Deregulation of Translational Control of the 65-kDa Regulatory Subunit (PR65α) of Protein Phosphatase 2A Leads to Multinucleated Cells*

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Stefaan Wera†ś, Anne Fernandez‡i, Ned J. C. Lamb†, Patric Turowski‡, Maja Hemmings-Mieszczak†, Regina E. Mayer-Jaekel†, and Brian A. Hemmings†**

Efficient translation of the mRNA encoding the 65-kDa regulatory subunit (PR65α) of protein phosphatase 2A (PP2A) is prevented by an out of frame upstream AUG and a stable stem-loop structure (ΔG = −55.9 kcal/mol) in the 5′-untranslated region (5′-UTR). Deletion of the 5′-UTR allows efficient translation of the PR65α message in vitro and overexpression in COS-1 cells. Insertion of the 5′-UTR into the β-galactosidase leader sequence dramatically inhibits translation of the β-galactosidase message in vitro and in vivo, confirming that this sequence functions as a potent translation regulatory sequence. Cells transfected or microinjected with a PR65α expression vector lacking the 5′-UTR, express high levels of PR65α, accumulating in both nucleus and cytoplasm. PR65α overexpressing rat embryo fibroblasts (REF-52 cells) become multinucleated. These data and previous results (Mayer-Jaekel, R. E., Ohkura, H., Gomes, R., Sunkel, C. E., Baumgartner, S., Hemmings, B. A., and Glover, D. M. (1993) Cell 72, 621-633) suggest that PP2A participates in the regulation of both mitosis and cytokinesis.

Protein phosphorylation/dephosphorylation is a crucial intracellular control mechanism. The phosphorylation state of a protein is the net result of the antagonistic activities of protein kinases and protein phosphatases. Protein phosphatase 2A (PP2A),1 one of the major Ser/Thr protein phosphatases, consists of a catalytic (C) subunit of 36 kDa, complexed to a "constant" regulatory subunit of 65 kDa (PR65). This core heterodimer further associates with a single "variable" third subunit to form multimeric holoenzymes. To date several classes of variable subunits with molecular sizes of 55 kDa (PR55), 72 kDa (PR72), 130 kDa (PR130), 54 kDa or 74 kDa have been identified (for reviews, see Refs. 1-3). Given this complexity of regulatory subunits it can be anticipated that many different control mechanisms are likely to operate to coordinate the synthesis and assembly of the different holoenzymes. These controls will most probably act at both transcriptional and post-transcriptional levels.

It appears that the level of PP2A catalytic subunit is tightly regulated because attempts to overexpress this protein have been unsuccessful even though it is possible to overexpress the mRNA (4, 5). Recently, Wadzinski et al. (6) succeeded in expressing an amino-terminal tagged form of PP2A catalytic subunit, but this expression probably occurs at the expense of the genomically derived catalytic subunit. Taken together these results indicate that some form of translational or post-translational control mechanism operates to control the amount of the catalytic subunit. Whether such mechanisms operate to coordinate the translation of the PP2A regulatory subunits has not been investigated.

Translational control is often exerted at the level of translation initiation. According to the scanning hypothesis of mRNA translation initiation (reviewed in Ref. 7), the complex between initiator Met-tRNA and the 40 S ribosomal subunit, binds at or near the m7GpppG cap structure at the 5′ end of the mRNA and scans in the + direction until an AUG codon is reached. The 60 S ribosomal subunit is subsequently recruited to the complex and translation starts. These steps are catalyzed by eukaryotic initiation factors. Two elements within the 5′-UTR of a mRNA are known to inhibit translation initiation. First, an AUG located upstream of the authentic start codon can inhibit translation initiation. If this upstream AUG is followed by a stop codon, the ribosome can resume scanning and reinitiate at the correct AUG start codon. The longer the distance between this upstream stop codon and the authentic start codon, the smaller the inhibitory effect of the upstream AUG. If the stop codon is located within the coding sequence (thus down stream of the authentic start AUG), inhibitory effects of the upstream AUG are maximal, since only ribosomes that ignore the upstream AUG will initiate at the correct start codon (8). Second, stable secondary structures in the 5′-UTR interfere with scanning of the ribosome and lead to inhibition of translation. Kauffman (9) showed that secondary structures in the 5′-UTR must have a free energy of more than −20 kcal/mol to inhibit translation in yeast. Besides these inhibitory mechanisms, other mechanisms for control of translation initiation exist, such as phosphorylation/dephosphorylation of initiation factors.
(7), but it is assumed that these have a more general effect on translation and are less messenger-specific.

In the present work we investigated whether expression of the PR65 subunit is controlled at the translational level. This subunit is common to all forms of PP2A characterized and therefore forms the scaffold for the assembly of the different trimeric holoenzymes. The PR65 subunit is encoded by two genes (11), termed PR65α and PR65β. The α-isofrom seems to be the most abundant in all tissues and cells examined, except in Xenopus oocytes, where the β-isofrom predominates (12). We show that the 5′-UTR of PR65α acts as a translational repressor, due to an upstream AUG and to a stable stem-loop structure. We further show that release of this translational inhibition leads to overexpression of PR65α and that this apparently causes defects in cytokinesis resulting in multinucleated cells.

MATERIALS AND METHODS

DNA Constructs—Full-length cDNAs encoding the human C subunit α-isofrom (14), human PR65α (11), human PR55α (15), and porcine PR55β (accession number Z39433) were subcloned into pECE (16) for transient transfection experiments and into pBluescript (PR55α, PR55β) or pGEM (PR65α, Cα) for in vitro transcription. The 5′-UTR regions of Cα, PR55α, PR55β, and PR65α were deleted by introducing (using PCR) a unique Sall site upstream of the ATG start codon (at −28 bp for Cα, −24 bp for PR55α), or a Xba site at −18 for PR55β at −29 for PR65α. The resulting products were subcloned into either pBluescript (Cα, PR55α, PR55β) or pGEM (PR65α) for in vitro transcriptions, or into pECE for transfections. The 5′-UTR of PR65α was isolated by PCR using a full-length cDNA (11) as template and the following primers (HindII sites underlined): ACAAGGCGACCTGTTCACTACT (primer 1, sense), ATTAAAGCTTCATTGGGGAGTCA (primer 2, antisense), and ATAAGCTGGTCCGTCCTTT (primer 3, antisense), resulting in 138- and 62-bp fragments, respectively. In the 62-bp fragment, the upstream ATG was mutated to ATT by PCR with primer 4 (ATAAGCTTCATTGGGGAGTCA, sense) and primer 3 (antisense). To introduce an ATG to ATT mutation in the 138-bp fragment, we performed PCR with primer 1 and primer 5 (AGTACTCCCCAAATGGA, antisense, HindII site underlined), and introduced the product into the 138-bp 5′-UTR using an internal HindII site. The PCR products are schematically presented in Fig. 4A. The resulting PR65α 5′-UTR constructs were subcloned in the 5′-UTR of the pSV-β-galactosidase vector (Promega) using an HindII site. An in-frame stop codon is present 11 residues downstream of the HindII site. The orientation of the 5′-UTR inserts was verified by dideoxy sequencing. Part of the coding sequence of β-galactosidase was excised from the pSV-β-galactosidase vector by digestion with HindII and EcoRV (thus encoding a polypeptide with a predicted molecular size of 49.8 kDa), and subcloned into the corresponding pBluescript sites to create pBS.β-galactosidase. The PR65α 5′-UTR PCR fragments described above were then subcloned into the 5′-UTR using the HindII site of pBS.β-galactosidase.

In Vitro Transcription and Translation—pGEM or pBluescript constructs were linearized using a unique restriction site located close to the 3′ end of the cDNA inserts, and used as templates for in vitro transcription from the T3, T7, or SP6 promoters. The reactions were performed in the presence of mGpppG cap analogue (New England Biolabs) using the Riboprobe kit (Promega) according to the manufacturer's instructions. In vitro transcription was performed as described previously (21, 22). Reactions were stopped by the addition of 7 M urea, 1 mM EDTA, 150 mM NaCl, 0.1% SDS, and 5% β-mercaptoethanol. Extracts were boiled for 10 min and analyzed by 10% SDS-PAGE and fluorography.

Cell Culture, Transient Transfection, Analysis of Cell Extracts, Microinjection, and Immunofluorescence—COS-1 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum as described previously (20). Prior to microinjection, cells were subcultured onto acid-washed glass coverslips. Micromanipulation was performed with a Leitz micromanipulator, using 0.1-μl pipettes. Extracellular micropipettes were pulled on a linear horizontal puller. The pECE PR65α expression plasmids were injected at a concentration of 0.5 mg/ml in a buffer containing 100 mM potassium glutamate, 40 mM potassium citrate, 1 mM MgCl₂ (pH 7.2), and 1 mg/ml mouse IgG (to act as a marker for injected cells). Cells were incubated for a further 24–48 h and then processed for immunofluorescence. The results presented are the mean of five independent microinjection experiments involving 15–30 cells per experiment.

For immunofluorescence, cells were fixed in 3.7% formaldehyde and further treated as described previously (13, 20). To mark the injected cells, cells were stained for the presence of mouse IgG using a fluorescein-conjugated anti-mouse IgG antibody (Organon Teknika).

Analysis of RNA Structure—Probing of the secondary structure of RNA was performed as described previously (21, 22). The full-length PR65α cDNA clone in pGEM2 was digested with Rsal and used as template for in vitro transcription from the SP6 promoter. The resulting RNA (5 μg) was 5′ end labeled to a specific activity of 0.2 μCi/μg with polyethylene glycol and [32P]ATP (Amersham, specific activity 3000 Ci/mmol), gel purified, and dissolved in water (10 μg/25 μl). For ribonuclease T₁ cleavage, RNA (1 μl) was mixed with 3 μl of denaturing buffer (7 M urea, 1 mM EDTA, 15 mM NaCl, 40 mM sodium acetate (pH 5), 0.4% xylene cyanol, 0.08% bromphenol blue), boiled for 1 min and cooled on ice. Subsequently 1 μl of ribonuclease T₁ (100 milliunits, Pharmacia) was added and the mixture incubated for 15 min at 55 °C. The reaction was terminated by the addition of 5 μl of stop solution (10 mM EDTA pH 7.4, 0.05% xylene cyanol, 0.01% bromphenol blue) and frozen on dry ice.

For renaturation, 15 μl of RNA was mixed with 45 μl of renaturation buffer (15 mM Tris-Cl (pH 7), 80 mM NaCl, 10 mM MgCl₂, and 2 μg/ml RNaseA), heated to 65 °C and allowed to cool slowly to room temperature. The mixture (3 μl) was incubated for 15 min at room temperature with distilled water (control) or with the indicated concentrations of enzyme (see below). Reactions were stopped by the addition of 7 μl of stop solution, boiled for 20 s at 80 °C, and frozen on dry ice. Reaction products were analyzed on a denaturing 6% polyacrylamide sequencing gel. Concentrations of the enzymes were as follows: ribonuclease A, 0.2 milliunits per reaction; nucleotide 5′-phosphatase S₁ (Pharmacia), 2.5, 5, 10 units per reaction; ribonuclease V₁ (Pharmacia), 2, 4, 8 milliunits per reaction; ribonuclease T₁, 18, 37, 75 milliunits per reaction. Lead acetate (pH 5.5) was used at a final concentration of 0.1, 0.25, or 0.5 mM.

RESULTS

Upstream AUGs Are a Common Element in the mRNAs of Various PP2A Subunits—Most PP2A subunit mRNAs carry one or more upstream AUG codons in their 5′-UTR (Fig. 1). This is the case for Cβ, PR65α, PR55β, PR55γ, PR72, and PR130 subunits. In the case of the Cβ subunit, the AUG is located just upstream of the major transcription initiation site (23), thus most Cβ messages will not contain this codon. To function as a start codon, an AUG should be located in the

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appropriate sequence context (24). Most efficient AUGs have a purine (most often A) in position 2 and a G in position 1 (relative to the A of AUG, which is +1). Upstream AUG codons are rather rare in the messengers of eukaryotic proteins. They occur in only 10% of all mRNAs, but in 70% of the mRNAs that encode proto-oncogenes (24). In this respect it is thought that they serve to regulate protein levels by down-regulating translation.

We deleted the 5′-UTR of Ca, PR55a, PR55b, and PR65a and compared in vitro translation efficiency of the full-length and 5′-UTR deleted mRNAs. Deletion of the 5′-UTR had no significant effect on the in vitro translation efficiency of Ca and PR55a messages because they lack upstream AUGs. In contrast, the translation efficiency increased dramatically after deletion of the 5′-UTR of PR65a (see below) and PR55b. These messages carry one and two upstream AUGs in their 5′-UTR, respectively. These data prompted us to further investigate the translational regulation of the PR65a subunit mRNA.

Analysis of the Secondary Structure of the PR65a 5′-UTR—Besides an upstream AUG, the 5′-UTR of the PR65a mRNA has another potential inhibitory element. As predicted by an RNA-fold program (25, 26), the 5′-UTR can form a stable (ΔG = −55.9 kcal/mol) stem-loop structure (Fig. 2A) with the potential to interfere with mRNA scanning by the ribosomal machinery. The predicted structure was obtained by analyzing the sequence spanning nucleotides 2140 to 11, covering the 5′-UTR of the PR65a messenger, using the RNA-fold program. If the first 36 nucleotides of the coding sequence are also included, the predicted structure of the 5′-UTR remains unchanged. This indicates that the predicted stem-loop might exist as a stable entity within the messenger. In addition to the stem-loop structure, we also found that nucleotides 2134 to 240 within the 5′-UTR of PR65a have the potential to base pair with nucleotides 517–612 within the coding sequence (see “Discussion”).

We checked whether a stem-loop structure occurred in the 5′-UTR of the Drosophila PR65a mRNA (27). Residues −194 to −47 have indeed the potential to fold as a stem-loop, but this structure is less stable (ΔG = −27.1 kcal/mol). The homology of this region with the stem-loop of human PR65a is 37.6% (gap...
COS-1 cells were transiently transfected with either the full-length PR65α, carbonic anhydrase (31 kDa), trypsin inhibitor (21 kDa), phosphorylase (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and comprised the 5′-UTR plus 31 nucleotides of the coding sequence. The in vitro transcribed RNA was 175 nucleotides long and comprised the 5′-UTR plus 31 nucleotides of the coding sequence. By comparing the effect of ribonuclease T1, (which cleaves single, but not double, stranded RNA at G residues) on denatured and renatured RNA (Fig. 2), we were able to elucidate the secondary structure of the 5′-UTR in solution. A stretch of 5 G residues starting at nucleotide −60 allowed us to locate the observed structures in the sequence. The structure was then further analyzed (not shown) with reagents that specifically cleave single-stranded RNA (lead acetate, ribonuclease A, and S1 nuclease) or double-stranded RNA (ribonuclease V1). Our analysis confirmed the predicted loop at residues −51 to −45. The predicted loop at position −76 to −71 was not detected, but an additional loop at position −64 to −59 was observed. Surprisingly, in the observed structure the upstream AUG (residue −62 to −60) is exposed in this loop. We used the RNA-fold program to calculate the stability ($ΔG = −39.0 \text{kcal/mol}$) of this observed structure (using the “prevent” command to insert a loop between residues −64 and −59). Taken together these results demonstrate that a stem-loop structure is indeed formed by the 5′-UTR of PR65α, albeit somewhat less stable than predicted, but within the range that is reported to inhibit mRNA scanning (9).

Deletion of the 5′-UTR of PR65α Allows Efficient Translation in Vitro and Overexpression in COS-1 Cells—The cDNA encoding PR65α was used as a template for in vitro transcription and subsequent translation in rabbit reticulocyte lysates. No protein product was observed with the full-length mRNA as template (Fig. 3A). However, when the 5′-UTR was deleted at nucleotide −25 relative to the start ATG codon, and used as template for in vitro transcription, the resulting mRNA was efficiently translated into a protein product of about 65 kDa. This translation was not observed with the antisense construct.

The full-length and 5′-UTR deleted cDNAs were subcloned in a mammalian expression vector (pECE) and used to transiently transfect COS-1 cells. Whereas transient transcription with both the full-length and the 5′-UTR deleted construct resulted in efficient overexpression of the PR65α message (analyzed by Northern blot, data not shown), only the 5′-UTR deleted construct resulted in efficient overexpression of PR65α protein as judged by immunoblotting (Fig. 3B). Overexpression was about 20-fold as judged from scanning of different exposures of the Western blot. (The data in Fig. 3B show a longer exposure which underestimates the level of overexpression relative to the control.) A small increase (approximately 2-fold) in the amount of PR65α was also observed with the full-length construct.

Fig. 3. Deletion of the 5′-UTR of PR65α allows efficient in vitro translation and overexpression in COS-1 cells. A, in vitro translation of 1 μg of either full-length (FL) or 5′-UTR deleted (ΔS) mRNA of PR65α in either the sense (S) or antisense (AS) orientation. A negative control (NC) lacking RNA was included. Protein products were resolved by SDS-PAGE and autoradiographed. Molecular mass markers were phosphorylase (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21 kDa). B, COS-1 cells were transiently transfected with either the full-length (FL) or the 5′-UTR deleted (ΔS) PR65α cDNAs in the pECE expression vector. Control transfections (C) were performed with the pECE vector without insert. 72 h after transfection total cell extracts were analyzed by SDS-PAGE and Western blotting with an affinity purified antibody against PR65α (Ab65α) and detected using enhanced chemiluminescence detection. Molecular weight markers are as in A. C-F, COS-1 cells were transfected with PR65α expression constructs as described under “Materials and Methods.” 48 h later they were fixed and immunostained with an affinity purified antibody against PR65α (Ab65α). Immunofluorescent micrographs of representative cells are shown after mock transfection (panel C), after transfection with a full-length (panel D), and with a 5′-UTR deleted PR65α expression construct (panels E and F). The images in panels C, D, and E were acquired at the same laser power, pinhole size, and photomultiplier sensitivity settings and are therefore directly comparable. Panel F shows the same field to that seen in panel E, but was collected with CLSM settings corresponding to a 10-fold reduced exposure. Cells in panels E and F with lower immunostaining (i.e. not overexpressing the PR65α) are indicated by arrowheads. Bar, 5 μm.
In a similar experiment COS-1 cells were transiently transfected with PR65α expression constructs with or without the 5′-UTR and subsequently analyzed by indirect immunofluorescence with PR65 specific antibodies as described under “Materials and Methods.” When cells transfected with the 5′-UTR deleted PR65α were analyzed about one out of 5 cells displayed an intense staining for PR65 (Fig. 3, E and F) which was approximately 10 times stronger than in the surrounding cells. This low staining of the surrounding cells corresponded to that observed in non-transfected (not shown) and mock transfected cells (Fig. 3C). Therefore we concluded that the intensely stained cells actively overexpressed PR65. COS-1 cells transfected with the full-length PR65α construct were indistinguishable from untransfected cells (Fig. 3D), again confirming that the 5′-UTR allowed only very low levels of expression. The observed immunofluorescence signal was specific for PR65α as it could be competed with the appropriate antigen (data not shown; see also Ref. 13). In addition, we obtained identical results with an antisera raised against the recombinant protein (Ab65 recom). The distribution of the overexpressed PR65 was found to change slightly when different time points after transfection were analyzed. In about 30% of the transfected cells (harvested 48 h after transfection), the overexpressed PR65α was almost exclusively nuclear, whereas in about 70% of the transfected cells, PR65α was present in both nucleus and cytoplasm. The reasons for this almost exclusive nuclear staining remain to be further investigated. By 72 h most if not all transfected cells stained homogeneously for PR65α in the cytoplasm and the nuclear compartment. This distribution corresponds to that found for endogenous PR65 (Fig. 3C and Ref. 13).

The 5′-UTR of PR65α Is a Translational Inhibitor—To directly demonstrate the importance of the 5′-UTR of PR65α, we fused several different constructs of the 5′-UTR (Fig. 4A) to the coding sequence of a reporter protein (a fragment of β-galactosidase) and compared the in vitro rate of translation of the chimeric messages. Translation of reporter mRNA was dramatically inhibited (89% compared to the control, as judged by PhosphorImager quantitation) if the 5′-UTR of PR65α was present as a leader sequence (Fig. 4B). Truncation of the 5′-UTR at nucleotide −63, results in a construct that is devoid of the stem-loop structure but still contains the upstream AUG and inhibits translation of the reporter by 72%. This inhibition is mainly due to the upstream AUG, since mutation of this AUG to AUU partially relieves the inhibition (30% inhibition compared to the control). To further investigate the importance of the secondary structure, we made a construct of the entire 5′-UTR in which the upstream AUG was mutated to AUU. Since we have shown above that the upstream AUG is present in a loop structure, one might expect that this mutation would

![Fig. 4. The 5′-UTR of PR65α is a translational inhibitor.](image)

A, schematic presentation of the various constructs of the 5′-UTR of PR65α. The constructs correspond to either the entire 5′-UTR (stem/loop with AUG), the entire 5′-UTR containing a point mutation in the upstream AUG (stem/loop with AUU), the 5′-UTR truncated at residue −63 and thus devoid of secondary structure (AUG), or the same construct containing a point mutation in the upstream AUG (AUU). The stability of each construct was calculated with the RNA-fold program (25, 26). B, in vitro translation of chimeric mRNA encoding a fragment of β-galactosidase fused to various constructs of the 5′-UTR of PR65α (see panel A). As a negative control no RNA was added. C, COS-1 cells were transiently transfected with either pSV-β-galactosidase or with the same vector containing various constructs of the 5′-UTR of PR65α (see panel A), inserted in the β-galactosidase leader sequence. Cells transfected with the pCE vector without insert were used as negative control. 48 h after transfection, cell extracts were assayed for β-galactosidase activity. The activity is expressed per microgram of protein in the cell extract. The figure shows means ± S.E. (n = 3), as indicated by the error bars. Transfection efficiency was tested by cotransfection of a pSV-chloramphenicol acetyltransferase plasmid. Variations in chloramphenicol acetyltransferase activity were less than 10%.
have no influence on the secondary structure. When fused to the reporter message this construct inhibited translation by 55%.

The same set of PR65\textsubscript{a} 5′-UTR sequences (see Fig. 4A) were ligated into the 5′-UTR of the β-galactosidase gene (in the pSV vector) and used to transiently transfect COS-1 cells (Fig. 4C). We first demonstrated by Northern blotting that insertion of the 5′-UTR in the expression plasmid did not influence translational efficiency (not shown). The 5′-UTR of PR65\textsubscript{a} inhibits translation of the β-galactosidase message dramatically (79 ± 4%). The secondary structure alone is responsible for a moderate inhibition of translation (17 ± 5%), whereas the AUG alone inhibits translation by 57% (±5%). In contrast to the in vitro data, mutation of the upstream AUG and disruption of the secondary structure, resulted in a slight stimulation of translation by 15% (±7%).

Taken together, the results demonstrate that the 5′-UTR of PR65\textsubscript{a} is a strong translational inhibitor, mainly due to the presence of an upstream AUG, and to a lesser (but significant) extent to the presence of a stem-loop structure. Apparently, the inhibitory effects of secondary structure and upstream AUG are additive.

Overexpression of PR65\textsubscript{a} Leads to Multinucleated Cells—The 5′-UTR of PR65\textsubscript{a} controls translation and prevents efficient overexpression of the protein. Deletion of the 5′-UTR overcomes this inhibition and allows overexpression of PR65\textsubscript{a}. The PP2A activity in total cell extracts (as measured with a peptide substrate, and thus reflecting the amount of catalytic subunit) is unchanged in the overexpressing cells (61 milliunits/mg compared to 58 milliunits/mg in mock-transfected cells). We used the DEAE-dextran method followed by a dimethyl sulfoxide shock treatment to ensure high transfection efficiency, as reported by Sussman and Milman (29). We found that PR65\textsubscript{a} overexpression caused no differences in the elution pattern of PP2A holoenzymes, when total cell extracts are chromatographed on a Mono-Q ion exchange column (not shown). The overexpressed PR65\textsubscript{a} elutes at around 230 mM NaCl, which is exactly the position where recombinant PR65\textsubscript{a} elutes,\textsuperscript{3} indicating that at least the majority of the overexpressed protein is apparently free and not sequestered to form PP2A holoenzymes.

COS-1 cells overexpressing PR65\textsubscript{a} show no apparent phenotype. This might be explained by the observation that the cells are essentially non-dividing in the 48–72-h period after DEAE-dextran transfection. (The presence of more than one nucleus or fragmented nuclei in the COS-1 cell transfection experiment did not correlate with PR65\textsubscript{a} overexpression. Indeed the non-transfected, mock transfected, and cells transfected with the full-length PR65\textsubscript{a} expression construct (Fig. 3D) showed the same proportion of abnormal nuclei compared to COS-1 cells overexpressing the PR65\textsubscript{a}).

To investigate possible effects of PR65\textsubscript{a} overexpression on cell division, we microinjected REF-52 cells with PR65\textsubscript{a} expression constructs. When cells were microinjected with a 5′-UTR deleted PR65\textsubscript{a} expression construct this resulted in a dramatic increase of PR65\textsubscript{a} protein detected in both the nucleus and the cytoplasm (data not shown). As determined by indirect immunofluorescence and confocal laser scanning microscopy this increase of PR65\textsubscript{a} protein was 10–20-fold compared to control uninjected cells. In contrast, microinjection of the full-length PR65\textsubscript{a} expression construct did not alter PR65\textsubscript{a} protein levels (data not shown). These results agree with those obtained using COS-1 cells (Fig. 3). Analysis of PR65\textsubscript{a} overexpressing cells 24 h after microinjection revealed major phenotypic changes during mitosis. The most marked effect observed was the formation of cells containing multiple nuclei (Fig. 5, A and B). Whereas only 3% of the non-injected control cells were binucleated, 65% of the PR65\textsubscript{a} overexpressing cells contained two or more nuclei. The remaining 35% of the overexpressing cells did not divide in the 24 h after microinjection. REF-52 cells microinjected with a full-length PR65\textsubscript{a} expression plasmid did not show any phenotypic changes (Fig. 5, C and D). These results indicate that cytokinesis is blocked in PR65\textsubscript{a} overexpressing cells without apparently affecting nuclear division.

**DISCUSSION**

In the present work we show that deregulation of the translational control of PR65\textsubscript{a} mRNA leads to overexpression of PR65\textsubscript{a}, which apparently disrupts cytokinesis and leads to binucleated cells. This translational control is mediated by the 5′-UTR of PR65\textsubscript{a}, which is a strong translational inhibitor, due to the presence of an upstream AUG and a stable stem-loop structure.

Translation of mRNAs with stable secondary structures in the 5′-UTR may be highly dependent on the helicase activities of eIF-4B and eIF-4F. The latter factor is a complex between eIF-4A, eIF-4E, and p220 (7). Phosphorylation of eIF-4E and eIF-4B and eIF-4F. The latter factor is a complex between eIF-4A, eIF-4E, and p220 (7). Phosphorylation of eIF-4E may lead to a more efficient unwinding of 5′-UTRs, and thereby up-regulate the translation of certain mRNAs. Furthermore, an eIF-4E interacting protein (4E-BP1...
Phosphorylation of 4E-BP1 in response to insulin causes its dissociation from eIF-4E, and relieves the inhibition.

In the case of PR65α, however, unwinding of the 5′-UTR may not be sufficient, since the upstream AUG alone inhibits translation (Fig. 4). The use of an alternative promoter, or alternative splicing, might produce messages that lack the inhibitory 5′-UTR and would therefore be efficiently translated (34). However, none of the PR65α cDNA clones isolated so far contain different 5′-UTR sequences, in fact the majority of the isolated cDNAs starts just downstream of the start ATG. This probably reflects the inability of reverse transcriptase to proceed through a stable stem-loop structure.

In addition to a stem-loop structure, nucleotides 134 to 40 of the 5′-UTR of PR65α have the potential to base pair with nucleotides 517–612 in the coding sequence. This potential base pairing and the stem-loop structure in the 5′-UTR are mutually exclusive. The ability of the 5′-UTR to base pair with an internal coding sequence is not a unique characteristic of PR65α, but is also found in the c-myc proto-oncogene transcripts. Saito et al. (35) suggest that a translocated c-myc gene, which lacks the exon encoding the 5′-UTR, is no longer under translational control and becomes oncogenic. Another example where abrogation of translation control leads to proto-oncogene activation is the lymphocyte-specific tyrosine kinase lck (36). The 5′-UTR of lck contains three upstream AUGs. Substitution of the 5′-UTR for retroviral sequences results in malignant transformation, as is observed in some murine lymphomas (36).

Another characteristic of certain proto-oncogenes transcripts, such as the c-myc transcript, is the presence of potential RNA-desaturating sequences in the 3′-UTR (37). The AUUUA sequence, which is thought to mediate rapid mRNA turnover, is also found in most PP2A subunit messages. Five copies of this motif are present in the 3′-UTR of Cα, six in Cβ, one in PR65, four in PR55α, seven in PR55β, one in PR72, and four in PR130. The striking exception is PR65α itself, which seems to be devoid of this motif. The absence of rapid turnover signals might explain why translation of the PR65α signal is tightly controlled. It therefore appears that the cell controls PP2A subunit transcripts in much the same way as mRNAs that encode proteins involved in growth control, such as proto-oncogenes.

Overexpression of PR65α leads to defects in cytokinesis and multinucleated cells. Cytokinesis is brought about by the contraction of actin-myosin fibers in the cleavage furrow. Injection of myosin antibodies specifically blocks cytokinesis, without affecting chromosome movement, and ultimately leads to multinucleated cells (38). Although speculative at the moment, one possible mechanism to explain our results is that overexpression of PR65α results in inefficient dephosphorylation and activation of myosin light chain kinase, a presumed PP2A substrate (1), resulting in hypophosphorylated myosin light chains which in turn could inhibit the contractile force in the cleavage furrow. The question that emerges from this study is, how does the overexpression of PR65α disrupt the normal regulation of PP2A? As judged from its elution on a Mono-Q column most of the overexpressed PR65α seems to be present as a free protein, i.e. not complexed with the catalytic or other regulatory subunits. Furthermore, neither the elution profile, nor the amount of the PP2A trimer (C/PP65/PR55) is influenced by overexpression of PR65α in COS-1 cells. Also the total PP2A activity (as measured with a peptide substrate) remains unchanged. Two possibilities exist to explain the results described in this article. First, the overexpression of PR65α could disrupt a PP2A holoenzyme which plays a specific role in cytokinesis. PR65α could do so by sequestering the catalytic or variable subunit. Although we do not detect such dimers in a Mono-Q profile, we cannot exclude that a low abundance PP2A trimer is dissociated upon PR65α overexpression. Second, the excess PR65α could act as an inhibitor of the catalytic subunit of PP2A, as predicted from in vitro data (39). In this model it is necessary to suggest that the catalytic subunit (or the complex of the catalytic subunit with a variable subunit) is released from the PR65 subunit at a certain point in the cell cycle to dephosphorylate specific targets. Free PR65α in overexpressing cells would in this model immediately capture the released catalytic subunit and suppress its activity. We have recently obtained evidence that indicates that PP2A undergoes subunit rearrangements (12, 13, 40). Interestingly, a very similar phenotype (defects in cytokinesis and multinucleated cells) is observed in budding yeast when the TPD3 gene, encoding the PR65 homologue, is mutated (41).

In summary, the data presented in this paper, and in other recent publications (19, 40–42) demonstrate the importance of PP2A in cell cycle regulation. In this context it should be pointed out that substrates of the cyclin-dependent protein kinase family need to be dephosphorylated prior to the subsequent round of cell division. This dephosphorylation is most likely not a stoichiastic process, but stringently regulated by the action of PP2A and other protein phosphatases.

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