Catalytic and Regulatory Domains of Doublecortin Kinase-1[†]

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ABSTRACT: Doublecortin kinase-1 (DCK1) is a newly described multidomain protein kinase with a sequence significantly similar to those of both CaM kinases (CaMKs) and doublecortin, the product of the gene mutated in X-linked lissencephaly/double cortex syndome, a severe developmental disorder of the nervous system. Functional studies have revealed microtubule binding and polymerization activities of the doublecortin domain, yet little is known regarding the enzymatic properties and regulation of the kinase catalytic domain. We have identified and report here notable similarities as well as differences between the catalytic and regulatory properties of DCK1 and those of the CaMKs. Using synthetic peptide substrates modeled on synapsin I, a substrate recognition motif for DCK1 of Hyd-Arg-Arg-X-X-Ser*/Thr*-Hyd was derived. The similarity of this motif to that of CaMKI [Lee, J. C., Kwon, Y.-G., Lawrence, D. S., and Edelman, A. M. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 6413−6417] is consistent with the 59% level of amino acid sequence similarity between their catalytic domains. DCK1 catalytic activity is enhanced by mutagenic introduction of negative charge at Thr-239, a residue in a position equivalent to that of Thr-177 of CaMKI, the activation loop site for regulation by CaM kinase kinase. Unlike CaMKs, DCK1 is not directly activated by Ca^{2+}-bound CaM. However, truncation of a pseudosubstrate-like sequence in the C-terminus of DCK1 results in an ∼6-fold enhancement of activity. Thus, DCK1 demonstrates the potential to be regulated by relief of autoinhibition in response to signal(s) distinct from Ca^{2+}-bound CaM and potentially by activation loop phosphorylation and to phosphorylate intracellular targets at sites similar to those recognized by CaMK pathways.

Protein kinase-catalyzed phosphorylation is thought to play a major role in the function of the fully differentiated nervous systems of both vertebrates and invertebrates (*1*, *2*); however relatively little is known about the role of protein kinases in nervous system development. Recently, a novel, neuron-specific kinase, termed here doublecortin kinase-1 (DCK1),[1] has been identified (*3*−*11*). DCK1 has two functional domains. Its NH_2-terminal segment is ∼70% identical to doublecortin (DCX), a microtubule-associated protein, mutated in X-lissencephaly/double cortex syndrome, a severe disorder of neuronal migration during cerebral cortical development (*12*−*16*). This doublecortin domain (DC) is "fused" to a C-terminal protein kinase catalytic domain related to those of calmodulin-dependent protein kinases (CaMKs). The presence in a single molecule of these two domains suggests that DCK1 may be uniquely positioned to respond to and transmit intracellular signals that are important for neuronal development.

A variety of isoforms with structural variations in the DC and/or kinase domains of DCK1 have been described (*5*, *17*, *18*). Among these modifications, the one having the most obvious functional consequences governs the length of the DC domain. A full-length (340-amino acid) DC domain confers upon DCK1 the ability to colocalize with, and promote, microtubule assembly (*10*, *11*). However, the existence of mature transcripts encoding DCK1 isoforms with short (31 amino acids) DC domains lacking microtubule binding activity additionally implies that the kinase domain may have physiological roles apart from modulation of the microtubule cytoskeleton. At present, detailed information regarding the catalytic and regulatory properties of the kinase domain in the context of any of the DCK1 isoforms is unavailable. In this study, we have sought to redress this lack of information by (1) defining the requirements for substrate recognition by DCK1, (2) identifying a COOH-terminal autoinhibitory domain (AID) capable of repressing activity under basal conditions, and (3) finding evidence of a possible activation loop-based mechanism.

EXPERIMENTAL PROCEDURES

Materials. Synthetic peptides used for the analysis of peptide phosphorylation rates were synthesized as previously described (*19*). Plasmid pET30a(+) was from Novagen. pcDNA3.1, modified to express inserts as N-terminal FLAG

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[1] Abbreviations: DCK1, doublecortin kinase-1, also termed CaMKLK, CPG16, DCLK, DCAMKL1, or KIAA0369; DCX, doublecortin; CaM, calmodulin; CaMK, calmodulin-dependent protein kinase; CaMKK, calmodulin-dependent protein kinase kinase; CARP, CaMK-related peptide; AID, autoinhibitory domain.

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fusions, was generously provided by H. Qin. Lipofectamine 2000 was from Invitrogen Life Technologies. Rat brain total RNA was purified by CsCl density gradient centrifugation (*20*). All other reagents and chemicals were from standard commercial sources.

Cloning and Sequence Analysis of DCK1-β2 cDNA. Rat brain total RNA was reverse transcribed into cDNA using MuLV reverse transcriptase at 42 °C for 1 h with random hexamers as a primer. The resultant cDNA was used as a template in a PCR with primers based on the coding sequence for DCK1-β2 (GenBank entry U78857) (coding region capitalized): 5′-gcgcggatccATGTTAGAACTCATAGAAGTT as the sense primer and 5′-gcgcgaattcTTATTAAAAGGGC-GAATTGGG as the antisense primer. The PCR was performed with *Pfu* DNA polymerase at 94 °C for 105 s, followed by 25 cycles (94 °C for 45 s, 56 °C for 60 s, and 72 °C for 180 s). The sample was incubated at 72 °C for an additional 14 min and the amplified product purified by sequential 1% agarose gel electrophoresis and a Wizard PCR purification kit (Promega). It was then digested with *Bam*HI and *Eco*RI and subcloned into pET30a(+) and pcDNA3.1/FLAG as in-frame six-His and FLAG fusions, respectively. The correct coding sequence and reading frames of DCK1-β2 (and all mutants, as described below) were confirmed by DNA sequencing. Comparisons of sequence relatedness were performed using the local homology algorithm of Smith and Waterman (*21*) (BESTFIT, GCG). PEST domains were predicted by the algorithm of Rechsteiner and Rogers (*22*) (PESTfind, http://www.at.embnet.org).

Mutagenesis of DCK1-β2. DCK1-β2 $Thr^{239} \rightarrow$ Glu (T239E) was created with an overlapping PCR as follows. The 5′-segment of the DCK1-β2 coding sequence was amplified with 5′-gcgcgaattcTTATTAAAAGGGCGAATTGGG as the sense primer and 5′-GCCACAGACTTCGTACAGGGGG as the antisense and mutagenesis primer (altered bases are underlined) and the 3′-segment with 5′-CTGTACGAAGTCT-GTGGCACCC as the sense and mutagenesis primer and 5′-gcgcgaattcTTATTAAAAGGGCGAATTGGG as the antisense primer. PCR was performed for 30 cycles (94 °C for 45 s, 53 °C for 60 s, and 72 °C for 120 s), and the amplified products were purified, annealed, and used as the template in a second round of PCR with 5′-gcgcggatccATGTTA-GAACTCATAGAAGTT and 5′-gcgcgaattcTTATTAAAA-GGGCGAATTGGG as sense and antisense primers, respectively, and with cycling parameters as for the wild-type enzyme. The final PCR product was digested with *Bam*HI and *Eco*RI and subcloned into pET30a(+) and pcDNA3.1/FLAG. The DCK1-$\beta2_{1-357}$ truncation mutant was created by PCR using 5′-gcgcgaattcTTATTAAAAGGGCGAATTGGG and 5′-gcgcgaattcCTAGATCTTGCCAGCTACTGA as sense and antisense primers, resepctively, and subcloned into pET30a(+) and pcDNA3.1/FLAG. The FLAG epitope-tagged mutants DCK1-$\beta2_{1-369}$, DCK1-$\beta2_{1-384}$, DCK1-$\beta2_{1-388}$, and DCK1-$\beta2_{1-397}$ were created by introduction of the appropriate stop codons using the QuickChange site-directed mutagenesis kit (Stratagene) per the manufacturer's instructions with pcDNA3.1/FLAG-DCK1-β2 as the template.

Bacterial Expression and Purification of DCK1-β2. Recombinant DCK1-β2 (wild type and the T239E mutant) enzymes were expressed as six-His fusions in *Escherichia coli* strain BL21(DE3)pLysS following induction with 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 30 °C for 2 h. The cells were lysed by sonication, and the cell extract was centrifuged at 100000*g* for 30 min at 4 °C followed by removal of the remaining particulate material by passage through a 0.45 μm filter. It was then chromatographed on a Ni^{2+}−NTA agarose (HIS-BIND, Novagen) column equilibrated with 20 mM Tris-HCl (pH 7.9), 0.5 M NaCl, and 5 mM imidazole, washed with 30 bed volumes of wash buffer [20 mM Tris-HCl (pH 7.9), 0.5 M NaCl, and 60 mM imidazole], and eluted with 4 bed volumes of elution buffer [20 mM Tris-HCl (pH 7.9), 0.5 M NaCl, 400 mM imidazole, and 0.1% Triton X-100]. The eluted protein was dialyzed against 50 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 0.5 mM EDTA, 10% glycerol, and 0.05% Triton X-100 and quantified with a modified Lowry protein assay as described previously (*23*).

Transfection and Transient Expression of DCK1-β2. HEK293 or -293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS), and penicillin/streptomycin at 37 °C in 5% CO_2. Cultures were transfected with Lipofectamine 2000 according to the protocol provided by the manufacturer (Life Technologies). Proteins were expressed for 24−48 h after transfection, after which cells were rinsed twice with PBS and lysed in a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 25 mM NaF, 5 mM EGTA, 5 mM EDTA, 1% Triton X-100, and 1% protease inhibitor cocktail (Sigma) for 20 min on ice. The lysate was centrifuged at 14000*g* for 15 min at 4 °C and the protein concentration of the supernatant determined using Bradford protein assay reagent (Bio-Rad) according to the manufacturer's instructions.

Western Blotting. Proteins from 2 μg of cellular extracts were separated by SDS−10% PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane at 100 V for 90 min. The membrane was blocked with 5% nonfat dry milk in PBS for 1 h, washed for 5 min twice in PBS, and probed for 30 min with the mouse anti-FLAG M2 antibody (Sigma) diluted 1:10000 with 1% BSA in PBS. It was then washed for 15 min, and then for 4 × 5 min in PBS, and incubated for 30 min in HRP-conjugated goat anti-mouse IgG diluted 1:10000 with 1% BSA in PBS. The membrane was washed with 0.1% Triton X-100 in PBS for 15 min and then for 4 × 5 min, and immunoreactive bands were detected with enhanced chemiluminescence using the Western Lightning Chemiluminescence Reagent Plus kit from NEN.

Peptide Kinase Assays. Bacterially expressed, purified DCK1-β2 wild-type and T239E mutant enzymes were assayed in solution as follows. Enzyme (0.5 μg) was incubated at 30 °C in 38 μL of a mixture containing 50 mM Tris-HCl (pH 7.5), 0.5 mM DTT, 10 mM $MgCl_2$, 200 μM [γ-^{32}P]ATP (∼100 cpm/pmol), and synthetic peptides as specified in the figure legends. Quantitation of the incorporation of ^{32}P into peptides was carried out by binding to phosphocellulose as described previously (*23*). Briefly, 15 μL of the reaction mixture was spotted onto 2 cm × 2 cm P81 phosphocellulose filter paper squares at two time points. The squares were washed four times for 10 min each in 75 mM H_3PO_4, rinsed in ethanol for 5 min, dried, and subjected to liquid scintillation counting. Kinetic parameters (K_m and V_{max}) were calculated by nonlinear curve fitting using the program ENZFITTER (Elsevier-Biosoft, Cambridge, U.K.) as previously described (*19*). For wild-type DCK1-β2 and

mutants expressed in mammalian cells, an immune complex assay was performed as follows. Extracts (typically, 100 or 300 μg) prepared from cells transfected with pcDNA3.1/FLAG-DCK1-β2 or pcDNA3.1/FLAG (empty vector control) were incubated with 30 μL of anti-FLAG M2 affinity gel overnight at 4 °C. Immune complexes were collected by centrifugation and washed twice with 1 mL of lysis buffer, twice with 1 mL of lysis buffer without protease inhibitor cocktail, and twice with 1 mL of 50 mM Tris-HCl (pH 7.5). The kinase activity of the immune complex was measured by resuspension and incubation at 30 °C in an assay mixture containing 50 mM Tris-HCl (pH 7.5), 0.5 mM DTT, 10 mM $MgCl_2$, 50 μM [γ-^{32}P]ATP (~400 cpm/pmol), and peptide substrate, LRRRLSLANF (25−100 μM), in a final volume of 76 μL for 5 min. The reaction was terminated by the addition of 4 μL of 100% TCA and the mixture centrifuged for 20 s at top speed in a microcentrifuge. The supernatant (40 μL) was spotted onto P81 paper and the level of ^{32}P incorporation was measured as described above for the solution assay. Specific DCK1-β2 kinase activity was calculated as that obtained after subtraction of that of the empty vector control.

DCK1-β2 Autophosphorylation. DCK1-β2 was incubated at 30 °C in 15 μL of autophosphorylation buffer containing 50 mM Tris-HCl (pH 7.5), 0.5 mM DTT, 10 mM $MgCl_2$, 20 μM [γ-^{32}P]ATP (~500 cpm/pmol), and 2 mM EGTA or 1 mM $CaCl_2$ with 1 μM CaM as indicated in the figure legends. The reaction was terminated by adding SDS−PAGE sample buffer [50 mM Tris-HCl (pH 6.8), 2% SDS, 5% glycerol, and 0.1 M DTT] and the sample boiled for 5 min. Following electrophoresis (200 V for 45 min), the gel was fixed in 10% H_3PO_4 and 10% 2-propanol for 1 h, the solution was discarded, and the gel was incubated in fresh fixing solution overnight. The gel was dried, and labeled proteins were detected by autoradiography.

RESULTS AND DISCUSSION

A variety of designations have been applied to this newly identified kinase. In rodents and humans, it has been variously termed CaMKLK (*24*), cpg16 (*4*, *8*), DCaMKL1 (*7*, *9*, *11*), DCLK (*6*), and KIAA0369 (*3*, *5*), and in *Caenorhabditis elegans*, it has been termed zyg-8 (*25*). As the presence of a domain closely related to DCX is its most distinctive feature, it will here be termed doublecortin kinase-1 (DCK1). Multiple DCK1 transcripts, including one lacking a kinase domain, CARP/Ania-4 (*26*, *27*), are generated from a single genetic locus mapping to human chromosome 13q12.3-q14.1 (*5*, *7*, *9*) distinct from that of doublecortin which maps to Xq22.3-q23 and that of a second doublecortin kinase gene, DCK2 (A. M. Edelman et al., manuscript in preparation). Sequence conservation between the respective DCK1 and CARP products was analyzed as described in Experimental Procedures and shown schematically in Figure 1.

Two promoters are used to generate DCK primary transcripts (*17*, *18*). They are designated here α and β, expressing either a full-length (340 amino acid residues) or truncated (31 residues) NH_2-terminal region, the DC domain, with a sequence 76 or 68% identical, respectively, to that of DCX. The COOH terminus of the DC domain has a segment rich in Ser, Thr, and Pro residues, the SP region, followed by an 18-residue tail shared by DCX and CARP but not DCK1. DCK1 also encodes a predicted protein kinase domain (*28*). The NH_2-terminal portion of this domain contains additional Ser and Thr residues and is followed by a domain (PEST sequence) rich in Pro, Glu, Ser, and Thr residues (*22*). The kinase catalytic domain is most closely related to those of CaM kinases, particularly to that of CaMKI, with which it is 59% similar and 46% identical over 299 amino acids. This region of CaMK homology includes a portion of the PEST domain followed by a "catalytic core" (*28*) with the canonical residues being diagnostic of Ser/Thr protein kinases and an additional 22 residues. Notably, the similarity in sequence to CaMKI ends prior to the CaM binding domain of the latter. Within the kinase domain, additional diversity is generated by alternate splicing of an exon encoding an in-frame Arg-rich sequence (R) and variable inclusion of a C-terminal exon creating two possible stop codons, designated here as forms −1 and −2, respectively, one of which (form −2), encodes a second predicted PEST domain.

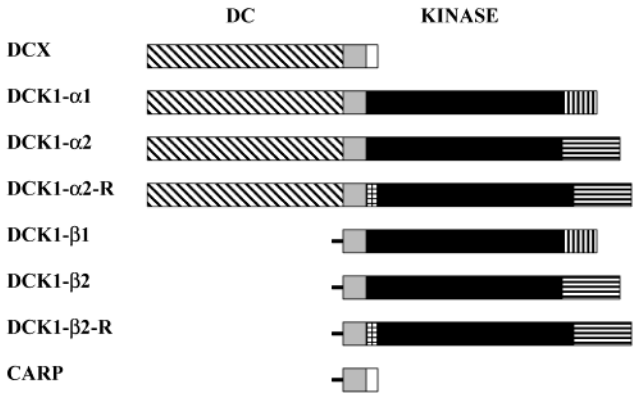

FIGURE 1: Schematic representation of DCK-related proteins. Similarly patterned boxes represent regions of sequence homology in the family of DCK-related proteins (shown at the left). DCK1 isoforms and CARP are generated by alternate splicing and promoter usage from a single genetic locus distinct from that of DCX. An additional mammalian DCK gene, DCK2 (A. M. Edelman et al., unpublished results) and the *C. elegans* DCK (zyg-8; *25*) are not shown. DC is the doublecortin domain, composed of microtubule-binding (diagonally striped), Ser-Pro-rich (gray), and COOH-terminal (white) segments. Kinase is the protein kinase domain, composed of an Arg-rich variably expressed exon (cross-hatched), a catalytic domain (black), and a COOH-terminal alternately expressed exon generating two possible stop codons (vertically and horizontally lined). Unique N-termini of β-forms and CARP are represented by short bars. Additional functional domains of DCK1 are not illustrated with shading but are described in the text and shown in Figure 6. The lengths of the domains are not drawn precisely to scale.

The roles of these various sequence elements in functional terms are, at present, poorly understood, with most of the current information relating to the function of the DC domain. As noted above, prior work has established that the full-length DC domain of DCK1-α is required for microtubule binding activity in vitro and DCK1 colocalization with microtubules in transfected cells (*10*, *11*). More recent studies have shown that the SP and PEST segments linking the DC and kinase domains in DCK1-α are susceptible to cleavage by calpain (*29*) or caspases (*24*) in response to calcium or apoptotic signals, respectively. Association of DCK1-α with the microtubule-based cytoskeleton and/or mediation of

Table 1: Kinetics of Phosphorylation of Peptides with Variation at the P+1 Position by Wild-Type and T239E DCK1-β2 Mutant Enzymes

	WT DCK1-β2			T239E DCK1-β2		
	V_{max}[a] (nmol min^{-1} mg^{-1})	K_m[a] (μM)	V_{max}/K_m ($\times 10^2$)	V_{max}[a] (nmol min^{-1} mg^{-1})	K_m[a] (μM)	V_{max}/K_m ($\times 10^2$)
LRRRLSDANF	4.0 $\pm$ 0.5	874 $\pm$ 147	0.46	27.2 $\pm$ 1.1	496 $\pm$ 58	5.5
LRRRLSLANF	7.8 $\pm$ 0.5	250 $\pm$ 16	3.1	47.9 $\pm$ 4.7	133 $\pm$ 17	36.0

[a] Kinetic parameters represent means $\pm$ the standard error of the mean of three separate assays of bacterially expressed and purified DCK1-β2 and DCK1-β2 T239E with each assay performed in duplicate.

apoptotic events may therefore be subject to regulation in vivo.

It is unclear, at present, why the microtubule binding doublecortin sequence is expressed in the same tissue (brain) both with (DCK1-α) and without (DCX) a kinase domain and, conversely, the rationale for the existence of a kinase (DCK1-β) with a truncated DC domain incapable of binding to microtubules . Answers to these questions have been delayed by a lack of information concerning the kinase domain, from both the standpoint of its substrate recognition properties and the mechanism of regulation of its catalytic activity. To begin to address these issues, we cloned and expressed DCK1-β2 in both bacterial and mammalian cells. As the kinase domain is identical between the α and β forms, characteristics of DCK1-β2 are likely to have applicability to DCK1-α isoforms. In addition, we are conducting separate studies with a DCK having a full-length, microtubule-binding DC domain, DCK2 (A. M. Edelman et al., unpublished results).

As shown in Figure 2A, DCK1-β2 was affinity purified to apparent homogeneity after expression as a six-His-tagged protein in *E. coli*. Preliminary studies conducted with this form of the enzyme and a variety of substrates demonstrated detectable, but low, levels of activity. For example, the enzyme was capable of slow autophosphorylation (WT, Figure 2C). Also, a peptide modeled on the sequence in the vicinity of Ser^9 of the synaptic vesicle-associated protein, synapsin, and known to be an effective substrate for CaMKI [LRRRLSDANF (*30*)] was phosphorylated by DCK1-β2, although with relatively poor kinetics (V_{max} = 4.0 nmol min^{-1} mg^{-1}, K_m = 874 μM) (Table 1). These data are in general agreement with those of Silverman and co-workers (*8*), who examined a variety of peptides and proteins, including crude brain extracts, as potential in vitro substrates for cpg16 (DCK1-β2) and reported autophosphorylation but little, if any, activity of the kinase toward exogenous substrates. In their study, the only exogenous substrate demonstrating detectable, albeit slow, in vitro phosphorylation (12 nmol min^{-1} mg^{-1}) was myelin basic protein, an observation leading to their conclusion that the kinase is characterized by a restricted substrate specificity.

Another possible explanation of the low apparent activity of DCK1-β2, however, is suggested by sequence comparison between DCK1-β2 and CaMKI (Figure 2B). In response to the elevation of the intracellular level of Ca^{2+}, CaMKI is phosphorylated at Thr^{177} within what is termed its "activation loop" (*31*) by the upstream enzyme CaM kinase kinase (CaMKK) (*32*−*36*). This results in massive activation (typically 20−50-fold), primarily due to a dramatic lowering of the K_m for the peptide substrate (*37*; A. M. Edelman et al., unpublished results). On the basis of the overall sequence similarity between the two proteins within this region and the presence of a correctly positioned "activating threonine"

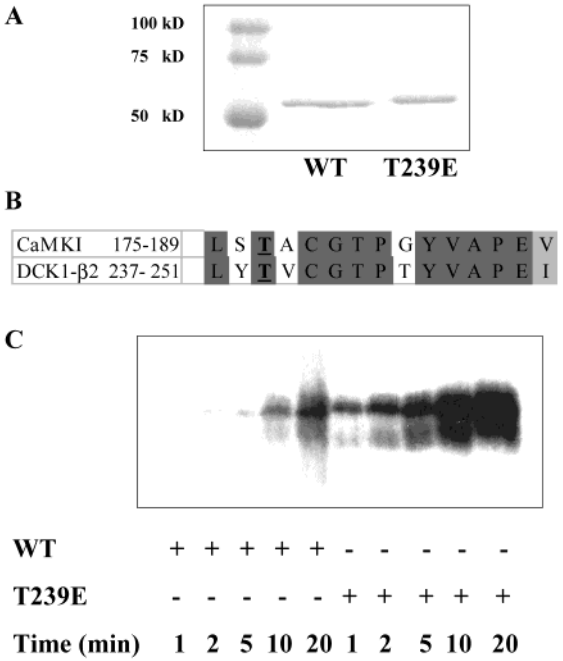

FIGURE 2: Enhancement of DCK1-β2 autophosphorylation by introduction of negative charge into its putative activation loop. (A) Wild-type DCK1-β2 (WT) and DCK1-β2 T239E (T239E) were expressed in *E. coli* and purified by Ni^{2+}−NTA agarose affinity column chromatography. The purity of the respective proteins is shown by SDS−PAGE and Coomassie staining. (B) Sequence comparison between the activation loop region of CaMKI (residues 175−189) and the corresponding region of DCK1-β2 (residues 237−251). Identical or similar residues between CaMKI and DCK1-β2 are darkly or lightly shaded, respectively. The phosphorylated activating Thr residue of CaMKI (*33*) and the aligned residue of DCK1-β2, T239, are in boldface and underlined. (C) Purified WT and T239E were separately incubated (1−20 min, as indicated, 30 °C) in the presence of EGTA and Mg-bound [γ-^{32}P]ATP and subjected to SDS−PAGE and autoradiography.

(Thr^{239} of DCK1-β2) (Figure 2B), we examined whether the activity of DCK1-β2 could be enhanced by phosphorylation. Incubation of the bacterially expressed and purified DCK1-β2 with CaMKK, either recombinant CaMKKβ (*38*) or CaMKKα or -β, purified from rat brain (*36*) and MgATP and in either the presence or absence of Ca^{2+}-bound CaM failed to activate DCK1-β2 (data not shown). In the case of CaMKI, we previously observed that introduction of negative charge into the activation loop via a T177D mutation led to a modest (2−6-fold) activation of the bacterially expressed enzyme (*33*). Although this enhancement is small compared to the effect of phosphorylation, we reasoned that it could, if observed, be diagnostic of phosphorylation in the case of DCK1-β2. We therefore expressed and purified a DCK1-β2 T239E mutant (Figure 2A). Relative to the wild type, the mutant demonstrated a 12-fold increase in V_{max}/K_m consisting of a 6.8-fold increase in V_{max} and 1.8-fold drop in K_m for

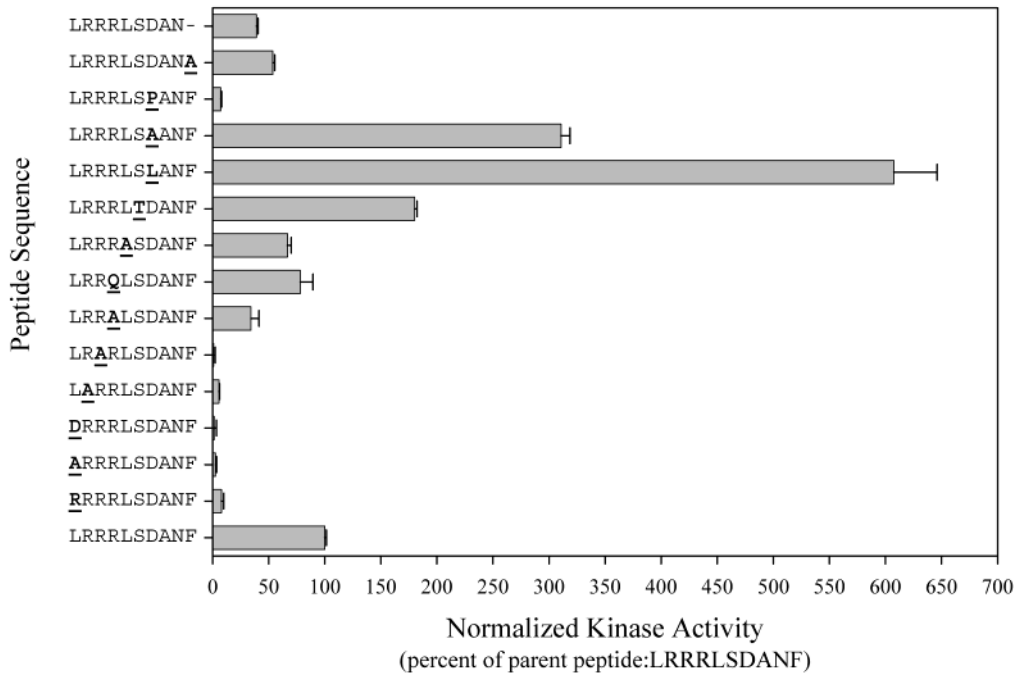

FIGURE 3: Synthetic peptide-mediated identification of substrate recognition determinants of DCK1-β2. The peptide kinase activity of bacterially expressed DCK1-β2 T239E, purified and quantified as described in Experimental Procedures, was determined under initial rate conditions using each peptide at 50 μM. Activities are calculated relative to the "parent peptide" LRRRLSDANF, which is arbitrarily set to 100, and represent the means $\pm$ the standard error of the mean of four to twelve independent determinations. For each peptide, the modified residue is in boldface and underlined, or with a dash in the case of the COOH-terminally deleted peptide (LRRRLSDAN).

the peptide substrate, LRRRLSDANF (Table 1). In addition, the mutation resulted in a markedly higher rate of autophosphorylation (Figure 2C). These results are consistent with DCK1-β2 being the target of an upstream kinase kinase distinct from CaMKK, although we cannot rule out the possibility that the mutation artificially induces an activating conformational change. Future studies will be required to distinguish between these alternatives. In any event, the DCK1-β2 T239E mutant was of sufficient activity to allow us to revisit the issue of DCK1-β2 substrate specificity.

Low rates of phosphorylation ($\sim$1 nmol min^{-1} mg^{-1}) were observed with the following proteins typically used as substrates for other protein kinases: myelin basic protein, protamine, histone IIa, and histone IIIs. Other typical kinase substrates (myosin light chain, phosvitin, and α-casein) were not phosphorylated at detectable rates (data not shown). Unlike a previous report (*8*), we did not find myelin basic protein to be a preferred substrate in comparison to the other proteins that were tested. Myelin basic protein, although a commonly used substrate for other kinases [e.g., MAPK (*39*)], is not a particularly good substrate for CaMKs such as CaMKI (*40*). This observation, coupled with the ability of DCK1-β2 to phosphorylate the CaMKI substrate, LRRRLSDANF (Table 1), and the fact that the region of homology between CaMKI and DCK1-β2 includes the entire kinase catalytic core (*28*), suggested that DCK1-β2 might respond to at least some of the primary sequence determinants identified as being important for substrate recognition by CaMKI (*30*).

The LRRRLSDANF peptide is an effective substrate for CaMKI such that the only alteration in the sequence which actually enhances substrate recognition is a Leu for Asp substitution at the P + 1 position (*30*). This is consistent with a preference, although not an absolute requirement, for a hydrophobic residue at this position. Accordingly, we used the LRRRLSLANF peptide as a potential substrate for wild-type DCK1-β2 and DCK1-β2 T239E. Increases in V_{max}/K_m of 6.7 and 6.5, relative to that of LRRRLSDANF, were observed for the two enzymes, respectively, primarily the result of K_m effects (Table 1). The similarity of this enhancement in DCK1-β2 activity to that shown by CaMKI in response to this substitution [6.3-fold (*30*)] is consistent with the possibility of overall similarity in their substrate specificities. It is also of note that an 11.6-fold increased V_{max}/K_m of the T239E mutant enzyme compared to that of wild-type DCK1-β2 was obtained using this modified peptide, a value similar to that found using the unmodified peptide (12-fold) (Table 1).

To test in greater detail the hypothesis of similar substrate specificities of CaMKI and DCK1-β2, we then examined the response of DCK1-β2 T239E to systematic alterations at other positions in the sequence of the peptide substrate. As shown in Figure 3, changes at all other positions resulted in either similar or lower rates of phosphorylation as had been observed for CaMKI (*30*). The slow turnover toward the majority of the peptides combined with the relatively low specific activity achieved even with the T239E mutant enzyme [$>$2 orders of magnitude lower than that of CaMKI (*30*, *33*)] precluded us from deriving kinetic parameters for all peptides, as in previous investigations which had been performed with fully activated forms of the CaMKs (*19*, *30*). Nonetheless, expressing initial rates of phosphorylation of modified peptides relative to that of LRRRLSDANF (the "parent peptide" included in each assay to normalize activities) as shown in Figure 3 yielded information regarding preferences at all of the positions previously studied with the CaMKs (*19*, *30*).

Peptides with basic (Arg), nonpolar and nonhydrophobic (Ala), or acidic (Asp) residues at the P−5 position[2] were phosphorylated at rates that were 1−8% of that of the parent peptide with Leu at the P−5 position. Thus, as for CaMKI, -II, and -IV, a hydrophobic residue is required at the P−5 position. At the P−4 position, an Arg residue is strongly preferred in that its substitution with Ala led to a phosphorylation rate that was 5.6% of that of the parent peptide having Arg at this position. Likewise, CaMKI responds to this substitution at the P−4 substitution with an 11.5-fold drop in V_{max}/K_m (*30*). Similar, although more modest, effects (decreases in V_{max}/K_m of 2.6- and 3.9-fold, respectively) are seen with CaMKII and CaMKIV (*19*).

As is the case for the CaMKs, the most important position for substrate recognition by DCK1-β2 is the P−3 position. Here, substitution of Arg with Ala led to almost undetectable phosphorylation (0.9% of the rate toward the parent peptide). Similarly, CaMKI, -II, and -IV phosphorylate the P−3 Ala-substituted peptide with V_{max}/K_m values that are 0.4, 1.0, and 0.3% of that of the parent peptide, respectively. Substitution of the P−2 Arg with Ala or Gln residues resulted in a rate of phosphorylation that is reduced to 34 or 78%, respectively, indicating that an Arg residue at the P−2 position is weakly preferred by DCK1-β2. A slight preference for Arg at the P−2 position is also seen with CaMKI and -IV, although not with CaMKII for which an Arg at the P−2 position is actually a negative determinant (*19*). The hydrophobic Leu residue at the P−1 position is not a specificity determinant for DCK1-β2 since its substitution with a nonhydrophobic Ala residue showed little effect. This is also true for CaMKI, although CaMKII and -IV show stronger preferences for Leu at the P−1 position in that its substitution with Ala led to decreases in V_{max}/K_m values of 4.3- and 5.1-fold, for the latter enzymes, respectively. At the phosphorylation position (P0), DCK1-β2 utilizes either Ser or Thr, consistent with its predicted Ser/Thr kinase catalytic core sequence (*28*) with threonine phosphorylated at a somewhat (1.8-fold) higher rate.

Substitution of a Leu residue for Asp at the P+1 position increased the rate of phosphorylation by 6.1-fold (Figure 3), in close agreement with the 6.5-fold increase in V_{max}/K_m (Table 1). Two additional substitutions were made at this position. Ala at the P+1 position also increased substrate efficacy but to a lesser extent (3.1-fold) than did Leu substitution, while a Pro residue at the P+1 position creates a dramatically poorer substrate, being phosphorylated at a rate that is only 7.2% of that of the parent peptide. Although this latter peptide has not been tested with CaMKI or CaMKIV, Stokoe and co-workers (*41*) reported a 100-fold drop in the rate of phosphorylation by CaMKII of a peptide substrate after incorporation of a position P+1 Pro residue. Thus, a hydrophobic residue at the P+1 position is clearly preferred by DCK1-β2, whereas proline is a negative determinant at this position. This observation, along with the deleterious effect of the substitution of Arg at the P−3 position, firmly places DCK1 in the group of "Arg-requiring" kinases, such as CaMKs, as opposed to "Pro-requiring kinases" such as MAPKs, cdks, etc. Finally, we used two alterations at the P+4 position, an Ala for Phe substitution and a deletion to test whether a hydrophobic residue is preferred at this position as is the case for CaMKI (*30*, *42*). The Ala-substituted and truncated peptides displayed phosphorylation rates that are 54 and 39%, respectively, of that exhibited by the Phe-containing parent peptide. These modestly lower phosphorylation rates are more similar to the lowered V_{max}/K_m values of CaMKII (46 and 76%, respectively, of the V_{max}/K_m toward the parent peptide) and CaMKIV (20 and 20%, respectively) than to those observed with CaMKI. For DCK1-β2, therefore, a hydrophobic residue at the P+4 position is only weakly preferred.

The data depicted in Figure 3, considered with respect to positions of greatest stringency, are compatible with a substrate recognition motif for DCK1-β2 of Hyd-Arg-Arg-X-X-Ser*/Thr*-Hyd.[3] At present, the only protein sequence to which this motif may be compared is a recently identified DCK1 autophosphorylation site (*18*). This site is found in the Arg-rich variably expressed exon (i.e., only in DCK1-α/β-R isoforms; see Figure 1) and therefore may have significance for regulation of either the localization or activity of these particular isoforms. The sequence context of this site, LGRRHS*L, incorporates most of the substrate determinants identified here, the P−3 Arg and the P−5 hydrophobic residues, as well as the hydrophobic amino acid at the P+1 position, with the only determinant of the motif not found in this autophosphorylation site being the P−4 Arg. It would therefore appear likely that phosphorylation sites of protein substrates of DCK1 will be found which incorporate the most important, although perhaps not all, of the determinants represented in this motif. The consistency between the sequence surrounding the site of autophosphorylation in DCK1-α/β-R and the peptide substrate recognition motif additionally implies that DCK1 utilizes the same site specificity for autophosphorylation as it does for phosphorylation of exogenous substrates. Although we used a form of DCK1 (DCK1-β2) in which this exon is spliced out, and no additional sites conforming closely to the motif are present, we also detected autophosphorylation (Figures 2C and 4A). It is therefore possible that DCK1-β2 autophosphorylation occurs slowly at poor or "atypical" sites. Autophosphorylation at atypical sites has been described for other kinases, for example, PKC (*43*). Although autophosphorylation represents an independent measure of DCK1-β2 activity, it may not by itself be regulatory since we have been unable to detect a change in peptide kinase activity after a 20 min preincubation under autophosphorylating conditions (consistent with the idea that in vivo Thr[239] may be targeted by an upstream kinase).

The substrate recognition motif of DCK1-β2 is strikingly similar to those of CaMKI, -II, and -IV using this family of peptides (*19*, *30*). For CaMKI, to which DCK1-β2 is most closely related, X-ray crystallographic data have provided insight into some of the active site residues capable of interaction with substrate determinants (*31*). For example, the substrate P−5 hydrophobic residue appears to be buried within a hydrophobic pocket formed by Phe[104], Ile[210], and Pro[216]. These residues are perfectly conserved in DCK1-β2,

[2] Positions of amino acid residues in synthetic peptide substrates are numbered negatively (P−) in the NH_2-terminal direction and positively (P+) in the COOH-terminal direction in relation to the phosphorylatable residue, designated the P0 position.

[3] Hyd designates a hydrophobic and X any amino acid residue. The asterisk denotes the site of phosphorylation.

and correspond to Phe^{167}, Ile^{272}, and Pro^{278}, respectively. Similarly, the substrate P−3 Arg of CaMKI is thought to electrostatically interact with Glu_{102} which is equivalent to Asp_{165} of DCK1-β2. Thus, it may be concluded that sequence relatedness of DCK1-β2 to the CaMKs within the catalytic cores of the respective kinases is predictive of similar substrate specificities, raising the possibility that DCKs and CaMKs could be directed to overlapping subsets of intracellular targets. Whereas we cannot eliminate the possibility that additional specificity determinants of DCK1 remain to be discovered, the dramatic kinetic changes seen in the response of the enzyme in vitro to the substrate determinants defined here suggest that they are important and may be of use in identification of its intracellular targets and prediction of specific sites of phosphorylation within those targets.

As discussed above, the intracellular signaling pathways by which the DCK1 family of enzymes is regulated remain to be identified, despite suggestive evidence for proteolysis (*24*, *29*) or activation loop phosphorylation (Table 1 and Figure 2C) as regulatory mechanisms. It was previously reported that treatment of cpg16 (DCK1-β2)-transfected COS7 cells with cAMP elevating agents (8-Br-cAMP and forskolin) provokes a 6−8-fold increase in its level of autophosphorylation when subsequently assayed in vitro, an observation leading to the suggestion that DCK1-β2/cpg16 is downstream of a PKA signaling cascade (*8*). We challenged DCK1-β2-transfected COS7L cells with forskolin (10 μM for 30 min) or 8-Br-cAMP (1 mM for 30 min) and also observed apparent increases in the extent of DCK1-β2 autophosphosphorylation, although of a more modest extent (1.9 ± 0.3-fold, $N = 4$, or 2.5 ± 0.4-fold, $N = 2$, respectively). The significance of these small increases is unclear. They do not appear to be due to enhanced DCK1-β2 expression (determined by immunoblotting, data not shown). Moreover, we have been unable to observe direct enhancement of the activity of purified DCK1-β2 by PKA in vitro (data not shown; see also ref *8*). We then tested whether cAMP-elevating signals applied to DCK1-β2-transfected cells are capable of regulating its activity toward an exogenous substrate using an immune complex kinase assay with the preferred peptide (LRRRLSLANF) serving as the substrate. For example, in transfected HEK293 cells, immunoprecipitated DCK1-β2 activities (after subtraction of the empty vector control activity, which represented <10% of the total activity and was unaffected by agonist) in forskolin-treated (10 μM for 30 min) and untreated cells were 67.7 ± 7.7 ($N = 4$) and 69.7 ± 4.2 ($N = 4$) pmol min^{-1} (mg of immunoprecipitated protein)$^{-1}$, respectively. Similar results were obtained using COS7L cells or with 8-bromo-cAMP treatment (data not shown). These results suggest that cAMP may not represent a primary mode of regulation of DCK1-β2 in a cellular context.

We also tested in this assay a variety of other agonists and extracellular signaling molecules. In neuroblastoma [SH-SY5Y, BE(2)-C] and/or non-neural (HEK293 and COS7L) cell lines, the following agents were found to be without effect on DCK1-β2 peptide kinase activity: forskolin, 8-Br-cAMP, ionomycin, serum, EGF, KCl, insulin, retinoic acid, TPA, and CNTF.

It is of note that despite the overall sequence similarity between DCKs and CaMKs, transient elevations of intracellular Ca^{2+} levels also appear to be unlikely to regulate

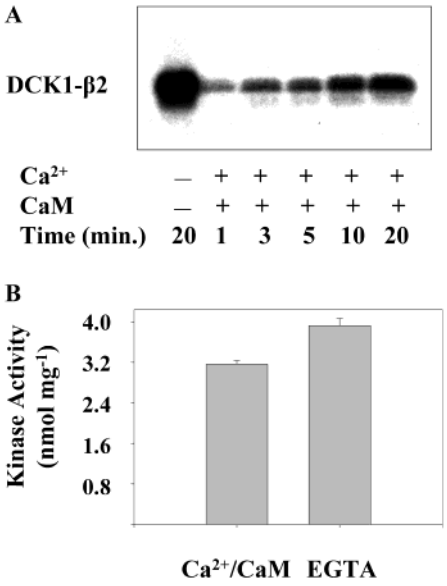

FIGURE 4: Lack of enhancement of DCK1-β2 kinase activity by Ca^{2+}-bound CaM. Bacterially expressed DCK1-β2 WT, purified and quantified as described in Experimental Procedures, was incubated (1−20 min, 30 °C) in the presence of Mg-bound [γ-^{32}P]-ATP and in the presence of Ca^{2+} (1 mM)-bound CaM (1 μM) or in its absence (and with 2 mM EGTA) as indicated and subjected to SDS−PAGE and autoradiography (A), or its peptide kinase activity was determined with LRRRLSLANF as the substrate and in the presence of Ca^{2+} (1 mM)-bound CaM (1 μM) or in its absence (and with 2 mM EGTA) as indicated (B). In panel B, peptide kinase activities represent the means ± the standard error of the mean of three independent determinations and are expressed as nanomoles of peptide phosphorylated per milligram in a 10 min reaction.

DCK1 activity as treatment with the Ca^{2+} ionophore, ionomycin, or KCl depolarization of neuroblastoma [SH-SY5Y, BE(2)C] cells was without effect on the DCK1-β2 activity. This latter conclusion is supported by an inability of Ca^{2+}-bound CaM to activate either the autophosphorylation (Figure 4A) or peptide kinase activity (Figure 4B) of bacterially expressed DCK1-β2 when assayed in vitro. In these assays, a small apparent inhibition of autophosphorylation and peptide kinase activity in the presence of Ca^{2+}-bound CaM was observed, although it is possible that CaM per se is not required for this effect. The lack of CaM activation is, however, consistent with the absence of significant sequence homology between DCK1-β2 and CaMKs within the latter's CaM-binding domain.

Although we cannot eliminate the possibility that DCK1-β2 is regulated by a transient allosteric activator, the association of which with the enzyme does not survive immunoprecipitation in this protocol, it is also possible that DCK1-β2 and possibly other DCK1 isoforms may be regulated by extra- and/or intracellular signals that are specific for this family of enzymes. To this end, we are currently pursuing two independent lines of experimentation, a yeast two-hybrid screen for DCK1-β2 interacting partners and an investigation of the role of the microtubule cytoskeleton in regulation of the activity and localization of DCK2, a DCK with an extended, microtubule-interacting DC domain.

We also considered the possibility that the DCK1 family is regulated, not by transient, enzyme activity-modulating stimuli but by longer-term influences directed, for example,

toward the control of DCK1 enzyme levels and/or localization. In fact, DCK1-β2 was discovered as a gene for which expression is upregulated by the glutamate analogue kainic acid (*4*). The possible existence of mechanisms for the regulation of levels and/or localization of DCK1, such as gene induction (*4*) or proteolysis (*24*, *29*), does not, however, preclude the possibility of more acute forms of regulation (for example, activation loop phosphorylation). In addition to the latter mechanism, to which only CaMKI and -IV are subject, all CaMKs are regulated by AIDs (recently reviewed in ref *44*). These domains are short, often resembling substrate ("pseudosubstrate") sequences, usually situated in a position COOH-terminal to the catalytic domain. Under basal conditions, the AID interacts with and occludes the catalytic site but is disengaged to allow substrate accessibility upon subsequent binding of CaM to the enzyme. Although it is not activated by Ca^{2+}-bound CaM (Figure 4), we examined DCK1-β2 for pseudosubstrate-like sequences which could serve as an AID, using as a guide its substrate recognition motif. A sequence was found [residues 389−397 (VFRRRRNQD)] (Figure 5A) which incorporates the most important of these determinants. If it is assumed that Asn^{395} occupies the position of the phosphorylatable Ser or Thr in substrates (i.e., the P0 position), there are Arg residues at positions P−3 (Arg^{392}) and P−4 (Arg^{391}) as well as a hydrophobic residue at position P−5 (Phe^{390}). The only determinant not incorporated into this sequence is a hydrophobic residue at the P+1 position, but as this is the least stringent of the requirements, it could in principle be offset by constraints imposed by tertiary structure and/or the other determinants.

Experimentally, the presence of an AID is typically revealed as enzyme activation upon its removal by either truncation mutagenesis (*33*) or limited proteolysis (*45*). As illustrated in Figure 5B, a series of successive COOH-terminal truncation mutants spanning residues 357−397 were prepared, and after we ascertained that they were correctly expressed (Figure 5C, bottom panel), they were assayed for immune complex peptide kinase activity (Figure 5C, top panel). Truncation COOH-terminal to residue 397 produced little change in activity, whereas four additional truncations NH_2-terminal to residue 389 were found to produce marked enzyme activation, on average 6.2-fold relative to that of the full-length enzyme (residues 1−433). These data indicate that DCK1-β2 exists in a basally autoinhibited state which can be relieved by removal of residues 389−397. As is the case for the enzyme activation that might occur as a result of T239 phosphorylation, the signals required to induce such activation remain to be determined. For CaMKI, these two modalities of activation are produced by different mechanisms, one allosteric (Ca^{2+}-bound CaM) and the other enzymatic (CaMKK). Interestingly, however, CaMKK-dependent activation of CaMKI itself requires the presence of Ca^{2+}-bound CaM (*32*, *33*, *46*). It will be of interest to determine whether distinct, but mechanistically interrelated, regulators exist for DCK1-β2 and its isoforms as well. It is also of note that in DCK1 isoforms with the second of the two alternate COOH termini (DCK1-α/β1; see Figure 1) there is a sequence with pseudosubstrate-like properties (IKRSGSLD) in a position similar to that of the AID sequence of DCK1-β2. As this site is potentially phosphorylated in autocatalytic fashion, it will be of interest to determine whether these isoforms are capable of autophosphorylation-dependent AID disengagement.

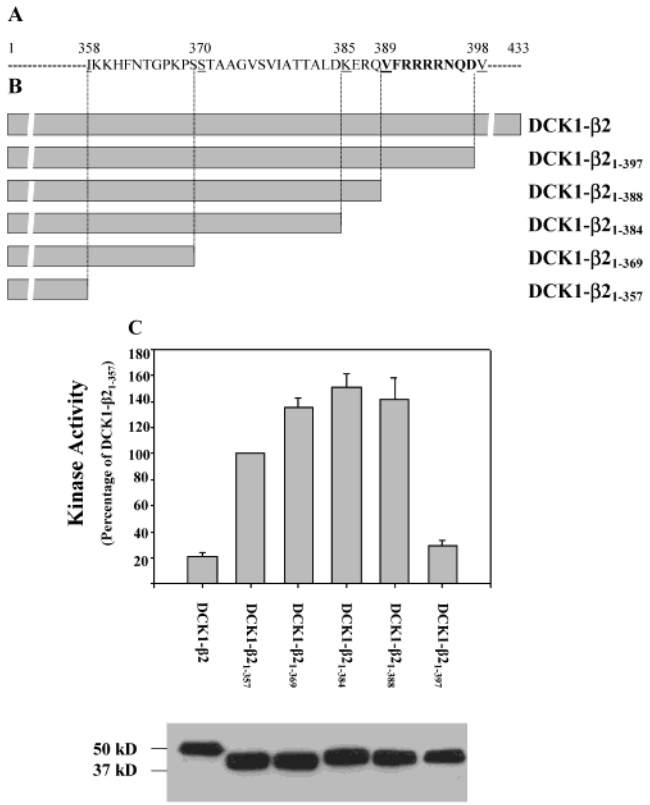

FIGURE 5: Identification of an autoinhibitory domain in DCK1-β2. Truncation mutations of DCK1-β2 were generated as described in Experimental Procedures. (A) The relevant sequence is shown with the amino acid residues replaced with stop codons underlined and numbered. A pseudosubstrate-like sequence (residues 389−397) is shown in bold. (B) Schematic representation of DCK1-β2 (full-length, residues 1−433) and truncation fragments. (C) FLAG epitope-tagged DCK1-β2 and truncation fragments were expressed in HEK239T cells immunoprecipitated with anti-FLAG M2 affinity gel and the respective kinase activities of the immune complexes measured using LRRRLSLANF as the substrate. The values represent the means ± the standard error of the mean (with the exception of DCK1-$\beta 2_{1-357}$ since activities are calculated relative to the latter being arbitrarily set to 100) of four independent assays. Activities are corrected for slight differences in the expression levels of the various forms by quantitative immunoblotting with a typical blot shown in the bottom panel.

FIGURE 6: Proposed domain structure of DCK1-β2. The relative positions of the SP region, the catalytic domain (catalytic core) within which is Thr^{239} in the putative activation loop, the autoinhibitory domain (AID), and the NH_2- and COOH-terminal PEST domains are indicated. Data used to identify residues delimiting these domains (shown below) are discussed in the text.

Figure 6 schematically illustrates the most likely domain structure for DCK1-β2. Close to its amino terminus is the SP region (residues 7−52) conserved in all DCK1 isoforms and DCX (Figure 1). Following this is a segment identified as a PEST sequence (residues 53−81) by virtue of its high score (9.52) using the PESTfind algorithm (*22*). In some isoforms, although not in DCK1-β2 (and thus not illustrated in Figure 6), there is the Arg-rich alternate exon with its autophosphorylation site (*18*). As noted above, the SP/PEST

region contains sites of proteolysis with proposed regulatory consequences for DCK1 function (*24*, *29*). The catalytic domain (residues 83−340) is defined following the convention for serine/threonine protein kinases established by sequence comparison with other members of this supergene family (*28*). It is delimited amino-terminally at a position seven residues upstream from the glycine-rich ATP-binding loop (Tyr^{83}) and carboxyl-terminally as the last residue of the conserved His-Pro-aromatic-hydrophobic sequence (Val^{340}) downstream of the invariant Arg residue (Arg^{328}). Within what would be predicted to be the large lower lobe of the catalytic domain based on the X-ray crystal structure of CaMKI (*31*) is a sequence (residues 237−251) homologous to the activation loop sequence of CaMKI (Figure 2B) and containing Thr^{239}, replacement of which with glutamic acid may mimic heterologous kinase-dependent DCK1-β2 activation (Figure 2C and Table 1). COOH-terminal to the catalytic domain is the AID (residues 389−397) (Figure 5) followed by a second predicted PEST domain (residues 403−426), the latter having an unknown function.

In conclusion, we have assessed the substrate specificity requirements and identified two potential regulatory domains of DCK1-β2. That these mechanisms may be generalizable to other DCK1 family members is suggested by the presence in all DCK1 isoforms of both the activation loop threonine and COOH-terminal pseudosubstrate-like sequences potentially capable of functioning as AIDs. Future studies will be required to identify the substrates and regulators of the DCK1 family operative in an in vivo context.

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