Alternative splicing variants of dual specificity tyrosine phosphorylated and regulated kinase 1B exhibit distinct patterns of expression and functional properties

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The dual specificity tyrosine phosphorylated and regulated kinase (DYRK) family of protein kinases is a group of evolutionarily conserved protein kinases that have been characterized as regulators of growth and development in mammals, *Drosophila* and lower eukaryotes. In the present study, we have characterized three splicing variants of DYRK1B (DYRK1B-p65, DYRK1B-p69 and DYRK1B-p75) with different expression patterns and enzymic activities. DYRK1B-p65 and DYRK1B-p69 exhibited similar, but not identical, patterns of expression in mouse tissues, with the highest protein levels found in the spleen, lung, brain, bladder, stomach and testis. In contrast, DYRK1B-p75 was detected specifically in skeletal muscles, in the neuronal cell line GT1-7 and also in differentiated, adipocyte-like 3T3-L1 cells, but not in undifferentiated 3T3-L1 preadipocytes. A comparison of the mouse and human *Dyrk1b* genomic and cDNA sequences defined the alternative splicing events that produce the variants of DYRK1B. In DYRK1B-p75, transcription starts with exon 1B instead of exon 1A, generating a new translation start, which extends the open reading frame by 60 codons. This gene structure suggests that alternative promoters direct the expression of DYRK1B-p69 and DYRK1B-p75. Both splicing variants exhibited kinase activity *in vitro* and contained phosphotyrosine when expressed in COS-7 cells. Owing to differential recognition of the 3′-splice site in exon 9, DYRK1B-p65 differs from DYRK1B-p69 by the absence of 40 amino acids within the catalytic domain. DYRK1B-p65 lacked kinase activity *in vitro* and did not contain phosphotyrosine. DYRK1B-p69 and DYRK1B-p75 stimulated reporter gene activity driven by the **f**or**kh**ead in **r**habdosarcoma (FKHR)-dependent glucose-6-phosphatase promoter more strongly when compared with DYRK1B-p65, indicating that the DYRK1B splicing variants exhibit functional differences.

Key words: alternative splicing, dual specificity tyrosine phosphorylated and regulated kinase-related kinase, expression pattern, gene structure.

INTRODUCTION

Dual specificity tyrosine phosphorylated and regulated kinase (DYRK) is a family of evolutionarily conserved protein kinases that are involved in the control of growth and development [1]. Five members of the DYRK family have been identified in humans (DYRK1A, DYRK1B, DYRK2, DYRK3 and DYRK4) [2,3]. The human *DYRK1A* gene has been suggested to be responsible for alterations in brain development associated with Down's syndrome. This hypothesis is based on the localization of *DYRK1A* in the 'Down's syndrome critical region' of chromosome 21 and its homology with the *minibrain* gene in *Drosophila*, whose mutation leads to defects in neurogenesis [4–7]. Transgenic mice that overexpress DYRK1A exhibit neurodevelopmental delay and cognitive deficits, supporting a role of DYRK1A in brain development [8].

DYRK1B shares 85 % identical amino acid residues with DYRK1A in the catalytic domain but displays no sequence similarity in the C-terminal domain [3]. Both kinases harbour a conserved nuclear localization signal in the N-terminal domain and are indeed targeted to the nucleus, whereas DYRK2 and DYRK3 are localized in the cytoplasm [2]. In contrast with the *DYRK1A* gene which is ubiquitously expressed in human and mouse tissues, *DYRK1B* exhibits a more restricted pattern of expression, with the highest mRNA levels in the testis and muscles [3]. DYRK1B has been described to support the survival of colon carcinoma cells in the absence of growth factors, and was found to be up-regulated in serum-free medium [9]. In these cells, transfection of DYRK1B caused an enhanced catalytic-centre activity (turnover) of cell-cycle regulators [10].

All members of the DYRK family are characterized by a conserved tyrosine residue in the activation loop of the catalytic domain, and phosphorylation of this tyrosine residue is required for the full activity of DYRK1A [11]. In contrast with the mitogen-activated protein kinases, tyrosine phosphorylation in the activation loop of DYRK appears to be due to autocatalysis, and its role in the regulation of DYRK1A is not yet clear. However, Yang et al. [12] reported that basic fibroblast growth factor stimulated tyrosine phosphorylation of DYRK1A and the DYRK1A-mediated phosphorylation of cAMP-response-element-binding protein (CREB) in an immortalized neuronal cell line. In addition to CREB, three other transcription factors [signal transducer and activator of transcription 3 (STAT3), **f**or**kh**ead in **r**habdosarcoma (FKHR) and GLI1 (glioma-associated oncoprotein)] [13–15] have

Abbreviations used: CREB, cAMP-response-element-binding protein; DYRK, dual specificity tyrosine phosphorylated and regulated kinase; EST, expressed sequence tag; FKHR, **f**or**kh**ead in **r**habdosarcoma (a transcription factor); NP40, Nonidet P40; ORF, open reading frame; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase.

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been identified as substrates of DYRK1A. DYRK1B has been described to phosphorylate and activate hepatic nuclear factor 1α [16]. However, the precise role of DYRK1A and DYRK1B in the regulation of transcription is not known.

In the present study, we have identified isoforms of DYRK1B that are generated by the use of separate promoters and alternative splicing. These splicing variants exhibit different patterns of expression in tissues and cell lines. Functional characterization showed that the splicing variants differ in their catalytic activity and in their ability to stimulate reporter gene activity driven by the glucose-6-phosphatase promoter.

MATERIALS AND METHODS

Antibodies

A peptide corresponding to the C-terminus of human or mouse DYRK1B (RLGLHGVPQSTAASS) was used to raise a DYRK1B-specific rabbit antiserum (BioScience, Göttingen, Germany). The phosphotyrosine-specific antibody PY99 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.).

Expression plasmids

To construct expression plasmids for human DYRK1B-p69, DYRK1B-p66 and DYRK1B-p65, the coding regions of these cDNAs were amplified from a human testis library and inserted into the vector pEGFP-C1 (Clontech, Palo Alto, CA, U.S.A.). The inserts were completely sequenced to ensure the fidelity of the PCR. In a second step, the green fluorescent protein-encoding cDNA was deleted with the appropriate restriction enzymes (*Nhe*I and *Bsr*GI). The human cDNA for DYRK1B-p75 was purchased as a full-length cDNA clone (clone ID CS0DC003YP16; GenBank® Nucleotide Sequence Database accession no. Al524061) from Research Genetics (Huntsville, AL, U.S.A.); this plasmid contained the full open reading frame (ORF) for DYRK1B-p75 in the mammalian expression vector pCMV-SPORT6. For direct comparison of different DYRK1B variants in promoter assays, DYRK1B-p69 and DYRK1B-p65 were also cloned into pCMV-SPORT6.

Cloning and sequencing of the mouse *Dyrk1b* gene

Genomic clones of *Dyrk1b* were isolated by screening a 129SvJ mouse genomic library (Stratagene, La Jolla, CA, U.S.A.). Overlapping restriction fragments from the inserts of three phage clones were subcloned into pGEM5Zf(+); the complete sequences of the inserts of these plasmids were determined with the aid of an automated sequencer (Li-COR, Lincoln, NE, U.S.A.).

Reverse transcriptase (RT)–PCR

Total RNA was isolated from 3T3-L1 cells by lysis in 4 M guanidinium thiocyanate and centrifugation through a cushion of CsCl (5.88 M). First-strand cDNA was synthesized from 5 μg of total RNA in a volume of 15 μl by oligo(dT) priming (First-strand cDNA Synthesis kit; Amersham Pharmacia, Freiburg, Germany). For specific detection of the transcripts for DYRK1B-p69 and DYRK1B-p75, we designed oligonucleotide primers that specifically matched exon 1A (forward primer 1A, 5′-CAGTGGGCAGGTCTGAGCTCG-3′), exon 1B (forward primer 1B, 5′-GAGCTAGCACCACCGCCGCCATGC-3′) and exon 3 (reverse primer 3, 5′-GGCAGTAGCCGCACATCAGG-3′) in both mouse and human genes. PCR was run for 35 cycles (60 s at 95 °C, 30 s at 65 °C and 30 s at 72 °C) with the JumpStart AccuTaq™ Polymerase (Sigma–Aldrich, Taufkirchen, Germany). The identity of the PCR products was verified by sequencing. Rapid amplification of cDNA ends (RACE) PCR was performed using mouse skeletal-muscle cDNA (Marathon-Ready cDNA; Clontech) and two nested gene-specific primers (reverse primer 3 and D1Bp75RACE2, 5′-GCCGCTGAGACCCGGGTCAG-3′).

Cell culture, transient transfections and cell lysis

Differentiation of the confluent 3T3-L1 fibroblasts (F_0) was induced by treatment with isobutylmethylxanthine (0.5 mM), dexamethasone (0.25 μM) and insulin (1 μg/ml) for 3 days followed by treatment with insulin for 4 days [17]. The mouse hypothalamic GT1-7 cell line was cultivated as described previously [18]. For Western blotting, cells were lysed at 96 °C in an SDS buffer (1 % SDS/20 mM Tris/HCl, pH 7.4), and lysates were then briefly sonicated (15 s) to reduce viscosity. For immunoprecipitations, cells were washed with cold PBS supplemented with 1 mM Na_3VO_4 and harvested in ice-cold lysis buffer [50 mM Hepes (pH 7.4), 150 mM NaCl, 2 mM EDTA, 30 mM sodium pyrophosphate, 25 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin and 1 % Nonidet P40 (NP40)].

Immunoblotting

For Western-blot analysis, mouse tissues and embryos were homogenized by 10 strokes in a 5 ml Potter homogenizer in a non-denaturing lysis buffer (20 mM Tris/HCl, pH 7.4). Commercial antibodies were used according to the supplier's instructions. The anti-DYRK1B antiserum was used at a dilution of 1:5000 for 2 h at room temperature (20–25 °C) or overnight at 4 °C. For detection of DYRK1B, the washing solution (Tris-buffered saline) was supplemented with 1 % NP40 and 0.1 % SDS to reduce non-specific binding. The bound primary antibodies were detected by chemiluminescence produced using peroxidase-conjugated secondary antibodies. If required (Figure 1), blots were prepared for reprobing by incubation in Tris-buffered saline supplemented with 100 mM 2-mercaptoethanol and 2 % SDS at 50 °C for 30 min.

Immunoprecipitation and kinase assay

Total cellular lysates were cleared by centrifugation at 13 000 ***g*** and incubated with 1 μl of crude antiserum for 1 h at 4 °C. After the addition of 15 μl of packed Protein A–Sepharose, the suspension was incubated at 4 °C for 1 h on a rotating shaker, briefly centrifuged and the supernatant discarded. The resin was washed twice with immunoprecipitation buffer with detergent [50 mM Hepes (pH 7.4)/150 mM NaCl/2 mM EDTA/0.1 % NP40] and twice without detergent. The resin was thereafter either incubated in Laemmli's sample buffer (5 min at 96 °C) to analyse the bound protein by Western blotting, or used for kinase assays. Kinase activity was assayed for 20 min at 30 °C in 20 μl of kinase buffer [25 mM Hepes (pH 7.4)/5 mM MgCl/5 mM MnCl/0.5 mM dithiothreitol], supplemented with 100 μM [γ-^{32}P]ATP using a synthetic peptide, termed DYRKtide (at 200 μM), as a substrate [19]. Incorporation of ^{32}P into the peptide was determined by the phosphocellulose method as described previously [19].

Luciferase assays

A DNA fragment derived from the 5′-flanking region of the mouse *Dyrk1b* gene (bp -245 to $+155$) was cloned upstream

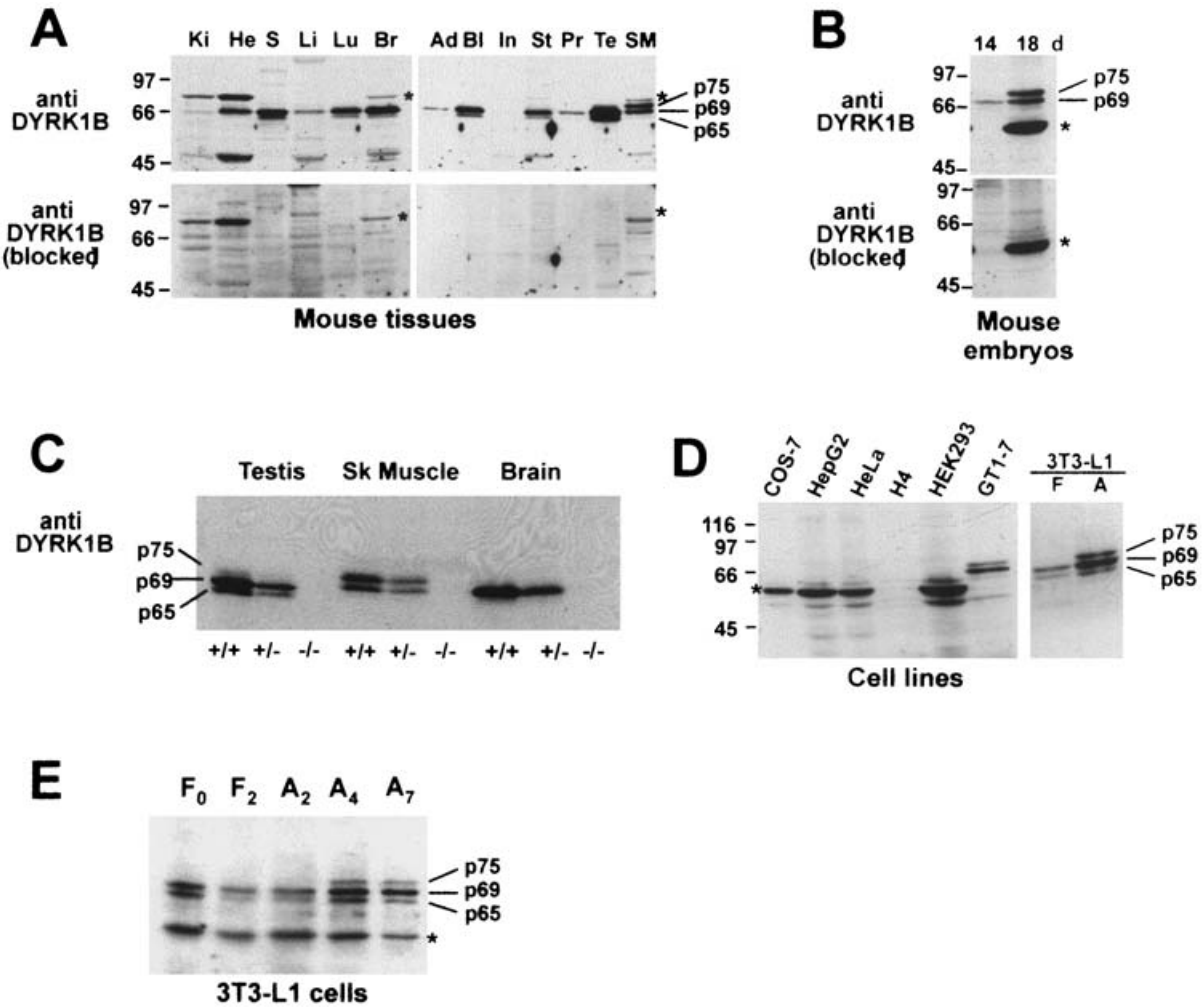

Figure 1 Western-blot analysis of DYRK1B in mouse tissues and clonal cell lines

(**A**, **B**) Western blots of total cellular protein from the indicated mouse tissues (**A**: Ki, kidney; He, heart; S, spleen; Li, liver; Lu, lung; Br, brain; Ad, adrenal, Bl, bladder; In, intestine; St, stomach; Pr, prostate; Te, testis; SM, skeletal muscles) or from total mouse embryo (**B**: 14 or 18 days post conception). (**C**) Western blot of tissues from a homozygous DYRK1B knockout mouse (−/−) in comparison with a wild-type (+/+) and a heterozygous mouse (+/−). (**D**, **E**) Western blots of cultivated cell lines. 3T3-L1 cells were differentiated as described in the Materials and methods section. Differentiation of the confluent fibroblasts (F_0) was initiated on day 0. Cells were treated according to the differentiation protocol for 2, 4 or 7 days (A_2, A_4 and A_7 respectively) or were cultured for 2 days under non-inducing conditions (F_2). Blots were developed with DYRK1B-specific antiserum or with blocked antiserum that was preincubated with the antigenic peptide (50 μg/ml). Asterisks indicate unspecific bands.

of the luciferase cDNA into the vector pGL3 (Promega, Madison, WI, U.S.A.). A luciferase reporter plasmid containing the human glucose-6-phosphatase promotor (bp − 1227 to + 57) was kindly provided by Dr Schmoll (University of Greifswald, Greifswald, Germany) [20]. For reporter gene assays, COS-7 cells, on 6-well plates, were co-transfected with 0.6 μg of the luciferase reporter construct, 0.2 μg of pEGFP-FKHR [21] and different amounts of the pCMV-SPORT6-DYRK1B expression plasmids. The total amount of pCMV-SPORT6 DNA was kept constant at 0.8 μg of DNA by adding the required amount of empty vector. Cells were lysed 42 h after transfection and luciferase activity was assayed using a commercial kit (Promega). Data were normalized to the total protein content of the lysates, and aliquots of the lysates were analysed by Western blotting for comparable expression of the DYRK1B variants. Luciferase assays in HepG2 cells were performed as described previously [26]; these data were normalized to co-expressed *Renilla* luciferase. Data were analysed for significance with the two-tailed Student's *t* test for paired samples, and differences were considered significant at $P < 0.05$.

RESULTS

Detection of three DYRK1B variants in mouse tissues and cell lines

To analyse the expression pattern of DYRK1B in mouse tissues, a DYRK1B-specific rabbit antiserum was raised against a synthetic peptide representing 15 C-terminal amino acids of DYRK1B. On a Western blot containing total cellular extracts from a panel of mouse tissues, two specific bands of approx. 65 and 70 kDa were detected in most tissues, with the highest levels in the testis, brain, spleen, lung, stomach, skeletal muscles and heart (Figure 1A). The electrophoretic mobilities of these proteins corresponded well to the deduced masses (64.9 and 69.2 kDa) of two splicing products of DYRK1B, which we have described previously [3]. We therefore tentatively refer to these proteins as p65 and p69

respectively. An additional band of approx. 75 kDa (p75) was detected exclusively in skeletal muscles. These three bands were considered specific because they did not react with the antiserum that was preincubated with an excess of the antigenic peptide. In total mouse embryos, only p69 was weakly detectable 14 days post conception, whereas both p69 and p75 were present at day 18 (Figure 1B). To verify directly that the p75 band detected by the DYRK1B-directed antiserum does indeed correspond to a gene product of *Dyrk1b*, we took advantage of a transgenic mouse strain with a targeted deletion of the *Dyrk1b* gene (S. Leder, B. Maenz, M. Moser, R. Kluge, H.-G. Joost and W. Becker, unpublished work). Western blotting showed the complete absence of p65, p69 and p75 in homozygous knockout mice, indicating that these three bands represent gene products of *Dyrk1b* (Figure 1C). An analysis of cell lines for the expression of DYRK1B identified the p75 form of DYRK1B in two cell lines from mouse, the hypothalamic GT1-7 cells and the adipocyte-like, differentiated 3T3-L1 cells. Undifferentiated 3T3-L1 preadipocytes contained only p65 and p69, and no specific bands were detected in the other cell lines tested (COS-7, HepG2, HeLa, H4 and HEK-293) (Figure 1D). To characterize further the differential expression of the DYRK1B variants, the time course of induction of p75 during differentiation of the 3T3-L1 cells was determined. As shown in Figure 1(E), p75 was detected on day 4 after the initiation of differentiation. The lag phase between administration of the differentiation-inducing agents (insulin, dexamethasone and isobutylmethylxanthine) and the appearance of p75 suggests that the expression of this protein species is not directly induced by the agents, but rather correlates with the transition of cells to the adipocyte-like phenotype.

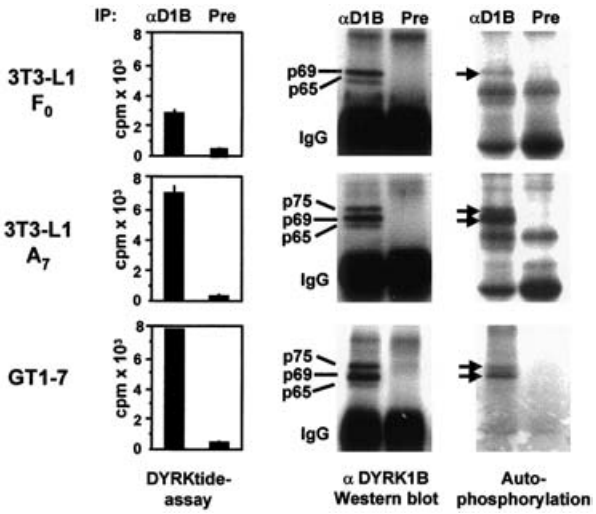

Figure 2 Kinase activity of DYRK1B in 3T3-L1 and GT1-7 cells

DYRKtide assays were performed with DYRK1B after immunoprecipitation with DYRK1B-specific antiserum (αD1B) or preimmune serum (Pre) from confluent 3T3-L1 fibroblasts (F_0), from fully differentiated, adipocyte-like 3T3-L1 cells (A_7) and from GT1-7 cells. Proteins bound to the Protein A–Sepharose were subjected to SDS/PAGE, blotted and analysed by autoradiography for incorporation of ^{32}P. Subsequently, bands corresponding to DYRK1B variants were identified by immunodetection.

Protein kinase activity of the DYRK1B variants

Immunocomplex kinase assays were performed to analyse the catalytic activity of the DYRK1B variants. After immunoprecipitation, the kinase activity was determined by assaying the incorporation of ^{32}P into the peptide substrate DYRKtide [20]. To analyse autophosphorylation of the kinases, the bound proteins were separated by SDS/PAGE, transferred to nitrocellulose membrane and radioactive bands were detected by autoradiography. The same blot was then subjected to immunodetection with the DYRK1B-specific antiserum. As shown in Figure 2, kinase activity was immunoprecipitated from undifferentiated and differentiated 3T3-L1 cells and from GT1-7 cells. Both p65 and p69 were immunoprecipitated from the undifferentiated cells, but only p69 was labelled with ^{32}P. In differentiated cells and GT1-7 cells, all three forms of DYRK1B were found in the pellet, and p69 and p75 contained ^{32}P. Quite probably, phosphate incorporation in this *in vitro* assay reflects an intramolecular autophosphorylation reaction. Therefore these results suggest that p69 and p75 represent active protein kinases, whereas p65 appears to be incapable of catalysing its autophosphorylation.

Structure of the mouse *Dyrk1B* gene

For a full characterization of the *DYRK1B* gene, the mouse gene was cloned and completely sequenced, and the exon–intron organization was defined by comparison with cDNA sequences (Figures 3A and 3B). Comparison of the genomic sequence with the mouse cDNA sequence described previously, which encodes a 69 kDa protein (GenBank® accession no. Y18280 [3]), defined exons 1A and 2–11. In addition, database searching identified two human expressed sequence tag (EST) sequences (GenBank® accession nos. N95477 and AI524061) with an alternative 5′-end that matches a sequence located between exon 1A and exon 2 (Figure 3B). This sequence was designated exon 1B. All attempts to detect transcripts with both exons 1A and 1B by PCR with specific primers failed, suggesting that the transcription of exon 1B-containing mRNA is driven by a separate promoter. Fusion of exon 1B with exon 2 introduces a new start codon and thereby extends the ORF by 60 amino acids (Figures 3B and 3C). The calculated mass of the protein encoded by this ORF is 75.1 kDa, corresponding exactly to the apparent molecular mass of the p75 variant identified by Western blotting. Thus these findings are consistent with the conclusion that p69 and p75 represent alternative splicing products that are generated by separate promoters.

By cDNA sequencing, we had identified previously [3] two splicing variants of 65 and 66 kDa that differ from p69 by the deletion of 28 or 40 amino acids in the catalytic domain. Comparison of these cDNA sequences with the gene sequence indicates that DYRK1B-p65 is generated by splicing of exon 8 to an alternative 3′ splice site within exon 9, whereas the excision of a short segment (74 bases) from exon 9 as a facultative intron results in the deletion of 28 amino acids in DYRK1B-p66 (Figure 3D). The corresponding splicing variants of the exon 1B-containing mRNA encode proteins of 70.9 and 72.2 kDa, which were designated DYRK1B-p71 and DYRK1B-p72 respectively. The existence of a transcript for DYRK1B-p72 was confirmed by PCR with specific primers on cDNA from 3T3-L1 adipocytes (results not shown).

The *Dyrk1b* gene spans approx. 10 kb and is located adjacent to the gene for fibrillarin (*Fbl*; Figure 3A). The mouse *Dyrk1b* gene and the human *DYRK1B* gene are identical with regard to exon number and size. Its exon–intron organization is similar to that of *DYRK1A* [22] except for the exons that encode the divergent N-termini of the kinases. Four exons are also conserved in the homologous *Drosophila* gene for the minibrain kinase (*mnb*) [7]; however, the C-terminal part of the catalytic domain of MNB is encoded by a single exon (exon 8) instead of

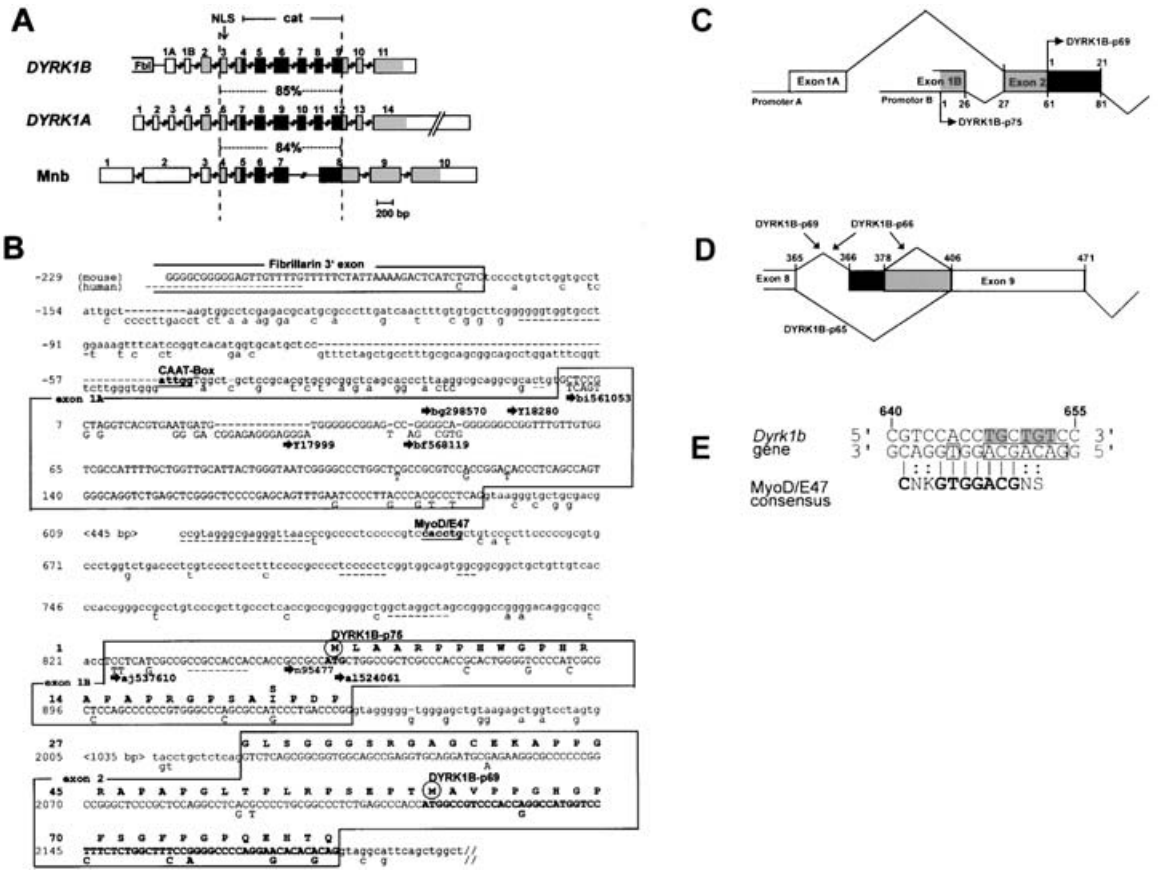

Figure 3 The mouse *Dyrk1b* gene and transcripts

(**A**) Schematic representation of the exon–intron structure of the *DYRK1B* gene in comparison with *DYRK1A* and *Drosophila mnb*. Boxes represent exons, identified by comparison with cDNA sequences; the coding sequences are shown as shaded boxes (non-catalytic domains) or filled boxes (catalytic domain). The region of sequence conservation between DYRK1A, DYRK1B and MNB is indicated, and the percentages of identical amino acids are given. Only exons are drawn to scale. Fbl, 3′-exon of the fibrillarin gene; NLS, nuclear localization signal. (**B**) Nucleotide and derived amino acid sequences of exons 1A, 1B and 2 and 5′-flanking regions of the mouse and human *DYRK1B* genes. The human nucleotide sequence (GenBank® accession no. Ac005393) is only shown where it differs from the mouse sequence (Aj252172). Gaps that were introduced to optimize the alignment are indicated by dashes. Exon sequences are shown as upper-case letters and highlighted by boxes. Arrows indicate the 5′-ends of the RACE clones that underlie the published cDNA sequences of mouse and human DYRK1B (Y17999 and Y18280 [3]) or those of ESTs as detected by database searching. The human EST that extended furthest to the 5′-end was assumed to define the start of exon 1A (bp + 1), and the 5′-end of exon 1B was determined by RACE PCR on mouse cDNA (Aj537610). Translation starts of DYRK1B-p69 and DYRK1B-p75 are circled. Putative promoter elements are underlined (CAAT box, MyoD/E47). (**C**) Alternative splicing in the N-terminus. Numbers refer to the amino acid sequence of DYRK1B-p69 and DYRK1B-p75 respectively. (**D**) Alternative splicing of exon 9. Numbers refer to the amino acid sequence of DYRK1B-p69. (**E**) Putative binding site for MyoD/E47 in the 5′-flanking region of exon 1B. Consensus sequences for MyoD (shaded), E47 (boxed) and MyoD/E47 heterodimers were taken from the Transfac matrix table (release 5.0; accession nos. M00002, M00071 and M99184).

three in the mammalian kinases (exons 7–9 in *Dyrk1b*). The putative transcription start of exon 1A, as deduced from the cDNA sequence at the 5′-end (human EST clone Bi561053), is located only 174 bp downstream of the 3′-exon of the *Fbl* gene, leaving space for only a very short promoter preceding exon 1A (Figure 3B). Reporter assays confirmed that this sequence (bp − 245 to + 155) indeed exhibited promoter activity in COS-7 cells (980-fold stimulation of luciferase activity compared with empty vector; results not shown). The 5′-end of the exon 1B-containing transcript (encoding DYRK1B-p75) was determined by RACE from mouse skeletal muscles (Figure 3B). No transcript was detected that contained both exons 1A and 1B, suggesting that exon 1B-containing mRNA is controlled by a distinct promoter. Notably, the 5′-flanking sequence of exon 1B is highly GC-rich (75 % G + C in bp 628–823) and is remarkably conserved in human and mouse genes (Figure 3B). A search for transcription factor-binding sites with the MatInspector software [23] detected matches for MyoD and E47 recognition sites (Figure 3E). MyoD is a well-characterized transcriptional activator of muscle-specific gene expression [24] and is known to function as a heterodimer with E47 [25].

Detection of exon 1B-containing mRNA by RT–PCR

Two pairs of PCR primers were designed to amplify specifically the transcripts containing either exon 1A or 1B (Figure 4A). Figure 4(B) shows that both the exon 1B-containing transcript (coding for DYRK1B-p75) and the exon 1A-containing mRNA (coding for DYRK1B-p69) were found in undifferentiated and differentiated 3T3-L1 cells. However, at higher dilutions of the cDNA, the exon 1B-containing transcript was detected only in the differentiated cells, consistent with the conclusion that the promoter driving the expression of DYRK1B-p75 is specifically activated in the adipocyte-like cell. The same pair of primers was used to identify exon 1B-containing transcripts in human tissues (results not shown). However, these results did not allow

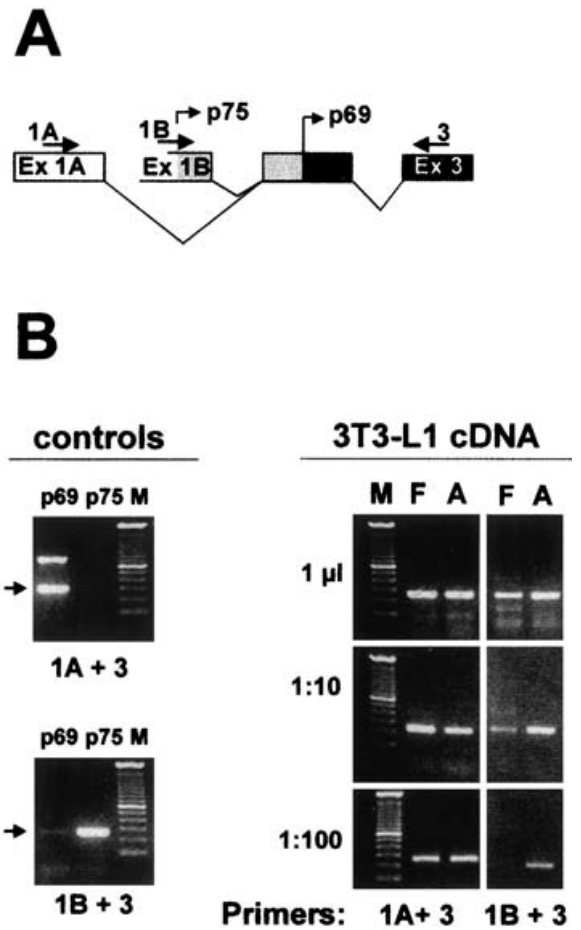

Figure 4 Detection of DYRK1B splicing variants by RT–PCR

(**A**) Scheme illustrating the localization of the primer sequences on exons 1A, 1B and 3. The selected primers match both the human and mouse *DYRK1B*/*Dyrk1b* genes. (**B**) Differential expression of DYRK1B splicing variants in 3T3-L1 cells. Control PCRs with plasmid clones for DYRK1B-p75 and DYRK1B-p69 as templates (1 pg of cDNA) are shown in the left panel. First-strand cDNA from 3T3-L1 fibroblasts (F) and from differentiated adipocytes (A) was diluted as indicated and used as template for PCR with the indicated primer pairs (right panel). One microlitre of cDNA corresponds to 300 ng of total RNA. M, molecular-mass marker (100 bp ladder); arrows point to the specific bands in the control PCR.

quantitative comparisons of mRNA levels between different tissues.

Comparative characterization of the DYRK1B splicing variants

For further characterization and comparison with the endogenous proteins, selected splicing variants of DYRK1B (DYRK1B-p65, DYRK1B-p69 and DYRK1B-p75) were ectopically expressed in COS-7 cells. Total cellular extracts of the transfected COS-7 cells and of 3T3-L1 cells, GT1-7 cells and mouse testis were analysed by Western blotting to compare the electrophoretic mobility of the recombinant proteins and the endogenous forms of DYRK1B in parallel samples. As shown in Figure 5(A), DYRK1B-p69 and DYRK1B-p75 co-migrate with the immunoreactive endogenous bands. It cannot be decided whether DYRK1B-p65 or DYRK1B-p66, or both, correspond to the smallest DYRK1B form detected *in vivo*. We have not constructed expression clones for DYRK1B-p71 and DYRK1B-p72, but obviously one or both of these proteins may contribute to the band at approx. 69/70 kDa in 3T3-L1 and GT1-7 cells.

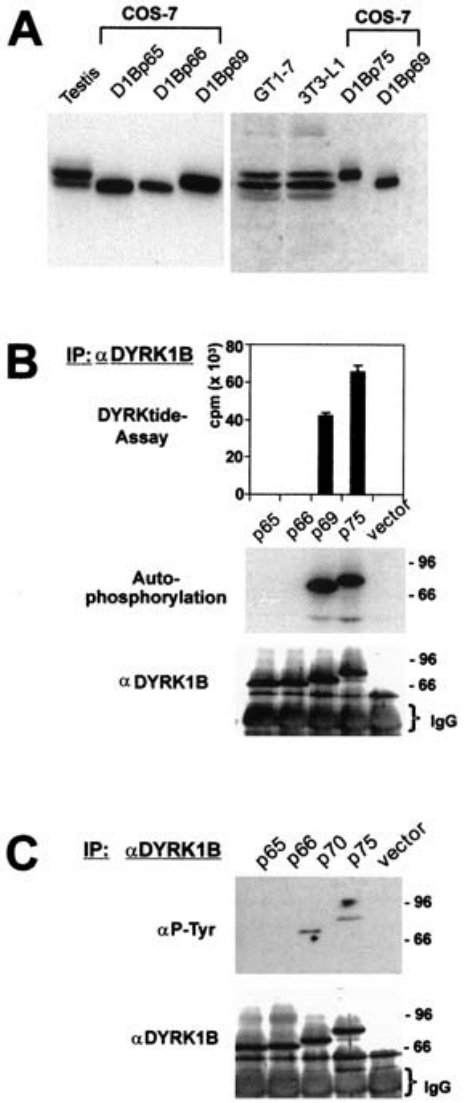

Figure 5 Characterization of recombinant DYRK1B splicing variants

(**A**) Electrophoretic mobility of endogenous and recombinant DYRK1B splicing variants. Total cellular lysates from transiently transfected COS-7 cells (D1B-p65, D1B-p66, D1B-p69 and D1B-p75), from GT1-7 cells, from 3T3-L1 adipocytes or from mouse testis were subjected to SDS/PAGE and Western-blot analysis with DYRK1B-specific antiserum. (**B**) Kinase activity. Recombinant DYRK1B splicing variants were immunoprecipitated from transiently transfected COS-7 cells with anti-DYRK1B antiserum. Kinase activity towards DYRKtide and autophosphorylation of the immunoprecipitated proteins were assayed as in Figure 2. (**C**) Phosphotyrosine Western blot. Tyrosine phosphorylation of DYRK1B splice variants was detected with a phosphotyrosine-specific antibody (PY99). IP, immunoprecipitation.

We next assayed the kinase activity of the DYRK1B splicing variants *in vitro* after immunoprecipitation from transiently transfected COS-7 cells (Figure 5B). This assay clearly showed that DYRK1B-p69 and DYRK1B-p75 catalysed the phosphorylation of DYRKtide and their autophosphorylation, whereas DYRK1B-p65 and DYRK1B-p66 were inactive. DYRK1B-p65 and DYRK1B-p66 were also found inactive when expressed in *E. coli*, whereas DYRK1B-p69 phosphorylated DYRKtide, histone H3 and myelin basic protein (results not shown). Furthermore, DYRK1B-p69 and DYRK1B-p75, but not DYRK1B-p65 and DYRK1B-p66, contained phosphotyrosine when immunoprecipitated from COS-7 cells (Figure 5C),

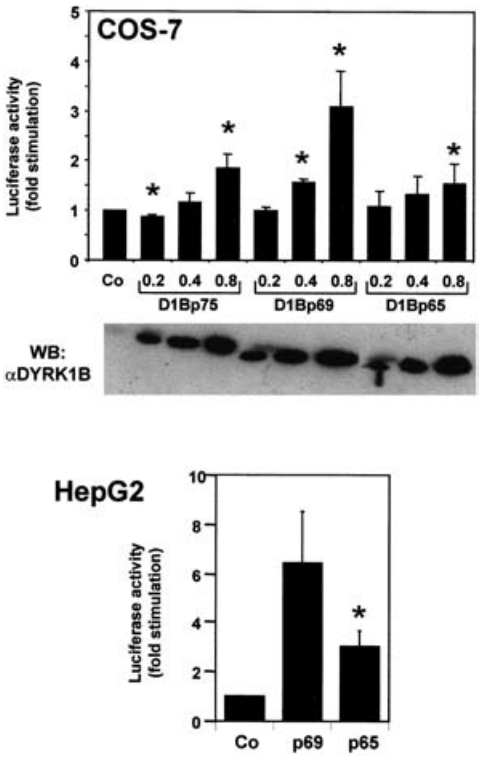

Figure 6 Stimulation of FKHR-dependent glucose-6-phosphatase promoter activity by DYRK1B splicing variants

Upper panel: COS-7 cells were co-transfected with the indicated amounts of the pCMV-SPORT6-DYRK1B constructs, 0.2 μg of the FKHR expression plasmid and a reporter gene construct with the glucose-6-phosphatase promoter (0.6 μg). Cells were lysed 42 h after transfection, and luciferase activity was determined and normalized to the total protein concentration of the lysates. For control of expression levels, aliquots of the lysates (pools of triplicate wells) were subjected to Western-blot analysis with a DYRK1B-specific antiserum (a representative blot is shown below the bar diagram). Lower panel: HepG2 cells were co-transfected with 1 μg of DYRK1B plasmid or vector control (Co), 0.5 μg of the reporter gene construct and 0.5 μg of pRL-TK control plasmid. Twenty-four hours after transfection, the cells were incubated for 18 h in serum-free medium. Luciferase activity was determined and normalized to the co-expressed *Renilla* luciferase. Data are expressed as fold stimulation relative to blank vector; scale bars represent means $\pm$ S.E.M. for 4–5 (COS-7) or 3 (HepG2) independent experiments. Asterisks indicate significant differences ($P < 0.05$) compared with vector control.

indicating that catalytic activity is required for tyrosine phosphorylation of DYRK1B.

DYRK1B splicing variants enhance the activity of FKHR in reporter gene assays

The transcription factor FKHR is known to interact with DYRK1A [15], and we have found recently that DYRK1A and DYRK1B-p69 increase FKHR-dependent reporter gene activity [26]. Therefore we have compared the capacity of the different DYRK1B splicing variants to enhance luciferase expression driven by the glucose-6-phosphatase promoter. As shown in Figure 6 (upper panel), co-transfection of DYRK1B-p69 or DYRK1B-p75 in COS-7 cells caused a dose-dependent increase in luciferase activity when compared with the control. A significant, but weaker, effect (1.5-fold) was produced by co-expression of DYRK1B-p65. DYRK1B-p65 was also capable of stimulating glucose-6-phosphatase promoter activity in HepG2 hepatoma cells (Figure 6, lower panel).

DISCUSSION

Results of the present study indicate that the *Dyrk1b* gene encodes at least three gene products that are differentially expressed in mouse tissues and cell lines and exhibit different functional characteristics. Several lines of evidence indicate that these variants of DYRK1B are not merely aberrant splicing products but represent proteins with physiologically relevant functions. First, comparable protein levels of DYRK1B-p75, DYRK1B-p69 and DYRK1B-p65/p66 were detected in tissues and cells that constitutively co-express these splicing variants (e.g. skeletal muscles, GT1-7 cells and differentiated 3T3-L1 cells). Secondly, the isoforms are generated both in mouse and in humans, and the extended N-terminal sequence of DYRK1B-p75 is highly conserved in these species. Thirdly, the regulated expression of DYRK1B-p75 during differentiation of 3T3-L1 fibroblasts into the adipocyte-like cells argues against the possibility that this protein is solely the product of an aberrant initiation of transcription.

The use of different sites for translation–initiation leads to the presence or absence respectively of an N-terminal region of 60 amino acids in the DYRK1B proteins. These variants are encoded by transcripts that contain either exon 1A or exon 1B, apparently due to the use of separate promoters. It is probable that the differential regulation of these promoters is the cause of the distinct patterns of expression of DYRK1B-p69 and DYRK1B-p75 in mouse tissues and in the 3T3-L1 cell line, although it cannot be excluded that post-transcriptional regulatory mechanisms are involved. The *in vitro* kinase assays showed that the additional N-terminal amino acids have no direct effect on the catalytic activity of DYRK1B. However, the high degree of sequence similarity of this segment between mouse and humans (98 % identical amino acids) argues for an evolutionarily conserved function. The additional sequence is rich in basic residues (10 arginine and lysine residues out of 60 residues) and small residues (8 alanine and 10 glycine residues) and contains 15 proline residues, which may constitute docking motifs for protein–protein interactions [27].

In addition to the N-terminus of DYRK1B, the catalytic domain is also affected by alternative splicing. Using PCR-based cloning methods, we have identified previously human cDNAs for splicing variants that lack 28 or 40 amino acids in the catalytic domain (DYRK1B-p66 and DYRK1B-p65). In the present study, we show by Western-blot analysis of mouse tissues and cell lines that at least one of these proteins is indeed expressed *in vivo*. These splicing variants lack detectable kinase activity *in vitro* and were not autophosphorylated *in vivo*, although the deletions affect none of the residues that are known to be essential for the catalytic activity of protein kinases [28]. However, sequence alignments and molecular modelling ([11]; Dr J. Grötzinger, Institute of Biochemistry, Kiel, Germany, personal communication) suggest that DYRK1B-p65 and DYRK1B-p66 lack three critical amino acids (amino acids 403–405 in DYRK1B-p69) that belong to the helix α_H, which is a conserved secondary structure element of the core domain of protein kinases. The observation that the catalytically inactive splicing variants of DYRK1B lack phosphotyrosine confirms and extends our previous finding that tyrosine phosphorylation of DYRK1A depends on its own kinase activity [11].

Western-blot and immunoprecipitation experiments clearly show that at least one of the inactive splicing variants is indeed expressed in mouse tissues, but the band at approx. 65 kDa could not be identified unambiguously as DYRK1B-p65 or DYRK1B-p66. However, a search of the EST databases revealed that out of 17 human EST sequences covering the alternatively spliced

region (exons 8 and 9), eight clones represented the longest version (DYRK1B-p69) and nine clones represented DYRK1B-p65, whereas none was found that corresponded to DYRK1B-p66. Consequently, the immunoreactive band of 65 kDa in mouse tissues and cell lines probably represents DYRK1B-p65. In a human colon carcinoma cell line, Lee et al. [9] identified two forms of DYRK1B (termed 'Mirk' by the authors) with apparent molecular masses of 70 and 67 kDa; these forms quite probably correspond to DYRK1B-p69 and DYRK1B-p65 as described in the present study.

At present, we can only speculate on the physiological role of the catalytically inactive variant of DYRK1B. First, an inactive splicing variant might act as a dominant-negative regulator of the active kinase. Secondly, some functions of DYRK1B may not require protein kinase activity. Consistent with this hypothesis, DYRK1B-p65 was capable of enhancing, although weakly, the FKHR-dependent promoter activity in reporter gene assays. We have shown previously that a kinase-negative DYRK1A mutant also increased the effect of FKHR on the promoter activity in this assay [26]. Similarly, a catalytically inactive mutant of DYRK3 has been described recently to stimulate CREB-dependent reporter gene activity, although to a lesser degree when compared with the wild-type kinase [29]. These results suggest that DYRK kinases can act as transcriptional regulators independent of their kinase activity. This effect may be a function of the non-catalytic domain that is structurally variable but consistently present in all members of the DYRK family.

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