The KH-Tudor Domain of A-Kinase Anchoring Protein 149 Mediates RNA-Dependent Self-Association†

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ABSTRACT: A-Kinase anchoring proteins (AKAPs) control the subcellular localization and temporal specificity of protein phosphorylation mediated by cAMP-dependent protein kinase. AKAP149 (AKAP1) is found in mitochondria and in the endoplasmic reticulum−nuclear envelope network where it anchors protein kinases, phosphatases, and a phosphodiesterase. AKAP149 harbors in its COOH-terminal part one KH and one Tudor domain, both known to be involved in RNA binding. We investigated the properties of the COOH-terminal domain of AKAP149. We show here that AKAP149 is a self-associating protein with RNA binding features. The KH domain of AKAP149 is sufficient for self-association in a RNA-dependent manner. The Tudor domain is not necessary for self-association, but it is required together with the KH domain for targeting to well-defined nuclear foci. These foci are spatially closely related to nucleolar subcompartments. We also show that the KH-Tudor-containing domain of AKAP149 binds RNA in vitro and in RNA coprecipitation experiments. AKAP149 emerges as a scaffolding protein involved in the integration of intracellular signals and possibly in RNA metabolism.

In the context of the wide array of intracellular effects mediated by cAMP signaling through cAMP-dependent protein kinase (PKA),[1] subcellular localization and temporal specificity of protein phosphorylation by PKA are mediated by proteins collectively termed A-kinase anchoring proteins (AKAPs) (*1*, *2*). AKAPs bind a PKA regulatory subunit dimer through a consensus sequence, whereas a targeting domain specifies subcellular localization. AKAPs also interact with other signaling molecules such as protein kinase C (PKC), phosphodiesterases, and protein phosphatases in a space- and time-regulated fashion (*2*, *3*).

AKAP149 (*4*), also designated AKAP1, is a human 149 kDa anchoring protein homologous to mouse AKAP121 identified in mitochondria (*5*−*7*) and in the endoplasmic reticulum−nuclear envelope (ER−NE) network (*7*, *8*). AKAP149 harbors the hallmarks of a protein phosphatase 1 (PP1) regulatory subunit and targets a fraction of nuclear PP1 to the vicinity of the NE upon nuclear reassembly in vitro and at the end of mitosis (*8*, *9*). AKAP149 has been shown to enhance PP1 phosphatase activity toward B-type lamins upon re-formation of NE at mitosis exit, thus promoting lamin dephosphorylation and polymerization (*8*, *9*). AKAP149 has also been shown to bind PKCα in immunoprecipitation experiments from NE fractions (*8*, *10*) and phosphodiesterase PDE4A (*11*). In addition, AKAP121 binds protein tyrosine phosphatase D1, a classical nonreceptor protein tyrosine phosphatase known to bind and activate the tyrosine kinase Src (*12*), and binds Src itself (*13*). These findings suggest that AKAP121 focuses PKA and Src signaling to mitochondria.

AKAP121/AKAP149 has been shown in two different instances to bind RNA. AKAP121 binds in vitro to the 3′ untranslated region (UTR) of two transcripts for mitochondrial proteins (*14*). AKAP121/AKAP149 also binds to the mRNA 3′ UTR of lipoprotein lipase, an extracellular enzyme hydrolyzing the triglyceride core of circulating chylomicrons (*15*, *16*). This interaction is critical for the decrease in the extent of LPL mRNA translation following PKA stimulation in white adipose tissue (*15*, *16*). In both cases, binding of AKAP121 to RNA is mediated by a single COOH-terminally situated K homology (KH) domain, which is a known RNA-binding motif (*17*, *18*). KH domains have also been implicated in the oligomerization of proteins such as fragile X mental retardation protein (FMRP), the expression of which is altered in fragile X syndrome (*19*). In addition to a KH domain, AKAP121/AKAP149 contains a single COOH-terminal Tudor domain, which is also found in many RNA-interacting proteins (*20*). Tudor domains may also be involved in DNA binding (*21*) and protein−protein interaction through binding to arginine-glycine (RG) motifs (*22*, *23*) and to methylated lysine (K4 or K79) in core histone

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[1] Abbreviations: AKAP, A-kinase anchoring protein; BSA, bovine serum albumin; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; NE, nuclear envelope; PKA, protein kinase A; PKC, protein kinase C; PP1, protein phosphatase 1; RFP, red fluorescent protein; UTR, untranslated region; KH, K homology; UBF, upstream binding factor.

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H3 (*24*, *25*). To our knowledge, AKAP149 and FMRP (*26*) are the only well-characterized proteins to date to contain a KH and a Tudor domain in combination.

We show here that AKAP149 is a self-associating protein with RNA binding features. The KH domain of AKAP149 is sufficient for self-association in a RNA-dependent manner. The Tudor domain is dispensable for self-association, but it is required together with the KH domain for targeting to defined nuclear foci. These foci are spatially close to nucleolar subcompartments. Furthermore, the KH-Tudor-containing fragment of AKAP149 binds RNA in vitro and in RNA coprecipitation experiments.

EXPERIMENTAL PROCEDURES

Expression and Purification of AKAP149 Fusion Proteins. Fragments of AKAP149 cDNA (full-length, nucleotides 1−2712; KH-Tudor, nucleotides 1736−2712; KH, nucleotides 1736−2040; Tudor, nucleotides 2040−2712) were amplified by PCR, using primers that appended a 5′ EcoRI site and a 3′ BamHI site. Amplified cDNA was digested with EcoRI and BamHI and cloned into the pEGFP-N3 expression plasmid (Clontech) to produce fragments of AKAP149 fused to the enhanced green fluorescent protein (EGFP). Resulting plasmids encoded AKAP149-EGFP, KH-Tudor(578−903)-EGFP (called KH-Tudor-EGFP), KH(578−680)-EGFP (called KH-EGFP), and Tudor(680−903)-EGFP (called Tudor-EGFP). To generate HA-tagged KH-Tudor fragments (HA-KH-Tudor), AKAP149(578−903) was subcloned into pEGFP-N1 (Clontech). The sequence encoding the HA tag (YPYDVPDYA) was inserted upstream of the insert, and EGFP transcription was prevented by introducing a stop codon. Coding sequences for NH_2-terminally His-tagged KH(578−686) and KH-Tudor(578−903) fragments were cloned into the pET16B vector (Novogen) as NdeI-BamHI and NdeI-XhoI fragments, respectively. Mutation in the KH domain was introduced using the Quick Change site-directed mutagenesis kit (Stratagene). The following primer was used, with the mutation in italics: 5′ GCAGGGGCGCTAT-G*A*GAGTTTTCTGAAGC 3′ (V629E sense primer).

Antibodies. Mouse monoclonal anti-AKAP149 antibodies (mAbs; Transduction Laboratories) were against residues 66−212 of human AKAP149. Anti-PKCλ antibodies were from Transduction Laboratories. Rabbit polyclonal and mouse monoclonal anti-GFP antibodies were from BD Biosciences. Anti-HA mAbs were from Nordic Biosite. Antibodies against PML (promyelocytic leukemia) were from R. Van Driel (University of Amsterdam, Amsterdam, The Netherlands) (*27*). Anti-coilin antibodies were from A. Lamond (University of Dundee, Dundee, U.K.). Anti-Sc35 antibodies were from M. Vincent (Université Laval, Quebec City, PQ). Anti-NO38/B23 antibodies were from T. Stokke (The Norwegian Radium Hospital-National Hospital, Oslo, Norway). Antibodies against UBF were from D. Hernandez-Verdun (Université Paris VII, Paris, France). Cy2-, Cy3-, and peroxidase-conjugated secondary antibodies were from Jackson Laboratories. Alexa Fluor 594-conjugated antibodies were from Molecular Probes.

Cell Culture and Transfection. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 1% glutamine, 1% sodium pyruvate, 1% nonessential amino acids, and 10% fetal calf serum. 293T cells were cultured in RPMI 1640 (Sigma-Aldrich) supplemented with 1% glutamine, 1% sodium pyruvate, 1% nonessential amino acids, and 10% fetal calf serum.

Cells were transfected using Lipofectamine 2000 as described by the manufacturer (Invitrogen). Briefly, for fluorescence analysis, cells were plated in DMEM on glass coverslips at a density of 0.7×10^6 cells per well of a six-well plate (Corning). After 48 h, 500 μL of OPTIMEM medium (Invitrogen) containing 10 μL of lipofectamine and 3−7 μg of DNA were added. Cells were incubated for 16−17 h and processed for microscopy analysis. For leptomycin B treatment, cells were exposed to 20 ng/mL leptomycin B (Sigma-Aldrich) for 3 h before microscopy analysis. For immunoprecipitation, cells were seeded in 75 cm^2 culture flasks 48 h before transfection.

Immunological Procedures. Western blotting analysis was performed as described previously (*28*) using anti-AKAP149 antibodies (1:1000 dilution), anti-GFP mAbs (1:5000), anti-HA antibodies (1:1000), and horseradish peroxidase-conjugated secondary antibodies (1:7500). For immunoprecipitation, HeLa cells were harvested, washed, resuspended in immunoprecipitation buffer [10 mM HEPES (pH 7.5), 10 mM KCl, 2 mM EDTA, and 1% Triton X-100], and allowed to swell for 30 min before sonication. In some experiments, RNase A (1 mg/mL) or DNase I (1 mg/mL) was added to the lysate and incubated for 30 min at 37 °C. The lysate was centrifuged at 15000*g* for 5 min. After preclearing, immunoprecipitation was carried out with relevant antibodies (1:50) at 4 °C for 2 h, followed by incubation with Protein A/G−Sepharose beads (Santa Cruz) at 4 °C for 1 h. Immune precipitates were washed three times in immunoprecipitation buffer adjusted to 100 mM KCl, and proteins were eluted in SDS sample buffer.

Immunofluorescence analysis was performed after paraformaldehyde fixation and permeabilization with 0.1% Triton X-100 as described previously (*28*). Cells were probed with anti-AKAP149 (1:300 dilution), anti-HA (1:400), anti-PML (1:500), anti-coilin (1:50), anti-NO38 (1:500), or anti-UBF (1:100) antibodies, followed by secondary antibodies conjugated to Cy3 (1:400), Cy2 (1:100), or Alexa Fluor 594 (1:5,000). DNA was labeled with 0.25 μg/mL DAPI. Samples were examined on an Olympus BX51 microscope under a 100× objective using AnalySIS (Soft Imaging Systems).

In Vitro RNA Binding. The oligoribonucleotide AU4 was radioactively labeled at the 5′ end with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP and 600 units/mL RNAsin as described previously (*29*). Labeled AU4 was extracted with a phenol/chloroform/isoamyl alcohol mixture (25:24:1), precipitated with 70% (v/v) ethanol and 100 mM NaAc, purified via 8.3 M urea−20% PAGE, and re-extracted as described above. Recombinant His-KH(578−686) or His-KH-Tudor(578−903) fragments (2 μg) were incubated with labeled AU4 (2 μCi/mL) in 20 μL of binding buffer [20 mM Tris-HCl (pH 7.5), 5 mM MgAc, 1 mM EDTA, 1 mM dithiothreitol, and 0.1 M NH_4Cl] and exposed to UV (254 nm, 250 mJ) in a Stratalinker UV apparatus. Samples were subjected to 12% Tricine-SDS−PAGE, and dried gels were autoradiographed. In competition experiments, 2 μM cold AU4 was added to the binding assay and samples were processed as described above.

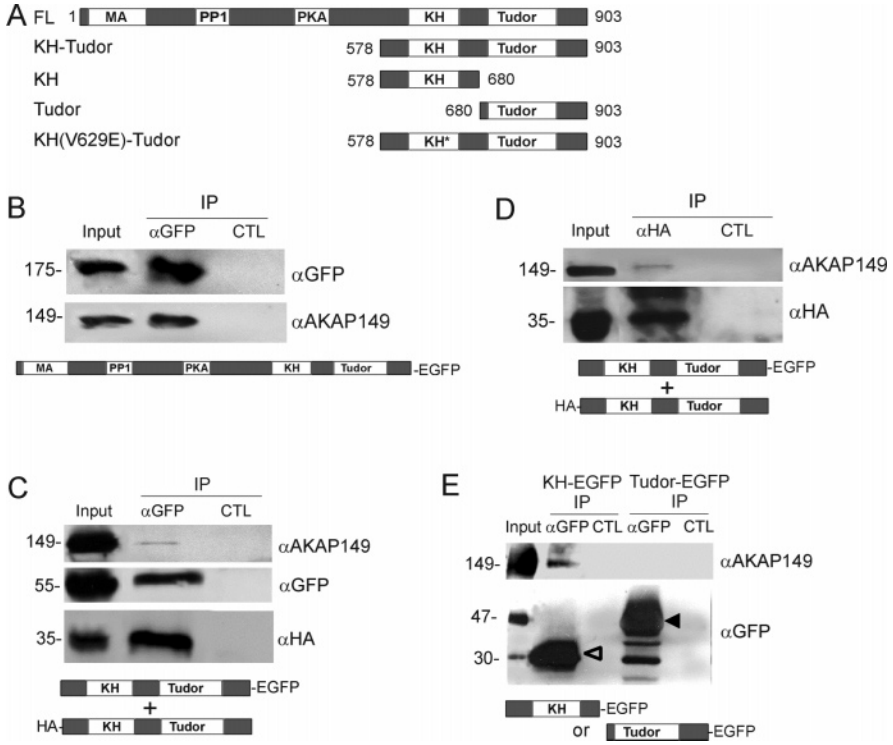

FIGURE 1: AKAP149 self-associates through the KH domain. (A) AKAP149 constructs used in this study. EGFP or mRFP1 tags were fused to the COOH terminus, and HA or His tags were fused to the NH_2-terminus of separate constructs, as described in the text. Positions of the membrane association (MA), PP1-binding, PKA-binding, KH, and Tudor domains are shown. KH* refers to a V629E mutation introduced into the KH domain. (B) HeLa cells transfected with full-length AKAP149-EGFP were subjected to immunoprecipitation (IP) using anti-GFP (αGFP) or preimmune control (CTL) antibodies. Proteins were immunoblotted using anti-GFP or anti-AKAP149 antibodies. (C) Cells were cotransfected with KH-Tudor-EGFP and HA-KH-Tudor, and lysates were immunoprecipitated using anti-GFP or control antibodies. Proteins were immunoblotted using anti-AKAP149, anti-GFP, or anti-HA antibodies. The low level of pulled down endogenous AKAP149 might result from displacement by the overexpressed HA-KH-Tudor fragment. (D) Cells were cotransfected with KH-Tudor-EGFP and HA-KH-Tudor, and lysates were immunoprecipitated using anti-HA (αHA) or control antibodies. Immune precipitates were immunoblotted using anti-AKAP149 or anti-HA antibodies. The KH-Tudor-EGFP fragment could not be resolved by SDS−PAGE as it migrated in the IgG heavy chain region. (E) Cells were transfected with either KH-EGFP or Tudor-EGFP, and lysates were immunoprecipitated using anti-GFP or control antibodies. Immune precipitates were immunoblotted using anti-AKAP149 or anti-EGFP antibodies. Black and white arrowheads point to Tudor-EGFP and KH-EGFP, respectively. In panels B−E, transfected tagged AKAP149 fragments are shown below the blots.

Association of RNA with the KH-Tudor-Containing Domain of AKAP149. Nickel−Sepharose beads coated with either His-KH-Tudor(578−903) or bovine serum albumin (BSA) were added to total RNA (1 μg/μL) purified from 293T cells using a total RNA purification kit (Sigma-Aldrich). Coated beads were added to undigested RNA or to RNA partially digested with T1 RNase (0.024 unit/μL for 10 min). After being washed, beads were digested with proteinase K (56 °C for 30 min). RNA was purified (RNAeasy, Qiagen) and end-labeled with [γ-^{32}P]ATP before separation in a 6% urea gel and autoradiography.

Immunoprecipitation of RNA with Endogenous AKAP149. 293T cells were resuspended in immunoprecipitation buffer [150 mM KCl, 10 mM HEPES (pH 7.5), 2 mM EDTA, and 1% Triton X-100] and allowed to swell for 30 min before sonication. The lysate was partially digested with T1 RNase (0.024 unit/μL for 10 min) before centrifugation (15000*g* at 4 °C for 5 min). Immunoprecipitation was carried out with either anti-AKAP149 antibodies or no antibodies as a control at 4 °C for 2 h, followed by incubation with Dynabeads Protein G magnetic beads (Dynal) at 4 °C for 1 h. Immune precipitates were washed three times in immunoprecipitation buffer and digested with proteinase K (56 °C for 30 min). RNA was purified (RNAeasy, Qiagen) andend-labeled with [γ-^{32}P]ATP before separation in a 6% urea gel and visualization of RNA by autoradiography.

RESULTS

The KH Domain of AKAP149 Is Sufficient for RNA-Dependent Self-Association. Many KH domain-containing proteins self-associate intra- or intermolecularly through their KH domain(s) (*30*). To determine whether AKAP149 oligomerized, we generated COOH-terminal fragments of AKAP149 containing either or both of the KH and Tudor domains (Figure 1A). These fragments were tagged NH_2-terminally with HA or COOH-terminally with EGFP and expressed in HeLa cells. Lysates of full-length AKAP149-EGFP-expressing cells were subjected to immunoprecipitation using anti-GFP or control antibodies. Immune precipitates and input lysate were immunoblotted using anti-AKAP149 and anti-GFP antibodies. Anti-GFP, but not control, immune precipitates pulled down endogenous AKAP149 (Figure 1B), indicating that AKAP149 directly or indirectly self-associates. Furthermore, in cells coexpressing KH-Tudor-EGFP and HA-tagged KH-Tudor (HA-KH-Tudor), anti-GFP immune precipitates harbored both endogenous AKAP149 and HA-KH-Tudor (Figure 1C). Anti-HA precipitates also contained endogenous AKAP149 (Figure 1D, top panel). Thus, the KH-Tudor domain of AKAP149

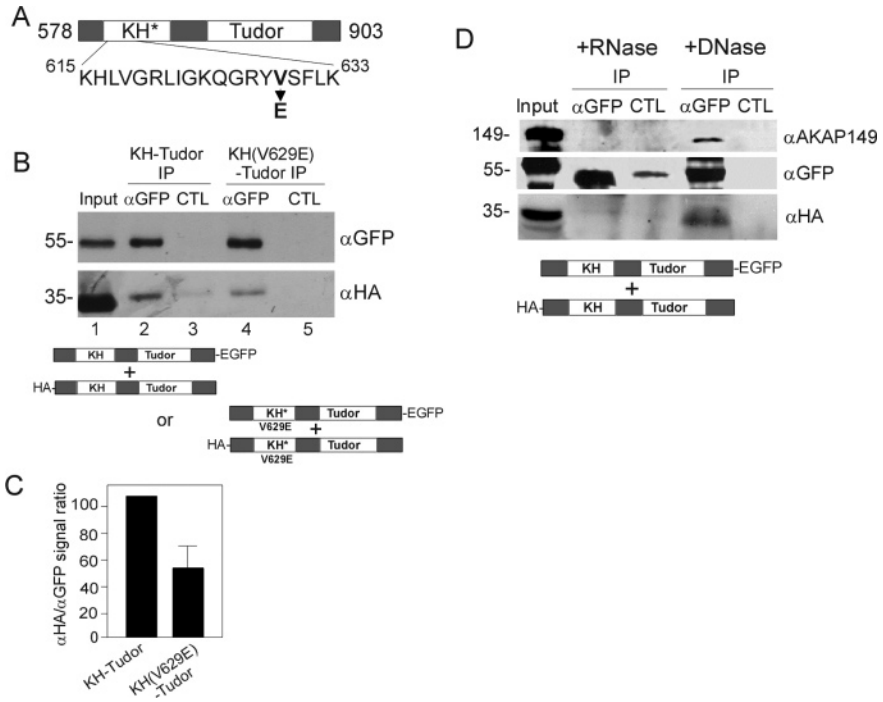

FIGURE 2: The KH-Tudor domain of AKAP149 self-associates in a RNA-dependent manner. (A) Amino acid sequence of the hydrophobic groove of the KH domain of AKAP149. The star denotes the V629E mutation. (B) Lysates of HeLa cells coexpressing wild-type KH-Tudor-EGFP and HA-KH-Tudor (lanes 2 and 3) or KH(V629E)-Tudor-EGFP with HA-KH(V629E)-Tudor (lanes 4 and 5) were immunoprecipitated using anti-GFP or control antibodies. Proteins were immunoblotted using anti-GFP or anti-HA antibodies. (C) Densitometric analysis of the ratio of anti-HA to corresponding anti-GFP signal intensity on three independent blots such as that shown in panel B. The mean anti-HA/anti-GFP signal intensity ratio for wild-type KH-Tudor-transfected cells was set to 100. (D) Cells were cotransfected with KH-Tudor-EGFP and HA-KH-Tudor. Lysates were treated with RNase A (1 mg/mL) or DNAse I (1 mg/mL) and immunoprecipitated (IP) using anti-GFP or control antibodies. Proteins were immunoblotted using anti-AKAP149, anti-GFP, or anti-HA antibodies. In panels B and D, transfected tagged AKAP149 fragments are shown below the blots.

self-associates and interacts with endogenous AKAP149. Additionally, anti-GFP immunoprecipitation from KH-EGFP-expressing cells, but not from Tudor-EGFP-expressing cells, coprecipitated endogenous AKAP149 (Figure 1E). This indicates that endogenous AKAP149 associates with KH-EGFP, but not Tudor-EGFP. Thus, self-association of AKAP149 involves the KH domain.

Structural studies have shown that the KH domain found in various proteins forms a hydrophobic groove (Figure 2A) involved in RNA binding and in self-association (*31*). Introduction of a V629E mutation disrupting the hydrophobic groove (*31*) of the KH domain of AKAP149 weakened the self-association of the KH-Tudor fragment in cells coexpressing HA-KH(V629E)-Tudor and KH(V629E)-Tudor-EGFP (in Figure 2B, compare lanes 2 and 4). Weakening of the interaction was confirmed by densitometry analysis of triplicate blots of immunoprecipitated wild-type and mutated HA-KH-Tudor fragments (Figure 2C).

To examine the role of RNA in AKAP149 self-association through the KH-Tudor domain, lysates of HeLa cells coexpressing KH-Tudor-EGFP and HA-KH-Tudor were treated with RNase A or DNase I before immunoprecipitation using anti-GFP antibodies. KH-Tudor-EGFP immunoprecipitation of RNase-treated extracts resulted in little, if any, coprecipitation of HA-KH-Tudor (Figure 2D, bottom panel) and no coprecipitation of endogenous AKAP149 (Figure 2D, top panel). In contrast, immunoprecipitation of KH-Tudor-EGFP from DNase-treated lysates coprecipitated HA-KH-Tudor as well as endogenous AKAP149 (Figure 2D). These results indicate that interaction between the KH-Tudor domains of AKAP149 is dependent on RNA.

The KH-Tudor Domain of AKAP149 Binds RNA in Vitro and in Vivo. To verify the RNA binding property of AKAP149, we examined RNA binding in vitro using an AU4 oligoribonucleotide probe previously shown to bind with high affinity to NIPP1, a nuclear RNA-binding protein interacting with PP1 (*29*). ^{32}P-labeled AU4 was incubated under UV cross-linking conditions without (control) or with an NH_2-terminal His-tagged KH-Tudor(578−903) AKAP149 fragment, or with a His-KH(578−686) fragment. Cross-linked samples were subjected to SDS−PAGE and visualized by autoradiography. Migration of the AU4 probe in the presence of His-KH(578−686) and His-KH-Tudor(578−903) (Figure 3A, white and black arrows, respectively) was retarded, indicating binding of the KH and KH-Tudor domains to RNA in vitro. Binding of His-KH-Tudor(578−903) to the radiolabeled AU4 probe was competed with a non-radiolabeled AU4 oligoribonucleotide (Figure 3B), illustrating the specificity of the interaction.

To further examine the RNA binding property of the KH-Tudor domain of AKAP149, RNA was purified from 293T cells and either partially digested with RNase T1 or left undigested. Undigested or digested RNA was added to Sepharose beads precoated with His-KH-Tudor(578−903) or with BSA as a control. After sedimentation and washes, RNA bound to beads was purified and radioactively labeled before separation in a 6% urea gel and autoradiography. Significant amounts of RNA were coprecipitated with the His-KH-Tudor(578−903) fragment (Figure 4A, lanes 5 and 6). In contrast, very little RNA was detected on BSA-coated beads (Figure 4A, lanes 3 and 4). Partial RNA digestion with RNase T1 generated several bands detected in the KH-Tudor-

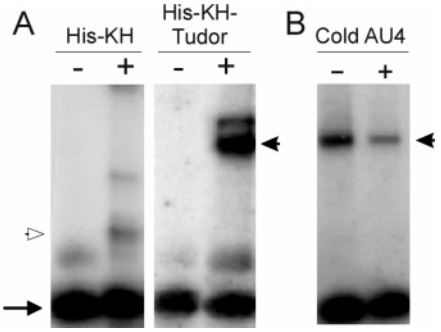

FIGURE 3: His-KH-Tudor and His-KH bind a RNA probe. (A) A ^{32}P-labeled oligoribonucleotide AU4 probe was incubated under UV cross-linking conditions with (+) or without (−) indicated tagged AKAP149 peptides. Samples were subjected to Tricine-SDS−PAGE and autoradiography. The bottom left arrow points to free labeled AU4; white and black arrowheads indicate migration of AU4 cross-linked to His-KH and His-KH-Tudor, respectively. (B) Binding of His-KH-Tudor to ^{32}P-labeled AU4 as in panel A (−) competed with cold AU4 RNA in the reaction mix (+). The arrowhead points to the His-KH-Tudor fragment bound to the AU4 probe.

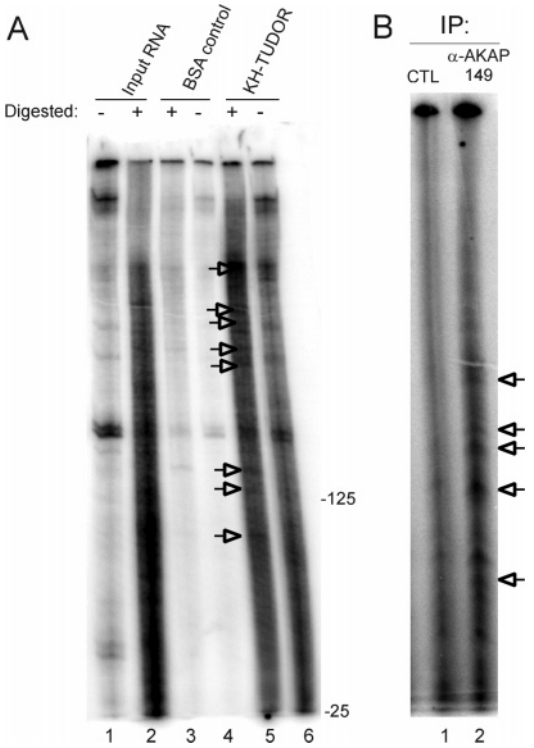

FIGURE 4: AKAP149 binds RNA. (A) The His-KH-Tudor fragments pull down purified RNAs. Total RNA purified from 293T cells (Input RNA) was subjected to RNAse T1 partial digestion (+) or left undigested (−) and incubated with Sepharose beads coated with His-KH-Tudor fragments or BSA as a control. RNA was precipitated by sedimentation of the Sepharose beads, ^{32}P-labeled, resolved in a urea gel, and visualized by autoradiography. Specific RNA products were enriched in the KH-Tudor pulldown from the RNase T1-digested material (arrows). (B) Endogenous AKAP149 coprecipitates with RNA. A lysate from 293T cells was partially digested with RNase T1 and immunoprecipitated (IP) using anti-AKAP149 antibodies coupled to Sepharose beads (lane 2) or using beads alone (lane 1). Precipitated RNA was ^{32}P-labeled, resolved in a urea gel, and visualized by autoradiography. RNA products enriched in the AKAP149 immune precipitate compared to control are denoted with arrows.

(578−903) precipitates (Figure 4A, lane 5, arrowheads). Because these bands were absent in undigested KH-Tudor-(578−903) pulldowns (Figure 4A, lane 6), we concluded that the products detected were RNA molecules. In a second experiment, whole 293T cell extracts were treated with T1 RNase to partially digest RNAs and endogenous AKAP149 was immunoprecipitated using anti-AKAP149 antibodies. Figure 4B shows the presence of T1 RNase-digested products in the anti-AKAP149 precipitates which were absent in control (beads alone) precipitates. We concluded from these experiments that endogenous AKAP149 also specifically coprecipitates RNA molecules.

The KH-Tudor Domain of AKAP149 Targets Intranuclear Foci Close to RNA Processing Sites. The mouse ortholog of human AKAP149, AKAP121, is known to have two NH_2-terminal (N0 and N1) splice variants directing membrane targeting either to mitochondria (N0) or to the NE−ER network (N1) (*7*). We have shown so far that the COOH-terminal part of AKAP149 is responsible for its self-association and for RNA binding. We then determined whether it also contained any subcellular targeting determinant. To this end, we examined the subcellular localization of overexpressed KH-Tudor-EGFP, which contains the KH and Tudor domains, and which lacks the 30 NH_2-terminal amino acids responsible for membrane localization of AKAP149 (*7*). KH-Tudor-EGFP localized predominantly inside the nucleus and displayed discrete foci over a faint nucleoplasmic background (Figure 5A, four left panels). In less than 40% of the cells, a proportion of the foci were concentrated in nucleoli (Figure 5A, arrow). In contrast, KH-EGFP exhibited a uniform cellular staining with some enrichment in the nucleus. Tudor-EGFP displayed a strong nucleoplasmic staining together with some accumulation at foci (Figure 5A). Thus, the KH and Tudor domains of AKAP149 accumulate in the nucleus, and both seem to be required for optimal targeting to foci. The Tudor domain is sufficient for enrichment in foci, contrary to the KH domain which, however, seems to act in conjunction with Tudor to strengthen and restrict the association with foci.

The nucleus is highly organized and contains numerous territories, domains, and bodies (*32*, *33*). Since transient overexpression of KH-Tudor-EGFP gives rise to distinct intranuclear foci, we determined the localization of these foci with respect to known nuclear bodies in cells fixed and labeled with relevant antibodies. No colocalization was detected with PML bodies, implicated in several functions, including transcriptional regulation (*34*), Cajal bodies, sites of ribonucleoprotein assembly and remodeling (*35*), or Sc35 splicing factor (*36*) (data not shown). We also investigated nucleolar fibrillar centers where the RNA−polymerase I transcription initiation complex forms, and the nucleolar granular component where maturation, processing, and storage of preribosomal particles take place (*37*−*39*). Fibrillar and granular centers are decorated with anti-UBF (upstream binding factor) and anti-NO38/B23 antibodies, respectively. Irrespective of whether they were localized within or outside nucleoli, KH-Tudor-EGFP foci were often found adjacent to the granular compartment decorated by anti-NO38/B23 antibodies, but they did not overlap with anti-NO38/B23 labeling (Figure 5B). However, KH-Tudor-EGFP foci often surrounded UBF-containing fibrillar centers and clearly exhibited colocalization with UBF in cells where KH-Tudor-EGFP foci were localized within nucleoli (Figure 5C).

Immunoprecipitation of KH-Tudor-EGFP using anti-GFP antibodies coprecipitated UBF (Figure 5D, left IP), whereas

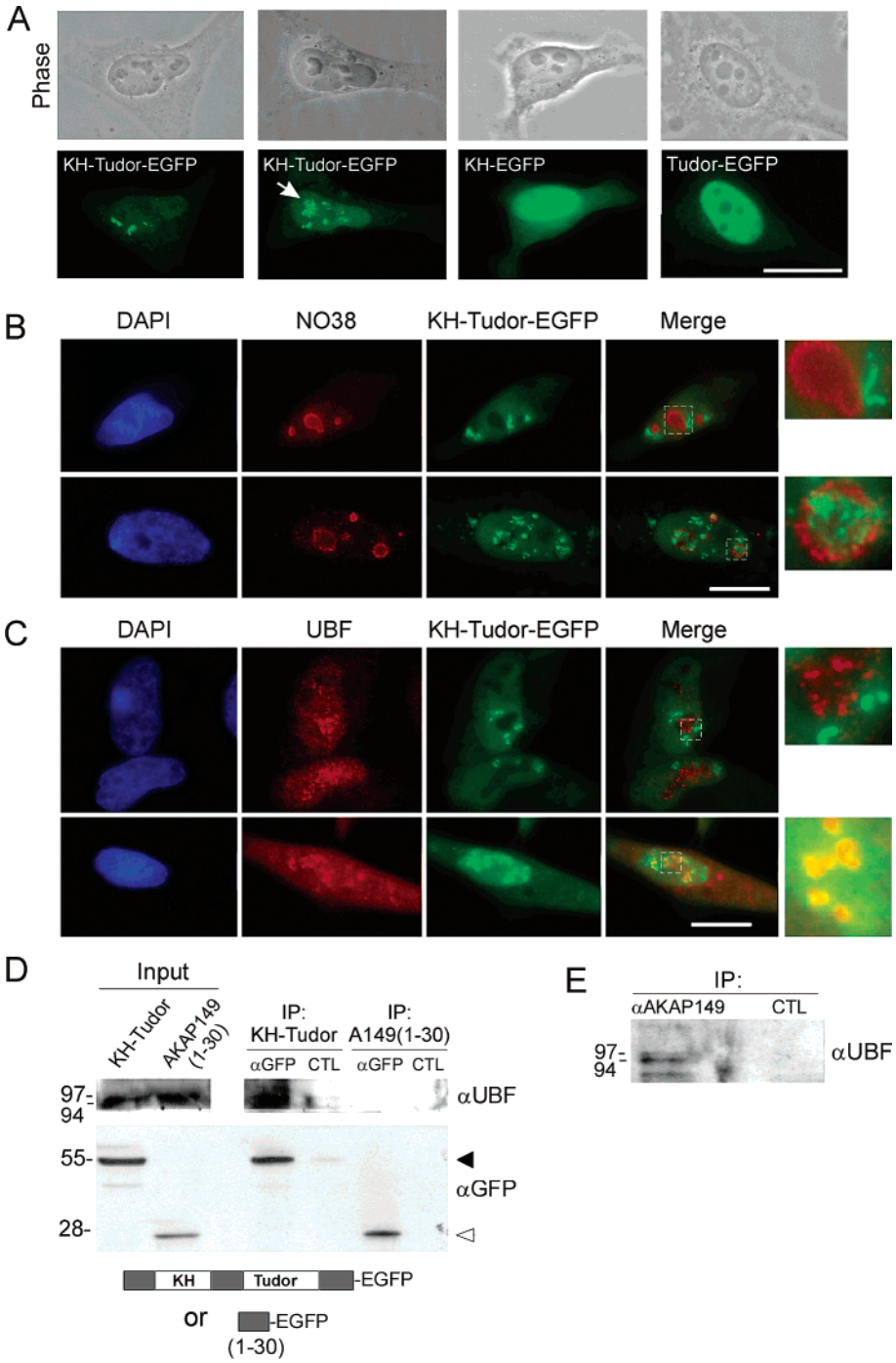

FIGURE 5: KH-Tudor-EGFP localizes in close association with nucleolar markers. (A) Distribution of KH-Tudor-EGFP, KH-EGFP, and Tudor-EGFP expressed in HeLa cells. Two patterns of KH-Tudor-EGFP labeling were detected (four left panels). Arrow points to nucleolar targeting of KH-Tudor EGFP. (B and C) HeLa cells transfected with KH-Tudor-EGFP were immunolabeled using (B) anti-NO38/B23 antibodies or (C) anti-UBF antibodies. Note the partial colocalization of KH-Tudor-EGFP-containing foci with the fibrillar compartment labeled with anti-UBF. DNA was stained with DAPI. Enlargements of the marked areas in the merge panels are shown at the right. Bars are 10 μm long. (D) KH-Tudor-EGFP or AKAP149(1−30)-EGFP was overexpressed in HeLa cells, and each fragment was immunoprecipitated using anti-GFP antibodies. Blots of immune and mock (CTL) precipitates were probed with anti-UBF and anti-GFP antibodies. The black arrowhead points to the KH-Tudor-EGFP fragment; the white arrowhead points to the AKAP149(1−30)-EGFP fragment. Transfected AKAP149 fragments are shown below the blots. (E) Endogenous AKAP149 was immunoprecipitated from untransfected HeLa cells using anti-AKAP149 antibodies, and the immune complex was immunoblotted using anti-UBF antibodies.

a control AKAP149 fragment harboring only the first 30 NH_2-terminal residues [AKAP149(1−30)-EGFP], which target the protein to mitochondrial membranes (data not shown), did not (Figure 5D, right IP). Immunoprecipitation of endogenous AKAP149 also coprecipitated UBF (Figure 5E), consistent with the partial colocalization of the KH-Tudor domain of AKAP149 with UBF. Collectively, these results suggest that a fragment of AKAP149 containing the KH-Tudor domain interacts with UBF or a UBF-containing complex in the nucleus.

Binding to RNA Is Required for Targeting of the KH-Tudor Domain of AKAP149 to Intranuclear Foci. To assess the importance of RNA binding by the KH domain in localizing the COOH-terminal part of AKAP149, we examined the

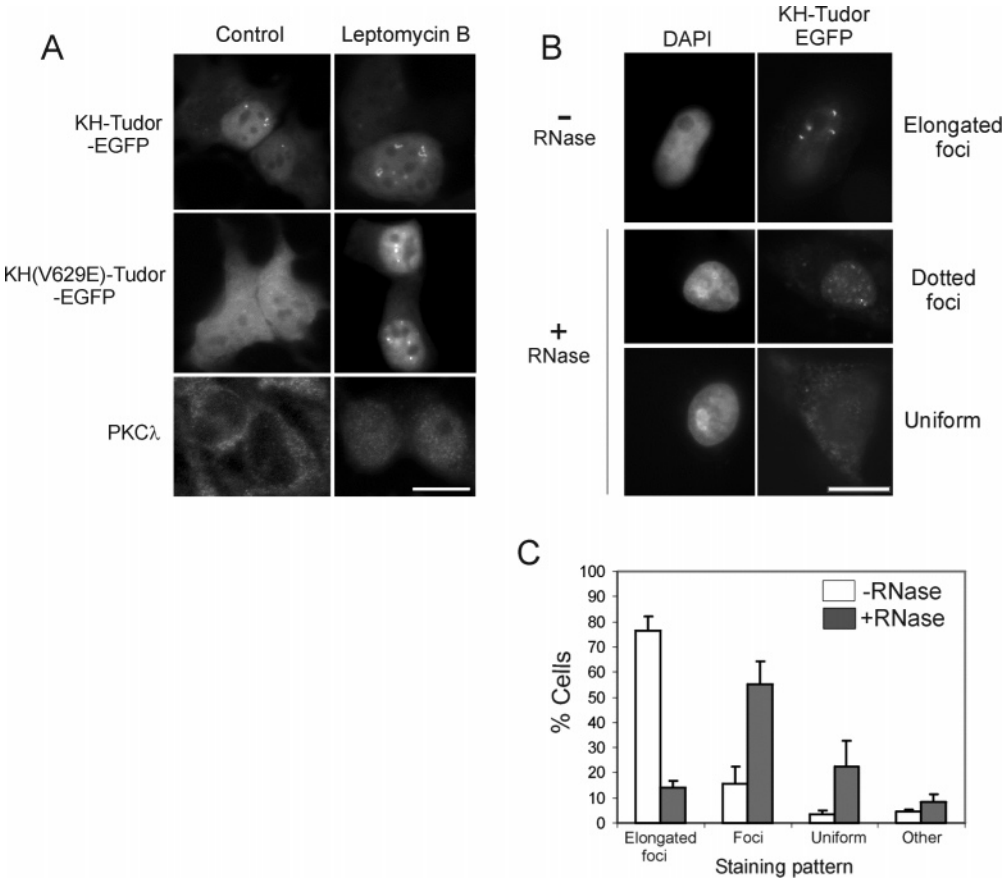

FIGURE 6: Intranuclear retention of KH-Tudor-EGFP. (A) KH(V629E)-Tudor-EGFP is retained in the nucleus after inhibition of nuclear export. HeLa cells expressing KH-Tudor-EGFP or KH(V629E)-Tudor-EGFP were treated with leptomycin B for 3 h before being fixed to inhibit nuclear export. Some cells were also immunolabeled with anti-PKCλ antibodies (bottom panels) to control for inhibition of nuclear export. (B) Distribution of KH-Tudor-EGFP in cells permeabilized with 0.1% Triton X-100 and 2% Tween 20 for 15 min, extracted with 1 mg/mL RNase A for 45 min, and fixed. DNA was stained with DAPI. The KH-Tudor-EGFP nuclear distribution was assayed as few elongated foci, numerous dotted foci, or uniform distribution. (C) Quantification of the distribution analysis shown in panel B (mean $\pm$ the standard deviation in percent of cells showing indicated phenotypes; three experiments). Other refers to mitotic figures and a mixture of the above staining patterns. Bars are 10 μm long.

subcellular localization of the KH(V629E)-Tudor-EGFP mutant. The V629E mutation caused a redistribution of KH-(V629E)-Tudor-EGFP throughout the cell, as compared to KH-Tudor-EGFP (Figure 6A). We hypothesized that the V629E mutation would result in diminished nuclear retention of KH(V629E)-Tudor-EGFP and, therefore, a loss of accumulation at foci and a decreased residence time in the nucleus. This effect should then be opposed by blocking nuclear export. Indeed, nuclear enrichment of KH(V629E)-Tudor-EGFP and formation of foci were restored after a 3 h exposure of cells to leptomycin B, an inhibitor of nuclear export (Figure 6A). In contrast, the wild-type KH-Tudor-EGFP distribution was not affected by leptomycin B treatment (Figure 6A). The λ isoform of protein kinase C (PKCλ), known to shuttle between the nucleus and cytoplasm (*40*), was used as a positive control for nuclear accumulation after leptomycin B treatment (Figure 6A). Collectively, these results indicate that enhanced intranuclear staining and formation of intranuclear KH-Tudor-EGFP foci require a functional KH domain.

To confirm an involvement of RNA in the intranuclear localization of the KH-Tudor domain, HeLa cells were permeabilized with 0.1% Triton X-100 and 2% Tween 20 at room temperature and exposed to 0 or 1 mg/mL RNase A. RNase treatment caused a redistribution of the few elongated foci characteristic of KH-Tudor-EGFP (Figure 6B, −RNase) into a pattern ranging from many evenly dispersed speckles to a uniform distribution without discernible speckles (Figure 6B, +Rnase; see quantification of the analysis in Figure 6C). These results reinforce the view that a RNA component is involved in the intranuclear localization of KH-Tudor-EGFP.

DISCUSSION

A large number of proteins with RNA binding features have been identified, and many of them contain specific domains responsible for protein−RNA interactions. Both KH and Tudor are such domains with amino acid sequences that are conserved throughout the eukaryotic realm (*41*). The KH domain is also found in eubacteria and archaea (*42*). In addition to AKAP149 and FMRP (*26*), only two other proteins of unknown function, *Caenorhabditis elegans* putative protein C56g2.1 and human protein TDRKH (*43*), harbor a KH domain and a Tudor domain in combination. It is thought that a single RNA-binding domain does not provide sufficiently high binding affinity; thus, RNA binding is promoted by multiple homotypic or heterotypic domains to increase affinity and determine selectivity. This is illustrated by heterogeneous nuclear ribonucleoprotein (hnRNP), the three KH domains of which bind RNA synergistically, whereas a single KH domain binds RNA weakly (*44*).

Another example is *Drosophila* P-element somatic inhibitor protein which contains four KH domains (*45*).

We show here that AKAP149 is a self-associating and RNA-binding protein and that the KH domain is critical for both features. The KH domain alone is sufficient for self-association and binds RNA in vitro. Digestion of RNA with RNase A prevents self-association of the KH-Tudor domain, suggesting that RNA binding is necessary for self-association. However, introducing a mutation (V629E) which disrupts the hydrophobic groove of the KH domain critical for RNA binding (*45*, *46*) only partially abolishes KH-Tudor self-association. This implies that the mutation does not completely abolish KH-mediated RNA binding or, more likely, that other regions in the COOH-terminal part of AKAP149 are also involved in RNA binding. A similar feature has been observed for the RNA binding properties of Vg1RBP/Vera, a protein localizing Vg1 mRNA to the vegetal cortex of *Xenopus laevis* oocytes during oogenesis (*47*). On the basis of these and our observations, we propose that the KH domain mediates self-association of AKAP149 and that this intermolecular association is stabilized by RNA. A stabilizing effect on KH−KH interaction by RNA has been observed for one of the KH domains of the Nova protein, although the KH domains could also interact, albeit at a reduced level, in the absence of RNA (*48*).

We also show a nuclear localization for the COOH-terminal part of AKAP149 when it is overexpressed in HeLa cells. This region consists mostly of the KH and Tudor domains. The KH-Tudor-EGFP fragment accumulates at discrete intranuclear foci, most of which associate with the nucleolar fibrillar center component UBF. An association with UBF was confirmed by co-immunoprecipitation of UBF with overexpressed KH-Tudor-EGFP and with endogenous AKAP149. This may suggest a role for AKAP149, mediated by its RNA binding properties, in ribosome biogenesis or in other nucleolar functions. Identification of the nature of RNA molecules associated with the KH-Tudor domain of AKAP149 would help in the identification of the role of AKAP149 in the vicinity of nucleoli. Interestingly, UBF is a phosphoprotein, and in vitro experiments suggest that UBF is more transcriptionally active when phosphorylated (*49*). This may imply a regulation of UBF activity by protein kinases and phosphatases, such as PKA, PKC, and PP1, all of which have been shown to be anchored by AKAP149 (*8*, *10*).

It is well-established that full-length AKAP149 binds to mitochondrial and ER−NE membranes (*7*, *8*), so one can speculate about the physiological significance of the nucleolar localization of the COOH-terminal KH-Tudor domain. One can envisage that AKAP149 anchored in the inner nuclear membrane through its NH_2-terminal membrane association motif interacts with nucleolar components situated at the nuclear periphery. In this scenario, it would be interesting to determine if this interaction is permanent or if it is context-regulated by, for example, phosphorylation/dephosphorylation events or by interaction with binding partners or other signaling molecules. An alternative explanation may be that NE-associated AKAP149 specifically interacts with nucleolar components at the level of the invaginations of the NE which have been shown to contact the nucleolus in mammalian cells (*38*, *50*).

Two lines of evidence suggest that RNA binding is critical for the localization of the COOH-terminal part of AKAP149 to nuclear foci. First, RNase treatment of cells results in a significant reduction in the size and intensity of the KH-Tudor-EGFP nuclear foci. Global RNA digestion might, however, cause a general perturbation of intranuclear structures, thus explaining our results. Second, the KH(V629E)-Tudor-EGFP mutant displays a uniform distribution throughout the cells, including throughout the nucleus, but with no visible nuclear foci. This phenotype results from a reduced level of nuclear retention because inhibition of nuclear export with leptomycin B rescued intranuclear accumulation of the mutant fragment. Therefore, KH domain-mediated RNA binding, in addition to promoting self-association of AKAP149, may also be involved in targeting or anchoring the COOH-terminal part of AKAP149 to subnuclear compartments. Because we also find that overexpressed KH-EGFP alone does not localize at foci, we conclude that a functional RNA-binding KH domain is necessary but not sufficient for targeting the COOH-terminal region of AKAP149 to discrete nuclear foci.

Accumulation of the COOH-terminal part of AKAP149 in nuclear foci is dependent on the Tudor domain, whereas KH-EGFP alone only shows nuclear enrichment without accumulation in discrete structures. Similarly, the 53BP1 protein, involved in DNA-damage-checkpoint signaling, relocalizes to sites of DNA damage following ionizing radiation in a manner requiring the tandem repeat of Tudor motifs of 53BP1 (*51*). Another example is survival motor neuron (SMN) protein, which plays a role in the assembly of small nuclear ribonucleoproteins (SnRNP) and mutations/deletions of which cause spinal muscular atrophy. The central Tudor domain of SMN protein has been shown to promote localization to Cajal bodies (*52*). Interestingly, this function necessitates two other domains, one involved in RNA binding (a K-rich sequence) and one implicated in self-association (a YG box) (*52*). In addition, the Tudor domain of the SMN protein interacts directly with coilin and Sm proteins, both of which are found in Cajal bodies (*52*). We show here that KH-Tudor-EGFP does not localize to Cajal bodies. KH-Tudor EGFP localizes to nucleoli in a proportion of the cells, possibly as a function of cell cycle stage or metabolic status. However, the identity of the extranucleolar compartment targeted by KH-Tudor-EGFP remains unknown. It would be interesting to identify the binding partner(s) of the KH and Tudor domains at these nuclear bodies. In addition to RNA, Tudor domains bind DNA (*21*) and proteins (*22*, *23*, *25*). Therefore, it is conceivable that targeting of the KH-Tudor domain of AKAP149 to nuclear foci results from interaction of Tudor with DNA. AKAP149 may also associate with nuclear proteins through its Tudor domain which, interestingly, in other proteins can bind to symmetrically dimethylated arginines in RG-rich sequences (*23*). Of note, RG repeats are abundant in proteins characteristic of nuclear bodies such as fibrillarin, nucleolin, Sm core proteins, or coilin (*53*).

Collectively, our results show that the KH-Tudor domain promotes self-association of AKAP149 and exhibits RNA binding properties. The KH domain is critical for both features and is also necessary but not sufficient for localization of the COOH-terminal part of AKAP149 to discrete nuclear foci. The Tudor domain is also necessary for foci accumulation, but whether this also requires RNA binding or whether another component is involved remains unknown.

Like many other AKAPs (*2*), AKAP149 anchors various signaling molecules (*10*, *11*, *54*). Further insight into the roles of AKAP149 in the context of RNA binding is expected to arise from the identification of the RNA molecules associated with AKAP149.

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REFERENCES

1. Feliciello, A., Gottesman, M. E., and Avvedimento, E. V. (2001) The biological functions of A-kinase anchor proteins, *J. Mol. Biol. 308*, 99−114.
2. Wong, W., and Scott, J. D. (2004) AKAP signalling complexes: Focal points in space and time, *Nat. Rev. Mol. Cell Biol. 5*, 959−970.
3. Tasken, K., and Aandahl, E. M. (2004) Localized effects of cAMP mediated by distinct routes of protein kinase A, *Physiol. Rev. 84*, 137−167.
4. Trendelenburg, G., Hummel, M., Riecken, E. O., and Hanski, C. (1996) Molecular characterization of AKAP149, a novel A kinase anchor protein with a KH domain, *Biochem. Biophys. Res. Commun. 225*, 313−319.
5. Chen, Q., Lin, R. Y., and Rubin, C. S. (1997) Organelle-specific targeting of protein kinase AII (PKAII). Molecular and in situ characterization of murine A kinase anchor proteins that recruit regulatory subunits of PKAII to the cytoplasmic surface of mitochondria, *J. Biol. Chem. 272*, 15247−15257.
6. Feliciello, A., Rubin, C. S., Avvedimento, E. V., and Gottesman, M. E. (1998) Expression of a kinase anchor protein 121 is regulated by hormones in thyroid and testicular germ cells, *J. Biol. Chem. 273*, 23361−23366.
7. Huang, L. J., Wang, L., Ma, Y., Durick, K., Perkins, G., Deerinck, T. J., Ellisman, M. H., and Taylor, S. S. (1999) NH2-Terminal targeting motifs direct dual specificity A-kinase-anchoring protein 1 (D-AKAP1) to either mitochondria or endoplasmic reticulum, *J. Cell Biol. 145*, 951−959.
8. Steen, R. L., Martins, S. B., Tasken, K., and Collas, P. (2000) Recruitment of protein phosphatase 1 to the nuclear envelope by A-kinase anchoring protein AKAP149 is a prerequisite for nuclear lamina assembly, *J. Cell Biol. 150*, 1251−1262.
9. Steen, R. L., and Collas, P. (2001) Mistargeting of B-type lamins at the end of mitosis: Implications on cell survival and regulation of lamins A/C expression, *J. Cell Biol. 153*, 621−626.
10. Küntziger, T., Rogne, M., Folstad, R. L., and Collas, P. (2006) Association of PP1 with Its Regulatory Subunit AKAP149 Is Regulated by Serine Phosphorylation Flanking the RVXF Motif of AKAP149, *Biochemistry 45*, 5868−5877.
11. Asirvatham, A. L., Galligan, S. G., Schillace, R. V., Davey, M. P., Vasta, V., Beavo, J. A., and Carr, D. W. (2004) A-kinase anchoring proteins interact with phosphodiesterases in T lymphocyte cell lines, *J. Immunol. 173*, 4806−4814.
12. Cardone, L., Carlucci, A., Affaitati, A., Livigni, A., DeCristofaro, T., Garbi, C., Varrone, S., Ullrich, A., Gottesman, M. E., Avvedimento, E. V., and Feliciello, A. (2004) Mitochondrial AKAP121 binds and targets protein tyrosine phosphatase D1, a novel positive regulator of src signaling, *Mol. Cell. Biol. 24*, 4613−4626.
13. Livigni, A., Scorziello, A., Agnese, S., Adornetto, A., Carlucci, A., Garbi, C., Castaldo, I., Annunziato, L., Avvedimento, E. V., and Feliciello, A. (2006) Mitochondrial AKAP121 links cAMP and src signaling to oxidative metabolism, *Mol. Biol. Cell 17*, 263−271.
14. Ginsberg, M. D., Feliciello, A., Jones, J. K., Avvedimento, E. V., and Gottesman, M. E. (2003) PKA-dependent binding of mRNA to the mitochondrial AKAP121 protein, *J. Mol. Biol. 327*, 885−897.
15. Ranganathan, G., Phan, D., Pokrovskaya, I. D., McEwen, J. E., Li, C., and Kern, P. A. (2002) The translational regulation of lipoprotein lipase by epinephrine involves an RNA binding complex including the catalytic subunit of protein kinase A, *J. Biol. Chem. 277*, 43281−43287.
16. Ranganathan, G., Pokrovskaya, I., Ranganathan, S., and Kern, P. A. (2005) Role of A kinase anchor proteins in the tissue-specific regulation of lipoprotein lipase, *Mol. Endocrinol. 19*, 2527−2534.
17. Siomi, H., and Dreyfuss, G. (1997) RNA-binding proteins as regulators of gene expression, *Curr. Opin. Genet. Dev. 7*, 345−353.
18. Adinolfi, S., Bagni, C., Castiglione Morelli, M. A., Fraternali, F., Musco, G., and Pastore, A. (1999) Novel RNA-binding motif: The KH module, *Biopolymers 51*, 153−164.
19. Laggerbauer, B., Ostareck, D., Keidel, E. M., Ostareck-Lederer, A., and Fischer, U. (2001) Evidence that fragile X mental retardation protein is a negative regulator of translation, *Hum. Mol. Genet. 10*, 329−338.
20. Ponting, C. P. (1997) Tudor domains in proteins that interact with RNA, *Trends Biochem. Sci. 22*, 51−52.
21. Charier, G., Couprie, J., pha-Bazin, B., Meyer, V., Quemeneur, E., Guerois, R., Callebaut, I., Gilquin, B., and Zinn-Justin, S. (2004) The Tudor tandem of 53BP1: A new structural motif involved in DNA and RG-rich peptide binding, *Structure 12*, 1551−1562.
22. Meister, G., Eggert, C., and Fischer, U. (2002) SMN-mediated assembly of RNPs: A complex story, *Trends Cell Biol. 12*, 472−478.
23. Selenko, P., Sprangers, R., Stier, G., Buhler, D., Fischer, U., and Sattler, M. (2001) SMN tudor domain structure and its interaction with the Sm proteins, *Nat. Struct. Biol. 8*, 27−31.
24. Huang, Y., Fang, J., Bedford, M. T., Zhang, Y., and Xu, R. M. (2006) Recognition of histone H3 lysine-4 methylation by the double tudor domain of JMJD2A, *Science 312*, 748−751.
25. Huyen, Y., Zgheib, O., Ditullio, R. A., Jr., Gorgoulis, V. G., Zacharatos, P., Petty, T. J., Sheston, E. A., Mellert, H. S., Stavridi, E. S., and Halazonetis, T. D. (2004) Methylated lysine 79 of histone H3 targets 53BP1 to DNA double-strand breaks, *Nature 432*, 406−411.
26. Ramos, A., Hollingworth, D., Adinolfi, S., Castets, M., Kelly, G., Frenkiel, T. A., Bardoni, B., and Pastore, A. (2006) The Structure of the N-Terminal Domain of the Fragile X Mental Retardation Protein: A Platform for Protein-Protein Interaction, *Structure 14*, 21−31.
27. Stuurman, N., de Graaf, A., Floore, A., Josso, A., Humbel, B., de Jong, L., and van Driel, R. (1992) A monoclonal antibody recognizing nuclear matrix-associated nuclear bodies, *J. Cell Sci. 101*, 773−784.
28. Collas, P., Le Guellec, K., and Tasken, K. (1999) The A-kinase-anchoring protein AKAP95 is a multivalent protein with a key role in chromatin condensation at mitosis, *J. Cell Biol. 147*, 1167−1180.
29. Jin, Q., Beullens, M., Jagiello, I., Van, E. A., Vulsteke, V., Stalmans, W., and Bollen, M. (1999) Mapping of the RNA-binding and endoribonuclease domains of NIPP1, a nuclear targeting subunit of protein phosphatase 1, *Biochem. J. 342*, 13−19.
30. Chen, T., Damaj, B. B., Herrera, C., Lasko, P., and Richard, S. (1997) Self-association of the single-KH-domain family members Sam68, GRP33, GLD-1, and Qk1: Role of the KH domain, *Mol. Cell. Biol. 17*, 5707−5718.
31. Ramos, A., Hollingworth, D., and Pastore, A. (2003) The role of a clinically important mutation in the fold and RNA-binding properties of KH motifs, *RNA 9*, 293−298.
32. Lamond, A. I., and Earnshaw, W. C. (1998) Structure and function in the nucleus, *Science 280*, 547−553.
33. Zimber, A., Nguyen, Q. D., and Gespach, C. (2004) Nuclear bodies and compartments: Functional roles and cellular signalling in health and disease, *Cell. Signalling 16*, 1085−1104.
34. Bernardi, R., and Pandolfi, P. P. (2003) Role of PML and the PML-nuclear body in the control of programmed cell death, *Oncogene 22*, 9048−9057.
35. Matera, A. G. (2003) Cajal bodies, *Curr. Biol. 13*, R503.
36. Spector, D. L., O'Keefe, R. T., and Jimenez-Garcia, L. F. (1993) Dynamics of transcription and pre-mRNA splicing within the mammalian cell nucleus, *Cold Spring Harbor Symp. Quant. Biol. 58*, 799−805.
37. De Angelis, P. M., Stokke, T., and Clausen, O. P. (1997) NO38 expression and nucleolar counts are correlated with cellular DNA content but not with proliferation parameters in colorectal carcinomas, *Mol. Pathol. 50*, 201−208.
38. Hernandez-Verdun, D., and Louvet, E. (2004) [The nucleolus: Structure, functions, amd associated diseases], *Med. Sci. (Paris) 20*, 37−44.

39. Prieto, J. L., and McStay, B. (2005) Nucleolar biogenesis: The first small steps, *Biochem. Soc. Trans. 33*, 1441−1443.

40. Perander, M., Bjorkoy, G., and Johansen, T. (2001) Nuclear import and export signals enable rapid nucleocytoplasmic shuttling of the atypical protein kinase C *λ*, *J. Biol. Chem. 276*, 13015−13024.

41. Maurer-Stroh, S., Dickens, N. J., Hughes-Davies, L., Kouzarides, T., Eisenhaber, F., and Ponting, C. P. (2003) The Tudor domain 'Royal Family': Tudor, plant Agenet, Chromo, PWWP and MBT domains, *Trends Biochem. Sci. 28*, 69−74.

42. Lewis, H. A., Musunuru, K., Jensen, K. B., Edo, C., Chen, H., Darnell, R. B., and Burley, S. K. (2000) Sequence-specific RNA binding by a Nova KH domain: Implications for paraneoplastic disease and the fragile X syndrome, *Cell 100*, 323−332.

43. Lamb, F. S., Barna, T. J., Goud, C., Marenholz, I., Mischke, D., and Schutte, B. C. (2000) Complex RNA processing of TDRKH, a novel gene encoding the putative RNA-binding tudor and KH domains, *Gene 246*, 209−218.

44. Paziewska, A., Wyrwicz, L. S., Bujnicki, J. M., Bomsztyk, K., and Ostrowski, J. (2004) Cooperative binding of the hnRNP K three KH domains to mRNA targets, *FEBS Lett. 577*, 134−140.

45. Chmiel, N. H., Rio, D. C., and Doudna, J. A. (2006) Distinct contributions of KH domains to substrate binding affinity of *Drosophila* P-element somatic inhibitor protein, *RNA 12*, 283−291.

46. Darnell, J. C., Fraser, C. E., Mostovetsky, O., Stefani, G., Jones, T. A., Eddy, S. R., and Darnell, R. B. (2005) Kissing complex RNAs mediate interaction between the Fragile-X mental retardation protein KH2 domain and brain polyribosomes, *Genes Dev. 19*, 903−918.

47. Git, A., and Standart, N. (2002) The KH domains of *Xenopus* Vg1RBP mediate RNA binding and self-association, *RNA 8*, 1319−1333.

48. Ramos, A., Hollingworth, D., Major, S. A., Adinolfi, S., Kelly, G., Muskett, F. W., and Pastore, A. (2002) Role of dimerization in KH/RNA complexes: The example of Nova KH3, *Biochemistry 41*, 4193−4201.

49. Voit, R., Schnapp, A., Kuhn, A., Rosenbauer, H., Hirschmann, P., Stunnenberg, H. G., and Grummt, I. (1992) The nucleolar transcription factor mUBF is phosphorylated by casein kinase II in the C-terminal hyperacidic tail which is essential for transactivation, *EMBO J. 11*, 2211−2218.

50. Lui, P. P. Y., Chan, F. L., Suen, Y. K., Kwok, T. T., and Kong, S. K. (2003) The nucleus of HeLa cells contains tubular structures for Ca^{2+} signaling with the involvement of mitochondria, *Biochem. Biophys. Res. Commun. 308*, 826−833.

51. Pryde, F., Khalili, S., Robertson, K., Selfridge, J., Ritchie, A. M., Melton, D. W., Jullien, D., and Adachi, Y. (2005) 53BP1 exchanges slowly at the sites of DNA damage and appears to require RNA for its association with chromatin, *J. Cell Sci. 118*, 2043−2055.

52. Renvoise, B., Khoobarry, K., Gendron, M. C., Cibert, C., Viollet, L., and Lefebvre, S. (2006) Distinct domains of the spinal muscular atrophy protein SMN are required for targeting to Cajal bodies in mammalian cells, *J. Cell Sci. 119*, 680−692.

53. Young, P. J., Francis, J. W., Lince, D., Coon, K., Androphy, E. J., and Lorson, C. L. (2003) The Ewing's sarcoma protein interacts with the Tudor domain of the survival motor neuron protein, *Mol. Brain Res. 119*, 37−49.

54. Steen, R. L., Beullens, M., Landsverk, H. B., Bollen, M., and Collas, P. (2003) AKAP149 is a novel PP1 specifier required to maintain nuclear envelope integrity in G1 phase, *J. Cell Sci. 116*, 2237−2246.

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