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In Vitro Protein Kinase C Phosphorylation Sites of Placental Lipocortin†

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ABSTRACT: Human placental lipocortin is a high-affinity substrate for rat brain protein kinase C in vitro with phosphorylation occurring on serine and threonine residues in a ratio of approximately 2 to 1. Comparison of the ability of various N-terminal-truncated derivatives of lipocortin to serve as phosphorylation substrates, and direct analysis of the N-terminal peptides cleaved from ^{32}P-labeled lipocortin, indicated that threonine-24, serine-27, and serine-28 were the phosphorylation sites. The possibility is discussed that a lysine residue near the carboxy side of the phosphorylation site was involved in lipocortin interaction with the catalytic site of protein kinase C.

Covalent modification of proteins by phosphorylation is one of the primary mechanisms through which extracellular signals modulate cellular function. Protein–tyrosine kinases and protein kinase C (PKC)[1] are implicated in a number of cellular events, including regulation of cell replication. The identification of cellular substrates for these kinases is an active area of current research. Recently, lipocortin (Wallner et al., 1986) has been identified as a high-affinity substrate for the EGF-stimulated protein–tyrosine kinase (De et al., 1986; Pepinsky & Sinclair, 1986; Haigler et al., 1987; Giugni et al., 1985; Sawyer & Cohen, 1985) and for PKC (Summers & Creutz, 1985; Michener et al., 1986; Khanna et al., 1986) both in vivo and in vitro.

The amino acid sequence of lipocortin (Wallner et al., 1986), which also has been called lipocortin I (Huang et al., 1986), p35 (De et al., 1986), calpactin II (Glenney, 1986a), and chromobindin 9 (Creutz et al., 1987), reveals that it belongs to a family of structurally related Ca^{2+}-binding proteins known as annexins (Geisow et al., 1987). This family of proteins, initially investigated as substrates for protein–tyrosine kinases, mediators of exocytosis, inhibitors of phospholipase A_2, or components of the cytoskeleton, undergo Ca^{2+}-dependent binding to certain phospholipids. The exact physiological roles of these proteins are not yet known. Lipocortin inhibits phospholipase A_2 in some in vitro assays (Waller et al., 1986), but this is by an indirect mechanism (Davidson et al., 1987; Haigler et al., 1987) and has not been shown to reflect a physiological function of the protein.

The complete amino acid sequences are known for four annexins: lipocortin (Wallner et al., 1986); calpactin I (Kristensen et al., 1986; Saris et al., 1986), which also has been called p36 (Saris et al., 1986), protein I (Gerke & Weber, 1984), and lipocortin II (Huang et al., 1986); protein II (Weber et al., 1987); and endonexin II (Schlaepfer et al., 1987; Kaplan et al., 1988), which also has been investigated as an anticoagulant protein (Funakoshi et al., 1987; Iwasaki et al., 1987). Each protein has two domains: a small N-terminal domain with only limited sequence similarity between proteins and a core domain with 40–60% sequence identity between proteins. The N-terminal and core domains are joined by a hinge region that is very sensitive to proteolysis (Haigler et al., 1987); Weber et al., 1987; Glenney, 1986b). The Ca^{2+} and phospholipid binding sites are located in the core domain (Glenney, 1986b; Schlaepfer & Haigler, 1987) while it is reasonable to speculate that the N-terminal domain confers a different biological activity to each protein.

Lipocortin and calpactin I are phosphorylated on tyrosine residues in the N-terminal domain by the EGF receptor/kinase (De et al., 1986; Haigler et al., 1987) and by pp60^{src} (Glenney & Tack, 1985), respectively. This raises the possibility that phosphorylation modulates an as yet undefined biological activity of these proteins. In addition, PKC phosphorylates serine-25 of calpactin I (Gould et al., 1986) and threonine-6 of protein II (Weber et al., 1987); both of these sites are in the N-terminal domains. Lipocortin has previously been shown to be a substrate for PKC in vitro (Summers & Creutz, 1985; Khanna et al., 1986) and in a stimulant-dependent manner in intact chromaffin cells (Michener et al., 1986). One study

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[1] Abbreviations: DNFB, 2,4-dinitrofluorobenzene; DNP, 2,4-dinitrophenyl; EGF, epidermal growth factor; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PKC, protein kinase C; PTH, phenylthiohydantoin; SDS, sodium dodecyl sulfate; TLC, thin-layer chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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found the in vitro phosphorylation to be predominantly on unidentified serine residues (Summers & Creutz, 1985), and another found it to be on threonine (Khanna et al., 1986). In this study, we showed that lipocortin was phosphorylated in vitro by rat brain PKC on a threonine residue and two serine residues located near the site of phosphorylation by the EGF-stimulated tyrosine kinase.

Experimental Procedures

Materials. [γ-^{32}P]ATP was synthesized from carrier-free [^{32}P]orthophosphate (ICN, Irvine, CA) using Gamma-Prep Synthesis Systems (Promega Biotec, Madison, WI). Lipocortin, des-1–12-lipocortin and des-1–26-lipocortin were isolated from human placenta (Haigler et al., 1987), and concentrations were calculated from A_{280} by using extinction coefficients of 18 600, 13 100, and 11 800 M^{-1}, respectively, which were determined on the basis of the known amino acid compositions. Des-1–29-lipocortin was prepared by plasmin digestion of native lipocortin (Huang et al., 1687) and maintained phospholipid binding properties indistinguishable from those of the native protein. Endonexin II was purified from human placenta as described (Haigler et al., 1987).

Phosphorylation Assay. PKC was purified from rat brain (Woodgett & Hunter, 1987). Unless otherwise indicated, PKC phosphorylation of lipocortin and its derivatives was measured as follows. The protein substrate (500 ng) was preincubated at 30 °C for 10 min in a solution (20 μL) containing Tris-HCl buffer (20 mM, pH 7.4), $MgCl_2$ (10 mM), $CaCl_2$ (0.5 mM), freshly sonicated phosphatidylserine (3.0 μg, Avanti Polar Lipids), diolein (0.3 μg, Sigma), and PKC. The reaction was initiated by the addition of [γ-^{32}P]ATP (20 μM, 10 Ci/mmol) to achieve a final volume of 25 μL. The reaction was allowed to proceed at 30 °C for 15 min and was terminated by the addition of 10 μL of a 4-fold concentrated solution of Laemmli SDS sample buffer followed by heating at 100 °C for 1 min. The reactions were analyzed by SDS–PAGE (Laemmli, 1970). Gels, calibrated by low molecular weight standards (Bio-Rad, Richmond, CA), were stained with Coomassie Blue, destained, dried, and exposed to Kodak X-Omat AR-5 film with intensifying screens at −70 °C. Gels of the standard phosphorylation assay required a 45-min exposure. Under the standard reaction conditions, incorporation of radioactivity into lipocortin was linear with respect to time. The stoichiometry of phosphorylation of lipocortin (500 ng) in the standard reaction was approximately 0.85 mol of phosphate/mol of protein.

Two-Dimensional SDS Gel Electrophoresis. Isoelectric focusing was performed in 5-mm tube gels (Garrels, 1979). The ampholytes were used at a 3% final concentration (40% Pharmacia pH 3–10, 30% Pharmacia pH 6.5–9, 20% LKB pH 5–7, and 10% Pharmacia pH 8–10.5). The pH profile of the first dimension was determined by Coomassie Blue staining of isoelectric focusing standards (Bio-Rad, Richmond, CA) with known p*I* values. The second dimension was 9% SDS–PAGE.

Phosphoamino Acid Analysis. Two-dimensional phosphoamino acid analysis was performed on proteins extracted from SDS–polyacrylamide gels after partial acid hydrolysis (Giugni et al., 1985). Phosphoamino acid standards were detected with ninhydrin, and the radioactive phosphoamino acids were detected by autoradiography. Radioactivity was quantitated by liquid scintillation counting of the excised radioactive spots.

Plasmin Digestion of ^{32}P-Labeled Lipocortin. Lipocortin (500 ng in each of 15 reactions) was phosphorylated by PKC in the standard phosphorylation assay. The reactions were stopped by the addition of EGTA to 10 mM and placed onto a G-25 column (Pharmacia PD-10) equilibrated in 200 mM Tris-HCl, pH 8.0, and 10 mM EGTA. The fractions containing the ^{32}P-labeled protein were concentrated by a Centricon-10 device (Amicon Inc.) to 750 μL (2500 cpm/μL). The ^{32}P-labeled lipocortin (125 000 cpm/50 μL) was added to 1 μg of native lipocortin and then incubated with varying amounts of plasmin in a total volume of 70 μL for 1 h at 30 °C. The plasmin digestion was stopped by the addition of 25 μL of a 4-fold concentrated solution of Laemmli SDS sample buffer and heated at 100 °C for 1 min, and the components were fractionated by 20% PAGE. The wet gel was covered with plastic wrap and exposed to X-ray film at −70 °C prior to staining/destaining. It was necessary to perform autoradiography prior to staining/destaining because this procedure extracted low molecular weight radioactive products of the digestion from the gel. The digestion generated two prominent radioactive bands with R_f values of 0.75 and 0.93. These were located, excised, and eluted from the gel and subjected to phosphoamino acid analysis as described.

Amino-Terminal Analysis by Dinitrophenyl Derivatization. (Dinitrophenyl)phosphoserine standard was prepared by adding 2,4-dinitrofluorobenzene (200 μL of a 10% solution in ethanol) to 1 mg of *O*-phosphoserine (Sigma) in carbonate buffer (200 mM, pH 8.8, 1 mL) and incubating at 40 °C for 90 min with stirring. The reaction was terminated by the addition of 100 μL of 6 N HCl, and the reactants were removed by ether extraction. The product, remaining in the aqueous phase, was lyophilized and extracted from the salt precipitate into acetone/1 N HCl (80:20 v/v). The yellow eluate was concentrated and analyzed by thin-layer chromatography (TLC) on silica gel 60 cellulose plates with 90% formic acid *n*-butyl alcohol (1:1 v/v). (Dinitrophenyl)-phosphoserine was detected by its yellow color while phosphoserine standard was visualized by (0.3%) ninhydrin staining. For amino-terminal analysis of proteins, ^{32}P-labeled proteins were reacted with 2,4-dinitrofluorobenzene in the same manner except that the total incubation volume was 200 μL and the reactions were terminated by the addition 20 μL of 6 N HCl. TLC of the protein hydrolysate was performed as above after acid hydrolysis (6 N HCl, 110 °C for 60 min) of the derivatized substrate. Control reactions showed that reaction of amino-terminal amino acids proceeded to completion.

Edman Degradation of ^{32}P-Labeled Des-1–26-lipocortin. Des-1–26-lipocortin (500 ng in each of eight reactions) was phosphorylated in the standard PKC phosphorylation assay. The reactions were stopped with the addition of EGTA to 10 mM and fractionated on a G-25 column (Pharmacia PD-10) equilibrated in 20 mM Hepes, pH 7.4. The fractions containing the ^{32}P-labeled protein were concentrated to 100 μL by a Centricon-10 device (Amicon Inc.), and trifluoroacetic acid was added to 0.5%. Equal aliquots (25 μL, 25 000 cpm) of the ^{32}P-labeled protein were applied to four different precycled Polybrene-treated filters. Edman degradation was performed by using an Applied Biosystems 470 instrument and a standard program supplied by the manufacturer. One filter was exposed to no Edman chemistry. A second filter was exposed to only one half-cycle; i.e., the PTH derivative was formed but was not exposed to the cleavage cycle, and the filter then was removed from the sequenator. A third filter was exposed to one full Edman cycle, resulting in the removal of the N-terminal PTH-serine. The fourth filter was exposed to two Edman cycles, resulting in the removal of the first and the second serine residues of des-1–26-lipocortin. After the above treatments, each filter was extracted by sonication in 200 μL of Laemmli SDS sample buffer, heating at 100 °C for 5 min, and centrifugation (10000*g* for 10 min). The su-

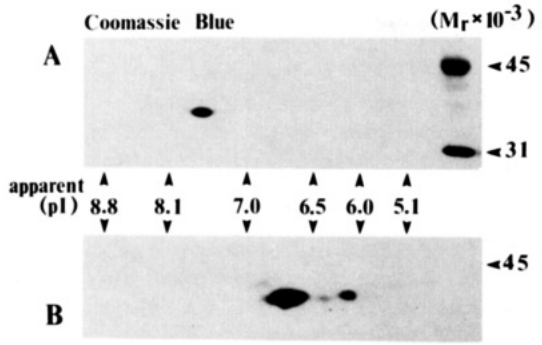

FIGURE 1: Two-dimensional gel analysis of lipocortin phosphorylated by PKC. Lipocortin (250 ng) was used as a substrate for PKC in the standard phosphorylation assay as described under Experimental Procedures. The reaction was stopped by the addition of 50 μL of Garrels SDS sample buffer containing ampholytes; 1 μg of native lipocortin was added as a marker, and the sample was fractionated on two-dimensional gels as described under Experimental Procedures. Panel A: Coomassie-stained gel. Panel B: Autoradiography of the same gel showing the ^{32}P-labeled lipocortin. Exposure time (at −70 °C with intensifying screen) was 2 h. The radioactivity incorporated into the protein was determined by Cerenkov counting of the excised spots and indicated that 1.1 mol of phosphate was incorporated per mole of protein in the reaction. Apparent p*I*'s are indicated as determined from the migration of isoelectric focusing standards (Bio-Rad, Richmond, CA).

pernatant was analyzed by 9% SDS–PAGE. Radioactivity in the protein band at M_r 32 000 was visualized by autoradiography and then excised and subjected to liquid scintillation counting. As a control, ^{32}P-labeled lipocortin, which has a blocked amino terminus, was applied to a precycled polybrene filter, exposed to five Edman cycles, and analyzed by SDS gel electrophoresis. Quantitative recovery of ^{32}P-labeled lipocortin was observed (data not shown).

RESULTS

Human placental lipocortin was an excellent substrate for rat brain PKC in vitro. Figure 1 shows a two-dimensional gel analysis of ^{32}P-labeled lipocortin prepared in our standard assay; native lipocortin was added after the reaction for Coomassie Blue visualization (Figure 1A), and the phosphorylated protein was visualized by autoradiography (Figure 1B). The major radioactive spot had an apparent isoelectric point (approximately 6.7) indistinguishable from that of lipocortin phosphorylated on tyrosine-21 by the EGF receptor/kinase (Haigler et al., 1987), indicating that this spot contained 1 mol of phosphate/mol of protein. A minor radioactive spot (5–10% of total) had an apparent p*I* of approximately 6.1, which is consistent with it being lipocortin containing 2 mol of phosphate/mol of protein. Phosphoamino acid analysis of the major spot with apparent isoelectric point of approximately 6.7 revealed that it contained phosphoserine (69%) and phosphothreonine (31%) but no detectable phosphotyrosine. Thus, the major spot of radioactivity was composed of lipocortin that was phosphorylated on either a single serine or a single threonine residue.

Analysis of lipocortin phosphorylation by PKC by one-dimensional SDS–PAGE showed that half-maximal phosphorylation of lipocortin occurred at approximately 50 nM lipocortin (Figure 2). At low substrate concentrations, greater than 1 mol of phosphate was incorporated per mole of lipocortin (not shown). The following experiments were performed to determine the sites of phosphorylation. We prepared lipocortin derivatives that were missing the N-terminal 12, 26, and 29 amino acid residues (Figure 2) and compared them with native lipocortin with regard to phosphorylation by PKC. Lipocortin and des-1–12-lipocortin were phosphorylated to a

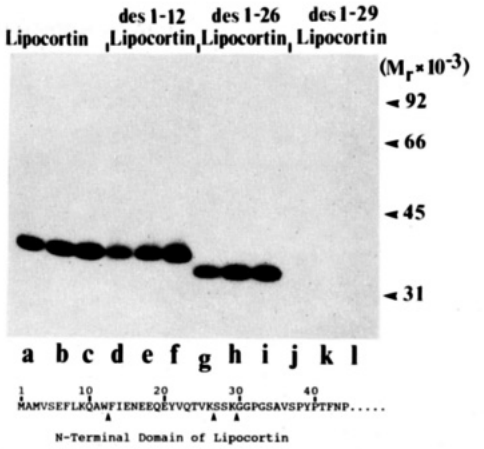

FIGURE 2: Phosphorylation of lipocortin and its N-terminal truncations by PKC. Lipocortin or lipocortin missing the N-terminal 12, 26, or 29 amino acid residues was tested as substrate for PKC in the assay described under Experimental Procedures. The reaction mixtures were resolved by SDS–PAGE, and proteins were visualized by Coomassie staining followed by autoradiography of the dried gel. The amount of substrate protein was as follows: lanes a, d, and g, 50 ng; lanes b, e, h, and j, 250 ng: lanes c, f, and i, 500 ng: lane k, 1000 ng; and lane l, 2500 ng. Exposure time (at −70 °C with an intensifying screen) was 30 min. Longer exposure times showed a radioactive band in each gel lane with an approximate M_r of 80 000. The amino acid sequence of the amino terminus of lipocortin as deduced from the cDNA nucleotide sequence (Wallner et al., 1986) is shown. The numbering of residues begins with the first methionine, although, by analogy with other proteins, cellular lipocortin probably begins with an acetylated alanine. The triangles indicate the sites of cleavage by chymotrypsin (residue 12), trypsin (residue 26), and plasmin (residue 29).

similar extent (Figure 2), and phosphoamino acid analysis revealed that both proteins contained phosphoserine and phosphothreonine ratio of approximately 2 to 1 (lipocortin = 72% phosphoserine, 28% phosphothreonine, and <1% phosphotyrosine; des-1–12-lipocortin = 66% phosphoserine, 34% phosphothreonine, and <1% phosphotyrosine). At the same protein concentration, des-1–26-lipocortin incorporated approximately as much phosphate as lipocortin (Figure 2), and all detectable radioactivity was incorporated into phosphoserine. Even at high substrate concentrations, des-1–29-lipocortin was not detectably phosphorylated by PKC (Figure 2).

The most simple interpretation of the above results is that the sole threonine in the 29-residue N-terminal domain of lipocortin, threonine-24, is a phosphorylation site. The results also suggest that either serine-27 or serine-28 are phosphorylation sites. The only other serine residue within the N-terminal 29 amino acids was serine-5; removal of this residue in the formation of des-1–12-lipocortin did not detectably alter the extent or relative ratio of serine phosphorylation, thereby suggesting that serine-5 was not a phosphorylation site.

The above interpretations are complicated by the possibility that N-terminal truncations of lipocortin could change the conformation of the remaining core protein and render residues that were phosphorylation sites in the native protein inaccessible to the kinase. To investigate this possibility, native lipocortin was phosphorylated and then digested with plasmin. Lysine-29 of lipocortin is extremely sensitive to plasmin cleavage (Huang et al., 1987). Lipocortin was phosphorylated by PKC, and the ^{32}P-labeled lipocortin was separated from the unreacted [γ-^{32}P]ATP by gel filtration. ^{32}P-Labeled lipocortin was digested with increasing concentrations of plasmin, and then the digest was analyzed by 20% SDS–PAGE. The locations of ^{32}P-labeled polypeptides were determined by autoradiography prior to staining and destaining

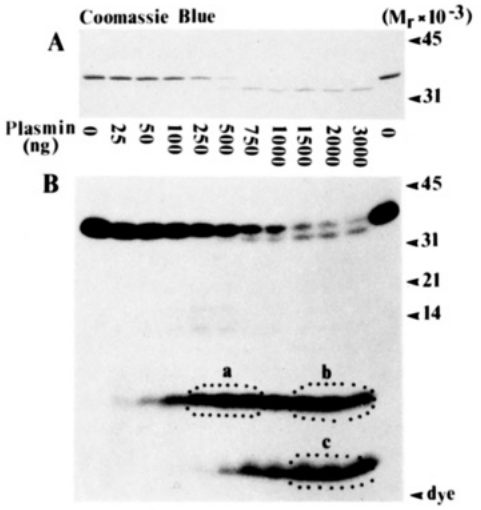

FIGURE 3: Digestion of lipocortin with plasmin. ^{32}P-Labeled lipocortin was prepared, mixed with 1 μg of native lipocortin, and incubated with the indicated amount of plasmin as described under Experimental Procedures. The reaction products were subjected to SDS–PAGE (20%), and the wet gel was analyzed by autoradiography (panel B), which identified radioactive digestion products with R_f values of 0.75 and 0.93. Exposure time (at −70 °C with intensifying screens) was 45 min. Areas a–c (see panel B) were excised from the gel and subjected to phosphoamino acid analysis. The remainder of the gel then was stained with Coomassie Blue (panel A).

the gel in order to detect small peptides that would have washed out of the gel. Low concentrations of plasmin caused a reduction in radioactivity that comigrated with native lipocortin with a concomitant appearance in a radioactive band with an R_f of 0.75 (Figure 3B). At higher concentrations of plasmin, a radioactive band with an R_f of 0.93 also appeared. Subsequent Coomassie Blue staining of the gel revealed the plasmin-dependent decrease in the native lipocortin band with the appearance of a band with an R_f corresponding to des-1–29-lipocortin (Figure 3A). Nonphosphorylated lipocortin and ^{32}P-labeled lipocortin showed equal sensitivity to plasmin cleavage. Only a minor amount of radioactivity (less than 5% of total) accumulated in the des-1–29-lipocortin band, indicating that the core domain of the protein is not phosphorylated to a significant extent by PKC. Phosphoamino acid analysis was performed on the radioactive bands of R_f 0.75 and 0.93. At lower plasmin concentrations, the R_f 0.75 band (region a of Figure 3B) contained phosphoserine (68%) and phosphothreonine (32%), approximately the same 2:1 ratio that was observed in the intact protein, while at higher plasmin concentrations the R_f 0.75 band (region b of Figure 3B) contained relatively less phosphoserine (42%) than phosphothreonine (58%). The only detectable phosphoamino acid in the R_f 0.93 band (region c of Figure 3B) was phosphoserine. These results indicate that low concentrations of plasmin removed the N-terminal 29 amino acid peptide containing the major phosphorylation sites. The size of this proposed peptide is consistent with the observed R_f of 0.75. At higher concentrations of plasmin, we propose that the protease cleaved at both lysine-29 and the less sensitive site, lysine-26, to generate the 26 amino acid peptide containing the phosphorylated threonine-24 (which still had an R_f of 0.75) and the phosphorylated tripeptide Ser-Ser-Lys, with an R_f of 0.93.

The R_f 0.93 band was eluted from the gel and reacted with DNFB as described under Experimental Procedures to determine which of the two serine residues was phosphorylated. If serine-27 were phosphorylated, partial acid hydrolysis of the tripeptide would yield DNP-phosphoserine while phosphorylated serine-28 would yield underivatized phosphoserine.

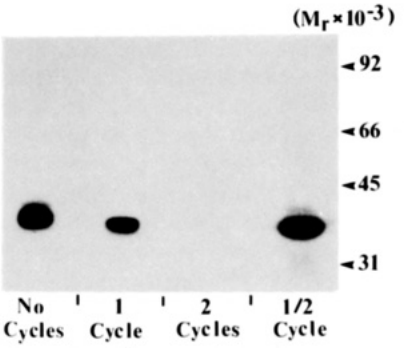

FIGURE 4: SDS–PAGE of ^{32}P-labeled des-1–26-lipocortin after Edman cleavage cycles. ^{32}P-Labeled des-1–26-lipocortin was prepared by the standard phosphorylation assay as described under Experimental Procedures. ^{32}P-Labeled des-1–26-lipocortin was applied to four different precycled Polybrene-treated filters and exposed to one or two cycles of degradation. As controls, one filter was exposed to the first coupling step but not the cleavage step, designated "$^1/_2$ cycle", and another filter was not exposed to Edman chemistry at all, designated "No cycles." After the above treatments, the filters were extracted and analyzed by SDS–PAGE as described under Experimental Procedures. Exposure time (at −70 °C with intensifying screen) was 10 h. The radioactive bands at M_r 3200 were excised and subjected to liquid scintillation counting: No cycles, 5890 cpm; $^1/_2$ cycle, 6184 cpm; one cycle, 3416 cpm; and two cycles, 390 cpm.

We observed DNP-phosphoserine and phosphoserine in approximately equal amounts, indicating that both serine residues were phosphorylated. Des-1–26-lipocortin also was phosphorylated by PKC in the standard reaction and analyzed by DNFB derivatization (see Experimental Procedures). Partial acid hydrolysis also released DNP-phosphoserine and phosphoserine in approximately equal amounts.

To obtain a more quantitative determination of the distribution of ^{32}P between serine-27 and serine-28, des-1–26-lipocortin was phosphorylated and then subjected to sequential Edman degradation. Because it is difficult to accurately measure the release of PTH-phosphoamino acids, the amount of radioactivity remaining associated with the protein after each cycle was determined. Figure 4 shows that approximately half of the radioactivity was removed in the first cycle and half in the second. Thus, serine-27 and serine-28 were phosphorylated to an equal extent.

Discussion

PKC is a Ca^{2+}-activated phospholipid-dependent enzyme that is stimulated in vivo by diacylglycerol and undergoes a translocation from the cytosol to the membrane in the presence of increasing intracellular Ca^{2+}. PKC modulates a number of cellular functions including cell replication. The intracellular protein, lipocortin, has no structural similarity to PKC but shares the functional properties of being an intracellular Ca^{2+}-dependent phospholipid-binding protein. It binds to phospholipids that are preferentially located on the cytosolic face of the plasma membrane. Lipocortin is a high-affinity substrate in vitro for both the EGF-stimulated protein–tyrosine kinase (De et al., 1986; Pepinsky & Sinclair, 1986; Haigler et al., 1987) and PKC (Summers & Creutz, 1985; Khanna et al., 1986). Since lipocortin is phosphorylated in intact cells by both of these enzymes, there is the possibility that rising intracellular Ca^{2+} concentrations are involved in promoting binding of the substrate to the plasma membrane so it can interact with these kinases. The physiological role of lipocortin and how this role is modulated by phosphorylation are not yet known. However, phosphorylation of tyrosine-21 is known to result in a decrease in the Ca^{2+} concentration needed for phospholipid binding (Schlaepfer & Haigler, 1987) and an

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                                    ▼  ▼▼
Lipocortin    MAMVSEFL*K**QAWFIENEEQEYVQTVKSSKGGPGSA...
Calpactin      ST-H-I-C-LSLEGDHSTPPSA-G*S--AYTNFDAER...
Protein II                          AAKGG---AAS-FNAAE...
Endonexin II                      MA-VLRG--TDFP-FDER-...
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FIGURE 5: Comparison of N-terminal amino acid sequences of annexins. The amino acid sequences of N-terminal regions of human lipocortin (Wallner et al., 1986), human calpactin I (Huang et al., 1986), bovine protein II (Weber et al., 1987), and human endonexin II (Kaplan et al., 1988) are represented by the single-letter amino acid code and are aligned with the inclusion of gaps ("*") to emphasize the similarity between the proteins. Residues that are identical with the corresponding residues in lipocortin are indicated ("-"). The arrows indicate the PKC phosphorylation sites of lipocortin. Calpactin and protein II are phosphorylated by PKC on Ser-25 and Thr-6, respectively. Lipocortin is phosphorylated by the EGF receptor/kinase on Tyr-21, and calpactin is phosphorylated by $pp60^{src}$ on Tyr-23.

increase in the sensitivity to tryptic cleavage at lysine-26 (Haigler et al., 1987).

Bovine lipocortin, also called chromobindin 9 (Creutz et al., 1987), has been reported to be phosphorylated predominantly on unidentified serine residues by the PKC in chromaffin granules (Summers & Creutz, 1985; Michener et al., 1986) while another report found bovine lipocortin to be phosphorylated predominantly on unidentified threonine residues by bovine brain PKC (Khanna et al., 1986). We found that human lipocortin was phosphorylated on threonine-24, serine-27, and serine-28 in an approximate relative molar ratio of 1:1:1 in our standard phosphorylation reaction. We found no evidence for significant phosphorylation of lipocortin in the core domain (Figures 2 and 3) as previously proposed on the basis of structural considerations (Wallner et al., 1986). Since multiple forms of PKC are known to exist (Woodget & Hunter, 1987), it is not known if the phosphorylation of each site in lipocortin was catalyzed by a separate form of the enzyme or if a single form had equal affinity for each site. Likewise, it is not known if phosphorylation of the three different sites serves the same the biological function. Since bovine (Glenney et al., 1987) and porcine (De et al., 1986) lipocortins have a glycine residue at the position analogous to the phosphorylation site, serine-27, in human lipocortin, it is unlikely that phosphorylation of serine-27 plays a unique and essential role.

Although the N-terminal domains of the annexins are composed of sequences with limited similarity, two other members of the annexin family of proteins, calpactin I (Gould et al., 1986) and protein II (Weber et al., 1987), also are phosphorylated in their N-terminal domains by PKC. As shown in Figure 5, the threonine phosphorylation site of lipocortin occurs at a position analogous to the phosphorylation sites of calpactin I (serine-25) and of protein II (threonine-6). All three substrate proteins contain the conserved sequence Thr(or Ser)-Val-Lys. A fourth member of the annexin family, endonexin II, contains the sequence Thr-Val-Thr at the analogous location but is not a substrate for PKC (Kaplan et al., 1988). All phosphorylation sites in the annexins have a Lys residue positioned near the carboxy side of the site. Previous studies of PKC phosphorylation of substrate proteins (Ikebe et al., 1987; Bengur et al., 1987; Hunter et al., 1984) and synthetic peptides (House et al., 1987; Woodgett et al., 1986) have found that basic residues are located adjacent to the phosphorylation sites, but the critical basic residues were located either to the amino side or to the carboxy side. However, for annexin substrates it is clear that a basic residue on the carboxy side is the determinant for annexin phosphorylation by PKC. This is supported by the fact that the basic residue on the amino side of threonine-8 of endonexin II (Arg-Gly-Thr-Val-Thr_{10}) was not sufficient for recognition by PKC (Kaplan et al., 1988). The importance of the basic residue to the carboxy side of the phosphorylation site is supported by the fact that des-1–26-lipocortin, whose amino-terminal sequence is Ser-Ser-Lys, was phosphorylated equally at the two N-terminal serine residues (Figure 4). However, direct experiments such as site-directed mutagenesis are required to clearly establish the role of this lysine residue.

Lipocortin, the EGF-stimulated protein–tyrosine kinase, and PKC are possibly interconnected biologically in several intriguing ways. PKC phosphorylates both the EGF receptor/kinase (Hunter et al., 1984) and lipocortin. The EGF receptor/kinase phosphorylates lipocortin and stimulates Ca^{2+} influx (Sawyer & Cohen, 1981), which could alter the cellular distribution and activity of both PKC and lipocortin. Evaluating a possible role of lipocortin as a link between the signaling pathways mediated by these two kinases must await a more clear description of the physiological function of lipocortin and a determination of how this function is modulated by phosphorylation.

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Deoxycytidine Kinase from Human Leukemic Spleen: Preparation and Characterization of the Homogeneous Enzyme†

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ABSTRACT: Deoxycytidine kinase from human leukemic spleen has been purified 6000-fold to apparent homogeneity with an overall yield of 10%. The purification was achieved by using DEAE chromatography, hydroxylapatite chromatography, and affinity chromatography on dTTP–Sepharose. Only one form of deoxycytidine kinase activity was found during all the chromatographic procedures. The subunit molecular mass, as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, was 30 kilodaltons. The pure enzyme phosphorylates deoxycytidine, deoxyadenosine, and deoxyguanosine, demonstrating for the first time that the same enzyme molecule has the capacity to use these three nucleosides as substrates. The apparent molecular weight of the active enzyme, determined by gel filtration and glycerol gradient centrifugation, was 60000. Thus, the active form of human deoxycytidine kinase is a dimer. The kinetic behavior of pure human deoxycytidine kinase was studied in detail with regard to four different phosphate acceptors and two different phosphate donors. The apparent K_m values were 1, 20, 150, and 120 μM for deoxycytidine, arabinosylcytosine, deoxyguanosine, and deoxyadenosine, respectively. The V_{max} values were 5-fold higher for the purine nucleosides as compared to the pyrimidine substrates. We observe competitive inhibition of the phosphorylation of one substrate by the presence of either of the three other substrates, but the apparent K_i values differed greatly from the corresponding K_m values, suggesting the existence of allosteric effects. The double-reciprocal plots for ATP·$MgCl_2$ as phosphate donor were convex, indicating negative cooperative effects. In contrast, plots with varying dTTP·$MgCl_2$ concentration as phosphate donor were linear with an apparent K_m of 2 μM. The enzyme activity was strongly inhibited by dCTP, in a noncompetitive way with deoxycytidine and in a competitive way with ATP·$MgCl_2$.

Deoxycytidine kinase (dCyd kinase) (NTP:deoxycytidine 5′-phosphotransferase, EC 2.7.1.74) catalyzes the phosphorylation of deoxycytidine to deoxycytidine 5′-phosphate in the presence of a nucleoside 5′-triphosphate phosphate donor. The enzyme has been isolated and purified from many sources (Kessel, 1968; Momparler & Fischer, 1968; Durham & Ives, 1969, 1970; Kazai & Sugino, 1971; Coleman et al., 1975; Cheng et al., 1977; Meyers & Kreis, 1976; Hurley et al., 1983; Sarup & Fridland, 1987), and most investigators found that the same enzyme fraction also phosphorylated purine deoxyribonucleosides but with lower efficiency. The best studied enzyme, isolated from calf thymus, showed a broad substrate specificity, phosphorylating both pyrimidine and purine nu-

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