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Short Communication

Proliferation-Associated Nuclear Antigen Ki-S1 Is Identical with Topoisomerase IIα

Delineation of a Carboxyl-Terminal Epitope with Peptide Antibodies

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Proliferation-linked expression of the nuclear Ki-S1 antigen is a significant prognostic indicator in mammary carcinomas. Here, we show staining of a protein of 170 kd by Ki-S1 antibody in immunoblots of* Saccharomyces cerevisiae *expressing human topoisomerase IIα but not in the parental strain. In HL-60 cells containing both isoforms of human topoisomerase II, Ki-S1 antibody binds selectively to the 170-kd isoenzyme in a similar fashion as peptide-antibodies directed against amino acid residues 1 to 15 or 1512 to 1530 of human topoisomerase IIα. Conversely, antibodies directed against carboxyl-terminal sequences of human topoisomerase IIβ selectively stain a 180-kd protein. The immunoreactive pattern of V8 endoproteinase restriction digests of human topoisomerase IIα was identical for Ki-S1-antibody and peptide-antibodies directed against residues 1512 to 1530 but different for peptide-antibodies directed against residues 1 to 15. The R_f values of the smallest fragment commonly recognized by Ki-S1 antibody and the carboxy terminus-specific peptide-antibody place the Ki-S1 epitope within the last 495 carboxyl-terminal amino acid residues of topoisomerase IIα. (Am J Pathol 1995, 146:1302–1308)

The monoclonal antibody Ki-S1 recognizes a nuclear protein of approximately 160 kd that is abundantly expressed by proliferating cells and is induced when cells leave G_0 and enter the cell cycle.[1,2] Because the epitope is resistant to conventional embedding procedures, the Ki-S1 antigen can be demonstrated in archival tissue samples, enabling retrospective studies. In mammary carcinomas, a high fraction of Ki-S1-bearing cells was significantly associated with a worse prognosis.[3–5] Tumors with a high Ki-S1 labeling index revealed frequently amplification of the c-*myc* oncogene.[6] Furthermore, the Ki-S1 antigen has been demonstrated in Hodgkin cells[7] and hematopoietic precursor cells.[8] Proliferating cells that have their growth arrested by serum starvation show a marked reduction in Ki-S1 antigen expression.[2] Up to now the Ki-S1 antigen has not been identified. In this study we show, using recombinant yeast strains, that the antigen is identical with human topoisomerase IIα and that the Ki-S1 antibody recognizes a carboxyl-terminal α-isoenzyme-specific epitope missing in topoisomerase IIβ.

Materials and Methods

Protease inhibitors and *Staphylococcus aureus* V8 endoproteinase (EC 3.4.21.19) were obtained from

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Boehringer (Mannheim, Germany). Rabbit anti-human topoisomerase II antibodies, cross-reacting with the α- and β-isoenzymes of human topoisomerase II, were a kind gift of Prof. L. F. Liu (Baltimore, MD). Rabbit peptide-antibodies, specific for the carboxyl-terminal amino acid residues (1512 to 1530) of human topoisomerase IIα[9] and the corresponding peptide were obtained from Cambridge Research Biochemicals (Cambridge, UK). Detergent-resistant endonuclease Benzonase was obtained from Merck (Darmstadt, Germany). A Hoefer Mighty Small II electrophoresis system was obtained from Serva (Heidelberg, Germany). Marker proteins were obtained from Pharmacia Biotech (Uppsala, Sweden). All chemicals were of the highest degree of purity commercially available.

Western Blots

For each lane, 10^6 exponentially growing HL-60 cells (American Type Culture Collection CCL240) were treated for 10 minutes with 50 µL of 50% dimethylsulfoxide/H_2O, containing 10 mmol/L dithiothreitol, 1 mmol/L phenylmethylsulfonyl fluoride, 1 µg/ml diisopropyl-fluorophosphate, and 10% glycerol. A 100-µl volume of 50 mmol/L Tris-HCL, pH 8.0, containing 10 mmol/L $MgCl_2$ and 250 U of Benzonase was then added. After a 10-minute incubation on ice, sodium dodecyl sulfate was added to a final concentration of 0.2%, and cells were lysed by vigorous mixing. To the lysate, potassium phosphate, pH 8.0, was added to a final concentration of 50 mmol/L. Nondigested DNA and cellular debris were sedimented (13,000 × *g*). The supernatant was precipitated with trichloroacetic acid (7.5% for 30 minutes at 4 C). Precipitates were dissolved in Laemmli's buffer, subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (8.5% gels), and finally transferred to nitrocellulose sheets. Immunostaining was carried out with gold-labeled secondary antibodies and silver enhancement.[10] Molecular weight marker proteins contained rabbit muscle myosin (212 kd), α_2-macroglobulin from bovine plasma (170 kd), β-galactosidase from *Escherichia coli* (116 kd), human transferrin (76 kd), and bovine liver glutamic dehydrogenase (53 kd).

Human topoisomerase IIα was heterologously produced in yeast, as described in ref. 11. Briefly, *Saccharomyces cerevisiae* strain Y100 containing the URA-selectable plasmid pSPT1 carrying the *S. pombe* topoisomerase II structural gene under the control of its own promoter was transformed with a 3.6-kb DNA fragment containing the *TRP1* gene flanked by terminal regions of the *S. cerevisiae TOP2* gene to allow homologous recombination. A *Top2* gene knock-out mutant in which the essential endogenous topoisomerase II was complemented by the plasmid-borne *S. pombe* enzyme (strain BJ 201) was isolated by URA/TRP selection. To obtain constitutive expression of human topoisomerase IIα, BJ 201 was transfected with plasmid pHT 300α, containing the human *TOP2α* gene under the control of a triose phosphate isomerase promoter. Transfectants containing pHT 300α but not pSPT1 were isolated by negative selection with 5′-fluoro-orotic acid. The disruption of the *S. cerevisiae TOP2* gene was confirmed by Southern blot analysis. For Western blot analysis, yeast cells were harvested in logarithmic growth phase and powdered under liquid nitrogen in a mortar, and 1 g of the yeast powder was rapidly mixed with 1 ml of 50 mmol/L potassium phosphate, pH 7.5, 2 mol/L NaCl, 10 mmol/L dithiothreitol, 1 mmol/L phenylmethylsulfonyl fluoride, 1 µg/ml diisopropyl-fluorophosphate, and 10% glycerol. After sedimentation of the debris (36,000 × *g* for 30 minutes at 4 C), the cell extract was subjected to trichloroacetic acid precipitation, followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blot analysis.

For proteolytic restriction analysis, 10 µg of topoisomerase IIα partially purified from BJ 201 transformed with pHT 300α by heparin-Sepharose chromatography[12] was digested with 30 µU of *S. aureus* V8 endoproteinase (EC 3.4.21.19) for 15 minutes at 25 C and pH 7.8 and subsequently subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (11% gels) and Western blot analysis. Molecular weight marker proteins contained phosphorylase b subunits (97 kd), bovine serum albumin (68 kd), ovalbumin (43 kd), carbonic anhydrase (30 kd), soybean trypsin inhibitor (20 kd), and lactalbumin (15 kd).

Production of Rabbit Anti-Peptide Immunoglobulin G

Rabbits were immunized with synthetic peptides, coupled to keyhole limpet hemocyanin. Peptides were synthesized according to human topoisomerase IIα, amino acid residues 1 to 15,[13] and human topoisomerase IIβ, amino acid residues 1586 to 1569 and 1611 to 1621,[14] respectively. Immune sera were tested by Western blot analysis, with partially pure human topoisomerase IIα. γ-Globulins were isolated by standard procedures.

Results

When probing Western blots of yeast strain BJ 201 transformed with pHT 300α, expressing human topoisomerase IIα, a single protein was stained by monoclonal antibody Ki-S1, but a negative result was obtained when probing the parental strain BJ 201, which expresses *S. pombe* topoisomerase II (Figure 1A, lanes 7 and 8). The apparent molecular mass of the immunoreactive band (170 kd) is in good agreement with the molecular mass of human topoisomerase IIα, as deduced from the primary sequence.[13] A protein of the same size was also stained in BJ 201 transformed with pHT 300α but not in BJ 201 expressing the *S. pombe* enzyme when probed by peptide-antibodies directed against carboxyl- (Figure 1A, lanes 3 and 4) or amino-terminal sequences of human topoisomerase IIα (Figure 1A, lanes 5 and 6) and by an antiserum raised against purified intact human topoisomerase II (Figure 1A, lanes 1 and 2). Taken together, these data show that Ki-S1 as well as the two peptide-antibodies targeting sequences of human topoisomerase IIα recognize the same protein as the rabbit antibody raised against the intact enzyme. Apparently, these antibodies are specific for the human enzyme and do not cross-react with the *S. pombe* topoisomerase II or any other yeast protein. The sequence specificity of the two peptide-antibodies directed against the termini of human topoisomerase IIα was further confirmed by the experiment shown in Figure 1B. The antiserum directed against the carboxyl-terminal amino acid residues (1512 to 1530 of the published sequence[13]) could be blocked by the corresponding peptide but not by the peptide corresponding to the 15 amino-terminal residues of human topoisomerase IIα (residues 1 to 15 of the published sequence[13]). Conversely, the antiserum directed against the 15 amino-terminal amino acid residues could be blocked by the corresponding amino-terminal but not by the carboxyl-terminal peptide.

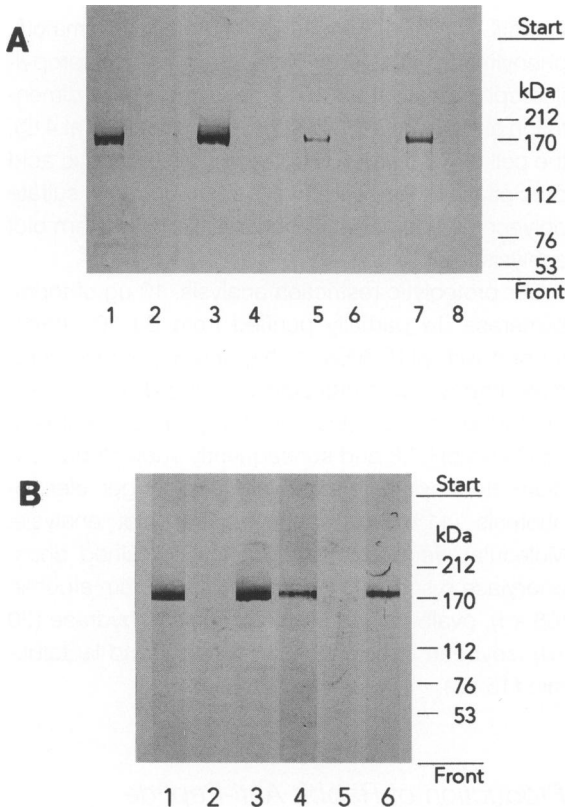

Figure 1. *Immunostaining of recombinant human topoisomerase IIα produced in* S. cerevisiae. **A:** *Western blots of yeast strain BJ 201 expressing* S. pombe *topoisomerase II* (lanes 2, 4, 6, *and* 8) *or yeast strain BJ 201 transformed with pHT 300α, expressing human topoisomerase IIα* (lanes 1, 3, 5, *and* 7) *were probed with rabbit antibodies raised against intact human topoisomerase II* (lanes 1 *and* 2) *or with rabbit peptide-antibodies, directed against amino acid residues 1512 to 1530* (lanes 3 *and* 4) *or 1 to 15* (lanes 5 *and* 6) *of human topoisomerase IIα, or with Ki-S1 monoclonal antibody* (lanes 7 *and* 8). **B:** *Western blots of yeast strain BJ 201 transformed with pHT 300α expressing human topoisomerase IIα were probed with rabbit peptide-antibodies directed against amino acid residues 1512 to 1530* (lanes 1 *to* 3) *or 1 to 15* (lanes 4 *to* 6) *of human topoisomerase IIα. The antibodies were preincubated for 30 minutes at 20 C with 100 ng/ml bovine serum albumin* (lanes 1 *and* 4) *or with the peptide corresponding to amino acid residues 1 to 15* (lanes 3 *and* 5) *or 1512 to 1530* (lanes 2 *and* 6).

In contrast to yeast, which has only one form of topoisomerase II, there are two isoforms of topoisomerase II known in mammals, which have molecular masses of 170 (α-form) and 180 kd (β-form).[14–18] To study the isoform specificity of Ki-S1, we probed Western blots of human HL-60 cell lysates. HL-60 cells contain approximately equal amounts of the α- and the β-form.[19] The two isoforms of topoisomerase II share a very high degree of sequence homology.[14–16,18] Consequently, the antiserum raised against the full-length enzyme reacts with both isoforms (Figure 2, lane 3). Major structural differences between topoisomerase IIα and -β exist only in the termini of the enzymes. Thus, antibodies raised against an α-form-specific peptide of the carboxy terminus (Figure 2, lane 4) or the amino terminus (Figure 2, lane 5) are specific for topoisomerase IIα and do not cross-react with the 180-kd form. Conversely, peptide-antibodies raised against two different β-form-specific carboxyl-terminal sequences are specific for the β-isoform of 180 kd and do not cross-react with the α-form of human topoisomerase II (Figure 2, lanes 1 and 2). When comparing the immunoreactive pattern obtained with Ki-S1 monoclonal antibody in HL-60 cells (Figure 2, lane 6) with that of the other antibodies, it becomes apparent that Ki-S1 does not react with the β-isoenzyme but has a similar selectivity for the 170-kd form as the two peptide-antibodies directed against the termini of the α-isoenzyme (compare Fig-

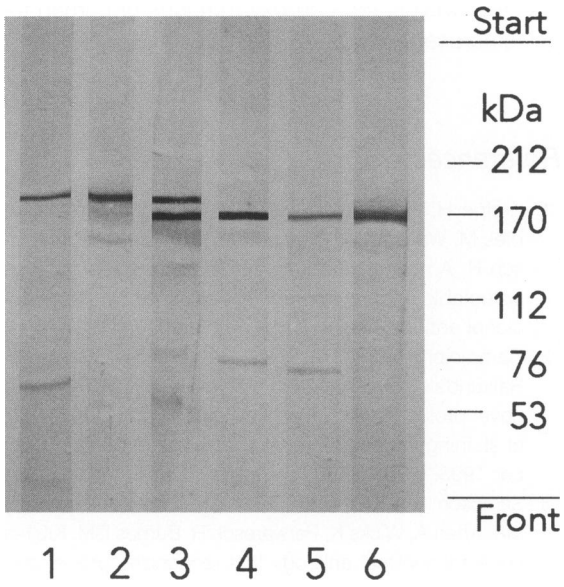

Figure 2. *Comparison of immunoreactive signals obtained with various topoisomerase II antibodies in HL-60 whole cell extracts.* **Lane 1**, *rabbit anti-human topoisomerase IIβ, amino acid residues 1586 to 1596;* **lane 2**, *rabbit anti-human topoisomerase IIβ, amino acid residues 1611 to 1621;* **lane 3**, *rabbit anti-human topoisomerase IIα and -β (raised against full-length enzyme);* **lane 4**, *rabbit anti-human topoisomerase IIα, amino acid residues 1512 to 1530;* **lane 5**, *rabbit anti-human topoisomerase IIα, amino acid residues 1 to 15;* **lane 6**, *mouse monoclonal anti-Ki-S1.*

ure 2, lanes 4 to 6). This finding suggests that the Ki-S1 antibody could be directed against an epitope located close to either end of human topoisomerase IIα. To test this hypothesis, we subjected partially pure recombinant human topoisomerase IIα to limited proteolysis by *S. aureus* V8 endoproteinase and compared the immunoreactive pattern of the peptide fragments obtained with Ki-S1 with those of the carboxyl- and amino-terminal-specific peptide-antibodies. As shown in Figure 3A, we obtained a set of five proteolytic fragments (R_f values of 0.47, 0.51, 0.68, 0.87, and 0.92) that could be stained by the amino-terminal-specific antibody but not by the carboxyl-terminal antibody (Figure 3A, lane 1) and a completely different set of another five proteolytic fragments (R_f values of 0.31, 0.34, 0.39, 0.41, and 0.49) that could be stained by the carboxyl-terminal antibody but not by the amino-terminal antibody (Figure 3A, lane 3). The staining pattern obtained with the Ki-S1 monoclonal antibody (Figure 3A, lane 2) was identical with that of the carboxyl-terminal-specific peptide-antibody. When comparing the R_f value of the smallest fragment commonly recognized by the Ki-S1 monoclonal antibody and the carboxyl-terminal-specific peptide antibody ($R_f = 0.49$) with the migration distance of molecular weight marker proteins (Figure 3B), it can be deduced that the epitope of Ki-S1 localizes within the 495 carboxyl-terminal

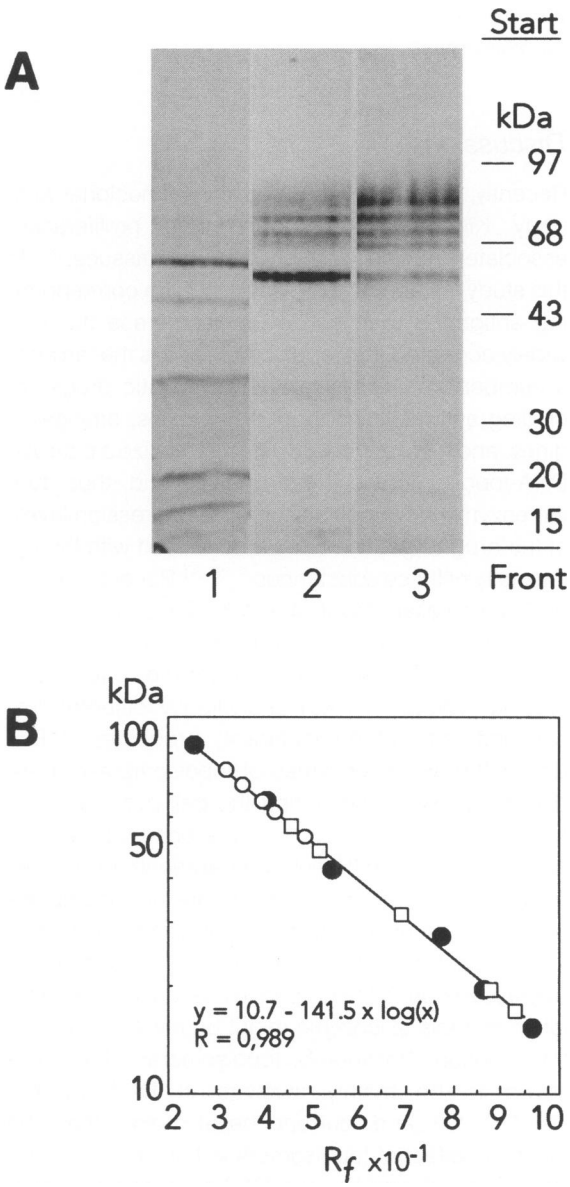

Figure 3. *Proteolytic restriction map of the Ki-S1 epitope. Recombinant human topoisomerase IIα was digested with* S. aureus *V8 endoproteinase (30 μU for 15 minutes at 25 C).* **A:** *Western blots were probed with rabbit anti-peptide immunoglobulin G directed against amino acid residues 1 to 15* (**lane 1**) *or 1512 to 1530* (**lane 3**) *of human topoisomerase IIα or with anti-Ki-S1 monoclonal antibody* (**lane 2**). **B:** *R_f plot of molecular weight marker proteins* (●) *and carboxyl-terminal* (○) *and amino-terminal* (□) *proteolytic fragments of human topoisomerase IIα.*

amino acid residues of human topoisomerase IIα. The only region within this domain that has a sufficient degree of sequence heterology between the α- and the β-isoform to allow for the observed isoenzyme selectivity of Ki-S1 antibody is located between amino acid residues 1501 and 1530. Therefore, it is reasonable to assume that the epitope of the Ki-S1 monoclonal antibody is located within the last 29

carboxyl-terminal amino acid residues of human topoiosmerase IIα.

Discussion

Recently, we have described the monoclonal antibody Ki-S1, which recognizes a proliferation-associated nuclear antigen in human tissues.[1–3] In this study, evidence is provided that the corresponding antigen is identical to topoisomerase IIα. It is widely accepted that topoisomerase II is the target of a number of clinically relevant cytostatic drugs, including anthracyclins, podophyllotoxins, aminoacridines, and mitoxantrone, which all stabilize a catalytic DNA-topoisomerase II intermediate and, thus, turn the enzyme into a cell poison.[20–24] Expression levels of the drug target seem to be correlated with the cytotoxicity of these substances.[9,25–31] Recently, a second mammalian topoisomerase II isoenzyme has been discovered that is encoded by a separate gene[14,15,18,32–34] and has a larger molecular mass (180 kd *versus* 170 kd) and slightly different biochemical and pharmacological properties.[15] Evidently, the two isoenzymes of topoisomerase II are differently expressed during the cell cycle and differentiation.[32,34] There are indications that they are also localized in different compartments of the cell nucleus. However, these results are not unambiguous.[35–41] The observation that topoisomerase IIβ is mainly located in the nucleolus and predominantly expressed during the G_2 phase has led to the speculation that this isoenzyme might be involved in gene transcription. Conversely, topoisomerase IIα is believed to be mainly involved in DNA synthesis,[17,32,35,38] and current belief holds that the cytotoxic effect of topoisomerase II inhibitors is predominantly effected during DNA synthesis.[21,23,24,42] With the help of topoisomerase II isoenzyme-specific antibodies suitable for immunohistochemistry, as described here, it should be possible to study further the subnuclear distribution of the isoenzymes and their co-localization with cytotoxic drugs.

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