Multiple C-terminal serine phosphorylation accompanies both protein kinase C-dependent and -independent activation of cytosolic 85 kDa phospholipase A₂ in macrophages

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Exposure of mouse macrophages to either phorbol ester or certain bacteria was previously shown to cause increased phosphorylation of the cytosolic 85 kDa phospholipase A₂ as well as a stable increase in its catalytic activity. We have now attempted to map the major phosphorylation sites on the enzyme in such cells. Phosphorylation occurred on serine residues without a detectable increase in either phosphothreonine or phosphotyrosine. After CNBr cleavage five fragments showed increased ³²P labelling. Among those the most heavily labelled fragment was identified as the most C-terminal (residues 698–749), containing six serine residues. This was true whether phorbol ester or bacteria, causing protein kinase C-independent phospholipase A₂ activation, was used as stimulus. The heavy phosphorylation of the most C-terminal fragment and an analysis of tryptic

peptides derived from it suggested that more than one of the six serine residues became phosphorylated. Smaller increases also occurred in other CNBr-cleaved fragments from the C-terminal part of the protein, including that carrying Ser-505, a known target of the mitogen-activated protein kinase ERK-2 (extracellular-signal regulated kinase). Dexamethasone treatment (1–100 nM for 20 h), which was earlier shown to dose-dependently down-regulate the 85 kDa phospholipase A₂ and its activation by phorbol ester and zymosan, was here shown also to counteract the protein kinase C-independent activation and arachidonate release elicited by bacteria. It remains to be determined whether all phosphorylation sites are equally affected under those conditions.

INTRODUCTION

The earliest step in the arachidonate cascade is that in which arachidonate is mobilized from its stores in membrane phospholipids. In macrophages there is considerable evidence [1–5] that this step is executed by the intracellular 85 kDa arachidonate-preferring phospholipase A₂ (PLA₂-85) [6–8]. This enzyme can be regulated by changes in [Ca²⁺] at submicromolar level [2,7,9–12] and by changes in phosphorylation [2–4,13,14]. Our earlier studies have shown that the latter activation can be achieved via one or more phorbol ester-sensitive isoforms of protein kinase C [1,2,15,16]. Evidence was also provided that the regulatory phosphorylation(s) is/are not performed directly by such a kinase [7]. Furthermore a signalling pathway independent of protein kinase C, by which certain bacteria cause increased phosphorylation and activation of PLA₂-85, was also demonstrated in macrophages [4]. In other cell types, too, signalling initiated by agents such as tumour necrosis factor α [17], thrombin [14] and epidermal growth factor [18] have been shown to lead to increased phosphorylation and activation of PLA₂-85; studies *in vitro* as well as in a transfected cell line have suggested that phosphorylation of Ser-505 by the mitogen-activated protein (MAP) kinase ERK-2 (extracellular-signal regulated kinase) accounts for this activation (reviewed in [19]).

The present report identifies serine residues in the C-terminal region of PLA₂-85, distinct from Ser-505, as being the most heavily phosphorylated in macrophages responding to either

phorbol ester or bacteria. It is also demonstrated by present and previous [20] data that dexamethasone treatment attenuates or abolishes arachidonate release and PLA₂-85 activation elicited by both types of agent.

EXPERIMENTAL

Isolation, culture and ³²P-labelling of macrophages

Resident macrophages were isolated from peritoneal cells obtained from female outbred NMRI mice (ALAB, Stockholm, Sweden, or Bommice, Copenhagen, Denmark) by adherence to plastic 80 cm² tissue culture flasks, with approx. 3.6 × 10⁷ peritoneal cells per flask. The cells were incubated in an atmosphere of air/CO₂ (19:1); non-adherent cells were removed 2 h after plating, as described previously [15]. To each flask was added 10 ml of Medium 199 containing 10% (v/v) heat-inactivated fetal bovine serum, and the cells were cultured for 18–20 h. The cells were thereafter washed with Dulbecco's PBS and equilibrated for 30 min in 10 ml of serum-free Medium 199, before being washed and transferred to phosphate-free medium [16] (10 ml per flask). After the addition of ³²PO₄ (30 mCi per flask), labelling was continued for 90 min. Where indicated, cultures were exposed during the latter part of the labelling period to either PMA (150 nM for 10 min) or bacteria (10⁷/ml for 60 min), specified in each case and obtained as described previously [4]. At the end of the experiment the cells were washed five times with Dulbecco's PBS and were then scraped off and homogenized in

Abbreviations used: MAP kinase, mitogen-activated protein kinase/microtubule-associated protein 2 kinase (p44/p42; ERK-1/ERK-2); PLA₂-85, 85 kDa cytosolic phospholipase A₂ (also called cPLA₂).

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buffer consisting of 80 mM KCl, 2.5 mM dithioerythritol, 1 mM EDTA, 10 mM Hepes, 10 mM NaF and 100 μ M ammonium vanadate, pH 7.4 (buffer A).

Cell fractionation and isolation of PLA₂-85

Homogenate from two flasks was combined and centrifuged at 700 *g* for 10 min and the resulting supernatant was further centrifuged at 100 000 *g* for 60 min to obtain a cytosol fraction and a membrane pellet. To the cytosol fraction was then added 10% (v/v) glycerol. The cytosol fraction was run on either a column (1 cm $\times$ 48 cm) of Sephadex G-200 superfine (Pharmacia), equilibrated in buffer B [buffer A containing 10% (v/v) glycerol but lacking dithioerythritol], or a Mono Q HR 5/5 column (Pharmacia), eluted with a non-linear gradient of NaCl (0–0.4 M) in buffer B. Aliquots of the cytosol fraction or of fractions from the gel, or ion-exchange, chromatography were assayed for PLA₂-85 activity (see below). Fractions containing PLA₂-85 were combined and subjected to hydrophobic interaction chromatography on a phenyl-Superose HR 5/5 column (Pharmacia), as described [7]. In this and the preceding chromatographic step, tubes used to collect fractions were first filled and preincubated with a solution of BSA (1 mg/ml) for 20 h; 0.1 mg of albumin was then added to each tube before use, to minimize losses of PLA₂-85 by adsorption. The recovery of PLA₂-85 enzyme activity was at least 80% in each of the chromatographic steps. From relevant fractions after the hydrophobic interaction, chromatography aliquots were taken for analysis by SDS/PAGE [6.5% (w/v) acrylamide gel] [21] and autoradiography, to assess the phosphoprotein purity of PLA₂-85 (see Figure 1, upper panel), while the major part of the fractions were combined and subjected to precipitation with trichloroacetic acid.

Separation and analysis of CNBr-cleaved fragments from PLA₂-85

Non-labelled PLA₂-85, isolated from J774 cells as described previously [7], was cleaved by treatment with CNBr [1% (w/v) CNBr in formic acid/water (7:3, v/v) for 20 h]. Fragments were separated by HPLC (System Gold, Beckman) in reverse-phase mode on a 4 mm $\times$ 250 mm LiChrocart RP-18 column (Merck) with a linear gradient (0–70%) of acetonitrile in trifluoroacetic acid/water (1:2000, v/v). After 10 initial fractions of 0.9 ml had been collected, the fraction volume was 0.3 ml. The N-terminal sequence of a number of separated peptides was then determined by automated sequential Edman degradation (BM unit, Lund University, Lund, Sweden) to identify their position within the protein. Some of these sequences have been briefly reported earlier [7]. Trichloroacetic acid-precipitated, ³²P-labelled PLA₂-85 from macrophages, carried through the two-step purification procedure described above, was also cleaved and the fragments were separated by the same procedure. ³²P-labelled CNBr fragments were further characterized by SDS/PAGE [16% (w/v) acrylamide gel] followed by phosphorimager analysis (FUJI Bas 2000) or autoradiography. The recovery of trichloroacetic acid-precipitable ³²P through CNBr cleavage and reverse-phase HPLC was 60–75%.

Analysis of tryptic peptides and phospho amino acids

Tryptic peptides were prepared from some of the ³²P-labelled CNBr fragments by treatment with trypsin [80 μ g in 0.1 M NH₄HCO₃/acetonitrile (8:2, v/v) for 20 h at 37 °C]. These peptides were separated by reverse-phase HPLC as described above (except that fractions of 0.5 ml were collected throughout) and their ³²P content was determined. The recovery of radioactivity was at least 80%.

For identification of phospho amino acids, trichloroacetic acid-precipitated, ³²P-labelled PLA₂-85 (with added phosphoserine, phosphothreonine and phosphotyrosine) was hydrolysed in 6 M HCl at 105 °C for 2.5 h, under vacuum. After freeze-drying, separation was achieved by isocratic HPLC on a strong-anion-exchange column (4 mm $\times$ 250 mm Nucleosil 5SB, Macherey-Nagel), with 40 mM KH₂PO₄, pH 2.8, as eluent. This is a modification of a method described previously [22] and led to excellent separation not only of the three phospho amino acids, but also between each of them and PO₄ (see Figure 1, lower panel).

Assay of PLA₂-85

PLA₂-85 was assayed by the method described earlier [2]. 1-Stearoyl-2-[1-¹⁴C]arachidonoyl phosphatidylcholine (Amersham International; 80–100 pmol per incubation) was used as substrate. Aliquots of the cytosol fraction containing PLA₂-85 were incubated in the presence of substrate, 1.4 mM CaCl₂ (approx. 400 μ M free Ca²⁺) and BSA (0.19 mg/ml) in a buffer containing 80 mM KCl, 10 mM Hepes and 1 mM EDTA, pH 7.4. The final volume was adjusted to 525 μ l. After incubation for 30 min at 37 °C the reaction was stopped by the addition of 1.5 ml of chloroform/methanol/HCl (2:1:0.1, by vol.), to which 0.05% carrier lipids had been added. After centrifugation the lipid phase was applied to a silicic acid column equilibrated in chloroform. Non-esterified fatty acids were eluted with 1.0 ml of chloroform, and phospholipids with 2.5 ml of methanol.

Release of [³H]arachidonate

During an overnight incubation of approx. 3×10^6 peritoneal macrophages per well, in 6-well 35 mm culture dishes, they were labelled with 0.5–1.0 μ Ci [³H]arachidonic acid (Amersham International; 212 Ci/mmol) per well and, when indicated, dexamethasone was included in the medium. At the end of the experiment the culture medium was collected and the cells were scraped off the dish in 0.1% (w/v) Triton X-100. The collected medium and the cell extract were centrifuged for 5 min at 2500 *g*. The release of incorporated [³H]arachidonate was determined from the radioactivity in the culture medium and the total radioactivity in the cell extract and medium, and was corrected for the basal release in control cultures. Dexamethasone by itself showed no detectable effect on the incorporation of [³H]arachidonate or the low (at most 2%) basal release in unstimulated cultures. Protein was determined by the method of Bradford [23], with BSA as standard.

RESULTS AND DISCUSSION

It was demonstrated previously that short-term treatment of macrophages with either PMA or zymosan leads to persistent activation of PLA₂-85 as well as an increase in its phosphorylation. Both of these effects could be reversed by phosphatase treatment of the enzyme, strongly indicating that the increase in phosphorylation was responsible for the increase in catalytic activity [2]. It was also shown in the same study that PLA₂-85 can be obtained by a two-step purification procedure as a virtually homogeneous phosphoprotein from a cytosolic fraction of ³²P-labelled peritoneal macrophages [2]. Figure 1 (upper panel) also confirms that the modified procedure employed in most of the current experiments yielded a single radiolabelled phosphoprotein, co-eluting with PLA₂ activity (results not shown) and co-migrating exactly with PLA₂-85 on SDS/PAGE as a 100 kDa protein.

Phospho amino acid analysis of PLA₂-85 from ³²P-labelled,

Figure 1 Phosphoprotein purity and phospho amino acid analysis of PLA₂-85

Upper panel: PLA₂-85 was purified from cytosol fractions prepared from untreated (lanes 1–4) or PMA-stimulated (lanes 5–8), ³²P-labelled macrophage cultures, by consecutive Mono Q and phenyl-Superose chromatography (see the Experimental section). Four consecutive phenyl-Superose fractions, containing the peak of PLA₂-85 activity, were analysed by SDS/PAGE [6.5% (w/v) acrylamide gel] and autoradiography. Lower panel: a mixture of phosphothreonine (P-Thr), phosphoserine (P-Ser), phosphotyrosine (P-Tyr) and [³²P]P_i (P) was subjected to isocratic ion-exchange HPLC as described in the Experimental section. Absorbance at 210 nm (solid line) and radioactivity (dotted line) were monitored continuously. Radioactivity data for collected fractions from a second run, where the sample contained phospho amino acid standards and a hydrolysate of isolated PLA₂-85 from ³²P-labelled and PMA-stimulated macrophages, are shown superimposed (▲).

PMA-stimulated cells showed that phosphorylation occurred on serine residues, with no detectable labelling of either phosphotyrosine or phosphothreonine residues (Figure 1, lower panel). To gain information on the number of phosphorylation sites and their localization, CNBr fragments from PLA₂-85 were prepared. From the amino acid sequence of the mouse enzyme [6] we can deduce that 25 fragments and one free serine residue would be formed. Of the fragments, 18 would carry one or more serine residues. Two of these fragments, both from the C-terminal part, consist of approx. 100 amino acid residues, whereas all others would be 53 residues long or less. CNBr fragments of PLA₂-85

Figure 2 ³²P content of CNBr fragments from PLA₂-85 and their positions in the linearized protein

Upper panel: PLA₂-85 was isolated from ³²P-labelled control (●) or PMA-stimulated (■) macrophages as described in the Experimental section. After CNBr cleavage fragments were separated by reverse-phase HPLC. Peaks are labelled A–F. Lower panel: a schematic representation of the primary structure of mouse PLA₂-85 [6] indicating CNBr cleavage sites (arrows). The labelling of some of the CNBr fragments (A, E, F) corresponds to peaks in the upper panel. The amino acid sequence of most of fragment E, encompassing the six C-terminal serine residues, which constitute major phosphorylation sites, is also depicted. Here the bars indicate tryptic cleavage sites. Residues 698–709 contain no serine and were therefore omitted from the lower panel.

isolated from J774 cells and from ³²P-labelled macrophages were both separated identically. The former were identified by N-terminal sequencing; the latter were analysed by SDS/PAGE or subjected to further cleavage.

As shown in Figure 2 (upper panel), six ³²P-labelled peaks were more or less well resolved by reverse-phase HPLC. Peak E was by far the largest in terms of ³²P content, but seemed unusually broad for a single homogeneous fragment. However, N-terminal sequencing revealed that the most C-terminal fragment in PLA₂-85 (residues 698–749) eluted broadly over several fractions and in

Table 1 SDS/PAGE and phosphorimager analysis of CNBr fragments

Selected fractions from the chromatography shown in Figure 2 (upper panel), in which CNBr fragments from ^{32}P -labelled PLA₂-85 from control and PMA-stimulated cultures were separated, were analysed by SDS/PAGE [16% (w/v) acrylamide gel] as described in the Experimental section; the gels were then dried and subjected to phosphorimager analysis. The apparent size of bands in the peak fractions, as judged from their electrophoretic mobility, were: Fraction 23 (lower band), 4 kDa; (upper band), 12 kDa; fraction 27, 6.5 kDa; fraction 36, 4.5 kDa; fraction 38, 15 kDa. Data show band intensities (arbitrary units) and represent means $\pm$ S.E.M. ($n = 3$).

Peak	Fraction	Apparent size (kDa)	Band intensity (arbitrary units)	
			Control	PMA
A	23 (lower)	4	25 $\pm$ 1	94 $\pm$ 6
	23 (upper)	12	90 $\pm$ 20	60 $\pm$ 1
B	27	6.5	16 $\pm$ 10	129 $\pm$ 22
E	36	4.5	75 $\pm$ 13	703 $\pm$ 98
F	38	15	148 $\pm$ 16	395 $\pm$ 11

Figure 3 Analysis of ^{32}P -containing tryptic peptides from the most heavily labelled CNBr fragment

Tryptic peptides were prepared from fractions 34–36 [peak E, Figure 2 (upper panel); PMA-stimulated sample] and were then separated by reverse-phase HPLC as described in the Experimental section. Peaks obtained are numbered 1–4. The results shown are representative of three separate experiments.

precisely the same position as peak E. Furthermore peak F was found to co-elute precisely, and as a sharp peak, with one of the two big CNBr fragments from the C-terminal part (residues 494–598). Sequence data also showed that one additional fragment from the C-terminal part of PLA₂-85 (residues 418–467) co-eluted with peak A. This is indicated schematically in Figure 2 (lower panel).

The separated peaks of CNBr fragments (Figure 2, upper panel) were then analysed by SDS/PAGE and phosphorimager analysis (Table 1). Peak A was found to contain two fragments differing in size, whereas peaks B, E and F showed one predominant band each. The size of the most C-terminal fragment (peak E; 6 kDa according to amino acid sequence) corresponded quite well to the migration of the major ^{32}P -labelled band on the gel, whereas the fragment in peak F migrated slightly more

Figure 4 A comparison of protein kinase C-dependent and -independent phosphorylation patterns of PLA₂-85

PLA₂-85 was isolated from ^{32}P -labelled macrophages stimulated with either PMA (■, broken line) or bacteria (*G. vaginalis*) (●, solid line), as described in the Experimental section. After CNBr cleavage fragments were separated by reverse-phase HPLC. Peaks are labelled as in Figure 2.

slowly than its expected size (12 kDa). The faster-moving band in peak A is very likely to represent fragment 418–467, in accordance with sequence data, whereas the slower-moving band is suspected, but not proved, to be fragment 600–696, the only additional fragment of appropriate size (Figure 2, lower panel). The former increased somewhat in response to PMA, whereas the latter band showed no increase in phosphorylation (Table 1). It is also evident that the most pronounced increases occurred in peaks B and E. Peak B presented as a single band whose identity is uncertain, whereas the sometimes less distinct and also less reproducible peaks C and D showed no distinct bands in the appropriate size range, and therefore might be the result of incomplete CNBr cleavage.

Peak E, containing the C-terminal fragment (698–749), was further cleaved by trypsin treatment. Such treatment would be expected to generate four serine-containing tryptic peptides (Figure 2, lower panel), three of which would contain a single serine residue and the fourth would carry three such residues. Analysis of the tryptic peptides led to the separation of four ^{32}P -labelled peaks, one of which was clearly more heavily labelled than the others (Figure 3). This would be consistent with the phosphorylation of more than one of the six C-terminal serine residues in PLA₂-85 (Figure 2, lower panel).

So far, only the phosphorylation pattern associated with protein kinase C-mediated activation had been investigated. Because certain bacteria, on interaction with macrophages, cause the activation of PLA₂-85 via signalling independent of phorbol ester-sensitive isoforms of protein kinase C [4], we decided to compare the phosphorylation pattern elicited by such activation with that elicited by PMA. Stimulation by either a Gram-positive (*Gardnerella vaginalis*) or a Gram-negative (*Fusobacterium nucleatum*) bacterial species was found to give results very similar to those obtained with PMA; i.e. only serine phosphorylation could be demonstrated, and the C-terminal CNBr fragment (peak E) showed the same predominance with respect to ^{32}P labelling (Figure 4). Thus great similarity in ^{32}P -labelling of

Table 2 Inhibition by dexamethasone of the activation of PLA₂-85 in response to bacteria

Macrophages cultured for 20 h in the presence or absence of 10 nM dexamethasone (Dex) were exposed to bacteria (10^7 /ml) for 45 min. After the removal of medium, cells from sets of three dishes were combined, homogenized and fractionated into a membrane pellet and a cytosol fraction. The cytosol fraction was then assayed for PLA₂-85 activity and the protein content was determined as described in the Experimental section. PLA₂-85 activity in non-treated cells (control, —Dex), which amounted to 27.6 ± 2.9 pmol/30 min per mg of protein, was set to 100% and the results represent means $\pm$ S.E.M. for four separate experiments.

	PLA ₂ -85 activity (% of control)	
	—Dex	+Dex
Control	100	36 $\pm$ 14
<i>F. nucleatum</i>	166 $\pm$ 23	38 $\pm$ 16
<i>P. acnes</i>	175 $\pm$ 19	34 $\pm$ 4

Figure 5 Dose-dependent inhibition by dexamethasone of arachidonate release in response to bacteria

Macrophages were incubated with dexamethasone for 18–20 h and were then exposed to either *F. nucleatum* (●) or *P. acnes* (○) at 10^7 bacteria/ml for 60 min. The release of radiolabel, corrected for the release in control cultures not exposed to bacteria, is expressed as a percentage of the release in the absence of dexamethasone. The latter amounted to approx. 15% of total cellular ^3H , or 21200 ± 2800 d.p.m./mg of protein (mean $\pm$ S.E.M.; $n = 7$). The results shown are means with positive or negative S.E.M. for seven separate experiments.

CNBr fragments in cells responding to PMA and bacteria respectively was a striking feature despite the clear difference in dependence on protein kinase C. This of course raises the possibility of converging signal chains. Additional evidence, consistent with such converging chains, was obtained by using macrophages pretreated for 20 h with dexamethasone. We have previously shown that this treatment not only down-regulates the PLA₂-85 protein but also greatly impairs its protein kinase C-mediated activation [20], with only very minor effects on the phorbol ester-binding isoforms of this kinase. Table 2 shows that the protein kinase C-independent activation elicited by bacteria was also severely inhibited. Furthermore, as shown in Figure 5, treatment with dexamethasone markedly and dose-dependently reduced the release of arachidonate elicited from intact cells by both a Gram-negative (*F. nucleatum*) and a Gram-positive (*Propionibacterium acnes*) bacterial species. These findings there-

fore suggest that the target for dexamethasone might be a downstream event involved in both PMA- and bacteria-induced signalling.

Previous studies have led to the proposal that Ser-505, surrounded by a consensus sequence for the p42/p44 proline-directed MAP kinases, would constitute a critical phosphorylation site for the regulation of PLA₂-85 [24,25]. Ser-505 is located in peak F, as one of 11 serine residues in this fragment. The same fragment also contains Ser-542, which is surrounded by basic residues and therefore is a potential substrate for other kinases such as protein kinase C. Peak F does indeed show increased labelling in PMA-stimulated cells, but it is also labelled in resting cells. Leslie and co-workers [3] previously provided evidence for multiple phosphorylation sites on PLA₂-85 in mouse macrophages, findings that are fully compatible with the results presented here. However, no information was provided in the earlier study [3] with regard to the localization of the sites. Our results indicate that the most prominent phosphorylation events occur among the six C-terminal serine residues. In addition the other fragments that we have identified and that show increased ^{32}P labelling in stimulated cells emanate from the C-terminal half of the protein. Recently, serine residues were mapped that show increased phosphorylation in response to the protein phosphatase inhibitor okadaic acid in recombinant human PLA₂-85 expressed in insect cells [26]. They were identified as Ser-437, Ser-454, Ser-505 and Ser-727. Interestingly, all of them are on CNBr fragments that in our study on mouse macrophages showed increased ^{32}P labelling in response to agonist stimulation; i.e. Ser-437 (peak A), Ser-505 (peak F) and Ser-727 (peak E). Ser-454 is not present in the mouse enzyme. Finally, whereas a severalfold increase in the phosphorylation of a single serine residue (Ser-727) among the six most C-terminal ones was reported for the recombinant human enzyme [26], our results suggest that several of these residues act as phosphorylation sites in mouse macrophages (compare Figures 2 (lower panel) and 3).

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