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# Ht31: the first protein kinase A anchoring protein to integrate protein kinase A and Rho signaling[1]

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**Abstract In an attempt to isolate protein kinase A anchoring proteins (AKAPs) involved in vasopressin-mediated water reabsorbtion, the complete sequence of the human AKAP Ht31 was determined and a partial cDNA of its rat orthologue (Rt31) was cloned. The Ht31 cDNA includes the estrogen receptor cofactor Brx and the RhoA GDP/GTP exchange factor proto-lymphoid blast crisis (Lbc) sequences. The Ht31 gene was assigned to chromosome 15 (region q24–q25). It encodes Ht31 and the smaller splice variants Brx and proto-Lbc. A protein of the predicted size of Ht31 (309 kDa) was detected in human mammary carcinoma and HeLa cells. Anti-Ht31/Rt31 antibodies immunoprecipitated RhoA from primary cultured rat renal inner medullary collecting duct cells, indicating an interaction between the AKAP and RhoA in vivo. These results suggest that Ht31/Rt31 represent a new type of AKAP, containing both an anchoring and a catalytic domain, which appears to be capable of modulating the activity of an interacting partner. Ht31/Rt31 have the potential to integrate Rho and protein kinase A signaling pathways, and thus, are prime candidates to regulate vasopressin-mediated water reabsorbtion.** 

*Key words:* Protein kinase A anchoring protein; Rho; Rho GTPase guanine nucleotide exchange factor; Ht31; Brx; Aquaporin

## 1. Introduction

Protein kinase A anchoring proteins (AKAPs) position protein kinase A (PKA) in the vicinity of its substrates and restrict its access to only a fraction of cellular substrates. The tethering of PKA to subcellular compartments was the original function attributed to AKAPs [1]. More recent evidence indicates that they also tether whole signaling complexes to specific sites. AKAP79, for example, interacts with the post-synaptic density proteins PSD95 and SAP97, which bind directly to glutamate receptors. Through this interaction, AKAP79 anchors PKA, PKC and calcineurin in close proximity of the receptors [2]. Herrgard et al. suggested a novel function of the $AKAP_{CE}$; this AKAP may interact with a TGFβ family receptor [3].

Amino acids 635–1012 of the previously identified partial open reading frame of the human AKAP Ht31 (1045 amino acids) [1,4,5] are identical with amino acids 4–381 of the human breast cancer nuclear receptor-binding auxiliary protein (Brx; Fig. 1A) [6]. Brx is an estrogen receptor cofactor which augments gene activation by the receptor [6]. Amino acids 536–1230 of Brx are identical with residues 1–693 of the RhoA-specific GDP/GTP exchange factor (GEF) proto-lymphoid blast crisis (proto-Lbc). In addition, amino acids 1264–1429 of Brx are identical with residues 727–893 of proto-Lbc. Proto-Lbc belongs to the Dbl-homology (DH) family of GEFs, characterized by the presence of tandem DH and pleckstrin-homology (PH) domains (Fig. 1A) [6–9].

In this study we determined the full length Ht31 cDNA sequence and cloned a partial cDNA of the rat orthologue (Rt31) of Ht31. The full length Ht31 cDNA includes Brx and proto-Lbc cDNA sequences. Rt31 interacts in vivo with RhoA in primary cultured rat inner medullary collecting duct (IMCD) cells, suggesting that Ht31/Rt31 may function not only as PKA anchor, but also as a modulator of Rho activity. Thus, Ht31/Rt31 may integrate PKA and Rho signals and are, therefore, prime candidates for regulating processes such as vasopressin-mediated water reabsorbtion which depend on both PKA and Rho signaling [10–12].

## 2. Materials and methods

### 2.1. Expression cDNA library screening, PCR, RACE-PCR, and DNA sequence analysis

A rat kidney Uni-ZAP XR expression cDNA library (Stratagene, La Jolla, CA, USA) was screened using the type II regulatory subunit of PKA (RII) overlay technique [13]. PCRs and RACE-PCRs were performed using human mammary gland Marathon-Ready cDNA and the Advantage cDNA polymerase mix according to the supplier's recommendations (Clontech, Heidelberg, Germany). Primer sequences used for the amplification of seven cDNA fragments (Fig. 2) encoding Ht31 are available upon request. Fragments 1 and 3–6 were cloned into the vector pGem-T Easy (Promega, Mannheim, Germany) and fragments 2 and 7 were cloned into the vector pCR-XL Topo (Invitrogen, Groningen, The Netherlands). Initially, sequencing was carried out using vector sequencing primers and subsequently by primer

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[1] The cDNA sequences encoding Ht31 and Rt31 were deposited in GenBank under the accession numbers AF387101 and AF387102 respectively.

*Abbreviations:* AKAP, A-kinase anchoring protein; AQP2, aquaporin-2; Lbc, lymphoid blast crisis; PKA, protein kinase A; IMCD, inner medullary collecting duct; RII, type II regulatory subunit of protein kinase A

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walking (primer sequences are available upon request; Dye Terminator FS 2.0 kit and ABI Prism 377 DNA sequencer, both Applied Biosystems, Weiterstadt, Germany). Homology searches were performed at GenBank, National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/blast), and at the Institute for Genomic Research (http://www.tigr.org). Sequence translations and alignments were carried out at the Human Genome Center, Baylor College of Medicine (BCM Search Launcher; http://dot.imgen.bcm.tmc.edu:9331/).

*2.2. Cell culture, cell fractionation, and Western blotting*

Primary cultured rat IMCD cells were prepared and cultured as described [10,11,18]. The breast cancer cell lines ZR75-1 (a kind gift of Prof. G. Schaller, Benjamin Franklin Klinikum, Free University of Berlin, Germany) and HeLa cells (a kind gift of Dr. U. Vinkemeier, Forschungsinstitut für Molekulare Pharmakologie, Berlin, Germany) were grown to confluency in Dulbecco's modified Eagle's medium containing 10% FCS. ZR75-1 cells were homogenized in phosphate-buffered saline using a glass/teflon homogenizer. Cell debris and nuclei were removed by centrifugation (4000×*g*, 10 min, 4°C). The supernatant was recentrifuged (150 000×*g*, 1 h, 4°C) to yield particulate (P; pellet) and soluble (S; supernatant) fractions. P fractions (200 000×*g* pellets) from HeLa cells were prepared as previously described for IMCD cells [10]. Ht31 was detected by Western blotting with affinity-purified antibodies 1965 or 3060 (dilutions 1:200; see below). Horseradish peroxidase-conjugated goat anti-rabbit $F(ab)_2$ fragments (Dianova, Hamburg, Germany) were used as secondary antibody (dilution 1:3000).

*2.3. Preparation of affinity-purified anti-Ht31 antibodies*

Rabbit polyclonal antiserum 1965 was raised against a peptide epitope present in Rt31 (amino acids 369–383), Ht31 (amino acids 1556–1570) and Brx (amino acids 172–186). Antiserum 3060 was raised against amino acids 688–709 of Ht31 (Biogenes, Berlin, Germany). Affinity-purified antibodies were prepared from the antisera by chromatography over the peptides used for immunization coupled to thiopropyl Sepharose 6B (Amersham Pharmacia Biotech, Freiburg, Germany).

*2.4. Immunoprecipitation*

IMCD cells were homogenized (20 strokes in a glass teflon homogenizer) in lysis buffer (20 mM Tris–HCl, pH 7.5, 1% Igepal, 1 mM EDTA, pH 8.0, 1 mM EGTA, pH 8.0, 1 mM dithiothreitol, 0.5% deoxycholate, 0.1% SDS, 1.5 mM $MgCl_2$, 0.15 M NaCl). The homogenates were centrifuged (30 000×*g*, 30 min, 4°C) and the supernatant from seven confluent 65 mm Petri dishes incubated overnight with the affinity-purified anti-Ht31 antibody 3060 (40 μg; see above) or preimmune serum (20 μl). Protein A-conjugated agarose beads (Sigma, Deisenhofen, Germany) were added (4 h). The beads were washed three times with 1 ml of lysis buffer. Bound proteins were eluted with Laemmli sample buffer (95°C) and subjected to Western blot analysis (see above) for the detection of RhoA with commercially available antibodies (Santa Cruz Biotechnology, Heidelberg, Germany).

## 3. Results

*3.1. Cloning of a fragment of Rt31 and identification of the Ht31 cDNA sequence*

A partial cDNA clone (1747 bp; GenBank accession number AF387102) encoding a protein with homology to AKAP Ht31 [4,5] was isolated from a rat kidney expression library. In analogy to Ht31, the new protein was named R(attus)t31 (Fig. 1A). The overall amino acid sequence (582 residues) identity between Rt31 and the corresponding region of Ht31 is 67.7%. Using Rt31 fragments and the RII overlay technique [4,13], the RII-binding site of Rt31 was mapped to amino acids 65–80 (SIEETATRIVEAVIKQ), corresponding to amino acids 494–507 of Ht31 (data not shown). The amino acid sequence identity in this region is 71.4%. Recombinant Rt31 protein bound PKA RIIβ subunits with a $K_d$ of 85 nM (data not shown).

The sequence identity between Ht31, Brx and proto-Lbc in the overlapping region (see Section 1) led to the hypothesis that the unidentified 3′ sequence of Ht31 includes the 3′ sequence of Brx and proto-Lbc. Using these known cDNA sequences as the basis, both the 3′ and 5′ ends of Ht31 were completed by a combination of conventional PCR, RACE-PCR and data base analysis (Fig. 2; see Section 2). The two large overlapping fragments 2 and 7 cover 8051 bp (Fig. 2). The total length of the composite Ht31 cDNA is 8442 bp from the start codon (contained within a Kozak consensus sequence) [14] to the stop codon (GenBank accession number

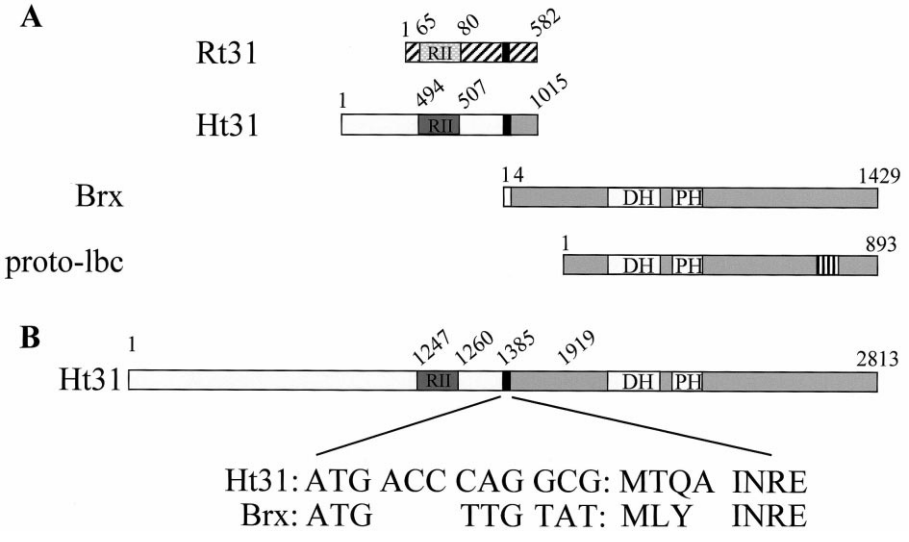


Fig. 1. A: Schematic representation of Rt31, Ht31, Brx and proto-Lbc proteins. Rt31 and Ht31 represent partial sequences. Open reading frames are indicated by boxes. Identical amino acid sequences are depicted by the same shading. RII, RII-binding site, DH and PH, dbl and pleckstrin homology domains. Numbers indicate amino acids. B: Schematic representation of the full length Ht31 which posseses a unique N-terminus and amino acids 4–1429 of Brx in its C-terminus. The first three amino acids of Brx are encoded by a GenBank clone which has not yet been assigned to a chromosome, but most likely represents a separate exon of the Ht31 gene. The differences between Ht31 and Brx in this region (highlighted) most likely result from alternative splicing.

AF387101). An Ht31-specific probe which neither hybridizes to Brx nor to proto-Lbc mRNAs detected several transcripts, including one >9.5 kb, in various human tissues (data not shown). The mRNA species >9.5 kb may encode the Ht31 protein (2813 amino acids; Fig. 1B). The protein has a calculated molecular weight of about 309 kDa and contains an RII-binding domain (amino acids 1247–1266) and DH (amino acids 1996–2226) and PH (amino acids 2240–2335) domains (Fig. 1B). As all three domains are present in a single cDNA, Ht31 may function not only as an AKAP, but also as an estrogen receptor cofactor and/or as a RhoA-specific GEF.

### 3.2. Analysis of Ht31 protein expression

The antibody 1965 is directed against a common epitope in Ht31 (amino acids 1556–1570; Fig. 1B), Rt31 and Brx. Brx has been identified in the human mammary carcinoma cell line ZR75-1 [6]. Therefore, this cell line was chosen to test whether the antibody detects Brx (about 170 kDa) and, additionally, a protein with the predicted size of Ht31 (309 kDa; Fig. 3A). In the S fraction, where Brx is expected [6], the antibody detected several proteins between 175 kDa and about 220 kDa. These proteins are either absent from the P fraction or present in the P fraction at much lower level. The 175 kDa species may represent Brx. In addition, a single protein significantly >205 kDa was detected. In the P fraction, the antibody detected one major protein <175 kDa and again the >205 kDa species, which may represent the full length Ht31 protein (Fig. 3A, left panel). None of these proteins was detected after preincubation of the antibody with the peptide used for immunization (Fig. 3A, right panel), indicating the specificity of the antibody and of the signals in the left panel.

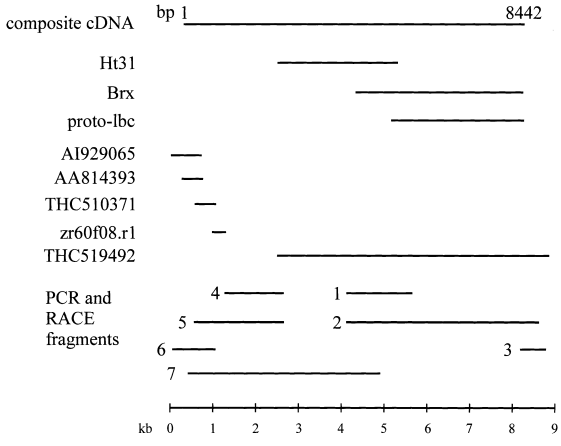


Fig. 2. The strategy to obtain the full length Ht31 cDNA sequence (8442 bp). The 3′ end of the published partial Ht31 sequence [4,5] shares identical sequences with the 5′ end of Brx [6], whose 3′ end shares identical sequences with proto-Lbc [6]. Using these sequences as the basis, the 3′ end of Ht31 was obtained by successive PCR amplifications (fragment 1: 1715 bp; fragment 2: 4527 bp; fragment 3: 444 bp; for details, see Section 2). The 3′ end, including an in-frame stop codon, was identical with GenBank clone THC519492. In an initial step for the identification of the 5′ end, a RACE-PCR product was obtained (fragment 4, 1656 bp) which was identical to the 3′ end of GenBank clone zr60f08.r1. The 5′ end of this clone was identical to the 3′ end of GenBank clone THC510371. Primers derived from this clone and from the Ht31 sequence allowed the amplification of fragment 5 (2275 bp). This sequence allowed the retrieval of GenBank clones AA814393 and AI929065, both containing an in-frame start codon and an upstream, in-frame stop codon. Primers derived from these sequences in combination with a primer derived from Ht31 were used for the amplification of fragment 6 (1265 bp), containing the start codon of Ht31. Fragment 7 (4878 bp) overlapping fragment 2 was amplified from the 5′ end to exclude possible PCR artifacts.

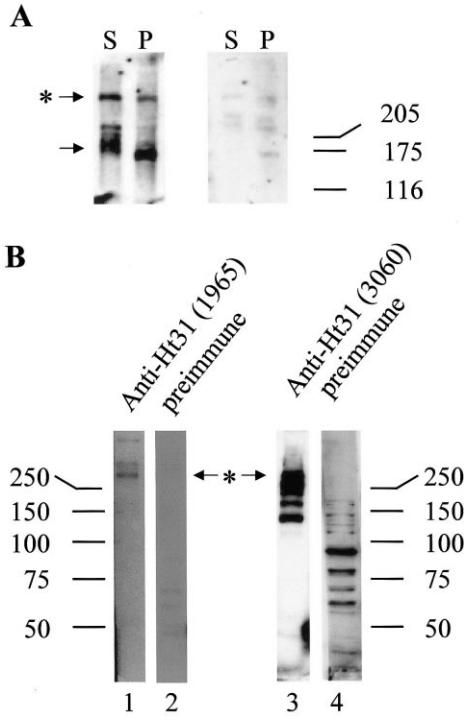


Fig. 3. A: Detection of Ht31 and Brx in S and P fractions (60 μg protein/lane) from the human mammary carcinoma cell lines ZR-75-1 by Western blotting using the antibody 1965 (left panel). In the right panel, the antibody was preincubated with the peptide used for immunization (1 h, 1000-fold excess). B: Detection of Ht31 in P fractions from HeLa cells (60 μg protein/lane) using antibody 1965 (lane 1) or 3060 (lane 3). In lanes 2 and 4, detection was carried out using preimmune sera 1965 and 3060 respectively. *→, potential full length Ht31 (calculated molecular weight of about 309 kDa); →, protein of about 175 kDa most likely representing Brx. Signals were visualized using the Lumi-light Western blot detection system and a Lumi-Imager F1 (Roche Diagnostics, Mannheim, Germany).

In HeLa cells, antibody 1965 detected several proteins >250 kDa in the P fraction (Fig. 3B). The antibody 3060, directed against an Ht31 epitope neither present in Brx nor in proto-Lbc, detected several proteins between 130 and >250 kDa (Fig. 3B). Both antibodies recognized one protein >250 kDa, suggesting that this may represent the full length Ht31, which includes Brx in its C-terminus (Fig. 3B). The preimmune sera 1965 and 3060 recognized several proteins <250 kDa, but none of the proteins >250 kDa were detected, indicating that the latter are specifically recognized by the antibodies 1965 and 3060 (Fig. 3B). A protein of the expected size of Ht31 was also detected in the mammary carcinoma cell line MCF7 and in IMCD cells (data not shown).

### 3.3. The gene encoding Ht31, Brx and proto-Lbc is located on chromosome 15 (region q24–q25)

Since Ht31, Brx and proto-Lbc cDNA share identical sequences, we investigated whether they are splice variants derived from the same gene. The chromosomal localization of the Ht31 gene was determined by a Blast search of the human genome data base at the NCBI. Using the 8442 bp Ht31

cDNA as query sequence, clone ref/NT 010343 was retrieved, representing 529261 bp of human chromosome 15 (region q24–q25) [15,16]. The analysis of ref/NT 010343 showed that the Ht31 cDNA fragments were 98–100% identical with sequences within ref/NT 010343, and covered 38 exons over about 250 000 bp (Fig. 4). The intron/exon splice junctions are conserved (data not shown). Since the complete Ht31 cDNA sequence, including the Brx- and proto-Lbc-specific sections, could be assigned to a single gene, Brx and proto-Lbc represent smaller splice variants of Ht31. In agreement with this finding, the Brx and proto-Lbc genes have previously been mapped to the same chromosomal region [8,17]. The first three amino acids of Brx are encoded by a GenBank clone which has not yet been assigned to a chromosome, but most likely represents a separate exon of the Ht31 gene (data not shown).

### 3.4. Rt31 and RhoA interact in vivo

A possible interaction of Rt31 with RhoA was investigated in immunoprecipitation experiments. Using the affinity-purified antibody 3060 directed against an N-terminal Ht31 epitope, Rt31 was immunoprecipitated from primary cultured IMCD cells (Fig. 5). RhoA was detected in the immunoprecipitate, indicating an interaction between Rt31 and RhoA in vivo. In precipitates obtained with preimmune serum, RhoA was not detected, indicating the specificity of the immunoprecipitation. It should be noted that the experiment was performed with non-overexpressing cells, underlining the significance of this finding.

## 4. Discussion

The full length Ht31 cDNA sequence presented here includes an RII-binding domain and both the Brx and proto-Lbc cDNA sequences. The multi-domain architecture of the Ht31 protein implies that it may not only function as an AKAP, but also as an estrogen receptor cofactor involved in transcriptional regulation (Brx) and/or as a RhoA-specific

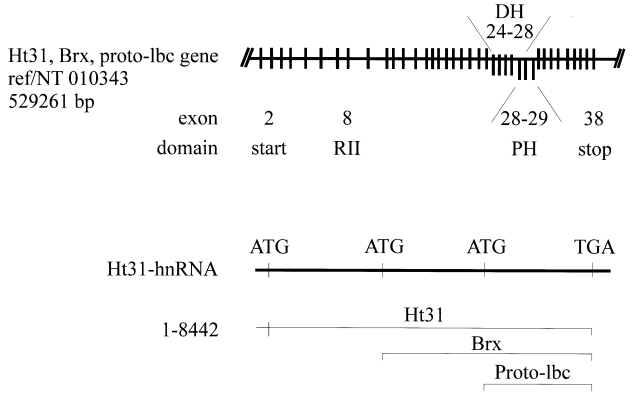


Fig. 4. Ht31, Brx and proto-Lbc are encoded by a single gene. Blast searches of the human genome using the full length Ht31 cDNA sequence (8442 bp) retrieved the genomic clone ref/NT 010343, representing 529 261 bp of chromosome 15 (region q24–q25). The cDNA was identical with 38 genomic DNA fragments representing 38 exons (vertical lines). Exons encoding the Ht31 start and stop codons, RII-binding, DH, and PH domains are highlighted. Since the 38 exons encode Ht31, Brx and proto-Lbc cDNA fragments, a heteronuclear RNA (hnRNA), including the different start codons and the stop codon, is most likely spliced into three separate transcripts.

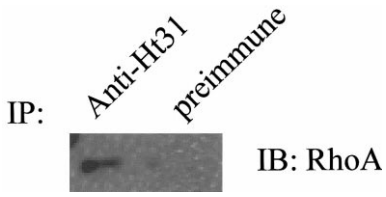


Fig. 5. Rt31 and RhoA interact in vivo. Homogenates from primary cultured IMCD cells were prepared and subjected to immunoprecipitation (IP) using affinity-purified anti-Ht31 antibodies (3060; for details see Section 2) or preimmune serum. RhoA was detected in the immunoprecipitates by immunoblot (IB; see legend to Fig. 3).

GEF (proto-Lbc). The latter function suggests that Ht31 is potentially capable of regulating cellular processes dependent on both PKA and Rho signaling pathways. An interaction of Rt31 and RhoA in vivo is indicated by the immunoprecipitation of RhoA from primary cultured IMCD cells using anti-Rt31/Ht31 antibodies (Fig. 5). This interaction seems to be of physiological significance since the experiments were performed in non-overexpressing cells. In renal inner medulla vasopressin exerts its antidiuretic action by a process involving the translocation of water channels (aquaporin-2 (AQP2)) from intracellular vesicles to the apical membranes of principal cells. Previous work using primary cultured IMCD cells has revealed that AQP2 translocation requires activation of PKA, anchoring of PKA to AKAPs and a decreased Rho activity [10–12]. Rt31/Ht31 may integrate PKA and Rho signaling pathways, and thus, are prime candidates for regulating the translocation of AQP2 in renal principal cells.

In summary, the data presented here suggest that Ht31/Rt31 does not only act as scaffold for PKA. Rather, it is the first AKAP capable of modulating the activity of the signaling protein Rho.

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