Advances in Brief

Rejection Antigen Peptides on BALB/c RLδ1 Leukemia Recognized by Cytotoxic T Lymphocytes: Derivation from the Normally Untranslated 5′ Region of the c-*Akt* Proto-Oncogene Activated by Long Terminal Repeat[1]

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Abstract

Tumor antigen peptides on BALB/c leukemia RLδ1 that were recognized by cytotoxic T lymphocytes were shown to be derived from a normally unstranslated region of the *akt* proto-oncogene (Uenaka, A. *et al.*, J. Exp. Med., *180*: 1599, 1994). We show here that the murine leukemia virus (MuLV) long terminal repeat (LTR) was inserted directly into the exon of c-*akt* in RLδ1 leukemia and that transcription started from the cap site of the LTR. Translation appeared to start from the ATG codon created in the six nucleotides of unknown origin, which were inserted into the LTR/akt junction. The deduced molecular size is approximately M_r 59,000 due to the addition of 33 amino acid residues to the normally expressed c-AKT protein. Western blot analysis demonstrated the presence of M_r 59,000 molecules in an RLδ1 lysate, and their expression at about ten times the level of normal AKT molecules of M_r 56,000, which is consistent with the increased expression of *akt* mRNA demonstrated by Northern blot analysis. The findings show that the molecular alteration of AKT protein by insertion of MuLV LTR is the mechanism for creating rejection antigen peptides derived from the untranslated region of *akt*.

Introduction

CTLs[3] have been shown to play a significant role in the eradication of certain human and mouse tumors (1). CTLs recognize antigen peptides in association with major histocompatibility complex molecules on the tumor cell surface (2). Recently, we identified the tumor rejection antigen peptide pRL1 recognized by CTLs on BALB/c radiation leukemia RLδ1 by using acid extraction, purification by high performance liquid chromatography, and direct sequencing (3). Bulk CTLs and six cloned CTL lines obtained from (BALB/c × C57BL/6)F_1 (hereafter referred to as CB6F_1) mice that had rejected RLδ1 recognized pRL1a (IPGLPLSL) bound to the H-2L^d molecule. Another peptide, pRL1b (SIIPGLPLSL), appeared to be the precursor peptide of pRL1a. Sequence homology analysis revealed that pRL1 peptides were derived from the untranslated 5′ region of the c-*akt* proto-oncogene (4, 5). The present study demonstrates that the presence of MuLV LTR juxtaposed to the first coding exon of *akt* results in enhanced production of an altered AKT protein and creation of tumor rejection antigen peptides derived from the normally untranslated region of the gene.

Materials and Methods

Mice. BALB/c and AKR mice were bred in our animal center.

Tumors. RLδ1 is a radiation-induced leukemia in a BALB/c mouse (3). AK2D is a spontaneous leukemia in an AKR mouse and was provided by Dr. Y. Obata (Aichi Cancer Center Research Institute, Nagoya, Japan).

5′ Rapid Amplification of cDNA Ends. 5′ RACE was performed by using a 5′ RACE kit (GIBCO-BRL, Gaithersburg, MD). For first-strand cDNA synthesis, the reaction was performed in 25 μl of reaction mixture containing 20 mM Tris, 50 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 100 nM primer 4-1 (Table 1), 1.6 mM dNTP, 1 μg total RNA from RLδ1, and 8 units/μl SuperScript II RT, at 42°C for 30 min. After isolation by using a GlassMax spin cartridge, cDNA was entailed with oligodeoxycytidine in 25 μl of reaction mixture containing 20 mM Tris, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dCTP, and 10 units terminal deoxynucleotidyl transferase, at 37°C for 10 min. For amplification of deoxycytidine-tailed cDNA, PCR was done in 50 μl of reaction mixture containing 20 mM Tris, 50 mM KCl, 1.5 mM $MgCl_2$, 400 nM primer 2-4 or primer 3-1, 400 nM anchor primer (5′-CUACUACUACUAGGC-CACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3′), 800 μM dNTP, and 0.04 unit/μl Taq DNA polymerase. The cycle profile was denaturation at 94°C for 30 s, annealing at 56°C for 1 min, and extension at 72°C for 2 min for 40 cycles in a DNA Thermal cycler (Perkin Elmer Cetus, Norwalk, CT).

Nucleotide Sequencing. BALB/c thymocytes and RLδ1 (1×10^7) were suspended in 1 ml ISOGEN (Nippon Gene, Tokyo, Japan) in a microcentrifuge tube and homogenized. After the addition of 0.2 ml of chloroform, the tubes were centrifuged at 12,000 × g for 15 min. Total RNA was precipitated by adding 0.5 ml of isopropyl alcohol. Reverse transcriptase-PCR was done with a GeneAmp RNA PCR kit (Perkin Elmer Cetus). Reverse transcription of RNA (1 μg) to cDNA was performed in 20 μl of PCR buffer containing 5 mM $MgCl_2$, 4 mM dNTP, 2.5 μM oligodeoxythymidylate$_{16}$ 1 unit/μl of RNase inhibitor and 2.5 units/μl of reverse transcriptase. The reaction mixture was incubated at 42°C for 60 min and 99°C for 5 min and then chilled on ice. PCR for the cDNA amplification was performed with the oligonucleotide primers 2-1 and 1-2. The cycle profile was denaturation at 94°C for 40 s, annealing at 60°C for 60 s, and extension at 72°C for 90 s, for 30 cycles in a DNA Thermal cycler (Perkin Elmer Cetus). PCR products were treated with T4 DNA polymerase (New England Biolabs [NEB], Beverley, MA) and T4 polynucleotide kinase (NEB), and then integrated into pBSKS (−) vector, which had been treated with EcoRV (NEB) and bovine alkaline phosphatase (NEB). Recombined DNA was introduced into *Escherichia coli* DH5α by electroporation. The plasmid was purified by using QIAGEN tips (QIAGEN, Chatsworth, CA). Direct cycle sequencing was performed in a Techne Thermal Cycler by using a PRISM Ready Reaction DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). The sequences were determined with an automated sequencer (Applied Biosystems). The primers used for sequencing were the same as those for amplification.

Southern Blot Analysis. DNA (10 μg) was digested with KpnI and electrophoresed in 0.8% agarose gel. The gel was soaked in 0.5 N NaOH 1.5 M NaCl for 45 min and in 0.5 M Tris-HCl (pH 7.0)/3 M NaCl for 45 min and blotted onto a nylon transfer membrane (Hybond-N+; Amersham International, Little Chalfont, United Kingdom). The membrane was baked at 80°C for 2 h and prehybridized at 42°C for 4 h in 0.75 M NaCl, 0.075 M sodium citrate, 2× Denhardt's solution, 0.5% SDS, 40 μg/ml denatured salmon sperm DNA and 50% formamide, and hybridized for 20 h with the ^{32}P-labeled c-*akt*

Table 1 *PCR primers used in this study*

Designation	Nucleotide sequence	Position in cAkt17b[a]
2-1 sense	5'-GCTCAGCCTACCGAGAAGA-3'	213–231
1-2 antisense	5'-CTACGTCGTTCATGGTATCCGT-3'	296–275
3-1 antisense	5'-TGTAGCCAATAAAGGTGCCA-3'	398–378
3-2 antisense	5'-GTGGGGACTCTCGCTGAT-3'	437–420
2-4 antisense	5'-GTCGTGGGTCTGGAATGAG-3'	946–928
4-1 antisense	5'-TCGAGACAGGTGGAAGAAGAGC-3'	1006–985
5-1 sense	5'-TTGACTGCCCAGCTTGGGGGT-3'[b]	

[a] See Ref. 5.
[b] The nucleotide sequence of sense primer 5-1 is that of nucleotides 499–519 of MuLV LTR (6).

5' probe at 42°C. A purified DNA fragment of the 5' portion (*SalI/PstI* digest of pcAkt17b; 542 bp) was labeled with ^{32}P and was used as a probe. The membrane was then washed with 2× SSCB (0.3 M NaCl-0.03 M Sodium citrate-0.05% SDS) at 42°C for 10 min, 2× SSCB at 65°C for 10 min, 1× SSCB at 65°C for 10 min, and 0.5× SSCB at 65°C for 10 min, and exposed to Kodak XAR-5 film.

Northern Blot Analysis. Total RNA (20 μg/lane) was electrophoresed in 1.4% agarose-formaldehyde gel. The material was then blotted onto a nylon membrane (Hybond-N⁺; Amersham International). The membrane was processed, hybridized, and washed and exposed as described above. Transcripts of GAPDH were used as controls for RNA loading.

Western Blot Analysis. BALB/c thymocytes (5 × 10⁷) and RLδ1 (1 × 10⁷) were incubated with lysis buffer consisting of 1% NP40, 0.2 mM 4-(2-aminoehtyl)-benzenesulfonylfluoride, 10 μM EDTA, 10 μM leupeptin, and 1 μM pepstatin A in PBS for 30 min at 4°C. The lysate was separated by SDS-PAGE, blotted onto a nitrocellulose membrane, and assayed with rabbit antiserum raised against a synthetic peptide composed of 16 amino acid residues (CVDSERRPHFPQFSYS) of the COOH-terminal of AKT protein coupled to keyhole limpet haemocyanine. Bound antibody was detected by goat antirabbit IgG Fc (Promega, Madison, WI) conjugated with alkaline phosphatase by using a Bio-Rad (Hercules, CA) substrate kit.

Results

Structural Analysis of the 5' Region of RLδ1 c-*akt* Transcript. We identified the antigen peptides pRL1a and pRL1b which are recognized by RLδ1-specific CTLs by acid extraction, HPLC purification, and direct sequencing. The amino acid sequences of pRL1a and pRL1b are IPGLPLSL and SIIPGLPLSL, respectively, and correspond to amino acid residues 271–278 and 269–278 of the v-AKT protein (5). This portion corresponds to the normally untranslated 5' region in the canonical c-*akt* proto-oncogene in mice. These findings indicated that the structure of the 5' region of the c-*akt* gene in RLδ1 is altered from the normal one, allowing the untranslated region to be translated.

We examined the structure of the 5'-terminal region of the RLδ1 c-*akt* (cRL*akt*) transcript by 5' RACE. The first-strand cDNA was synthesized by using an antisense primer 3-2 (Table 1). An anchor sequence was then added to the 3' end of the cDNA and amplified by PCR by using a synthetic anchor primer and another nested antisense primer 3-1. PCR products were then sequenced. As shown in Fig. 1, the MuLV LTR sequence was fused to the *akt* transcript. The LTR sequence was colinear with the published MuLV LTR sequence (6) from the cap site, nucleotide 384, to nucleotide 526, one before the end of U5. Downstream of the LTR, a stretch of six nucleotides of unknown origin was entailed, in which an ATG codon was formed in frame with the c-*akt* open-reading frame starting from nucleotide 150 (nucleotide 190 in the cAkt17b sequence).

Genomic Change of RLδ1 c-*akt*. For analysis of the relationship between LTR and c-*akt* sequences in the genomic structure, the genomic DNA was amplified by using the sense primer 5-1, which has the sequence of nucleotides 499–519 of the LTR sequence and an antisense primer 1-2. The nucleotide sequence of the PCR product of

146 nucleotides was identical to that obtained with mRNA shown in Fig. 1.

For confirmation of the chromosomal rearrangement, Southern blot analysis was performed with the 5' 520-bp fragment from a plasmid of cDNA clone cAkt17b (5) that was obtained from AKR thymocytes as a probe after digesting genomic DNAs with *KpnI*, which cleaves the LTR sequence once. As shown in Fig. 2a, a rearranged band was observed in genomic DNA from RLδ1 but not from BALB/c thymocytes. These findings indicate that an LTR sequence was juxtaposed to or inserted within the first coding exon of the c-*akt* gene in RLδ1 leukemia cells. As a consequence, transcription of the rearranged c-*akt* gene seems to be accelerated by the strong promotor activity of the LTR sequence.

Analysis of Expression of *akt* in RLδ1 Leukemia. Northern blot analysis demonstrated higher expression of *akt* mRNA in RLδ1 cells than in BALB/c thymocytes (Fig. 2b). Densitometric scanning revealed about a 5-fold increase calculated after normalization with an internal control GAPDH transcript.

We raised rabbit antiserum against a synthetic peptide consisting of 16 hydrophilic amino acid residues of the carboxyl terminus of the AKT protein. On Western blot analysis, the antiserum reacted with both M_r 59,000 and M_r 56,000 molecules in the RLδ1 lysate but with only M_r 56,000 molecules in the lysate from BALB/c thymocytes. The expression of the 59,000 molecules was about ten times that of normal M_r 56,000 AKT molecules as judged by densitometric analysis (Fig. 2c). No significant difference was observed between the levels of expression of M_r 56,000 molecules in RLδ1 and BALB/c thymocytes.

Polymorphic Change of the Amino Terminal Ser Residue of pRL1b. The amino acid sequence of the antigen peptide pRL1b, a probable precursor of the pRL1a, is SIIPGLPLSL. The amino terminal residue of the pRL1b is Ser, whereas that of the v-AKT protein is Ile. As shown in Fig. 3, at this position the cDNA sequences of both BALB/c thymocytes and RLδ1 had the AGC codon (Ser), whereas those of AKR thymocytes and AKR-derived leukemia (AK2D) had
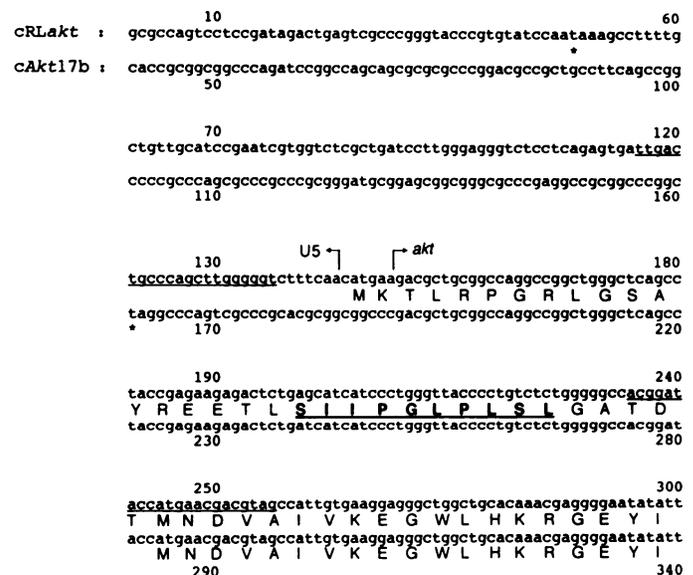
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                10                                              60
cRLakt :  gcgccagtcctccgatagactgagtcgcccgggtacccgtgtatccaataaagccttttg
                                                            *
cAkt17b :  caccgcggcggcccagatccggccagcagcgcgcgcccggacgccgctgccttcagccgg
                50                                             100

                70                                             120
          ctgttgcatccgaatcgtggtctcgctgatccttgggagggtctcctcagagtgattgac
          ccccgcccagcgcccgcccgcgggatgcggagcggcgggcgcccgaggccgcggcccggc
                110                                            160

                130        U5¬     ¬akt                         180
          tgcccagcttgggggtctttcaacatgaagacgctgcggccaggccggctgggctcagcc
                                    M  K  T  L  R  P  G  R  L  G  S  A
          taggcccagtcgcccgcacgcggcggcccgacgctgcggccaggccggctgggctcagcc
          *      170                                            220

                190                                            240
          taccgagaagagactctgagcatcatccctgggttacccctgtctctggggccacggat
          Y  R  E  E  T  L  S  I  I  P  G  L  P  L  S  L  G  A  T  D
          taccgagaagagagctctgatcatcatccctgggttacccctgtctctggggccacggat
                230                                            280

                250                                            300
          accatgaacgacgtagccattgtgaaggagggctggctgcacaaacgaggggaatatatt
          T  M  N  D  V  A  I  V  K  E  G  W  L  H  K  R  G  E  Y  I
          accatgaacgacgtagccattgtgaaggagggctggctgcacaaacgaggggaatatatt
             M  N  D  V  A  I  V  K  E  G  W  L  H  K  R  G  E  Y  I
                290                                            340
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Fig. 1. Nucleotide sequence of the 5' portion of the RLδ1 c-*akt* transcript. LTR was fused to *akt* sequences. LTR was identified from the cap site (nucleotide 384 of the LTR sequence) to nucleotide 526, one before the end of U5. In this figure, nucleotides 1–143 are from LTR, nucleotides 144–149 are of unknown origin and nucleotides 150– are from c-*akt*. Deduced amino acid sequences are shown starting with the methionine (*M*) residue following the nearest termination codon (*). Bold letters with *underlining*, amino acid sequence of pRL1b. *Underlined* sequences in *normal lettering*, primers for PCR, as shown in Table 1, used to investigate the genomic structure.
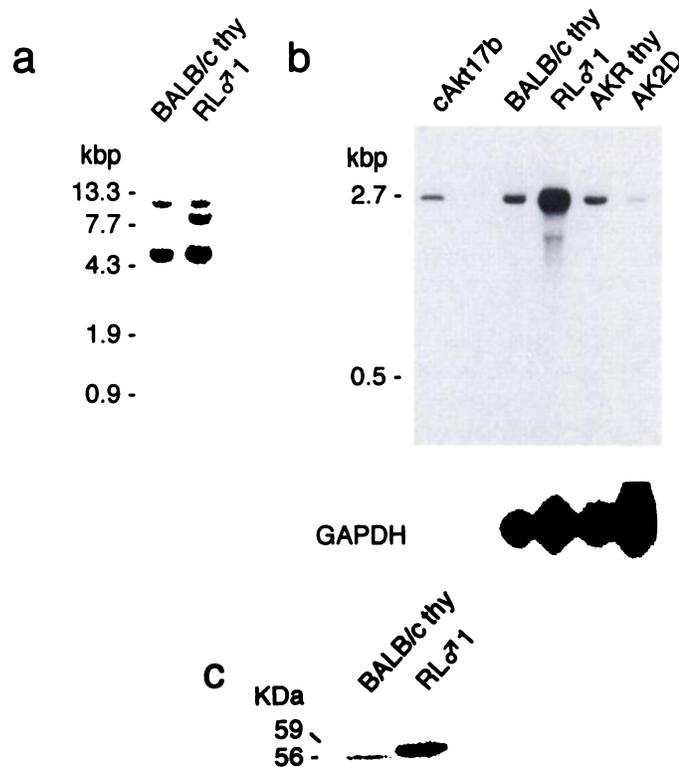
4781

Fig. 2. a, Southern blot analysis of the c-akt gene. Genomic DNA from BALB/c thymocytes or RLδ1 cells was digested with KpnI. A rearranged band (~8 kb) was observed in RLδ1 cells. b, Northern blot analysis. Expression of akt mRNA in RLδ1 cells was five times that in BALB/c cells as determined by densitometry. c, Western blot analysis. Expression of altered 59 KDa AKT molecules was detected in a lysate of RLδ1 cells but not of BALB/c thymocytes. kbp, kilobase pairs; KDa, kilodalton.

the ATC codon (Ile), indicating polymorphic divergence between the BALB/c and AKR mouse strains.

Discussion

Tumor rejection antigen peptides recognized by CTLs have been reported from several laboratories (7–19). These peptides are encoded by genes expressed in various tumors, but are silent in most normal tissues (7, 10), being expressed both in tumor and normal cell counterparts (e.g., melanoma and melanocyte, Refs. 12–18) or as mutated forms (19). Tumor antigen pRL1 peptides derived from an untrans-

lated region of the normally expressed akt gene represent a new class of tumor antigens.

Our findings show that the MuLV LTR sequence is inserted directly into the exon of c-akt in RLδ1 leukemia and transcription starts from the cap site of the LTR. Translation appeared to start from the ATG codon created in the six nucleotides of unknown origin which had been inserted into the LTR/akt junction. The deduced molecular size is approximately M_r 59,000 due to the addition of 33 amino acid residues to the normally expressed c-AKT protein. Western blot analysis demonstrated the presence of M_r 59,000 molecules in an RLδ1 lysate and their expression at about ten times the level of normal AKT molecules of M_r 56,000, consistent with the increased expression of akt mRNA demonstrated by Northern blot analysis. Thus, the LTR-driven expression of the altered form of c-AKT protein is the main mechanism for creation of the tumor rejection antigen peptides derived from the untranslated region of akt. The finding that M_r 56,000 AKT molecules were also present in RLδ1 indicated the hemiallelic occurrence of LTR insertion. This clearly demonstrated the involvement of a proto-oncogene product in the production of tumor rejection antigen peptides.

Many studies have shown that the LTR region of the viral genome is responsible for aberrant transcription of chromosomal proto-oncogenes during insertional activation (20–25). In particular, in a significant proportion of spontaneous AKR leukemias, retroviral insertion has been shown to occur near c-myc (23). Such abnormal gene expression is presumably involved in tumorigenesis. Thus, high expression of the altered form of c-AKT protein is likely to be one cause of leukemogenesis in RLδ1.

AKT protein has an NH₂-terminal domain (AKT homology domain), part of which is related to the Pleckstrin homology domain and a Ser/Thr protein kinase domain in its COOH-terminal half (4, 26). The physiological role of this kinase is presently unknown, but it may be involved in signal transduction through PI3 - kinase (26). Clarification of the role of this protein should be helpful for understanding the mechanisms of tumorigenesis and immunological responses to tumors.

We also demonstrated that codon AGC for the amino terminal Ser residue in pRL1b (SIIPGLPLSL) in RLδ1 is present in the corresponding position of mRNA from BALB/c thymocytes. In both thymocytes and leukemia (AK2D) cells from AKR mice, codon ATC for the Ile residue was detected at the corresponding site (data not shown), consistent with the published sequence of cDNA clone cAkt17b obtained from AKR thymocytes. Thus, the replacement of Ile by Ser at this site is a polymorphic difference between BALB/c and AKR mouse strains. The finding of no difference in sensitization activity for
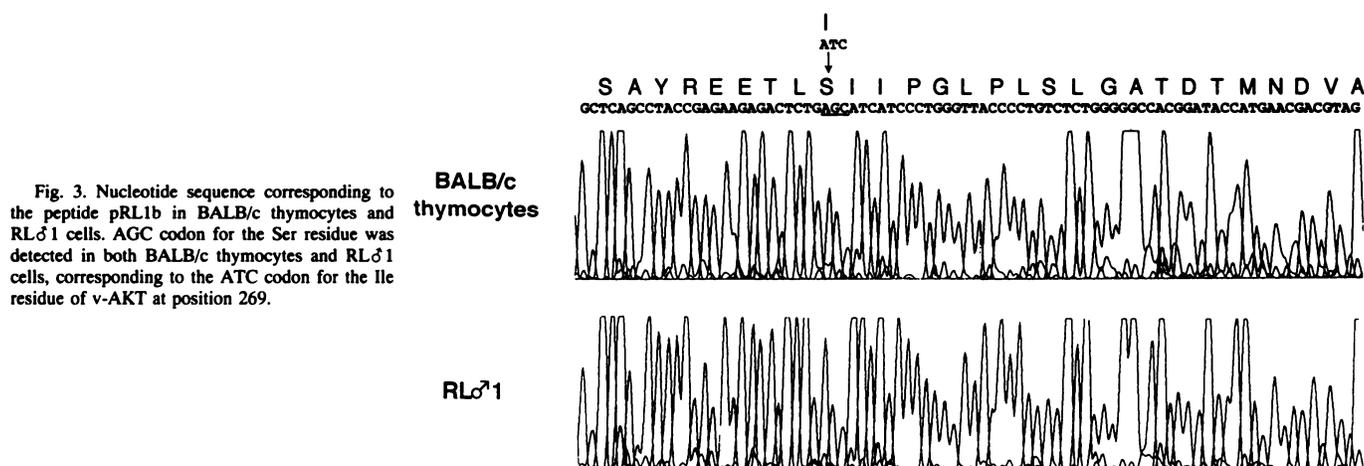

Fig. 3. Nucleotide sequence corresponding to the peptide pRL1b in BALB/c thymocytes and RLδ1 cells. AGC codon for the Ser residue was detected in both BALB/c thymocytes and RLδ1 cells, corresponding to the ATC codon for the Ile residue of v-AKT at position 269.

CTL recognition between SIIPGLPLSL and IIIPGLPLSL (3) indicates that the amino terminal Ser residue in pRL1b plays no significant role in creating an antigenic epitope. However, we do not know whether the Ser residue is involved in processing and creating the pRL1b peptide from altered AKT protein in RL♂1 cells.

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