Alternative splicing of mRNA transcripts expands the range of protein products from a single gene locus. Several splice variants of DCLK (doublecortin-like kinase) have previously been reported. Here, we report the genomic organization underlying the splice variants of DCLK and examine the expression profile of two splice variants affecting the kinase domain of DCLK and CPG16 (candidate plasticity gene 16), one containing an Arg-rich domain and the other affecting the C terminus of the protein. These splice alternatives were differentially expressed in embryonic and adult brain. Both splice variants disrupted DCLK PEST domains; however, all splice variants remained sensitive to proteolysis by calpain. The adult-specific C-terminal splice variant of DCLK had reduced autophosphorylation activity, but similar kinase activity for myelin basic protein relative to the embryonic splice variant. The splice variant adding an Arg-rich domain gained an autophosphorylation site at Ser-382. Although this protein isoform was expressed mainly in the adult brain, the phosphorylated form was strongly enriched in embryonic brain and adult olfactory bulb, suggesting a possible role in migrating neurons.

The majority of neuronal cells in the central nervous system are generated during embryogenesis and persist throughout life without further rounds of division and differentiation. Individual neurons accomplish an array of distinct tasks during their life span, including cellular migration, axon extension and pathfinding, synaptogenesis, and participation in mature nervous system function. It has been suggested that to deal with these diverse demands on cellular function, the repertoire of neuronal gene products is enhanced by alternative splicing and RNA editing. Following the description of differential RNA processing of the calcitonin gene (1), alternative splice forms of many gene products have been detected in the central nervous system (2, 3).

Doublecortin-like kinase (DCLK) is a serine-threonine kinase expressed in the central nervous system. The N-terminal domain of DCLK is very similar to the doublecortin protein and mediates microtubule localization (4–6). The C-terminal kinase domain of DCLK resembles members of the family of calcium/calmodulin-dependent protein kinases, but lacks a canonical calmodulin-binding site (7). The human gene was named DCAMKL1 (doublecortin- and Ca2+/calmodulin-dependent protein kinase-like protein-1) (8); however, biochemical evidence does not indicate that calcium/calmodulin modulates kinase activity (7).

The DCLK locus gives rise to several transcripts through differential splicing and use of alternative promoters (see Fig. 1). The DCL product includes the doublecortin domain, but lacks the kinase domain. DCLKEX2 is a full-length transcript embracing both doublecortin and kinase domains and includes an additional 16 amino acids enriched in arginine residues (the Arg-rich domain), between the doublecortin and kinase domains (9). Two splice variants affecting the final coding exon of DCLK have been described (9–11). A second promoter following the doublecortin domain gives rise to two distinct transcripts: CPG16, which represents the kinase domain with six unique amino acids at the N terminus (7, 9–11); and a second transcript encoding a small protein referred to CARP (Ca2+/calmodulin-dependent protein kinase-related peptide) (11) or Ania-4 (12), the N terminus of which is identical to CPG16 over 38 amino acids, whereas the C terminus consists of 17 unique amino acids.

DCLKα is susceptible to cleavage by the calcium-dependent protease calpain (13). This event releases an active kinase domain from the DCLK microtubule anchorage domain and may therefore represent a mechanism for regulating the localization of the kinase domain in response to neuronal calcium transients. Although no consensus sequence for calpain substrates has been identified, many proteins cleaved by calpain contain hydrophilic motifs enriched in proline, glutamic acid, serine, and threonine (PEST domains) (14, 15). Splice forms of DCLK disrupt the PEST domains, potentially altering its susceptibility to proteolysis. Differential splicing potentially modulates kinase activity. The C-terminal tail of calmodulin kinase I has been shown to fold across the active site of the kinase (16), and the homologous segment modulates kinase activity in several serine-threonine kinases (17, 18). We therefore sought to determine whether kinase activity was altered in splice variants of DCLK.

**EXPERIMENTAL PROCEDURES**

*Exon Structure Determination*—To determine the exon structure of DCLK underlying different splice variants, we used the BLAT search tool (19) to align human and mouse cDNAs representing DCLK, DCL, CPG16, and CARP against the human genome sequence. Where non-human cDNAs were used, the alignment was manually curated to determine exact intron/exon boundaries. A list of shared and unique

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*Available at genome.ucsc.edu/*.
exons was compiled and is schematically represented in Fig. 1. We used the same process to determine the exon structure of DCX.

Isolation of the Mouse Intron between Exons 9 and 10—Intron/exon boundaries are highly conserved in human and mouse (20). We therefore designed primers for amplifying the mouse intron following exon 9 based on mouse cDNA sequence corresponding to human exons 9 and 10. Primer sequences were 5'-TCTCTCCTTTTGAAGCGTTGANGAGGAGAGGATGGTTTGGCTTAAAGAATACCGCCGAGT. The RT-PCR conditions included. Reaction products were separated on 2% agarose gels. In all cases, control reactions containing no RNA and no reverse transcriptase were included. Reaction products were separated on 2% agarose gels.

**Antibodies and Western Blotting**—Antisera raised against the DCLK C and N termini have been characterized previously (4, 9). For anti-DCLK Arg domain antibodies, a peptide (CLGRRHSLQRGWR) was purchased coupled to keyhole limpet hemocyanin (Genemed Synthesis, San Francisco, CA) and used for production of polyclonal antibodies (Antibody Unit, Weizmann Institute of Science). The antibodies were tested by Western blotting on extracts of 293-T cells transfected with FLAG-DCLKR° or FLAG-DCLKR°-D527A constructs (4). For anti-DCLK phospho-Ser-382 antibodies, a peptide (CLGRRHSLQRGWR with a phosphorylated serine residue) was purchased and used for

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**Expression and Kinase Activity of Splice Variants of DCLK**

**Fig. 1.** Exons of the human DCLK gene homolog Dcamkli. Exons were determined by alignment of DCLK, DCL, CPG16, and CARP cDNAs against human genomic sequence. For comparison, the exon organization of human doublecortin is also illustrated. Exons colored black encode the same segments of DCX and Dcamkli proteins as the corresponding vertically aligned exon (e.g. DCX exon 6 is very similar to Dcamkli exon 7). Exons marked with α indicate alternative splice sites. DCX exon 5a adds the five amino acids GDELG following Glu-374; Dcamkli exon 9a in mouse adds the Arg-rich insert discussed under “Results”; and for the DCLK transcript, a cryptic 5' donor site within Dcamkli exon 13a is spliced to a 3' splice acceptor in exon 20a, within the untranslated region of other transcripts depicted. Gene products arising from differential splicing and/or promoter use are indicated along with the designation used in this study.

**Fig. 2.** Differential splicing alters the protein coding sequence of DCLK transcripts. A, inclusion of exon 19 in DCLKβ causes a shift in the reading frame of exon 20. Exons 18–20 are indicated along with the human-mouse nucleotide identity. The splice organization of DCLKα and DCLKβ is illustrated along with cognate amino acids. B, use of an alternative 5'-splice donor site after exon 9 generates the 16-amino-acid insert constituting the Arg-rich domain in DCLKR°. Human and mouse sequences at the end of and following exon 9 are aligned; nucleotides contributing to the coding region are underlined; and the corresponding protein sequences are shown. Although experimental evidence supports the use of the distal 5'-splice donor site in mouse transcripts, there is no evidence for a corresponding splice variant in human. Moreover, the human putative protein sequence (in italics) is not similar to the mouse protein sequence in the Arg-rich domain.

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**RT-PCRs—**RT-PCR was carried out in all cases using the Access RT-PCR kit (Promega, Madison, WI) with 65 pmol of each primer and 100 ng of total mRNA isolated from adult mouse heart, testes, and brain and embryonic day 13, 15, and 17 mouse brain isolated using TriReagent (Sigma, Rehovot, Israel) according to the manufacturer’s protocol. The primer pairs used in each reaction are shown in Fig. 3. The sequences of the primers used are as follows: 40310, 5'-CGCTCGACTTACATTACATTACATTAC; 47745, 5'-GGCTCTCGACTTACATTACITTCC; 47746, 5'-CAGCAAAATTCGCTCTCT; 48044, 5'-AGGGCTGGTGGCTGCTGCTGCTG; 48045, 5'-CTTCGGAGGAAGCAAGTC; 48046, 5'-CCTTCGAACAGCTCATCAC; and 49139, 5'-TCCAAAGAATACCGCCGAGT. The RT-PCR conditions were 48°C for 45 min; 94°C for 2 min; and then 10 cycles of 94°C for 30 s, touchdown at 68°C to 58°C for 45 s, and 66°C for 30 s; and then 25 cycles of 94°C for 30 s, 58°C for 45 s, and 68°C for 30 s. In all cases, control reactions containing no RNA and no reverse transcriptase were included. Reaction products were separated on 2% agarose gels.

**Antibodies and Western Blotting**—Antisera raised against the DCLK C and N termini have been characterized previously (4, 9). For anti-DCLK Arg domain antibodies, a peptide (CLGRRHSLQRGWR) was purchased coupled to keyhole limpet hemocyanin (Genemed Synthesis, San Francisco, CA) and used for production of polyclonal antibodies (Antibody Unit, Weizmann Institute of Science). The antibodies were tested by Western blotting on extracts of 293-T cells transfected with FLAG-DCLKα or FLAG-DCLKα-D527A constructs (4). For anti-DCLK phospho-Ser-382 antibodies, a peptide (CLGRRHSLQRGWR with a phosphorylated serine residue) was purchased and used for
Expression and Kinase Activity of Splice Variants of DCLK

immunization the same way. Sera that tested positive for reactivity to DCLK on transfected cell extract were affinity-purified to extract a phospho-specific component. The phosphopeptide and an unphosphorylated sample of the same peptide were each dissolved in Me2SO. 10 g/ml aprotinin, 5 g/ml leupeptin). Protein concentration was assessed with the Bradford reagent (Bio-Rad). Western blotting was carried out using the Laemmli system on 10 or 12% SDS, 1 mM EDTA, 0.3 M NaCl, and 50 mM Tris-HCl (pH 8.0)) supplemented with protease inhibitors (10 mg/ml aprotinin, 5 mM pepstatin, 10 

**Plasmid Construction and Recombinant Protein Production—Splice forms of DCLK were constructed as follows. For GST-DCLKα, first a C-terminal EcoRI-SauI fragment from FLAG-DCLKα was subcloned into FLAG-DCL, which lacks the Arg-rich domain, to create FLAG-DCLKα. The open reading frame of DCLKα was then subcloned into pGEX-2TK, creating GST-DCLKα. For GST-DCLKβ and GST-DCLKβ, a partial DCLK cDNA containing the DCLKβ C terminus was cloned during screening of a cDNA library (9). The C terminus was amplified using primers 5'-CAGAAGCATTTCAACACAGG and 5'-TT-CTCGAGGATCCTGGTTGCGTCTTA and subcloned into pCPG16-C.**

**Blots were developed using Supersignal chemiluminescence (Pierce).**

**FIG. 3. RT-PCR shows differential expression of alternative splice forms in embryonic and adult brain. DCLK gene products are schematized on the right, together with the positions of the primers used. The sizes of the expected PCR products N1 to N8 are indicated and aligned below the corresponding primers.**

**A.**, RT-PCR with primers 47745 and 47746 detected the Arg-rich domain (R) splice variant (product N1) in embryonic and adult brain. Note that product N1 was potentially amplified from either DCLK or CPG16 (see schematic on right). **B.**, RT-PCR with primers 48045 and 48046 demonstrated the inclusion of the Arg-rich domain specifically in DCLK (as opposed to CPG16) transcripts. **C.**, RT-PCR with primers 48044 and 47746 detected a splice variant of CPG16 including the Arg-rich domain in adult brain (product N4). **D.**, RT-PCR with primers 48044 and 49139 detected CARP expression exclusively in adult brain (product N6), similar to CPG16 (product N5 in C). **E.**, RT-PCR with primers 40310 and 40311 demonstrated predominance of α-splice forms skipping exon 19 in embryonic brain (product N7) and β-splice forms including it in adult brain (product N8).

MQ, MilliQ water; E13, E15, and E17, embryonic days 13, 15, and 17, respectively; Ad.-RT, no reverse transcriptase; C-ter, C terminus.
Expression and Kinase Activity of Splice Variants of DCLK

GENOMIC ORGANIZATION UNDERLYING ALTERNATIVE SPlice FORMS OF DCLK—We first sought to clarify the genomic structure underlying the set of DCLK gene products. We aligned the various protein products of the DCLK gene against the human genome (Fig. 1). Our analysis concurs with the intron/exon organization reported by Matsumoto et al. (21) and Vreugdenhil et al. (22); however, adding the first exon of both CPG16 and CARP (exon 6) and the third exon of CARP (exon 8) to generate a full representation of DCLK exons means that our exon numbering system is not identical to theirs. As the N terminus of DCLK is ~80% identical to DCX, we also used BLAT to determine the intron/exon organization of the DCX gene. The intron/exon structures of the doublecortin domains of the two genes are extremely similar. The first exon of both genes is noncoding. Exons 2–4 of DCX and DCLK are homologous, encoding coordinate sets of amino acids. Exon 5 of the two genes is very similar, with the DCLK exon extending for an additional two codons and the DCX gene having an alternative splice donor site allowing for the inclusion of an additional five amino acids (GNDQD) after serine 310. DCLK exon 6 specifies six N-terminal amino acids unique to CPG16 and CARP. DCLK exon 7 and DCX exon 6 encode homologous amino acids. Interestingly, DCLK exon 8, used only in CARP, is also homologous to DCX exon 7.

In the course of cloning mouse DCLK transcripts, we encountered several splice variants (9). Exon 19 is differentially incorporated, giving rise to the DCLKα and DCLKβ transcripts (Fig. 1). As exon 19 is 74 bases long, its inclusion in DCLKβ transcripts causes exon 20 to be translated in an alternative reading frame with a premature stop codon (Fig. 2A). Despite the early stop codon, DCLKβ is only 11 amino acids shorter than DCLKα due to the inclusion of exon 19. A second splice variant, DCLKK*, incorporated an additional 48 nucleotides following exon 9, encoding for an Arg-rich domain. We searched for a homologous coding region between exons 9 and 10 in the human genome sequence, but found only partial homology to this sequence in the intron immediately after exon 9. We therefore amplified mouse genomic DNA using primers from exons flanking exon 9. Sequencing revealed that the nucleotides encoding the Arg-rich domain follow immediately from exon 9 in mouse. This splice variant therefore arises from alternative 5′-splice site utilization in mouse, but does not exist in human, although, interestingly, nucleotides encoding the first five amino acids of this domain are identical in human. The alternative splice sites in mouse both match five of the nine nucleotides in the splice donor consensus sequence (2).

ALTERNATIVE SPlice FORMS OF DCLK ARE DIFFERENTIALLY Expressed—To determine whether all the described splice products could be detected in mouse tissue, we undertook a series of RT-PCR and Western blot experiments using antisera raised against peptides from different splice forms of DCLK. RT-PCR experiments demonstrated that splice forms of both CPG16 and DCLK exist that contain the Arg-rich domain. From total mRNA, experiments performed with primers designed to co-amplify splice forms with and without the Arg-rich domain indicated that although the splice form lacking the Arg-rich
whereas only scores above 5.0 are considered to be true PEST domains. Scores of significant PEST domains are marked. Higher numbers represent putatively stronger domains, whereas only scores above 5.0 are considered to be true PEST domains. 

**Domain was more readily amplified and therefore probably more abundant, the Arg-rich domain was detected in both embryonic and adult mouse brain** (Fig. 3A). Primers designed to specifically amplify the Arg-rich domain from DCLK but not CPG16 detected DCLKRγ at all ages, most strongly during embryogenesis, consistent with down-regulation of DCLK in adult brain (Fig. 3B). In contrast, CPG16-specific primers revealed expression of both CPG16 and CPG16Rγ in adult brain only (Fig. 3C). Expression of a second product of the CPG16 promoter (CARP) was also confined to adult brain (Fig. 3D). Experiments designed to differentially amplify transcripts encoding the alternative C termini of DCLK demonstrated a switch in utilization from the α-splice form during embryogenesis to the β-splice form in the adult (Fig. 3E).

To confirm these results, we raised antibodies against a peptide representing part of the amino acid sequence of the Arg-rich domain (Fig. 4A). Immunoblot analysis using an antibody against the N terminus of DCLK, which should recognize all splicing forms of DCLK, revealed expression in both adult and developing brain. The anti-DCLK Arg domain antisera reacted with a band of the same size mainly in adult brain (Fig. 4B). Expression of DCLKRγ mRNA has been reported in adult hippocampus (22). As previously reported (9), α-specific antisera revealed strong expression only in embryonic brain lysate, consistent with the switch from α- to β-splice form utilization seen by RT-PCR.

**Splice Variants Have Altered Kinase Activity, but Not Calpain Susceptibility**—Both the Arg-rich domain and α/β-splice variants alter PEST domains within DCLK (Fig. 5A). The β-splice form lacks a C-terminal PEST domain altogether, whereas the Arg-rich domain disrupts a strong PEST domain preceding the kinase domain, producing two weak adjacent PEST domains. As PEST domains have been considered to be calpain-targeting signals, we tested whether all splice forms of DCLK remained sensitive to cleavage by calpain in vitro. Recombinant forms of all splice forms of DCLK were cleaved by calpain within 10 min of incubation, indicating that splicing does not render DCLK immune to proteolytic attack (Fig. 5B).

We next tested whether splice forms of DCLK demonstrated different kinase activity in vitro, as assayed by the rate of autophosphorylation and phosphorylation of an exogenous substrate, myelin basic protein. Whereas all splice forms showed similar kinase activity for myelin basic protein (Fig. 6A), the rate of autophosphorylation of DCLKβ was only 50% of that of DCLKα or DCLKRγ α (Fig. 6B). We therefore sought to determine sites of autophosphorylation of DCLK and subjected recombinant DCLKRγ α autophosphorylated in vitro to analysis by mass spectrometry.

**DCLK Autophosphorylates at Ser-382**—Mass spectrometry of tryptic fragments of DCLK revealed a major peptide peak with a molecular mass exactly representing a phosphorylated form of Ser-382, which lies in the Arg-rich domain (Fig. 7A). This peptide was eliminated by treatment of DCLK with alkaline phosphatase (Fig. 7B). Furthermore, it was retained on metal columns designed to enrich for phosphopeptides (Fig. 7, C and D), but not following treatment with alkaline phosphatase (Fig. 7, E and F). These results strongly indicate that one site of autophosphorylation of DCLK is in its Arg-rich domain, at Ser-382.

We raised antisera against a synthetic peptide phosphorylated at Ser-382 and affinity-purified a phospho-specific component. The affinity-purified antisera reacted strongly with recombinant DCLKRγ α purified from bacteria, which appeared to autophosphorylate during expression. The specificity for phosphorylated Ser-382 was indicated by the presence of only faint cross-reactivity with a kinase mutant form of the DCLKγ α-D527A protein (Fig. 8A). Similar results were obtained with recombinant DCLKRγ β (data not shown).

To further test the specificity of the antisera, 293-T cells were transfected with FLAG-DCLKγ α, FLAG-DCLKα, or a FLAG-DCLKγ α-S382A construct carrying a point mutation changing serine 382 to alanine, eliminating the putative autophosphorylation site. Western blotting of protein extracts from transfected cells with antisera against the DCLK N terminus demonstrated expression of all three constructs (Fig. 8B, upper panel). The affinity-purified antisera recognized the FLAG-DCLKγ α protein, but showed no immunoreactivity with the FLAG-DCLKα protein (which lacks the phosphorylation site) or FLAG-DCLKγ α-S382A (in which the epitope carries a point mutation) (Fig. 8B, lower panel).

Either autophosphorylation at Ser-382 is saturated during expression, or Ser-382 is only poorly phosphorylated in vitro, as immunoreactivity during in vitro autophosphorylation increased by 3.7-fold (quantitation of immunoblot in Fig. 8C). The affinity-purified antisera were used to study the extent of endogenous autophosphorylation in mouse tissue. Protein extract was made in buffer with inhibitors of serine-threonine phosphatases and subjected to immunoblotting (Fig. 8D). Again, we
noted the pronounced expression of the DCLKR protein in adult brain. Surprisingly, phosphorylation at Ser-382 was most easily detected in embryonic brain and olfactory bulb, suggesting that although expression of DCLKR is very low, it is more likely to be phosphorylated at Ser-382.

**DISCUSSION**

**Regulated Expression of DCLK Splice Forms**—Here, we have reported the differential expression of alternative splice products of the DCLK gene. Splice forms of DCLK vary in autophosphorylation activity, but not in activity for an *in vitro* substrate, myelin basic protein. We have demonstrated that the β-splice variant of the DCLK C terminus has only 50% of the autophosphorylation activity of the α-splice variant. A second splice variant incorporating an Arg-rich domain before the kinase domain is autophosphorylated at Ser-382, as assessed by mass spectrometry and anti-DCLK phospho-Ser-382 antibodies.

It has been predicted that one-third to one-half of human mRNAs are subject to alternative splicing (23, 24). Only ~20% of alternatively spliced genes have variations in the coding region (24). In 40% of the cases, variant protein sequences are generated by splice alternatives utilizing exons in different reading frames. The use of an exon in two reading frames may result from the use of alternative 5′- or 3′-splice sites; from the retention of an intron; or, as in the case of DCLK, from the differential inclusion of an exon encoding an odd number of nucleotides (25).

Alternative 5′-splice site selection of DCLK exon 9 accounts for a splice variant of mouse DCLK in which an Arg-rich domain precedes the kinase domain. A second splice variant of DCLK arises from differential incorporation of exon 19. The first 67 nucleotides of DCAMKL1 exon 20, which are utilized in two reading frames, are identical in mouse and human, compared with a 90% nucleotide identity in other exons, including the region of exon 20 that is not translated in two frames. This may reflect constraints on mutation due to the necessity of preserving function in two amino acid sequences. However, as exon 19 is also almost identical in mouse and human (73 of 74 nucleotides), mutation may be constrained by other factors, including sequence motifs regulating alternative splicing. A high degree of human-mouse nucleotide sequence similarity in alternatively spliced exons has previously been reported for the myotonic dystrophy kinase gene (26).

Splice variants encompassing the Arg-rich domain were enriched in adult mouse brain. This probably reflects the adult-specific expression of CPG16 and CPG16R+. As the DCLKR protein (but not mRNA) was also enriched in adult brain, there may be differences in mRNA stability in embryonic and adult tissues. We demonstrated that the Arg-rich domain of DCLK is specific to mouse. The RT-PCR results, although not strictly quantitative, suggest that the DCLKR+ splice form is either a minor transcript or expressed in a small subset of neurons.

In addition, we observed a switch from mRNA transcripts encoding the α-splice variant in embryonic tissue to the β-splice variant in adult tissue. Western blot analysis confirmed that DCLKo expression is lost following embryogenesis and showed that total DCLK expression (presumably reflecting DCLKβ expression) is somewhat reduced in adult tissue. The main splice forms of DCLK are therefore DCLKo during embryogenesis and DCLKβ in adult brain. As CPG16 expression is confined to adult brain, this indicates that the main splice form of CPG16 is CPG16β, a conclusion supported by additional RT-PCR experiments (data not shown). Previous *in situ* hybridization experiments have demonstrated expression of both α- and β-splice forms in adult hippocampus; however, it is unclear whether the transcripts detected represent CPG16 or DCLK (22).

RNA splicing involves the recruitment of spliceosomal proteins and small nuclear ribonucleoproteins to pre-mRNA at 5′- and 3′-splice sites. Splice alternatives arise from competition between spliceosomes forming at potential splice sites, and control of splice decisions may therefore be achieved by regulation of spliceosome formation. Spliceosome formation is regulated by several mechanisms, including phosphorylation of the SR splicing factor ASF/SF2 (27) and the ratio of spliceosome heterogeneous nuclear ribonucleoprotein A1 to ASF/SF2 proteins (28). A large number of neuron-specific splicing events have been described (2), making it likely that neuronal cells...
Mass spectrometry reveals autophosphorylation by DCLK in the Arg-rich domain at serine 382. A, recombinant DCLK<sup>ΔH1100/H9251</sup> was allowed to autophosphorylate in vitro before tryptic digestion and analysis by mass spectrometry. The molecular mass (876.42 Da) of a prominent peak (indicated by the arrow) corresponds closely to the expected molecular mass of a tryptic fragment (RHSLQR) with a phosphorylated serine residue. B, when the mixture of tryptic peptides was previously subjected to treatment with alkaline phosphatase (AP), the peak at 876.42 Da was eliminated. Instead, a peak was observed at 796.48 Da, corresponding to the expected molecular mass of the same but now unphosphorylated peptide (arrow). C–F, metal affinity columns selectively retained a phosphorylated peptide at 876.4 Da. After in vitro autophosphorylation, the protein was subjected to tryptic digestion, purification on metal affinity columns, and analysis by mass spectrometry. The peptide at 876.4 Da (marked by an arrow in each panel), potentially representing phospho-RHSLQR, was retained on both ferric (C) and nickel (D) metal affinity columns, suggesting that it bears a negative charge. Such peptides are often phosphopeptides. Retention of the 876.4-Da band on metal affinity columns was eliminated by pretreatment of the peptide mixture with alkaline phosphatase (compare the peak exactly under the tip of the arrow before alkaline phosphatase treatment in E and after treatment in F). This demonstrates that the negative charge on the 876.4-Da peptide is indeed derived from a phosphate moiety.
contain factors that orchestrate RNA processing in a neuron-specific manner (29–31).

Splicing decisions are also controlled by the combinatorial action of multiple RNA elements residing in both exons and introns (3, 32, 33). Intronic polypyrimidine tracts at the 3′/H11032-splice site of exons constitute negative splicing control elements. In the large conductance calcium-activated potassium (BK) channel and NMDAR1, these act to exclude the incorporation of exons in response to calcium signaling through Ca2+/H11001/calmodulin-dependent protein kinase IV (33). The polypyrimidine tract-binding protein represses utilization of the neuron-specific N1 exon in c-src by binding CUCUCU motifs in the flanking introns (34). The polypyrimidine tract within the 3′/H11032-splice acceptor of DCLK exon 19 contains such a motif, potentially contributing to repression of this exon during embryogenesis.

Regulated splice sites often display a poor match to splice consensus sequences, facilitating regulation of the site (2). The alternative 5′/H11032-splice sites following exon 9 are equally poor matches to the splice consensus sequence, potentially making it prone to differential splicing.

DCLK Splice Variants Are Susceptible to Cleavage by Calpain—Control of susceptibility to cleavage by calpain by alternative splicing has been previously demonstrated for IkBβ (35) and prointerleukin-1α (36). As PEST domains are disrupted by splice alternatives of DCLK, we examined the significance of the DCLK PEST domains for calpain-mediated proteolysis.

It has been suggested that by sequestering calcium, negatively charged residues clustered in PEST domains could raise its local concentration sufficiently to activate calpain (14). Evidence supports the original contention that PEST domains target proteins for rapid degradation (for example, see Ref. 37).

FIG. 8. DCLK is phosphorylated at serine 382 in mouse brain lysate. A, antisera that specifically recognize Ser-382-phosphorylated DCLK were prepared by immunization with a synthetic phosphopeptide. These antisera react with recombinant DCLK in vitro only when the protein is competent to autophosphorylate; the kinase mutant form of DCLK with a D527A mutation (4) is not recognized by these antisera. The antisera against the DCLK C terminus, which are insensitive to the phosphorylation state, revealed similar amounts of DCLK in each lane. B, in protein extract from transfected 293-T cells (40 μg/lane), the anti-DCLK phospho-Ser-382 antibody (lower panel) recognized only FLAG-DCLKRα–α (containing the phosphorylated epitope), but not FLAG-DCLKα–αS382A (in which the phosphorylated serine is replaced by alanine) or FLAG-DCLKα (the splice form lacking this autophosphorylation site altogether). As a transfection control, the antibody against the DCLK N terminus demonstrated expression of all three proteins (upper panel). C, autophosphorylation of GST-DCLKRα–α in vitro enhanced immunoreactivity with the anti-DCLK phospho-Ser-382 antisera (upper panel). Anti-GST antibodies were used as a loading control (lower panel). Quantitation of the signal by scanning densitometry, normalized to anti-GST immunoreactivity, demonstrated a 3.7-fold increase in signal after autophosphorylation. D, the anti-DCLK phospho-Ser-382 antibody was used to study whether Ser-382-phosphorylated DCLK exists in brain lysate. The antibody against the DCLK N terminus showed expression of DCLK in embryonic and adult brain and minor expression in olfactory bulbs (first panel). Although the splice form of DCLK that includes this residue was most expressed in adult brain (second panel), the phosphorylated form was detected most strongly in embryonic brain extract and in adult olfactory bulb (third panel). The blot was also probed with anti-tubulin antibody as a loading control (fourth panel). WB, Western blotting; E16 and E18, embryonic days 16 and 18, respectively.
The strongest evidence for PEST domains conferring susceptibility to calpain comes from a study in which the PEST domain of IxBa was shown to be required for binding to and cleavage by calpain (38). This domain also acted as a transferable calpain susceptibility module. Other results do not support a function for PEST domains as calpain susceptibility motifs (39, 40). Moreover, some calpain substrates lack strong PEST signatures (40–42), whereas some PEST domain-containing proteins are either not susceptible or resistant to calpain (15, 40).

Despite significant variations in the strength of PEST motifs in splice forms of DCLK, all splice forms of DCLK were cleaved by calpain in vitro. Our results therefore lend support to a body of evidence arguing that PEST motifs are not required for proteolytic cleavage of substrates by calpain. Possibly calmodulin-binding motifs interact with the calmodulin-like domain of calpain and act as targeting signals (15, 39, 43). Calmodulin often alters the cleavage pattern of calpain substrates (44, 45), however, the calmodulin-binding domain itself is not required for calpain proteolysis in several proteins (15).

Research into the role of PEST domains in targeting calpain may be confounded by two problems. The algorithm that scores PEST domains may not be optimal for recognizing biologically significant motifs. Its performance may be compromised by the requirement for positive residues bracketing (but not within) the PEST domain (15). Additionally, as the PEST domain is not the site of proteolysis by calpain, but rather was proposed to target calpain to protein substrates (14), the use of in vitro assays to determine cleavage susceptibility may be inappropriate.

**DCLK Splice Variants Affect Kinase Activity**—In characterizing autophosphorylation sites of DCLK, we found a strong mass spectrometry signal for a peptide with mass compatible with phosphorylation at Ser-382. The residues immediately surrounding Ser-382 are RRHSLQR. Arg or Lys residues are commonly found closely preceding target phosphorylated residues in substrates of serine-threonine kinases. Ser-382 lies in the differentially spliced Arg-rich domain. Modulation of kinase activity by Arg-rich domains has been previously described (46, 47). Using anti-DCLK phospho-Ser-382 antisera, we detected phosphorylation of this residue in mouse tissue. An unexpected finding was that phosphorylated Ser-382 was more strongly detected in embryonic brain and adult olfactory bulb than in adult brain despite higher expression of the Arg-rich domain in adult brain. This may be due to greater autophosphorylation activity of DCLKa, which is the main embryonic splice form of DCLK. Alternatively, this residue may be phosphorylated by another kinase in embryonic brain. Autophosphorylation has been previously linked to regulation of serine-threonine kinase activity. Autophosphorylation by the closely related kinase Ca2+/calmodulin-dependent protein kinase II leads to calcium-independent activity and is necessary for the establishment of long term potentiation (48).

We note with interest that a feature common to embryonic brain and adult olfactory bulb is ongoing neuronal migration. In the developing cortex, DCLK is most strongly expressed in cortical plate neurons (4), whereas some reports show expression in migrating neurons (5, 49). We plan to use the anti-DCLK phospho-Ser-382 antibodies to study the distribution of phosphorylated DCLK within the brain and to study the pattern of phosphorylation of this residue in response to stimulation protocols in cultured neurons.

**Acknowledgments**—We thank Dr. Ort Leitner and Alon Levy (Anti-body Unit, Weizmann Institute of Science) for help in producing phosho-specific antibodies. We also gratefully acknowledge the expert assistance of Dr. Alla Shainskaya and Tehve Mehlman (Mass Spectrometry Unit, Weizmann Institute of Science).
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J. Biol. Chem. 2002, 277:17696-17705.
doi: 10.1074/jbc.M111981200 originally published online March 7, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M111981200

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