analyzed using the ImageQuant software (Molecular Dynamics).

RESULTS

Expression and purification of recombinant p100, PABP and PARN in *E. coli*

*E. coli* cells transformed with plasmids containing the human genes encoding p100 and PABP were analyzed for total protein content after induction. Fig 5 shows that the recombinant proteins were successfully over-expressed in the cells after growth in LB and IPTG induction for 2h. No recombinant proteins were seen in the non-induced controls.

Larger cultures producing p100 and PABP were grown, and the soluble fractions were purified by metal ion affinity chromatography (Talon, Clontech). The reason for this choice was that the recombinant proteins contain an N-terminal His-tag. The expression of soluble PABP after growing bacteria in LB medium at 37°C was successful (fig 6), but unfortunately we did not manage to get enough p100 in soluble form (fig 6). Most of the p100 was expressed as inclusion bodies under these conditions. Therefore, different expression conditions were tried for the cells to produce as much soluble p100 as possible (table 1). First, LB-medium was much more efficient than TB-medium. Second, slow growth at room temperature was better than faster growth at 30 and 37°C. Third, the best result was achieved when bacteria was left to grow over night at room temperature before inducing with IPTG. The concentration of IPTG did not have much influence on the result. p100 after purification by Talon His bind affinity chromatography at optimal conditions is shown in fig 7.

![Fig 5: Total content of recombinant PABP and p100 after induction. Coomassie stained SDS-gel. 10µl of 1 ml processed culture was applied to each lane. Lane 1: PABP-non-induced control. Lane 2: PABP after induction with IPTG (1 mM final concentration). Lane 3: p100 after induction with IPTG (1 mM final concentration). Lane 4: p100-non-induced control. Arrows indicate PABP and p100, respectively. The reason why the p100-band appears even above the 116 kDa marker is that p100 is fused to a 109 aa long thioredoxin tag when cloned into pET 32.](image)

![Fig 6: Amount of PABP and p100 in soluble fractions after purification by Talon metal ion affinity chromatography according to materials and methods. Silver stained SDS-gel. 5 µl loaded in each lane. Lane 1-6 represents PABP and lane 7-12 p100. Flow through (lane 1 and 7), wash (lane 2 and 8), eluted fractions (lanes 3,4,5,6 and 9,10,11,12). Arrows indicate PABP and p100 respectively.](image)
Table 1. Different expression conditions to produce p100 in a soluble form. The best conditions are indicated in bold.

<table>
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<th>Medium</th>
<th>37°C</th>
<th>30°C</th>
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<td>Concentration of IPTG</td>
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Table 1. Different expression conditions to produce p100 in a soluble form. The best conditions are indicated in bold.

Fig 7: Soluble part of p100 purified by Talon metal affinity resin after expression at optimal conditions (shown in fat text in table 1). Silver stained SDS gel. 5 µl loaded in each lane. Lane 1-4 represents elution fractions 1-4. p100 is indicated with an arrow.

However, the purity of the proteins after only one purification step using Talon His bind affinity chromatography was quite low, see figures 6 and 7. To purify the proteins further, the elution fractions were pooled, diluted into Buffer D (see Materials and Methods) and loaded on a poly(A)-sepharose column. We had reason to believe that PABP and recombinant p100 should bind efficiently to the resin in the column. The result is shown in fig 8. An improvement in purity was achieved (compare fig 6 and 7 with 8), although the proteins did not become 100% pure after this second step. A western blot was made to determine that the purified p100 really was the correct protein. Fig 8C shows that the antibody also detected some bands that migrated faster, suggesting that these bands were degradation products of p100 as result of proteolytic activity. Attempts to purify p100 further using AMP-sepharose, Blue sepharose and Heparin sepharose were unsuccessful (data not shown).

PARN (74kDa) was expressed in *E. coli* (done by project worker P.Nilsson in the lab) and purified by two affinity chromatography steps. First, Talon metal ion affinity matrix was used followed by 7-Me-GTP sepharose chromatography. Fig 10 shows that an almost 100% pure preparation of PARN (74kDa) was obtained by this procedure.
Fig 8: Analysis of PABP and p100 after purification on poly(A) sepharose column. A. PABP analyzed on silver stained SDS gel. 5 µl was loaded in each lane. Flow through (lane 1), wash (lane 2), elution fractions (lanes 3, 4, 5 and 6). B. Fraction 2 of p100 after poly(A) sepharose column (silver stained SDS gel). C. Western blot of the same fraction. The membrane was probed with anti-p100 antibody, 1:500. Notice that some bands further down in gel (B) also are visible on the western blot (C). That suggests that those bands are degradation products of p100.

Fig 9: Purification in two steps of recombinant PARN (74 kDa). A. Silver stained SDS gel showing PARN after Talon metal ion chromatography. Lane 1-4 represents elution fractions 1-4. 10 µl loaded in each lane. B. PARN after 7-Me-GTP sepharose chromatography. Silver stained SDS gel. 10 µl loaded in each lane. Lane 1 shows input consisting of eluted fractions after Talon purification (see A). Flow through (lane 2), wash (lane 3), eluted fractions (lanes 4, 5, 6 and 7).

Expression of recombinant p100 in insect cells with baculovirus

p100 was also expressed in insect cells using bacmids (thankfully provided by Y. Ren). Unfortunately, after the first step, when the cells were transfected with bacmids to produce virus particles, no protein of right size was detected neither on SDS silver gel nor western blot with anti-p100 antibody (data not shown). Then the virus particles were used to infect a larger cell colony, and the soluble fraction was purified by Talon His bind affinity chromatography. An unexpected band close to the 66 kDa marker was identified,
which was absent in the non-infected control (fig 10). Unfortunately, another attempt to express p100 in insect cells was not within the timeframe of this project.

**Fig 10:** Talon His bind affinity chromatography of p100 expressed in insect cells using baculovirus expression vector. Silver stained SDS gels. 10 µl was loaded in each lane. A. p100. Input (lane 1), flow through (lane 2), wash steps (lane 3 and 4), elution fractions (lanes 5, 6, 7 and 8). * Band around 66 kDa not present in the negative control. # Expected position of p100. B. Negative control (non infected cells). The numbers refer to the same fractions as in A.

PABP binds in vitro transcribed RNA. p100 expressed in E. coli does not show any binding property. p100s expressed in insect cells with baculovirus binds poorly to RNA.

To check the poly(A) binding capacity of the purified p100 and PABP proteins, electrophoretic mobility shift assays were performed according to materials and methods. In this assay RNA/protein complexes can be detected. They migrate slower than naked RNA, and can be visualized as bands higher up on the gel. The pattern is called a gel shift. Gel shift experiments shown in fig 11 revealed that PABP binds the RNA substrate, while p100 did not form a stable RNA/protein complex.

Material already expressed in insect cells with baculovirus vector, and purified on Ni²⁺ column by Y.Ren (a PhD student in the lab) was also tested. This shorter version of p100 (p100s) was pure and could also be detected on western blot (fig 12). Unfortunately this protein (which had been stored in cold room (4°C) for three years) did not give a distinct shift either, even though a small tendency of shifting was recognized at 310 nM (fig 12).

**Fig 11:** Electrophoretic mobility assays with PABP (A), and p100 (B) expressed in E. coli. Reactions were performed as described in materials and methods. Capped substrate (0.3 nM in A and 0.5 nM in B) was used. N=Negative control without protein. Numbers indicate amount of protein in nM.
Fig 12: Analysis of the purity and RNA-binding property of p100s expressed in insect cells using Baculovirus expression vector A. Pure p100s analyzed on SDS gel (silver stained). Lane 1-3 shows three different preparations of p100 after Ni²⁺ affinity chromatography. 10 µl was loaded in each lane. B. Western blot of the same gel. The membrane was probed with anti-p100 antibody (1:500). Arrows indicate p100s. C. Gel shift experiment with p100s (the prep from lane 2 in A and B) according to the conditions described in materials and methods. Capped substrate (0.5 nM) was used. N=Negative control without protein. Numbers indicate amount of protein in nM.

PABP affect deadenylation

We decided to study the effect of PABP on PARN's deadenylating activity. This choice was made because PABP was the only recombinant protein in this study that showed a clear RNA binding capacity. In a previous study (7) it was seen that at 100 mM salt concentration, PABP showed an inhibitory effect on PARN, but at 10 mM salt concentration, the effect was slightly stimulatory. To verify these results, we repeated these experiments in the in vitro system described in materials and methods using tail-labeled mRNA. Fig 13 shows the result of a typical TLC experiment, and the data of that experiment is plotted in fig 14.

Surprisingly enough, when the substrate concentration was raised from 0.8 nM to 20 nM, and the experiment was repeated in Buffer D containing 100 mM KCl, PABP had a small activating effect at 5 nM concentration, but became inhibitory when the concentration was raised to 20 nM (fig 15).

From the results presented in figures 14 and 15 we conclude that:

1. PARN deadenylates more efficiently in 100 mM KCl than in 10 mM.
2. PABP is inhibitory in both 100 mM and 10 mM KCl at low substrate concentration.
3. At high substrate concentration, PABP showed an activating effect on PARN. However, when the PABP concentration was raised keeping the substrate concentration high, the initial rate of the enzyme was lowered.

Thus, the relative concentration of PABP, PARN and RNA affect the outcome.
**Fig 13:** The effect of PABP on PARN's activity visualized by a TLC experiment. The free AMP's move with the buffer front and are shown as spots at the top of the filter. The substrate and RNA-body product left after the reaction is shown as spots at the bottom of the filter. Experimental conditions: 0.8 nM mRNA substrate, 0, 5, 20 nM PABP respectively and 0.3 nM PARN. 10 mM KCl in BufferD was used in (A) and 100 mM KCl in (B).

**Fig 14:** Percent radioactivity as free AMP as a function of time. The diagrams plotted are the results of the experiments in fig. 13. It can clearly be seen that the overall activity of PARN is lower when 10 mM KCl is used compared to 100 mM. Further, PABP has an inhibitory effect in both cases.
DISCUSSION

We showed that PABP, bound to in vitro transcribed RNA, inhibited the action of PARN in both 100 mM and 10 mM KCl concentration (fig 14). This is a different result than in the previous study (7), where a stimulatory effect could be seen in the case with low salt concentration. We have to emphasize that this is just a preliminary result. Repeated experiments have to be made to draw any final conclusions. We could conclude, however, that a lower concentration of PARN compared to substrate has to be used to calculate any kinetics of the reaction. A rule of thumb used when TLC data is analyzed is that no more than 10% of the total radioactivity should be in the form of free AMP:s. As seen in fig 13 and 14 this was only the case after 1 min at 10 mM KCl. The enzyme concentration was so high that after a while, the RNA body was degraded as well. When the substrate concentration was raised from 0.8 to 20 nM, initial rates could be calculated after 1 min (fig 15). At these conditions, PABP had a significant rate activating effect when provided at 5 nM concentration. Repetitions of the experiments have to be done to draw any final conclusions, but here the preliminary results show that in some “window” PABP has an activating effect on the initial rate of PARN. That finding is interesting when taken what is previously found in yeast into account (3,4,5).

We established a procedure to produce p100 in a soluble form in E. coli. When grown over night at room temperature, the cells have the opportunity to reach an OD600 well above 1, enough to produce a large amount of recombinant protein. When induced at room temperature, the p100 produced obviously had a chance to stay in soluble form without forming inclusion bodies to a great extent. Most of the contaminating proteins after the second purification step by poly(A) sepharose affinity chromatography (fig 8B and C) were detected as degradation products of p100. Therefore, the conclusion could be made that the two purification steps were enough to purify p100. To reduce the action of proteases during the purification procedure, protease inhibitors can be added after cell lysis.

The reason why we didn’t get any p100 successfully expressed in insect cells with baculovirus might be that the bacmids were not intact or that the cells didn’t take up the bacmids at all. The solution would
have been to produce new bacmids, but that lay beyond the timeframe of this project. It was a bit puzzling, however, that a band around 66 kDa appeared after Talon purification (fig10A). It could correspond to PARN (74 kDa) as a result of wrong bacmid being used, but when examining the gel in fig 10, that band was not present in the input (lane 1). Therefore, most likely it is a contamination from the column. Insect cells did not produce background proteins that bound to the Talon matrix to the same extent as the background from E. coli. Therefore we found that only one purification step is needed when the recombinant protein is expressed using the eucaryotic host.

p100 expressed in E. coli did not bind to the in vitro transcribed RNA at all. The reasons for this can be several. First, post-translational modifications are missing in a procaryotic expression system like E. coli. These modifications, for example phosphorylation, can be crucial for the RNA binding capacity of p100. Second, the protein can be wrongly folded and therefore not work properly. Third, if p100 is naturally active in an oligomeric form, the protein can be present as an inhomogeneous form after expression, a so-called “conformational hell” (29). Fourth, p100 cloned in the pET 32 plasmid contains a 109 amino acids long thioredoxin tag in the N-terminal. This tag might also affect the RNA-binding capacity. It was a bit inconsistent that p100 bound to the immobilized poly(A) in the second purification step, but not to the substrate in the gel shift assays. It can be explained by the fact that at pH 5, the protein most likely has a positive net-charge and therefore interacts electrostatically with the negatively charged poly(A). The gel shift experiment was however tested in pH 5 as well, without any positive outcome.

We managed to get a tendency of a shift when highest concentration of p100s was used (fig 12C). Previous results (Martinez, personal communication) showed that p100s gave a visible gel shift at a lower concentration and a more distinct band higher up on the gel. The prep of p100s had been stored in the cold-room for almost three years, and that might explain the low RNA-binding capacity. The result was a bit puzzling, because almost no degradation products were seen on the SDS-silver gel (fig 12A). Further, the Western blot in fig 12B verified that the band corresponded to the right protein. The reason why we didn’t continue with this shorter version of p100 was that a clear band (corresponding to the complex between protein and RNA) must be seen to calculate the K_D- value of the interaction. The K_D-value is of importance when the conditions for the in vitro deadenylation are to be chosen.

It would be very beneficial if one could understand the roles of RNA-binding proteins in RNA metabolism. This degree project was a step in this direction, because the effect of PABP on PARN activity was investigated. The choice of relative concentrations of the different components in the in vitro deadenylation turned out to be important for the result. It is not unlikely that p100 might have an additional role on PARN in mammals, as the proteins caf1 and ccr4 in yeast (9). Therefore, investigation of p100, and its effect on deadenylation is a thing to be dealt with more in the future.
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