Thesis for the Master's degree in biochemistry

Heterologous expression in bacterial and mammalian cells of PRNPIP: a protein that interacts with the prion protein PrP^C

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Abstract

Transmissible spongiform encephalopathies (TSEs) are fatal, neurodegenerative diseases in human and animals, caused by misfolding and aggregation of the host-encoded prion protein (PrP). The functions of PrP and the basis of these diseases are still under debate. In the present study characteristics of a possible PrP binding parter (prion protein interactor PINT), have been studied.

Four ovine PINT constructs were used for expression of protein in prokaryotic (*E.coli*) and eukaryotic cell lines (murine neuroblastoma cells (N2a) and human neuroblastoma cells (SH-SY5Y)). Overexpression of PINT-His_6 protein in *E.coli* led to the formation of insoluble inclusion bodies, which did not depend on growth temperature (16- 37ºC was used). Transient and stable transfections in mammalian cells, and fluorescence protease protection (FPP) assay allowed a detailed study of the cellular localization of PINT protein. PINT was shown to be localized both in cytosol and in the cell nucleus; some of the cytosolic PINT-DsRed-Ex appeared also to be bound to membranes. It was shown that PINT-DsRed-Ex is resistant to trypsin digestion, but not to proteinase K. Application of unboiled lysates on the gel showed that PINT-DsRed-Ex tends to form oligomers in transiently transfected cells. To study the possible interaction between PINT and PrP, immunoprecipitation assay was performed. Lysates of transfected N2a-PrP-EGFP cells were added anti-RFP (directed against PINT-DsRed-Ex) or P4 (directed against PrP) antibodies and antibody binding proteins such as Protein A or Protein G. Signals were detected with antibodies directed against both proteins. Immunoprecipitation assay confirmed weak interaction between PrP and PINT protein.

Abbreviations

ALP	alkaline phosphatase
Amp	ampicilin
ATP	adenosine triphosphate
BHK cells	baculovirus- transducted baby hamster kidney cells
bp	base pair
BSA	bovine serum albumin
CBB	Coomasie Brilliant Blue
CBP	carbohydrate binding protein
Chl	chloramphenicol
Co-IP	co-immunoprecipitation
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
dNTP	deoxyribonucleotide
ECL	enhanced chemiliuminescence
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
ER	endoplasmic reticulum
FBS	foetal bovine serum
FPP	fluorescence protease protection
GB1	protein G domain
GFAP	glial fibrillary acidic protein
GPI	glycosyl-phosphatidylinositol
GST	glutathione-S-transferase
HRP	horse radish peroxidase
HSP	heat shock protein
ICH	immunohistochemistry
IDA	iminodiacetate
IP	immunoprecipitation
IPTG	isopropyl β-D-1-thiogalactopyranoside
kDa	kilo daltons
LB	Lysogeny broth medium

MBP	maltose binding protein
MEM	minimum essential medium
mRNA	messenger RNA
MW	molecular weight
N-CAM	cell adhesion molecule
NLS	nuclear localization signal
NTA	nitrilotriacetic acid
NusA	*E.coli* protein
ORF	open reading frame
PBS	phosphate buffered saline
PBS-T	PBS-tween
PCR	polymerase chain reaction
PINT	prion interactor
PNGase F	peptide N- glycosidase F
PrP^{C}	the cellular form of prion protein
PrP^{SC}	disease related form of prion protein
psi	pound-force per square inch
RFP	red fluorescence protein
RT	room temperature
SDS	sodium dodecyl sulphate
Tg	transgenic
TBS	tris buffered saline
TBS-T	TBS-tween
TRX	thioredoxin
TSEs	transmissible spongiform encephalopathies
ZZ	IgG binding protein

I. Introduction

Transmissible spongiform encephalopathies (TSEs), also known as prion diseases, are fatal, neurodegenerative diseases in human and animals caused by prions. TSEs include scrapie, bovine spongiform encephalophaty, kuru, Creutzfeldt-Jacob disease and some other disorders (Table 1). Prion diseases can be classified in to genetic, infectious, or sporadic, all of which involve modification of the cellular prion protein (PrP^C).Genetic forms were traced to several mutations in PrP^C, while, infectious forms were traced to previous infections. In contrast, most cases of prion diseases, known as sporadic forms, can neither be attributed to a previous infection nor mutation in a gene, and are supposed to occur by stochastic conversion of PrP^C into abnormal form (reviewed by Prusiner, 1998). The normal function of PrP is still under discussion; the pathogenesis of prion disease requires its expression (Prusiner et al., 1993) and is often accompanied by accumulation in the brain of an abnormal isoform (PrP^{SC}).

1. Cellular prion protein (PrP^C)

1.1 Structure of prion protein and some characteristics

PrP^C is a soluble, glycosyl-phosphatidylinositol (GPI) anchored protein of 256 amino acids (sheep protein) with a molecular mass of 33-40 kDa, or 27 kDa in its unglycosylated form. This protein is highly conserved. Structurally, the protein has 5 glycine/proline rich octapeptide repeat regions, two strongly hydrophobic segments that can span the phospholipid layer, two N-linked glycosylation sites, a single disulphide bond, and signalling peptides at both termini (Bazan et al., 1987; Stahl et al., 1987).

PrP exists in two conformational isoforms: cellular- normal (PrP^C) and disease associated-scrapie (PrP^{SC}) isoform. The isoforms differ just three-dimmensional folding with different content of secondary structures conformation. PrP^C is contains about 40% α-helical structures and less than 10% β-sheet. Yet, the content of β-sheet structures increases up to 60% with con-comitant decrease in α-helical structures in the misfolded disease-associated form of the protein. (Martins, 1999) (Figure 1). Cellular prion protein is easily degraded by proteinase K, while abnormal form is partly resistant to proteinase K treatment and physiol-chemical

manipulations such as, high temperature, formaldehyde treatment, UV and X-ray irradiation (reviewed by Harris, 2003; Prusiner, 1998).

PrP^{C} internalization is mediated either by clathrin-coated pits or by caveolae-like membranous domains. However, both pathways seem to require an additional factor X to convert PrP^{C} into PrP^{SC} (Campana et al., 2005).

Table 1: The prion disease (Prusiner, 1998)

Disease	Host	Mechanism of pathogenesis
Kuru	Fore people	Infection through ritualistic cannibalism
iCJD	Humans	Infection from prion-contaminated HGH, dura mater grafts, etc.
vCJD	Humans	Infection from bovine prions?
fCJD	Humans	Germ-line mutations in PrP gene
GSS	Humans	Germ-line mutations in PrP gene
FFI	Humans	Germ-line mutation in PrP gene (D178N, M129)
sCJD	Humans	Somatic mutation or spontaneous conversion of PrP^{C} into PrP^{Sc}?
FSI	Humans	Somatic mutation or spontaneous conversion of PrP^{C} into PrP^{Sc}?
Scrapie	Sheep	Infection in genetically susceptible sheep
BSE	Cattle	Infection with prion-contaminated MBM
TME	Mink	Infection with prions from sheep or cattle
CWD	Mule deer, elk	Unknown
FSE	Cats	Infection with prion-contaminated bovine tissues or MBM
Exotic ungulate encephalopathy	Greater kudu, nyala, oryx	Infection with prion-contaminated MBM

iCJD, iatrogenic CJD; vCJD, variant CJD; fCJD, familial CJD; sCJD, sporadic CJD; GSS, Gerstmann–Sträussler–Sheinker disease; FFI, fatal familial insomnia; FSI, fatal sporadic insomnia; BSE, bovine spongiform encephalopathy; TME, transmissible mink encephalopathy; CWD, chronic wasting disease; FSE, feline spongiform encephalopathy; HGH, human growth hormone; MBM, meat and bone meal.

Resistance of the one species to infection with prion particles from another was also observed (Kocisko et al., 1995). Mice are normally resistant to hamster or human prions, and they become ill only when hamster/human transgene is introduced (hamster/human PrP gene is microinfected into fertilized murine embryos) (Scott, 1989; Telling et al., 1995); these findings argue that a species-specific molecule participates in prion formation.

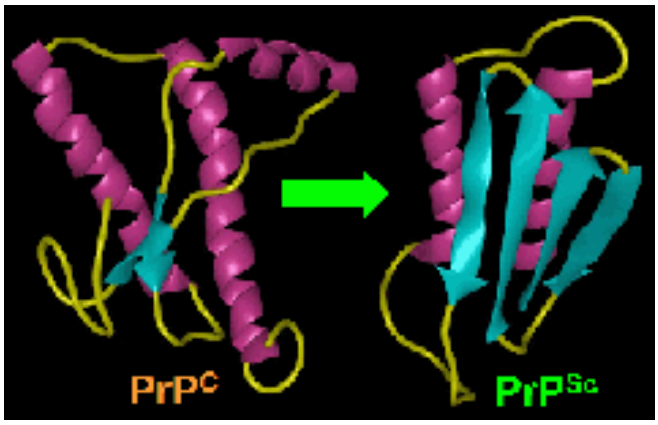


Figure 1: Two isoforms of secretory prion protein (α-helix in red, β-sheet in blue).
(Taken from http://molvirology.huji.ac.il/research5.htm**)**

1.2 Functions of cellular prion protein

The suggested physiological functions of prion protein are contrasting and include: function as a membrane receptor, regulation of apoptosis (Chiarini et al., 2002), carrier or binding protein for copper and/or zinc ions, effector in signal transduction mechanisms, protection from oxidative stress, neuritogenesis, regulator of synaptic transmission and transcription factor (reviewed by Campana et al., 2005; Griffoni et al., 2003). All these functions are supposedly achieved through the interaction with other proteins at various localizations in the cell (Table 2).

1.3 Biosynthesis, different isoforms and cellular localization of PrP

Like other membrane proteins, PrP^C is synthesized in the rough endoplasmic reticulum (ER) and travels through the Golgi apparatus to the plasma membrane. During its biosynthesis cellular prion protein undergoes a number of posttranslational modifications, such as cleavage

of the amino N-terminal signal peptide, addition of N-linked oligosaccharide chains, formation of a disulphide bonds and attachment of GPI anchor (Harris, 2003), which attach PrP to the outher leaflet of the plasma membrane. At the ER membrane, the prion protein can be synthesized in several topological forms: one that is fully translocated (^{sec}PrP) (located on plasma membrane) and two transmembrane forms; ^{Ctm}PrP and ^{Ntm}PrP, with the C-terminus facing ER lumen or cytosol, respectively (reviewed by Griffoni et al., 2003; Hegde et al., 1998). Mironov and co-worke (Mironov et al., 2003) demonstrated that in neurons PrP^C can be localized on all biosynthetic and endocytic transport membranous structures, and is expressed with the same frequency on presynaptic and postsynaptic membranes and in synapse, but almost no was found in synaptic vesicles. In a subset of neurons in the hippocampus and thalamus PrP is located predominantly in cytoplasm (Mironov et al., 2003).

The presice cellular location for the transformation of PrP^C to PrP^{SC} is still unknown. Campana and co-workers (Campana et al., 2005) concluded, that both, the plasma membrane and ER, are important, but they might be differently involved in prion formation. It is most likely, that the first contact between the physiological and pathological forms of PrP occurs at the plasma membrane, and the subsequent contact could occur either directly on the plasma membrane or after internalization. Campana and colleagues also reviewed the possible involvement of caveolae and clathrin-coated pits in internalization of PrP^C.

1.4 Factor X

At the beginning factor X was suggested to explain the conversion of PrP^C to PrP^{SC} (it was unknown what kind of molecule it might be), because this conversion was difficult to achieve *in vitro* (Prusiner, 1998); indeed, just recently it has been possible to generate the infectious agent *in vitro* (Legname et al., 2004).

Studies performed with transgenic (Tg) mice provided evidence about existence of protein X, which is involved in the conversion of the normal cellular prion protein into the scrapie isoform and which can function as a molecular chaperone in the formation of PrP^{SC} (Telling et al., 1995). Transgenic mice expressing human prion proteins genes were inoculated with brain extracts from humans with prion disease. Although the mice expressed high levels of human PrP^C, they were resistant to human prions, and only upon ablation of mouse PrP gene they became susceptible to human prions. Scott and colleagues (Scott et al., 1989) worked with a Tg mouse expressing hamster PrP; their findings indicate that the PrP gene modulates

scrapie susceptibility and incubation times. A species barrier between hamster and mice is maintained by differences in the primary structure of the PrP molecules of the donor and recipient host (14 amino acid substitutions out of 254) (Scott et al., 1989). By introduction of various substitutions in PrP protein it was demonstrated that binding of PrP^C to protein X is likely to occur through specific side chains of amino acids located on the C-terminal of PrP^C (Kaneko et al., 1997; Yehiely et al., 1997). Yehiely and colleagues postulated that PrP^C first binds to protein X, and then PrP^{SC} binds to PrP^C resulting in a ternary complex (if PrP^C and PrP^{SC} are monomers or dimers are unknown).

Propositions that factor X is a protein, was required to explain the prion species barrier, but lately it was also suggested that factor X might be a RNA (Gabus et al., 2001; Weiss et al., 1997) or DNA (Cordeiro et al., 2001; Nandi et al., 1999) molecule. Cordeiro and colleagues demonstrated that some nucleic acid sequences bind with high affinity to the murine PrP converting it to the β-sheet isoform, and that binding is sequence specific. They claimed that DNA stabilizes a soluble β-sheet structure, and that a macromolecular complex of prion-DNA may act as an intermediate for the formation of the growing fibber. At the same time, Gabus and coworkers showed that the prion protein has RNA binding properties (Gabus et al., 2001).

The nucleic acid can be the entire part of factor X or only a part of it. Supattapone and colleagues (Supattapone et al., 1999) demonstrated that polyamines (known to interact with nucleic acids) eliminate PrP^{SC} from scrapie infected neuroblastoma cells.

1.5 Protein-protein interactions (in general)

Protein-protein interactions refer to the association of protein molecules and the study of these associations from the perspective of biochemistry and networks. Interactions between proteins are important for many biological functions. Specific interactions are operative in almost every level of cell function, in the structure of subcellular organelles, the transport machinery across the various biological membranes, packaging of chromatin, the network of sub-membrane filaments, muscle contractions, signal transduction from the exterior of a cell to the inside , and regulation of gene expression, to name a few.

Protein-protein interactions can be classified according to the function of the interaction, or by the time-scale of the interaction, which are related. Proteins might interact for a long time to form part of a protein complex, a protein may be carrying another protein (for example, from cytoplasm to nucleus or vice versa in the case of the nuclear pore importins), or a protein

may interact briefly with another protein just to modify it (for example, a protein kinase will add a phosphate to a target protein).

Four different types of protein complexes are possible (reviewed by Jones et al., 1996): homodimeric (they are usually permanent and optimized) and heterodimeric protein complexes (these complexes can have the same properties as homodimeric, but can also be nonobligatory, being made and broken according to the environment or external factors and involve proteins that must also exist independently), enzyme-inhibitor complex, and antibody-protein interactions. Several fundamental properties that characterize a protein-protein interface are: size and shape of interfaces; electrostatic complementary between surfaces; residue interface propensities; hydrophobicity including hydrogen bonds (interaction through hydrophobic patches on protein surfaces most common, but polar interactions are possible too); segmentation and secondary structure; and conformational changes on complex formation (Jones et al., 1996).

1.6 Interaction of PrP^c with other proteins

Cellular prion protein is a quite well characterized protein, but its functions still remains enigmatic. Search for molecules that are able to interact specifically with the prion protein is a powerful tool in the hope to identify interactors that play an important role in the life of prions, or that could be developed into potent prion diseases therapeutics. Binding partners can help to elucidate functions of PrP, different subcellular localizations and the basis of diseases caused by the prion protein.

As mentioned before, prion protein can exist in different topological forms: secretory form, which is located on plasma membrane attached by a GPI anchor; 2 transmembrane forms with C- or N-terminal facing the cytosol. Some PrP is also found in the cytosol. There are a number of structural features within PrP that might allow it to interact with other proteins. Near the middle of the molecule, there is an amphiphatic helix (Bazan et al., 1987); in other proteins such helices take part in protein-protein interactions (Kaiser et al., 1984). The GPI anchor can also take part in interactions.

The prion protein has been reported to bind to or interacts with a large number of diverse proteins (Table 2), both intra- and extra cellular.

The first interacting proteins identified were a pair of PrP ligands: pli 45 (later identified as GFAP (glial fibrillary acidic protein) and pli 110 from hamster brain extract (Oesch et al., 1990).

Later, a number of other putative PrP^C binding proteins have been identified using the yeast two- hybrid system (PrP was used as bait to search mouse or hamster brain cDNA; both bait and pray were derived from the same host in order to insure putative interactions); these include the anti-apoptotic protein Bcl-2 (Kurschner et al., 1995); the synaptic vesicle marker synapsin 1, the adaptor protein Grb2 and the prion interactor 1 (PINT1) (Spielhaupter et al., 2001), for which no function was determined; the cellular chaperone heat shock protein- Hsp 60, Hsp 104 and GroEL (Edenhofer et al., 1996); the 37- kDa/67- kDa laminin receptor (Gauczynski et al., 2001; Hundt et al., 2001). The interaction in yeast does not prove that this association exists in mammalian cells. In order to prove *in vivo* interaction of PrP^C with other proteins, Co-IP was performed with recombinant and authentic proteins expressed in different mammalian cell lines. And all interactions were confirmed independently of yeast.

PrP^C also binds to Nrf2 transcription factor (NF-E2 related factor) and Aplp1 (amyloid precursor like protein) (Yehiely et al., 1997); different therapeutics (reviewed by Gauczynski et al., 2001); dystroglycan complex and synaptophysin, a synaptic vesicle protein (Keshet et al., 2000); laminin in PC 12 cells and rodent primary neurons, and this interaction promotes neurite growth in these cells (Graner et al., 2000). Caveolin-1 a principal component of the caveolar coat and a regulator of caveolae-dependent signaling and endocytosis binds to prion protein and FYN kinase in neuronal cells (Massimino et al., 2002; Mouillet-Richard et al., 2000); CBP70 molecule (nuclear lectin-carbohydrate binding protein (CBP), most of witch are plurifunctional and regulates various physiological activities) co-localizes with PrP in the nucleus of NB4 cells. Immunoprecipitation revealed that these proteins are co-precipitated with PrP^C, and interact via sugar dependent binding moiety (Rybner et al., 2002). By using an in situ crosslinking method that maintains the microenvironment of PrP^C Schmitt-Ulms and colleagues (Schmitt-Ulms et al., 2001) demonstrated that neural cell adhesion molecules, N-CAMs, interact with prion protein and that the interaction occurs through the amino acid side chains, since enzymatic removal of N- linked sugar moieties did not disrupt the complexes. Nuclear riboprotein A2/B1 (functions in various aspects of RNA metabolism) and aldolase C are also proteins that interact with prion protein *in vivo* and vitro (Strom et al., 2006). Aldolase is an enzyme which catalyses the aldol reaction: the substrate, fructose 1.6-bisphosphate (F-1.6-BP) is broken down into glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (this reaction is a part of glycolysis).

Table 2: Proteins interacting with cellular PrP

Molecule	Function	Possible binding site	Method of identification
Aldolase C	Enzyme(aldolase reaction)	(*)	Protein overlay assay
Aplp1	Regulation of neurite growth	Cell surface	PrP-AP screening
Bcl2	Apoptosis	(*)	Yeast-Two-Hybrid screening
Caveolin 1	Signaling	Caveolae raft	Antibody-mediated cross linking
CBP70	Plurifunctional	Nucleus	Immuniprecipitation
Dystroglycan complex	Links excracellular matrix to cutoskeleton	Cell surface	Detergent-dependent immunoprecipitation
FYN kinase	Enzyme(transfer of phosphate)	(*)	Antibody-mediated cross linking
GFAP	protective barrier in the cell	(*)	Ligand blots
Grb2	Intracellular signal transduction	Intracellular vesicles	Yeast-Two-Hybrid screening
GroEL	Chaperone	(*)	Yeast-Two-Hybrid screening
Hsp60	Chaperone	(*)	Yeast-Two-Hybrid screening
Laminin	Neurogenesis	Cell surface	Ligand radiolabeling
Laminin receptor (37/67 kDa)	Laminin binding	Cell surface	Different assays
N-CAMs	Adhesion	Caveolae-like domain	Formaldehyde cross linking
Nrf2 (Transcription factor)	Apoptosis inhibitor	Unknown	PrP-AP screening
Nuclear riboprotein A2/B1	RNA binding	(*)	Protein overlay assay
PINT1	Unknown	Unknown	Yeast-Two-Hybrid screening
pli 110	(*)	(*)	Ligans blots
STI1	HSP related	Cell surface	Complementary hydrophaty
Synapsin Ib	Regulation of neurotransmitter	Intracellular vesicles	Yeast-Two-Hybrid screening

(*) no information given

Finally, complementary hydropathy, a technique that lets to generate a hypothetical molecule which is a complementary mirror image of the target protein, identified a 66 kDa protein, the stress inducible protein STI-1, with binds to PrP^C on the cell surface and may be involved in neuroprotection (Martins, 1999; Zanata et al., 2002).

2. PINT, a possible prion interactor

The only evidence that the PINT protein (also called PRNPIP) might interact with cellular prion protein was published by Spielhaupter and co-workers (Spielhaupter et al., 2001). Initially, they used yeast-two-hybrid screen based on Gal4 system. Murine PrP^C (amino acids 23-231) was fused in the frame to Gal4-binding domain as bait to search a mouse brain cDNA expression library fused to the Gal4-transactivation domain, and several interaction partners were sequenced and identified. One of them, not characterized before, was named PINT1 (prion interactor 1).

To confirm the interaction of the PINT1 with PrP by a method completely unrelated to the yeast interaction trap, Spielhaupter and co-workers used a vaccinia virus-based expression system in mammalian cells. The interaction led to high expression of the desired protein in the cytoplasm. Lysates were subjected to co-immunoprecipitation and the co-precipitated PrP was detected by immunoblot.

For detection of a possible region of interaction, PINT1 was subjected to binding assay with two truncated constructs of PrP (N-terminal construct (amino acids 23-100) and C-terminal construct (amino acids 90-231)), and the ability to interact was tested in BHK cells by co-immunoprecipitation. Results revealed that PINT1 interacts just with C-terminal part of prion protein.

To identify the organs where PINT1 is expressed, Spielhaupter and colleagues examined an mRNA dot blot representing different murine tissues by probing it with a PINT1 DNA fragment. A strong expression was detectable in brain as well as in heart, thyroid, and testis. A faint signal was visible in muscle cells, liver, pancreas, and kidney. No transcripts were detectable in embryonic development stages and in ovary, uterus, eye, lung, and spleen. The tissue distribution of PrP mRNA has been published previously by Yehiely and colleagues (Yehiely et al., 1997). They reported a high expression of PrP^C in brain, lung, kidney, and heart, and a weaker expression was seen in liver, spleen, and some other organs. The pattern of PINT expression partially overlaps with the known mRNA tissue distribution of PrP^C

indicating co-expression of proteins in these organs, and that these two proteins physically can interact with each other.

The complete open reading frame of murine PINT1 was not fully identified. Spielhaupter and colleagues were working with a part PINT1 of protein (120 amino acid long), which did not have an original start codon.

2.1 Sequence of PINT protein

In the present study PINT protein cloned from sheep was studied. The sequence of this protein is presented in Figure 2. PINT from sheep is a 259 amino acid protein with size of about 30 kDa. This protein has a very similar sequence to the homologous from man, rat and mouse (Figure 2): prion protein interacting protein from man shows 99% sequence similarity (has 2 substitutions), rat protein shows 98% sequence similarity (with 4 substitutions) and mouse PRNPIP1 has 97% sequence similarity (7 substitutions) relative to PINT protein from sheep. The sequence has a striking similarity to some other hypothetical proteins from chimpanzee and rhesus monkey, which have an additional 28 amino acid sequence and one amino acid substitution at the position 58 (data not shown) (for distance tree of homologous proteins see Appendix 4).

PINT protein contains several conserved domains, which are common to variety of proteins (Figure 3).

Exonuc_X-T conserved domain is common to different exonucleases. This family includes a variety of exonuclease proteins, such as ribonuclease T (ribonuclease is a nuclease that catalyzes the breakdown of RNA into smaller components. They can be divided into endonucleases and exonucleases. RNase T is an exoribonuclease that initiates attack at the 3' hydroxyl terminus of tRNA and releases AMP in a random mode of hydrolysis. The possible involvement of RNase T in end-turnover of tRNA and in RNA metabolism in general is discussed by Deutscher and colleagues (Deutscher et al., 1984)) and the epsilon subunit of DNA polymerase III (main prokaryotic polymerase that are responsible for DNA elongation).

EXOIII is an exonuclease domain in DNA-polymerase alpha and epsilon chain, ribonuclease T and other exonucleases (exonucleases are enzymes that cleave nucleotides one at a time from an end of a polynucleotide chain. These enzymes hydrolyze phosphodiester bonds from either the 3'- or 5'-terminus of polynucleotide molecules).

KapD domain, also found in inhibitor of the KinA pathway to sporulation, is predicted exonuclease.

PolC is alpha subunit of DNA polymerase III (gram-positive type), responsible for DNA replication, recombination, and repair.

DNAQ- DNA polymerase III, epsilon subunit and related 3'-5' exonucleases, takes part in DNA replication, recombination, and repair.

PINT protein sequence shows 30%-40% similarity to different nucleases (exoribonuclease, DNA polymerase and others). It is likely that the PINT protein might also have nuclease activity, but this has to be confirmed by further studies.

A) PINT reading frame (sheep)

```
1   MLAPLQTGAA RFSSYLLSRA RKVLGSHLFS PCGVPEFCSI STRKLAAHSF GASMAAMMSF  60
61  PPQRYHYFLV LDFEATCDKP QIHPQEIIEF PILKLNGRTM EIESTFHMYV QPVVHPQLTP  120
121 FCTELTGIIQ AMVDGQPSLQ QVLERVDEWM AKEGLLDPNV KSIFVTCGDW DLKVMLPGQC  180
181 QYLGLPVADY FKQWINLKKA YSFAMGCWPK NGLLDMNKGL SLQHIGRPHS GIDDCKNIAN  240
241 IMKTLAYRGF IFKQTSKPF  259
```

B) Alignment of homologous regions

```
PINT: MLAPLQTGAA RFSSYLLSRA RKVLGSHLFS PCGVPEFCSI STRKLAAHSF GASMAAMMSF 60
(SHEEP)
MAN : ********** ********** ********** ********** ********G* *******V** 60
RAT : ********** ********** ********** ********** ********G* *******VP* 316
MOUSE:********** ********** ********L* ******L*** ********G* **A****VP* 65

PINT: PPQRYHYFLV LDFEATCDKP QIHPQEIIEF PILKLNGRTM EIESTFHMYV QPVVHPQLTP 120
(SHEEP)
MAN : ********** ********** ********** ********** ********** ********** 120
RAT : ********** ********** ********** ********** ********** ********** 376
MOUSE:********** ********** ********** ********** ********** ********** 125

PINT: FCTELTGIIQ AMVDGQPSLQ QVLERVDEWM AKEGLLDPNV KSIFVTCGDW DLKVMLPGQC 180
(SHEEP)
MAN : ********** ********** ********** ********** ********** ********** 180
RAT : ********** ********** ********** ********** ********** ********** 436
MOUSE:********** ********** ********** ********** ********** ********** 185

PINT: QYLGLPVADY…….259
(SHEEP)
MAN : **********…….259
RAT : H*********…….515
MOUSE:H*********…….264
```

Figure 2: Animo acid sequence of PINT (A) and alignments (B) (data from NCBI blast).
* indicates identical amino acids (for one letter symbols of amino acids see Appendix 5).

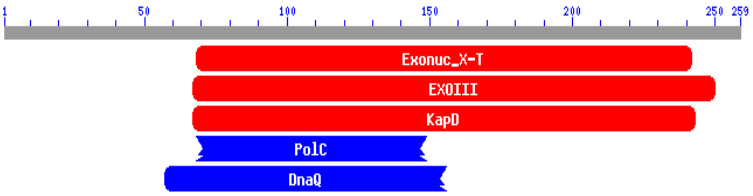


Figure 3: Conserved domains found in PINT protein (data from NCBI blast).

2.2 Alternative splicing (in general)

Alternative splicing is the process that occurs in eukaryotes in which the splicing process of a pre-mRNA transcribed from one gene can lead to different mature mRNA molecules and therefore to different gene products with various functions. When the pre-mRNA has been transcribed from the DNA, it includes several introns (sections of DNA that will be spliced out after transcription) and exons (any region of DNA within a gene, which is transcribed to the final messenger RNA (mRNA) molecule). But introns and exons are not yet determined at this stage. This decision is made during the splicing process. The types of alternative splicing alteration that have been observed include constitutive splicing, exon skipping, intron retention, manually exclusive exons, and alternative 5' and 3' splice sites (Figure 4). Brett and colleagues (Brett et al., 2002) showed that among several eukaryotes the amount of alternative splicing is comparable, with no larger differences between humans and other animals.

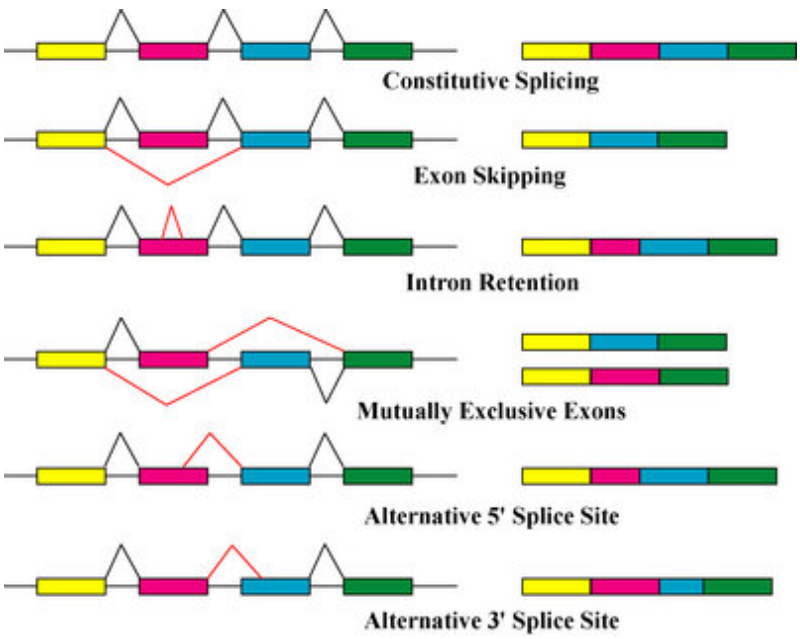


Figure 4: Types of alternative splicing (taken from http://lexikon.freenet.de/Alternatives_Splicing)

2.3 Splicing forms and polymorphism of PINT1 gene

Different splicing forms of sheep and human genes are presented in Figure 5. The human gene has at least ten exons, the ovine gene has at least nine exons. The mRNAs in both species show a high degree of differential splicing. The high degree of conservation of exonic nucleotide sequences enables mapping of sheep exons onto the human gene sequence. Most of the sheep cDNA sequences map exactly to tentative human exons. There are homologies between transcripts II & IV and I & VI. The main difference is that sheep exon 1 is small, only 29 bp, and is included in the tentative human exon 1a, but with a consensus splice donor site in the 3' flank. Two of the cDNA variants of sheep give rise to the same protein (transcripts IV and V) (this protein was used in the present study), while the third (transcript VI) code for a truncated version. Both protein variants correspond to variants described in man, cow and dog. All currently known mRNAs encode part or the entire putative PrP binding site, encoded in sheep by exons 4 to 7.

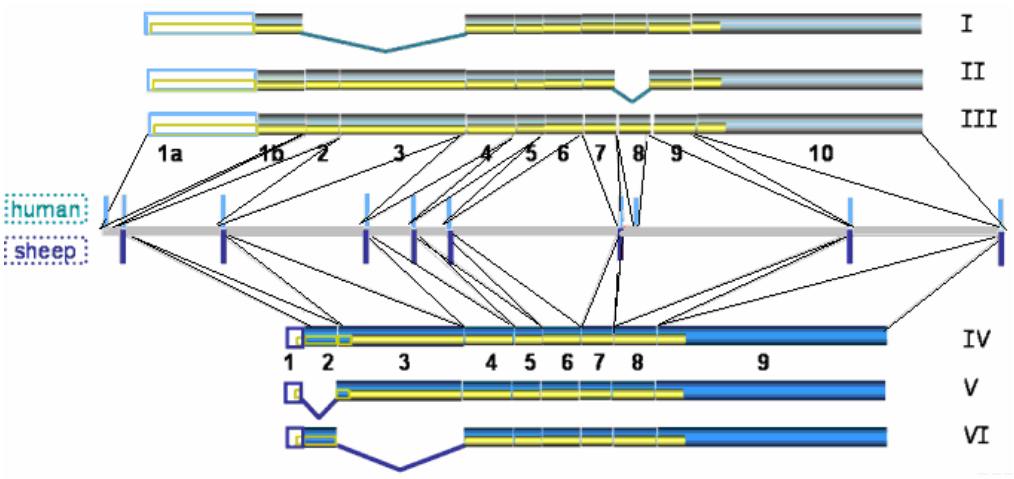


Figure 5: Gene structure of human and sheep PINT1 gene (modified, (Gilliland, et al., 2006)). (Blue, exons; yellow, open reading frame).

Gilliland and coworkers (Gilliland et al., 2006) also demonstrated polymorphisms in ovine PINT1 gene. Two polymorphisms were detected in intron 6 at positions -233 (G/A) and -115 (G/A).

3. Affinity tags

Expression of recombinant proteins is a standard, widely used technique in molecular biology. The limiting steps in this technique usually are synthesis of proteins which are poorly translated in the host cell, and purification of active and correctly folded recombinant protein of interest. In order to make this procedure easier different affinity tags are used.

Affinity tags can be divided into two groups according to their size: small affinity tags (His_6, Arg_5, FLAG) and large tags (glutathione-S-transferase (GST), maltose binding protein (MBP), gb1 domain of protein G (GB1), different fluorescent proteins).

The procedure of tagging with large tags is quite simple: a gene encoding for a large tag protein is fused in frame to the gene encoding the protein of interest (either to the N- or C-terminus) and the resulting chimera is expressed in different cell lines (Baneyx, 1999). Often wild type tag proteins have to be modified by introducing different substitutions in order to engineer a tag protein with the best characteristics (Bevis et al., 2002; Zolotukhin et al., 1996).

Small tags are often introduced in the cDNA using either PCR or insertion into an existing expression vector using a small fragment encoding the tag (Schmitt et al., 1993).

Several cloning vectors for different expression systems are available (Qiagen, Invitrogen). Vectors are designed to permit fusion of a small or large tag (to both termini) with or without a protease cleavage site, which allows the removal of the foreign amino acids.

In addition to their obvious utility in protein purification, affinity tags have also been observed to have many other positive affects on proteins they are fused with. Tags may improve the yield of recombinant proteins (Nilsson et al., 1997); help to protect them from intracellular proteolysis (Nilsson et al., 1997; Smyth et al., 2003), and influence crystal formation (Bucher et al., 2002). Bucher and colleagues demonstrated the effect of affinity tags on crystallization of Pyrococcuc furiosus maltodextrin-binding protein and on its ability to diffract X-rays. The amino acid sequence of the tag seems to have major effect on both, crystal formation and X-ray diffraction; small tags interfere less with crystal formation than large tags. Tags can help to synthesize otherwise poorly translated polypeptides (Baneyx, 1999). Fusion proteins may also enhance the solubility of whole chimera (Hammarstrom et al., 2002; Nilsson et al., 1997; Smyth et al., 2003). Hammarstrom and colleagues showed that

large fusion proteins may increase expression and solubility of the protein. They tested how different tags (His_6, GST, NusA, ZZ, GB1, MBP and thioredoxin (TRX)) affect the solubility of small human proteins (only the His_6 tag did not have any positive effect). Solubilisation of inclusion bodies and then refolding of protein in order to get active product can be a very difficult task to do. The best way out of this problem is to make a fusion to a protein that is known to have high solubility.

However, affinity tags have a potential to interfere with the biological activity of the target protein. Large tags can decrease crystal growth (Smyth et al., 2003), and affect cellular localization (Campbell et al., 2002). Removal of large tags is almost always necessary, but it requires expensive proteases and can cause precipitation of protein (Baneyx 1999) or formation of inactive product (Baneyx 1999; Bucher et al., 2002). Cleavage is rarely complete, this leads to reduction in yields; additional steps may be required to obtain active products, and solubility is newer guaranteed. Overexpression leads to the disappearance of signal and can be also toxic to the host cell.

To decide which tag to use, is an important decision which can influence the results of different assays. Large tags offer an advantage over a small tag when expressing soluble and active recombinant proteins, but small tags often improve purification. Fluorescent tags help to verify successful transfection, to report gene expression, and to monitor the subcellular localization of protein.

3.1 Histidine tag (His_6)

His_6 (sequence: HHHHHH) is one of the smallest tags used in molecular biology. The biggest advantage of this tag is that His_6 can be used in immobilized metal-affinity chromatography (IMAC) (Arnold, 1991), which is based on interactions between chelated metals (usually Ni^{2+}, Zn^{2+}, Cu^{2+}, Fe^{3+}) and the side chains of Glu, Tyr, Cys, His, Arg, Lys, Asp, and Met. Metals are chelated by iminodiacetate (IDA; has three chelating sites), nitrilotriacetic acid (NTA; has four chelating sites) and some other substances.

The most popular ligand for His_6 tag is Ni-NTA (Arnold 1991; Schmitt et al., 1993). (This ligand was also used in the present study). NTA has four chelating sites, which allow a stable interaction between Ni^{+} and column matrix leaving two metal coordination sites free to interact with functional groups of protein. Bound proteins are eluted from the resign by the displacement with another metal binding ligand, such as imidazole (when protein purification

is performed under native conditions), or by protonation of the active amino acid side chains by changing pH (denaturing conditions).

Metal chelate affinity chromatography using Ni-NTA resin offers a number of advantages: cysteine is relatively rare amino acid, so unspecific interactions are atypical; very diluted solutions of proteins can be used (Schmitt et al., 1993); purification under native conditions preserves protein-protein interactions allowing studies of bindings partners; the His_6 tag usually do not interact with the activity of the protein (Janknecht et al., 1991; Janknecht et al., 1992; Schmitt et al., 1993); quantitative binding and elution of tagged protein is possible; and denaturing conditions can be also used (if the tag is inaccessible from the surface) (Nilsson et al., 1997).

Metal chelate affinity chromatography also has some limitations (Arnold, 1991): proteins bind to column according to the number of accessible His (two histidines separated by three amino acids in an α- helix exhibit a very high affinity for metals); proteins may not interact with the resign in its native form (when folded, tag lies inside the structure); certain proteins have a high affinity for the column and needs higher concentration of imidazole for elution.

3.2 Red fluorescent protein (DsRed)

DsRed, a bright red fluorescent protein cloned from the coral *Discosoma*, is a 28 kDa polypeptide. The wild type of this protein has broad excitation and emission bands with maxima at 558 and 583 nm, respectively, with a minor peak at 494 nm and a significant tryptophan peak at 280 nm (Yarbrough et al., 2001) (Figure 9 in Materials and Methods).

DsRed display many properties of great utility for cell biological studies: protein exhibits a bright red signal; fluorescence emission is separable from GFP; the absorbance and fluorescence signals remain unchanged at pH from 5 to 12 and are relatively resistant to photobleaching (Baird et al., 2000). At the same time this protein has a number of negative characteristics, such as slow maturation (takes several days to mature at room temperature (Baird et al., 2000; Yarbrough et al., 2001)), incomplete chromophore formation and an mandatory green-emitting intermediate (Baird et al., 2000), and obligatory oligomerization *in vitro* and in living cells (Baird et al., 2000; Campbell et al., 2002) followed by further aggregation (Robinson et al., 2005). Campbell and colleagues (Campbell et al., 2002) showed that wild type DsRed exists as monomer (32 kDa), but at higher concentrations tends to form

dimers (60 kDa), and tetramers (120 kDa); at very high concentrations DsRed might form even octamers (Baird et al., 2000).

An important question is to what extent oligomerization affects transport and folding of the tagged proteins. Unlike the case of GFP, proteins tagged with DsRed are often found to aggregate within the cell. Aggregation is caused by strong tendency of tetramerization and not by the proteins this fluorescent tag is fused with (Lauf et al., 2001). At the same time, some studies reported that the influence of both EGFP and DsRed on transport and localization is minimal: transport of the cell envelope proteins E1 and E2 of rubella virus in BHK cells, was not influenced by these tags (Ojala et al., 2004), and no effect on Epstein-barr virus membrane protein B2 was either reported (Lynch et al., 2002).

In order to change some of characteristics, several DsRed mutants were created (mutant, which was used in the present study, is called DsRed-Express). In some cases just one substitution can cause mutant to mature much faster if compared to wild type protein (Robinson et al., 2005); by introduction of more substitutions mutants with increased solubility and decreased level of residual green emission were formed (Baneyx 1999; Robinson et al., 2005).

3.3 Green fluorescent protein (GFP)

Wild type GFP is a spontaneously fluorescent protein isolated from Pacific jellyfish, *Aequorea Victoria,* a 238 amino acid protein with excitation and emission maxima at 395 and 508 nm, respectively, with a minor excitation peak at 475 nm (Yang et al., 1996) (Figure 9 in Materials and Methods).

Wild type GFP can form dimers in some crystals (Yang et al., 1996) or in solution at high protein concentration (Tsien, 1998) (it is not known if dimers appear under physiological conditions). GFP is one of the most often used fluorescent tag because of its minimal toxicity to the cell (Billinton et al., 2001), and stability (if matured properly at low temperature this tag is stable and fluorescent at temperature up to 65 °C, while, higher temperature causes denaturation); loses fluorescent signal disappears just at very high pH (11-12) (Tsien, 1998). GFP was successfully fused and targeted to different organelles all over the cell (reviewed by

Tsien, 1998). This tag is very resistant to denaturation, partial or near total renaturation is achieved after removal of denaturing conditions (Yang et al., 1996).

Although native GFP produces significant fluorescence and is extremely stable, the excitation maximum is close to the ultraviolet range. Because UV light requires special optical considerations and can damage living cells, it is generally not well suited for live cell imaging with optical microscopy. Another problem working with wild type GFP is autofluorescence, which contaminates the GFP signal (cellular metabolites, flavins, NAD(P)H, lipofuscins, protoporphyrin IX causes autofluorescence in the cell (Billinton et al., 2001)). In order to solve all these problems, several GFP mutants were created. The excitation maximum of GFP is readily shifted to 488 nm by introducing a single point mutation. This mutation is featured in the most popular variant of GFP, termed enhanced GFP (EGFP; also used in the present study).

4. The aim of this study

PINT is a new protein cloned from sheep that might be a prion protein binding partner. The only thing known about this protein is conserved nuclease domain and possible nuclease activity (data from protein sequence).

The aim of the present study was:

- to make three PINT constructs with different plasmids (pDsRed-Express-N1, pZeoSV and pET16B);

- to transform *E.coli* with pET-PINT and express PINT protein in bacterial cells; purify and analyze it;

- to transfect different mammalian cell lines with pDsRed-Express-PINT and pZeoSv-PINT; and by using different assays learn as much as possible about PINT protein (cellular localization, behaviour in the cell and so on);

- to co-transfect PINT and PrP in order to study co-localization of these proteins and possible interaction.

II. Materials and methods

Each method and some related theory is presented in brief, followed by a short protocol. Plasmid maps, list of reagents and their manufacturers, different buffers and media used in this work are presented in the Appendix 1-3, respectively.

1. General techniques for working with DNA and bacterial cultures

The purpose of the first part of this study was to make three different PINT constructs by running polymerase chain reaction (PCR), cutting and ligating vectors and PCR products, multiply and isolate them. Later these constructs were used for transformation of *E.coli* or transfection of mammalian cells in order to express PINT protein.

1.1 DNA constructs

The primers used in cloning of PINT constructs are listed in Table 3. In this study three different constructs were made: pDsRed-Express-PINT, pZeoSv-PINT and pET-PINT. Those constructs were later used for expression of PINT in *E.coli* (construct pET-PINT) and for transfection into mammalian cells (constructs pDsRed-Express-PINT and pZeoSv-PINT). All plasmids were sequenced to confirm correct amplification and cloning. Primers used for sequencing are presented in Table 3.

The pDsred-Express-N1 vector (Appendix 1) is a mammalian expression vector (4700 bp) that contains a gene for canamycin resistance and encodes DsRed-Express, a variant of *Discosoma sp*. red fluorescent protein. DsRed-Express contains nine amino acid substitutions, which improve the solubility of the protein and reduce the time from transfection to detection of red fluorescence (the native form of this protein is not very soluble and has a long maturation time (Campbell et al., 2002)). In addition, these substitutions reduce the level of residual green emission (Bevis et al., 2002). When DsRed-Express is expressed in mammalian cell cultures, red-emitting cells can be detected by either fluorescence microscopy or flow cytometry 8–12 hours after transfection (DsRed-Express excitation and emission maxima is at

557 nm and 579 nm, respectively). For more information about this vector see www.bdbiosciences.com.

The pZeoSv vector (Appendix 1) is a constitutive mammalian expression vector (3500 bp) containing a gene that confers resistance to the novel antibiotic zeocin, which can be used to select for the pZeoSv vector in bacterial and mammalian cells. Genes cloned into this vector are expressed from the Simian Virus 40 (SV40) early enhancer/promoter for high level transient and stable expression in mammalian cell lines. For more information about this vector see www.invitrogen.com.

The pET system is a powerful system for the cloning and expression of recombinant proteins in *E.coli*. Target genes are cloned in pET plasmids under control of strong bacteriophage T7 transcription and translation signals. Once established in a non-expression host, target protein expression may be initiated by transferring the plasmid into an expression host containing a chromosomal copy of T7 RNA polymerase under control of the lac Z promoter. Expression is then induced by adding IPTG to the bacterial culture; and the desired product can comprise more than 50% of the total cell protein a few hours after induction. Another important benefit of this system is its ability to maintain target genes transcriptionally silent in the uninduced state.

The pET-16b vector (5711 bp) (Appendix 1) carries an N-terminal His-tag sequence followed by Factor Xa site (protease site) and three cloning sites. For more information about this vector see www.novagen.com.

Table 3. Primers used in PCR and sequencing

	Primers	Sequence	Restriction enzymes
1	PINTRedF	5'- GATGCTTCT**AGATCT**CAATGCTAGCACCT-3'	BglII
2	PINTRedR	5'- TCCTCA**GGATCC**CCGAACGGCTTTGACGT-3'	BamHI
3	PINT-Z-F	5'- GGCTTTCC**ACCGGT**CACTGAACCT-3'	AgeI
4	PINT-Z-R	5'-CTCATC**ACGCGT**CAATGATGATGATGATGATG GAACGGCTTTGACGT-3'	MluI
5	PINTlongF	5'-TGGATGT**CATAT**GCTAGCACCTTTACAGACT-3'	NdeI
6	PINT-pETcorrR	5'- GCAGCC**GGATCC**CTCAGAACGGCTTT-3'	BamHI
7	DsRedF	5'- TACGGTGGGAGGTCTATAT-3'	
8	DsRedR	5'- TCCATGCGCACCTTGAAGC-3'	
9	SP6	5'- CATTTAGGTGACACTATAG- 3'	
10	T3	5'- AATTAACCCTCACTAAAGGG- 3'	
11	T7promotor(F)	5'- TAATACGACTCAATATAGGG- 3'	
10	T7terminator(R)	5'- CTAGTTATTGCTCAGCGGT- 3'	

Forward (F) and reverse (R) primers used in the construction of PINT-expression plasmids (1-6), primers used for sequencing pDsRed-Express-PINT (7-8), primers used for sequencing pZeoSv-PINT (9-10) and primers used for sequencing pET-PINT (11-12). Restriction sites are indicated in bold. Sequence coding for 6xHis (in reverse) is underlined (4). All primers were produced by MWG-Biotech AG.

1.1.1 Polymerase chain reaction (PCR)

The PCR method enables amplification of nucleic acid sequence fragments *in vitro* by primer extension (Vosberg, 1989). This can be a single gene, or just a part of a gene. As opposed to living organisms, the PCR process can copy only short DNA fragments, usually up to 40 kb. The PCR process usually consists of a series of 20 to 35 cycles. Each cycle consists of three major steps. The double-stranded DNA has to be heated at 94-96 °C (or 98°C if extremely thermostable polymerases are used) in order to separate the strands. This step is called denaturing. It breaks apart the hydrogen bonds that connect the two DNA strands. Prior to the first cycle, the DNA is often denatured for an extended time to ensure that both the template DNA ant the primers have completely separated and are now single-stranded. After separating the DNA strands, the temperature is lowered, so the primers can hybridize to the single template DNA strands. This step is called annealing. The temperature of this stage depends on the primers and is usually 5 °C below their melting temperature (45-60 °C). Finally, the DNA polymerase has to copy the DNA strands. It starts at the annealed primer and works its way along the DNA strand. This step is called elongation. The elongation temperature depends on the DNA polymerase that was used in the reaction.

In this study all polymerase chain reactions were performed as followed:

5x Phusion HF buffer	**10 µl**
2 mM dNTP	**5 µl**
Forward and reverse primers	**0.25 pmol (**each)
Template DNA	**10 ng** (max)
Phusion DNA polymerase	**0.5 µl**
H_2O	**to the end volume of 50 µl**

Reaction conditions are presented in Table 4.

Table 4. PCR conditions

Name of the step	Temperature (°C)	Time (sec)	Repetitions
First denaturing	98	30	1
Denaturing	98	5	30
Annealing	60*	30	
Elongation	72	30	
Last elongation	72	600	1

* annealing temperature was 55 °C in PCR amplification with PINT-Z-F and PINT-Z-R primers.

1.1.2 Agarose Electrophoresis

Gel electrophoresis is a method that separates macromolecules (nucleic acids or proteins) on the basis of size, electric charge, and other physical properties.

Agarose gels are used for DNA separation. Agarose is a natural colloid extracted from sea weed, a linear polysaccharide (average molecular mass about 12.000) made up of the basic repeat unit agarobiose, which comprises alternating units of galactose and 3.6-anhydrogalactose.

An agarose gel is loaded with the DNA fragments and current is passed through the gel. Since DNA is negatively charged, it will migrate towards the positive pole. The DNA will not migrate at the same rate, however. Larger pieces of DNA collide with the gel matrix more often and are slowed down, while smaller pieces of DNA move through more quickly. By using gels with different concentrations of Agarose (1-3%), one can resolve different sizes of

DNA fragments. Higher concentrations of agarose facilitate separation of small DNAs, while lower concentrations allow resolution of larger DNAs.

In the present study agarose was used at the concentration of 1%. Agarose gels were formed by suspending dry agarose in 1x TAE buffer, then autoclaving the mixture (for 20 min at 120 °C) to melt agarose. Mixture was poured into forms, added ethidium bromide (a potent mutagen, fluorescent dye that intercalates between bases of nucleic acids and allows very convenient detection of DNA fragments at UV light) and allowed to cool at room temperature to form a rigid gel (to make one gel, 50 ml 1% agarose were added 5 µl ethidium bromide solution (1% in water)). Gel was loaded with DNA samples mixed with 6x Mass Ruler Loading Dye Solution and current were passed through it (100 V) for about 1hour, using 1x TAE as electrophoresis buffer. Different standard DNA ladders (Appendix 2) were used to estimate the sizes of DNA fragments.

1.1.3 Purification of DNA from agarose gel slices

Isolation of DNA fragments from agarose gel slices was performed using 2 kits: Geneclean Spin Kit from Q-Bio Gene (while working with pDsRed-Express-PINT construct) and E.Z.N.A Gel Extraction Kit from Omega Bio-tek (while working with pZeoSv-PINT and pET-PINT constructs).

In general, both kits are based on the same principle, just a little bit different columns and buffers are used: DNA binds to a silica-based membrane at high concentrations of salt, is washed a few times to get rid of various impurities and is eluted when the salt concentration is lowered. The fact that DNA binds in high salt and elutes in low salt makes this method especially useful as a purification procedure. DNA is eluted with either water or a low salt buffer. For detailed protocols, consult the manufacturer's handbooks.

1.1.4 Cutting of DNA with restriction enzymes

A restriction enzyme is an enzyme that cuts double-stranded DNA. The enzyme makes two incisions, one through each of the phosphate backbones of the double helix without damaging the bases. Rather than cutting DNA indiscriminately, a restriction enzyme cuts only double-helical segments that contain a particular nucleotide sequence, and it makes its incisions only within that sequence, known as a "recognition sequence", always in the same way. Some enzymes make strand incisions immediately opposite one another, producing "blunt end" DNA fragments. Most enzymes make slightly staggered incisions, resulting in "sticky ends", out of which one strand protrudes.

All restriction enzymes used in this study (Table 3) for making the PINT constructs produce "sticky ends". It was possible to cut with two restriction enzymes at the same time by choosing a suitable buffer. Enzymes and suitable buffers are presented in Table 5.

Restriction enzyme cutting reactions were performed as followed:

10x buffer	**5 µl**
DNA (PCR-product or vector)	**0.5-1 µg**
Restriction enzyme 1 and 2	**1 µl** (each)
H_2O	**to the end volume of 50 µl**

Reaction mix was incubated at 37 °C for one hour.

Table 5. Restriction enzymes and buffers

Name of the construct	Restriction enzymes	Buffer
pDsRed-Express-PINT	BglII	10x BamHI buffer
	BamHI	
pZeoSv-PINT	AgeI	10x R-buffer with BSA
	MluI	
pET-PINT	NdeI	10x R-buffer with BSA
	BamHI	

1.1.5 Ligation of DNA fragments

The enzyme used to ligate DNA fragments is T4 DNA ligase, which originates from the T4 bacteriophage. This enzyme will ligate DNA fragments having overhanging, cohesive ends that are annealed together, as well as blunt-ended fragments. A ligation reaction requires three ingredients in addition to water: two or more fragments of DNA that have either blunt or compatible cohesive ("sticky") ends, buffer which contains ATP, and T4 DNA ligase. A typical reaction for inserting a fragment into a plasmid vector (subcloning) would utilize about 0.01 (sticky ends) to 1 (blunt ends) units of ligase.

Ligation reactions were performed as followed:

2x Rapid ligation buffer	**5 µl**
Vector cutted with restriction enzymes	**50 ng**
PINT-fragment cutted with the same restriction enzymes	**35 ng**
T4 DNA ligase	**1 µl**
H_2O	**to the end volume of 10 µl**

Reaction mix was incubated at 16 °C over night or at RT for 2-3 hours.
Before the transformation of *E.coli* T4 DNA ligase was inactivated by heating reaction mix at 70 °C for 15 min.

1.2 Transformation of *E.coli*

Among many systems available for heterologous protein production, the gram-negative bacterium *Escherichia coli* remains one of the most attractive because of its ability to grow rapidly and at high density, its well characterized genetics, and the availability of large number of cloning vectors and mutant host strains.

Transformation is the process of getting the recombinant vector from a reaction mixture or vector solution into E. coli cells. To enable the cells to take up circular vector DNA they have to be made competent. Competent cells are cells which can accept extra-chromosomal DNA or plasmids. Cells can be made competent by chemical treatment or by transfer to a salt-free medium for electroporation. The method for the preparation of competent cells depends on the transformation method used and transformation efficiency required.

The choice of the E. coli host strain depends on the goal of the transformation. The transformation of a vector for multiplication should be done in a recA$^-$ strain, such as DH5α, NovaBlue or XL1-Blue. The transformation of a vector for protein expression should be done in the appropriate expression host too (BL21(DE3)LysS, AD94(DE3)LysS or others).

In this study *E.coli* strain DH5α was used for multiplication of plasmids. Simple and efficient method for making these cells competent was published by Inoue and co-workers (Inoue et al, 1990).

For expression of PINT protein *E.coli* strain BL21(DE 3)LysS was used. BL21 cells produces lower levels of proteases and thus are more suitable for expression of recombinant proteins, and also contain a T7 RNA polymerase gene under control of a lac promoter. Prior transformation of plasmid into BL21 cells, plasmid was multiplied in non-expressional host, DH5α cells.

1.2.1 Transformation of competent DH5α competent *E.coli* cells

Competent DH5α *E.coli* cells (these cells are quite stable and can be stored in a competent state at -80 °C) were thawed on ice. 50-100 µl of cell suspension was added 2 µl of ligation reaction mix and cells stayed on ice for 30 min. After that cells were given a heat shock at 42°C for 45 sec. Then cells were left to stay on ice for 5 more minutes and streaked out on preheated LB-plates with suitable antibiotic (different PINT expression plasmids and suitable media with antibiotic for growing over night cultures or making plates are presented in Table 6). Plates were incubated at 37 °C over night.

Table 6. PINT expression plasmids, media and antibiotics

Expression plasmid	*E.coli* strain	Medium	Antibiotic (concentration)	Purpose of the cells
pDsRed-Express-PINT	DH5α	LB, high salt	Canamycin(50 μg/ml)	Multiplication of plasmid
pZeoSv-PINT	DH5α	LB, low salt	Zeocin(50 μg/ml)	Multiplication of plasmid
pET-PINT	DH5α	LB, high salt	Ampicilin(50 μg/ml)	Multiplication of plasmid
	BL21(DE3) LysS	LB, high salt	Ampicilin/ Chloramphenicol (50/20 μg/ml)	Expression of recombinant protein

1.2.2 $CaCl_2$ method for making competent BL21 cells

Before each transformation BL21(DE 3)LysS *E.coli* cells have to be made competent by $CaCl_2$ treatment, since the Inoue method used for DH5α does not work on these cells.

BL21(DE3)LysS cells (frozen) were streaked out on LB/Chl (50 μg/ml) plate (this *E.coli* strain has chloramphenicol resistance gene (chl^r) on LysS plasmid) and incubated over night at 37 °C. Next day some colonies that were formed on the plate were inoculated in 100 ml LB/Chl medium until OD_{550} reached about 0.05-0.1; cells were grown to OD_{550} about 0.3 and were incubated on ice for 10 min. After that, the cells were spinned down at 5000x g for 10 min at 4 °C and supernatant was removed, while the cell pellet were resuspended in 20 ml cold 100 mM $CaCl_2$ solution. After staying on ice for 5 min cells were spinned down one more time and resuspended in 4 ml of cold $CaCl_2$ solution; cells were left to stay on ice for minimum one hour, eventually over night (the longer they stay on ice, the more competent they become). 200 μl of cell suspension was used for each transfection.

1.2.3 Transformation of competent BL21(DE3)LysS *E.coli* cells

To 200 μl of competent BL21(DE3)LysS cells were added 50 ng of plasmid, and cells were incubated on ice for 30 min. After that cells were given a heat shock at 42 °C for 45 sec. Then cells were left to stay on ice for 2 more minutes, added 800 μl LB-medium and 2μl sterile 2 M glucose, mixture was incubated at 37 °C for 30min. Cells were streaked out on LB/Amp/Chl plates and incubated over night at 37 °C.

1.3 Isolation of multiplied DNA

For isolation of plasmid DNA two different kits were used: Wizard Plus Sv Minipreps DNA Purification System from Promega and Pure Yield Plasmid Midiprep System also from Promega (gives higher DNA concentration).

The silica-based Wizard Plus Minipreps DNA Purification System provides a simple and reliable method for rapid isolation of plasmid DNA, with no organic extractions or ethanol precipitations. This system can be used for isolation of any plasmid, but works most efficiently when the plasmid is < 20,000 bp. Isolation of DNA consists of a few main steps: making clear lysate (by sedimentation, resuspendation, lysis and neutralization of cell suspension. Alkaline protease solution are used for inactivation of endonucleases and other proteins that can affect the quality of isolated DNA), transfer of lysate on the column which binds DNA, wash of the column, removal of solution and elution of DNA in nuclease-free water. For detailed protocol see www.promega.com.

The Pure Yield Plasmid Midiprep System provides high-speed purification of plasmid DNA using a newly developed silica-membrane-based column. This design allows purification of 100–200 µg of plasmid DNA from 50 ml of bacterial culture. The method of isolation is similar to one described above, except that Endotoxin Removal Wash is used to reduce endotoxin, protein and RNA contamination, improving results for eukaryotic cell transfection; also improving *in vitro* transcription/translation reactions and fluorescent DNA sequencing. Elution is performed in a small volume of water, providing concentrated DNA for chosen application. In this study DNA purification by centrifugation was performed. For a detailed protocol see www.promega.com.

1.4 Quick-check of plasmid-containing cells

In order to quick-check the size of *E.coli* plasmids, the procedure described by Akada (Akada, 2004) was modified and used in this study:

27 µl of over night cultures were mixed with 3 µl 6x loading buffer. Then 25 µl of phenol-chloroform mixture were added, and the sample was vortexed for 30 seconds; then centrifuged for 3 min at 13000 rpm. 15 µl of the upper water phase was loaded on an agarose

gel. The strongest bands on the gel were ribosomal RNA. Plasmids formed weaker bands above these. In addition to MW standards, reference plasmids were also added to the gel.

2. General techniques for working with proteins

The purpose of the second part of this study was to work with recombinant protein: to express PINT protein in BL21(DE3)LysS *E.coli* cells, purify and analyze it.

2.1 Expression of recombinant protein in bacterial cells

In order to grow bacterial culture (BL21(DE3)LysS cells containing the expression plasmid pET-PINT) and later express recombinant PINT protein, the procedure described in Ni-NTA Spin Handbok by Qiagen was slightly modified and used in this study:

3 ml of LB medium containing 50 μg/ml ampicilin and 20 μg/ml chloramphenicol was inoculated with a fresh bacterial colony containing the expression plasmid and grown at 37 °C overnight. Next day 50 ml medium containing antibiotics was inoculated with 800 μl of overnight culture and grown at 37 °C with vigorous shaking until the OD_{550} reaches about 0.3. Later IPTG (isopropyl β-D-1-thiogalactopyranoside, a inducer of the lac promoter) was added to the cells to a final concentration of 1 mM and the culture was grown at 37 °C* vigorously shaking over night**. After induction cells were harvested by centrifugation at 4000x g for 15 min at RT and used in different assays or stored at -20 °C.

* In some cases lower temperatures were used (16-30 °C)
** In some cases the duration of this growth was shortened to 5-6 hours or increased up to 30 hours, depending on the temperature

2.2 Recombinant protein purification

PINT protein expressed from pET-PINT vector has His_6 tag on the N-terminal end. This tag was used for purification of PINT from cells lysates by using the Ni-NTA Spin Kit from Qiagen. Ni-NTA Spin Kit is based on unique and versatile metal chelate chromatography material, NTA ligand, packaged in spin column. NTA has a tetradentate chelating group that occupies for of six sites in the nickel coordination sphere. The metal is bound very tight and as the result the proteins are very strongly bound to the resign. The high affinity of the Ni-NTA resins for His_6-tagged proteins or peptides is due to both the specificity of the interaction between histidine residues and immobilized nickel ions, and to the strength with which these ions are held to the NTA resin. Ni-NTA silica material has been modified to provide a hydrophilic surface; non-specific hydrophobic interactions are kept to a minimum, while the silica support allows efficient microspin technology.

In this study protein purification under native and denaturing conditions were performed, since the interaction between Ni-NTA and the His_6 tag of the recombinant protein does not depend on proteins tertiary structure. For detailed protocols see the manufacturer's handbook.

Protein purification under native conditions was performed exactly following the manufacturer's protocol in the handbook; except that in some cases, a French press was used instead of sonication. When using French press, cells were resuspended in 3 ml lysis buffer and lysozyme was not added to resuspended cells.

French press is a procedure that is used for disruption of cell walls by using high hydrostatic pressures without adding detergents (cells are sheared by forcing them through a narrow space) first described by Deckmann and coworkers (Deckmann et al., 1985). The pressure disrupts the cellular walls of a sample while leaving the cell nucleus undisturbed. This technique results in uniform and complete disruption. The two models of working cells give a choice of cell volume and working pressures. The 40K cell has a maximum working pressure of 40.000 psi and a cell capacity of 35 ml. The miniature cell used for work with small sample volumes holds from 1.4 to 3.7 ml and has a maximum working pressure of 20.000 psi (in the present study the second one was used).

The protocol for purification under denaturing condition was slightly modified, mostly the lysis of cells. In this study cells were resuspended in 2 ml buffer B, incubated at room

temperature gently shaking for 3 hours, centrifuged at 13000x g for 20 min and supernatant was collected. Further, the protocol from the handbook was followed.

In this study, in addition to Ni-NTA columns, His Gravi Trap (GE Healthcare) columns were also used for purification of recombinant proteins. For detailed protocol see the manufacturer's handbook.

2.3 Inclusion bodies

It is often difficult to obtain soluble and active proteins from expression of eukaryotic proteins in prokaryotes. Often, the overexpression leads to the production of inclusion bodies, insoluble aggregates of misfolded protein. Formation of inclusion bodies protects proteins from proteolytic attack and toxic proteins can not inhibit cell growth when presented in inactive form. Inclusion bodies can be easily purified. However, the challenge is to convert the inactive and insoluble inclusion body protein aggregates into soluble, correctly folded and biologically active products (Clark, 1989) (kinetic competition between folding and aggregation always takes place in the cell (De Bernardez et al., 1998)).

2.3.1 Purification of inclusion bodies

Cells from 250 ml culture were sedimented by centrifugation at 7000x g for 10 min, resuspended in 25 ml NaCl-Tris buffer pH 8.0 (for the composition of all buffers see Appendix 3), added lysozyme to a final concentration of 0.3 mg/ml and frozen at -20 °C. The cell membranes were disrupted by thawing the frozen cell suspension at 30-50 °C. After dilution with 25 ml NaCl-Tris buffer, and addition of $MgCl_2$ and DNaseI (to the final concentration of 5 mM and 7 μg/ml, respectively), the suspension was stirred with a glass rod and drawn through an 18G syringe until the viscosity disappeared. The inclusion bodies were sedimented at 11000x g for 30 min at 4 °C, and washed twice with 600 ml NaCl-Tris buffer containing 1% Triton X-100 by magnetic stirring for 1 hour. The final pellet was resuspended in 25 ml 10mM NaCl-Tris buffer with 1 mM EDTA pH 8.0 and stored at -20 °C.

2.3.2 Solubilisation and refolding of inclusion bodies

In the present study a few different protocols for solubilisation of purified inclusion bodies and for refolding of recombinant protein were used (for the composition of all buffers see Appendix 3).

Protocol 1: inclusion bodies from 10 ml suspension (check 2.3.1 Purification of inclusion bodies) were pelleted at 18000x g for 15 min, dissolved in 30 ml refolding buffer containing 6-8 M guanidine hydrochloride or urea by vortexing, and stirred over night at 4 °C*. Then the protein solution was filtered through a 0.2 μm filter before loading on Ni-NTA (Qiagen) or His Gravi Trap (GE Healthcare) column equilibrated with refolding buffer at 4 °C*. Buffer change from refolding to phosphate buffer was performed by washing column with 2 ml of refolding/phosphate buffers mixed in different ratio (starting from, refolding buffer: phosphate buffer 5:0, and finishing with: refolding buffer: phosphate buffer 0:5). After washing the column with 10 ml phosphate buffer, protein was eluted in phosphate buffer containing 300-500 mM imidazole.

* in some cases room temperature was used.

Protocol 2: this method is similar to one described in protocol one, just after dissolving of inclusion bodies in 30 ml buffer containing 6 M guanidine hydrochloride, suspension was stirred for 1 hour at RT before loading the protein solution on His Gravi Trap column (GE Healthcare); column was washed with 10 ml washing buffer containing 6 M urea and proteins were refolded by slowly washing the column with buffers containing different urea concentrations (from 6 M urea to 0 M, using 10 ml of buffer at the time). Protein was eluted in buffer without urea, containing 300 mM imidazole.

Protocol 3: dilution of the solubilised protein directly into renaturation buffer is the most commonly used method, however, the protein concentration has to be carefully controlled to prevent aggregation. In this study inclusion bodies were dissolved in 30 ml refolding buffer (protocol 1) and diluted 10x with buffer containing no detergent, but 10% glycerol at 4 °C or room temperature. Mixture was stirred slowly for 3 hours at 4 °C or RT, centrifuged at 24000x g for 15 min at 4 °C to get rid of impurities, filtrated through 0.2 μm filter and loaded

on His Gravi Trap column (GE Healthcare) equilibrated with 10 ml dilution buffer. Protein was eluted with buffer containing 300 mM imidazole.

Protocol 4: method is similar to one described in protocol 3, just refolding buffer containing 8 M urea was used for solubilisation of washed inclusion bodies and then slowly diluted 10 times with buffer containing no urea.

2.4 Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide is a polymer formed from acrylamide subunits that is readily cross-linked. Gel electrophoresis of proteins is usually done in an SDS (sodium dodecyl sulphate) polyacrylamide gel (for DNA, polyacrylamide is used for separating fragments of less than about 500 bp). SDS is an anionic detergent which denatures secondary and non-disulphide-linked tertiary protein structures and applies a negative charge to each protein in proportion to its mass, making the protein mass the main factor that influences migration during electrophoresis. The pore size of the gel may be varied to produce different molecular effects for separating proteins of different sizes. In this way, the percentage of polyacrylmide can be controlled in a given gel. By controlling the percentage (from 3% to 30%), precise pore sizes can be obtained, usually from 5 to 2.000 kDa. This is the ideal range for gene sequencing, protein, polypeptide, and enzyme analysis. The proteins move into the gel when an electric field is applied. The gel minimizes convection currents caused by small temperature gradients, and it minimizes protein movements other than those induced by the electric field. After electrophoresis proteins can be visualized by treating the gel with different stains.

In this study Criterion XT Precast Gels from BioRad were used (10-12% Bis-Tris). Electrophoresis was runned at 200 V for about 20-60 min, using XT MES or MOPS as running buffer.

Protein containing pellet was resuspended in 1x XT Sample buffer; supernatant with proteins were added 2x XT Sample buffer. Sample buffer contained $MgCl_2$ (to lower the viscosity of the sample) and reducing agent, which reduce disulphide linkage. All protein samples and MW standards were boiled at 100 °C for 3 minutes before loading them in wells.

The amount of each sample that was applies to the gel was calculated by using the equation:

$$\textbf{amount of sample, µl} = (X * 0.03)/ OD_{550}$$

where X is the amount of sample buffer used for resuspension of the pellet (µl); 0.03 is a constant, and OD_{550} is the optical density of the cell culture at 550 nm.

2.5 Staining of polyacrylamide gels

Proteins separated by electrophoresis were visualized by treating the gel with 2 different stains: Coomasie Brilliant Blue (CBB) and Silver (for the composition of all buffers see Appendix 3).

CBB, an aminotriarylmethane dye, forms strong, but not covalent complexes with protein, most probably by a combination of van der Waals forces and electrostatic interactions with NH_3^+. This stain binds to the proteins but not to the gel itself. Each band on the gel represents a different protein (or a protein subunit). After electrophoresis, the gels were transferred to a bath with the staining solution and incubated at room temperature, gently shaking for 30 min. Then the staining solution was exchanged with destaining solution and the gel was incubated at room temperature for 24 hours.

Silver staining: there are two major classes of silver reagents used to stain proteins in PAGE gels: ammonial silver solutions and those that use silver nitrate (in this study the second one was used). The process relies on differential reduction of silver ions that are bound to the side chains of amino acids. This staining method is about 100-1000 fold more sensitive than staining with CBB. There are several different protocols for silver staining of the gels. The one used in this study is:

The gel was kept gently shaking in solution A for 2 hours, RT (solution was changed into fresh one each 30 min.). After that solution B was added to the gel for 45 min, RT. Later gel was washed with water for 1 hour (changing water every 10 min.). Solution C was added and gel was incubated for 1.5 hours at RT. After that solution D was added to the gel and gel was observed all the time. When protein bands became bright enough solution D was changed into solution E for 30 min. Later gel can be stored in the water up to 6 months.

3. General techniques for working with mammalian cells

The purpose of the last part of this study was to express PINT protein in mammalian cells. When expressed with a tag, different assays can be performed in order to characterize that protein, study its cellular localization, binding partners and so on.

In this study the neuroblastoma cell lines N2a (murine) and SH-SY5Y (human) and stable clones such as N2a-DsRed-Ex, N2a-PrP-EGFP, SH-DsRed-Ex were used. N2a cells were grown in minimum essential medium (MEM) added 10% foetal bovine serum (FBS), non-essential amino-acids, 1% penicillin/streptomycin, 1% glutamine and 1% pyruvate. SH-SY5Y were grown in Dulbecco's modified Eagle's medium (DMEM) with addition of 1% glutamine, 10% FBS and 1% penicillin/streptomycin. All cells were grown at 37ºC with 5% CO_2 and saturated humidity.

3.1 Transfection

Transfection is the introduction of foreign DNA into eukaryotic cells, such as animal cells. Transfection typically involves opening transient "holes" or gates in cell's plasma membrane to allow the entry of extracellular molecules, typically supercoiled plasmid DNA.

Cells were washed 1x with room tempered PBS and trypsinated until almost all of the cells became lose from the flask; suitable amount of the cells (depending on the assay) was transferred to a new plate and let grow over night. Introduction of foreign DNA into a cell was performed by liposomes, i.e. small, membrane-bounded bodies, which fuse with the cell membrane, releasing the DNA into the cell. N2a and SH-SY5Y cells were transfected with lipofectamine and Plus reagents (Invitrogen) according to the manufacturer's instructions in Opti-MEM medium. Usually, transient transfected cells were harvested 24 hours after transfection (see 3.3 Western blot analysis).

Stably transfected cells (SH-SY5Y) were selected with G-418-sulphate at a concentration of 0. 5 mg/ml. Several colonies were initially selected from this cell line. After screening for fluorescence intensity (Olympus IX81 fluorescence microscope) and PINT signals on Western blots, one clone was selected for further studies.

Some stable clones were freezed and stored in nitrogen tanks. Before freezing cells were centrifuged at 1200 rpm for 5 min, resuspended in 3 ml medium with 10% DMSO, freezed down first at -80 °C over night and later transferred into nitrogen tank.

3.2 Antibodies

Monoclonal (Mab) mouse-anti-His, C-terminal anti-His, N-terminal P4 and polyclonal rabbit anti-RFP were used in this study as primary antibodies. In Western blots, primary antibodies were used in concentrations of 0.05-0.2 µg/ml. For immunoprecipitation 1- 2 µg of antibodies were used.

Secondary antibodies, goat-anti-rabbit, goat-anti-mouse and anti-mouse true blot conjugated with horseradish peroxidase (HRP), goat-anti-mouse, conjugated with alkaline phosphatase (ALP) were used in 0.005-0.3 µg/ml concentrations. For detection enhanced chemiluminescence (HRP) or fluorescence (ALP) were used.

3.3 Western blot analysis

Cells were harvested 24 hours after transfection. Before the lysis, cells were washed twice with ice cold PBS and scraped in RIPA buffer (all buffers are listed in Appendix 3) (N2a and SH-SY5Y transfected and untrasfected cells) or IP-lysis buffer (N2a-PrP-EGFP cells). For one million cells 200 µl buffer containing 15 µl protease inhibitor cocktail were used. Then cells were left to stay on ice for 30 min, and spinned down in a centrifuge for 5 min at 13000 rpm; supernatant was collected.

60 µl supernatant was added 20 µl NuPAGE LDS Sample buffer, 8 µl NuPAGE Sample Reducing agent and mixture was boiled for 5 min before applying on the gel.

For Western blots, proteins were separated by SDS-gel electrophoresis (see 2.4 Polyacrylamide gel electrophoresis) (at 200V for 1 hour; using MOPS as the running buffer; Criterion XT precast gel, 12% Bis-Tris (Biorad)) and electroblotted onto polyvinylidene difluoride membranes with a semi-dry blotter (BioRad); proteins were blotted for 45 min at 25 V with Tris/CAPS transfer buffer as recommended by the supplier). To reduce unspecific binding of antibodies, membranes were blocked by incubation with 5% (w/v) fat-free dried milk in TBS-T for 1 hour at room temperature. Incubation with primary antibodies was

performed in TBS-T and 1% (w/v) fat-free dried milk at 4 °C over night, while with secondary antibodies for 1 hour at room temperature. After both incubations membranes were washed 4x 15 min with TBS-T. Visualization of bands were achieved with enhanced chemiliuminescence (ECL Plus, Amersham Biosciences), captured by ECL Hyperfilm (Amersham Biosciences) or by fluorescence scanning with a variable mode imager (Typhoon 9200, Amersham Biosciences), when secondary antibodies labelled with ALP.

It is possible to strip the membrane, completely remove primary and secondary antibodies and reprobe the same membrane several times. In order to do that, membrane was added striping buffer and incubated at 60 °C 2x 15 min; washed 2x 10 min with large amounts of TBS-T at RT and blocked with 5% fat-free dried milk in TBS for 1 hour at RT; later membrane was incubated again with primary and secondary antibodies.

3.4 Immunoprecipitation

Immunoprecipitation (IP) is a procedure by which peptides or proteins that react specifically with an antibody are removed from solution and examined for quantity or physical characteristics. Antibody-antigen complexes are removed from solution by addition of an insoluble form of an antibody binding protein such as Protein A, Protein G or second antibody. The procedure can be divided into several stages: sample preparation; preclearing; antibody incubation/formation of antibody-antigen complexes and precipitation. The principle of immunoprecipitation is presented in Figure 6.

Co-immunoprecipitation (Co-IP) is a popular technique for protein interaction discovery. Co-IP is conducted in essentially the same manner as an IP. However, in a co-IP the interacting protein is bound to the target antigen, which becomes bound by the antibody that becomes captured on the Protein A or G gel support. The principle of Co-IP is presented in Figure 7.

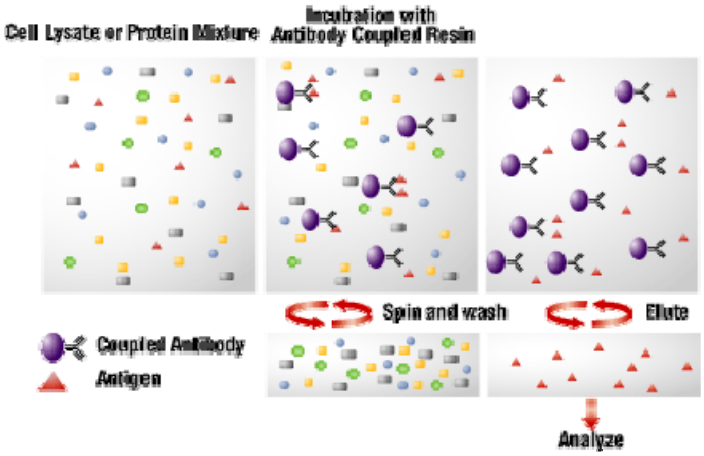


Figure 6: Principle of IP
(Taken from http://www.piercenet.com/Products/Browse.cfm?fldID=9C471132-0F72-4F39-8DF0-455FB515718F)

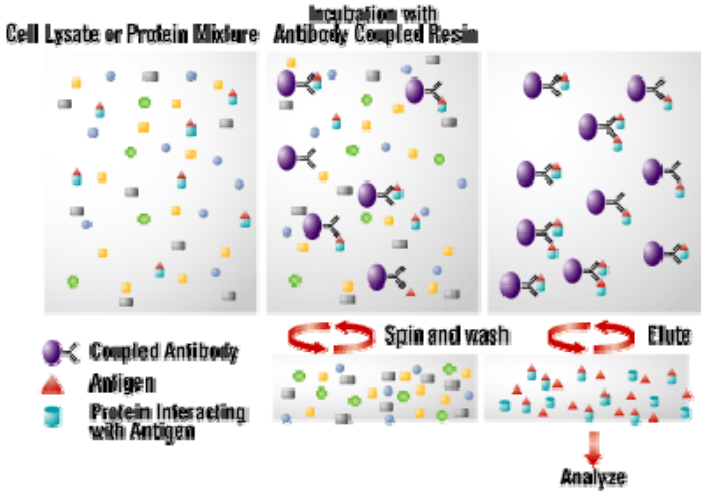


Figure 7: Principle of Co-IP
(Taken from http://www.piercenet.com/Products/Browse.cfm?fldID=9C471132-0F72-4F39-8DF0-455FB515718F)

In this study both IP and Co-IP were used (all buffers are listed in Appendix 3).

While working with anti-His (C-term) antibody, the IP procedure recommended by the manufacturer (Invitrogen) was followed. For a detailed protocol see the manufacturer's handbook.

For other antibodies slightly modified IP protocol was used. 150 µl of cell lysate (se 3.3 Western Blot analysis) was precleared for 30 minutes at 4 °C with 20 µl of washed (3x for 1 min with IP-wash solution and 3x for 1 min with IP-wash solution with 1% BSA) protein A or G beads. Beads were removed by centrifugation at 13000 rpm for 1 min. 1-2 µg of primary antibody were added to the lysate and rolled at 4 °C over night. At the same time a new

portion of beads were washed 3x for 1 min with IP-wash solution, added 1ml IP-wash solution with 1% BSA and rolled at 4 °C over night. Next day 20 µl of washed beads were added to lysate and rolled for 3 hours at 4 °C. Beads were sedimented at 13000 rpm for 1 min, and washed 3x with buffer containing BSA and 3x with buffer without BSA; beads were added sample buffer with reducing agent, boiled for 5 min and sedimented one more time, to avoid application of beads on the gel.

To perform Co-IP, 24 hours after transient transfection, N2a-PrP-EGFP cells were gently lysed using IP-lysis buffer; then lysates were added anti-RFP (directed against DsRed-Ex) or P4 (directed against PrP) and incubated over night at 4 ºC. Next day A or G beads were added to lysates containing anti-RFP or P4 antibodies, respectively, and incubated for additional 3 hours at 4 ºC. Samples were added sample buffer containing reducing agent, boiled for 5 min and applied on the gel. For detection of prion protein, P4 (1:10.000) and mouse true blot HRP (1:1.000) were used as primary and secondary antibodies, respectively. In order to detect PINT-DsRed-Ex, anti-RFP (1:16.000) and goat anti-rabbit HRP (1:15.000) were used.

3.5 Other assays performed in this study

In this study a few other assays were used in order to study cellular localization, signal sequences and other properties of PINT protein.

3.5.1 Leptomycin B studies of nuclear export

Small proteins and other molecules constantly move into and out of the cell nuclei. A number of such proteins have nuclear import and export leucine rich sequences. Leptomycin B is an unsatured, branched-chain fatty acid, an antibiotic with anti-fungal and anti-tumour activity (Hamamoto et al., 1983; Hamamoto et al., 1985), and a specific inhibitor of nuclear export signal. The suggested inhibition mechanism involves the direct binding of Leptomycin B to CRM1 (exportin 1), which blocks binding of CRM1 to proteins containing the nuclear export signal (Henderson et al., 2000; Nishi et al., 1994), via interaction with a cysteine residue in the CRM1 central conserved region (Kudo et al., 1999).

Before the treatment with leptomycin B, cells were synchronized, in order to ensure that cells progress through the cell cycle in a synchronous manner. To synchronize cells, several

methods are used. Commonly, cells are induced to quiescence by serum starvation before releasing them from this state, or cells are treated with chemical inhibitors which arrest cells in distinct phases of the cycle.

In this study cells were grown in medium containing 0.5% FBS (normal medium contains 10% FBS) for 2-3 days. This deprives the cells of the nutrients required to proliferate and forces the cells into quiescence, namely G_0-phase. The cells were released from quiescence by adding back serum to the medium.

Cells were added leptomycin B to the end concentration of 20 mM in Opti-MEM or DMEM with 10% serum and incubated at 37 °C. After 2.5 hours cells were fixed with formaldehyde and mounted with DacoCytomation fluorescent mounting medium and screened for fluorescence intensity.

3.5.2 PNGase F treatment

Glycosylation is one of many post-translational modifications of proteins. Two types of glycosylation exist: O-linked glycosylation to the hydroxy oxygen of serine and threonine side chains and N-linked glycans, which are attached to the protein backbone via an amide bond to an asparagine residue in an Asn-Xaa-Ser/Thr motif, where X can be any amino acid, except Pro (Maley et al., 1989). The most frequent enzyme used for deglycosylation of glycoproteins is N-glycosidase F (PNGase F). This enzyme hydrolyzes nearly all types of N-glycans chains from glycoproteins.

For enzymatic deglycosylation samples were treated with PNGase F, for 1 hour at 37 °C, as recommended by the manufacturer. For detailed protocol see the manufacturer's handbook.

3.5.3 Fluorescence protease protection (FPP) assay

FPP is a fluorescence based technique for investigating the topology of proteins and for localizing protein subpopulations within the complex environment of the living cell. The assay provides fluorescence readout in response to enzyme (trypsin or proteinase K) induced destruction of fluorescent tag attached to a protein of interest before and after plasma membrane permeabilization. This technique was published by Lorenz and colleagues (Lorenz et al., 2006). By this assay it is possible to determine whether a protein is membrane

associated, cytoplasmic or luminal and which part of a membrane protein faces the lumen (or cell exterior) and cytoplasm.

Treatment with trypsin of nonpermeabilized cells should readily extinguish fluorescence associated with molecule facing the cell exterior, but have no effect on fluorescence associated with molecule facing the cell interior. In order to permeabilize cell walls, digitonin was added to the cells. When added to the cells, digitonin, a small toxin, intercalates into cholesterol-rich membranes by forming a complex with hydroxysterols, causing the plasma membrane to become perforated (Plutner et al., 2002). Small molecules can then diffuse out of the cell from cytoplasm and nucleus, while, small external molecules such as trypsin can diffuse into the cell. Trypsin is a serine proteases found in the digestive system, where it breaks down proteins by cleaving peptide bonds in proteins. When in the cell, trypsin extinguishes fluorescence from bound molecules facing the cytosol, which can not diffuse out of the cell. However, molecules localized in different organelles such as Golgi, ER, mitochondria, peroxisomes and others, are protected from trypsin and fluorescent signal is not affected.

The similar results can be achieved by using proteinase K instead of trypsin, which is also a serine protease that cleaves peptide bonds at the carboxylic sides of aliphatic, aromatic or hydrophobic amino acids.

In this study digitonin concentration of 40 mM was sufficient to release DsRed-Ex from the cytosol in N2a and SH-SY5Y cell lines; trypsin was applied directly to the cells at concentration of 4 mM, proteinase K was used at final concentration of 50 µg/ml.

Cells were grown in glass chamber slide (Lab-Tec, NUNC), and transfected as previous described. After 24 hours cells were washed with room temperatured KHM buffer and added digitonin, later added KHM buffer with trypsin or proteinase K, and occurred changes in fluorescent signal was captured with microscope.

Biochemical protease protection assay: for this assay the protocol published by Lorenz and colleagues (Lorenz et al., 2006) was slightly modified and used in the present study.

Transiently transfected N2a cells 24 hours after transfection were washed 1x with PBS, trypsinated, washed 1x with MEM with 10% serum to stop trypsin action, and then 1x with KHM buffer (Appendix 3). Washes were performed using 5 min centrifugations at 4 °C, 500x g. 25% of washed cells were added SDS-buffer and boiled at 100 °C for 5 min (fraction: homogenate of whole cells). Rest of the cells were added digitonin to the end concentration of

40 μM, incubated on ice for 10 min and divided into three tubes. One tube was centrifuged at 4 °C for 30 min, 50000x g (supernatant-cytosol fraction; pellet-membrane fraction). Cells in the second tube were added trypsin or proteinase K to a final concentration of 4 mM and 50 μg/ml, respectively; the third tube was added trypsin/proteinase K to a final concentration of 4 mM/50 μg/ml and 1% (vol/vol) triton X-100. Both tubes were incubated on ice for 30 min and then centrifuged at 4 °C for 30 min, 50000x g. Pellet was carefully washed with KHM buffer and treated with benzonase for the removal of nucleic acids from protein samples (pellet was added benzonase buffer (Appendix 3) and boiled for 5 min, later 1μl of benzonase (344 units) was added to each sample and induced at 37 ºC for 1 hour). Samples were added sample buffer, boiled for 5 min and loaded on a gel.

4. Fluorescence microscopy

Fluorescence microscopy is used to detect structures, molecules or proteins within the cell. Fluorescent molecules absorb light at one wavelength and emit light at another, longer wavelength. When fluorescent molecules absorb a specific absorption wavelength for an electron in a given orbital, the electron rises to a higher energy level (the excited) state. Electrons in this state are unstable and will return to the ground state, releasing energy in the form of light and heat. This emission of energy is fluorescence. Because some energy is lost as heat, the emitted light contains less energy and therefore is a longer wavelength than the absorbed (or excitation) light.

In fluorescence microscopy, a cell is stained with a dye and the dye is illuminated with filtered light at the absorbing wavelength; the light emitted from the dye is viewed through a filter that allows only the emitted wavelength to be seen. The dye glows brightly against a dark background because only the emitted wavelength is allowed to reach the eyepieces or camera port of the microscope. Most microscopes are designed using epi-illumination. In epi-illumination excitation, light goes through the objective lens and illuminates the object. Light emitted from the specimen is collected by the same objective lens (Figure 8).

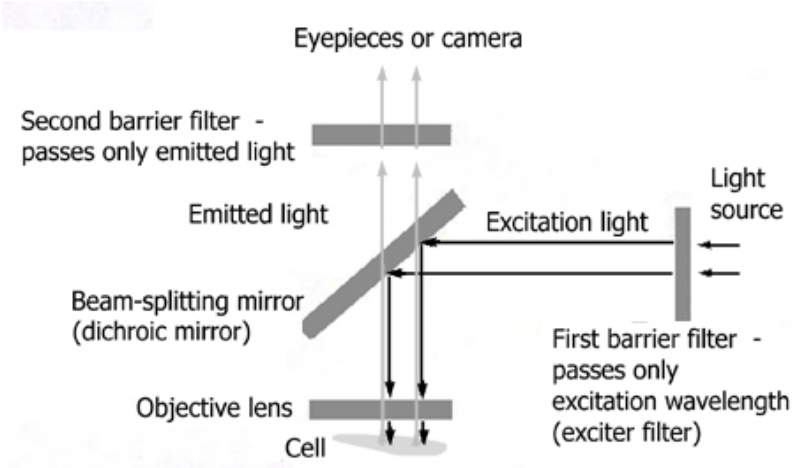


Figure 8: The optical system of an epi-fluorescence upright microscope (taken from http://dept.kent.edu/projects/cell/fluoro.htm)

In this study Olympus IX81 fluorescence microscope was used for imaging and capturing of signals.

PINT protein was fused with red fluorescent protein, while one of stable N2a cell lines, N2a-PrP-EGFP, had enhanced green fluorescent protein (EGFP) fused to PrP (emission and excitation spectra for EGFP and DsRed are presented in Figure 9) (Billinton et al., 2001).

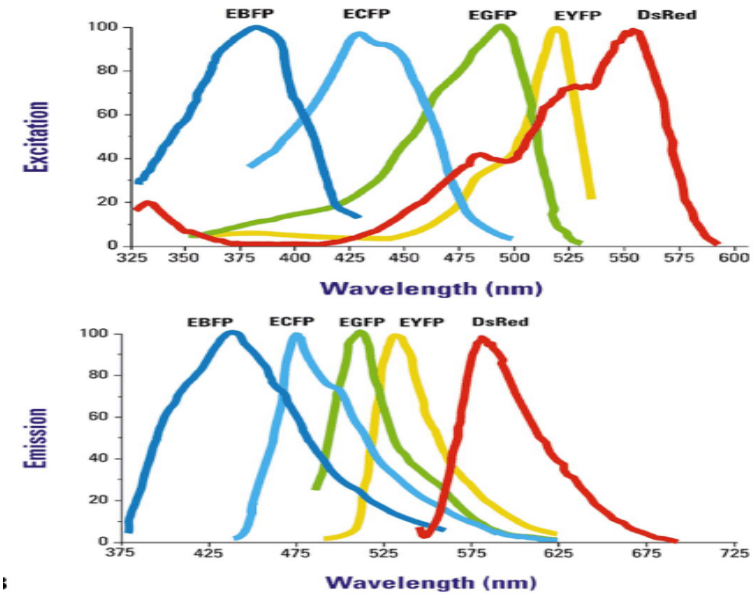


Figure 9: Excitation and emission spectra for EGPR and DsRed (Billinton et al., 2001)

III. Results

1. Cloning

1.1 pDsRed-Express-PINT construct

For production of the pDsRed-Express-PINT construct, PCR was runned using PINTRedR and PINTRedF primers (Table 3) and pGEM-T-Easy-PINT 1B clone as template DNA to amplify PINT fragment. Later this PINT fragment and pDsRed-Express-N1 vector were cut with the restriction enzymes BglII and BamHI in BamHI buffer; after ligation with T4 DNA ligase, a new opened reading frame was formed (Appendix 1) comprising whole PINT protein connected to N-terminal of DsRed-Express (variant of red fluorescent protein). Competent DH5α cells were added aliquots ligation reaction mix, streaked out on preheated LB plates containing 50 μg/ml canamycin and incubated at 37 °C over night. Next day a few of the colonies were picked, inoculated in 2 ml LB medium containing canamycin and grown over night with vigorous shaking at 37 °C. Plasmid DNA was isolated with Plus Sv Minipreps DNA Purification System from Promega.

Restriction fragment analysis was used to verify a successful introduction of insert into vector (Figure 10). Plasmid cut with BglII and BamHI (lane 1) showed two DNA fragments of about 5000 and 800 bp, (vector and PINT fragment, respectively), because PINT fragment has restriction sites of these enzymes on the both sides. Plasmid containing PINT insert and empty vector were also analysed with BsaHI restriction enzyme in NE Buffer 4 (Figure 10). This analysis also indicated that plasmid contains the PINT fragment. BsaHI enzyme has a few restriction sites in pDsRed-Express-N1 vector, and one in PINT fragment. Because of that, restriction analysis of pDsRed-Express-PINT (lane 2) showed one additional fragment on the agarose gel if compared to analysis of empty vector (lane 3).

Before starting other experiments with this construct, pDsRed-Express-PINT was sequenced to confirm correct amplification and cloning using DsRedR and DsRedF primers (Table 3). Later this construct was used for expression of PINT-DsRed-Express protein (PINT-DsRed-Ex) in mammalian cells.

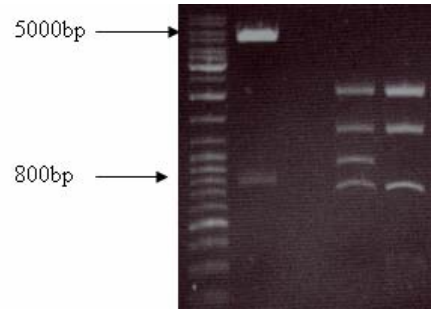


Figure 10: Restriction analysis of pDsRed-Express-PINT: 1) pDsRed-Express-PINT cut with BglII and BamHI; 2) pDsRed-Express-PINT cut with BsaHI; 3) pDsRed-Express-N1 vector cut with BsaHI; 1% agarose gel.

1.2 pZeoSV-PINT construct

To make pZeoSV-PINT construct, PCR was runned using PINT-Z-R and PINT-Z-F primers (Table 3) and pGEM-T-Easy-PINT 1B clone as template DNA to amplify PINT fragment. Amplified fragment and pZeoSV vector were cut with two restriction enzymes, AgeI and MluI, in R-buffer containing BSA; after ligation a new open reading frame was formed containing the whole PINT ORF with C-terminal His_6-tag (Appendix 1).

Transformed DH5α *E.coli* colonies were grown on LB plates containing 50 μg/ml zeocin over night. Next day a few of the colonies were picked, inoculated in 2 ml LB medium containing zeocin and grown over night with vigorous shaking at 37 °C. Plasmid DNA was isolated with Plus Sv Minipreps DNA Purification System from Promega.

Restriction analysis was performed to confirm that the insert was successfully introduced into the vector (Figure 11). After cutting plasmid with AgeI and MluI, two DNA fragments were formed (lane 1), since PINT-His_6 fragment contains restriction sites of both enzymes. The larger fragment (≈ 3500 bp) matched pZeoSv vector, while smaller fragment (≈ 850 bp) matched PINT-His_6.

Prior to further studies of this construct, pZeoSV-PINT was sequenced to confirm correct amplification and cloning using SP6 and T3 primers (Table 3). Later this construct was used for the expression of PINT-His_6 protein in mammalian cells.

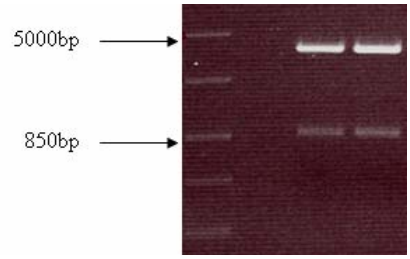


Figure 11: Restriction analysis of pZeoSv-PINT construct: 1) plasmid cut with AgeI and MluI; 2) parallel experiment using different *E.coli* colony; 1% agarose gel.

1.3 pET-PINT construct

To construct pET-PINT, primers PINTlongF and PINT-pETcorrR (Table 3) and pET-PINT-16b as template DNA were used to run PCR. After amplification, PINT fragment and pET16B vector were cut with two restriction enzymes, NdeI and BamHI, in R-buffer containing BSA; after ligation a new plasmid, coding for PINT with N-terminal His_6-tag, was formed (Appendix 1). DH5α *E.coli* were streaked out LB-plates containing 50 µg/ml ampilicin and grown over night. A few colonies were inoculated in 2 ml LB medium, grown over night and DNA was purified with Plus Sv Minipreps DNA Purification System from Promega.

Restriction analysis with NdeI and BamHI enzymes was performed (Figure 12), because PINT fragment has restriction sites of both enzymes. After this cut two DNA fragments were seen on the agarose gel: one, that matched pET16B vector, and second, slightly smaller than 850 bp, matching PINT fragment.

Prior to further studies of this construct, pET-PINT was sequenced to confirm correct amplification and cloning using T7R and T7R primers (Table 3). Later this construct was used for the expression of His_6-PINT protein in bacterial cells.

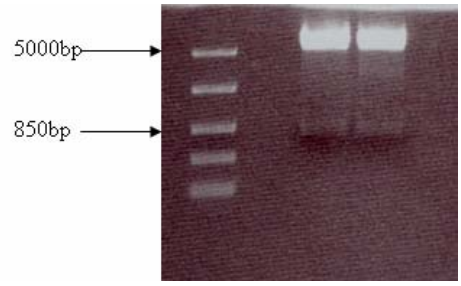


Figure 12: Restriction analysis of pET- PINT: 1) pET-PINT cut with NdeI and BamHI; 2) parallel experiment with different E. coli colony; 1% agarose gel.

2. Results of PINT protein expression in bacterial cells

2.1 Protein expression at 37 °C in *E.coli*

The pET system is a powerful system for cloning and expression of recombinant proteins in bacterial cells. In order to express recombinant protein, target genes have to be induced with IPTG; after induction it takes just a few hours and desired protein can comprise more than 50% of the total cell proteins. In the present study pET-PINT construct was used for expression and purification of His_6-PINT protein in *E.coli* BL21 cells. His_6-PINT is about 35 kDa protein.

First, cell growth and protein purification were performed as recommended by the manufacturer by using Ni-NTA Spin kit from Qiagen. To 50 ml of NZCYM medium containing ampicilin and Chl were added 800 µl cell suspension (*E.coli* BL21 cells containing pET-PINT plasmid); cells were grown until optical density of suspension reached 0.3 at 550 nm, induced with IPTG and grown for about 5 hours with vigorous shaking at 37 °C. Some samples were taken before and after induction and analysed by SDS-PAGE (Figure 13). His_6-PINT was observed on the gel already after 1 hour of induction (lane 3); after 5 hours, 35 kDa band was very strong (lane 6), indicating high concentration of His_6-PINT protein.

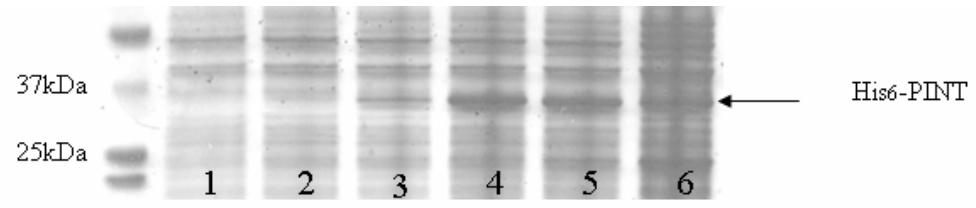


Figure 13: Induction of cell suspension with IPTG: 1) sample before adding IPTG; 2-6) samples after 0.5, 1, 2, 3 and 5 hours after induction, respectively; 10% polyacrylamide gel stained with CBB.

After five hour induction cells were harvested by centrifugation and later used in purification assays.

For purification under native conditions, cells were added lysis buffer and lysozyme, incubated on ice for 30 min; cell walls were disrupted by sonication or French press, and after 0.5 hour centrifugation at 10000x g the collected supernatant was loaded on a Ni-NTA spin

column. The column was washed twice and PINT was eluted in elution buffer. Samples after each step were analysed by SDS-PAGE (Figure 14).

Purification under denaturing conditions ensures that all His_6-tagged proteins in the cell are solubilised and that His_6 tag is fully exposed, which leads to more efficient purification. After lysis with strong denaturants, cells were centrifuged for 0.5 hour at 13000x g and the collected supernatant was loaded on a Ni-NTA spin column. His_6-PINT was eluted in elution buffer after two washes. Samples of each step were analysed by SDS-PAGE (Figure 14).

His_6-PINT was not eluted neither by native nor denaturing conditions (lanes 5-6 and 11-12, respectively), no bands were seen at the expected 35 kDa size; PINT protein was not seen in cell lysates either (lanes 1 and 7) (just very small amount of protein at size of 35 kDa were seen on the gel in cell lysates when purification was performed under denaturing conditions (lane 7), indicating that denaturing conditions as well as native ones are too mild for purification of His_6-PINT). This result showed that His_6-PINT protein is not soluble when expressed in E. coli at 37 °C, probably due to formation of inclusion bodies

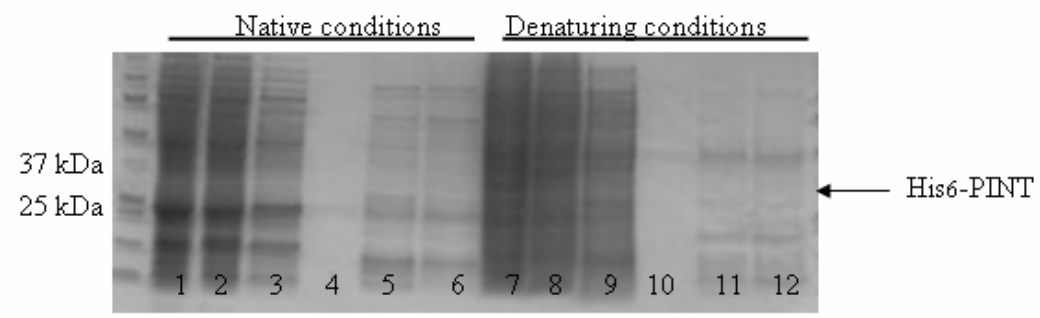


Figure 14: His_6-PINT purification: native conditions: 1) cell lysate; 2) flow-through; 3-4) washes; 5-6)eluates; denaturing conditions: 7) cell lysate; 8) flow-through; 9-10) washes; 11-12) eluates; 10% polyacrylamide gel stained with CBB.

2.2 PINT expression and purification under different conditions

Inclusion bodies are a common problem when expressing recombinant proteins in prokaryotes. It is often difficult to obtain soluble and active proteins. Often, overexpression of recombinant protein leads to the production of inclusion bodies- insoluble aggregates of misfolded protein.

In order to avoid the formation of inclusion bodies, different growth conditions of bacterial cell culture were applied in the present study.

Bacterial culture was grown at 16-25 °C temperature until optimal density of cell suspension at 550 nm reached about 0.3, and then was induced with IPTG at the final concentration of 1mM. Because of lower temperature induction time was increased up to 35 hours (16 °C). Afterwards, cells were centrifuged and purification at native and denaturing conditions was performed as described above. No effect of lower temperature was observed; even when expressed at 16 °C, His_6-PINT remained in its insoluble form (data not shown).

2.3 Inclusion bodies

After all unsuccessful attempts to avoid formation of inclusion bodies, the best conditions to wash and solubilise inclusion bodies, to refold and elute His_6-PINT protein has were studied.

E.coli BL21 cells were grown at 37 °C as described in 2.1 chapter in Materials and Methods. After 4 hour induction with IPTG cells were sedimented, and His_6-PINT inclusion bodies were washed following protocol described in 2.3.1 chapter of Materials and Methods. Sample containing washed inclusion bodies was analysed by SDS-PAGE (Figure 15). After the washes PINT inclusion bodies were quite pure, just minor impurities were seen on the gel.

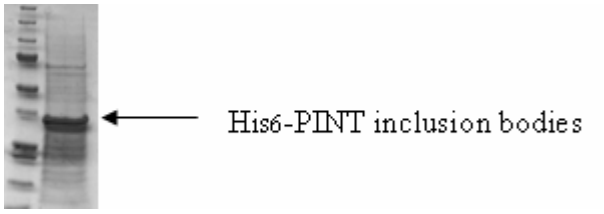


Figure 15: Washed inclusion bodies; 10% polyacrylamide gel stained with CBB.

From this stage on, different methods were tried out to solubilise and refold PINT protein, because in order to obtain soluble and active protein, the protein has to be refolded after solubilisation of inclusion bodies.

2.4 Refolding of PINT protein on the column

Inclusion bodies can be solubilised in buffers containing high concentrations of strong detergents. In this study buffers containing 6-8 M guanidine hydrochloride or urea were used (for composition of all buffers used to refold PINT protein on the column see Appendix 3; for detailed protocols see 2.3.2 chapter in Materials and Methods).

Washed inclusion bodies were dissolved in refolding buffer containing 6 M guanidine hydrochloride by sonication and vortexing, and were stirred over night at 4 °C. Solubilised protein was filtered through 0.2 μm filter before loading onto His Gravi Trap column. Buffer change from refolding to phosphate buffer was performed by slowly washing the column with refolding/phosphate buffers in different ratio. Protein was eluted in phosphate buffer containing 300 mM imidazole (Figure 16 A). It was clear that at 4 °C solubilisation was unsuccessful; protein was lost in the filter (lane 3) and no protein was eluted (Figure 16A, lanes 10 and 11).

To solve this problem, solubilisation and refolding of His_6-PINT again was performed in guanidine hydrochloride buffer, but this time at room temperature (Figure 16 B). Still, PINT was not eluted from the column (lane 11), but inclusion bodies were solubilised (after filtration, supernatant gave a strong band of about 35 kDa (Figure 16B, lane 4)) and PINT protein was bound to the column, because no protein was detected in flow-through (Figure 16B, lane 5).

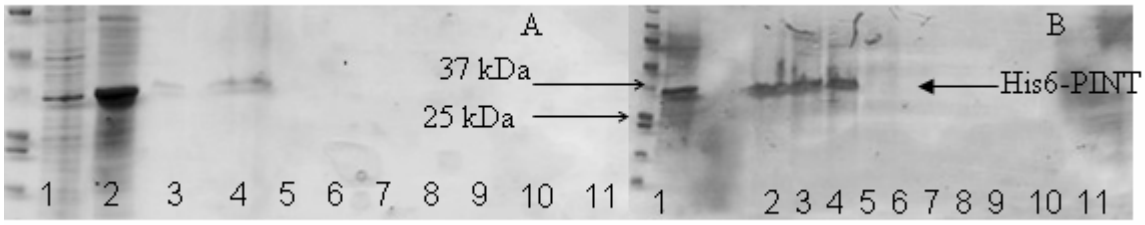


Figure 16: A) inclusion bodies solubilised at 4 °C in guanidine hydrochloride buffer:
1) sample after 4 hour induction with IPTG; 2) inclusion bodies in refolding buffer; 3) supernatant after filtration; 4) flow-through; 5-8) buffer change (refolding: phosphate buffer- 4:1, 3:2: 2:3 and 1:4 ratio, respectively); 9) wash; 10-11) eluates; **B)** inclusion bodies solubilised at room temperature in guanidine hydrochloride buffer: 1) washed inclusion bodies; 2) inclusion bodies in refolding buffer; 3) supernatant after centrifugation; 4) supernatant after filtration; 5) flow-through; 6-9) buffer change (refolding: phosphate buffer- 4:1, 3:2: 2:3 and 1:4 ratio, respectively); 10) wash; 11) eluate.

Afterwards another strong detergent, urea, was also used for solubilisation of inclusion bodies and refolding of protein at 4 °C or RT. All attempts were unsuccessful. Inclusion bodies were not dissolved at all (4 °C) or protein was not eluted (RT) from the column (data not shown).

The same negative results were obtained in attempt to solubilise and refold His_6-PINT protein combining two buffers containing both detergents. In order to do that, washed inclusion bodies were dissolved in buffer containing 6 M guanidine hydrochloride by vortexing, and suspension was stirred at RT for 1 hour. After loading suspension onto the His Gravi trap column, the column was slowly washed with buffer containing 6 M urea. PINT was refolded by very slowly washing column with buffers containing different urea concentrations (from 6 M urea to 0 M); but anyway, no His_6-PINT protein was eluted in buffer containing 300 mM imidazole (Figure 17).

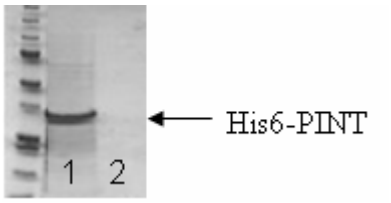


Figure 17: Refolding of inclusion bodies by combining buffers containing guanidine hydrochloride and urea: 1) washed inclusion bodies; 2) eluate; 10% polyacrylamide gel stained with silver stain.

2.5 Refolding of PINT by dilution

Dilution is one of the most popular methods for refolding of inactive proteins. In the present study a few different protocols were used (see 2.3.2 chapter in Materials and Methods). Washed inclusion bodies were solubilised in refolding buffer containing strong detergent, stirred over night at RT, very slowly diluted ten times with buffer containing no detergent (dilution was performed at 4 °C or RT) and stirred with magnet for 3 hours (4 °C or RT). Afterwards, mixture was centrifuged for 15 min, filtered through 0.2 μm filter and loaded onto the column. Protein was eluted in elution buffer containing 300 mM imidazole (Figure 18). Lanes 6-7 indicated that His_6-PINT protein was not eluted from the column.

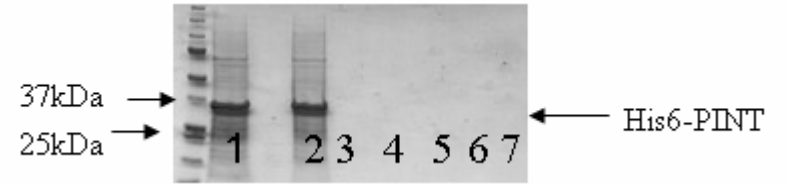


Figure 18: Refolding of PINT protein by dilution: 1) washed inclusion bodies; 2) inclusion bodies in refolding buffer; 3) supernatant after dilution; 4) supernatant after filtration; 5) flow-through; 6-7) eluates; 10% polyacrylamide gel stained with CBB.

It is possible that His_6-PINT protein did not bind to the column at all (just because of high dilution no protein was detected in flow-through (Figure 18, lane 5)), or bound column so strong that was not eluted from the column.

2.6 Second pET-PINT construct

All the experiments described in chapter 2 in Results were performed using pET-PINT construct coding for His_6-PINT protein with twenty additional amino acids on its C-terminal end (if compared to the longest protein form *in vivo*; see Alternative splicing in Introduction). To check if this additional tail was influencing the solubility of PINT protein, a new construct with the correct C-terminal end was made (see 1.3 pET-PINT construct in the Results). Plasmid was amplified in DH5α *E.coli* cells; purified using Plus Sv Minipreps DNA Purification System from Promega, and then used for transformation of competent *E.coli* BL21 cells. Transformed cells were grown on NZCYM-plates containing ampicilin/Chl over night. Some colonies were picked, inoculated with 2ml NZCYM medium containing both antibiotics and grown over night at 37 °C with vigorous shaking. Further purification under native conditions was performed exactly as described above. Samples after each step was analysed by SDS-PAGE (Figure 19).

Already after one hour induction with IPTG cells started to synthesize His_6-PINT protein (lane 2); concentration of PINT was high after over night induction with IPTG (lane 5). After lysis and centrifugation both, supernatant and pellet were applied on a gel (lanes 6 and 7, respectively). Lane 7 indicated inclusion bodies of His_6-PINT, because all protein remained in the pellet, no protein was seen in supernatant, and no bands were observed in eluates at the expected size (lanes 11-12). It seems that removal of the additional amino acid tail had no effect on the solubility of His_6-PINT protein.

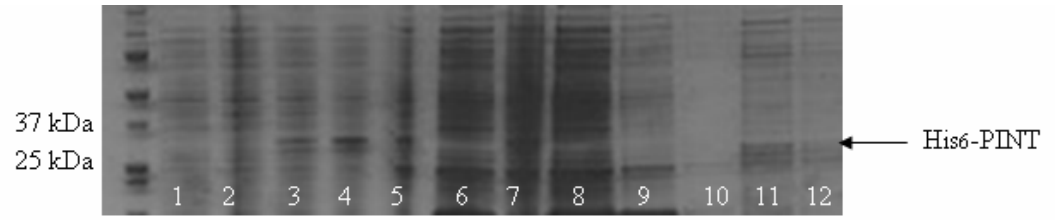


Figure 19: Purification of correct size His_6-PINT protein under native conditions: 1) sample before induction with IPTG; 2-5) samples after 0.5, 1, 2 hours and over night induction, respectively; 6) supernatant after lysis and centrifugation; 7) pellet after lysis and centrifugation; 8) flow-through; 9-10) washes; 11-12) eluates; 10%polyacrylamide gel stained with CBB.

3. PINT protein expression in mammalian cells

3.1 Expression of PINT-His_6 in mammalian cells

To study the localization of PINT protein in mammalian cells, pZeoSV-PINT construct, coding for PINT-His_6 protein, was used. PINT-His_6 is about 30 kDa protein. His_6 tag is a small, not fluorescent tag, so success of transfection can not be confirmed by fluorescent microscope.

N2a cells were transiently transfected with pZeoSV-PINT. 24 hours after transfection, cells were lysed and analyzed in western blot. As a control, untransfected cells were used. Two different anti-His antibodies (Invitrogen and Amersham Bioscience) were used as primary antibodies (1:5000). Both goat-anti-mouse ALP- (1:5000) and goat-anti-mouse HRP- (1:20000) conjugated secondary antibodies were tested, and signals were captured by Thyfoon scanning and hyperfilm, respectively. Western blot analysis did not show any difference between untransfected control (Figure 20, lane 1) and transfected cells (Figure 20, lanes 2-3); no bands were seen at the size of about 30 kDa.

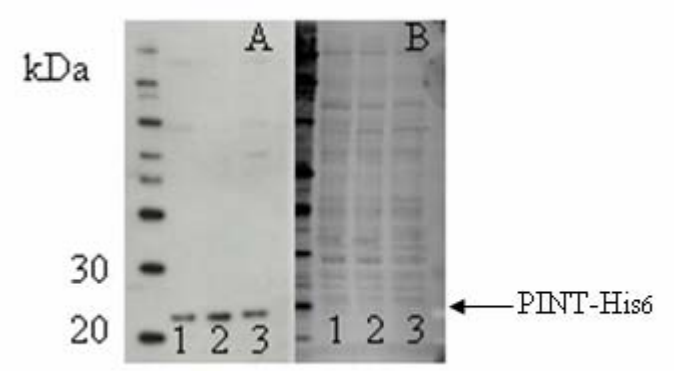


Figure 20: Western blot analysis of PINT-His_6: 1) untransfected N2a cells; 2-3) N2a cells transiently transfected with pZeoSV-PINT; A) signal detected with anti-His (Invitrogen) and goat-anti-mouse HRP; B) signal detected with anti-His (Amersham Bioscience) and goat-anti-mouse ALP; 12% polyacrylamide gel.

Immunoprecipitation with two different anti-His antibodies of PINT protein from lysates did not give positive results either (results not shown).

From this stage on, PINT fused with DsRed-Express protein (coded by pDsRed-Express-PINT) was used for further studies, because of all unsuccessful attempts to detect PINT-His_6 protein in murine N2a cells by analyzing lysates directly or after immunoprecipitation in western blots.

3.2 PINT protein cellular localization

To study cellular localization of PINT protein, two assays were used in the present study: transfection of N2a and SH-SY5Y cells with pDsRed-Express-PINT and fluorescence protease protection (FPP) assay.

First both cell lines were transiently transfected with pDsRed-Express-PINT, which codes for PINT-DsRed-Express (PINT-DsRed-Ex) protein, using lipofectamine and Plus reagents. Successful transformation was confirmed by fluorescence microscope 24 hours after transfection. A strong red signal was seen in both cell lines (Figure 21).

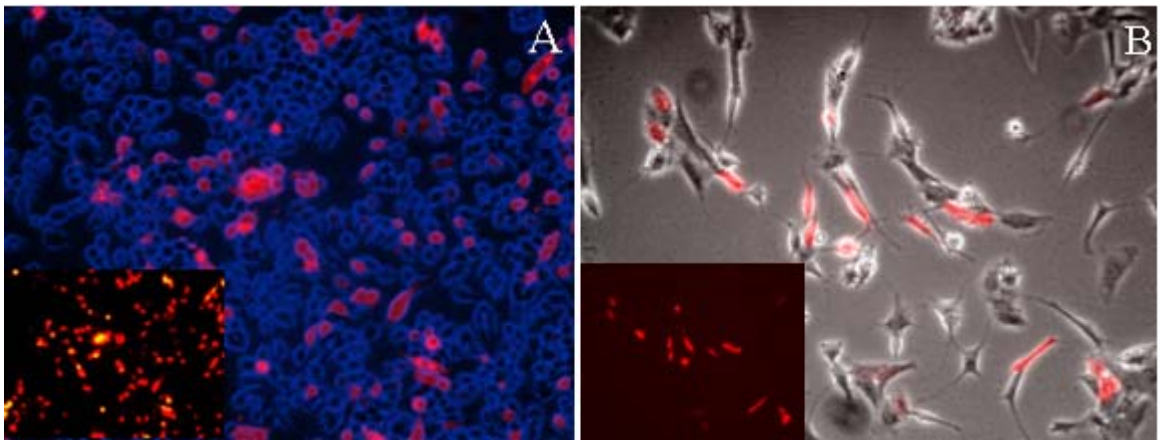


Figure 21: PINT-DsRed-Ex 24 hours after transfection, expressed in: A) N2a cells; B) SH-SY5Y cells.

In order to compare the signal from the PINT-DsRed-Ex protein to DsRed-Ex alone, both cell lines were also transiently transfected with pDsRed-Ex (Figure 22). It was clearly seen that the localization of signal in the cells transfected with pDsRed-Ex (Figure 22 A and C) is quite different if compared to cells transfected with pDsRed-Express-PINT (Figure 22 B and D). DsRed-Ex is a small 28 kDa protein, which is localized mostly in cytoplasm and nucleus, and which gives an even signal all over the cell when red filter in fluorescent microscope is used. When cells were transfected with pDsRed-Express-PINT, the red signal, in most cases, was localized around the nucleus of the cell. In some cells the signal was also detected in nucleus in different patterns, and other cells had so strong and bright signal that it was impossible to decide the cellular localization.

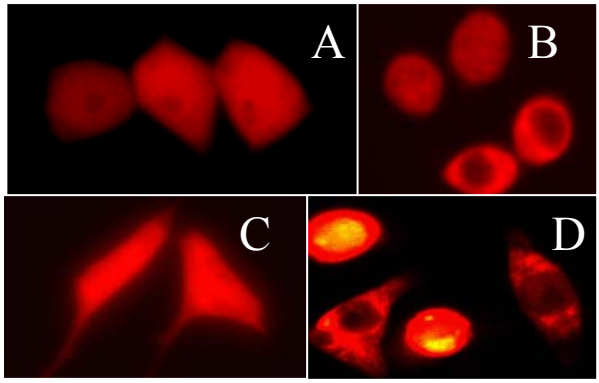


Figure 22: Transiently transfected cells (24 hours after transfection): A-B) N2a cells transfected with pDsRed-Express and pDsRed-Express-PINT, respectively; C-D) SH-SH5Y cells transfected with pDsRed-Express and pDsRed-Express-PINT, respectively.

Western blot analysis of transfected cells confirmed that PINT-DsRed-Ex protein was expressed in the cells (Figure 23). Transiently transfected cells (N2a and SH-SY5Y) were harvested 24, 48 and 72 hours after transfection for detection of PINT-DsRed-Ex protein (about 50 kDa). Unfortunately, the anti-RFP antibody from Chemicon gave unspecific bands at the same size as PINT-DsRed-Ex protein in untransfected N2a and SH-SY5Y cells (Figure 23, lanes e and h, respectively) making it difficult to confirm the presence of PINT protein in transfected cells. The unspecific bands disappeared when reducing agent was omitted from the samples (Figure 23, lanes D and H). PINT was synthesized in both cell lines 24, 48 and 72 hours after transfection.

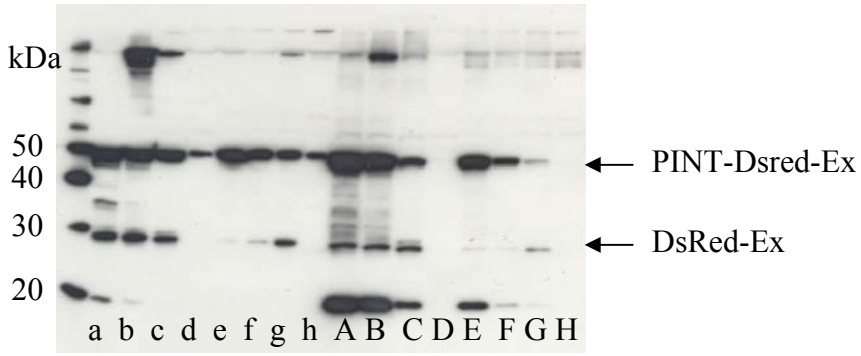


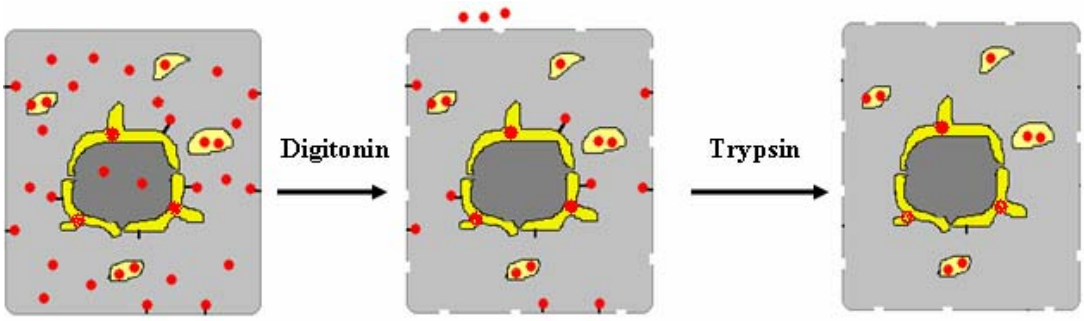
Figure 23: Western blot analysis of transiently transfected N2a and SH-SY5Y cells: samples added reducing agent: a-c) transfected N2a cells harvested after 24, 48 and 72 hours; d) untrasfected N2a cells; e-g) transfected SH-SY5Y cells harvested after 24, 48 and 72 hours; h) untrasfected SH-SY5Y cells; samples without reducing agent: A-C) transfected N2a cells harvested after 24, 48 and 72 hours; D) untrasfected N2a cells; E-G) transfected SH-SY5Y cells harvested after 24, 48 and 72 hours; H) untrasfected SH-SY5Y cells; 12% polyacrylamide gel, signal detected with anti-RFP (1:500) (Chemicon) and goat-anti-rabbit HRP (1:15000).

Fluorescence protease protection (FPP) assay was used to study the localization of PINT protein in detail. FPP provided fluorescence readout in response to trypsin or proteinase K induced destruction of DsRed-Ex attached to PINT protein before and after plasma membrane permeabilization.

N2a cells transiently transfected with pDsRed-Express-PINT were washed once with KHM buffer 24 hours after transfection (Figure 24II A), after removing washing buffer, trypsin at a final concentration of 4 mM was added to the cells. After 3 min incubation, the signal was preserved. No effect of trypsin on nonpermeabilized cells indicated that PINT

protein is localized internally. After removal of trypsin, digitonin was added (40 µM solution) and incubated for 3 min (Figure 24II B). Digitonin permeabilizes the plasma membrane and releases first cytosolic and later nuclear DsRed-Ex. Before this treatment the signal was bright, but after 3 min with digitonin it became weaker, showing that some of PINT-DsRed-Ex was localized freely in cytoplasm and nucleus. After digitonin was removed, trypsin was again added to the cells. 3 min later the signal was even weaker (Figure 24II C), demonstrating that trypsin entered digitonin permeabilized cells and extinguished fluorescence from bound DsRed-Ex molecules facing the cytosol. The still remaining signal after trypsin treatment showed, that PINT-DsRed-Ex might be localized in mitochondria or ER (cartoon illustrating a possible cellular localization of PINT-DsRed-Ex is presented in Figure 24 I). After treatment with triton X-100 the signal totally vanished (Figure 24II D). Still, in some cases the red signal did not disappear, this was observer mostly in the cells that at the beginning gave very strong signal (Figure 24II E).

I



II

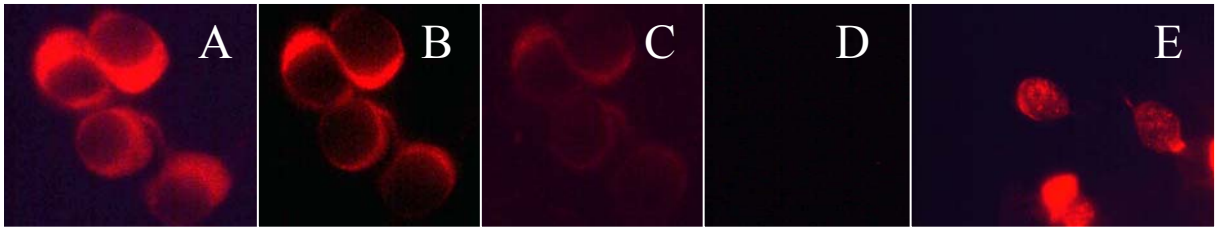
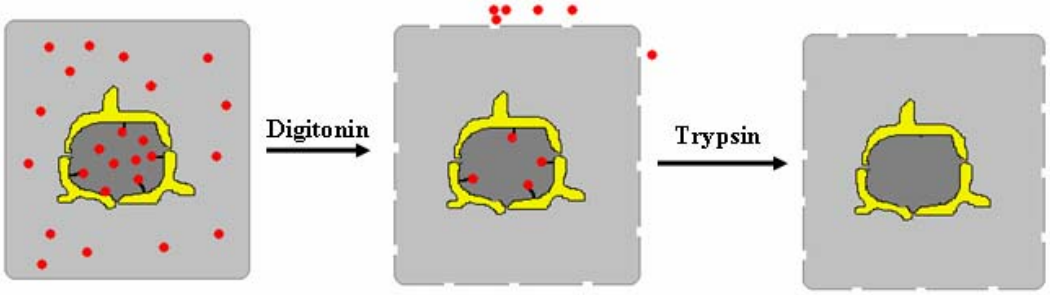


Figure 24: FPP assay (using trypsin) of N2a cells transiently transfected with pDeRed-Express-PINT: **I**. Cartoon of the FPP assay illustrating the cellular localization of PINT-DsRed-Ex; **II**. A) Control cells washed with KHM buffer; B) the same cells after 3 min treatment with digitonin; C) cells after 3 min treatment with trypsin; D) cells after treatment with triton X-100; E) some other cells after triton X-100 treatment.

Parallel experiment was performed with stable N2a-DsRed-Ex clones (Figure 25II A), and large differences between cells transfected with pDsRed-Express alone and pDsRed-Express-PINT were observed. After 5 min treatment with digitonin almost all the signal had disappeared, only in the nucleus a weak signal remained (Figure 25II B), which disappeared 30 seconds after trypsin addition to the cell. It seems that DsRed-Ex is bound to some structures in the nucleus of the cell. Cartoon showing cellular localization of Dsred-Ex is presented in Figure 25I.

I



II

Figure 25: FPP assay (using trypsin) of sable N2a-DsRed-Ex cells: **I**. Cartoon of the FPP assay illustrating the cellular localization of DsRed-Ex; **II**. A) Control cells washed with KHM buffer; B) the same cells after 3 min treatment with digitonin; C) cells after 30 seconds treatment with trypsin.

Slightly different results were obtained when N2a cells transiently transfected with pDsRed-Express-PINT were treated with proteinase K instead of trypsin (Figure 26). Proteinase K is another serine protease that cleaves peptide bonds at the carboxylic sides of aliphatic, aromatic or hydrophobic amino acids. Transfected N2a cells were washed with KHM buffer (Figure 26 A) and incubated with digitonin at a final concentration of 40 µM for 3 min (Figure 26 B). When digitonin was removed, proteinase K at a concentration of 50 µg/ml was added. After 3 min incubation all signal had disappeared from some cells (Figure

26 C). Other cells were not affected by proteinase K and still gave a quite strong red signal, which did not vanish even after triton X-100 was added to the cells (Figure 26 D).

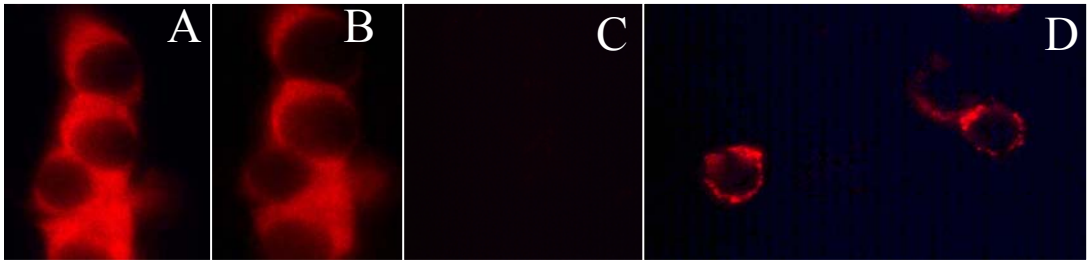


Figure 26: FPP assay (using proteinase K) of N2a cells transiently transfected with pDsRed-Express-PINT using proteinase K: A) control cells washed with KHM buffer; B) the same cells after 3 min treatment with digitonin; C) cells after 3 min treatment with proteinase K; D) other cells after proteinase K treatment.

To confirm the results of PINT protein localization from FPP assay, biochemical protease protection assay was also performed using trypsin and proteinase K (Figure 27). It seems, that DsRed-Ex is mostly localized freely in cytosol (lane b, band of 28 kDa) and some can be seen in membrane fraction after digitonin treatment (lane c). A band of 28 kDa is also seen in samples which were treated with trypsin and very weak band was seen after proteinase K treatment (lanes d and f, respectively). It might be that some of DsRed-Ex is protected from trypsin. Cells transiently transfected with pDsRed-Express-PINT gave a quite different picture. PINT-DsRed-Ex is seen in whole cell homogenate (Figure 27, lane A); some in cytosol just in oligomeric form (Figure 27, lane B, band heavier than 100 kDa) and a strong band at a size of 50 kDa was present in the membrane fraction after digitonin treatment (Figure 27, lane C). No difference was seen in lanes D and E (Figure 27) presenting samples added trypsin with and without triton X-100, respectively. As mentioned before, quite a few cells gave a fluorescent signal even after triton X-100 was added (Figure 24 E). It seems, that PINT-DsRed-Ex might form aggregates, and that proteinase K managed to penetrate those PINT-DsRed-Ex aggregates much better than trypsin (Figure 27, lane F), but still some signal remained even after triton X-100 addition (Figure 27, lane G).

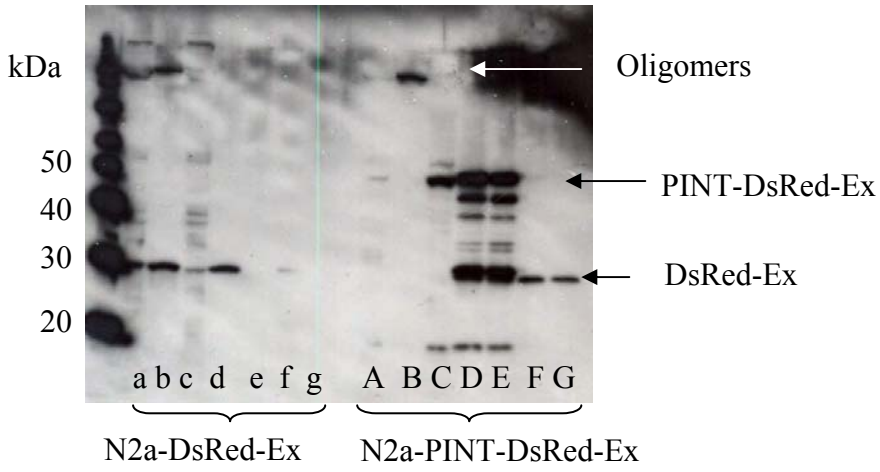


Figure 27: Biochemical protease protection assay: N2a-DsRed-Ex cells: a) whole cell homogenate; b) cytosol fraction after digitonin treatment; c) membrane fraction after digitonin treatment; d) membrane fraction after trypsin; e) membrane fraction after trypsin and triton X-100; f) membrane fraction after proteinase K; g) membrane fraction after proteinase K and triton X-100; N2a-PINT-DsRed-Ex cells: A) whole cell homogenate; B) cytosol fraction after digitonin treatment; C) membrane fraction after digitonin treatment; D) membrane fraction after trypsin; E) membrane fraction after trypsin and triton X-100; F) membrane fraction after proteinase K; G) membrane fraction after proteinase K and triton X-100; 12% polyacrylamide gel, signal detected with anti-RFP (1:16000) (Clontech) and goat-anti-rabbit HRP (1:15000).

3.2 Stable transfection of SH-SY5Y cells

To achieve more physiological levels of PINT-DsRed-Ex protein expression in cells, and to avoid aggregate formation, stable transfection of SH-SY5Y cells was performed. In this kind of transfection cells that have inserted the foreign genetic material into their genome are selected. After applying selection pressure with G418 (drug for selection of stable mammalian cell lines) for some time, only the cells with a stable transfection remained. Stably transfected cells synthesize smaller amount of recombinant protein, making it easier to study cellular localization of PINT-DsRed-Ex protein (Figure 28).

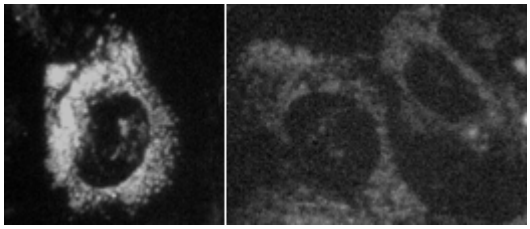


Figure 28: Stable transfected SH-SY-PINT-DsRed-Ex clones

Western blot analysis was also performed to verify if PINT-DsRed-Ex was synthesized in stable clones (Figure 29). Lanes 3-6 confirmed that PINT was synthesized in stable clones, and that the amount of PINT protein is much smaller than in transiently transfected cells. Different stable clones gave somewhat different bands (lanes 3 and 5).

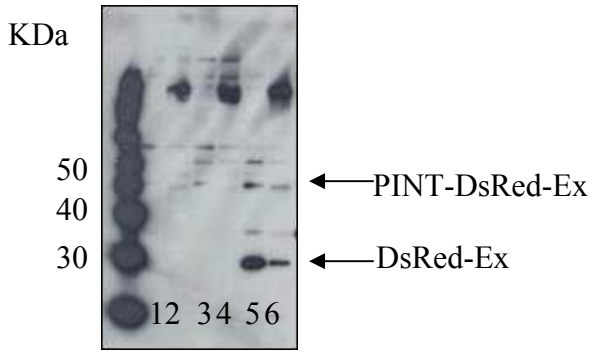


Figure 29: Western blot analysis of stable clones: 1- 2) untransfected SH-SY5Y cells with and without reducing agent, respectively; 3- 4) stable PINT-DsRed-Ex clone with and without reducing agent, respectively; 5- 6) another stable PINT-DsRed-Ex clone with and without reducing agent, respectively; 12% acrylamide gel, signal detected with anti-RFP (1:500) (Chemicon) and goat-anti-rabbit HRP (1:15000).

Stable clones were inspected in microscope. The clones in which cells looked mostly alike and gave even signal, and which gave relatively strong 50 kDa bands in western blots, were chosen for further studies of PINT protein.

3.3 Glycosylation of PINT protein

To check if PINT is a glycoprotein, PINT-DsRed-Ex lysate from stable SH-SY-PINT-DsRed-Ex clone was treated with PNGase F (Figure 30). No difference in bands sizes before and after treatment with PNGase F was observed, indicating that the PINT protein does not have attached carbohydrate residues attached and consequently is not a glycoprotein. An additional unspecific very strong band around 35 kDa appeared after PNGase F treatment in all transfected and untransfected samples.

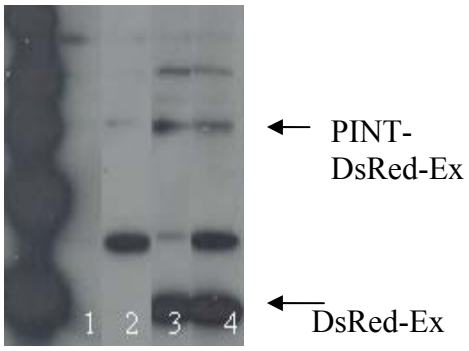


Figure 30: Treatment of stable SH-SY-PINT-DsRed-Ex clones with PNGase F: 1-2) untransfected SH-SY5Y cells before and after adding PNGase F, respectively; 3-4) stable SH-SY-PINT-DsRed-Ex clone before and after PNGase F, respectively; 12% polyacrylamide gel, signal detected using anti-RFP (1:500) (Chemicon) and goat-anti-rabbit HRP (1:15000).

3.4 Oligomerization of PINT-DsRed-Express

DsRed protein tends to form dimers, tetramers or even octamers at very high concentrations. This oligomerization can affect the behavior and localization of proteins that DsRed is fused to. Baird and colleagues (Baird et al., 2000) showed the multimeric nature of DsRed protein by various lines of evidence. One of the evidence was slow migration on SDS-PAGE of DsRed protein unless sample was preboiled before application.

To analyze in what form PINT-DsRed-Ex is present in cells, the experiment published by Baird and colleagues was performed. Transiently transfected N2a cells were lysed with gentle IP-lysis buffer (se chapter 3.3 in Materials and Methods); lysates were added sample buffer with reducing agent, and analyzed in western blots (preboiled and not boiled before application). Results of this experiment are presented in Figure 31. Antibodies detected bands that were about 200 kDa and heavier in unboiled samples (Figure 31A, lanes 1 and 2), while band at about 50 kDa also appeared in boiled sample in addition to some heavy bands (Figure 31 A, lane 3). When DsRed-Ex fluorescence was scanned directly in the gel, the not boiled samples gave well defined and sharp bands at about 200 kDa, while no signal was seen in boiled sample (Figure 31B, lanes1-2 and 3, respectively). It seems that PINT-DsRed-Ex in transiently transfected cells mostly forms oligomers.

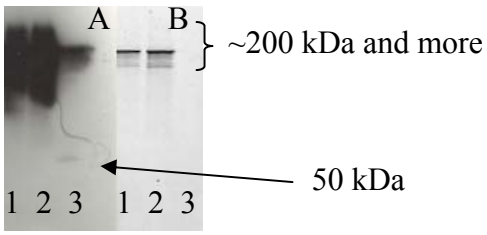


Figure 31: Oligomerization of PINT-DsRed-Ex protein in transiently transfected N2a cells: **A)** Western blot using anti-RFP (1:16000) (Clontech) and goat-anti-rabbit HRP (1:15000); **B)** DsRed-Ex signal detected by direct fluorescence scanning of the gel with a variable mode imager (Typhoon 9200, Amersham Biosciences); 1-2) not boiled lysates, 3) boiled lysate; 12% polyacrylamide gel.

3.5 Leptomycin B experiment

Leptomycin B is a specific inhibitor of classical nuclear export, which is usually involving a leucine rich sequence. In the sequence of PINT protein quite a few leucines are presented. To check if PINT protein has an export signal, stable SH-SY-PINT clones were serum starved for 4 days before incubating with leptomycin B for 2.5 hours at 37 ºC in serum containing medium. Then the cells were fixed with formaldehyde and screened for fluorescence intensity (Figure 32). After leptomycin B treatment a slight difference between signal localization was observed, signal was seen in the nucleus as well as in cytoplasm (Figure 32B), while before treatment no signal was observed in the nucleus (Figure 32A). It seems that PINT-DsRed-Ex might be localized in nucleus, but on the other hand, the difference is quite small and inconclusive, so it is difficult to conclude if PINT export is exportin dependent without further studies.

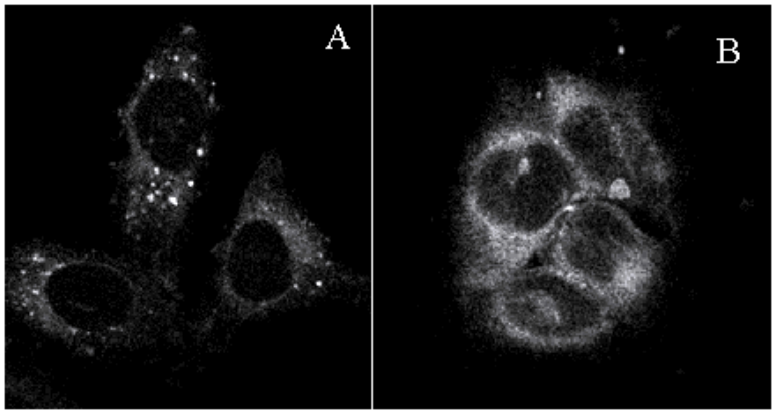


Figure 32: Leptomycin B experiment, stable SH-SY-PINT-DsRed-Ex clones: A) control cells; B) cells added Leptomycin B.

3.6 PINT and PrP co-localization

To study the interaction between PINT and PrP protein N2a-PrP-EGFP cell line was used (N2a cells stably expressing prion protein tagged with GFP).

PrP-EGFP cells were transiently transfected with DsRed-Ex alone or PINT-DsRed-Ex and pictures were taken 24 hours after transfection using red and green filters in the fluorescence microscope (Figure 33). When control cells (transfected with DsRed-Ex) (Figure 33A and B) were compared to cells tranfected with PINT (Figure 33C and D) a clear difference in the signal distribution was seen. However, it was difficult to decide if those proteins are localized in the same cell compartments because of overlapping excitation and emission wave lengths of EGFP and DsRed-Ex (Figure 9), which give a lot of false positive signal; red and green signals were seen together through FITC filter.

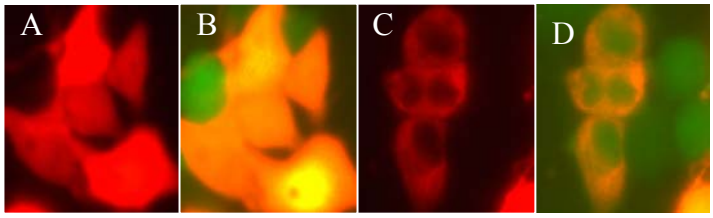


Figure 33: Stable N2a-PrP-EGFP cells transiently transfected with: A-B) pDsRed-Express alone; or C-D) pDsRed-Express-PINT. A,C) TRITC filter; B,D) FITC filter, respectively.

Another way to check the interaction between proteins is by immunoprecipitation.

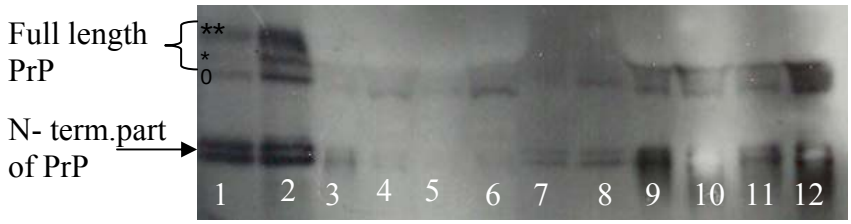


Figure 34: Western blot of immunoprecipitation, anti-PrP P4 (1:10000) and anti-mouse true blot (1:100) were used as primary and secondary antibodies, respectively: 1) PrP-EGFP IP P4; 2) PrP-EGFP-PINT IP P4; 3-4) PrP-EGFP and PrP-EGFP-PINT lysates after IP with P4, respectively; 5-6) PrP-EGFP and PrP-EGFP-PINT lysates before IP with P4, respectively; 7) PrP-EGFP IP anti-RFP; 8) PrP-EGFP-PINT IP anti-RFP; 9- 10) PrP-EGFP and PrP-EGFP-PINT lysates after IP with anti-RFP, respectively; 11- 12) PrP-EGFP and PrP-EGFP-PINT lysates before IP with anti-RFP, respectively; 12% acrylamide gel.

Figure 34 gave some positive evidence about interaction of PINT and PrP. Prion protein (full length and N-terminal part) was immunoprecipitated with P4 from PrP-EGFP and PrP-EGFP-PINT lysates (lanes 1 and 2) and with anti-RFP from PrP-EGFP-PINT lysate (lane 8). P4 antibody was more suitable for precipitation than anti-RFP. After precipitation with P4, almost no PrP was detected in lysates (lanes 3 and 4), while in PrP-EGFP-PINT lysate PrP signal was still quite strong (lane 10). Lane 7 also gave a weak signal of N-terminal part of PrP, but no full length PrP. It is possible that N-terminal part of PrP is so "sticky" that it bound A beads unspecificly and was detected.

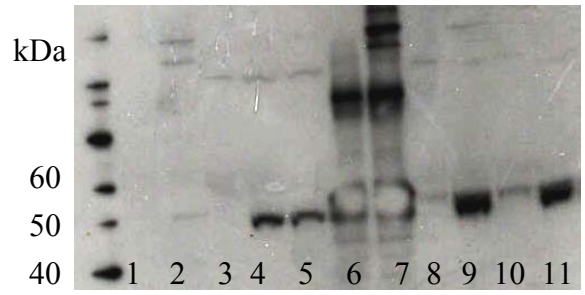


Figure 35: Western blot of immunoprecipitation, anti-RFP (1:16000) (Clontech) and goat-anti-rabbit HRP (1:15000) were used as primary and secondary antibodies, respectively: 1) PrP-EGFP IP P4; 2) PrP-EGFP-PINT IP P4; 3-4) PrP-EGFP and PrP-EGFP-PINT lysates after IP with P4, respectively; 5) PrP-EGFP-PINT lysate before IP with P4; 6) PrP-EGFP IP anti-RFP; 7) PrP-EGFP-PINT IP anti-RFP; 8- 9) PrP-EGFP and PrP-EGFP-PINT lysates after IP with anti-RFP, respectively; 10-11) PrP-EGFP and PrP-EGFP-PINT lysates before IP with anti-RFP, respectively; 12% polyacrylamide gel.

Figure 35 also showed some possible interaction between PINT and PrP. When P4 antibody was used for precipitation, a weak signal of PINT-DsRed-Ex (50 kDa) was detected only in the PrP-EGFP-PINT lysate (lane 2) but not in the PrP-EGFP lysate (lane 1). Anyhow, a strong signal of PINT-DsRed-Ex was seen in the lysate after precipitation (lane 5), showing that just a tiny fraction of protein had been precipitated. Immunoprecipitation with anti-RFP (lanes 6- 7) did not give any information about interaction, except for two heavy bands around 120-220 kDa which also appeared in lane 2 IP with P4. There were no differences between lysates of PrP-EGFP and PrP-EGFP-PINT before and after immunoprecipitation with anti-RFP (lanes 8-11, respectively).

IV. Discussion

Studies of PINT protein is of great interest, because of the possible interaction between PINT and PrP^C, which functions still remains enigmatic. The main purpose of the present study was expression (in mammalian and bacterial cells) and characterization of PINT protein.

1. Expression of PINT protein in bacterial cells

Expression of proteins in bacterial systems, especially in *E.coli*, plays a major role for the efficient production of genetically engineered proteins when their biological function does not depend on posttranslational modifications (Li et al., 2003). However, not every gene can be expressed efficiently in *E.coli*. This may be due to the unique and subtle structural features of the gene sequence encoding protein, the stability and translational efficiency of mRNA, the ease of protein folding, degradation of the protein by host cell proteases and potential toxicity of the protein to the host (reviewed by Makrides, 1996). The major drawbacks of *E.coli* as en expression system include the inability to perform many of the posttranslational modifications found in eukaryotic proteins, the lack for secretion mechanism for the efficient release of protein into the cultural medium, and the limited ability to facilitate extensive disulphide bond formation (reviewed by Makrides, 1996). All these downsides might lead to the formation of inactive aggregates of some proteins.

This study confirmed that His_6-PINT protein is one of the proteins that forms insoluble aggregates when expressed in *E.coli* under optimal growth conditions (37 ºC) (Figure 19).

There are numerous single cases reported, which shows increased solubility of recombinant proteins at lower cultivation temperatures (reviewed by Hammarstrom et al., 2002), but Hammarstrom and colleagues did not se the same effect on their set of proteins. No effect of lower growth temperatures (16-30 ºC) was observed on the expression of His_6-PINT protein in the present study either. It is reasonable to expect that larger proteins are more likely to have complex folding mechanisms, which render them more vulnerable to the aggregation associated with very high transcription rates at optimal growth temperatures. Lower growth temperature decreases formation of inclusion bodies, by making the growth cycle longer and giving more time for correct folding of large protein. Impaired solubility of smaller proteins (such as PINT protein) could, on the other hand, be an effect of less

temperature dependent factors such as missing cofactors, post-translational modifications, or folding partners.

1.1 Advantages and disadvantages of inclusion bodies

Expression of proteins as inclusion bodies has certain advantages (reviewed by Li et al., 2003): the expression levels of protein are often very high; the proteins are often protected from proteolytic degradation by host cell enzymes; the inclusion body protein can easily be separated from soluble proteins of the host cell by centrifugation, filtration or size-exclusion chromatography; if the expression product is toxic to the host, the formation of inactive protein can increase the viability of the cells and the yield of the target protein. Inclusion bodies often contain almost exclusively the over-expressed protein.

In the present study cells synthesized large amounts of His_6-PINT protein (Figure 13). After cell lysis and several washes, SDS-PAGE analysis showed quite pure samples containing mostly His_6-PINT protein (Figure 15), except for minor contaminants. Major contaminants of inclusion body material are most probably outer membrane proteins, which are themselves not part of the inclusion body particles but co-purify as non-solubilised protein with the inclusion body fraction. Separation of these membrane proteins from inclusion body material can be achieved by extensive washing with detergents (Lilie et al., 1998).

Disadvantages of aggregate formation are: highly problematic refolding of aggregated protein, refolded protein may not regain its biological activity, and reduction in final protein yield.

1.2 Reasons for formation of inclusion bodies

Why some proteins tend to form inclusion bodies when expressed in *E.coli*, while others do not? What are the main factors that influence aggregate formation? To answer these questions, several studies have been performed during the years. Specific support vector machine based algorithms for prediction of protein solubility were also recently created. The composition and arrangement of amino acids in the protein seems to be a major influencing factor in deciding its aggregation propensity (Idicula-Thomas et al., 2006). The role of the primary structure was demonstrated by introduction of various point mutations into DNA

sequence coding for various proteins, and then observing their expression under identical conditions (Dale et al., 1994; Jenkins et al., 1995).

Wilkinson and colleagues observed that inclusion body formation is correlated to net charge, turn forming residue fraction, cysteine fraction, proline fraction, hydrophilicity and total number of residues (the first two parameters show the strongest correlation) (Wilkinson et al., 1991). Ser percentage composition is also shown to be an important factor in determining solubility of recombinant proteins (Goh et al., 2003).

Amongst the physicochemical properties considered, thermostability of proteins (represented by its aliphatic index) is found to be the most crucial determinant of solubility. A higher instability index also increases the solubility of proteins. This observation suggests that a protein with a lower *in vivo* half-life has a lesser propensity to form inclusion bodies, because of shorter lived partially folded intermadiates of a protein (reviewed by Idicula-Thomas et al., 2006).

In summary, positive effect on solubility of recombinant protein have a high aliphatic index, the presence of some amino acids such as Glu, Arg, Gln, Ile, Leu, some dipeptides (His-His, Arg-Ala, Gly-Ala and Arg-Gly), net charge (positive or negative), and the instability index of the entire protein and of its N-terminal. On the other hand, Gly, Cys, Met, Phe, Ser, Pro and Asn-Thr have negative effects (for amino acid letter code check Appendix 5).

To find out if the primary structure of the His_6-PINT protein might also favour formation of insoluble inclusion bodies, the ProtParam program (Wilkins et al., 1999) on the ExPaSy proteomics Server was used for calculation of different physicochemical properties. Results showed that the estimated half- life (time it takes for half of the amount of protein in a cell to disappear after its synthesis) of PINT is about 30 hours (mammalian reticulocytes *in vitro*), more than 20 hours (yeast *in vivo*) and more than 10 (*E.coli in vivo*). Instability index is 44.66 (protein is unstable); aliphatic index is 84.36. Grand average of hydropathicity (sum of hydropathy values of all the amino acids, divided by the number of residues in the sequence) is -0.027. Proteins half-life and high aliphatic and instability indexes most probably decreases PINT inclusion body formation. On the other hand, the fraction of amino acids in PINT sequence that favours aggregation is higher than the fraction of amino acids that disfavours it.

It seems that primary structure of His_6-PINT protein might favour formation of inclusion bodies, but it is not clear to what extent. Aggregation is a complicated process, which depends on many factors, not only on primary structure of proteins.

Sequence-independent factors such as the kinetics of translations (Cortazzo et al., 2005; Komar et al., 1999), absence of certain post-translational modifications and reducing environment of the cytoplasm (bacterial proteins contain few Cys residues and few disulphide bonds, so most proteins with stable disulphide bonds are exported from the cytoplasm. Thus, mammalian proteins which 3D structure depends in part on disulphide bond formation are not synthesized in the correct conformation in bacterial cytosol) (Makrides, 1996; Wilkinson et al., 1991) might also influence the solubility of proteins. A lot of aspect has to be considered in order to achieve expression of soluble His_6-PINT protein.

1.3 Refolding of PINT protein

Protein express as inclusion bodies later has to be solubilised and refolded to facilitate active and soluble protein. The mechanism of *in vivo* protein folding remains one of the most intriguing problems to be elucidated in molecular biology. There is no single refolding technique or method that satisfies all the refolding requirements of a protein. The proper chemical conditions also vary from protein to protein; several experiments are required in the search for the optimum refolding process. The efficiency of refolding depends on the equlibrium between correct folding and aggregation. To be successful in refolding one have to think about suitable protein concentration (not too high), conditions must be carefully optimized regarding external parameters, such as temperature, pH or ionic strength.

After all unsuccessful attempts to synthesize soluble PINT protein, a few different refolding methods have been performed in the present study. After several washes inclusion bodies were solubilised by a strong detergent. His_6 tag retains its binding capabilities also in the presence of denaturing agents, so this tag was still used for purification of PINT with help of Ni-NTA columns. For solubilisation two strong detergents, guanidine hydrochloride and urea, were used. When solubilisation was performed at 4 ºC (with both detergents), all the protein was lost in the filter (Figure 16A) (the solution has to be filtered before loading onto the column, to avoid application of impurities), while protein solubilised at RT was pressed through the filter (Figure 16B). It was clear that RT is the most suitable temperature for solubilisation of PINT aggregates. Solubilised PINT protein containing a His_6 tag was loaded onto the column and refolded by slowly washing away detergent. The elution of PINT was unsuccessful. The possible reasons might be very strong interaction between His and Ni ions,

precipitation of protein in the column caused by too high PINT concentration, or non-specific hydrophobic interactions.

The same negative results were obtained after dilution of solubilised PINT inclusion bodies (Figure 18). It is possible that protein was not bound to the column at all; or very strong interaction between His and Ni ions and non-specific hydrophobic interactions might be the problem; but most likely aggregation of PINT on the column was the main difficulty.

1.4 Increasing solubility; further studies

Refolding of PINT protein in the present study has been problematic. All attempts to refold and obtain soluble and active PINT protein were negative. It seems that in this case attempts to reduce the aggregation and misfolded configuration might be the better solution than solubilisation and refolding of protein. In further studies several factors could be changed:

Firstly, another expression system and the effect of several different fusion partners have to be studied. Hammarstrom and co-workers (Hammarstrom et al., 2001) observed large differences in the effects of fusion protein on the expression and solubility, but the combination of several fusion possibilities is still the key to the high overall success. His tag alone gives a lower chance of obtaining soluble product than with any other fusion, but can be kept for purification reasons.

The change of *E.coli* host strain or medium (Luan et al., 2004) might also increase solubility of recombinant proteins; introduction of amino acid substitutions might also be very helpful. Moreover, co-expression of molecular chaperones (mediates posttranslational folding and localization of proteins) (Makrides, 1996), addition of low molecular weight molecules in refolding buffer (Zn^{2+}, Ca^{2+} stabilise intermediates, low concentrations of urea (1- 2 M) and guanidine hydrochloride (0, 5- 1, 5 M) inhibit the intermolecular interactions that cause aggregation) (Li at al., 2003; Lilie et al., 1998), altered pH, sucrose and rafinose in growth medium might help to avoid incorrect folding of proteins.

2. Expression of PINT protein in the mammalian cells

To study expression, cellular localization and other characteristics of PINT protein, mammalian cells (N2a and SH-SY5Y were transfected with pDsRed-Express-PINT and pZeoSv-PINT, coding for PINT-DsRed-Express and PINT-His_6 proteins, respectively.

2.1 Cellular localization of PINT protein

Transient transfection of N2a and SH-SY5Y cells with pDsRed-Express-PINT revealed two different localisation patterns. Some cells had strictly cytosolic PINT-DsRed-Ex, others had PINT-DsRed-Ex in the nucleus (Figure 22). This was very different from cells transfected with pDsRed-Express alone, which had a homogenous distribution of DsRed-Ex in the whole cell.

To do more thorough investigation of PINT-DsRed topology, a fluorescence protease protection assay (FPP) was used. FPP assay provides a new approach for determining protein topology in the cells. After the attachment of DsRed-Ex to the C-terminus of a PINT protein, cells expressing the fusion protein were exposed to trypsin or proteinase K, either before or after plasma membrane permeabilisation by digitonin. If the fluorescent protein moiety faces the environment exposed to trypsin or proteinase K (that it, the cytoplasm), then its fluorescent signal should be lost. Conversely, if the fluorescent protein moiety faces the environment protected from trypsin (that is, lumen of a compartment) then its fluorescence should persist. Treatment of nonpermeabilized cells with trypsin or proteinase K should readily extinguish fluorescence associated with molecule facing the exterior of the cell, and have no effect on molecules facing the interior (Lorenz et al., 2006).

In the present study the intracellular signal remained after nonpermeabilized cells were treated with trypsin, showing that PINT is entirely localized internally. After the addition of digitonin, cytosolic content of the cell diffuses out, and small molecules such as trypsin/proteinase K can diffuse in to the cell. Notably, the permeabilizing effect of digitonin can be limited to the cholesterol-rich plasma membrane when used in the right concentration. Membranes of intracellular organelles have lower cholesterol concentration and are unaffected by digitonin concentrations permeabilizing the plasma membrane. Weaker fluorescent signal after digitonin treatment showed that some of PINT-DsRed-Ex was localized freely in cytoplasm (most probably as dimmers, showed by biochemical FPP assay

(Figure 27)). Addition of trypsin led to even fainter signal. It seems that some PINT-DsRed-Ex was exposed to trypsin (bound and facing cytosol). Still remaining signal demonstrated that a small fraction of PINT-DsRed-Ex might be localized inside internal organelles (mitochondria or ER). Signal was entirely lost after addition of triton X-100, which permeabilize all membranes (Figure 24).

Interestingly, Lorenz and coworkers (Lorenz et al., 2006) also used proteinase K on their set of proteins, and obtained the same results, while in the present study some differences concerning PINT-DsRed-Ex were observed. The entire fluorescent PINT-DsRed-Ex signal was lost in chosen cells after addition of proteinase K (Figure 26). It seems that PINT-DsRed-Ex might be somehow protected from trypsin (interestingly, PeptideCutter (one of ExPaSy tools) predicted that proteinase K might cut PINT protein into 111 peptides, while trypsin is only predicted to cut PINT in 22).

To check if FPP results are reliable the same experiment was performed on stable N2a-DsRed-Ex cells (Figure 254). After permeabilization, as expected, almost all the fluorescent signal disappeared, except for minor signal in the nucleus, which fainted soon after trypsin addition. DsRed-Ex in soluble form is localized freely in the cytoplasm and nucleus, and just minor fraction is bound to some structures in the nucleus of the cell.

Biochemical FPP assay and western blot analysis (Figure 27) showed interesting results. After trypsin and triton X-100 treatment, strong bands were still detected with anti-RFP antibodies in the membrane fraction, while cells in the microscope had lost all signal. Only the DsRed-Ex signal was preserved, while the gel stained with coomasie brilliant blue was almost clear, indicating that trypsin had digested all other proteins (results not shown). After proteinase K and triton X-100 treatment, just a minor band was seen in the western blot, and no signal was seen in the microscope. This might be explained by a large amount of PINT protein synthesized during transient transfection that cells packed very tightly. Transiently transfected cells expressing PINT-DsRed-Ex were quite different. For microscope studies, only cells giving a nice and not too strong fluorescence signal were chosen, while some other transiently transfected cells synthesized so much PINT-DsRed-Ex protein, which gave very strong fluorescent signal. It seems that to deal with such large amounts of foreign protein, cells were trying to pack it very tightly. Those cells formed a lot of aggregates, which were protected from trypsin (Figure 24E) much better than from proteinase K (Figure 26D).

Based on these results it can be concluded that PINT-DsRed is localized in cytoplasm (free and bound), and some might be localized in intracellular organelles such as mitochondria or ER. These results confirm theoretical predictions of some ExPaSy tools (CELLO, Subloc and others). From the PINT sequence it was predicted that PINT might be localized in the cytoplasm and/or might be exported to mitochondria. On the other hand, as mentioned above, in some transiently transfected cells PINT-DsRed-Ex signal was detected in the nucleus of the cells, while most of the stable SH-SY-PINT-DsRed-Ex clones showed both nuclear and cytosolic localisation in the same cells, most probably because of a lower amount of PINT-DsRed-Ex in the cells. Maybe the cellular localization of PINT protein is cell cycle dependent, so that PINT enters the nucleus only at a certain point in the cycle, while otherwise being localized in the cytoplasm. Further studies are needed to examine this phenomenon, for instance by using synchronized cells and time-lapsed confocal microscopy to follow a complete cycle of cell division, or by using other methods that allow observation of cell cycle position and studies of the PINT-DsRed-Ex signal at the same time.

2.2 Import and export from nucleus (Leptomycin B experiment)

Transient transfections showed cells with and without nuclear PINT-DsRed-Ex. A number of proteins travelling in and out of the cell nucleus have nuclear import (localizing) and export sequences. A nuclear localizing sequence (NLS) is an amino acid sequence which acts like a 'tag' on the exposed surface of a protein. This sequence is used to confine the protein to the cell nucleus through the nuclear pore complex and to direct a newly synthesized protein into the nucleus via its recognition by cytosolic nuclear transport receptors. Typically, this signal consists of a few short sequences of positively charged lysines or arginines ((NH2)-Pro-Pro-Lys-Lys-Lys-Arg-Lys-Val-(COOH)). PINT protein does not have typical NLS.

An NLS is the opposite of a nuclear export signal, which confines proteins to the cytosolic face of the nuclear membrane. Many export sequences contain leucins separated by one or a few amino acids. In the PINT sequence, leucine constitutes more than 10% of the amino acids. Several streches of the primary sequence have repeated leucins separeted only by one amino acid, because of that the possibility of export signal was investigated.

Leptomycin B is a specific inhibitor of the nuclear export signal. The suggested inhibition mechanism involves the direct binding of Leptomycin B to CRM1 (exportin 1), which blocks binding of CRM1 to proteins containing the nuclear export signal. After treatment with

leptomycin B, some differences were observed in fluorescent signal localization, but no convincing accumulation in the nucleus (Figure 32). Small particles (< 50 kDa) are able to pass through the nuclear pore complex by passive diffusion. PINT-DsRed-Ex seems to be on the limit between proteins that can and can not passively diffuse in and out of the nucleus. However, larger particles are also able to pass through the large diameter of the pore but at almost negligible rates. So it is also possible, that PINT-DsRed-Ex depend on passive diffusion, rather than on exportin 1 dependent export. However, many proteins known to switch between cytosol and nucleus are independent of exportin 1 (Kumar et al., 2006), so other transport proteins could be involved in PINT trafficking out of nucleus.

2.3 Can PINT be an exonuclease?

It is difficult to answer question if PINT might have exonuclease activity, based only on the results obtained from this study. But there are two points that supports this suggestion. Firstly, NCBI blast predicted that PINT protein has conservative Exonuc_X-T domain that is common to different exonucleases, ribonuclease T and different subunits of DNA polymerase. And secondly, based on the information from MisPred database, Exonuc_X-T domains were predicted as strongly multilocale, localized in nucleus and/or cytoplasm, and some in ER, as seen for the PINT protein. Further studies are needed for determining biological activity of PINT protein.

2.4 The use of DsRed as fluorescent tag

Wild type DsRed has many positive characteristics as tag-protein: bright fluorescence, stability against pH and photobleaching, but on the other hand, DsRed also proves to be at least obligate tetramers, which may weakly associate into octamer (Baird et al., 2000; Gross et al., 2000). Gross and colleagues reported that not boiled DsRed samples in SDS sample buffer gave heavy bands of 100 kDa and more in SDS-PAGE analysis, proving the obligate tetramer and/or possible octamer. The high molecular mass DsRed disappeared, however, when the sample was briefly boiled before electrophoresis. DsRed samples boiled before SDS-PAGE always showed two fragment bands of apparent masses 15 and 22 kDa; one band of about 35 kDa was also formed. It seems that DsRed samples undergo cleavage during boiling. In some western blots in this study a band around 20 kDa was also observed (Figure 23, 27) which

could indicate that some of the DsRed-Ex (mutant of wild type DsRed) was also cleaved by boiling.

DsRed-Express used in the present study contains 9 amino acid substitutions to increase the solubility of the protein, reduce time from transfection to detection of red fluorescence and reduce the level or residual green emission. DsRed-Ex expressed alone was soluble and distributed throughout the entire cytoplasm and nucleus of expressing cells (Figure 22, 25), on the other hand, PINT-DsRed-Ex seems to tend to form oligomers (at least dimers and/or tetramers). PINT-DsRed-Ex aggregates first were observed in fluorescent microscope, and later confirmed by western blotting (Figure 31). The aggregates were packed so tightly that even boiling of the sample for 5 min could not destroy them; cells with aggregates were also not affected by trypsin and proteinase K treatment.

The question still remains to what extent fusion with this protein will affect location and functions of a protein of interest? In rare cases, addition of DsRed interferes with proper functioning and localization of the fusion protein (Lauf et al., 2001), a matter that needs to be considered and addressed. Some other studies, however, have demonstrated that DsRed chimeras retain the characteristics of the untagged protein (Lee at al., 2002). To answer this question, PINT protein has to be fused to smaller tags, because large tags interfere with proper protein localization much more often than small tags. After expression in the same cell lines, the cellular localization of PINT has to be compared to localization of PINT-DsRed-Ex. If localizations match, DsRed-Ex does not affect PINT behaviour, but if they do not match, one of the tags influences PINT cellular localization and behaviour.

2.5 Posttranslational modifications

In some cases, mostly when stable SH-SY-PINT-DsRed-Ex clones were analysed by western blots, anti-RFP antibodies recognized multiple bands between 50 and 60 kDa. To check if those bands were the result of glycosylation, lysates were treated with PNGase F. No difference between samples added and not added PNGase F was observed (Figure 30). This indicates that PINT is not a glycoprotein.

For prediction of other posttranslational modifications different ExPaCy tools were used. No posttranslational modifications such as acetylation, O- and N- glycosylation, and oxidation

were predicted for PINT protein except for possible phosphorylation of four Ser and four Thr residues and one N-myristoylation site. No disulphide bridges between protein molecules seem to be present in PINT-DsRed-Ex since no additional heavy bands appeared when no reducing agent was added before SDS-PAGE (Figure 23). This was as predicted, since PINT-DsRed-Ex was shown to be localized in cytosol which is a reducing environment.

2.6 Interaction of PINT and PrP

The majority of PrP is imported into the ER lumen and attached to the luminal ER membrane and later the plasma membrane via a GPI anchor. Nevertheless, transmembrane forms with either the N- or the C-terminus in the ER lumen and the other end facing the cytosol, and cytosolic forms of PrP have also been identified (reviewed by Griffoni at al., 2003; Mironiv et al., 2003). Therefore, interaction of PrP^C with PINT protein, which is mainly present in cytosol, is possible.

Spielhaupter and colleagues (Spielhaupter et al., 2001) reported the tissue distribution of murine PINT protein. A strong expression was detectable in brain as well as in heart, thyroid, and testis. A faint signal was visible in muscle cells, liver, pancreas, and kidney. No transcripts were detectable in embryonic development stages and in ovary, uterus, eye, lung, and spleen. The tissue distribution of PrP mRNA has been published previously by Yehiely and colleagues (Yehiely et al., 1997). They reported a high expression of PrP^C in brain, lung, kidney, and heart, and a weaker expression was seen in liver, spleen, and some other organs. The pattern of PINT expression partially overlaps with that of PrP^C, indicating co-expression of proteins in these organs, and that these two proteins physically can interact with each other. Spielhaupter and colleagues identified murine PINT as possible PrP binding partner and demonstrated that the interaction is very strong. In the present study results about interaction were rather inconclusive.

Cells stably expressing PrP-EGFP were transfected with pDsRed-Express-PINT and 24 hours after transfection images with fluorescent microscope were taken (Figure 33). However, the expression of both PINT-DsRed-Ex and PrP-EGFP was very widely in the cells making it difficult to see if the two proteins were co-localized in some particular compartments. Another factor making it difficult to decide if those proteins are localized in the same cell compartments was the FITC filter on our microscope, which let much of the DsRed-Ex signal

through. Because of overlapping excitation and emission wave lengths of EGFP and DsRed-Ex, red and green signals were seen together through the FITC filter; which then gave a lot of false positive signal. Perhaps confocal microscope with appropriate filter sets which let through narrow fixed wave length could give better resolution when studying co-localization of DsRed-Ex and EGFP-tagged proteins.

Co-immunoprecipitation of PrP-EGFP::PINT-DsRed-Ex complex with P4 (directed against PrP) and anti-RFP (directed against DsRed-Ex) was performed to study the interaction between the two proteins.

PrP (full length and N-terminal end) was detected after IP with P4 in lysates from untransfected PrP-EGFP cells as well as from the same cells transfected with pDsRed-Express-PINT (Figure 34). Little PrP was left in the lysate after immunoprecipitation with P4 (lanes 3-4) which show that P4 is an efficient IP-antibody. In contrast, after immunoprecipitation with anti-RFP strong PrP signal was still detected in cell lysate (lanes 9-10), indicating that only a very little fraction of PrP was immunoprecipitated with anti-RFP. Weak PrP bands seen after immunoprecipitation with anti-RFP (lane 8) might show interaction between PrP and PINT, on the other hand, in untransfected cell lysate N-terminal end of PrP was also detected (lane 7). It is possible that N-terminal part of PrP is so "sticky" that it bound protein A beads unspecificly and was detected, or that weak bands of PrP immunoprecipitated with anti-RFP could indicate only unspecific binding of PrP to beads.

PINT protein was also detected in the same lysates (Figure 35). A lot of PINT was left in the lysates after immunoprecipitation with both P4 and anti-RFP (lanes 4 and 9, respectively), indicating that just a little fraction of PINT was immunoprecipitated and that these antibodies are inefficient IP-antibodies. A faint PINT signal was detected in transfected cell lysate after IP with P4 (lane 2) and no signal in untransfected lysate (lane 1); this might indicates some kind of weak interaction between the proteins. Results from immunoprecipitation with anti-RFP (lanes 6-7) are inconclusive when it comes to a study of protein interaction.

In order to prove the interaction between PrP and PINT, other antibodies, more suitable for IP and directed against PrP, EGFP, DsRed-Ex or PINT will have to be used, since the antibodies used in the present study are too inefficient.

2.7 anti-RFP antibody

In the present study two polyclonal anti-RFP antibodies from Chemicon and Clontech were used for immunoprecipitation and detection of PINT-DsRed-Ex signal. None of the RFP-antibodies were suitable for immunoprecipitation of PINT-DsRed-Ex from cell lysates, especially when the same antibody was used for both precipitation and detection of signal. It is possible that both these anti-RFP antibodies recognize several unspecific proteins in addition to DsRed-Ex.

Anti-RFP from Chemicon recognizes a protein in untransfected N2a and SH-SY5Y cells with the same size as PINT-DsRed-Ex (Figure 23), making in difficult to study PINT-DsRed-Ex in the cells. The antibody from Clontech shows less unspecific binding, but still is not the optimal antibody for detection of DsRed-Ex.

2.8 Expression of PINT-His_6

As mentioned before, DsRed-Ex could affect cellular localization and behaviour of PINT. To check this fact, expression of PINT protein has to be studied under same conditions, in the same cell lines, but with other fusion partners. To eliminate the effect of DsRed-Ex on PINT protein localization, six His residues were added to the C-terminal end of PINT and expressed in N2a cells. Two different anti-His tag antibodies were used for detection of signal in western blots, but all attempts were negative. One of the reasons can be that cells expressed too little protein and PINT was not detected, but PINT was not detected after upconcentration by immunoprecipitation with anti-His antibody either (both anti-His antibodies were checked with positive controls).

Some possible reasons why PINT-His_6 was not detected or expressed in the cells might be: plasmid instability or plasmid loss; proteolytic degradation of the protein product caused by formation of incomplete polypeptides, post-translational damage and culture growth parameters such as nutrient composition of media, growth temperature, and pH (Makrides, 1996); different translation issues (Baneyx, 1996); and instability of mRNA (Makrides, 1996).

Further studies will be needed for expression of PINT with His_6-tag. Expression conditions have to be carefully checked and optimized, or perhaps some other small tags can be used for successful expression of PINT protein in mammalian cells to double-check the cellular localization of this protein.

3. Future perspectives

Before this study not much was known about the PINT protein. Lorenz and colleagues (Lorenz et al., 2001) suggested that PINT is a possible PrP binding partner in mice. From the sequence it was known that PINT probably has nuclease activity.

In the present study, new information was gained about this protein, but still it is a very long way to go in order to characterize it properly.

First of all, in order to study the nuclease activity and other physicochemical properties, active and correctly folded protein is required. Further work would consist of finding suitable conditions for solubilisation of inactive inclusion bodies. The protein has to be refolded and the active product analyzed for nuclease activity and substrate specificity (DNA or RNA, single- or double-stranded and so on).

Secondly, specific antibodies can be made by injecting for instance rabbits with suitable amounts of PINT protein. Detection of PINT protein in western blots would become easier; it would be possible to perform immunoprecipitation and immunohistochemistry and to study localization without using fusion tags that may affect the behaviour of proteins.

Further work with co-transfection of PINT and PrP and co-localization is also needed. The anti-RFP antibody used in this study was not good because of unspecific bands at the same size as PINT-DsRed-Ex. It would be useful to examine other anti-RFP antibodies, or perhaps change fusion tags, to allow the use of other antibodies.

By using DsRed-Express and EGFP tags it is difficult to observe co-localization in the fluorescent microscope because of false positive signal (DsRed-Express absorbs light at the same wave length as EGFP). Confocal microscope should be used, or perhaps another tag can be fused to the PINT protein.

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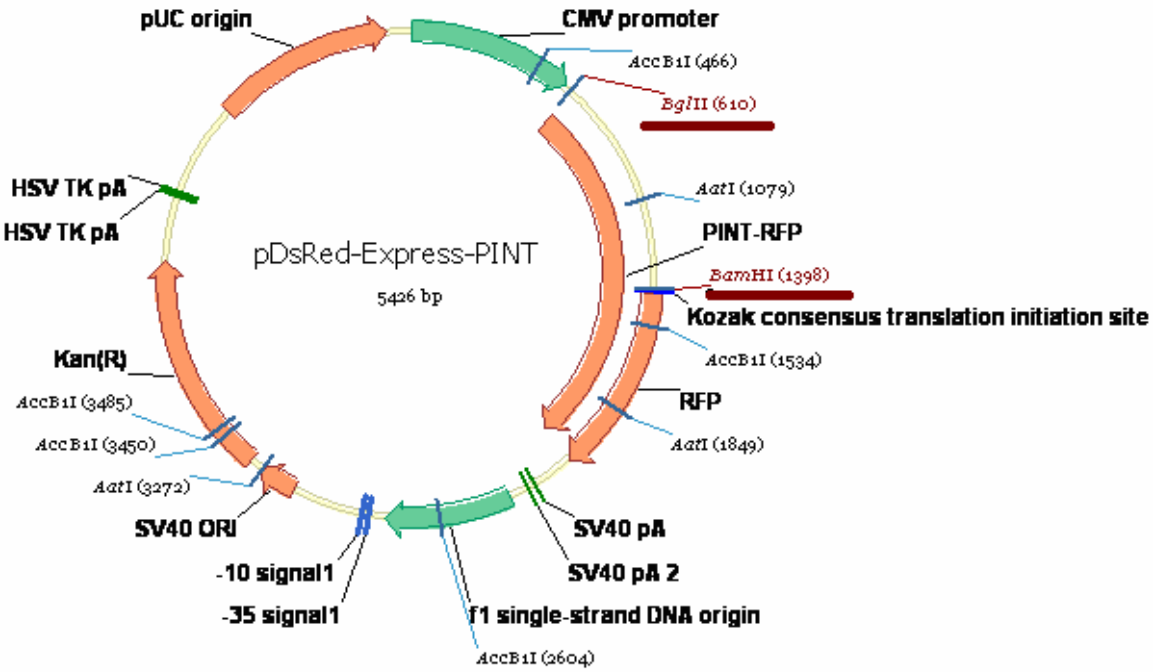
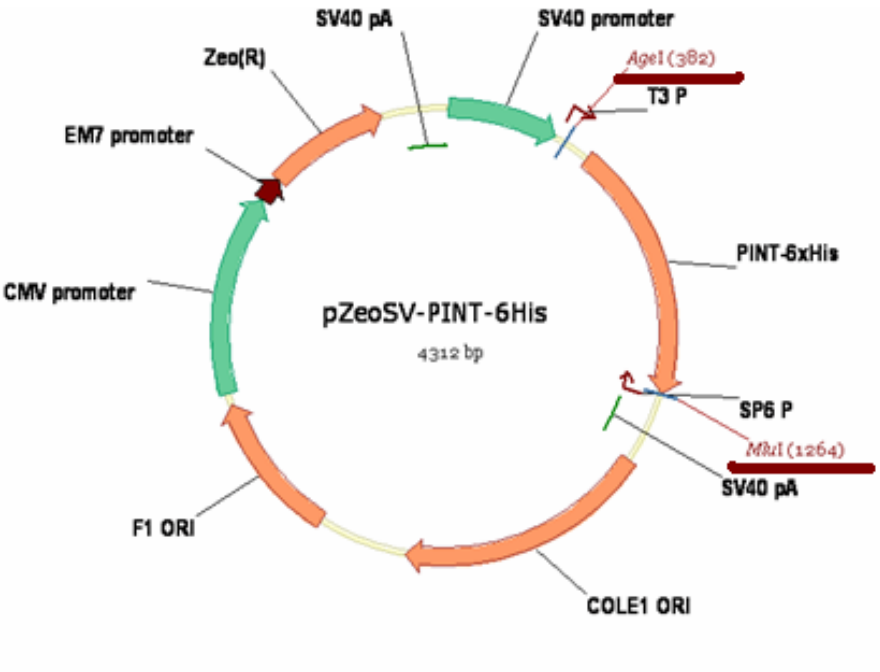
Appendix 1

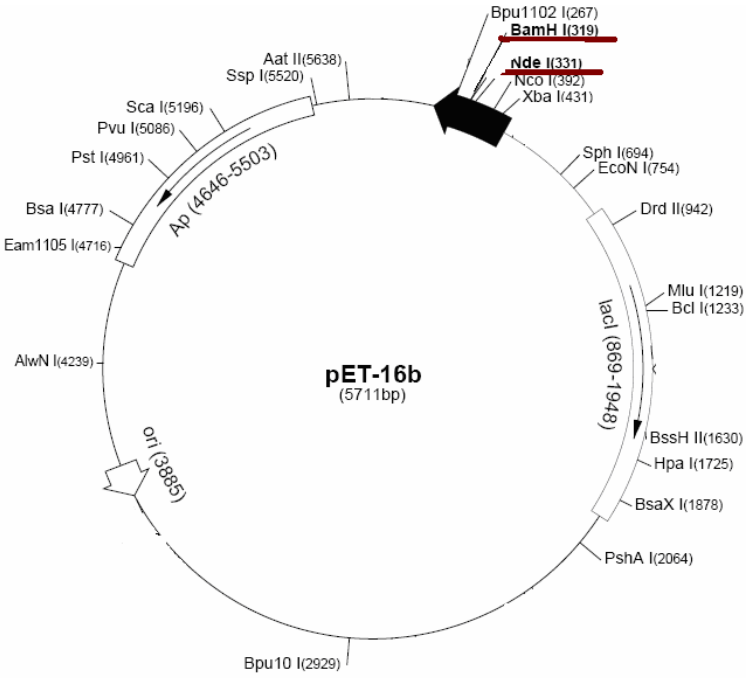
Maps of different constructs used in this study. Restriction enzymes used for cutting vector and PCR- product are underlined in red.



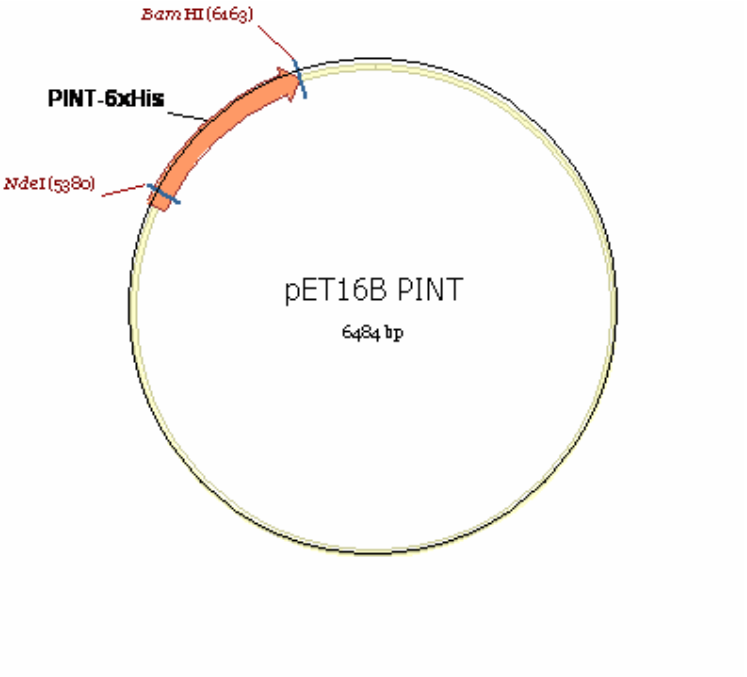
A. pDsRed-Express-PINT construct



B. pZeoSv-PINT construct



C. pET- 16b vector



D. pET- PINT construct

Appendix 2

List of reagents and their manufacturers used in this study (in alphabetical order)

Name of the reagent	Manufacturer
Reagents*	
Agarose	Cambrex
BSA	Sigma
$CaCl_2$	Merck
Chloroform	Merck
DacoCytomation fluorescent mounting med.	Dako cytomation
Digitonin	Sigma
DMSO	Sigma
dNTP	Fermentas
Ethidium bromide	Quantum
Glycerol	Merck
IPTG	Saveen
Lipofectamine	Invitrogen
Non Fat Dry Milk	BioRad
OPTI- MEM	Invitrogen
Phenol	Applichem
Plus reagents	Invitrogen
Protein A Sepharose	Amersham Biosciences
Protein G Agarose	KLP
Buffers**	
10x BamHI buffer	Fermentas
10x MOPS Running buffer	BioRad
10x NE Buffer 4	New Englad Biolabs
5x Phusion HF buffer	Finnzymes
10x R buffer with BSA	Fermentas
2x Rapid ligation buffer	Promega
20 x XT MES Running buffer	BioRad
Enzymes	
AgeI (BshTI)	Fermentas
BamHI	Promega
BglII	New England Biolabs
Benzonase	Sigma
BSA HI	New England Biolabs
DNase I	TaKaRa
Lysozyme	Sigma
MluI	Fermentas
NdeI	Fermentas
Phusion DNA polymerase	Finnzymes
PNGase F	New England Biolabs
Protease inhibitor cocktail	Sigma

Proteinase K	Fluka, Biochemica
T4 DNA ligase	Promega
Trypsin	PAA Laboratories
MW standards and dies	
6x Loading dye	Fermentas
6x Mass Ruler Loading Die	Fermentas
Bench Mark	Invitrogen
Fast ruler DNA ladder, high range	Fermentas
Fast ruler DNA ladder, low range	Fermentas
Fast ruler DNA ladder, middle range	Fermentas
Gene ruler 1 kb DNA ladder	Fermentas
Magic Mark	Invitrogen
Mass Ruler DNA ladder mix, ready to use	Fermentas
NuPAGE LDS Sample buffer (4x)	Invitrogen
NuPAGE Sample Reducing agen	Invitrogen
Page Ruler protein ladder	Fermentas
Precision Plus All Blue Protein Standards	BioRad
See Blue	Invitrogen
Trypan Blue solution (0, 4%)	Sigma
Antibiotics	
Ampicilin	Duchefa Biochemie, Sigma
Canamycin	Sigma
Chloramphenicol	Duchefa Biochemie
G- 418	PAA LAboratories
Leptomycin B	Sigma
Zeocin	Invitrogen
Antibodies	
Anti-His(C-term)	Amersham Bioscience, Invitrogen
Anti- RFP	Chemicon, Clontech
Goat anti- rabbit HRP	BioRad
Goat anti- mouse ALP	Amersham Biosciences
Goat anti- mouse HRP	Molecular probes
Mouse True Blot HRP	eBioscience
P4	R.Biopharm
Kits	
E.Z.N.A Gel Extraction Kit	Omega Bio- tek
Geneclean Spin Kit	Q- Bio Gene
Ni-NTA Spin	Qiagen
Pure Yield Plasmid Midiprep System	Promega
Wizard Plus Sv Minipreps DNA Purification System	Promega

*- chemicals that were used for making of buffers are listed in Appendix 3
**- buffers that were bought already made, not from kits

Appendix 3

List of different buffers and media used in this study (buffers that were included in different kits are not listed). Buffers marked with (*) - were already made by other colleagues.

1M Tris- HCl buffer
60. 55 g Tris base (Angus)
H_2O to the end volume of 500 ml
(Adjust pH with HCl before adjusting the volume)

1x Sample buffer (for proteins)
250 µl 4x XT Sample Buffer (BioRad)
600 µl H_2O
50 µl DTT (20x Reducing agent) (BioRad)
100 µl 1M $MgCl_2$ (Sigma)

1x TE- buffer
10 ml 0, 1 M EDTA (Sigma, Bio Chemica)
10 ml 1M Tris- HCl
H_2O to the end volume of 1 l; pH 8.0

50 x TAE- buffer
242 g Tris base (BioRad)
5. 71 ml acetic acid (Merck)
10 ml 0, 5 M EDTA (Sigma)
70 ml H_2O

Benzonase buffer*
20 mM Tris- HCl, pH 8.0
2 mM $MgCl_2$
0. 02 % SDS
0. 02 % DTT

Destaining solution (for CBB staining)
810 ml MQ H_2O
120 ml Methanol (Merck)
70 ml Acetic acid (Merck)

Different solutions for silver staining
Solution A: 600 ml ethanol (Arcus)
200 ml Acetic acid (Merck)
1200 ml H_2O

Solution B: 6g potassium tetrathionate (Merck)
98 g calcium acetate (Merck)
600 ml ethanol (Arcus)
1400 ml H_2O

Solution C: 2g $AgNO_3$ (Merck)
1000 ml H_2O

Solution D*: 60 g potassium carbonate
600 µl phormaldehyde
250 µl 10% sodium thiosulphate pentadydrat
H_2O to the end volume of 2 l

Solution E: 80 g Tris base (Angus)
40 ml Acetic acid (Merck)
H_2O to the end volume of 2 l

IP-lysis buffer
100 mM Tris (Sigma)
1% Triton- X 100 (Sigma)
2 mM NaCl (Chemi Teknik)
Adjust pH to 8.0

IP-wash buffer
50 mM Tris (Sigma)
150 mM NaCl (Prolabo)
0. 05% Triton- X 100 (Sigma)
Adjust pH to 7.4

KHM buffer*
110 mM potassium acetate
20 mM HEPES
2 mM $MgCl_2$

LB- medium
10/ 12. 5 g LB Broth (low/ high salt) (Duchefa Biochemie)
H_2O to and volume of 0. 5 l
Adjust pH to 7.2

N2a medium
450 ml MEM (Sigma)
5 ml FBS (Euroclone)
5 ml puruvate and non- essential amino acids (Cambrex)
5 ml L- glutamine, penicillin/ streptomycin (PAA Laboratories)

4. 33 ml Bicarbonate solution (Cambrex)
NaCl-Tris buffer (solubilisation of inclusion bodies)
150 mM NaCl (Merck)
50 mM Tris- HCl (pH 8.0)

NZCYM- medium
11. 29 g NZCYM BROTH EZ MIX (Sigma)
H_2O to end volume of 1 l
Autoclave for 20 min and add antibiotic

PBS* and PBS- T buffer
11. 5 g Di- sodium hydrogen orthophosphate, anhydrous
2. 96 g sodium dihydrogen orthophosphate
5. 84 g NaCl
H_2O to the and volume of 1 l (Adjust pH to 7.5)
To make PBS-T to PBS buffer add Tween 20 to give 0, 1% (v/ v) solution (USB)

Phosphate buffer (inclusion bodies)
20 mM Na phosphate buffer (8x) (DE Healthcare)
0. 5 M NaCl (Merck)
10 mM imidazole (pH 7.4) (GE Healthcare)

Refolding buffer (inclusion bodies)
10 mM Tris- HCl (pH 8.0)
100 mM Na_2HPO_4 (J.T.Baker)
10 / 1 mM reduced/ oxidized glutathione (Sigma)
Protease inhibitor cocktail (1 ml to 100 ml of cell lysate) (Sigma)
And suitable concentration of guanidine hydrochloride (Fluka BioChemica) or urea (Invitrogen)

RIPA buffer*
150 mM NaCl
1% NP- 40
0. 5% DOC
0. 1 SDS
50 mM Tris (pH 7.5)

SH-SY5Y medium
450 ml DMEM (Cambrex)
5 ml FBS (Euroclone)
5 ml L- glutamine (PAA Laboratories)
5 ml penicillin/ streptomycin (PAA Laboratories)

Staining solution (for CBB)
400 ml H_2O
500 ml Methanol (Merck)
100 ml Acetic acid (Merck)
1 g CBR- 250 (BioRad)
Shake for 4 hours/ over night to dissolve the powder

Striping buffer*
100 mM β- mercaptoethanol
2% (w/ v) SDS
62. 5 mM Tris- HCl (pH 6.7)

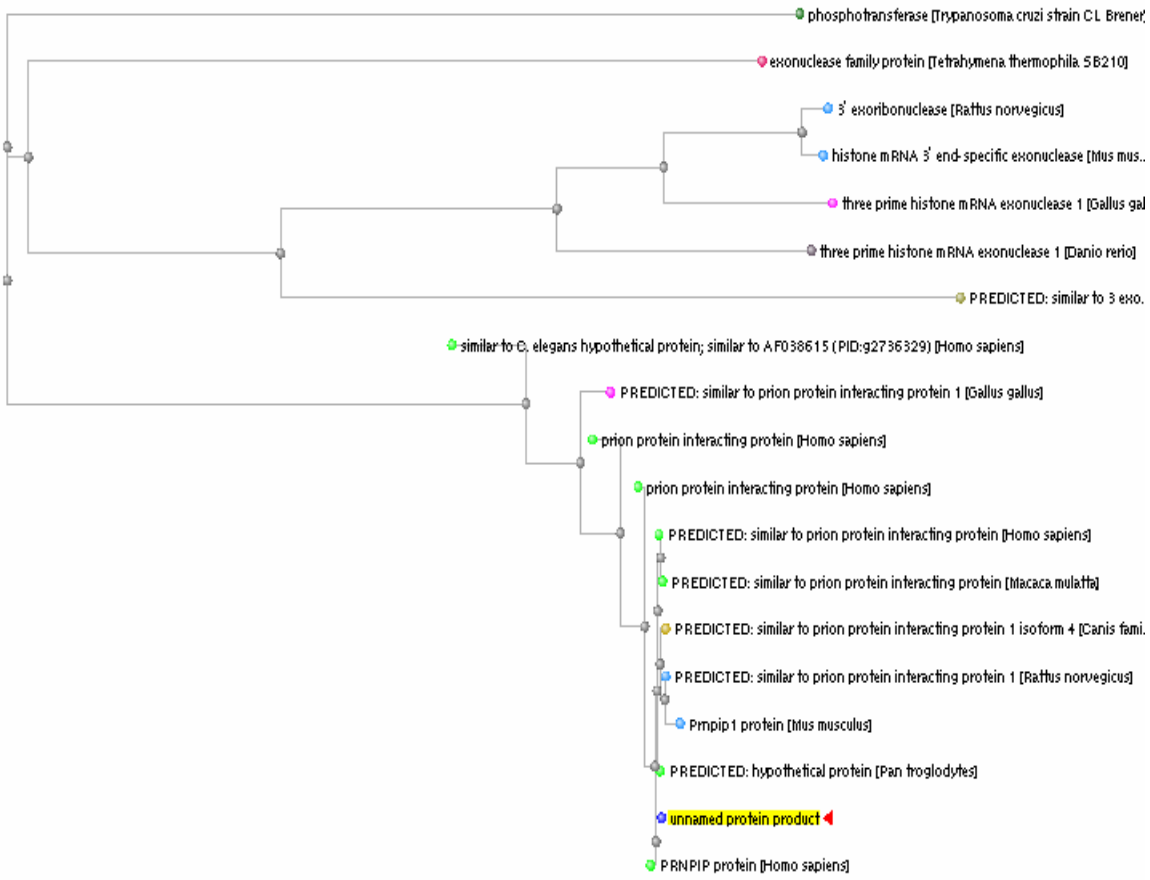
TBS* and TBS- T buffer
12. 1 g Trizma- base
40 g NaCl
H_2O to the end volume of 5 l; (Adjust pH to 7.6)
To make TBS-T to TBS buffer add Tween 20 to give 0, 1% (v/ v) solution (USB)

Washing buffer (inclusion bodies)
20 mM Tris- HCl (pH 8.0)
0. 5 NaCl (Merck)
5 mM imidazole (GE Healthcare)
10 / 1 mM reduced/ oxidized glutathione (Sigma)
And suitable concentration of urea (0- 6 M) (Invitrogen)

Appendix 4

(A) The distance tree of PINT (sheep) and a few homologous proteins (ovine PINT is marked in yellow); (B) colour code for homologous proteins from (A).

(A)



(B)

Blast names color map	
	unknown
	primates
	mammals
	rodents
	birds
	ciliates
	bony fishes
	echinoderms
	kinetoplastids

Appendix 5

One and three letter symbols for amino acids:

One letter code	Three letter code	Full name
A	**Ala**	Alanine
B	**Asx**	Asparagine or aspartic acid
C	**Cys**	Cysteine
D	**Asp**	Aspartic acid
E	**Glu**	Glutamic acid
F	**Phe**	Plenylalanine
G	**Gly**	Glycine
H	**His**	Histidine
I	**Ile**	Isoleucine
K	**Lys**	Lysine
L	**Leu**	Leucine
M	**Met**	Methionine
N	**Ans**	Asparagine
P	**Pro**	Proline
Q	**Gln**	Glutamine
R	**Arg**	Arginine
S	**Ser**	Serine
T	**Thr**	Threonine
V	**Val**	Valine
W	**Trp**	Tryptophan
Y	**Tyr**	Tyrosine
Z	**Glx**	Glutamine or glutamic acis