Yeast two-hybrid screening for proteins that interact with α_1-adrenergic receptors[1]

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ABSTRACT

AIM: To find novel proteins that may bind to α_{1A}-adrenergic receptor (α_{1A}-AR) and investigate their interactions with the other two α_1-AR subtypes (α_{1B}-AR and α_{1D}-AR) with an expectation to provide new leads for the function study of the receptors. **METHODS:** Yeast two-hybrid assay was performed to screen a human brain cDNA library using the C terminus of α_{1A}-AR (α_{1A}-AR-CT) as bait. X-Gal assay and *o*-nitrophenyl-beta-*D*-galactopyranoside (ONPG) assay were subsequently conducted to further qualitatively or quantitatively confirm the interactions between receptors and the three identified proteins. **RESULTS:** (1) Selection medium screening identified segments of bone morphogenetic protein-1 (BMP-1), active Bcr-related protein (Abr), and filamin-C as binding partners of α_{1A}-AR-CT in yeast cells respectively. Besides, protein segments of BMP-1 and Abr could only specifically interact with α_{1A}-AR-CT while filamin-C segment interacted with all three α_1-AR subtypes. (2) In X-Gal assay, the co-transformants of α_{1A}-AR-CT and BMP-1 segments turned strong blue at about 30 min while other positive transformants only developed weak blue at about 5-6 h. (3) In ONPG assay, interaction (shown in β-galactosidase activity) between α_{1A}-AR-CT and BMP-1 segments was about 30 times stronger than that of control (P<0.01), while other positive interactions were only about 2-5 times as strong as those of controls (P<0.05). **CONCLUSION:** In yeast cells BMP-1, Abr and/or filamin-C could interact with three α_1-AR subtypes, among which, interaction between BMP-1 and α_{1A}-AR was the strongest while other interactions between proteins and receptors were relatively weak.

INTRODUCTION

α_1-Adrenergic receptors (α_1-ARs) were members of G-protein-coupled receptor (GPCR) superfamily and consisted of α_{1A}-AR, α_{1B}-AR, and α_{1D}-AR subtypes. α_1-AR subtypes were important mediators of several cardiovascular actions regulated by the sympathetic nervous systems such as vasoconstriction, hypertrophy, cardiac inotropy, and remodeling[1]. α_1-ARs were encoded by separate genes and they were different in structure, G protein coupling, tissue distribution, signaling, regulation, and functions. Among the three subtypes, it was clear that there was a high sequence homology (about 70 %) within the putative transmem-

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brane domain. On the contrary, there was almost no sequence homology in the cytoplasmic C-terminal region. Recently, a variety of proteins have been found to regulate physiological functions of several adrenergic receptors by directly binding to their C-terminal regions. For example, gc1q-R, a multifunctional protein, was found to bind with the C-terminal cytoplasmic domain of the α_{1B}-AR[2] and β_1-AR was found to be associated with the PSD-95, which facilitated the formation of a complex between β_1-AR and the *N*-methyl-*D*-aspartate receptors[3]. Thus, from structural difference, we might explain functional differences existed among all the α_1-AR subtypes.

Considering the striking observations described above, we supposed that there might be a high possibility that some latent cytoplasmic proteins interacted with other ARs, including the α_1-AR. In all the three α_1-AR subtypes, it was reported that the vascular α_{1A}-AR played a role in the regulation of peripheral resistance and systemic arterial pressure, while α_{1B}-AR and α_{1D}-AR played minor roles in acute arterial pressure regulation. Besides, it was noted that α_{1A}-AR was the most efficient as to stimulate calcium release and inositol phosphate production whereas α_{1D}-AR was poorly coupled to intracellular signaling pathways [4]. So α_{1A}-AR played an important role in the regulation of cardiovascular functions. In this experiment, we screened a human brain cDNA library by yeast two-hybrid assay using α_{1A}-AR C-terminal domain as a bait, with an expectation to find new binding partners of α_{1A}-AR and thus provide clues for the functional study of α_{1A}-AR as well as the related proteins.

MATERIALS AND METHODS

Reagents Advantage 2 PCR kit, pGBKT7, Matchmaker 3 pretransformed human brain cDNA library, X-α-Gal and all other yeast two-hybrid components were purchased from Clontech. *ECoR* I, *BamH* I, T4 DNA ligase and ampicillin were purchased from Promega. PCR Product Purification Kit was obtained from Qiagen.

Plasmids construction The C-terminal domain of human α_{1A}-AR (amino acids 322-466), α_{1B}-AR (amino acids 344-519), and α_{1D}-AR (amino acids 398-572) was amplified respectively by polymerase chain reaction (PCR) from the full length human α_{1A}-AR, α_{1B}-AR, and α_{1D}-AR accordingly, using primer sets containing *Eco*R and *Bam*H restriction sites (Tab 1), and was inserted downstream of the Gal4 DNA-binding domain in the (bait) vector pGBKT7 (CLONTECH Laboratories) using T4 DNA ligase respectively. All the three constructs (α_{1A}-AR-CT/pGBKT7, α_{1B}-AR-CT/pGBKT7, and α_{1D}-AR-CT/pGBKT7) were sequenced and the inserts were found to be in frame with the yeast Gal4 DNA-binding domain.

Yeast two-hybrid screening All assays were carried out according to the protocols described in the Pretransformed MATCHMAKER Libraries User Manual (PT3183-1). In brief, the α_{1A}-AR-CT/pGBKT7 plasmids were initially introduced into the AH109 yeast strains using a modified lithium acetate protocol and the transformants were selected on SD/-Trp plates. The mating between the selected AH109 and Y187 yeast cells were performed and co-transformants were selected on agar plates SD/-Leu-Trp-His and SD/-Leu-His-Ade-Trp (QDO) with or without X-α-Gal to detect the initiation of reporter gene (HIS, LEU, TRP, ADE, and MEL1) transcription. Colonies grew at 30 ºC for about 5 d and those turned blue were at last selected as positive clones. Plasmids from positive clones were extracted, introduced parts of the cDNA were amplified by PCR using sequencing primers (CLONTECH Libraries), then PCR products were sequenced and blasted on Genebank.

X-Gal assay and ONPG assay In order to confirm the positive reactions both of the assays were per-

Tab 1. PCR primers used in this work.

α_{1A}-AR-CT 5′F	5′-CCG GAATTC AAC CCC ATC ATA TAC CCA TGC TCC A-3′
α_{1A}-AR-CT 3′R	5′-CGC GGATCC CCT GTC CTA GAC TTC CTC CCC GTT C-3′
α_{1B}-AR-CT 5′F	5′-CG GAATTC AGG GGC ATG TTG GTT TTG AAG-3′
α_{1B}-AR-CT 3′R	5′-CG GGATCC AAC CCC ATC ATC CCA TGC TC-3′
α_{1D}-AR-CT 5′F	5′-CG GAATTC AAC CCG CTC ATC TAC CCC TGT TC-3′
α_{1D}-AR-CT 3′R	5′-CG GGATCC AGG GAC ACA GCC TCC ACC TCT GA-3′

The underlined bases indicate restriction sites.

formed in the Y187 yeast strains to detect the initiation of LacZ reporter gene transcription qualitatively and quantitatively as described before[5,6].

In X-Gal assay, Colony-lift Filter assay was used to check the activity of β-galactosidase. Briefly, fresh colonies grown to about 1-3 mm in diameter were transferred completely to a sterile filter and submerged in a pool of liquid nitrogen for 10 s and thawed at room temperature, then it was put on a pre-soaked filter in the Z buffer/X-Gal solution (100 mL Z buffer, 0.27 mL β-mercaptoethanol, 1.67 mL X-gal stock solution; Z buffer: $Na_2HPO_4 \cdot 7H_2O$ 16.1 g/L, $NaH_2PO_4 \cdot H_2O$ 5.50 g/L, KCl 0.75 g/L, $MgSO_4 \cdot H_2O$ 0.246 g/L, pH=7.0), then the filters were incubated at 30 ºC and the colors of colonies were checked periodically.

In ONPG assay, ONPG was used as the substrate of β-galactosidase for the liquid culture assay. In brief, at least three independent clones were selected, grown, harvested, centrifuged, and resuspended in Z buffer, frozen in liquid nitrogen, and thawed at 37 ºC in a water bath. Then the reaction systems (ONPG+Z buffer+β-mercaptoethanol+yeast cells resuspension) were placed in a 30 ºC incubator. After the yellow color developed $Na_2CO_3$1 mol/L was added to the reaction and blank tubes. Relapsed time was recorded in minutes. Reaction tubes were centrifuged at 14 000 r/min for 10 min and supernatants were carefully transferred to clean cuvettes and OD_{420} of the samples relative to the blank was recorded. At last, the β-galactosidase units were calculated as:

β-galactosidase units=$1000 \times OD_{420}/(t \times V \times OD_{600})$

where t =elapsed time (in min) of incubation, V=0.1 mL×concentration factor (the concentration factor is 5), OD_{600}=Opital density at 600 nm of 1 mL of culture.

Statistical analysis All data were presented as mean±SD and processed with the SPSS v 11.0 statistical software package. Dunnett *t*-test was used for two-sided statistical analysis. The level of statistical significance was set at $P<0.05$.

RESULTS

Screening the binding partner of α_{1A}-AR C terminal domain in human brain cDNA library To identify novel proteins that interact with the α_{1A}-AR C terminal domain *in vivo*, we screened a human brain cDNA library subcloned into the bait vector pACT2 with the C-terminal domain of the α_{1A}-AR. Initially, to ensure that the α_{1A}-AR-CT/pGBKT7 clone alone was not able to activate the yeast reporter genes, the bait construct was co-transformed into the yeast AH109 strains with the library prey vector (pACT2) alone and tested for the transcription initiation of HIS and MEL1 reporter gene. The resulted clone was negative for self-activation and therefore, it was used as negative control in library screening.

Mating of the yeast Y187 strains was pre-transformed with a human brain library cDNA and the AH109 yeast strains containing α_{1A}-AR-CT/pGBKT7 plasmids was performed and approximately 2×10^6 colonies among all the 2.3×10^7 human brain cDNA library colonies were screened for α_{1A}-AR C-terminus. The library screening produced seven individual clones that were HIS^+ and $MEL1^+$ and could grow on the SD-Leu-His-Thr-Trp (QDO) agar plates and then they were subjected to sequence analysis. The blasting results showed that three of them encoded the C-terminal part of BMP-1 (Gene bank, GI: 5902809), Abr (Gene bank, GI: 15316553), and filamin-C (Gene bank, GI: 14744091) respectively. Co-transformation of α_{1A}-AR-CT/pGBKT7 with pACT2 alone was also performed at the same time and the co-transformants could not grow on QDO selection medium, so the co-transformants of α_{1A}-AR-CT/pGBKT7 with pACT2 alone (1A-T) was used as control in this work. To eliminate false positive reactions, we co-transformed pGBKT7 with BMP-1/pACT2, Abr/pACT2, and filamin-C/pACT2 respectively, no transcription initiation of reporter gene was detected (Tab 2).

Identification of the interactions between the α_{1B}-AR-CT or α_{1D}-AR-CT and the three cloned protein segments In order to identify whether the three protein segments interacted with the α_{1A}-AR subtypes specifically or not, the interactions between the other two α_1-AR-CTs and the three cloned protein segments were further tested. As performed for the α_{1A}-AR, we co-transformed BMP-1/pACT2, Abr/pACT2, and filamin-C/pACT2 with α_{1B}-AR-CT/pGBKT7 and α_{1D}-AR-CT/pGBKT7, respectively. Strikingly, both α_{1B}- and α_{1D}-AR C-terminal domains showed positive interactions with filamin-C, whereas neither of them interacted with BMP-1 or Abr. Co-transformation of α_{1B}-AR-CT/pGBKT7 or α_{1D}-AR-CT/pGBKT7 with pACT2 alone (1B-T or 1D-T) was not able to activate the yeast reporter genes and was used as control (Tab 3).

X-Gal and ONPG assay In the following experiments, we performed the co-transformation of

Tab 2. Yeast two-hybrid analysis of the interactions between the α_{1A}-AR C-terminus and the three cloned protein segments.

Cotransformants	HIS^+	$MEL1^+$	Growth on QDO plates
pGBKT7 + BMP-1/pACT2	–	–	–
pGBKT7 + Abr/pACT2	–	–	–
pGBKT7 + filamin-C/pACT2	–	–	–
α_{1A}-AR-CT/ pGBKT7+pACT2 (1A-T)	–	–	–
α_{1A}-AR-CT/pGBKT7+BMP-1/pACT2	+	+	+
α_{1A}-AR-CT/pGBKT7+Abr/pACT2	+	+	+
α_{1A}-AR-CT/pGBKT7+filamin-C/pACT2	+	+	+

HIS^+, the reporter gene HIS was activated; $MEL1^+$, the reporter gene MEL1 was activated; QDO, SD/-Leu-His-Ade-Trp selection culture.

Tab 3. Yeast two-hybrid analysis of the interactions between the α_{1B}-AR or α_{1D}-AR C-terminus and the three cloned protein segments.

Cotransformants	HIS^+	$MEL1^+$	Growth on QDO plates
α_{1B}-AR-CT/pGBKT7+pACT2 (1B-T)	–	–	–
α_{1B}-AR-CT/pGBKT7+Abr /pACT2	–	–	–
α_{1B}-AR-CT/pGBKT7+BMP-1/pACT2	–	–	–
α_{1B}-AR-CT/pGBKT7+filamin-C/pACT2	+	+	+
α_{1D}-AR-CT/pGBKT7+pACT2 (1D-T)	–	–	–
α_{1D}-AR-CT/pGBKT7+Abr/pACT2	–	–	–
α_{1D}-AR-CT/pGBKT7+BMP-1/pACT2	–	–	–
α_{1D}-AR-CT/pGBKT7+filamin-C/pACT2	+	+	+

HIS^+, the reporter gene HIS was activated; $MEL1^+$, the reporter gene MEL1 was activated; QDO, SD/-Leu-His-Ade-Trp selection culture.

different bait and prey constructs into the yeast Y187 cells carrying the β-galactosidase reporter genes to further confirm the positive reactions. The activity of β -galactosidase was measured qualitatively by the filter assay (X-Gal assay) and quantitatively by liquid culture assay (ONPG assay).

X-Gal assay In this assay, at least three independent clones were tested and all the positive co-transformants developed weak or strong blue color on the filter within about 6 h at 37 ºC, among which co-transformants of α_{1A}-AR-CT/pGBKT7 and BMP-1/pACT2 (1A-B) showed strong interactions (clones turned deep blue at about 30 min) while other positive transformants (α_{1A}-AR-CT/pGBKT7 and filamin-C/pACT2, 1A-F; α_{1A}-AR-CT/pGBKT7 and Abr/pACT2, 1A-A; α_{1B}-AR-CT/pGBKT7 and filamin-C/pACT2 , 1B-F; α_{1D}-AR-CT/pGBKT7 and filamin-C/pACT2, 1D-F) only showed weak but apparent blue at about 5-6 h, suggesting relatively weak interactions occurred (Fig 1). This assay was performed for more than three times.

ONPG assay In this assay, the release of *o*-nitrophenol from the substrate ONPG was recorded at 420 nm and normalized to cell density measured photometrically at 600 nm; the β-galactosidase activity was presented in Miller units. Quantitatively, the interaction between α_{1A}-AR-CT and BMP-1 protein segment (1A-B) was about 30 times stronger than that of the control (1A-T) (P<0.01), while β-galactosidase activity of 1A-F or 1A-A were only about 4-5 times as strong as that of the control (P<0.05) (Fig 2A). β-galactosidase activity of 1B-F (P<0.05) or 1D-F (P<0.01) was about 2-3 times stronger than the control respectively (Fig 2B and 2C). The results obtained by the two methods were fully compatible.

DISCUSSION

In this work, we identified novel interactions between the C-terminal tail of α_1-ARs and three non-G-

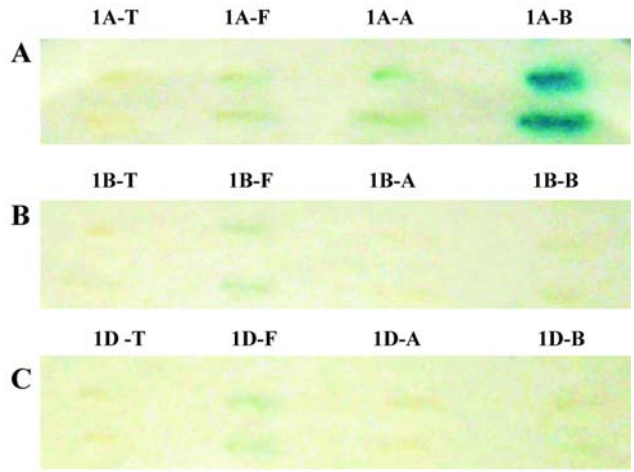

Fig 1. X-Gal assay of interactions between three α_1-AR C-terminal tails and the three identified protein segments in yeast Y187 cells. (A) The interactions between α_{1A}-AR-CT/pGBKT7 and pACT2 alone (1A-T), filamin-C/pGBKT7 (1A-F), Abr/pGBKT7 (1A-A), or BMP-1/pGBKT7 (1A-B). The blue colors on the filter were recorded at 6 h after the transformants were transferred onto the filter and kept at 37 ºC. (B) and (C) indicated the results of the two methods carried out for the α_{1B}-AR-CT/pGBKT7 and α_{1D}-AR-CT/pGBKT7, respectively.

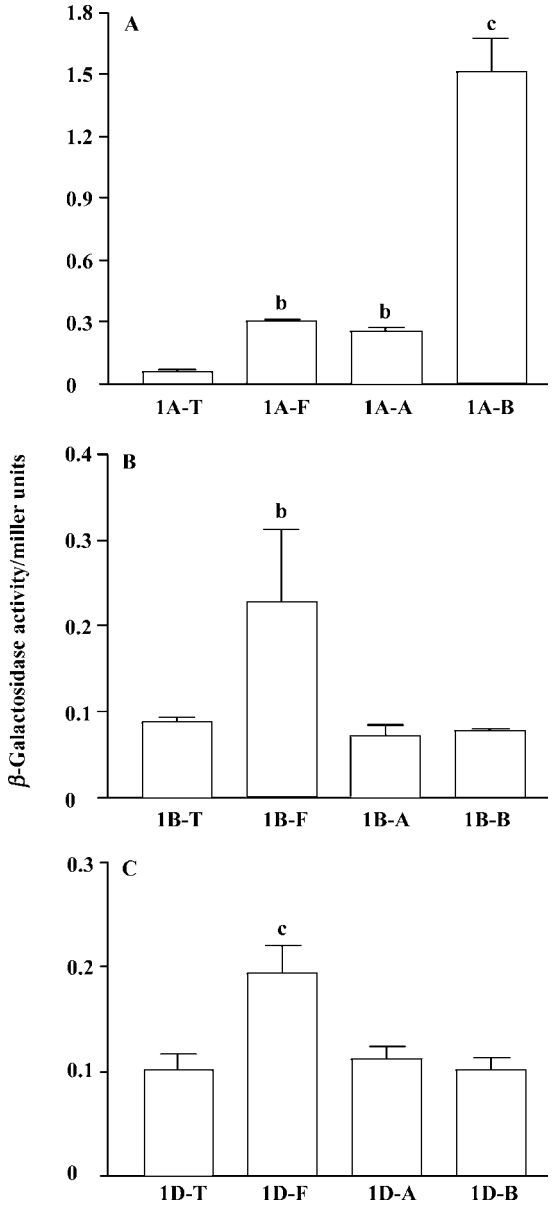

Fig 2. Plasmids carrying the bait and constructed prey protein segments were co-introduced into yeast Y187 by the lithium acetate method. Transformants of pACT2 alone and pGBKT7 fused with each of the α-AR C-terminus were used as controls (1A-T, 1B-T, and 1D-T). The β-galactosidase activity in each transformant was monitored by the liquid ONPG assay and shown in miller units. At least three independent transformants were carried out in ONPG assay. Mean±SD. (A) The interactions between α_{1A}-AR-CT/pGBKT7 and pACT2 alone (1A-T), filamin-C/pGBKT7 (1A-F), Abr/pGBKT7 (1A-A), or BMP-1/pGBKT7 (1A-B). (B, C) The two methods carried out for the α_{1B}-AR-CT/pGBKT7 and α_{1D}-AR-CT/pGBKT7, respectively. $^bP<0.05$, $^cP<0.01$ *vs* control.

proteins in the yeast two-hybrid systems. Of all the three proteins, α_{1A}-AR C-terminus showed a strong interaction with BMP-1 segments but interacted with filamin-C or Abr segments weakly, while the C-terminal tail of α_{1B}-AR or α_{1D}-AR only interacted with filamin-C weakly.

Yeast two-hybrid assay was an effective method for the identification of interaction between two proteins. Although it had the limitation that there maybe false-positives exist, compared with other methods, such as protein affinity chromatography, affinity blotting, immunoprecipitation and cross-linking, yeast two-hybrid assay had the advantage of screening all the protein-protein interactions *in vivo*, under which conditions the proteins observed were more likely to be in their native conformations[7]. So, it has been widely used for the study of protein-protein interactions.

ARs were classical G-protein-coupled receptors (GPCR), alias, seven-transmembrane receptors. Recently, some reports came that the cytoplasmic C-terminal tails of ARs could interact with other proteins besides G-proteins. It meant that ARs could accomplish their biological functions in a G-protein-dependent or G-protein-independent way. Several proteins were found to interact with the C-terminal domain of ARs by yeast two-hybrid analysis. For instance, the α-subunit of eukaryotic initiation factor 2B (Eif-2b), a guanine

nucleotide exchange protein that was involved in regulation of translation, was observed to associate with the C-terminal cytoplasmic domains of α_{2A}- and α_{2B}-AR in a yeast two-hybrid screen of a cDNA library prepared from HEK293 cell[8]. In the following years, gC1q-R, a multifunctional protein, and PSD-95, a multiple PDZ domain-containing scaffolding protein, were identified as the binding partner of α_{1B}- and β_1-AR C-terminal tails respectively[2,3].

Of all the subtypes of AR, α_1-AR, especially the α_{1A}-AR subtype, played a potential important role in the physiological or pathological processes, including the cell growth regulation, cardiac inotropy, hypertrophy, and so on. Considering the important roles that α_{1A}-AR played, it was imperative to find possible partners that bound to it and so that may affect its functions. In our study the C-terminal tail of α_{1A}-AR was used as a bait to screen a human brain cDNA library with an expectation to find novel proteins that might interact with α_{1A}-AR through its C-terminal domain. Fortunately, we found three novel and interesting proteins interacting with the α_{1A}-AR C-terminal domain in the yeast cells.

One of the proteins identified in our experiment is bone morphogenetic protein-1 (BMP-1), a zinc metalloproteinase whose metalloproteinase domain shared 39 % sequence identity with that of astacin, the digestive proteinase from crayfish[9]. It was increasingly apparent that in addition to procollagen processing, BMP-1 could cleave several other substrate[10-12] and thus played other roles central to extracellular matrix formation and early morphogenesis like embryo ventralization[13-15]. Besides, BMP-1 was reported to process the homotrimer of type V procollagen[16]. Structurally, the domains in BMP-1 included an N-terminal astacin-like zinc-binding metalloendopeptidase domain followed by varying numbers of epidermal growth factor-like (EGF-like) motifs and internal repeats termed CUB domains that might mediate binding to other extracellular proteins[17-19]. However, until now, few intracellular proteins were reported to interact with BMP-1, let alone α_{1A}-AR. We detected a strong positive interaction (the activity of β-galactosidase was about 300 times stronger than the control) between the fifth CUB domain of BMP-1 and α_{1A}-AR, which indicated there might exist strong or significant protein-protein interactions between α_{1A}-AR and BMP-1 in eukaryotic cells. In fact, we confirmed the interaction between BMP-1 and α_{1A}-AR by ELISA assay in HEK293 cells [20]. Based on our present findings, we supposed that there might exist intracellular interactions between α_{1A}-AR and BMP-1 before it was secreted outside of the cell, which might lead to novel functional findings of the two proteins, such as α_{1A}-AR cell signaling or BMP-1 secretion.

The second protein was filamin-C, which belonged to the filamins family of high molecular mass cytoskeletal proteins that organize filamentous actin in networks and stress fibers. In humans three filamin genes were cloned: FLNA, FLNB, and FLNC, encoding three filamin proteins (filamin-A, B, and C) respectively. Different from other two filamins, filamin-C exists only in cytoskeletal muscles and myocardiac muscles. Further more, structurally, filamin-C assembled into tail-to-tail, non-covalently associated dimers. Their amino-termini encode an ABD (actin binding domain), which was followed by a rod-domain composed of 24 100-residue repetitive segments including a carboxy-terminal dimerization domain. It was clear that there existed an 81 amino acid insertion in repeat 20, which was not present in filamin-A or -B. So far, 20 plus proteins were found to bind to filamins, and more were expected[21]. To this end, filamins were proposed to integrate cell mechanics as well as cell signaling. They not only interacted with peripheral actin networks but also bound to a variety of other ligand proteins through their C-terminal β-sheet repeats. Among those identified binding partners, six of them bound specifically to filamin-C C-terminal regions, which had been identified as γ,δ-sarcoglycan, androgen receptor, FATZ, myotilin, LL5b and PKCα[22-27]. We also observed interactions among part region of filamin-C C terminus (repeats 24) and the C-terminal domain of the three subtypes of α_1-AR, which indicated that filamin-C played important roles in α_1-AR cellular localization and cell signaling. It was very interesting to find that filamin-C segments could nonspecifically interact with each of the C-terminal domain of the three α_1-AR subtypes, but whether these nonspecific interactions also existed in mammalian cells and whether their interactions played different roles still need to be further studied.

The other protein identified in our work was Abr, encoded by the active BCR-related gene (ABR). Interestingly, the Bcr-related protein Abr contained both Dbl- and GAP-homology domains analogous to those in Bcr, suggesting that it also might function as a bi-directional modulator of Rho family function[28-31]. Until now, the molecular mechanisms underlying the Rho GTPases functioning were still not very clear. Using

library screening, we detected that the C-terminus of α_{1A}-AR could bind to the C-terminal region of Abr, which might function as a bi-directional modulator of Rho family and had never been reported to bind to α_{1A}-AR.

In conclusion, by yeast two-hybrid assay, we found three novel protein partners of the C-terminus of α_1-AR. Although all the interactions were found only in yeast cells and took place just between parts of the proteins, it was sure that there indeed existed interactions between the three proteins and the C-terminal tails of the receptors. Besides, no matter strong or weak, the interactions were observed *in vivo*. This greatly enhanced the possibility that the interactions might also exist between the entire proteins under natural conditions and might be of great biological significance. However, other methods such as GST-pull down assay or co-immunoprecipitation assay should be done to further confirm their interactions in mammalian cells. Anyway, considering the physiological functions of the three proteins, we had the reason to expect that our findings in the yeast cells might provide new clues for the study of functional relationships between them and α_1-AR. We also demonstrated that yeast two-hybrid assay was really a useful method for detection of protein-protein interactions, and we were sure that more proteins were to be identified to bind with ARs using this method.

REFERENCES

1 Michael TP, Dianne MP. α_1-adrenergic receptors: new insights and directions. J Pharmacol Exp Ther 2001; 298: 403-10.

2 Hirasawa A, Awaji T, Xu Z, Shinoura H, Tsujimoto G. Regulation of subcellular localization of α_1-adrenoceptor subtypes. Life Sci 2001; 68: 2259-67.

3 Xu J, Paquet M, Lau AG, Wood JD, Ross CA, Hall RA. β_1-Adrenergic receptor association with the synaptic scaffolding protein membrane-associated guanylate kinase inverted-2 (MAGI-2). Differential regulation of receptor internalization by MAGI-2 and PSD-95. J Biol Chem 2001; 276: 41310-7.

4 Theroux TL, Esbenshade TA, Peavy RD, Minneman KP. Coupling efficiencies of human alpha 1-adrenergic receptor subtypes: titration of receptor density and responsiveness with inducible and repressible expression vectors. Mol Pharmacol 1996; 50: 1376-87.

5 Klein U, Ramirez MT, Kobilka BK, von Zastrow M. A novel interaction between adrenergic receptors and the alpha-subunit of eukaryotic initiation factor 2B. J Biol Chem 1997; 272: 19099-102.

6 Hirasawa A, Awaji T, Xu Z, Shinoura H, Tsujimoto G. Regulation of subcellular localization of α1-adrenoceptor subtypes. Life Sci 2001; 68: 2259-67.

7 Phizicky EM, Fields S. Protein-protein interactions: methods for detection and analysis. Microbiol Rev 1995; 59: 94-123.

8 Klein U, Ramirez MT, Kobilka BK, von Zastrow M. A novel interaction between adrenergic receptors and the alpha-subunit of eukaryotic initiation factor 2B. J Biol Chem 1997; 272: 19099-102.

9 Titani K, Torff HJ, Hormel S, Kumar S, Walsh KA, Rodl J, *et al*. Amino acid sequence of a unique protease from the crayfish *Astacus fluviatilis*. Biochemistry 1987; 26: 222-6.

10 Nicholls AC, Oliver JE, McCarron S, Harrison JB, Greenspan DS, Pope FM. An exon skipping mutation of a type V collagen gene (COL5A1) in Ehlers-Danlos syndrome. J Med Genet 1996; 33: 940-6.

11 Toriello HV, Glover TW, Takahara K, Byers PH, Miller DE, Higgins JV, *et al*. A translocation interrupts the COL5A1 gene in a patient with Ehlers-Danlos syndrome and hypomelanosis of I_{to}. Nat Genet 1996; 13: 361-5.

12 Wenstrup RJ, Langland GT, Willing MC, D'Souza VN, Cole WG. A splice-junction mutation in the region of COL5A1 that codes for the carboxyl propeptide of pro alpha 1 (V) chains results in the gravis form of the Ehlers-Danlos syndrome (type I). Hum Mol Genet 1996; 5: 1733-6.

13 Mendler M, Eich-Bender SG, Vaughn L, Winterhalter KH, Bruckner P. Cartilage contains missed fibrils of collagen types II, IX, and XI. J Cell Biol 1989; 108: 191-7.

14 Li Y, Lacerda DA, Warman ML, Beier DR, Yoshioka H, Ramirez F, *et al*. A fibrillar collagen gene, Col11al, is essential for skeletal morphogenesis. Cell 1995; 80: 423-30.

15 Fessler JH, Fessler LI. In: Mayne R, Burgeson RE, editors. Structure and function of collagen types. Orlando (FL): Academic Press; p 81-103.

16 Moradi-Ameli M, Rousseau JC, Kleman JP, Champliaud MF, Boutillon MM, Bernillon J, *et al*. Diversity in the processing events at the *N*-terminus of type-V collagen. Eur J Biochem 1994; 221: 987-95.

17 Prockop DJ, Sieron AL, Li SW. Procollagen *N*-proteinase and procollagen C-proteinase. Two unusual metalloproteinases that are essential for procollagen processing probably have important roles in development and cell signaling. Matrix Biol 1998; 16: 399-408.

18 Hulmes DJ, Mould AP, Kessler E. The CUB domains of procollagen C-proteinase enhancer control collagen assembly solely by their effect on procollagen C-proteinase/bone morphogenetic protein-1. Matrix Biol 1997; 16: 41-5.

19 Bork P, Beckmann G. The CUB domain. A widespread module in developmentally regulated proteins. J Mol Biol 1993; 231: 539-45.

20 Xu Q, Zhang T, Han QD, Zhang YY. Binding between α_{1A} (1A)-adrenergic receptor and segment of bone morphogenetic protein-1 in human embryonic cell 293. Acta Physiol Sin 2003; 55: 692-8.

21 Stossel TP, Condeelis J, Cooley L, Hartwig JH, Angelika N, Schleicher M, *et al*. Filamins as integrators of cell mechanics

and cell signaling. Nature Rev Mol Cell Biol 2001; 2: 138-45.
22 Van der Ven PF, Wiesner S, Salmikangas P, Auerbach D, Himmel M, Kempa S, *et al*. Indications for a novel muscular dystrophy pathway. γ-filamin, the muscle-specific filamin isoform, interacts with myotilin. J Cell Biol 2000; 151: 235-48.
23 Thompson TG, Chan YM, Hack AA, Brosius M, Rajala M, Lidov HG, *et al*. Filamin 2 (FLN2): A muscle-specific sarcoglycan interacting protein. J Cell Biol 2000; 148: 115-26.
24 Fauldner G, Pallavicini A, Comelli A, Salamon M, Bortoletto G, Ievolella C, *et al*. FATZ, a filamin-, actinin-, and telethonin-binding protein of the Z-disc of skeletal muscle. J Biol Chem 2000; 275: 41234-42.
25 Ozanne DM, Brady ME, Cook S, Gaughan L, Neal DE, Robson CN. Androgen receptor nuclear translocation is facilitated by the f-actin cross-linking protein filamin. Mol Endocrinol 2000; 14: 1618-26.
26 Paranavitane V, Coadwell WJ, Eguinoa A, Hawkins PT, Stephens L. LL5b is a phosphatidylinositol (3,4,5)-trisphosphate sensor that can bind the cytoskeletal adaptor, gamma-filamin. J Biol Chem 2003; 278: 1328-35.
27 Tigges U, Koch B, Wissing J, Jockusch BM, Ziegler WH. The F-actin cross-linking and focal adhesion protein filamin A is a ligand and *in vivo* substrate for protein kinase Ca. J Biol Chem 2003; 278: 23561-69.
28 Hart MJ, Eva A, Evans T, Aronson SA, Cerione RA. Catalysis of guanine nucleotide exchange on the CDC42Hs protein by the db1 oncogene product. Nature 1991; 354: 311-5.
29 Hart MJ, Eva A, Zangrilli D, Aaronson SA, Evan T, Cerione RA, *et al*. Cellular transformation and guanine nucleotide exchange activity are catalyzed by a common domain on the db1 oncogene product. J Biol Chem 1994; 269: 62-5.
30 Diekmann D, Brill S, Garret MD, Totty N, Hsuan J, Monther C, *et al*. Bcr encodes a GTPase-activating protein for p21rac. Nature 1992; 351: 400-2.
31 Heisterkamp N, Morris C, Groffen J. ABR, an active BCR-related gene. Nucleic Acids Res 1989; 17: 8821-31.