Dysbindin Structural Homologue CK1BP Is an Isoform-Selective Binding Partner of Human Casein Kinase-1[†]

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ABSTRACT: Casein kinase-1 is a family of ubiquitous eukaryotic protein kinases that frequently function in tandem with the ubiquitin modification system to modulate protein turnover and trafficking. In Alzheimer's disease, these enzymes colocalize with ubiquitinated lesions, including neurofibrillary tangles and granulovacuolar degeneration bodies, suggesting they also play a role in disease pathogenesis. To identify binding partners that potentially regulate or recruit these enzymes toward disease lesions, a Sos-recruitment yeast two-hybrid screen was performed with human Ckiδ (the casein kinase-1 isoform most closely linked to granulovacuolar degeneration bodies) and a human brain cDNA library. All interacting clones contained a single open reading frame termed casein kinase-1 binding protein (CK1BP). On the basis of sequence alignments, CK1BP was a structural homologue of the acidic domain of dysbindin, a component of the dystrophin-associated protein complex and the biogenesis of lysosome-related organelles complex-1. CK1BP interacted with full-length Ckiδ, the isolated Ckiδ catalytic domain, Ckiγ2, -γ3, and -ϵ in the yeast two-hybrid system, and bound Ckiδ and -ϵ in pulldown assays but did not interact with Ckiα. Interaction with the Ckiδ catalytic domain led to concentration-dependent inhibition of protein kinase activity in the presence of protein substrates tau and α-synuclein. Although intact dysbindin did not bind any CK1 isoform, deletion of its coiled-coil domain yielded a protein fragment that behaved much like CK1BP in two-hybrid screens. These data suggest that the acidic domain of dysbindin and its paralogs in humans may function to recruit casein kinase-1 isoforms to protein complexes involved in multiple biological functions.

Casein kinase-1 (CK1)[1] is a family of structurally conserved protein kinases characterized by a monomeric structure, an unusually high isoelectric point, and a constitutive phosphotransferase activity selective for Ser/Thr residues located in or adjacent to anionic segments of substrate proteins (reviewed in refs *1* and *2*). CK1 enzymes are found in all eukaryotes and frequently function in tandem with the ubiquitin system to modulate turnover and trafficking of substrate proteins (*3*−*6*). Each CK1 family member consists of a highly conserved N-terminal catalytic domain linked to C-terminal segments of differing length and amino acid sequence. In humans, CK1 is encoded by six distinct genes (Ckiα, -γ1, -γ2, -γ3, -δ, and -ϵ), with further structural complexity attained by alternative splicing. Access to substrates is controlled in part by the subcellular distribution of individual CK1 isoforms. For example, in budding yeast, CK1 homologues Yck1p and Yck2p selectively associate with cell surface membranes (*7*) where they gain access to membrane-bound substrates, including mating-type receptors Ste2p and Ste3p (*3*, *4*) and components of the permeases and sensors involved in the detection and transport of extracellular nutrients (*5*, *6*, *8*). In each case, phosphorylation precedes ubiquitination and subsequent trafficking or proteasome-mediated turnover of substrate.

In the case of Yck1p/Yck2p, membrane localization is essential for cell viability (*7*) and is mediated by acylation of their C-terminal segments (*3*, *9*, *10*). The mechanisms underlying the subcellular distribution of other isoforms, including those found in humans, are less clear. For example, mammalian Ckiα associates with various membrane fractions in mature neurons, but this interaction may be mediated by exogenous factors other than or in addition to direct modification of the Ckiα polypeptide chain (*1*). In addition to activity at cellular membranes, metazoan CK1 homologues function in cytoplasm to mediate phosphorylation-dependent turnover of proteins involved in circadian rhythm (*11*, *12*) and Wnt (*13*) and Hedgehog (*14*) signaling pathways. In these examples, the interactions that recruit or modulate CK1 activity are unknown.

Interaction of CK1 with protein turnover machinery may also contribute to neurodegenerative diseases that accumulate misfolded protein within affected neurons. In AD tissue, CK1 isoforms colocalize with ubiquitinated cytoplasmic lesions, including both granulovacuolar degeneration bodies and the tau filaments of neurofibrillary tangles (*15*). The appearance

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[1] Abbreviations: AD, Alzheimer's disease; CD, circular dichroism; CK1, casein kinase-1; CK1BP, casein kinase-1 binding protein; HEK, human embryonic kidney; IMAC, immobilized metal affinity chromatography; Ni^{2+}−NTA, nickel−nitrilotriacetic acid.

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of CK1 in these lesions correlates strongly with cognitive decline in longitudinal studies of AD progression (*16*), suggesting that CK1 may contribute to hyperphosphorylation and subsequent aggregation of resident proteins. Consistent with this observation, tau filaments isolated from AD brain contain up to 0.3% CK1 on a weight basis (*17*).

These data suggest that modulation of CK1 subcellular localization and constitutive phosphotransferase activity may be important in disease as well as in normal biology. To identify proteins capable of performing these functions, a human brain cDNA library was subjected to yeast two-hybrid screening using the human Ckiδ isoform as bait. All positive clones encoded a previously uncharacterized protein we term CK1-binding protein (CK1BP). Here we describe the identification and characterization of CK1BP, a homologue of dysbindin (a protein involved in protein trafficking and lysosome biosynthesis and implicated in muscular dystrophy and schizophrenia).

EXPERIMENTAL PROCEDURES

Materials. Enhanced chemiluminescence (ECL) detection reagents and [γ-^{32}P]ATP were from GE Healthcare (Waukesha, WI) and Ni^{2+}−NTA agarose beads from Qiagen (Valencia, CA), and protein G Plus-Agarose was from Santa Cruz Biotechnology (Santa Cruz, CA). Lipofectamine 2000 was from Invitrogen (Carlsbad, CA). Partially hydrolyzed casein (5% solution) and protease inhibitors containing (final concentrations) 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 0.8 μM aprotinin, 40 μM bestatin, 20 μM leupeptin, 15 μM pepstatin A, 14 μM L-transepoxysuccinyl-leucyl-amido-[4-guanidine]butane, and 26 μM *N*-acetyl-Leu-Leu-Norleu-Val were obtained from Sigma (St. Louis, MO). The RRDDEEDEEMSETADGER synthetic peptide substrate [corresponding to residues 166−180 of rabbit skeletal muscle phosphatase inhibitor-2 with terminal Arg residues added to the natural peptide (*18*)] was a gift from ICOS Corp. (Bothell, WA). Full-length cDNAs encoding human Ckiα (CSNK1A lacking L and S inserts, described in ref *19*), Ckiδ [CSNK1D variant 1 (*20*)], Ckiε [CSNK1E (*21*)], Ckiγ2 [CSNK1G2 (*22*)], Ckiγ3 [CSNK1G3 (*23*)], and dysbindin (DTNBP1 variant *c*; GenBank accession number NM183041) were obtained by PCR amplification of an adult human brain cDNA library prepared in the pMyr vector (Stratagene, La Jolla, CA).

Antibodies. Monoclonal antibodies 128A (anti-Ckiδ) and 94.1 (anti-Ckiα) were the gifts of ICOS Corp., whereas anti-Ckiε and 12CA5 were from BD Biosciences (San Jose, CA) and Roche Molecular Biochemicals (Indianapolis, IN), respectively. Peroxidase-labeled affinity-purified anti-mouse secondary antibody was from KPL (Gaithersburg, MD).

Yeast Two-Hybrid Screen. Screening was performed with an hSos/Ras recruitment system in Cdc25Hα cells (MATα *ura*3−52 *his*3−200 *ade*2−101 *lys*2−801 *trp*1−901 *leu*2−3 112 *cdc*25−2 Gal^+) (*24*, *25*) according to the manufacturer's instructions (Stratagene). The coding region of full-length Ckiδ was amplified by PCR, cloned into the *Bam*HI site of the bait plasmid pSos, and cotransformed with an adult human brain cDNA library into *Saccharomyces cerevisiae* cdc25Hα cells. Yeast clones that grew on glucose minimal medium at 25 °C and galactose minimal medium at 37 °C, but not on glucose minimal medium at 37 °C, were judged positive for interaction (*24*, *26*). Positive prey plasmids were isolated from yeast and sequenced in both directions using vector-specific primers at the 5′ and 3′ ends.

For directed two-hybrid analysis, CK1BP, dysbindin, or dysbindin Δ1−83 cDNA was isolated and cloned into the pMyr vector at its *Eco*RI−*Xho*I sites. Human Ckiα, -γ2, and -γ3 and Ckiδ-Δ317 (*27*) and Ckiε were amplified by PCR and cloned into pSos. Interaction between each of these CK1 constructs and pMyr-CK1BP was then tested in yeast cdc25Hα cells.

Recombinant Proteins. All His_6-tagged proteins (CK1BP, htau40, α-synuclein, dysbindin, and Ckiδ-Δ317) were expressed in *Escherichia coli* using the pT7C vector and purified as described previously (*28*−*30*). CK1BP without the His_6 tag was expressed in *E. coli* using vector pT7II (*31*). Recombinant, nontagged protein was purified after extract preparation (*31*) by liquid chromatography. The extract was loaded directly onto a Sepharose-Q fast flow column equilibrated in buffer A [10 mM MOPS (pH 7.0) and 0.1 mM EGTA] containing 50 mM NaCl, washed with 5 bed volumes of buffer A containing 150 mM NaCl, and eluted with buffer A containing 300 mM NaCl. Fractions containing CK1BP were pooled and brought to 25% saturation with solid $(NH_4)_2SO_4$, loaded onto a phenyl-Sepharose column equilibrated in buffer B [50 mM sodium phosphate (pH 7.0) and 0.1 mM EGTA] containing 1 M $(NH_4)_2SO_4$, washed with 5 bed volumes of buffer B containing 800 mM $(NH_4)_2SO_4$, and eluted with buffer B containing 400 mM $(NH_4)_2SO_4$. Fractions containing CK1BP were pooled, diluted with buffer C [10 mM HEPES (pH 7.4), 150 mM NaCl, and 0.1 mM EGTA] to lower the salt concentration, concentrated to 4 mL by centrifugal filtration (Centricon-30; Amicon), and finally loaded directly onto a 180 mL column (1.6 cm × 91 cm) of Sephacryl S-200 HR equilibrated and run (30 mL/h) in buffer C. Fractions containing CK1BP were pooled, concentrated, and stored at −20 °C until they were used.

Cell Culture and Transfection. A triple-HA-tagged CK1BP construct was created by taking the triple HA tag from pSLF173 (*32*) as a *Xho*I−*Not*I fragment and ligating it into the pcDNA3.1 (Invitrogen) vector. CK1BP was amplified by PCR and cloned directly into the PCR-BluntII-TOPO vector (Invitrogen). The construct was then digested with *Not*I and *Eco*RI and ligated into the pcDNA3.1 in frame with the HA tag. HEK-293 cells, which were grown as described previously (*30*), were transiently transfected with this plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

His_6 Pulldown Assay. HEK-293 cells were harvested and disrupted in lysis buffer [20 mM Tris-HCl (pH 7.4), 500 mM NaCl, 20 mM imidazole, 1 mM PMSF, 1% (v/v) Nonidet-40, and 1 mM EDTA] containing 1× complete protease inhibitors (*30*). Cell debris was removed by centrifugation (10 min at 10000*g*) at 4 °C. Ni^{2+}−NTA−agarose beads were resuspended in lysis buffer in a ratio of 1:1 (v/v), and 40 μL of this slurry was incubated with 20 μg of His_6-CK1BP or His_6-α-synuclein at 4 °C for 1 h. The beads were pelleted by centrifugation and washed three times with lysis buffer. HEK-293 extracts (500 μg) were then added to the beads and incubated (2 h at 4 °C). The beads were sedimented a second time, washed five times with lysis buffer, and finally resuspended in SDS−PAGE sample buffer. Proteins were separated by SDS−PAGE and either

stained with Coomassie brilliant blue R-250 or subjected to immunoblotting using monoclonal antibody 128A, 94.1, or anti-Ckiϵ as described previously (*30*).

Immunoprecipitation. HEK-293 cells were harvested in RIPA buffer [150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1% NP-40, 5 mM EDTA, and 0.5% sodium deoxycholate] containing 1× complete protease inhibitors and disrupted by sonication. After removal of cell debris by centrifugation (10000*g* for 15 min), extracts (500 μg) were incubated (2 h at 4 °C) with 2 μg of monoclonal antibody 128A (anti-Ckiδ) or 12CA5 (anti-HA tag). Protein G−agarose beads [60 μL of a 25% (w/v) slurry] were then added to the samples, and incubation was continued for 1 h at 4 °C. The resultant immunocomplexes were collected by centrifugation, washed three times with RIPA buffer, and subjected to immunoblot or in vitro protein kinase assays as described below.

In Vitro Kinase Reactions. Protein kinase activity of purified Ckiδ-Δ317 or of immunocomplexes derived from immunoprecipitation experiments was estimated by incubating each with various protein substrates in phosphorylation buffer [10 mM MOPS (pH 7.0), 50 mM NaCl, and 4 mM $MgCl_2$]. When present, reaction mixtures contained varying amounts of CK1BP (0−2 μM). After the mixtures had been preincubated for 15 min, reactions were initiated by the addition of 100 μM [γ-^{32}P]ATP (500 cpm/pmol), the mixtures incubated for 30 min at 37 °C, and the reactions terminated with SDS sample buffer. Following SDS−PAGE, the stained and dried gels were subjected to autoradiography on a film or with a Molecular Imager FX Pro Plus multiimager system (Bio-Rad).

Circular Dichroism. Samples were prepared for CD analysis by desalting into 100 mM $NaClO_4$ and 20 mM H_3BO_3 (pH 7.4). These buffer components were employed because of their transparency at far-UV wavelengths (*33*). Spectra were collected (178−260 nm) at 25 °C using an AVIV model 202 CD spectrometer and a quartz cuvette with a path length of 0.01 cm. Four repetitive scans (integration time of 4 s, step size of 1 nm, and bandwidth of 1 nm) were recorded, averaged, and corrected for buffer-only blank without additional filtering or smoothing. Raw CD signals (in millidegrees) were converted to mean residue molar ellipticity $[\theta]_{MRW}$ in degrees square centimeters per decimole using the formula $[\theta]_{MRW} = [\theta]_{obs}/(10lcn)$, where $[\theta]_{obs}$ is the observed ellipticity in millidegrees, l is the path length in centimeters, c is the molar concentration of protein, and n is the number of residues in the protein (*35*). The secondary structure composition of α-helix (normal and distorted), β-strand (normal and distorted), turns, and random coil was estimated from net spectra using CONTIN/LL (*34*), CDSSTR (*35*), and SELCON3 (*36*) algorithms. Resultant estimates were then averaged and presented ± the standard deviation (*37*).

Hydrodynamic Analysis. Analytical size-exclusion chromatography was performed (4 °C) on a 15 mL (7.8 mm × 300 mm) TSK 3000 SWXL column equilibrated with 10 mM HEPES (pH 7.0) and 100 mM NaCl and operated at 0.5 mL/min. The hydrodynamic radius of CK1BP was estimated from calibration standards, including bovine serum albumin (3.62 nm), ovalbumin (2.83 nm), bovine erythrocyte carbonic anhydrase (2.01 nm), and lysozyme (1.90 nm) as described previously (*38*).

Velocity sedimentation measurements were performed in linear 4 to 20% sucrose gradients containing 10 mM HEPES (pH 7.4) and 150 mM NaCl. Standards included ovalbumin (4.3 s), bovine erythrocyte carbon anhydrase (3.2 s), myoglobin (1.9 s), and cytochrome *c* (1.6 s). After centrifugation (200000*g* for 20 h at 4 °C), gradients were fractionated, and sedimentation coefficients were estimated as described previously (*38*). The native molecular weight and frictional ratio were calculated from hydrodynamic parameters as described previously (*39*) using partial specific volumes calculated from the amino acid sequence (*40*). Theoretical hydrodynamic radii for CK1BP in native, molten globule, pre-molten globule, random-coil, and denatured states were calculated from molecular weight using the empirical equations deduced as described previously (*41*).

Analytical Methods. Hyperbolic inhibition curves were fit to the rectangular hyperbola

$$y = ax/(b + x) \quad (1)$$

where y is kinase activity determined at inhibitor concentration x and constant b corresponds to x at $0.50y_{max}$ (i.e., IC_{50}).

Probability values were determined via a Student's t test for single comparison and one-way ANOVA with Tukey's post hoc test for multiple comparisons. All analyses were performed using InStat.

Protein concentrations were determined for extracts using the Coomassie blue binding method with bovine serum albumin as a standard (*42*) and for purified proteins by absorbance using extinction coefficients calculated from amino acid composition (*40*).

Immunoblots were performed as described previously using peroxidase-labeled goat anti-mouse IgG as a secondary antibody (*30*) and developed using the enhanced chemiluminescence.

RESULTS

Identification of CK1BP as a Ckiδ Binding Protein. To identify binding partners of human Ckiδ, a human brain cDNA library was screened in a Sos recruitment yeast two-hybrid system. This screening paradigm was employed because of its ability to capture interactions occurring at membranes, the natural intracellular location of several CK1 isoforms (*2*). Eighteen clones confirmed positive for interaction were obtained from 2.5×10^6 independent transformants. DNA sequence analysis revealed that all 18 positive clones were derived from a single open reading frame located on chromosome 20 at position 20q13.12 (C20orf35). Two variant transcripts have been identified for this locus. One transcript (GenBank accession number NM018478), identified in cDNA libraries prepared from human hypothalamus (*43*), encodes a protein predicted to contain 261 amino acid residues (27 830 Da; pI = 4.5). The second transcript (GenBank accession number BC012818) was also identified in hypothalamus as well as in signet-ring cell carcinoma (NEDO human cDNA sequencing project), in skeletal muscle (Telethon project B41), in placenta, and in colon adenocarcinoma (*44*). Isoform 2 does not encode the first 100 N-terminal amino acids of isoform 1, yielding only the C-terminal 161 residues of this protein (17 493 Da; pI = 4.13). To clarify the relationship between these known transcripts and the isolated Ckiδ interacting clones, sequence

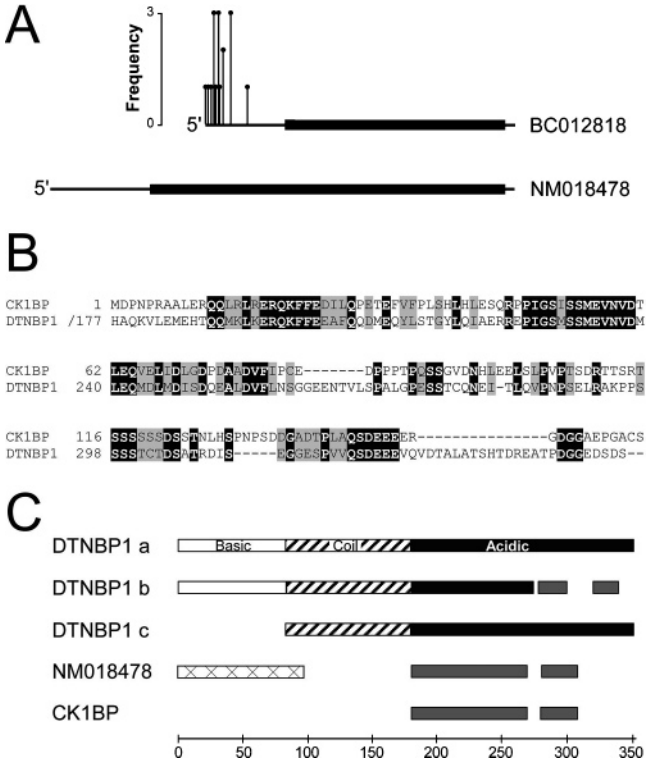

FIGURE 1: Identification of CK1BP. A two-hybrid screen for proteins interacting with human Ckiδ bait yielded 18 positive clones derived from a single open reading frame located on chromosome 20. (A) Frequency and location of 5′-ends of all positive clones (consisting of multiple copies of 11 unique sequences) relative to long (1.5 kb, GenBank accession number NM018478) and short (1.0 kb, GenBank accession number BC012818) transcripts associated with the open reading frame in the GenBank database (black bars denote coding sequences). All 5′-ends were consistent with the latter transcript being the source of Ckiδ interacting activity. The protein encoded by this transcript was named CK1BP. (B) Alignment of the CK1BP amino acid sequence with C-terminal domain of dysbindin (DTNBP1 residue numbering corresponds to variant *a*). Identical residues have a black background, whereas similar residues have a gray background. (C) Protein domains of CK1BP relative to dysbindin. Full-length dysbindins are derived from the DTNBP1 gene and consist of a weakly basic N-terminal domain (white bar), followed by a coiled-coil domain (striped bar), and finally a strongly acidic C-terminal segment (black bar). Dysbindin variant *c* lacks the N-terminal basic domain, whereas variant *b* contains an alternatively spliced C-terminus. CK1BP shares homology with the strongly acidic, C-terminal domain of dysbindin variants (this domain is depicted with dark shading). Although the protein product of transcript NM018478 contains a weakly basic N-terminal segment, it is unrelated to the N-terminal sequence found in dysbindin and so is represented with a crosshatched pattern. The numbered scale at the bottom corresponds to dysbindin variant *a*.

alignments were performed. Results revealed that the 18 interacting clones consisted of 11 unique sequences related to each other by small differences in their 5′ ends (Figure 1A) and that all were consistent with the shorter transcript being the source of Ckiδ binding activity. The encoded 161-residue protein was therefore termed CK1BP (casein kinase-1 binding protein).

CK1BP Structure. The primary structure of human CK1BP is shown in Figure 1B. It consists of a strongly acidic sequence that is 39% identical and 63% similar to the C-terminal domain of dysbindin, a ubiquitous protein that has been implicated in the pathogenesis of Hermansky-Pudlak syndrome type 7 (*45*) and familial schizophrenia (*46*).

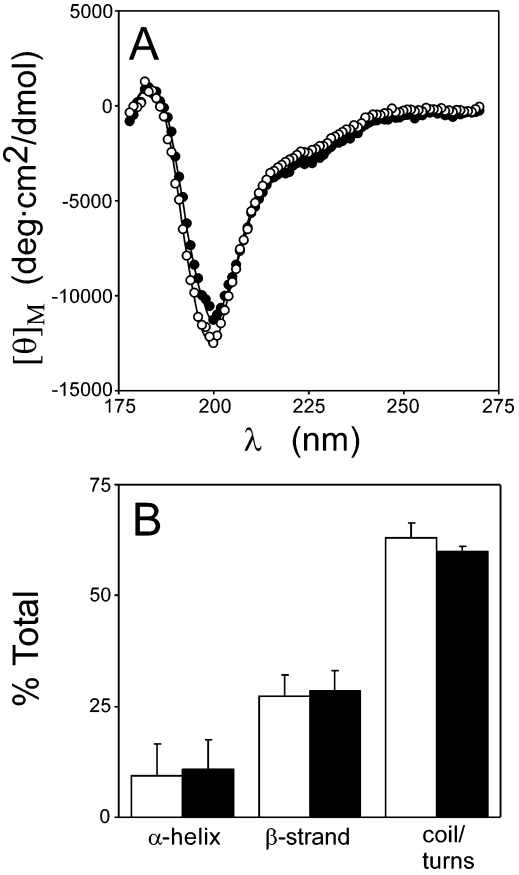

FIGURE 2: CD spectra of CK1BP and His_6-tagged CK1BP. (A) CK1BP (○) and His_6-tagged CK1BP (●) were prepared in 100 mM sodium perchlorate and 20 mM boric acid (pH 7.4) and subjected to far-UV CD spectroscopy (25 °C). (B) CD spectra were individually fit with CDSTTR, SELCON3, and CONTIN/LL algorithms to estimate total α-helical, β-strand, and random coil/turn conformations. The three estimates were then averaged and plotted ± the standard deviation for CK1BP (black bars) and His_6-tagged CK1BP (white bars). Both preparations contained mostly disordered secondary structure and did not differ significantly from each other at $p < 0.05$.

Three splice variants of human dysbindin have been identified from cDNA clones, each containing a coiled-coil domain that may mediate association with itself or other proteins containing coiled coils (*47*, *48*). Variants differ by the presence or absence of a basic N-terminal domain and by alternative splicing of sequences in the acidic domain (Figure 1C). Similarly, CK1BP and the sequence derived from the longer transcript (GenBank accession number NM018478) differ by the presence or absence of a weakly basic N-terminal extension. Thus, on the basis of the primary structure, CK1BP appears to be a member of the dysbindin protein family.

To assess CK1BP secondary structure content, recombinant CK1BP and His_6-tagged CK1BP were prepared from bacterial expression systems as described in Experimental Procedures and subjected to circular dichroism spectroscopy. Both preparations yielded spectra typical of proteins with substantial random-coil character (i.e., broad minima of ellipticity centered at 200 nm; Figure 2A). To quantify relative amounts of secondary structure, CD spectra were deconvoluted by three independent algorithms as described in Experimental Procedures (Figure 2B). On the basis of the secondary structure content calculated from averages of the

Table 1: CK1BP Physical Properties

hydrodynamic radius (nm)	3.0 ± 0.1
sedimentation coefficient ($s_{20,w}$)	1.6 ± 0.1
partial specific volume (mL/g)[a]	0.699
native molecular mass (Da)[b]	18000 ± 1000
native structure	monomer
$(f/f_o)_{obs}$	1.76
$(f/f_o)_{shape}$[c]	1.47
axial ratio (prolate ellipsoid)	8.7:1

[a] Calculated from the amino acid composition. [b] Estimated from hydrodynamic parameters. [c] Corrected for hydration assuming 0.5 g of water/g of protein.

three fits, the two preparations contained ≤39% α-helix and β-strand conformation and did not differ significantly in secondary structure content from each other ($p < 0.05$). These data suggest that CK1BP contained limited secondary structure in solution and that the presence of a polyhistidine tag did not significantly modify its folding.

To assess higher-order structure, recombinant CK1BP was subjected to hydrodynamic analysis using a combination of gel filtration chromatography and velocity sedimentation. Results showed that under the near-physiological buffer conditions employed in the analysis, CK1BP migrated as a monomer with a hydrodynamic radius of 3.0 ± 0.1 nm and an axial ratio of ~8.7:1 (Table 1). Empirical formulas relating hydrodynamic radii to chain lengths have been derived for different folding states (*49*). Conversion of these theoretical hydrodynamic radii into frictional ratios [$(f/f_o)_{obs}$] (*41*) facilitates comparison among folding states on the basis of shape. Comparison of the experimentally determined $(f/f_o)_{obs}$ value for recombinant CK1BP with these theoretical predictions indicated that the recombinant preparation resides in a pre-molten globule-like folding state. This state is characterized by loosely packed tertiary structure (*50*). The molar ellipticities for CK1BP at 200 and 222 nm (−12522 and −2611, respectively) also were consistent with this assignment (*51*). Together these data indicate that CK1BP is a monomeric structural homologue of dysbindin that adopts only partially folded secondary structure and limited tertiary structure when free in solution.

CK1BP Is CK1 Isoform-Selective. Human CK1 is composed of six distinct gene products, with further diversity created by alternative splicing. To determine whether CK1BP selectively bound individual CK1 isoforms, directed two-hybrid assays were performed with human pSos-Ckiα, -δ, -ε, -γ2, and -γ3. The shortest Ckiα splice variant was used for this experiment because it lacked the L insert that mediates nuclear localization (*52*, *53*), whereas Ckiγ1 was not analyzed because of its similarity to the sequences of Ckiγ2 and Ckiγ3 (*54*). Results confirmed that neither pMyr-CK1BP cotransformed with the pSos empty vector nor pSos-Ckiδ cotransformed with the pMyr empty vector rescued the growth defect of Cdc25Hα cells (Figure 3). In contrast, growth was restored when pMyr-CK1BP was cotransformed with pSos-Ckiγ2, -γ3, and -ε, but not Ckiα (Figure 3). These data suggest that interactions between CK1BP and CK1 family members are isoform-selective.

CK1 family members are composed of homologous N-terminal catalytic domains fused to isoform-specific C-terminal extensions. To determine which domain mediated interactions between CK1BP and Ckiδ, C-terminal truncation mutant Ckiδ-Δ317 was prepared and included in the directed

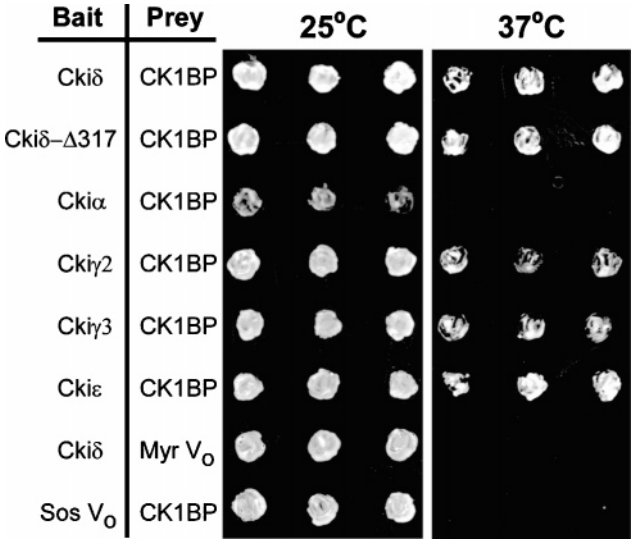

FIGURE 3: CK1BP selectively interacts with CK1 isoforms. Cdc25Hα cells transformed with different combinations of bait and prey plasmids were grown under conditions that were nonselective (glucose minimal medium at 25 °C) or selective (galactose minimal medium at 37 °C) for interaction. Sos-V_o and Myr-V_o denote empty bait and prey vectors, respectively. CK1BP supported growth of Cdc25Hα cells in the presence of all CK1 isoforms that were examined except Ckiα. CK1BP also supported growth in the presence of Ckiδ-Δ317, indicating that interaction was mediated through the protein kinase catalytic domain or a short segment C-terminal to it.

two-hybrid screen. This mutant was chosen for analysis because its protein kinase domain is intact (spanning residues 9−290) and folds properly in heterologous expression systems (*27*), whereas the bulk of the C-terminal extension (residues 318−415) is absent. Results showed that CK1BP interacted with Ckiδ-Δ317 in a manner similar to that of full-length Ckiδ (Figure 3), suggesting that its principal binding target is composed of the protein kinase catalytic domain or the short segment C-terminal to it.

CK1BP Forms a Complex with CK1 in Vitro. To confirm findings from two-hybrid screens, the ability of purified recombinant CK1BP to bind selected CK1 isoforms in cell lysates was determined in vitro. His_6-tagged CK1BP protein was used for these studies because it retained the folding properties of nontagged recombinant CK1BP (Figure 2) while avidly binding Ni^{2+}−NTA beads. Analysis was limited to human Ckiα, -δ, and -ε because of the availability of specific monoclonal antibody probes for these three isoforms. Results showed that uncharged Ni^{2+}−NTA beads alone could not pull down any of these three CK1 isoforms (Figure 4). Similarly, beads charged with His_6-tagged α-synuclein (a protein unrelated in structure to CK1BP) did not selectively pull down CK1 isoforms. In contrast, beads charged with His_6-tagged CK1BP effectively pulled down Ckiδ and Ckiε but not Ckiα (Figure 4). These data recapitulate the interactions observed in yeast two-hybrid experiments and demonstrate that CK1BP is a CK1 binding protein in vitro. Moreover, they confirm that CK1BP does not directly bind Ckiα, suggesting that the failure of Ckiα to suppress the growth defect of Cdc25Hα cells in the presence of pMyr-CK1BP did not arise solely from deficiencies in the expression or folding of Sos−Ckiα fusion protein.

Full-Length Dysbindin Does Not Interact with CK1 Isoforms. To determine whether dysbindin, a CK1BP struc-

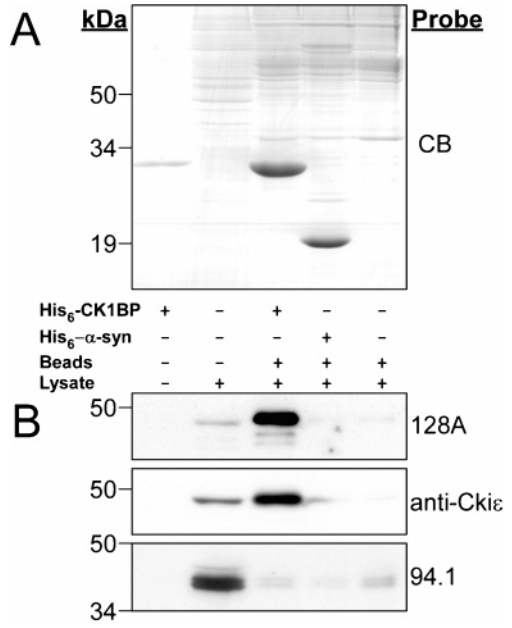

FIGURE 4: CK1BP binds CK1 isoforms in vitro. The ability of CK1 isoforms to bind His_6-tagged CK1BP immobilized on Ni^{2+}−NTA beads was assessed with pulldown assays performed as described in Experimental Procedures using HEK-293 cell lysate as the source of endogenous CK1 immunoreactivity. Ni^{2+}−NTA beads alone or charged with His_6-tagged α-synuclein were included to control for nonspecific binding. Complex components were detected after beads were boiled and subjected to SDS−PAGE and immunoblot analysis. (A) Coomassie blue stain after SDS−PAGE showing the presence or absence of input bait proteins. His_6-tagged CK1BP but not α-synuclein migrates anomalously on SDS−PAGE. (B) Immunoblot analysis performed using monoclonal antibodies raised against Ckiα (94.1), Ckiδ (128A), and Ckiε. Endogenous levels of all three of these CK1 isoforms were detectable in cell lysates. Both Ckiδ and Ckiε selectively bound immobilized His_6-tagged CK1BP relative to immobilized His_6-tagged α-synuclein or uncharged Ni^{2+}−NTA beads alone, but Ckiα did not.

tural homologue, retained CK1 binding activity, directed two-hybrid screens were performed with pSos-Ckiα, -δ, -ε, -γ2, or -γ3 or empty pSos vector as bait and pMyr-dysbindin (human variant *c*) or empty pMyr vector as prey. Surprisingly, full-length dysbindin was incapable of rescuing the growth defect of Cdc25Hα cells in the presence of any CK1 isoform (Figure 5). The result was not an artifact of yeast expression, because recombinant His_6-tagged dysbindin was incapable of interacting with CK1 isoforms in pulldown assays performed as described above for CK1BP (data not shown). These results suggested that either the dysbindin acidic domain was not functionally homologous to CK1BP or the coiled-coil domain common to all dysbindin variants but absent in CK1BP interfered with the ability of the acidic domain to interact with CK1. To distinguish between these possibilities, the isolated acidic domain of dysbindin (i.e., the C-terminal sequence beginning with Met^{184} of dysbindin variant *a* shown in Figure 1B) was tested as prey in directed two-hybrid screens with human Ckiα, -δ, -ε, -γ2, and -γ3 as bait. Results show that the acidic domain of dysbindin could in fact rescue the growth defect of Cdc25Hα cells in the presence of Ckiγ2, -γ3, -δ, and -ε, but not Ckiα (Figure 5). Together, these results suggest that the acidic domain of dysbindin could selectively interact with CK1 isoforms and was a functional homologue of the acidic domain found in CK1BP.

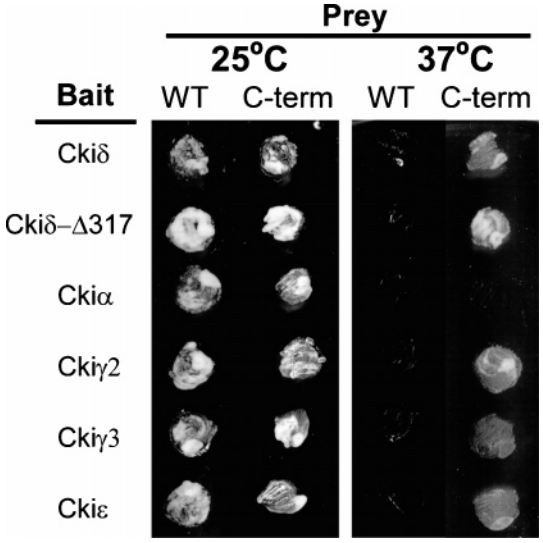

FIGURE 5: Dysbindin acidic domain selectively binds CK1 isoforms. Cdc25Hα cells transformed with different combinations of bait and prey plasmids were grown under conditions that were nonselective (glucose minimal medium at 25 °C) or selective (galactose minimal medium at 37 °C) for interaction. The C-terminal acidic domain of dysbindin (C-term) composed of residues 184−351 of DTNBP1 variant 1 (see Figure 1 for the amino acid sequence) supported growth of Cdc25Hα cells in the presence of all CK1 isoforms examined except Ckiα, whereas full-length dysbindin variant *c* (WT) did not.

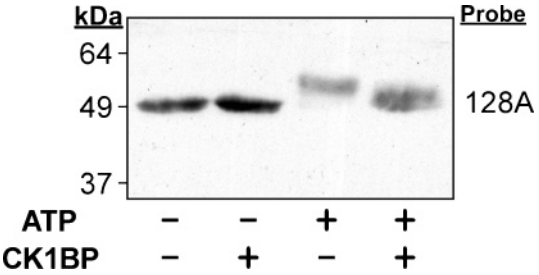

FIGURE 6: CK1BP inhibits Ckiδ autophosphorylation. HEK-293 lysates (25 μg of total protein) were prepared and incubated in vitro (30 min at 37 °C) with phosphorylation buffer in the presence or absence of 1 mM ATP and recombinant CK1BP (250 nM). Reaction products were separated by SDS−PAGE and detected on immunoblots with anti-Ckiδ antibody 128A. In the presence of nucleotide substrate, full-length Ckiδ underwent a band shift consistent with autophosphorylation. CK1BP inhibited the substrate-dependent band shift, consistent with an ability to antagonize autophosphorylation.

CK1BP Inhibits CK1 Phosphotransferase Activity. To determine whether CK1BP binding affected the protein kinase activity of CK1, HEK-293 cell lysates were subjected to in vitro autophosphorylation assays in the presence and absence of recombinant CK1BP. HEK-293 cells were chosen as the source of CK1 activity because they contain high basal levels of full-length human Ckiδ (*30*). Incubation of lysates with nucleotide substrate in vitro gave rise to a pronounced Ckiδ band shift on immunoblots (Figure 6), consistent with the established ability of autophosphorylation to modulate Ckiδ migration during SDS−PAGE (*27*). The presence of CK1BP antagonized this nucleotide-dependent band shift (Figure 6).

These data suggest that CK1BP was capable of inhibiting Ckiδ autophosphorylation. To determine whether this reflected direct inhibition of Ckiδ catalytic activity, purified recombinant Ckiδ-Δ317 was incubated with various

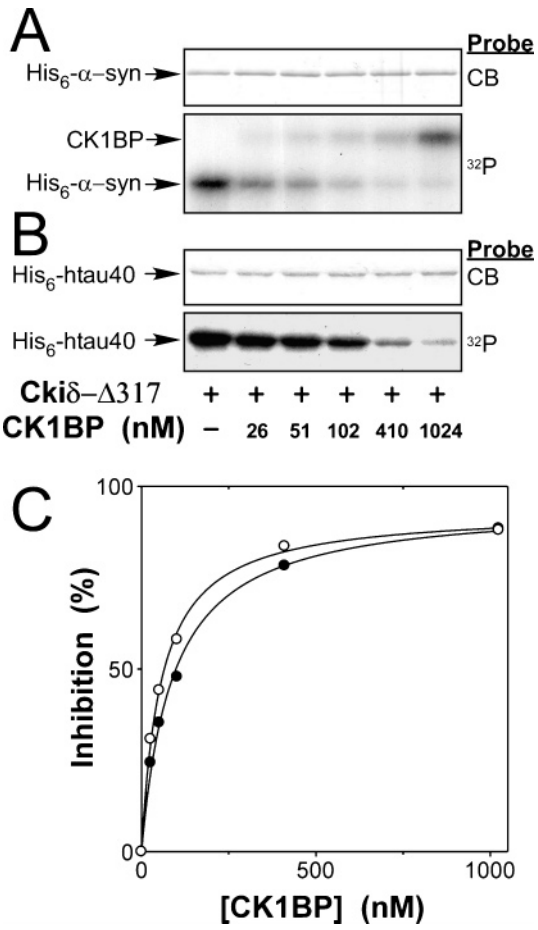

FIGURE 7: CK1BP inhibits Ckiδ-Δ317 activity in vitro. Purified recombinant Ckiδ-Δ317 (34 nM) was incubated (30 min at 37 °C) under phosphorylating conditions in the presence of 4 μM protein substrates, 100 μM [γ-^{32}P]ATP, and varying concentrations of CK1BP (0−1024 nM). Reaction products were separated by SDS−PAGE and then fixed and stained with Coomassie blue (CB). Incorporation of ^{32}P label was then analyzed by autoradiography. Increasing concentrations of CK1BP led to a dose-dependent inhibition of (A) His_6-α-synuclein and (B) His_6-htau40 phosphorylation. (C) Phosphorylation levels shown in panels A and B were quantified by densitometry and plotted as the percent inhibition (i.e., 0% inhibition corresponds to ^{32}P incorporation in the absence of CK1BP). The line represents the best fit of the data to a rectangular hyperbola (eq 1). CK1BP inhibition of Ckiδ-Δ317-mediated phosphorylation of His_6-α-synuclein (○) and His_6-htau40 (●) was saturable with IC_{50} values of 57 ± 3 and 90 ± 8 nM, respectively.

exogenous protein substrates in the presence of varying concentrations of recombinant CK1BP. Ckiδ-Δ317 was employed for this experiment because it is constitutively active, retains CK1BP binding activity, and is readily prepared from *E. coli* expression systems for solution-based assays (*27*). In addition, C-terminal truncation of Ckiδ eliminates most of its autoregulatory region, thereby reducing the modulatory effect of autophosphorylation on catalytic activity (*27*). Htau40 [i.e., full-length four-repeat human tau protein (*55*)] and human α-synuclein were used as protein substrate, because the former is a substrate for Ckiδ in situ (*30*), whereas the latter is phosphorylated by CK1 at a residue (Ser^{129}) thought to be important for protein aggregation in neurodegenerative disease (*56*, *57*). Both substrates were phosphorylated by Ckiδ-Δ317 in vitro (Figure 7A,B). However, the phosphorylation of both substrates was inhibited by the presence of CK1BP, which was itself a substrate

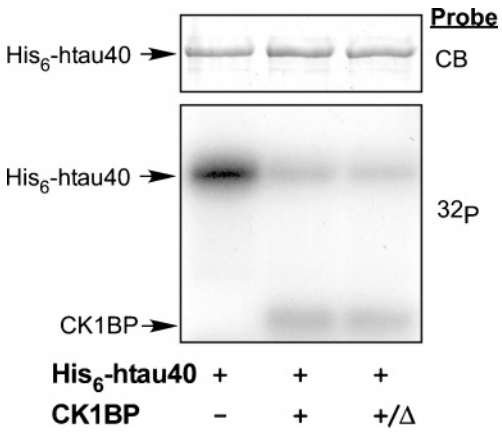

FIGURE 8: CK1BP-mediated inhibition is heat stable. Purified recombinant Ckiδ-Δ317 (34 nM) was incubated (30 min at 37 °C) under phosphorylating conditions in the presence of 4 μM His_6-htau40 and 100 μM [γ-^{32}P]ATP and in the presence or absence of 250 nM CK1BP (~3-fold above the IC_{50}). Reaction products were separated by SDS−PAGE and then fixed and stained with Coomassie blue (CB). Incorporation of the ^{32}P label was then analyzed by autoradiography. Tau phosphorylation was inhibited by CK1BP, and this inhibition was retained even in the presence of heat-treated CK1BP (100 °C for 3 min).

for Ckiδ-Δ317 (Figure 7A). Inhibition of phosphorylation was dose-dependent, with IC_{50} values of 90 ± 8 and 57 ± 3 nM for tau and α-synuclein, respectively (Figure 7C). In the case of tau substrate, CK1BP-mediated inhibition was heat stable, suggesting that the small amount of higher-order structure present in native CK1BP was not crucial for inhibitory activity (Figure 8). CK1BP at 1 μM was also capable of completely inhibiting phosphorylation of ~400 μM synthetic peptide substrate derived from phosphatase inhibitor-2 [residues 166−180 (*18*)] (data not shown). However, inhibition of casein phosphorylation was far less potent, requiring ~2.5 μM CK1BP to inhibit phosphorylation of 4 μM casein by 50% (data not shown). These data suggest that the association between CK1BP and Ckiδ-Δ317 is a high-affinity association but that the ability of this association to inhibit protein kinase activity in vitro may depend on the nature and concentration of the protein substrate.

CK1BP Modulates CK1 Phosphotransferase Activity in Cell Cultures. To determine whether CK1BP could inhibit full-length Ckiδ in the presence of endogenous substrates within mammalian cells, Ckiδ from HEK-293 cells transiently transfected with either pcDNA-HA-CK1BP or pcDNA empty vector was immunoprecipitated with monoclonal antibody 128A and subjected to in vitro kinase assays. Immunoblots performed using an anti-HA monoclonal antibody confirmed that HA-tagged CK1BP was present in lysates prepared from cells transfected with pcDNA-CK1BP but not empty vector (Figure 9A). On the basis of immunoblot analysis with anti-Ckiδ monoclonal antibody 128A, the expression of endogenous Ckiδ was similar in lysates of cells transfected with either pcDNA-HA-CK1BP or vector alone (Figure 9A), indicating that transient expression of CK1BP did not alter levels of endogenous Ckiδ. Ckiδ was then immunoprecipitated from cell lysates using monoclonal antibody 128A and subjected to in vitro kinase assays using htau40 and [γ-^{32}P]ATP as substrates. Radioactive reaction products were separated by SDS−PAGE and quantified with a phosphorimager. Immunoblot analysis performed with monoclonal antibody 128A showed that the amount of Ckiδ

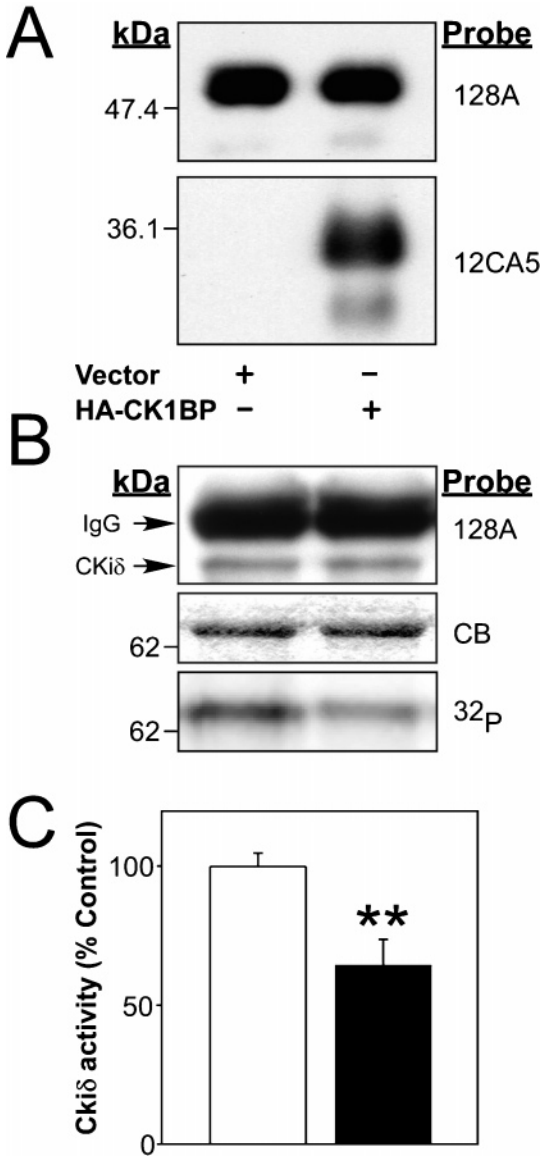

FIGURE 9: CK1BP inhibits Ckiδ activity in situ. HEK-293 cells transiently transfected (48 h) with pcDNA-HA-CK1BP or empty vector were harvested, lysed, and subjected to immunoprecipitation with anti-Ckiδ antibody 128A. Immunocomplexes from each condition were then incubated in vitro (30 min) with protein kinase substrates His_6-htau40 and [γ-^{32}P]ATP. Reaction products were separated by SDS−PAGE and viewed in a phosphorimager. (A) Immunoblot analysis performed on cell lysates with monoclonal antibodies 128A and 12CA5. Whereas levels of endogenous Ckiδ detected with monoclonal antibody 128A did not vary between the two conditions, HA-CK1BP was detected only in lysates prepared from cells transfected with pcDNA-HA-CK1BP. (B) Although levels of Ckiδ protein in the immunoprecipitates (detected with monoclonal antibody 128A) and of His_6-htau40 substrate (detected by Coomasie blue staining; CB) were indistinguishable, the amount of detectable phospho-tau product (^{32}P) decreased in immunoprecipitates from cells expressing HA-CK1BP relative to empty vector control. (C) Quantitative data from three independent experiments presented as means ± the standard error. Asterisks denote a p of <0.01 compared with the empty vector control.

in each immunoprecipitate was the same (Figure 9B). Moreover, the total amount of htau40 remaining after protein kinase assay was constant (Figure 9B). Nonetheless, Ckiδ immunoprecipitates prepared from cells transfected with pcDNA-CK1BP had significantly less tau kinase than immunoprecipitates prepared from cells transfected with pcDNA vector alone (Figure 9BC). These results suggest that CK1BP is capable of modulating basal Ckiδ activity in HEK-293 cells as well as in vitro.

DISCUSSION

Structure. CK1BP is a novel binding partner of the CK1 family in human brain. It and its putative longer variant derived by alternative splicing are paralogs of dysbindin, a ubiquitously expressed protein named for its ability to bind components of the dystrophin-associated protein complex (i.e., α- and β-dystrobrevins) in both muscle and non-muscle cells (*47*). Nonetheless, little is known about the molecular activity of any dysbindin homologue beyond participation in macromolecular complexes in part because its protein domains are poorly characterized. Family members contain different combinations of up to three distinct segments: an N-terminal basic domain, a central coiled-coil domain, and a highly acidic C-terminal domain. Although the coiled-coil region functions to mediate complex formation with other coiled-coil-containing binding partners (*48*) or potentially with itself, the function of the remaining protein segments has been unclear. Here we found that the acidic domain in isolation can bind CK1 isoforms. Because the acidic domains of both CK1BP and dysbindin bind truncation mutant Ckiδ-Δ317 and because the C-terminal extension of each CK1 isoform differs, it is likely that binding to intact CK1 isoforms is mediated primarily through their catalytic domains. Consistent with this proposed interaction, CK1BP can inhibit both Ckiδ autophosphorylation and Ckiδ-Δ317-mediated phosphorylation of exogenous substrates such as tau and α-synuclein. Moreover, CK1BP itself is an in vitro substrate of Ckiδ-Δ317. CK1 catalytic domains contain abundant positive surface charge and are highly conserved among isoforms (*58*), and so electrostatic interactions may play a role in binding. Nonetheless, nonspecific charge−charge interactions alone are insufficient for high-affinity binding, because neither CK1BP nor the acidic domain of dysbindin bound Ckiα, despite it having a high isoelectric point and being >75% identical with other CK1 isoforms. On the basis of sequence alignments and the established crystal structures of fission yeast Cki1 and rabbit Ckiδ catalytic domains (*58*, *59*), the origin of this selectivity is not clear. None of the CK1BP or dysbindin variants contains docking motifs discovered in some CK1 substrate proteins, such as PER and NFAT (*60*), beyond having clusters of acidic amino acid residues, and these are not preceded by SLS motifs as found in other CK1 substrates (*61*). Nonetheless, CK1BP may bind competitively with exogenous substrates because it is phosphorylated by Ck1δ-Δ317 and because its inhibitory potency varies with the protein substrate used to assay activity. Moreover, the heat stability of CK1BP-mediated CK1 inhibition suggests that its interaction with CK1 isoforms is mediated mostly by primary structure. The Ckiα variant employed here may lack a small number of residues required to support high-affinity binding of CK1BP primary structure. Indeed, individual CK1 isoforms can differ in their rates of phosphorylation of peptide substrates by up to 2 orders of magnitude despite a high degree of sequence similarity among their catalytic domains (*62*). The binding free energy in protein−protein interactions is not equally distributed at the binding interface. Rather, there are "hot spots" of binding energy consisting of a subset

of residues at the interface (*63*, *64*). For example, the sequences of TEM-1 and SHV-1 β-lactamases are 68% identical, and their atomic structures are nearly superimposable (*65*); however, TEM-1 binds to the β-lactamase inhibitor protein ~10000-fold more efficiently. It has been suggested that the weaker binding of SHV-1 β-lactamases with inhibitor results from only four residue changes in SHV-1 (*65*, *66*). Clearly, small changes in hot spot residues may produce large changes in binding specificity, and this may be the case for CK1 isoforms. It is also possible that the short C-terminal extension of Ckiα, which differs among CK1 isoforms, interferes with binding.

The ability of the acidic domain of CK1BP and dysbindin to bind CK1 also depends on its sequence context. When isolated and in monomeric form, as exemplified by CK1BP, the acidic domain displayed CK1 binding activity. However, when presented in the context of dysbindin variant *c*, which contains a coiled-coil domain N-terminal to the acidic domain, CK1 binding activity could not be detected either in yeast using a two-hybrid screen or in vitro using pulldown assays. Binding activity was unmasked only after removal of the coiled-coil domain. This suggests that the coiled-coil domain interferes with the binding activity of the acidic domain either directly (by interacting with it) or perhaps indirectly (by fostering homodimerization or oligomerization). It is conceivable that conditions that disrupt the former, such as participation in higher-order complexes (see below), or that disrupt coiled coils, such as posttranslational modifications (*67*, *68*), including proteolysis, may unmask CK1 binding activity in full-length dysbindin variants.

Function. In addition to its ability to bind the dystrophin-associated protein complex, dysbindin is a component of BLOC-1 (biogenesis of lysosome-related organelles complex-1), a protein complex involved in the generation or trafficking of lysosome-related organelles. These organelles are cell-type-specific modifications of the post-Golgi membrane system that share various characteristics with lysosomes but that are specialized for storage and secretion rather than degradation. They include melanosomes, platelet dense granules, lamellar bodies of type II alveolar epithelial cells, and lytic granules of cytotoxic T lymphocytes and natural killer cells, mast cell basophilic granules, Weibel Palade bodies of endothelial cells, azurophilic granules of neutrophils and eosinophils, osteoclast granules, and rennin granules of juxtaglomerular cells. Misfunction of dysbindin and other members of the BLOC-1 complex can lead to a syndrome (i.e., Hermansky-Pudlak syndrome) characterized by deficient skin pigmentation (albinism), blood platelet dysfunction (prolonged bleeding), visual impairment, and abnormal storage of a fatty-like substance (ceroid lipofuscin) in various tissues of the body (*69*). Lysosome/vacuole formation and function is linked to the secretory and endocytic pathways used in protein and lipid trafficking where CK1 is known to localize in postmitotic cells such as neurons (*70*). Moreover, some CK1 isoforms, such as Ckiδ, are major markers of granulovacuolar degeneration bodies found in AD and other neurodegenerative diseases (*15*). In yeast, at least one CK1 isoform (Yck3p) resides in part with the vacuole (*71*) where it regulates budding and fusion reactions of this organelle (*72*). Because CK1 frequently functions in tandem with the ubiquitin system, its potential interaction with dysbindin family members may be important for turnover and trafficking of protein complexes involved in formation of lysosome-related organelles. Such a function would be specific to metazoans because unlike CK1, dysbindin homologues are not found in lower eukaryotes.

Whether CK1BP functions in secretory or endocytic pathways as part of macromolecular complexes such as dysbindin remains to be determined. When CK1BP is free in solution, its binding of CK1 results in inhibition of protein kinase activity. The IC_{50} for this effect is ~60−90 nM for htau40 and α-synuclein (at a bulk concentration of 4 μM). However, IC_{50} values depend on the ratio of substrate concentration to K_M (*73*), so higher CK1BP concentrations are required to inhibit the phosphorylation of efficient substrates such as casein. K_i values, which do not depend on substrate concentration, are predicted to be significantly lower than the estimated IC_{50}, and well within 2 orders of magnitude of the affinity of established protein kinase inhibitors, including the cAMP-dependent protein kinase inhibitor (PKI; *74*). The functions of inhibitors such as PKI are not entirely clear but may involve control of basal levels of protein kinase activity. Although some CK1 isoforms such as Ckiδ and -ϵ are regulated in part via autophosphorylation of C-terminal domains (*27*, *75*), phosphotransferase activity remains constitutively active. Free CK1BP may function to inhibit basal CK1 activity in cytoplasm.

Comparison with Previous Studies. The yeast two-hybrid system is a widely used genetic approach for detecting protein−protein interactions, accounting for almost 40% of identified interactions (*76*). The Sos recruitment paradigm employed here indicated that CK1BP was the only interacting species that can be detected by this method in a human brain library. Two prior screens employing Ckiδ as bait and GAL4 transcriptional activation assays identified scaffolding protein CG-NAP/AKAP450 and the light chain of microtubule-associated protein 1A from human testis and bone marrow libraries, respectively (*77*, *78*). The emphasis on libraries from different mitotic cell types could account for differences observed between them and the neuronal library employed here. For example, Ckiδ colocalizes with the spindle in mitotic cells (*79*), but the microtubules of neuronal cells no longer organize their microtubules into spindles, instead forming more complicated structures that function in the context of cellular processes (axons and dendrites). Thus, although CK1 isoforms are ancient and conserved among eukaryotes, it is not surprising that different binding partners would be discovered in different cell types. However, each of the GAL4-based screens generated additional positives beyond those reported, and these may contain dysbindin/CK1BP family members. Because isolated full-length dysbindin does not bind CK1, two-hybrid screens employing full-length dysbindin as bait (*48*) would not be expected to identify any CK1 isoform unless accompanied by proteolysis or other post-translational alterations.

CK1 isoforms have been detected as prey in a number of two-hybrid screens using various proteins as bait, including the SH2/SH3 domain-containing adapter protein Nck (*80*), metastatic tumor antigen 1 (*81*), and components of Wnt signaling pathways (*82*). However, Ckiδ was not among the CK1 isoforms identified in these screens. These data are consistent with tight binding interactions among proteins being CK1 isoform-selective.

Finally, binding partners for Ckiα have been sought by affinity chromatography, revealing various interacting partners, including protein phosphatase 1 inhibitor CPI-17, centaurin-α_1, and other proteins (*83*−*85*). CK1BP does not bind Ckiα and so should not appear in screens such as these.

In summary, CK1BP and the acidic domain of its paralog, dysbindin, can function as binding partners for CK1 isoforms. The finding suggests a mechanism for generating additional CK1 functionality in the variants generated by alternative splicing of multiple genes.

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