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ARP2 a novel protein involved in apoptosis of LNCaP cells shares a high degree homology with splicing factor Prp8

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Abstract

The mechanism of apoptosis has been recognized as an important event in processes such as cellular development and homeostasis, as well as degenerative conditions like cancer. Prostate cancer during its advanced stages develops androgen independent cells that ultimately overgrow and promote metastatic events. Our group employing androgen independent LNCaP cells have previously proposed, based on electrophysiological findings, that apoptosis induced cells overexpress a cell death calcium channel-like molecule. Here we report the cloning and expression in *Xenopus laevis* oocytes of apoptosis regulated protein 2 (ARP2), a protein overexpressed in apoptosis induced LNCaP cells capable to induce calcium inward currents and apoptosis typical morphology changes in oocytes injected with *arp*2 mRNA. Our results also indicate that clone *arp*2 cDNA (1.3Kb) shares a 99% homology with a small fragment that corresponds to 18% of the complete sequence of Prp8 cDNA (7.0 Kb), a molecule that codifies for an important protein in the assembly of the spliceosome. We propose that protein ARP2 as a fragment of protein Prp8, corresponds to a molecule with a new function in apoptosis related phenomena. (Mol Cell Biochem **269:** 189–201, 2005)

Key words: cancer, apoptosis, membrane, channel, calcium, spliceosome

Introduction

Programmed cell death or apoptosis [1] executes an important function in processes such as embryonic development, cell homeostasis, and in diseases such as cancer [2]. The mechanism of apoptosis originates from activation of a suicide program proper to each cell [3]. The process of programmed cell death is divided into several phases [4], the earliest one associated with the stimulus that triggers the apoptotic response. The second phase is related to the mechanisms of signal transduction, while the third phase corresponds to the effectors mechanisms, in which the apoptotic machinery activates a series of caspases [5]. The fourth phase of apoptotic cell death involves chromatin condensation, DNA degradation and eventually cell death [4].

Tumor cells possess the inability to promote apoptosis in response to several physiological stimuli [6]. Nonetheless, due to the fact that calcium is an intracellular regulator [7], its increased and sustained levels can activate a series of cytotoxic mechanisms associated with apoptosis in various cell types [7]. Since apoptosis has been proposed as a control mechanism of tumor growth, the modulation of its activation mechanisms has been considered as an adequate way to find a novel pathway to study and counteract carcinogenesis [8–10]. In human prostate cancer [11], elimination of androgens generates increased intracellular levels of Ca^{2+} and cells die via apoptosis [11]. However, in advanced stages, the disease develops a group of androgen-independent cells [12] unresponsive to chemotherapeutic agents. Our group, demonstrated the activation of a

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Ca^{2+}-permeable, non-selective cationic channel or channel-associated molecule in androgen-independent lymphoid nodule prostate cancer cells (LNCaP), by two different inductors of apoptosis: ionomycin and elimination of serum from cell culture media [13]. Several reports have previously shown that other types of ionic channels might be also directly or indirectly involved with the process of apoptosis [13–15]. Either the activation or *de novo* synthesis of these channels during the initiation of an apoptotic event has been proposed to be of central importance when defining the survival rates of a cancer cell. In this study using androgen-independent LNCaP cells, we carried out an analysis of expression of different Ca^{2+}-permeable membrane channels, and based on this analysis cloned two cDNAs of molecules synthesized during an apoptosis-induced event such as serum elimination during cell culture. Expression of one of these clones in *Xenopus laevis* oocytes suggests that ARP2 (for apoptosis regulated protein 2) a TRP channel-like protein related in structure to splicing factor Prp8 [16], increases the influx of calcium through the plasma membrane and induces the process of apoptosis in the oocyte itself.

Materials and methods

Materials

The human lymphoid nodule prostate cancer cell line LNCaP (hormone-independent) [17] was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, U.S.A.). RPMI 1640 medium containing glutamine and 0.2% (w/v) sodium bicarbonate, and the bovine fetal serum (BFS) were obtained from GIBCO BRL (Gaithersburg, MD, U.S.A.). Disposable materials for cell culture were obtained from Nunc (Roskilade, Denmark). Acrylamide and nitrocellulose membranes were obtained from Bio-Rad Laboratories (Hercules, CA, U.S.A.). Bis-Acrylamide, NP40 detergent, ethidium bromide, aprotinin, phenylmethylsulphonyl fluoride (PMSF), benzamidine, dithiothreitol (DTT), diethyl pyrocarbonate (DEPC), thapsigargin and acridine orange were obtained from Sigma (St. Louis, MO, U.S.A.). Supersignal chemiluminescent substrate and bicinchoninic acid (BCA) protein reagent were obtained from Pierce (Rockford, IL, U.S.A.). OMAT auto X-ray material was purchased from Kodak (Rochester, NY U.S.A.). Guanidine thiocyanate from Fluka Chemie (Switzerland). Additional materials included RNA PCR Core Kit, r *Tth* DNA polymerase XL from Roche Molecular Systems (Branchburg, NJ, U.S.A.), and deoxyribonucleotide dNTPs from Boehringer Mannheim (GmbH, Germany). Oligonucleotides utilized were synthesized at the Synthesis Unit, Institute of Biotechnology (UNAM). In addition, we employed the T7 mMESSAGE mMACHINE High Yield Capped RNA Transcription Kit and EF-1α from Ambion (Austin, TX, U.S.A.).

Cell culture

LNCaP cells were cultured in RPMI 1640 medium with glutamine and 0.2% (w/v) sodium bicarbonate, supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin. These cells reached high confluence after 48 h of incubation. Androgen-independent LNCaP cells were induced to apoptosis by serum elimination from the culture medium [13, 18].

Flow cytometry

A FACScan flow cytometry apparatus (Becton Dickinson) was used. We employed 1 000 000 cells either induced to apoptosis by serum elimination from the culture media or control cells (induction times were 16 and 72 h). We considered the following parameters: physical changes such as increased granularity in cells, cell shrinkage, and cell fragmentation [19]. The control used in these assays was serum-maintained cells.

Cell viability experiments

Cell viability experiments were carried out to follow exclusion of trypan blue by control cells and cells induced to apoptosis by serum elimination during cell culture for 16, 24, 48, 72, 96, 120, and 144 h.

DNA fragmentation

LNCaP cells at high confluence were induced to apoptosis by serum elimination during 16, 24, 72 h. These cells were washed with versene and lysed in 0.5 mL of lysis buffer (140 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris/HCl, pH 7.4, 0.5% (w/v) NP40 detergent) for 30 min at 4 °C. Nuclei were obtained in the pellets after centrifugation at 10 000 g for 10 min at 4 °C and resuspended in hypertonic solution (350 mM NaCl, 1.5 mM $MgCl_2$, 1 mM DTT, 10 mM Tris/HCl, pH 7.4) for 30 min at 4 °C. DNA extraction was carried out with phenol-chloroform-iso-amyl alcohol and precipitated with 7.5 M ammonium acetate and absolute ethanol. Washed in 70% (v/v) ethanol and separated by electrophoresis in 2% (w/v) agarose gels using ethidium bromide.

Western blots

A group of membrane proteins involved in apoptosis and related with the influx of Ca^{2+} were selected. The Swiss Prot

Table 1. Selected peptides for polyclonal antibody production

Epitope	Molecular weight (kDa)	No. of residues	Dilutions	Reference
Purinergic receptor P2X1	44.980	380–399	1:25	[22]
Purinergic receptor P2X7	68.539	576–595	1:100	[23]
Transient receptor potential TRPC1	92.376	774–793	1:75	[24]
Proapoptotic protein Bax	21.184	80–98	1:100	Commercial

data base [20] and the FASTA [21] programs were utilized for the selection of peptides from these proteins. The selected peptides were employed to produce the polyclonal antibodies shown in Table 1 [22–24]. Peptide synthesis and polyclonal antibody production in rabbits were carried out by Alpha Diagnostic International (San Antonio, TX). Anti-bax was obtained as a commercial antibody from Santa Cruz Biotechnology (Santa Cruz, California). Dr. Manuel Hernández, CINVESTAV, México provided the anti-actin antibody employed in this study. After being incubated for 16 h in medium with or without serum, cells were harvested in a lysis buffer (100 mM NaCl, 10 mM Tris/HCl, pH 8.0, 1 mM EDTA, pH 8.0, 1 μg/mL aprotinin, 100 μg/mL phenylmethylsulfonyl fluoride [PMSF], 1 μg/mL benzamidine) and sonicated. Protein quantification was carried out using bicinchoninic acid (BCA) and concentrations of 100 μg used throughout. 12.5% (w/v) polyacrylamide gels were utilized and transferred to nitrocellulose. Membranes were saturated with Tris-buffered saline (TBS), pH 7.6 (20 mM Tris/HCl, pH 7.4, 100 mM NaCl, 0.05% (v/v) Tween 20, 2.5% (w/v) skimmed milk) and incubated with primary antibodies (Table 1). The secondary antibody used was a goat-produced anti-rabbit antibody labelled with peroxidase at a dilution of 1:5000. In the case of the experiments where actin was used as a loading control, we used as secondary antibody an anti-mouse antibody produced in goat in a dilution of 1:5000. The reaction was visualized by means of the chemiluminescent substrate super signal system.

arp1 and arp2 cloning

Templates for PCR reactions consisted of a mixture of cDNAs of LNCaP cells maintained with or without serum during 16 h. Total RNA from LNCaP cells maintained with or without serum during 16 h was extracted using the guanidinium/phenol/chloroform method [25], and used to synthesize cDNA with a MuLV reverse transcription system. Synthesized cDNAs were amplified [26] with the following sense primer 5′-TGACAGTGATGCGGGAGAAGG-3′ and an antisense degenerated primer that corresponds to a conserved region of the Trp family of proteins with sequence EWKFAR [24, 27] 5′TGY-TCK-MGC-AAA-YTT-CCA-YTC 3′. These sequences were selected with the Mac Vector Analysis 6.5.3. [28]. PCR was performed 30 cycles at 94 °C using 45 s/cycle, 60 °C for 45 s, and 72 °C for 2 min and one cycle at 72 °C for 10 min. PCR products were visualized in 1% (w/v) agarose gels and purified with the Concert gel extraction system from Gibco BRL (Gaithersburg Maryland). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used as an internal standard, where sense oligonucleotide 5′-TGAAGGTCGGAGTCAACGGATTTGGT3′ (position 71–95) and antisense oligonucleotide 5′-CATGTGGGCCATG-AGGTCCACCAC-3′ were chosen. These products were cloned in TOPO 4 vector (zero blunt TOPO PCR cloning kit for sequencing invitrogen, life technologies) for sequence determination. We also carried out the cloning of these products in the pxenex1 vector kindly provided by Dr. Michael Jeziorski (Instituto de Neurobiología, Universidad Nacional Autónoma de México) in order to be able to add posttranscriptional modifications such as the tail of poly A, designed to obtain optimal expression when using *Xenopus laevis* oocytes.

Expression experiments

Transient expressions were carried out using *Xenopus laevis* oocytes [29]. Techniques for injection of mRNA for electrophysiological recordings from oocytes have been previously described [30–32]. Briefly, ovaries were dissected from *Xenopus laevis* frogs (obtained from Ann Arbor Michigan) by ventral incision in donors anesthetized by hypothermia. The oocytes (stage VI) were removed and maintained in Normal Barth's solution (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.33 mM $CaNO_3$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 5 mM HEPES, 70 μg/mL gentamicine, pH 7.4 with NaOH). Oocytes were treated with collagenase 0.5 mg/mL in a normal Ringer solution (115 mM NaCl, 1 mM KCl, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.0 with NaOH) for 45 min, and all follicular layers were removed using forceps. cRNAs were prepared using the high production T7 RNA transcription mMESSAGE mMACHINE system, and dissolved in H_2O (c.a. 1 ng/nL). Oocytes injected with 50 nL were maintained in Barth's medium at room temperature for 36 h. Ion currents were registered and morphological changes photographed. After this time had elapsed, oocytes were separated in three groups and incubated in: (1) Normal Barth's medium, (2) Barths Ca^{2+}-free solution (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 4.2 mM $MgCl_2$, 5 mM HEPES, 70 μg/mL gentamicine, pH 7.4 with NaOH) and (3) Barth's Ca^{2+}-free solution with 5 μM thapsigargin.

In addition, groups of control oocytes included oocytes that were not subjected to injections.

For each of the previously mentioned conditions, three oocytes in each group were fixed in 4% (v/v) glutaraldehyde in Barth's solution at different times and visualized with a digital stereoscopic microscope. In order to evaluate Ca^{2+} influx, electrophysiological recordings of oocytes from each group were performed after 2–5 h incubation in thapsigargin using the two-electrode voltage-clamp technique [33] and applying pulses of 10 mM $CaCl_2$ substituted by $MgCl_2$ in Ringer-Mg^{2+} solution (75 mM $MgCl_2$, 5 mM HEPES pH 5). All data are presented as mean ± SEM and, differences between two groups were analyzed for statistical significance using paired *t*-test statistical. p values ≥ 0.05 were accepted to indicate statistical significance.

Acridine orange staining

Oocytes obtained from the different experimental conditions were fixed in normal Barth's solution containing 4% (v/v) glutaraldehide and cryofractured. Samples were placed in slides, stained with acridine orange (200 (μg/mL) [34] and fixed with Dako. Images were obtained using a digital stereoscopic microscope (Motic).

Caspases 3 and 7 activity measurements

Oocytes injected with the *Xenopus laevis* elongation factor (EF-1α) [35] shown to be involved in apoptosis [36, 37], were used as a positive apoptotic control. Briefly, after oocyte injection and thapsigargin treatment, 12 oocytes from each group were frozen at −70 °C. After homogenization in lysis buffer containing Triton X-100, samples were allowed to thaw and maintained on ice. Samples were centrifugated in a refrigerated microfuge at 5000–7000 rpm for 5 min at 4 °C and the supernatant diluted 10 times with PBS adding DTT (1 mM final concentration). Reagent Z-DEVD-AFC thawed and placed on ice was added to each sample (30 μM final concentration) mixed and incubated 30 min at 37 °C. Fluorescence was measured according to a Molecular Probes protocol using 400 nm absorption and 505 nm emission. Maximal absorption wavelength was found at 488 nm.

Results

Apoptosis in LNCaP cells

Taking into account the fact that elimination of nutrients generates programmed cell death [13, 18]; this procedure has proven to be an effective way to activate apoptosis. Therefore, in the present study we have continued to use this stimulus in order to induce apoptosis in LNCaP cells. This event was evidenced using the following parameters: cell viability, ladder patterns for fragmented DNA, morphological cell changes studied by flow cytometry, *Xenopus laevis* oocyte morphological analysis, electrophysiological recordings, and caspases activation measurements.

Cell viability almost immediately began to fade when cells were incubated in the absence of serum (Fig. 1A). Nevertheless, the typical fragmented DNA pattern usually associated to apoptosis was only evident after 16 h or more (Fig. 1B). Employing flow cytometry assays and incubation times without serum that ranged from 16 up to 72 h, apoptosis-induced cells in suspension clearly showed a decrease at forward scatter (FSC-H) and an increase at side light scatter (SSC-H) (Fig. 1D) which were related to shrinkage and cellular fragmentation compared with control samples (Fig. 1C). Both morphological changes have been associated with mechanisms related to apoptotic cell death [19]. Although cells incubated without serum for 72 h demonstrated a population of undamaged cells, they presented low viability values due to a grater fragility shown during handling. Therefore, our results show

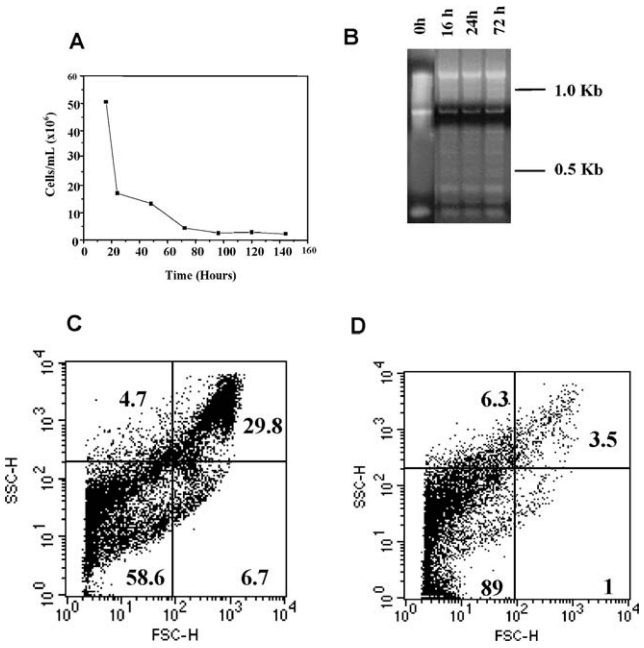

Fig. 1. Progression of apoptosis in LNCaP cells. (**A**) Cellular viability of apoptosis induced cells by removal of serum during cell culture for 16, 24, 48, 72, 96, 120 and 144 h. (**B**) 2% (w/v) agarose gel electrophoresis showing DNA degradation. Control (lane 1); serum removal from LNCaP cell culture media at 16, 24, 72 h (lanes 2–4). Size standards (GIBCO BRL DNA). (**C**) Flow cytometry of control cells. (**D**) Flow cytometry of cells maintained in culture without serum during 16 h.

that LNCaP cells generated a well-defined process of programmed cell death at 16 h in culture in the absence of serum.

Recognition experiments with antibodies

In order to detect representative members of proposed apoptosis associated molecules with similar structures to calcium permeable channels involved in apoptosis [13], LNCaP cells incubated during 16 h in the absence of serum were used to study the levels of expression of different membrane proteins associated with apoptosis. Expression levels for the P2X1 purinergic receptor showed no difference in expression between apoptosis-induced and control cells (Fig. 2A). In contrast, the purinergic receptor P2X7 showed a larger expression in control cells than in apoptosis induced cells (Fig. 2B). These results are in agreement with previous reports showing that P2X7, 2 and 5 are negatively regulated during apoptosis in the ageing rat prostate [38]. Although there are reports showing that P2X7 receptors might be involved in the induction of apoptosis in several other cell types [39, 40], according to our results employing LNCaP cells, P2X7 might be of importance only during the first hours of the apoptotic event. Protein bax showed a slight increase in expression when analyzed in apoptotic LNCaP cells (Fig. 2C), and TRPC1 a representative molecule of the capacitative calcium channels family [24] showed a dramatic difference with the control and the highest increase in expression among proteins apoptotic related produced by serum removal (Fig. 2D). This suggested an important participation of TRPC1-like channels in the genesis and development of apoptosis in androgen-independent LNCaP cells; therefore, experiments were designed in order to further test this possibility.

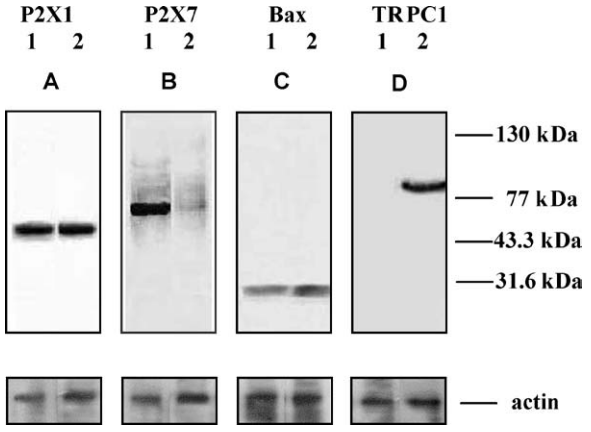

Fig. 2. Recognition experiments using antibodies raised against apoptosis related molecules. (**A**) Anti- P2X1 antibody. (**B**) Anti-P2X7 antibody. (**C**) Anti-bax antibody. (**D**) Anti-TRPC1 antibody. Lower panels correspond to actin used as a loading control. Lane 1; Cell lysate of LNCaP cells incubated in RPMI 1640 medium containing calf fetal serum. Lane 2; Cell lysate of LNCaP cells incubated in RPMI 1640 medium without serum during 16 h.

Cloning

On the basis of our Western blot experiments, and the use of both specific and degenerated oligonucleotides chosen from the previously reported family of capacitative calcium uptake channels [27], several PCR products were amplified. Two PCR products of approximately 1.3 Kb denominated *apoptosis regulated protein* 1 and 2 (*arp*1 and 2) were isolated and sequenced. Originally the two isolated clones were thought to correspond to the same molecule, since from the technical point of view it was not possible to appreciate any changes using agarose gels due to the minimal sequence changes between *arp*1 and *arp*2. Sequencing gave us the possibility to study the small but critical changes between *arp*1 and *arp*2. Although *arp*1 levels did not change between control and apoptotic condition, *arp*2 showed a concentration increase when an apoptotic condition was employed (Fig. 3A). Proteins encoded by these cDNAs were named ARP1 and ARP2. The GenBank accession number for Apoptosis Regulated Protein 2 (ARP2) is AY486134 and Apoptosis Regulated Protein 1 (ARP1), AY486135. Using FASTA program [21], clones *arp*1 and *arp*2 showed homology with a fragment of human splicing factor Prp8 (7.0 Kb) that corresponds to 18% of the complete nucleotide sequence of Prp8 cDNA. This specifically corresponds from nucleotide 13 of *arp*1 and *arp*2 with nucleotide 4591 of the splicing factor Prp8 [16] (Appendix A and B). Homology ends with nucleotide 1309 of our clones and nucleotide 5887 of human Prp8 (Fig. 3B) (Appendix A and B). Within this interval that roughly corresponds to 18% of the whole Prp8 molecule, *arp*1 and *arp*2 share a 99% homology (Fig. 3B) (Appendix B). Although homology between clones *arp*1 and *arp*2 corresponds to 99%, several changes in their nucleotide sequence (Fig. 3B) cause the proteins to present differences that seem to be relevant when function is studied (Appendix C). Interestingly, the amino acid sequence analysis encoded by the third reading frame of these specific cloned sequences also showed a 99% homology with the same small fragment of the human splicing factor Prp8 [16]. Transmembrane helix prediction and hydrophilicity analysis of ARP1 and ARP2 using the third reading frame, allowed us to reveal specific regions rich in hydrophobic α-helices that might associate to the cell membrane in a similar way channels do (Figs. 3C and 3D). Although there is no direct correlation between potential membrane associated regions with a channel protein such as htrp3, the transmembrane helix prediction and hydrophilicity analysis of ARP proteins show several

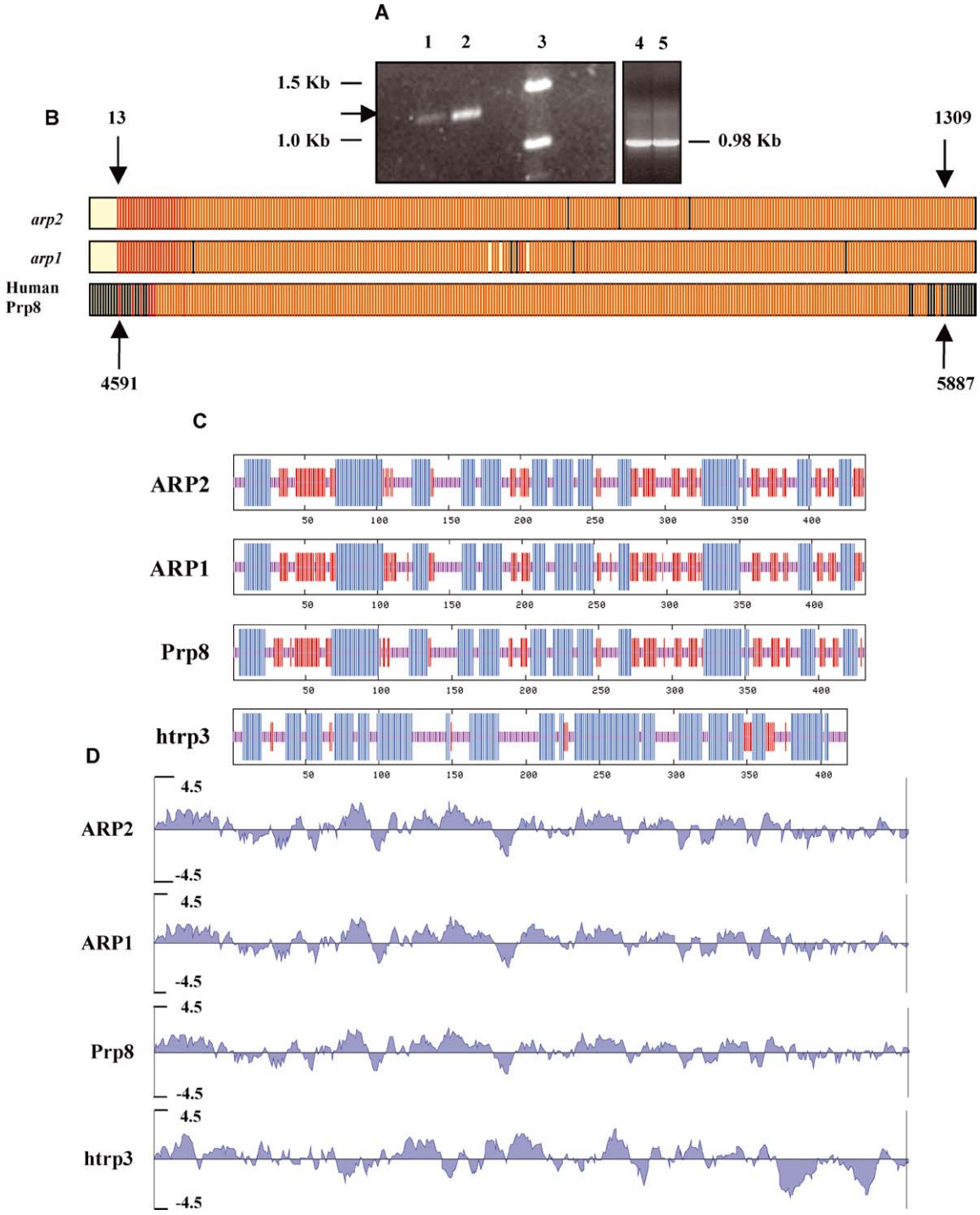

Fig. 3. Cloning of *arp*1 and *arp*2. (**A**) 1% (w/v) agarose PCR products of *arp*2 clones. Lane 1; PCR product of *arp*2 from cells grown in the presence of serum (control). Lane 2; PCR product of *arp*2 from cells grown in the absence of serum. Lane 3; Molecular size standards (GIBCO BRL, DNA 1Kb). Lanes 4 and 5 correspond to GAPDH controls. (**B**) Multiple alignment of *arp*1 and *arp*2 cDNAs with human Prp8 cDNA. Nucleotides 4591–5887 correspond to the region of human Prp8 that overlaps with sequences from *arp*1 and *arp*2 cDNAs. Black lines indicate nucleotides that do not show homology between cDNAs. Yellow spaces in between red lines correspond to nucleotide gaps between cDNAs. Black arrows point nucleotides 13 and 1309 from *arp*1 and *arp*2 and nucleotides 4591 and 5887 from Prp8. (**C**) Secondary structure prediction analysis and sequence alignment for ARP2, ARP1, Prp8 and htrp3. Blue bars (α helix), red bars (β-sheet) and purple bars (random structure). (**D**) Hydrophilicity analysis of proteins ARP2, ARP1, Prp8 and trp3. Regions with aminoacid residues showing values between 0 and 4.5 are considered hydrophobic, while aminoacid residues between values of 0 and –4.5 are considered hydrophilic.

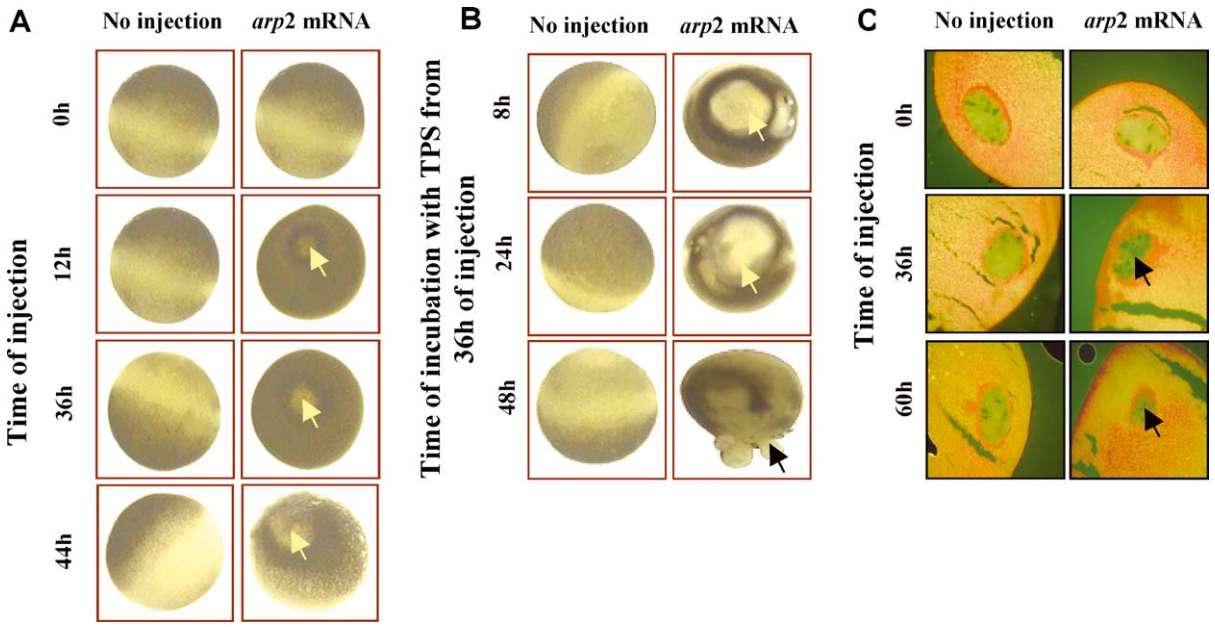

Fig. 4. Morphological changes of *Xenopus laevis* oocytes related to apoptosis after the injection of *arp*2 mRNA. (**A**) Induction of cell death in *Xenopus laevis* oocytes by microinjection of *arp*2 mRNA. Vertical columns correspond to oocytes that received no injection and oocytes injected with *arp*2 mRNA. Horizontal lines show times of injection (0, 12, 36, 44 h). Yellow arrows show the formation of blisters. (**B**) Progression of cell death in oocytes of *Xenopus laevis* injected with the *arp*2 mRNA and incubation with thapsigargin (5 μM). Vertical columns show control oocytes that received no injection, and injected oocytes with *arp*2 mRNA. Horizontal lines show times of incubation with thapsigargin 5 μM from 36 h of injection and 8, 24 and 48 h of incubation with TPS. Arrows show the formation of blisters. (**C**) Morphological changes in the nucleus of *Xenopus laevis* oocytes observed after the injection of *arp*2 mRNA. Vertical columns show histological sections of non-injected oocytes, and oocytes injected with *arp*2 mRNA. Horizontal lines show incubation time after injection of mRNAs (0, 36, 60 h). Black arrows show condensation of chromatin.

sequence segments that might be compatible to a channel protein.

Functional expression

Morphological changes were consistently observed in all groups of *arp*2 mRNA–injected oocytes (Fig. 4A) and resulted to be more evident in treated oocytes with thapsigargin (Fig. 4B). Concomitantly to these morphological changes, oocytes presented a strong decrease in resting membrane potential, changing from a control value of -46.8 ± 6.6 mV to -5.9 ± 3.4 mV (5–8 oocytes, 2 frogs). In general, morphological changes in oocytes included the formation of blisters, which were evident from 12 h after injection (Fig. 4A). A group of oocytes ($n = 3$) injected with *arp*1 mRNA did not show neither decrease in membrane potential (-43 ± 5 mV) nor morphological changes (data not shown). These changes observed in *arp*2 mRNA injected-oocytes were potentiated when cells were incubated with thapsigargin, accelerating the damage process evident by the loss of definition of the animal and vegetable poles occurring around 36 h after *arp*2 mRNA injection and 8 h of incubation with thapsigargin (Fig. 4B). Similar morphological changes and cell membrane depolarization results have been described in *Xenopus* oocytes, when injected with cytochrome *c* as a measure to trigger the apoptotic process [41] as well as in oocytes injected with the proapoptotic molecule Bcl-xs [42]. It is important that injection of *arp*1 mRNA did not show the same effects on the oocytes compared with those generated by *arp*2 mRNA, this included both morphological or electrical changes. In fact, despite the important sequence homology observed between ARP2 and ARP1, from a secondary structure point of view, the few changes found between them resulted to be critical, since ARP1 seems not to develop the phenomenon of apoptosis as efficiently as ARP2 (data not shown). As shown in Fig. 4C, using acridine orange staining [34], revealed that morphological changes were also observed in the nuclei of oocytes injected with *arp*2 mRNA. These nuclei showed elongated forms and condensation of chromatin from 36 up to 60 h of injection, while control oocytes from the same frogs, non-injected oocytes did not show these characteristics ($n = 2$, 2 frogs).

Injection of *arp*2 mRNA in *Xenopus laevis* oocytes, produced a slightly increase in calcium influx through the plasma membrane when the intracellular reservoirs were depleted

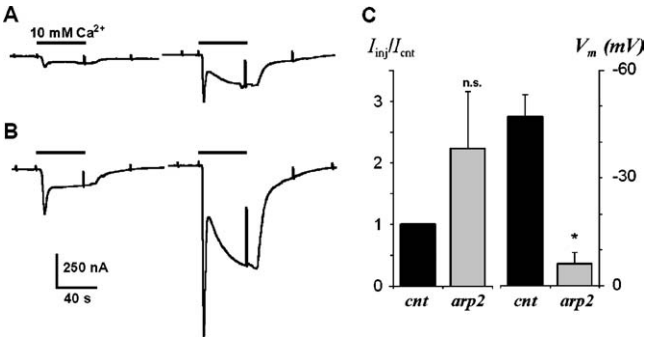

Fig. 5. Functional expression of *arp*2 mRNA in *Xenopus laevis* oocytes. Ionic currents generated by two consecutive pulses of 10 mM Ca^{2+} in Ringer-Mg^{2+} solution after oocyte incubation in absence of calcium and the presence of 5 μM thapsigargin. (**A**) Inward current generated in a control oocyte (non-injected). (**B**) Current in an oocyte injected with *arp*2 mRNA. (**C**) Histogram showing the mean (±S.E.) current generated in control oocytes ($n = 3$) and *arp*2 mRNA-injected oocytes ($n = 6$). Also is shown the change in resting potential (Vm) observed between the different groups of oocytes (8 control oocytes, 6 injected oocytes). All oocytes in this figure were from the same donor, and similar results were obtained in oocytes from a second frog. (n.s.: not significant, $^{*}p \leq 0.05$).

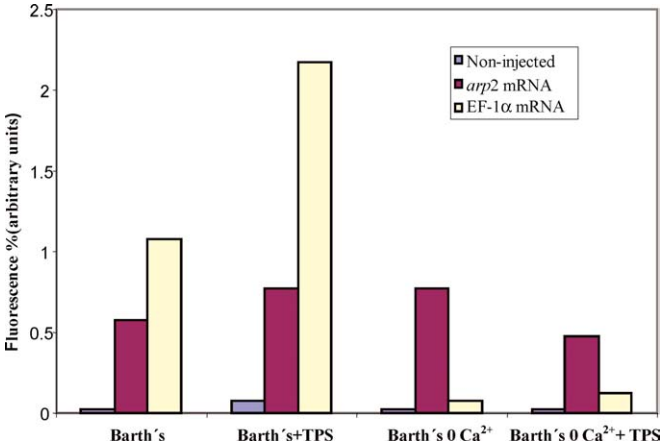

Fig. 6. Activity of caspases 3 and 7 in *Xenopus laevis* oocytes induced to apoptosis by injection of *arp*2 mRNA and EF-1α mRNA. Average of two experiments using 3 oocytes in each condition. Experiments were performed in Barth's medium with or without the presence of calcium and thapsigargin.

by incubation with thapsigargin. Followed by the application of calcium pulses, *arp*2 mRNA injected oocytes generated membrane currents that were shown to be slightly increased to $122 \pm 90\%$ when compared with control oocytes (Fig. 5). The injected oocytes with *arp*1 mRNA in these experiments did not show any difference with respect to the non-injected control oocytes (data not shown). Currents observed were apparently due to the activation of Ca^{2+}-dependent Cl^{-} channels, suggesting that thapsigargin increased the activation of capacitative currents in injected oocytes. These results support the idea that *arp*2 codifies for a Trp channel-like molecule associated to depletion of endoplasmic reticulum stores [27, 43], or that ARP2 might correspond to a molecule that indirectly modulates a native channel. Since this current increase was statistically not significant with the number of experiments performed, the effect might be also related to an ancillary change produced by the triggering of the apoptosis process.

A further analysis in order to know the metabolic state of injected oocytes consisted in the measurement of the activity displayed by caspases 3 and 7, well known to be involved in the process of apoptosis [44]. Fig. 6 shows the activity of caspases 3 and 7 in non-injected oocytes, and oocytes injected with *arp*2 mRNA, as well as mRNA corresponding to the *Xenopus laevis* elongation factor EF-1α [35–37]. As expected, non-injected oocytes independently of being in the absence or the presence of calcium or thapsigargin, do not express any caspase activity. Oocytes injected with *arp*2 mRNA showed enzyme activation independently of the incubation protocol (thapsigargin, calcium, etc). In contrast, EF-1α mRNA injected oocytes, showed the highest caspase activation sensitive to both thapsigargin and calcium. Since in the absence of calcium the injection of EF-1α mRNA totally losses the capability to activate caspases (Fig. 6), calcium sensitivity must probably show that the apoptotic process caused by the expression of ARP2 and the protein expressed by the EF-1α mRNA might be related to differences in their apoptotic transduction pathways. In this respect, it has been reported that overexpression of transcription factor E2F-1 promotes apoptosis in several cell types [45, 46], and proposed to regulate Apaf-1 [47], an important molecule critical in the expression of the apoptotic pathway triggered by cytochrome *c* [48].

Discussion

The molecular search of a channel-like protein was stimulated by previous findings from our laboratory obtained when combining electrophysiological recordings with simultaneous intracellular calcium measurements in single human prostate cancer cells (hormone independent LNCaP cell line). These results originally allowed us to identify using electrophysiological evidence, a non-selective cationic channel-like protein that was calcium permeable, and only activated when two unrelated inducers of apoptosis were employed [13]. In this report, a novel channel-like protein (ARP2) from prostate cancer cells has been cloned and successfully expressed in *Xenopus laevis* oocytes. *arp*2 was identified mostly in cells that had become apoptotic, since control cells under

normal culture conditions seem to express it in very low concentrations. *arp*1 in either control or apoptotic conditions was shown to be expressed also in very low concentrations. *arp*2 presented a high homology with a fragment that corresponds to 18% of the cDNA nucleotide sequence of human splicing factor Prp8. Prp8 recognizes U5 snRNA, and since it binds to RNA in the spliceosome, it has been suggested to be critical in the catalytic arrangements of RNA [16, 49].

Homology of clones *arp*1 and *arp*2 with the fragment of Prp8 from different species such as *Homo sapiens*, *Mus musculus* and *Xenopus laevis,* was achieved from nucleotide 13 of our clones and nucleotide 4591 of Prp8; ending with nucleotide 1309 of our clones and nucleotide 5887 of Prp8 (Appendix B). Since *arp*1 and *arp*2 were obtained using as primers specific sequences and conserved degenerated sequences of human trp3 (htrp3), thus the possibility exists that the channel-like protein sharing an important similarity to a fragment of Prp8 as well as to a trp3 is directly responsible for the increase in our calcium dependent chloride currents observed after oocyte injection. Alternatively, ARP2 could modulate an endogenous protein that in turn might activate the apoptosis mechanism. It is also possible to think that the increase in Ca^{2+} influx observed is in fact an ancillary effect to this process.

Supporting results include: Secondary structures for ARP1 and ARP2 using the third reading frame, are highly related to calcium binding proteins and in general to plasma membrane associated proteins. A series of hydropathy plots demonstrated that ARP2 presented several hydrophobic regions compatible in general to secondary structure and aminoacid characteristics to transmembrane sequences in a similar fashion as found in channels. Also, it is important to note that, influx of calcium in *arp*2 mRNA injected oocytes might have been underestimated due to the fact that analyzed cells were those in which it was technically possible to perform electrical recordings, i.e., oocytes that necessarily were still alive, due to a low ARP2 expression.

In conclusion, our results are consistent with the fact that protein ARP2 corresponds to a truncated form of Prp8 that presents a secondary structure compatible to a channel-like protein or membrane associated protein. It remains to be proven if this molecule functions as an independent channel or as a membrane associated protein that in turn modulates an oocyte endogenous channel. Nevertheless, independently of this last possibility, oocytes injected with *arp*2 mRNA actively promoted important apoptosis associated morphology and electrophysiological changes believed to be favoured by inward calcium currents as well as the activation of caspases, both associated to the progression of apoptosis. In contrast, injection of *arp*1 mRNA into oocytes was not capable to promote inward calcium currents, membrane potential decrease, apoptosis associated morphology changes, nor caspases activation. From the point of view of the sequence changes observed that most probably led to secondary structure rearrangements, we suggest that ARP1 lacks the ability to be functional. These data together with the fact that elongation factor EF-1α employed by us as a positive control has been proposed to modulate protein synthesis, suggest that synergistic effects of ARP2 might be also achieved: first, through the formation of a membrane channel, or a complex between ARP2 and a membrane associated molecule; and second, through RNA splicing mechanisms associated to the progression of apoptotic cell death, due to the high homology degree found between ARP2 and a fragment of Prp8. We believe ARP2 derived from Prp8 corresponds to a molecule with a new function in membrane associated events and apoptosis related phenomena.

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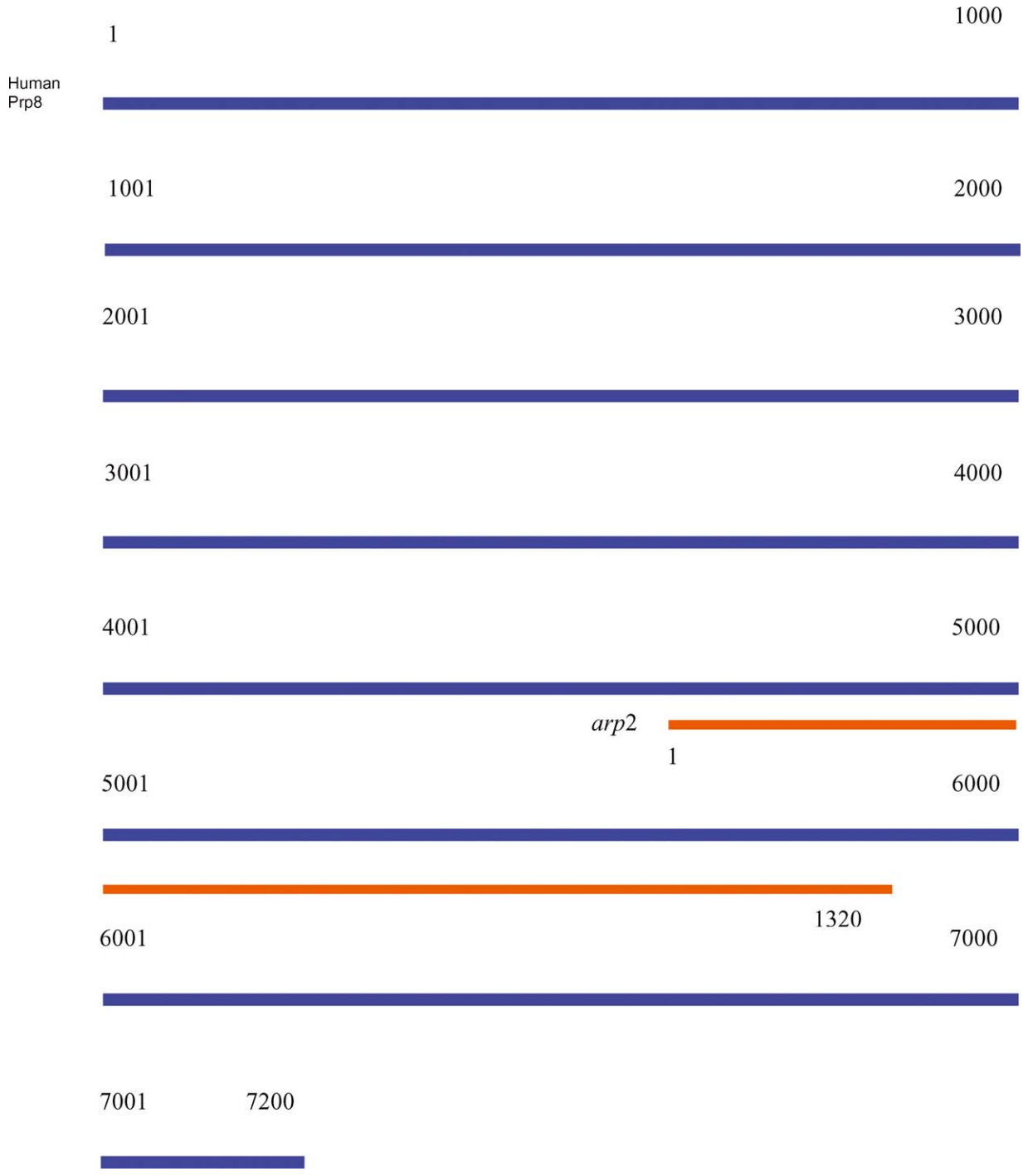
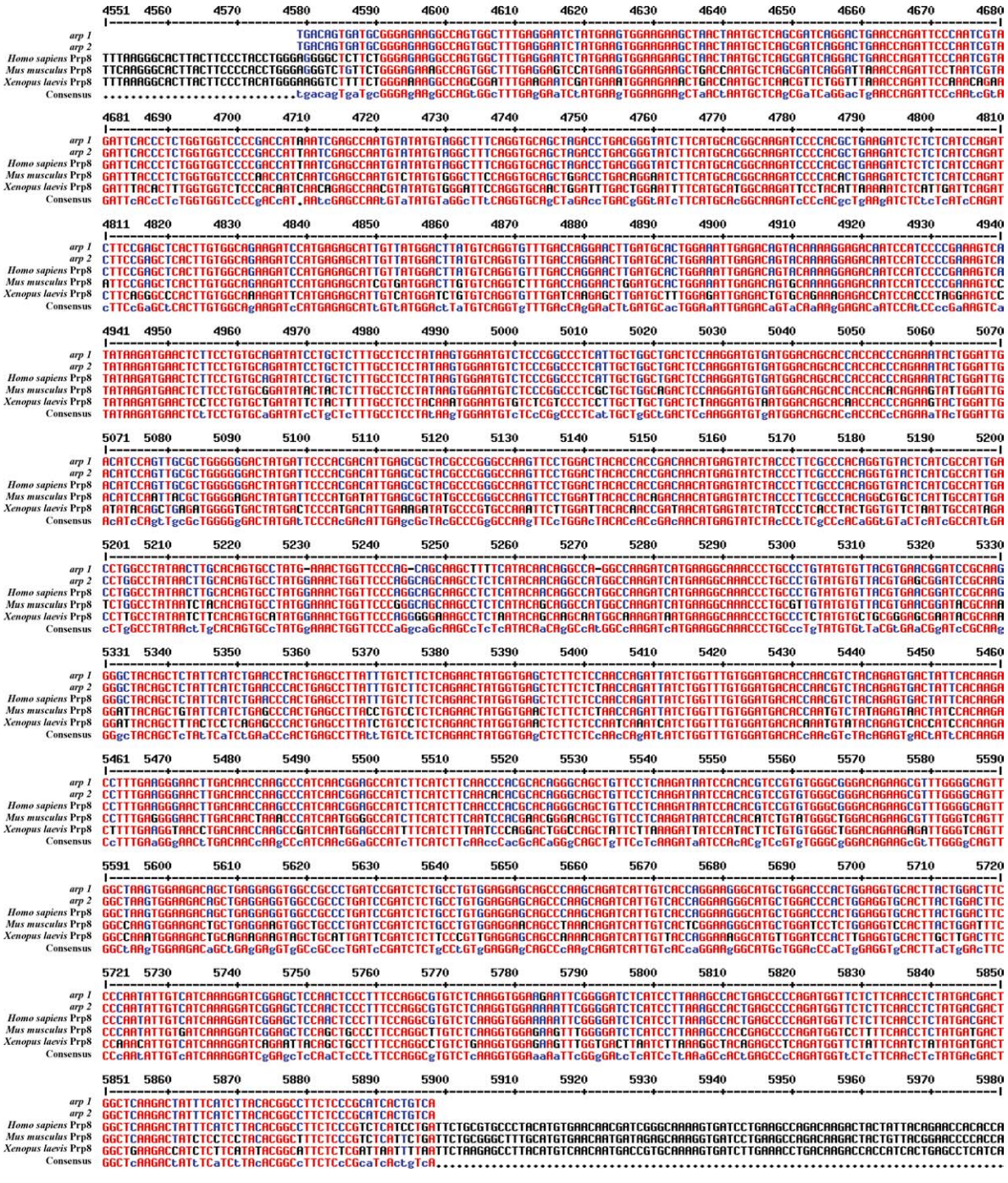
Appendix A

Nucleotide sequence alignment for *arp*2 and Prp8 presented to stress the fact that *arp*2 corresponds to a small fragment of Prp8.



Appendix B

Nucleotide sequence alignment for *arp*1, *arp*2 and Prp8 from *Homo sapiens*, *Mus musculus* and *Xenopus laevis*. Region presented in red represents a perfect match between sequences. Regions presented in blue show nucleotides partially similar between sequences, and regions shown in black represent different individual nucleotides.



Appendix C

Aminoacid alignment between ARP1 and ARP2. Sequences shown in blue represent a region of 20 aminoacids where the sequences present no similarities.

```
ARP1   1  MREKASGFEESMKWKKLTNAQRSGLNQIPNRRFTLWWSPTINRANVYVGFQVQLDLTGIF 60
          MREKASGFEESMKWKKLTNAQRSGLNQIPNRRFTLWWSPTINRANVYVGFQVQLDLTGIF
ARP2   1  MREKASGFEESMKWKKLTNAQRSGLNQIPNRRFTLWWSPTINRANVYVGFQVQLDLTGIF 60

ARP1  61 MHGKIPTLKISLIQIFRAHLWQKIHESIVMDLCQVFDQELDALEIETVQKETIHPRKSYK 120
          MHGKIPTLKISLIQIFRAHLWQKIHESIVMDLCQVFDQELDALEIETVQKETIHPRKSYK
ARP2  61 MHGKIPTLKISLIQIFRAHLWQKIHESIVMDLCQVFDQELDALEIETVQKETIHPRKSYK 120

ARP1 121 MNSSCADILLFASYKWNVSRPSLLADSKDVMDSTTTQKYWIDIQLRWGDYDSHDIERYAR 180
          MNSSCADILLFASYKWNVSRPSLLADSKDVMDSTTTQKYWIDIQLRWGDYDSHDIERYAR
ARP2 121 MNSSCADILLFASYKWNVSRPSLLADSKDVMDSTTTQKYWIDIQLRWGDYDSHDIERYAR 180

ARP1 181 AKFLDYTTDNMSIYPSPTGVLIAIDLAYNLHSAYET---GSQ---QQAFHTTGQAKIMKA 234
          AKFLDYTTDNMSIYPSPTGVLIAIDLAYNLHSAY     GS+   QQA      AKIMKA
ARP2 181 AKFLDYTTDNMSIYPSPTGVLIAIDLAYNLHSAYGNWFPGSKPLIQQA-----MAKIMKA 235

ARP1 235 NPALYVLRERIRKGLQLYSSEPTEPYLSSQNYGELFSNQIIWFVDDTNVYRVTIHKTFEG 294
          NPALYVLRERIRKGLQLYSSEPTEPYLSSQNYGELFSNQIIWFVDDTNVYRVTIHKTFEG
ARP2 236 NPALYVLRERIRKGLQLYSSEPTEPYLSSQNYGELFSNQIIWFVDDTNVYRVTIHKTFEG 295

ARP1 295 NLTTKPINGAIFIFNPRTGQLFLKIIHTSVWAGQKRLGQLAKWKTAEEVAALIRSLPVEE 354
          NLTTKPINGAIFIFN RTGQLFLKIIHTSVWAGQKRLGQLAKWKTAEEVAALIRSLPVEE
ARP2 296 NLTTKPINGAIFIFNTRTGQLFLKIIHTSVWAGQKRLGQLAKWKTAEEVAALIRSLPVEE 355

ARP1 355 QPKQIIVTRKGMLDPLEVHLLDFPNIVIKGSELQLPFQACLKVEEFGDLILKATEPQMVL 414
          QPKQIIVTRKGMLDPLEVHLLDFPNIVIKGSELQLPFQACLKVE+FGDLILKATEPQMVL
ARP2 356 QPKQIIVTRKGMLDPLEVHLLDFPNIVIKGSELQLPFQACLKVEKFGDLILKATEPQMVL 415

ARP1 415 FNLYDDWLKTISSYTAFSRITV 436
          FNLYDDWLKTISSYTAFSRITV
ARP2 416 FNLYDDWLKTISSYTAFSRITV 437
```