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Accelerated Publications

α- and β-Forms of the 65-kDa Subunit of Protein Phosphatase 2A Have a Similar 39 Amino Acid Repeating Structure[†,‡]

Brian A. Hemmings,[*,§] Carolyn Adams-Pearson,[§] Francisca Maurer,[§] Peter Müller,[§] Jozef Goris,[∥] Wilfried Merlevede,[∥] Jan Hofsteenge,[§] and Stuart R. Stone[§]

Friedrich Miescher-Institut, P.O. Box 2543, CH-4002 Basel, Switzerland, and Afdeling Biochemie, Faculteit Geneeskunde, Katholieke Universiteit te Leuven, Leuven, Belgium

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ABSTRACT: Protein phosphatase 2A (polycation-stimulated protein phosphatase L) was purified from porcine kidney and skeletal muscle. The 36-kDa catalytic and the 65-kDa putative regulatory (hereafter termed PR65) subunits of protein phosphatase 2A2 were separated by reverse-phase HPLC. Partial amino acid sequence data (300 residues) was obtained for PR65. Molecular cloning showed that two distinct mRNAs (termed α and β) encoded the PR65 subunit. The cDNA encoding the α-isotype spanned 2.2 kilobases (kb) and contained an open reading frame of 1767 bases predicting a protein of 65 kDa, which was in good agreement with the size of the purified protein. The cDNAs encoding the β-isotype contained an open reading frame of size similar to that of α-form but lacked an initiator ATG. Northern analysis, using RNA isolated from several human cell lines, indicated that the α-isotype was encoded by a mRNA of 2.4 kb that was much more abundant than the β mRNA of 4.0 kb. Comparison of the predicted amino acid sequences of the two isotypes revealed 87% identity. The deduced protein sequences of the α- and β-isotypes were found to be made up of 15 imperfect repeating units consisting of 39 amino acids. This repeating structure was conserved between species.

Protein phosphorylation plays a central role in regulating a diverse number of cellular processes. For phosphorylation of specific target proteins to function as an effective and dynamic control mechanism, it requires the coordinated action of both protein kinases and phosphatases. The role of hormones, growth factors, andd neurotransmitters in regulating protein kinase activity has been extensively investigated [reviewed by Edelman et al. (1987) and Yardin and Ullrich (1988)]. However, the influence of different signal transduction pathways on the regulation of protein phosphatases is less well established.

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* Author to whom correspondence should be addressed.
[§] Friedrich Miescher-Institut.
[∥] Katholieke Universiteit te Leuven.

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Several different forms of serine/threonine-specific protein phosphatases have been purified (termed type 1, 2A, 2B, and 2C) and characterized on the basis of their mode of regulation and substrate specificity [reviewed in Ballou and Fischer (1986) and Cohen (1989)]. Four oligomeric forms of protein phosphatase 2A have been purified to apparent homogeneity (Crouch & Safer, 1980; Tamura & Tsuiki, 1980; Tamura et al., 1980; Pato & Adelstein, 1980, 1983; Li, 1981; Paris et al., 1984; Tung et al., 1985; Mumby et al., 1985; Waelkens et al., 1987; Usui et al., 1988). All isoforms contain a 36-kDa catalytic subunit associated with a common regulatory subunit of 62–68 kDa (hereafter termed PR65). Additional subunits of 55 or 74 kDa can also be found associated with the 36- and 65-kDa subunits. Biochemical characterization of the protein phosphatase 2A holoenzymes has revealed that they have different substrate specificities (Werth et al., 1982; Pato & Adelstein, 1983; Imaoka et al., 1983; Mumby et al., 1987; Agostinis et al., 1987) and regulatory properties (Goris et al., 1986, 1989a,b; Waelkens et al., 1987; Usui et al., 1988). Moreover, reconstitution experiments of the catalytic subunit with the PR65 subunit from pig heart (Imaoka et al., 1983), human erythrocytes (Takeda et al., 1985), and rabbit reticulocytes (Chen et al., 1989) have demonstrated changes in the activity of the catalytic subunit upon combination with PR65.

In order to understand the function of the protein phosphatase 2A regulatory subunits more fully, we have isolated cDNA clones encoding the 65-kDa subunit which is the common form of regulatory subunit found in all purified preparations of protein phosphatase 2A. Sequence analysis revealed that at least two distinct genes encode the PR65 that were expressed in all cell lines examined. Both isotypes of PR65 subunit consisted of 15 imperfect repeating units of 39 amino acids.

Experimental Procedures

Purification of the Protein Phosphatase 2A Holoenzyme. Protein phosphatase 2A holoenzyme was purified from pig kidney and skeletal muscle according to published procedures (Tung et al., 1985; Waelkens et al., 1987) with the following modifications. Two kilograms of tissue was homogenized in 25 mM Bis-Tris buffer, pH 7.2, containing 1 mM EDTA, 1 mM DTT, and 150 mM NaCl (buffer A). Following ammonium sulfate precipitation (30–50% of saturation) and dialysis into buffer A, the protein was applied to a DEAE-Sepharose CL-6B (5 × 40 cm) column equilibrated with buffer A and eluted with a 2-L gradient of 0.2–0.4 M NaCl in buffer A. Phosphatase activity eluting at 0.22–0.3 M NaCl was pooled, diluted 1:1 with buffer A, and then applied to a polylysine column (2.5 × 7.5 cm) previously equilibrated with buffer A. Protein phosphatase 2A activity, eluted with a 400-mL linear gradient of 0.15–0.60 M NaCl in buffer A, was concentrated to less than 10 mL and applied to an Ultrogel AcA-34 column (2.5 × 95 cm). To obtain homogeneous preparations, protein phosphatase 2A was further purified by chromatography on a Mono-Q column as described by Waelkens et al. (1987). In addition, a second Mono-Q purification step was run with buffer A without EDTA; chromatography under these conditions resulted in a shift in the peak of activity away from contaminating proteins. The protein phosphatase 2A purified from porcine kidney and muscle was judged to be pure by SDS–PAGE and consisted of two polypeptides of 36 and 65 kDa, respectively (data not shown).

Purification of the PR65 Subunit, Isolation of the N-Terminal Peptide, Protein Sequence Determination, and Amino Acid Analysis. To separate the PR65 subunit from the catalytic subunit, the holoenzyme (250 μg in 200 μL) was diluted with 2.0 mL of 10% formic acid, incubated at room temperature for 30 min, centrifuged, and loaded onto a Vydac C4 column (Hesperia, CA) equilibrated with 0.1% (v/v) trifluoroacetic acid (TFA). The column was washed with 0.1% (v/v) TFA and developed with a gradient of 0–28% (v/v) actonitrile in 0.1% TFA over 30 min followed by a gradient of 28–63% acetonitrile over 90 min with a flow rate of 1.0 mL/min. Aliquots of the eluted fractions were electrophoresed on 10% SDS–polyacryamide gels, and proteins were visualized by staining with Coomassie Blue. The catalytic and PR65 subunits eluted at about 48% and 55% acetonitrile, respectively.

Tryptic digests (Hofsteenge et al., 1988) of PR65 were fractionated on a C18 reverse-phase column with a linear gradient of acetonitrile (0–50% in 360 min) in 0.1% (v/v) TFA. For the isolation of the N-terminal peptide, CNBr fragments were separated by reverse-phase HPLC on a C4 column with an acetonitrile gradient. The N-terminal peptide was identified by its resistance to Edman degradation and amino acid composition. To isolate the N-terminal tryptic peptide, the CNBr fragment was digested with trypsin (2% w/w) at 37 °C for 2 h. The subdigest was then fractionated on a C18 column with a gradient of acetonitrile (0–50% in 210 min). The N-terminal peptide was identified by amino acid composition and resistance to Edman degradation.

Amino acid sequence analysis was performed on an Applied Biosystems gas-phase sequencer equipped with an on-line PTH analyzer. Amino acid analysis of purified peptides was carried out following 24-h hydrolysis at 110 °C in the vapor of 6 N HCl, using the method of Knecht and Chang (1987).

Fast atom bombardment mass spectroscopy of the N-terminal typtic peptide was carried out by M Scan, Berks, U.K.

Assay Methods. Protein phosphatase activity was determined according to standard procedures (Cohen et al., 1988); activity was measured with phosphorylase as substrate in the early stages of purification and with *p*-nitrophenyl phosphate in the later steps (Waelkens et al., 1987). Protein determination was by the dye binding method (Bradford, 1976).

Molecular Cloning. Screening of LLC-PK_1 cDNA libraries with oligonucleotide probes based on codon preference (Lathe, 1985) was carried out as follows. Two overlapping 36-mers, spanning a 19-residue peptide (AIIEYMPLLAGQLQVEFFE), were annealed and filled-in with [α-^{32}P]dATP and the Klenow fragment of DNA polymerase. Filters were prehybridized, hybridized, and washed as previously described (Stone et al., 1987a,b). Following the positive isolation of a clone (λPPR65-C7) encoding the PR65 subunit, further cDNAs were identified with the *Eco*RI fragment from this clone by screening other LLC-PK_1 libraries as previously described (Stone et al., 1987a,b).

Isolation of human cDNAs was initially achieved by screening a human lung fibroblast cell line (IMR-90) cDNA library (Clontech) with the porcine probes (λPPR65-A1). Subsequently, several human cDNA libraries [lung tissue and cell lines WI38 (Stratagene), HeLa (obtained from Peter Nielson, University of Basel), and MCF7 (Prof. Pierre Chambon, INSERM, Strasbourg)] were screened with probes for the α- and β-isotypes as described above.

Sequencing of the cDNAs. cDNA sequences were determined by the dideoxy method (Sanger et al., 1977) using Sequenase according to the manufacturer's protocols with the universal primer or specific oligonucleotides. All clones were

sequenced on both DNA strands; difficult (high G/C content) regions were sequenced with dITP. DNA sequences were analyzed with the GCG software package (Deveroux et al., 1984) and FASTA and TFASTA programs (Lipman & Pearson, 1988).

RNA Isolation and Northern Analysis. Total RNA was isolated by the method of Chomsczynski and Sacchi (1987) as described by Khew-Goodall and Hemmings (1988). For Northern analysis 20 μg of total RNA was fractionated on 1% formaldehyde–agarose gels and electrotransferred at 30 V for 16 h to Zetaprobe membranes in 10 mM Tris–acetate/5 mM EDTA, pH 7.8, buffer. Hybridization and washing of blots was as previously described (Khew-Goodall & Hemmings, 1988).

Results

Isolation and Separation of the Catalytic and Regulatory Subunits of Protein Phosphatse 2A. The dimeric form of protein phosphatase 2A was purified from porcine skeletal muscle and kidney as described under Experimental Procedures. The two subunits were separated by reverse-phase HPLC on a C4 column (Figure 1a). As can be seen, the catalytic and PR65 subunits were well resolved with the former eluting at 48% and the latter at 55% acetonitrile; the first peak eluting immediately in front of the catalytic subunit did not contain material that stained with Coomassie Blue after SDS–PAGE (data not shown).

Purified PR65 was reduced, carboxymethylated, and treated with trypsin or CNBr. The peptides were separated by reverse-phase HPLC with an acetonitrile gradient and used for amino acid sequence analysis. In total, 300 residues distributed throughout the PR65 sequence were determined (see Figure 2).

Isolation of cDNAs Encoding PR65 from Porcine Libraries. To determine the complete structure of PR65, we isolated a series of overlapping clones from several cDNA libraries derived from the porcine cell line LLC-PK_1 (Figure 1b). Initially, two overlapping 36-mer oligonucleotide probes corresponding to the tryptic peptide AIIEYMPLLAGQLQVEFFE were synthesized on the basis of codon preference (Lathe, 1985). From initial screening with the filled-in oligonucleotide probes several positive clones were identified, purified, and sequenced (see Figure 1b). Sequence analysis confirmed that the cDNAs encoded a protein homologous to that isolated from porcine kidney and muscle (see below). Further cDNAs were isolated from other LLC-PK_1 libraries by screening with the original clone (λPPR65-C7). One of the cDNAs (λPPR65-A1) was found to span approximately 2000 bp. In addition, a cDNA (λPPR65-52) extending the 3′ end was isolated.

DNA Sequence Analysis of the Porcine PR65 Clones. Complete sequence analysis (data not presented) of the *Eco*RI fragments of λPPR65-C7, λPPR65-A1, and λPPR65-52 and of a *Kpn–Sst*I fragment of λPPR65-A1 containing the complete set of *Eco*RI fragments revealed an open reading frame of 602 amino acids starting at nucleotide 2. However, the cDNA lacked an initiator ATG. During the course of this study, we isolated three other independent cDNAs that had the same 5′ start point as λPPR60-A1. Two possible explanations for these results are (a) all the cDNAs lack the 5′ end of the mRNA and start at the same internal *Eco*RI site or (b) the initiator codon for PR65 is not ATG. The molecular mass of the protein predicted by the open reading frame was approximately 66 kDa, which is somewhat larger than that determined by SDS–PAGE. The predicted amino acid sequence of the open reading frame of the cDNA showed about 87% identity with the protein sequence data indicating that

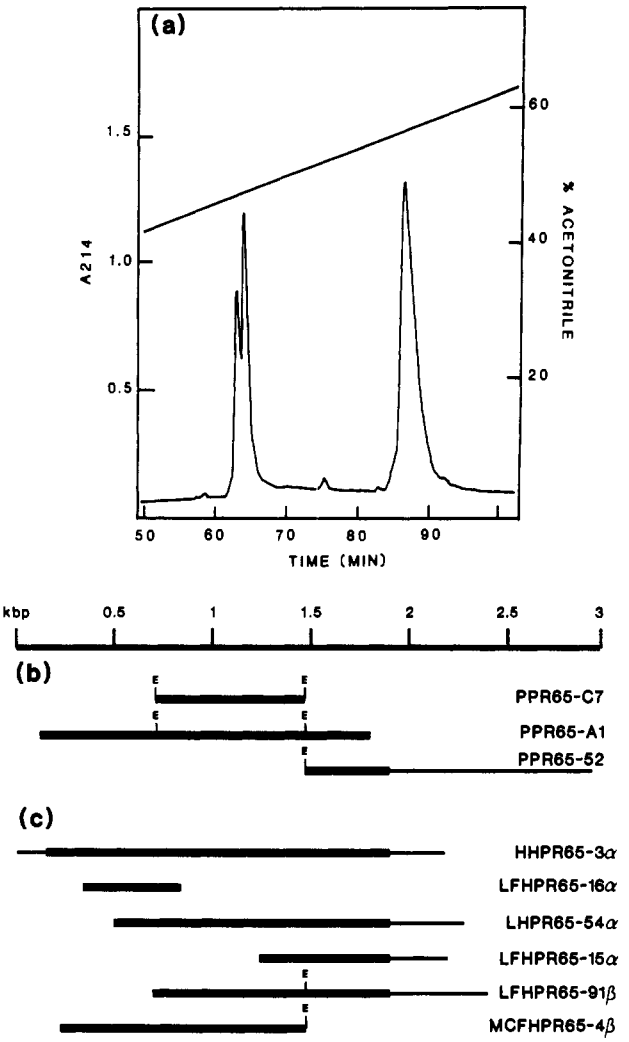

FIGURE 1: (a) Separation of the catalytic subunit and the regulatory subunit (PR65) of protein phosphatase 2A. Purified protein phosphatase 2A (260 μg) was adjusted to 10% formic acid and then fractionated on a C4 column in an acetonitrile gradient. Purified PR65, eluting at 55% acetonitrile, was subsequently fragmented by trypsin, and peptides were purified by HPLC on a C18 column. (b) Porcine PR65 cDNAs isolated from LLC-PK_1 libraries. (c) Human PR65 cDNAs encoding the α- and β-isotypes. Solid lines delineate the coding regions and the thin lines the noncoding sequences. Source of RNA used for library construction: H, HeLa; LF, lung fibroblast cells, IMR-90; L, lung tissue; MCF, MCF7 cells. E = *Eco*RI sites. Only the clones used for DNA sequence analysis are shown.

we had cloned the cDNA for a protein homologous to the one purified (see Figure 3). This form of PR65 has been denoted βPR65 and the form corresponding to the protein sequence αPR65.

[It appears that the β-isoform mRNA was very highly represented in the LLC-PK_1 cDNA library probably due to the internal *Eco*RI sites leading to an enrichment of these sequences in the library. Recently, we have isolated partial cDNAs from a porcine brain library corresponding to the α-isoform (Hemmings et al., unpublished data).]

Two Distinct Genes Encode PR65 in Human Cells. Since our initial attempts to isolate the cDNA corresponding to the α-isoform of PR65 from porcine libraries were unsuccessful, we screened several human cDNA libraries prepared from RNA isolated from lung tissue, two lung fibroblast cell lines (IMR-90 and WI38) and two epithelial-derived cell lines (HeLa and MCF7). Sequence analysis of several clones (Figure 1c) isolated from the lung libraries confirmed that two distinct mRNA species encoded the human PR65 (termed α

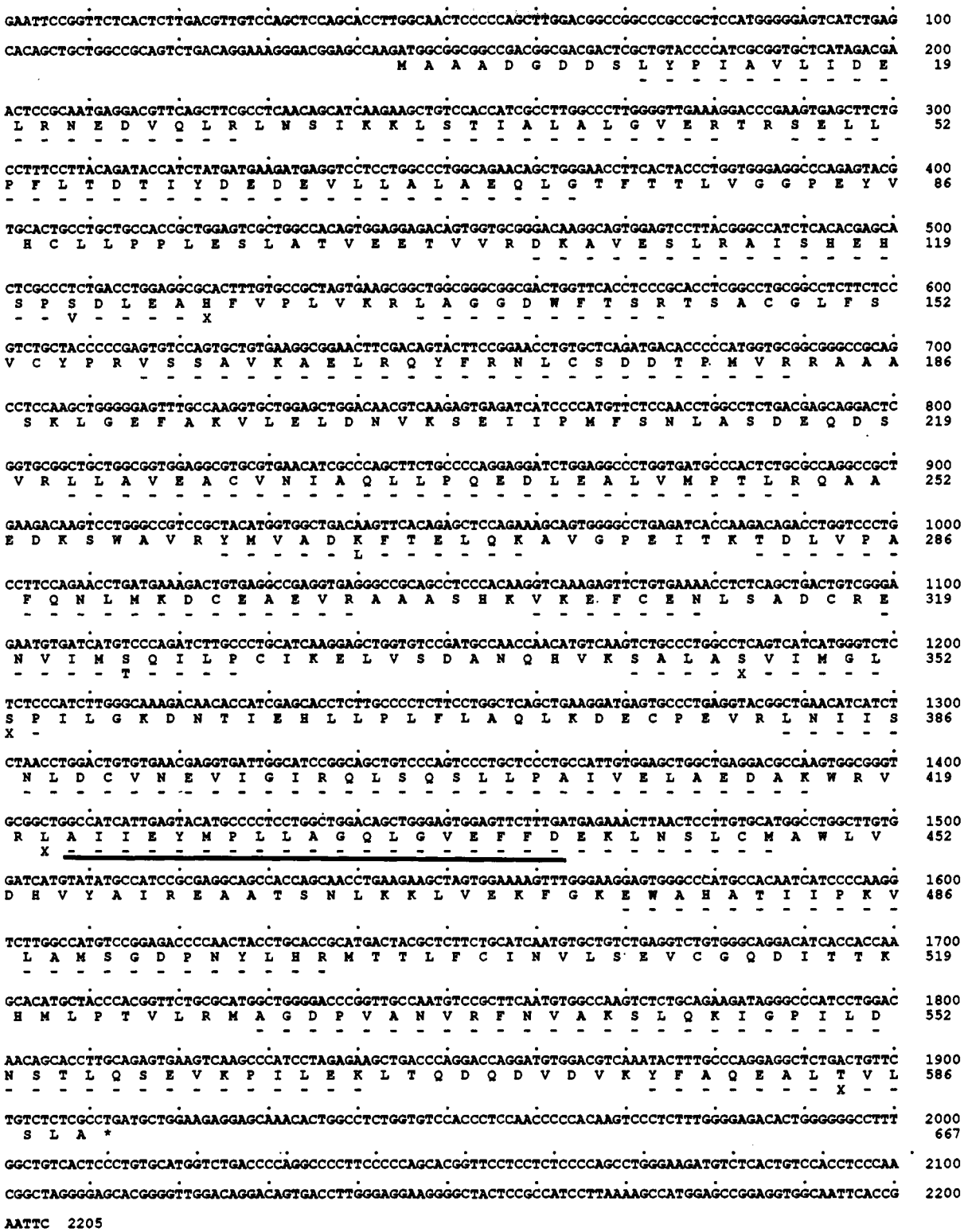

FIGURE 2: Nucleotide and deduced amino acid sequence for the human (HeLa) α-isotype of PR65. The complete nucleotide sequence was obtained from λHHPR65-3; the cDNAs derived from the lung libraries λLFHPR65-16, λLHPR65-54, and λLFHPR65-15 were also sequenced (data not presented), and a single base change at nucleotide 404 (A to G) resulting in a conservative amino acid change from His to Arg was found. The amino acid sequence of the longest open reading frame beginning at nucleotide 145 is translated. Data obtained by peptide sequencing of the porcine αPR65 are aligned with the open reading frame. Identities are shown as a (–), while differences are shown in the single-letter code for amino acids; residues that could not be unambiguously assigned are designated as X. The peptide sequence used for synthesis of oligonucleotide probes is underlined; (*) indicates the position of the stop codon.

and β). The α-type corresponded to the porcine protein sequence while the β-type was homologous to the porcine cDNA described above.

The complete nucleotide sequence of the human αPR65 (Figure 2) was obtained from the HeLa cDNA (λHHPR65-3); attempts to isolate full-length clones from the lung libraries were unsuccessful. The HeLA clone contained 145 5′-noncoding bases, an open reading frame of 1767 bases, and 289 3′-noncoding bases. The open reading frame encoded a protein of 589 residues (see Figure 2) with a molecular mass of 65 kDa which is in good agreement with that determined for the purified protein from porcine kidney and muscle. The proposed

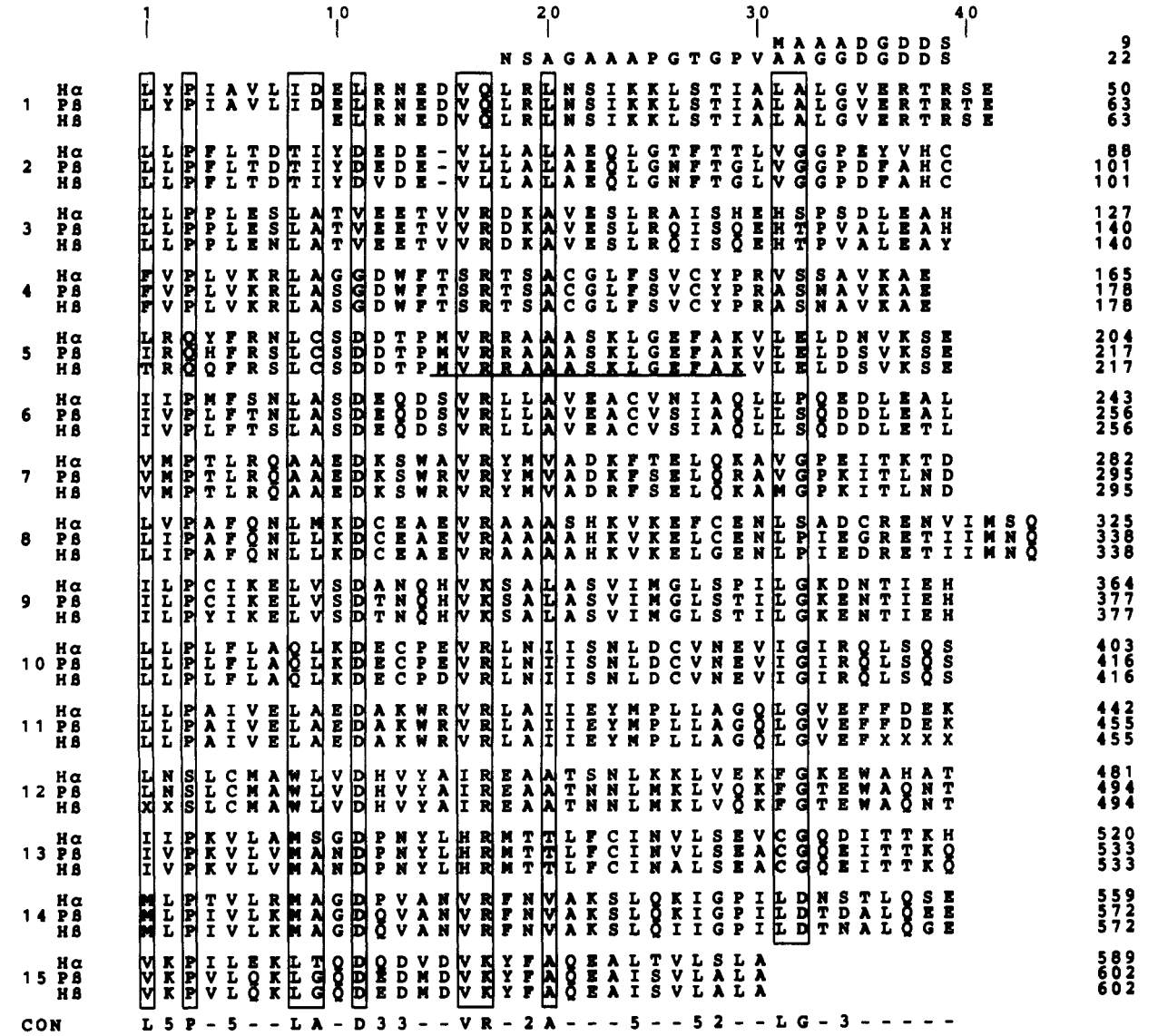

FIGURE 3: Internal homology of the α- and β-forms of PR65 protein. The amino acid sequence has been aligned to show the 15 repeats of 39 amino acids of the human α- and β-isotypes and the porcine β-isotype. The numbers at the left side indicate the repeat unit, and those on the right side indicate the position of the last amino acid of each repeat in the protein, while the numbers across the top indicate the position in the repeat. The human β cDNA was not full length, and therefore, the numbering of the porcine clone has been used; in addition, residues 451–457 were not found, presumably because of two adjacent *Eco*RI sites 18 bp apart. The Gap program (Devereux et al., 1984) was used to define the alignments; the Gap weight used was 5 and the gap-length weight was 0.3. Conserved residues in each repeat are boxed. If seven of the repeat units contain the same amino acid residue, this amino acid is listed in the consensus. If the sequences contain residues from the same group, then the group is referred to in the consensus [conservation substitutions are defined as (1) C; (2) S, T, P, A, G; (3) N, D. E, Q; (4) H, R, K; (5) M, I, L, V; and (6) F, Y, W].

reading frame contained sequences homologous to all those obtained for the porcine protein by amino acid sequencing. Over 300 residues of peptide sequence was determined for the porcine αPR65, and only three definite differences were found between the porcine peptide sequence and that deduced from the human cDNA for the α-isotype. The proposed initiator ATG codon was preceded by a G/C-rich region and conformed well with the consensus for initiation of translation in vertebrates (Kozak, 1987). The composition of the N-terminal peptide isolated from the porcine muscle PR65 was in complete agreement with translation starting at the proposed initiator ATG (see below).

The nucleotide sequence (not presented) of the human β cDNAs showed 95% conservation in the coding region and 84% in the 3′-noncoding region compared with the porcine cDNA. The 3′-noncoding sequence showed no homology with the human α-isotype, confirming that they were the products of two distinct genes. The deduced amino acid sequence of the human β-isotype showed 98% identity with the porcine β sequence (see Figure 3).

Identification of the N-Terminal Peptide of PR65. The primary structure of the N-terminus of porcine αPR65 was established by protein chemical techniques. PR65 was resistant to Edman degradation, presumably due to the presence of a blocking group. In order to determine the structure of the N-terminus, αPR65 subunit was cleaved with CNBr, and the peptides were fractionated by reverse-phase HPLC on a C4 column. Examination of the deduced amino acid sequence of PR65 (α and β) indicated that the N-terminal CNBr fragment would be the largest. The last peptide to elute from the column (at about 60% acetonitrile) was found to be resistant to Edman degradation and was, thus, identified as the N-terminal peptide. This CNBr fragment was further digested with trypsin, and several of the resulting peptides were sequenced. A peptide resistant to Edman degradation, which eluted at 35% acetonitrile, was identified. The amino acid composition was de-

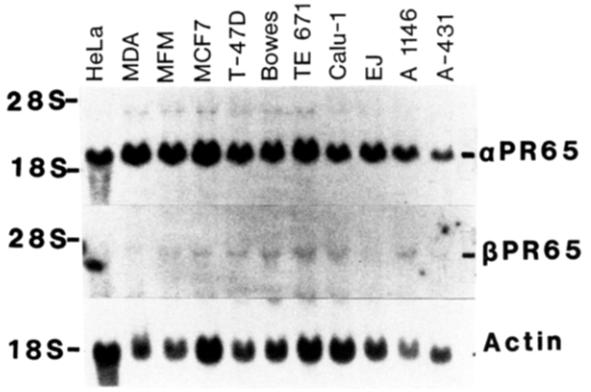

FIGURE 4: PR65 mRNA analysis. Total RNA isolated from the human cell lines was analyzed with α- or β-specific probes labeled by the random priming method to a specific activity of 0.88×10^9 and 1.32×10^9 cpm/μg, respectively. The α- and β-specific probes used were *Eco*RI fragments isolated from λLFHPR65-15 and λLFHPR65-91. Autoradiograms were exposed for 72 h at −70 °C except for the actin probe which was for 6 h.

termined [Asp 3.4, Glu 1.3, Ser 1.1, Gly 1.1, Ala 3.5, Arg 1.0, Pro 1.0, Val 1.1, Ile 1.7, Leu 2.9, Tyr 0.6 (mol/mol of peptide)] and corresponded well with that calculated for the peptide from residues 2–23 of the human α-isotype. The same N-terminally blocked peptide was also isolated from a tryptic digestion of native αPR65. Separate subdigestions of the N-terminal tryptic peptide with Asp-N, thermolysin, *N*-bromosuccinamide, chymotrypsin, and prolylendopeptidase established that this peptide contained the sequence -Y-P-I-A-V-L-I-D-E-L-R. Analysis of the entire tryptic peptide by mass spectroscopy indicated that its molecular mass was 2057 Da. This value compared well with the molecular mass of 2058 Da that can be calculated for the N-terminal tryptic peptide of the human α-isotype in which the initiator methionine has been removed and the adjacent alanine acetylated. The fact that no methionine was detected in the N-terminal peptide and its resistance to Edman degradation were in accordance with this proposal.

Northern Analysis of PR65 Expression. To ascertain both the size and abundance of the α- and βPR65 transcripts (Figure 4), total RNA from a number of human cell lines was fractionated and hybridized with gene-specific probes, λLFHPR65-15 and λLFHPR65-91 (see Figure 1c). From comparison with the ribosomal 28S and 18S RNA the length of the α-transcript was determined to be approximately 2400 nucleotides. The β-transcript size was approximately 4000 nucleotides. Quantitation of the ratio of α- to β-transcripts by densitometric scanning of autoradiographs revealed that the α mRNA was about 40 times more abundant in most cell lines.

Discussion

In this paper we describe the molecular cloning of human and porcine cDNAs encoding two forms of the 65-kDa regulatory subunit of protein phosphatase 2A that are, apparently, products of separate genes as indicated by nucleotide sequence differences. Comparison of the deduced amino acid sequences of the two isotypes reveals that they are 87% homologous. The sequence conservation for a particular isotype between species is quite remarkable. With the α-isotypes, over the 300 residues where sequence is available, only three differences were found between human and porcine sequences. Similarly, comparison of the deduced amino acid sequences of the β-isotypes shows 97% homology. Similarly, high sequence conservation has been reported for the catalytic subunits of protein phosphatase 1 and 2A gene families [see Cohen and Dombardi (1989)].

Previously, we found two distinct genes encoding the catalytic subunit of protein phosphatase 2A (Stone et al., 1987a,b). Thus, the structure of phosphatases of the 2A group is complex. So far, at least four different holoenzymes assembled from the catalytic subunit and at least three or four different regulatory subunits have been identified at the biochemical level. Molecular cloning indicates, however, that this complexity is likely to be somewhat greater because both the catalytic and PR65 subunits are products of two distinct genes. Western analysis of the purified protein phosphatase 2A, using peptide-specific antisera, indicated that both the α- and β-isotypes of the catalytic subunit were associated with the PR65 isolated in these studies (Hemmings et al., unpublished observations). It should, however, be noted that protein sequence analysis of the muscle and kidney PR65 did not reveal any sequence corresponding to the β-protein. This suggests two possibilities: either the levels of the β-isoform at the protein level are very low, or the β-isoform is specifically associated with a form of protein phosphatase 2A different from the one isolated in this study. Recently, we have detected the β-isotype of PR65 in partially purified preparations of protein phosphatase 2A using peptide-specific antisera (P. Hendrix et al., unpublished data); interestingly, the levels of βPR65 in protein phosphatase 2A isolated from *Xenopus* oocytes were, apparently, higher than the αPR65.

The nucleotide sequence of the β-isoform cDNA predicted a protein with a molecular mass in the region of 66 kDa, which is somewhat large than that determined by SDS gel electrophoresis. However, the cDNA lacks an initiator ATG codon. Two possibilities exist to explain these observations: (a) The 5′ *Eco*RI site is an internal site, and therefore, all the cDNAs isolated had terminated prematurely. In total, three different cDNAs were isolated that started at exactly the same site. (b) The 5′ end of the PR65 β mRNA could coincide with the cDNAs isolated, thereby explaining why they all terminated at the same 5′ site. In this case, the initiator codon of the PR65 mRNA would have to be a codon other than ATG. Some examples have been documented where mRNAs use an initiator codon other than ATG. For example, in mammalian cells a CTG triplet upstream of the initiator ATG is employed to direct the synthesis of a slightly larger c-myc protein (Hann et al., 1988). Similar observations have been reported for the capsid protein of adeno-associated virus (Becerra et al., 1985) and Sendai virus C′ protein (Curran & Kolakofsky, 1988; Gupta & Patwardhau, 1988). Recently, Peabody (1988) demonstrated that other codons could substitute for ATG, when located in the same position, both in vitro and in vivo. It was concluded that the non-ATG initiator should reside in a favorable sequence context to function effectively (Kozak, 1984, 1989). Examination of the 5′ sequences of the PR65 mRNA reveals a very high GC content, a property of many mRNAs. A GTG codon was found at a position equivalent to that of the initiator ATG of the human α-isotype, and the surrounding nucleotides conformed to the consensus sequence for initiator codons (Kozak, 1989). Currently, we are attempting to resolve the question of the initiator codon of the β-isotype by isolation of genomic clones and cDNAs from primer extension libraries. More definitive proof, however, will come from the isolation of the β-protein and determination of the structure of its N-terminal peptide.

Whether PR65 shows any homology with other proteins that regulate phosphatase function remains to be established. Comparison with inhibitor-1 and inhibitor-2, both potent inhibitors of the type 1 protein phosphatase, did not reveal any

significant similarities. Searches, using the programs of Lipman and Pearson (1985, 1988), of the EMBL and NBRF data banks did not reveal any homologies. However, analysis of the α- and βPR65 protein sequence using the Compare and DotPlot program (Devereux et al., 1984) showed considerable internal homology (Figure 3). It was possible to identify 15 repeats on the basis of several residues that were found in identical positions in each unit as defined in Figure 3. Significantly, this structure was conserved between isotypes and across species. The consensus repeat unit sequence is shown at the bottom of the figure. If a residue occurred in seven or more of the repeats, it was then assigned to that position in the consensus. If seven of the sequences contained amino acids from the same closely related group (see figure legend for amino acid groupings), the appropriate group number was used in the consensus. The Gap program (Devereux et al., 1984) was used to calculate the percent similarity between each unit; the sequence between residues 404 and 442 of the human α-isotype was chosen as reference because it conformed most closely to the consensus. The degree of similarity ranged from 34% to 60%. Another interesting feature of the protein is the high leucine/isoleucine content (20 mol %), and that several of the conserved residues are leucyl (or isoleucyl) residues; it is possible that these hydrophobic residues are important for the association of PR65 with the catalytic subunit. Furthermore, a pseudophosphorylation site (VRRAAASKLGEFAKV) in the fifth repeat unit (underlined in Figure 3) could also be an important determinant in the mechanism of inhibition of the catalytic subunit by PR65.

The sequence data presented in this paper and the recently published structure of the catalytic subunits form the structural basis for the recent observation made by Pallas et al. (personal communication) that two of the major proteins that complex with middle T antigen, the transforming protein of polyoma virus, are the protein phosphatase 2A catalytic and PR65 subunits. Further experiments are needed to investigate whether middle T suppresses or activates the activity of the protein phosphatase. Furthermore, Virshup and Kelly (1989) found that protein phosphatase 2A is involved in the early stages of SV40 DNA replication. Whether these two observations of the involvement of protein phosphatase in viral replication and transformation are in some way connected remains to be established, but they certainly point to the significance of protein phosphatases in cellular growth control.

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Articles

Cholesterol Oxidase Catalyzed Oxidation of Cholesterol in Mixed Lipid Monolayers: Effects of Surface Pressure and Phospholipid Composition on Catalytic Activity†

Lotte Grönberg and J. Peter Slotte*
Department of Biochemistry and Pharmacy and Department of Physical Chemistry, Åbo Akademi University, SF-20500 Turku, Finland

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ABSTRACT: The catalytic activity of cholesterol oxidase from *Streptomyces* sp. in mixed monolayers of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), *N*-oleoylsphingomyelin (O-SPM), and cholesterol (CHL) has been determined at lateral surface pressures between 10 and 30 mN/m. The highest cholesterol oxidase activity (determined at 37 °C) was observed at surface pressures around 20 mN/m in a POPC/CHL monolayer (50:50 mol %). Above and below this surface pressure, the enzyme activity decreased markedly. A similar optimal activity vs surface pressure relationship was observed also for an O-SPM/CHL monolayer (50:50 mol %). The activity of cholesterol oxidase toward cholesterol in the O-SPM/CHL monolayer was, however, less than in the corresponding POPC mixed monolayer. The surface activity of cholesterol oxidase decreased markedly when the temperature was lowered to 20 °C, and hardly any enzyme activity was observed in an O-SPM/CHL monolayer at 25 mN/m or above. With a monolayer containing POPC/O-SPM/CHL (42:18:40 mol %), maximal cholesterol oxidase activity was observed at the lowest surface pressure tested (i.e., 10 mN/m), and the catalytic activity decreased markedly with increasing lateral surface pressures in the monolayer. The results of this study show (i) that the activity of cholesterol oxidase in general is highly dependent on the lateral surface pressure in the substrate membranes and (ii) that sphingomyelin, by interacting tightly with cholesterol, can prevent or restrain the accessibility of cholesterol for oxidation by cholesterol oxidase.

The characterization of the distribution of unesterified cholesterol between structures of intact cells has been a challenging and difficult task. One approach has utilized the enzyme cholesterol oxidase, which under controlled conditions is believed to oxidize cell surface cholesterol without oxidizing cholesterol located in intracellular membranes (Gottlieb, 1977; Lange & Ramos, 1983). However, it has been found that cholesterol in native membranes of cultured cells and erythrocytes is not readily accessible for oxidation by the enzyme (Gottlieb, 1977; Patzer et al., 1978; Lange & Ramos, 1983). In order to make cell cholesterol susceptible to oxidation by cholesterol oxidase, different manipulations (e.g., phospholipase C treatment, exposure to low ionic strength buffers, or sphingomyelinase treatment) have been necessary (Patzer et al., 1978; Lange & Ramos, 1983; Slotte et al., 1989). The resistance of cholesterol in native membranes to enzymatic oxidation could be due to at least two different mechanisms, both of which could operate simultaneously.

First, the resistance to oxidation could be due to the tight interaction of cholesterol with some classes of membrane

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*Address correspondence to this author at the Department of Biochemistry and Pharmacy, Åbo Akademi University.

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