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Interaction of the regulatory subunit of the cAMP-dependent protein kinase with PATZ1 (ZNF278)

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ABSTRACT

The effects of cAMP in cell are predominantly mediated by the cAMP-dependent protein kinase (PKA), which is composed of two genetically distinct subunits, catalytic (C) and regulatory (R), forming a tetrameric holoenzyme R₂C₂. The only known function for the R subunit is that of inhibiting the activity of the C subunit kinase. It has been shown that overexpression of RI α , but not the C subunit kinase, is associated with neoplastic transformation. In addition, it has also been demonstrated that mutation in the RI α , but not the C subunit is associated with increased resistance to the DNA-damaging anticancer drug cisplatin, thus suggesting that the RI α subunit of PKA may have functions independent of the kinase. We show here that the RI α subunit interacts with a BTB/POZ domain zinc-finger transcription factor, PATZ1 (ZNF278), and co-expression with RI α results in its sequestration in the cytoplasm. The cytoplasmic/nuclear translocation is inducible by cAMP. C-terminus deletion abolishes PATZ1 interaction with RI α and results in its localization in the nucleus. PATZ1 transactivates the cMyc promoter and the presence of cAMP and co-expression with RI α modulates its transactivation. Moreover, PATZ1 is aberrantly expressed in cancer. Taken together, our results showed a potentially novel mechanism of cAMP signaling mediated through the interaction of RI α with PATZ1 that is independent of the kinase activity of PKA, and the aberrant expression of PATZ1 in cancer point to its role in cell growth regulation.

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Introduction

Phosphorylation mediated by the cAMP signal transduction pathway is involved in the regulation of metabolism, cell growth and differentiation, apoptosis, and gene expression [1,2]. In mammalian cells, PKA is a tetrameric holoenzyme of two catalytic (C) and two regulatory (R) subunits (R₂C₂), encoded by four genetically distinct R subunit isoforms designated as RI α and RI β , and RII α and RII β , and three isoforms of the C subunit, C α , C β , and C γ [1,3–5].

The principal mechanism of cAMP signaling involves the binding of cAMP to the R subunit, which causes dissociation of the holoenzyme and the activation of the C subunit kinase. However, recent discoveries have shown that there are other receptors for cAMP in addition to the R subunit, as well as alternative signaling pathways activatable by the C subunit including the direct activation of a class of cyclic nucleotide-gated ion channels by cAMP in the central

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nervous system [6–8]. It was also demonstrated that the C subunit could be activated in a ternary complex of NF κ B–I κ B–C subunit, independent of cAMP and the R subunit, and degradation of I κ B following the exposure to inducers of NF κ B activates the C subunit, which then phosphorylates NF κ B [9]. It was further shown that a family of novel cAMP-binding guanine nucleotide exchange factors can selectively activate the Ras superfamily of guanine nucleotide binding protein, Rap1, in a cAMP-dependent but PKA-independent manner [10,11]. Moreover, it has also been reported that a cAMP-bound RII subunit complex inhibits phosphatase activity [12].

Defects in cAMP response and differential expression of RI and RII has been correlated with cell differentiation and neoplastic transformation, with RI preferentially expressed in transformed cells, and increased RII expression in terminally differentiated tissues, which cannot be attributed to the kinase activity of PKA [3]. That the R subunit may have function independent of the C subunit kinase activity is further implicated in the report that, PKA genetic mutants derived from CHO and the mouse Y1 adrenocortical carcinoma cells having mutations in the RI α but not the C subunit, exhibit increased resistance to cisplatin, a DNA-damaging anticancer drug [13,14]. Subsequently, it was shown that the RI α subunit interacts with the subunit Vb of cytochrome c oxidase (CoxVb) that is regulated by cAMP, and elevation of cAMP levels inhibits

cytochrome *c* oxidase activity in CHO cells [15]. More notably, the R1 α subunit is a genetic target for alteration in the inherited autosomal dominant Carney complex (CNC) disorder, a multiple neoplasia syndrome, suggesting its role as a tumor suppressor [16–18]. These results together suggest that the cAMP/PKA signaling mechanism is more complex and diverse than previously appreciated and this signaling network warrants further exploration.

We show here that a BTB-POZ domain zinc-finger transcription factor, PAZT1 (ZNF278) interacts with the R1 α subunit, and translocation of PAZT1 from the cytoplasm into the nucleus is regulated by cAMP. We showed further that PAZT1 is a transcription activator and is aberrantly expressed in cancer. Our results suggest that in addition to inhibiting the C subunit kinase activity, the R1 α subunit may have novel function by interacting with and sequestering PAZT1 in the cytoplasm, thereby, regulating its transcriptional activity and function in cell growth control in response to cAMP.

Materials and methods

R1 α interactions by the yeast two-hybrid cloning assay. Yeast two-hybrid interaction cloning experiments with R1 α were conducted as described before [15,19]. Full-length coding sequence of R1 α fused to the Gal4 DNA-binding domain in plasmid pAS2-1 (pAS2-R1 α) was used as bait to screen a human liver cDNA library fused to the Gal4 activation domain in the pGAD-GH vector. Positive clones were detected by β -galactosidase assay followed by confirmation with the mating assay using pAS2-R1 α cells.

GST fusion, R1 α deletion, and pull down assay. Expression of the glutathione S-transferase (GST)-R1 α fusion protein and its partial purification with glutathione beads were conducted as previously described [15]. In brief, R1 α cloned into pGEX-4T-1 vector was expressed in the *Escherichia coli* DH5 α cells with IPTG, lysed by sonication, and the lysates were incubated with glutathione resin to immobilize the GST fusion proteins. GST-R1 α beads were then incubated with either yeast lysates overexpressing clone, which harbors the R1 α interacting protein domain, or *in vitro* translated PATZ1. The R1 α amino-terminal (GST-R1 α (Δ 1–76)) and the carboxyl-terminal (GST-R1 α (Δ 77–380)) deletion mutants were constructed as described previously [15]. Deletion mutants were expressed and bound to glutathione resin and then incubated with yeast lysates containing clone pACT-A14, comprising of PATZ1, separated on SDS-PAGE and immunoblotted with anti-GAD antibody.

Cell cultures and green fluorescent protein analysis. Cells were obtained from American Type Culture Collection (Manassas, VA) and grown in 100 mm Petri dishes at 37 °C, in 5% CO₂, in the appropriate media supplemented with 10% or 15% serum. Cells were then washed and replenished in Opti-MEM (Invitrogen, Carlsbad, CA) for transfection using Lipofectamine (Invitrogen) with various plasmid constructs as indicated for approximately 4 h according to manufacturer's specifications. Transfected cells were analyzed 36 h later and subcellular localization of GFP/PATZ1 was imaged using a Zeiss Axioskop fluorescence microscope. Transfected cells were also treated with 8-bromo-cAMP (100 μ M) and the GFP/PATZ1 subcellular localization was analyzed as above.

RNA blot analysis. Normal human tissue RNA blots (BD Biosciences, Palo Alto, CA) were probed with the 1.5 kb insert encompassing the 3'-end of PATZ1, derived from clone pACT-A14. RNAs from normal human breast tissue and breast cancer cell lines were prepared using the Qiagen RNeasy kit according to manufacturer's specification, fractionated on denaturing formaldehyde agarose gel, and then transferred to nitrocellulose for probing with the 1.5 kb PATZ1 cDNA fragment as above.

Construction of PATZ1. Full-length PATZ1 cDNA isolated from the human spleen cDNA library was cloned into the pBlueScript vector

(Stratagene, La Jolla, CA) to yield pBPATZ1. The plasmid was further digested with HindIII and SgrA1 to remove 5 upstream ATGs in the 5' leader sequence, and then religated to yield Δ PATZ1. A 1.9 kb Sall/HincII fragment of PATZ1, containing the coding sequence, was excised from Δ PATZ1 and ligated in frame into pLEGFP-C1 (BD Biosciences) digested with Sall and SmaI. The same fragment was also ligated in frame into the pLEGFP-N2 vector. C-terminus deletion mutant of PATZ1 was made by PCR using the forward primer 5'-AAATAAGCTTCCATGGAGCGG-GTAAAC and the reverse primer 5'-GCGGTCTCTCACTCAGCTGATT and cloned as a HindIII/Sall fragment, in frame into pLEGFP-C1. The forward and reverse primers included the linkers sequence.

Transfection and reporter assay. Approximately 1×10^5 cells were plated per well in 12-well cluster dishes for overnight, and then washed, replenished with Opti-MEM and transfected using Lipofectamine as with the human cMyc promoter luciferase reporter plasmid and various plasmid constructs as indicated. The *Renilla* luciferase expression plasmid pRL-CMV (Promega) (0.5 μ g) was included in the cotransfections as internal standard for normalization. Luciferase activity was determined using the Turner Designs Luminometer Model TD-20/20 (Promega) according to manufacturer's specifications.

Results

Identification of R1 α interaction with PATZ1

To further understand the function of R1 α that is independent of PKA, we performed yeast two-hybrid interaction experiments using the full-length R1 α as bait and screened a human liver two-hybrid cDNA library, and found interaction of R1 α with PATZ1 (ZNF278), a member of the superfamily of BTB-POZ domain zinc finger proteins [20,21]. Yeast mating assay confirmed the interaction of PATZ1 with R1 α (Fig. 1A). PATZ1 encodes a BTB-POZ domain zinc finger protein of 641 amino acids with an estimated molecular mass of 69 kDa. Computational analysis of the intron–exon boundaries of the genomic sequence of PATZ1 on chromosome 22 predicted four splice variants, which include PATZ [21], ZSG (Zinc finger Sarcoma Gene) [22], and the variant identified here that interacts with R1 α . The splice variant PATZ retains most of the features of PATZ1 except for the three zinc finger motifs and the R1 α interacting domain that are absent at the C-terminus of PATZ [21]. In Ewing sarcoma (EWS), all four splice variants are targeted for rearrangement and form a chimera with the EWS gene on 22q12, that is thought to activate EWS in soft tissue sarcoma [22].

In vitro translation and interaction of PATZ1 with R1 α

To further characterize the above interaction, PATZ1 was translated and labeled *in vitro* with [³⁵S]methionine. Gel electrophoresis showed a product with molecular size of approximately 67–70 kDa (Fig. 1C), corresponding closely to the calculated molecular weight from the open reading frame of PATZ1. Deleting a cluster of upstream ATGs in the 5'-end of the PATZ1 cDNA (Fig. 1B) seemed to enhance translation from the 3'-most ATG start codon (Fig. 1C). *In vitro* translated PATZ1 interacted with GST-R1 α but not GST (Fig. 1D).

Coimmunoprecipitation using GST-R1 α showed interaction with PATZ1 from yeast lysates (Fig. 1F). Deleting R1 α C-terminus, GST-R1 α (Δ 77–380) (Fig. 1E), which includes the autoinhibitory region and the two cAMP binding sites, did not affect its association with PATZ1 (Fig. 1F), while the deletion of R1 α N-terminus, GST-R1 α (Δ 1–76) (Fig. 1E), containing the dimerization as well as the A-kinase anchoring protein (AKAP) binding domains abolished its interaction with PATZ1 (Fig. 1F).

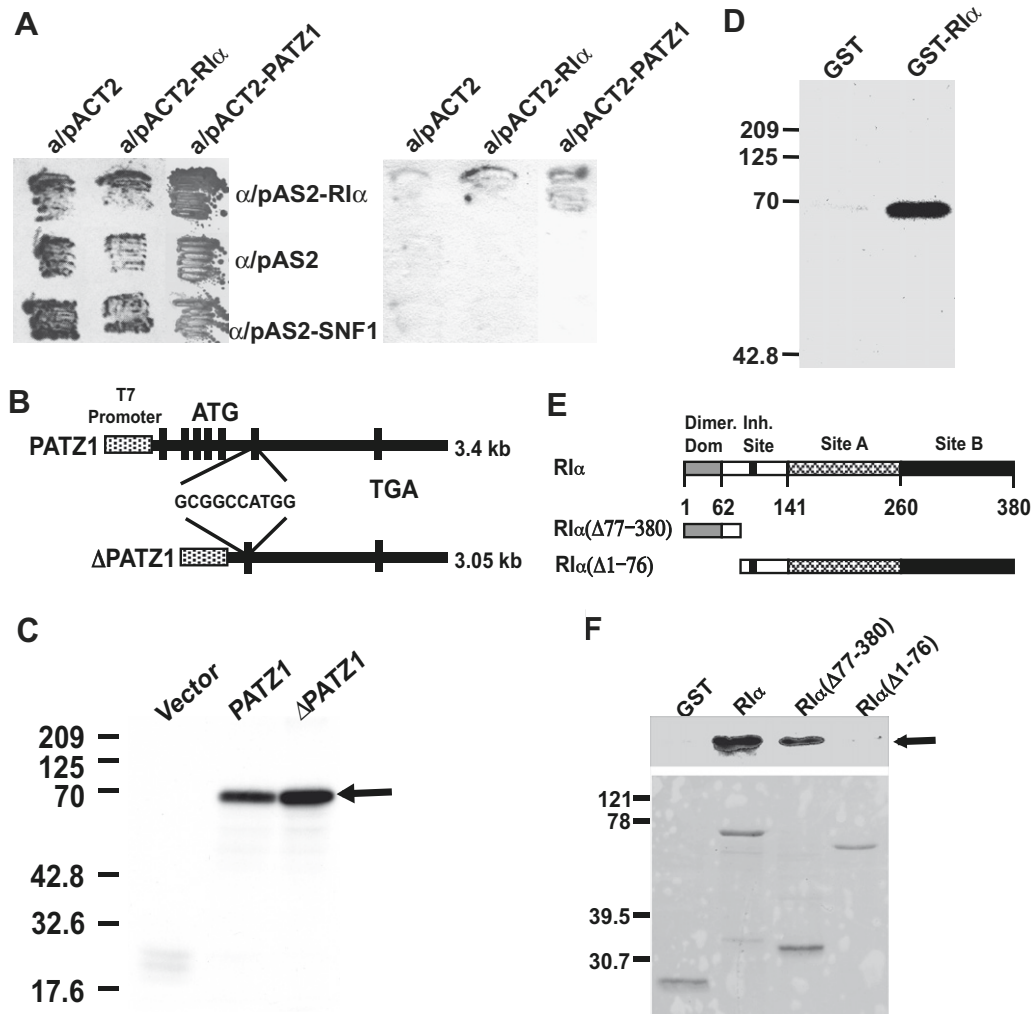


Fig. 1. Interaction of RI α with PATZ1. (A) Yeast two-hybrid interaction experiment showing the association of RI α with PATZ1. (B) Schematic representation of the full length and the upstream ATGs-deletion mutant of PATZ1. Approximately 350 bp from the 5'-end of PATZ1 cDNA, containing a cluster of upstream ATGs, was deleted. (C) *In vitro* transcription and translation was performed with the full-length PATZ1 or its upstream ATGs-deletion mutant (Δ PATZ1) compared to the pBluescript II SK(-) vector control. (D) The *in vitro*-[35 S]methionine-labeled translated PATZ1 was interacted with either GST or GST-RI α immobilized on glutathione resins. (E) Schematic representation of wild-type and deletion mutants of RI α . Dimer. Dom., the N-terminal dimerization domain; Inh. Site, inhibitory site; sites A and B are the two cAMP binding domains. (F) Top panel: interaction of PATZ1 from yeast extracts with either recombinant GST, GST-RI α , GST-RI α (Δ 1-76), and GST-RI α (Δ 77-380); bottom panel: bacterial lysates containing the respective expressed proteins GST, GST-RI α ; GST-RI α (Δ 77-380) and GST-RI α (Δ 1-76).

Subcellular distribution of PATZ1 and its interaction with RI α

Type I PKA is predominantly localized in the cytoplasm [23] and RI α subunit has been shown to bind tightly to the plasma membrane [24]. We examined whether interaction of PATZ1 with RI α might influence its localization. PATZ1 was fused to the green fluorescence protein (GFP) at either the N- (GFP/PATZ1) or the C-terminus (PATZ1/GFP) and a speckled/punctate distribution in the nucleus was observed in either PC3M prostate carcinoma cells (Fig. 2B), or the normal fibroblasts, GM38A (Fig. 2F). Fusion of GFP to the N- or the C-terminus of PATZ1 did not alter its nuclear distribution (Fig. 2K and L). Cotransfection of GFP/PATZ1 with RI α in these cells resulted in the redistribution of PATZ1 in both the cytoplasm and the nucleus (Fig. 2D, H and M); suggesting that RI α may sequester PATZ1 in the cytoplasm. RI α did not alter GFP localization (Fig. 2I and J). Addition of cAMP caused the translocation of PATZ1 from the cytoplasm to the nucleus (Fig. 3N).

Effects of PATZ1 C-terminus deletion on its interaction with RI α

As shown above, the dimerization and the AKAP binding domains of RI α is required for interaction with PATZ1 (Fig. 1F).

We found further that deleting the C-terminus of PATZ1(Δ 461-641) did not affect its nuclear localization compared to wild-type (Fig. 3A and B). Cotransfection with RI α led to a redistribution of PATZ1 to both the cytoplasm and the nucleus (Fig. 3C). In contrast, cotransfecting RI α with PATZ1(Δ 461-641) showed only nuclear distribution (Fig. 3D); thus suggesting that PATZ1 C-terminus deletion abolishes its interaction with RI α and sequestration in the cytoplasm.

Expression of RIAZ in normal tissues and breast cancer

Previous studies suggest that the various splice variants of PATZ1 might be targeted for genetic alterations in cancer and have potential roles in growth control [22,25,26]. To gain insight into its pathological role in cancer, we determined PATZ1 expression by Northern blot analysis in normal tissues and found its ubiquitous expression in many human tissues with the highest abundance in the spleen, ovary, and heart, and significant levels in the brain, prostate, testis, pancreas, and leukocyte (Fig. 4A). Compared to the normal human fibroblasts, GM388, aberrant PATZ1 expression was found in some cancer cells of the breast (MCF-7) and cervix (C33A), as well as the Jurkat lymphoma, JEG1 cells (Fig. 4B). PATZ1

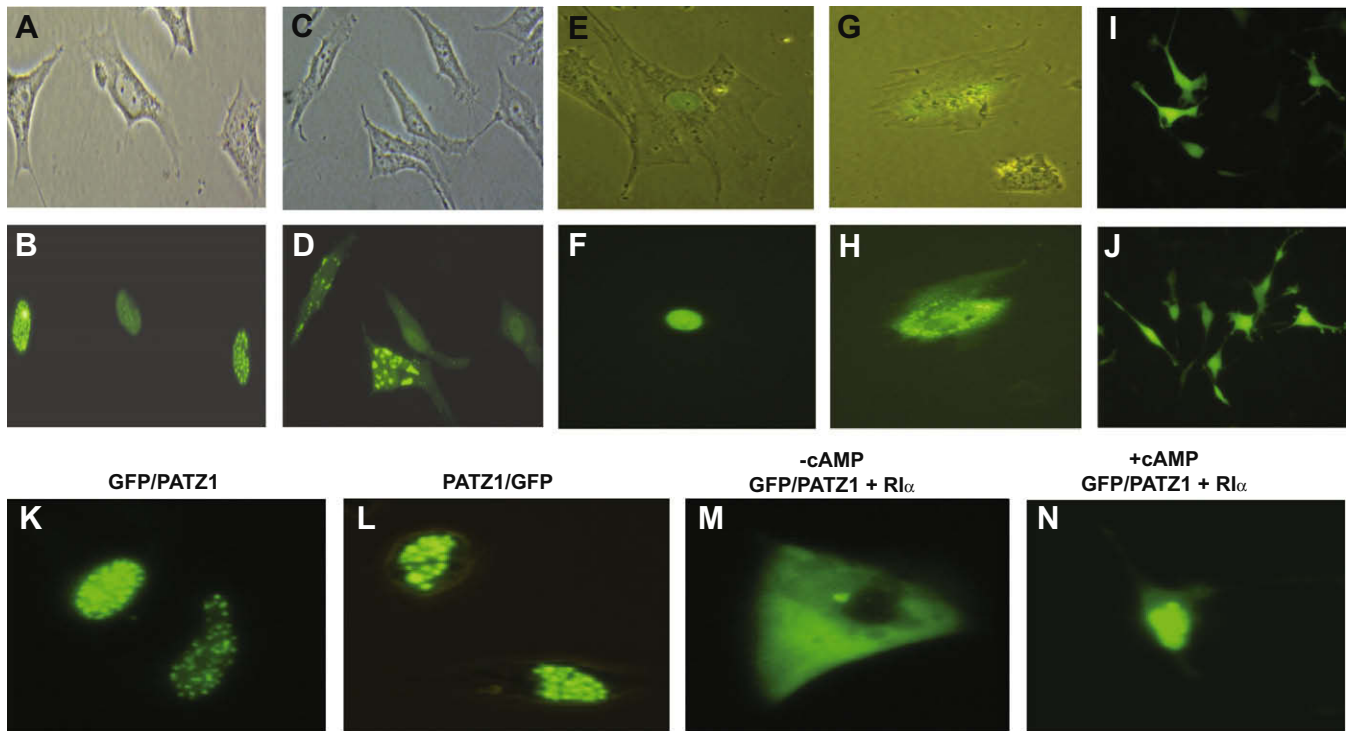


Fig. 2. Subcellular localization of RIAZ. (A, C, E, and G) Light micrograph of PC3M cells (A, C) or normal fibroblasts (E, G) either transfected with GFP/PATZ1 (A, B, E, and F) or cotransfected with GFP/PATZ1 and RI α (C, D, G, and H). (B, D, F, and H) Fluorescence image of GFP/PATZ1 localization. (I) Fluorescence image of PC3M cells transfected with GFP alone and (J) cotransfected with GFP and RI α . (K, L) Localization of either GFP/PATZ1 or PATZ1/GFP in HTB-46 cells; (L) transfected with RIAZ/GFP; (M) cotransfected with GFP/RIAZ and RI α in the absence of 8-Br-cAMP; (N) cotransfected with GFP/RIAZ and RI α in the presence of 1 mM 8-Br-cAMP. Subcellular localization of GFP/RIAZ was visualized using a Zeiss Axioskop fluorescence microscope.

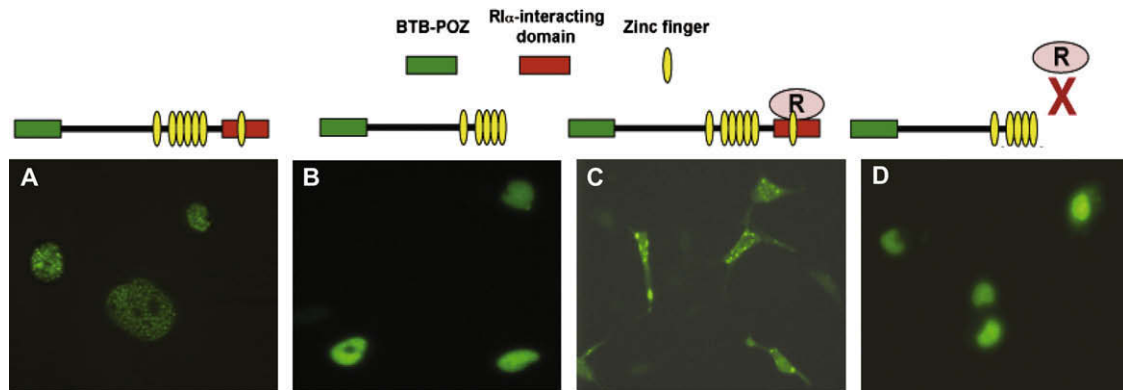


Fig. 3. Interaction with RI α with PATZ1 deletion mutant. PC3M cells were transfected with (A) GFP/PATZ1; (B) GFP/PATZ1 and RI α ; (C) GFP fusion of C-terminus deletion mutant of PATZ1 (GFP/ Δ PATZ1); or (D) GFP/ Δ PATZ1 and RI α . Subcellular localization of GFP/PATZ1 was visualized 24 h after transfection.

overexpression was also observed in a panel of breast tumor cell lines including MCF-7, BT474 and SKBr3, compared to normal breast tissue. Notably, we further observed approximately 16% (22 of 119 cases) of breast cancer specimens from patients in a previous microarray study [27] exhibited elevated levels of PATZ1 expression.

Discussion

The cAMP signal transduction system is one of the best-characterized biochemical pathways in mammalian cells. Until recently, the R subunit has been the only known receptor for cAMP in cells and cAMP binding to the holoenzyme has been the accepted mechanism that regulates PKA activity. The only known function for the R subunit is that of inhibiting the C subunit kinase activity in the

holoenzyme complex. Previous work from our laboratory using mammalian genetic mutant cell lines implicates potential functions for the R subunit that is independent of the C subunit kinase [17,28,29]. In this report, we showed by interaction cloning experiment the association of the RI α subunit of PKA with a BTB-POZ domain zinc-finger transcription factor PATZ1, and regulation by cAMP (Figs. 1 and 2). We further showed that PATZ1 is aberrantly expressed in about 16% of breast cancer cases (Fig. 4).

The role of cAMP in cell growth has been intensely investigated [28]. In a large number of human cancer as well as cancer cell lines, only type I PKA or the RI subunit is detected. Moreover, overexpression of RI α in CHO cells confers growth advantage in monolayer and soft agar conditions, but overexpression of the C subunit does not [29]. It was further observed that overexpression of RI α , but not the C subunit, in MCF-10A cells confers the ability to

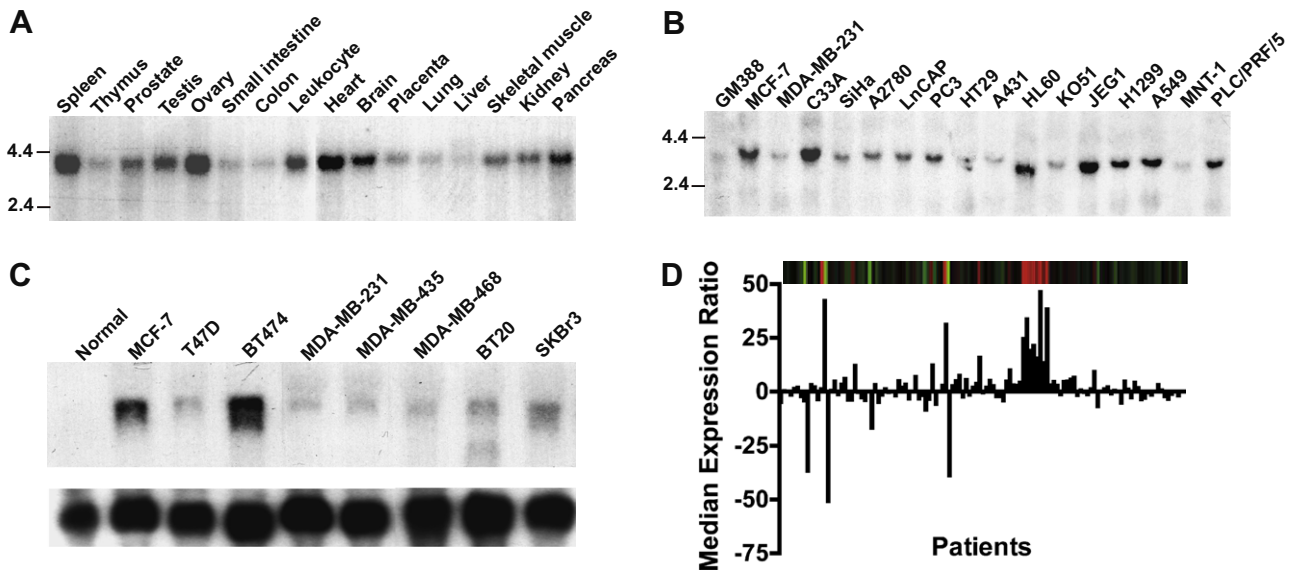


Fig. 4. Expression of PATZ1 in normal human tissue, cancer cell lines, and clinical cancer specimens. (A) RNA blot analysis of PATZ1 in normal human tissues. Northern blot analysis of PATZ1 in various (C) human cancer cell lines, and a panel of (C) breast cancer cells. (D) PATZ1 expression in breast cancer specimens from 119 patients. Red bars in dendrogram showed increased PATZ1 levels, and green bar, decreased levels compared with reference to mean. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

grow in serum and growth factor free conditions [30]. It is intriguing that the cAMP-mediated cell growth is independent of the kinase activity, thus raising the possibility that the R subunit may have hitherto unknown function that contribute to cell growth. Such a possibility is supported by the finding that the $R1\alpha$ subunit gene is targeted for genetic alterations in the inherited autosomal dominant Carney complex (CNC) disorder, which is a multiple neoplasia syndrome characterized by spotty skin pigmentation, cardiac and other myxomas, endocrine tumors and psammomatous melanotic schwannomas [16,17,31]. The germline mutations in $R1\alpha$ suggest that it is a tumor suppressor gene and have potential role in cell growth regulation. This and the aberrant expression of PATZ1 in cancer (Fig. 4) and its abnormal translocation in Ewing sarcoma, suggest an important synergy between the interaction of $R1\alpha$ with PATZ1 in tumorigenesis. Moreover, the splice variant PATZ has been shown to transcriptionally repress the *c-myc*, *CDC6* and *galectin-1* promoters [21]. In addition, MAZR, the mouse homolog of PATZ1, exhibit transactivation potential from the SV40 and the *c-myc* promoter [32], thus suggesting that PATZ1 may have transcriptional potential.

The precise mechanism by which $R1\alpha$ regulates cell growth that is independent of PKA, remains unclear. Our observation here suggest a novel signaling mechanism in which the sequestration of PATZ1 in the cytoplasm through its interaction with $R1\alpha$ is regulated by cAMP, thus enabling PATZ1 to translocate into nucleus and transactivate its target genes upon activation of the cAMP pathway. Therefore, further understanding of the interaction of $R1\alpha$ with RIAZ and their effects in growth regulation may offer insights on the mechanisms of cAMP mediated growth control.

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