Lipocortins 1 and 2 as Substrates for the Insulin Receptor Kinase in Rat Liver

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Lipocortins 1 and 2 are major substrates for the epidermal growth factor receptor and the pp60c-src tyrosine kinases in transformed cells. In the present study, we have characterized the phosphorylation of lipocortins 1 and 2 by the insulin receptor tyrosine kinase in vitro and in vivo. In vitro, the solubilized insulin receptor, partially purified from rat liver, catalyzed phosphorylation of human recombinant lipocortin 1 and purified bovine lipocortin 2. Phosphorylation of lipocortin 1 was increased 15-fold upon stimulation with 10^{-7} M insulin. The apparent K_m of the reaction was 3.3 μM and was not affected by insulin stimulation. Insulin stimulated phosphate incorporation into lipocortin 2 by 20-fold (apparent K_m > 20 μM). Both lipocortins were phosphorylated exclusively on tyrosine residues as judged by phosphoamino acid analysis. Based upon peptide mapping, lipocortin 1 was phosphorylated on Tyr-21, a site phosphorylated by other tyrosine kinases. Polyclonal anti-phosphotyrosine antibodies recognized the tyrosine-phosphorylated lipocortin 2, but not lipocortin 1 in its phosphorylated form.

In hepatocytes from normal and dexamethasone-treated rats, lipocortin 1 content was less than 50 ng/10^6 cells. Insulin-induced phosphorylation of lipocortin 1 was detected in intact hepatocytes from corticosterone-treated animals but not in cells from normal rats. No phosphorylation of lipocortin 2 was found, although its content was ~100 ng/10^6 cells from normal animals and increased to ~1 μg/10^6 cells following treatment of rats with dexamethasone for 4 days. Thus, although lipocortins 1 and 2 are in vitro substrates of the insulin receptor kinase, only lipocortin 1 is phosphorylated in an insulin-dependent manner in intact hepatocytes, and this is only observed after dexamethasone treatment of the rats.

The abbreviation used are: EGF, epidermal growth factor; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

EXPERIMENTAL PROCEDURES

Materials—[32P]Orthophosphate, [γ-32P]ATP, and Triton X-100 were obtained from New England Nuclear; wheat germ agglutinin agarose from Vector Laboratories; reagents for SDS/PAGE from Bio-Rad Laboratories; dexamethasone, HEPES, phenylmethylsulfonyl fluoride, aprotinin, N-acetyl-D-glucosamine, and tosylphenylalanyl chloromethyl ketone-treated trypsin from Sigma. Porcine insulin was from Eli Lilly and Pansorbin was from Calbiochem. Human recombinant lipocortin 1 was produced in Escherichia coli (25). Lipocortin 2 was purified from bovine intestine mucosa (18). Anti-lipocortin 1 and anti-lipocortin 2 sera were generated in rabbits as previously described (18). Anti-phosphotyrosine antibody was purified from the sera of rabbits immunized with phosphotyramine coupled to keyhole limpet hemocyanin by affinity chromatography (26).

Male Sprague-Dawley rats weighing 150–175 g were purchased from Charles River, and used for insulin receptor preparation. Hep-

Lipocortins 1 and 2 are the best characterized endogenous substrates for receptor- and oncogene-encoded tyrosine kinases. Lipocortin 1 is a substrate for the EGF receptor kinase in A431 cells and fibroblasts (1–3), while lipocortin 2 undergoes phosphorylation by pp60c-src in Rous sarcoma virus-transformed cells (4–6). The lipocortins are a family of intracellular proteins that are located at the membrane cytoplasm interface and found in many tissues (7–10). They bind to phospholipid in the presence of calcium and have been shown to inhibit phospholipase A_2 activity in vitro assays (11–15). These properties have been shown to decrease upon phosphorylation of lipocortin (16, 17). Both lipocortins are 36–38-kDa proteins, and their deduced amino acid sequences are 50% identical (6, 13, 18). The homology is concentrated in four internal repeating units which account for the calcium-dependent lipid binding and phospholipase A_2 inhibition properties of these proteins.

Like the EGF receptor and pp60c-src, the insulin receptor is a tyrosine kinase (19). Upon insulin binding, the kinase is activated, resulting in phosphorylation of tyrosine residues on the receptor itself, as well as on other proteins (20). These sequential phosphorylation reactions are believed to be crucial for transduction of the insulin signal (21, 22). The similarities between the receptors for EGF and insulin suggest that lipocortin, which is a substrate for the former, may also be phosphorylated by the latter. In addition, lipocortin’s role in insulin signal transduction is suggested by the observation that corticosteroid treatment which induces insulin resistance also results in induction of lipocortins (14). In vitro, at high substrate concentration, substrate inhibition of receptor kinase activity has been shown to occur (23, 24), and this may occur in cells as well. Steroid-induced increase in the availability of intracellular lipocortins may thus attenuate the insulin response rather than enhance it. In this study, we addressed the issue of lipocortins 1 and 2 as substrates for the insulin receptor kinase activity in both a cell-free system using partially purified insulin receptor from rat liver, and in intact cells using isolated hepatocytes from normal and corticosteroid-treated animals.
Phosphorylation of Lipocortins by the Insulin Receptor

Preparation of Solubilized Insulin Receptor—Rats were killed with carbon monoxide, and livers were removed, washed with ice-cold saline, weighed, and minced with scissors. Ten volumes of 50 mM HEPES (pH 7.4), 0.25 M sucrose supplemented with aprotinin (1 mg/dl) and 1 mM N-acetyl-D-glucosamine were added to the liver, and this was homogenized in a Dounce homogenizer with a loose pestle. The homogenate was centrifuged at 1,200 rpm in a Sorvall centrifuge to remove large clumps, and Triton X-100 was added to the supernatant to a final concentration of 1%. The resulting suspension was stirred for 60 min, centrifuged at 100,000 × g for 60 min, and the supernatant was applied to a 2-ml column of 6% blue exclusion, exceeded 90% upon isolation of cells and there was a further loss up to 10% of the viable cells at the end of the 2-h incubations for phosphorylation of hepatocytes.

Labeling of the hepatocytes was achieved by incubating 0.5-ml aliquots of suspended cells for 90 min with 100 mM dithiothreitol, separated by SDS-PAGE, and subjected to enzymatic digestion was continued for an additional 16 h. The supernatant solutions were used for immunoprecipitation with anti-phosphotyrosine or anti-lipocortin antisera. Proteins phosphorylated in experiments with solubilized insulin receptor aliquots (10 μg of protein) were diluted in a volume of 50 μl; final concentrations were 50 mM HEPES (pH 7.4), 0.1% Triton X-100, 4 mM Mn2+, 10 mM Mg2+, 0.5 mM Ca2+. These mixtures were preincubated with or without insulin (10−7 M) at 37 °C for 30 min and then incubated at 22 °C for 6 min with 25 μM ATP to allow receptor phosphorylation followed by an addition of 5% [γ32P]ATP and lipocortins 1 or 2 in the concentration indicated. Reactions were allowed to continue for another 10 min and stopped by adding 5× electrophoresis sample buffer containing 100 mM dithiothreitol (27). For immunoprecipitation experiments, the reactions were stopped by adding 500 μl of ice-cold stopping buffer (50 mM HEPES (pH 7.4), 10 mM Tris, 0.5 mM CaCl2, 0.1% Triton X-100, and 2 mM sodium fluoride, 4 mM EDTA, and 2 mM sodium vanadate) and proteins of interest were immunoprecipitated with anti-phosphotyrosine or anti-lipocortin antibody as previously described (28).

Isolation and 32P-Labeling of Cellular Proteins—Hepatocytes were isolated from rats treated with 1 mg of dexamethasone intraperitoneally for 4 days and from control rats using a modification of the method of Berry and Friend (29). The cells were washed with 137 mM NaCl supplemented with 2.7 mM KCl and 20 mM HEPES (pH 7.4) and resuspended in phosphate-free RPMI 1640 medium to yield 2 × 107 cells/ml. Viability of isolated hepatocytes, judged by trypan blue exclusion, exceeded 90% upon isolation of cells and there was a further loss up to 10% of the viable cells at the end of the 2-h incubations for phosphorylation of hepatocytes.

Labeling of the hepatocytes was achieved by incubating 0.5-ml aliquots of suspended cells for 90 min with 100 mM dithiothreitol, separated by SDS-PAGE, and subjected to enzymatic digestion was continued for an additional 16 h. The supernatant solutions were used for immunoprecipitation with anti-phosphotyrosine or anti-lipocortin antisera. Proteins phosphorylated in experiments with solubilized insulin receptors were dissolved directly in sample buffer (27) or immunoprecipitated prior to preparation in sample buffer. Proteins phosphorylated in intact cells were always immunoprecipitated prior to sample preparation for SDS-PAGE. Phosphoproteins, in sample buffer with 100 mM dithiothreitol, were separated on 7.5% or 10% resolving polyacrylamide gel. The gels were stained with Coomassie Blue in 50% trichloroacetic acid, destained in 7% acetic acid, dried, and autoradiographed with Kodak X-OMat film.

Phosphoamino Acid Analysis—Tryptic phosphopeptides were obtained from the protein bands in polyacrylamide gel fragments as previously described (30). After the positions of the phosphorylated proteins in the gel were determined by autoradiography, the corresponding segments of the gel were excised, washed for 12 h at 37 °C in 20% methanol, dried at 80 °C for 2 h, and digested with 2 ml of 50 μg/ml of trypsin (pH 8.0). After a 6-h incubation at 37 °C, another 100 μg of trypsin was added, and the enzymatic digestion was continued for an additional 16 h. The supernatant solution was lyophilized, and the phosphopeptides were dissolved in 100 μl of 6 N HCl and hydrolyzed for 2 h at 110 °C. The phosphoamino acids were separated by high voltage electrophoresis on thin layer plates (Avicel, Analtech, Newark, DE; 250 μm) using a solution of H2O-acetic acid-pyridine (89:10:1). Unlabeled phosphoserine, phosphothreonine, and phosphoserylserine standards were added to all samples and identified by reaction with ninhydrin, and the radioactivity was located by autoradiography.

RESULTS AND DISCUSSION

Phosphorylation of Lipocortins 1 and 2 by the Insulin Receptor Kinase—In the presence of Mn2+ and [γ-32P]ATP, insulin stimulated phosphorylation of the β-subunit of the partially purified insulin receptor from rat liver which appears as a 95-kDa band on the autoradiograms of SDS gels (Fig. 1). Addition of human recombinant lipocortin 1 in concentrations from 0.5 to 5 μM to this mixture in the absence of insulin resulted in minor phosphorylation of this protein substrate (Fig. 1, lanes a–c). However, in the presence of insulin, lipocortin 1 was phosphorylated in a concentration-dependent manner (Fig. 1, lanes d–f). Purified bovine lipocortin 2 was also phosphorylated by the insulin receptor kinase in an insulin-dependent way (Fig. 1, lanes g–j). Like the EGF receptor and pp60c-src, the insulin receptor catalyzes lipocortin phosphorylation. The potential to undergo in vitro phosphorylation by more than one tyrosine kinase is not unique to lipocortins. Lactate dehydrogenase, enolase, and angiotensin II are shared substrates by the insulin receptor and pp60c-src kinases. Grassin, myosin light chain, and casein are phosphorylated by both the insulin and EGF receptor kinases, while src-like peptide, anti-src antibody, and histone are common substrates for all three tyrosine kinases (32–37). Although, these proteins are good substrates in vitro, they are not known to be substrates in vivo. Previous studies have shown that lipocortin 1 is a substrate for the EGF receptor (1–3), while lipocortin 2

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Fig. 1. Phosphorylation of lipocortins 1 and 2 in a cell-free system. Aliquots of wheat germ-perfused insulin receptor (10 μg of protein) were incubated without (lane a, b, c, g, h) or with (lanes d, e, f, i, j) insulin (10−7 M) for 30 min at 22 °C. Phosphorylation was initiated by adding 25 μM ATP followed by a concurrent addition of 20 μCi of [γ-32P]ATP per tube and the indicated amount of lipocortin 1 or lipocortin 2. After stopping the reaction, the mixture was reduced with 100 mM dithiothreitol, separated by SDS-PAGE, and subjected to autoradiography.
is an intracellular substrate for pp60^src (4-6) in intact cells.

**Characteristics of Phosphorylation of Lipocortins 1 and 2 by the Insulin Receptor**—For a quantitative determination of the amounts of 32P incorporated into lipocortin by the insulin receptor kinase, the bands corresponding to the lipocortins were cut from the dried gels and counted. The incorporation of 32P into lipocortin 1 was linearly proportional to the duration of incubation for 30 min (data not shown). Insulin increased the $V_{max}$ of the phosphorylation reaction 15-fold, (Fig. 2, upper panel). The value for $K_m$ for lipocortin 1 phosphorylation, estimated using a sigmoid fit according to the nonlinear least squares method described by Cleland (38) was 3.3 ± 0.6 μM. This value for $K_m$ (app) was not significantly changed upon insulin stimulation. This value was somewhat higher than that described for lipocortin 1 phosphorylation by the EGF receptor (50 nM) (39), but is among the lowest described for phosphorylation of any substrate by the insulin receptor kinase (20). Since the lipocortin content in some cells is as high as 0.5% of total cellular protein (31), the apparent $K_m$ seems to fall in the physiological concentration range. Whether variations in $K_m$ reflect actual differences in the various kinases or in the method of presentation of the substrate to the kinases remains to be determined. Since lipocortins become membrane-associated in the presence of Ca$^{2+}$, a lower $K_m$ may result from use of the EGF kinase receptor in its membrane-bound form. The extent of lipocortin 1 phosphorylation increased 15-fold by the insulin receptor kinase (40). Phosphorylation of lipocortin 2 by pp60^src (40).

To determine the sites and nature of phosphorylation of lipocortins 1 and 2, the corresponding labeled bands were eluted from the gel by trypic digestion and hydrolyzed by 6 N HCl at 110 °C for 2 h. Phosphoamino acid content was analyzed by thin layer chromatography. Both proteins were phosphorylated by the insulin receptor exclusively on tyrosine residues (Fig. 3). A trypic peptide map of lipocortin 1 phosphorylated by the insulin receptor kinase was performed by reverse phase high performance liquid chromatography. All of the radioactivity was found in two closely eluting peaks at 40 and 42 min (Fig. 4). This map of labeled peptides is identical with that observed when lipocortin 1 is phosphorylated by the Abelson sarcoma virus tyrosine kinase or by the EGF receptor kinase (41). Previous studies have indicated that this peak corresponds to the trypic peptide containing tyrosine 21 of lipocortin 1. Thus, it is likely that tyrosine 21 on lipocortin 1 is also phosphorylated by the insulin receptor. The peptide map of fragments labeled by the tyrosine kinases differs from that observed following lipocortin 1 phosphorylation by protein kinases A or C (indicated by arrows in Fig. 4). Protein kinase A phosphorylates lipocortin at threonine 216, while protein kinase C labels 2 residues near the amino terminus of lipocortin 1 (41).

Although both lipocortins 1 and 2 undergo tyrosine phosphorylation by the insulin receptor, only phosphorylated lipocortin 2 is recognized by the anti-phosphotyrosine antibody. These results are shown in Fig. 5 using lipocortin 1 and 2 preparations that were phosphorylated by the insulin receptor in vitro as described above. Lanes a and b show Coomassie

![Fig. 2. Phosphorylation of lipocortins 1 and 2 in a cell-free system. Bands corresponding to lipocortins 1 and 2 from gels shown in Fig. 1 were cut, and the amount of 32P incorporated into lipocortin was quantitated. (57 and 46 cpm/nmol of 32P incorporated into lipocortins 1 and 2 respectively). The amount incorporated is plotted against the concentration of the respective lipocortin with and without insulin for lipocortin 1 in the upper panel and for lipocortin 2 in the lower panel.](image1)

![Fig. 3. Phosphoamino acid analysis of lipocortins 1 and 2. Lipocortins 1 and 2 were phosphorylated by the wheat germ agglutinin-purified insulin receptor in a cell-free system and analyzed by SDS-PAGE as described under ‘‘Experimental Procedures.’’ Gel pieces containing lipocortins were excised, eluted by digestion with trypsin, and hydrolyzed with 6 N HCl. The hydrolyzed phosphoamino acids were separated by high voltage electrophoresis on thin layer plates and identified by the migration of known standards.](image2)
Phosphorylation of Lipocortins by the Insulin Receptor

Blue-stained SDS gels, and lanes c and d show the autoradiograms of the same immunoprecipitates. Profiles of immunoprecipitates using either anti-phosphotyrosine or anti-lipocortin antibody of lipocortin 1 (upper panel) and lipocortin 2 (lower panel) are depicted. Anti-phosphotyrosine antibody failed to recognize phosphorylated lipocortin 1 (Fig. 5, upper panel, lanes a and c), but precipitated the autophosphorylated β-subunit of the insulin receptor (Fig. 5, upper panel, lane c). Lipocortin 2 in its phosphorylated form was recognized by the anti-phosphotyrosine antibody (Fig. 5, lower panel, lanes a and c), as was the autophosphorylated β-subunit which acted as the kinase (Fig. 5, lower panel, lane c). By contrast, anti-lipocortin 1 and anti-lipocortin 2 antibodies recognized lipocortin 1 and lipocortin 2, respectively (Fig. 5, upper and lower panels, lanes b and d).

Although a high degree of structural identity exists between lipocortins 1 and 2, sequence conservation is confined to the areas of the four calcium phospholipid binding internal repeats and is not present in the area where the tyrosine phosphorylation occurs (6, 18). There is a tyrosine at residue 23 in lipocortin 2 which corresponds positionally to Tyr-21 in lipocortin 1 and is the presumed site of tyrosine phosphorylation by the tyrosine kinases (40). Differences in sequence between the two lipocortins in the areas around the phosphorylation sites may lead to conformational changes accounting for the differential recognition of tyrosine-phosphorylated lipocortin 2, but not tyrosine-phosphorylated lipocortin 1 by anti-phosphotyrosine antibody. Alternatively, lipocortin 2 may be phosphorylated in more than one site and that multiple phosphorylation sites may be necessary for recognition by the anti-phosphotyrosine antibody.

Phosphorylation of Lipocortins 1 and 2 in Intact Hepatocytes—To determine if lipocortins 1 and 2 are phosphorylated in vivo, hepatocytes were isolated from dexamethasone-treated and untreated male CD rats, labeled with [32P]orthophosphate, and stimulated with insulin. Lipocortins 1 and 2 were immunoprecipitated using anti-lipocortin 1 and anti-lipocortin 2 antisera. Coomassie Blue staining of the gels containing the immunoprecipitates from these experiments did not detect any lipocortin 1 in cells from either normal or dexamethasone-treated animals (Fig. 5, lower panel, lanes 1 and d). In contrast, lipocortin 2 was observed by Coomassie Blue staining in extracts from normal hepatocytes (lane c), and its amount was increased 10-fold by dexamethasone treatment. Based on Coomassie Blue staining of standards, these data indicate that there is less than 50 ng of lipocortin 1 per 10^6 hepatocytes from either nontreated or steroid-treated animals. On the
other hand, hepatocytes from control rats contain about 100 ng of lipocortin 2/10^6 cells and ~1 µg/10^6 cells in corticosteroid-treated animals. This is in agreement with a previous tissue survey which has revealed low levels of lipocortin 1 in rat liver (9), but higher than previously reported for lipocortin 2 (10).

In spite of the very low concentration of lipocortin 1 in hepatocytes, insulin induced phosphorylation of this protein in intact cells from dexamethasone-treated rats (Fig. 7, lanes c and d). On the other hand, lipocortin 2, whose concentration was found to be higher in hepatocytes, was not phosphorylated by insulin in either normal (Fig. 7, lanes e and f) or dexamethasone-treated rats (Fig. 7, lanes g and h). This differential phosphorylation by the insulin receptor of lipocortin 1, but not 2, in spite of the abundance of lipocortin 2 and the small amounts of lipocortin 1 in hepatocytes, could be attributed to the lower K_m of lipocortin 2 for phosphorylation or some compartmentalization of these proteins within the cell. Also, lipocortin 2 has been found to appear partly as a heterotetramer in most cells, while lipocortin 1 is a monomer (18). This oligomerization could prevent access of the former to the catalytic site of the kinase or could hide the phosphorylation site.

Implications of the Data—Our data show that lipocortin 1 is a low K_m substrate for the insulin receptor in a cell-free system and undergoes phosphorylation in intact hepatocytes from a corticosteroid-treated animal. This represents the first description of a well characterized, sequenced protein which is a substrate for insulin-induced tyrosine phosphorylation in intact cells. Whether this has a functional role in insulin signal transduction is uncertain. Lipocortins bind to phospholipids and inhibit their hydrolysis by phospholipases A_2 and phospholipase C. This property has been shown to be regulated by phosphorylation. If indeed this is the case, it offers a possible link between the insulin receptor and insulin-stimulated hydrolysis of phospholipids which recently have been suggested to play a role as modulators of insulin action (42). On the other hand, the overall stoichiometry of lipocortin phosphorylation is low (<0.1 mol/mol), and its role as a phospholipase inhibitor remains debated (15, 43). Like other substrates of the insulin receptor, lipocortin may inhibit phosphorylation-mediated kinase activation and thereby inhibit substrate phosphorylation. In such a case, an increase in lipocortin amount could lead to inhibition of phosphorylation of other less abundant substrates which in turn results in a reduction of insulin signal. This would provide one plausible explanation for corticosteroid induced insulin resistance. Further investigation of the influence of phosphorylation on lipocortin function and its role in insulin signal transduction are indicated.

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