The murine telomerase catalytic subunit shares the PAb-240 mutant specific epitope of the p53 protein

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Abstract **Many tumorigenic p53 mutants gain a common antigenic epitope that is recognized by the PAb-240 antibody. Database search identified the presence of this epitope in several other proteins, including several antibodies and the catalytic subunit of mouse telomerase, mTERT. These antibodies may represent a part of the previously demonstrated anti-idiotypic network built around p53. In the present study we demonstrate that the PAb-240 antibody was able to inhibit telomerase activity in extracts from both mouse and human tumor cells. The recognition of mTERT by PAb-240 is demonstrated by Western blotting and by using blocking peptides derived from mTERT. The existence of a shared epitope between mutant p53 and telomerase may suggest that the two proteins contribute to malignant transformation through a common pathway.**

Key words: Mutant p53; Telomerase; Epitope; PAb-240

1. Introduction

It is well accepted that inactivation of the p53 tumor suppressor gene plays a central role in the induction and progression of malignant transformation [1]. Tumorigenic mutations in the p53 gene occur at a high frequency mostly at the core domain of the molecule [2]. The majority of mutations in p53 are missense, and result in a full length, albeit mutant, protein. The observation that a high percentage of primary tumors overexpress various forms of mutant p53 implies that its presence may confer some selective advantage to cancer cells. It was suggested that mutant p53 might act by a dominant negative mechanism or by gain of function. The possibility that mutant p53 acts by gain of function was mostly based on the observation that transfection of mutant p53 into p53 null cells conferred a more highly malignant phenotype. Recent studies have suggested that mutant p53 function is associated with transcriptional activity [3]; however, the molecular mechanisms and the pathways associated with the oncogenic gain of function of p53 mutants are still unknown [4,5].

The integrity of the protein conformation of wild type p53 is central for its activity. Although tumorigenic mutations occur at various parts of the molecule, many of them gain a common antigenic epitope that is recognized by the PAb-240 anti-p53 monoclonal antibody [6]. The PAb-240 linear epitope spans five amino acids that are located at the middle of the central core domain of p53 (amino acids 213–217) and is inaccessible to the antibody in the wild type p53 conformation [7]. It was assumed that many mutations cause partial unfolding of the core domain leading to the exposure of this epitope. Thus, the PAb-240 epitope is considered to be mutant specific [6]. The existence of a common mutant specific epitope may imply that the various mutant p53 forms share a specific conformational structure that is important for their malignancy associated activity. If indeed mutant p53 acts by gain of function, then one possible mechanism that may underlie its activity could be that the PAb-240 antigenic epitope is also shared by other cellular protein(s). Once mutant p53 is overproduced in tumor cells it may modulate the regulation of important growth control pathways. This hypothesis prompted us to conduct a database search for cellular proteins which may express the PAb-240 antigenic epitope.

2. Materials and methods

2.1. Blocking of PAb-240 by peptides derived from human or mouse TERT

PAb-240 antibody was preincubated either with 200 μg of peptides 1, 2, 3, 4 or with 40 μg of peptides 5, 6, and 7 (Fig. 1A) for 1 h at room temperature. Eight identical samples each containing 1 μg of baculoviral derived p53 protein were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). After transfer to nitrocellulose membrane lanes were excised and reacted with the PAb-240 antibody, preincubated with the indicated peptides. The protein–antibody complexes were detected by a horseradish peroxidase (HRP)-conjugated secondary antibody (Boehringer Mannheim) using the enhanced chemiluminescence system (Amersham).

2.2. Telomerase activity assays

Telomerase activity determinations were performed using a commercial TRAPeze kit (Intergene) according to the manufacturer's non-radioactive protocol. However, the cycling conditions were modified as follows: 30°C 30 min, 94°C 3 min, and 29 cycles of amplification: 94°C for 30 s, 56°C for 30 s, 72°C for 30 s. 200 ng lysate per TRAP reaction was used.

For the immunodepletion assays, 20 μg of cell lysates prepared according to the TRAPeze kit instructions was incubated with either PAb-240 or PAb-421 antibodies overnight in 150 μl IP buffer (50 mM Tris pH 8, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40 and a protease inhibitor cocktail (Roche)). Then, aliquots were taken and 2 μl was used in TRAP assay (designated binding). 30 μl of protein A beads was added to the rest. After 2 h rolling at 4°C and centrifugation the supernatant was removed and 2 μl was used for the TRAP assay (designated immunodepletion).

2.3. Western blot analysis

For Western blotting of TERT protein, 100 μg of nuclear extracts prepared according to Dignam [8] was separated by 7.5% SDS–PAGE and transferred to nitrocellulose membrane. Membranes were blocked

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overnight with 5% dry skimmed milk in phosphate buffered saline–Tween (PBST) and TERT protein was detected using either commercial anti-TERT polyclonal antibody (Calbiochem, Cat. No. 581400) diluted 1:500 in 5% milk–PBST or PAb-240. The protein–antibody complexes were detected using an HRP-conjugated secondary antibody (Boehringer Mannheim) by enhanced chemiluminescence system (Amersham).

3. Results and discussion

The non-redundant GenBank database was searched for mammalian proteins containing the amino acid sequence RHSVV (PAb-240 epitope) [7] using the BLASTP 2.2.3 program for short nearly exact matches [9]. Table 1 presents all perfect matches obtained from such a Blast search. It should be noted that the PAb-240 epitope is highly conserved in p53 orthologs and therefore those were not included in this table. The data obtained by our search can be divided into three main categories.

The first category identified in our Blast search consists of hypothetical proteins and proteins with unknown function. The significance of sharing the PAb-240 epitope with p53 by these proteins will be resolved once their function is understood. The fact that so many cellular proteins share this antigenic epitope emphasizes the problematics of using PAb-240 monoclonal antibody as a single reagent for the identification of p53.

The second category of proteins identified in our search consists of 17 hits of mouse antibodies that contain the PAb-240 epitope sequence in their variable regions. In his idiotypic network theory Jerne [10] suggested that a given antibody (Ab1) does not only react with an epitope on a target antigen, but that it is also subject to recognition itself. Thus, Ab1 may induce other, anti-idiotypic antibodies (Ab2), some of which could mimic the antigenic determinant recognized by Ab1. The idiotype of Ab2 also can be recognized by a set of antibodies (Ab3), of which some may again manifest the specificity of Ab1.

Indeed, it was shown previously that mice immunized with Ab1 PAb-240 spontaneously produce Ab3 anti-p53 antibodies that reflect the specificity of their Ab1 inducer; PAb-240 induced Ab3 specific for mutant p53. This study suggested that an anti-idiotypic network built around certain domains of p53 seems to be programmed within the immune system and can be associated with the resistance of the host to tumor development [11,12]. It is possible that some of the antibodies identified in our search represent the Ab2 antibodies, which can mimic the DNA binding domain of p53.

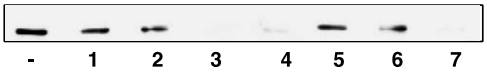
The third category consists of known characterized proteins that include the mouse transcription factor IIIA that was already predicted to contain the PAb-240 epitope [7]. Another interesting member identified in this category is the catalytic subunit of telomerase. Since both p53 and telomerase are involved in malignant transformation we have further focused in this report on the possible relationship between the two.

Telomerase, a specialized RNA directed DNA polymerase that extends telomeres of eukaryotic chromosomes, has been

A.

peptide number	Sequence modification	Protein	Peptide sequence
1	Scrambled	mouse TERT	SLFDFFLHFLRMAVAKIGDRCYTQC
2	wild type	human TERT	GLFDVFLRFMCHHAVRIRGKSYVQC
3	wild type	mouse p53	PEYLEDRQTF**RHSVV**VPYEPPEAGS
4	wild type	mouse TERT	SLFDFFLHFL**RHSVV**KIGDRCYTQC
5	mismatch	mouse p53	FRMSVVV
6	scrambled	mouse p53	RVFMVSV
7	wild type	mouse p53	F**RHSVV**V

B.



C.

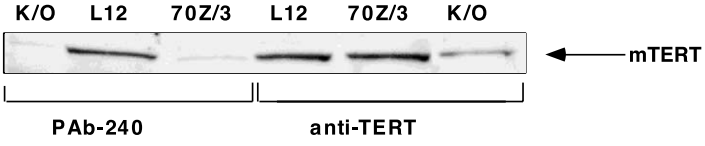


Fig. 1. Blocking of PAb-240 by peptides derived from human or mouse TERT and recognition of endogenous mTERT by PAb-240. A: Amino acid sequences of the peptides used for blocking the PAb-240 antibody. The PAb-240 epitope is in bold. B: Blocking of PAb-240 by peptides derived from human or mouse TERT. C: Western blot of endogenous mTERT.

demonstrated to have an important role in aging, immortalization and transformation. The activity of telomerase is repressed in most normal human somatic tissues, whereas the enzyme is activated during tumor progression in about 90% of human cancers [13]. However, the mechanism of its reactivation in the process of human carcinogenesis is still unclear. It should be noted that most normal mouse adult tissues, in contrast to human, show detectable levels of telomerase activity [14]. Nevertheless, similarly to human, the telomerase activity is elevated in tumors of mice [14–16]. The functional telomerase minimally consists of the RNA subunit TR [17], containing the template for telomere synthesis, and the catalytic subunit TERT [18]. Interestingly, most tumor cells that show telomerase activity have lost wild type p53 and usually express mutant p53 [19,20]. Our working hypothesis was that telomerase and mutant p53, which exhibit the common antigenic epitope, may share structural elements that are also functionally related.

Table 1
Mammalian proteins containing the RHSVV amino acid motif

Accession number	Protein description
	Unknown function
20849231	RIKEN cDNA 5330403M05 [*Mus musculus*]
3882183	KIAA0731 protein [*Homo sapiens*]
10047213	KIAA1574 protein [*Homo sapiens*]
20540646	hypothetical protein XP_170183 [*Homo sapiens*]
12655205	similar to hypothetical protein FLJ10378 [*Homo sapiens*]
20555810	hypothetical protein XP_166331 [*Homo sapiens*]
8923530	hypothetical protein FLJ20559 [*Homo sapiens*]
6453438	hypothetical protein DKFZp727K171.1, human (fragment) [*Homo sapiens*]
10434817	unnamed protein product [*Homo sapiens*]
10434659	unnamed protein product [*Homo sapiens*]
20856117	hypothetical protein XP_151622 [*Mus musculus*]
	Immunoglobulins
13171716	immunoglobulin heavy chain variable region [*Homo sapiens*]
20346796	immunoglobulin heavy chain [*Mus musculus*]
20860485	similar to Ig heavy chain V region VH558 A1/A4 precursor [*Mus musculus*]
20343054	similar to Ig H chain [*Mus musculus*]
20346788	similar to Ig mu chain V-D-JH1 region (B2) precursor
20860419	similar to monoclonal antibody heavy chain [*Mus musculus*]
20346794	similar to Ig H chain V-JH3 region [*Mus musculus*]
XM_138370	similar to AHT107 VH region [*Mus musculus*]
20860479	similar to Ig heavy chain V region VH558 A1/A4 precursor [*Mus musculus*]
20859952	similar to Ig heavy chain V region 3 precursor [*Mus musculus*]
XM_111335	similar to Ig heavy chain V region 3 precursor [*Mus musculus*]
20859814	similar to anti-poly(dC) monoclonal antibody heavy chain [*Mus musculus*]
XM_111323	similar to Ig heavy chain V region 3 precursor [*Mus musculus*]
XM_138369	similar to monoclonal antibody heavy chain [*Mus musculus*]
20951123	similar to Ig heavy chain (myeloma M104E), mouse (fragment) [*Mus musculus*]
XM_111348	similar to immunoglobulin heavy chain variable region [*Mus musculus*]
XM_138375	similar to Ig heavy chain precursor V region (IdB5.7), mouse (fragment) [*Mus musculus*]
	Known function
6226781	telomerase reverse transcriptase; mTERT [*Mus musculus*]
7407653	telomerase catalytic subunit [*Rattus norvegicus*]
20900718	mannosidase 2, α1 [*Mus musculus*]
18448380	transcription factor IIIA [*Rattus norvegicus*]
18448382	transcription factor IIIA [*Mus musculus*]
12849777	homolog to transcription factor IIIA [*Mus musculus*]
20888083	similar to carboxylesterase 2; intestinal carboxylesterase; liver carboxylesterase-2 [*Mus musculus*]
20888779	similar to MBD2 (methyl-CpG-binding protein) interacting zinc finger protein; DKFZP434F162 protein [*Mus musculus*]
20845998	similar to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [*Mus musculus*]

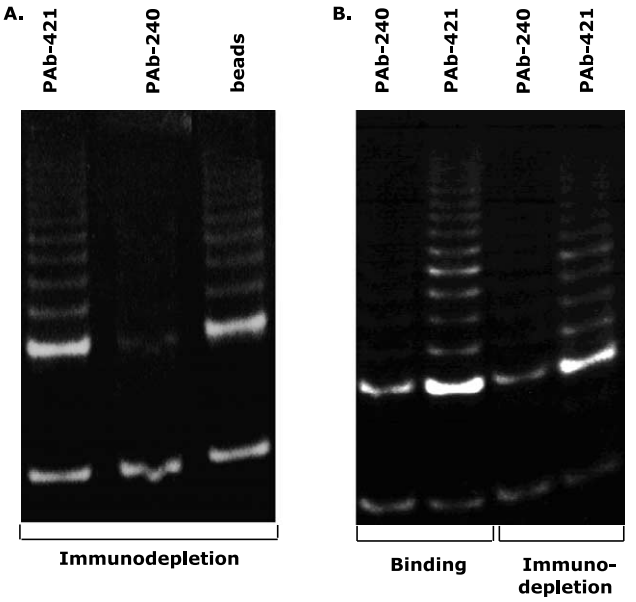


Fig. 2. Inhibition of telomerase activity by PAb-240. A: Telomerase activity in M1/2 lysates after immunodepletion with PAb-240, PAb-421 or beads only. B: Telomerase activity in H1299 lysates after binding or immunodepletion with PAb-240 or PAb-421. The intensity of the ladder pattern relative to the internal standard (faster migrating band) is a measure of telomerase activity.

To confirm experimentally the existence of the PAb-240 epitope in the telomerase protein we measured the actual binding of the monoclonal antibody PAb-240 to hTERT and mTERT. In our first experiment we examined whether peptides derived from the region containing the predicted PAb-240 epitope in telomerase will block the binding of this monoclonal antibody to the p53 protein. PAb-240 was preincubated with equimolar amounts of the corresponding peptides and then its ability to recognize p53 was examined by Western blotting. This indirect approach was used to enable solution contact between PAb-240 and the peptides, while providing conformational freedom for the peptides. For the same reason, relatively long (25 amino acids) peptides were used. Fig. 1A,B shows that as expected, PAb-240 recognized the baculoviral expressed p53 protein. It should be stressed that under denaturing conditions of SDS–PAGE, the PAb-240 epitope is exposed and detected in both wild type and mutant p53 protein forms. Peptides derived from the sequence of mouse TERT (No. 4) but not from human TERT (No. 2) blocked PAb-240 binding. Peptides derived from the sequence of mouse p53 PAb-240 epitope (Nos. 3 and 7) served as positive controls. The specificity of binding is demonstrated by

the inability of mutated peptides (Nos. 5 and 6) to block PAb-240 binding.

To test the ability of PAb-240 to bind endogenous mTERT we performed a Western blot for mTERT using different mouse cell lines. Two equivalent samples from each cell line were loaded on the gel. After transfer to nitrocellulose membrane, half of it was reacted with a commercial anti-TERT antibody and the other half with PAb-240. The results shown in Fig. 1C demonstrate that PAb-240 recognized telomerase in cell extracts obtained from fibroblasts derived from p53 knocked-out mice (K/O) as well as in cell extracts of p53 null early pre-B cell lines (L12 and 70Z/3). Indeed, both antibodies detected similar size bands at the expected molecular weight of 125 kDa, albeit with different affinities. The strongest signal evident with PAb-240 was obtained with the L12 cell line extract. With the anti-TERT antibodies, again, the signal in L12 was the most intense. Nevertheless, a strong signal was also evident with the other cell lines used. These variations in the ratio of signal intensities may reflect cell type specific post-translational modifications, or polymorphism in the sequence of mTERT protein at the PAb-240 epitope region.

In the following experiment we examined the effect of PAb-240 on the enzymatic activity of telomerase as measured by the TRAP assay. Cell extracts were incubated with PAb-240 or with another anti-p53 antibody, PAb-421, which served as a negative control. This antibody recognizes the C-terminal epitope of p53, consisting of amino acids 371–380 [21]. Since the mere binding of PAb-240 and telomerase might not necessarily interfere with telomerase enzymatic activity, we also performed immunodepletion of the cell lysates with protein A beads to physically remove the complex from the solution. To avoid potential multiprotein complexes, which would pose problems in interpreting the results, all the cell lines used in these experiments were p53 negative.

As seen in Fig. 2A, a significant inhibition of telomerase activity by immunodepletion with PAb-240 in the M1/2 mouse myeloid cell line was observed. Interestingly, an even more striking inhibition was evident in human H1299 cells not only in the case of immunodepletion but also by the mere addition of PAb-240 (Fig. 2B). The specificity of the inhibition is demonstrated by the lack of effect of PAb-421 antibody.

In summary, our results show that PAb-240 binds to the linear epitope of the mTERT catalytic subunit. Of note is the fact that PAb-240 does not recognize human TERT in either native or denatured conformation. Nevertheless, telomerase activity of H1299 cells is strongly inhibited by PAb-240. One intriguing possibility is that PAb-240 binds to and inactivates some other protein(s), which are necessary for telomerase activity. The existence of a common epitope between mutant p53 and telomerase may suggest that the two proteins contribute to malignant transformation through a common pathway. Furthermore, our search yielded a number of additional human proteins with yet unknown functions containing this epitope that may be associated with the same biological pathway.

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