DNA binding protein dbpA binds Cdk5 and inhibits its activity

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Abstract Progress in the cell cycle is governed by the activity of cyclin dependent kinases (Cdks). Unlike other Cdks, the Cdk5 catalytic subunit is found mostly in differentiated neurons. Interestingly, the only known protein that activates Cdk5 (i.e. p35) is expressed solely in the brain. It has been suggested that, besides its requirement in neuronal differentiation, Cdk5 activity is induced during myogenesis. However, it is not clear how this activity is regulated in the pathway that leads proliferative cells to differentiation. In order to find if there exists any Cdk5-interacting protein, the yeast two-hybrid system was used to screen a HeLa cDNA library. We have determined that a C-terminal 172 amino acid domain of the DNA binding protein, dbpA, binds to Cdk5. Biochemical analyses reveal that this fragment (dbpA(CΔ)) strongly inhibits p35-activated Cdk5 kinase. The protein also interacts with Cdk4 and inhibits the Cdk4/cyclin D1 enzyme. Surprisingly, dbpA(CΔ) does not bind Cdk2 in the two-hybrid assay nor does it inhibit Cdk2 activated by cyclin A. It could be that dbpA's ability to inhibit Cdk5 and Cdk4 reflects an apparent cross-talk between distinct signal transduction pathways controlled by dbpA on the one hand and Cdk5 or Cdk4 on the other.

Key words: Cyclin dependant kinase 5; DNA binding protein A; Kinase inhibitor; Yeast two-hybrid system

1. Introduction

Cyclin dependent kinases (Cdks) are a class of serine/threonine protein kinases which regulate important transitions in the cell cycle. Cdk5 was initially identified on the basis of its sequence similarity to the family of cyclin dependent kinases [1,2]. However, Cdk5 is thought be a cyclin dependent kinase with neuron-specific function only, since its catalytic subunit is predominantly expressed in post-mitotic cells of the nervous system, cells which have permanently exited the cell cycle [2,3]. Moreover, the protein p35 which activates the Cdk5 enzyme is exclusively expressed in the central nervous system (CNS) [3,4].

Nevertheless, the catalytic subunit of Cdk5 is widely expressed at basal levels, in both cycling and non-cycling cells, in most human tissues [2,3]. Interestingly, cyclins D and E, activator molecules which regulate Cdk activity in proliferating cells, bind Cdk5 ([5,6]; our own observations). It could be argued that Cdk5 bound to cyclin D or cyclin E phosphorylates substrates which are not yet known.

The 35 kDa p35 protein, the only known activator of Cdk5, which was originally isolated as a smaller 25 kDa proteolytic product, bears no significant homology to the family of cyclins [3,4]. As a Cdk activator, p35 is unique since it has no influence on the activity of other Cdks [2,3].

Histone H1 and the retinoblastoma protein (pRb) are substrates that are frequently used to confirm in vitro activity of the Cdks. Heterodimeric Cdk5/p35 and Cdk5/p25 complexes can phosphorylate both histone H1 and pRb [3,7]. Activated Cdk5 also phosphorylates its activating partner p35 [8]. Besides, Cdk5 is known to phosphorylate in vitro a number of neuron-specific cytoskeletal proteins that includes the neurofilament proteins NF-M, NF-H, the microtubule associated protein tau and the actin binding protein caldesmon [2,3]. Phosphorylation of cytoskeletal proteins is thought to play an important role in the polymerization and assembly of cytoskeletal elements which, in turn, may affect growth of neurites. Indeed, Cdk5 activity has been demonstrated to play a key role in neurite outgrowth [9] and neuronal migration during differentiation of neurons [10]. Recently, Cdk5 has been shown to participate in the regulation of myogenesis in the early embryo [11,12]. These observations may provide new insight into the possible function of Cdk5 during differentiation.

Cdk5 is known to phosphorylate neurofilament proteins exclusively at sites phosphorylated in Lewy body pathologies, i.e. diffuse Lewy body disease (dementia), Parkinson's disease [13,14] and amyotrophic lateral sclerosis [2,15], and tau protein at sites phosphorylated in Alzheimer's disease [2,3,16,17]. Moreover, it has been reported that the kinase activity of Cdk5 correlates with the extent of differentiation of neuronal cells [2,3,18] and colocalizes with neurofilament tracts in the axons of neuronal cells in culture.

It is therefore possible that Cdk5 activity is tightly regulated in pathways that lead from cell proliferation towards differentiation. Hence, we have inquired whether if there are any proteins that could be involved in the regulation of Cdk5 (i.e. may activate or inhibit the kinase) in cycling cells. With this in mind, a cDNA library, constructed from total RNA obtained from HeLa cells, was screened using the yeast two-hybrid system. Amongst other proteins (unpublished observations), we find that a C-terminal fragment of the DNA binding protein dbpA (in this communication referred to as dbpA(CΔ)) interacts with the bait protein Cdk5, in the two-hybrid assay. Conversely, when dbpA(CΔ) was used as bait, the interaction between dbpA(CΔ) and Cdk5 was equally efficient. The validity of these interactions was corroborated using the *Escherichia coli* expressed GST-dbpA(CΔ) fusion protein. The purified protein precipitates not only in vitro transcribed/translated Cdk5 but also the protein which is expressed in

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Abbreviations: β-gal, β-galactosidase; Cdk, human cyclin dependent kinase; CSD, cold shock domain; DBD, DNA binding domain; GSH-Sepharose, glutathione Sepharose; GST, glutathione *S*-transferase; p35, 35 kDa protein which activates Cdk5; PCR, polymerase chain reaction; pRb, human retinoblastoma protein; RT, room temperature; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TAD, transcriptional activation domain

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COS-1 cells. Similar experiments reveal that dbpA(CΔ) can bind Cdk4 as well, but not Cdk2 or Cdk1. Surprisingly, dbpA(CΔ) appears to be an efficient inhibitor of Cdk5/p35 and Cdk4/cyclin D1 kinase activities but fails to inhibit Cdk2/cyclin A.

2. Materials and methods

2.1. Plasmid constructs for yeast expression

*Eco*RI-*Bgl*II PCR fragments of human *Cdk1*, *Cdk2* and *Cdk4* and *Eco*RI-*Sal*I PCR fragments of bovine *Cdk5* and human *p35* were cloned in the pLEX-a vector [19] downstream of the *LEX-a* DNA binding domain (DBD). Expression in yeast from pLEX-a plasmids is controlled by the constitutive alcohol dehydrogenase promoter (*ADH1*), and the plasmid encodes the *TRP1* and *ADE2* genes for stable propagation in yeast. Furthermore, the *Eco*RI-*Sal*I fragments of *Cdk5* and *p35* were also cloned in the vector pGAD424 (Clontech) in such a way that they lie directly downstream of the *GAL4* transcriptional activation domain (TAD). Expression from the pGAD424 plasmid, which encodes *LEU2* as a marker gene, is also driven by the *ADH1* promoter.

A *Bam*HI-*Sal*I PCR fragment of *dbpA*(*C*Δ) (the isolated HeLa cDNA) was cloned in pLEX-a for expression in yeast. Furthermore, a *Bam*HI-*Xho*I fragment of the *dbpA*(*C*Δ) was cloned in pACT2 (Clontech) whereas a *Bam*HI-*Not*I fragment was cloned in pVP16 [19]. The pACT2 plasmid encodes $GAL4_{TAD}$ whereas the pVP16 plasmid encodes $VP16_{TAD}$. The TADs in both plasmids are under the control of the constitutive *ADH1* promoter. The plasmids encode *LEU2* for selection in yeast. The *dbpA*(*C*Δ) fragment has been cloned directly downstream of $GAL4_{TAD}$ and $VP16_{TAD}$.

Plasmids pLEX-a/lamin and pLEX-a/MyoD were gifts from S.M. Hollenberg [19].

2.2. Plasmid constructs for E. coli expression

A *Bam*HI-*Sal*I fragment of the *dbpA*(*C*Δ) cDNA was cloned in (a) pGEX4T-1 (Amersham-Pharmacia Biotech) for expression of a GST-dbpA(CΔ) fusion protein and (b) pQE32 (Qiagen) for expression of a His-tagged dbpA(CΔ) protein.

2.3. Plasmid constructs for COS-1 cell expression and in vitro transcription/translation

A *Bam*HI-*Sal*I fragment of *Cdk5* was cloned in pcDNA3.1− (Invitrogen). The *Eco*RI-*Xho*I fragments of *Cdk1*, *Cdk2* and *Cdk4* were cloned into pcDNA3.1+ (Invitrogen). The plasmid pcMV-β-gal was obtained from H.-J. Keller (Novartis).

2.4. Plasmid constructs for insect cell expression

A *Bam*HI-*Sal*I fragment of *Cdk5* was cloned in pFastBac1 (Gibco BRL) for construction of a recombinant baculovirus. The pFastBac1 vector has a baculovirus-specific promoter from *Autographa californica* nuclear polyhedrosis virus (AcNPV) for heterologous expression of proteins in insect cells. Similarly, *Bam*HI-*Eco*RI fragments of human *Cdk2* and *Cdk4* were cloned in pFastBac1 for construction of recombinant baculoviruses.

*Eco*RI-*Sal*I fragments of human *p35*, human *cyclin A*, murine *cyclin D2* and murine *cyclin D3* were cloned in pFastBac1 downstream of a *Bgl*II-*Eco*RI fragment of GST (PCR fragment from pGEX4T-1; Amersham-Pharmacia Biotech) for construction of recombinant baculoviruses. Similarly, an *Eco*RI-*Xho*I fragment of human *cyclin D1* and a *Bam*HI-*Sal*I fragment of *dbpA*(*C*Δ) were cloned as *GST* fusions in pFastBac1.

2.5. Yeast strains and media

The *Saccharomyces cerevisiae* strains L40 (Mata, *his*3Δ200, *trp*1-901, *leu*2-3,112, *ade*2, *lys*2-801am, *URA3*::$(lexAop)_8$-lacZ, *LYS*::$(lexAop)_4$-*his*3) and AMR70 (Matα, *his*3Δ200, *trp*1-901, *leu*2-3,112, *ade*2, *lys*2-801am, *URA3*::$(lexAop)_8$-*lacZ*) [19] were used for yeast transformation and for mating assays. In order to perform the yeast two-hybrid screen, L40 was transformed sequentially with (a) pLEX-a/cdk5 and (b) a human HeLa S3 Matchmaker cDNA library cloned unidirectionally into the *Eco*RI and *Xho*I sites of pGADGH (Clontech, HL4000AA). The pGADGH vector contains the $GAL4_{TAD}$ under the control of the *ADH1* promoter and also encodes *LEU2* as a marker gene. In order to mate a L40 strain that was cured of bait plasmid but still contains the prey plasmid (from the cDNA library), transformants of AMR70 (bearing plasmids pLEX-a/cdk5 or pLEX-a/lamin) were used. Both L40 and AMR70 contain the *lacZ* reporter gene linked to the *lexA* operon. Besides, L40 also contains the *HIS3* reporter gene downstream of the *lexA* operon. Untransformed yeast strains were grown in YPAD whereas transformed yeast strains were grown in minimal medium that allow maintenance of plasmids in a particular strain.

The *S. cerevisiae* strain L40 was also used for transformation of pLEX-a plasmids that encode *Cdk1, Cdk2, Cdk4, Cdk5*, *p35*, *dbpA*(*C*Δ), *lamin* or *MyoD*. A pACT2 or pVP16 plasmid that encodes *dbpA*(*C*Δ) or pGAD424 plasmids that encode *Cdk5* and *p35* were used to perform yeast two-hybrid assays on selected transformants.

2.6. Yeast two-hybrid screen

A pLEX-a/cdk5 plasmid-bearing strain of L40 was transformed with the HeLa cDNA library (Clontech) and transformants were selected exactly as described earlier [19]. His^+ colonies were lysed in liquid nitrogen and assayed for β-galactosidase activity on filters. Only those colonies, which do not express β-galactosidase after loss of the pLEX-a/cdk5 bait plasmid, were selected for further analysis. The plasmids pLEX-a/cdk5 or pLEX-a/lamin were reintroduced into L40 (a) by direct transformation of L40 strains that already contain the prey plasmid or (b) by mating prey plasmid-containing L40 strains with AMR70 transformants of pLEX-a/cdk5 or pLEX-a/lamin. Later, the prey plasmids were isolated by transforming total yeast DNA into HB101 and by selecting for leucine prototrophy on minimal medium plates (manufacturer's protocol; Clontech). Plasmids, that contain defined inserts, were then retransformed into L40 strains that already harbor pLEX-a/cdk5 or pLex-a/lamin. Plasmids, which express proteins that do not bind lamin but reproducibly interact with Cdk5, were sequenced using an automated DNA sequencer (LiCor). Inserts were identified by comparing translated DNA sequences with the SWISS PROT database.

2.7. Pull-down assay with [^{35}S]methionine-labeled Cdk1, Cdk2, Cdk4 and Cdk5

The pcDNA3.1 plasmids (1 μg) carrying the genes *Cdk1*, *Cdk2*, *Cdk4* or *Cdk5* were linearized uniquely at the 3′-end of the gene inserts. The linearized plasmids were used as templates for in vitro transcription/translation. The Cdk proteins were radioactively labeled with [^{35}S]methionine (20 μCi of an in vivo cell labeling grade; Amersham-Pharmacia Biotech). The transcription and translation was performed using the TNT-T7 in vitro transcription/translation kit (Promega).

Bacterially expressed glutathione *S*-transferase (GST) and GST-dbpA(CΔ) fusion protein were bound to glutathione Sepharose 4B (Amersham-Pharmacia Biotech) by incubating protein and beads at RT for 1 h. After four washing steps with 10 volumes of ice-cold PBS, the beads were resuspended in 200 μl 1% w/v BSA-PBS solution and kept on ice. 5 μl of the [^{35}S]methionine-labeled Cdk proteins (from 100 μl of an in vitro transcribed/translated product) diluted in 200 μl 1% w/v BSA-PBS solution, which contained 4 μl of a 100× solution of universal protease inhibitors (tablet dissolved in redistilled H_2O; Boehringer Mannheim), was kept on ice for 15 min. GSH-Sepharose 4B beads, bound to GST or GST-dbpA(CΔ), were incubated with labeled Cdks at 4°C for 2 h with gentle mixing. The beads were washed three times with 1 ml of bead-binding buffer (50 mM potassium phosphate pH 7.5, 150 mM KCl, 1 mM $MgCl_2$, 10% v/v glycerol, 1% v/v Triton X-100) containing universal protease inhibitors (Boehringer Mannheim). The pelleted bead-bound protein complexes were denatured by boiling in Laemmli sample buffer (4% w/v SDS, 0.1 M Tris, 4 mM EDTA, 20% v/v glycerol, 33% w/v bromophenol blue) containing 50 mM DTT, and were analyzed by 12.5% SDS-PAGE. The gels were fixed (10% v/v glacial acetic acid, 30% v/v methanol solution) for 10 min, and the signal was enhanced by soaking the gel in EN^{3}HANCE (NEN) for 1 h at RT. The dried gel was exposed to Kodak X-OMAT AR film.

2.8. Transfection of pcDNA 3.1+/cdk2, pcDNA 3.1+/cdk4 and pcDNA 3.1-/cdk5 in COS-1 cells and precipitation of COS-1 expressed Cdks by GST-dbpA(CΔ)

COS-1 cells in six-well plates, grown in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) that contained 10% v/v fetal bovine serum (FBS, Gibco BRL), were transfected with 2 μg of pcDNA 3.1

plasmids that carry *Cdk2*, *Cdk4* or *Cdk5*, using calcium phosphate. In order to control transformation efficiency, pcMV-β-gal was used.

Harvested cells ($\sim 5\times10^5$) were washed once with PBS and then lysed in 1 ml of hypotonic lysis buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% v/v Nonidet P-40) that contained 1 mM DTT and universal protease inhibitors (Boehringer Mannheim). Total protein in cell lysates was measured by the Bradford assay (Bio-Rad) and was stored at −70°C before use.

400 μl of a COS-1 cell lysate was first preincubated for 2 h at 4°C with 30 μl of Sepharose 4B (to remove unspecific binding of proteins to beads). This was followed by incubation with 30 μl of GSH-Sepharose 4B that was already bound to bacterially expressed GST-dbpA(CΔ). Pull-down of the Cdks in COS-1 cell lysates was performed as described above for the [^{35}S]methionine-labeled Cdks. The pelleted bead-bound protein complexes were denatured by boiling in Laemmli sample buffer and were analyzed by Western blotting. The blots were probed with polyclonal antibodies raised against Cdk2 and N-terminal fragments of Cdk4 and Cdk5 and were detected by ECL (Amersham-Pharmacia Biotech).

2.9. Northern blotting

25 ng of pcDNA3.1+ encoding the *dbpA*(*C*Δ) gene was used to make a labeled probe for Northern blotting (1.9×10^9 dpm/μg) using the rediprime DNA labeling system (Amersham-Pharmacia Biotech, RPN1633) with Redivue [^{32}P]dCTP (specific activity = 3000 Ci/mmol; Amersham-Pharmacia Biotech). The probe was used to hybridize a human multiple tissue Northern blot according to the manufacturer's protocol (Clontech). The blot was quantified using a phosphorimager (Molecular Devices).

2.10. Expression of baculoviruses

Recombinant baculoviruses encoding different genes were constructed using protocols provided by the manufacturer (Gibco BRL). The viruses were harvested and amplified until a desired titer was reached (viz. 1×10^8 pfu/ml).

2×10^7 Sf9 cells were used to co-infect baculoviruses carrying *Cdk5*, *Cdk4* or *Cdk2* with *GST-p35*, *GST-cyclin A*, *GST-cyclin D1*, *GST-cyclin D2* or *GST-cyclin D3* in 25 ml of SF900 II SFM medium (Gibco BRL). Triple infections were performed with the above mentioned viral combinations and baculoviruses carrying *GST-dbpA*(*C*Δ). After 72 h, cells were harvested and lysed by sonication in NETN buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 05% v/v NP-40, 5 mM NaF, 30 mM p-NPP, 1 mM PMSF, 1 μg/ml antipain) that contained universal protease inhibitors (Boehringer Mannheim). HBT buffer (50 mM HEPES pH 7.6, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1% v/v Tween 20, 1 mM DTT, 5 mM NaF, 30 mM p-NPP, 25 mM β-glycerolphosphate, 1 mM PMSF, 1 μg/ml antipain) was used to lyse cells that were co-infected with *GST-cyclin D1*, *D2* or *D3* viruses. Heterodimeric enzyme complexes were isolated by binding to 100 μl of GSH-Sepharose 4B, overnight at 4°C. The beads were washed seven times with ice-cold NETN buffer (or HBT buffer) and three times with ice-cold kinase buffer (20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$). The beads were resuspended in 100 μl kinase buffer and kept on ice before a kinase assay was performed.

2.11. Kinase assays

Phosphorylation of 0.1 μg histone H1 (type III-S: from calf thymus, Sigma H-5505) or 0.25 μg GST-Rb152 (i.e. C-terminal 152 amino acid residues of pRb covalently linked to GST; Santa Cruz Biotechnology) was performed in kinase buffer for 40 min at 30°C using 2 μl of beads, 10 μM ATP and 3 μCi [γ-^{33}P]ATP (specific activity = 3000 Ci/mmol; Amersham-Pharmacia Biotech) in a total volume of 10 μl. The reactions were boiled in Laemmli sample buffer and were analyzed by 12.5% SDS-PAGE. The gel was fixed and the phosphorylation was quantified using a phosphorimager (Molecular Devices).

3. Results

3.1. A C-terminal fragment of dbpA binds to Cdk5 in a yeast two-hybrid screen

Cdk5 has been used as a bait in the yeast two-hybrid system to screen for proteins that may interact with Cdk5. Since Cdk5 is expressed in both cycling and non-cycling cells, we thought it would be interesting to know if there are any Cdk5 binding proteins in cycling cells. Hence, a HeLa cDNA library in the yeast vector pGADGH was transformed into the yeast strain L40 [19] that already contained the bait plasmid pLEX-a/cdk5 (which encodes $Lexa_{DBD}$-Cdk5). Genes encoding interacting proteins were identified by selecting for His$^+$ prototrophs and subsequently through induction of β-galactosidase (β-gal) activity. Colonies that were His$^+$ and blue were considered positive and were used for further analysis (results not shown). It was later found that one such positive yeast colony harbored a cDNA library plasmid (i.e. the prey plasmid) encoding $GAL4_{TAD}$ fused to a C-terminal fragment of the human gene for the DNA binding protein, dbpA (in this communication referred to as dbpA(CΔ); Fig. 2A).

In order to eliminate the possibility that an identified positive colony was an artifact of the two-hybrid system (frequently referred to as 'false positives') [20], the yeast strain was cured of the bait plasmid pLEX-a/cdk5 by growing the strain in a non-selective medium (50 generations of growth in minimal medium that contained tryptophan and adenine but lacked leucine; the prey plasmid contains *LEU2*). The resulting L40 strain (MATa) that contains the prey plasmid, pGADGH/dbpA(CΔ), was mated with strains of AMR70 (MATα) that had been transformed either with pLEX-a/cdk5 or with pLEX-a/lamin. β-Gal assays performed on mated diploid strains showed that dbpA(CΔ) specifically interacts with Cdk5 but not with lamin (Fig. 1A).

At this stage, the pGADGH/dbpA(CΔ) plasmid was isolated from yeast (via transformation of total yeast DNA in *E. coli*; see Section 2). The insert was sequenced and it revealed that the cloned gene encoded 172 amino acids of the C-terminal fragment of human dbpA (Fig. 2A). The *dbpA*(*C*Δ) cDNA was amplified by PCR from the pGADGH plasmid and was subcloned into pACT2, another yeast vector often used for two-hybrid assays. The plasmid pACT2/dbpA(CΔ) (which encodes $GAL4_{TAD}$-dbpA(CΔ) fusion protein) was transformed into the strain L40 that already contained pLEX-a/cdk5, pLEX-a/lamin, pLEX-a/MyoD or pLEX-a/p35 (all plasmids code for $Lexa_{DBD}$ fusion proteins). The results reiterate that, at least in the two-hybrid system, Cdk5 not only binds p35 (Cdk5's known activator) but also interacts with dbpA(CΔ) (Fig. 1B, left and right panels). It was also found that interaction of dbpA(CΔ) with Cdk5 does not depend on the specific TAD to which dbpA(CΔ) is linked. This was supported by the observation that a $VP16_{TAD}$-dbpA(CΔ) fusion protein binds as well as $GAL4_{TAD}$-dbpA(CΔ) to $Lexa_{DBD}$-Cdk5, in the two-hybrid assay (data not shown). Furthermore, the *dbpA*(*C*Δ) was cloned in pLEX-a and *cdk5* in pGAD424. It was seen that interchanging the two domains, TAD and DBD, did not affect the interaction between dbpA(CΔ) and Cdk5 (i.e. $Lexa_{DBD}$-dbpA(CΔ) fusion protein bound strongly to $GAL4_{TAD}$-Cdk5; Fig. 1C). All these observations prove beyond doubt that neither a TAD nor a DBD has any influence on the Cdk5-dbpA(CΔ) interaction.

We also wanted to find out if Cdk5 was the only Cdk to which dbpA(CΔ) has affinity. In order to test this, yeast strain L40 was first transformed with pLEX-a/cdk5, pLEX-a/cdk4, pLEX-a/cdk2 or pLEX-a/cdk1. The plasmid pACT2/dbpA(CΔ) (which encodes $GAL4_{TAD}$-dbpA(CΔ)) was then transformed into L40 strains that already had the ability to express Lexa-Cdk5, Lexa-Cdk4, Lexa-Cdk2 or Lexa-Cdk1 fusion proteins. A β-gal assay (Fig. 1D) depicts that, in the yeast-two-hybrid

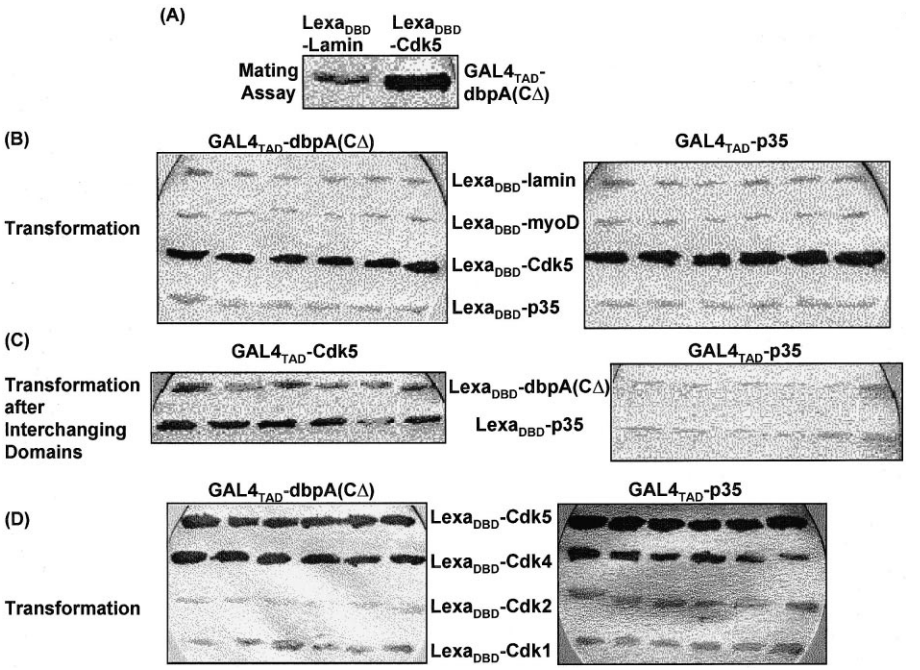

Fig. 1. In a yeast two-hybrid assay the C-terminal fragment of DNA binding protein dbpA binds to Cdk5 and Cdk4. Interactions were monitored by analyzing six individual colonies by colorimetric β-galactosidase assays. A: After curing the bait plasmid, a L40 strain that retains the prey plasmid (which encodes $GAL4_{TAD}$-dbpA(CΔ) fusion protein) was mated with strain AMR70 that had already been transformed with pLEX-a/lamin or pLEX-a/Cdk5 (these plasmids express Lex-a-lamin and Lex-a-Cdk5 fusion proteins, respectively). The results show that dbpA(CΔ) interacts with Cdk5 but not with the unspecific protein, lamin. B: Left panel, plasmid pACT2/dbpA(CΔ) (which expresses $GAL4_{TAD}$-dbpA(CΔ)) was transformed in strain L40 that already contains pLEX-a/lamin, pLEX-a/MyoD, pLEX-a/Cdk5 or pLEX-a/p35. The results show that dbpA(CΔ) interacts specifically with Cdk5. Right panel, plasmid pGAD424/p35 (expressing $GAL4_{TAD}$-p35) was transformed in the same L40 yeast strains (see left panel), as a control. C: Plasmids pGAD424/Cdk5 (left panel) and pGAD424/p35 (right panel, as negative control) were transformed into L40 strains that already harbor pLEX-a/dbpA(CΔ) or pLEX-a/p35. Results show that Cdk5 and dbpA(CΔ) can still interact with each other even after interchanging the two domains ($GAL4_{TAD}$ and Lex-a_{DBD}) which has to be linked to two putative interacting proteins, in the two-hybrid assay. D: Plasmids pACT2/dbpA(CΔ) (left panel) and pACT2/p35 (right panel, as control), which express $GAL4_{TAD}$-dbpA(CΔ) and $GAL4_{TAD}$-p35 respectively, were transformed in L40 strains that already harbor pLEX-a/Cdk5, pLEX-a/Cdk4, pLEX-a/Cdk2 or pLEX-a/Cdk1. Results show that dbpA(CΔ) interacts specifically with Cdk5 and Cdk4.

system, dbpA(CΔ) can interact with Cdk5 and Cdk4 but not with Cdk2 and Cdk1.

3.2. The dbpA(CΔ) protein precipitates ^{35}S-labeled Cdk5 and Cdk4

In order to confirm some of the yeast two-hybrid data, a set of in vitro pull-down experiments were employed. We found that bacterially expressed GST-dbpA(CΔ) fusion protein (Fig. 2B) was able to pull down [^{35}S]methionine-labeled Cdk5 and Cdk4 but not Cdk2 and Cdk1 (all proteins were transcribed/translated in vitro). We were sure that the GST moiety in GST-dbpA(CΔ) did not play a role in the precipitation of Cdk5 or Cdk4 since the GST protein alone is not able to pull down Cdk5 (Fig. 2C).

3.3. The dbpA(CΔ) protein precipitates Cdk5 and Cdk4 expressed in COS-1 cells

As an alternative to the in vitro transcription/translation procedure to obtain desired proteins, Cdk5, Cdk4 and Cdk2 were expressed in COS-1 cells. Lysates from transfectants were incubated with the *E. coli* expressed GST-dbpA(CΔ) fusion protein. The bound proteins were detected by Western blotting, using antibodies specific to Cdk5, Cdk4 or Cdk2. Similar to our earlier observations with labeled proteins, we observed that GST-dbpA(CΔ) was able to bind COS-1-expressed Cdk5 and Cdk4 whereas it was unable to pull-down Cdk2 (Fig. 2D).

3.4. Tissue specific expression of dbpA

The *dbpA*(*C*Δ) cDNA was labeled with ^{32}P and a human multiple tissue Northern blot was probed. The *dbpA* mRNA was relatively abundant in skeletal muscle tissue and the heart (Fig. 3), corroborating earlier findings of Kudo et al. [21]. Since Cdk5 has been demonstrated to play a role in muscle differentiation [11,12] we were interested to find out what effect dbpA(CΔ) would have on the Cdk5 kinase.

3.5. Inhibition of the Cdk5 kinase by bacterially expressed GST-dbpA(CΔ) and His-dbpA(CΔ)

The Cdk5/GST-p35 heterodimeric complex was expressed in insect cells using the baculovirus expression system. The enzyme was purified by binding to GSH-Sepharose 4B. The bead-bound enzyme was tested for activity (see Section 2) using histone H1 as a substrate. Fig. 4A (lane 1) portrays the phosphorylation of histone H1 by GST-p35 activated Cdk5 kinase. Kinase assays performed on GST-dbpA(CΔ) demonstrate that dbpA(CΔ) is not phosphorylated by Cdk5 and therefore cannot be a Cdk5 substrate (Fig. 4A, lane 3). We then asked if dbpA(CΔ) could be an inhibitor of Cdk5. Fig. 4B shows a dose-dependent inhibition of Cdk5 by GST-dbpA(CΔ). It should be noted that, whereas 10 μg of GST did not have any effect on the kinase (Fig. 4B, lane 8), 0.4 μg of GST-dbpA(CΔ) distinctly inhibited the complex ($>50\%$; Fig. 4B, lane 5). Staurosporine (CGP39360, Novartis), which

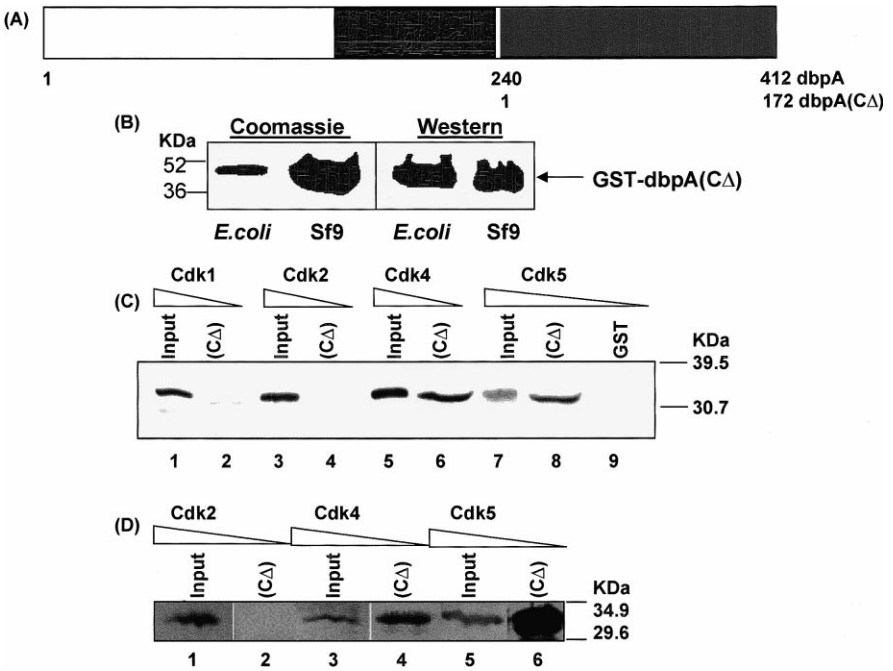

Fig. 2. A: Model of DNA binding protein dbpA. The gray area is the C-terminal fragment of dbpA (172 aa) which we have found to interact with Cdk5 and Cdk4. This area contains α-helices, basic and acidic stretches which are important in protein-protein interactions. The black area is the cold shock domain, this region is homologous in all Y-box binding proteins and is presumed to be the DNA binding domain [24]. B: Coomassie stain of 0.1 μg *E. coli* and 1 μg insect cell (Sf9) expressed GST-dbpA(CΔ) and Western blot of 0.1 μg *E. coli* and 0.1 μg insect cell (Sf9) expressed GST-dbpA(CΔ) using a monoclonal antibody against GST (Clontech). C: GST-dbpA(CΔ) (referred to as (CΔ)) binds to ^{35}S-labeled Cdk4 and Cdk5 and not to ^{35}S-labeled Cdk1 and Cdk2. Lane 1, 10% of the ^{35}S-labeled Cdk1 input used for the pull-down assay with (CΔ) (as in lane 2); lane 3, 10% of the ^{35}S-labeled Cdk2 input used for the pull-down assay with (CΔ) (as in lane 4); lane 5, 10% of the ^{35}S-labeled Cdk4 input used for the pull-down assay with (CΔ) (as in lane 6); lane 7, 10% of the ^{35}S-labeled Cdk5 input used for the pull-down assay with (CΔ) (as in lane 8) and the pull-down assay with GST alone (as in lane 9). D: GST-dbpA (CΔ) binds to Cdk4 and Cdk5 and not to Cdk2 overexpressed in COS-1 cells. Each lane shows a Western blot of proteins fractionated on a 10% SDS-polyacrylamide gel. Polyclonal antibodies raised against Cdk2 and the N-termini of Cdk4 and Cdk5 were used to detect the respective proteins. Lanes 1, 3 and 5, 15 μl of lysates from COS-1 cells expressing Cdk2, Cdk4 or Cdk5. Lanes 2, 4 and 6, 400 μl of same cell lysates, used in pull-down assays with GST-dbpA(CΔ) (referred to as (CΔ)).

strongly inhibits most Cdks including Cdk5, was used as a control inhibitor [22].

It can be argued that the relatively large GST moiety causes a steric hindrance in the fusion protein so that it interferes with dbpA(CΔ)'s ability to act as a substrate for Cdk5 or that the presence of GST causes an artifactual inhibition of Cdk5. Therefore, dbpA(CΔ) was expressed in *E. coli* as a His-tagged protein (six consecutive histidines attached to the protein at its N-terminus, as in pQE32; see Section 2). His-dbpA(CΔ) was used to probe if it is a substrate or an inhibitor of the Cdk5/p35 kinase. Like GST-dbpA(CΔ), His-dbpA(CΔ) is not phosphorylated by Cdk5/GST-p35 (Fig. 4D, right panel, lane 4). Again similar to GST-dbpA(CΔ), His-dbpA(CΔ) inhibits the Cdk5/GST-p35 complex in a dose-dependent manner. His-dbpA(CΔ) completely abolishes the Cdk5-mediated phosphorylation of histone H1 (Fig. 4C, left panel). The His-tagged protein can also completely inhibit the phosphorylation of GST-Rb152 by the Cdk5/GST-p35 complex (Fig. 4C, right panel, lane 4 and 5). Kinase assays performed with Cdk5/GST-p35 enzyme eluted from the GSH-Sepharose 4B beads gave identical results (results not shown).

3.6. Activation of Cdk5 with D-type cyclins

Since D-type cyclins are reported to bind Cdk5 [5], we wanted to find out if cyclin D formed a complex with Cdk5 when both proteins are expressed in insect cells, and if it did, whether the complex formed an active kinase by phosphorylating either histone H1 or GST-Rb152. One could speculate that an active cyclin D/Cdk5 enzyme, with no known cellular substrate, may phosphorylate the Cdk5 binding protein, dbpA(CΔ). Hence, Sf9 insect cells were co-infected with baculoviruses encoding Cdk5 and GST-cyclin D1, GST-cyclin D2 or GST-cyclin D3. The cyclin D-bound Cdk5 proteins obtained from insect cells were precipitated with GSH-Sepharose 4B and the bead-bound complexes (as confirmed by Western blotting using antibodies specific for Cdk5 and D-type cyclins; results not shown) were used directly for kinase assays. We observed that neither GST-cyclin D1 nor GST-cyclin D3 can activate Cdk5 (results not shown). Surprisingly, like cyclin D2-activated Cdk2 (Fig. 4D, left panel, lane 2) and Cdk4 (Fig. 4D, left panel, lane 4), kinases that are known to phos-

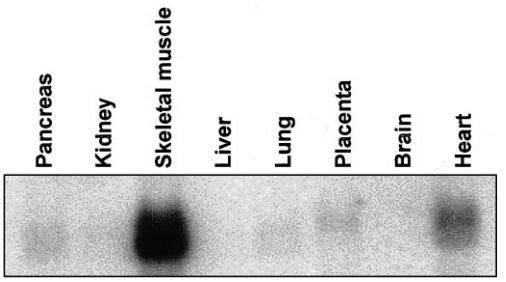

Fig. 3. Tissue-specific expression of dbpA. Human multiple tissue Northern blot (Clontech) hybridized with dbpA(CΔ).

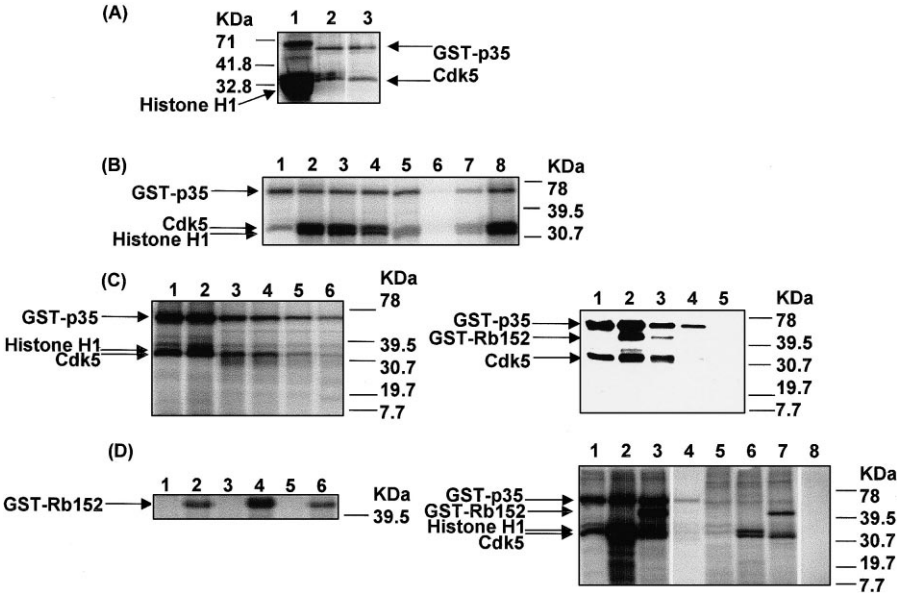

Fig. 4. Kinase assays with histone H1 and GST-Rb152, as substrates, show that both GST-dbpA(CΔ) and His-dbpA(CΔ) proteins are not substrates for Cdk5 that is activated by p35 or cyclin D2. However, GST-dbpA(CΔ) and His-dbpA(CΔ) inhibit Cdk5-mediated phosphorylation of histone H1 and GST-Rb152 in a dose-responsive manner. A: GST-dbpA(CΔ) is not phosphorylated by p35 activated Cdk5 kinase. Lane 1, Cdk5/GST-p35+10 μg histone H1 as substrate; lane 2, Cdk5/GST-p35+10 μg GST as substrate; lane 3, Cdk5/GST-p35+0.4 μg GST-dbpA(CΔ) as substrate. B: Dose response to GST-dbpA(CΔ) in the inhibition of Cdk5-mediated phosphorylation of histone H1 (1 μg). Lane 1, Cdk5/GST-p35+no substrate; lane 2, Cdk5/GST-p35+substrate; lane 3, Cdk5/GST-p35+substrate+0.4 μg GST-dbpA(CΔ); lane 4, Cdk5/GST-p35+substrate+0.8 μg GST-dbpA(CΔ); lane 5, Cdk5/GST-p35+substrate+1.6 μg GST-dbpA(CΔ); lane 6, Cdk5/GST-p35+substrate+300 nM staurosporine; lane 7, Cdk5/GST-p35+substrate+40 nM staurosporine; lane 8, Cdk5/GST-p35+substrate+10 μg GST. C: Left panel, dose response to His-dbpA(CΔ) in the inhibition of Cdk5-mediated phosphorylation of histone H1 (0.1 μg). Lane 1, Cdk5/GST-p35+no substrate; lane 2, Cdk5/GST-p35+substrate; lane 3, Cdk5/GST-p35+substrate+0.5 μg His-dbpA(CΔ); lane 4, Cdk5/GST-p35+substrate+1 μg His-dbpA(CΔ); lane 5, Cdk5/GST-p35+substrate+3 μg His-dbpA(CΔ); lane 6, Cdk5/GST-p35+substrate+6 μg His-dbpA(CΔ). Right panel, dose response to His-dbpA(CΔ) in the inhibition of Cdk5-mediated phosphorylation of GST-Rb152 (0.25 μg). Lane 1, Cdk5/GST-p35+no substrate; lane 2, Cdk5/GST-p35+substrate; lane 3, Cdk5/GST-p35+substrate+0.5 μg His-dbpA(CΔ); lane 4, Cdk5/GST-p35+substrate+1 μg His-dbpA(CΔ); lane 5, Cdk5/GST-p35+substrate+6 μg His-dbpA(CΔ). D: His-dbpA(CΔ) is not a substrate for Cdk5/GST-p35 and Cdk5/GST-cyclin D2 kinases. Left panel, GST-cyclin D2-activated kinases were prepared and were tested for their ability to phosphorylate 0.25 μg GST-Rb152. Lane 1, Cdk2/GST-cyclin D2+no substrate; lane 2, Cdk2/GST-cyclin D2+substrate; lane 3, Cdk4/GST-cyclin D2+no substrate; lane 4, Cdk4/GST-cyclin D2+substrate; lane 5, Cdk5/GST-cyclin D2+no substrate; lane 6, Cdk5/GST-cyclin D2+substrate. Right panel, both Cdk5/GST-p35 and Cdk5/GST-cyclin D2 were tested for their ability to phosphorylate histone H1, GST-Rb152 and His-dbpA(CΔ). Lane 1, Cdk5/GST-p35+no substrate; lane 2, Cdk5/GST-p35 incubated with 1 μg histone H1; lane 3, Cdk5/GST-p35 kinase incubated with 0.25 μg GST-Rb152; lane 4, Cdk5/GST-p35 incubated with 5 μg His-dbpA(CΔ); lane 5, Cdk5/GST-cyclin D2+no substrate; lane 6, Cdk5/GST-cyclin D2 incubated with 1 μg histone H1; lane 7, Cdk5/GST-cyclin D2 incubated with 0.25 μg GST-Rb152; lane 8, Cdk5/GST-cyclin D2 incubated with 5 μg His-dbpA(CΔ).

phorylate pRb, cyclin D2-activated Cdk5 also has the ability to phosphorylate GST-Rb152 (Fig. 4D, left panel, lane 6). However, His-dbpA(CΔ) is not phosphorylated by cyclin D2 and p35 activated Cdk5 (Fig. 4D, right panel, lanes 4 and 8).

3.7. *Inhibition of the Cdk4 kinase by bacterially expressed His-dbpA(CΔ)*

Since dbpA(CΔ) interacts not only with Cdk5 but also with Cdk4 in both two-hybrid and in vitro binding assays, the effect of His-dbpA(CΔ) on active Cdk4/cyclin D1 complex was investigated. We see that the His-tagged protein completely inhibits Cdk4/GST-cyclin D1 mediated phosphorylation of GST-Rb152 (Fig. 5A, lane 4). Furthermore, dbpA(CΔ) is definitely not a substrate for the Cdk4/GST-cyclin D1 enzyme (Fig. 5A, lane 5).

3.8. *Specificity of inhibition of the Cdk5 kinase compared to the Cdk2 kinase*

Although His-dbpA(CΔ) inhibits the Cdk5/GST-p35 mediated phosphorylation of histone H1 (there is at least a 20-fold decrease in activity; compare lanes 3 and 4 in Fig. 5B), it seems that phosphorylation of the same substrate by Cdk2/GST-cyclin A is not affected at all (Fig. 5B, compare lanes 1 and 2). This seems to corroborate our earlier two-hybrid data which indicated that dbpA(CΔ) does not interact with Cdk2 but binds Cdk4 and Cdk5.

3.9. *Triple infections with GST-DbpA(CΔ) baculoviruses do not yield active Cdk5 or Cdk4 kinases*

Sf9 insect cells were infected with three baculoviruses encoding (a) Cdk5, GST-p35 and GST-dbpA(CΔ), (b) Cdk4, GST-cyclin D1 and GST-dbpA(CΔ), or (c) Cdk2, GST-cyclin A and GST-dbpA(CΔ). Proteins were precipitated using GSH-Sepharose. Western blot analysis showed that all three proteins were expressed in each precipitate. Kinase assays performed on the GSH-Sepharose purified enzymes (according to Section 2) yielded inactive kinases for Cdk5 and Cdk4 whereas the Cdk2 kinase was still active (results not shown).

3.10. *Inhibition of the Cdk5 and Cdk4 kinase by insect cell expressed GST-DbpA(CΔ)*

GST-dbpA(CΔ) was purified from Sf9 insect cells using the baculovirus expression system (Fig. 2B) and used in Cdk5/GST-p35, Cdk4/GST-cyclin D1 and Cdk2/GST-cyclin A kin-

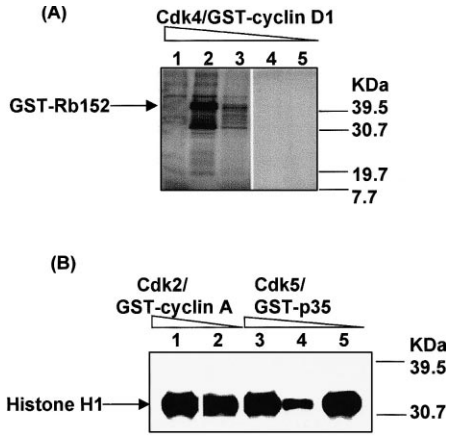

Fig. 5. His-dbpA(CΔ) is not a substrate for Cdk4/GST-cyclin D1 but instead inhibits the kinase. Comparison of the inhibition of Cdk5/GST-p35 and Cdk2/GST-cyclin A kinases by His-dbpA(CΔ). A: His-dbpA(CΔ) inhibits Cdk4/GST-cyclin D1-mediated phosphorylation of GST-Rb152 (0.5 μg) and is not phosphorylated by the kinase. Lane 1, Cdk4/GST-cyclin D1+no substrate; lane 2, Cdk4/GST-cyclin D1+substrate (the lower band is a breakdown product of GST-Rb152); lane 3, Cdk4/GST-cyclin D1+substrate+1 μg His-dbpA(CΔ); lane 4, Cdk4/GST-cyclin D1+substrate+6 μg His-dbpA(CΔ); lane 5, Cdk4/GST-cyclin D1+6 μg His-dbpA(CΔ). B: Comparison of inhibition of the Cdk5/GST-p35 and Cdk2/GST-cyclin A-mediated phosphorylation of histone H1 (1 μg) by His-dbpA(CΔ). Lane 1, Cdk2/GST-cyclin A+substrate; lane 2, Cdk2/GST-cyclin A+substrate+0.5 μg His-dbpA(CΔ); lane 3, Cdk5/GST-p35+substrate; lane 4, Cdk5/GST-p35+substrate+0.5 μg His-dbpA(CΔ); lane 5, Cdk5/GST-p35+substrate+6 μg His-FKBP12 (an unspecific protein). The results show that His-dbpA(CΔ) inhibits Cdk5/GST-p35 but not the Cdk2/GST-cyclin A kinase.

ase assays. The insect cell expressed GST-dbpA(CΔ) protein gave the same results as the bacterially expressed GST-dbpA(CΔ) in its inhibition of the Cdk5/GST-p35 and the Cdk4/GST-cyclin D1 kinase and not the Cdk2/cyclin A kinase, whereas it was not a substrate for any of the kinases tested (results not shown). Eukaryotic expression of GST-dbpA(CΔ) did therefore not give any drastic difference in the folding or post-translational modification of the polypeptide.

4. Discussion

DNA binding protein DbpA belongs to the Y-box protein family. These proteins contain three domains: N-terminal domain, cold shock domain (CSD) and C-terminal domain. The cold shock domain, comprising of 70 amino acids, is highly conserved from prokaryotes to eukaryotes [23] and can bind so called Y-boxes on DNA. The DNA binding depends on the presence of inverted CCAAT motifs and flanking bases in these Y-boxes. The N-terminal region of dbpA is rich in proline and alanine residues which could potentially function as a transcriptional regulation domain [24]. Several eukaryotic genes contain a Y-box in their regulatory region. Ishii and colleagues were the first to isolate dbpA via interaction with the enhancer of the human epidermal growth factor receptor gene and the promoter of the human *c-erbB-2* gene [25]. The dbpA protein has also been found to bind to the promoter sequence of the leukosialin gene [21]. Furthermore, the dbpA protein represses the expression of the *I-Aβ* gene of the major histocompatibility complex [24] and the expression of the stress-inducible gene *grp78* [26]. The C-terminal domain of dbpA is hydrophilic due to an alteration of groups of basic and acidic amino acids, termed the charged zipper domain, that presumably contributes to interactions with other proteins [24]. This is the region we have isolated using the yeast two-hybrid system (Fig. 2A). Surprisingly, it inhibits both Cdk5 and Cdk4 activity. Further experiments should reveal whether full length dbpA has the same effect on Cdk5 and Cdk4 as its C-terminal fragment.

We have not found the consensus sequence (X-S/T-P-X-K/R-X-) [27], which is typical of a site phosphorylated by a Cdk, in dbpA(CΔ) (EMBL-PROSITE database). Our data confirm that dbpA(CΔ) is neither a substrate for Cdk5 nor for Cdk4 (Fig. 4D, right panel, lane 4 and Fig. 5A, lane 5 respectively). However, it should be noted that the protein has three potential protein kinase C phosphorylation sites. We do not know if these sites are utilized at all.

Although Cdk5 is expressed in both proliferative and differentiated cells, its expression is relatively abundant in nerve and muscle cells. Since dbpA mRNA is present in HeLa cells, the dbpA protein must also be present in proliferating cells. DbpA may therefore play a role in inhibiting Cdk5 during the cell cycle.

The p35 protein, the only known activator of Cdk5, is solely expressed in differentiated nerve cells. Cdk5 kinase activity (with p35 as its activator) could therefore be responsible for the onset of differentiation of nerve cells and possibly of muscle cells [9–12]. However, Cdk5 kinase activity should be blocked during terminal differentiation. Since both Cdk5 and p35 are present in terminally differentiated cells, other proteins should be involved in preventing this complex to be active in these cells. We would like to propose that dbpA is probably a specific inhibitor of Cdk5. This assumption is supported by results from Northern blot analyses which show that dbpA is highly expressed in skeletal muscle and heart as compared to other tissues ([21]; and our results, Fig. 3). Cdk5 is reported to phosphorylate neurofilament proteins and tau protein in vitro. Neurofilament proteins are also hyperphosphorylated in patients suffering from Lewy body pathologies, whereas the tau protein is hyperphosphorylated in patients suffering from Alzheimer's disease. Since the dbpA transcript was not detected in the brain ([21]; and our results, Fig. 3) other proteins may be responsible for inhibition of Cdk5 in the brain. It is also possible that dbpA is expressed in a particular section of the brain whose mRNA was not present in the commercial blot used for Northern analysis.

It is known that the Cdk4 kinase is responsible for phosphorylation of pRb early in the G1 phase of the cell cycle and has a very important function in cycling cells [28]. One can speculate that binding of dbpA to the Cdk4 protein allows inhibition of its kinase activity which thereby causes cell cycle arrest. Probably, this is what occurs in differentiated cells where it is essential that Cdk4 activity is inhibited. In cycling cells however, dbpA should be bound to Cdk5 to prevent the premature onset of differentiation. When Cdk5 is activated during differentiation, dbpA may switch its binding allegiance to Cdk4, resulting in the latter's inhibition and causing a cell cycle block. Later, terminally differentiated cells may again have dbpA bound to Cdk5 to maintain the kinase in its inactive form.

It has been reported that dbpA activates the transcription of thymidine kinase [29]. This may also suggest that dbpA is expressed at the beginning of S phase of the cell cycle and

may thus block Cdk4 activity at a point in the cell cycle where Cdk4 activity is no longer needed. This assumption may be equally valid if Cdk5 were to be responsible for the onset of differentiation. It is noteworthy that interactions between dbpA and Cdk5 or Cdk4 provide a link between unique signal transduction pathways and allow for alteration in gene expression. However, the true role of dbpA in cycling and post-mitotic cells needs to be evaluated through further cellular analyses.

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