TRBP, a regulator of cellular PKR and HIV-1 virus expression, interacts with Dicer and functions in RNA silencing

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SUPPLEMENTARY MATERIAL

MATERIALS AND METHODS

cDNA cloning and construction of plasmids

Cloning of TRBP cDNAs. TRBP2 cDNA was cloned by reverse transcription (RT) from HEK293 total RNA using GAGGACGGATCCTGGGCTGGCTGAGG as a primer and Superscript II Reverse Transcriptase kit (Invitrogen). The cDNA generated was used as a template for PCR using CCTAGCTCGTCGGCTGTGTATTGG as a sense primer and GTACCAGAGCTGCGCAGATACATGAGC as an antisense primer. PCR products were subcloned into PCRII-TOPO (Invitrogen) and sequenced. Among these clones cDNAs encoding TRBP2, TRBP1 and a new splice variant, referred to as TRBP3, were found. TRBP3 cDNA is missing exon 7, which changes the reading frame of the following exons and results in a translational stop signal within exon 8 (Fig 3D). Database searches revealed human ESTs gi|45695934, gi|46281253, gi|24041628, gi|12601190, gi|19091656 supporting the existence of such a splicing variant.

Cloning of pT-REx-DEST30-HA-TRBP2. Sequences encoding a hemagglutinin (HA) tag flanked by XhoI and SalI sites, and a NotI site, were introduced upstream and downstream of the coding region of TRBP2 by PCR, respectively. HA-TRBP2 was cloned into modified pT-REx-DEST30 (Invitrogen), using SalI and NotI restriction sites introduced into this plasmid.

Renilla luciferase (RL) reporter constructs, pRL-miR-17-Perf and pRL-control. pRL-miR-17-Perf is a derivative of pRL-control (obtained from R. Pillai of this laboratory). pRL-control expresses humanized RL, originating from phRLTK (Promega), under the control of a CMV promoter. To generate pRL-miR-17-Perf, oligonucleotides CTAGAACTACCTGCACTGTAAGCACTTTGC and TCGAGCAAAGTGCTTACAGTGCAGGTAGTT were annealed and ligated into XbaI and NotI sites within pRL-Con to create a perfect complementary site for miR-17-5p (referred to hereafter as miR-17) within the 3'UTR of RL (underlined is the sequence complementary to miR-17).

Constructs expressing anti-TRBP and control short hairpins (sh). Plasmids pTER-TRBPsh1 and pTER-TRBPsh2 were generated by cloning annealed synthetic 63-mer oligonucleotides into BglII and HindII sites within pTER (a kind gift of M. van de Wetering (van de Wetering et al., 2003)). Oligonucleotides GATCCCGCCTGGATGGTCTTCGAAATTCAAGAGATTTCGAAGACCATCCAGG CTTTTTGGAAA and AGCTTTTCCAAAAA GCCTGGATGGTCTTCGAAATCTCTTGAATTTCGAAGACCATCCAGGCGG were

used to generate pTER-TRBPsh1. pTER-TRBPsh2 was cloned using oligonucleotides GATCCCGGATTCTCTACGAAATTCATTCAAGAGATGAATTTCGTAGAGAATCC TTTTTGGAAA and AGCTTTTCCAAAAAGGATTCTCTACGAAATTCATCTCTTGAATGAATTTCGTA GAGAATCCGG. pTER-control-hairpin, cloned in an analogous way using GATCCCATTCTCCGAACGTGTCACGTTCAAGAGACGTGACACGTTCGGAGAA TTTTTTGGAAA and AGCTTTTCCAAAAAATTCTCCGAACGTGTCACGTCTCTTGAACGTGACACGTT CGGAGAATG oligonucleotides was kindly provided by K. Tang of this laboratory. Sense and antisense siRNA sequences within the hairpins are underlined.

Plasmids for two hybrid assays. pGADGH-CyclinT1, -TRBP, and -Tat and pGBT9-TRBP, -TRBP fragments, and -Tat have been described (Dorin et al., 2002; Battisti et al., 2003). To construct pGADGH-Dicer, the SalI-NotI (Klenow filled) fragment of pBluescript-Dicer-His_6 was inserted into SalI-XhoI (filled in) sites of pGADGH. The frame was restored by amplifying the SalI-DraIII fragment by PCR using CACGCGTCGACCATGAAAAGCCCTGCTTTGCATGGC as sense primer and GGTCAAGTGAGGCAGGTGAG as antisense primer to insert an additional C between the SalI site and the ATG. This SalI-DraIII fragment was inserted in the previous vector to obtain pGADGH-Dicer.

Other plasmids. Plasmids encoding Dicer-His_6, MBP-TRBP2, and MBP were described previously (Zhang et al., 2002; Dorin et al., 2003). pCIneo-HA-LacZ was kindly

provided by R. Pillai of this laboratory. pCMV-FL, encoding firefly luciferase (FL) was previously described (Pillai et al., 2004).

Cell culture and preparation of stable cell lines

Cell culture. HEK293 human embryonic kidney cells and P19 mouse teratoma cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL) supplemented with L-glutamine (2mM) and 10% heat inactivated fetal calf serum (FCS). HEK293-T-REx (Invitrogen) cells were grown similarly but in the presence of blasticidin (Invitrogen), according to the manufactures protocol.

293/HA-TRBP2, 293/HA-LacZ, 293/TRBPkd1, 293/TRBPkd2, 293/Ago2kd and 293/control-hairpin cell lines. To generate stably transfected tetracycline (Tet) inducible cell lines, HEK293-T-REx cells were co-transfected with a pBABE-puro (Clontech), encoding a puromycin resistance marker, and pT-REx-DEST30-HA-TRBP2, pTER-TRBPsh1 or pTER-TRBPsh2. Cells were grown in the presence of puromycin and blasticidin according to the manufacturers protocol to select stably transfected clones. Single clones were selected to generate monoclonal cell lines. 293/Ago2kd, expressing a short hairpin targeting human Ago2 was constructed similarly and kindly provided by D. Schmitter of this laboratory. Following treatment with Tet, expression of Ago2 was reduced approximately 80% in this cell line. To generate 293/HA-LacZ and 293/control-hairpin cells, pCIneo-HA-LacZ and pTER-control-hairpin, respectively, were co-transfected with pBABE-puro into HEK293-T-REx cells and a pool of stable transformants was selected.

SiRNA transfection. siCONTROL Lamin A/C siRNA (Dharmacon) was transfected into 293/TRBPkd, 293/Ago2kd and 293/control-hairpin cells using Lipofectamine 2000 (Invitrogen) at a final concentration of 20 nM according to the manufactures protocol. Cells were harvested 72 h after transfection. Whole cell extracts were separated by SDS-polyacrylamide gel electophoresis (SDS-PAGE) and analyzed by western blotting for lamin A/C, Dicer and α-tubulin.

Antibodies

Generation of anti-Dicer mAbs. A recombinant human Dicer His_6-tagged at the C-terminus was purified as described by Zhang et al., 2002. Final eluate was dialysed against immunization buffer (20 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM $MgCl_2$, 20% glycerol). Immunization of mice and isolation of hybridoma followed standard procedures (Harlow and Lane, 1998). Initial immunization (53 μg of Dicer per injection/per mouse) was followed by three boosts (25 μg each). Splenocytes were prepared, fused with myeloma (P3-X63-Ag8.653) cells and selected in HAT-medium. Hybridomas were subcloned and screened by ELISA and western blotting. Positive clones were selected and expanded in IMEM medium containing 10% IgG reduced FCS. mAbs were purified using protein G-sepharose (Amersham) and concentrated in PBS buffer with 50% glycerol. To map Dicer regions recognized by individual mAbs they were tested by western blotting using *Escherichia coli* cell extracts overexpressing deletion fragments of Dicer. mAbs 33 and 73 recognize the C-terminal part of Dicer

containing two RNaseIII domains and dsRBD. mAb 83 recognizes the central domain of Dicer encompassing the DUF283 and PAZ domains (data not shown).

Other antibodies. Following Abs were used for western analysis: anti-Dicer Ab347 (Billy et al., 2001), anti-TRBP672 (Duarte et al., 2000), anti-HA High Affinity (3F10) (Roche), anti-Lamin A/C (H-110) (Santa Cruz Biotechnology), and anti-α-tubulin (DM1A) mAb (Labvision/Neomarkers). For western analysis all antibodies except anti-α-tubulin were used at 1:1000 dilution; anti-α-tubulin was used at 1: 3000 dilution. Following Abs, in addition to anti-Dicer mAbs, were used for immunoprecipitations: anti-Dicer Ab347 (Billy et al., 2001), anti-HA (12CA5) mAb, anti-Myc (9E10) mAb. All co-immunoprecipitations were performed as described for anti-Dicer mAbs.

Two dimensional PAGE analysis and liquid chromatography tandem mass spectrometry (LC-MSMS) analysis

For LC-MSMS analyses, immunoprecipitates were analysed by one dimensional (1D)-PAGE gels, which were stained with a Colloidal Blue staining kit (Invitrogen). For two dimensional (2D)-PAGE analysis followed by LC-MSMS, immunoprecipitates were resuspended in 7M urea, 2M thiourea, 4% CHAPS, 1% DTT, 0.3% Pharmalytes 3-10. After isoelectric focusing on Immobile DryStrips (pI 4.0-7, linear) (Amersham Bioscience) using IPGphor, proteins were separated by 10% SDS PAGE. The gels were stained using Silver Quest silver staining kit (Invitrogen) and analyzed by Proteome Weaver (Definiens). Protein containing gel fragments, were digested with trypsin according to Schrimpf et al. (2001) and analyzed by LC-MSMS (LCQ Deca XP, Thermo

Finnigan). Proteins were identified using Turbo Sequest and MASCOT, searching SwissProt database restricted to human proteins.

Northern blotting and real time PCR

miRNA northern blots. Oligonucleotide probes complementary to miR-17 CTAGAACTACCTGCACTGTAAGCACTTTGC and miR-16 CTAGAACTACCTGCACTGTAAGCACTTTGC (miRNA-specific sequences are underlined), were 5′-end-labeled, using T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP. U6 snRNA was quantified using CGTTCCAATTTTAGTATATGTGCTGCCGAAGCGAGCAC oligonucleotide probe to normalize signals for equal loading. Total cellular RNA was isolated using TRIZOL reagent (Invitrogen), separated by 12% Urea-PAGE and transferred to Hybond N+ membranes (Amersham). Prehybridisation and hybridization solutions contained 2x SSPE, 5x Denhardt's solution, 0.1% SDS and 20% formamide. After prehybridisation for 3 h, hybridization with specific labeled antisense oligonucleotides was performed at 37 °C for at least 5 h. Membranes were washed 5 times with 2x SSPE, 0.2% SDS. Quantifications were done using Storm 860 PhosphorImager and ImageQuant (Molecular Dynamics). To determine miRNA abundance in 293/TRBPkd cell lines, two independent RNA preparations have been analyzed in quadruplicates with different probes and P-values have been calculated (T-test: 1-tailed; equal variance).

Real Time PCR. Knock down and control cells were induced with 2 μg/ml Tet for 1 day. Total RNA was isolated using TRIZOL reagent. Reverse transcription was performed with random hexamers and oligo-dT primers using Taq Man Reverse transcription

Reagents (Roche). For detection of mRNAs, Hs00366328_m1 and GAPDH Hs99999905_m1 Taq Man Gene Expression Assays (Applied Biosystems) were used in combination with Taq Man universal PCR Mix. ABI Prism 7000 (Applied Biosystems) was used for analysis and quantification. TRBP mRNA levels were normalized to GAPDH mRNA levels.

Recombinant proteins and *in vitro* interaction

Protein expression and purification. Recombinant Dicer was purified as described by Zhang et al., (2002). MBP-TRBP2 and MBP were expressed in *Escherichia coli* BL21(DE3) cells and purified as described by Dorin et al., (2002). Purity of the proteins was analysed by 8% SDS-PAGE. Protein concentration was determined by a Bradford method.

In vitro protein interaction. Purified recombinant proteins were mixed in buffer containing 30 mM Tris-HCl, pH7.5, 1 mM $MgCl_2$, 150 mM NaCl, 0.2% NP-40 and 10% glycerol and incubated at 25 °C for 1 h. The reaction was split in two and to one half anti-Dicer antibody was added, incubated at 25 °C for 30 min and applied to protein G-sepharose (Amersham). Similar incubation with anti-HA antibody served as a control. To the second half, amylose beads (New England Biolabs) were added. Samples were incubated for additional 1 h at 25 °C. Reactions were spun down, washed 4 times with binding buffer and eluted in a SDS-PAGE sample buffer. Eluates were resolved by 8% SDS-PAGE and analysed by western blotting.

Preparation of RNA Substrates and RNA processing assays

Preparation of RNA substrates. The internally ^{32}P-labeled 30-bp dsRNA containing 2-nt 3' protruding ends was prepared essentially as described before (Zhang et al., 2002). RNAs were synthesised by the T7 polymerase *in vitro* transcription, using the Ambion T7 MaxiScript transcription kit and [α-^{32}P]UTP. After transcription, samples were treated with DNase I, extracted with phenol, and RNA purified by denaturating 8% PAGE. Following dephosphorylation by calf intestine phosphatase (CIP), RNAs were 5′-end-phosphorylated, using T4 polynucleotide kinase and ATP. Complementary RNA strands were annealed at 95 °C for 3 min in 20 mM NaCl, transferred to 75 °C, and then slowly cooled down to 20 °C. The internally ^{32}P-labeled pre-let-7 RNA bearing authentic 5' and 3' termini was prepared as described (Kolb et al., 2005; Zhang et al., 2004) Prior to use, pre-let-7 RNA was dissolved in water and renaturated by incubation at 90 °C for 1 min, followed by incubation at 25 °C for 15 min in 30 mM Tris-HCl, pH 6.8 containing 50 mM NaCl, 2 mM $MgCl_2$ and 10% glycerol.

RNA Processing Assays. For preparation of S10 cell extracts used in pre-let7 RNA processing, HEK293 cells were lysed in buffer containing 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.25% NP40 and 1.5 mM $MgCl_2$, and centrifuged at 10,000 x g for 10 min at 4 °C. Protein concentration in the extracts was adjusted to 5 mg/ml using the lysis buffer. Processing assays (50 µl) were carried out as previously described (Zhang et al. 2004, 2002). 3-5 fmol of ^{32}P-labeled substrate was incubated with a cytoplasmic S10 extract (final concentration 2.5 mg/ml) in buffer containing 20 mM Tris-HCl pH 7.5, 2 mM $MgCl_2$, 75 mM NaCl and 10% glycerol at 37 °C. RNA was extracted with

phenol/chloroform and analyzed by 10% denaturing PAGE. Quantification was done using the Storm 860 PhosphorImager.

Gradient sedimentation, luciferase reporter assays and yeast two hybrid analysis

Glycerol gradients. S10 extracts, prepared as described above, were separated on 10-30% glycerol gradients by centrifugation at 36,000 rpm for 20 h in a Beckman SW41 Ti rotor at 4 °C, according to Zhang et al., (2004). Fractions were collected and processed for western analysis with specific anti-Dicer and anti-TRBP antibodies. For northern blot analysis gradient fractions were extracted with phenol and chloroform, precipitated with sodium acetate and ethanol, separated by 12% Urea-PAGE, and transferred to Hybond N+ membranes for hybridization with specific oligonucleotide probes. For Dicer cleavage activity assays, Dicer was immunoprecipitated from pooled gradient fractions using anti-Dicer mAb 73 cross-linked to Protein G-Sepharose beads. Immunoprecipitates were washed with 20 mM Tris-HCl, pH 7.5, 300 mM NaCl, 0.5% NP40, 2.5 mM $MgCl_2$ and resuspended in 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2.5 mM $MgCl_2$. Dicer cleavage activity was measured using internally ^{32}P-labeled 30-bp dsRNA containing 2-nt 3' protruding ends, as described above. Catalase and ferritin (HMW Gel Filtration Calibration kit, Amersham Bioscience) were sedimented on parallel gradients as molecular mass markers.

Luciferase reporter assays. Stable cell lines were induced for 3 days with 2 μg/ml Tet and transfected in 24-well plates with indicated RL constructs and pCMV-FL using Lipofectamine PLUS reagent (Invitrogen). 5 ng of pRL-miR-17 or 5 ng of pRL-control

together with 20 ng of pCMV-FL were co-transfected into one 24-well-plate. pRL-control, lacking the binding site for miRNA, was used to measure RL expression from plasmids devoid of miRNA-binding sites. Cells were lysed 48 h after transfection and luciferase activities were measured using the Dual-Luciferase Reporter System (Promega) as recommended in the manufactures instructions. All RL activities were normalized to FL activites to correct for transfection efficiency (RL/FL). Four transfections were performed per each individual experiment. P-values were calculated (T-test: one-tailed; equal variance).

Yeast two-hybrid assay. Yeast expression plasmids were introduced into the yeast reporter strain SFY526. The double transformants were selected and screened for β-galactosidase activity as described by Daher et al., (2001).

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Supplementary figure legends

Fig S1. Specificity of anti-Dicer mAbs (A) and identification of TRBP in Dicer immunoprecipitates by LC-MSMS analysis (B and C). (**A**) anti-Dicer mAbs 33, 73 and 83 recognize Dicer in HeLa cell S10 extracts. Hybridoma supernatants were used at 1:10 dilution for western blotting. (**B**) Silver staining of two-dimensional PAGE demonstrating that TRBP is specifically co-immunoprecipitated by anti-Dicer mAb (left upper panel) but not control anti-Myc mAb (right upper panel). Spots yielding Dicer and TRBP peptides as well as IgG spots are indicated. (**C**) Sequence of TRBP2 (Swiss-Prot Q9BRY2) with TRBP peptides from three independent LC-MSMS experiments shown in bold.

Fig S2. Levels of TRBP mRNA in HEK293 TRBP knock down and 293T-REx control cell lines. RNA levels were determined by real time PCR. Values of the bars are means of two experiments, with the range indicated.

Fig S3. Sequence alignment (**A**) and phylogenetic tree (**B**) of dsRBDs of human TRBP2 and related proteins. PRBP is mouse homolog of TRBP2. The two proteins are 93% identical and 94% similar. In (A) dsRDB regions are indicated by black bars. Abbreviations used: hs, *Homo sapiens*; mm, *Mus musculus*; dm, *Drosophila melanogaster*; ce, *Caenorhabditis elegans*. Alignment and phylogenetic tree were prepared using ClustalW (http://www.ebi.ac.uk/clustalw/).

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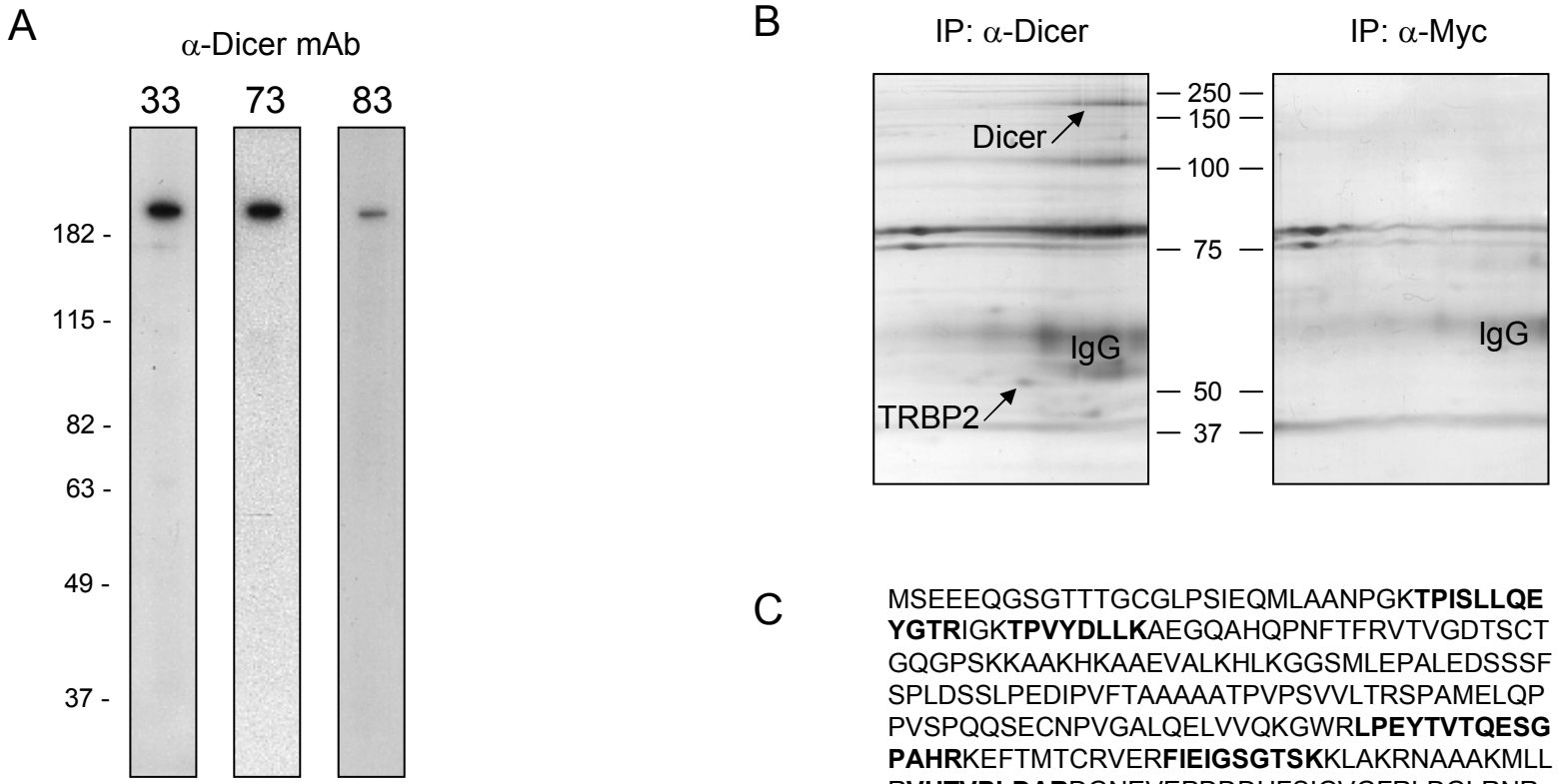

C

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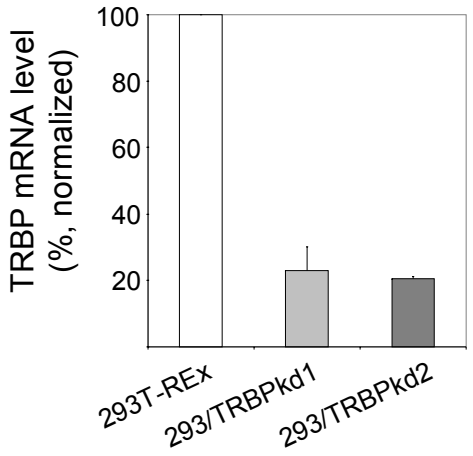

Fig. S3
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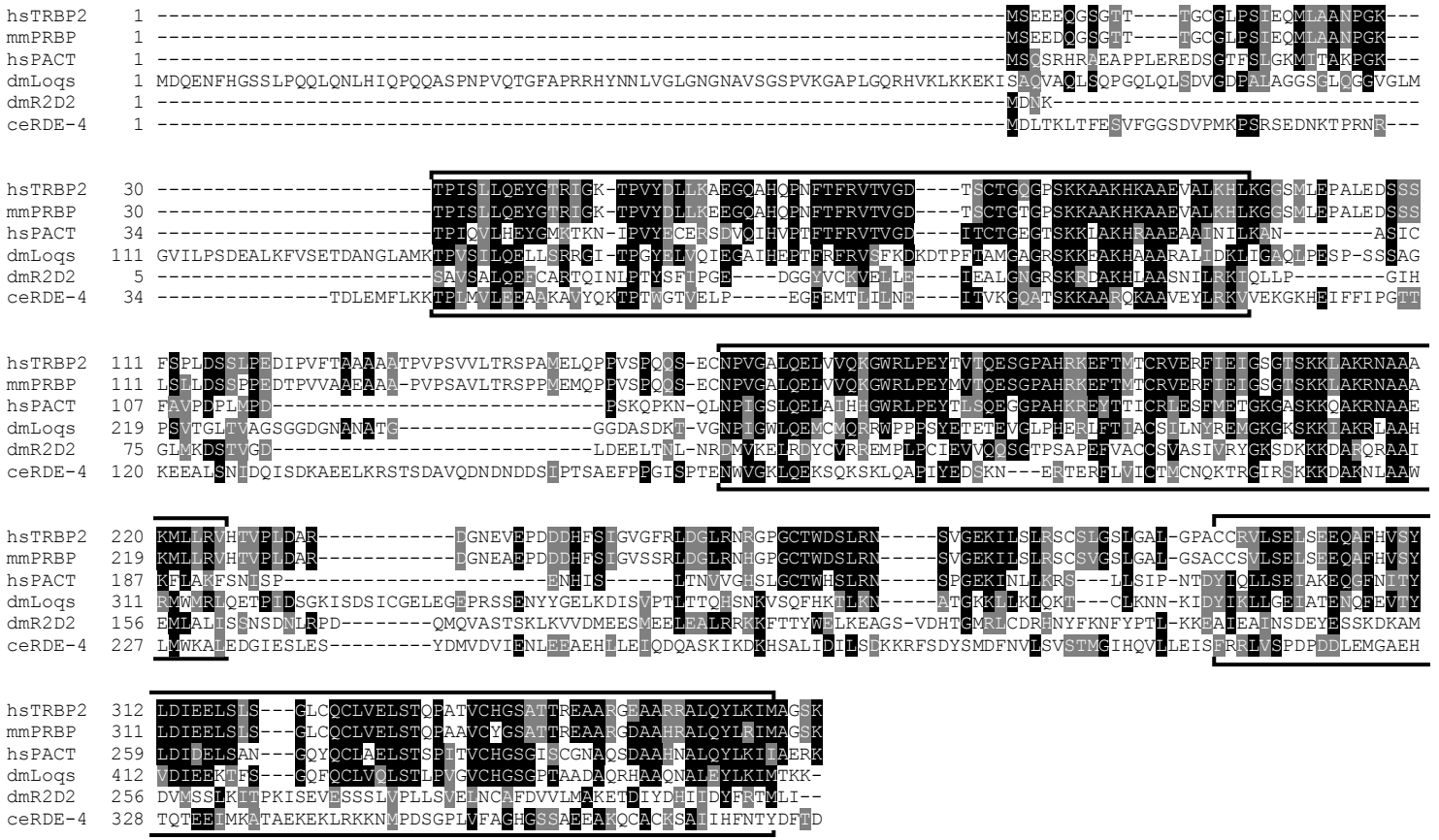
A



B