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*i*PABP, an Inducible Poly(A)-Binding Protein Detected in Activated Human T Cells

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The poly(A)-binding protein (PABP) binds to the poly(A) tail present at the 3′ ends of most eukaryotic mRNAs. PABP is thought to play a role in both translation and mRNA stability. Here we describe the molecular cloning and characterization of an inducible PABP, *i*PABP, from a cDNA library prepared from activated T cells. *i*PABP shows 79% sequence identity to PABP at the amino acid level. The RNA binding domains of *i*PABP and PABP are nearly identical, while their C termini are more divergent. Like PABP, *i*PABP is primarily localized to the cytoplasm. *i*PABP is expressed at low levels in resting normal human T cells; following T-cell activation, however, *i*PABP mRNA levels are rapidly up-regulated. In contrast, PABP is constitutively expressed in both resting and activated T cells. *i*PABP mRNA was also expressed at much higher levels than PABP mRNA in heart and skeletal muscle tissue. These data suggest that the regulation of cytoplasmic poly(A)-binding activity is more complex than previously believed. In most tissues, poly(A)-binding activity is likely to be the result of the combined effects of constitutively expressed PABP and *i*PABP, whose expression is subject to more complex regulation.

A T-cell immune response is initiated when a quiescent T cell encounters an antigenic peptide presented in the context of self major histocompatibility complex on an antigen-presenting cell. As a result of antigen activation, quiescent T cells are induced to transcribe an array of lymphokine genes. Concomitant with transcriptional activation, T cells also dramatically up-regulate their rate of translation. The combined effects of transcriptional and translational activation allow the activated T cells to produce high levels of secreted proteins, lymphokines, that act by paracrine mechanisms to regulate an immune response. Lymphokine production can be enhanced posttranscriptionally by costimulation via the CD28 receptor (21).

In order to isolate activation-induced genes from T cells, a cDNA library was constructed from normal human T cells which had been activated for 6 h with phorbol 12-myristate 13-acetate (PMA), ionomycin, and anti-CD28. One clone which fulfilled the criteria of being induced following activation was found to be related to poly(A)-binding protein (PABP).

PABP binds to the poly(A) tail of mRNA and has a reported size of 68 to 72 kDa (5, 9). PABP has been isolated from a wide range of species and is highly conserved. Several reports have demonstrated cytoplasmic localization of PABP in both mammals and *Saccharomyces cerevisiae* (1, 2, 8), although one group has identified a nuclear 50-kDa form in *S. cerevisiae* (16). The nuclear and cytoplasmic forms are encoded by the same gene, the nuclear form of PABP being derived by proteolytic cleavage of the longer cytoplasmic form. The function of PABP is still undefined. In *S. cerevisiae* it is required for viability. PABP has been shown to be necessary for the initial shortening of the poly(A) tail, an event which occurs in the cytoplasm following transport of mRNA from the nucleus (17, 18). PABP is also required in *S. cerevisiae* for translational initiation, where it allows 60S ribosomal subunit joining (17). PABP is also important for mRNA stability, since the poly(A) tail has been shown to degrade very rapidly once the PABP is removed (4). Recently, a second PABP, PAB II, with a molecular mass of 49 kDa, was isolated from human cells (22). The nucleotide sequence of PAB II has been reported to contain two regions with homology to the ribonucleoprotein 1 (RNP1) and RNP2 consensus motifs found in RNA-binding proteins, but otherwise its sequence is unrelated to that of PABP. In addition, PAB II is a nuclear protein involved in nuclear polyadenylation (11). Specifically, PAB II binds to the growing poly(A) tail and stimulates its elongation by poly(A) polymerase (22).

We now describe a third PABP, *i*PABP. Like PABP, *i*PABP appears to accumulate primarily in the cytoplasm. In contrast to PABP mRNA, however, *i*PABP mRNA can be rapidly induced in response to cellular activation. *i*PABP is also expressed at much higher levels than PABP in heart and skeletal muscle tissue. The RNA binding properties of *i*PABP appear to be identical to those of PABP. The presence of an inducible PABP provides a potentially important mechanism for the differential regulation of gene expression in activated and resting T cells.

MATERIALS AND METHODS

Library construction and screening. To screen for genes induced during activation, CD28-positive T lymphocytes were purified from the peripheral blood of healthy subjects by a previously described negative-selection method (10). The purified T cells were stimulated for 6 h with PMA at a final concentration of 10 ng/ml, ionomycin at 0.4 μg/ml, and the monoclonal anti-CD28 antibody 9.3 (12) at 1 μg/ml. Following stimulation, the cells were harvested and RNA was prepared as described previously (7). A custom cDNA library with this RNA was prepared in the vector lambda ZAP II by using both random and oligo(dT) primers (Stratagene). The initial *i*PABP clone was isolated as part of a composite clone which displayed higher levels of mRNA expression in activated T cells than in resting T cells. The screening was aimed at obtaining activation-induced genes containing multimers of the sequence motif AUUUA, using a PCR-based approach (5a). The *i*PABP clone obtained was not a full-length clone, and therefore the library was screened again. Two clones were obtained: 1E and 2B. 1E contained an initiation codon in a position in the cDNA identical to the position of the initiation methionine in PABP. However, this clone did not produce a stable product when translated in vitro. Clone 2B fell 104 bp short of the initiation codon found in clone 1E. Clone 1E contains an 88-bp deletion from

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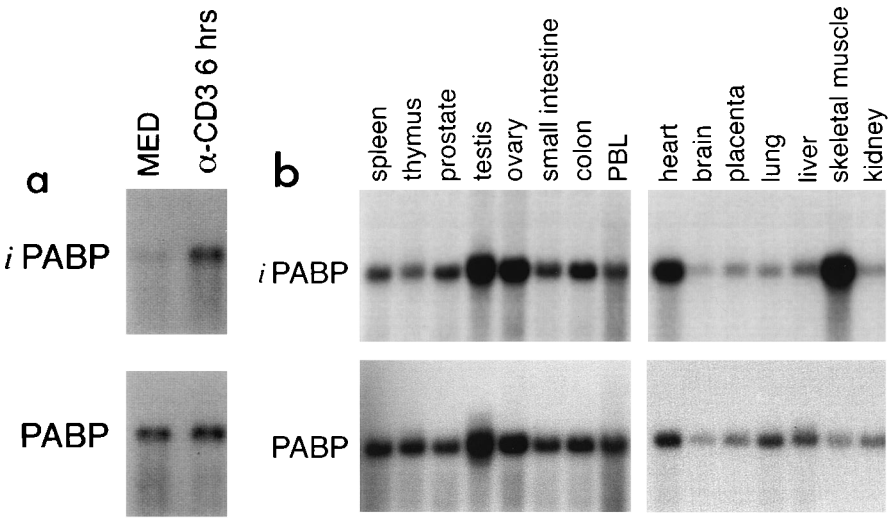

FIG. 1. mRNA expression of *i*PABP. (a) *i*PABP mRNA expression in human T cells. Normal human T cells were either left in culture medium (MED) or stimulated for 6 h with immobilized anti-CD3. RNA was harvested and equalized on the basis of the intensity of ethidium bromide staining of the 28S rRNA, as described in Materials and Methods. (b) Tissue distribution of *i*PABP. The Northern blot was sequentially hybridized with probes specific for *i*PABP and PABP. The size of the mRNA for *i*PABP is 3.2 kb. PBL, peripheral blood lymphocytes.

position 1399 to position 1487, and in addition there is a 43-bp insertion of novel sequence at position 1558, leading to the insertion of a premature stop codon. To obtain a full-length clone, a fragment spanning the region from the *Pst*I site at position 50 to the *Bam*HI site at position 420 in clone 1E was ligated to clone 2B. This clone translated in vitro to an 80-kDa product (see Fig. 3). DNA sequencing by the dideoxy chain termination method was performed with a Sequenase kit (United States Biochemical). Sequence analysis was done by using the DNAStar programs and the Genetics Computer Group (University of Wisconsin, Madison) package.

Northern (RNA) blots. Human CD28-positive cells obtained as described above were stimulated either with PMA at 10 ng/ml and ionomycin at 0.4 μg/ml or with an immobilized monoclonal antibody, G19.4 (13), directed against the CD3 portion of the T-cell receptor–CD3 complex as previously described (10). Following stimulation, the cells were harvested and RNA was prepared and equalized to 28S rRNA on nondenaturing agarose gels (7, 10). Following equalization the RNAs were separated on denaturing formaldehyde-agarose gels and transferred to nitrocellulose. Multiple-tissue Northern blots were obtained from Clontech and probed according to the manufacturer's instructions. A DNA fragment extending from the *Eco*RI site at position 718 to the *Eco*RI site at 2129 was used as a probe for *i*PABP. A probe spanning the 3′ untranslated region of *i*PABP gave identical results. A probe specific for the 3′ untranslated region of PABP, extending from bp 2407 to 2795 (9), was obtained by PCR, using a Jurkat T-cell cDNA library as a template.

In vitro translation and RNA-binding assays. In vitro translation of 1 μg of full-length *i*PABP was performed with the TNT-coupled reticulocyte lysate systems (Promega) by following the manufacturer's instructions. The in vitro translation was performed in the presence of 40 μCi of in vitro-translation-grade [^{35}S]methionine (ICN). Binding of in vitro-translated, [^{35}S]methionine-labelled *i*PABP to RNA homopolymers coupled to Sepharose was performed as previously described (6). Briefly, poly(A)-Sepharose 4B and poly(U)-Sepharose 4B (Pharmacia) were prepared according to the manufacturer's protocol and resuspended at a concentration corresponding to 100 μg (dry weight) per ml in binding buffer (10 mM Tris [pH 7.4], 2.5 mM $MgCl_2$, 100 mM KCl, and 0.5% Triton X-100). Poly(C)-agarose was obtained from Sigma. Twenty-five microliters of poly(A)- and poly(U)-bound Sepharose or poly(C)-agarose was mixed with 5 μl of the in vitro-translated product in a total of 300 μl of binding buffer. In competition experiments, poly(U), poly(A), and poly(C) (Pharmacia) were added to the binding reaction mixtures at concentrations indicated in the figure legends. Heparin at a final concentration of 1 mg/ml or yeast tRNA at 0.5 μg/ml was added as a nonspecific competitor. The binding reactions were carried out at 4°C for 10 min, and the Sepharose beads were then washed four times in binding buffer. The samples were resuspended in sample buffer, boiled for 2 min, and loaded on a sodium dodecyl sulfate (SDS)–9.5% polyacrylamide gel. Following electrophoresis the gels were fixed in 40% methanol–10% glacial acetic acid, fluorographed in Amplify (Amersham), dried, and exposed to XAR film overnight at room temperature.

Epitope tagging. In order to determine the subcellular localization of *i*PABP, a chimeric clone was made by fusing *i*PABP to the sequence encoding the nonapeptide YPYDVPDYA from the influenza virus hemagglutinin [HA] protein, which is recognized by monoclonal antibody 12CA5 (Boehringer Mannheim). The 3′ end of *i*PABP was tagged with this epitope by a PCR-based approach. Specifically, the two primers used were a sense primer, CAGCC CGGGCTGTTGCCCCCTACAAATA, spanning the *Sma*I site (underlined) at position 1703 in Fig. 2a, and an antisense primer, GCTCTAGAGC[CTA]**G GCATAGTCAGGGACGTCATAAGGATA***AGAGGTAGCAGCAGCAACAGCG CCCAC*. In the latter primer the first 10 bases constitute the *Xba*I restriction site (underlined), followed by the stop codon (in brackets) and 27 bases of HA-specific sequence (boldface); the remainder of the primer is homologous to the 27 bases of *i*PABP immediately in front of the stop codon (italics). These two primers were used in a PCR with the full-length *i*PABP clone as a template, and a 400-bp product was obtained. Next, the *Sma*I-*Xba*I fragment, which comprises the 3′ part of the wild-type *i*PABP clone, was removed and replaced with the 400-bp PCR product containing the epitope tag. This new clone, *i*PABP-HA, was sequenced to confirm that correct ligation had taken place and subsequently was subcloned into the eukaryotic expression vector pcDNA3 (Invitrogen).

Transfection. *i*PABP-HA in pcDNA3 was transfected into COS-7 cells with DEAE-dextran by standard protocols (19). Briefly, COS-7 cells were diluted to 10^5 cells per ml in Dulbecco modified Eagle medium with 10% fetal calf serum, 2 mM L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml 20 h prior to transfection. The cells were transfected with 10 μg of *i*PABP DNA at a concentration of 250 μg/ml of DEAE-dextran and in the presence of chloroquine. The cells were incubated for 3 h and then washed; these steps were followed by a short exposure to 10% dimethyl sulfoxide. After two washes, the cells were allowed to grow for 48 h and then assayed for expression.

Immunoprecipitation and immunofluorescence. Immunoprecipitation of [^{35}S]methionine-labelled, in vitro-translated *i*PABP-HA was performed in 100 mM NaCl–20 mM Tris–1 mM EDTA–0.2% Nonidet P-40. The in vitro-translated *i*PABP-HA was precleared with Pansorbin (Calbiochem) before addition of the monoclonal anti-HA antibody 12CA5 (Boehringer-Mannheim) at a final concentration of 4 μg/ml. After a 1-h incubation at 4°C, protein G-agarose (Gibco BRL) was added. Purified mouse immunoglobulin G2b (PharMingen) was used as a control antibody. The immunoprecipitates were washed three times in the above-mentioned buffer, resuspended in sample buffer, and loaded on an SDS–9.5% polyacrylamide gel. Immunofluorescence was performed to identify the subcellular localization of *i*PABP in COS-7 cells transiently transfected with *i*PABP-HA. The cells were harvested, spun in a cytocentrifuge onto glass slides, fixed in 2% paraformaldehyde, and permeabilized in 0.1% saponin. The cells were then incubated with the anti-HA antibody 12CA5 at a final concentration of 10 μg/ml, washed, and stained with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Sigma) at a final concentration of 20 μg/ml. The cells were then inspected under a Leitz DMRB fluorescence microscope, and photographs were taken with a Nikon N6006 camera.

Nucleotide sequence accession number. The GenBank accession number of the sequence reported in this paper is U33818.

RESULTS

***i*PABP is an inducible PABP.** To screen for activation-induced genes, a cDNA library was constructed from normal

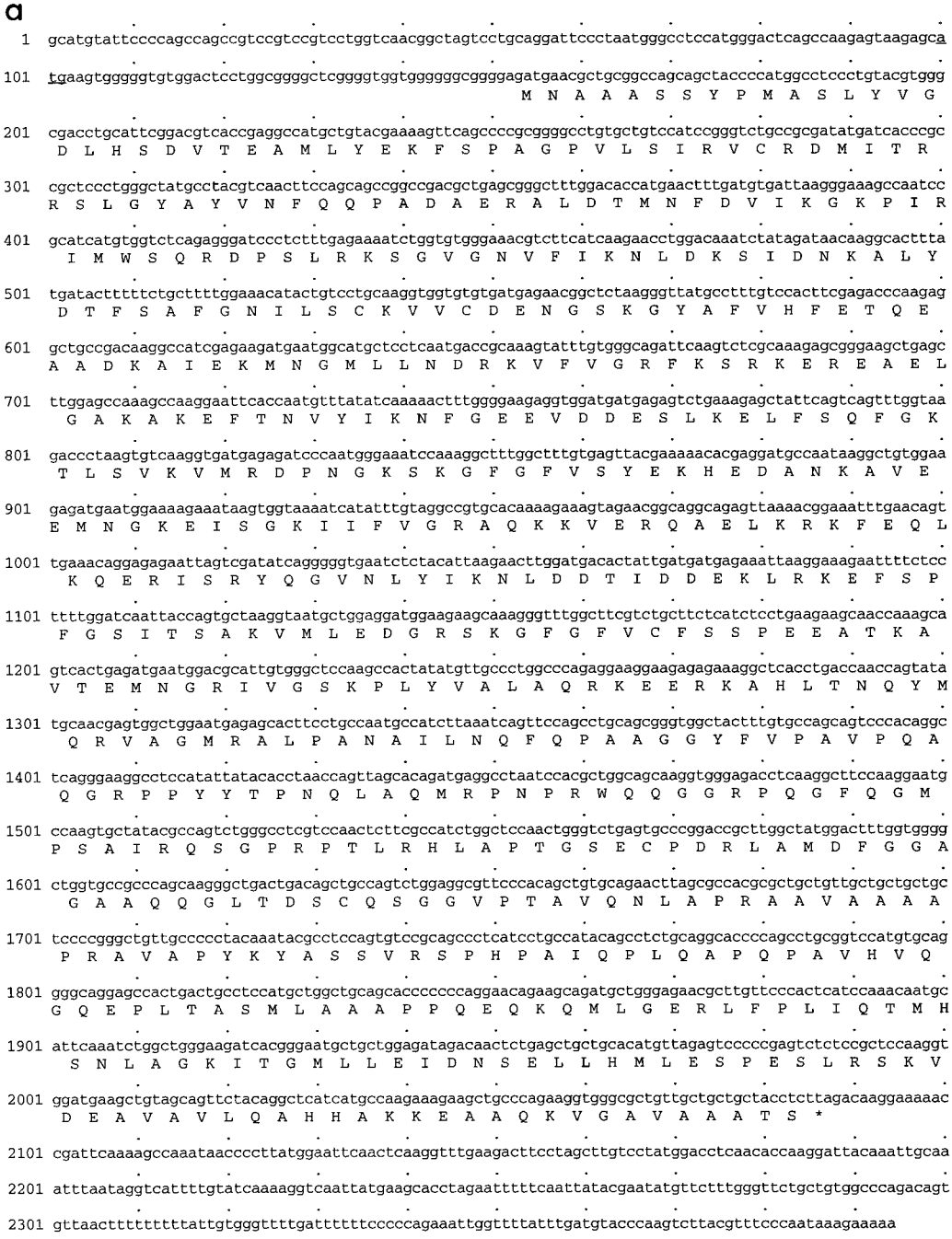

FIG. 2. DNA and predicted amino acid sequence of *i*PABP. (a) Nucleotide sequence and predicted amino acid sequence of *i*PABP. Underlined is an in-frame ATG located upstream of the predicted initiation codon (see the text). The asterisk indicates the stop codon. (b) Comparison of the amino acid sequences of *i*PABP and PABP. The shaded areas indicate RBD I through IV. The solid underline marks the RNP1 consensus sequence, and the dashed underline marks the RNP2 consensus sequence. The box shows an area of the sequence which is conserved in a variety of species. Vertical lines indicate identical amino acids; dots and colons indicate conserved amino acid changes.

human T cells which had been stimulated for 6 h with PMA–ionomycin–anti-CD28. This combination of stimuli is known to mediate maximal induction of lymphokines in normal T cells (21). One clone which displays approximately fivefold-higher levels of expression in activated than in resting T cells showed sequence similarity to PABP (Fig. 1a; the sequence is shown in Fig. 2). However, this expression pattern is distinct from that of PABP, as PABP is constitutively expressed in both resting and activated T-cell populations (Fig. 1a). We have named this novel gene *i*PABP. Stimulation with PMA and ionomycin, which mimics the signal transduction events taking place during cross-linking of the T-cell receptor–CD3 complex, was found to be sufficient to induce the expression of *i*PABP (data not shown). However, neither PMA nor ionomycin alone induced *i*PABP. The size of the *i*PABP mRNA is 3.2 kb, slightly larger than the 2.9-kb mRNA reported for PABP (9). *i*PABP

b

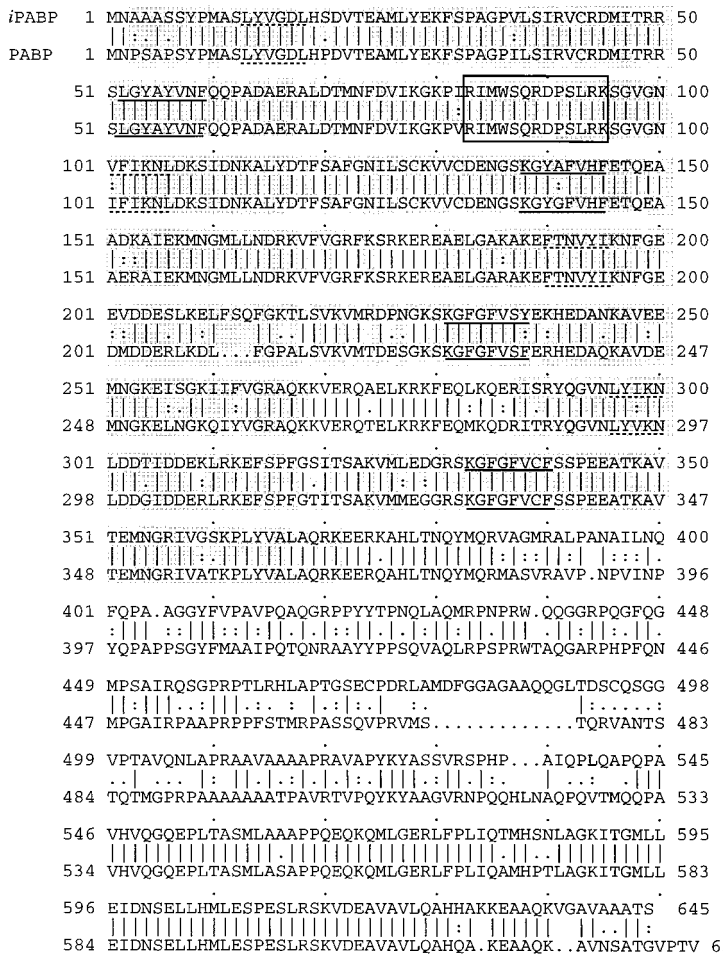

FIG. 2—*Continued.*

expression was not restricted to T cells; Fig. 1b shows that *i*PABP was expressed in all tissues examined. Interestingly, *i*PABP is expressed at much higher levels than PABP in heart and skeletal muscle tissue.

Isolation and sequence analysis of *i*PABP. The complete cDNA and predicted amino acid sequences of the full-length *i*PABP clone are shown in Fig. 2a. In comparing the sequence to that of PABP, a 73.6% identity at the DNA level can be noted. There are two possible in-frame initiation codons for *i*PABP, one at position 154 and a second at position 100. The downstream ATG is identical in the flanking sequence surrounding the ATG to the initiator ATG found in PABP. In addition, the homology at the amino acid level between *i*PABP and PABP begins at this methionine. Deletion of the upstream ATG does not affect the size of the protein produced by in vitro translation of the *i*PABP cDNA (data not shown). It is therefore most probable that the downstream in-frame ATG is the initiation codon. The open reading frame is 1,934 bp long, in contrast to 1,901 bp for PABP. The overall level of homology between *i*PABP and PABP is greatest in the 5′ ends of the genes, whereas the 3′ ends show more divergence. There are a number of examples of third-base-pair wobble in *i*PABP compared with PABP which lead to the same amino acid being translated, thus keeping the amino acid sequences identical despite differences in nucleotide sequence. This excludes the possibility that *i*PABP is a differentially spliced form of PABP. In Fig. 2b is shown a comparison of the amino acid sequences of *i*PABP and PABP. Overall, the two proteins are 79% identical and 88% similar because of conserved amino acid substitutions.

The four RNA binding domains, RBD I to IV, previously defined for PABP by Burd et al. (6), are also found in *i*PABP. A comparison of *i*PABP and PABP shows that RBD I and II have the highest degrees of identity (94 and 96%, respectively). RBD III shows 78% and RBD IV shows 88% identity between the two proteins. It is interesting that it has been shown that the poly(A)-binding ability of PABP resides within RBD I and II. Also indicated in Fig. 2 are RNP1 and RNP2 consensus sequences. In RBD I the RNP1 and RNP2 sequences are identical for *i*PABP and PABP. In RBD II there is one conserved amino acid substitution for each of the RNPs. Similarly, RBD III and IV contain conservative single amino acid substitutions between *i*PABP and PABP.

It has previously been shown that the boxed region bridging RBD I and RBD II is completely conserved in human, *S. cerevisiae*, *Schizosaccharomyces pombe*, and *Xenopus laevis* PABPs (6). Figure 2b shows complete identity in this region between *i*PABP and PABP. Another region which has been

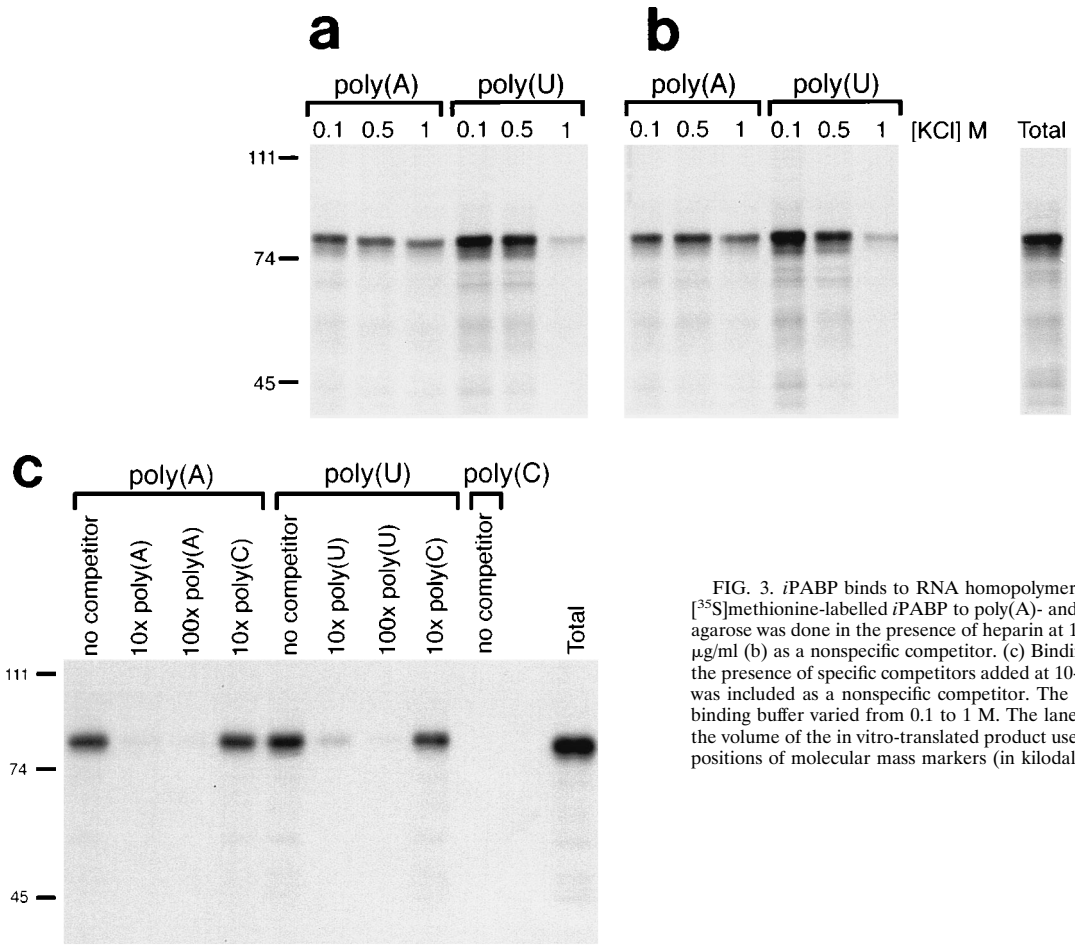

FIG. 3. *i*PABP binds to RNA homopolymers. Binding of in vitro-translated [^{35}S]methionine-labelled *i*PABP to poly(A)- and poly(U)-Sepharose or poly(C)-agarose was done in the presence of heparin at 1 mg/ml (a) or yeast tRNA at 0.5 μg/ml (b) as a nonspecific competitor. (c) Binding reactions were carried out in the presence of specific competitors added at 10- and 100-fold excesses. Heparin was included as a nonspecific competitor. The KCl concentration of the RNA binding buffer varied from 0.1 to 1 M. The lane marked "Total" shows 25% of the volume of the in vitro-translated product used for the binding reactions. The positions of molecular mass markers (in kilodaltons) are indicated on the left.

shown to be conserved among different species is the proline-rich carboxyl terminus. Although there are some nonconserved amino acid differences in this region between *i*PABP and PABP, *i*PABP also has a proline-rich carboxyl terminus (residues 510 to 645). However, there is considerable divergence between *i*PABP and PABP in the region between RBD IV and the proline-rich region. The sequenced 5′ and 3′ untranslated regions between these two PABPs display no significant similarities.

***i*PABP binds to RNA homopolymers.** Sequence analysis of *i*PABP would predict that the RNA binding properties of *i*PABP are very similar to those of PABP. To test this directly, the properties of *i*PABP binding to RNA homopolymers were studied. *i*PABP translated in vitro in the presence of [^{35}S]methionine was incubated with Sepharose-bound poly(A) or poly(U); the results are shown in Fig. 3. Figure 3a shows binding in the presence of heparin as a nonspecific competitor, whereas for Fig. 3b yeast tRNA was used as a competitor. Binding was performed in KCl at concentrations varying from 0.1 to 1 M. *i*PABP efficiently binds both poly(A) and poly(U) in the presence of either competitor. As has previously been shown for PABP, binding to poly(U) is more efficient than binding to poly(A) at low KCl concentrations (6, 20). It is only when the KCl concentration is raised to 1 M that specific binding to poly(A) over poly(U) is seen. Similar findings have been reported for PABP (20). To confirm binding specificity, binding to poly(A)- and poly(U)-Sepharose was also performed in the presence of 10- and 100-fold excesses of soluble poly(A) and poly(U), respectively. As can be seen in Fig. 3c, the binding is specific. In addition, as has been shown for PABP, *i*PABP does not bind to poly(C). Nor can binding to poly(A)- and poly(U)-Sepharose be inhibited by soluble poly(C). Thus, *i*PABP has RNA binding properties very similar to those of PABP.

Subcellular localization of *i*PABP. We also sought to determine the subcellular localization of *i*PABP. PABP is a cytoplasmic protein (1, 2, 8), whereas the second PABP, PAB II, described for humans is nuclear (11). One report states that *S. cerevisiae* displays nuclear and cytoplasmic PABPs that are encoded by the same gene (16). To determine the cytoplasmic localization of *i*PABP, the full-length sequence was epitope tagged with a sequence specific for the influenza virus HA protein as outlined in Materials and Methods. The potential validity of this approach has been demonstrated by Görlach et al. (8) in experiments in which yeast PABP was transfected into COS cells and cytoplasmic localization was shown, confirming results obtained with a PABP-specific antibody. To confirm that the HA tag was expressed, the *i*PABP-HA clone was translated in vitro and immunoprecipitated with an anti-HA antibody. As can be seen in Fig. 4a, the in vitro-translated product of *i*PABP-HA is efficiently immunoprecipitated by the anti-HA antibody. Next the *i*PABP-HA clone was transfected into COS-7 cells. The cells were harvested 45 h following transfection, fixed, solubilized, and stained with the anti-HA

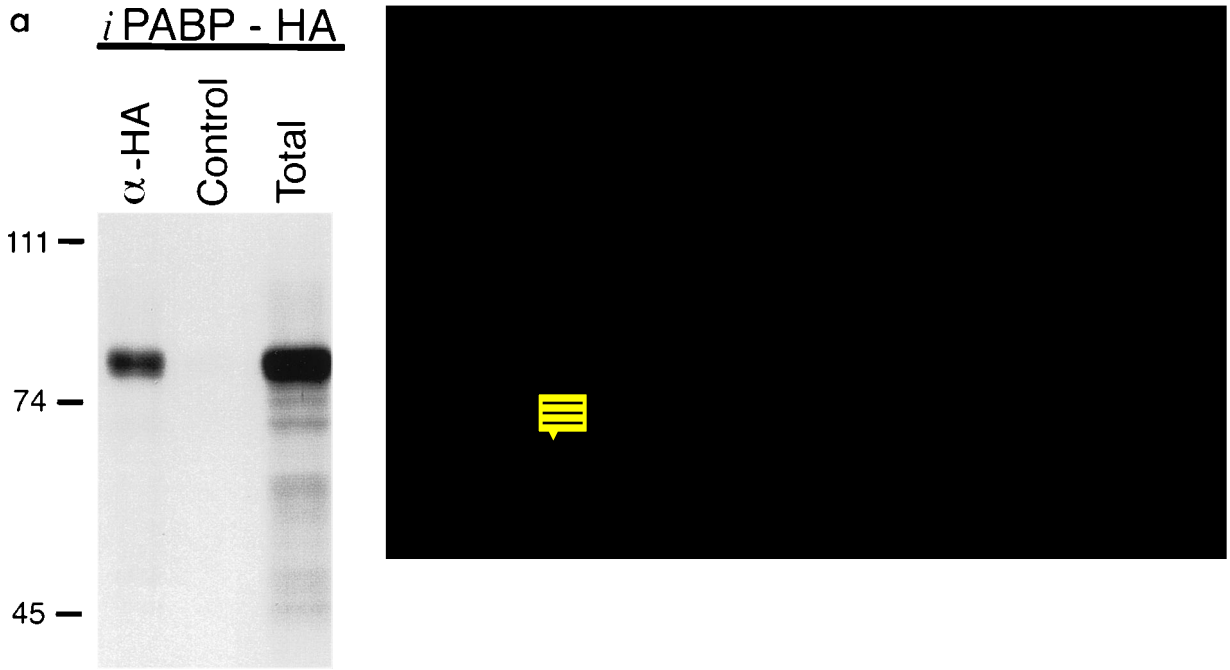

FIG. 4. *i*PABP localizes to the cytoplasm. (a) In vitro-translated *i*PABP tagged with a nonapeptide specific for influenza virus HA is immunoprecipitated by the anti-HA monoclonal antibody 12CA5 but not by a control antibody. The lane marked "Total" shows 10% of the volume of the in vitro-translated product used in the immunoprecipitations. (b) COS-7 cells transfected with the HA-tagged *i*PABP show cytoplasmic staining with the 12CA5 monoclonal antibody.

antibody. As can be seen in Fig. 4b, the staining is localized to the cytoplasm. No specific staining was seen either with a control antibody or with the anti-HA antibody on mock-transfected cells.

DISCUSSION

This paper describes the isolation and characterization of a novel cytoplasmic PABP, *i*PABP. *i*PABP is expressed at low levels in resting normal T cells but is induced within 6 h following activation. Although sequence analysis shows a high degree of homology to PABP and the properties of binding to ribohomopolymers are similar, there are regions of divergence which may imply a novel function for *i*PABP. For example, the fact that the induction of *i*PABP coincides with the induction of lymphokine mRNA in activated T cells suggests that perhaps *i*PABP is necessary for regulation of stability of labile mRNA species. This could result either from *i*PABP providing a generalized increase in poly(A)-binding activity or from a specific role of *i*PABP distinct from that of PABP.

The role of PABP in mRNA stability is still not fully delineated (for a review, see reference 15). In *S. cerevisiae* a mutation which depletes PABP results in accumulation of long poly(A) tails, indicating that in fact PABP targets poly(A) tail shortening (17). On the other hand, a function for PABP in mRNA stability has been suggested by Bernstein et al. on the basis of in vitro degradation experiments (4). In those experiments it was shown that when mammalian cell extracts were depleted of PABP, β-globin mRNA degraded rapidly. The stability of β-globin was restored when the reaction mixtures were reconstituted with purified PABP. These seemingly contradictory findings could be due to the fact that initial PABP-dependent poly(A) tail shortening is a step in the maturation of the final mRNA species and thus is independent of the process of deadenylation and mRNA degradation. As *i*PABP expression is induced 6 h following stimulation at the same time as peak lymphokine mRNA expression is seen in activated T cells, it is possible that *i*PABP displaces PABP binding to the poly(A) tail of lymphokine mRNAs. Once *i*PABP binds to the poly(A) tail, it could exert its specific function. Potentially, either *i*PABP could target labile lymphokine mRNA species for rapid degradation or *i*PABP could have a protective effect on such sequences. Delineation of the different roles of *i*PABP and PABP awaits the development of selective reagents to individually manipulate the levels of these two proteins in vitro and in vivo.

The selective overexpression of *i*PABP in skeletal and heart muscle tissue also remains to be explained. It is not immediately obvious why high levels of poly(A)-binding activity might be necessary in these tissues. However, there is a precedent for PABPs with distinct patterns of expression in plants. It has been shown that *Arabidopsis thaliana* has at least three distinct PABPs which show differential expression in distinct tissues (3). Furthermore, *X. laevis* also appears to have a family of PABPs (14). In conclusion, our data suggest that like the activity of many other critical control proteins, poly(A)-binding activity is provided by a family of related proteins with similar functions but distinct patterns of expression.

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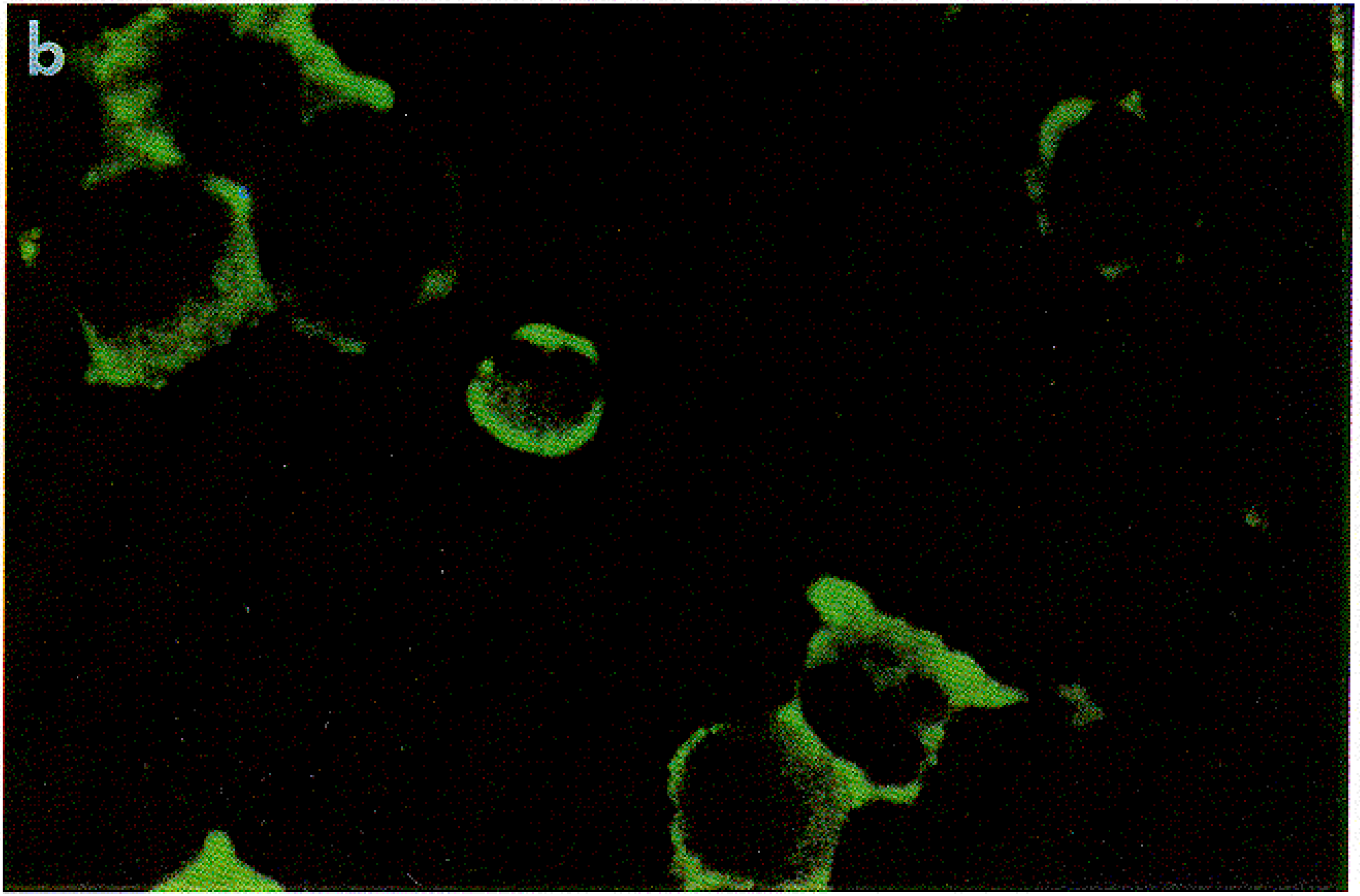
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