Characterization of the Tubulin-Tyrosine Ligase

Klaus Ersfeld, Jürgen Wehland, Uwe Plessmann, Huub Dodemont, Volker Gerke, and Klaus Weber

Max-Planck-Institute for Biophysical Chemistry, Department of Biochemistry, D-3400 Goettingen, Germany

Abstract. The sequence of tubulin-tyrosine ligase (TTL), the enzyme catalyzing the ATP-dependent posttranslational addition of a tyrosine to the carboxy-terminal end of detyrosinated α-tubulin, has been determined. TTL from bovine and porcine brain was purified by immunoaffinity chromatography and extensively characterized by protein sequencing. Oligonucleotides derived from the protein sequence were synthesized and partial cDNA sequences were obtained using reversed transcribed brain mRNA in polymerase chain reactions. Polymerase chain reaction fragments were used to isolate a full-length cDNA clone from a randomly primed λgt10 cDNA library obtained from embryonic porcine brain mRNA. Porcine TTL is encoded by 1,137 nucleotides corresponding to 379 amino acid residues. It has a molecular weight of 43,425 and a calculated isoelectric point of 6.51. Northern blot analysis revealed a surprisingly long mRNA (~ 6 kb in embryonic porcine brain). The protein sequence of TTL shares no extended homology with the sequences in the data banks. TTL contains a potential serine phosphorylation site for cAMP-dependent protein kinase (RKAS at positions 73 to 76). Residues 244 to 258 lie at the surface of the molecule. A rabbit antibody raised against a synthetic peptide corresponding to this sequence binds to native TTL. The same sequence contains the cleavage site for endoproteinase Glu-C (residue 248) previously shown to convert TTL into a nicked derivative in which the two fragments still form a tight complex but don't display enzymatic activity.

TUBULINS and microtubules are subject to several posttranslational modifications such as acetylation (L'Hernault and Rosenbaum, 1985), polyglutamylation (Edde et al., 1990), and phosphorylation (Eipper, 1974). The reversible detyrosination/tyrosination (Barra et al., 1973) of the carboxy-terminal end of most α-tubulins is an additional posttranslational modification of tubulin (for a recent review see Greer and Rosenbaum, 1989). Even though the carboxy-terminal tyrosine is encoded by the α-tubulin mRNA, it can be removed by a specific carboxypeptidase (Hallak et al., 1977; Argarana et al., 1978, 1980) and subsequently added again due to the activity of the tubulin-tyrosine ligase (Barra et al., 1973; Arce et al., 1975). This highly specific modification system has been detected in a wide range of eukaryotic organisms. These include Trypanosomes (Sherwin et al., 1987; Russell and Gull, 1984), *Caenorhabditis elegans* (Gabius et al., 1983), *Drosophila* (Warn et al., 1990), and various vertebrates (for a recent review see Greer and Rosenbaum, 1989). It seems, however, absent from the fission yeast *Schizosaccharomyces pombe* (Alfa and Hyams, 1991).

Poly- and monoclonal antibodies specific for either detyrosinated or tyrosinated α-tubulin allow a discrimination between the two α-tubulin forms and have been used to describe their relative distribution in various cells and tissues. Detyrosinated tubulin seems restricted to more stable microtubule subpopulations such as those present in axons, dendrites, and centriolar structures, while tyrosinated tubulin predominates in more dynamic microtubule subpopulations (Gundersen and Bulinski, 1986; Kreis, 1987; Wehland and Weber, 1987*b*; Schulze et al., 1987; Prescott et al., 1989). In spite of the correlation between microtubule stability and elevated levels of detyrosinated tubulin, detyrosination itself does not seem to mediate this stability. Instead, stable microtubules seem to be the preferred substrate for tubulin-tyrosine carboxypeptidase (Khawaja et al., 1988; Webster et al., 1990), while tubulin-tyrosine ligase (TTL)[1] acts on the αβ-tubulin dimer itself.

Due to the difficulty in purifying the enzyme, the tubulin-tyrosine carboxypeptidase is still poorly characterized. Many of the in vitro properties of TTL are well described since the enzyme can be purified to homogeneity by biochemical methods (Murofushi, 1980) or more conveniently by immunoaffinity purification using immobilized mAbs (Schröder et al., 1985).

TTL requires K^+, Mg^{2+}, ATP, and αβ-tubulin containing detyrosinated α-tubulin. TTL from pig brain is a monomeric protein with a molecular weight close to 43,000. It forms a tight complex with αβ-tubulin, which can be monitored by

Dr. Ersfeld's present address is University of Salford, Department of Biological Sciences, Manchester M5 4WT, England.

Dr. Wehland's present address is Gesellschaft für Biotechnologische Forschung, Mascheroder Weg 1, D-3300 Braunschweig, Germany.

1. *Abbreviations used in this paper:* CDTA,1, 2-cyclohexylenedinitrotetraacetic acid; PCR, polymerase chain reaction; TTL, tubulin-tyrosine ligase.

glycerol gradient centrifugation. In addition to the carboxyl end of α-tubulin, where the enzyme acts, a second TTL-binding site has been detected by chemical cross-linking on β-tubulin. TTL exposed to endoproteinase Glu-C (V8-protease) is converted to a nicked derivative. Although devoid of enzymatic activity it still forms the complex with αβ-tubulin. The nicked TTL contains two fragments with apparent molecular weights of 30,000 and 14,000, respectively, which interact tightly under physiological conditions. The 30,000 molecular weight fragment carries the binding sites for ATP and β-tubulin. The 14,000 molecular weight fragment can possibly cover part of the catalytic site, since it harbors the epitope for the mAb ID3. This antibody inhibits the enzymatic activity of TTL but not the formation of the ligase–tubulin complex (Wehland and Weber, 1987*a*). Although the true physiological function of TTL has so far not been established (Webster et al., 1990), TTL as an α-tubulin specific protein ligase poses several interesting enzymological questions. To approach these problems, we have characterized porcine brain TTL by protein sequencing and cDNA cloning.

Materials and Methods

Materials

Enzymes for molecular biology were from Boehringer Mannheim GmbH (Mannheim, Germany) and New England Biolabs (Schwalbach, Germany). Taq-polymerase was obtained from Perkin Elmer/Cetus (Überlingen, Germany). Radiochemicals were from Amersham Corp. (Braunschweig, Germany). Other chemicals were purchased from Merck (Darmstadt, Germany) and Sigma (Deisenhofen, Germany), respectively. Phages, plasmids, and *E. coli* strains were from Stratagene (Heidelberg, Germany). Tris-HCl buffers were titrated at 22°C.

Purification of the TTL

TTL was purified from porcine and bovine brain by immunoaffinity chromatography using the TTL mAb LA-C4 coupled to CNBr-Sepharose 4B (Pharmacia, Freiburg, Germany) (Wehland et al., 1986). The following modifications of the procedure were made: during the incubation of the antibody matrix with the brain extracts the buffer was made 0.1% (vol/vol) in Triton X-100. The column filled with the matrix was washed first with 10 vol of buffer containing 0.1% Triton X-100 and then with 5 vol of buffer without detergent. The enzyme was eluted with 3 M $MgCl_2$ in extraction buffer without ATP and dialyzed for 18 h at 4°C against 100 vol of extraction buffer 15% (vol/vol) in glycerol. The addition of Triton X-100 significantly reduced unspecific protein binding to the antibody matrix.

Proteinchemical Procedures

Alkylation of TTL with 4-vinylpyridine (Friedman et al., 1970) was in 6 M guanidine-HCl originally 10 mM in 2-mercaptoethanol. After extensive dialysis against water the protein was lyophilized and subjected to chemical cleavage by CNBr using standard conditions. Fragments were separated by HPLC on a Vydac C_{18} column using a gradient from 7 to 72% acetonitrile in 0.1% trifluoroacetic acid. Fragments were characterized by automated sequencing using a gas phase sequencer (model A470; Applied Biosystems, Foster City, CA) and a sequenator (model 810; Knauer, Berlin, Germany). Both instruments were equipped with an on-line PTH-amino acid analyzer. Some of the CNBr fragments were subjected to one or more proteolytic digests (see Results). Endoproteinases Asp-N, Glu-C, and Lys-C were from Boehringer Mannheim GmbH, while chymotrypsin was from Sigma. Proteolytic fragments were purified by HPLC (see above) and subjected to extensive sequence analysis. For convenience CNBr fragments are numbered according to their position from the amino terminal end.

Native bovine and porcine TTL were treated with endoproteinase Glu-C (Boehringer Mannheim GmbH) to obtain the nicked protein (Wehland and Weber, 1987*a*). The resulting two fragments of apparent molecular weights of 30,000 and 14,000, respectively, were separated by gel filtration on a Sephadex S-200 column equilibriated in 9 M urea, 100 mM NaCl, 2 mM NaN_3, 1 mM EGTA, 5 mM dithiothreitol, 20 mM Tris-HCl, pH 7.5.

Oligonucleotides used in this study (5'→3'):

TTL1: GA(T/C) AA(T/C) CA(A/G) GG(T/G/C/A) CA(A/G) GT (T/G/C/A) CA(T/C) GT(T/G/C/A) AT

TTL2: TG (A/G)CA (A/G/C/A)GT (T/C)TT (A/G)TC (T/C)TG (A/G)AA (A/G)TT (A/G)TC

TTL3: GAG CCA GGC CAT CGC AAG TTT GAC AT

TTL4: AT (T/G/C/A)GC (A/G/T)AT (A/G)TC (T/G/C/A)AC (A/G/T) AT (T/G/C/A)CC (C/T)TG (A/G)CA

TTL5: GTA GAT GTT ATA CTG ATG ATC CAC CA

TTL6: GT(T/G/C/A) AA(T/C) TA(T/C) TA(T/C) (C/A)G(T/G/C/A) GG (T/G/C/A) GC(T/G/C/A) GA(T/C) AA

TTL7: CTC CGG GAA CCA CGT GCA GGA CTC AGC

TTL8: ATG TA(T/C) ACI TT(T/C) GTI GTI (C/A)GI GA(T/C) GA (A/G) AA

A, adenine; *C*, cytosine; *G*, guanine; *T*, thymine; *I*, inosine. The positions of the oligonucleotides are given in the TTL cDNA sequence (see below).

Purification of Poly A⁺ mRNA

Poly A^+ mRNA for the construction of a cDNA library was isolated from prenatal day 85 embryonic porcine brain using the Fast-Track mRNA isolation kit (Invitrogen, San Diego, CA). Polysomal poly A^+ mRNA from prenatal day 85 embryonic porcine brain used for reverse transcribed PCR and Northern blotting was prepared according to Dodemont et al. (1990). Briefly, 1 g of frozen brain was homogenized at 0°C in 20 ml of 50 mM Tris-HCl, pH 7.5, 25 mM NaCl, 5 mM $MgCl_2$, 2% (vol/vol) Triton X-100 with a Polytron homogenizer. After removing nuclei and cellular debris by a short spin at 25,000 *g*, the supernatant was adjusted to 1 mg/ml heparin and 100 mM $MgCl_2$ and incubated on ice for 1 h. The resulting precipitate was pelleted by centrifugation at 40,000 *g* for 30 min. The pellet was dissolved in 10 ml of 50 mM Tris-HCl, pH 7.3, 50 mM CDTA (1,2-cyclohexylenedinitrotetraacetic acid-monohydrate; Sigma), 0.5% Na-sarcosyl, and 100 μg/ml proteinase K. After a 1-h incubation at 37°C the solution was adjusted to 0.5 M NaCl, 50 mg of preequilibrated oligo(dT)-cellulose (Pharmacia) were added and the incubation was continued for another 1 h. Subsequently, the cellulose was filled into Ultrafree-MC filtration units (Millipore Continental Water Systems, Bedford, MA) and washed twice with 20 mM Tris-HCl, pH 7.6, 0.5 M NaCl, 1 mM EDTA, and 0.05% SDS by short spins in a table top centrifuge. Poly A^+ mRNA was eluted with 10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.05% SDS, and precipitated with ethanol. After centrifugation, the RNA-pellet was dissolved in 20 μl TE (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA).

Polymerase Chain Reaction

cDNA-mRNA hybrids used as substrate for polymerase chain reactions (PCR) were prepared as follows: For random primed cDNA, 1 μg embryonic pig brain polyA^+ mRNA was reverse transcribed by the Superscript reverse transcriptase (BRL-Gibco, Gaithersburg, MD) under the conditions recommended by the manufacturer. 100 ng of random hexanucleotides (Pharmacia) were used as primers. Oligo(dT)-primed cDNA was prepared using the λZAP cDNA synthesis kit (Stratagene).

For synthesis of specifically primed cDNA, 1 μg mRNA and 8 fmol of oligonucleotide TTL5 were incubated at 80°C for 4 min in 0.3 M NaCl, 10 mM Tris-HCl, pH 7.5, 2 mM EDTA in a total volume of 10 μl and then cooled to 42°C over a period of 2 h. After addition of 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, and 400 U of Superscript-reverse transcriptase the reaction was incubated for 30 min at 42°C in a final volume of 50 μl. 10% of the different cDNA preparations were used directly in the individual PCR.

Conditions for PCR with the different oligonucleotide pairs were as follows:

TTL1/TTL2. Oligo(dT) cDNA as substrate, 30 cycles: 1 min 95°C (template denaturation), 1 min 45°C (primer annealing), 1.5 min 70°C (primer extension).

TTL3/TTL4. Oligo(dT) cDNA as substrate, first cycle: 1.5 min at 94°C, 1.5 min at 35°C, 2 min at 72°C. Second cycle: same as first but annealing at 45°C; cycles 3–37: same as first but annealing at 50°C.

TTL5/TTL6. TTL5 primed cDNA as substrate, 30 cycles: 1 min at 95°C, 2 min at 37°C, 2 min at 72°C.

TTL7/TTL8. Random-primed cDNA as substrate, 35 cycles: 1 min at 95°C, 1 min at 55°C, 4 min at 72°C.

The reactions were carried out in 10 mM Tris-HCl, ph 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (wt/vol) gelatin, and 200 μM of each dNTP containing 50 pmole of each oligonucleotide and 2.5 U Taq-polymerase in a total volume of 100 μl (Saiki et al., 1988). The mixture was overlaid with 50 μl mineral oil and the reactions were performed in a Thermal Cycler (Perkin-Elmer/Cetus, Berkeley, CA). Before the addition of enzyme, the mixture was heated to 95°C for 10 min. The final polymerization step was extended to 15 min to ensure a complete extension of the PCR products. 10 μl of each reaction were analyzed by agarose gel electrophoresis (Sambrook et al., 1989). DNA fragments of expected length were isolated from preparative gels by squeeze freezing (Tautz and Rentz, 1983). In some cases the identity of the DNA fragments was confirmed by Southern blotting using internal oligonucleotides as probes which were 5′ end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP.

Isolated fragments were treated with Klenow DNA-polymerase to create blunt ends, phosphorylated with T4 polynucleotide kinase and ligated into the EcoRV cloning site of the pBluescript KS+ phagemid vector (Stratagene). Transformation of XL 1 Blue cells with plasmid DNA was done using the $CaCl_2$ procedure (Ausubel et al., 1989). Plasmid DNA preparation and doublestranded sequencing was done as described below. The selection of recombinant clones was carried out by colony hybridization using end-labeled oligonucleotides as probes. Hybridization was in 5× SSC, 0.05% BLOTTO (1× BLOTTO is 5% dry milk powder in H_2O, 0.02% NaN_3), 5 × 10^5 cpm/ml for 6 h at 50°C. The nitrocellulose filters were washed twice with 5× SSC at 50°C for 10 min and autoradiographed for 4-5 h.

Northern Blot Analysis

2 and 5 μg of glyoxylated embryonic porcine brain poly A^+ mRNA were separated in a 1% agarose gel in 10 mM Na-phosphate buffer, pH 7.0. RNA was transferred to a nitrocellulose filter (Schleicher & Schüll, Dassel, Germany) by capillary transfer in 20× SSC (Sambrook et al., 1989). After transfer the filter was baked at 80°C for 3 h in a vacuum oven and then subjected to prehybridization without further treatment (Thomas, 1980). Hybridization was carried out using the randomly labeled PCR fragment 3/4. Hybridization conditions were identical to those described for the cDNA library screening procedure.

Construction and Screening of a cDNA Library

2 μg poly A^+ mRNA were reversed transcribed using the You-Prime cDNA synthesis kit (Pharmacia). 200 ng of random hexanucleotides were added as primers for the first strand synthesis. The double stranded cDNA equipped with EcoRI/NotI adaptors was ligated to the EcoRI cloning site of λgt10 (Stratagene) and packaged into phage particles using the Gigapack II Gold packaging extract (Stratagene). 1.2 × 10^6 pfu of the unamplified library were plated on *E. coli* strain C−600 hfl^-. The phage DNA was transferred by plaque lifting onto Nytran filters (Schleicher & Schüll), denatured as described (Sambrook et al., 1989) and fixed by baking for 2 h at 80°C in a vacuum oven. The filters were screened sequentially with the PCR fragments 5/6 and 7/8, labeled by random priming to a specific activity of 6 × 10^8 cpm/μg DNA with [^{32}P]-dCTP and the Oligolabeling kit (Pharmacia). Hybridization was in 5× SSC, 5× Denhardt's solution (Denhardt, 1966), 50 mM Tris-HCl, pH 7.4, 50% formamide, 1% SDS, 100 μg/ml denatured herring sperm DNA, and 5 × 10^5 cpm/ml heat-denatured, labeled DNA probe for 18 h at 42°C. Filters were washed once in 2× SSC, 1% SDS at RT and twice in 0.1× SSC, 1% SDS at 65°C for 20 min each and then exposed on a Kodak XAR film (Eastman Kodak Co., Rochester, NY) for 20 h at −70°C using intensifying screens. Positive clones were recovered, diluted in SM (100 nM NaCl, 10 nM $MgSO_4$, 50 mM Tris-HCl pH 7.5, 0.01% gelatin), and plaque purified in a second screening round performed under the same conditions. Phage DNA was purified from plate lysates by chromatography on a DEAE column (DE-52; Whatman Laboratories Products, Inc., Clifton, NJ) and phenol extraction (Meese et al., 1990). DNA inserts were excised by digestion of the phage DNA with EcoRI and cloned into the EcoRI site of pBluescript KS+ (Stratagene). Plasmid DNA was purified on Qiagen columns (Diagen, Düsseldorf, Germany) and sequenced using the T7 sequencing kit (Pharmacia) and the T3 or T7 universal primer. In addition, internal oligonucleotides served as specific sequencing primers.

Peptide–Protein Conjugates

Peptides were coupled via the sulfhydryl group of the NH_2-terminal cysteine to free amino groups of ovalbumin by the bifunctional crosslinker sulfo-MBS (m-maleimidobenzoyle-*N*-hydroxysulfosuccinimide-ester; Pierce Chemicals) following the instructions of the manufacturer.

The following peptides were used in this study:

Peptide 2: CDEREFFLTSYNKKKEDG
(TTL amino acid residues 126-143)

Peptide 1: CIQKEYSKNYGKYEE
(TTL amino acid residues 244-258)

The underlined C in peptide 2 indicates an additional cysteine added to the NH_2-terminus to allow cross-linking to the carrier protein (Kitagawa and Aikawa, 1976).

Immunological Procedures

New Zealand white rabbits were injected with 1 mg of the peptide–protein conjugate three times at 3-wk intervals with Freund's complete adjuvans in the first and incomplete adjuvans in the subsequent injections. Sera were tested by immunoblotting using purified TTL as antigen. Transfer of proteins from SDS–polyacrylamide gels to nitrocellulose (Schleicher & Schüll) was performed according to Towbin et al. (1979). Antigens were detected by indirect immuno-labeling using the diluted peptide antisera (1:50 in PBS) and the corresponding presera as controls and a peroxidase-labeled second antibody (Dako, Hamburg, Germany).

Rabbit sera diluted one to ten with phosphate buffered saline (PBS) were incubated with equal volumes of a 50% suspension of protein A-Sepharose (Pharmacia) for 12 h at 4°C. The Sepharose was washed three times with PBS and suspended in half of the original volume. 10 μl of the suspension was added to 10 μl of TTL (different dilutions in dialysis buffer, see above) and incubated for 1 h at 4°C. After a short centrifugation 10 μl of the supernatant were assayed for TTL activity. The enzyme assay was performed as described (Schröder et al., 1985).

Results

Amino Acid Sequence of Tyrosine–Tubulin Ligase

Automated sequencing of the protein isolated from adult porcine brain established the first 30 residues. After alkylation of its cysteine residues with 4-vinyl-pyridine the protein was treated with CNBr. The five fragments isolated by HPLC were characterized by direct automated sequencing and by the sequences of their proteolytic fragments obtained with several endoproteinases. Proteolytic fragments purified by HPLC were again subjected to automated sequencing. CNBr fragment 1 (residues 2 to 40) was directly sequenced for positions 2 to 30 in the final sequence of Fig. 1. Endoproteinase Glu-C yielded residues 17 to 40. The short CNBr fragment 2 was directly sequenced (residues 41 to 59). The sequence of the large fragment 3 (residues 60 to 262) was obtained from an NH_2-terminal sequence (residues 60 to 91) and three sets of overlapping peptides obtained from enzymatic digests. Endoproteinase Lys-C provided residues 83 to 106, 107 to 137, 138 to 150, 157 to 184, 185 to 198, 199 to 236, 237 to 247, and 252 to 262. Endoproteinase Glu-C yielded residues 97 to 113, 130 to 158, 171 to 203, 219 to 248, and 249 to 262. Endoproteinase Asp-N provided in addition residues 171 to 200 and 201 to 229. Thus, the entire stretch of residues 60 to 262 was established. Because of technical difficulties, CNBr fragment 4 (residues 263 to 320 in Fig. 1) was only characterized by its first 33 residues (positions 263 to 295) which were obtained by direct sequencing. The sequence of CNBr 5 (residues 321 to 379) was obtained from an NH_2-terminal sequence (residues 321 to 364) and a fragment obtained by endoproteinase Asp-N (residues 352 to 379). This sequence of CNBr 5 was confirmed by chymotryptic fragments covering residues 321 to 328, 329 to 340, 358 to 376, and 376 to 379 (Fig. 1).

The linear arrangement of the five CNBr fragments was

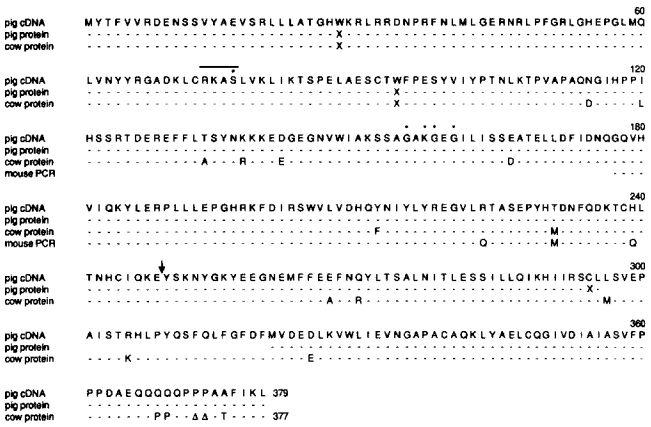

Figure 1. Amino acid sequences of TTL from porcine and bovine brain. The topline gives the sequence later deduced from porcine cDNA clones (see Results and Figs. 2 and 3). The second line shows the proteinchemically determined sequences from porcine TLL. Residues identical with the sequence deduced from the cDNA are marked by dashes. Note a gap for residues 296 to 319 in the protein sequence and three non-identified residues (positions 27, 93 and 294) marked by X (second line). The third line shows the sequence of bovine TTL established by protein chemistry. Only residues differing from the porcine sequence are given; identical residues are marked by dashes. Note that the two proteins differ only by 16-amino acid replacements and a two-residue deletion (marked by triangles) close to the carboxyterminal end of the bovine enzyme. The two non-identified residues (positions 27 and 93) marked by X are occupied by tryptophan in the sequence deduced from the porcine cDNA clones. A short sequence of 63 residues for murine TTL is included. This sequence (fourth line) was deduced from a murine PCR probe (see Results). The sequence RKAS (residues 73 to 76) possibly serving as a phosphorylation site for protein kinase A at serine 76 (marked by a star) is indicated above the top line. A cluster of three closely spaced glycine residues (positions 154, 157, and 159) containing a lysine (position 156) is marked by dots above the glycine residues (see Discussion). Native TTL treated with endoproteinase Glu-C is nicked at glutamic acid 248 (arrow; see Results). The two fragments of apparent molecular weights 30,000 and 14,000, respectively, remain under native conditions as an enzymatically inactive complex (Wehland and Weber, 1987*a*). The cleavage site is contained in peptide 1 (residues 244 to 258) which elicits a rabbit antibody able to bind native TTL (see Results and Fig. 6).

obtained by the following results. Extensive automated sequencing of the intact protein covered residues 1 to 50. This result showed that the NH_2-terminal methionine residue of TTL is followed by fragment 1 (residues 2 to 40) and fragment 2 (residues 41 to 59). A peptide obtained in low yield from the digest of CNBr fragment 3 with endoproteinase Lys-C (residues 41 to 63) overlapped the short CNBr fragment 2 with the NH_2-terminal sequence of CNBr fragment 3 (residues 60 to 262). Thus, the order of the first three fragments was clearly established. For the following assignments we made use of the mild treatment of native ligase with endoproteinase Glu-C previously found to cleave the protein into two fragments with apparent molecular weights of 30,000 and 14,000, respectively (Wehland and Weber, 1987*a*). These were separated by gel filtration on Sephadex G200 using a buffer containing 9M urea and 5 mM DTT. Direct sequencing showed that the fragment of molecular weight 30,000 starts at residue 1 and covers the NH_2-

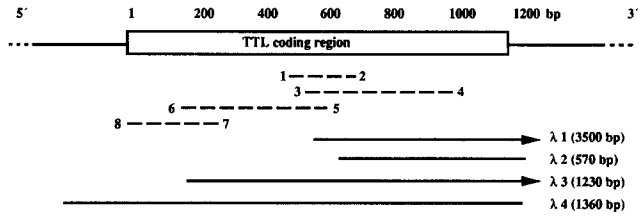

Figure 2. Overview of the position of PCR-fragments and λgt10-clones of the randomly primed embryonic porcine brain cDNA library containing TTL cDNA. The position of the PCR fragments is indicated by interrupted lines. The oligonucleotide primers used for the PCR fragments are designated by numbers according to Materials and Methods. The position and approximate length of the λ-clones is shown by the thick lines. Arrowheads indicate that clones λ1 and λ3 extend further into the 3'-noncoding region.

terminal portion of the TTL molecule. The smaller fragment of molecular weight 14,000 starts with tyrosine 249 located 13 residues before the carboxy-terminal end of CNBr fragment 3. Since CNBr fragment 5 carries a carboxy-terminal leucine rather than a homoserine residue, this fragment was assigned to the carboxy-terminal end of the TTL polypeptide chain. The remaining CNBr fragment 4 lies therefore between fragments 3 and 5. The combined results provide a sequence proposal (Fig. 1) which lacks residues 296 to 320. These are located in CNBr fragment 4.

Using a similar approach on bovine TTL from adult brain a complete sequence was established (Fig. 1). Bovine and porcine TTL are highly homologous proteins. Among the few conservative amino acid exchanges are two additional methionine residues at positions 230 and 296 of the bovine protein which helped to consolidate the sequence. Thus the region which remained undetermined in the porcine sequence (residues 296 to 320) could be directly sequenced as a CNBr fragment of the bovine protein. The sequence of the bovine TTL (Fig. 1) contains two non-identified residues at positions 27 and 93, which are indicated by X. The same two positions also remained unidentified in the porcine protein. The porcine DNA sequences later obtained by PCR and cDNA cloning (see below and Figs. 2 and 3) show that positions 27 and 93 are occupied by tryptophan, an amino acid easily destroyed during the CNBr treatment used in our protein sequence approach.

Cloning of TTL

After the porcine TTL had been sequenced on the protein level, two degenerate oligonucleotides (TTL1 and TTL2; see Fig. 2 for positions of the oligonucleotides) were synthesized and used as primers in the polymerase chain reaction. Due to the primer degeneracy and the low stringency amplification conditions chosen, several PCR products were found when the reaction was analyzed by agarose gel electrophoresis and ethidium bromide staining. Therefore the PCR products ranging from 150 to 300 bp and thus covering the size of the expected TTL fragment (210bp) were size-fractioned by preparative PAGE. Each fraction was re-electrophoresed, blotted onto a nylon membrane, and probed with an end-labeled, internal oligonucleotide. The fraction with the strongest hybridization signal was cloned into pBluescript and analyzed by dideoxy sequencing. One of the sequenced inserts corresponded to the TTL protein sequence (Fig. 2).

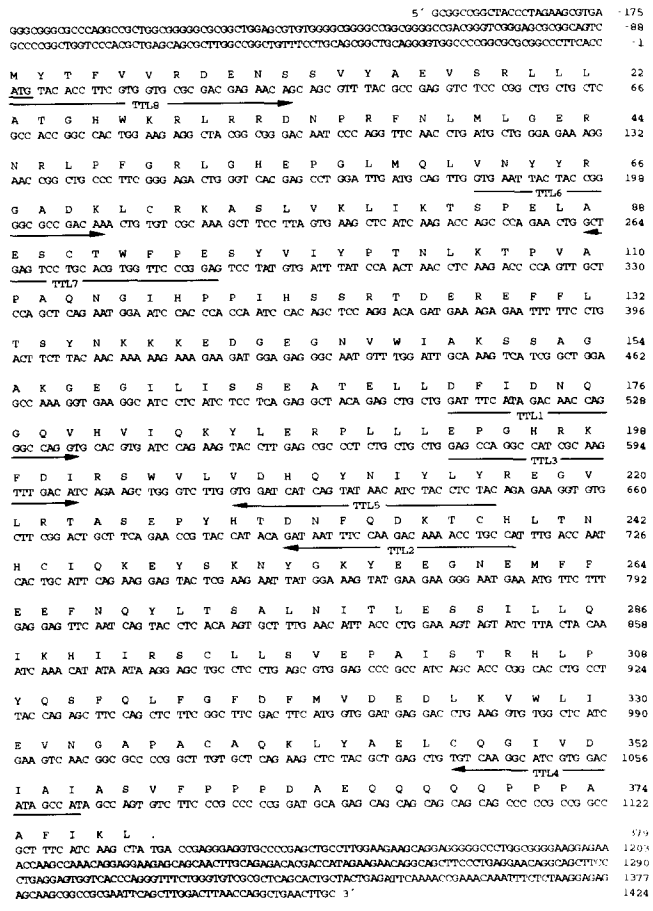

Figure 3. The cDNA sequence of pig TTL. The start codon ATG is underlined. The arrows indicate the position and orientation of the oligonucleotide primers used for PCR. The numbers refer to the designation of the oligonucleotides in Materials and Methods. The single letter code was used for the translation of the DNA sequence. These sequence data are available from EMBL/GenBank/DDBJ under accession number X68453.

Based on this first nucleotide sequence, homologous oligonucleotide primers (TTL3 and TTL5) were prepared and used in an additional round of PCR. In these reactions, the second primer was a degenerate oligonucleotide derived again from the known protein sequence (TTL4, TTL6) (see Figs. 2 and 3 for location of the primers). Because the two expected PCR fragments partially overlap each other, the homologous primers could also be used as internal hybridization probes (Fig. 2). An oligo(dT)-primed first strand cDNA was used as substrate for the polymerase chain reactions with the primer pair TTL3/TTL4 while the reaction with the primer pair TTL5/TTL6 was carried out with a cDNA obtained by specific priming with the oligonucleotide TTL5. DNA amplification with primers TTL5/TTL6 was performed under low-stringency conditions (annealing at 37°C) without giving rise to unspecific PCR products. However, in the reaction with primers TTL3/TTL4 it was essential to raise the annealing temperature stepwise from 35°C in the first to 45°C in the second and 50°C in the third cycle to suppress the amplification of unspecific DNA fragments. Under these conditions, the PCR with both primer pairs resulted in a single product which was again cloned into pBluescript and characterized by sequence analysis. The sequence obtained indeed encodes porcine TTL and closes the gap at amino acid position 296 which remained after direct protein sequencing (Fig. 1).

To obtain the cDNA sequence still missing at the 5′ end, a fourth pair of oligonucleotide primers was synthesized: one homologous primer (TTL7) whose sequence corresponded to a region in PCR fragment 5/6 and one degenerate primer (TTL8) whose sequence was based on the NH_2-terminal protein sequence. In TTL8 the third position of codons with fourfold degeneracy was replaced by deoxyinositol (Patil and Dekker, 1990). The cDNA substrate used in this PCR was obtained by priming with random hexamers. Agarose gel analysis of the amplification reaction revealed a number of different DNA fragments, one of which hybridized with the internal oligonucleotide TTL6. The sequence of this PCR fragment represented the portion of the TTL cDNA coding for the NH_2-terminal 117 amino acid residues of the enzyme (Fig. 3).

Northern blot analysis of poly A^+ mRNA of embryonic pig brain revealed that TTL is encoded by a transcript of approximately 6 kb (Fig. 4). Because the coding region of the TTL is only 1137 bases in length, a long 3′-untranslated region is likely to account for the large size of the TTL transcript. The TTL mRNA seems to be a rare cellular transcript, because a reasonable signal in the Northern blot analysis of 2 μg poly A^+ mRNA was obtained only after prolonged exposure on X-ray film.

To obtain the missing cDNA sequence information at the 3′ end of the TTL coding region and to confirm the PCR results, we isolated TTL clones from a randomly primed λgt10 cDNA library derived from embryonic porcine brain mRNA. This randomly primed library was used instead of the oligo(dT) primed cDNA since our Northern blot analysis predicted a long 3′-untranslated region. In a first round, ~1.2 $\times$ 10^6 phages of the nonamplified library were screened using the PCR-fragment 3/4 as a hybridization probe. Three positive clones were identified. All contained the 3′ end of the TTL coding sequence (Fig. 2, λ1 to λ3). One of them also comprised 3 kb of the 3′-noncoding region. Because none of these clones comprised the 5′-end of the TTL, filters were again screened with PCR-fragment 7/8 as probe. One additional clone was obtained, which contained the entire protein-coding region plus 175 nucleotides of the 5′-nontranslated sequence (Fig. 3, λ4). Porcine TTL has a molecular weight of 43,425 and a calculated isoelectric point of 6.51.

Peptide Antibodies

To prove that the sequence obtained was indeed that of TTL, we raised polyclonal rabbit antibodies against two hydrophilic peptides present in the sequence. Peptides corresponding to residues 126 to 143 (peptide 2) and 244 to 258 (peptide 1) were synthesized and purified by HPLC. An aliquot was used to document purity by automated sequencing. Peptides coupled to ovalbumin were used as antigens. Both antisera obtained detected TTL in immunoblotting (Fig. 5) at dilutions up to 1:50. In direct immunoprecipitation assays using protein A coupled to Sepharose only antiserum 1 removed the enzymatic activity from TTL containing solutions (Fig. 6). That residues 244 to 258 used to raise antiserum 1 lie at the surface of the TTL molecule is also shown by the mild treatment of native TTL with endoproteinase Glu-C (Wehland and Weber, 1987*a*). The enzyme cleaves the peptide

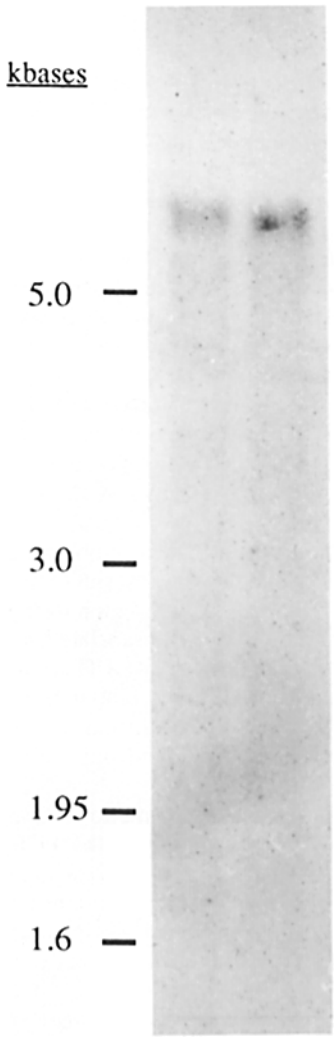

Figure 4. Northern blot analysis of embryonic pig brain mRNA probed with PCR fragment 3/4. The left lane contains 2 μg of poly A^+ mRNA and the right lane contains 5 μg. The blot was exposed for 7 d.

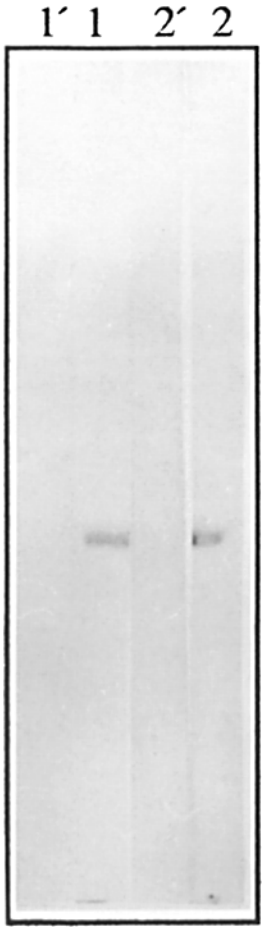

Figure 5. Peptide antibodies in immunoblotting. TTL was subjected to electrophoresis and electrophoretically transferred to nitrocellulose. Peptide antisera 1 and 2 bind to the TTL polypeptide (lanes *1* and *2*) while the corresponding preimmune-sera (lanes *1′* and *2′*) do not bind. Binding of rabbit antibodies was detected by a peroxidase-labeled second antibody and chloronaphthol/H_2O_2 as substrates. All rabbit sera were diluted 1 to 50.

bond between glutamic acid 248 and tyrosine 249 (see above).

Discussion

The original aim of this study was to obtain a complete cDNA clone for mammalian brain TTL and to ascertain its identity by a few sequences directly obtained on the purified enzyme. While the molecular biological approach was frustrated by an unexpected property of the cDNA clone, which was only discovered much later, protein sequencing proceeded very well since immunoaffinity purification allowed a rapid isolation of an enzyme present in relatively low abundance in adult brain. Since first attempts to clone TTL from various expression libraries with the monoclonal and polyclonal antibodies available led to cDNA sequences unrelated to the partial protein sequences established, we aimed at a complete protein sequence of TTL. This was achieved for the bovine brain enzyme, which is highly homologous to its porcine counterpart. In parallel experiments we explored PCR procedures based on degenerate oligonucleotides deduced from the protein sequence. Using oligo (dT)-primed cDNA obtained from embryonic pig brain mRNA, we isolated PCR probes encoding the corresponding protein sequences. Use of these PCR probes in Northern blot analysis suggested that TTL mRNA is with 6 kb unexpectedly large and contains a long 3′-noncoding region. This probably explains our unsuccessful attempts to clone a low-abundance mRNA from expression libraries. Therefore, we prepared randomly primed cDNA and established a λgt10 library for embryonic porcine brain. Screening of the library with the PCR probes identified four positive clones in 1.2×10^6 phages. One of these clones, λ4, contained the entire coding region for TTL. Another clone (λ1) covered only part of the coding region but showed a 3′-untranslated region of 3 kb. Thus the mRNA for TTL from embryonic porcine brain indeed has a very long 3′-untranslated region. During an earlier phase of this work, we also cloned a short PCR fragment of 189 bp of mouse cDNA using the primers based on the porcine protein sequence.

The ligase sequence shows no extended obvious similarity with any protein sequence present in the data banks. It clearly lacks the presence of the typical repeat elements found in MAP 2 and Tau (Lewis et al., 1988; Lee et al., 1989), two proteins known to interact with microtubules (Sloboda et al., 1975; Weingarten et al., 1975). The lack of sequence homology between these two proteins and ligase is probably not surprising as the former bind only to microtubules (Sloboda et al., 1975; Weingarten et al., 1975) while TTL forms a tight complex with unpolymerized $\alpha\beta$ tubulin

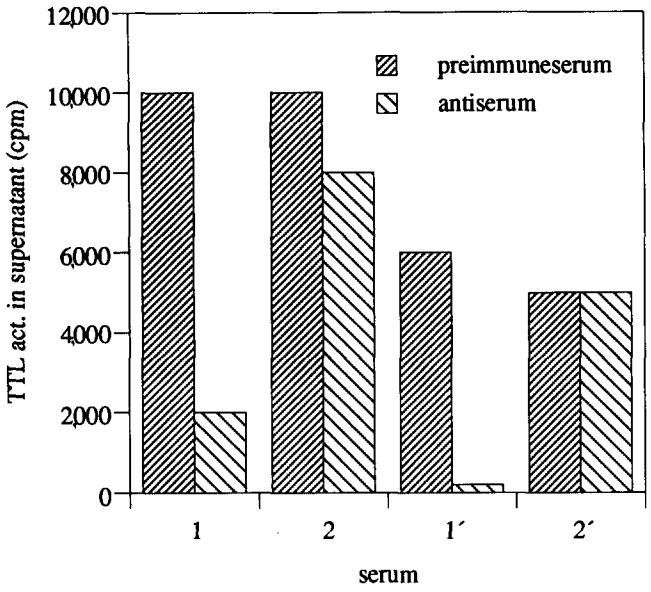

Figure 6. Indirect immunoprecipitation of the enzymatic activity by anti-peptide sera. Rabbit antibodies from peptide-sera 1 and 2 (diluted 1 to 10 in PBS) were bound to protein A-Sepharose. They were incubated with a TTL solution and then removed by centrifugation. TTL activity was measured in the supernatants. Antibodies from pre-immune sera served as controls. 1' and 2' show the corresponding results when a more dilute TTL solution was used. Note that antibodies from anti-peptide serum 1 (residues 244 to 258) remove the TTL activity while corresponding antibodies to peptide 2 (residues 126 to 143) do not.

(Wehland and Weber, 1987*a*). To exclude the remote possibility that the actual ligase activity is only a minor contamination of our protein preparations, we have raised antibodies in rabbits to two hydrophilic peptides synthesized according to the sequence. Although both antibodies detect the ligase protein in Western blots, the enzymatically defined activity of the ligase is removed by centrifugation after the addition of protein A-Sepharose only by the antibodies directed against residues 244 to 258 (serum 1) and not by antibodies against residues 126 to 143 (serum 2). While the latter region may not be accessible in the native protein, the former region is not only available to the antibodies but also to endoproteinase Glu-C. We have previously shown that this enzyme converts native ligase into a nicked molecule with the two resulting fragments of molecular weight 30,000 and 14,000 still forming a tight complex (Wehland and Weber, 1987*a*). The protein sequence work now shows that Glu-C opens the peptide bond between glutamic acid 248 and tyrosine 249, which are contained in the peptide used to raise antibodies able to bind the ligase activity (serum 1). In agreement, computer predictions on surface accessibility show that this region should be exposed to the surface.

Secondary structure prediction programs show that the ligase most likely contains α helix, β structure and turns compatible with a globular molecule as also deduced earlier by hydrodynamic properties (Schröder et al., 1985). One potentially more interesting feature of the ligase sequence is the presence of the motif RKAS at residues 73 to 76. It indicates a phosphorylation site for protein kinase A at serine 76 (Kemp and Pearson, 1990). Future experiments have to decide whether this site has any physiological importance for ligase activity and/or turnover. Many ATP binding sites have been mapped in the past. Most have a pronounced P-loop harboring several glycine residues which often conform with the consensus sequence GXXXXGK(T/S) but others like actin, aminoacyl-tRNA-synthetases and hexokinase lack a strict relation to this sequence (Walker et al., 1982; Saraste et al., 1990). Inspection of the ligase sequence does not reveal a typical P-loop sequence. However, a cluster of three glycines containing a lysine residue is present at residues 154 to 159. Whether this sequence is related to the ATP binding site of ligase is not known. Previous results using ultraviolet light induced cross-linking of radioactive ATP showed labeling of ligase. This label was located in the nicked enzyme to the 30,000 molecular weight fragment (Wehland and Weber, 1987*a*), which we have now shown to span residues 1 to 248, thus covering the glycine cluster. Use of smaller fragments should allow in the future a finer mapping of the ATP binding site.

The ligase sequences are well conserved across mammalian species. Fig. 1 shows that porcine and bovine ligase are highly conserved in sequence. Over the region covering residues 1 to 367 there are only 13 conservative amino acid exchanges, all compatible with single nucleotide exchanges of the corresponding codons. The very short carboxyterminal region covering residues 368 to 379 shows much greater variability due to three amino acid replacements and a two-residue deletion in the bovine enzyme. The sequence identity over the entire molecules is 95%. This high homology value is also supported by the short sequence currently available for the murine protein (Fig. 1).

Although the mechanism of the reversible detyrosination of α-tubulin has been known for almost twenty years (Barra et al., 1973), the physiological function of this posttranslational modification remains unclear. Based mainly on immunological studies, it has been suggested that the detyrosination of α-tubulin is somehow linked to the maturation and differentiation of certain cells (see for example Wehland and Weber, 1987*b*; Cumming et al., 1984; Cambray-Deakin and Burgoyne, 1987). But whether a change in the degree of tyrosination can trigger a differentiation of the cellular cytoskeleton is not known. The TTL cDNA should be a helpful tool to elucidate some aspects of the function of tubulin detyrosination/tyrosination. Treatment of inducible cultured cell lines by TTL antisense oligonucleotides or by stable transfection with antisense mRNA-producing vectors may lead to an inactivation of TTL in vivo over much longer time periods than previously reached by injection of antibodies (Webster et al., 1990; Wehland and Weber, 1987*b*). Similar experiments have been successfully performed by suppressing microtubule-associated protein 2 expression by transfection of cells with an antisense mRNA-vector (Dinsmore and Solomon, 1991) or by suppression of Tau protein expression by antisense oligonucleotides (Caceres and Kosik, 1990). Perhaps even more promising is the possibility of disruption of the TTL gene by gene targeting.

Although the physiological consequences of the reversible tyrosination of α-tubulin for microtubular function(s) are not yet known, the cDNA clone and the protein sequence of TTL are valuable tools for the molecular analysis of the enzymatic mechanism of a tRNA-independent protein ligase. Future experiments have to decide whether the enzymatic mechanism of TTL shares some molecular aspects with other systems in-

volving the tRNA-independent formation of a peptide bond. Well documented examples include the first step of glutathione synthesis catalyzed by the γ-glutamyl-cysteine synthetase (Meister, 1974) and the synthesis of some bacterial antibiotics (Hash, 1975).

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