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Biochemical and Biophysical Research Communications 318 (2004) 415–421
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βPix-b_L, a novel isoform of βPix, is generated by alternative translation

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Received 25 March 2004
Available online 22 April 2004

Abstract

βPix (Pak-interacting exchange factor) isoforms are recently identified guanine nucleotide exchange factors (GEFs) for Rho family GTPases, Rac/Cdc42, that are key players in the regulation of actin dynamics. Here we show that a novel 105-kDa βPix isoform, βPix-b_L, is generated by alternative translation of βPix-b mRNA. Translation of βPix-b_L starts at an atypical initiation site, GTG, that is located 57 nucleotides downstream from the newly identified 5′ end of βPix-b cDNA. The expression of two isoforms, βPix-b and βPix-b_L, from βPix-b mRNA is controlled by an internal ribosome entry site (IRES)-driven mechanism. Comparing to βPix-b, βPix-b_L contains additional 105 amino acids composed of a calponin homology (CH) domain and a serine-rich sequence in the N-terminus. The expression of βPix-b_L in rat brain is developmentally regulated and high in the embryonic stages, suggesting that the function of βPix-b_L is more heavily required during the early stages of brain development.
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Keywords: βPix; Calponin homology; Internal ribosome entry site; Alternative translation

Actin filament rearrangement is required in many cellular processes such as cell movement, endocytosis, cytokinesis, etc. In these processes, small GTPases of the Rho family serve as key regulators in the regulation of actin dynamics [1,2]. GTPases regulate molecular events by cycling between inactive GDP-bound and active GTP-bound forms. This GDP/GTP cycle is regulated by the interaction of the GTPases with guanine nucleotide exchange factors (GEFs) [3]. The p21-activated protein kinase (Pak)-interacting exchange factor (Pix/COOL/p85SPR) was cloned and identified as a putative GEF for Cdc42/Rac1 by several groups [4–6]. Expression of βPix in fibroblasts enhanced Rac-mediated membrane ruffling. Like other DH protein family of GEFs, βPix possesses several protein modules that mediate protein–protein interactions, thus enabling modulation of multiple signaling pathways. Furthermore, βPix exists in several splice variants, named βPix-a, -b, -c, and -d [7,8]. Different modular structures and tissue distributions of the splice variants suggest that each variant might have a distinct function [9].

In the analysis of βPix gene structure, it was noticed that the 5′ untranslated region (UTR) of βPix-b mRNA is unusually long and predicted to fold into a complex secondary structure with several stable hairpins. This feature indicates that the 5′UTR of βPix-b mRNA is incompatible with efficient ribosomal scanning to synthesize βPix-b. Moreover, a presumptive primary translation product of the 5′UTR of βPix-b mRNA is in-frame with the previously reported βPix-b open reading frame (ORF) without any stop codons. These observations led us to investigate the possibilities that translation of βPix-b might be regulated by an unconventional translation mechanism and furthermore, that the deposited βPix-b cDNA may be incomplete and another isoform, including an additional sequence in the N-terminus, may be synthesized by alternative initiation of translation.

In this study, we demonstrate the existence of a novel isoform of βPix, designated βPix-b_L, which is generated through an alternative translation initiation of βPix-b mRNA. We also show that βPix-b is synthesized by an internal ribosome entry site (IRES)-dependent initiation mechanism.

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doi:10.1016/j.bbrc.2004.04.039

Materials and methods

5′Rapid amplification of cDNA end (RACE)-PCR. Oligo(dT)-primed cDNA was made from 1 μg of total RNA of a 4-week-old mouse brain using Superscript II reverse transcriptase (Life Technologies) in a 20 μl reaction. Negative controls for PCR were processed as above without Superscript II. The SMART RACE cDNA amplification kit (Clontech) was used for all RACE-PCRs, following the manufacturer's protocol. The cDNA template was amplified with antisense βPix primer 5′-GCTCTTGTTGATGGCAGTCGTATCG-3′ and 5′ universal primer. To obtain complete cDNA of βPix-b, 3′-RACE-PCR was performed with sense primer corresponding to the end sequence of 5′-RACE product 5′-CTAGGAAAGGGGGAGCGG ATGAAGCG-3′ and 3′ universal primer. The RACE-PCR products were confirmed by Southern blotting using a probe for the βPix-b C-terminal region. Then, the nucleotide sequences of RACE-PCR products were determined using Autoread DNA sequencing kit (Amersham–Pharmacia Biotech).

Plasmid construction. The various expression constructs used in this study were cloned by PCR-based strategy. The cDNA fragments with or without 5′UTR were amplified by PCR using oligonucleotide primers corresponding to the appropriate sites and were introduced into pcDNA 3.1/Myc-His expression vector (Invitrogen). To construct the bicistronic vector, GFP and RFP coding sequences were amplified from pEGFP-N1 and pDsRed1-mito (Clontech) by PCR and were inserted into a pcDNA3.1 expression vector (pRG). The EMCV IRES sequence from pIRES-EGFP (Clontech) and the 5′UTR sequence of βPix-b_L were amplified by PCR, and those fragments were inserted between the RFP and the GFP of the pRG bicistronic vector. A 150-base pair-long spacer separates the first cistron from inserted cDNA. This spacer contains eight stop codons, which are in-frame with the first cistron. Site-directed mutagenesis was performed using QuikChange site-directed mutagenesis kit (Stratagene), following the manufacturer's instruction. The mutation was verified by automatic DNA sequencing.

In vitro transcription and translation. In vitro transcription and translation was performed by TNT reticulocyte lysate system (Promega). One microgram of supercoiled plasmid was used according to the manufacturer's protocol. The 25 μl reaction mixture contained 12.5 μl TNT rabbit reticulocyte lysate, TNT reaction buffer, T7 RNA polymerase, 20 U RNasin, and an amino acid mixture without methionine, supplemented with [^{35}S]methionine as the radioactive precursor (1000 Ci/mmol, NEN). After 90 min incubation at 30 °C, the reaction mixture was analyzed on a 12% SDS–polyacrylamide gel electrophoresis and visualized by fluorography.

Cell culture, transfection, and antibodies. All materials for cell culture were purchased from Gibco-BRL (Life Technologies). COS7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and maintained in 10% CO_2 at 37 °C. Transient transfection of cells with mammalian expression vectors was performed using LipofectAMINE PLUS reagent according to the manufacturer's instruction (Life Technologies). Anti-CH antibody was raised in rabbit by immunizing with the synthetic peptide NH_2-GSQSSHSRTSKL-COOH. This sequence was deduced from the nucleotide sequence of 5′UTR of βPix-b cDNA from positions −63 to −28 and synthesized by Research Genetics. The peptide was coupled to keyhole limpet hemocyanin by using glutaraldehyde, and the coupled product was used for immunization. Affinity-purified anti-CH antibody was used for immunoblotting. The βPix SH3 domain antibody (anti-SH3) was prepared as described previously [4]. The anti-Myc monoclonal antibody, anti-tubulin monoclonal antibody, and anti-GFP monoclonal antibody were purchased from Santa Cruz, Sigma, and Clontech, respectively.

Western blot analysis. COS7 cells were washed three times with ice-cold PBS and lysed with lysis buffer (0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 50 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium vanadate, 10 μM leupeptin, and 1.5 μM pepstatin) at 4 °C for 20 min. Lysates were clarified by centrifugation at 15,000*g* for 10 min. Rat brains were homogenized using Ultra TURRAX polytron (T25, IKA Labortechnik) in five volumes of lysis buffer and incubated at 4 °C for 1 h. Lysates were clarified by centrifugation at 90,000*g* for 20 min. Protein concentration was determined with the BCA reagent (Pierce). Equal amounts of protein (30 μg for COS7 cell lysates and 40 μg for brain lysates) were subjected to electrophoresis on 10% SDS–polyacrylamide gels, transferred onto a polyvinylidine difluoride (PVDF) membrane (Millipore), and immunoblotted using indicated antibody. Horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories. Immunoblots were visualized using enhanced chemiluminescence detection system (Amersham).

Results

βPix-b_L is generated by alternative translation of βPix-b mRNA

One of the unusual features of the previously reported βPix-b cDNA is that the presumptive translation product of the 5′UTR of 297 nucleotides is in-frame with the ORF of βPix-b without any stop codons. Besides, comparison of the amino acid sequence deduced from this 5′UTR sequence with other proteins in the GenBank Database revealed that the predicted amino acid sequence contains a putative CH domain; this sequence is 50%, 43%, 44%, 41%, 40%, and 44% identical to the homologous region of αPix, Vav1, Vav2, Vav3, α-calponin, and β-calponin, respectively. These observations suggest a possibility that alternative initiation of translation may occur in the 5′UTR, to make another protein containing an extended N-terminal sequence. Thus, we performed 5′-RACE to identify the 5′ end of βPix-b mRNA. The 5′-RACE-PCR product containing additional 75 nucleotides to the released sequence (GenBank Accession No. AF247654) was obtained (Fig. 1A). Furthermore, the newly obtained nucleotide sequence from 5′-RACE was identical to a sequence in a mouse genomic clone deposited in the mouse genome sequencing database (Trace archive) on the NCBI website (http://www.ncbi.nlm.nih.gov/genome/seq/MmBlast.html). Analysis of this genomic clone reveals that the newly obtained sequence of the 5′ end with the flanking downstream 78 nucleotides, previously reported as the 5′ end of βPix-b cDNA, may comprise an exon encompassing 153 base pairs. In addition, RT-PCR using a sense primer positioned at the 5′ end of the newly obtained sequence and an antisense primer corresponding to the C-terminal region of βPix-b amplified a single transcript with the predicted size (data not shown). Taken together, these results suggest that βPix-b mRNA contains additional sequences to the previously reported one at the 5′ end in vivo.

Because the 5′UTR of βPix-b mRNA has a UGA stop codon at −345 but no AUG codon, we first

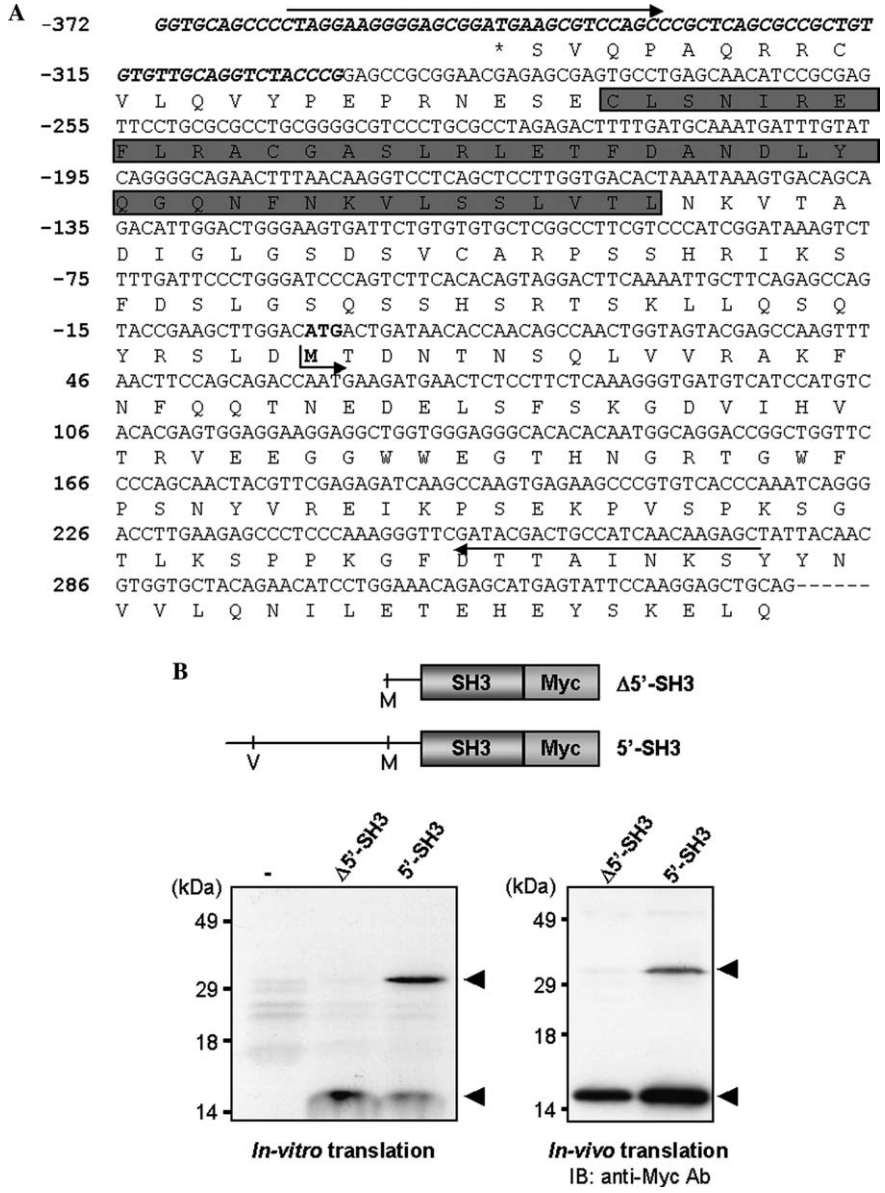

Fig. 1. Identification of the 5′UTR of βPix-b cDNA. (A) The extended 5′UTR of βPix-b cDNA and predicted amino acid sequence. The criterion of numbering is relative to ATG, the putative initiation codon of βPix-b as position +1. The extended sequence, which is added to the previously reported βPix-b cDNA, is shown in italics. Arrows indicate the primer sites for 5′ and 3′ RACE-PCR. The gray shading box indicates the CH conserved region. The nucleotide sequence data are available from GenBank/EMBL/DDBJ under Accession No. AY220301. (B) Schematic representation of constructs used for in vitro and in vivo translations (top). A fluorograph of in vitro translated products and an immunoblot of the transfected COS7 cell lysates with anti-Myc antibody are shown (bottom panels).

examined whether the 5′UTR of βPix-b mRNA is able to synthesize another protein that contains extended amino acids. Translation of the βPix-b SH3 domain construct either with (5′-SH3) or without (Δ5′-SH3) the complete 5′UTR sequence was analyzed either by in vitro translation using rabbit reticulocyte lysate or by transfection into COS7 cells (Fig. 1B). Compared to the Δ5′-SH3, which yields a single product of the predicted size, the 5′-SH3 produced two translational products both in in vitro and cell-transfection experiments. These results strongly suggest that a novel βPix isoform containing a putative CH domain can be produced by alternative initiation of translation at a start codon located upstream from the AUG start codon of βPix-b mRNA.

To confirm the presence of the CH domain-containing βPix isoform in tissues, the expression of the isoform in rat brain was examined by immunoblotting with an antibody raised against a peptide sequence in the putative CH domain (Fig. 2A). As shown in Fig. 2B, the antibody recognized the 105-kDa protein in the brain lysates. The expression level of the protein was high in

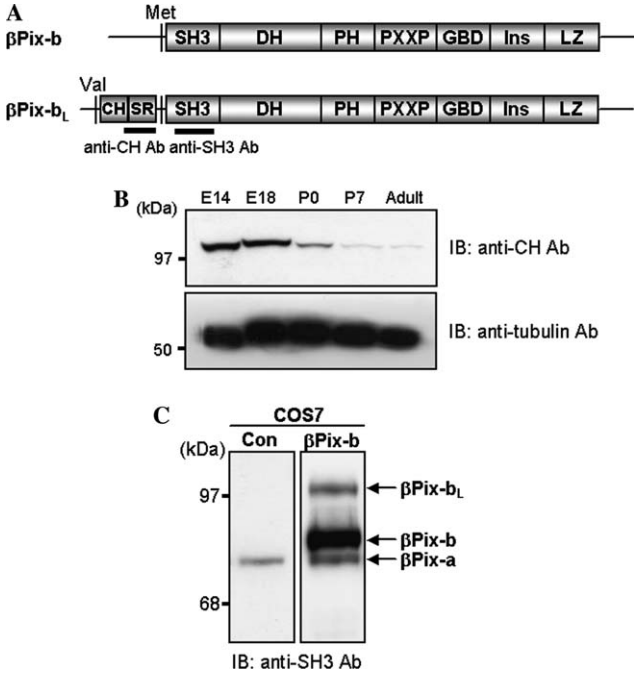

Fig. 2. Transfection of βPix-b cDNA in COS7 cells produces two proteins, βPix-b and βPix-b_L. (A) Comparison of domain structures of βPix-b and βPix-b_L. CH, calponin homology; SR, serine-rich region; SH3, src homology 3; DH, dbl homology; PH, pleckstrin homology; PXXP, proline-rich region; GBD, Git-1 binding domain; Ins, insert region; and LZ, leucine zipper motif. Domains that are recognized by the anti-CH, anti-SH3 polyclonal antibodies are indicated. (B) Expression of βPix-b_L in different developmental stages (E14, E18, P0, P7, and adult) of rat brain was analyzed by immunoblotting with anti-CH antibody. The same membrane was deprobed and reprobed with anti-tubulin antibody to show that an equal amount of protein was loaded in all lanes. (C) Immunoblotting analysis of COS7 cells transfected with βPix-b cDNA (βPix-b). Control COS7 cells exhibit only the endogenous βPix-a protein (Con).

the embryonic stages, gradually declined during the postnatal stages with age, and almost diminished in the adult stage. Furthermore, the expression in COS7 cells of the full-length βPix-b cDNA produced the 105-kDa protein in addition to the 95-kDa βPix-b protein (Fig. 2C). These results indicate that the 105-kDa protein is generated by alternative translation initiation of βPix-b mRNA. Therefore, we designated this protein as βPix-b_L.

As no additional AUG codon exists upstream of the $^{+1}$AUG initiation codon of βPix-b mRNA, we considered the GUG at position −315, UUG at −312, or CUG at −273 as putative alternative start codons of βPix-b_L. Usages of these atypical codons as an alternative start site were reported in other proteins [10–12]. To determine which codon was used as the alternative translational initiation site in βPix-b mRNA, a point mutation of each codon was introduced into the chimeric 5′UTR-SH3Myc construct (Fig. 3A). In vitro and in vivo translation of the wild type as well as mt2($^{-312}$TTG to TCC) and mt3($^{-273}$CTG to CCC) mutations gave rise to a double band at 16- and 30-kDa. However, the mt1($^{-315}$GTG to GTA) or mt4($^{+1}$ATG to AAG) mutation resulted in disappearance of the upper or lower band, respectively (Fig. 3B and C). These data suggest that βPix-b_L is generated by initiation at the $^{-315}$GUG codon and βPix-b at the $^{+1}$AUG codon in vitro and in vivo.

An IRES-driven mechanism regulates the alternative translation of βPix mRNA

The above results indicate that two proteins, βPix-b and βPix-b_L, are generated from a single mRNA by alternative initiation of translation. It has been reported that more than one alternative translation product can be synthesized from a single mRNA by an IRES-dependent mechanism in several mammalian proteins [13,14]. To investigate whether the context of 5′UTR sequence (from −1 to −303) of βPix-b mRNA is critical for the translation of βPix-b, the expression of βPix-b SH3Myc construct with sense (pM-5′SH3) or antisense

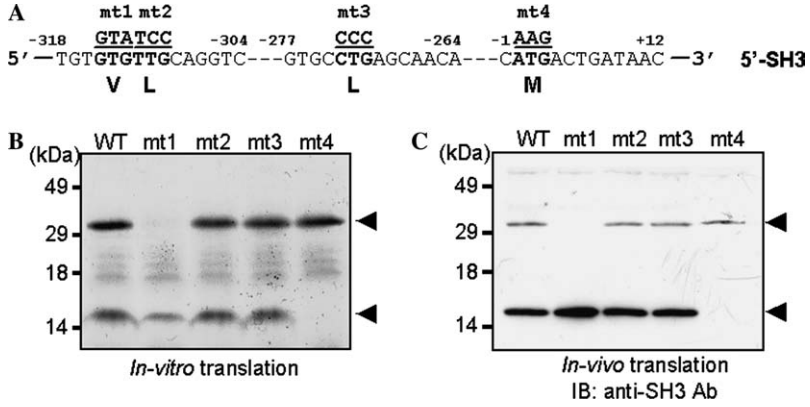

Fig. 3. Identification of the alternative translation initiation site of βPix-b_L. (A) Four possible translation initiation sites (GTG, TTG, CTG, and ATG) are indicated by boldface in the 5′UTR sequence of the 5′-SH3 construct. Each one of these four sites was replaced with nucleotides shown above the original sequence. Each mutant is named mt1, mt2, mt3, and mt4, respectively. (B,C) A fluorograph of in vitro translated products (B) and an immunoblot of the transfected COS7 cell lysates with anti-SH3 antibody (C) are shown. Wild type (WT) and mutant constructs used in the experiments are indicated at the top of each panel.

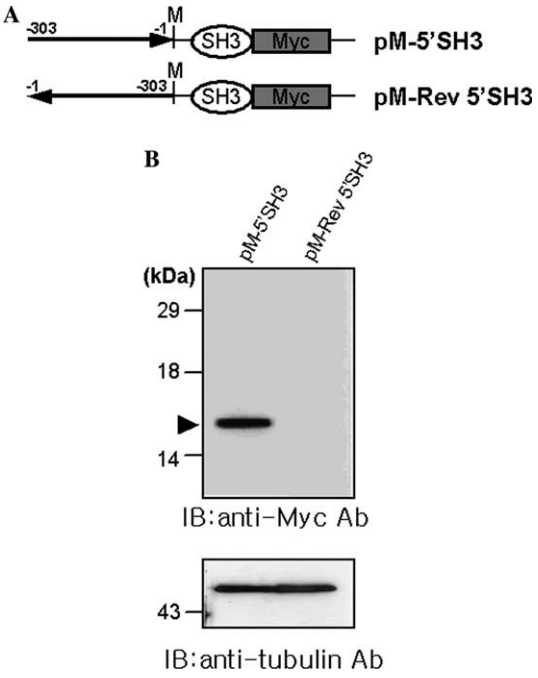

Fig. 4. Translation of βPix-b is sensitive to the orientation of 5′UTR sequence. (A) Schematic representation of the chimeric constructs with sense (pM-5′SH3) or antisense (pM-Rev5′SH3) orientation of the 5′UTR of βPix-b. (B) COS7 cells were transfected with pM-5′SH3 or pM-Rev5′SH3 and the protein expression is analyzed by immunoblotting with anti-Myc antibody. The same membrane was deprobed and reprobed with anti-tubulin antibody to show that an equal amount of protein was loaded in all lanes.

(pM-Rev5′SH3) orientation of the 5′UTR was examined in the transfected COS7 cells (Fig. 4). The pM-5′SH3 construct produced a single band of protein because it contains the methionine initiation site for βPix-b at +1 position but not the alternative translation start site for βPix-b_L at −315 (Fig. 4B, pM-5′SH3). However, the inversion of the 5′UTR sequence in front of the initiating AUG codon completely abolished translation of the reporter gene (Fig. 4B, pM-Rev 5′SH3), indicating that translation of βPix-b mRNA might be dependent on a *cis*-acting element, such as an IRES, rather than a cap-dependent scanning mechanism. To examine the existence of any possible IRES in the 5′UTR of βPix-b mRNA, we employed bicistronic vectors that have two open reading frames in a single mRNA. In eukaryotic cells, the second cistron cannot be translated unless there is an IRES between the first and second cistrons [15]. To monitor the IRES activity in the 5′UTR of βPix-b mRNA in vivo, we constructed bicistronic vectors with red fluorescent protein (RFP) as the first cistron and SH3-green fluorescent protein (GFP) with or without the 5′UTR as the second cistron and analyzed its expression by fluorescence microscopy in the transfected COS7 cells (Fig. 5A). While both the RFP and GFP were expressed from the bicistronic vector containing the 5′UTR of βPix-b mRNA, only the

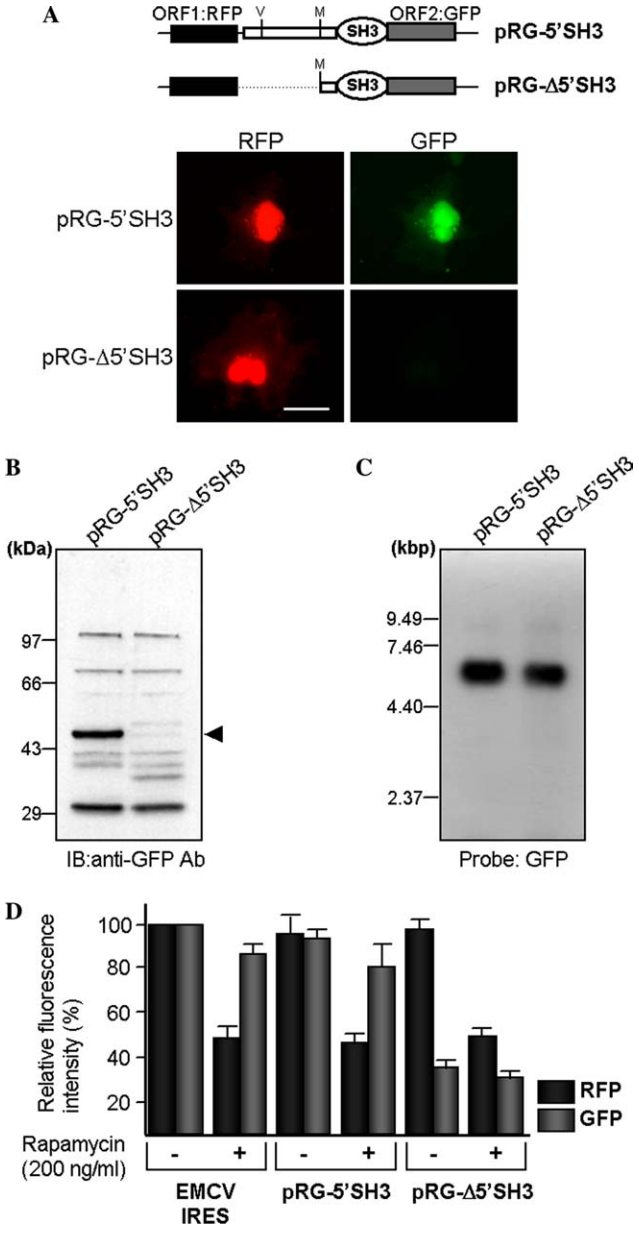

Fig. 5. 5′UTR of βPix-b mRNA contains IRES. (A) Schematic representation of bicistronic vectors (top). Bicistronic constructs were transfected into COS7 cells and visualized for the expression of either the first cistron or the second cistron under a fluorescence microscope (bottom panels). Scale bar, 30 μm. (B,C) Total RNAs and cell lysates were prepared from the COS7 cells transfected with the bicistronic vectors. Expression of the second cistron was analyzed by immunoblotting using anti-GFP antibody (B). Northern blot analysis was performed with GFP cDNA probe using 20 μg of total RNA (C). (D) COS7 cells expressing indicated bicistronic vectors were maintained for 18 h in the absence or presence of 200 ng/ml rapamycin. Translation efficiency of each cistron was quantified by luminescence spectrofluorometry. Bicistronic construct containing EMCV-IRES sequence was used as a positive control. Values are means ± SDV for five independent experiments.

RFP was expressed from the one without 5′UTR (Fig. 5A, bottom panels). Expression of the pRG-5′SH3 construct was also analyzed by immunoblotting with an

anti-GFP antibody. The results showed that the pRG-5′SH3 construct efficiently produced the SH3-GFP protein in COS7 cells, while the pRG-Δ5′SH3 construct did not (Fig. 5B). Northern blotting enabled us to confirm that bicistronic mRNAs of the expected sizes were produced in both cases (Fig. 5C). In addition, the absence of any additional band allowed us to rule out the presence of an active internal promoter. To further demonstrate that the translation of βPix-b was cap-independent in vivo, we monitored the expression levels of RFP and GFP from the bicistronic vectors upon rapamycin treatment. This compound partially inhibits cap-dependent translation by promoting the dephosphorylation and activation of 4E-BP1, a repressor of the cap-binding protein 4E [16]. Rapamycin treatment caused about 60% decrease of RFP intensity in the pRG-5′SH3 transfected cells, but there was only a small decrease in the level of GFP intensity (Fig. 5D, pRG-5′SH3). Similar results were obtained using a well-known IRES-containing construct of encephalomyocarditis virus (EMCV) (Fig. 5D, EMCV-IRES). These results demonstrate that the 5′UTR of βPix-b mRNA contains an IRES, which serves as a regulator of alternative translation.

Discussion

In this study, we demonstrate that two βPix isoforms, βPix-b and βPix-b_L, are generated from a single mRNA, and the expression of two isoforms can be controlled by alternative initiation of translation and IRES-mediated translational regulation. According to the classical cap-dependent scanning model, such long 5′UTR sequences as in βPix-b mRNA are expected to impair translation initiation by preventing ribosome scanning from the capped 5′-end of mRNA [15]. Actually, when the 5′UTR of βPix-b mRNA was in antisense orientation, the expression of the chimeric construct was completely abolished (Fig. 4). This result strongly suggests that the translation of βPix-b may be regulated by a *cis*-acting element such as IRES rather than a cap-dependent scanning mechanism. Indeed, analyses using bicistronic vector system, the most valid test to prove the existence of IRES, indicate that βPix-b is synthesized by an IRES-driven mechanism. One criticism with respect to our result that still remains is that some form of reinitiation may have occurred at the second cistron after translation of the first cistron. However, evidence shown in Fig. 5 disproves this possibility. First of all, the second cistron was still being expressed when the cap-dependent translation was blocked by rapamycin treatment. In addition, abrogation of the expression of second cistron in the bicistronic construct without a 5′UTR sequence cannot be explained by a reinitiating mechanism since the inter-cistron in this construct (Fig. 5A, pRG-Δ5′SH3) still contains a 150-nucleotide spacer at the end of the first cistron.

Alternative initiation of translation can greatly amplify potential functions of a gene, because the alternative forms of the protein may have co-operative, alternative or even opposite effects [17]. Thereby, most of the cellular mRNAs, known to regulate protein synthesis by alternative initiation of translation, express their translational products at very specific stages and/or in response to different stimuli. For example, a CUG-initiated isoform of human fibroblast growth factor 2 is synthesized by alternative initiation of translation in transformed cells and behaves as a survival factor [18]. In the case of βPix-b_L, we observed that the expression of βPix-b_L in rat brain is developmentally regulated. It exhibited high levels of expression in the embryonic stages and almost diminished in the adult stage (Fig. 2B). This observation suggests that the function of βPix-b_L might be more heavily required during the early stages of brain development. It is also worthy to note that the expression level of βPix-b_L was much lower than that of βPix-b in the transfected COS7 cells unlike in in vitro translation experiments where similar levels of βPix-b and βPix-b_L were expressed (Figs. 2C and 3). This result indicates that the expression level of βPix-b_L is tightly regulated in vivo. Further studies on the search for specific cues to regulate the expression of βPix-b_L will provide additional important insights into identifying the physiological role of βPix-b_L in the early stages of brain development.

Acknowledgments

This work was supported by Grants M1-0203-00-0072 under the National Research Laboratory Program from the Ministry of Science and Technology and KRF-2002-015-CS0043 from the Ministry of Education and Human Resources Development, the Republic of Korea. S.J.L. and S.J.Y. were supported by BK21 fellowship.

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