THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 51, Issue of December 18, pp. 34341–34348, 1998

Printed in U.S.A.

The Cellular Localization of the Murine Serine/Arginine-rich Protein Kinase CLK2 Is Regulated by Serine 141 Autophosphorylation*

(Received for publication, August 19, 1998)

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Pre-mRNA splicing is catalyzed by a multitude of proteins including serine/arginine-rich (SR) proteins, which are thought to play a crucial role in the formation of spliceosomes and in the regulation of alternative splicing. SR proteins are highly phosphorylated, and their kinases are believed to regulate the recruitment of SR proteins from nuclear storage compartments known as speckles. Recently, a family of autophosphorylating kinases termed CLK (CDC2/CDC28-like kinases) was shown to phosphorylate SR proteins and to influence alternative splicing in overexpression systems. Here we used endogenous CLK2 protein to demonstrate that it displays different biochemical characteristics compared with its overexpressed protein and that it is differentially phosphorylated *in vivo*. Furthermore, CLK2 changed its nuclear localization upon treatment with the kinase inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole. We have also identified a CLK2 autophosphorylation site, which is highly conserved among all CLK proteins, and we show by site-directed mutagenesis that its phosphorylation influences the subnuclear localization of CLK2. Our data suggest that CLK2 localization and possibly activity are influenced by a balance of CLK2 autophosphorylation and the regulation by CLK2 kinases and phosphatases.

Pre-mRNA splicing is catalyzed by a multimolecular complex, the spliceosome, whose major components are small nuclear ribonucleoproteins, heteronuclear RNA-associated proteins, and serine/arginine-rich (SR)[1] proteins. SR proteins are composed of at least one RNA recognition motif and an SR domain. SR protein-depleted extracts are splicing-deficient, although the addition of any SR protein will restore the catalytic activity (1–5). However, splice site choice is significantly influenced by the concentration of SR proteins in splicing assays using cellular extracts or overexpression systems (2, 6–8), suggesting that the control of the local SR protein concentration in the nucleoplasm regulates alternative splicing. SR proteins are enriched in nuclear structures called speckles, to which some (SC35 and SRp20) are targeted by means of the SR domain (9, 10). Speckles are sensitive to kinase and phosphatase inhibitors, and their size responds dynamically to changes in transcriptional activity (10–12). Furthermore, phosphorylation of splicing components was previously demonstrated to be crucial for the formation of spliceosomes, whereas subsequent dephosphorylation was necessary to complete catalysis (13, 14). Together, these results suggest that the reversible phosphorylation of speckle components, *i.e.* SR proteins, controls their release into the nucleoplasm, and the resulting changes in local SR protein concentrations then regulate alternative splicing (10).

A number of SR protein kinases have been described, including a U1 70-kDa associated kinase (15), SRPK1 (16), SRPK2 (17), lamin B-receptor kinase (18), and topoisomerase I (19). Recently, a member of a family of kinases termed CLK (CDC2-like kinases) or LAMMER kinases was shown to phosphorylate ASF/SF2 *in vitro* (20, 21), and subsequently, it was demonstrated that all CLK family members phosphorylate SR proteins *in vitro* and ASF/SF2 *in vivo* (22). CLK proteins contain an SR domain and autophosphorylate on tyrosine, serine, and threonine residues in overexpression systems and *in vitro* (22–25). When overexpressed, the catalytically inactive mutant kinases localize to nuclear speckles, whereas the wild-type enzymes distribute throughout the nucleus and cause speckles to dissolve (20). This has led to the current model that CLK family members may regulate SR protein activity and consequently splicing (10). Further support was provided in a recent report that demonstrated the influence of overexpressed CLK1/STY, human CLK2, and human CLK3 on the splicing pattern of an E1A minigene construct (26, 27). We have now investigated endogenous CLK2 in Friend murine erythroleukemic cells (F-MEL) and compared some of its properties with previous reports on the overexpressed CLK kinases. Here we demonstrate that endogenous CLK2 is submaximally phosphorylated under physiological conditions and that it is associated with other nuclear proteins. Furthermore, in contrast to the overexpressed protein, endogenous CLK2 displayed undetectable tyrosine phosphorylation *in vivo*. CLK2 phosphorylation levels were sensitive to okadaic acid and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) treatment, and our data suggest that CLK2 may be regulated by a number of kinases and phosphatases. Concomitant with decreased phosphorylation levels induced by DRB, we observed that CLK2 and SR proteins translocated from the nucleoplasm to enlarged speckles, and we show that DRB inhibits CLK2 autophosphorylation *in vivo*. All CLK protein family members contain a highly conserved serine residue within an alternatively spliced exon (22, 26, 28). We identified this residue as a CLK2 autophosphorylation site (Ser-141) using *in vitro* kinase assays and demonstrate by site-directed mutagenesis that it participates in the control of CLK2 subnuclear localization.

* This work was supported by a grant from the Schweizerischer Nationalfonds (to O. N.), the Max-Planck Society, and Sugen Inc. 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.

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[1] The abbreviations used are: SR, serine/arginine-rich; F-MEL, Friend murine erythroleukemic; DRB, 5,6-dichloro-1-β-ribofuranosylbenzimidazole; RIPA, radioimmune precipitation assay; GST, glutathione *S*-transferase; GFP, green fluorescent protein.

Together, our data suggest that the nuclear localization of CLK2 is regulated in a combined mechanism by an interplay of kinases and phosphatases in conjunction with CLK2 autophosphorylation. This process might thereby influence the interaction of CLK2 with its substrates, the SR proteins, leading to a change in alternative splicing.

EXPERIMENTAL PROCEDURES

Cell Culture—F-MEL cells were grown in suspension in RPMI 1640 medium, 10% fetal calf serum, and 2 mM glutamine. Cells were seeded at 2×10^5/ml (20 ml) and grown for 24 h before treatment and subsequent lysis. Okadaic acid (BIOMOL Research Laboratories Inc.) was dissolved in Me_2SO and used at various concentrations for 12–24 h as indicated. Sodium orthovanadate was used at 50 μM for 24 h or at 1 mM for 1 h. Cells were arrested at the G_2/M phase with 158 nM demecolcine (Sigma) for 9.5 h or were differentiated in 2% Me_2SO for 6 days (29). DRB (BIOMOL Research Laboratories Inc.) was used for 1–2 h (12). Actinomycin D (Sigma) was used at the indicated concentrations for 1–4 h. Quercetin (Sigma) was used at the indicated concentrations for 1–2 h.

Cell Lysis—Cells (1×10^7) were centrifuged at $800 \times g$ for 5 min and lysed on ice for 30 min in 200 μl of HNTG buffer and 1% Triton X-100 (22) or in 200 μl of RIPA lysis buffer (1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM sodium phosphate (pH 7.2), 2 mM EDTA, 50 mM NaF, 5 mM β-glycerophosphate, and freshly added 4 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, and 100 units/ml benzonase unless otherwise stated). Lysates were centrifuged at 4 °C in a microcentrifuge for 15 min or 20 min at $100{,}000 \times g$ in a Beckman TLA-100 centrifuge (HNTG buffer and RIPA lysis buffer without benzonase). The supernatants were diluted 4-fold in HNTG buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM EDTA, 20 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 100 mM NaF, 5 mM β-glycerol phosphate, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin) and 0.1% Triton X-100 (HNTG lysis) or in RIPA rescue buffer (10 mM sodium phosphate (pH 7.2), 1 mM NaF, 5 mM β-glycerophosphate, 20 mM NaCl, and freshly added 2 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 20 μg/ml aprotinin). Immunoprecipitations were carried out using anti-CLK2 antibodies (22) and protein A-Sepharose overnight at 4 °C. The beads were washed three times in HNTG buffer and 0.1% Triton X-100 and resuspended in 30 μl of 2× SDS sample buffer. The samples were analyzed using 10% SDS-polyacrylamide gel electrophoresis and Western blotting as described (22).

In Vitro Phosphorylation Assays—GST-CLK2 was expressed in yeast and purified using GSH-Sepharose (Amersham Pharmacia Biotech). CLK2 mutagenesis was carried out as described previously (22). A fragment corresponding to the alternatively spliced exon EB (26, 28) was polymerase chain reaction-amplified from CLK2, CLK2-S141A, or CLK2-S141E using the EB sense (CGC GAA TTC AGC AGC CGG AGA GCC AAG) and EB antisense (CGC GGA TCC ATA TCG CTC TTG TAG CCA) primers and cloned into a pGEX vector (Amersham Pharmacia Biotech). The resulting GST-EB-wt, GST-EB-S141A, and GST-EB-S141E fusion proteins were expressed in *Escherichia coli* and purified using GSH-Sepharose beads. 1 μg of fusion protein and 100 ng of GST-CLK2 were used in *in vitro* kinase assays as described (22).

Immunofluorescence—HEK 293 cells were grown on gelatin-coated coverslips and transfected using 100 ng of plasmid DNA encoding N-terminal green fluorescent protein-CLK2, CLK2KR, CLK2-S141A, or CLK2-S141E fusion constructs (enhanced GFP-C2, CLONTECH) as described (22). 24 h after transfection, the cells were incubated with 75 μM DRB for 1 h, washed once in cold phosphate-buffered saline, and fixed in 3.7% formaldehyde/phosphate-buffered saline for 15 min at room temperature. Cells were then treated with 0.5% Triton X-100/phosphate-buffered saline for 30 min on ice. Coverslips were mounted in Gelmount (Dianova). EGFP-ASF/SF2 and EGFP-SC35 constructs were used to generate stable HEK 293 cell lines. Cells were grown, fixed, and prepared for microscopy as described above. Immunofluorescence was performed using confocal scanning microscopy (Leica).

RESULTS

Endogenous Murine CLK2 Is Associated with Nuclear Structures, and Its Phosphorylation Level Is Influenced by Okadaic Acid—We (22) and others (20) have previously investigated CLK proteins in overexpression systems such as HEK 293 and COS-7 cell lines. There, the kinases were largely found in the soluble fraction of a Triton X-100-based cell lysis buffer, whereas the catalytically inactive mutant kinases were found almost exclusively in the pellet. Furthermore, the overexpressed CLK kinases were autophosphorylated on serine, threonine, and tyrosine residues (23–25, 30). To investigate the regulation of CLK2 under more physiological conditions, we chose F-MEL cells. These cells express sufficient amounts of endogenous CLK2 protein (22) to reevaluate and extend previous overexpression results.

To investigate the solubility of endogenous CLK2 protein, equal amounts of F-MEL cells were lysed in HNTG or RIPA buffer, and CLK2 was immunoprecipitated and analyzed by Western blotting using anti-CLK2 antibodies. As shown in Fig. 1*A*, a reduced solubility of endogenous CLK2 was observed in HNTG buffer compared with the harsher RIPA lysis buffer, and only trace amounts of CLK2 were detectable in the pellet fraction following RIPA lysis. Control experiments using recombinant GST-CLK2 revealed no differences in the efficacy of immunoprecipitations between both buffers (data not shown). This reduced solubility of endogenous CLK2 in a Triton X-100-based buffer contradicts the previously described solubility of overexpressed CLK2 or CLK1/STY (20, 22) and suggests that a significant amount of CLK2 is associated with other nuclear proteins that are not easily solubilized. Based on these initial experiments, RIPA lysis was employed, although we included benzonase in our standard lysis protocol as it facilitated sample handling.

It was recently proposed that CLK proteins could be targets of regulatory kinases influencing their catalytic activity, localization, or substrate affinity (26). To test this, we treated F-MEL cells with the serine/threonine phosphatase inhibitor okadaic acid and observed a dose-dependent increase in the molecular mass of immunoprecipitated CLK2 (Fig. 1*B*). As phosphorylation or dephosphorylation of proteins is often visualized by a change of apparent molecular mass following SDS-polyacrylamide gel electrophoresis, our results indicated that endogenous CLK2 was submaximally phosphorylated and could be a potential substrate of other kinases. Since okadaic acid can arrest cells at the G_2/M phase (31), we investigated cell cycle-dependent phosphorylation of CLK2 and compared CLK2 molecular mass shifts from okadaic acid-, sodium orthovanadate-, okadaic acid/sodium orthovanadate-treated and cell cycle-arrested cells. Cells were arrested at the G_2/M phase using demecolcine or were arrested in G_0 following differentiation for 6 days in 2% Me_2SO (29). As shown in Fig. 1*C*, a clear increase in molecular mass was observed following okadaic acid treatment, but neither differentiated nor G_2/M phase arrested cells showed any visible change in CLK2 mobility. It is noteworthy that the amount of protein was drastically reduced in okadaic acid-treated or differentiated cells and slightly reduced in demecolcine-arrested cells. This suggests that CLK2 is engaged in actively proliferating but not quiescent cells and that CLK2 is not specifically activated during G_2/M phase.

Endogenous CLK2 Is Not Tyrosine-phosphorylated—Overexpressed CLK2 autophosphorylates on tyrosine residues in sodium orthovanadate-treated cells (22). Furthermore, when CLK2 is expressed in yeast and purified, it autophosphorylates on serine, threonine, and tyrosine residues *in vitro* (data not shown). These observations suggested that several CLK2 characteristics resemble the properties described for CLK1/STY (23–25, 30). We therefore assumed that endogenous CLK2 tyrosine phosphorylation could serve as a marker for autophosphorylation. However, when F-MEL cells were incubated with okadaic acid, sodium orthovanadate, or both, no CLK2 tyrosine phosphorylation signal was observed in Western blot experiments using anti-phosphotyrosine antibodies, even after pro-

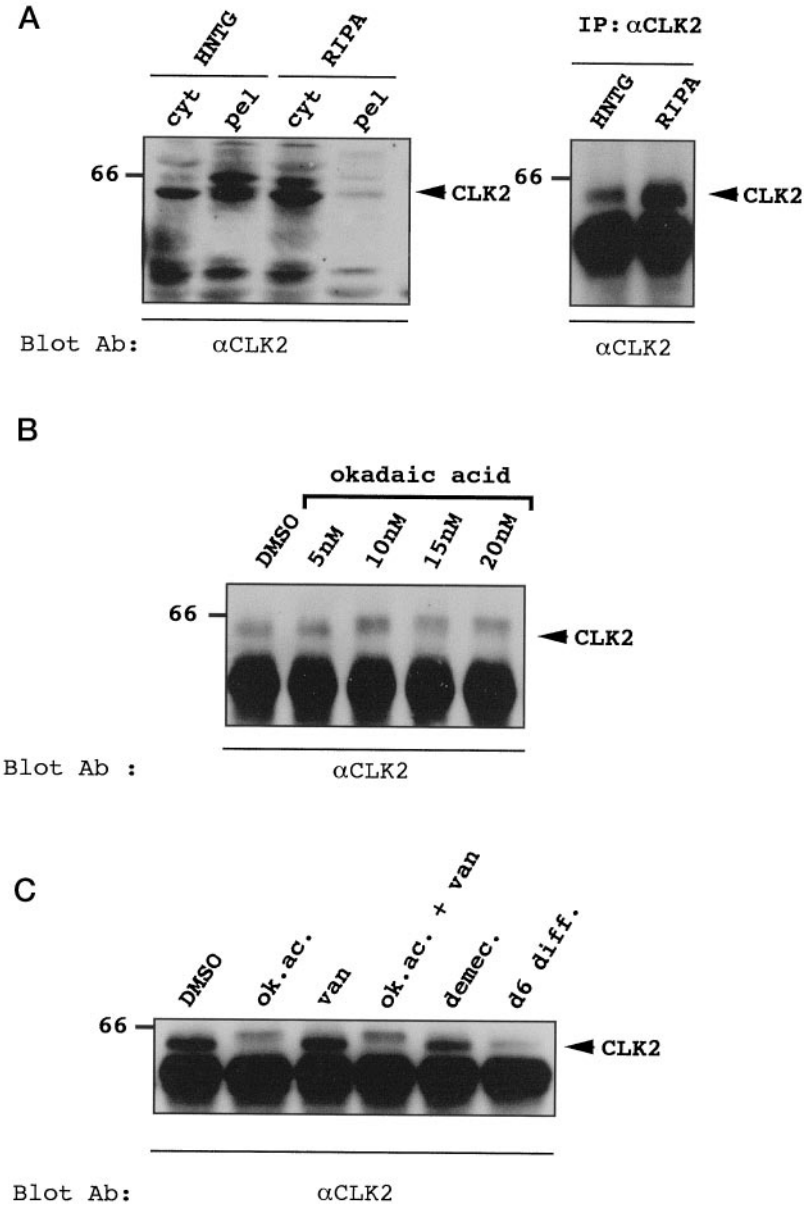

FIG. 1. **Solubility and okadaic acid-induced hyperphosphorylation of endogenous CLK2.** *A*, F-MEL cells were lysed in HNTG or RIPA buffer (no benzonase), and equal volumes of supernatant (*cyt*) and re-solubilized pellet (*pel*) fractions were loaded and analyzed by Western blotting using anti-CLK2 antibodies (*Ab*) (*left panel*). CLK2 was immunoprecipitated (*IP*) with anti-CLK2 antibodies from the remaining supernatant fractions and analyzed by Western blotting using anti-CLK2 antibodies (*right panel*). *B*, F-MEL cells were incubated with increasing amounts of okadaic acid for 12 h and lysed in RIPA buffer containing benzonase. CLK2 was immunoprecipitated with anti-CLK2 antibodies and analyzed by Western blotting using anti-CLK2 antibodies. *C*, F-MEL cells were incubated with 20 nM okadaic acid (*ok.ac.*), 50 μM sodium orthovanadate (*van*), or both for 24 h. Dimethyl sulfoxide (*DMSO*; 0.02%) was used as a control. G_2/M phase arrested cells were incubated with demecolcine (*demec.*), and G_0 phase arrested cells were differentiated in 2% Me_2SO for 6 days (*d6 diff.*). Cells were lysed in RIPA buffer containing benzonase, and CLK2 was immunoprecipitated with anti-CLK2 antibodies. CLK2 protein was analyzed by Western blotting using anti-CLK2 antibodies. The position of CLK2 in untreated cells is indicated by *arrowheads*. The molecular mass marker is indicated in kilodaltons.

longed ECL exposure (Fig. 2*A*). Furthermore, using *in vivo* labeling experiments with [^{32}P]orthophosphate and phosphoamino acid analysis of immunoprecipitated CLK2, we could not detect an increased tyrosine phosphorylation signal following sodium orthovanadate treatment (data not shown). To investigate whether CLK2 had lost its capability of tyrosine autophosphorylation, CLK2 was immunoprecipitated from F-MEL cells, and *in vitro* kinase assays were performed in the presence or absence of ATP followed by Western blot analysis. As shown in Fig. 2*B*, CLK2 was considerably shifted in molecular mass and was heavily tyrosine-phosphorylated in the presence of ATP. The tyrosine phosphorylation signal was evident after a few seconds of exposure despite the low amounts of CLK2 used in this experiment. This indicates that CLK2 may not be tyrosine-phosphorylated *in vivo,* although its autophosphorylation capability is not impaired. Furthermore, the strong shift in molecular mass seen after an *in vitro* phosphorylation of immunoprecipitated CLK2 supports our view that endogenous CLK2 is hypophosphorylated *in vivo.*

CLK2 Autophosphorylation Is Inhibited by DRB in Vivo—To assess the existence of CLK2 kinases, we incubated F-MEL cells with a variety of known kinase inhibitors and examined the effects on the molecular mass of immunoprecipitated CLK2. The kinase inhibitor DRB, which was previously shown to inhibit casein kinase II (32), tightened the CLK2 signal and shifted it to a lower molecular mass in a dose-dependent manner (Fig. 3*A*), corresponding to a decrease in the CLK2 phosphorylation level. Since DRB also inhibits transcriptional activity (11), we tested a different transcription inhibitor, actinomycin D, at various concentrations. This treatment did not lead to an equivalent shift in the molecular mass of CLK2 (Fig. 3*A*), suggesting that the observed effects of DRB were directly linked to a kinase inhibition. We next addressed whether the DRB-inhibited kinase was responsible for the hyperphosphorylation of CLK2 alone and incubated cells with okadaic acid in the presence of DRB. However, the okadaic acid-induced shift in the molecular mass of CLK2 was not fully inhibited by DRB (Fig. 3*B*), indicating that other CLK2 kinases or CLK2 autophosphorylation was still active. The inhibition of CLK2 phosphorylation by DRB was fully reversible, as CLK2 regained its normal phosphorylation status within 1.5 h after washing out the inhibitor (data not shown).

To examine whether the DRB-induced decrease in phosphorylation was due to the inhibition of CLK2 autophosphoryla-

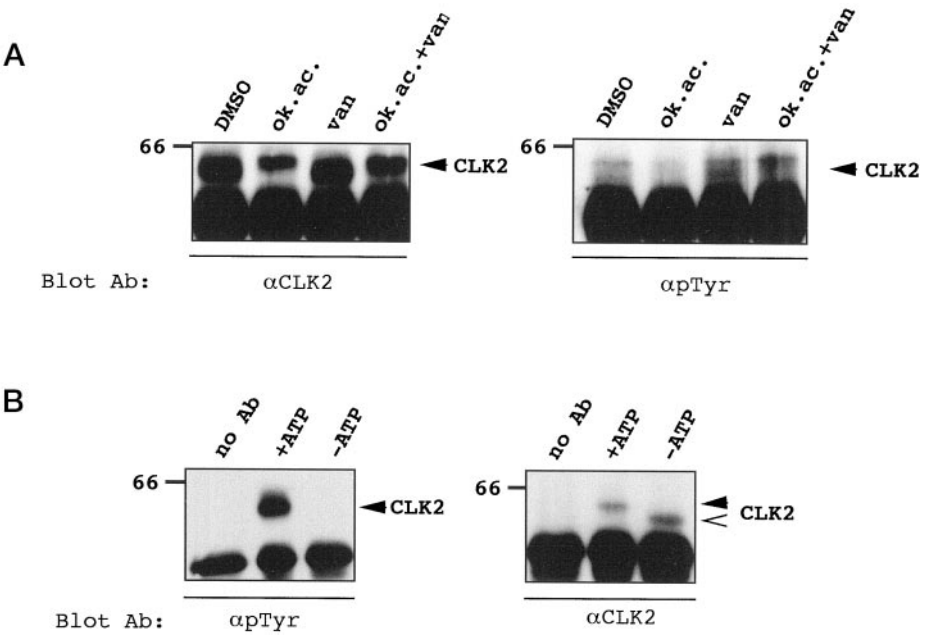

FIG. 2. **Endogenous CLK2 is tyrosine-phosphorylated *in vitro*, but not *in vivo*.** *A*, F-MEL cells were incubated with 20 nM okadaic acid (*ok.ac.*), 50 μM sodium orthovanadate (*van*), or both for 24 h. CLK2 was immunoprecipitated from benzonase-containing RIPA buffer lysates, analyzed by Western blotting using anti-phosphotyrosine (*αpTyr*) antibodies (*Ab*) (*right panel*), and reprobed with anti-CLK2 antibodies (*left panel*). *B*, F-MEL cells were lysed in RIPA buffer containing benzonase, and CLK2 was immunoprecipitated with anti-CLK2 antibodies. The immunoprecipitate was divided in half and used for *in vitro* kinase assays in the presence or absence of ATP (as indicated). As a control, an immunoprecipitation was carried out without antibodies (*no Ab*). Anti-CLK2 immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting using anti-phosphotyrosine antibodies (*left panel*) and reprobed with anti-CLK2 antibodies (*right panel*). The position of CLK2 is indicated by *arrowheads*. The molecular mass marker is indicated in kilodaltons. *DMSO*, dimethyl sulfoxide.

tion, we performed *in vivo* assays using overexpressed GFP-CLK2 in HEK 293 cells. As it was previously shown that overexpressed CLK2 autophosphorylates on tyrosine residues in the presence of orthovanadate (22), we treated the cells with DRB, orthovanadate, or both. Following immunoprecipitation of CLK2, we analyzed tyrosine phosphorylation using Western blotting and anti-phosphotyrosine antibodies. Fig. 3*C* shows that the tyrosine-autophosphorylating activity of CLK2 was significantly reduced by DRB. Furthermore, the tyrosine phosphorylation signal was completely abolished when the cells were pretreated with DRB for 1 h and then incubated with orthovanadate/DRB as described above (data not shown). In addition, GST-CLK2 was also inhibited by 75 μM DRB (data not shown) using previously described *in vitro* assay conditions (32). These results suggest that DRB directly inhibits the autophosphorylation activity of CLK2 *in vivo*, thereby inducing the observed reduction in the molecular mass of the CLK2 protein. Furthermore, since DRB only partially reduced the observed increase in molecular mass after okadaic acid treatment (see above), we assume that other CLK2 kinases remained active.

The Alternatively Spliced Exon EB Contains a Crucial CLK2 Autophosphorylation Site—Recent reports suggested that CLK proteins are regulated by phosphorylation and alternative splicing, and it emerged that all CLK family members contain a conserved, alternatively spliced exon previously termed exon EB (30). Omission of exon EB creates a frameshift and causes the expression of truncated, catalytically inactive proteins (28). Furthermore, it was recently shown that exon EB splicing of the *clk1/sty* gene is autoregulated by CLK1 (26). Interestingly, all CLK family members contain a highly conserved serine residue in exon EB (22, 28). As the inclusion of a phosphorylation site, as a consequence of an alternate splice, is frequently reflected in a change of substrate specificity (33) or oncogenic potential (34), we examined the role of this conserved serine residue in CLK2. First, we mutated serine 141 in CLK2 to alanine (S141A) or glutamate (S141E) and created GST-EB domain fusion proteins, which we used in *in vitro* kinase assays with GST-CLK2. In these experiments, strong phosphorylation of GST-EB-wt, but not of GST-EB-S141A or GST-EB-S141E (Fig. 3*D*), was observed, which suggests that Ser-141 of CLK2 is an autophosphorylation site.

DRB Induces a Subnuclear Translocation of CLK2 and SR Proteins—DRB inhibits transcription (32), and several reports have shown the effects of DRB on the subnuclear localization of SR proteins using immunofluorescence microscopy (11, 12, 35). However, the molecular mechanisms underlying the DRB-induced effects are still unclear. As DRB is a known kinase inhibitor (32), these effects could be attributed to a change in the phosphorylation levels of SR proteins, in agreement with previously shown effects of other kinase and phosphatase inhibitors on SR protein localization (10).

CLK2 phosphorylates SR proteins (22), and our observations that DRB inhibited CLK2 activity suggested that CLK2 inhibition might be partially involved in the DRB-induced enlargement of speckles. To address this, we created stable HEK 293 cell lines expressing GFP-ASF/SF2 or GFP-SC35 fusion proteins and investigated the subnuclear localization of these splice factors. GFP-ASF was recently shown to correspond to its wild-type protein both functionally and in its subnuclear localization (10). In contrast to the ubiquitously expressed GFP (Fig. 4*A*, *panels a* and *b*), GFP-tagged SR proteins were clearly nuclear and were partially localized in speckles (*panels c* and *e*). Upon DRB treatment, GFP-ASF/SF2 (*panel d*) and GFP-SC35 (*panel f*) accumulated in large aggregates, which is in agreement with a previous report using immunofluorescence (11). We next applied the same approach to GFP-tagged CLK2 and its catalytically inactive mutant, CLK2KR (22). In transient transfection assays, we observed that GFP-CLK2 was predominantly nucleoplasmic, although small speckled structures were occasionally observed (Fig. 4*B*, *panel a*). In contrast, GFP-CLK2KR was clearly concentrated in speckles (*panel c*) and co-localized with SR proteins (data not shown). When the cells were treated with DRB, we observed a translocation of GFP-CLK2 into aggregates, concomitant with a reduction in nucleoplasmic staining (*panel b*), whereas little change was seen for GFP-CLK2KR. Based on our previous observations (see above), we suspect that the DRB-induced translocation of GFP-CLK2 is caused by a reduction of the CLK2 phosphorylation level due to the inhibition of CLK2 autophosphorylation. Furthermore, as our *in vitro* kinase experiments identified Ser-141 as a CLK2 autophosphorylation site, we examined the effects of the S141A and S141E mutations on the nuclear localization of GFP-CLK2. Interestingly, we observed an increased speckled appearance for GFP-CLK2-S141A, which was exacerbated upon DRB treatment (*panels e* and *f*), whereas

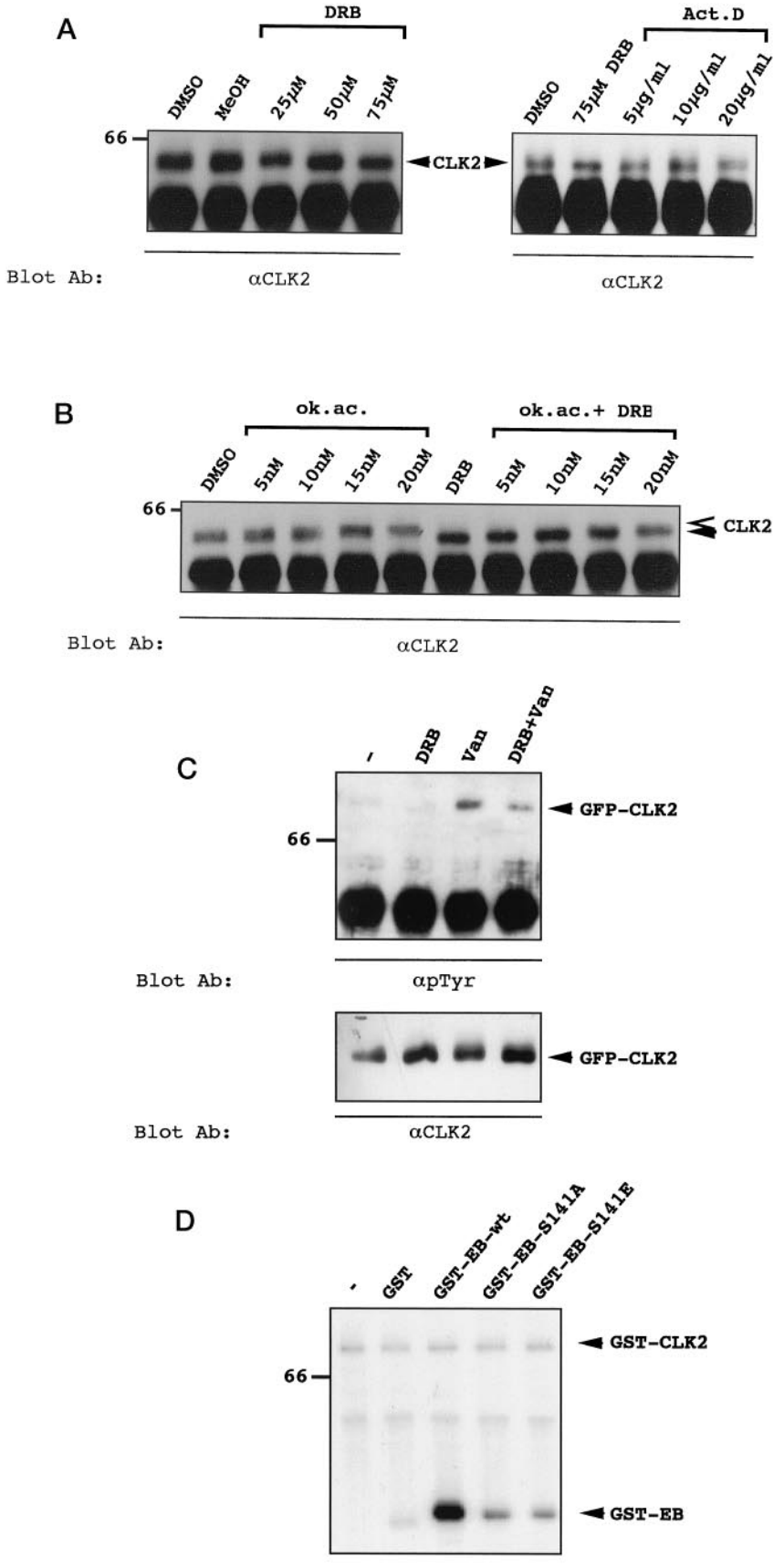

FIG. 3. **CLK2 phosphorylation levels are reduced by DRB.** *A*, F-MEL cells were incubated in the presence of DRB or actinomycin D (*Act.D*) for 1 h and lysed in RIPA buffer containing benzonase. Dimethyl sulfoxide (*DMSO*; 0.02%) or 0.5% MeOH was used as a control. Anti-CLK2 immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting using anti-CLK2 antibodies (*Ab*). *B*, DRB only partially inhibits the okadaic acid-induced CLK2 hyperphosphorylation. F-MEL cells were incubated with okadaic acid (*ok.ac.*) alone or with 75 μM DRB for 12 h. Cells were lysed in RIPA buffer containing benzonase, and CLK2 was immunoprecipitated using anti-CLK2 antibodies. Anti-CLK2 immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting using anti-CLK2 antibodies. CLK2 is marked with an *open arrowhead* (maximally hyperphosphorylated form) or with a *closed arrowhead* (after partial inhibition by DRB). *C*, DRB inhibits CLK2 autophosphorylation *in vivo*. GFP-CLK2 constructs were transiently transfected in HEK 293 cells. Cells were stimulated with Me_2SO (−), 75 μM DRB, 1 mM sodium orthovanadate (*Van*), or 75 μM DRB and 1 mM sodium orthovanadate (*DRB+Van*) for 1 h. Cells were lysed in RIPA buffer containing benzonase, and CLK2 was immunoprecipitated using anti-CLK2 antibodies. Immunoprecipitates were analyzed by Western blotting using anti-phosphotyrosine (*αpTyr*)antibodies (*upper panel*) and reprobed with anti-CLK2 antibodies (*lower panel*). *D*, CLK2 phosphorylates serine 141 *in vitro*. Purified GST-CLK2 (100 ng) was incubated with 1 μg of GST, GST-EB-wt, GST-EB-S141A, or GST-EB-S141E in an *in vitro* kinase assay. The proteins were separated by SDS-polyacrylamide gel electrophoresis, blotted on nitrocellulose, and analyzed by autoradiography. The position of CLK2 is indicated by an *arrowhead*. The molecular mass marker is indicated in kilodaltons.

GFP-CLK2-S141E, in which the glutamate residue mimics the charge of the phosphorylated serine residue, remained predominantly nucleoplasmic and largely resisted DRB treatment (*panels g* and h). Since both GFP-CLK2-S141A and GFP-CLK2-S141E were tyrosine-phosphorylated upon orthovanadate treatment (data not shown), we conclude that the autophosphorylation activities of both mutants were unaffected by the introduced mutations.

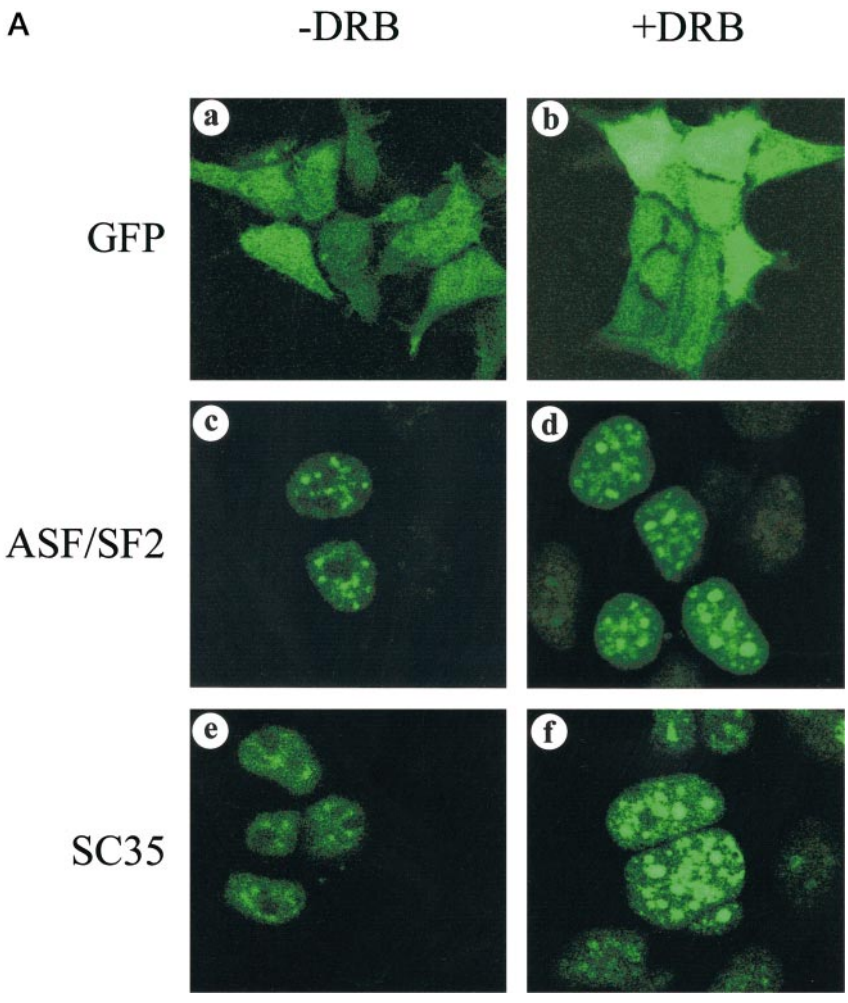

FIG. 4. **DRB induces the redistribution of SR proteins and CLK2.** *A*, HEK 293 cells expressing GFP, GFP-ASF, or GFP-SC35 were incubated with 75 μM DRB for 1 h (*panels b*, *d*, and *f*) or were left untreated (*panels a*, *c*, and *e*). *B*, HEK 293 cells were transiently transfected with 100 ng of GFP-CLK2, GFP-CLK2KR, GFP-CLK2-S141A (*CLK2SA*), or GFP-CLK2-S141E (*CLK2SE*) construct. Cells were incubated for 1 h with 75 μM DRB 24 h after transfection (*panels b*, *d*, *f*, and *h*) or were left untreated (*panels a*, *c*, *e*, and *g*).

Together, these data indicate that the intranuclear distribution of CLK2 is regulated by the CLK2 phosphorylation status and that phosphorylation of Ser-141 within the alternatively spliced exon EB is an important step to release CLK2 from speckles. Furthermore, it is likely that the inhibition of several CLK family members by DRB contributes to the observed accumulation of SR and CLK proteins in enlarged speckles.

DISCUSSION

Despite numerous examples of alternative splicing as a means to control gene expression (36), the mechanisms that govern alternative splice site selection are still poorly understood. SR proteins have been identified as *trans*-acting factors that play an important role in splice site selection. They influence alternative splicing by binding to splicing enhancers and by mediating protein-protein interactions (21, 37). It is currently believed that SR proteins are stored in nuclear structures called speckles, and their nucleoplasmic concentration is thought to be regulated by kinases, which mobilize them by phosphorylating the SR domain (10). SR proteins are phosphorylated by several different kinases, including an uncloned U1 70-kDa associated kinase (15), SRPK1 (16), SRPK2 (17), Lamin B receptor kinase (18), topoisomerase I (19), and a family of kinases named CLK or LAMMER kinases (20, 22–24, 38, 39). All these kinases could act in concert to control SR protein activity. However, little is known about signaling pathways leading to the modulation of SR protein kinase activity.

We have investigated endogenous CLK2 and found a reduced solubility in a Triton X-100-based lysis buffer, which suggests that CLK2 may be associated with other nuclear proteins. This result is further supported by a recent report demonstrating that CLK2 associates with nuclear scaffold attachment factor B (40). We also observed changes in endogenous CLK2 phosphorylation levels using kinase and phosphatase inhibitors, suggesting that CLK2 may be a substrate for other kinases and phosphatases. Overexpressed CLK proteins autophosphorylate on tyrosine, serine, and threonine residues in sodium orthovanadate-treated cells and were hence considered dual specificity kinases (23–25). To our surprise, we were not able to detect any endogenous tyrosine phosphorylation of CLK2 in F-MEL cells even after prolonged orthovanadate treatment or pervanadate stimulation (data not shown), although immunopurified CLK2 was still capable of tyrosine autophosphorylation *in vitro* and was considerably shifted in molecular mass. These results imply that endogenous CLK2 is not fully autophosphorylated and question whether tyrosine autophosphorylation is physiologically relevant. However, the lack of detectable tyrosine phosphorylation could be equally explained by steric inhibition or a very tightly associated tyrosine phosphatase, which could be released upon immunopurification of CLK2. It would be interesting to know whether other endogenous CLK members also lack tyrosine phosphorylation *in vivo*.

The kinase inhibitor DRB induced a tightening of the CLK2 band and a decrease in molecular mass *in vivo*. DRB was previously reported to be a specific casein kinase II inhibitor (32), although a more recent report also found inhibition of a transcription factor IIH-associated kinase (41). We have tested the effects of DRB on the catalytic activity of CLK2 *in vivo* and found a significant inhibition of tyrosine autophosphorylation.

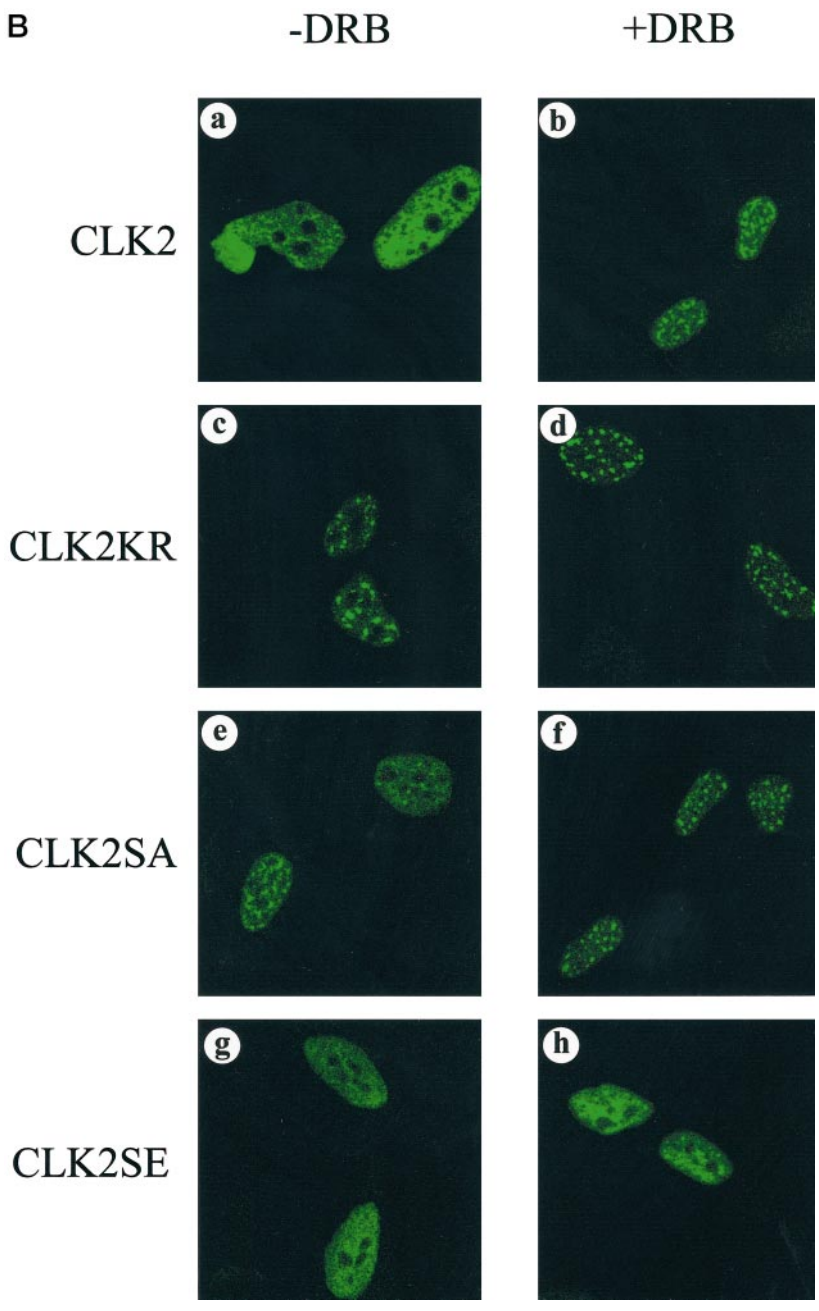

FIG. 4—*continued*

However, as DRB only partially inhibited the okadaic acid-induced increase in CLK2 molecular mass, we investigated other potential CLK2 kinases and found that protein kinase C and casein kinase II, but not protein kinase A or CDC2, which all contain consensus phosphorylation sites in the CLK2 N-terminal domain, phosphorylated CLK2 *in vitro* (data not shown). Currently, we have no evidence supporting a cell cycle-regulated phosphorylation of CLK2, and we believe, in accordance with a model proposed for CLK1/STY (21), that SRPK1 and SRPK2 (16, 17) could be responsible for the hyperphosphorylation of SR proteins during mitosis, whereas CLK2 could participate in the regulation of SR protein activity during interphase.

CLK proteins were previously shown to change their nuclear localization depending on their catalytic activity. Catalytically inactive mutants localized to nuclear speckles, whereas the catalytically active proteins dissolved speckles and were distributed throughout the nucleoplasm (20, 22). These overexpression results show that the control of CLK2 phosphorylation can influence its localization, although the precise nature of the phosphorylation sites and the kinases involved are currently unknown. We have shown here that DRB inhibits CLK2 phosphorylation *in vivo* and that this induces a change in CLK2 subnuclear localization. We have also mapped a CLK2 autophosphorylation site and show by site-directed mutagenesis that the highly conserved serine 141 plays a critical but possibly not exclusive role in this process. Interestingly, the subnuclear localization of GFP-CLK2-S141A resembled the observed localization of CLK1 in which the entire alternatively spliced EB domain was deleted (26). Since Ser-141 is located within the EB domain of CLK2, this suggests that the lack of this phosphorylation site alone can mimic the effect of a missing EB domain.

DRB was previously shown to inhibit SR protein mobilization from speckles to sites of viral transcriptional activity (12), to increase the size of SR protein aggregates (11), and to affect the splicing pattern of adenovirus 2 transcripts (42). Our experiments suggest that the catalytic inhibition of CLK2 and

possibly other CLK family members by DRB can lead to an accumulation of SR and CLK proteins in enlarged speckles. This is further supported by the fact that the dose response of the DRB-induced re-localization of speckle components correlated with CLK2 inhibition (Fig. 3*A* and data not shown). However, other mechanisms such as transcriptional inhibition alone can also induce SR protein aggregation (11), but possibly in a CLK-independent way, as we did not observe CLK2 re-localization upon actinomycin D treatment (data not shown).

Based on our fluorescence data and biochemical studies, we suggest that CLK2 autophosphorylation and phosphorylation by other kinases could control CLK2 localization and that this possibly affects its substrate interaction properties. Our experiments highlight some important biochemical differences between overexpressed and endogenous CLK proteins, and this will provide an important basis to identify CLK2 kinases. This will help to reach an understanding of the regulation of alternative pre-mRNA splicing through signal transduction pathways.

Acknowledgment—We thank M. Parry for reading the manuscript and helpful discussions.

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