Location of Sites in Human Lipocortin I That Are Phosphorylated by Protein Tyrosine Kinases and Protein Kinases A and C†

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ABSTRACT: Lipocortin I is a 39-kilodalton membrane-associated protein that in A431 cells is phosphorylated on tyrosine in response to epidermal growth factor (EGF). We have used recombinant human lipocortin I as a substrate for several protein kinases and identified phosphorylated residues by a combination of peptide mapping and sequence analysis. Lipocortin I was phosphorylated near the amino terminus at Tyr-21 by recombinant $pp60^{c\text{-}src}$. The same tyrosine residue was phosphorylated by polyoma middle T/$pp60^{c\text{-}src}$ complex, by recombinant $pp50^{v\text{-}abl}$, and with A431 cell membranes by the EGF receptor/kinase. The primary site of phosphorylation by protein kinase C was also near the amino terminus at Ser-27. The major site of phosphorylation by adenosine cyclic 3′,5′-phosphate dependent protein kinase was on the carboxy-terminal half of the molecule at Thr-216. These sites are compared to the phosphorylation sites previously located in the structurally related protein lipocortin II.

Lipocortins are a family of steroid-induced proteins that inhibit phospholipase A_2 activity [for reviews, see Flower et al. (1984) and Hirata (1984)]. On the basis of their inhibitory activity, numerous related proteins with masses of 15, 30, 40, 55, and 70 kilodaltons (kDa)[1] have been detected; however, the mechanism of inhibition is controversial (Davidson et al., 1987). Recently, two lipocortin genes were cloned (Huang et al., 1986; Saris et al., 1986). Both encode 39-kDa proteins with 50% sequence identity. By peptide mapping and sequencing, lipocortin I was identified as pp35, a known substrate for the EGF receptor/kinase in A431 cells (Fava & Cohen, 1984). Similarly, lipocortin II was identified as pp36 (the 39-kDa subunit of calpactin I), a substrate for $pp60^{v\text{-}src}$ in Rous sarcoma virus transformed cells (Erikson & Erikson, 1980; Radke et al., 1980). The lipocortins are members of a larger family of calcium and phospholipid binding proteins referred to as annexins (Geisow & Walker, 1986), which contain a variety of related proteins termed endonexins, calelectrins, calcimedins, calpactins, chromobindins, and proteins I–III. Although lipocortin-like proteins contain hydrophilic sequences, they bind tightly to phospholipid vesicles in the presence of micromolar concentrations of calcium (Fava & Cohen, 1984; Gerke & Weber, 1984; Glenney, 1986a). These proteins all have a common conserved sequence, which is repeated 4 times in each molecule (Geisow & Walker, 1986) and is believed to be involved in calcium binding.

The physiological function of the lipocortins is unclear. The tendency of lipocortins to form nonspecific aggregates in the presence of calcium has complicated experiments designed to determine their specific associations with various cellular components. In intact cells, the lipocortins are phosphorylated by a variety of stimuli. Lipocortin I is phosphorylated near its amino terminus in A431 cells by the EGF receptor/kinase (De et al., 1986; Pepinsky & Sinclair, 1986). Lipocortin II is phosphorylated near its amino terminus by $pp60^{v\text{-}src}$ in Rous sarcoma virus transformed cells (Glenney & Tack, 1985). Both proteins are also substrates for protein kinase C (Khanna et al., 1986; Gould et al., 1986). Other reports have shown that a lipocortin is phosphorylated by cAMP-dependent protein kinase in vitro (Hirata, 1981) and by protein tyrosine kinases in mitogen-stimulated murine thymocytes (Hirata et al., 1984).

In this study, we characterized sites within human recombinant lipocortin I which were phosphorylated in vitro by several purified protein tyrosine kinases and protein kinases A and C. These sites are compared to phosphorylation sites previously reported for lipocortin II.

Experimental Procedures

Materials. Recombinant human lipocortin I was expressed and purified from *Escherichia coli* (Wallner et al., 1986). Recombinant avian $pp60^{c\text{-}src}$, expressed in *E. coli*, was a gift from Helen Piwnica-Worms and Thomas Roberts (Dana Farber Cancer Research Institute). Preparations of NIH 3T3 cells transformed with polyoma middle T and of rabbit anti-middle T antiserum were previously described (Kaplan et al., 1986; Whitman et al., 1985). Recombinant Abelson tyrosine kinase, $pp50^{v\text{-}abl}$, produced and purified from *E. coli*, was a gift from Gordon Foulkes (Foulkes et al., 1985). Protein kinase C was purified from human red cells as described by Faquin et al. (1986). Human platelet protein kinase C was a gift from Resai A. Bangur (Laboratory of Molecular Cardiology, NIH). The catalytic subunit of cAMP-dependent protein kinase from bovine heart was obtained from Sigma (47 pM units/μg of protein).

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[1] Abbreviations: cAMP, adenosine cyclic 3′,5′-phosphate; ATP, adenosine 5′-triphosphate; EGF, epidermal growth factor; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; kDa, kilodalton(s); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TLC, thin-layer chromatography.

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Truncated des-30 lipocortin was generated by limited proteolysis with plasmin as previously described (Huang et al., 1987). Lipocortin I variants containing single amino acid substitutions (Tyr-21 to Phe-21, Thr-216 to Ala-216, Met-125 to Leu-125, and Met-55 to Leu-55) were generated by site-directed mutagenesis and expressed in *E. coli* (D. Schindler and L. Vitto, unpublished data). The various point mutations had no effect on the biochemical properties or on the phospholipase inhibitory activity of lipocortin I.

In Vitro Phosphorylation of Lipocortin I. Recombinant lipocortin I was phosphorylated with an immune complex of polyoma middle T/pp60$^{c\text{-}src}$ as described previously for phosphorylation of enolase (Kaplan et al., 1986). Briefly, NIH 3T3 cells transformed with the middle T gene were grown to 90% confluency in a 100-mm dish. Cells were lysed in detergent-containing buffer, and 2 μL of anti-middle T antiserum was added. The immune complex was collected with 35 μL of protein A–Sepharose beads (Whitman et al., 1985). The kinase-containing complex was suspended in 20 mM Tris-HCl, pH 7.4, 10 mM $MnCl_2$, 2 μM ATP with 50 μCi of [γ-^{32}P]-ATP, and either 4 μg of lipocortin I or acid-denatured enolase in a final volume of 60 μL. The mixture was incubated at 37 °C for 10 min.

A similar procedure was used for phosphorylation of lipocortin I by recombinant avian pp60$^{c\text{-}src}$ (Piwnica-Worms et al., 1986) except that the recombinant protein was immunoprecipitated directly from disrupted bacteria with monoclonal antibody GD11 (Parsons et al., 1984) directed against pp60$^{c\text{-}src}$ and subsequently added to protein A–Sepharose beads.

EGF-dependent phosphorylation of lipocortin I was performed using a membrane fraction of A431 cells exposed to 0.2 μM EGF for 30 min as described (Pepinsky & Sinclair, 1986). Lipocortin I (4 μg) was added to 5 μg (based on a Bio-Rad protein assay) of membrane fraction suspended in 20 mM HEPES, pH 7.5, 2 mM $MgCl_2$, and 10 μM ammonium vanadate, and the reaction was carried out for 10 min on ice in the presence of 5 μM ATP, 50 μCi of [γ-^{32}P]ATP, and 2 μM EGF.

For phosphorylation of lipocortin I by pp50$^{v\text{-}abl}$, 2–5 μL of purified protein kinase was suspended in 50 μL of 50 mM HEPES, pH 7.5, 5 mM $MgCl_2$, and 0.1 mM EDTA, and the mixture was incubated for 10 min on ice in the presence of 2 μM ATP, 50 μCi of [γ-^{32}P]ATP, and 4 μg of lipocortin I.

Protein kinase A phosphorylation of lipocortin was performed using 230 pM units of catalytic subunit of protein kinase A (Sigma) for 30 min at 37 °C in 25 mM sodium acetate, pH 6.0, 2.5 mM $MgCl_2$, 100 mM dithiothreitol with 300 μCi of [γ-^{32}P]ATP, and 30 μg of lipocortin in a total volume of 40 μL.

Lipocortin was phosphorylated by protein kinase C as described by Uchida and Filburn (1979). Reactions contained 25 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 50 μg/mL phosphatidylserine, 0.5 μg/mL diolein, 20 μM ATP with 300 μCi of [γ-^{32}P]ATP, 10 μL of 2000-fold-purified protein kinase C from red cells (Faquin et al., 1986) or from platelets, and 4 μg of lipocortin I in a total volume of 60 μL. Samples were incubated for 10 min at 37 °C. Control reactions were performed without calcium and phospholipid in the presence of 1 mM EGTA.

Reactions were stopped by adding electrophoresis sample buffer containing 1% sodium dodecyl sulfate and 2% 2-mercaptoethanol. Samples were heated for 10 min at 65 °C and subjected to SDS–PAGE using a Laemmli discontinuous buffer system with 10% acrylamide in the running gel (Laemmli, 1970).

Peptide Mapping and HPLC Analysis. Cyanogen bromide (CNBr) mapping of phosphorylated lipocortin I was performed as previously described (Pepinsky & Sinclair, 1986). Phosphorylated proteins were subjected to SDS–PAGE, and the appropriate regions of the gel were excised. The gel slices were incubated with the indicated amounts of CNBr in 0.1 M HCl and 0.1% 2-mercaptoethanol for 1 h at room temperature and washed. Cleavage fragments were separated by SDS–PAGE [15% acrylamide/0.18% bis(acrylamide)] and stained with either silver or Coomassie brillant blue, or dried and analyzed by autoradiography.

Tryptic digestion of lipocortin was performed on protein that had been electroeluted from SDS gels into 10 mM NH_4HCO_3 and 0.1% SDS (Hunkapillar et al., 1983). To improve yields, 60 μg of purified lipocortin I was added as carrier to each of the electroeluted preparations. Samples were treated with trichloroacetic acid, washed with acetone, dried, and suspended in 400 μL of 0.1 M NH_4HCO_3 containing 0.5 mM $CaCl_2$ as previously described (Pepinsky et al., 1986). Trypsin was added in three equal aliquots of 2 μg/sample each: the first was at time zero, the second after 3 h, and the third after 12 h. The samples were incubated for a total of 16 h at 37 °C. The digest was acidified with formic acid to 20% (v/v) and subjected to reverse-phase HPLC at 40 °C on a C_{18} column (Spectra Physics, column dimensions 0.46 × 25 cm) equilibrated with 0.1% trifluoroacetic acid. Peptides were eluted with a 95-min gradient of acetonitrile (0–75%) in 0.1% trifluoroacetic acid at a flow rate of 1.4 mL/min; 200 0.5-min fractions were collected [see Huang et al. (1987) for sequence analysis of tryptic fractions]. The column eluates were monitored at 214 and 280 nm. When analyzing phosphorylated protein, the absorbance profile was used as an internal control for reproducibility of each HPLC run, and ^{32}P-labeled peptides were detected by scintillation counting. Slight variations in relative retention times observed between these and previously published profiles reflect changes in column performance.

Limited Proteolysis with Plasmin. Preparations containing 50 μg of lipocortin I were phosphorylated with recombinant pp50$^{v\text{-}abl}$ or protein kinase C as described. Unincorporated ATP was removed by desalting on Bio-Rad P6DG resin in 100 mM Tris-HCl, pH 7.7, 5 mM EDTA, and 1 mM NaF. The labeled samples were incubated for 1 h at room temperature with 5 μg of bovine plasmin (Sigma), and aliquots were analyzed by SDS–PAGE. SDS gels were stained with Coomassie blue and analyzed by autoradiography. The remainder of the samples was subjected to reverse-phase HPLC on a C_4 column (Vydac, column dimensions 0.46 × 25 cm) as described (Huang et al., 1987). Polypeptides were eluted with a 45-min gradient of acetonitrile (0–75%) in 0.1% trifluoroacetic acid at a flow rate of 1.0 mL/min; 90 0.5-min fractions were collected.

Phosphoamino Acid Analysis and Sequencing. For phosphoamino acid analysis, gel-purified protein or HPLC-purified peptides were hydrolyzed in 6 N HCl for 1 h at 110 °C. The products were spotted on cellulose Machery Nagel TLC plates (Kodak), and subjected to electrophoresis in pyridine buffer, pH 3.5, at 50 V/cm as described (Cooper et al., 1983). Unlabeled *O*-phosphoserine, *O*-phosphothreonine, and *O*-phosphotyrosine (Sigma) were used as standards. Phosphoamino acid markers were detected by staining with ninhydrin spray, and radioactive phosphoamino acids were identified by autoradiography. Radioactivity in each spot was quantitated by scintillation counting.

Protein sequencing was performed by automated Edman degradation in a Beckman spinning-cup solid-phase sequencer in the presence of polybrene with myoglobin as carrier. Samples were concentrated to 0.8 mL by lyophilization and injected into the sequencer. Repetitive yields during sequencing (88–90% at each cycle) were evaluated by monitoring the appearance of leucine after cycles 2, 8, and 11, and alanine after cycles 15 and 19.

Manual Amino-Terminal Sequencing of Phosphorylated Peptide. Dansylation of the small phosphopeptide derived from the tryptic digest of protein kinase C labeled lipocortin was performed by using a modification of a published procedure (Hartley, 1970). The peptide was dissolved in 0.2 M $NaHCO_3$, pH 10.5, with 10 nmol of melanocyte-stimulating hormone (4–10)-heptapeptide (Sigma) as a nonradioactive carrier or with 10 nmol of free *O*-phosphoserine. Dansyl chloride in acetone was added to a final concentration of 1.4 mg/mL, and the samples were incubated at 37 °C for 1 h. The samples were lyophilized and hydrolyzed for 1 h at 110 °C in 6 N HCl, and the products were separated on the same cellulose TLC plates used for the separation of phosphoamino acids. *N*-Dansylserine (Sigma) and *N*-dansyl-*O*-phosphoserine, prepared by the method described above, were used as markers. The products were visualized under UV light and/or by autoradiography when appropriate.

RESULTS

CNBr Peptide Maps of Lipocortin I. When gel slices containing human recombinant lipocortin I were treated with CNBr and cleavage products separated by SDS–PAGE, 23 cleavage products were observed. The relative intensities of the fragments varied as a function of CNBr concentration and reaction time, but no additional fragments were detected. Lanes a and b in Figure 1 show cleavage profiles obtained with different concentrations of CNBr. After a 1-h incubation, 10% of lipocortin I remained intact with 7 mg/mL CNBr, while only 2% remained intact with 21 mg/mL CNBr. These concentrations span useful conditions for visualizing the 23 cleavage products simultaneously.

To identify fragment compositions of specific cleavage products, we used a CNBr mapping procedure which has been described in detail previously (Pepinsky, 1983). The mapping technique is based on nearest-neighbor analysis of fragments released after CNBr treatment of partial cleavage products and relies on the fact that each complete CNBr fragment is represented by a distinct subset of partial cleavage products. To assist in the analysis, we also characterized peptide maps for three structural variants of human lipocortin I and determined the effect of each modification on the normal digestion profile. This type of analysis by difference has been described previously (Pepinsky & Vogt, 1984).

Figure 2 shows two-dimensional silver-stained cleavage profiles for lipocortin I (panel A) and for the three structural variants (panels B–D). Each variant produced changes in the profile which served to identify specific subsets of cleavage products. Panel B shows the cleavage profile for lipocortin in which Met-126 was replaced by leucine. The Met-126 replacement resulted in fusion of fragments 2 and 3 and thus eliminated cleavage products derived from either fragment alone (2, 3, 1–2, 3–4, 3–5, 3–6, 3–7, and 3–8). Panel C shows the profile for the Met-55/Met-126 double mutant where both methionines were replaced by leucines. In addition to elimination of cleavage products derived from either fragment 2 or fragment 3, the double mutation also eliminated products dependent on the release of fragment 1 (1, 2–3, 2–4, 2–5, 2–6, 2–7, and 2–8).

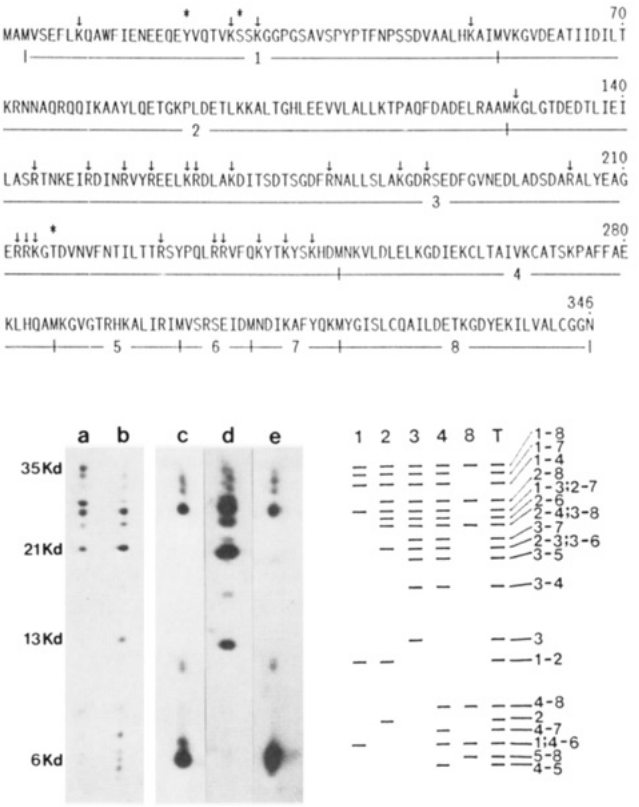

FIGURE 1: Identification of phosphorylated fragments of lipocortin I by cyanogen bromide mapping. The top panel shows the organization of CNBr fragments within the lipocortin I sequence. Arrows indicate trypsin cleavage sites within CNBr fragments 1 and 3. The bottom panel shows CNBr mapping results for lipocortin I. Preparations of phosphorylated lipocortin I were subjected to SDS–PAGE and the appropriate regions excised. The gel slices were incubated with CNBr and washed, and the fragments were subjected to SDS gel analysis. Lanes a and b show Coomassie blue stained profiles for protein treated with 7 and 21 mg/mL CNBr, respectively. Lanes c–e show autoradiographs of cleavage profiles of lipocortin I (treated with 21 mg/mL CNBr) phosphorylated ([γ-^{32}P]ATP) by recombinant pp60$^{c\text{-}src}$ (c), by the catalytic subunit of protein kinase A (d), and by protein kinase C from red cells (e). Lanes 1–4, 8, and T show schematic diagrams summarizing the CNBr mapping data for lipocortin I. Numbers at the right reflect fragment compositions where 1 refers to the amino-terminal CNBr fragment and 8 to the carboxy-terminal fragment. Lanes 1–4 and 8 are subsets of the digest representing specific CNBr fragments. Lane T represents a profile of the total digest. Apparent molecular masses of specific fragments are indicated at the left.

The simplified profile shown in panel C was used to identify fragment 1 containing cleavage products. Because of the large size difference between the fused fragment 1–3 (26 kDa) and the remaining fragment 4–8 (13 kDa), all of the large products detected must contain fragment 1 and differ by sequences at their C-terminus. Fragments 1–5 and 1–6 were omitted from the analysis because of the low yield of these products under the cleavage conditions used. Similarly, differences between profiles in panels B and C were used to identify cleavage products that contain fragment 2, and differences between profiles in panels A and B were used to assign the cleavage products that contain fragment 3. Panel D shows the cleavage profile for a fragment of lipocortin I which lacks 30 amino acids from its amino terminus (des-30 variant). In the truncated form, mobilities of all fragment 1 containing products are affected. The modified fragment 1 is referred to as °1. The cleavage profile of the des-30 variant confirms the orientation of cleavage products.

Figure 1 summarizes the mapping information. For reference, the entire sequence of lipocortin I has been included and the positions of methionines noted. The seven internal

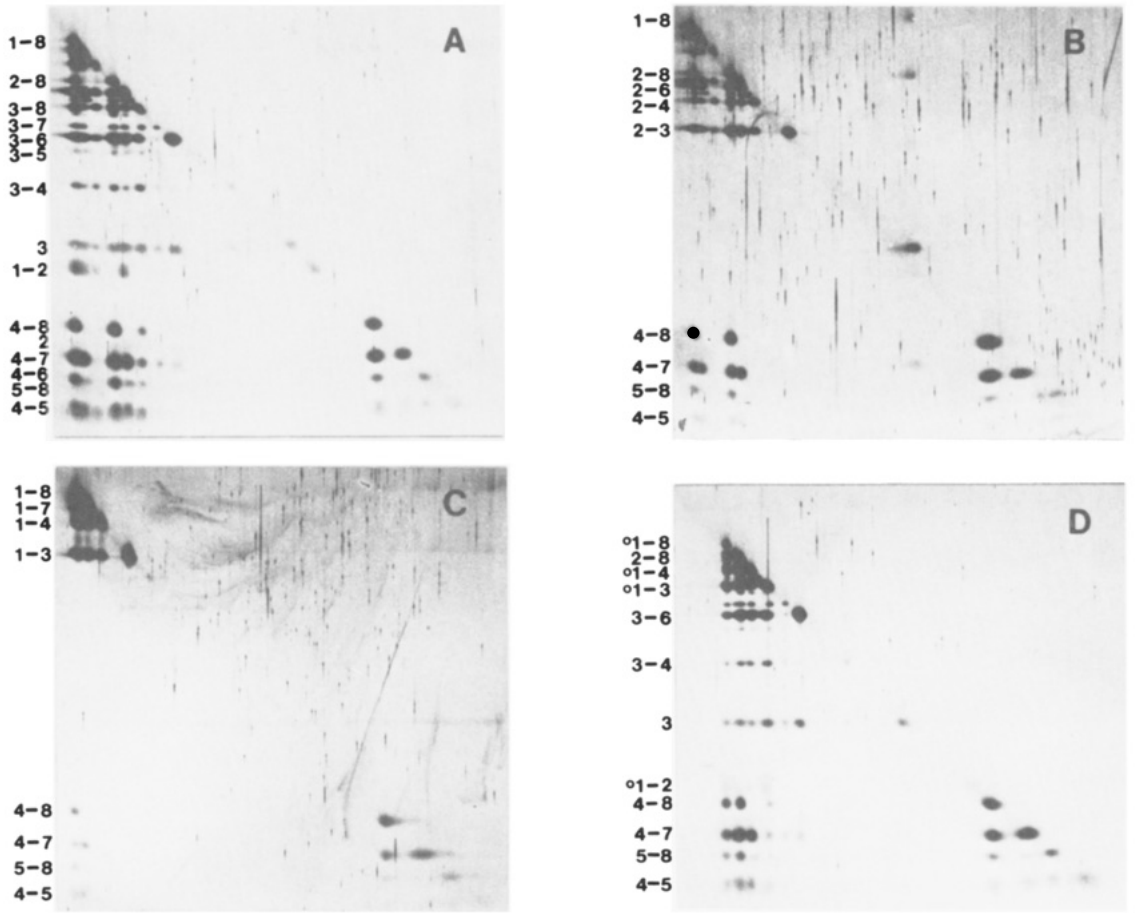

FIGURE 2: Two-dimensional electrophoretic analysis of CNBr fragments derived from lipocortin I. Gel slices containing either 1 μg of purified lipocortin I or 1 μg of each of the lipocortin I structural variants noted were treated with 7 mg/mL CNBr in 0.1 N HCl/0.1% 2-mercaptoethanol, and cleavage products were separated by SDS–PAGE as described previously (Pepinsky & Sinclair, 1986). Longitudinal gel strips, containing the entire cleavage profile, were excised and treated with 14 mg/mL CNBr, and the resulting cleavage products were electrophoresed orthoginally out of the gel strips into slab gels. The two-dimentional profiles were visualized by silver staining. (A) Lipocortin I; (B) Met-126 mutant; (C) Met-55/Met-126 double mutant; (D) des-30 variant. Designations at the left of the panels indicate subsets of cleavage products that are identified by the specific digest.

methionines upon cleavage with CNBr can be used to separate the protein into five large and three small CNBr fragments. Lane T in Figure 1 shows a schematic representation of the mapping data. Instead of 36 expected cleavage products (8 complete and 28 partials), only 23 were detected. Ten of the missing cleavage products (4, 5, 6, 7, 8, 5–6, 5–7, 6–7, 6–8, and 7–8) were too small to be detected by SDS–PAGE. The three others were undetected because they comigrate with other partials (1–5, 1–6, and 2–5). Of the complete cleavage products, fragment 3 was the only one identified by silver staining; fragments 1 and 2 were detected by staining with Coomassie blue. The schematic profiles at the bottom right of this figure (Figure 1) show one-dimensional cleavage maps that correspond to each of the five large CNBr fragments (lanes 1, 2, 3, 4, and 8). Each fragment is represented by a specific subset of cleavage products.

CNBr Peptide Maps of Phosphorylated Lipocortin I. Recombinant human lipocortin I was phosphorylated by recombinant pp60$^{c\text{-}src}$, immune complex of polyoma middle T/pp60$^{c\text{-}src}$, and recombinant pp50$^{v\text{-}abl}$ in vitro, and the phosphoproteins were analyzed by CNBr mapping. Lane c in Figure 1 shows an autoradiograph of the CNBr cleavage profile for lipocortin I which was phosphorylated by recombinant pp60$^{c\text{-}src}$. Phosphorylation by pp50$^{v\text{-}abl}$ or polyoma middle T/pp60$^{c\text{-}src}$ produced the same CNBr cleavage profile (not shown). The profile of radiolabeled fragments is identical with the one described previously for naturally occurring and recombinant human lipocortins I which were phosphorylated with EGF-dependent kinase (Pepinsky & Sinclair, 1986). In each instance, only six labeled products were obtained. According to the analysis of CNBr cleavage products outlined in the previous section, the pattern of labeled fragments indicates phosphorylation within CNBr fragment 1, the 55 amino acid fragment located near the amino terminus of lipocortin. Phosphoamino acid analysis of lipocortin I phosphorylated by recombinant pp60$^{c\text{-}src}$, pp50$^{v\text{-}abl}$, EGF-dependent protein kinase, or immune complex of polyoma middle T/pp60$^{c\text{-}src}$ revealed phosphotyrosine exclusively (shown for recombinant pp50$^{v\text{-}abl}$-phosphorylated lipocortin I in Figure 3, lane A).

Protein kinase C also catalyzed phosphorylation of lipocortin I within CNBr fragment 1. The cleavage profile of lipocortin I phosphorylated by protein kinase C (Figure 1, lane e) is identical with that shown in Figure 1, lane c, for lipocortin I phosphorylated by recombinant pp50$^{v\text{-}abl}$. Phosphoamino acid analysis showed that serine and threonine residues were labeled, indicating multiple sites of phosphorylation (Figure 3, lane B). Phosphoserine was the most prominent species, accounting for about two-thirds of total radioactivity. Only a trace of radioactivity was released as inorganic phosphate.

The catalytic subunit of cAMP-dependent protein kinase phosphorylated lipocortin I with an apparent K_m of 0.2 μM and with a V_{max} similar to the one for partially dephosphorylated casein obtained under the conditions of the assay described under Experimental Procedures (data not shown). The CNBr map of phosphopeptides was distinct from that of the tyrosine kinase or protein kinase C phosphorylated species

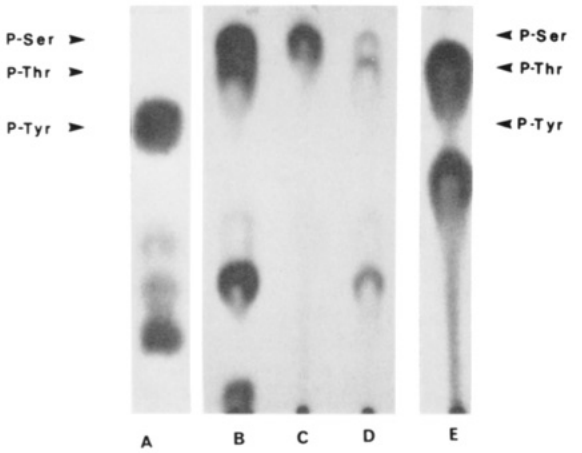

FIGURE 3: Phosphoamino acid analysis of phosphorylated lipocortin I. Samples containing either phosphorylated lipocortin I that was gel purified or phosphorylated tryptic fragments purified by reverse-phase HPLC were acid hydrolyzed. Phosphoamino acids were separated by high-voltage electrophoresis on TLC plates and visualized by autoradiography. (Lane A) pp50$^{v\text{-}abl}$-phosphorylated lipocortin; (lane B) erythrocyte protein kinase C treated lipocortin; lanes C (HPLC fraction 5) and D (HPLC fraction 89) were peptides derived from the tryptic map of protein kinase C phosphorylated lipocortin shown in Figure 4C; (lane E) protein kinase A treated lipocortin. The base of each lane represents the origin. Unlabeled phosphoamino acid standards were added to the radioactive samples and visualized with ninhydrin; their migration positions are indicated by the arrows.

(Figure 1, lane d). Analysis of the pattern of labeled fragments indicates that phosphorylation was restricted to CNBr fragment 3. Phosphoamino acid analysis of protein kinase A phosphorylated lipocortin I revealed phosphothreonine exclusively (Figure 3, lane E).

Tryptic Peptide Maps of Phosphorylated Lipocortin I. To further localize specific phosphorylation sites, lipocortin I was subjected to tryptic peptide mapping, using reverse-phase HPLC to separate cleavage products. The amino acid sequence information for 36 peaks from the tryptic map of lipocortin I was published previously (Huang et al., 1987) and accounts for about 85% of lipocortin's primary structure. Most of the missing tryptic fragments were small and eluted with the flow-through peak. Panel A in Figure 4 shows the elution profile of lipocortin I tryptic fragments monitored at 214 nm. Panels B, C, and D in Figure 4 show radioactive profiles from peptide maps of lipocortin I which were phosphorylated by recombinant pp60$^{c\text{-}src}$, protein kinase C, and protein kinase A, respectively. Profiles for lipocortin I phosphorylated in vitro by recombinant or immune complex containing pp60$^{c\text{-}src}$ were identical with profiles obtained with recombinant Abelson kinase or EGF-dependent kinase (not shown). Since phosphorylation affected migration of the peptides on HPLC, identification of a specific phosphorylated residue was made with the aid of CNBr mapping and sequence analysis.

The assignment of the tyrosine phosphorylation site to CNBr fragment 1 restricted the possible phosphorylated residues to Tyr-21 or Tyr-39. Tyr-21 was identified as the phosphorylation site on the basis of the following information: (i) All tryptic peptides derived from CNBr fragment 1 have been identified by sequence analysis (Huang et al., 1987). The fragment containing Tyr-39 elutes as a single peak at fraction 100 while the fragment containing Tyr-21 elutes as two peaks at fractions 99 and 103 due to incomplete cleavage at Lys-9 (Figure 4, panel A). The two *O*-phosphotyrosine-containing tryptic peptides migrated at fractions 87 and 91 (Figure 4, panel B) with similar differential retention times to two peptides which contain Tyr-21. (ii) Sequence analysis of fraction 87 revealed radioactivity at cycle 12, consistent with the correct

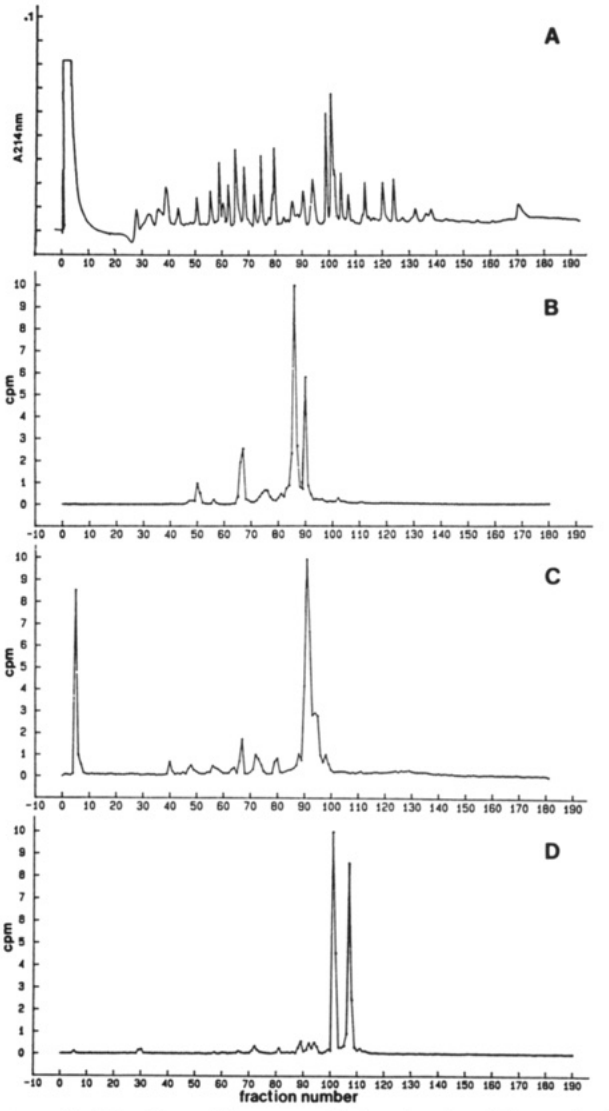

FIGURE 4: Tryptic peptide maps of phosphorylated lipocortin I. Preparations of phosphorylated lipocortin I were subjected to SDS–PAGE. Labeled protein was electroeluted and digested with trypsin. The digests were analyzed by reverse-phase HPLC on a C_{18} column. The column eluate was monitored for protein by absorbance at 214 nm. Column fractions were monitored for radioactivity by scintillation counting. (Panel A) Tryptic digest of nonphosphorylated lipocortin I. Panels B–D show peptide maps of labeled fragments where lipocortin was phosphorylated by pp60$^{c\text{-}src}$ (B), by protein kinase C (C), and by protein kinase A (D). For recombinant pp60$^{c\text{-}src}$ kinase, 10 = 120 000 cpm; for protein kinase C, 10 = 570 000 cpm; for protein kinase A, 10 = 730 000 cpm.

assignment of the tyrosine residue to the tryptic peptide which begins with Gln-10 (Table I). Tyr-39 was excluded as a possible phosphorylation site since it is the tenth residue in the tryptic peptide which begins with Gly-30. (iii) A mutant of lipocortin I was generated by site-directed mutagenesis in which Tyr-21 was replaced by phenylalanine. The phenylalanine variant failed to be phosphorylated by recombinant pp50$^{v\text{-}abl}$ kinase (Figure 5, lanes c and d).

Analysis of the tryptic phosphopeptides from protein kinase A phosphorylated lipocortin I indicates that Thr-216 is the major phosphorylation site. Although several lipocortin I tryptic fragments located in the region included by CNBr fragment 3 contain threonine residues, only two of these peptides migrate on reverse-phase HPLC near the two phosphopeptides obtained from protein kinase A phosphorylated protein (Figure 4, panel D). These two peptides result from alternative cleavages after Arg-213 or Lys-214, and migrate

Table I: Results of Automated Edman Degradation of Lipocortin I Phosphorylated by Recombinant pp60^{c-src} and Protein Kinase A[a]

fraction 87 from HPLC of pp60^{c-src}-phosphorylated lipocortin I			fraction 99 from HPLC of protein kinase A phosphorylated lipocortin I	
cycle	amino acid	radioact. (cpm)	amino acid	radioact. (cpm)
1	Gln-10	230	Lys-214	670
2	Ala	180	Gly	420
3	Trp	190	Thr-216	2270
4	Phe	260	Asp	1240
5	Ile	190	Val	1000
6	Glu	200	Asp	840
7	Asn	150	Val	660
8	Glu	200	Phe	640
9	Glu	180	Asn	590
10	Gln	200	Thr-223	1630
11	Glu	120	Ile	1360
12	Tyr-21	490	Leu	510
13	Val	750	Thr	380
14	Gln	640	Thr	380
15	Thr	610	Arg	370
16	Val	430	Ser	390
17	Lys	450	Tyr	310
18			Pro	345
19			Gln	335
20			Leu	310

[a] Fraction 87 from HPLC of pp60^{c-src}-phosphorylated lipocortin I contained 200 000 cpm, and fraction 99 from HPLC of protein kinase A phosphorylated protein contained 280 000 cpm. Edman degradation was carried out in a Beckman spinning-cup sequencer.

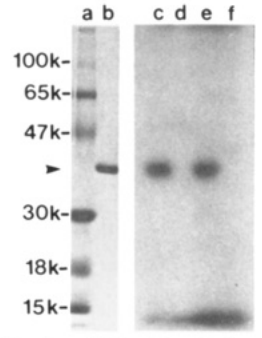

FIGURE 5: Characterization of phosphorylation sites by site-directed mutagenesis. Lipocortin I and its mutants containing amino acid substitutions for Tyr-21 and Thr-216 were analyzed for their ability to be phosphorylated by pp50^{v-abl} and protein kinase A, respectively. Phosphorylated samples were subjected to SDS–PAGE and analyzed by staining with Coomassie blue and by autoradiography. Radioactive bands were excised and quantified by scintillation counting. Lanes c and d show lipocortin treated with pp50^{v-abl} and lanes e and f with protein kinase A. Lane a, BRL prestained high molecular weight markers, lane b, Coomassie-stained lipocortin I; lane c, lipocortin I (2760 cpm); lane d, Tyr-21 mutant (50 cpm); lane e, lipocortin I (2840 cpm); lane f, Thr-216 mutant (90 cpm).

at fractions 108 and 114 when not phosphorylated. Sequence analysis of fraction 99 from the experiment described in Figure 4D revealed radioactivity in the third cycle, consistent with phosphorylation at Thr-216 of the peptide beginning with Lys-214. Phosphate was also detected at the tenth cycle, suggesting a partial phosphorylation of Thr-223 (Table I). None of the other tryptic peptides from this region contains threonine at the third and tenth residues (see tryptic cleavage sites depicted in the top panel of Figure 1). Analysis of ^{32}P release during automated Edman degradation of the phosphopeptide obtained from HPLC fraction 104 revealed phosphate in the second cycle. Although the total recovery of the radioactivity from sequencing this peptide was much lower than that obtained from fraction 99 (data not shown),

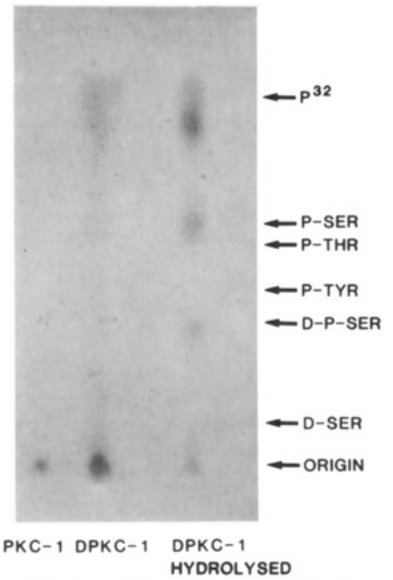

FIGURE 6: Identification of Ser-27 as a protein kinase C phosphorylation site. Lipocortin phosphorylated by protein kinase C was digested with trypsin, and cleavage products were separated by reverse-phase HPLC (see Figure 4C). Fraction 5, which contained a small phosphorylated peptide, was treated with dansyl chloride and subjected to phosphoamino acid analysis with or without prior acid hydrolysis. ^{32}P-Labeled species were detected by autoradiography. Dansylserine and dansyl-*O*-phosphoserine standards were visualized by UV light. PKC-1, unhydrolyzed peptide; DPKC-1, same peptide, after dansylation; DPKC-1 hydrolyzed, dansylated peptide, after acid hydrolysis. The arrows indicate the migration positions of free ^{32}P, phosphoamino acids, dansylserine, and dansyl-*O*-phosphoserine.

the results are consistent with a peptide obtained by an alternative cleavage after Arg-213 and phosphorylated at Thr-216. The assignment was further supported by the experiment shown in Figure 5 (lanes e and f) in which a recombinant lipocortin I variant with Thr-216 replaced by alanine failed to be phosphorylated by protein kinase A.

Identification of Sites Phosphorylated by Protein Kinase C. HPLC analysis of tryptic digests of protein kinase C phosphorylated lipocortin I revealed two major peaks at fractions 5 and 90 with a shoulder on the latter peak (Figure 4, panel C). The phosphopeptides obtained by tryptic digestion of lipocortin I phosphorylated by protein kinase C from human platelets were indistinguishable from those obtained from phosphorylation by protein kinase C from human red cells. Phosphoamino acid analysis of fraction 5 revealed phosphoserine exclusively; fraction 90 contained both phosphoserine (60%) and phosphothreonine (40%), as seen in Figure 3, lanes C and D. The short retention time of fraction 5 suggests a small, hydrophilic phosphopeptide. Since phosphorylation by protein kinase C occurs within CNBr fragment 1 (see Figure 1, lane e), the only candidate for such a peptide is the sequence Ser-Ser-Lys which begins at Ser-27. Because of the difficulty in sequencing such a small hydrophilic peptide, we devised a simple manual procedure for determining if Ser-27 was phosphorylated. We used dansyl chloride to label the amino terminus of this peptide and thus distinguish between the two serines. The intact peptide was dansylated and subjected to acid hydrolysis: the appearance of dansylated phosphoserine would indicate that the amino terminus contains the phosphorylated residue. No *N*-dansyl-*O*-phosphoserine was detected without the acid hydrolysis step, confirming the absence of free labeled *O*-phosphoserine in the flow-through or hydrolysis of the peptide before or during dansylation. After acid hydrolysis, a significant amount of *N*-dansyl-*O*-phosphoserine was detected, indicating that at least 40% of phosphoserine

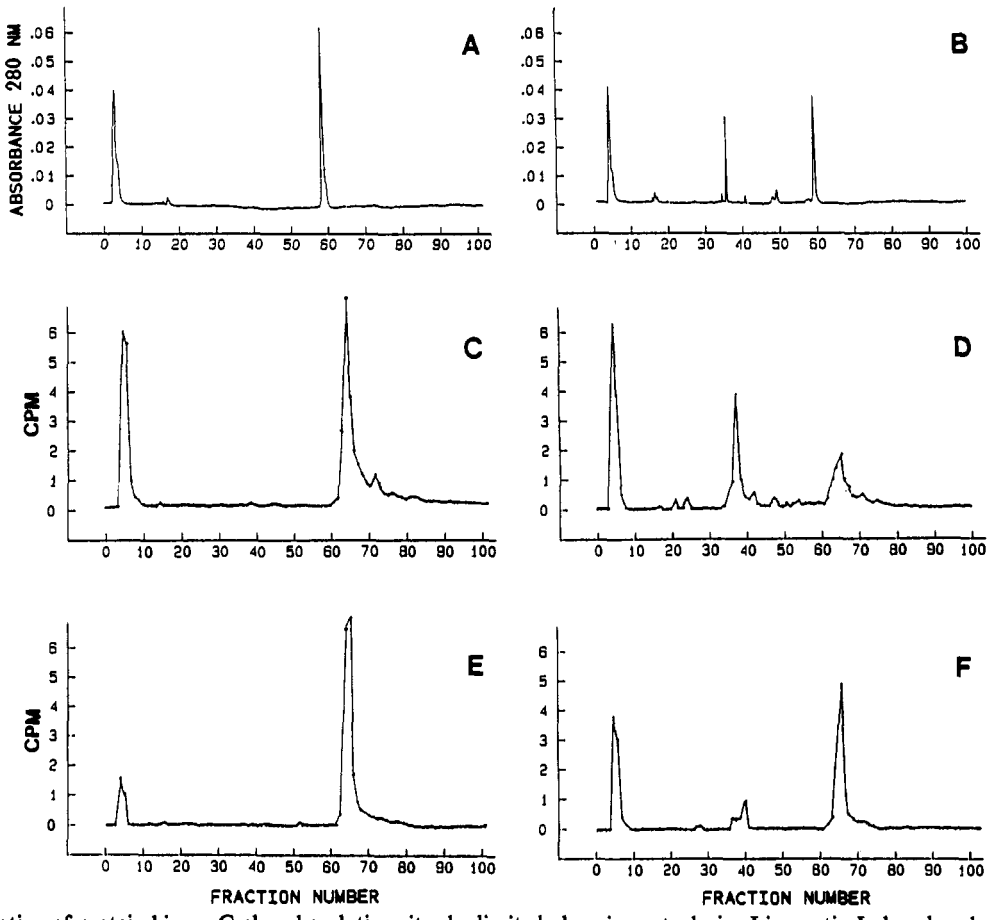

FIGURE 7: Localization of protein kinase C phosphorylation sites by limited plasmin proteolysis. Lipocortin I phosphorylated by pp50$^{v\text{-}abl}$ or by protein kinase C was partially digested with plasmin as described under Experimental Procedures. The products were analyzed by reverse-phase HPLC on a C_4 column. Fractions were monitored for absorbance at 280 nm and for radioactivity by scintillation counting. Panels A and B show absorbance profiles of lipocortin I before and after digestion with plasmin, respectively. Panels C and D and panels E and F show similarly paired profiles of labeled products for lipocortin I that was phosphorylated by pp50$^{v\text{-}abl}$ (C and D) and protein kinase C (E and F).

was at position 27 (Figure 6). Under these conditions, most of the label was converted to inorganic phosphate. The failure to obtain 100% conversion of phosphoserine to its dansyl derivative does not necessarily indicate that phosphorylation also occurred at the second internal site (Ser-28). The charged phosphate group raises the pK of the amino terminus of this peptide, reducing its reactivity to dansyl chloride. This effect could also reduce reactivity to phenyl isothiocyanate, which together with the instability of the phenylthiohydantoin (PTH) derivative of *O*-phosphoserine due to β-elimination of phosphate could explain the low yields of PTH-phosphoserine obtained during attempted manual Edman degradation (data not shown). Improved yields of *N*-dansyl-*O*-phosphoserine were obtained when the pH of the reaction was raised to 10.5.

Fraction 90, the larger phosphopeptide from the tryptic digest of kinase C phosphorylated lipocortin I, was subjected to solid-phase sequencing. The peptide was obtained and sequenced from three separate phosphorylation reactions: twice with protein kinase C purified from red cells and once with human platelet enzyme. In all three cases, a small peak of radioactivity appeared at the 15th cycle. All other cycles (20 cycles analyzed) gave counts near background. Phosphorylation by protein kinase C in CNBr fragment 1 limits the number of potential sites to Thr-24 or Thr-41. The 15th residue of the peptide which begins with Gly-30 is Thr-41. The recovery of radioactivity was only 0.02% from 300 000 cpm incorporated into the peptide. This low recovery may be due to a blocked amino terminus or because the 15th residue is near the end of the peptide. The presence of serine in this fraction (Figure 3, lane D) could result from a peptide beginning with Ser-27 due to incomplete cleavage at Lys-26 or a random phosphorylation at any of the five serines present in the peptide containing Thr-41.

The assignment of Thr-41 as the only phosphorylated threonine residue present within CNBr fragment 1 was confirmed by results obtained after plasmin digestion of lipocortin I. As shown previously (Huang et al., 1987), digestion with plasmin cleaves lipocortin I at Ser-27 and Gly-30, separating the 3-kDa amino-terminal tail region from the remainder of the protein. Since Thr-24 and Thr-41 fall on opposite sides of the cleavage site, plasmin digestion provides a simple method for distinguishing these sites. Figure 7, panel B, shows an HPLC analysis of lipocortin I subjected to digestion with plasmin. The 3-kDa amino-terminal fragment migrates at fraction 38, and the 32-kDa fragment (fraction 65 for phosphorylated lipocortin; fraction 60 for unphosphorylated lipocortin) migrates near the nondigested protein. Panel F in Figure 7 shows the results for protein kinase C phosphorylated lipocortin. Most of the counts were detected in the flow-through (40%) and the large 32-kDa peptide (50%). Very little (less than 10% of total) was detected in the 3-kDa peak. Phosphoamino acid analysis of the flow-through and the 3-kDa peak fraction revealed phosphoserine exclusively. All detected phosphothreonine remained in the 32-kDa peptide (not shown). SDS–PAGE analysis of the phosphorylated protein verified that no intact lipocortin remained after plasmin digestion as judged by autoradiography and Coomassie blue staining. As a control for the experiment, lipocortin I which had been phosphorylated by pp50$^{v\text{-}abl}$ (panels C and D) was analyzed. Fifty percent of the label was recovered in the 3-kDa fragment.

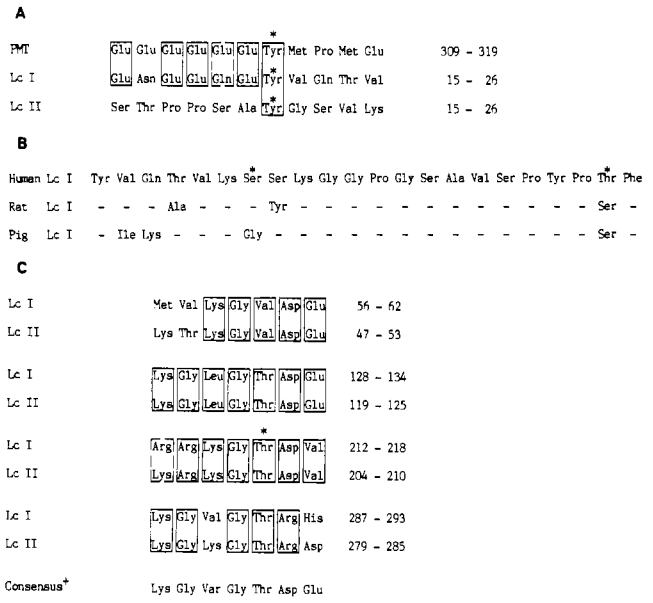

FIGURE 8: Comparison of homologous phosphorylation sites on lipocortins. (A) Comparison of amino-terminal sequence of lipocortins I and II (Lc I and Lc II) with the major site of phosphorylation of polyoma middle T (PMT). Solid lines indicate identity; dashed lines indicate conservative substitutions. (B) Amino acid sequence of human lipocortin I between residues 15 and 42 is compared to rat and pig proteins. Sequence differences are indicated. (C) Sequence of protein kinase A phosphorylation site in lipocortin I and homology with internal repeats in lipocortins I and II. Phosphorylation sites are indicated by asterisks. In (A) and (C), solid lines indicate identity; dashed lines indicate conservative substitutions.

While a significant portion of the counts eluted with the 32-kDa fragment, SDS–PAGE analysis revealed that only 10% of the label in the intact protein remained in the 32-kDa fragment generated by plasmin. No intact lipocortin was detected, indicating that the digestion went to completion. Phosphoamino acid analysis revealed *O*-phosphotyrosine exclusively (not shown).

Discussion

We have used peptide mapping and protein sequence analysis to identify sites within lipocortin I that are phosphorylated by several purified protein tyrosine kinases and protein kinases A and C. For tyrosine kinases and protein kinase A, these sites were verified by characterizing mutants in which the phosphorylated sites had been altered by site-directed mutagenesis. Tyr-21 is the major site of phosphorylation by recombinant $pp60^{c\text{-}src}$, polyoma middle T/$pp60^{c\text{-}src}$ complex, and recombinant $pp50^{v\text{-}abl}$. Previous work (Pepinsky & Sinclair, 1986; De et al., 1986) showed that this is the same site which is phosphorylated in response to EGF in intact A431 cells and in membranes from A431 cells. The major sites of phosphorylation by protein kinase C are Ser-27, located six residues from the tyrosine kinase phosphorylation site, and Thr-41. The major site of phosphorylation by cAMP-dependent protein kinase is Thr-216.

The sequence around the phosphorylation site in lipocortin I (tyrosine preceded by a series of glutamate residues) is consistent with a consensus sequence for preferred sites of protein tyrosine kinases. Figure 8A compares the sequence of lipocortin I to that of the major site for phosphorylation of polyoma middle T by $pp60^{c\text{-}src}$. This is the only unambiguous substrate for $pp60^{c\text{-}src}$ in vivo which has been sequenced (Schaffhausen & Benjamin, 1981). The amino-terminal sequence of lipocortin II is also presented for comparison. In lipocortin II, Tyr-23 is phosphorylated (Glenney & Tack, 1985). The fact that both proteins are phosphorylated at approximately the same position even though little sequence similarity exists between lipocortins I and II in this region suggests that this phosphorylation is determined by structural features other than the primary sequence surrounding the target tyrosine. Since the calcium binding of lipocortin II is affected by association with p10, phosphorylation also may be influenced its association with p10 (Powell & Glenney, 1987). Although a lot of information exists regarding phosphorylation of lipocortin in vivo, the significance of tyrosine phosphorylation is not clear. Recently, Schlaepfer and Haigler (1987) have reported that tyrosine phosphorylation lowered the Ca^{2+} requirements for association of lipocortin with membrane vesicles, while Powell and Glenney (1987) suggested that phosphorylation of lipocortin II by $pp60^{c\text{-}src}$ decreases its affinity for negatively charged phospholipid vesicles. Further studies are needed to evaluate which of the effects are physiologically significant.

The sites on lipocortin I which are phosphorylated by protein kinase C are also located in the N-terminal tail region. Figure 8A illustrates that only weak similarity exists between the two proteins in this region with Thr-24 of lipocortin I near Ser-25, the reported protein kinase C phosphorylation site of lipocortin II. We did not observe phosphorylation of Thr-24 in human lipocortin I. In the rat lipocortin I sequence, Thr-24 is replaced by alanine and thus does not represent a conserved phosphorylation site. Although we detected phosphorylation at Thr-41, the major site of phosphorylation appears to be the Ser-27. Ser-27 also satisfies the criterion for a preferred phosphorylation site by protein kinase C, with a lysine following two residues later (Kishimoto et al., 1985; Woodgett et al., 1986). Panel B in Figure 8 compares the human lipocortin I sequence with the ones corresponding to rat and pig (Pepinsky et al., 1986; De et al., 1986; Tamaki et al., 1987). It is of interest that in lipocortin I from pig Ser-27 is replaced by Gly and in the rat Ser-28 is replaced by Tyr. In the rat and pig sequences, Thr-41 is replaced by Ser, representing the most conservative substitution. Other than those amino acid substitutions, the sequences for rat, pig, and human lipocortin I are nearly identical. It seems that phosphorylation of lipocortin I by protein kinase C involves multiple sites which are not entirely conserved between species. The phosphorylation sites for protein kinase C, like those for the protein tyrosine kinases, are in a region where little similarity exists between lipocortins I and II. This may indicate functional differences between the two proteins. Following the 30–40 amino acid amino-terminal tail, the remainder of the protein exists as four 74–86-residue stretches with approximately 30% sequence similarity within a given protein and with 40–70% positional identity between the two lipocortins (Huang et al., 1986; Saris et al., 1986). This structural organization suggests that the highly conserved repeated domain confers similar function to the proteins, while the amino-terminal domain may give diversity in either regulation or target. The location of the phosphorylation sites near the boundary of these two domains also may affect whether the protein is proteolytically cleaved.

Thr-216 phosphorylated by cAMP-dependent protein kinase is near the beginning of the third repeated sequence of the conserved domain. This threonine is in the most highly conserved region of the repeated domain and is also conserved in lipocortin II (Figure 8C). The sequence preceding Thr-216, Arg-Arg-Lys-Gly, is positively charged as expected for a protein kinase A phosphorylation site. Interestingly, although two of the other three repeat sequences have a Thr residue which aligns with Thr-216, these sequences do not have pos-

itively charged residues at amino acid positions 2 and 3 preceding the threonine; no phosphorylation occurs at these other sites. The fact that cAMP-dependent protein kinase phosphorylates at a site distant from the other kinase phosphorylation sites argues against the amino terminus being merely an adventitious site for nonspecific phosphorylation.

It has been proposed that the amino acid sequence, Thr-Asp-Glu, at the end of the highly conserved consensus sequence (bottom of Figure 8C) is part of a calcium binding site (Kretsinger & Creutz, 1986). The stoichiometry of calcium binding of lipocortin I has been estimated as 4 Ca^{2+} per molecule, with an apparent K_d of 75 μM (Haigler et al., 1987; Schlaepfer & Haigler, 1987). The effect of phosphorylation by protein kinase A on calcium binding needs to be determined. Phosphorylation of a lipocortin by protein kinase A has been reported to reduce its phospholipase A_2 inhibitory activity (Hirata, 1981). The effect of phosphorylation at a specific site of a well-characterized recombinant protein on this inhibitory activity can now be investigated.

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