2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

All chemicals were purchased from the following companies:
Sigma-Aldrich Chemie (Deisenhofen), Roth GmbH & Co. (Karlsruhe), Serva Feinbiochemica (Heidelberg), Merck AG (Darmstadt), Fluka (Neu-Ulm). If not mentioned, all chemicals used were of analytical grade. Protein A-Sepharose was from Amersham Biosciences (Amersham-Pharmacia) (Freiburg). Nitrocellulose membranes were obtained from Schleicher & Schuell (Dassel) and Polyvinylidenfluorid (PVDF) transfer membranes were from Millipore Corporation (Bedford, MA). Secondary antibodies were obtained from Sigma. Radiochemicals were purchased from Amersham Biosciences and ICN Biomedicals GmbH (Mechenheim).

2.1.2 Marker

DNA standard	1kb Ladder	Gibco BRL (Eggenstein)
Protein standard	SDS-7L	Sigma-Aldrich
	SDS-prestained	Fermentas
	HMW (high molecular weight)	Amersham Biosciences

2.1.3 cDNA clones

For construction of the chimeric proteins, a collection of plasmids (23/23, 16/16, PC/PC, 16/23, 16/EGFP etc.) available from the previous works in our laboratory has been used. These plasmids contain cDNA sequences encoding either the authentic precursors of various polypeptides of chloroplast, or the cassettes encoding fusion proteins composed of one transit peptide and one mature portion from different precursor proteins. These plasmids have been described previously (Berghöfer, J., 1998; Hou, B., 2005; Molik, S., 2005) and, if desirable, are specified in detail in the text.

2.1.4 Bacterial strains and Vectors

Bacterial strains		
	E.coli DH5α	Hanahan, 1983
	E.coli strain BL21 (DE3)	Studier and Moffat, 1986
Vectors		
	pGEM-T easy	Promega
	pBluescript II KS-	Stratagene, San Diego
	pBAT	Annweiler et al., 1991

2.1.5 Enzymes

Molecular biology enzymes were purchased from: Roche Diagnostics GmbH (Mannheim), MBI Fermentas (Vilnius, Lithuania), New England Biolabs (Schwalgach), Stratagene (La Jolla, CA) and USB (Cleveland, OH). Proteases and protease inhibitors were from Sigma. RNase inhibitors were from MBI Fermentas.

2.1.6 Oligonucleotides

Oligonucleotides used for polymerase chain reaction (PCR) and mutagenesis were synthesized by Metabion GmbH (Planegg-Martinsried).

For construction of the "train-like" chimera, the linker plus EGFP part was directly taken from the pEGFP-N2 plasmid (GenBank Accession #: U57608) by use of the enzymes SmaI and NotI. The first parts (i.e. 16/23, 23/23, PC/PC) of the chimera were cloned by use of either T3 or T7 primer as forward primers and the reverse primers were as following:

16/23 reverse:	GCCGGCAACACTGAAAGAACTGGTAGC
PC reverse:	AATATTGACAGTTACTTTTCCCACCATAC

For construction of the "tandem-substrate" as well as its derivatives, the primers for the first part (i.e. 16/23, 23/23) were the same as used for the "train-like" chimera. For the second part, 16/EGFP is the template and T7 primer was used as reverse primer. The forward primers were used as following:
For construction of 23-16$_{LTD}$/EGFP, the 16/23-16$_{LTD}$/EGFP was used as template and the forward primer, 23mATG: ATGGCCTATGGAGAAGCTGCTAATG, and T7 primer was used as reverse primer.

PC_{LTD} forward:	CCCGGGGCTTCCTTGAAGAATGTCGG
16_{LTD} forward:	CCCGGGGCTCAGCAAGTGTCAGCTGAG
$16_{LTD-\Delta(1-5)}$ forward:	CCCGGGGCTGAGGCTGAGACTAGCC
$16_{LTD-\Delta(1-11)}$ forward:	CCCGGGCGCCGAGCTATGTTGGGCTTC
$16_{LTD-\Delta(1-13)}$ forward:	CCCGGGGCTATGTTGGGCTTCGTCGC
$16_{LTD-\Delta(1-20)}$ forward:	CCCGGGCTGGTTTGGCTTCTGGTTC

For construction of 16/23-16_{LTD} and its derivatives, the 16/23-16_{LTD}/EGFP was used as template, the T3 primer was used as forward primer and the reverse primer were as following:

16_{LTD} reverse:	TTAAGCAAGAACAGCCTTAAC
16_{LTD-L} reverse:	TTATAAAAGAACAGCCTTAAC
$16_{LTD-His}$ reverse:	AGCAAGAACAGCCTTAAC
PC_{LTD} reverse:	TTAGGCCATGGCGTTTCCGGCTAG

For the relative mutations, either 16/23 or 16/EGFP were used as template as indicated in the name of primer and the following pairs of primers were used:
16/23(A83L):
GGTTCGTTTGTTAAGGCTGTTCTTTTAGCCTATGGAGAAGCTGCTAATG
CATTAGCAGCTTCTCCATAGGCTAAAAGAACAGCCTTAACAAACGAACC(antisense)

16/EGFP(A83L) :
GTTCGTTTGTTAAGGCTGTTCTTTTAGGGATCCACCGGCCGGTCG
CGACCGGCCGGTGGATCCCTAAAAGAACAGCCTTAACAAACGAAC(antisense)

16/EGFP(AA69LL):
GAGCTATGTTGGGCTTCGTCCTGCTTGGTTTGGCTTCTGGTTCG
CGAACCAGAAGCCAAACCAAGCAGGACGAAGCCCAACATAGCTC(antisense)

16/EGFP(SG74LL):
CGTCGCAGCTGGTTTGGCTTTGCTTTCGTTTGTTAAGGCTGTTC
GAACAGCCTTAACAAACGAAAGCAAAGCCAAACCAGCTGCGACG(antisense)

2.1.7 Plant materials

Pea (*Pisum sativum*) seedlings were grown in green house at 25 °C with long day light (10 hours per day), and harvested on the 7th-10th day after sowing.

2.2 Methods

2.2.1 Standard methods

Basic molecular methods were performed according to Sambrook et al. (1989). Ligation, plasmid transformation in *E. coli*, DNA isolation from plasmid were performed according to Birnboim & Doly (1979). DNA-Restriction, Agarose gel electrophoresis, Plasmid preparation, DNA-extraction were made as detailed in the instructions given in the kits. DNA sequencing was performed either by using the dideoxynucleotide chain termination method (Sanger et al., 1977) or by use of ABI PRISM method (Applied Biosystems).

2.2.2 Construction scheme of the train-like protein

For construction of the "train-like" protein (16/23-EGFP), the DNA templates of 16/23 (Clausmeyer et al., 1993) and EGFP from pEGFP-N2 plasmid have been prepared. The fragment of 16/23 part was amplified by PCR by use of T7 and 23-resverse primers.

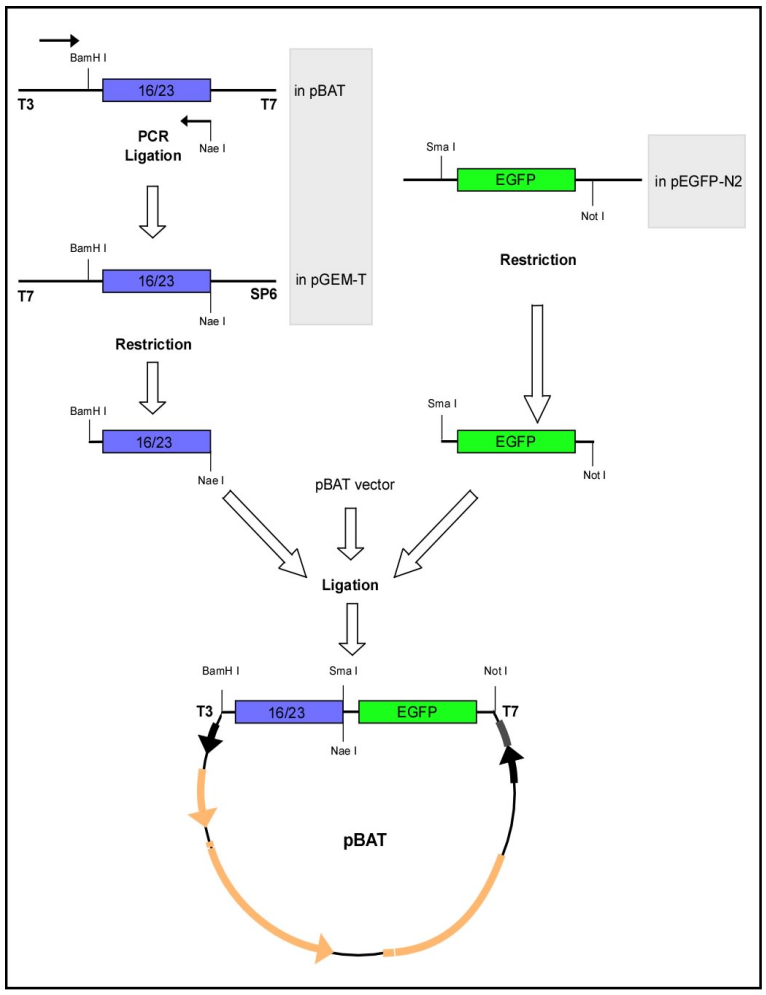

Fig 2.1: **Construction outline of the "train-like" chimera.**

Then the PCR products were ligated into the pGEM-T easy vector for amplification. The fragment was digested with restriction endonucleases BamHI and NaeI. The linker plus EGFP fragment was digested by use of SmaI and NotI directly from pEGFP-N2 plasmid. The isolated 16/23 and EGFP fragments were ligated into pBAT vector which contains the BamHI and NotI recognition site. The resulting recombinant fusions were verified by DNA sequence analysis. The construction procedure was summarized in Fig. 2. 1.

The same procedure was applied for construction of the "tandem substrate". The mutation has been performed according to the instruction manual from Stratagene (QuikChange Site-Directed Mutagenesis Kit, Catalog #200518). The respective "tandem substrates" then were used as templates for generation of derivatives used for the analysis of the fate of Tat signal peptide.

2.2.3 *In vitro* transcription and *in vitro* translation

In order to produce analytical amounts of radioactively labelled proteins, *in vitro* transcription and translation of respective cDNA clones encoding original, chimeric or mutant proteins were performed.

In vitro transcription

In vitro transcription of cDNAs was performed from gene cassettes cloned in either pBluescript KS or pBAT vectors. After linearization of the plasmid DNAs downstream of the gene sequence by proper restriction enzymes, the linearized plasmid DNAs were subjected for transcription reactions, using either T3 or T7 RNA polymerase according to manufactural recommendations (Stratagene and New England Biolabs, respectively).

Composition of the *in vitro* transcription reaction was as following:

H_2O (DEPC-treated)	6.5 μl
5 x reaction buffer	5.0 μl
2.5 mM rNTP mixture (GTP: 0.25 mM)	5.0 μl
100 mM DTT	2.5 μl
5 mM m^7GpppG (capping nucleotide)	2.5 μl
40 U/μl RNase inhibitor	0.5 μl
linerarized plasmid DNA (2 μg)	2.5 μl
RNA polymerase (40 U/l)	0.5 μl
Total volume	25 μl

The reaction mixture was pre-incubated at 37 °C for 30 min to allow the formation of

the cap structure. Incubation was continued for additional 1 h at 37 °C after adding 1 μl of 11.25 mM rGTP to the reaction, then the reaction was terminated by addition of 100 μl ice-cold DEPC-treated H_2O. To check the transcription products, 4 μl was taken out and subjected to 1% Agarose gel (1×MOPS buffer: 5 mM Na-Acetate pH 7,0, 20 mM MOPS, 1 mM EDTA). Finally, all the rest of the synthesized RNAs were precipitated by addition of 0.1 volume of 4 M NH_4OAc and 3 volumes of EtOH. RNA-ethanol suspension can be stored at −20 °C for years. RNAs were collected by centrifugation at 15,000 rpm for 30 min before subjected to *in vitro* translation reaction.

***In vitro* translation**

Synthesis of radioactively labelled proteins was performed by *in vitro* translation of mRNA obtained from *in vitro* transcription in the presence of ^{35}[S] -methionine (Amersham) using an reticulocyte lysate-based cell-free translation system.

DEPC-H_2O	4.65 μl
1 M KCl	0.6 μl
Amino acids mixture (-Met)	0.25 μl
100 mM DTT	2.5 μl
^{35}[S]-Met	0.5 μl
Reticulocyte lysate	0.5 μl
Total volume	12.5 μl

The reaction was carried out for 60-90 min at 30 °C. The resulting *in vitro* translation products were used for import experiments directly or stored at −80 °C for up to one week.

2.2.4 Isolation of chloroplasts from pea leaves

Green house-grown pea seedlings were harvested 7-10 days after germination; the leaves were homogenized in 400 ml of ice-cold SIM buffer by use of a Waring Blendor. Homogenate was filtered through two layers of Miracloth and centrifuged in a Serva SLC-250T rotor for 2 min at 4,000 rpm. The crude chloroplast pellet was resuspended with approx. 8 ml of SRM and loaded onto a 35% Percoll cushion. After centrifugation for 7 min at 4,000 rpm in a Serva SL-50T rotor, the pellet of intact chloroplasts was washed twice with 1 x SRM, and the chloroplasts were collected by centrifugation for 2 min at 3,000 rpm in a SL-50T rotor. The chloroplasts were finally resuspended in 2 ml 1x SRM.

The concentration of the chloroplast suspension was defined by its chlorophyll concentration. Chlorophyll was extracted from 10 μl of the chloroplast resuspension with 1 ml 80% acetone, and the solution was subjected to a Schimadzu spectrophotometer.

The total concentration of chlorophyll a and b was obtained according to the formula (Arnon, 1949): $C_{(Chlorophyll)}[\mu g/\mu l]=(A_{663}$ x 8.02 + A_{645} x 20.2)/10

1x SIM	Hepes/KOH, pH 7,6	25 mM
	EDTA	2 mM
	Sucrose	350 mM
5x SRM	Sorbitol	1,65 M
	Hepes/KOH pH 8,0	250 mM
35% Percoll solution	5 x SRM	2 ml
	Percoll	3.5 ml
	H_2O	4.5 ml
HM buffer	Hepes/KOH, pH 8.0	10 mM
	$MgCl_2$	5 mM

2.2.5 Import of proteins into intact chloroplasts

The standard *in organello* import reaction was performed as following:

	volume	final concentration
chloroplasts	equal to 60 μg chlorophyll	
250 mM methionine	3 μl	5 mM
100 mM Mg-ATP	12 μl	2 mM
1 M $MgCl_2$	1.5 μl	10 mM
in vitro translation product	12.5μ l	
1 x SRM	to 150 μl	

Import reaction with Nigericin

chloroplasts	equal to 60 μg chlorophyll	
250 mM methionine	3 μl	5 mM
100 mM Mg-ATP	12 μl	2 mM
1 M $MgCl_2$	1.5 μl	10 mM
250 mM KCl	6.0 μl	10 mM
0,3 mM Nigericin	3,0 μl	6 μM
in vitro translation product	12.5μ l	
1 x SRM	to 150 μl	

Complete assays without the *in vitro* translation products were briefly preincubated at 25 °C, and the import reactions were initiated by addition of *in vitro* translation product and carried out for 30 min at 25 °C in the light. After incubation, samples were

transferred onto ice and diluted with 350 μl of ice-cold 1x SRM buffer. Chloroplasts were collected from a 50 μl aliquot of the sample by centrifugation at 6,000 rpm for 3 minutes, and denatured with 2 x Laemmli buffer (C^- fraction). Chloroplasts collected from the rest of the sample were resuspended in 1x SRM containing 150 μg/ml thermolysin, and the reaction mixture was chased on ice for 20 min to remove the envelope-bound radioactive protein. Thermolysin treatment was terminated by addition of EDTA to 25 mM. Chloroplasts collected from one tenth aliquot of the assay were denatured with 2 x Laemmli buffer (C^+ fraction), and the rest of the chloroplasts were reisolated by centrifugation through a 35% Percoll cushion at 8,000rpm for 8 min. The intact chloroplasts were thoroughly washed with 1 ml of 1 x SRM buffer supplemented with 10 mM EDTA, and collected by centrifugation for 1 min at 6,000 rpm. Stroma and thylakoid fractions were separated by osmotically lysing chloroplasts in 100 μl of HM buffer containing 10 mM EDTA for 5 min, followed by centrifugation at 10,000 rpm for 5 min. Stromal proteins in the supernatant were supplemented with equal volume of 4 x Laemmli buffer (S fraction). Thylakoid membranes were washed with HM buffer and resuspended in 200 μl of the same buffer. One half of the thylakoids were mock-treated directly (T^- fraction), and the other half was treated with 200 μg/ml thermolysin for 30 min on ice to remove proteins exposed at the surface of the thylakoid membrane. The thermolysin treatment was terminated by addition of EDTA to 10 mM, and the resulted thylakoids were collected by centrifugation at 10,000 rpm for 4 min followed by denaturing with 2 x Laemmli (T^+ fraction). Protein samples were analyzed by SDS-PAGE and autoradiography.

2.2.6 Import experiments with isolated thylakoids

In thylakoido import experiments were generally carried out using thylakoids obtained from pea chloroplasts. To isolate thylakoids, chloroplasts were lyzed in HM buffer at a concentration of 0.75 mg/ml chlorophyll for 5 min on ice and then centrifuged for 5 min at 10,000 rpm at 4 °C. The supernatant containing stroma was separated from thylakoid pellets, collected and stored on ice. Thylakoids were washed twice with HM buffer by centrifugation (5 min, 10,000 rpm) and finally resuspended either in HM buffer or in stroma fraction at a chlorophyll concentration of 0.75 mg/ml. Routinely, thylakoid import reactions were conducted for 30 min at 25 °C in the light. The standard *in thylakoido* import assay includes the following compounds:

thylakoid suspension	40 μl
in vitro translation product	5 μl
HM buffer	5 μl
Total volume	50μl

The resulting thylakoid vesicles were re-isolated by centrifugation for 5 min at 10,000 rpm at 4 °C and washed twice with HM buffer. Then, half of the thylakoid vesicles were resuspended in 2x Laemmli sample buffer, while the second half were resuspended in HM buffer containing 200 μg/ml thermolysin. After incubation for 30 min on ice, protease treatment was terminated by addition of HME (10 mM Hepes/KOH, pH 8.0, 5 mM $MgCl_2$, 10 mM EDTA) buffer. Thylakoids were collected by centrifugation at 10,000 rpm for 5 min and resuspended in 2x Laemmli sample buffer. After denaturation by heating for 3 min at 100 °C, samples were analyzed by gel electrophoresis followed by autoradiography.

To examine the influence of inhibitors on the thylakoid translocation of proteins, assays were supplemented with nigericin (to 2 μM) or sodium azide (10 μM). To examine the role of NTPs, apyrase was added (1 U per 50 μl Assay). Competitor proteins were added to the concentration indicated in the respective assays. For characterization of the unknown protease cleaving cleaving within the signal peptides, respective inhibitors were incubated with thylakoids on ice for 10 min, then proceeded with standard import procedure.

2.2.7 Electrophoresis of proteins

SDS polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis of SDS-denatured proteins was performed according to Laemmli (Laemmli, 1970). As a rule, polyacrylamide gels with an acrylamide gradient from 10-15% were used except being mentioned (Hou, 2005; Molik, 2005).

Non-denaturing electrophoresis of membrane proteins (Blue Native electrophoresis (BN-PAGE))

To isolate the photosynthetic complexes from the thylakoid membrane, the blue native gel electrophoresis was used (Schäger and Jagow, 1991; Schäger et al., 1994; Karnauchov, 1998). By use of the mild detergent digitonin, the thylakoid membrane could be solubilized and the oligomeric complexes of the thylakoid membrane could be reproducibly separated with high-resolution.

- Preparation of samples

  To solubilize the membrane protein complexes, thylakoid membranes (equivalent to 30 μg of chlorophyll) were resuspended with 15 μl of lysis buffer and 7.5 μl of 5% digitonin (freshly prepared and 98 °C dissolved). After incubation for 30-60 min at 4 °C under agitation, nonsolubilized membrane materials were spun down at 40,000 g for 1 h at 4 °C. The supernatant was supplemented with 1.5 μl of

5% Coomassie Briliant Blue G-250 in lysis buffer, bound on ice for 10 min, and centrifuged at 40,000 g for 3 min. The supernatant of this centrifugation was used for loading onto the blue native gel.

Lysis buffer:

Stock solutions	Volume	Final concentration
0.5 M Bistris, pH7,0	500 μl	50 mM
2M aminocaproic acid	2500 μl	1 M
0.5 M EDTA, pH 8.0	50 μl	5 mM
0.1 M $MgCl_2$	25 μl	0.5 mM
0.1 M PMSF (in isopropanol)	50 μl	1 mM
0.1 M DTT	50 μl	1 mM
H_2O	add to 5 ml	

- Gel electrophoresis:

  10 x running buffer:

  0.5M tricine, 0.15 M Bistris, pH 7,0

  For preparation of the blue native gel:

Component	Stacking gel 4%	Separation gel 13,5%	Separation gel 10%
H_2O	5,1 ml	1,38 ml	3,08 ml
10 x Bistris (0,5M, pH 7,0)	1,0 ml	1,5 ml	1,5 ml
30% acrylamide/bisacrylamide	1,3 ml	6,75 ml	5 ml
2M ε-aminocaproic acid	2,5 ml	3,75 ml	3,75 ml
87,5% glycerol	–	1,5 ml	1,5 ml
5% digitonin	60 μl	90 μl	90 μl
10% APS	90 μl	46 μl	50 μl
TEMED	9 μl	4,6 μl	5 μl
Total volume	10 ml	15 ml	15 ml

  Protein samples were resolved at approx. 10 mA (with power restricted at 280 V) at 4 °C. After separation, the gel was fixed with 50% Methanol and 12% Acetic acid for about 15 min before drying. 1x running buffer contained 0,0075% of Coomassie G250. Detection of proteins in these gels was performed by either silver staining or autoradiography.

Immunodetection of proteins - Western blot

For detection of proteins using specific antisera, immediately after electrophoresis, the gels were incubated in transfer buffer (150 mM glycine, 20 mM Tris, 10% methanol)

for 30 min. Then the proteins were blotted onto a PVDF-Membrane (Immobilon-P, Millipore) with a semi-dry transfer apparatus (Gibco-BRL) following the manufacturer instructions (Immobilion-P Transfer Membrane User Guide). The transfer time is 1 h at 2 mA/cm^2 membrane. Prehybridization, hybridization with primary and secondary antisera were carried out in 1 x PBS containing 1% v/v Tween 20 and 5% dry skimmed milk. Specific antisera were used usually in a 1:1,000 dilution. The incubation time is 1-2 h. After 4 x 10 min washing with milk buffer, the secondary antisera conjugated to horseradish peroxidase (Anti-Rabbit-IgG Peroxidase-Conjugate, Sigma-Aldrich) were used in a 1:30,000 dilution for 1-2 h incubation. After this hybridization with the secondary antisera, the PVDF membranes were washed three times with 1 xPBS containing 0.1% v/v Tween 20. For visualization of protein bands, the secondary antibodies were developed with ECL (enhanced chemiluminescence) reaction (Voelker and Barkan, 1995). The developing reagent was set up before the reaction freshly from stock solution. After incubation for 1 min in the developing reagent, PVDF membranes were wrapped in plastic foil and exposed to an X-ray film for an appropriate time.

10 x PBS	NaCl	750 mM
	KCl	30 mM
	Na_2HPO_4	45 mM
	KH_2PO_4	5 mM

ECL reagent

stock solution	volume	concentration
1 M Tris-HCl, pH 8.5	500 μl	50 mM
250 mM luminol (in DMSO)	50 μl	1.25 mM
90 mM p-coumaric acid (in DMSO)	22 μl	200 μM
30% H_2O_2	3 μl	2.7 mM
H_2O bidist.	Add to 10 ml	

Immunoprecipitation

Washed thylakoid membranes (30μg chlorophyll) were solubilized in 100 μl resuspension buffer with Triton-X 100 (50 mM Hepes/KOH pH 8.0, 100 mM NaCl, 0.5 mM $MgCl_2$, 0.05% BSA, 1 mM PMSF, 25 mM EDTA and 1% Triton-X 100). After incubation for 45 min at 4 °C with agitation, the solubilized membrane material was gained from the supernatant of centrifugation at 13,000 rpm for 20 min, and was mixed with 1-5 μg IgG. 20 μl of 10% protein A-Sepharose CL4B (Pharmacia) was then added to the mixture, and the suspension was incubated for 1 h at 4 °C with agitation. The unbound proteins

were recovered by centrifugation for 5 min at 10,000 rpm, and the protein A-Sepharose beads were washed with resuspension buffer as described above, except containing only 0.1% Triton-X 100. Bound proteins were recovered by incubation in SDS-sample loading buffer at 100 °C for 5 min then followed by centrifugation. Protein samples were separated by SDS-PAGE followed by autoradiography.

Coomassie Staining of proteins

Staining buffer	45% (v/v)	Methanol
	9% (v/v)	Acetic acid
	0,25% (w/v)	Coomassie G-250
Destaining buffer I	20% (v/v)	Methanol
	7% (v/v)	Acetic acid
Destaining buffer II	50% (v/v)	Methanol
	10% (v/v)	Acetic acid

For detection of protein bands on gels, staining with Coomassie Brilliant Blue R-250 was routinely used. The gel was firstly stained in the staining buffer for 30 min at 50 °C and stopped by incubation in destaining buffer I at 50 °C until a better overview of the protein bands (approx. 1 h). After incubation with destaining buffer II for 10 min at room temperature, the gel was dried for 2 h at 80 °C in vacuum and exposed to Phosphorimaging plate (FUJIFILM). Phosphorimage analyzer Fujifilm FLA-3000 and the Programm AIDA (advanced image data analyzer, RAYTEST/FUJIFILM) were used for data analysis.