

Confirmation of the existence of a third family among peptidyl-prolyl *cis/trans* isomerases

Amino acid sequence and recombinant production of parvulin

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Abstract In addition to the major cyclophilin-like peptidyl-prolyl *cis/trans* isomerases (PPIases) of *Escherichia coli* an enzyme of very low relative molecular mass (10.1 kDa) was discovered in this organism which gave first indication of the existence of a novel family in this enzyme class [1994, FEBS Lett. 343, 65–69]. In the present report we describe the chemically determined amino acid sequence of four peptides derived from the 10.1 kDa protein by the treatment with either cyanogen bromide or endoproteinase Lys-C. Together with a continuous run of 75 amino acids starting N-terminally, the sequence of the mature enzyme, 92 residues in length, was elucidated. Cloning and determination of the primary structure of a DNA fragment encoding this enzyme were also performed. Overexpression of the enzyme by using multicopies of plasmid pSEP38 in *E. coli* and detecting an enhanced PPIase activity attributed to the 10.1 kDa enzyme provided additional proof that the 92 amino acid protein was a PPIase. The enzyme was called parvulin (lat.: parvulus, very small). Homology analyses indicated that several parvulin-like proteins could be found in the database screened. To further elucidate the functional role of PPIases it might be of some importance that homologous proteins like the PrtM protein of *Lactococcus lactis* and the PrsA lipoprotein of *Bacillus subtilis* are known to be involved in the protein export and maturation machinery of the bacteria.

Key words: Peptidyl-prolyl *cis/trans* isomerase; Parvulin; *Escherichia coli*; Amino acid sequence; Sequence homology

1. Introduction

Presently peptidyl prolyl *cis/trans* isomerases (PPIases, EC 5.2.1.8), the enzymes which catalyze the *cis/trans* isomerization in oligopeptides and certain conformational states of proteins, are subdivided into two different, sequence-unrelated families. Due to their specific interaction with cyclosporin A and FK506, these enzymes are termed cyclophilins (Cyp) and FK506 binding proteins (FKBPs), respectively (for reviews see [1–3]). Different PPIases are found with relative molecular masses ranging between 11.8 and 158 kDa. Usually the enzymes contain a conserved core region common to all PPIases of a family, with additional C- and N-terminal extensions bearing sequence motifs of mainly unknown function. Particularly for cyclophilins, this core region was found to be highly conserved during evolution from bacteria to humans.

However, separation of cellular homogenates by either gel filtration or continuous free flow electrophoresis gave rise to the assumption of additional PPIase enzymes being less sensitive to cyclosporin A and FK506 [1].

An example for the detection in *E. coli* of a PPIase resistant to nanomolar concentrations of both types of inhibitors is an enzyme with a molecular mass of $10,101 \pm 2$ Da [4]. In addition, the N-terminal 21 amino acid residues of the enzyme did not show any similarity to other members of PPIase families. These 21 amino acids comprise already about 25% of the total chain length of the novel enzyme. Therefore, the presence of sequence

portions involved in activity should have been highly probable. However, residues thought to be involved in the expression of catalytic activity and thus strongly conserved throughout all of the FKFBPs and cyclophilins were missing. For this reason, the results were an important indication for the existence of a third family of PPIases. It may be a characteristic of PPIases that, with a few exceptions, members of the cyclophilin or FKBP protein family established by convincing sequence similarity were mostly found to express enzyme activity. One of the exceptions in the FKBP family is the metal ion binding FKBP with a molecular mass of 22 kDa from *E. coli* [5,6] which failed to show PPIase activity [5] when assayed with the standard enzyme test [7]. Although the predicted fold resembled that of FKBP12, only 7 out of 13 amino acid residues, strongly conserved in all of the other FKFBPs, were fully retained in this protein.

In cyclophilins the residues 53–57 (human Cyp18cy numbering) were found throughout as FHR(IV) motif. In each case tested this sequence motif was connected with PPIase properties of the respective protein in the standard PPIase assay mentioned above. Under easy terms, a database search utilizing the amino acid sequence of the entire polypeptide chain of the 10.1 kDa PPIase from *E. coli* may help to identify homologous proteins with putative PPIase function.

In this study the complete sequence, overexpression and partial characterization of the secondary structure by CD spectroscopy of the 10.1 kDa PPIase, named as parvulin, is described. Sequence comparisons identified other possible parvulins which together may form a third class of PPIases different from FKFBPs and cyclophilins.

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2. Experimental

2.1. Amino acid sequence analysis

A sample of the purified protein was cleaved in 75% formic acid by addition of a small crystal of CNBr and incubation at 23°C for 24 h under nitrogen in the dark. The cleavage products were separated by reversed phase HPLC on a C4 column (0.46 × 25 cm, Vydac 214 Tp54, 5 µm) using 0.1% trifluoroacetic acid (solvent A) and 70% acetonitrile in 0.1% trifluoroacetic acid (solvent B) in a gradient from 0 to 90% B in 60 min at a flow rate of 0.5 ml/min. Another sample of protein was cleaved with endoproteinase Lys-C (Boehringer Mannheim) at an enzyme/substrate ratio of 1:50 in 0.1 M ammonium hydrogen carbonate containing 2 M urea for 4 h at 25°C. The resulting peptides were separated by reversed phase HPLC on a C18 column (0.3 × 25 cm, Vydac 218 Tp54, 5 µm) using the same solvent system as above and a gradient of 0–60% B in 160 min at a flow rate of 0.25 ml/min. For cysteine determination, purified protein (100 pmol) was reduced with 2-mercaptoethanol in 0.25 M Tris-buffer, pH 8.5, containing 6 M guanidine hydrochloride and 1 mM EDTA by incubation at 22°C for 2 h under argon in the dark and subsequently alkylated with 4-vinylpyridine by incubation as above. The modified protein was desalted by reversed phase HPLC on a C4 column (0.3 × 5 cm, Nucleosil 300-5) at 40°C using 0.1% trifluoroacetic acid (solvent A) and acetonitrile containing 0.08% trifluoroacetic acid (solvent B) in a gradient of 1–70% B in 10 min at a flow rate of 1 ml/min. Amino acid sequences were determined using Applied Biosystems sequences 470A and 476A according to the manufacturer's instructions.

2.2. Molecular cloning techniques, DNA sequence analysis and gene expression

E. coli K-12 strain HB 101 was used for the amplification of the parvulin gene *parvA*. The host strain used for transformation with recombinant plasmid derived from the vector pUC18 (Pharmacia, Freiburg) was *E. coli* K-12 strain DH5α.

Isolation of plasmid DNA was performed by the alkaline extraction procedure [8]. DNA fragments were extracted from agarose gel using the Sephaglas BandPrep Kit (Pharmacia, Freiburg). *E. coli* cells were transformed with hybrid-plasmids as in [9].

The complete parvulin gene was amplified by PCR using chromosomal DNA of *E. coli* K-12 strain HB101 and the following primers: 5'-GCACGACGTATTACGATGGC-3' and 5'-CTGTTCTCTCTTACGCTTGTGTG-3'. The primers were purchased from Pharmacia (Freiburg). After an initial denaturation step (2 min at 94°C), 35 cycles were performed (1 min at 94°C, 1 min at 52°C and 1 min at 72°C) and completed by a final elongation step at 72°C for 5 min.

The vector pUC18 was digested with *Sma*I (Boehringer, Mannheim) and dephosphorylated. The PCR fragments were cloned into pUC18. The recombinant plasmid pSEP38 was obtained with an insert of 1,134 bp.

Sequencing was carried out with a T7 polymerase sequencing kit (Pharmacia, Freiburg) as described by the manufacturer. Both strands were sequenced using a pUC18-universal primer followed by the sequence primers SP4 to SP6 and a pUC18 reverse primer followed by the sequence primers SP1 to SP3. The composition of the primers were: SP1, 5'-GATCCAGCAGATTCGTTTGGGC-3'; SP2, 5'-GTACCAAGCCACTCCCTGACTG-3'; SP3, 5'-GGTGCTGTACCGCAACTAATAGC-3'; SP4, 5'-GAGCAGGTAGGTAAGAACTGCG-3'; SP5, 5'-GTCAGTCAGGAGTGGCTGGTAC-3'; and SP6, 5'-CGACTGACTTGTGTGACCAAG-3'.

2.3. Purification of overexpressed parvulin from *E. coli*

An overnight culture (5 ml) of *E. coli* strain K-12 DH5α harbouring plasmid pSEP38 was used to inoculate several 1 l portions LB broth medium (10 g bactotryptone, 5 g NaCl, 5 g yeast extract) in parallel. After growth to late logarithmic phase at 37°C one of the parallel cultures was treated with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) to induce enhanced expression of the *lacZ* promoted parvulin. Cells were harvested and treated as described [4]. The enzymatic assay for parvulin was performed as described [4]. The last step of the purification procedure (cation-exchange chromatography on a Fractogel EMD SO³-650(M) column) was omitted. For amino acid sequence analysis and electrospray mass spectroscopy the protein was applied to a C4 narrowbore reversed-phase HPLC column (50 × 3 mm, Nucleosil 300-5, Macherey-Nagel (Düren)) and eluted with a gradient of 0 to 60%

acetonitrile in 0.1% aqueous trifluoroacetic acid within 12 min at a flow-rate of 1 ml/min.

2.4. Protein analytical methods

The molecular mass of the recombinant protein was determined by electrospray mass spectrometry as described [4].

SDS-PAGE was performed as in [4]. For Western immunoblotting the semi-dry electroblotting system Fast Blot B32 from Biometra (Göttingen) was used. A polyclonal antiserum specific to the 10.1 kDa PPIase was obtained from rabbit. HPLC purified protein (600 µg) was taken for immunisation using established procedures (pab productions, Hebertshausen).

Circular dichroism was measured on a JASCO J-710 spectrophotometer (Jasco, Tokyo) with the protein dialysed against 10 mM sodium phosphate, pH 6.5. The resulting spectra were the average of 8 scans, recorded in a 0.1 cm cell at 20°C. For subtraction of the buffer baseline and smoothing the resulting spectrum we used the software provided by JASCO (Tokyo).

2.5. Computer methods

Database searches were performed using the UWGCG (University of Wisconsin Genetics Computer Group) and PIR (Protein Identification Resource) program packages. The FASTA and TFASTA algorithms [10] were used for similarity searches. Multiple sequence alignments were generated using CLUSTAL [11], pairwise similarity comparisons were performed using BESTFIT.

3. Results and discussion

The N-terminal amino acid sequence analysis of the PPIase yielded the first 75 amino acids of the protein (Fig. 1). This revealed the presence of a single methionine which was subsequently used to generate peptide fragments by CNBr treatment. The resulting two fragments could be separated by reversed phase HPLC on a C4 column and showed the expected N-terminal sequences. However, it was not possible to sequence the complete C-terminal fragment because of the presence of some unfavourable bonds. Therefore, the protein was also cleaved with endoproteinase Lys-C and the peptides were separated by C18 reversed phase HPLC. Screening of the major peaks yielded among others the C-terminal peptides K1 and K2, completing the sequence (Fig. 1). At this stage the sequence still contained two unidentified amino acid residues at positions 41 and 69. The difference in mass between the measured value and the sum of identified residues was 206 Da, indicating the presence of cysteine residues at these positions. Final proof for this assignment was obtained by treating the protein with 4-vinylpyridine. The mass difference between modified and unmodified protein was found to be 210 Da, corresponding exactly to two pyridylethyl residues. The calculated relative molecular mass of 10,100.44 Da for the 92 amino acid-long sequence was in excellent agreement with the value of

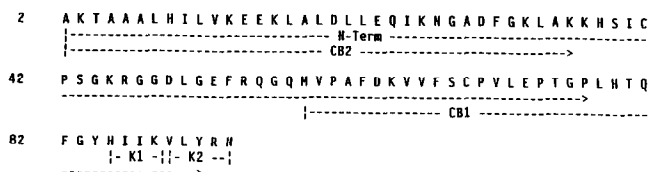


Fig. 1. The sequence of mature parvulin as derived from N-terminal sequence analysis (N-Term) and sequence analyses of peptides generated by cleavage with CNBr (CB) and endoproteinase Lys-C (K). Only K-peptides important for sequence determination are shown. The cysteines were determined by mass spectrometry of the unmodified and the pyridylethylated protein. Amino acid residues were numbered according to the gene sequence (Fig. 2).

10,101.3 $\pm$ 2 Da determined previously by electrospray mass spectrometry [4]. The protein sequence was then backtranslated into a DNA sequence using the coding preference of *E. coli*. Searching the GenEMBL database with this DNA sequence using the TFASTA program identified a region between 84.5 to 86.5 min in the *E. coli* genome [12] which contained this previously uncharacterized sequence. However, the open reading frame contained two frameshift errors due to the lack of cytosines after bases 12,717 and 12,776. When this was corrected, the identity was 100% except for the starting Met which was not present in the mature protein. For resequencing of this region a 1,134 bp fragment was isolated by the PCR method using primers corresponding to the region of 12,370 to 12,390 and 12,482 to 12,501 of the *E. coli* sequence locus ECOUW85U. Both strands of the 1,134 bp fragment were sequenced after cloning into the pUC18 vector using the *Sma*I restriction site and the transformation of competent *E. coli* K-12 DH5 α cells (Fig. 2).

This construct was also used for recombinant production of parvulin in order to ascertain that the polypeptide chain of

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1  GCACGACGTATTACGATGGCGCATTCTCTGAAATGATAAAAAACAGGTTAGTTGTCTG
61  TGCTCAACGGAAAAAATTAATGGCAACGCATGCATCGTCTCCATTATCAACCACCTCGCG
121  ACCAGACAAGTTAAACAGTGAATGCATGTTGAAATAGCCAGTAATGTTATCAATGACCT
181  GCAATTCTTTGATCAGCAGATTCTGTTGGGCCAAGCGACCTGTTACACAAGTCAGTCG
241  AAACAGATTAAACAGAGCAAGGACTTACAGAGGGTATCTTAATCAATGTTTGCACATGA
301  GTTGGTTGCAGACATACAGAGGCGGCTCATCAAGTCAATCAATGCCATATCGCTCATAAA
361  TTGACATAAAATAACCATCCCGGATTAATGACTTTGTAGCCATAGAGGCCCTAAGTTT
      -35          -10
421  CTCCCCTTGAGGACCTGCGAGCAGGATTTTCGGGCTTTTGCATATATGTACCGCCAC
481  TCCCTGACTGACAAATTACCTTAGCATCTCGGATCATGCTAAATCGCCGCTGACAATT
541  ATTCACCTCAAGGCAACGATCATGGCAAAACAGCAGCAGCACTGCATATCTTGTAAGA
      rbs
1      M A K T A A A L H I L V K E
601  AGAGAAACTGGCTCTGGATCTTCTCGAGCAGATTAAAGACGGGCGGATTCGGCAAGCT
15     E K L A L D L L E Q I K N G A D F G K L
661  GCGGAAGAAACACTCCATTTCGCCATCAGGCAACGCGCGGTGATTAGGTGAATTCGG
35     A K K H S I C P S G K R G G D L G E F R
721  CCAGGGTCAGATGGTTCCGGCGTTCGATAAAGTGGTTTCTCTTTCGGTACTGGAGCC
55     Q G Q M V P A F D K V V F S C P V L E P
781  GACCGGCGCGCTGCACACCCAGTTTCGATATCACATCAATTAAGGTGCTGTACCGCAACTA
75     T G P L H T Q F G Y H I I K V L Y R N *
841  ATAGCAAGGCTTCTCCAGGAGAAGGCTTGAGTGTCTTCTCCCTCTCCCTGTGGAGAGT
901  CGGGGTGAGGGCATCAGCGCGCACTTAACCGCAACAGCAATACGTTTCATATCTGTCTAT
961  ATAGCCGCGAGTTTCTTACCTACCTGCTCAATCGCATGGCTGCGAATCGCTTCGTTTAC
1021  ATCAGCGAGTTGCGCGTTATCTACCGCGCTTCCGGAATAGCTTTACCGAGGTCGCCCG
1081  TTGCAGCTCTGCCATAAAGCGTTTCAGCAACGGCACACAAGCGTAAGAGACAGA

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Fig. 2. Nucleotide and deduced amino acid sequence of the *parvA* gene and the neighbouring regions. Nucleotides and amino acids are numbered on the left hand side. The potential ribosome binding site (rbs) is underlined. A possible promoter sequence (indicated by -35 and -10) is underlined and printed in italics. Two putative terminator regions are indicated by two arrows.

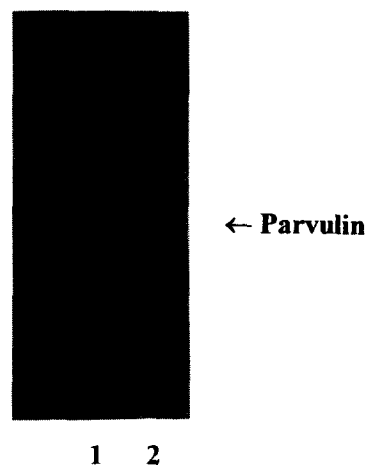


Fig. 3. Western immunoblot analysis of proteins from *E. coli* K-12 cells. The immunoblot was performed after running an SDS-PAGE (17.5%). Lane 1, strain DH5 α with pSEP38, 0.5 μ g protein; lane 2, HB101, 0.5 μ g protein.

92 amino acids did indeed contain the full information necessary for the catalytic machinery of a PPIase.

In fact, as monitored by Western blot experiments the amount of the protein was significantly enhanced in the transformed cells (Fig. 3). However, IPTG induced expression did not lead to a further enhancement of the protein yield, indicating that the used construct already contained a promoter read with maximal efficiency. In order to confirm that the observed higher yield of the protein lead to an increased PPIase activity as well, purification and enzymatic assay of the wild-type *E. coli* and the transformed strain were performed in parallel. Obviously, the data shown in Fig. 4 indicated that the overproduction of parvulin also caused an 40–50-fold increase of PPIase activity. The purified parvulin of the overexpression strain was composed of the house-keeping level of the enzyme and the major amount resulting from the plasmid information. The purified protein with a specific activity similar to the wild type enzyme showed the same N-terminal sequence as authentic parvulin with no indication of a contaminating polypeptide and had the same molecular mass of 10,100.66 $\pm$ 1.64 Da as determined by electrospray mass spectrometry. Furthermore, substrate specificity and avidity to react with polyclonal antibodies raised against the wild type parvulin were indistinguishable from that of the authentic protein. These results unequivocally show that recombinant parvulin is the cyclosporin A and FK506-insensitive PPIase previously isolated from wild type *E. coli*. The data also show that parvulin did not represent a proteolytic fragment of a larger polypeptide chain and exerts its catalytic activity without the help of other factors. With respect to molecular size, a polypeptide chain of 92 amino acids belongs to the extremes among enzymes. Thus, monomers of a tetrameric dihydrofolate reductase have 78 amino acids [13]. HIV-1 protease with 99 residues performs catalysis as a dimer [14] and very small subunits of the 4-oxalocrotonate tautomerase comprising only 62 amino acid residues assemble to a pentameric enzyme [15]. From the pattern of the protein peak in gel filtration studies there was no indication for the presence of oligomeric parvulin molecules.

The amino acid sequence of parvulin is very hydrophilic as

charged residues make up 28% of the protein. The pI value, as estimated by isoelectric focusing on a pH 5–10 IEF gel, was >9.5, approaching the calculated pI of 9.65.

Application of the Chou-Fasman-Rose algorithm [17,18] to the sequence of parvulin predicted the presence of an extended α -helical region of about 30 residues at the N-terminus which turned out to have amphiphilic features in the helical wheel projection [19], and only few β -sheet. This was confirmed by analyzing the far UV-CD spectrum of parvulin with the protein secondary structure estimation program SSE-338 (Fig. 5). Thus, parvulin was calculated to have 75.8% α -helix and 10.5% β -sheet and 13.8% β -turn. This is in contrast to human cytosolic cyclophilin [20] and human FKBP12 [21,22] the 3D-structure of which show much less α -helix and more β -sheet.

As already expected from the 21 amino acid fragment [4], the entire sequence of parvulin did not share convincing similarity with either FKBP12cy or cyclophilin 18cy when aligned utilizing the BESTFIT program (Fig. 6B). Identities were calculated as 12.7% and 23.1%, respectively, with only minor contributions of strongly conserved amino acids and introduction of several gaps. These data underlined the existence of a PPIase unrelated in its primary structure to the members of known families of this enzyme class.

Comparison of the sequence of parvulin to sequences con-

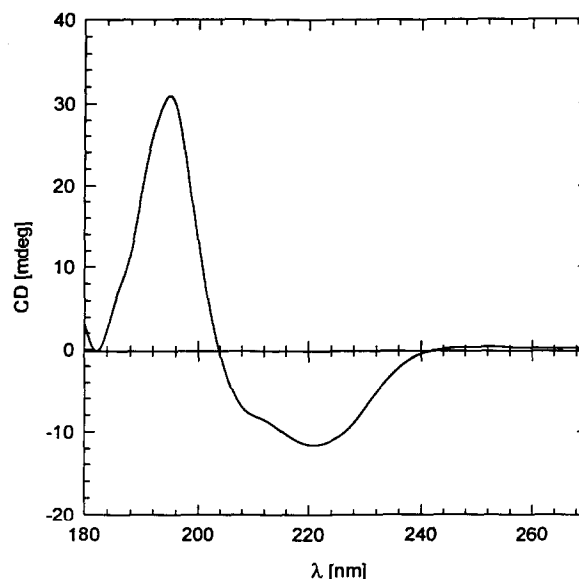


Fig. 5. Far UV-CD spectrum of parvulin. The spectrum was monitored in 10 mM sodium phosphate, pH 6.5 at 20°C. The protein concentration was 0.18 mg/ml.

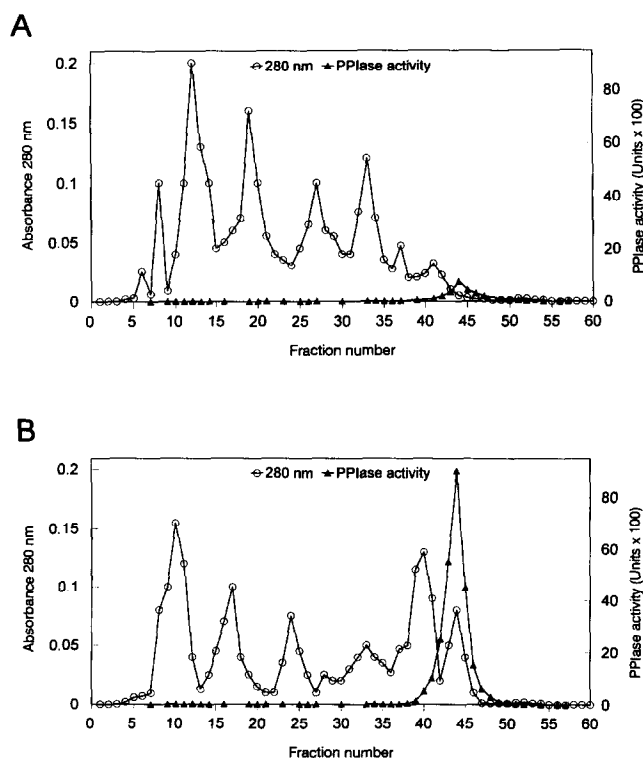


Fig. 4. Comparison of gel filtration profiles of the purification of authentic and recombinant parvulin. Samples of 1 ml protein solution derived from *E. coli* K-12 strain HB101 (A) and from *E. coli* K-12 strain DH5 α with pSEP38 (B) after performing anion exchange chromatography (Fractogel EMD DEAE-650(M)), affinity chromatography (Fractogel TSK AF-Blue) and concentration with a Filttron OMEGACELL, 500 Da were applied to a HiLoad 16/60 Superdex 75 gel filtration column. The gel filtration was performed in 10 mM HEPES buffer, pH 7.8, containing 150 mM KCl and 0.5 mM DTT at a flowrate of 1 ml/min.

tained in the combined protein database (Pir, Mipsown, Patchx, Swissprot) by FASTA revealed interesting similarities. Three microbial proteins were found to have a highly significant level of identity: PrsA from *Bacillus subtilis* (43.8%) [23], SurA from *E. coli* (32.0%) [24], and PrtM from *Lactococcus lactis* (26.8%) [25]. Based on the CLUSTAL multiple alignment shown in Fig. 6A the percentage of fully conserved residues of parvulin (15.2%) allowed to define a family of related proteins which may probably exhibit the enzymatic function of PPIases.

Because the homologous proteins are much larger than parvulin (PrsA, 33 kDa; PrtM, 33.1 kDa and SurA, 47.4 kDa), they may contain domains not involved in the suggested PPIase activity. The parvulin sequence was found to contain the motif DFG (pos. 29–31), the triad required in protein kinases for catalysis of phosphate transfer [16]. We do not know, however, whether this has any biological relevance, because this motif is not conserved in all of the homologous proteins (Fig. 6A).

Interestingly, two out of three homologous proteins mentioned above appear to be involved in maturation of proteins. In *prsA* mutants of *Bacillus subtilis* the level of certain secreted exoproteins is decreased by blocking the protecting effect of PrsA on events which lead to misfolded proteins [23]. Keeping in mind that PPIases are devoid of true chaperone-like effects [26], PPIase activity may also simulate the postulated chaperone capability [27,28] of PrsA by kinetic control of folding intermediates.

A more specific action has been established for the PrtM protein of *Lactococcus lactis* SK11, because a serine proteinase of the cell wall was produced as inactive precursor in the absence of the protein [29].

It remains to be seen whether these proteins have indeed PPIase activity and thus belong to the same newly identified PPIase family as parvulin.

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Fig. 6. (A) Sequence alignment of parvulin from *E. coli*, PrsA from *B. subtilis* (Accession Number Pir3:S15269), SurA from *E. coli* (Accession Number Pir3:S40574), and PrtM from *L. lactis* (Accession Number Pir2:A32313), as performed by the multiple alignment program CLUSTAL. Identities are printed in bold letters and are indicated by an asterisk in the last line. Similarities are presented as a period. (B) The alignments of the amino acid sequences of *E. coli* parvulin with stretches from human FKBPcy (Accession Number Pir3:S11089), and human cyclophilin A (Accession Number Pirl:CSHUA), as calculated using the program BESTFIT are presented. Identities are shown as |, weak and strong similarities as . and :, respectively. Residues being strongly conserved in FKBP and cyclophilins are printed in bold letters.

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