# RFX-1, a putative alpha Adducin interacting protein in a human kidney library

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**Abstract** **Adducin regulates tubular absorption of sodium by modulating the expression levels of the sodium–potassium-ATPase in renal tubular cells. Adducin is a candidate gene in the pathogenesis of hypertension. Yeast two hybrid screen showed a specific interaction between human alpha Adducin and the regulatory factor for X box (RFX-1), a nuclear protein that down regulates the expression of several proteins in non neuronal cells. The interaction was confirmed in cells through coimmunoprecipitation and colocalization experiments. The binding of alpha Adducin to RFX-I and their nuclear co-localization suggests that Adducin can have a role in modulating the transcriptional regulating activity of RFX-I.**


*Keywords:* Adducin; Hypertension; RFX-1; Sodium absorption; Yeast two hybrid; Kidney library

## 1. Introduction

Essential hypertension is a multifactorial disease due to genetic and environmental factors. It is a common disease with a prevalence ranging from 25% to 30%, with wide variations depending on the ethnical and racial composition of the population [1].

Recent studies suggest that Adducin plays a leading role in the pathogenesis of human hypertension. Mutations in the alpha-Adducin gene have been associated with increased renal sodium retention and with the development of high blood pressure in humans and rodents [2,3], although this association has not been found in all the populations studied [4,5]. Alpha Adducin is an ubiquitously expressed cytoskeleton protein that is involved in cell-to-cell contact [6] and regulates both actin dynamics [7] and the expression of the sodium–potassium-ATPase on the basolateral side of renal tubular cells, thus modulating the complex tubular re-absorption of sodium. The activity of the sodium–potassium-ATPase pump is regulated by hormonal factors, like dopamine, aldosterone and vasopressin as well as by non hormonal factors. The sodium retentive effect has been attributed to an increase in the rate of actin polymerization and in the number of Na, K-ATPase units in the plasma membrane of renal tubular cells [8].

We performed yeast two hybrid screening using a kidney library as a source of prey cDNA. A clone coding for a putative Adducin interactive molecule was identified, as the COOH terminal sequence of regulatory factor for X box (RFX-I), a non neuronal cell-specific transcription factor that inactivates the transcription of the microtubule-associated protein MAP1A [9].

We present evidence, based upon colocalization and coimmunoprecipitation experiments, that the interaction is specific and occurs in yeast as well as in eukaryotic cells. These findings suggest that Adducin can have a role in modulating the transcriptional regulating activity of RFX-I, with possible consequences on the establishment of an actin-microtubule cytoskeleton network, regulating the internalization of Na, K-ATPase subunits.

## 2. Materials and methods

### 2.1. Constructs

*pCMVneoHA-Adducin*. The full length human Adducin cDNA, cloned into pCMVneoHA, was a generous gift of Prof. Bianchi.

*pBridge-Adducin*. Polymerase chain reaction (Klen.Taq; BD Biosciences/Clontech, Palo Alto, CA) was used to amplify alpha Adducin from pCMVneoHA-Adducin. Primers introducing EcoRI restriction site (CAGAATTCATGAATG GTGATTCTCGTGCTG) and *Bam*HI restriction site (CTGGATCCTCAGGAGTCACTCTTCT) were used.

The amplified fragment, flanked by an *Eco*RI site upstream and a *Bam*HI site downstream, was ligated into pGEM-T Easy vector (Promega, Madison, WI, USA) and subcloned into the multiple cloning sites of pBridge (Clontech laboratories, Inc. Palo Alto, CA ).

*pCDNA3 Myc-RFX-I*. A pCMV SPORT 6 vector containing the full length cDNA sequence of RFX1 was obtained from Research Genetics through Invitrogen (Invitrogen, Carlsbad, CA).

The coding sequence of RFX-I was amplified from pCMV SPORT 6 RFX-1 by a PCR based method (Klen.Taq).

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The following primers were used:

(1) (ATGCGGAATTCCATGGAACAAAAACTCATCTCAGAC-GAGGATCTGGCAACACAGGCG TAT) introduced an *Eco*RI restriction site and a Myc tag 5′ to the sequence coding for RFX-1.

(2) (GGCACTCGAG TTAGCTGGAGGGCAGCGCCT) introduced a *Xho*I site 3′ to the sequence coding for RFX-1.

The amplified fragment, was subcloned into pcDNA3 (Invitrogen). This vector was used in all the experiments requiring the expression of RFX-1 in eukaryotic cells.

### 2.2. Yeast two hybrid screening

To identify Adducin binding proteins, a yeast two hybrid system was used (MATCHMAKER Gal4 Two-Hybrid Clontech Laboratories Inc.) [10]. The bait plasmid pBridge-Adducin, with full length human alpha Adducin expressed as a fusion protein with the DNA binding domain (BD) of the yeast transcription factor Gal 4, was used to transform a suitable yeast strain (*Saccharomyces cerevisae* AH109). The yeasts transformed by pBridge-Adducin were used for mating with a *Saccharomices cerevisiae* host strain Y187 (Clontech laboratories, Inc.) pretransformed by a MATCHMAKER human Kidney cDNA pACT2 derived library, cloned into a yeast GAL4 activation domain (AD) expressing proteins containing a HA-tag at the N terminus. After 20 h of mating we spread the mating mixture on SD/-Ade/-His/-Leu/-Trp plates. Yeast colonies that demonstrated activation of both reporters conferring galactose-dependent blue staining in the presence of X-Gal (5-bromo-4chloro-3-indolylbeta-D-galactopyranoside) and adenine and histidine-independent growth were selected and considered for further evaluation to screen for putative alpha Adducin interacting proteins.

Library plasmid DNA was isolated from this selection of clones in presence of lyticase solution and then rescued into HB101 *Escherichia coli* strain by the $CaCl_2$ method (Invitrogen.). The transformants were recovered on minimal M9 selective medium lacking leucine for nutritional selection. The specificity of the interaction was tested for several clones by retransforming the interactor plasmid into yeast expressing pBridge-Adducin bait, as well as in yeast strain transformed with empty vector, pBridge and two unrelated bait plasmids: pBridge-HMG and pBridge-SGK. The growth was then assayed on plates SD/-Ade/-His/-Leu/-Trp. The cDNA encoding specific alpha Adducin interacting proteins were sequenced and studied with BLAST analysis.

### 2.3. Expression in COS 7

COS 7 cells were plated at a density of $3.5 \times 10^5$ cells/ml in six well plates. For the immunofluorescence studies cells were plated at a density of $2.0 \times 10^5$ cells/ml on glass cover slips in individual 35 mm plates. Cells were cultured overnight in DMEM containing 10% fetal bovine serum (Invitrogen). The following day the cells were transfected with eukaryotic expression vectors using lipofectamine plus (Invitrogen.) following the manufacturer's instructions. We used pCMVneoHA-Adducin (600 ng/ml) and pCDNA3 Myc-RFX-1 (600 ng/well) for the coimmunoprecipitation experiments and for the confocal microscopy experiments. Empty vectors were transfected in the sham transfected cells.

Five hours after transfecting the cells, the transfection medium was changed with DMEM medium containing fetal bovine serum (10%) and antibiotics (penicillin, 100 U/ml, and streptomycin, 100 μg/ml). Thirty-six hours after transfection, the cells were used for studying the coimmunoprecipitation and the colocalization experiments. Confocal microscopy experiments showed that the efficiency of transfection reached approximately 30%.

### 2.4. Coimmunoprecipitation experiments

The transfected cells were solubilized for 20 min, at 4 °C, in a solubilization buffer (250 μl/well) containing: 25 mM Hepes, 100 mM KCl, 12.5 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, 0.1%, Nonidet P-40, pH 7.9, with protease inhibitors complete TM (Roche Molecular Biochemicals, Mannheim, Germany) and phosphatase inhibitors (100 mM NaF; 5 mM sodium pyrophosphate; 2 mM sodium orthovanadate, 5 mM EDTA). Protein extracts were quantified by means of a Bradford based assay (Bio-Rad, Hercules, CA), and an aliquot (20μg) of protein extracts was analyzed by immunoblotting with rabbit anti-HA (Roche Diagnostic) and rabbit anti-Myc (Santa Cruz Biotechnology, CA) to assess the expression of HA-Adducin and Myc-RFX-1. Four hundred micrograms of proteins were immunoprecipitated with rabbit anti-HA antibodies, (8 μl, Roche Diagnostic S.p.A., Italy) at 4 °C, overnight. The antibody was bound to protein G-Ultralink (15 ml, Pierce) at 4 °C for 60 min. The immune complexes were sedimented, washed three times with a washing buffer containing 25 mM Hepes, 100 mM KCl, 12.5 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, 0.1%, Nonidet P-40, pH 7.9 [4]. The pellets were then resuspended in Laemmli sample buffer containing dithiothreitol (1 mM), boiled for 5 min, and separated by SDS polyacrylamide gel electrophoresis using a 10% gel. The proteins were transferred to nitrocellulose, blocked in 5% non fat dry milk in TTBS and incubated with rabbit anti-HA antibody at a dilution of 1:1000 in 5% non fat dry milk in TTBS, to detect immunoprecipitated Adducin, rabbit anti Myc antibody at a dilution of 1:1000 in 5% non fat dry milk in TTBS for the detection of RFX-1.

### 2.5. Immunofluorescence for Adducin, RFX-1 and spekles SC-35

For the immunofluorescence experiments, cells were plated and transfected with the appropriate vectors. After 24 h of incubation with the transfection mixture, the cells were fixed with 4% formaldehyde in PBS at 4 °C for 15 min and then permeabilized with 0.5% Triton X-100 in PBS. In the Adducin/RFX-1 colocalization experiments Adducin was visualized with a mouse anti-HA antibody (Santa Cruz Biotechnology Inc.). In the Adducin/speckles sc-35 colocalization experiments



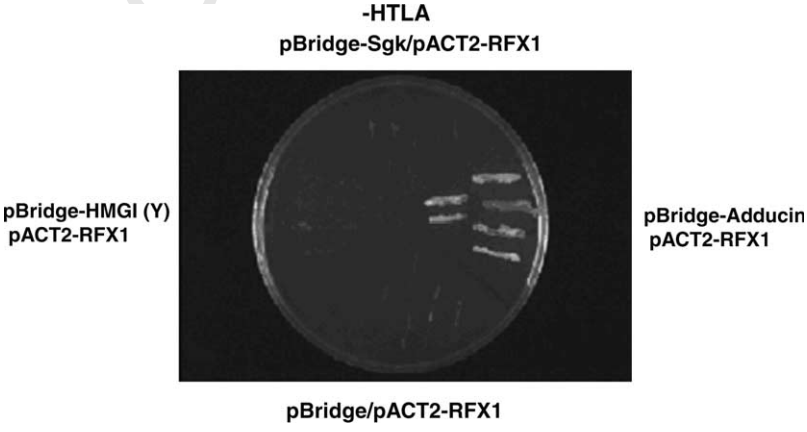


Fig. 1. Identification of regulatory factor for box X-I (RFX-I) as Adducin interacting protein using yeast two-hybrid assay. AH 109 yeast strain cells cotrasformed with p-Bridge-Add and pACT2-RFX-I were able to grow in selective medium lacking histidine, tryptophan, leucine, and adenine. The interaction was specific because yeasts cotransformed by vectors expressing different molecules, pACT2-RFX-I with p-Bridge SgK, pBridge HMG(Y) and p-Bridge empty vector, were unable to grow in selective medium.

Adducin was visualized with rabbit anti HA antibodies (Santa Cruz). RFX-1 was visualized with a rabbit anti-Myc antibody (Santa Cruz). sc-35 speckles were visualized with a mouse monoclonal anti sc-35 antibody (Sigma–Aldrich, Milan, Italy). All the primary antibodies were diluted 1:200 in Blocking solution (BSA 1%, Triton 0.1% in PBS). Adducin was detected, with 1:800 Alexa Fluor 568 goat anti mouse IgG (Molecular Probes) in the Adducin/RFX-1 colocalization experiments, and with 1:800 Alexa Fluor 488 goat anti rabbit IgG (Molecular Probes) in the Adducin/speckles sc-35 colocalization experiments. RFX-1 was detected with 1:800 Alexa Fluor 488 goat anti rabbit IgG (Molecular Probes). sc-35 speckles were detected with 1:800 Alexa Fluor 568 goat anti mouse IgG (Molecular Probes). All the incubations were performed in a humidified chamber. After being washed with PBS, cells were mounted in Prolong anti fade reagent (Molecular Probes) and visualized using a confocal microscope (Leica Microsystems, Wetzlar, Germany).

## 3. Results

### 3.1. Yeast two-hybrid screening reveals specific Adducin/RFX-1 interaction

Two hybrid screening allowed us the identification of different independent clones, interacting specifically with Adducin. Approximately, 10 million yeast transformants were screened. We found only one putative interacting clone. Blast analysis of the corresponding cDNA revealed that the library clone contained a partial sequence coding for the COOH terminal region, from Leucine 866 to the end of the molecule, of the regulatory factor for X box (RFX-I) (Fig. 1). The interaction was specific since the growth on plates SD/-Ade/-His/-Leu/-Trp was observed only when the interactor plasmid pACT2-RFX-1 was retransformed into yeast expressing pBridge-Adducin bait. No growth was observed when the interactor plasmid pACT2-RFX-1 was retransformed into yeast containing the empty vector pBridge or two unrelated bait plasmid: pBridge-HMG(Y) and pBridge-PTP$\eta$ (Fig. 1).

### 3.2. Coimmunoprecipitation of Adducin and RFX-1 in COS-7 cells

Rabbit anti Myc and anti HA immunoglobulins allowed the detection of HA-alpha Adducin (Fig. 2 panel A, lanes 3) and Myc-RFX-1 (Fig. 2 panel A, lanes 2 and 3) in cell extracts. Rabbit anti HA immunoglobulins were used to immunoprecipitate HA-Adducin, detected by blotting with rabbit anti HA antibodies as expected (Fig. 2 panel B, lanes 3). RFX-1 was detected, by blotting with a rabbit anti Myc antibody (Fig. 2 panel C, lane 3), only in HA immunoprecipitates from cells transfected with vectors coding for HA Adducin and Myc RFX-1, thus proving that the interaction between alpha Adducin and RFX-1 occurs in eukaryotic cells. As positive control Myc-RFX-1 was detected by rabbit Myc antibodies in extracts from cells transfected with pCDN3- Myc RFX-1 (Fig. 2 panel C, lane 4).

### 3.3. Adducin colocalizes with RFX-1 in specific subnuclear domains

The localization of alpha Adducin, RFX-1 was studied in COS7 cells by confocal microscopy (Fig. 3 panels A and B).

Both the proteins localized in subnuclear structures, similar to the subnuclear domains, previously described as typical of RFX-1. Colocalization of alpha Adducin and RFX-1 was demonstrated by the appearance of the yellow color in the alpha Adducin, RFX-1 overlay (Fig. 3, panel A). Interestingly, alpha Adducin showed no colocalization with other subnuclear structures (speckles) identified by the mouse sc-35 antibody in separate experiments, thus confirming the specificity of the subnuclear colocalization between alpha Adducin and RFX-1 (Fig. 3, panel B).

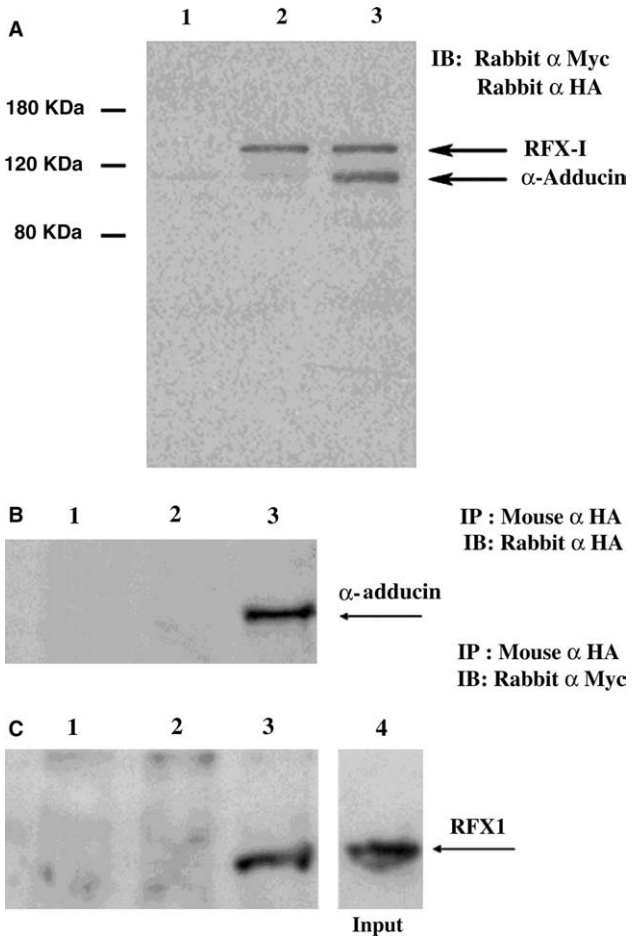


Fig. 2. Panel A, B and C. Coimmunoprecipitation of Add and RFX-I. COS-7 cells were transfected with empty vector (A, B, lane 1), pCMVneo Ha Add (A, B, lane 2), pcDNA3Myc RFX-I more pCMVneo Ha Add (panel A, B, lane 3 ) and only pcDNA3Myc RFX-I as control (panel B, lane 4). Rabbit anti Myc and anti HA immunoglobulins allowed the detection of HA-alpha Adducin (panel A, lanes 2 and 4) and Myc RFX 1 (panel A, lanes 3 and 4) in cell extracts. Rabbit anti HA were used to immunoprecipitate HA-Adducin, detected by blotting with rabbit anti HA antibodies as expected (panel B, lanes 3 and 4). RFX1 was detected by blotting with a rabbit anti Myc antibody (panel B, lane 3), only in HA immunoprecipitates from cells transfected with vectors coding for HA Adducin and Myc RFX1, thus proving that the interaction between alpha Adducing and RFX1 occurs in euk aryotic cells. Lane 4 shows the Myc RFX1 was detected with rabbit anti myc antibodies in extracts from cells transfected with pCMVneoMyc RFX-I.

## 4. Discussion

The understanding of the mechanisms underlying renal tubular sodium reabsorption is very important in enlightening the genetic mechanisms of human hypertension. Alpha-Adducin is a cytoskeleton protein [11] involved in the complex renal

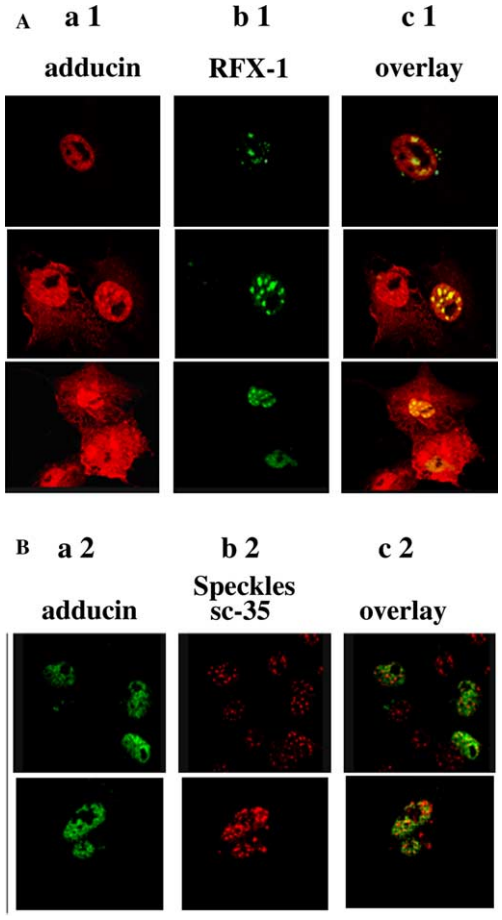


Fig. 3. Panels A and B. Colocalization of alpha Adducin and RFX1 in COS7 cells (panel A) and immunofluorescence of Adducin-S-35 by confocal microscopy (panel B). COS 7 cells grown on glass coverslips at ~50% confluence were transfected with expression vectors encoding the full-length Ha-tagged Adducin (pCMVneo-Ha-Adducin) and the full-length Myc-tagged RFX-I (pcDNA3-Myc-RFX-I). Serum-starved cells were fixed as indicated in Section 2. Panel A: HA-Adducin was detected by mouse anti-HA antibodies and visualized by anti mouse antibody (ALEXA FLUOR 568 goat anti-mouse IgG-) (panel A, a1), Myc RFX-1 was detected by rabbit anti-Myc antibodies and visualized by ALEXA FLUOR 488 goat anti-rabbit Ig G (panel A, b1). Panel B: HA-Adducin was detected by rabbit anti HA antibodies and visualized by ALEXA FLUOR 488 goat anti-rabbit Ig G (panel B, a2). The Sc-35, detected by mouse anti-Sc-35 antibodies and visualized by Alexia Fluor 568 goat anti-mouseIgG] (panel B, b2). Images shown in c1 and c2 represent the overlay of a1 and b1, a2 and b2, respectively.

tubular re-absorption of sodium by regulating the expression levels of the sodium–potassium-ATPase on the basolateral side of renal tubular cells [8].

In the present paper, we present evidences of structural interaction between alpha Adducin and a regulatory factor X-I box (RFX-I), a non neuronal cell-specific transcription factor that inactivates the transcription of the microtubule-associated protein MAP 1A [9]. The interaction was first discovered in yeast, and then confirmed in eukaryotic cells. Interestingly alpha-Adducin colocalized with RFX-1 in specific subnuclear structures, as previously described for RFX-1 [12]. On the contrary a very weak, if any colocalization at all, was found between alpha Adducin and other transcriptionally active subnuclear structures, named speckles [13]. The activity of RFX-I as a transcription factor is regulated in a complex manner. RFX-I belongs to a protein family sharing a DNA-binding domain and a conserved C-terminal region. In RFX-I, the C terminal region mediates the dimerization, and is followed by a terminal tail, containing a highly acidic stretch. The adjacent "acidic region" potentiates the functions of the NLS such as the nuclear import and DNA-binding activity of RFX-1. In HL-60 cells nuclear translocation of RFX-1 is regulated by protein Kinase C through a poorly characterized mechanism [12]. RFX-1 has been shown to be able to bind to regulatory elements of several genes. It associates with a Myc intron binding factor (MIBP1) to activate myc expression [14] as well as with a B cell specific activity protein (BSAP/Pax5) that regulates B spell specificity of Epstein Barr virus growth transforming function [15]. RFX-1 also interacts with and activates cAbl kinase, a non receptor tyrosine kinase activated in the nucleus during S phase [16]. RFX-1 homodimers, RFX-1/RFX-2 heterodimers and RFX5 can form complexes on methylated as well as unmethylated collagen transcription factor start site, thereby controlling collagen expression [17]. This activity can explain some of RFX1 function in the light of the mechanisms of inflammation were INFγ decreases and TGFβ increases collagen transcription. Moreover a RFX1 transcription factor binding site has been located in the promoter region of the gene coding for 11β-hydroxysteroid dehydrogenase, type 2 (11βHSD2) [18], thus suggesting that RFX1 can indeed modulate the expression of an enzyme involved in regulating the sodium retentive activity of steroids. On the other hand, a gene transcription regulating activity has recently been described for nuclear actin-binding proteins [19].

Although a functional meaning of the interaction described in the present paper is still lacking, the binding of alpha Adducin to RFX-1 and their specific nuclear co-localization strongly suggests that Adducin can have a role in modulating the transcription regulating activity of RFX-1. Interestingly adducin colocalizes with RFX-1, a negative regulator of the transcription of specific genes, not with the proteins identified by the sc-35 anti speckles antibody, which detects transcriptionally active subnuclear domains [20]. Taken together these data suggest that alpha adducin can be involved in modulating the negative regulation of transcription through RFX-1.

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