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EXPRESSION, PURIFICATION AND BIOCHEMICAL COMPARISON OF NATURAL AND RECOMBINANT HUMAN NON-PANCREATIC PHOSPHOLIPASE A2

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The gene coding for human non-pancreatic phospholipase A2 (npPLA2) was cloned in a eukaryotic expression vector and transfected into chinese hamster ovary (CHO) cells. A number of cell lines stably expressing npPLA2 were obtained. Northern analysis of these cell lines showed an abundant transcript of expected size 1200 nt. The recombinant enzyme was efficiently secreted in quantities up to 400 µg npPLA2 per liter culture medium in the most productive cell lines. npPLA2 was purified to homogeneity from conditioned medium as previously described (1). The recombinant npPLA2 migrated by SDS- PAGE as a single band with an apparent mass of 14,000. The recombinant enzyme displayed the pH-optimum, calcium dependence and substrate preference that were characteristic of the human platelet and synovial fluid enzymes.

Phospholipases A2 (PLA2) are a ubiquitous family of enzymes that hydrolyze the sn-2 fatty acyl ester bond of phospholipids producing free fatty acids and lysophospholipids. PLA2s are abundant as digestive enzymes in pancreatic secretions and in the venoms of snakes and bees (2, 3). Detailed studies of these enzymes have provided the basis for our understanding of the structural and biochemical properties of PLA2s (4). On the other hand, most other PLA2s occur in trace amounts and thus to date have not been available in quantities necessary for such analysis. However, it is the non-digestive PLA2s that are fundamental to phospholipid turnover, tailoring of phospholipids, regulation of eicosanoid production, protection of membranes from peroxidation damage, and certain aspects of signal transduction, and they are therefore the focal points for many areas of research (5).

We have previously cloned the gene for a human non-pancreatic PLA2 (1). The enzyme is the major PLA2 in rheumatoid arthritic synovial fluid and is also found in human platelets

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where it is secreted when the cells are activated with physiological stimuli such as thrombin or collagen. Elevated levels of this enzyme correlates well with state of the disease in inflammatory diseases, indicating that PLA2 is involved in the pathogenesis of inflammatory diseases. This makes it an important target for drug development (6). Here we describe the production of a stable CHO cell line that secretes the PLA2 in a mature, native form. The Ca2+-dependence, pH- optimum and substrate specificity of the recombinant, platelet and synovial fluid PLA2 were identical. The ability to produce large amounts of npPLA2 should assist in our understanding of the biology and biochemistry of this enzyme, and also play a key role in the design and discovery of specific PLA2-inhibitors as potential drugs.

MATERIALS AND METHODS

Expression of npPLA2 in CHO cells Construction of the mammalian expression vectors for npPLA2 (plasmid pBG341::3.8; (1)) and for dhfr (plasmid pAdD26-1; (7)) were previously described. CHO cells deficient in the dihydrofolate reductase (*dhfr)* gene (CHO^-; (8)) were maintained at 37° C in α-MEM (minimal essential medium, GIBCO) supplemented with 10% fetal bovine serum. The cells were transfected with DNA (20μg/10^6 cells) by the $CaPO_4$ precipitation method (9) with the two plasmids pAdD26-1 : pBG341::3.8 at a ratio of 1:10. Prior to the transfection, the plasmids were linearized with restriction endonucleases *Stu*1 or *Xma*1, respectively. Cells that had incorporated DNA (expressing dhfr) were selected for growth in α-MEM without ribonucleo-sides and deoxyribonucleosides supplemented with 10% dialyzed fetal bovine serum, and then selected for amplification of the *dhfr* gene by growth in the same medium containing 30 nM methotrexate (MTX) (9). Clones derived from single cells were generated by limiting dilution.

Assay of npPLA2 activity Two assays were used to measure PLA2 activity.

Method no. 1: npPLA2 activity was routinely assayed by measuring (^3H) oleic acid released from phospholipids in labelled, autoclaved *Escherichia coli* (10). Samples were incubated with 100,000 cpm of substrate, which based on lipid analysis contained approximately 0.5 nmol of phosphatidyl-ethanolamine + phosphatidylglycerol. Using this assay we previously demonstrated that the human npPLA2 exhibits the same specific activity towards the *E. coli* substrate as the porcine pancreatic PLA2 (eg. 300 μmol/min/mg). Therefore, expression levels of the npPLA2 protein were estimated using porcine pancreatic PLA2 as a standard.

Method no. 2: npPLA2 assays using sonicated liposomes or detergent/phospholipid mixtures were performed using the Dole`s reaction procedure as previously described (11). The ^{14}C-labeled phospholipids (1-palmitoyl-2-(1-^{14}C)arachidonoyl-PC, 55 mCi/mmol or 1-palmitoyl-2-(1-^{14}C)arachidonoyl-phosphatidyl etanolamine-, 55 mCi/mmol; from NEN Research Products) were dispersed at 50 μM by either sonication in 250 mM Tris-HCl, pH 9.0 or using deoxycholate (2.5 mg/ml) in the same buffer and 10 μl of the lipid dispersion was added to the standard incubation mixture.

Northern Analysis RNA was extracted from lysed cells (12) and electrophoresed in denaturing formaldehyde agarose gels and then transferred to nylon membranes as described (1). Hybridization with random primed probes was performed as described (1). The relative amounts of transcripts were determined by densitometry (data not shown). Northern blot hybridization with a restriction fragment of the pyruvate kinase (PK) gene gives a signal in a position

corresponding to a 2400 nt transcript. The PK (housekeeping gene) transcript was used to quantify the relative amounts of RNA in each lane.

Genomic Southern Analysis High molecular weight genomic DNA was prepared from cells grown in monolayer cultures, using standard techniques. Genomic DNA was analysed as described, as well as hybridization with random primed probes (1).

Peptide mapping of natural and recombinant npPLA2s Synovial fluid and recombinant human npPLA2s were iodinated by the chloramine T method in the presence of 0.1% SDS using carrier-free ^{125}iodine. Free iodine was removed by desalting on a Bio-Gel P-6DG (Bio-Rad) column pre-equilibrated in 0.1% SDS. The specific radioactivities of both the labeled proteins were approximately 5000 Ci/mmol. The labeled proteins (12 µCi) were concentrated to dryness in a Speed Vac Concentrator. Each sample was suspended in 50 µl of 10 mM dithiothreitol (DTT) in 8 M urea, 0.2 M N-ethyl morpholine acetate, pH 8.6 and incubated at 37° C for 1h. 11 µl of 100 mM iodoacetic acid in 4 M urea, 0.2 M N-ethyl morpholine acetate pH 8.6 was added and samples were incubated at room temperature for 30 min in the dark. The alkylation reaction was quenched with electrophoresis sample buffer containing 2% 2- mercaptoethanol and samples subjected to SDS-PAGE on 14% gels. Labeled proteins were visualized by autoradiography and gel pieces corresponding to the proteins of interest excised with a razor blade. Gel slices containing labeled protein were subjected to further analysis by Cleveland mapping (13). Each gel slice was overlayed with 20 µl of 0.5 mg/ml protease (*Staph. aureus* V8 protease, chymotrypsin or papain) and cleavage products separated by SDS-PAGE on 16% gels. Gels were dried and radioactivity visualized by autoradiography.

RESULTS AND DISCUSSION

Expression of the npPLA2 gene in CHO cells Recently we purified npPLA2 from blood platelets and synovial fluid from rheumatoid arthritis pasients to homogeneity and sequenced the aminoterminal amino acids. This information was used to clone the gene from a genomic library using degenerate probes based on the sequence from the two proteins, which were identical. The gene was contained within a 3.8 kb *Eag*1/*Hin*d3 fragment and was shown to be functional using transient expression in simian virus 40 (SV40) transformed African Green monkey kidney (COS) cells as a test system. PLA2 was secreted into the medium and levels of activity corresponding to approx. 3 ng per ml medium were observed (1).

To obtain enough homogeneous enzyme for more extensive biochemical characterization, we produced a number of transfected cell lines that expressed 5-100 times as much of recombinant enzyme, thus providing an adequate source for its purification. The expression vector used for transfection experiments, pBG341::3.8, uses the adenovirus-2 major late promoter (AMLP) with the SV40 enhancer (14). Along with pBG341::3.8, CHO$^-$ cells were cotransfected with a plasmid that contains the *dhfr* gene, using the dhfr system first as a method for selecting cells that had incorporated plasmid DNA, and then as a method for amplifying the gene. 96 colonies from the initial transformation were picked and grown in 48-well tissue culture dishes. As cells reached confluence, the supernatants were collected and 5 µl aliquots tested for PLA2 activity (method 1). Observed levels of activity ranged from background which corresponded to about 3.5% of the input counts to the high producing lines that hydrolyzed 40 % of the input counts. Among the first 66 samples that were assayed, 25 showed activities ranging

from background (bg) to 3 times bg, 24 showed activities that were 4-6 times bg, 15 were 7-9 times bg and 2 were 12 times bg. The five highest producing cell lines (I-B2, II-A8, II-B1, II-D7, II-F7) were used for further experiments. By diluting the samples into linear range of the PLA2 assay and standardizing the counts with values observed for known amounts of pancreatic PLA2, we determined that clones II-A8, II-D7 and II-F7 produced 300, II-B1 produced 105, and I-B2 produced 95 ng of PLA2 per ml of culture supernatant.

The five highest producing lines were subjected to a second round of selection using MTX to drive amplification of the *dhfr* and PLA2 genes. 180 cells from each line were plated in individual wells of 96-well tissue culture plates in selective medium containing 30 nM MTX. The medium was changed every 5-7 days. Surviving cells after four weeks of treatment, were subcloned and again tested for PLA2 production. Activity measurements of stable cell lines after amplification indicated that the levels of PLA2 activity had increased 2-3 fold over levels observed in the parental lines.

After the amplification process only 3-17% of the wells contained viable cells, with one of the five lines being unable to grow in 30 nM MTX. One explanation for the low survival rate is that the number of cells plated (180/line) is close to the statistical limit for amplification of transfected DNA to occur. This assumption is based on previous estimates which indicated that the amplification event takes place at a frequency of about 10^{-3} in CHO cells (15).

Northern analysis of npPLA2 and dhfr transcripts A stable CHO cell line (II-F7) expressing the recombinant npPLA2 and the *dhfr* gene (before amplification), was used for isolation of total RNA. The RNA was separated in denaturing agarose gels, transferred to nylon membranes, and subjected to Northern analysis. Fig. 1A shows the result of hybridization with ^{32}P-labelled npPLA2 cDNA (1).

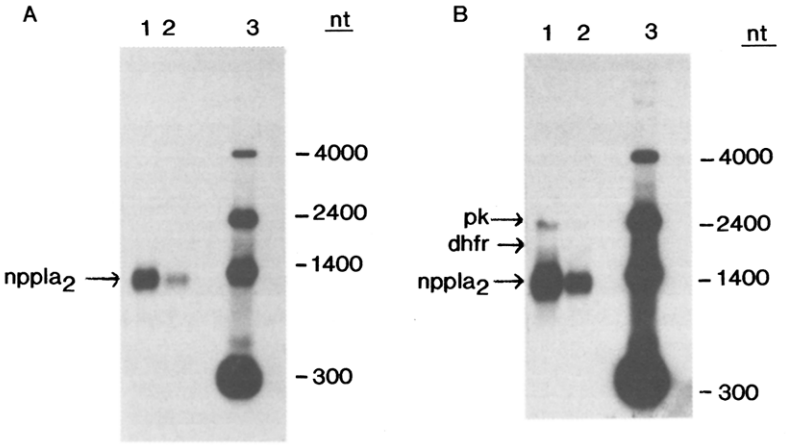

FIGURE 1: *Hybridization analysis of RNA extracted from CHO cells using npPLA2, dhfr and PK probes.* Total RNA was isolated from CHO cells transfected with pBG341::3.8/pAdD26-1 (lanes 1 and 2) and molecular weight marker (lane 3, RNA ladder, BRL). Amount of total RNA in each lane, lane 1:2.5 µg and in lane 2: 0.5 µg. A, Northern blot probed with ^{32}P-labeled npPLA2 cDNA. B, Northern blot probed with ^{32}P-labeled npPLA2 cDNA, *dhfr* and PK.

A single 1200 nt transcript was observed, which by size is consistent with it starting from the adenovirus late promoter and ending with termination sequences from within the npPLA2 gene. Fig. 1B shows the result of hybridization with a ^{32}P-labelled 400 bp *dhfr* restriction fragment and a ^{32}P-labelled PK restriction fragment, in addition to the labelled npPLA2 gene. The *dhfr* and PK probes hybridized to 1800 nt and 2400 nt RNAs, respectively, which agree with the known sizes of the *dhfr* and PK transcripts. The more intense signal for npPLA2 RNA relative to the signal we observed for the PK RNA, indicates that the npPLA2 gene is efficiently transcribed. Densitometer scans of the exposed films showed that the npPLA2 transcript was more than 50 times as abundant as the *dhfr* transcript. This result is consistent with the large molar excess of npPLA2 DNA used in the transfection, plus the fact that the npPLA2 construct contained the SV40 enhancer, which may have further increased its expression.

Genomic DNA analyses Genomic DNA was isolated from the same cell line used for quantification of npPLA2 mRNA. The DNA was digested with restriction endonucleases *Bam*H1 or *Eco*R1 and subjected to Southern blotting. The DNA fragments were separated by agarose gel electrophoresis, transferred to a nylon membrane and probed with ^{32}P-labelled npPLA2-cDNA. In the *Bam*H1 digest, a single 3400 bp fragment hybridized with the probe. This fragment can be assigned to the transfected DNA, since the genomic copy of the DNA would give rise to two fragments of sizes 2200 bp and 4300 bp (1). In the *Eco*R1-digest two fragments (1800 and 1000 bp) were recognized by the labelled probe. These fragments also originate from the transfected DNA, since *Eco*R1 digestion of the genomic DNA should yield a single fragment of 2500 bp.

Purification and characterization of recombinant npPLA2 Recombinant npPLA2 was purified from conditioned medium of CHO cells that were transfected with pBG341::3.8 essentially as described previously for the purification of the platelet and synovial fluid enzymes. Briefly, the conditioned medium was acidified with H_2SO_4 and acid soluble proteins dialyzed overnight against 200 mM NaCl, 50 mM sodium acetate, pH 4,5. The dialysate was subjected to sequential chromatography steps on ion-exchange (Fast S Sepharose), gel filtration (Sephadex G50) and reverse phase HPLC (C4) columns. The enzyme at each step behaved like the natural product, eluting from Fast S with 1 M salt, from gel filtration with an apparent mass of 13,000, and from the HPLC column with 35% acetonitrile. The overall yield was 40%. As previously observed with the platelet and synovial fluid enzymes, the recombinant npPLA2 exhibited fluctuations in activity as a result of the state of the protein. Acid treatment resulted in a slight increase in activity of about 35%. High salt treatment produced a transient loss of 80% of the activity which was restored after gel filtration. The purified protein migrates on SDS-gels with an apparent mass of 14,000.

The activity of the recombinant npPLA2 and of the corresponding natural human PLA2s from platelets and synovial fluid were characterized with respect to pH, calcium and substrate preferences using the PLA2 assay. Fig. 2A shows the effect of pH on activity. All three preparations exhibited hyperbolic responses with optimal activity at a pH of 8-10. Interestingly,

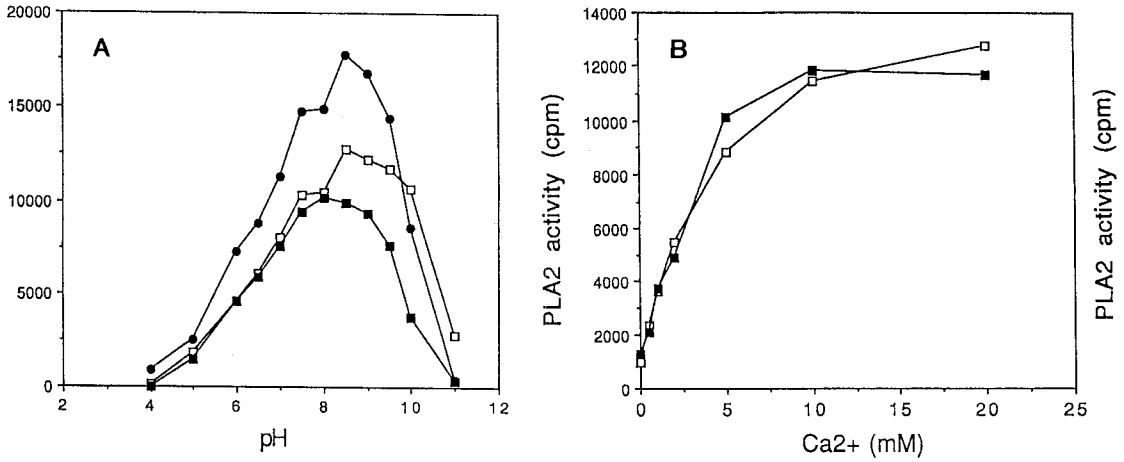

FIGURE 2: *pH and Ca2+-dependence of natural and recombinant npPLA2.* HPLC-purified recombinant (■), platelet (□) or RA synovial fluid (●) npPLA2 (10-20 pg) was incubated with (3H)oleatelabeled *E. coli* in 10 mM CaCl2, 100 mM NaCl, 25 mM piperazine, 25 mM glycylglycine at indicated pH values (A) or in 250 mM Tris-HCl, pH 9.0, with increasing amounts of CaCl2 (B) using the standard incubation conditions described in MATERIALS AND METHODS.

the profile we observed for the synovial fluid enzyme differed from the data published (16) for a synovial fluid PLA2 they characterized after purification by CM-Sephadex, gel filtration on G75, and preparative SDS-PAGE. Their final preparation had a specific activity of 4 μmol/min/mg protein. While the sample was gel purified, the complexity of their pH profile suggested that the preparation might be a mixture of PLA2s. This possibility was supported in a separate study where multiple PLA2s were detected in synovial fluid (17). In this latter study, the ratios of the isozymes were found to be dependent on the type of arthritis and the disease state.

Fig. 2B shows the calcium dependence of the platelet and recombinant PLA2s. Both enzymes displayed a dose dependent increase in activity as a function of calcium concentration that started to level off at 5 mM Ca^{2+} and reached maximal activity with 10 mM Ca^{2+}. The requirement of high levels of calcium for activity supports an extracellular role for the enzyme. While the same results were obtained for the synovial fluid enzyme, the experiment was run separately and thus the data is not included.

The most striking property of the recombinant npPLA2 which was also common to the platelet and synovial fluid enzymes was its unusual substrate preference (see Table 1). In the four assay sytems tested, we observed a 600-fold span in apparent activities. The enzyme showed a great preference for the *E. coli* substrate where the apparent activity was 300 μmol/min/mg protein and was least active in phosphatidyl choline (PC) liposomes where the apparent specific activity of the PLA2 was only 0.5 μmol/min/mg protein. In a direct comparison of its activity towards phosphatidyl etanolamine (PE) and PC in deoxycholate containing mixed micelles we observed activities of 25 and 8 μmol/min/mg, indicating a preference for PE.

TABLE 1

Hydrolysis of various phospholipid substrates by human platelet, RA synovial fluid and recombinant npPLA2

PLA2 source	Substrate			
	*E. coli**	PE/DOC	PC/DOC	PC liposomes
			μ mol/mg/min	
Human platelet	300	29	7	0.4
RA synovial fluid	300	20	9	0.6
Recombinant	300	25	8	0.5

DOC, sodium deoxycholate; PC, phosphatidylcholine; PE, phosphatidylethanolamine

* The protein concentration of HPLC-purified human platelet, rheumatoid arthritic (RA) synovial fluid and recombinant npPLA2 (prepared for the present study in amounts of less than 0.2 μg of total protein) was estimated from *E. coli*PLA2 assays using porcine pancreatic PLA2 as a standard.

To evaluate the structural identity of recombinant npPLA2 with natural npPLA2, ^{125}I-labeled npPLA2s were digested with proteases and the cleavage products separated by SDS-PAGE. As shown in Fig. 3, the cleavage profiles for *Staph. aureus* V8 protease, chymotrypsin and papain were identical for both the natural and the recombinant npPLA2 confirming that they are the same protein.

In conclusion, we have shown that the genomic sequence of the npPLA2 gene can be expressed in CHO cells to produce mature npPLA2 that is secreted from the cells. Clones have been isolated that produce over 400 μg of enzyme per liter culture medium, providing a simple source from which large quantities of enzyme can be obtained. The biochemical and structural characteristics of recombinant and native npPLA2 were identical.

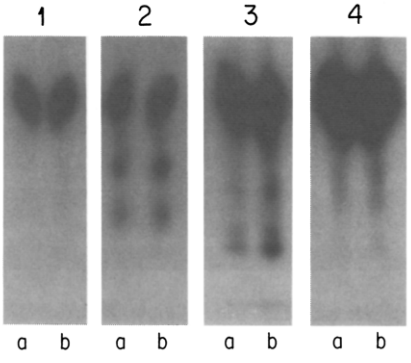

FIGURE 3: *Peptide mapping of human synovial fluid (a) and recombinant (b) npPLA2s.* npPLA2s were labeled with ^{125}iodine and subjected to Cleveland mapping using control buffer (1), *S. aureus* protease (2), chymotrypsin (3) and papain (4).

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