THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 42, pp. 32048–32056, October 20, 2006

Functional Consequences of Mutations in *CDKL5*, an X-linked Gene Involved in Infantile Spasms and Mental Retardation*

Received for publication, July 3, 2006, and in revised form, August 17, 2006 Published, JBC Papers in Press, August 24, 2006, DOI 10.1074/jbc.M606325200

Ilaria Bertani[‡], Laura Rusconi[‡], Fabrizio Bolognese[‡], Greta Forlani[‡], Barbara Conca[‡], Lucia De Monte[§], Gianfranco Badaracco[‡], Nicoletta Landsberger[‡1], and Charlotte Kilstrup-Nielsen[‡2]

From the [‡]Dipartimento di Biologia Strutturale e Funzionale, Università dell'Insubria, 21052 Busto Arsizio (VA), Italy and [§]Tumour Immunology, San Raffaele Scientific Institute, Via Olgettina 58, 20132 Milan, Italy

Mutations in the X-linked cyclin-dependent kinase-like 5 (*CDKL5*) gene have been identified in patients with Rett syndrome, West syndrome, and X-linked infantile spasms sharing the common features of generally intractable early seizures and mental retardation. Disease-causing mutations are distributed in both the catalytic domain and in the large COOH terminus. In this report, we examine the functional consequences of some Rett mutations of *CDKL5* together with some synthetically designed derivatives useful to underline the functional domains of the protein. The mutated CDKL5 derivatives have been subjected to *in vitro* kinase assays and analyzed for phosphorylation of the TEY (Thr-Glu-Tyr) motif within the activation loop, their subcellular localization, and the capacity of CDKL5 to interact with itself. Whereas wild-type CDKL5 autophosphorylates and mediates the phosphorylation of the methyl-CpG-binding protein 2 (MeCP2) *in vitro*, Rett-mutated proteins show both impaired and increased catalytic activity suggesting that a tight regulation of CDKL5 is required for correct brain functions. Furthermore, we show that CDKL5 can self-associate and mediate the phosphorylation of its own TEY (Thr-Glu-Tyr) motif. Eventually, we show that the COOH terminus regulates CDKL5 properties; in particular, it negatively influences the catalytic activity and is required for its proper sub-nuclear localization. We propose a model in which CDKL5 phosphorylation is required for its entrance into the nucleus whereas a portion of the COOH-terminal domain is responsible for a stable residency in this cellular compartment probably through protein-protein interactions.

X-linked cyclin-dependent kinase-like 5 (*CDKL5*,[3] previously named *STK9*) was originally identified in a transcriptional mapping project focused on the human chromosome Xp22.3-p21.3, spanning a region critical for several diseases. Expression studies demonstrated that *CDKL5* was transcribed in several tissues, including brain (1). However, the possible link between *CDKL5* and human diseases was drawn only few years later when balanced translocating events disrupting the gene were identified in two female patients affected by severe infantile spasms and mental retardation (2). Retrospectively, a previous publication had identified a large deletion involving *CDKL5* in a male patient with X-linked retinoschisis and seizure (3); it has recently been hypothesized that retinoschisis is due to deletion of the *XLRS1* gene, whereas epilepsy is caused by truncation of at least the last exon of *CDKL5* (2). The importance of *CDKL5* in early onset seizures and severe mental retardation in females has been further reinforced by five recent reports linking mutations in *CDKL5* to patients with Rett syndrome but only in those affected by a variant form characterized by seizure onset before 6 months of age (4–8). Very recently the frequency of *CDKL5* mutations in patients affected by infantile spasms or early onset epilepsy of unknown cause has been investigated. The identification of several novel likely pathogenic mutations led the authors to propose that *CDKL5* mutations in females are a significant cause of severe mental retardation and of forms of early epilepsy that are generally intractable (9). Despite the clear involvement of *CDKL5* in human health, this protein remains almost completely uncharacterized; presently, we are missing its functions in the nervous system as well as the molecular consequences of its pathogenic mutations.

Given its amino acids sequence, CDKL5 was supposed to be a proline-directed serine/threonine kinase sharing homology with members of the mitogen-activated protein (MAP) kinase and cyclin-dependent kinase (CDK) families (1). Moreover, the similar Rett phenotypes in patients carrying mutations in the gene coding for the methyl-CpG-binding protein 2 (*MECP2*) and *CDKL5*, together with recent reports demonstrating the importance of MeCP2 phosphorylation for selective binding to DNA (10), opened the possibility that the two proteins might operate in a common developmental pathway. In favor of this hypothesis, we have recently shown that generally the two genes show spatial and temporal overlapping expression pattern that is simultaneously activated by neural maturation and synaptogenesis. Moreover, we demonstrated that CDKL5 and MeCP2 interact and that immunopurified CDKL5 leads, directly or indirectly, to the phosphorylation of the methyl-binding protein (8). The interaction between MeCP2 and CDKL5 has also been confirmed by Lin and colleagues; however, the failure in reproducing the MeCP2 phosphorylation led

* This work was supported by Telethon (to N. L.), the Fondo per gli Investimenti della Ricerca di Base (FIRB) from Ministero dell'Istruzione, dell'Università e della Ricerca (to N. L), the Rett Syndrome Research Foundation (to C. K.-N.), and by the Fondazione Cariplo (to G. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom correspondence may be addressed. Tel.: 39-0331-339-406; Fax: 39-0331-339-459; E-mail: landsben@uninsubria.it.

[2] To whom correspondence may be addressed. Tel.: 39-0331-339-406; Fax 39-0331-339-459; E-mail: c.kilstrup-nielsen@uninsubria.it.

[3] The abbreviations used are: CDKL5, cyclin-dependent kinase-like 5; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; MeCP2, methyl-CpG-binding protein 2; GFP, green fluorescent protein; ERK, extracellular signal-regulated kinase.

the authors to propose that MeCP2 might target CDKL5 to DNA-binding complexes containing a different functional substrate of the enzyme (11).

Integrating all data so far available, the emerging picture is that, in fact, CDKL5 and MeCP2 work in common molecular pathways. However, the demonstration that *CDKL5* mutations are an important etiological factor for neurodevelopmental disorders in addition to Rett syndrome (9), together with our previous expression studies showing that in some specific cerebellar domains the two genes are independently regulated (8), suggest that the kinase might also have functions not connected to the methyl-binding protein.

In this study we characterize several CDKL5-mutated derivatives, including some of those identified in Rett patients. Phosphorylation assays performed with the wild-type protein confirm its capability to mediate the modification of MeCP2 *in vitro*, whereas Rett missense mutations within the conserved catalytic domain abrogate or significantly impair the enzymatic activity. Interestingly, none of these mutants maintains the capability to influence the phosphorylation status of the methyl-binding protein. Frameshift mutations, which generate truncated proteins in patients, have been found both in the amino- and carboxyl-terminal regions of CDKL5. We therefore analyzed two derivatives interrupting the unusually large COOH-terminal region. Our results suggest that the COOH terminus of the protein has regulatory functions that can influence either the catalytic activity or the subcellular localization. We conclude that both a change in CDKL5 activity and its mislocalization can be responsible for the onset of neurodevelopmental disorders and believe that these studies should help in drawing some speculations on the CDKL5 mutation genotype/phenotype correlation.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Recombinant hMeCP2E2 was produced from the pTYB1-hMeCP2 vector containing the entire cDNA of MeCP2 (486 amino acids) cloned into the NdeI and XhoI sites of pTYB1 (New England Biolabs) generating a chitin-binding fusion protein. Recombinant hCDKL5 encompassing amino acids 301–751 for immunizing rabbits was produced as a chitin-binding fusion protein from pTYB1-CDKL5$_{301-751}$. The corresponding cDNA was generated by PCR and cloned into the NdeI and SapI sites of pTYB1.

pGFP-CDKL5 contains the entire cDNA of hCDKL5 (1030 amino acids) generated by PCR and cloned into the BspEI-BamHI sites of pEGFP-C1 (Clontech). Subsequently, an extra BamHI site, allowing the excision of the *CDKL5* cDNA with BamHI, has been introduced downstream of the BspEI site. The C152F, R175S, and K42R derivatives were generated by site-directed mutagenesis using the QuikChange® site-directed mutagenesis kit (Stratagene). ΔN contains amino acids 298–1030, Δ781 amino acids 1–780, and Δ525 1–524. The analogous FLAG-CDKL5 derivatives (NH_2-terminal FLAG-tag) were expressed from the pCMV-Tag-2B vector (Stratagene) into which the cDNAs were cloned into BamHI.

All PCR-generated constructs were verified by sequencing.

Antibodies—A rabbit anti-CDKL5 anti-serum (Covance Research Products Inc.) was raised against hCDKL5 amino acids 301–751 expressed in *Escherichia coli* as a fusion protein containing a chitin-binding domain that was eliminated during the purification procedure.

For immunopurification and Western blots the following antibodies were used: monoclonal anti-FLAG (Sigma), polyclonal anti-MeCP2 (Sigma), monoclonal anti-GFP (Roche), and polyclonal anti-phospho-p44/42 MAP kinase antibody (Cell Signaling).

Recombinant Protein Purification—Recombinant full-length hMeCP2E2 and hCDKL5 amino acids 301–751 were expressed in *E. coli* ER2566 and induced with 0.5 mM isopropyl β-D-thiogalactopyranoside at 30 °C for 5 h. Following induction, extracts were prepared by resuspending the bacteria in lysis buffer (750 mM NaCl, 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.1% Triton X-100, protease inhibitor mixture (Sigma P4380)) and sonicated. Following centrifugation at 40,000 rpm for 10 min, the cleared lysate was applied to chitin-agarose (New England Biolabs Inc.) pre-equilibrated with lysis buffer, after which the column was washed with 20 column volumes of lysis buffer. Fusion proteins were cleaved on the column overnight by incubation with lysis buffer containing 50 mM dithiothreitol. Eluted fractions containing the bulk of MeCP2 or CDKL5 were pooled. Recombinant purified MeCP2 was dialyzed against the kinase buffer used in the phosphorylation assays whereas CDKL5 was dialyzed against phosphate-buffered saline.

Cell Culture and Transfection—The human embryo-derived kidney cell line HEK293 and mouse fibroblasts, NIH3T3, were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37 °C with 5% CO_2. Transient transfections were performed with calcium phosphate or Lipofectamine 2000 (Invitrogen) according to the manufacturers instructions and cells harvested 20–36 h post-transfection.

In Vitro Phosphorylation Assays—For the detection of CDKL5 autophosphorylation, FLAG-CDKL5 or its mutated derivatives were immunoprecipitated from overexpressing HEK293 cells and incubated with [γ-^{33}P]ATP. Briefly, total cell extracts were prepared from ~10^6 transiently transfected cells with high salt lysis buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.1% Nonidet P-40, 1 mM dithiothreitol, phenylmethylsulfonyl fluoride, and protease inhibitor mixture (Sigma P4380)). Equal amounts of protein extract were precleared for 1 h with 20 μl of 100% mouse IgG-agarose beads at 4 °C; 40 μl of 100% EZviewRed Anti-FLAG M2 Affinity gel (Sigma) was added to the cleared extract and the mixture incubated for 2 h at 4 °C. Immunocomplexes were collected by centrifugation, washed five times with the above lysis buffer containing 700 mM NaCl, and 85% of the pellet was used in kinase assays as described previously (8).

For the phosphorylation of MeCP2, 3 μg of recombinant hMeCP2E2 were added to the FLAG-CDKL5 pellet resuspended in kinase buffer and subjected to phosphorylation as previously described (8). 85% of the labeled proteins were separated by SDS-PAGE, visualized by autoradiography or quantitated by PhosphorImager analysis (GE Healthcare). To detect the immunopurified CDKL5 derivatives and MeCP2, 15% of the immunocomplexes were assayed by Western blot. A Kodak Image Station 2000R was used to quantitate the signals in the

Western blot. CDKL5 autophosphorylation activity was expressed as the ratio between incorporated ^{33}P and the corresponding immunoreactive signal.

Fractionated Extracts and Immunoprecipitation Experiments—For fractionation experiments, 5×10^5 HEK293 were collected 36 h after transfection, washed twice with phosphate-buffered saline, and lysed in hypotonic lysis buffer (20 mM Hepes, pH 8.0, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA) supplemented with phosphatase and protease inhibitors (1 mM Na_3VO_4, 1 mM dithiothreitol, 5 mM NaF, phenylmethylsulfonyl fluoride, and protease inhibitor mixture) by incubation on ice for 45 min. After centrifugation at 500 × *g* for 5 min the supernatant was kept as the cytosolic fraction. The pelleted nuclei were washed once in the phosphate-buffered saline, resuspended in nuclear lysis buffer (10 mM Hepes, pH 8.0, 1.5 mM $MgCl_2$, 1 M KCl, 0.2 mM EDTA, 1% Nonidet P-40, 25% glycerol) supplemented with the above mentioned inhibitors, incubated for 30 min on ice, and subsequently sonicated to destroy genomic DNA. Cellular debris were removed by centrifugation for 30 min at 18,000 × *g* at 4 °C and the protein extracts analyzed by immunoblotting.

For coimmunoprecipitation experiments, 1×10^6 HEK293 cells coexpressing GFP-CDKL5 and FLAG-CDKL5 derivatives were collected, resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton, 2 mM EDTA, 10% glycerol) supplemented with protease and phophatase inhibitors (1 mM Na_3VO_4, 1 mM dithiothreitol, 5 mM NaF, phenylmethylsulfonyl fluoride, and protease inhibitor mixture), sonicated, and centrifuged for 15 min at 18,000 × *g* at 4 °C. The extract was cleared and immunoprecipitated with an anti-FLAG resin as described for phophorylation assays and the immunocomplexes washed four times with the lysis buffer and analyzed by Western blot.

For the phospho-TEY assay, total extracts from 1×10^6 transiently transfected HEK293 cells were prepared as for coimmunoprecipitation experiments and extracted proteins purified with either anti-FLAG resin as described above or with anti-GFP antibodies. The extracts were incubated for 1 h with the primary antibody, after which protein-G-Sepharose (KPL) was added, the mixture incubated for 2 h and immunocomplexes washed four times with the lysis buffer and analyzed by Western blot.

Immunofluorescence—Mouse fibroblasts, NIH3T3, were seeded on gelatin-coated glass coverslips and transiently transfected with Lipofectamine 2000 (Invitrogen). 20 h post-transfection the cells were fixed with 4% paraformaldehyde, stained with Hoechst 33258 (Sigma) and analyzed with an Olympus BX51 fluorescence microscope.

RESULTS

Disease-causing Mutations in CDKL5—Mutations in the *CDKL5* gene have recently been identified in patients suffering from Rett syndrome, West syndrome, and infantile spasms (2, 4–9), which share the common features of early-onset seizures and mental retardation, suggesting an important role of CDKL5 in the nervous system. CDKL5 is a large protein of 1030 amino acids with an estimated molecular mass of 116 kDa (Fig. 1*A*) containing a conserved serine-threonine kinase domain within its NH_2 terminus and a large COOH-terminal region to which

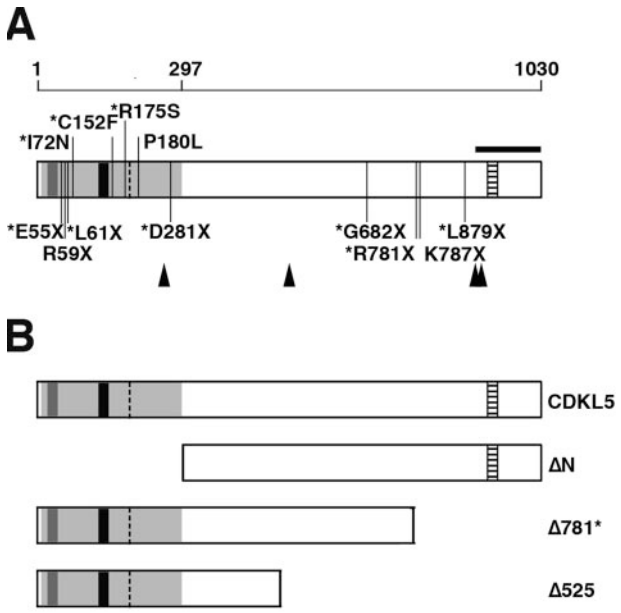

FIGURE 1. **Schematic illustration of CDKL5 with disease-causing mutations within the open reading frame.** *A*, full-length human CDKL5 is 1030 amino acids long and contains within the catalytic domain (*light gray box*) the ATP-binding site (*dark gray box*), the serine-threonine kinase active site (*black box*), and the conserved TEY motif (*dotted line*). A putative signal peptidase I serine active site is present in the carboxyl terminal region (*striped box*). Four P*xx*P sequences, constituting possible binding sites for Src homology 3-containing proteins, are indicated with *arrowheads*. Disease-causing mutations are shown with missense mutations on the top and frameshift mutations on the *bottom*. The *black bar* above the COOH terminus of CDKL5 indicates the region deleted in some retinoschisis patients with epilepsy. Rett causing mutations are indicated with an *asterisk*. *B*, schematic representation of the utilized CDKL5 derivatives fused to either FLAG or GFP. The disease-causing mutations are named according to Den Dunnen *et al.* (12).

no function has yet been assigned (1). Even if the number of patients is still rather limited, the mutations identified so far are located in various regions of the protein. Four missense mutations have been identified in the catalytic domain of the protein; among these, the C152F and R175S mutations have been suggested to interfere with phosphotransfer and either kinase activation or substrate specificity, respectively (4, 11). Frameshift mutations causing the premature deletion of the protein have been found both in the catalytic domain and in a region spanning almost 400 amino acids of the very carboxyl terminus, suggesting an important role of this region for CDKL5 functions. To map the functional domains of the protein and reveal how its activities are regulated, we decided to analyze various CDKL5 derivatives in functional assays; besides performing *in vitro* phosphorylation assays, we also assayed the subcellular localization as well as the capacity of CDKL5 to interact with itself. We chose to characterize the two Rett derivatives C152F and R175S mutated within the catalytic domain as well as two COOH-terminally truncated proteins (Fig. 1*B*). Whereas the Δ781 derivative carries a 249 amino acids deletion identified in a Rett patient, the synthetic Δ525 derivative is deleted of most

of the COOH terminus and lacks the three PxxP sites within this region, which constitutes binding sites for Src homology 3-domain proteins (11). Finally, as a control, we also analyzed a CDKL5 derivative lacking the entire kinase domain (ΔN) as well as a kinase-dead mutant, K42R, that has been mutated in the highly conserved ATP-binding site (11, 13).

Rett Mutations in the Catalytic Domain Influences CDKL5 Catalytic Activity—The catalytic activity of CDKL5 has been reported to be involved in its autophosphorylation *in vitro* (8, 11). We decided to analyze the various mutated derivatives for autophosphorylation activity and to this aim the mutated proteins carrying an NH_2-terminal FLAG epitope were immunopurified from overexpressing mammalian cells and the extensively washed pellet incubated with [γ-^{33}P]ATP. 85% of the labeled proteins were separated by SDS-PAGE and visualized by autoradiography (Fig. 2*A*, *upper panel*). To normalize for the amount of protein used in these assays, the remaining 15% of the labeled proteins were analyzed by Western blot using anti-CDKL5 antibodies (Fig. 2*A*, *lower panel*). As shown in the autoradiogram in Fig. 2*A*, a band corresponding to wild-type CDKL5 is present (*lane 2*); the absence of this signal in the reaction with FLAG alone (*lane 1*) shows that the signal is specific for CDKL5. Furthermore, the absence of phosphorylation of the two CDKL5 derivatives lacking catalytic activity allows us to conclude that no other kinase co-purifying with CDKL5 causes its post-translational modification. In fact, the lanes corresponding to ΔN and K42R are devoid of signals (*lanes 3* and *7*, respectively). The C152F mutation precedes the conserved DFG triplet involved in phosphoryl transfer and has accordingly been predicted to interfere with the catalytic activity of CDKL5 (4, 11, 13). In agreement with this hypothesis, we observe that the C152F derivative is completely devoid of autophosphorylation activity (*lane 5*). Conversely, the R175S mutant, which is located downstream of the dual phosphorylation site within the activation loop (4, 13), maintains the capacity of autophosphorylation even if it appears to have a reduced activity (*lane 6*), corresponding to ~55% of wild-type CDKL5 (Fig. 2*B*) (henceforth referred to as hypomorphic mutant). Deletion of the last 249 amino acids confers a higher autophosphorylation activity to CDKL5; in fact, the Δ781 derivative appears ~1.5-fold more active than the wild-type protein (Fig. 2*A*, *lane 4*, and Fig. 2*B*). Lin *et al.* (11) have recently proposed that the COOH terminus of CDKL5 has a negative regulatory function since a derivative of the kinase corresponding basically to the catalytic domain alone had higher autophosphorylation activity; our data suggest that the very COOH-terminal region of the protein contributes to this regulation.

CDKL5 Is Capable of Phosphorylating the TEY Motif in Its Activation Loop—CDKL5 contains the TEY sequence within its activation loop (residues 169–171); this motif has been extensively characterized in the extracellular signal-regulated kinases (ERKs), and its dual phosphorylation is required for activation of these protein kinases (14). This mechanism of activation is in most cases mediated by other upstream kinases; however, one member of the ERK family, ERK7, has been shown to be constitutively active due to its ability to autophosphorylate the TEY motif (15). CDKL5 has previously been reported to be phosphorylated on its TEY motif; in fact, the

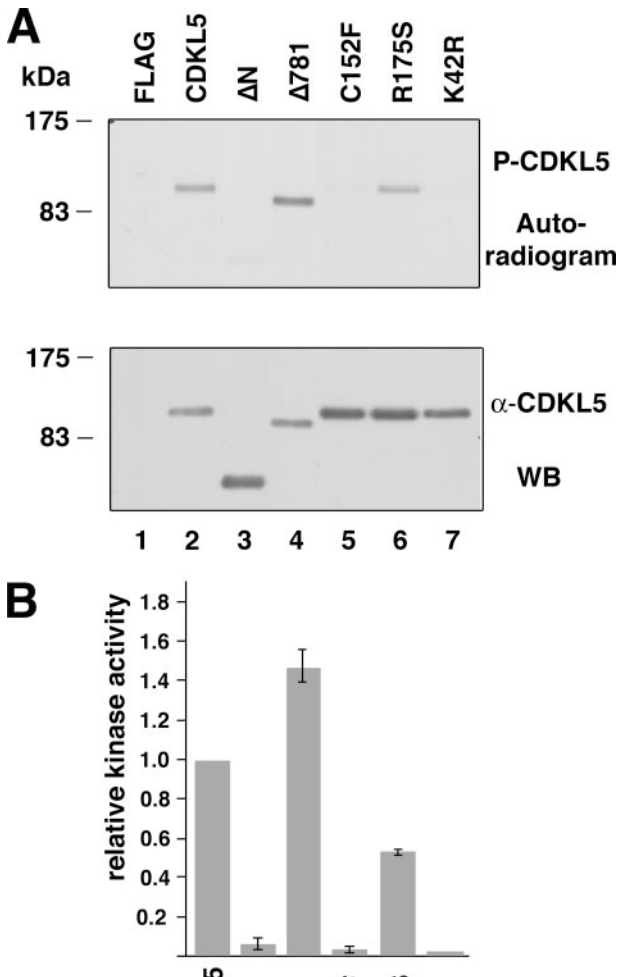

FIGURE 2. **Two disease-causing mutations within the catalytic domain interfere differently with CDKL5 autocatalytic activity.** *A*, autophosphorylation assay in which FLAG-CDKL5 and its mutated derivatives were immunopurified from overexpressing HEK293 cells and, after extensive washes, incubated with [γ-^{33}P]ATP for 30 min at 30 °C. Labeled proteins were divided in two fractions; 85% was separated by SDS-PAGE and visualized by autoradiography (*upper panel*), whereas the remaining 15% were subjected to Western blot analysis with anti-CDKL5 antibodies (*lower panel*). Molecular masses are indicated to the *left*. *B*, graphic illustration showing the quantitation of CDKL5 autophosphorylation activity from three independent experiments. The data show the ratio of the radioactive signal (quantified with a PhosphorImager) to the amount of immunoreactive proteins (estimated with a Kodak Image Station 2000R) for each CDKL5 derivative. CDKL5 autophosphorylation activity was arbitrarily set to 1.0 and the activity of its mutated derivatives expressed as values relative to this.

anti-phospho-MAPK antibody recognizes overexpressed CDKL5 immunopurified from mammalian cells (11). We wanted to investigate whether an altered autophosphorylation activity could affect the modification of the TEY motif. To this aim, we performed a Western blot analysis of the various FLAG-tagged immunopurified CDKL5 derivatives with the anti-phosphoMAPK antibody (Fig. 3*A*, *upper panel*). 15% of the immunoprecipitated material was analyzed with an anti-CDKL5 antibody to assay the amount of immunopurified CDKL5 (Fig. 3*A*, *lower panel*). As illustrated in Fig. 3*A*, wild-type CDKL5 is recognized in this assay, confirming that it is indeed phosphorylated on its TEY motif (*lane 2*). The CDKL5

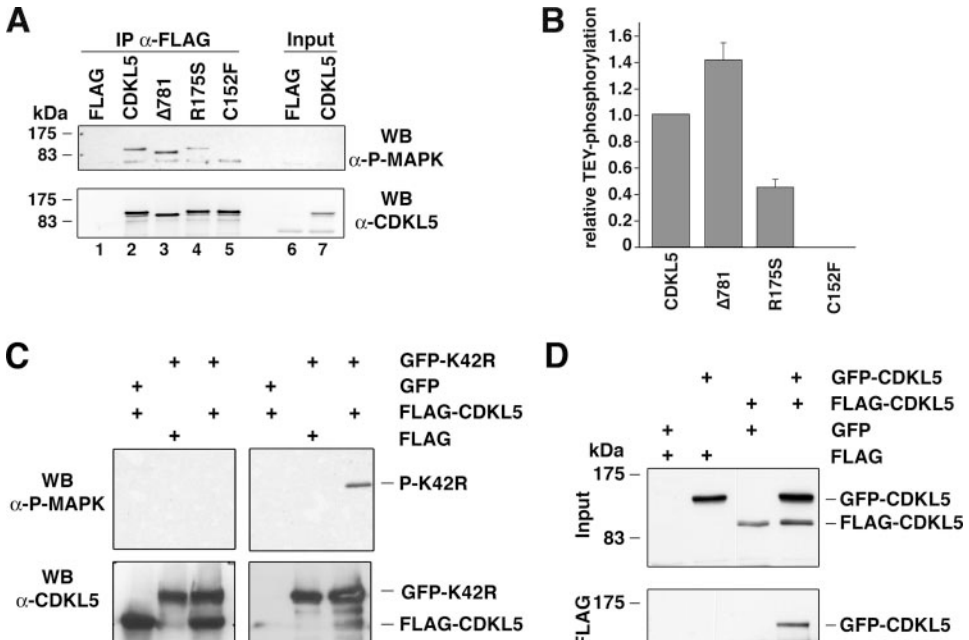

FIGURE 3. **Phosphorylation of the TEY motif correlates with the autocatalytic activity of CDKL5 and is mediated by intermolecular phosphoryation.** *A*, phosphorylation of the TEY motif was analyzed subjecting immunopurified FLAG-CDKL5 or its mutated derivatives to Western blot with anti-phospho-MAPK antibodies (*upper panel*). 15% of the immunoprecipitated material was analyzed by Western blot with anti-CDKL5 antibodies (*lower panel*). Molecular masses are indicated to the *left*. *B*, graphic illustration showing the mean relative TEY-phosphorylation from three individual experiments. The amounts of TEY-phosphorylation and immunopurified CDKL5 were quantified with a Kodak Image Station 2000R. TEY phosphorylation of wild-type CDKL5 was arbitrarily set to 1.0 and the degree of phosphorylation of its mutated derivatives expressed as values relative to this. *C*, autophosphorylation of the TEY motif was examined by coexpressing GFP-K42R or GFP with FLAG-CDKL5 or FLAG in HEK293 cells, immunopurifying the proteins with anti-GFP and anlyzing the precipitated material with anti-P-MAPK (*upper panel*) or anti-CDKL5 antibodies (*lower panel*). *Lanes 1–3* and *lanes 4–6* show the input and immunoprecipitate, respectively. *D*, CDKL5 autointeraction was analyzed by coimmunopurification of GFP-CDKL5 and FLAG-CDKL5 from overexpressing HEK293 cells. Overexpressed proteins were purified with an anti-FLAG resin and analyzed with anti-CDKL5. The *upper panel* shows the input whereas the precipitated material is shown in the *lower panel*. Molecular masses are indicated to the *left*.

derivative lacking the region spanning the 249 most COOH-terminal amino acids is also phosphorylated (*lane 3*), whereas the kinase-dead mutant, K42R, is not immunoreactive with the anti-phosphoMAPK antibody (data not shown). In accordance with the autophosphorylation data, we observed that while the C152F mutant was not recognized at all (*lane 5*), R175S was less reactive in the Western blot (*lane 4*) even if the analysis with the anti-CDKL5 antibody shows that comparable amounts of protein was immunopurified in all lanes. The graphic illustration in Fig. 3*B*, obtained normalizing the results of three independent experiments, confirms that indeed the Rett 175 missense mutation leads to a hypoactive CDKL5 protein, whereas the Δ781 truncation has exactly the opposite effect making the enzyme more effective. Moreover, these experiments show a correlation between the ability to autophosphorylate and to be phosphorylated on the TEY motif suggesting that CDKL5 is capable of autoactivating by modifying the dual phosphorylation site within the activation loop. These results, however, do not reveal if this is an intermolecular autophosphorylation or if it occurs in *cis*. We examined this point by investigating whether the kinase-dead mutant, K42R, might be TEY-phosphorylated when overexpressed in the presence of wild-type CDKL5. To this aim, K42R fused to the green fluorescent protein, GFP, was overexpressed together with FLAG-CDKL5 or, as a negative control, FLAG alone. The extracted proteins were immunopurified with anti-GFP and analyzed with anti-phosphoMAPK antibodies. Interestingly, as demonstrated in Fig. 3*C*, GFP-K42R was recognized by the anti-phophoMAPK antibody when it was immunopurified from cells overexpressing also the wild-type FLAG-tagged protein but not FLAG alone (*lanes 6* and *5*, respectively). The specificity was confirmed by the fact that no signal was present when only GFP was expressed with FLAG-CDKL5 (*lane 4*). This result allows us to establish that CDKL5 is indeed capable of phosphorylating its own TEY motif and the fact that the phosphorylation in this experiment occurs *in trans* implies that CDKL5 interacts with itself, either directly or indirectly. This point was addressed by assaying if GFP-CDKL5 coprecipitated with immunopurified FLAG-CDKL5. As demonstrated in Fig. 3*D*, GFP-CDKL5 was indeed present in the material that coimmunopurified with FLAG-CDKL5 (*lane 4*) confirming that CDKL5 is able to phosphorylate in *trans* because the molecules are contacting each other; whether CDKL5 is also able to *cis*-phosphorylate as suggested by Lin *et al.* (11) cannot be excluded from these data. Eventually, we observed that kinase dead CDKL5 molecules are able to interact suggesting that TEY phosphorylation is not required for this property; in fact, GFP-K42R and FLAG-K42R coprecipitate (data not shown).

MeCP2 Phosphorylation Is Impaired in Two Disease-causing Mutations within the Catalytic Domain—We have previously shown that immunopurified CDKL5 mediates the phosphorylation, directly or indirectly, of MeCP2 (8), and presently this remains the only heterologous substrate of CDKL5. We wanted to test the impact of the various mutations in CDKL5 on its capacity to mediate MeCP2 phosphorylation; in particular, we found it challenging to analyze if the hypomorphic R175S mutant might be defective in phosphorylating the methyl-binding protein. To this aim, recombinant purified MeCP2E2 was incubated with immunopurified FLAG-tagged CDKL5 or its mutated derivatives in the presence of [γ-^{33}P]ATP. Labeled proteins were separated by SDS-PAGE and visualized by autoradiography. As described above (Fig. 2), CDKL5 and MeCP2 amounts were detected by Western blot. As seen in the autoradiogram in Fig. 4, MeCP2 is readily phosphorylated in the presence of wild-type CDKL5 (*lane 4*), whereas no signal is present when the methyl-binding protein is incubated with the resin

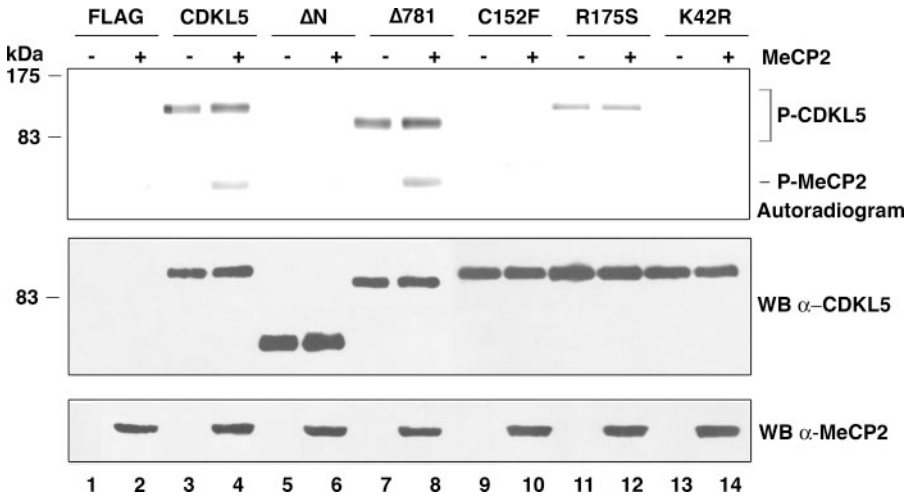

FIGURE 4. **Disease-causing mutations within the CDKL5 kinase domain interfere with MeCP2 phosphorylation.** Immobilized FLAG-CDKL5, or its mutated derivatives, was incubated with or without recombinant MeCP2 and [γ-^{33}P]ATP for 30 min at 30 °C. Labeled proteins were divided in two fractions; 85% were separated by SDS-PAGE and visualized by autoradiography (*upper panel*), while the remaining 15% was subjected to Western blot analysis with anti-CDKL5 (*middle panel*) or anti-MeCP2 antibodies (*lower panel*). The position of phosphorylated CDKL5 derivatives and MeCP2 in the *upper panel* is indicated to the *right*. Molecular mass markers are indicated to the *left*. Recombinant MeCP2 was added to *lanes 2*, *4*, *6*, *8*, *10*, *12*, and *14*.

containing only FLAG or the kinase dead controls, ΔN and K42R (*lanes 2*, *6*, and *14*, respectively). The Δ781 derivative has maintained its MeCP2 interaction domain, which is located between amino acids 450–550. Accordingly, as can be seen in Fig. 4, Δ781 is capable of mediating MeCP2 phosphorylation (*lane 8*). On the contrary, the two missense mutations in the catalytic domain, C152F and R175S, are impaired in phosphorylating MeCP2. This means that in our experimental conditions the R175S mutant is unable to mediate the modification of MeCP2, even if it is characterized by a low autocatalytic activity and TEY phosphorylation. As expected, the C152F mutant, devoid of autocatalytic activity, does not induce any modification of MeCP2.

The CDKL5 COOH-terminal Region Is Important for a Proper Subcellular Localization—The lowered catalytic activity of mutations within the NH_2-terminal kinase domain can provide an explanation for disease onset in patients. Conversely, the COOH-terminal truncation of CDKL5 represented by the Δ781 derivative, which is a Rett causing mutation mapping in the region often mutated in patients with mental retardation and infantile spasms, showed better performance in our autophosphorylation assays. Even though it is possible to speculate that a higher catalytic activity might be detrimental for brain function, we wanted to analyze if this region or the catalytic activity are important for other regulatory aspects of the protein such as its subcellular localization. To this aim, we compared the subcellular distribution of the various overexpressed CDKL5 derivatives fused to GFP in mouse fibroblasts, NIH3T3, and human HEK293 cells. Fig. 5*A* shows a typical localization pattern of each CDKL5 derivative in NIH3T3 cells (GFP, *upper row*, *A*–*G*) where the cell nucleus was identified with Hoechst staining (Hoechst, *middle row*, *A′*–*G′*). Wild-type CDKL5 was predominantly nuclear with a particular dotted staining (*A*; Ref. 11). Even if the nature of these dots remains unknown, it appears from the lack of overlap between GFP and Hoechst (merge, *lower row*, *A″*–*G″*) that they do not correspond to the intensely Hoechst-stained pericentric foci rich in methylated DNA and MeCP2 (16). We observed, however, that these nuclear dots appeared bigger in size when the protein was heavily overexpressed, and we have therefore paid attention on assaying cells expressing the exogenous proteins only at moderate levels. The same nuclear dotted staining was also observed with the Δ781 and R175S derivatives (*B* and *E*, respectively) and also in few cells expressing the C152F mutant (~25%; *D*). On the contrary the kinase-dead K42R mutant and in large part the Rett C152F derivative were characterized by the complete lack of nuclear dots; a similar result was obtained with the Δ525 mutant. Furthermore, we noticed that Δ525, C152F, and K42R had some cytoplasmic staining. There is a significant difference between the Δ781 and Δ525 derivatives; in fact, the Δ781 mutant behaves like the wild-type protein, whereas the Δ525 mutant has completely lost the dotted staining and localizes partially within the cytoplasm. This suggests that the COOH-terminal region plays an important role in localizing the kinase properly within the nuclear compartment, possibly by its interaction with other nuclear factors. It is worthwhile mentioning that identical results were obtained in human HEK293 cells, where the heterochromatic dots are not identifiable with Hoechst staining (data not shown).

Since our data suggest that both the catalytic activity of CDKL5 and residues comprised between 526 and 780 might regulate the localization of the kinase within the nucleus, we decided to investigate this point further and performed a Western blot analysis using fractionated cell extracts. We therefore compared the full-length CDKL5 derivatives as well as the COOH-terminal deletions, Δ781 and Δ525. As seen in Fig. 5*B*, wild-type CDKL5, Δ781, and R175S are found predominantly in the nuclear fraction (*lanes 3*, *6*, and *15*, respectively), whereas the Δ525 as well as the C152F and K42R mutants were present also in the cytoplasmic fractions (*lanes 8*, *11*, and *17*, respectively). A good correlation thus exists between the immunofluorescence and Western blot analysis and the obtained results indicate that the nucleocytoplasmic distribution of CDKL5 may indeed depend on the catalytic activity of the kinase and sequences within its COOH-terminal domain; we cannot exclude the opposite situation, however, that the nuclear translocation depends on a correct domain configuration and is important for CDKL5 catalytic activity.

DISCUSSION

The human *CDKL5* gene has been mapped to Xp22 through an exon trapping screening aimed at identifying novel genes associated with human disorders (1). Indeed, several recent publications reported the involvement of *CDKL5* in some neurodevelopmental disorders, and mutations in *CDKL5* have been isolated from patients affected by West Syndrome, the

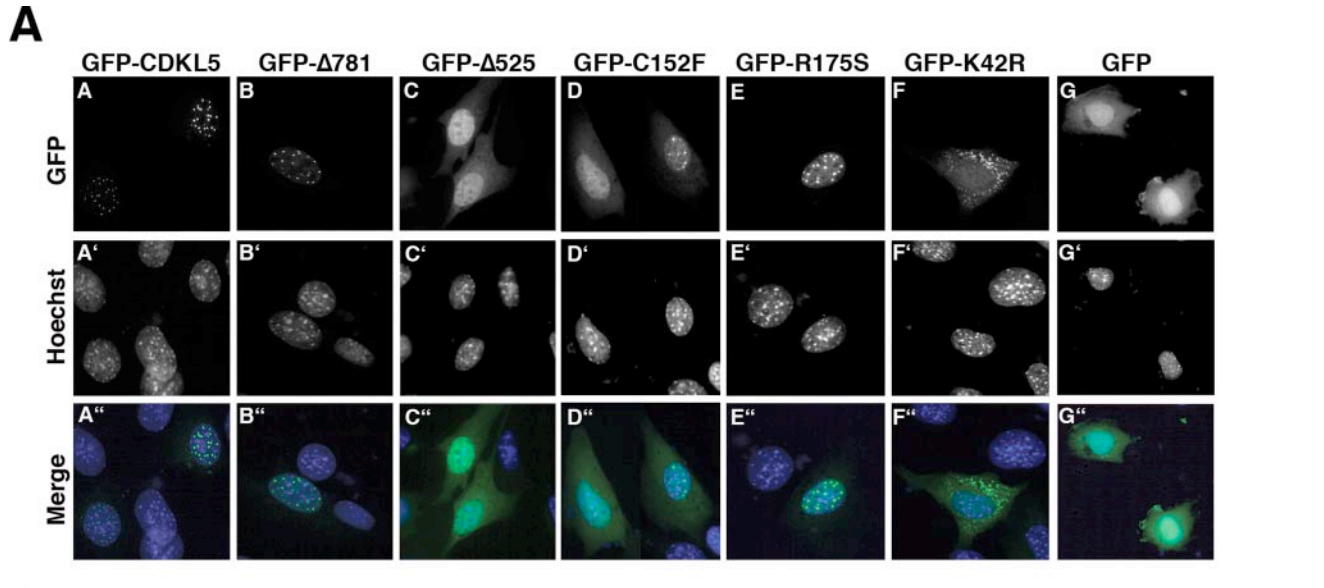

FIGURE 5. **CDKL5 subcellular localization is influenced by its catalytic activity and protein-protein interactions.** *A*, CDKL5 and its mutated derivatives were fused to GFP and transiently overexpressed in NIH3T3 cells. Fixed cells were stained with Hoechst and the subcellular localization of the exogenous protein analyzed. The *upper row* shows GFP-expressing cells (*A–G*), the *middle row* shows the Hoechst stained nuclei (*A′–G′*), and the *lower row* shows the merge with GFP in *green* and Hoechst in *blue* (*A″–G″*). *B*, Western blot of fractionated HEK293 cells overexpressing GFP-tagged CDKL5 or its mutated derivatives analyzed by anti-CDKL5. *T* = total extract; *n* = nuclear extract, *C* = cytoplasmic extract.

Hanefeld variant of Rett Syndrome, and Infantile Spasms (2–9). Integrating all the data, *CDKL5* seems to be an important cause in females of clinical manifestations characterized by severe mental retardation and seizures starting within the first 6 months of life and largely resistant to treatment. According to its involvement in brain function, CDKL5 is a ubiquitous protein particularly expressed in the brain (1, 11). Moreover, studies on *Cdkl5* activation during brain development have shown that its transcription is first observed in neural cells that have reached their final position in the cortical plate. In the first postnatal stages its expression correlates with neuronal maturation and synaptogenesis (8).

The analysis of the primary sequence of CDKL5 revealed homologies with the catalytic domain of different members of proline directed serine-threonine kinases (1). In fact, it has recently been demonstrated that CDKL5 owns a phosphorylating activity (8, 11). In analogy to all the other members of the family of ser/thr kinases, 12 conserved subdomains can be identified in the 284-amino acid kinase domain of CDKL5 (12). Interestingly, most of the pathogenic missense mutations identified so far hit the catalytic domain suggesting that the enzymatic activity is required for a normal neurodevelopment. In this paper we have compared the activity of wild-type CDKL5 to that of two point mutants causing a variant form of Rett. Cysteine 152, located immediately upstream the conserved DFG motif involved in phosphotransfer, has been substituted with phenylalanine (4, 12). Tao and colleagues (4) have recently observed that there are no other deposited sequences characterized by a phenylalanine residue in this position and proposed that this mutation might modify the proper orientation of the DFG signature important for a proper catalytic activity. In our assays of auto- and heterophosphorylation this Rett derivative does not show any catalytic activity (Figs. 2 and 4, respectively). Furthermore, the immunoprecipitated C152F mutant is not recognized by the antibody specific for the activated TEY (Fig. 3). Therefore, in these assays, this mutant behaves as the K42R that, by definition, is a kinase-dead protein. However, the comparison of the cellular distribution of this mutant with the "dead" derivative revealed some differences (Fig. 5). In fact, microscopy and biochemical assays show that wild-type CDKL5 is characterized by a nuclear dotted staining and remains almost undetectable in the cytoplasm. On the contrary, the kinase dead mutant discloses an almost equal distribution between the nucleus and the cytoplasm; concerning the subnuclear distribution, the exogenous protein has lost the punctuated pattern. Interestingly, the C152F mutant appears as an intermediate between the wild-type and the K42R mutant; in fact, whereas ~25% of the cells show a dotted nuclear staining, almost 75% present a diffuse distribution of the mutated CDKL5. Eventually, a weak signal is detectable in the cytoplasm. With this in mind, we suggest that a correct subcellular distribution of this kinase requires a threshold catalytic activity and that the C152F mutant retains some residual phosphorylating activity that, even if too weak to be detected in our enzymatic assays, is testified by its distribution in the cell. We have also analyzed the R175S Rett mutation that, considering

its autophsphorylation (Fig. 2) and TEY modification (Fig. 3), appears active but hypomorphic. In accordance with our model, its cellular distribution appears indistinguishable from the wild-type CDKL5 (Fig. 5) reinforcing the hypothesis that a certain catalytic activity is required for the nuclear localization. The mutated Arg-175 lies within a highly conserved sequence context important for the recognition of substrates containing a proline immediately after the Thr/Ser residue acceptor of the phosphate (17, 18). It has been hypothesized that the R175S mutation might change substrate specificity (4). Our data seem to confirm this; in fact, the R175S derivative is unable to mediate MeCP2 modification even though it is capable of interacting with the methyl-binding protein (Fig. 4 and data not shown). Although, so far, there are no proofs of the capability of CDKL5 to directly modify the methyl-binding protein, our results suggest that *in vitro* the immunopurified kinase is capable of doing so, therefore strengthening their belonging to the same molecular pathway (Fig. 4 and Ref. 8). Linking the obtained data to Rett syndrome, it appears that impairment of the kinase activity and/or of its substrate specificity might be pathogenic. Moreover, analyzing the phenotype of the patients described by Tao and colleagues (4) we can speculate that a correlation exists between the severity of the neurological disorder and the degree of the catalytic impairment. Eventually, we are aware that Lin *et al.* (11) have recently published that the two missense mutations lead to a completely inactive enzyme. However, the authors have analyzed the mutated derivatives in the context of a 352-residue peptide, missing all the amino acids COOH-terminal to the kinase domain. We believe that this, as well as a difference in the sensitivity of the assays, might justify the apparent contradictory results. Other two missense mutations have been described (Fig. 1). The first, I72N, causing Rett syndrome, consists in the substitution of an isoleucine residue with an asparagine; interestingly, this isoleucine is strictly maintained in CDKL5 and has a conservative substitution into leucine in other CDKL family members (7). The second mutation, responsible for severe mental retardation, marked hypotonia, and serious epileptic seizures, leads to the substitution of a highly conserved proline residue (P180L). In the future, it will be interesting to analyze the molecular consequences of these alterations.

As already mentioned, truncating mutations have also been reported (5–8). While it is easy to understand the pathogenic effects of mutations terminating the protein in the NH_2-terminal end, therefore abolishing the kinase activity, it is not known what the consequences are of later truncations that terminate the protein in the long, still uncharacterized COOH-terminal region. We have therefore decided to analyze a mutation, which causes the truncation of CDKL5 at R781 and causes the Hanefeld variant of Rett syndrome (8). It is worthwhile to note that this is one of the earliest COOH-terminal truncations reported so far. As for the other mutants, the Δ781 derivative has been challenged in auto- and heterophosphorylation assays (Figs. 2–4) and the obtained results suggest that the protein has a slightly augmented catalytic activity. Since the cellular distribution of this mutant resembles that of the wild-type protein, and we do not observe a reproducible higher expression (data not shown) we suggest that, in this case, the neurological disease might be explained by the hyperactivity of the kinase. A possible confirmation of this working hypothesis with future experiments might suggest that a tight regulation of CDKL5 levels and/or activity is essential for the proper function of the central nervous system therefore making the search for pathogenic gene duplications relevant. In line with this, it is worth mentioning that also MeCP2 protein levels need to be strictly regulated to maintain proper brain functions; in fact, male Rett patients with *MECP2* gene duplications have been described in accordance with the observation that too much MeCP2 protein causes Rett like phenotypes in mice (19). Moreover, the identification of the physiological targets of CDKL5 will permit us to disclose if a hypermorphic mutant is capable of modifying novel proteins or if it is responsible for an excess of activity on correct substrates. We want to mention that even if this is just a working hypothesis, it has already been published that a hyperactivity of protein kinases, such as CDK5, might lead to neuronal pathologies (20). However, we cannot exclude that the molecular cause of this pathogenic mutation resides in protein-protein interactions defects.

Albeit CDKL5 remains largely uncharacterized, our data together with two previous publications (8, 11) provide additional information about CDKL5 functional domains and regulation. In particular, we have demonstrated that CDKL5 is capable of associating with itself and that this interaction occurs independently of an active catalytic domain (Fig. 3 and data not shown). Conversely, we still do not know if this interaction is direct or mediated through a yet unknown factor. The activation loop of CDKL5 contains a TEY motif whose phosphorylation seems to be required for the kinase activity (Ref. 11 and this paper). Exogenously expressed CDKL5 leads to an intermolecular autophosphorylation of this activating signature (Fig. 3); to our knowledge, the capability of autoactivating the TEY motif has, so far, only been described for ERK7 (21).

CDKL5 is a rather big kinase due to a remarkably large COOH-terminal extension of almost 700 amino acids that harbors several functions. First, the tail seems to act as a negative regulator of the catalytic activity of CDKL5 (11), and our results suggest that this function is enclosed in the very last 240 residues (Figs. 2–4). Interestingly, also the long COOH-terminal extension of ERK5 stabilizes it in an inactive form and this occurs through the phosphorylation of a serine residue (22). Moreover, a region of CDKL5 encompassing residues 526–780 is necessary for the subnuclear localization. In fact, whereas the wild-type protein as well as the Δ781 derivative show a typical speckled staining, a further truncation at amino acid 525 leads to a diffuse staining (Fig. 5); we suggest that this mutant has lost a region important for protein-protein interactions. Importantly, analyzing the primary structure of the protein (Fig. 1*A*) it appears that the Δ525 mutants lacks three of the four Src homology 3-binding sites. Furthermore studies will define which and how many other regulatory functions are carried out by the COOH-terminal domain. Eventually, as already mentioned, regarding the CDKL5 subcellular localization our working model is that its entry into the nucleus depends on its kinase activity; however, in analogy with ERK7, its permanence in this cellular compartment depends on specific protein-protein interactions.

Acknowledgments—We sincerely thank Massimo Alessio, Mauro Mengoni, Alessandro Carnelli, and Elisa Binda for contributing to the experimental part of this research. Furthermore, we are grateful to Mario Costa for critical suggestions, and finally, we thank ProRET-Tricerca for their continuous and encouraging trust in the laboratory and for supporting Ilaria Bertani.

REFERENCES

1. Montini, E., Andolfi, G., Caruso, A., Buchner, G., Walpole, S. M., Mariani, M., Consalez, G., Trump, D., Ballabio, A., and Franco, B. (1998) *Genomics* **51,** 427–433
2. Kalscheuer, V. M., Tao, J., Donnelly, A., Hollway, G., Schwinger, E., Kubart, S., Menzel, C., Hoeltzenbein, M., Tommerup, N., Eyre, H., Harbord, M., Haan, E., Sutherland, G. R., Ropers, H. H., and Gécz, J. (2003) *Am. J. Hum. Genet.* **72,** 1401–1411
3. Huopaniemi, L., Tyynismaa, H., Rantala, A., Rosenberg, T., and Alitalo, T. (2000) *Hum. Mutat.* **16,** 307–314
4. Tao, J., Van Esch, H., Hagedorn-Greiwe, M., Hoffmann, K., Moser, B., Raynaud, M., Sperner, J., Fryns, J.-P., Schwinger, E., Gécz, J., Ropers, H.-H., and Kalscheuer, V. M. (2004) *Am. J. Hum. Genet.* **75,** 1149–1154
5. Weaving, L. S., Christodoulou, J., Williamson, S. L., Friend, K. L., McKenzie, O. L. D., Archer, H., Evans, J., Clarke, A., Pelka, G. J., Tam, P. P. L., Watson, C., Lahooti, H., Ellaway, C. J., Bennetts, B., Leonard, H., and Gécz, J. (2004) *Am. J. Hum. Genet.* **75,** 1079–1093
6. Scala, E., Ariani, F., Mari, F., Caselli, R., Pescucci, C., Longo, I., Meloni, I., Giachino, D., Bruttini, M., Hayek, G., Zappella, M., and Renieri, A. (2005) *J. Med. Genet.* **42,** 103–107
7. Evans, J. C., Archer, H. L., Colley, J. P., Ravn, K., Nielsen, J. B., Kerr, A., Williams, E., Christodoulou, J., Gecz, J., Jardine, P. E., Wright, M. J., Pilz, D. T., Lazarou, L., Cooper, D. N., Sampson, J. R., Butler, R., Whatley, S. D., and Clarke, A. J. (2005) *Eur. J. Hum. Genet.* **13,** 1113–1120
8. Mari, F., Azimonti, S., Bertani, I., Bolognese, F., Colombo, E., Caselli, R., Scala, E., Longo, I., Grosso, S., Pescucci, C., Ariani, F., Hayek, G., Balestri, P., Bergo, A., Badaracco, G., Zappella, M., Broccoli, V., Renieri, A., Kilstrup-Nielsen, C., and Landsberger, N. (2005) *Hum. Mol. Genet.* **14,** 1935–1946
9. Archer, H. L., Evans, J., Edwards, S., Colley, J., Newbury-Ecob, R., O'Callaghan, F., Huyton, M., O'Regan, M., Tolmie, J., Sampson, J., Clarke, A., and Osborne, J. (2006) *J. Med. Genet.* **43,** 729–734
10. Chen, W. G., Chang, Q., Lin, Y., Meissner, A., West, A. E., Griffith, E. C., Jaenisch, R., and Greenberg, M. E. (2003) *Science* **302,** 885–889
11. Lin, C., Franco, B., and Rosner, M. R. (2005) *Hum. Mol. Genet.* **14,** 3775–3786
12. Den Dunnen, J. T., and Antonarakis, S. E. (2000) *Hum. Mut.* **15,** 7–12
13. Hanks, S. K., and Hunter, T. (1995) *FASEB J.* **9,** 576–596
14. Pearson, G., Robinson, F., Gibson, T. B., Xu, B.-E., Karandikar, M., Berman, K., and Cobb, M. H. (2005) *Endocr. Rev.* **22,** 153–183
15. Abe, M. K., Kahle K. T., Saelzler, M. P. Orth, K., Dixon, J. E., and Rosner, M. R. (2001) *J. Biol. Chem.* **276,** 21272–21279
16. Nan, X., Campoy, F. J., and Bird, A. (1997) *Cell* **88,** 471–481
17. Russo, A. A., Jeffrey, P. D., and Pavletich, N. P. (1996) *Nat. Struct. Biol.* **3,** 696–700
18. Canagarajah, B. J., Khokhlatchev, A., Cobb, M. H., and Goldsmith, E. J. (1997) *Cell* **90,** 859–869
19. Van Esch, H., Bauters, M., Ignatius, J., Jansen, M., Raynaud, M., Hollanders, K., Lugtenberg, D., Nienvenu, T., Jensen, L. R., Gecz, J., Moraine, C., Marynen, P., Fryns, J. P., and Froyen, G. (2005) *Am. J. Hum. Genet.* **77,** 442–453
20. Ubeda, M., Kemp, D. M., and Habener, J. F. (2004) *Endocrinology* **145,** 3023–3031
21. Abe, M. K., Kuo, W. L., Hershenson, M. B., and Rosner, M. R. (1999) *Mol. Cell. Biol.* **19,** 1301–1312
22. Zheng, Q., Yin, G., Yan, C., Cavet, M., and Berk, B. C. (2004) *J. Biol. Chem.* **279,** 8787–8791