

# Localization and retention of p90 ribosomal S6 kinase 1 in the nucleus: implications for its function

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**ABSTRACT** Ribosomal S6 kinase 1 (RSK1) belongs to a family of proteins with two kinase domains. Following activation in the cytoplasm by extracellular signal-regulated kinases (ERK1/2), it mediates the cell-proliferative, cell-growth, and survival-promoting actions of a number of growth factors and other agonists. These diverse biological actions of RSK1 involve regulation of both cytoplasmic and nuclear events. However, the mechanisms that permit nuclear accumulation of RSK1 remain unknown. Here, we show that phosphorylation of RSK1 on S221 is important for its dissociation from the type I $\alpha$  regulatory subunit of protein kinase A (PKA) in the cytoplasm and that RSK1 contains a bipartite nuclear localization sequence that is necessary for its nuclear entry. Once inside, the active RSK1 is retained in the nucleus via its interactions with PKA catalytic subunit and AKAP95. Mutations of RSK1 that do not affect its activity but disrupt its entry into the nucleus or expression of AKAP95 forms that do not enter the nucleus inhibit the ability of active RSK1 to stimulate DNA synthesis. Our findings identify novel mechanisms by which active RSK1 accumulates in the nucleus and also provide new insights into how AKAP95 orchestrates cell cycle progression.

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## INTRODUCTION

Ribosomal S6 kinase 1 (RSK1) is a member of a family of proteins with two kinase domains (Frodin and Gammeltoft, 1999; Anjum and Blenis, 2008). Among the four isoforms of RSKs that are products of different genes, RSK1, RSK2, and RSK3 share significant sequence similarity; RSK4 is longer than the other isoforms and may also be functionally different (Frodin and Gammeltoft, 1999; Dummmler *et al.*, 2005; Anjum and Blenis, 2008). Despite their similarity, RSK1, RSK2, and RSK3 are not functionally redundant. Thus, although RSK1 and RSK3 show normal overlapping expression patterns with RSK2 in

brains of patients with Coffin–Lowry syndrome, in which RSK2 is mutated, the functions of the mutant RSK2 are not replaced by the other isoforms (Jacquot *et al.*, 1998). Likewise, whereas RSK1 can differentiate PC12 cells, RSK2 is not effective (Silverman *et al.*, 2004), and RSK1, but not the other isoforms, regulates lung cancer cell motility and metastasis (Lara *et al.*, 2011).

The RSKs are the immediate downstream effectors of extracellular signal-regulated kinases 1/2 (ERK1/2) and mediate the growth-promoting, proliferative, and antiapoptotic actions of growth factors and agonists that activate this pathway (Frodin and Gammeltoft, 1999; Anjum and Blenis, 2008; Cargnello and Roux, 2011). In keeping with their role as positive modulators of cell proliferation and growth, RSK1 and RSK2 levels are elevated in ductal and lobular human breast cancer tissues, as well as in 50% of prostate tumors, and inhibition of RSK1 and RSK2 activities results in decreased cell growth (Clark *et al.*, 2005; Smith *et al.*, 2005). RSK1 also plays a profound role in neuronal differentiation, as demonstrated by the finding that constitutively active RSK1 is sufficient to elicit neuronal differentiation of PC12 cells (Silverman *et al.*, 2004). This observation also suggests that the prolonged ERK1/2 activation that is necessary for PC12 cell differentiation (Vaudry *et al.*, 2002) is mediated by activating the downstream kinase RSK1. The role of RSK1 in promoting cell survival has been demonstrated using dominant-negative forms

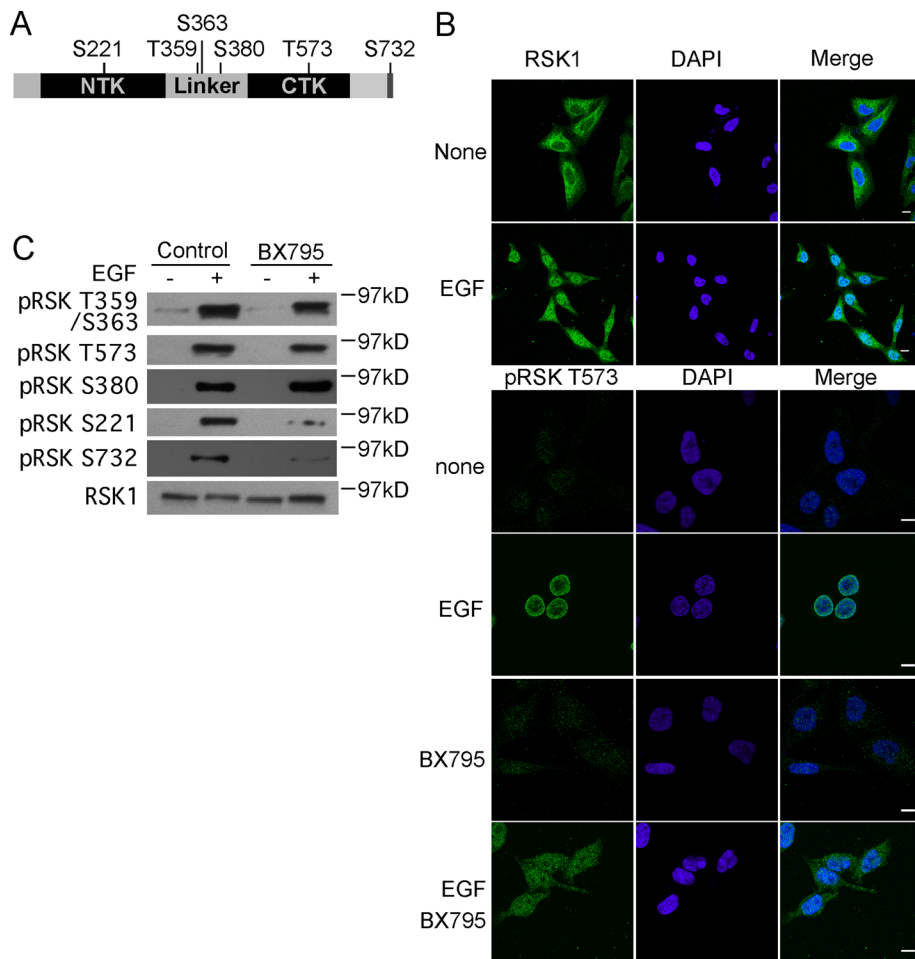
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Abbreviations used: AKAP, PKA-anchoring proteins; BrdU, bromodeoxyuridine; CTK, C-terminal kinase domain; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; GFP, green fluorescent protein; HA, hemagglutinin; LMB, leptomycin B; NLS, nuclear localization sequence; NTK, N-terminal kinase domain; PDK1, phosphoinositide-dependent kinase-1; PKA, protein kinase A; PKAc, catalytic subunit of PKA; PKAR1 $\alpha$ , type I $\alpha$  regulatory subunit of PKA; RSK, p90 ribosomal S6 kinase; siRNA, small interfering RNA.

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**FIGURE 1:** RSK1 phosphorylation by PDK1 is required for its nuclear translocation. (A) Schematic of phosphorylation sites on rat RSK1. (B) Top two rows, serum-starved HeLa cells were treated with and without EGF (100 nM) for 10 min and the localization of total RSK1 was monitored with anti-RSK1 antibody; 4',6-diamidino-2-phenylindole staining shows nuclei. Bottom four rows, the PDK1 inhibitor BX795 inhibits nuclear translocation of RSK1. HeLa cells were treated as described above with and without overnight preincubation with 0.5  $\mu$ M BX795. The localization of active RSK1 was monitored with anti-phospho-T573-RSK antibody. (C) BX795 inhibits phosphorylation of RSK1 on S221 and S732. Cells were treated as in B. After immunoprecipitation with anti-RSK1 antibody, Western analyses of the immune complexes were performed with anti-phospho RSK antibodies. Scale bar, 10  $\mu$ m. Representative data from three independent experiments are shown.

of RSK1 to antagonize the cell survival that was promoted by active forms of mitogen-activated protein kinase kinase alleles (Bonni *et al.*, 1999; Shimamura *et al.*, 2000), as well as some of our recent studies (Chaturvedi *et al.*, 2006, 2009; Gao *et al.*, 2010).

The activation of RSK1 depends on the docking of ERK1/2 to the C-terminus (Gavin and Nebreda, 1999; Smith *et al.*, 1999; Figure 1A). Following docking onto RSK1, ERK1/2 phosphorylates T573 in the activation loop of the C-terminal kinase (CTK), as well as T359 and S363 in the linker region that joins the N-terminal kinase (NTK) and CTK (Frodin and Gammeltoft, 1999). Phosphorylation of T573 activates the CTK, and this domain can then phosphorylate S380 in the linker region (Frodin and Gammeltoft, 1999). Phosphorylation of S380 provides a docking site for phosphoinositide-dependent kinase 1 (PDK1) (Frodin *et al.*, 2000, 2002), which can then phosphorylate S221 in the activation loop of the NTK (Jensen *et al.*, 1999; Richards *et al.*, 1999) and permits RSK1 to phosphorylate its downstream substrates (Frodin and Gammeltoft, 1999). Serine 732 (S749 in avian RSK1) in the ERK1/2 docking site also represents a RSK1 consensus

sequence (RKXXS/T) and is autophosphorylated by the NTK with a resultant dissociation of ERK (Roux *et al.*, 2003). On activation of RSK1 in the cytoplasm and at the plasma membrane, the fully active RSK1 localizes to the nucleus (Richards *et al.*, 2001).

In recent studies from our laboratory, we showed that RSK1 forms a complex with the type I cAMP-dependent protein kinase (PKA). By binding the pseudosubstrate site on the type I $\alpha$  regulatory subunit of PKA (PKAR1 $\alpha$ ), the inactive RSK1 competes with the catalytic subunit of PKA (PKAc) for interactions with PKAR1 $\alpha$ , and, thereby, inactive RSK1 can activate PKA in a cAMP-independent manner (Chaturvedi *et al.*, 2006; Gao *et al.*, 2010). On the other hand, active RSK1, via its C-terminal 13 residues, binds to PKAc, increasing the interactions of PKAc with PKAR1 $\alpha$  and decreasing the ability of cAMP to activate PKA (Chaturvedi *et al.*, 2006; Gao and Patel, 2009). This latter interaction requires phosphorylation of Ser-732 on RSK1 (Gao and Patel, 2009). The phosphorylation of Ser 732 on RSK1 also results in the dissociation of ERK1/2 (Roux *et al.*, 2003; Gao and Patel, 2009). By associating with the subunits of PKA, the inactive and active RSK1 ensure their indirect interactions with A-kinase-anchoring proteins (AKAPs) such as D-AKAP1 (dual-specificity AKAP1; Chaturvedi *et al.*, 2006, 2009). Of interest, dissociation of the RSK1 from its complex with AKAPs by different approaches, including silencing of PKAR1 $\alpha$  or use of cell-permeable peptides that interfere with PKAR1 $\alpha$ /AKAP interactions, decreases the nuclear content of active RSK1 and increases its amounts in the cytosol, with a resultant increase in phosphorylation of its cytosolic substrates, such as Bcl-xL/Bcl-2-associated death promoter, and augmenting of its anti-apoptotic actions (Chaturvedi *et al.*, 2006, 2009; Gao *et al.*, 2010). However, the mech-

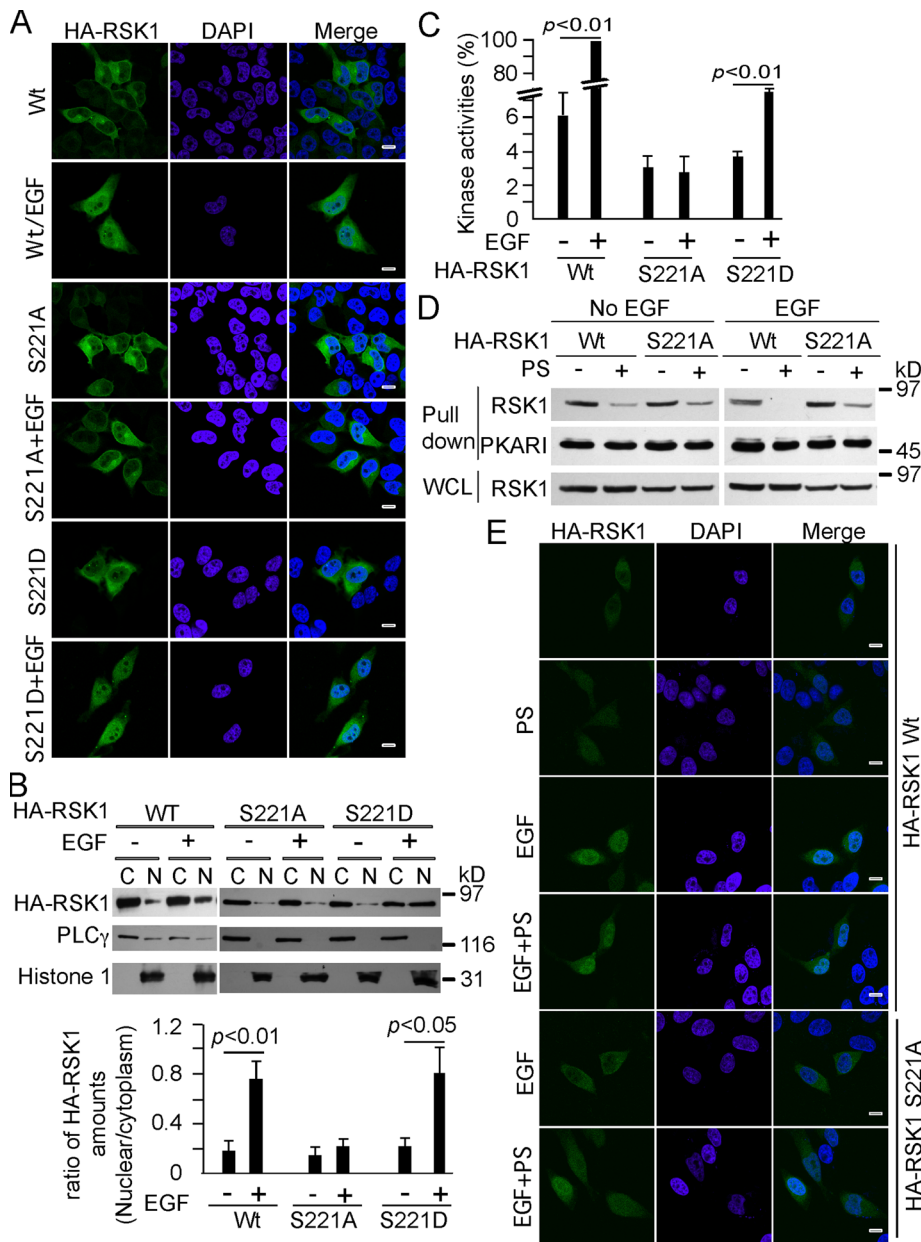
anisms that regulate the nuclear localization of RSK1, and for that matter, its other isoforms, remain unknown.

Therefore the aims of this study were 1) to elucidate the mechanisms by which active RSK1 enters the nucleus, 2) to identify the AKAP(s) that retain active RSK1 in the nucleus, and 3) to understand the functional consequences of impeding active RSK1 entry or retention in the nucleus.

## RESULTS

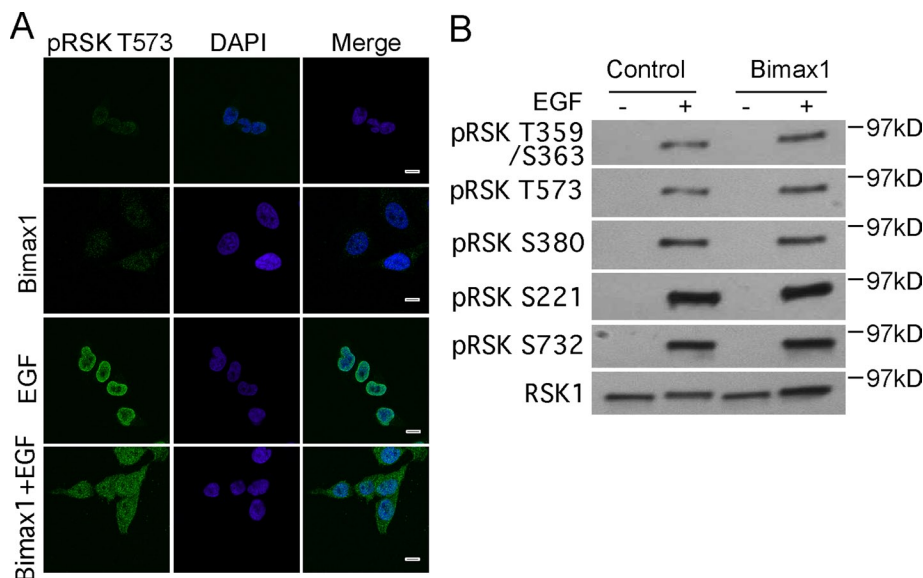
### Phosphorylation of Ser-221 on RSK1 is necessary to dissociate it from PKAR1 $\alpha$ and permit its nuclear localization

Because previous reports suggested that the fully active RSK1 is localized to the nucleus, initially, we determined whether the last phosphorylation (Ser-221 in the NTK; Figure 1A) that fully activates RSK1 is necessary for its nuclear localization. Ser-221 is phosphorylated by PDK1 (Jensen *et al.*, 1999). Therefore, as the first approach, we determined whether inhibition of PDK1 attenuated phosphorylation of S221 and altered the nuclear localization of RSK1. As shown



**FIGURE 2:** Phosphorylation of RSK1 on S221 dissociates it from PKARI $\alpha$  to permit its nuclear translocation. (A, B) Wild-type and S221D mutant, but not the S221A form, of RSK1 translocate to nuclei after EGF treatment as shown by immunocytochemistry (A) and cellular fractionation (B). HeLa cells were transfected to express HA-RSK1 or its mutants. After serum starvation, cells were treated with or without 100 nM EGF for 10 min. The HA-RSK1 localization was monitored with anti-HA antibody (A). In B, the amounts of HA-RSK1 or its mutants in cytosolic fraction (C) or nuclear fraction (N) were analyzed by Western blotting. PLC $\gamma$ , cytosol marker; histone 1, nuclear marker. (C) Kinase activities of HA-RSK1 or its mutants. HA-tagged wild-type and its mutant RSK1 forms were immunoprecipitated with anti-HA antibody from HeLa cells treated with or without EGF for 10 min and then were subjected to the *in vitro* kinase assays. (D) Cell-permeable peptide PS, corresponding to the pseudosubstrate region of PKARI $\alpha$ , dissociates wild-type and S221A mutant of RSK1 from their association with PKARI $\alpha$ . Serum-starved HeLa cells were preincubated with or without 2  $\mu$ M peptide PS for 10 min and then treated with or without EGF for 10 min. PKARI $\alpha$  in the lysates was pulled down with cAMP-agarose, and presence of HA-RSK1 or its mutant was monitored. (E) Peptide PS allows RSK1 S221A to translocate to the nuclei in the presence of EGF. HeLa cells were treated as in D. HA-RSK1 localization was monitored with anti-HA antibody. All data presented are representatives from three independent experiments. Values in B and C are the mean  $\pm$  SE ( $n = 3$ ). Statistical significance was determined by Student's *t* test analyses. Scale bar, 10  $\mu$ m.

in Figure 1B (top two rows), in the absence of epidermal growth factor (EGF), endogenous RSK1, detected by the anti-RSK1 antibody, was localized mainly in the cytoplasm, with very little being present in the nucleus. However, after EGF treatment, considerable increase in the nuclear localization of the endogenous RSK1 was observed (Figure 1B, top two rows). Similarly, use of anti-phospho-Thr-573 antibody showed that there is very little active phospho-Thr-573 RSK1 in serum-starved HeLa cells (Figure 1B, third row). On stimulation with EGF, the endogenous phospho-RSK1 in HeLa cells was localized mainly in the nucleus, as reported before (Richards *et al.*, 2001), and exposure of cells to the PDK1 inhibitor BX795 decreased the EGF-mediated nuclear localization of RSK1 (Figure 1B, bottom three rows). Of note, the PDK1 inhibitor did not affect RSK1 phosphorylation in the activation loop (Thr-573) of the C-terminal kinase, the linker region (Thr-359/Ser-363), and Ser-380, which forms the PDK1-binding site (Figure 1C). However, consistent with its role as PDK1 inhibitor, BX795 inhibited the phosphorylation of Ser-221 (the PDK1 site) and Ser-732, the site on RSK1 that is autophosphorylated by its NTK (Roux *et al.*, 2003; Figure 1C). These data suggest that Ser-221 phosphorylation is necessary for nuclear localization of RSK1. To more carefully examine the role of Ser-221 in nuclear localization of RSK1, we expressed hemagglutinin (HA)-tagged wild-type RSK1 and HA-RSK1 harboring substitutions of Ser-221 with either Ala (S221A), which cannot be phosphorylated, or Asp (S221D) to mimic phosphorylation at this site. As shown in Figure 2, A and B, upon EGF treatment of HeLa cells, the wild-type RSK1 localized to the nucleus. However, EGF did not enhance nuclear accumulation of the S221A mutant of RSK1. On the other hand, like its wild-type counterpart, the S221D form of RSK1 was localized to the nucleus after EGF treatment of HeLa cells (Figure 2, A and B). Consistent with the role of S221 phosphorylation in the activation of RSK1, *in vitro* kinase assays, the S221A form of RSK1 was inactive and did not exhibit any kinase activity (Figure 2C). In contrast, the S221D form of RSK1 was activated upon EGF addition but was not active without EGF treatment of HeLa cells (Figure 2C). This is consistent with the findings that in addition to phosphorylation of S221 (or a negative charge at this position) in the NTK, the phosphorylation of other sites in the linker region on RSK1 is necessary for activation of the NTK (Dalby *et al.*, 1998; Gross *et al.*, 2001). Of note, by immunocytochemistry, the active endogenous RSK1 (detected



**FIGURE 3:** Nuclear translocation of RSK1 requires importin-mediated transport. (A) The importin inhibitor peptide Bimax1 decreases nuclear localization of RSK1. After overnight serum starvation, HeLa cells were incubated with or without 20  $\mu$ M Bimax1 for 20 min prior to treatment with 100 nM EGF for 10 min. (B) Bimax1 does not alter EGF-mediated phosphorylation of RSK1 on the various sites. RSK1 from lysates of cells treated as in A was immunoprecipitated with anti-RSK1 antibody, and the immune complexes were probed with anti-phospho-RSK antibodies. Scale bar, 10  $\mu$ m. Representative data from three independent experiments are shown.

by anti-phospho-Thr-573 antibody) is localized mainly in the nucleus, whereas significant amounts of total endogenous or expressed RSK1 are still observed in the cytoplasm after EGF treatment (Figure 1B, top two rows, and Figure 2A). This is because the amount of active RSK1 is only a fraction of the total RSK1 and its translocation to the nucleus creates a higher concentration in the nucleus than the larger cytoplasmic compartment. Because of this concentration difference, it is difficult to clearly see the cytoplasmic active RSK1 by immunocytochemistry with anti-phospho-T573 antibody. However, by cell fractionation (see Figure 6C later in this paper) and ImageJ analyses (see Figure 5 later in this paper), active endogenous RSK1 is detectable in the cytoplasm.

Although, at first sight, the data in Figure 1, as well as in Figure 2, A–C, suggest that phosphorylation of S221 on RSK1 is necessary for its nuclear localization, we recently showed that S221 phosphorylation or substitution of Ser-221 with Asp on RSK1 also dissociates RSK1 from its interactions with the pseudosubstrate region of PKAR1 $\alpha$  (Gao *et al.*, 2010). Thus it is possible that phosphorylation of Ser-221 on RSK1 is required to release the enzyme from its interactions with PKAR1 $\alpha$  in the cytoplasm to then permit its entry into the nucleus. To address this possibility, we took advantage of our previously published findings that a cell-permeable, palmitoylated peptide corresponding to the pseudosubstrate region of PKAR1 $\alpha$  (peptide PS) dissociates it from RSK1 (Gao *et al.*, 2010). Essentially, we determined whether the cell permeable peptide PS that interferes with RSK1/PKAR1 $\alpha$  interactions dissociates the S221A form of RSK1 from PKAR1 $\alpha$  and permits its entry into the nucleus. As shown in Figure 2D, preincubation of HeLa cells with peptide PS decreased the association of RSK1 with both wild-type and S221A forms of RSK1. Moreover, whereas EGF treatment of cells did not result in nuclear localization of S221A RSK1, pretreatment of cells expressing this form of RSK1 with peptide PS permitted its nuclear localization upon EGF addition (Figure 2E). Collectively, the data in Figure 2 demonstrate that phos-

phorylation of Ser-221 on RSK1 is necessary for its dissociation from PKAR1 $\alpha$  and that, once released from this interaction, RSK1 can enter the nucleus.

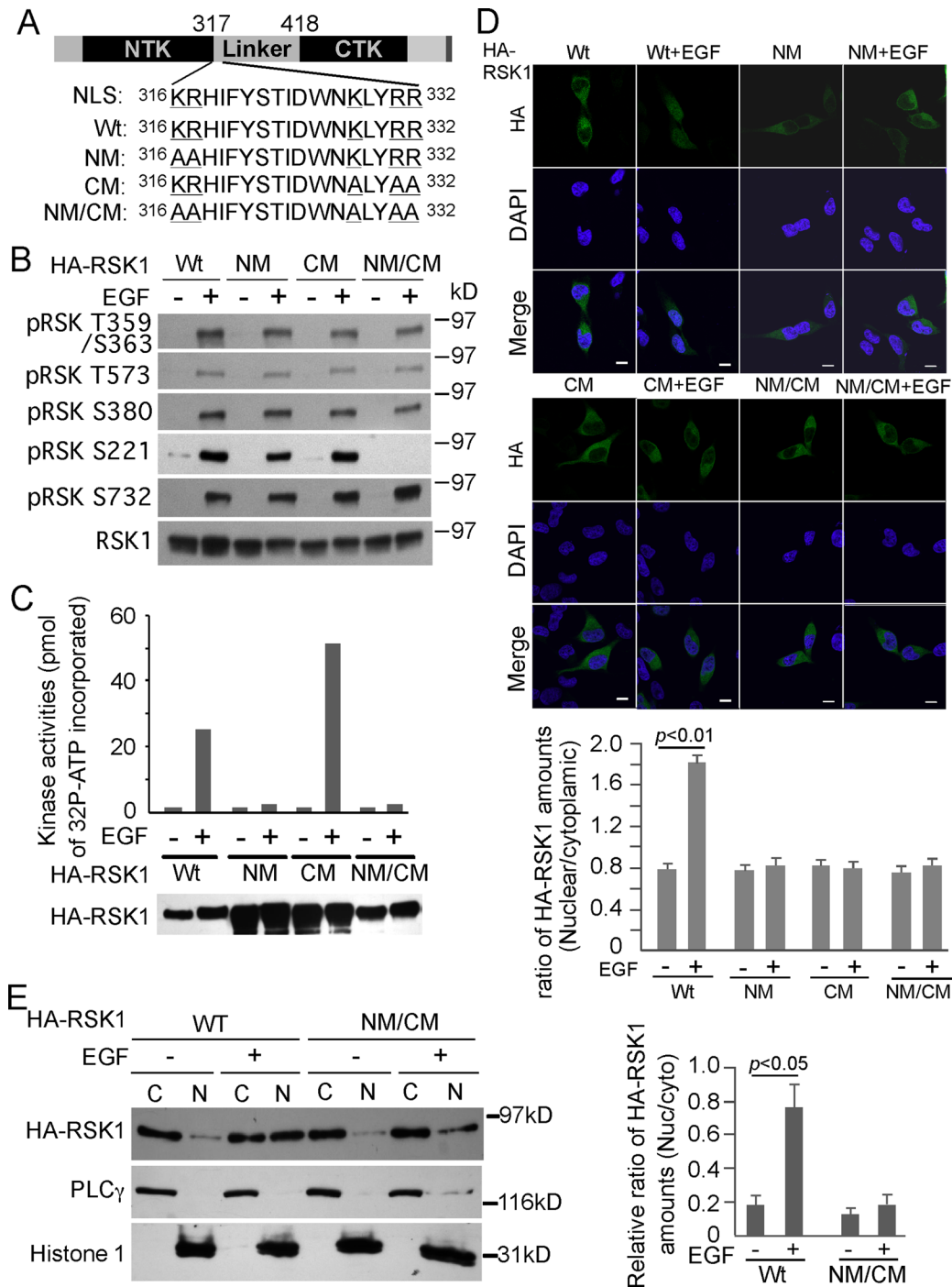
### Nuclear localization of RSK1 requires importin-mediated transport

Because a number of large proteins translocate to the nucleus via importin  $\alpha/\beta$ -mediated transport, we determined whether this mechanism was involved in the nuclear localization of endogenous, active RSK1 in HeLa cells. For this purpose, we used the recently described cell-permeable peptide Bimax1 (sequence, RRRRPRKRPLEWDEEPPRKRKRLW), which binds with importin  $\alpha$  and inhibits nuclear localization of proteins via an importin  $\alpha/\beta$ -mediated pathway (Kosugi *et al.*, 2008). Pretreatment of HeLa cells with Bimax1 for 20 min abrogated the nuclear localization of RSK1 (Figure 3A). The lack of endogenous RSK1 localization to the nucleus in the presence of Bimax1 was not due to changes in the ability of EGF to phosphorylate RSK1 on its various sites and activate it (Figure 3B). These data suggested that nuclear localization of RSK1 requires importin  $\alpha/\beta$ -mediated nuclear transport.

### RSK1 contains a bipartite nuclear localization sequence

Importin  $\alpha/\beta$ -mediated transport of proteins requires a nuclear localization sequence (NLS). A close examination of the RSK1 sequence revealed the presence of a 17-residue-long “putative” bipartite NLS (K<sub>316</sub>RHIFYSTIDWNLKLYRR<sub>332</sub>) at the interface of the NTK and linker region on RSK1 (Figure 4A). Because the basic residues on the N- and C-termini of the bipartite NLS are functionally important, we made substitutions of the N-terminal basic residues K316/R317 with Ala (NM) or the C-terminal basic residues K328/R331/R332 with Ala (CM), as well as substitutions of all basic residues in both the N- and C-terminals in the “putative” NLS with Ala (NM/CM; Figure 4A). In response to EGF treatment of cells, the phosphorylation of T573, T359/S363, or S380 on the various mutant forms of RSK1 was not altered (Figure 4B). However, the NM/CM form of RSK1 was not phosphorylated on S221 (Figure 4B). Consistent with the requirement for S221 phosphorylation being important for activation of RSK1, the NM/CM form of RSK1 that was immunoprecipitated from cells treated with EGF did not exhibit elevation in kinase activity (Figure 4C). On the other hand, although the NM mutant of RSK1 was phosphorylated on S221, it was devoid of kinase activity (Figure 4C), suggesting that the substitution of K316/R317 with Ala in the extreme C-terminus of NTK (Figure 4A) disrupted the structure of this kinase domain, rendering it inactive. The kinase activity of the CM mutant of RSK1 was enhanced by EGF treatment (Figure 4C). Another interesting finding in these experiments is the observation that EGF increased phosphorylation of S732 on the NM/CM mutant of RSK1 that is not phosphorylated on S221 and is also not activated by the growth factor (Figure 4, B and C). This suggests that phosphorylation of S732 on RSK1 can occur *in-trans* and, in the case of the NM/CM RSK1, possibly via activation by EGF of the endogenous RSK1.

Next, by confocal microscopy, we tested the ability of the HA-tagged wild-type or mutant forms of RSK1 harboring substitutions



**FIGURE 4:** Nuclear translocation of RSK1 requires its intact NLS sequence. (A) Schematic of the NLS sequences of RSK1 and its mutants. (B) Phosphorylation of wild-type and NLS mutants of RSK1 on the various sites. HeLa cells were transfected to express HA-RSK1 or its mutants and then treated with or without EGF (100 nM) for 10 min. Cell lysates were immunoprecipitated with anti-HA antibody, and then the IP complex was probed with anti-phospho-RSK antibodies. Representative data from three similar experiments are shown. (C) Kinase activity of the wild-type and NLS mutants of RSK1. After treatment of cells as in B, and following immunoprecipitation of HA-tagged RSK1 forms, the RSK1 kinase activity was determined in *in vitro* kinase activity assays. The blot below shows the amounts of HA-RSK1 or its mutants in the immune complex. The results shown are representative of three experiments performed in triplicates. (D) Mutations in the NLS of RSK1 abolish its nuclear translocation. HeLa cells were transfected to express HA-RSK1 or its mutant forms harboring substitutions in its NLS. After starvation, cells were treated with or without EGF (100 nM) for 10 min. The localization of HA-RSK1 was monitored with anti-HA antibody. Lower panel, the quantification of the amount of HA-RSK1 in the nucleus as a function of that in the cytoplasm (mean  $\pm$  SE) from 30–36 cells per each condition from three independent experiments. (E) Cellular fractionation of HeLa cells treated as in D shows that mutations in the NLS of RSK1 decrease its amounts in the nuclei. C, cytosolic; N, nuclear. PLC $\gamma$ , cytosol marker; histone 1, nuclear marker. Right, the quantitative data shown are the mean  $\pm$  SE (n = 3). Statistical significance was determined by Student's *t* test analyses. Scale bar, 10  $\mu$ m.

in the putative NLS sequence to localize in the nucleus upon EGF treatment. As shown in Figure 4D, treatment of HeLa cells with EGF resulted in the nuclear localization of the wild-type RSK1 but none of the forms of RSK1 harboring mutations in the putative NLS. The findings in Figure 4D were also confirmed by monitoring the cytoplasmic and nuclear fractions of HeLa cells expressing either the wild-type or the NLS mutant forms of RSK1 (Figure 4E). The inability of the CM mutant of RSK1 to localize to the nucleus in response to EGF is not due to the lack of either its kinase activity or its phosphorylation on S221 (Figure 4, B and C), strongly suggesting that neither the phosphorylation of S221 nor kinase activity of RSK1 by itself is sufficient for nuclear localization. Moreover, these data (Figure 4, D and E) strongly suggest that residues K328/R331/R332 form part of the NLS on RSK1.

### Retention of active RSK1 in the nucleus requires its association with AKAP(s)

We previously showed that active RSK1 interacts with PKAc, which binds to its regulatory subunit PKAR1 $\alpha$ , which in turn associates with AKAPs, and that disruption of the indirect (via PKA subunit) interaction of RSK1 with AKAPs by different approaches disrupts the nuclear localization of active RSK1 (Chaturvedi *et al.*, 2006, 2009; Gao *et al.*, 2010). Therefore we asked whether, once localized to the nucleus, RSK1 requires its indirect interactions with AKAPs to be retained in the nucleus. To address this question, we first treated HeLa cells with EGF for 10 min and then added steared-Ht31, a cell-permeable peptide that disrupts the interactions of PKA regulatory subunits with AKAPs (Carr *et al.*, 1991; Vijayaraghavan *et al.*, 1997). At different times thereafter, we examined the localization of the endogenous active RSK1 in HeLa cells. As shown in Figure 5A (right panel), EGF treatment resulted in the nuclear localization of RSK1 over the time span (20 min) of the experiment. However, when Ht31 was added after pretreatment with EGF, the amount of the nuclear RSK1 decreased over time, such that 5 min after Ht31 addition, the amount of active RSK1 in the cytoplasm was markedly increased. These data (Figure 5A) show that the retention of the active RSK1 in the nucleus requires its association in a complex with AKAP(s). Of interest, exposure of HeLa cells to the nuclear export inhibitor leptomycin B (LMB) abolished the Ht31-mediated decrease in nuclear accumulation of active RSK1 (Figure 5B), suggesting that RSK1 may also contain a nuclear export sequence (see *Discussion*). The data in Figure 5B also confirm that the loss of active RSK1 in the nucleus after Ht31 treatment was due to efflux of RSK1 from the nucleus.

### AKAP95 retains RSK1 in the nucleus

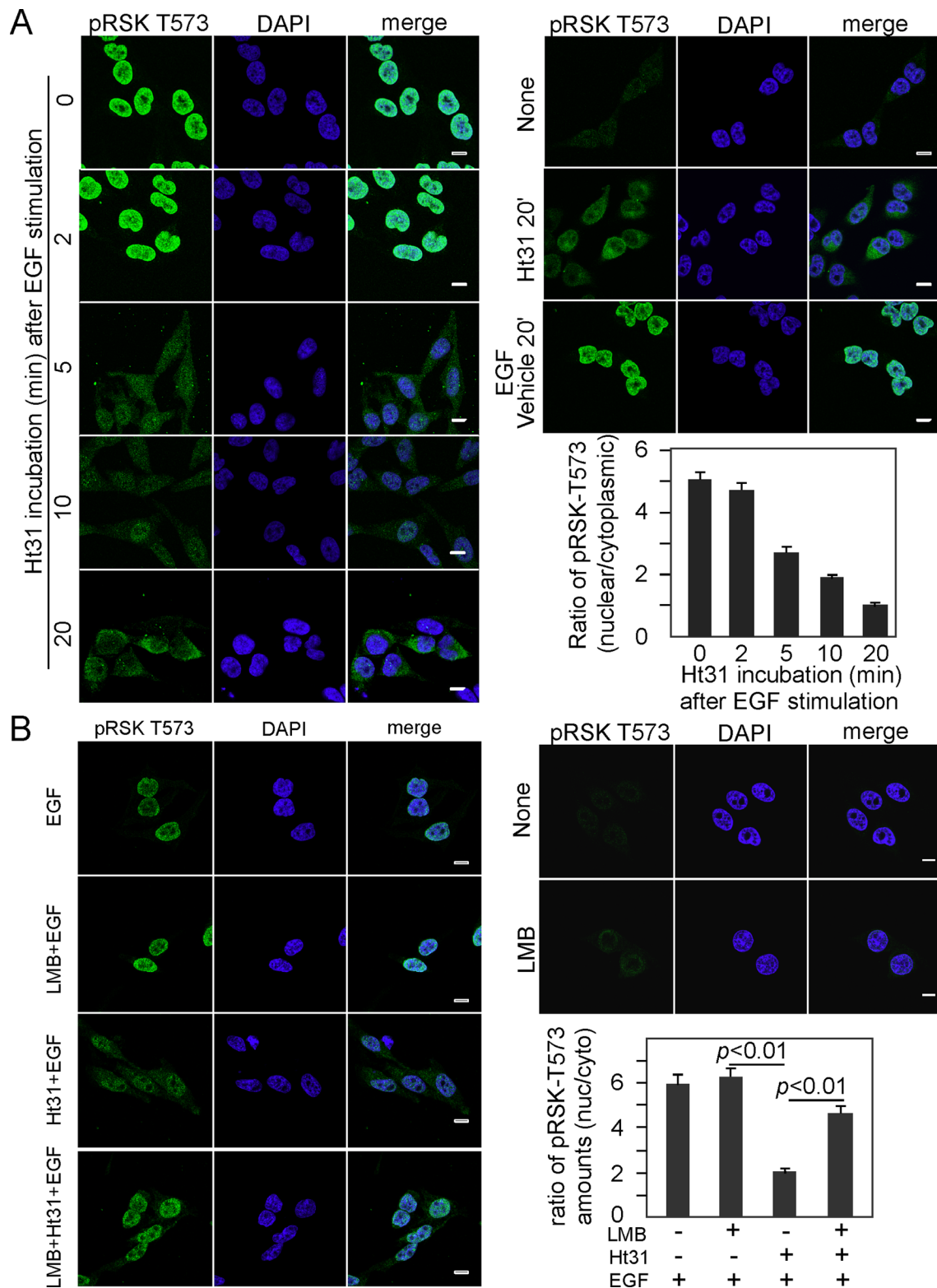
To identify the AKAP(s) that retain active RSK1 in the nucleus, we treated B82L cells with cell-permeable, steared Ht31 or control Ht31P that does not dissociate PKA regulatory subunits from AKAPs (Carr *et al.*, 1991; Vijayaraghavan *et al.*, 1997) for 20 min and then treated cells with EGF for 10 min. Following immunoprecipitation of RSK1, we analyzed the associated proteins by SDS-PAGE and gel code blue staining. Ht31 decreased a few protein bands associated with RSK1 (Supplemental Figure S1A). Liquid chromatography–mass spectrometer/mass spectrometer analyses of the ~100-kDa band in control and Ht31P yielded peptides corresponding to several proteins, including AKAP95 (Supplemental Figure S1B). AKAP95 was shown to be a nuclear protein (Coghlan *et al.*, 1994; Eide *et al.*, 1998; Akileswaran *et al.*, 2001). Therefore we investigated whether RSK1 and AKAP95 existed in the same complex. As shown in Figure 6A, immunoprecipitation of AKAP95 from HeLa cells treated with EGF resulted in the coimmunoprecipitation of RSK1. In controls with NIH3T3 cells that do not express endogenous RSK1 but do express

RSK2 and RSK3 (Supplemental Figure S2), this interaction was not observed (Figure 6A). Of note, silencing of AKAP95 did not alter the ability of EGF to activate RSK1 in HeLa cells (Figure 6B). Moreover, as assessed by cell fractionation and fluorescence microscopy, silencing of AKAP95 in HeLa cells, respectively, resulted in a decrease in the nuclear content of active RSK1 and increase in its cytosolic amounts (Figure 6, C and D). Similar results (not shown) were also found in B82L mouse lung fibroblasts, in which RSK1 interacts with PKA subunits as shown here with HeLa cells (Chaturvedi *et al.*, 2006, 2009; Gao *et al.*, 2010). Thus the inability of phospho-RSK1 to localize to the nucleus is not due to altered RSK1 activation. Together these data suggest that AKAP95 is one of the AKAPs that retain active RSK1 in the nucleus.

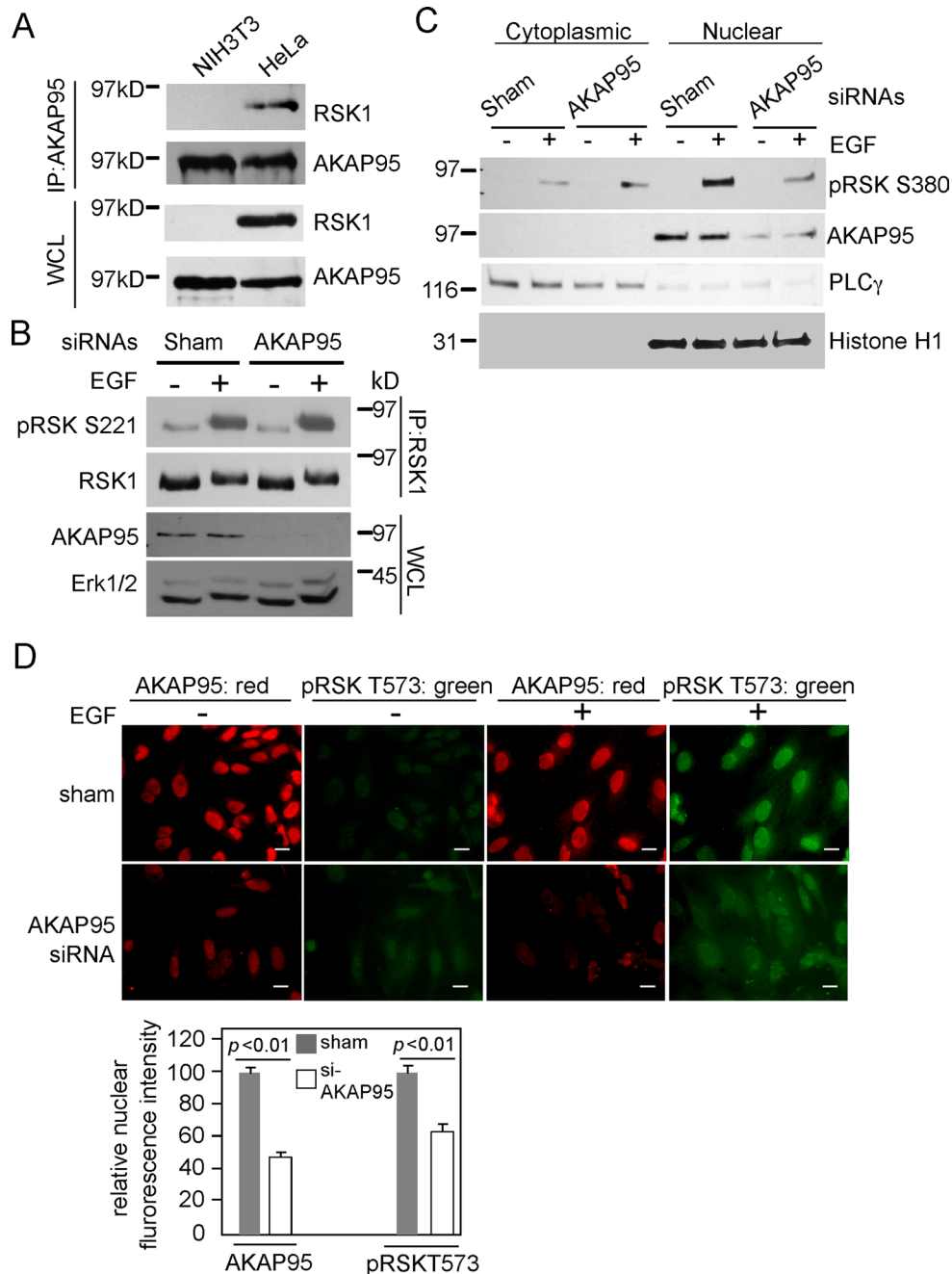
To further confirm the notion that AKAP95 retains active RSK1 in the nucleus, we expressed either the green fluorescent protein (GFP)-tagged wild-type AKAP95 or its mutant forms that contain substitutions of one (1M; R290S), two (2M; K304S/R305S), or three (3M; R290S/K304S/R305S) residues in its NLS and, therefore, do not affect (1M), attenuate (2M), or abolish (3M) the nuclear localization of AKAP95 (Kamada *et al.*, 2005). Consistent with the original report with these forms of AKAP95 (Kamada *et al.*, 2005), as monitored by immunocytochemistry in HEK293 cells, the wild-type and 1M forms were mainly nuclear, the 2M form was distributed in both the nucleus and cytoplasm, and the 3M mutant was cytoplasmic (Supplemental Figure S3, A–D). In addition, after expressing wild-type and mutant forms of GFP-AKAP95 in HEK 293 cells and treating them with EGF, we determined by cell fractionation studies the localization of the endogenous RSK1 in the nucleus and cytosolic fractions of the cells. As shown in Figure 7A, in the presence of EGF, more phospho-RSK1 was present in the nuclear fractions than in the cytosolic fractions from cells transfected with the wild-type and 1M mutant forms of AKAP95. The distribution of phospho-RSK1 was approximately similar in the cytosolic and nuclear fractions from cells transfected with GFP-AKAP95 2M and mostly cytosolic in cells expressing GFP-AKAP95 3M (Figure 7A). Similarly, in HeLa cells, the expressed wild-type GFP-AKAP95 was localized to the nucleus together with active (phospho-380) RSK1, whereas expression of the 3M mutant of GFP-AKAP95 resulted in colocalization of the active RSK1 with the 3M mutant in the cytoplasm (Figure 7B). These findings were also corroborated by immunolocalization of the wild-type AKAP95 or its 3M mutant and RSK1 (Figure 7C). Of note, mutations of the NLS in AKAP95 did not alter its ability to be coimmunoprecipitated with RSK1 (Supplemental Figure S3E). Together, the data in Figure 7 demonstrate that active RSK1 interacts with AKAP95 in cells and the 3M mutant of AKAP95, which cannot be localized to the nucleus, sequesters active RSK1 in the cytoplasm. In this latter respect, the overexpressed 3M mutant of AKAP95 acts as a “trap” for RSK1 and precludes its localization into the nucleus.

### Nuclear localization of RSK1 is required for stimulation of DNA synthesis

Because RSK1 was shown to play an important role in cell proliferation by promoting G1  $\rightarrow$  S progression (Fujita *et al.*, 2003; Cargnello and Roux, 2011), using two different approaches we investigated whether its translocation to the nucleus is important to initiate DNA synthesis. In the first approach, we investigated whether the nuclear function of the CM mutant form of RSK1 that is activated by EGF but does not localize to the nucleus (Figure 4) is different from its wild-type counterpart. For this purpose, we monitored bromodeoxyuridine (BrdU) incorporation in NIH3T3 cells, which express RSK2 and RSK3 but do not express any detectable amounts of endogenous RSK1 (Supplemental Figure S2). To



**FIGURE 5:** Retention of active RSK1 in the nucleus requires its indirect (via PKA) interactions with AKAPs. (A) Ht31 decreases the nuclear retention of active RSK1 in the nucleus. After serum starvation, HeLa cells were treated with or without 100 nM EGF for 10 min, followed by incubation with 20  $\mu$ M Ht31 for the indicated times to dissociate PKA from AKAP(s). Immunocytochemical analyses were performed with anti-phospho-T573 RSK antibody. Right, controls with Ht31 or EGF alone for 20 min. The bar graph shows quantification (mean  $\pm$  SE) of the nuclear phospho-T573 RSK as a ratio of that in the cytoplasm from 45–51 cells per each condition from three separate experiments. (B) LMB abolishes Ht31-mediated efflux of RSK1 from the nucleus. After serum starvation, HeLa cells were incubated with or without LMB (20 ng/ml) for 2 h, followed by treatment with or without 20  $\mu$ M Ht31 for 15 min. Subsequently, cells were stimulated with or without 100 nM EGF for 10 min. RSK1 translocation was monitored with anti-pRSK T573 antibody. Scale bar, 10  $\mu$ m. Data from 41–44 cells per each condition from three experiments were quantified as in A.

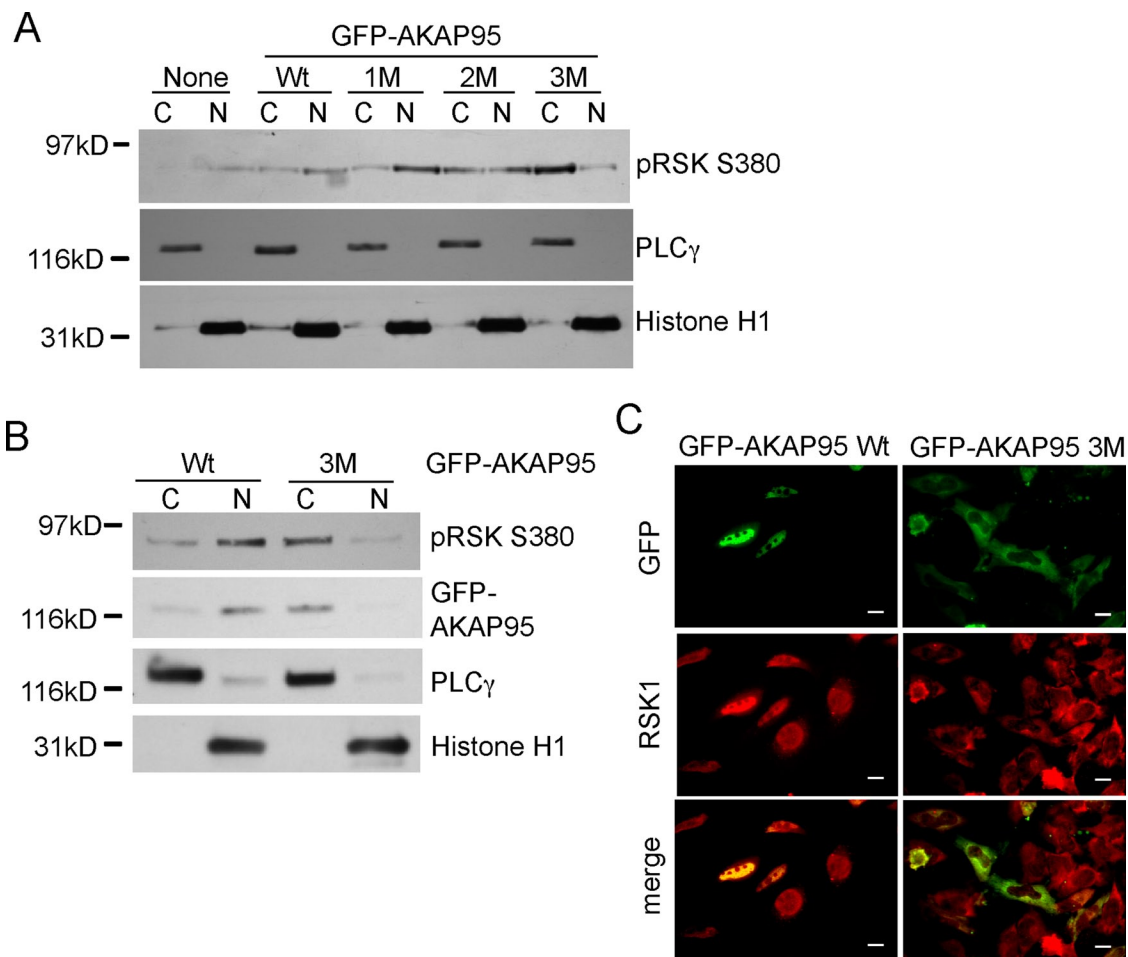


**FIGURE 6:** RSK1 is present in a complex with AKAP95, and silencing of AKAP95 decreases phospho-RSK1 in the nucleus. (A) Immunoprecipitates of AKAP95 from HeLa cells, but not NIH3T3 cells, contain RSK1. NIH3T3 cells used as controls do not express any detectable amounts of RSK1. AKAP95 was immunoprecipitated from HeLa and NIH3T3 cells, and the presence of endogenous RSK1 in the immunocomplex was monitored using anti-RSK1 antibody. WCL, whole-cell lysate. (B) Silencing of AKAP95 in HeLa cells does not alter activation of RSK1. The phosphorylation of RSK1 was detected after immunoprecipitation with anti-RSK1 specific antibody from control and AKAP95-specific small interfering RNA (siRNA)-treated cells. (C) Silencing of AKAP95 in HeLa cells alters the distribution of phospho-RSK in cytoplasmic and nuclear fractions. After transfection with AKAP95 siRNA for 48 h and overnight serum starvation, HeLa cells were stimulated with EGF and processed for the isolation of cytosolic and nuclear fractions. Western analyses of the fractions was performed with anti-phospho-S380 RSK and AKAP95 antibodies. PLC $\gamma$ , cytoplasmic marker; Histone H1, nuclear marker. (D) Silencing of AKAP95 decreases nuclear localization of phosphorylated endogenous RSK. HeLa cells were treated with or without EGF after transfection with AKAP95 siRNA for 48 h as in C. Immunocytochemical analyses with anti-AKAP95 and anti-phospho-Thr573 RSK antibodies were performed. Quantification of nuclear fluorescence of AKAP95 and phospho-RSK (mean  $\pm$  SEM) from 41–49 cells per condition from three independent experiments is shown in the lower panel. Scale bar, 10  $\mu$ m. The data in A–C are representatives of three independent experiments.

facilitate interpretation, the data were quantified to reflect BrdU-positive cells as a ratio of the total number of cells that expressed the desired wild-type or mutant forms of either RSK1 or AKAP95.

The BrdU staining of untransfected cells in the same fields served as internal controls. As shown in Figure 8A, in NIH3T3 cells, EGF activated both the wild-type and CM forms of RSK1 to similar





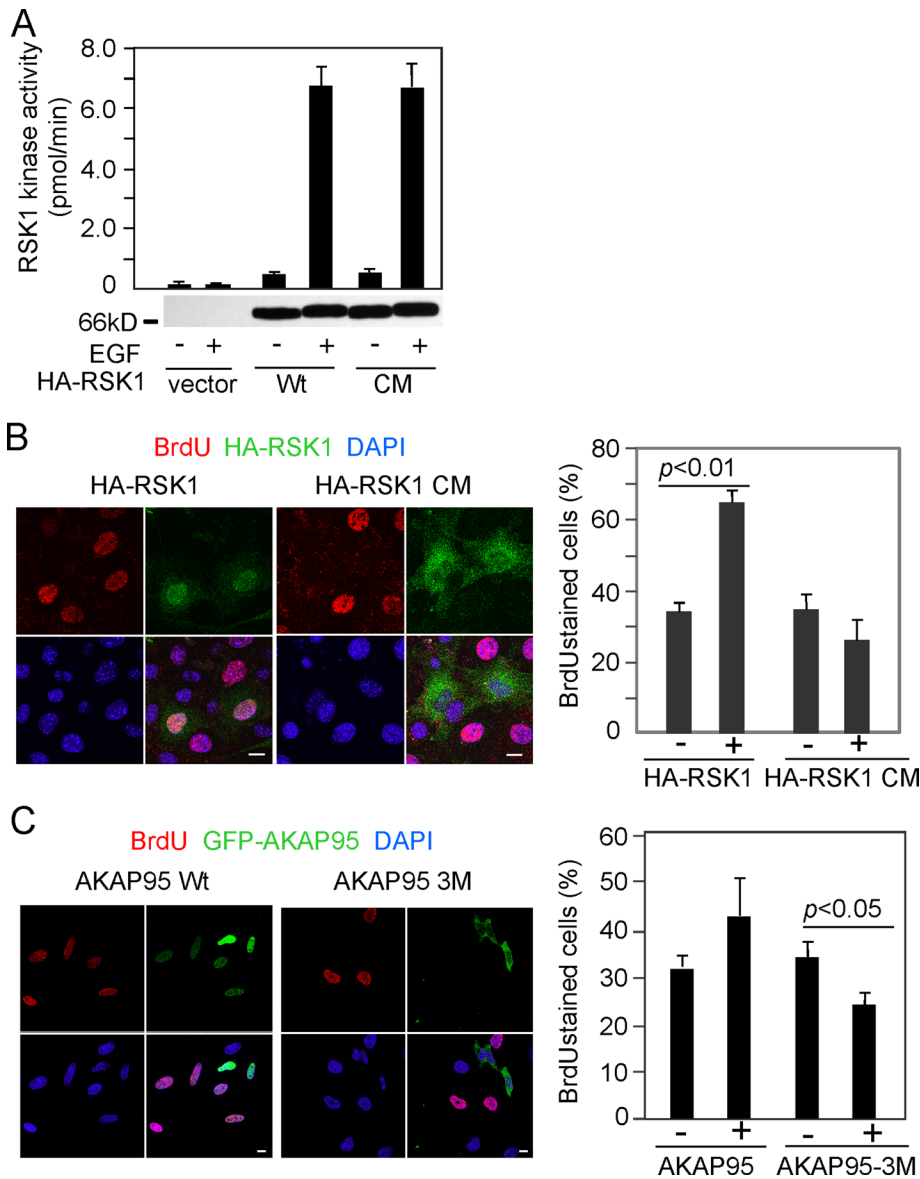
**FIGURE 7:** Overexpression of wild-type and NLS mutant forms of GFP-AKAP95 alter the cellular localization of endogenous RSK1. (A) HEK293T cells transfected with wild-type AKAP95 or its 1M, 2M, or 3M mutants were serum starved and treated with EGF. The cytosolic and nuclear fractions were isolated for Western blotting. Phospho-RSK1 was detected with anti-pRSK S380 antibody. C, cytosolic; N, nuclear. Histone H1, nuclear marker; PLC $\gamma$ , cytosolic marker. (B) HeLa cells were transfected with the wild-type and 3M mutant of AKAP95 and treated as in (A). Following cell fractionation, the active RSK1 and AKAP95 were monitored. (C) More of the endogenous RSK1 is present in the nuclei of HeLa cells expressing wild-type GFP-AKAP95 than those expressing the 3M form of GFP-AKAP95. After 48 h of transfection, cells were serum starved overnight and stimulated with EGF. RSK1 was visualized with anti-RSK1 specific antibody. Data shown are representative of three independent experiments.

extents. However, the wild-type RSK1 enhanced BrdU incorporation, but the CM mutant did not (Figure 8B). As a second approach, we expressed either wild-type or 3M mutant of AKAP95 in HeLa cells to determine whether permitting (with wild-type AKAP95) or restricting (with 3M-AKAP95) the nuclear localization of endogenous RSK1 altered BrdU incorporation into cells. Whereas the expression of wild-type AKAP95 did not alter BrdU incorporation, the 3M mutant of AKAP95, which sequesters active RSK1 in the cytoplasm, decreased BrdU incorporation (Figure 8C). These data demonstrate that disruption of the nuclear localization of active RSK1 by either mutation of its NLS or “trapping” of endogenous RSK1 in the cytoplasm by the nuclear localization-deficient 3M mutant of AKAP95 attenuates the nuclear functions of RSK1.

## DISCUSSION

Besides elucidating how active RSK1 is localized and retained in the nucleus, we unraveled some additional salient features concerning how this enzyme is regulated. First, as previously suggested (Gao *et al.*, 2010), although the phosphorylation of Ser-221 is necessary for nuclear localization of RSK1, we demonstrated that this phos-

phorylation is required to dissociate the active RSK1 from PKAR1 $\alpha$  in the cytoplasm. Hence the S221A mutant of RSK1, which does not localize to the nucleus upon EGF treatment of cells, can localize to the nucleus if its interaction with PKAR1 $\alpha$  is disrupted by low concentrations of a cell-permeable peptide (peptide PS) corresponding to the pseudosubstrate region of PKAR1 $\alpha$ , the inactive RSK1-binding site (Gao *et al.*, 2010). At the low concentrations of peptide PS used in this study, PKAc and PKAR1 $\alpha$  interactions are not affected (Gao *et al.*, 2010). Because the S221A mutant of RSK1 is not active, our findings with peptide PS also demonstrate that the inactive RSK1 can enter the nucleus. However, because nuclear localization of the S221A mutant of RSK1 is EGF dependent, our findings suggest that the other sites on RSK1 have to be phosphorylated for its nuclear entry. Overall, therefore, although phosphorylation of S221 is required to release RSK1 from PKAR1 $\alpha$  and permit its nuclear translocation, the activity of the NTK is not necessary for its nuclear translocation. Second, supporting the notion that phosphorylation of sites in the linker region of RSK1 are necessary for its activity (Dalby *et al.*, 1998; Gross *et al.*, 2001), we showed that the S221D mutant of RSK1 also requires EGF for activation of the NTK and that a negative



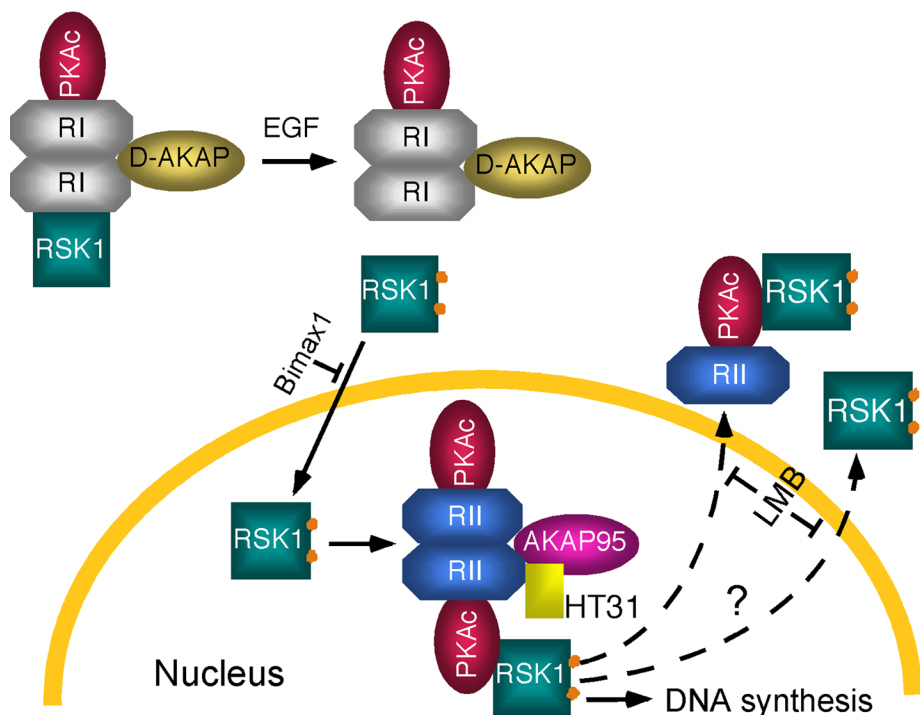
**FIGURE 8:** Nuclear translocation of RSK1 is required to stimulate DNA synthesis. (A) HA-RSK1 and its CM mutant form demonstrate similar kinase activities. NIH3T3 cells were transfected to express HA-RSK1 or its CM mutant. After serum starvation, cells were treated with or without 100 nM EGF for 10 min. HA-RSK1 and HA-RSK1 CM were immunoprecipitated with anti-HA antibody, and the IP complex was subjected to the *in vitro* kinase assays. The amounts of HA-RSK1 in the IP were monitored with Western blotting (bottom). Data are mean  $\pm$  SE ( $n = 3$  independent experiments). (B) HA-RSK1, but not its CM mutant, stimulate BrdU incorporation. NIH3T3 cells were transfected to express HA-RSK1 or HA-RSK1 CM. After serum starvation, cells were incubated with 10% serum/DMEM for 18 h, followed by the addition of BrdU for 4 h. (C) Overexpression of GFP-AKAP95 3M, but not wild-type GFP-AKAP95, decreases BrdU incorporation in HeLa cells. After transfection to express wild-type GFP-AKAP95 or its 3M mutant in HeLa cells, experiments were performed as in B. The quantified data in B and C reflect the number of BrdU-positive cells as a ratio of the total number of cells that expressed the different transfected constructs. The BrdU-positive staining in *untransfected* cells *within the same fields* provided the control ratios. The data are mean  $\pm$  SE from at least 102 cells from four separate experiments (B) and at least 181 cells from three experiments (C). Statistical significance was determined by Student's *t* test analyses. Scale bar, 10  $\mu$ m.

charge at this position by itself is not sufficient to activate the enzyme. A third interesting aspect is that the substitution of K316/R317 with Ala abrogated the activity of the NTK without altering its phosphorylation on Ser-221. Thus these two basic residues in the extreme C-terminus of the NTK, which lie on its surface (Ikuta *et al.*,

2007), are important for activity, and their substitution perhaps alters the conformation of the NTK to abrogate its activity.

We also demonstrated that RSK1 contains a bipartite NLS, and substitution of basic residues in its N- or C-terminus abrogates its translocation to the nucleus after EGF addition. Because substitution of both the N-terminal (K316/R317) and C-terminal (K328/R331/R332) basic residues in the NLS with Ala (RSK1-NM/CM) also disrupts its phosphorylation on S221, this form of RSK1 most likely does not translocate to the nucleus because it cannot dissociate from PKAR1 $\alpha$ . However, substitutions of basic residues in only the N-terminal (K316/R317, RSK1-NM) or C-terminal (K328/R331/R332, RSK1-CM) regions of the NLS that permit the phosphorylation of S221 on these mutant forms also do not translocate to the nucleus irrespective of whether the activity of the NTK can be augmented by EGF (RSK1-CM) or not (RSK1-NM). These findings, coupled with the observations that phosphorylation of S221 or a negative charge at this position, but not the activity of the NTK, is required for nuclear translocation of RSK1 demonstrate that the 17-residue-long region comprising K316-R332 is the NLS. This region is encompassed by the  $\alpha$ I- $\alpha$ J loop as well as the  $\alpha$ J helix at the surface of the large helical lobe of the NTK (Ikuta *et al.*, 2007) and is conserved in RSK1 in different species such as mouse, rat, and human. In addition, cognate regions comprising residues K313-K329 and K322-R338 on RSK2 and RSK3, respectively, also have significant similarity to the NLS on RSK1. Thus it is possible that these regions on RSK2 and RSK3 are also their respective NLSs. However, in one study, the expression of the N-terminal half of RSK2, which included residues K313-K329, did not localize to the nucleus, and that study suggested that the C-terminal half of RSK2 is important for its nuclear localization (Eisinger-Mathason *et al.*, 2008), raising the possibility that the NLS containing K313-329 is functional in the context of the full-length RSK2 when the autophosphorylation sites on the linker region are phosphorylated.

We previously showed that the active RSK1 via its extreme C-terminal 13 residues, which include Ser-732, associates with PKAc, and this interaction requires the phosphorylation of Ser-732 (Gao and Patel, 2009). This interaction permits the indirect (via the regulatory subunit of PKA) association of the active RSK1 with AKAPs, and disruption of the RSK/PKA complex from AKAPs results in decreased nuclear localization of RSK1. The decreased nuclear localization could result from either export of the RSK1 from the nucleus or the lack of RSK1 entry into the nucleus. Here we showed that



**FIGURE 9:** Schematic representing the mechanisms of nuclear localization and retention of RSK1 following its activation. Inactive RSK1 is associated with PKAR1 $\alpha$  (RI) in the cytoplasm. Activation of RSK1 dissociates it from PKAR1 $\alpha$  and permits its entry into the nucleus, where, via interactions with catalytic subunit of PKA (PKAc) and AKAP95, it is retained in the nucleus. Disruption of the PKA/AKAP95 interactions with Ht31 result in decreased RSK1 retention in the nucleus, an event that is LMB sensitive. Whether the RSK1 is exported from the nucleus in complex with PKAc or by itself is not currently known and is depicted by a question mark. D-AKAP, dual-specificity AKAPI known to be present in the RSK1/PKAR1 $\alpha$  complex (see the text).

once the active RSK1 is in the nucleus, the addition of Ht31, which disrupts PKA interactions with AKAPs, results in a time-dependent export of the active RSK1 from the nucleus. Hence the interaction of active RSK1 with PKAc and, therefore, AKAP(s) is necessary for its retention in the nucleus. The need for RSK1 interactions with PKA subunits and AKAP(s) for nuclear retention is akin to the nuclear retention of RSK2 via its interactions with TIA-1 (Eisinger-Mathason *et al.*, 2008).

Our studies also identified AKAP95 as one of the AKAPs that is necessary for the retention of RSK1 in the nucleus. Of interest, AKAP95 associates with the RII subunits of PKA (Herberg *et al.*, 2000). Previously, we demonstrated that the inactive RSK1 interacts with RI but not RII subunits (unpublished data). However, since the active RSK1 interacts with PKAc, it is possible that in the nucleus the RSK1, via PKAc and PKARII, is tethered to AKAP95 (see schematic in Figure 9). This would be consistent with the notion that PKAR1 $\alpha$  is cytosolic in its localization whereas nuclear PKARII is responsible for association of PKAc with nuclear AKAPs (Day *et al.*, 2011). Also interesting are the previous findings that AKAP95 is important in G1  $\rightarrow$  S phase transition and is associated with proteins that regulate this process (Arsenijevic *et al.*, 2004, 2006). In addition, AKAP95 is involved in chromosome condensation at mitosis (Collas *et al.*, 1999). Similarly, RSK1 is also important for G1  $\rightarrow$  S progression (Fujita *et al.*, 2003; Cargnello and Roux, 2011). Thus, it appears that AKAP95, by associating with RSK1 and the other proteins, facilitates G1  $\rightarrow$  S progression, DNA synthesis, chromosome condensation, mitosis, and orchestrating of overall cell cycle progression.

The disruption of the RSK1 interactions with AKAPs results in its export from the nucleus (Figure 5). It is not clear whether the RSK1

is exported out of the nucleus while it is still attached to PKAc and the regulatory subunit of PKA (Figure 9). Because leptomycin B blocked the Ht31-mediated efflux of RSK1 from the nucleus (Figure 5B), it is possible that RSK1 contains a nuclear export signal. However, a nuclear export signal for RSK1 has not been identified. From its primary structure, it appears that residues Leu-173 to Ser-180 on RSK1 qualify as a putative nuclear export sequence. These residues in the  $\alpha$ E helix are adjacent to the NLS on the surface of the large helical loop of the NTK on RSK1 (Ikuta *et al.*, 2007). Because substitutions of K316 and R317 that are adjacent to the putative nuclear export signal (L173-S180) on the surface of the NTK disrupt its activity, we have not endeavored to mutate the putative RSK1 nuclear export signal.

In NIH3T3 cells, which do not express appreciable amounts of RSK1, expression of wild-type RSK1, but not its NLS-deficient CM mutant, increased DNA synthesis as monitored by BrdU staining. These data are consistent with the role of RSK1 as a promoter of cell proliferation (Smith *et al.*, 2005) and also demonstrate that the NLS mutant of RSK1 loses its nuclear function. Similarly, overexpression of GFP-AKAP 3M, which traps endogenous RSK1 in the cytoplasm, also diminished incorporation of BrdU, further demonstrating the necessity for nuclear RSK1 in this process.

Overall, our findings demonstrate that RSK1 contains an NLS (K316-R332) that is necessary for its import into the nucleus and that phosphorylation of Ser-221 in the NTK is required to dissociate it from PKAR1 $\alpha$ /AKAP complex in the cytoplasm. The activation of the NTK is not necessary for nuclear translocation of RSK1, and besides S221, other phospho sites on RSK1 are also important for both its activity and nuclear import. Once inside the nucleus, RSK1 requires indirect (via PKA) interactions with AKAP95 to be retained in the nucleus. Moreover, mutations of the NLS on RSK1 that do not affect its kinase activity but interfere with its nuclear localization or trapping of RSK1 in the cytoplasm by an NLS-deficient mutant of AKAP95 diminishes BrdU incorporation in cells.

## MATERIALS AND METHODS

### Reagents

Anti-RSK1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-RSK1/2 (S221) from R&D Systems (Minneapolis, MN), anti-phospho-RSK (T359/S363) from Cell Signaling (Beverly, MA), anti-phospho-RSK (S380) from Epitomics (Burlingame, CA), anti-phospho-RSK (T573) from Cell Signaling, and anti-phospho-RSK S732 raised by Rockland (Gilbertsville, PA) were used. Anti-PKAR1 $\alpha$  was from BD Biosciences (Palo Alto, CA). Anti-Erk1/2 was from Upstate Biotechnology (Lake Placid, NY). Anti-HA-horseradish peroxidase was from Roche Applied Science (Indianapolis, IN), and the anti-HA polyclonal antibody was from Covance (Berkeley, CA). Anti-BrdU was from Calbiochem (La Jolla, CA). N-terminally palmitoylated PKAR1 $\alpha$  peptide (PS), corresponding to PKAR1 $\alpha$  pseudosubstrate region (amino acids 91–99), was synthesized by New England Peptide (Gardner, MA). N-terminally

palmitoylated Bimax1 peptide, which inhibits the importin  $\alpha/\beta$  nuclear import pathway (Kosugi *et al.*, 2008), was synthesized by Neo Peptide (Cambridge, MA). Kemptide was from Sigma-Aldrich (St Louis, MO). BX795 was from Axon Medchem (Groningen, Netherlands).

### Plasmids

pHM6-RSK1 (rat) wild-type or S221 mutants expressing HA-tagged RSK1 were described previously (Gao *et al.*, 2010). Mutations in the nuclear localization signal sequence of RSK1 were inserted using mutagenic primers and universal PCR of pHM6-RSK1. GFP-tagged AKAP95 and its mutant forms (1M, 2M, and 3M) harboring mutations in its nuclear localization sequence (Kamada *et al.*, 2005) were obtained from Shinji Kamada and Tony Hunter (Salk Institute, San Diego, CA).

### Cell culture and transfection

Both HeLa cells and NIH3T3 cells were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and penicillin/streptomycin. HeLa cells were plated in 60-mm dishes at  $4 \times 10^5$ /dish and transfected with plasmids using a TransIT HeLaMONSTER-Transfection Kit (Mirus Bio, Madison, WI) following the manufacturer's protocol. NIH3T3 cells were plated in 60-mm dishes at  $2 \times 10^5$ /dish and transfected with Transit 2020 Transfection Reagent (Mirus Bio).

### Immunoprecipitation

HeLa cells were serum starved overnight and then were treated with or without 100 nM EGF for 10 min. Cells were washed twice with cold phosphate-buffered saline and scraped into lysis buffer containing 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 8.0, 150 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 1 mM ethylene glycol tetraacetic acid, 50 mM NaF, 1% Triton X-100, 0.1% (vol/vol)  $\beta$ -mercaptoethanol, and protease inhibitor mixture (Roche Diagnostics). A total of 500  $\mu$ g of cleared cell lysates was incubated for 2 h at 4°C with 0.8  $\mu$ g of anti-RSK1 antibody or anti-HA monoclonal antibody (1:250) together with 15  $\mu$ l of protein G-conjugated agarose beads. After three washes with lysis buffer, the immunoprecipitates were eluted with Laemmli sample buffer and subjected to Western analyses.

### Pulldown of PKAR1 $\alpha$ with cAMP-agarose

Serum-starved HeLa cells were preincubated with or without 2  $\mu$ M PKAR1 $\alpha$  peptide PS for 10 min and then treated with or without EGF for 10 min. Cleared cell lysates (500  $\mu$ g of protein) were mixed with cAMP-agarose (Sigma-Aldrich) for 2 h at 4°C. The beads were then washed twice with high-salt buffer (10 mM HEPES, pH 7.4, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 M NaCl, 0.1% Igepal CA-630, 1 mM dithiothreitol, and proteinase inhibitor mixture) followed by two washes with no-salt buffer (same as high-salt buffer but without NaCl). Proteins in the complex were resuspended in Laemmli buffer and subjected to Western analyses using anti-PKAR1 $\alpha$  and anti-HA horseradish peroxidase.

### In vitro kinase activity assays

The assays were performed as described previously (Gao *et al.*, 2010). Essentially, HeLa cells were transfected to express HA-RSK1 or its mutants. After overnight serum starvation, cells were treated with or without 100 nM EGF for 10 min. HA-RSK1 or its mutants were immunoprecipitated as described, and then the immunoprecipitate (IP) complex was resuspended in 20 mM HEPES, pH 7.5, 0.1% mercaptoethanol, 1 mM sodium orthovanadate, 1 mM NaF,

25 mM glycerophosphate, and 5 mM MgSO<sub>4</sub>. The reaction was started with addition of 200  $\mu$ M Kemptide, 125  $\mu$ M ATP, and 2.5  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP and stopped after 15 min of incubation at room temperature. One parallel set of IP complex was resuspended in Laemmli sample buffer for Western blotting to monitor the amounts of HA-RSK1 or its mutants in the complex.

### Immunocytochemistry

HeLa cells were plated in eight-chamber slides at 10,000 cells/well. After serum starvation, cells underwent different treatments as indicated in the figure legends. After fixation with 4% formaldehyde in phosphate-buffered saline (PBS) for 10 min, cells were permeabilized with 0.3% Triton X-100 in PBS for 10 min, followed by 1 h of blocking with 5% bovine serum albumin in PBS. Cells were stained with primary antibodies, rabbit anti-RSK1, rabbit anti-phospho-T573 RSK, rabbit anti-AKAP95, or mouse anti-HA antibody overnight at 4°C. Fluorescence staining was performed using Alexa 488-conjugated goat anti-rabbit or anti-mouse antibody (green) or Alexa 594-conjugated goat anti-rabbit antibody (red). Confocal microscopy was performed using a multiphoton Zeiss LSM-510 laser scanning microscope (Carl Zeiss, Jena, Germany) equipped with a C-Apo 40 $\times$ /1.2 water lens. Confocal images were acquired with LSM 510 software. Nonconfocal images were acquired with an Olympus IX81 microscope (Olympus, Center Valley, PA) equipped with 40 $\times$ /0.60 lens and Retiga EXi digital camera (QImaging, Surrey, Canada). For quantification, the fluorescence densities in the nucleus or cytoplasm were analyzed with ImageJ (National Institutes of Health, Bethesda, MD).

### Cell fractionation

Cells were homogenized after incubation in hypotonic buffer from the nuclear extraction kit (Active Motif, Carlsbad, CA) on ice for 15 min. The cell homogenates were centrifuged at 200  $\times$  g to remove intact cells and cell debris. The supernatant was centrifuged at 1000  $\times$  g for 10 min to pellet cell nuclei. The nuclear pellet was washed and centrifuged again. The 1000  $\times$  g supernatants were then centrifuged at 100,000  $\times$  g to generate the cytosolic fraction. The proteins in nuclear and cytosolic fractions were subjected to Western analysis.

### BrdU incorporation

NIH3T3 cells were plated in eight-chamber slides at 10,000 cells/well. Cells were transfected with plasmids expressing HA-RSK1 or its mutant using Metafectene Pro transfection reagent (Biontex Laboratories GmbH, Martinsried, Germany). Similarly, HeLa cells were transfected with GFP-AKAP95 or its 3M mutant, which does not localize inside the nucleus, using TransIT-HeLaMONSTER Transfection Kit. One day after transfection, cells were serum starved for 24 h and then replaced with DMEM containing 10% fetal bovine serum for 18 h, followed by addition of 10  $\mu$ M BrdU and 4 h of incubation. The cells were fixed with 4% formaldehyde/PBS for 10 min at room temperature and then were permeabilized with 0.3% Triton X-100/PBS for 10 min. To denature DNA, cells were incubated with 2 N HCl at room temperature for 30 min. After extensive washes with PBS and 5% bovine serum albumin/PBS blocking, cells were incubated with anti-BrdU antibody (mouse monoclonal) and anti-HA antibody (rabbit polyclonal) at 4°C overnight. The secondary antibodies were Alexa 594-conjugated goat anti-mouse antibody or Alexa 488-conjugated goat anti-rabbit antibody. The images were obtained by a multiphoton Zeiss LSM-510 laser-scanning microscope as described. The results are expressed as the percentage of cells showing nuclear staining with BrdU.

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