Phosphoregulation of the RNA-binding Protein Hu Antigen R (HuR) by Cdk5 Affects Centrosome Function*§

Received for publication, February 23, 2012, and in revised form, July 17, 2012. Published, JBC Papers in Press, July 24, 2012, DOI 10.1074/jbc.M112.353912

Natalia Filippova†, Xiuhua Yang‡, Peter King§, and L. Burt Nabors††

From the †Department of Neurology, University of Alabama at Birmingham, Birmingham, Alabama 35294 and the ‡Veterans Affairs Medical Center, Birmingham, Alabama 35233

Background: Regulation of protein function by phosphorylation is an important mechanism to control many cellular processes.

Results: We found that the mRNA-binding protein HuR is phosphorylated by Cdk5 at the serine 202 residue.

Conclusion: The aberrant phosphorylation of HuR at Ser-202 affects centrosome function and induces arrest of cell cycle progression.

Significance: This work emphasizes HuR phosphorylation as a novel molecular target in cancer.

Hu antigen R (HuR) is an mRNA-binding protein belonging to the ELAV family. It is highly expressed in cancer and involved in cell survival and proliferation. The impact of post-translational regulation of HuR and resulting cellular effects are poorly understood. In the current report, we describe a direct interaction between HuR and Cdk5 in glioma. We determined that Cdk5 specifically phosphorylates HuR at the serine 202 residue in the unique hinge region. The molecular consequences of this interaction are an altered HuR ability to bind, stabilize, and promote translation of mRNAs. At the cellular level, the anomalous HuR phosphorylation at this site evokes robust defects in centrosome duplication and cohesion as well as arrest of cell cycle progression. Subcellular fractionation and immunofluorescence technique confirm a direct integration of HuR and Cdk5 with centrosomes. We propose that HuR stores mRNA in the centrosome and that HuR phosphorylation by Cdk5 controls de novo protein synthesis in near proximity to centrosomes and, thus, impacts centrosome function.

The cyclin-dependent kinase (Cdk) family is implicated in cell cycle progression, cell differentiation, and migration. The Cdk family contains two types of members: first a the common one, which must bind to cyclin proteins to become active (Cdk1,-2,-3,-4,-6,-7,-8, and -11), and a unique, cyclin-independent member (Cdk5) activated by p39, p35, and p25 co-factors (1–4). The expression of cyclin-dependent Cdk members is not tissue-specific; however, the unique Cdk5 is mainly expressed in the central nervous system (CNS) (4, 5). Cdk5 is regulated by both growth factor (PDGF, EGF/βEGF)-dependent and -independent mechanisms. Phosphorylation-independent Cdk5 activation by p35 and p25 is strictly controlled during development and aberrant in tumor tissue (6–8).

Many CNS functions, including memory, movements, and plasticity, have been reported as controlled by Cdk5 (9). As a result, a number of CNS diseases, such as Huntington disease, Alzheimer disease, and Parkinson disease have been associated with aberrant expression or regulation of Cdk5 (10). During early stages of development, the depletion of Cdk5 from neuronal stem cells abolishes neurogenesis (11, 12). The homozygote Cdk5 gene deletion results in global abnormalities of neuronal migration, affects differentiation, and is lethal for the mouse embryo (13). In mature neurons, Cdk5 subcellular localization controls cell cycle initiation and consequent neuronal death (14, 15). Cdk5 is required for synapses formation and is important in local protein synthesis and synaptic transmission (16, 17). In tumor cells, Cdk5 overexpression or activation by p35 and growth factors promotes an aggressive metastatic behavior, resistance to the chemotherapeutic drugs, and, in some cases, alterations of cell cycle progression (7, 8, 18, 19). The mechanisms implicated in Cdk5 function include phosphorylation and activation of transcriptional factors, ion channels, cytoskeleton proteins, and kinases (19–21).

In the present paper, we report a novel Cdk5 target, the mRNA-binding protein HuR. HuR is highly conserved during development, is required for angiogenesis and neurogenesis in normal tissues, and is involved in inflammation, migration, proliferation, and survival of different types of transformed cells (22–28). HuR is essential for cell cycle progression and may influence cytoskeleton architecture (22–28). At the molecular level, HuR directs mRNA stabilization, localization, splicing, and translation. Several kinases (Cdk1, PKCα, PKCδ, and Chk2) phosphorylate HuR and influence HuR function and subcellular localization (29, 30).

This study provides evidence of HuR and Cdk5 interaction in cancer cells. We determine that HuR is phosphorylated by Cdk5 at serine 202. Centrosomes were discovered as sites of functional significance of HuR and Cdk5 interaction. At the cellular level, the prolonged disruption of the balance between phosphorylated and dephosphorylated HuR at serine 202 provokes an arrest of cell cycle progression in glioma cells.
DNA Constructs and Stable Cell Line Creation—mRNA was isolated from primary GBM with TRIzol reagent and converted to cDNA by RT-PCR for cloning. Cdk5 was cloned using CDK5-FORW (5′-GGCAATGCAGAGAAATACGAGAAC) and CDK5-Rev (5′-CTAGGGCCGACAGAATCGGG) primers (Sigma) and ligated in pCR-2.1TOPO vector (Invitrogen). Cdk5 was recloned in pDsRed2-C1 (Clontech) (HindIII/BamHI) by using Forw/CDK5/ Hind3 (5′-GAAGTACGAGCTCGAGAAATACGAGAACGACAGGGAAGATTGAG) and Rev/CDK5/BamHI (5′-GGCGGTGATCATCTAGGGCCGACAGAATCGGG) primers. HuR S202A, HuR S221A, and HuR S202D constructs were achieved by point mutagenesis on FLAG-HuR wild type (WT) in pTre2Hyg vector. HuR WT, HuR S202A, and HuR S221A were cloned in pGEX-6P vector for recombinant protein expression and purification from bacteria. The PACT domain of pericentrin attached to the red fluorescence protein (mKO1) under the Ta promoter was a gift from Dr. Akira Sakakibara. PACT-mKO1 was recloned under the CMV promoter. All constructs were confirmed by sequencing.

Cell cycle analysis by PI and EDU incorporation was performed using the Click-it EDU 6/47 flow cytometry assay kit (Invitrogen). Construct expression was induced by cell treatment with DOX (for HuR) or by transfection (for p35) (transfection kit from LONZA) for 1–2 days, and then 10 μM EDU was incorporated for 5–8 h, and cells were washed and fixed in 100% ethanol and stored at −20 °C. The following week, EDU was developed and PI was incorporated by using the Invitrogen protocol, and cells were analyzed by a flow cytometry technique. The parental TETO cell line without construct expression was used as a control.

Fluorescence microscopy was performed by using an Olympus DP71 fluorescence microscope with a DP controller 3.3.1.294 system and Nikon Eclipse Ti fluorescence microscope with NIS-Elements software. Polyclonal DKO/DDDDK tag (Alexa® 488) antibody (Cell Signaling Technology) was employed for subcellular detection of FLAG-HuR S202A, FLAG-HuR S202D, and FLAG-HuR WT proteins. Cells were fixed and permeabilized by using a protocol from Invitrogen, to immunoprecipitation, Universal Buffer was used to block nonspecific binding, and cells were incubated with antibody (1:500) overnight at 4 °C and washed in PBS. HuR-EGFP, Cdk5-DesRed, and PACT-mKO1 constructs were transfected to cells by using a transfection kit from LONZA.

Protein/mRNA Pull-down Assay—FLAG-tagged HuR was immunoprecipitated with A/G beads coated with FLAG antibodies, co-precipitated mRNA was purified by using TRizol reagent, and mRNA targets (GAPDH, hey1, cyclin A1, and jagged-1; primer/probes Hs00266705_g1, Hs01114115_m1, Hs00171105_m1, Hs00164982_m1, respectively: Applied Biosystems) were analyzed by using TAQMAN. Cdk5/HuR co-immunoprecipitation was performed using the immunoprecipitation method as described (Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), ExtraCruz C, dilution in kinase buffer) with monoclonal Cdk5 and HuR 3A2 (Santa Cruz Biotechnology, Inc.) antibodies for immunoprecipitation and polyclonal Cdk5 (Cell Signaling Technology) and HuR-H280 (Santa Cruz Biotechnology, Inc.) antibodies for immunoblotting. Protein fractionation was performed using the nuclear/cytoplasmic fractionation kit (Pierce).

RNA kinetics were analyzed following 12 h of actinomycin D (ACDM) treatment. Each graph point (see Fig. 3C) represents mRNA values normalized to the corresponding initial mRNA level at the 0 h time point. A one-phase exponential decay equation was fitted using Prism 5 (GraphPad, La Jolla, CA).

In vitro recombinant HuR protein phosphorylation by recombinant Cdk5/p35 (Invitrogen) was performed at 30 °C for 30 min in kinase buffer (Cell Signaling Technology) supplemented with ATP. Phosphorylated HuR-GST or HuR (GST was truncated with thrombin) was detected by using p-S-Cdk substrate-specific antibody (Cell Signaling Technology). For in vitro experiments, HuR-GST proteins was purified by using 5-ml GSTrap HP columns (HiTrap), dialyzed against PBS in 3–12-ml Slide-A-Lyzer dialysis cassettes (Thermo Scientific) and verified by Western blot.

The α screen assay was developed using glutathione α donor beads (PerkinElmer Life Sciences) (which bind to HuR-GST protein) and protein A acceptor beads (PerkinElmer Life Sciences) (which bind to phospho-Cdk substrate antibody). In vitro phosphorylation of recombinant HuR-GST by Cdk5/p35 was performed at 24–28 °C (room temperature) for 1 h, and beads were added for the next 1 h. Acceptor beads were preincubated with phospho-Cdk substrate antibody at room temperature for 1 h before the addition to the reaction. The signal was recorded on a PerkinElmer Life Sciences Envision Multilabel Plate Reader. The titrations of Cdk5/p35, HuR-GST, and Cdk5 family inhibitor alsterpaullone (Alexis Biochemicals) were performed at room temperature.

In Vitro mRNA/HuR Binding—High protein binding 96-well plates (Nunc) were loaded with equal amounts (1 μg) of GST, HuR-GST, and HuR-GST phosphorylated by Cdk5/p35. Cyclin A RNA was in vitro synthesized (Ambion T7 kit) and loaded in binding buffer. Ribogreen was added, and the fluorescence signal was read. All fluorescence readings were compared with signal from corresponding protein wells with 0 nM RNA, normalized to the maximum signal representing HuR-GST/cyclin A binding, and fit with a dose-response function using Origin-Pro software. Binding buffer contained 25 mM KCl, 5 mM Heps, 1.25 MgCl2, 3.8% glycerol, 0.02 mM DTT, 1 mM EDTA.

Centrosome purification and fractionation was performed as described by Meigs and Kaplan (31). The purity of the centrosome fractions was confirmed by staining with γ-tubulin. Briefly, the centrosome fractions for each clone were purified from cells induced or non-induced by DOX. The initial step of purification included separation of nuclear fraction from centrosome/cytoplasmic fractions and was verified by lamin A/C.
distribution in fractions by Western blot (supplemental Fig. 5A). The crude centrosome fractions were isolated and collected following centrifugation in Ficoll cushion solution. A total of 15–17 fractions were collected for each clone after the second step of purification (centrifugation and fractionation in a discontinuous 62.5–20% sucrose gradient). The purity of the centrosomal fractions was determined by the appearance of γ-tubulin, which was observed in 2–3 of 15 fractions at ~48% of sucrose gradient level for each clone (disregarding the first two fractions, which probably contain cell debris) (31). The presence of two specific centrosomal proteins Cdk5rap2 and γ-tubulin and the absence of non-centrosomal protein GAPDH in centrosome fractions confirm the purity of the purification (supplemental Fig. 5B).

**HuR**

**mRNA Co-immunoprecipitate from Cytoplasmic Fraction**—The cells were electroporated with constructs as control, p35 cDNA for p35 overexpression, p35 for p35 overexpression, and siRNA of Cdk5 for Cdk5 inhibition. Following p35 overexpression, cells were expanded in the absence or presence of the its co-activator p35 are localized mainly in the cytoplasmic fraction (supplemental Fig. 1). The details of Cdk5/HuR interaction (supplemental Fig. 1). The statistically significant data are labeled by an asterisk in the graphs.

**RESULTS**

The cytoplasmic localization of Cdk5 has been reported as crucial for cell cycle initiation and proliferation (36). An increase in the cytoplasmic localization of HuR is noted in highly aggressive cancers and is essential for cell survival, migration, and proliferation (22–28). To support our hypothesis that unique proline-directed serines in HuR serve as points of regulatory control by Cdk5, we provide the following results.

**Cdk5 Interacts with HuR**—To determine if HuR serves as a substrate for Cdk5, we examined the expression, interaction, and subcellular distribution of both proteins in glioma tumor samples and established cell lines. Fig. 1A illustrates the expression of HuR and Cdk5 in all samples of brain tumors with a clear increase in the cytoplasmic localization of HuR is noted in highly aggressive cancers and is essential for cell survival, migration, and proliferation (22–28). To support our hypothesis that unique proline-directed serines in HuR serve as points of regulatory control by Cdk5, we provide the following results.

**FIGURE 1. Cdk5 is expressed and interacts with HuR in glioma tumor samples and cell lines.** A, expression of Cdk5 and HuR in samples of brain tumors. A (a), lanes 1–4, control brain; lane 5, pilocytic astrocytoma (WHO I); lanes 6–8, newly diagnosed GBM (WHO IV); lanes 9 and 10, recurrent GBM (WHO IV); lane 11, U251 cell line. A (b), established glioma cell lines. Lane 1, U251; lane 2, U118; lane 3, U138; lane 4, SF188; lane 5, LN319, lane 6, SNB19; lane 7, MD59K; lane 8, D37. B, co-immunoprecipitation (IP) of Cdk5 and HuR from two primary GBM xenolines. C, expression (a) and co-immunoprecipitation (b) of Cdk5 and HuR from total (T), nuclear (N), and cytoplasmic (C) fractions of U251. Lamin C1/C2 and α-tubulin were chosen as indicators of nuclear and cytoplasmic fractions, respectively. D, localization of Cdk5-DsRed (a) and HuR-EGFP (b) and co-localization of Cdk5-DsRed and HuR-EGFP constructs (c) in U251. Nuclei were stained with DAPI.

The co-immunoprecipitation of HuR and Cdk5 in all samples of brain tumors with a clear increase of HuR in the most aggressive forms (WHO IV). The established glioma cell lines also exhibited expression of both Cdk5 and HuR proteins uniformly (Fig. 1, bottom). The co-immunoprecipitation of HuR and Cdk5 in two primary glioma xenolines confirmed a direct interaction between these proteins (Fig. 1B). The RNase treatment of cell lysates prior to co-immunoprecipitation did not diminish the HuR and Cdk5 interaction (supplemental Fig. 1). The details of Cdk5/HuR protein interaction were investigated in the established U251 line. Nuclear/cytoplasmic fractionation revealed that Cdk5 and its co-activator p35 are localized mainly in the cytoplasmic fraction, with HuR levels greatest in the nucleus and less in the cytoplasm (Fig. 1C (a)). A direct interaction between Cdk5 and HuR was confirmed in total and cytoplasmic fractions by using a co-immunoprecipitation assay (Fig. 1C (b)). We did not observe a significant Cdk5/HuR interaction in the nuclear fraction. Expression of HuR-EGFP and Cdk5-DS-Red constructs in U251 cells verified a mostly cytoplasmic localization of Cdk5...
Cdk5 Interacts with HuR

(Fig. 1D(a)), nuclear and cytoplasmic localization of HuR (Fig. 1D(b)), and co-localization of HuR and Cdk5 in discrete cytoplasmic areas (Fig. 1D(c)). Our data confirm a direct interaction between HuR and Cdk5 in glioma tumor samples and established cell lines.

Cdk5 Directly Phosphorylates the Serine 202 Residue in HuR Hinge Region—The hinge region of HuR contains two proline-directed serine residues (amino acids 202 and 221) that may serve as potential Cdk5 phosphorylation sites. Recombinant wild type, S202A, and S221A HuR-GST proteins were created, and an in vitro Cdk5/HuR phosphorylation assay was employed to evaluate HuR phosphorylation by Cdk5. Fig. 2A illustrates HuR-wild type (GST-cleaved) phosphorylation by Cdk5 detected by p-S-Cdk substrate-specific antibody in a kinase assay. Substitution of the serine 221 with alanine had no significant effect on Cdk5-dependent HuR phosphorylation (Fig. 2B). However, the substitution of serine 202 to alanine in HuR completely abolished HuR phosphorylation by Cdk5 (Fig. 2C). To check if Cdk5/p35 may phosphorylate alternative sites to serine Ser-202 in HuR, we examined the phosphorylation of recombinant GST, GST-HuR wild type, and GST-HuR S202A (2.5 μg of each) proteins by Cdk5/p35 (50 ng) with an in vitro assay to detect the generation of phosphoproteins (Pro-Q phosphoprotein gel staining; Invitrogen) by Western blot (supplemental Fig. 2). The experiment was performed three times, suggesting that Cdk5/p35 preferentially phosphorylates the Ser-202 residue of HuR because the mutation of Ser-202 to alanine in HuR abolished the phosphofluorescence signal induced by Cdk5/p35 phosphorylation. This finding suggests that the serine 202 in the HuR hinge region is a unique phosphorylation site regulated by Cdk5.

An α screen assay was developed and employed to analyze details of the Cdk5/HuR interaction. Fig. 2D(a) illustrates a concentration-dependent phosphorylation of HuR wild type and HuR S202A mutant by Cdk5. Phosphorylation of HuR S202A was not detectable over a wide concentration range. The ratio of luminescence signals of phosphorylated HuR-GST wild type (500 ng) to phosphorylated HuR S202A-GST mutant (500 ng) by Cdk5 was 14 ± 1-fold greater (n = 8 experiments) (Fig. 2D(a), inset), suggesting specific serine 202 phosphorylation by

FIGURE 2. Serine 202 is the unique residue in HuR phosphorylated by Cdk5. A, phosphorylation of recombinant HuR WT protein (GST cleaved) by Cdk5 detected in Western blot by p-S-Cdk substrate-specific antibody. B, phosphorylation of HuR S221A-GST at two different concentrations (150 and 300 ng) by Cdk5 detected by p-S-Cdk substrate-specific antibody. C, phosphorylation of recombinant HuR WT-GST and HuR S202A-GST mutant are exhibited at two different concentrations (200 and 400 ng). D(a), an example of a screen signal from recombinant HuR WT-GST and HuR S202A-GST mutant proteins phosphorylated by Cdk5. Inset, average signal from phosphorylated HuR WT-GST (0 and 500 ng) and HuR-S202A-GST mutant (0 and 500 ng) normalized to the maximum of HuR WT-GST phosphorylated signal (n = 5). D(b), Cdk5/p35 titration in HuR WT-GST (500 ng) phosphorylation reaction. Inset, average data normalized to the maximum of phosphorylation signal in each experiment (n = 3). D(c), the Cdk family ATP competitive inhibitor, alsterpaullone, blocks Cdk5 phosphorylation of HuR in a dose- and ATP-dependent manner.
Cdk5. Fig. 2 (b) illustrates titration of Cdk5 in a phosphorylation reaction performed with a constant HuR wild type amount (500 ng). The $K_{1/2}$ of Cdk5 for HuR phosphorylation was 17 ng. The ATP competitive inhibitor of the Cdk family (alsterpaullone) induced a decline of HuR phosphorylation by Cdk5 in an ATP-dependent manner (Fig. 2 (c)). Thus, our data confirms that Cdk5 specifically phosphorylates serine 202 in HuR.

Phosphorylation of HuR at Ser-202 Alters HuR/mRNA Interaction—U251 clones were engineered to conditionally express WT, S202A, or S202D FLAG-tagged HuR proteins in order to evaluate the cellular and molecular consequences of HuR phosphorylation at the Ser-202 residue (Fig. 3 A). Previously, it has been reported that HuR binds to the 3′-UTRs of mRNAs for the transcriptional regulator hey1, angiogenesis-promoting factor jagged-1, and cell cycle regulator cyclin A. To investigate the impact of HuR Ser-202 phosphorylation on stabilization of mRNA targets, we evaluated hey1, jagged-1, and cyclin A mRNA degradation following ACMD treatment in cells with conditional expression of HuR Ser-202 mutants. Mutants were induced to the same level for HuR S202A and HuR S202D clones by the same DOX concentration (0.5 mg/ml) (data not shown). Fig. 3B illustrates the kinetics of hey1, jagged-1, and cyclin A mRNAs in TETON control, HuR S202A, and HuR S202D clones before and after construct induction with DOX (two experiments in triplicate). Table 1 provides a summary of the RNA half-lives for the above targets and c-myc. c-myc was included due to its rapid decay and as a positive control for the inhibition of transcription. The declines in mRNA signals of TETON, HuR S202A, and HuR S202D cell lines following ACMD treatment were fitted by a single phase exponential decay function for all mRNA targets. The control TETON clones demonstrated similar RNA half-lives with or without DOX induction, supporting a lack of influence for DOX. The non-induced mutant clones for both S202A and S202D demonstrated half-lives greater than the TETON controls but similar to each other, suggesting consistency with the system but a possible effect of the antibiotics utilized to maintain the clone stability. Following induction of the mutant clones with DOX, a significant alteration in target RNA half-life was noted. With induction of the S202D mutant, the half-lives were increased 2–3 fold; however, following induction of the S202A mutant, a half-life was determined only for the c-myc target due to lack of decay within the experimental time window. These data suggest that the phosphorylation state of HuR influences target RNA binding affinity.

To assess the effect of serine Ser-202 phosphorylation on HuR/mRNA binding, a HuR/mRNA co-immunoprecipitation assay was employed on HuR S202A and HuR S202D induced clones. The mRNAs co-precipitated with HuR mutants (by using FLAG antibody) normalized to the corresponding mRNA targets co-immunoprecipitated with control mouse IgG-coated beads from induced clones (n = 3). Error bars, S.D.

**TABLE 1**

| mRNA   | TETON | TETON DOX | S202A DOX | S202A DOX | S202D DOX | S202D DOX |
|--------|-------|-----------|-----------|-----------|-----------|-----------|
| hey1   | 4.68  | 5.67      | 8.5       | ND        | 7.56      | 16.47     |
| jagged-1| 11.24 | 9.24      | 15.02     | ND        | 8.82      | 20.24     |
| Cyclin A | 8.26  | 9.53      | 16.25     | ND        | 11.46     | 35.29     |
| c-myc  | 0.38  | 0.40      | 0.49      | 6.7       | 0.39      | 10.0      |

ND, not determined.
Cdk5 Interacts with HuR

**In Vitro HuR binding to cyclin A RNA.** Cyclin A mRNA binding curves to HuR-GST in non-phosphorylated and phosphorylated by Cdk5/p35 conditions are illustrated in the graph. The binding values for each mRNA concentration have been normalized to the maximum HuR-GST/cyclin A binding observed at 10 nM in vitro synthesized cyclin A RNA. The maximum cyclin A RNA binding to HuR-GST in a phosphorylated by Cdk5/p35 condition was diminished by 85 ± 8% (n = 4) compared with the maximum binding to non-phosphorylated HuR-GST. No significant mRNA binding was detected to GST alone (n = 4). The averaged EC50 = 0.8 ± 0.1 nM (n = 4) for the cyclin A RNA/HuR binding affinity. Error bars, S.D.

**In Vitro mRNA/HuR Binding—**To clarify the mechanism of HuR/mRNA interaction under regulation by Cdk5/p35, we performed cyclin A RNA binding to recombinant Hu-R-GST under non-phosphorylated and phosphorylated by Cdk5/p35 conditions in vitro (Fig. 4). Cyclin A RNA containing 3′-UTR was synthesized in vitro from human cyclin A clone. Fig. 4 illustrates the binding signal of cyclin A RNA (from 0 to 10 nM) to non-phosphorylated HuR-GST, phosphorylated HuR-GST, and GST. The averaged EC50 of cyclin A RNA to HuR was 0.8 ± 0.1 nM. The maximum of cyclin A binding to phosphorylated HuR reached only 15–20% of maximum cyclin A binding to non-phosphorylated HuR-GST. GST alone did not exhibit any binding ability to cyclin A. These data suggest that HuR phosphorylated by Cdk5/p35 has an unfavorable conformation for cyclin A binding.

**Prolonged Alteration of HuR Phosphorylation at Ser-202 Induces Centrosome Abnormalities and Arrest of Cell Cycle Progression—**HuR is involved in proliferation of several types of cancer (25–28, 32, 33). An EDU incorporation assay was employed to evaluate the influence of HuR phosphorylation at Ser-202 on cell cycle progression. A significant decrease in EDU incorporation was observed in cells containing Ser-202 mutant clones associated with an accumulation of cells in the G1/M phases. This finding suggests that consecutive phosphorylation and dephosphorylation of serine 202 in HuR are essential for cell cycle progression.

We hypothesized that abnormalities in cell cycle progression resulting from induced HuR S202A and S202D may be tied to defects in centrosome duplication and cohesion caused by aberrant HuR-dependent regulation of cyclin A levels. We marked centrosomes with the conserved PACT domain of pericentrin attached to the red fluorescence protein and analyzed their structure in HuR WT-, S202A-, or S202D-overexpressing cells. Fig. 5B represents distribution (in percentage) of cells with different centrosome number per nuclei in control and induced clones. Robust defects in centrosome duplication and cohesion were observed in HuR mutant clones (Fig. 5B, diagrams). The majority of HuR S202A-overexpressing cells exhibited amplification of centrosome number (Fig. 5B); however, the majority ofHuR S202D-overexpressing cells displayed a decrease in centrosome number as well as abnormal centrosome cohesion (Fig. 5B).

To detail the HuR influence on centrosome function, we employed an immunostaining technique and immunoblotting analysis of purified centrosome fractions (31). The immunostaining approach revealed HuR co-localization with centrosomes marked by PACT-miko.1 fluorescence construct (Fig. 5C (ai)). The centrosome purification by subcellular fractionation confirmed that HuR and Cdk5 proteins were integrated with centrosomes (Fig. 5C (b) and supplemental Fig. 4). The centrosome protein fraction obtained by using a discontinuous (60–20%) sucrose gradient was identified with γ-tubulin at ~48–53% sucrose level and exhibited a significant amount of endogenous HuR and Cdk5 proteins (Fig. 5C (b)). The analysis of purified centrosome fractions from HuR Ser-202 mutant clones revealed that the mutations of serine 202 to A or D did not prohibit the ability of HuR to co-localize with the centro-
some fraction marked with γ-tubulin (Fig. 5D (a and b)). Our data suggest that HuR may integrate in centrosomes and that the unbalanced HuR phosphorylation at serine 202 distorts centrosome function and cell cycle progression.

**Phosphorylation of Serine 202 in HuR and Overactivation of Cdk5 Have a Common Impact on Cyclin A Protein Level—Cyclin A oscillation in centrosomes is essential for normal duplication and cohesion** (34, 35). We hypothesized that abnormal serine 202 phosphorylation in HuR may affect cyclin A levels in the centrosomes. The comparison of crude centrosome content from different clones revealed that the level of cyclin A protein correlates positively with the amount of HuR S202A mutant and inversely correlates with the mimicking serine S202 phosphorylation of HuR S202D mutant (Fig. 6A). The average value of cyclin A was 2.1 ± 0.5 (n = 3)-fold higher in crude centrosome fractions from the induced HuR S202A clone compared with the cyclin A value in crude centrosomes of the HuR S202D induced clone; the value of cyclin A in crude centrosome fractions of non-induced HuR S202A and HuR S202D clones were not significantly different (1 ± 0.2, n = 3). The difference was statistically significant for the induced HuR S202A compared with S202D (p = 0.04). To check if HuR phosphorylation influences cyclin A translation, we performed fluorescence labeling of de novo synthesizing proteins by using modified glycine in U251 cells. Fluorescence labeling confirms spots of de novo protein synthesis in proximity (0–3 μm) to centrosomes, suggesting possible attachment of translational apparatus to centrosomes (supplemental Fig. 5). The flow cytometry analysis...
of de novo protein synthesis revealed that overexpression of HuR S202D mutant was associated with a decrease of de novo protein synthesis compared with protein synthesis in the HuR wild type- or HuR S202A mutant-overexpressing cells. (Fig. 6B, n = 4). The immunoprecipitation of de novo synthesized cyclin A (6 h of labeled translation) confirmed a decrease in cyclin A protein level in HuR S202D-overexpressing cells compared with HuR S202A-overexpressing cells (Fig. 6B (b)). To further support the role of HuR phosphorylation on cyclin A expression, a decrease of cyclin A protein level was observed following p35 overexpression in U251 cells (Fig. 6C (a)). The total decrease of protein synthesis reached 19 ± 5% (n = 4) compared with control (vector) overexpressing cells (Fig. 6C (b and c)). This finding highlights a common impact of HuR Ser-202 phosphorylation and Cdk5 activation by p35 on cyclin A protein translation in U251 cells.

Further confirmation of centrosome regulation by HuR/Cdk5 interaction was achieved by centrosome analysis following inhibition of the Cdk5/HuR interaction. The knockdown of Cdk5 co-activator p35 (Fig. 7A) provoked a decrease in the HuR/Cdk5 interaction confirmed by a co-immunoprecipitation assay (Fig. 7B) and was associated with robust defects in centrosome duplication. The amplification in centrosome

**FIGURE 6.** Serine 202 phosphorylation in HuR affects protein translation. A, the protein content of crude centrosome fractions from induced and non-induced HuR Ser-202 mutant clones. The chemiluminescence values of cyclin A bands are normalized to the corresponding values of γ-tubulin bands. B (a), averages of de novo protein synthesis in induced clones normalized to the corresponding values from non-induced clones obtained by flow cytometry (n = 4). Note the significant reduction of protein synthesis in HuR S202D induced clone. B (b), de novo synthesized cyclin A protein (during 6 h of labeled translation) immunoprecipitated (IP) from HuR S202D and HuR S202A induced clones. C, overexpression of p35 was associated with a decrease in cyclin A protein levels (a) and a reduction of de novo protein synthesis (b and c). De novo protein synthesis is illustrated in the flow cytometry plots (b) and in quantified graph (c) (n = 4) following 24 and 48 h of p35 overexpression. Error bars, S.D.

**FIGURE 7.** Knockdown of p35 by siRNA reduces HuR/Cdk5 interaction and provokes amplification of centrosome number. A, reduction of p35 expression by siRNA (a) and reduction of Cdk5 co-immunoprecipitation with HuR (b). B, illustration of amplification of centrosome number per nuclei following p35 knockdown. Nuclei were stained with DAPI, and centrosomes were marked with the PACT domain of pericentrin attached to the red fluorescence protein. Bar, 50 μm. IP, immunoprecipitation; IB, immunoblot.
number was observed in more than 50% of cells after p35 inhibition (Fig. 7C) versus only 5–10% of control cells following transfection with scrambled siRNA.

**DISCUSSION**

In the current report, we have provided several lines of evidence supporting a direct Cdk5/HuR interaction: 1) co-immunoprecipitation of both proteins from several glioma lines; 2) co-localization by fluorescence microscopy; and 3) direct interaction in an in vitro phosphorylation assay. We confirm HuR integration in centrosomes and a phosphorylation-dependent influence on centrosome function and cell cycle progression.

The role of HuR in cell cycle progression via regulation of cyclins and growth factors is well documented (25–27, 32). The phosphorylation-dependent HuR shuttling between nucleus and cytoplasm, mRNA stabilization, and up- and down-regulation of protein synthesis underlie HuR function during cell cycle progression (30, 37–39). Several kinases (Cdk1 and PKCα) have been reported to regulate HuR function during the cell cycle (29, 30). We found that HuR phosphorylation by Cdk5 may influence cell function via alteration of cyclin A protein level. The oscillation of cyclin A protein levels is essential for mitosis progression through activation of DNA replication and for centrosome duplication through cyclin A/Cdk2 phosphorylation-dependent regulation of Msp1 kinase (34, 35, 40). The range of cyclin A oscillation is determined by both translation and degradation and may have positive and negative impacts on cell cycle progression. We observed an inverse correlation between HuR phosphorylation at Ser-202 and cyclin A protein level in centrosomes. In agreement with previous observations, we found that elevation of cyclin A level in centrosomes is associated with amplification of centrosome number in HuR S202A induced clone, and a decline of cyclin A protein level in HuR S202D induced clone is linked to a decrease in centrosome duplication and aberrant cohesion (35). The centrosome’s role in mitotic orientation of progenitors is well documented (41). Recently, centrosomes have emerged as key regulators of mitosis in cancer cells with abnormalities in centrosome function tightly related to genomic instability and aberrant cell cycle progression (42–44). The arrest of cell cycle progression observed in HuR S202A/D induced clones could be well explained by 1) disruption of centrosome duplication, 2) inadequate cyclin A protein level during G2-M phase of cell cycle progression, or 3) both.

The direct integration and regulation of centrosome function by HuR highlights a new, active contribution for this mRNA-binding protein in cell cycle progression. Numerous key proteins that are involved in centrosome function, including cyclins, growth factors, β-actin, β-catenin, and connexin, are controlled by HuR at the mRNA and translational levels (28, 45–48). We hypothesize that HuR may store mRNA in centrosomes and facilitate local translation during mitosis. In support of our hypothesis, we observed spots of de novo protein synthesis attached to the centrosomes and the presence of translation initiation factors in crude centrosomes (supplemental Fig. 1). In agreement with our theory, several authors have reported a correlation between local de novo protein synthesis and centrosome formation, supporting a dependence of centrosome structure on components of the translational machinery (49, 50). We found that HuR phosphorylation by Cdk5 influences cyclin A mRNA stabilization and translation in proximity to the centrosome. However, other proline-directed kinases, such as Aurora A, Nek2, SIK2, and Polo-like kinase 1, have also been reported in proximity to centrosomes and may play an active function in promoting cell cycle progression (51–55). The particular type of proline-directed kinases involved in HuR-phosphorylation at Ser-202 may depend on the cell line or cellular fate.

The function of HuR is heavily dependent on the phosphorylation status of its hinge region (29, 30, 56–58). The hinge domain is directly or indirectly responsible for HuR subcellular localization, binding affinity with mRNA, and regulation of translational efficiency (29, 30, 56–58). The main consequence of aberrant serine Ser-202 phosphorylation in the HuR hinge region is the arrest of cell cycle progression. Overall, our data suggest that HuR S202A has a more favorable role in mRNA stabilization and processing compared with HuR S202D, specifically in the regulation of cyclin A mRNA delivery and processing in proximity to centrosomes. In addition, the alterations of mRNA levels and the decrease in protein synthesis following induction of HuR Ser-202 mutants point to an important role of hinge region phosphorylation in HuR mRNA binding function. The mechanism of this effect is not clear and could be partially explained by different HuR Ser-202 mutant affinities to mRNA, alterations in subcellular location or trafficking ability of HuR Ser-202 mutants, or direct and indirect HuR influences on the translational machinery. This certainly situates the HuR hinge domain as a potential novel therapeutic target in cancer. Given these results, HuR and Cdk5 with emphasis on the phosphorylation of the hinge region may be considered as unique molecular targets of a novel class in cancer therapeutic.

Acknowledgments—We greatly appreciate the space and support provided by the Southern Research Institute. We thank Dr. Lynn Rasmussen for great advice and Robert E McHugh for technical assistance.

**REFERENCES**

1. Satyanarayana, A., and Kaldis, P. (2009) Mammalian cell-cycle regulation. Several Cdks, numerous cyclins, and diverse compensatory mechanisms. Oncogene 28, 2925–2939
2. Lew, J., Huang, Q. Q., Qi, Z., Winkfein, R. J., Aebersold, R., Hunt, T., and Wang, J. H. (1994) A brain-specific activator of cyclin-dependent kinase 5. Nature 371, 423–426
3. Nebreda, A. R. (2006) Cdk activation by non-cyclin proteins. Curr. Opin. Cell Biol. 18, 192–198
4. Tsai, L. H., Delalle, I., Caviness, V. S., Jr., Chae, T., and Harlow, E. (1994) p35 is a neural-specific regulatory subunit of cyclin-dependent kinase 5. Nature 371, 419–423
5. Tsai, L. H., Takahashi, T., Caviness, V. S., Jr., and Harlow, E. (1993) Activity and expression pattern of cyclin-dependent kinase 5 in the embryonic mouse nervous system. Development 119, 1029–1040
6. Sasaki, Y., Cheng, C., Uchida, Y., Nakajima, O., Ohshima, T., Yagi, T., Taniguchi, M., Nakayama, T., Kishida, R., Kudo, Y., Ohno, S., Nakamura, F., and Goshima, Y. (2002) Fyn and Cdk5 mediate semaphorin-3A signaling, which is involved in regulation of dendrite orientation in cerebral cortex. Neuron 35, 907–920
7. Feldmann, G., Mishra, A., Hong, S. M., Bais, S., Strock, C. J., Ball, D. W.,
Cdk5 Interacts with HuR

Goggins, M., Maitra, A., and Nelkin, B. D. (2010) Inhibiting the cyclin-dependent kinase Cdk5 blocks pancreatic cancer formation and progression through the suppression of Ras-Ral signaling. Cancer Res. 70, 4460–4469

8. Goodyear, S., and Sharma, M. C. (2007) Roscovitine regulates invasive breast cancer cell (MDA-MB231) proliferation and survival through cell cycle regulatory protein Cdk5. Exp. Mol. Pathol. 82, 25–32

9. Lalioti, V., Pulido, D., and Sandoval, I. V. (2010) Cdk5, the multifunctional surveyor. Cell Cycle 9, 284–311

23. Hinman, M. N., and Lou, H. (2008) Diverse molecular functions of HuR and mRNA stability.

25. López de Silanes, I., Lal, A., and Gorospe, M. (2005) HuR. Post-transcriptional paths to malignancy.

28. Dormoy-Raclet, V., Ménard, I., Clair, E., Kurban, G., Mazroui, R., Di Marco, S., von Roretz, C., Pause, A., and Gallouzi, I. E. (2007) The RNA-binding protein HuR promotes cell migration and cell invasion by stabilizing the β-actin mRNA in a U-rich element-dependent manner. Mol. Cell Biol. 27, 5365–5380

29. Kim, H. H., Abdelmohsen, K., Lai, A., Pullmann, R., Jr., Yang, X., Galban, S., Srikantan, S., Martinez, J. L., B lethrow, J., Shokat, K. M., and Gorospe, M. (2008) Nuclear HuR accumulation through phosphorylation by Cdk1. Genes Dev. 22, 1804–1815

32. Dormoy-Raclet, V., Ménard, I., Clair, E., Kurban, G., Mazroui, R., Di Marco, S., von Roretz, C., Pause, A., and Gallouzi, I. E. (2007) The RNA-binding protein HuR promotes cell migration and cell invasion by stabilizing the β-actin mRNA in a U-rich element-dependent manner. Mol. Cell Biol. 27, 5365–5380

35. Taylor, S. M., Nevis, K. R., Park, H. L., Rogers, G. C., Rogers, S. L., Cook, J. G., and Bautch, V. L. (2010) Angiogenic factor signaling regulates centrosome homeostasis.
E. T., Winey, M., Salmon, E. D., Casey, P. J., Nelson, W. J., and Barth, A. I. (2008) β-Catenin is a Nek2 substrate involved in centrosome separation. *Genes Dev.* **22**, 91–105
48. Bahmanyar, S., Guiney, E. L., Hatch, E. M., Nelson, W. J., and Barth, A. I. (2010) Formation of extra centrosomal structures is dependent on β-catenin. *J. Cell Sci.* **123**, 3125–3135
49. Müller, H., Schmidt, D., Steinbrink, S., Mirgorodskaya, E., Lehmann, V., Habermann, K., Dreher, F., Gustavsson, N., Kessler, T., Lehra, H., Herwig, R., Gobom, J., Ploubidou, A., Boutros, M., and Lange, B. M. (2010) Proteomic and functional analysis of the mitotic *Drosophila* centrosome. *EMBO J.* **29**, 3344–3357
50. Vijayakumar, S., Shah, N., Fernandez-Garcia, I., and Barcellos-Hoff, M. (2011) Origins and consequences of radiation in induced centrosome aberrations. *Low Dose Radiation Research Investigators’ Workshop, Bethesda, MD, May 9–11, 2011,* United States Department of Energy, Washington, D. C.
51. Dutertre, S., Descamps, S., and Prigent, C. (2002) On the role of aurora-A in centrosome function. *Oncogene* **21**, 6175–6183
52. Qin, L., Tong, T., Song, Y., Xue, L., Fan, F., and Zhan, Q. (2009) Aurora-A interacts with cyclin B1 and enhances its stability. *Cancer Lett.* **275**, 77–85
53. Rosales, J. L., Rattner, J. B., and Lee, K. Y. (2010) The primary microcephaly 3 (MCPH3)-interacting protein, p35, and its catalytic subunit, Cdk5, are centrosomal proteins. *Cell Cycle* **9**, 618–620
54. Kishi, K., van Vugt, M. A., Okamoto, K., Hayashi, Y., and Yaffe, M. B. (2009) Functional dynamics of Polo-like kinase 1 at the centrosome. *Mol. Cell Biol.* **29**, 3134–3150
55. Fry, A. M. (2002) The Nek2 protein kinase. A novel regulator of centrosome structure. *Oncogene* **21**, 6184–6194
56. Kim, H. H., Yang, X., Kuwano, Y., and Gorospe, M. (2008) Modification at HuR(S242) alters HuR localization and proliferative influence. *Cell Cycle* **7**, 3371–3377
57. Li, H., Park, S., Kilburn, B., Jelinek, M. A., Henschen-Edman, A., Aswad, D. W., Stallcup, M. R., and Laird-Offringa, I. A. (2002) Lipopolysaccharide-induced modification of HuR. *J. Biol. Chem.* **277**, 44623–44630
58. Mazroui, R., Di Marco, S., Clair, E., von Roretz, C., Tenenbaum, S. A., Keene, J. D., Saleh, M., and Gallouzi, I. E. (2008) Caspase-mediated cleavage of HuR in the cytoplasm contributes to pp32/PHAP-I regulation of apoptosis. *J. Cell Biol.* **180**, 113–127