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JOHNNY NILSSON

Possible use of bacteriophage lambda CIII protein in anti-cancer gene therapy: Introductory studies

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Author

Johnny Nilsson

Title (English)

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Abstract

Paraplegin, a recently found human mitochondrial protein, is highly homologous to the essential *Escherichia coli* protease FtsH. The bacteriophage lambda CIII protein is known to inhibit FtsH and the main objective with this project is to investigate its abilities to inhibit the human homologue of FtsH. If Paraplegin is inhibited by CIII that will probably induce apoptosis and CIII could thus be used in a gene therapy model against cancer. In this report, CD studies confirm that CIII contains alpha helix secondary structure, which is thought to be of importance in the inhibition mechanism. In the structural studies severe problems with decreasing concentrations of CIII occurred. It is here suggested that this is due to very strong interaction between the hydrophobic parts of CIII and the plastic eppendorf tube. To test the inhibition of Paraplegin *in vivo*, a eukaryotic vector containing the *cIII* gene for transfection of mammalian cells was cloned. The performed transfection experiments on CV1 cells failed to give any detectable results. Computer analysis of the CIII sequence revealed high homology with a crystallised *E. coli* protein, which was utilised to make the first 3D models of the bacteriophage lambda CIII protein.

Keywords

CIII, FtsH, HflB, Bacteriophage lambda, structure

Supervisors

Amos Oppenheim

Examiner

Elles Steensma

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Biology Education Centre Biomedical Center Husargatan 3 Uppsala
Box 592 S-75124 Uppsala Tel +46 (0)18 4710000 Fax +46 (0)18 555217

Possible use of bacteriophage lambda CIII protein in anti-cancer gene therapy: Introductory studies

Johnny Nilsson

Sammanfattning

I ett virus som angriper bakterier har man hittat ett protein som kallas CIII. Det här arbetet undersöker möjligheterna att använda CIII i en metod för att behandla cancer.

Examensarbete 20 p i Molekylär bioteknikprogrammet
Uppsala universitet Juni 2001

Table of contents

1. Introduction

When a phage, like e.g. bacteriophage lambda (see figure 1.1), infects bacteria it can lead to either lysis of the host cell or integration of the phage DNA into the host chromosome. The lambda CIII protein plays an important role in the decision between these two options.[1, 2] This is due to the inhibition of an important host factor in the process, the *Escherichia coli* protease FtsH.[3, 4] The bacterial protease is involved in a multitude of activities and is of vital importance for bacterial cell growth and division, without it cell growth cannot be sustained.[5, 6] FtsH is a highly conserved protease also present in higher organisms. Recently, mutations in the gene coding for Paraplegin, a human homologue of FtsH, were demonstrated to be responsible for hereditary spastic paraplegia.[7] The mutations lead to mitochondrial defects suggesting that Paraplegin is a nuclear-encoded mitochondrial protein.[7, 8] Paraplegin is identified to belong to a subclass of the AAA-protein family, *ATPases associated with diverse cellular activities*. Members of this subclass are membrane integrated metalloproteases, in yeast they are active at the inner mitochondrial membrane.[9]

One question that arises is whether CIII will have the ability to inhibit the human FtsH homologue and thereby to induce apoptosis in human cells. As CIII inhibits FtsH, and overexpression of the phage encoded protein leads to cell death, it is already proposed for antibiotic use. If the small phage encoded inhibitor also induces apoptosis in mammalian cells, another application might be to utilise the protein to kill cancer cells by developing a gene therapy model against cancer. In this study, the structure of CIII will be analysed, and a transfection of mammalian cells *in vivo* will be carried out to examine the induction of

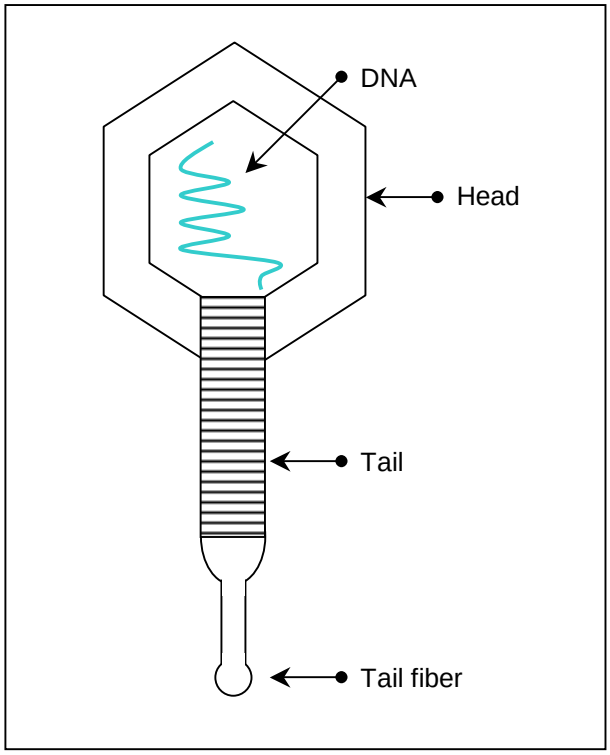

Figure 1.1 Bacteriophage lambda

apoptosis. The possibilities of creating an assay for *in vitro* inhibition of Paraplegin will also be investigated.

As the mechanism of the protease inhibition is not known, structural studies of CIII might give some clues to this mystery. If the structure, and the mechanism of inhibition, can be determined, the possibilities of understanding the effects on eukaryotic cells will improve. Protein engineering studies to accomplish or increase such inhibition will also be discussed. The structural studies will be done mainly with Circular dichroism (CD) spectroscopy, a method to measure how polarised light is absorbed which gives information on the secondary structure of the protein. The structure will also be investigated by computer analysis of the CIII sequence. To get some indications about the CIII´s induction of apoptosis *in vivo*, I will create a eukaryotic vector carrying the *cIII* gene for transfection of mammalian cells. The cells will be cotransfected with a vector for a fluorescence protein that will be synthesised together with CIII. This experimental set-up will provide the possibility for simple analysis of cell death with FACS analysis (fluorescence activated cell sorter). Finally an *in vitro* assay for analysis of the CIII-Paraplegin interaction will be performed.

2. Background

2.1 CIII

The lambda CIII protein is a small protein, 54 amino acids long, produced in bacteriophage lambda.[10] Homologues of the protein seem to be present in all phages and the sequence is highly conserved in a region of 24 amino acids.[11] CIII plays a crucial role in the decision of the lytic or lysogenic pathway in the phage life cycle.

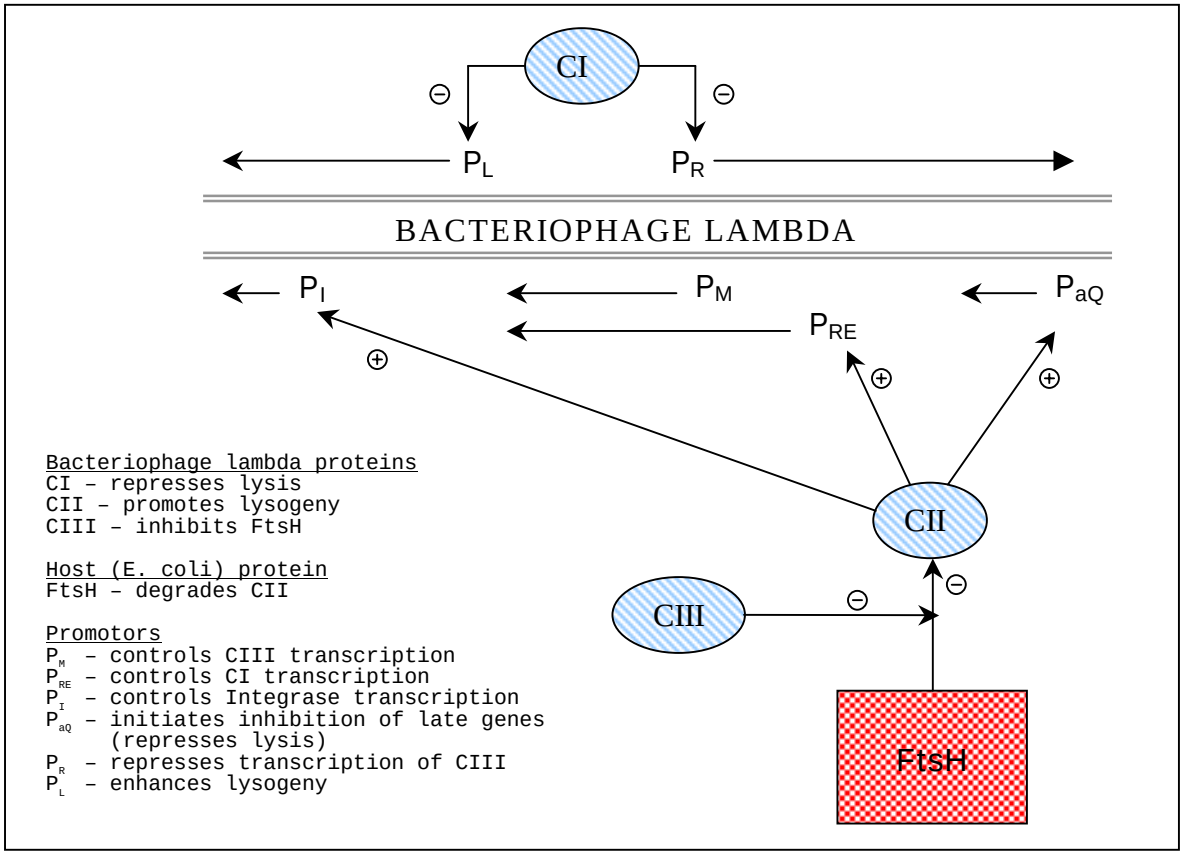

Figure 2.1 Genetic map of key lambda genes involved in the lysis-lysogeny decision
Following infection of bacteriophage lambda, the CII protein stimulates promotors P_{RE}, P_{aQ} and P_I. P_{RE} and P_I control the transcription of repressor (*cI*) and integrase (*int*) genes, which are required for site directed recombinant integration of the phage DNA into the host genome and its maintenance in a dormant state. CI represses the expression of Cro, a protein that represses lysogeny, in addition this repression enhances transcription in the left hand direction of the cI gene itself, though strong binding stops this transcription as a feedback mechanism. The P_{aQ} promotor is thought to direct the synthesis of an antisense RNA that reduces expression of all phage late genes during lytic growth. CII is rapidly degraded by the host protease FtsH. CIII inhibits FtsH, thereby extending the half-life of CII, which prolongs CI repressor synthesis, which in turn promotes the lysogenic pathway.

In its life cycle bacteriophage lambda infects *E. coli* and, after injection of the viral DNA, enters either the lytic or the lysogenic pathway. The lytic cycle leads to the lysis of the host cell and the release of new phage particles. For lytic growth the injected DNA has to express the late genes that are essential for phage replication and cell lysis. In the lysogenic

pathway the injected phage DNA is integrated into the *E. coli* chromosome as a prophage, and can be maintained more or less indefinitely as a benign guest. In lysogeny, the phage functions required for lytic growth are repressed, and the genes required for integration of the phage genome are turned on.[12] Under conditions of nutritional or environmental stress the integrated lambda DNA can be excised and enter a lytic cycle.

It is in this decision between the lytic or lysogenic pathway the bacteriophage lambda CIII protein has an important role. The decision is based on the action of several phage-encoded proteins that interact with host factors, see figure 2.1. The lysogenic pathway is governed by another phage protein, lambda CII, that acts as a transcriptional activator of repressor (*cI*) and integrase (*int*) genes, and represses transcription of the late genes. The CII protein is rapidly degraded by a host factor, the *E. coli* protease FtsH.[1, 11] By inhibiting FtsH lambda CIII extends the half-life of CII. Thereby prolonging CI repressor synthesis, which in turn promotes the lysogenic pathway.[1, 11]

CIII is known to protect two regulatory proteins from degradation by the essential *E. coli* protease FtsH - the lambda CII protein mentioned above and the heat shock sigma factor σ^{32}. Lambda CIII is an unstable protein, and it has been suggested that CIII is a substrate of FtsH, acting as a competitive inhibitor.[13] Overexpression of CIII inhibits cell growth and is relieved by simultaneous overexpression by FtsH.[3, 4]

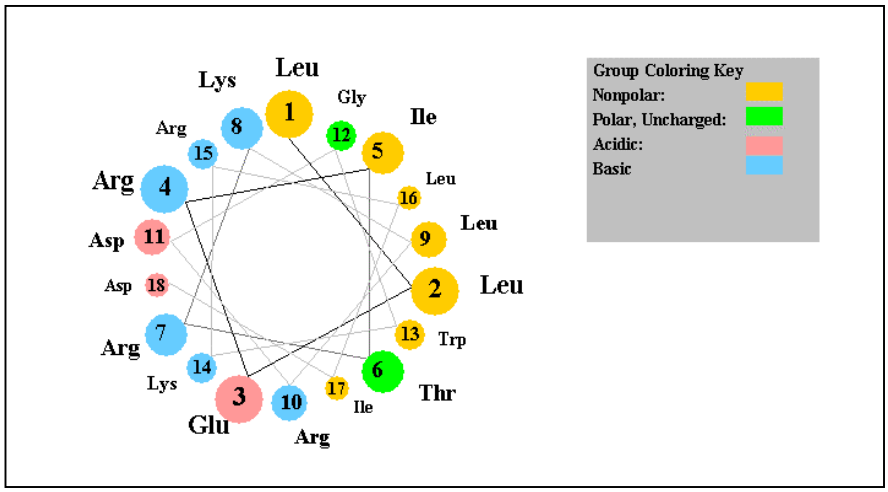

Figure 2.2 Proposed amphipatic helix
Helical wheel representation of a segment of the conserved amino acid sequence in CIII (from Leu18, marked "1", to Arg32, marked "15"). The hydrophobic residues are shown in yellow and the charged in red or blue.

It has been suggested that CIII forms a helical structure[2] and multimers are found in solution. The region in CIII from residue 16 to 37 is proposed to form an amphipathic

helix,[11] see figure 2.2. Other CIII homologues appear to have similar amphipathic helices and mutations in this region lead to loss of activity.[11] A purified in vitro system for the study of the function of CIII has established that a 22 amino acid polypeptide within this central region inhibits the proteolysis of CII and σ^{32}. This inhibition by CIII is not due to the inhibition of FtsH ATPase activity.[13]

2.2 FtsH and Paraplegin

The bacterial protein FtsH (also known as HflB) is known to be of vital importance for bacterial cell growth and division, without it cell growth cannot be sustained. FtsH is an integral membrane protease belonging to a large class of ATPase-dependent proteins known as the AAA-protein family.[14] Over 100 members of this protein family are now known. The AAA-proteins are found in both bacteria and eukaryotes as e.g. *Saccharomyces cerevisiae*, *Xenopus laevis* and mammals including humans. They are involved in a multitude of activities e.g. protein secretion, protein assembly and proteolysis and in cell cycle control. All members have a large domain of about 200 amino acids containing an ATP-binding site.

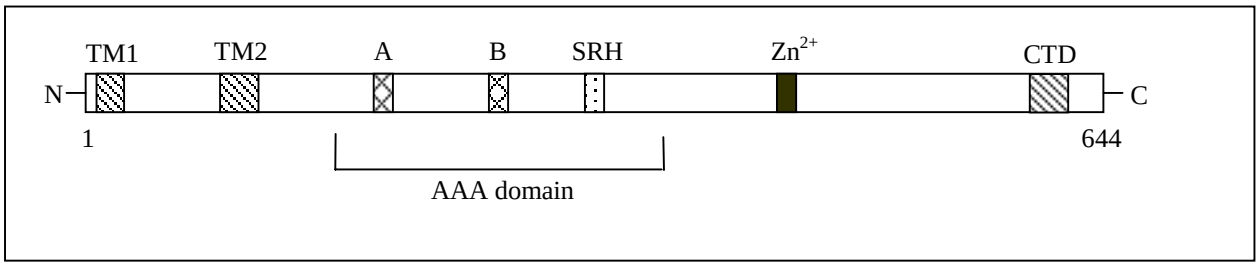

Figure 2.3 Domain structure of FtsH
The homology shared between prokaryotic FtsH proteins is substantial. The domains shown in the figure are:
Transmembrane anchors (TM1 and TM2) – All FtsH homologues contain two hydrophobic segments at the N-terminus. These segments anchor the protein to the phospholipid membrane, exposing the C-terminal AAA-domain to the cytoplasm.
ATP-binding motifs (A and B) – The transmembrane segments are followed by an ATP-binding consensus sequence. Binding of ATP has been shown to induce a conformational change in FtsH.[17]
SRH (second region of homology) – Identified in all AAA proteins. Function unknown.
AAA-domain – The 200 amino acid region including the ATP binding site and the SRH.
Proteolytic domain (Zn^{2+}) – A conserved C-terminal domain containing a characteristic motif of Zn^{2+}-metalloproteases.
CTD (C-terminal domain) – Alpha helix structure, essential for protease activity but is not involved in the ATPase activity.

FtsH is a membrane bound Zn^{2+} metalloprotease first identified as an *hflB* mutation (high frequency of lysogenisation by phage lambda).[15] It is highly conserved in the identified regions (see figure 2.3) and is the only known essential protease in *E. coli*.[16] FtsH participates in the phage lambda lysis-lysogeny decision by its ability to rapidly degrade the lambda CII transcriptional activator and by its response to the *cIII* gene product, see figure 2.1.[17] The protease is highly specific and ATP dependent. A small number of other substrates have been identified including CIII, the heat shock sigma factor σ^{32} and Xis proteins.[18] Electron microscopy has revealed that purified FtsH form hexameric ring-shaped structures with a diameter of 6-7 nm suggesting that the protease active site is hidden in the central cavity.[18] The active site is accessible via a narrow channel preventing non-specific degradation.[19, 20]

The role of the ATPase activity and the mechanism of selecting proteins for degradation is still unknown. It has been found that FtsH acts as an endopeptidase degrading substrates with limited specificity at the cleavage site.[19] A motif in the C-terminal domain (CTD) of FtsH has been identified as being essential for protease activity.[14] In the CTD a short alpha helix predicted to form a coiled coil, leucine zipper, structure was identified. This structure appears to be highly conserved, although conservation is restricted to a few key residues. Mutants with changed conserved leucine residues in this domain were unable to degrade σ^{32} and lambda CII, however the ATPase activity was not interfered with.

In higher organisms, FtsH homologues are found in mitochondria and chloroplasts. A human homologue was recently identified in the search for the genetic basis of heredity spastic paraplegia.[7] The protein, called Paraplegin, shows high homology with a subclass of the AAA-protein family - metalloproteases with both proteolytic and chaperone-like functions. Members of this subclass in yeast are known to be active at the inner mitochondrial membrane.[9] A second mitochondrial feature is the presence of an N-terminal leading sequence typical for targeting proteins for mitochondrial transport. Experiments with immunohistochemical localisation revealed a mitochondrial pattern, providing clear morphological evidence of its mitochondrial nature. Heredity spastic paraplegia is caused by mutations in the *paraplegin* gene that lead to disfunctional mitochondria, thus implying that Paraplegin is essential for mitochondrial activity.

Paraplegin is larger than FtsH, 795 amino acids versus 647. In a large segment, 487 residues long, the two proteins share 40% identical residues (62% similarity) with very few gaps (3%),[18] see figure 2.4. The key residues for the important CTD coiled coil alpha helix in FtsH are also present in Paraplegin.

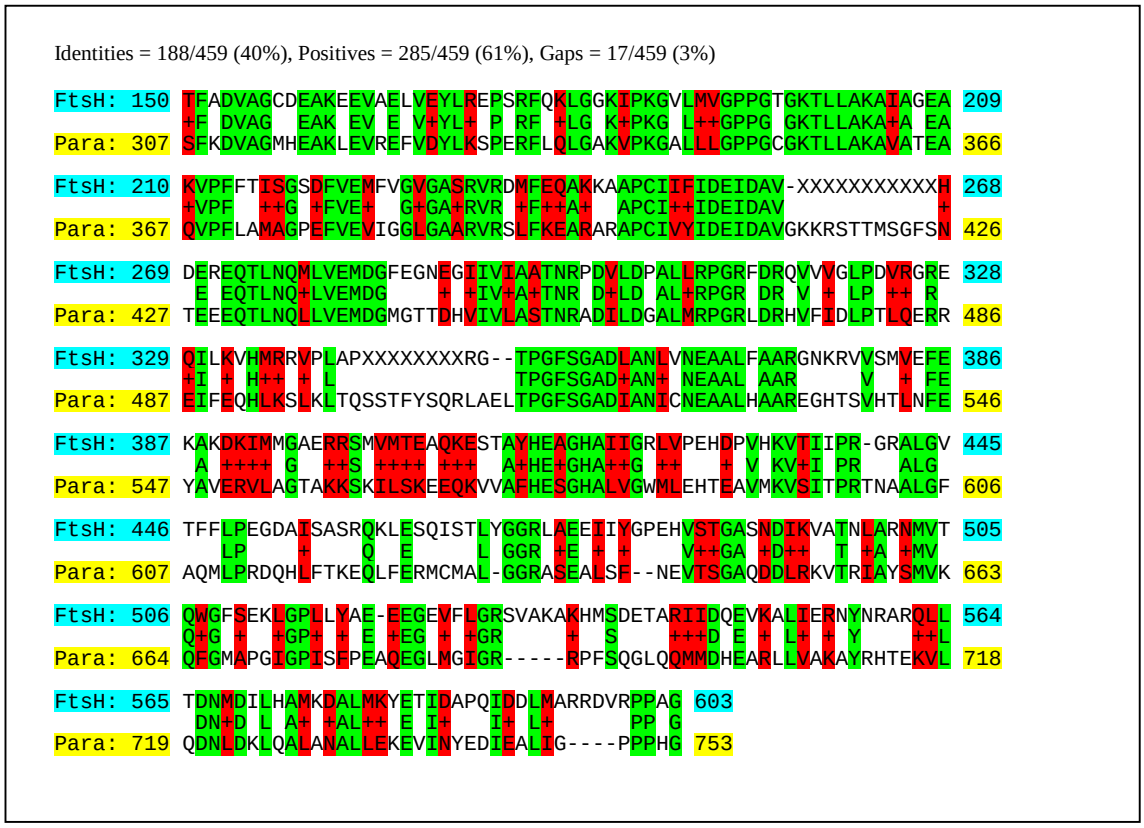

Figure 2.4 Homology of Paraplegin and FtsH

3. Materials and methods

3.1 Strains, plasmids and media

The *E. coli* strains used as hosts for plasmids were A5039 [K37 *lacZ*::Tn*5 lacI*^{q1}] (our collection), XL2-Blue [*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*; F'::*proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^{r}) Amy Cam^{r}] (Stratagene) and SCS1 [*recA1 endA1 gyrA96 thi-1 hsdR17(rK*$^{-}$ *mK*$^{+}$*) supE44 relA1*] (Stratagene). The transfection experiments were done with CV1 cells. The plasmid pOK1 (kindly provided by Oren Kobilar) carries the *cIII* gene under control of the *lac* promotor. The plasmid was constructed by cloning the *cIII* gene from phage λ into pQE30 (Qiagen) using the primers:

A84: 5' - CGGGATCCATGCAATATGCCATTGCAG - 3'
a 5'-primer for cloning cIII into pQE30 at BamHI site

A85: 5' - GGGAAGCTTAGTCTGGATAGCCATAAG - 3'
a 3'-primer for cloning cIII into pQE30 at HindIII site

The plasmids pEGFP-C1 (Clontech) and pCKU (pEGFP-C1 with deleted EGFP region; kindly provided by the lab of Ariella Oppenheim) were used for transfection experiments.
Bacteria were propagated in Luria-Bertani (LB) broth supplemented with antibiotics (50 µg/ml ampicillin or 40 µg/ml kanamycin) when required. The CV1 cells were grown on Dulbeccos minimum essential medium (DMEM)+10% FCS.

3.2 Computer analysis

The bacteriophage lambda *cIII* sequence was used in a FASTA sequence homology search on the PredictProtein website, (URL: http://dodo.cpmc.columbia.edu/predictprotein). Information was also obtained about secondary structure and accuracy of prediction (Profile network prediction Heidelberg program, PHD), plausible motifs (PROSITE search; A. Bairoch; P. Bucher and K. Hofmann), domain homologues (ProDom domain search), MAXHOM alignment and prediction of globarity (GLOBE). Multiple alignment, with all protein sequences sharing a region of similarity, was done with the Clustal program. Modelling was done with the SWISS-PROT Model program (URL: http://www.expasy.ch/) using homologues with known structures as templates. Pictures were generated using the program RasMac 2.6 (R. Sayle, August 1995).

3.3 Expression and purification of CIII

The production of CIII was carried out using the QIAexpress Expression System (Qiagen) for expression of recombinant proteins with a His-tag at the N-terminal end, see figure 3.1.

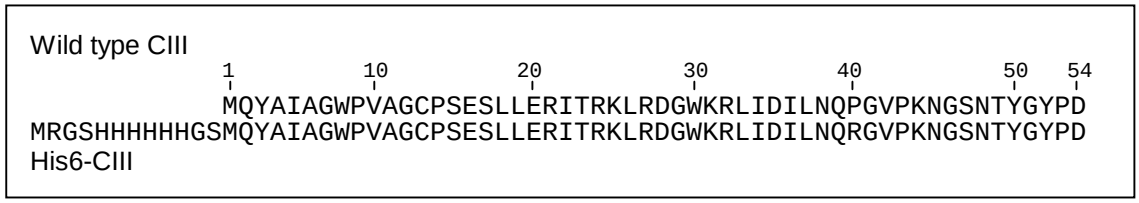

Figure 3.1 Comparison between wild type CIII and His-tagged CIII
The His-tagged CIII used in this study has 64 amino acids, compared to native CIII's 54, and a Pro40Arg mutation.

The pOK1 plasmid was transformed into competent A5039 cells by electroporation and plated on LB ampicillin plates. Cultures of single colonies were grown at 25°C over night in LB medium supplemented with ampicillin. The culture was diluted 1:50 and the bacteria were grown into mid-log phase (OD600 0.4-0.6). Production of 6xHis-CIII was provided by induction of the lac promotor with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After 90 minutes incubation at 30°C on shakers, the cells were harvested.

The cells were washed with phosphate buffered saline (PBS) and resuspended in buffer A for denaturing conditions (6 M guanidine, 20 mM Tris pH 8.0, 50 mM NaCl). Lysis was ascertained by sonication at an amplitude of 12 microns for 10 seconds repeated six times with 20 seconds breaks. After centrifugation at 12 000 r.p.m. for 15 minutes at 4°C the supernatant was collected. A 50% slurry of Ni-NTA beads (previously washed in PBS and buffer A) was added to the solution, the added volume corresponding to the volume of the starting culture according to the manufacturer's suggestions. The binding was carried out at 4°C overnight with gentle agitation. The beads were washed by centrifugation at 3 000 r.p.m. for 10 seconds, at least three times, with wash buffer (6M Urea, 20 mM Tris pH 8.0, 500 mM NaCl, 20 mM Imidazole). The protein was refolded on the beads in PBS at 4°C over night. Elution was carried out for 1 h in 25°C, or 20 minutes in 70°C (see below), with gentle agitation in 500 mM Imidazole, 150 mM NaCl and 20 mM Tris pH 8.0. Four to five consecutive eluates were collected. The eluates were desalted on Sephadex G-25 columns (MicroSpin or PD-10) according to the manufacturer's instruc-tions (Pharmacia Biotech). The purity of the protein was determined by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and the protein concentration was assayed as described below. The protein solutions were stored at –20°C.

3.3.1 Optimisation of elution conditions

To increase the yield of eluted protein, elutions were carried out at 37°C in pH 3.25, 4, 5, 6 and 7 with the buffer and other conditions as described above. Elutions with 1.5 M imidazole were also performed.

Elution of the protein by chemical cleavage at the methionine site was tried by treatment with cyanogen bromide. The beads were dried on a SpeedVac, treated with cyanogen bromide in 100 folded excess and incubated in the dark at room temperature over night. The mix was then vaporised with N_2 and finally dried on a SpeedVac and dissolved in H_2O. The beads were spinned down and the supernatant was collected.

The effects of high temperature were also studied. Elutions with 500 mM imidazole were carried out for 10 minutes at 50, 60, 70, and 80°C under continuous shaking. Under the same conditions, elutions with the elution buffer supplemented with 6 M Guanidine were performed.

3.3.2 Determination of protein concentration

The protein concentration was determined by Bradford analysis or by absorbance at 280 nm under denaturing conditions. Bradford dye reagent (Bio-Rad) was diluted five times in water. A standard curve was made by adding 2, 4, 6, 8 and 16 µg Bovine Serum Albumin (BSA; New England Biolabs) in duplicates. Samples of 5 µl were added to 1 ml diluted Bradford reagent till the readings were in the range of the standard curve. The absorbance was read in a spectrophotometer at 595 nm. A diluted reagent with no additions was used as a reference. The final concentration of the sample was determined by comparison with the standard curve.

The absorbance at 280 nm was measured for up to 20 times diluted sample in 6 M Guanidine, 20 mM Phosphate buffer, final pH 6.5. The formula used for calculating the concentration was:

$A = \varepsilon \times c \times l$

A= Absorbance

ε = Extinction coefficient [M^{-1} cm^{-1}]

c = Concentration [M]

l = Cuvette length [cm]

The extinction coefficient for 6xHis-CIII was calculated with the ProtParam tool at URL: www.expasy.ch/tools/protparam.html, and was 15220 at 280 nm.

3.4 Circular dichroism (CD) spectroscopy

CD spectra of the 6xHis-CIII protein (10 µM protein in 100 mM NaCl and 10 mM phosphate buffer) were recorded using a 0.5 mm path length cuvette at 25°C on a Jobin et Yvon CD 6 Spectropolarimeter. Spectra were obtained from 195 to 250 nm. Typically, the average of six scans, taken at a scan rate of 20 nm/min with sampling intervals of 0.5 nm was used. The fractional helicity was calculated as:

$$f_h = \frac{[\theta]_{222} - [\theta]^0_{222}}{[\theta]^{100}_{222} - [\theta]^0_{222}}$$

where $[\theta]_{222}$ is the experimentally observed mean residue ellipticity at 222 nm and in which the values for $[\theta]^0_{222}$ and $[\theta]^{100}_{222}$ correspond to 0% and 100% α-helix content at 222 nm, estimated as -2000 and -32000 deg×cm^2/dmol respectively.

Temperature gradient studies to investigate the denaturation of the 6xHis-CIII protein were performed from 25°C to 75°C in steps of 5°C.

3.5 Homotypic crosslinking of CIII

The oligomeric state of the protein was investigated by chemical crosslinking with the bifunctional crosslinking reagent EGS [Ethylene glycolbis(succinimidylsuccinate)]. Homotypic crosslinking of 6xHis-CIII was carried out in 100 mM NaCl and 10 mM phosphate buffer containing 1 mM EGS (Pierce) for 15 minutes at 25°C and 42°C, the reaction was quenched with glycin. The experiment was done with 10, 100, 500 and 1000 ng of 6xHis-CIII. After cracking buffer was added the crosslinked products were resolved by NuPage 4-12% precast gel (Novex). Western blotting was performed as described,[21] except that immunoblots were developed by an ECL Western blotting detection kit (Pierce) according to the manufacturer's instructions. The membranes were exposed to RX films (Fuji Film).

3.6 Cloning of cIII into a eukaryotic vector

3.6.1 Amplification and purification of 6xHis-CIII fragment

The 6xHis-CIII fragment was amplified using the primers A88, a 5'-primer for cloning pQE30 inserts, and A85, a 3'-primer for cloning CIII into a Hind III site. The primers had the following sequences:

A88: 5' - CGGATAACAATTTCACACAG - 3'

A85: 5' - GGGAAGCTTAGTCTGGATAGCCATAAG - 3'

The PCR protocol (table 3.1) was adapted from the manufacturer's instructions (Boehringer Mannheim). The PCR was performed on a PTC-100 (MJ Research, Inc).

Table 3.1a&b PCR protocol

a) Reaction mix	
Tris-HCl	10 mM
KCl	25 mM
$(NH_4)_2SO_4$	5 mM
$MgSO_4$	2 mM
dNTP	200 µM
A88	600 nM
A85	600 nM
pOK1	1 µl (0.1-0.75µg)
Pwo	2.5 units
pH	8.85
Volume	50 µl

b) PCR Program
1. 94°C for 2 min.
2. 94°C for 1 min.
3. 56°C for 1 min.
4. 72°C for 40 sec.
5. 25 cycles to step 2
6. 72°C for 3 min.

The product was checked with electrophoresis on a 1.3 % agarose gel stained with ethidium bromide, using 2.5 µl of the reaction mix. The PCR product was purified using the High Pure PCR product purification kit (Boehringer Mannheim) according to the manufacturer's instructions. The reaction mix was diluted to 100 µl before purification and after the purification concentrated on a SpeedVac Concentrator to a volume of about 40 µl. The product was then checked with electrophoresis using 5 µl of the sample.

3.6.2 Restriction and ligation

The purified PCR product was restricted with Hind III (Boehringer Mannheim). The protocol (table 3.2) was adapted from the manufacturer's suggestions.

Table 3.2 Restriction protocol

Restriction Mix	
PCR product	10 µl
Hind III	1.5 µl
Tris	1 mM
MgCl	1 mM
NaCl	5 mM
DTT	100 µM
Volume	20 µl
The reaction mix was incubated over night at 37°C and the reaction was stopped at 65°C for 20 min.	

The pEGFP was restricted with Hind III and Eco47 III (Fermentas). The protocol (table 3.3) was adapted from the manufacturer's suggestions.

Table 3.3 Restriction protocol

Restriction mix 1a	
PEGFP	5 µl
Hind III	1.5 µl
Tris	1 mM
MgCl	1 mM
NaCl	5 mM
DTT	100 µM
Volume	15 µl
The reaction mix 1a was incubated at 37°C for 1 h and the reaction was continued in mix 1b.	
Restriction mix 1b	
Eco47 III	1.5 µl
Tris	30 mM
MgCl	10 mM
NaCl	100 mM
BSA	0.05 mg/ml
NaCl (3M)	0.5 µl
DTT	50 µM
Total volume	30 µl
The reaction mix was incubated at 37°C for 1.5 h and the reaction was stopped at 65°C for 20 min.	

The product was checked with electrophoresis on a 0.8% agarose gel stained with ethidium bromide, using 3 µl of the reaction mix. A part, 18 µl of total 30 µl, of the reaction mix was run on a 0.8% agarose gel. The fragment of interest was cut out and extracted with the Agarose Gel DNA Extraction Kit (Boehringer Mannheim) according to the manufacturer's instructions. The size of the vector was approximately 18 times the size of the fragment. The ligation was conducted with at least three-fold excess of the *6xHis-cIII* fragment. The concentrations were determined by optical analysis of the agarose gels. A self-ligation was made as a negative control. The protocol (table 3.4) was adapted from the manufacturer's suggestions (Fermentas). The ligation mixes were incubated over night at 16°C.

Table 3.4 Ligation

	Ligation mix	**Self ligation mix**
Vector	5 µl	5 µl
Insert	1 µl	–
T4 Ligase	2 µl	2 µl
Tris	40 mM	40 mM
$MgCl_2$	10 mM	10 mM
DTT	10 mM	10 mM
ATP	100 µM	100 µM
PH	7.8	7.8
Volume	20 µl	20 µl

The ligated plasmids were transformed to SCS1 cells by calcium chloride transformation and plated on LB plates supplemented with kanamycin. To check that the desired insert was present, DNA from the growing colonies was extracted by lysing the cells in H_2O, followed by boiling and centrifugation. Samples were collected from the supernatant. PCR was carried out as described above. As an additional control the cells were plated on

ampicillin plates to reassure that the ampicillin resistance from the template plasmids was not retained. The plasmids were purified with the Wizard *Plus* SV Minipreps DNA Purification System (Promega) according to the manufacturer's instructions. The plasmids were further purified with phenol and sent away for sequencing using the oligos A85 and A88 as primers.

3.7 Transfection of CV1 cells with cIII vector

The transfection experiments were done by cotransfection of CV1 cells with pEGFP and pJNcIII carrying the *cIII* gene for expression in eukaryotes, see plasmid maps in figure 3.2.

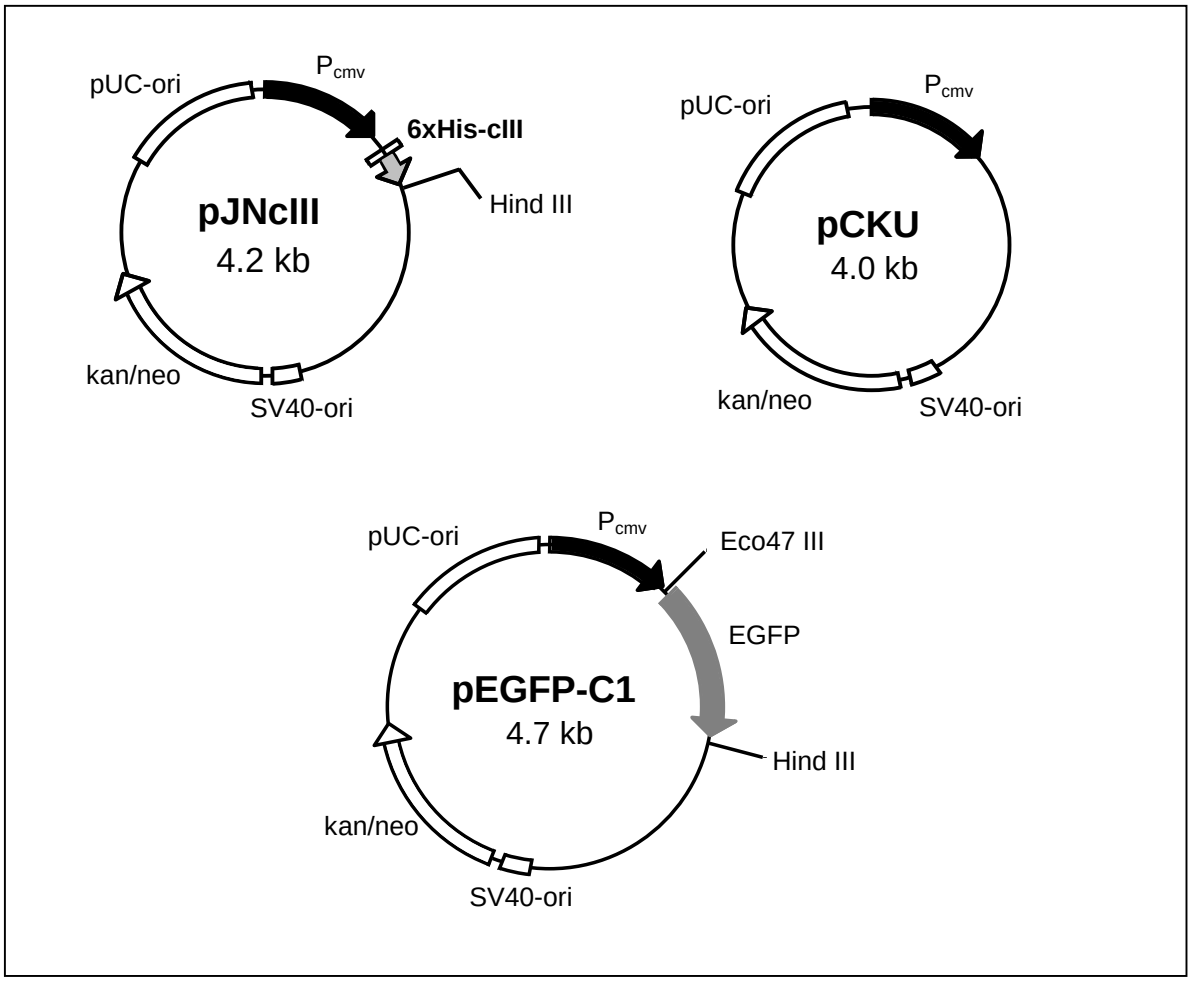

Figure 3.2 Plasmid maps pEGFP-C1, pCKU and pJNcIII
pEGFP expresses green fluorescent protein under control of the CMV promotor, pCKU is an empty vector and pJNcIII is designed to express 6xHis-CIII.

A transfection with pEGFP was used as a reference and as a control one cotransfection was made with pEGFP and an empty vector (pCKU, figure 3.2). The plasmid concentrations were determined by reading the absorbance at 260 nm. Each transfection was done with 500 ng of plasmid DNA, with the vectors in four-fold excess of the pEGFP. The results were obtained by FACS analysis on FACScan (Becton-Dickson). In an experiment without any transfection with pEGFP the morphology of the cells was studied with microscopy. The experimental set-up is shown in table 3.4 and 3.5.

One day before transfection the cells were divided with a transformation of 1.2×10^5 cells to each well. Prior to transfection the cells were 30-40% confluent. They were washed three times with PBS and then overlaid with the transfection solution (500 ng DNA, 250 µl 2x DEAE-Dextran [Pharmacia], 250 µl H_2O) followed by incubation for three hours at 37°C in 5% CO_2. Then the washes were repeated and 2 ml of DMEM+10% FCS was added to each well.

Table 3.5 Transfection experiment, fluorescence studies

	PEGFP	**pCKU**	**PJNcIII**
	100 ng	400 ng	400 ng
Set 1	–	–	–
Set 2*	**+**	–	–
Set 3	**+**	**+**	–
Set 4	**+**	–	**+**

*cotransfected with 400 ng pGEM – a bacterial vector without any promotors for eukaryotic expression

Table 3.6 Transfection experiment, morphology studies

	pEGFP	**pCKU**	**PJNcIII**
	–	500 ng	500 ng
Set 1	–	–	–
Set 2	–	**+**	–
Set 3	–	–	**+**

4. Results

4.1 Computer analysis

The FASTA search at the ProteinPredict website revealed that CIII has large similarities to *E. coli* proteins involved in polyamine transport. A periplasmic protein, PotD (polyamine transport D), of this family had been crystallised and was utilised as a template for 3D models of CIII, the alignment is shown in figure 4.1. PotD is a rather large protein compared to CIII, 348 amino acids versus 54, but in a region of 38 residues the identity is 36.8 %. The homology gave a score of 58 in a program used to evaluate the significance of sequence similarities (PRSS; www.expasy.ch/cgi-bin/call_PRSS.pl?/work/expasy/tmp/http/ July 1999). The probability for this score is below 0.07%.

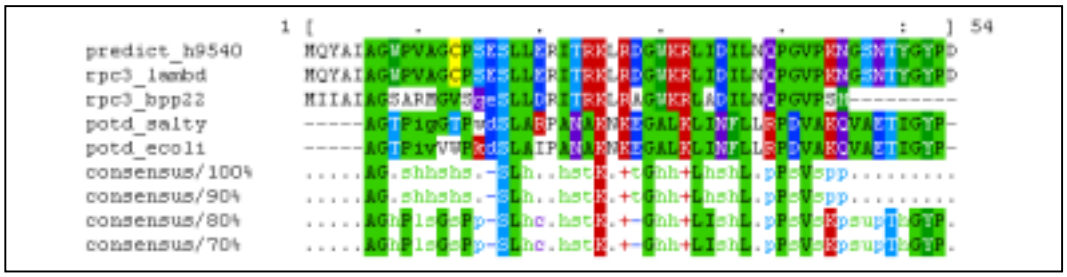

Figure 4.1 Alignment of CIII and PotD proteins
Homologues to the CIII sequence (called predict_h9540 in the figure) were found in a PredictProtein (http://dodo.cpmc.columbia.edu/predictprotein/) search. CIII is 100% identical to lambda CIII (rpc3_lambd) and has very high homology with CIII from other phages (rpc3_bpp22). Surprisingly, high homology was also found with PotD (polyamine transport D) proteins from Salty and *E. coli* (potd_salty respectivly potd_ecoli). The figure also shows the level of consensus between the conserved amino acids.

The modelling of CIII was done with the SWISS-PROT modelling program. The model shows two alpha helices in the regions from Arg27 to Asn38 and from Pro40 to Thr49. The conserved region from Glu16 to Leu37, which is proposed to form one amphipathic helix[11] (see figure 2.2) or two alpha helices, begins in the model with a beta sheet like region followed by a loop prior to the first helix at residue 27, see figure 4.2. The alpha helix at the C-terminal end of CIII has previously not been predicted.

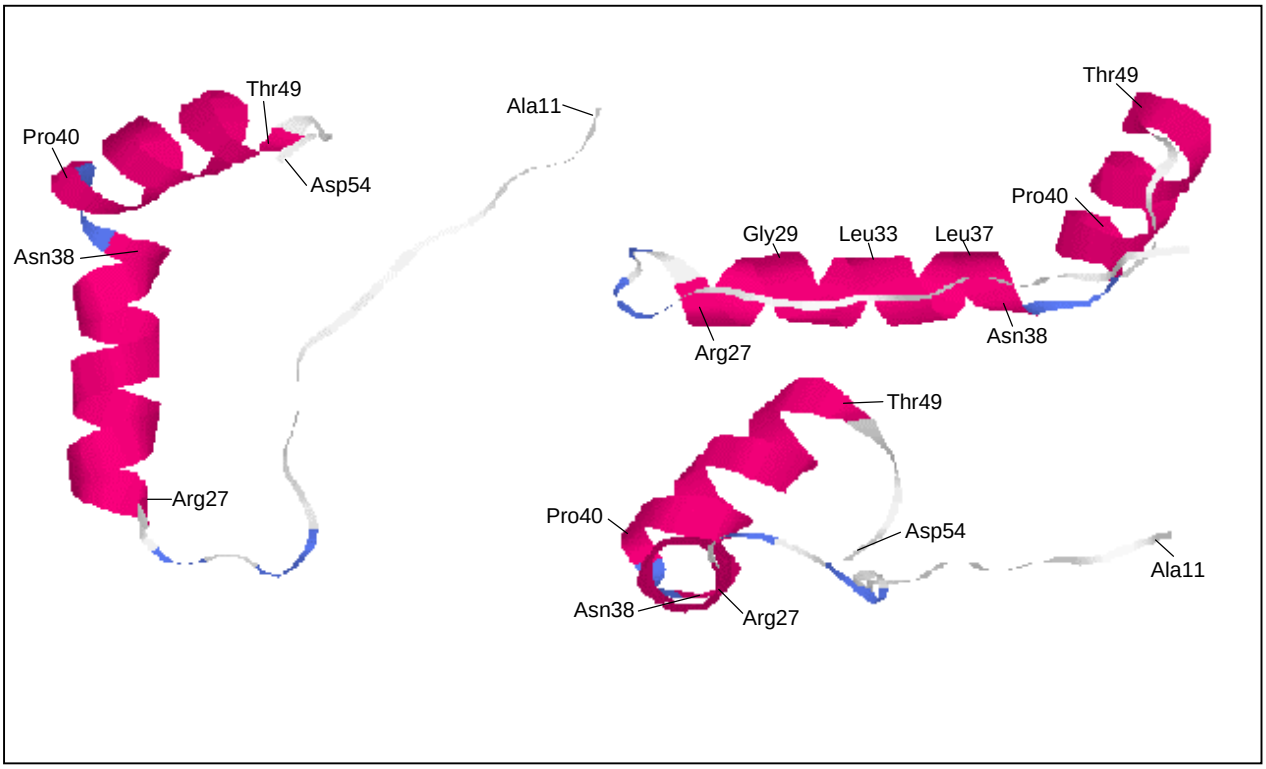

Figure 4.2 3D model of CIII
CIII was modelled with SWISS-MODEL, using pdb-files frof polyamine transport protein (PotD) as a template. In a region of 38 amino acids the identity between CIII and the template is 36,8%. The alpha helix from Arg27 to Asn38 has a high content of hydrophobic residues, e.g. Leu33 and Leu37 – compare figure 2.2.

4.2 Expression and purification of CIII

CIII was produced in *E. coli* using a vector containing a His-tag at the N-terminal end. The produced protein has 64 amino acids compared to native lambda CIII's 54 amino acids. The CIII clone contained a mutation at Pro40, which was mutated to an arginine.

After one successful culture, the concentration of CIII was determined to 0.37 mg/ml by measuring the absorbance at 280 nm in 6 M Guanidine. This gives a yield of about 1.8 mg 6xHis-CIII from 400 ml starting culture. The 6xHis-CIII was judged to be at least 90% pure (data not shown).

Several growth experiments were performed and occasionally some problems occurred. In one batch there were problems with eluting the His-tagged CIII from the Ni beads (see also *4.2.1 Optimisation of elution conditions* below). There were also some difficulties with determination of the protein concentration and loss of protein occurred after desalting with G-25 columns. Figure 4.3 shows an example of eluated CIII on SDS-PAGE.

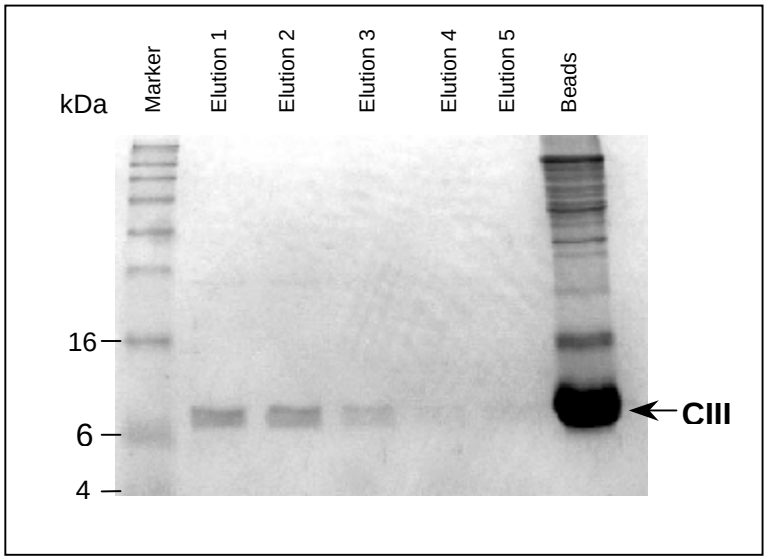

Figure 4.3 Purified CIII
Elutions of the His-tagged CIII from Ni-NTA beads in 500 mM imidazole, 150 mM NaCl and 20 mM Tris pH 8.0. Each elution was carried out for 20 min in 70°C. Most CIII is still stuck on the beads.

4.2.1 Optimisation of elution conditions

To optimise the yield of eluated of His-tagged CIII, elutions were carried out under several different conditions. Elutions with 500 mM imidazole left most of the protein on the beads so elutions with higher concentrations of imidazole were used, but no higher yield of eluted CIII was detected. Other strategies used were:

- to elute in a pH-gradient,
- using a temperature gradient and
- utilise denaturing conditions.

When using high temperature and denaturing conditions, a higher yield of eluted protein was indicated. Attempts to cleave CIII of the beads by treating the beads with cyanogen bromide for cleavage at the single methionine site were made. This approach did not lead to a higher yield of CIII after elution.

4.3 Circular dichroism (CD) spectroscopy

One CD spectra of the produced 6xHis-CIII was taken at a concentration of 10 µM CIII in 10% TFE, 100 mM NaCl, 10 mM NaH_2PO_4, see figure 4.4. The ellipticity at 222 nm showed comparable values with prior CD studies of CIII made by Oren Kobilar and colleagues.

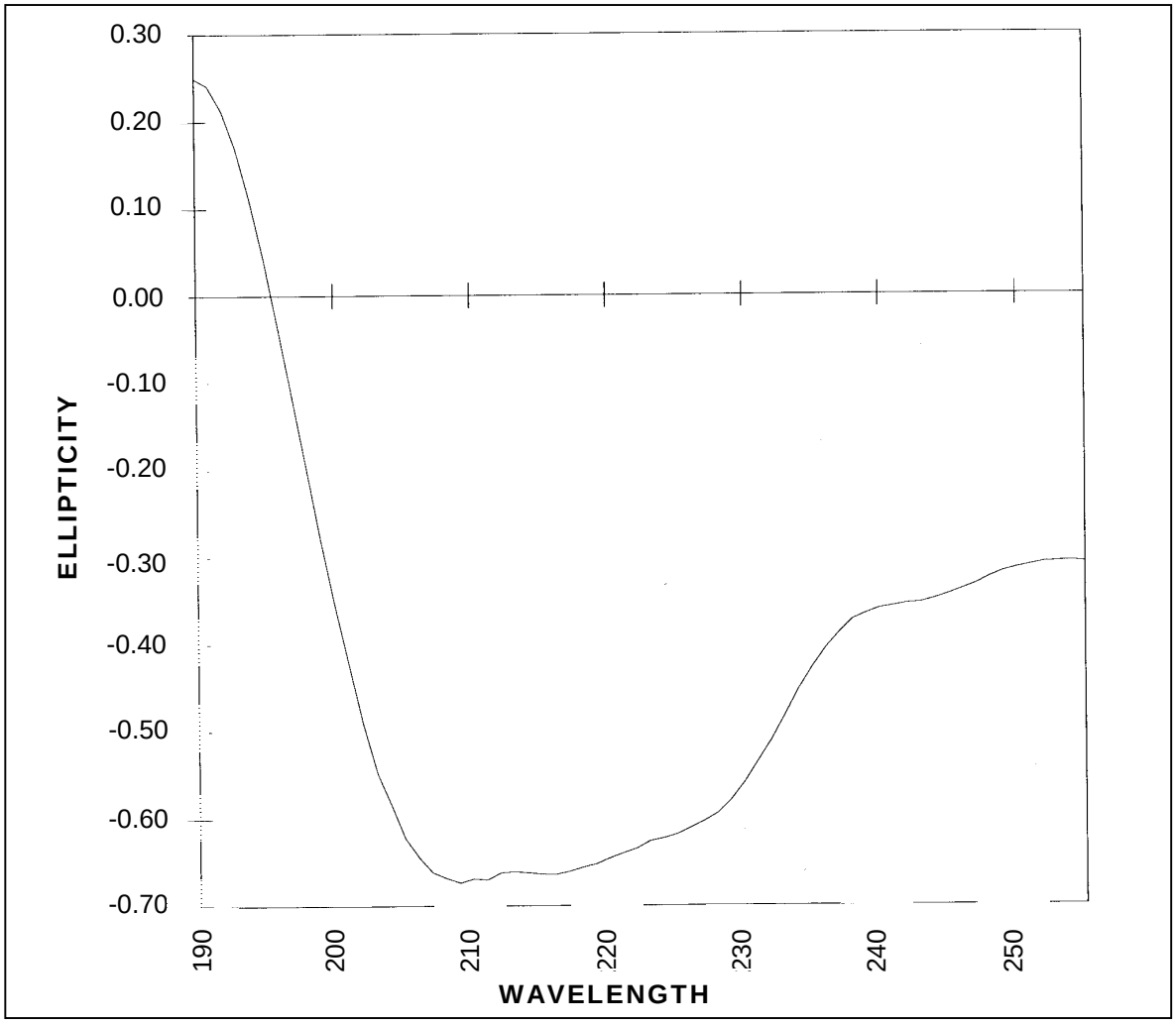

Figure 4.4 CD spectra of His_6-CIII
10 µM His_6-CIII in 10%TFE, 100 mM NaCl and 10 mM NaH_2PO_4

The CD studies of the temperature effects on the secondary structure failed. The detected ellipticities were not consistent with the experimental set-up or with previous results.

4.4 Homotypic crosslinking of CIII

The homotypic crosslinking was performed at different temperatures and with different concentrations of 6xHis-CIII. After the exposure of the western blotting no visible marks of any crosslinking products could be detected. The immunoblotting was repeated and exposure times were prolonged but there were still no detectable products.

When the original protein sample was examined, it showed almost no presence of CIII on a gel. We hypothesised that the amphipathic CIII was stuck on the plastic eppendorf tube walls and experiments for recovery of the protein were carried out at high temperature and in high molarity of guanidine but without any success. To prevent

further problems with disappearing CIII, siliconised eppendorf tubes were used for all storage of CIII.

4.5 Cloning of 6xHis-cIII into pEGFP

The cloning of *cIII* into pEGFP-C1 (kan^r) was accomplished by amplification of the *6xHis-cIII* fragment from the pOK1 (amp^r) plasmid by PCR followed by restriction of the fragment and the vector, and ligation of the restricted products. The ligation was first performed with the fragment in three-fold excess over the vector and then transformed into competent XL2-B cells. As this did not give any colonies when plated on LB kanamycin plates, a new experiment was done with higher excess of fragment while the SCS1 strain was used for transformation. This gave some colonies on LB kanamycin plates. On the control plate with the self-ligation also a few colonies grew.

The presence of the desired *6xHis-cIII* fragment was tested by PCR using the same program as for the previous amplification. Four of the 20 picked colonies were shown to have a band indicating the insert. The self-ligation did not give any PCR product.

When plated on an ampicillin plate the clones did not grow, showing that the template plasmid containing ampicillin resistance was not present.

Two of the clones were sent off for sequencing using the oligomers as primers. The results showed that *6xHis-cIII* was correctly inserted, flanked by the cleavage sites for EcoR 47 and Hind III.

4.6 Transfection of CV1 cells with the cIII vector

Plasmid concentrations were determined by measuring the absorbance at 260 nm. For each transfection 500 ng of plasmids was used. The cells were 30-40% confluent prior transfection and analysis by microscopy showed healthy and dividing cells. After the transfection and incubation for three hours, the cells in the transfection media looked suffering. The cells were grown in DMEM+10%FCS.

One day after transfection the cells looked healthy. They were about 30% confluent, a little denser in the control plate, which was not transfected, and a little less on the plates transfected with both pEGFP and pJNcIII. FACS analysis was carried out although the number of cells counted was less than the planned 5000. Unfortunatly, the analysis of the cells that were counted did not indicate any production of EGFP.

5. Discussion

This project consisted of three different approaches to explore the possibilities to use the CIII protein in gene therapy against cancer. The three proposed paths were:

- *in vitro* inhibition of Paraplegin,
- *in vivo* studies of effects of CIII on mammalian cells and
- structural studies of CIII.

The approach to create an *in vitro* assay for studying Paraplegin was soon abandoned as the attempts to clone *paraplegin* for expression failed. An *in vitro* system for studying the inhibition of FtsH had recently been accomplished in the laboratory and a similar approach was planned for investigation of the CIII-Paraplegin interaction. I had anticipated problems in the protein purification as Paraplegin had not prior been produced *in vitro* and the structure with the transmembrane part was suspected to cause problems. Therefore it was discussed to produce a FtsH/Paraplegin hybrid, keeping the proposed active site from Paraplegin but fusing it to the tail of FtsH, which we know can be purified. The difficulties in the early cloning step were probably due to laboratory inexperience as this was the first experiment made in this study. To study the *in vivo* effect of CIII on mammalian cells, a new eukaryotic vector was constructed. CV1 cells were transfected and analysed with FACS. The structure studies performed were CD spectroscopy, homotypic crosslinking of CIII and computer analysis of the CIII protein sequence.

5.1 Computer analysis

The proteins homologous to CIII found in this study had not been previously reported. The fact that the structure of one of these proteins, called PotD (polyamine transport proteins), had been determined by crystallisation provided unexpected possibilities to create a 3D homology model of CIII with the PotD structure as a template. As the identity is 36.8% in a 38-residue region of the 54-reidue CIII protein, the model is believed to be adequate. The generated model consists of the amino acids 11-54 in CIII. The most interesting features in the model are the two alpha helices in the regions 27-38 and 40-49. Previous secondary structure predictions have shown high probability of alpha helix from residue 16 to 37. Thus the built model supports the hypothesis of a helical structure in the end of this region. Furthermore, it suggests the presence of an alpha helix close to the C-terminal end.

As the PotD protein is a much larger protein than CIII, the structure of the former protein is a result of interactions not present in CIII. It is thus difficult to draw general conclusions about the accuracy of the model. This is especially true with the regions suspected to have high interaction with other parts of the PotD molecule as e.g. beta sheets. The part of the model consisting the alpha helices are considered to be more accurate, as they are a result of interaction between residues present in both the template and CIII.

Investigations of the polyamine transport proteins indicate that the region homologous to CIII is not crucial for protein function, thus implying that although the similarity between CIII and the PotD region is very high it cannot give us any further understanding about the function of CIII.

5.2 Expression and purification of CIII

The produced His-tagged CIII protein has 64 amino acids compared to the native lambda CIII's 54 amino acids. The CIII clone contained a mutation at amino acid position 40 (Pro40Arg). As the mutated CIII previously had been proven to be active *in vivo* (unpublished studies) this was not considered to be crucial to our studies.

The production of CIII is complicated by the fact that overexpression of CIII is toxic to *E. coli*. However, the consequences were minimised as long as the production was carried out according to the developed protocol. Other problems that occurred were difficulties in eluting 6xHis-CIII from the Ni beads, determining the concentration of the eluat and loss of protein in the purification through G-25 columns.

The problems with eluting CIII and the loss of protein in G-25 columns are believed to be due to strong aggregate formation by CIII (see also 5.2.1 below). The problems with determining the concentration could be due to aggregate formation, or that the amphipathic CIII binds to the plastic tubes, or both. In the most successful growth experiment the obtained CIII was determined to be at least 90% pure, and could be used to perform structural experiments.

5.2.1 Optimisation of elution conditions

The problems with eluting the His-tagged CIII were probably due to aggregate formation on the beads that had to be desolved before the protein could be eluted by traditional means. This hypothesis is consistent with the facts that elution with high concentration of imidazole or by chemical cleavage with cyanogen bromide failed. For comparison, CIII

was obviously detached from the beads by the cracking buffer used before running samples on a gel. The cracking procedure includes boiling the samples and the buffer provides in addition to high salt concentration very strong denaturing conditions.

5.3 Circular dichroism (CD) spectroscopy

The performed CD studies were consistent with prior results in the same concentration range.[22] Previous CD spectra of CIII in high concentrations have shown clear peaks at 222 nm and 208 nm strongly suggesting that the protein has a high alhpa helical content.

The failure of later attempts to investigate the temperature effects on the secondary structure by CD studies was the first indications of the coming problems with the decreasing CIII concentration. At first the inconsistent results were believed to occur from insufficient cleaning of the cuvette prior to taking the spectra. When this hypothesis was shown not to be true and the crosslinking experiments also failed, a simple sample analysis unravelled that almost no protein was present in the solution.

5.4 Homotypic crosslinking of CIII

The crosslinking experiment was an attempt to determine CIII´s oligomeric state in solution. The experiment was conducted so that the bifunctional crosslinkning reagent EGS was added to the protein samples. Present oligomers of the protein should then be trapped together by the reagent and would be detected after immunoblotting for the His-tag. As the immunoblotting did not show any detectable products, the original protein sample was re-examined and it showed almost no presence of CIII on a gel. This is suggested to depend on the high hydrophobicity of CIII, and that storage of CIII in eppendorf tubes led to strong interaction with the plastic walls. Experiments to recover the protein were carried out at high temperature and in high molarity of Guanidine but without any success. To prevent further problems with disappearing CIII, siliconised eppendorf tubes were used for all storage of CIII protein.

5.5 Cloning of 6xHis-cIII into pEGFP

To be able to study the effect of CIII on mammalian cells *in vivo*, a vector carrying the *cIII* gene for expression in eukaryotic cells was needed. The choice of pEGFP as the carrying vector was based on good experiences from working with this vector and from the presence of a strong promotor on this plasmid, which would lead to high production of CIII. Another

objective was to choose a simple approach that would generate a general picture that later could be investigated more thoroughly. The use of the His-tagged CIII as insert secured the possibility to detect expressed protein by affinity chromatography means. The cloning was straightforward and the resulting vector was sequenced in two directions to confirm that the *cIII* gene was inserted correctly.

5.6 Transfection of CV1 cells with cIII vector

The transfection was carried out on CV1 cells that were 30-40% confluent prior transfection. The experiment, performed with appropriate controls, was made so that every set was transfected with the same amount of vector. Although the transfection was performed according to a familiar protocol, no production of EGFP could be detected after transfection, suggesting that the transfection failed.

5.7 The future

To determine the structure of CIII it is probably necessary to use X-ray crystallography. There might occur some difficulties as CIII is a rather small protein and, according to previous research, forms multimers in solution. However, this study has resulted in a first homology model of the CIII structure. It has also highlighted some problems that can arise when working with CIII. These problems can hopefully be eliminated with the methods suggested in this report i.e. usage of siliconised eppendorf tubes and elutions made in high temperature and strong denaturing conditions.

The investigations of CIII´s action *in vivo* are now in progress. As a vector for transfection of eukaryotic cells is cloned and an experimental set-up is planned one natural and simple continuation is to repeat the transfection experiment. This will at least show whether the vector and the experimental set-up are adequate. According to the theory, CIII is unlikely to inhibit the mitochondrial protein Paraplegin without transport to the mitochondria. However it is possible that CIII will affect the cells anyway, and this must be taken into consideration in further experiments.

The proposed approach of making an assay for CIII´s inhibition of Paraplegin *in vitro* is another natural way to proceed. This is probably an essential experiment for full understanding of the suspected CIII - Paraplegin inhibition.

6. Table of figures

7. Glossary

AAA-protein family	-	ATPases associated with diverse cellular activities
Bacteriophage lambda	-	Virus that infects *E. coli*
CD	-	Circular dichroism
CI	-	Protein encoded by Bacteriophage lambda. Function: inhibits lysis, thus enhances lysogeny
CII	-	Protein encoded by Bacteriophage lambda. Function: promotes lysogeny.
CIII	-	Protein encoded by Bacteriophage lambda. Function: inhibits FtsH.
CTD	-	Carboxy terminal domain
DMEM	-	Dulbeccos minimum essential medium
E. coli	-	*Escherichia coli*, bacteria
EGS	-	Ethylene glycolbis(succinimidylsuccinate)
FACS	-	Fluorescence activated cell sorter
FCS	-	
FtsH	-	Protein encoded by E. coli. Function: protease essential for bacterial life, degrades e.g. CII. Also known as HflB
HflB	-	Synonymous to FtsH, high frequency of lysogenisation by phage lambda
IPTG	-	Isopropyl-b-D-thiogalactopyranoside
LB medium	-	Laura-Bertani medium
Lysis	-	The destruction or breakage of cells by viruses or chemical- or physical treatment
Lysogeny	-	A condition in which a bacteriophage genome (prophage) survives within a host bacterium either integrated in the host genome or in an extrachromosomal element and repression of lytic functions
Lytic cycle	-	The steps in viral production that usually lead to cell lysis
PCR	-	Polymerase chain reaction
PBS	-	Phosphate-buffered saline
SDS-PAGE	-	Sodium dodecyl sulfate-polyacrylamide gel

8. Acknowledgements

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