Comparison of the Two Murine Deoxynucleotidyltransferase Terminal Isoforms

A 20-AMINO ACID INSERTION IN THE HIGHLY CONSERVED CARBOXYL-TERMINAL REGION MODIFIES THE THERMOSENSITIVITY BUT NOT THE CATALYTIC ACTIVITY

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Terminal deoxynucleotidyltransferase (TdT, EC 2.7.7.31) is the only known enzyme to catalyze the nontemplated addition of nucleotides to 3'-hydroxyl ends of DNA strands in a template-independent manner and has been shown to add N-regions to gene segment junctions during V(D)J recombination. TdT is highly conserved in all vertebrate species, with a second isoform, characterized by a 20-amino acid insertion near the COOH-terminal end, described only in the mouse. The two murine isoforms differ in their subcellular localization, and the long isoform (TdTL) has previously been found to be unable to add N-regions. Using purified protein produced in a high level expression system in Escherichia coli, we were able to carry out detailed catalytic comparisons of these two TdT isoforms. We discovered that TdTL exhibits terminal transferase activity with kinetic parameters similar to those of the conserved TdT isoform (TdTS). We observed, however, that TdTL is inactivated at physiologic temperature but stable at lower temperatures. This thermal sensitivity of TdTL polymerase activity is not correlated with a significant change in the circular dichroism spectrum of the protein. Thus, the 20-amino acid insertion in TdTL does not affect the catalytic activity but modifies the thermosensitivity.

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† The abbreviations used are: TdT, terminal deoxynucleotidyltransferase; aa, amino acids.
led to an estimation of the purity of the two proteins superior to 80%.

Protein concentration was calculated using a theoretical extinction coefficient of 54,870M$^{2}$cm$^{-1}$ (12).

**TdT Enzymatic Assays and Determination of Kinetic Parameters—** Two substrates are involved in the terminal transferase nucleotidyl transfer reaction: a single stranded DNA (initiator) and a deoxynucleotide triphosphate. Michaelis-Menten kinetic parameters (K$_{m_1}$ dATP, K$_{m_2}$ (dA)$_{10}$, and k$_{cat}$) were obtained by titrating each substrate in the presence of a saturating concentration of a second substrate, as described previously (11). (dA)$_{10}$ and (dA)$_{50}$ oligonucleotide primers were purchased from Genset (Paris, France). Ultrapure deoxyribonucleotide solutions were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden).

Terminal transferase activity was detected by incorporation of dATP into single-stranded DNA using the following standard assay: recombinant proteins were incubated at 35 °C in 200 mM potassium cacodylate, 25 mM Tris-HCl, pH 6.6, 0.25 mg/ml bovine serum albumin, 4 mM MgCl$_2$, 4 mM ZnSO$_4$, 1 mM [$^{32}$P]dATP (70 cpm/pmol), and 100 µM oligonucleotide primer unless otherwise specified. Reactions were stopped at different times with 15 mM EDTA. Aliquots were spotted onto Whatman DE81 discs. Unpolymerized nucleotides were washed away by immersing the discs three times for 5 min in NH$_4$COOH/Na$_4$P$_2$O$_7\cdot 10$H$_2$O (300 mM/10 mM) and then in H$_2$O and in EtOH. Dried discs were counted in a scintillation fluid (Econofluor from Packard, Göthenburg, The Netherlands). Kinetic curves show the percentage of total dATP present in the reaction incorporated over time.

Owing to the distributive mechanism of TdT polymerization, analysis of chain length distribution was done at low oligonucleotide primer concentration. 5 mM 5'-$^{32}$P-labeled (dA)$_{10}$ primer was used as the radioactive substrate. Aliquots were withdrawn at different times, supplemented with a formamide dye mix (10 mM NaOH, 95% formamide, 0.05% bromphenol blue, 0.05% xylene cyanole), and electrophoresed, after heat denaturation, on a 16% acrylamide denaturing gel. TdT products were visualized after exposure of the wet gel under a Kodak film (Biomax MR) at 270 °C.

**Comparison of the Two Murine TdT Isoforms**

An amino acid sequence alignment of the two murine TdT isoforms with seven other vertebrate TdT was drawn using the Pileup program (Genetics Computer Group version 9.0), and a consensus sequence was deduced manually. Functionally equivalent amino acids were considered as follows: Leu, Ile, Met, and Val; Ala and G; Tyr, Trp, and Phe; Asp, Glu, Gln, and Asn; Lys, Arg, and His; Ser and Thr and are shown in italics. Strictly conserved amino acids are in plain text. Strictly conserved aspartate residues involved in catalysis (Asp$^{343}$, Asp$^{345}$, Asp$^{434}$) are underlined in the consensus sequence. In the murine TdTS sequence, the b peptide comprises amino acids 131–421 and the a peptide amino acids 422–510. Accession numbers in the Swiss Protein Data Bank are as follows: mouse, P09838; cattle, P06526; human, P04053; opossum, O02789; chicken, P36195; Xenopus, P42118; axolotl, O57486; and rainbow trout, Q92089.
Thermostability—To measure the thermostability of TdT isoforms, proteins at 3 mM in the storage buffer (25 mM Hepes, pH 7.0, 25 mM MgOAc, 25 mM (NH₄)₂SO₄, and 100 mM NaCl, 50% glycerol) were preincubated at various temperatures ranging from 20 to 60 °C for 10 min or at 35 °C for various times and chilled on ice. The residual activity was measured at 35 °C in the standard kinetic assay buffer with 100 μM (dA)₁₀, [α-³²P]dATP at 70 cpm/pmol, and 200 nM TdTS or TdTL.

Circular Dichroism (CD) Spectrum—Secondary structures of recombinant proteins were measured using circular dichroism on a Jobin-Yvon CD6 spectrometer (Longjumeau, France). Measurements were done at wavelengths from 195 to 260 nm, at two temperatures. First measurement was done at 25 °C. The temperature was then raised progressively to 35 °C and proteins incubated further for 20 min, before the second measurement was done. For each temperature three independent measurements were performed. Concentrations of protein solutions were 1 mg/ml in 2× storage buffer without glycerol.

RESULTS

Comparison of the Two Murine TdT Isoforms

The 20-Amino Acid Insertion in the Long Isoform of Murine TdT Is Localized in the Highly Conserved Carboxyl-terminal Region—A multiple alignment of all known TdT sequences was drawn using the Pileup program (Genetics Computer Group version 9.0), and a consensus sequence was deduced (Fig. 1). 153 amino acids are shared by all the sequences analyzed, corresponding to 30% identity, and 71 amino acids are functionally equivalent, leading to an overall similarity of 44%. TdT protein can be divided into three regions: the NH₂-terminal domain, which is dispensable for TdT activity (13), the so-called β and α peptides (14). Positions are given for the

### TABLE I

| Specific activity | Kₘ(dATP) | kₗ(dATP) | kₗ(dATP)/Kₘ(dATP) | Kₗ(dA)₁₀ | kₗ(dA)₁₀ | kₗ(dA)₁₀/Kₗ(dA)₁₀ |
|------------------|----------|----------|-------------------|----------|----------|-------------------|
| TdTS             | 110 ± 6  | 300 ± 6  | 180 ± 5           | 0.6 ± 0.02 | 16 ± 2.8 | 150 ± 7.6 |
| TdTL             | 67 ± 37  | 374 ± 43 | 98 ± 4.3          | 0.3 ± 0.05 | 6.7 ± 1.9 | 58 ± 4   |

a One unit of enzyme activity is defined as 1 nmol of nucleotide polymerized per h.

b Kₘ and kₗ mean values ± S.D. were obtained from a nonlinear least squares fit of the kinetic data to the Michaelis-Menten equation.

The 20-Amino Acid Insertion in the Long Isoform of Murine TdT Is Localized in the Highly Conserved Carboxyl-terminal Region—A multiple alignment of all known TdT sequences was drawn using the Pileup program (Genetics Computer Group version 9.0), and a consensus sequence was deduced (Fig. 1). 153 amino acids are shared by all the sequences analyzed, corresponding to 30% identity, and 71 amino acids are functionally equivalent, leading to an overall similarity of 44%. TdT protein can be divided into three regions: the NH₂-terminal domain, which is dispensable for TdT activity (13), and the so-called β and α peptides (14). Positions are given for the

2 J. B. Boulé, F. Rougeon, and C. Papanicolaou, unpublished result.
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murine TdT. The NH₂-terminal region (aa 1–131, 37% similarity) contains a consensus nuclear localization sequence (aa 11–17) (13) and a conserved BRCA1 carboxyl terminus-like sequence (aa 27–124), which is a protein-protein interaction domain first identified in the COOH-terminal region of the BRCA1 breast cancer suppressor protein (15) and in many other proteins, including some involved in DNA damage repair and recombination (16, 17). It was recently demonstrated that, in vitro, this domain in TdT interacts with the Ku70/86 heterodimer that binds DNA and is involved in double-strand break repair and V(D)J recombination (18). Mutagenesis experiments of human TdT showed that two amino acids analogous to Asp⁴₂⁴ and Asp⁴₃⁵ in murine TdT are essential for catalysis (19). We have shown that Asp⁴₃⁵ is also required for catalysis. The catalytic center of the protein thus contains amino acids from both β peptide (aa 132–421, 41% similarity) and α peptide (aa 422–510, 67% similarity). The 20-amino acid insertion is located at position 482 in the α peptide, which is the most conserved region in all the analyzed sequences (46% identity).

**TdT** is a True Terminal Deoxynucleotidyltransferase—Catalytic activities of purified TdTS and TdT were measured using a standard assay (described under “Experimental Procedures”) with dATP and (dA)₁₀ as substrates. Initial velocities using a standard assay (described under “Experimental Procedures.”) were measured at wavelengths from 195 to 260 nm, at 25 °C (solid line) and 35 °C (dashed line), as described under “Experimental Procedures.” The curves represent the mean of three independent measurements. The mean molar ellipticity [θ] was calculated with the formula: [θ] = (MRW·c/λ/10 x L x C), where MRW is the mean residue weight in dalton, θ the circular dichroism in millidegrees, L the path length (0.1 cm), and C the concentration of the enzyme (1 mg/ml).

TdT is indeed unstable around the temperature (35 °C) commonly used. This was further confirmed by measuring the enzymes residual activities after preincubation at 35°C over varying periods of time from 15 to 60 min. As can be seen in Fig. 3B, the residual activity was 60% for TdTS but only 20% for TdT after 60 min of preincubation at 35°C. We verified, using SDS-polyacrylamide gel electrophoresis analysis, that there was no significant protein degradation even after an hour of incubation at 35 °C. Furthermore, addition to the reaction buffer of bacterial protease inhibitors did not prevent the inactivation of TdT (data not shown). These observations led us to assess the retention of secondary structures of the two proteins by comparing their CD spectra. The analyses were first done at 25 °C, where both isoforms are stable. At this temperature, TdTS and TdT CD spectra were similar with a mean molar ellipticity at 210 nm around −15,000 degree cm² dmol⁻¹. This low value reflects the abundance of α-helices and β-sheets. The proteins were then slowly brought to 35 °C and further incubated for 20 min at this temperature. Both isoforms retained a low molar ellipticity (Fig. 4). The thermosensitivity of TdT catalytic activity is thus not correlated with a loss of secondary structure.

To test the possibility that the two isoforms could interact in vitro and exert an influence on each other, we compared the activity of a mixture of the two enzymes to the activity of each enzyme tested separately. As can be seen in Fig. 5, with a standard kinetic assay for incorporation of α-³²P-labeled dATP on a (dA)₅₀ primer, the activities of TdT and TdTL were additive. The same result was obtained when the experiment was carried out with different ratios of the two enzymes (data not shown).

**Discussion**

TdT has attracted great interest because of its unique catalytic capability, role in V(D)J recombination, unexplained evo-
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FIG. 5. Activity of TdTS and TdTL mixture. Terminal transferase activity was measured by incorporation of α-32P-labeled dATP on a (dA)10 primer in our standard enzymatic assay. Reactions were carried out with 290 nM TdTS (black circles), 290 nM TdTL (black squares), or 290 nM amounts of each enzyme (black triangles). The percentage of total dATP incorporated was measured between 0 and 40 min. The curve corresponding to the addition of dATP incorporated by TdTS and TdTL tested in separate assays is also displayed (open triangles). Each experiment was repeated several times, and results were averaged. Error bars represent the S.D.

Temperature sensitivity acquired by spontaneous mutations has been described for several proteins. A defect in the cellular trafficking of thermosensitive mutants of human tyrosinase and cystic fibrosis transmembrane conductance regulator has been correlated with a loss of activity in vivo (21, 22). It remains to be determined whether cultivating transfected cells at lower temperatures can modify the subcellular localization of TdTL and rescue its putative cellular function. The possibility that TdTL in vivo plays a role distinct from N-nucleotide addition will need to be explored.

TdT has only been found in vertebrates and no homolog to murine TdTL has been reported. Genomic data are yet to be collected to allow the search for an exon homologous to the murine TdT exon X-bis. Murine TdTL could represent an ancestral, perhaps vestigial, form of the enzyme (23) or may result from an evolutionary happenstance, such as the late capture of an additional exon. The evolutionary origin of TdT is a matter of interest and debate. Based upon some sequence similarity with polymerase β, TdT has been classified in the family X polymerases (24), a subclass of an ancient nucleotidylyltransferase superfamily whose members share a common signature in the active site and catalyze the same chemical reaction but have diverse biological roles (25, 26). Confirmation of this filiation awaits the elucidation of TdT tertiary structure.

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Nic1p, a relative of bacterial transition metal permeases in Schizosaccharomyces pombe, provides nickel ion for urease biosynthesis.

Thomas Eitinger, Olaf Degen, Ute Böhnke, and Marion Müller

Page 18031: The values for nickel accumulation of the parental strain and the mutant should be 389 pmol/10^9 cells and 32 pmol/10^9 cells, respectively, rather than 48 pmol/10^9 cells and 4 pmol/10^9 cells.

Page 18032: All nickel accumulation values in Table I, Fig. 3, and Fig. 5 should be multiplied by a factor of 8.1.

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The title was printed incorrectly. The correct title is shown above.