Insulin Receptor Tyrosine Kinase-catalyzed Phosphorylation of 422(aP2) Protein

SUBSTRATE ACTIVATION BY LONG-CHAIN FATTY ACID*

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Richard C. Hresko*, Robert D. Hoffman†, Jaime R. Flores-Riveros†, and M. Daniel Lane‡

From the Department of Biological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

The major pathways for energy storage and mobilization in animal cells are under strict hormonal control, most notably by insulin and glucagon. A large body of evidence indicates that the pleiotropic response to insulin, which includes the stimulation of glucose and fatty acid uptake, glycogenesis, and lipogenesis, is initiated by the binding of the hormone to its specific cell surface receptors on the plasma membrane (1). This insulin receptor interaction stimulates autophosphorylation of specific tyrosine residues in the β-subunit of the receptor (1–6) and thereby the activity of the receptor’s intrinsic tyrosine kinase toward protein substrates (6–9). The importance of the receptor’s tyrosine kinase activity was demonstrated by experiments in which insulin action was inhibited by site-directed mutagenesis of either a critical autophosphorylation site (12). Other experiments have revealed that insulin action can be blocked by introducing antibodies into cells that inhibit insulin-induced activation of the tyrosine kinase (13). Other studies have suggested that several monoclonal antibodies directed against the extracellular domain of the receptor are insulin mimetic without activating the tyrosine kinase activity of the receptor (14, 15). A recent examination (16), however, using a more sensitive assay revealed that these same antibodies do stimulate kinase activity and that this stimulation correlates with their ability to elicit a biological response.

Several years ago we identified a 15-kDa protein (pp15) that is phosphorylated on tyrosine in 3T3-L1 adipocytes treated with insulin and phenylarsine oxide (PAO), an agent that forms stable ring complexes with vicinal or neighboring dithiols (17). Previous studies had shown that PAO blocks insulin-stimulated glucose uptake without affecting insulin binding to its receptor, insulin receptor autophosphorylation, or the receptor’s capacity to catalyze protein substrate phosphorylation (17–19). The site of PAO action that leads to accumulation of pp15 may be a tyrosine-specific phosphatase (20, 21). Preliminary experiments have confirmed the existence of two membrane-bound enzymes which catalyze the dephosphorylation of pp15 (22). Both enzymes are inhibited by PAO and have been extensively purified in our laboratory from 3T3-L1 adipocytes. The formation of pp15 is insulin-specific, epidermal growth factor, platelet-derived growth factor, and insulin-like growth factor 1 being inactive despite the occurrence of receptors for these factors in 3T3-L1 adipocytes (17).

The cellular function of pp15 is not known. Our earlier experiments suggested that pp15 may serve an intermediary role in insulin-activated glucose uptake (17, 21). The reciprocal effects of PAO, i.e. the accumulation of pp15 and the inhibition of glucose uptake, are both rapidly reversed by the vicinal dithiol, 2,3-dimercaptopropanol but not by the monothiol, 2-mercaptoethanol. The temporal relationship between insulin receptor autophosphorylation, pp15 phosphorylation, and insulin-activated glucose uptake is also consistent with an intermediary role for pp15.

Recently, pp15 was purified to homogeneity from 3T3-L1 adipocytes (23). Amino acid sequence analysis of phosphotyrosine-containing peptides generated by trypsin cleavage of pp15 revealed that pp15 is the phosphorylated product of 422(aP2) protein, i.e. O-phospho-Tyr19-422(aP2) protein (23).

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† Supported by National Research Service Awards from the National Institutes of Health.

‡ To whom correspondence should be addressed.

The abbreviations used are: PAO, phenylarsine oxide; PAGE, polyacrylamide gel electrophoresis; TPCK, tosylphenylethyl chloromethyl ketone; DTT, dithiothreitol; HAP-dextran, hydroxalkoxypropyl derivative of sephadex; WGA, wheat germ agglutinin; GC, gas chromatography; HPLC, high performance liquid chromatography; FAD*, fatty acid-binding protein; SDS, sodium dodecyl sulfate.
By coincidence, the full-length cDNA corresponding to 422(aP2) protein had already been cloned and sequenced in this laboratory (24). By its striking amino acid sequence homology to myelin P2 protein (60%), 422(aP2) was recognized as a member of a family of related 15-kDa proteins known as fatty acid-binding proteins (FABP) (25–27). These proteins have no known enzymatic activity but appear to bind straight chain lipophilic ligands such as fatty acids or retinoids (28, 29, 29). Even so their natural ligands and the biological consequences of binding, if any, are not known. When studied crystallographically, relatively diverse members of the family have been shown to conform to a distinctive tertiary structure described as a "β-clam" with two parallel planes of perpendicular strands of β-pleated sheet forming a ligand cavity closed by a short helix-turn-helix "door" (27, 30, see Fig. 15). Of most significance, it was shown in the structure of bovine myelin P2 protein that the tyrosine residue analogous to Tyr\(^{\text{195}}\) of 422(aP2) is oriented inside the ligand cavity, a state apparently incompatible with access to the active site of a macromolecular catalyst. Consistent with the "sequestered tyrosine" model, our early attempts to phosphorylate 422(aP2) with the insulin receptor, or to iodinate 422(aP2) by standard methods resulted in unexpectedly low yields. On this basis we began to explore the possibility of ligand-induced conformational activation of 422(aP2) protein as a substrate for the insulin receptor.

**Experimental Procedures**

**RESULTS**

**Purification of 422(aP2) Protein**—Since earlier studies (17, 21, 23) indicated that 422(aP2) protein is phosphorylated by the insulin receptor tyrosine kinase in the intact 3T3-L1 adipocytes, it was important to characterize this enzymatic phosphorylation reaction in vitro with purified components. As our previous method of purifying the phosphorylated form of 422(aP2) protein (O-phospho-Tyr\(^{\text{195}}\)-422(aP2) protein) involved steps that denature the protein with urea and SDS (23), a less drastic procedure was sought to obtain native homogeneous 422(aP2) protein. Fully differentiated 3T3-L1 adipocytes in monolayer culture were used as starting material for the purification. Progress of the purification was monitored by a radioimmunossay which utilized polyclonal antibodies directed against a synthetic peptide corresponding to the COOH-terminal amino acids of 422(aP2) protein (see "Experimental Procedures").

The first two steps of the purification procedure involved perforating the plasma membranes of cells with dilute buffered digitonin followed by ammonium sulfate fractionation of the released cytosolic proteins (23). Since 422(aP2) protein, like pp15 (23), is localized in the cytosol, cell lysis with digitonin releases 422(aP2) protein leaving the cytoskeletal, organelar proteins, and triacylglycerol droplets within the lysed cell monolayer (32). Ammonium sulfate fractionation of the digitonin supernate revealed that, whereas pp15 is precipitated by 60% saturated ammonium sulfate, 422(aP2) protein remains soluble in 70% saturated ammonium sulfate. This step resulted in a 6-fold purification of 422(aP2) protein (Table I). After reducing the volume and ammonium sulfate concentration of the supernate by ultrafiltration (Amicon YM5 filter) and dialysis, 422(aP2) protein was further purified by HPLC (MONO S cation exchange column) using a NaCl gradient. The elution profile shown in Fig. 1 reveals one major and several minor protein peaks. The major and one minor peak (peaks 1 and 2, respectively) react with antibodies directed against the COOH-terminal sequence of 422(aP2) protein. One-dimensional SDS-PAGE and two-dimensional (nonequilibrium isoelectric focusing SDS/PAGE) gel electrophoresis (Fig. 2) carried out on the major protein peak showed a single Coomassie Blue-staining protein. Consistent with the properties of pp15 (O-phospho-Tyr\(^{\text{195}}\)-422(aP2) protein), this protein migrates with an apparent molecular mass of 15 kDa, but with a more basic pI (about 8.5) than pp15 (pI = 6.3).

Two-dimensional gel analysis of the minor peak (peak 2) (results not shown) reveals that it is composed of approximately equal amounts of two 15-kDa proteins that may be associated as a heterodimer. One of the two proteins migrates identically to the major protein in peak 1 by two-dimensional gel analysis (nonequilibrium isoelectric focusing SDS/PAGE), while the other protein has a more basic pI. Both proteins react by Western blot analysis with antibodies against the COOH terminus of 422(aP2) protein (data not shown) and thus, are at least partially related. The more basic protein found in peak 2 is probably a minor isoform of 422(aP2) protein. The protein in the major peak 1, presumably 422(aP2) protein, was further characterized.

**Identification and Characterization of Peak 1 Protein as 422(aP2) Protein**—Several studies were conducted to verify that the protein (peak 1) purified to homogeneity was in fact 422(aP2) protein. It was shown above (Fig. 1) that the purified protein reacts with antibodies directed against the COOH terminus of 422(aP2) protein. In addition, Western blot analysis (results not shown) revealed that the purified protein migrates upon two-dimensional (nonequilibrium isoelectric focusing SDS/PAGE) gel electrophoresis as a protein with an apparent molecular mass of 15 kDa and a basic pI (approximately 8.5). The amino acid composition of the purified protein was found to be virtually identical to that predicted by the deduced amino acid sequence from 422(aP2) cDNA (Table II).

The purified protein was subjected to limited proteolytic digestion with trypsin after which the tryptic fragments were purified by reverse-phase HPLC on a C\(_{6}\) column. The tryptic peptide profile (Fig. 3) is similar to that reported by Matarese and Bernlohr (25) using a C\(_{6}\) reverse-phase column. Three of the major tryptic peptides (A–C in Fig. 3) were subjected to mass-phase amino acid sequencing. The sequences of the three peptides matched exactly those of tryptic peptides predicted from the amino acid sequence from 422(aP2) cDNA (Table III). It is of interest that Peptide C corresponds to the tryptic peptide of pp15 which is phosphorylated in the intact cell (23). Based on these findings we conclude that the purified protein (peak 1) is 422(aP2) protein.

**Verification That pp15 Is Phosphorylated 422(aP2) Protein**—Our previous investigation showed that the amino acid sequence of the phosphotyrosine-containing tryptic peptide derived from pp15 corresponded to an amino acid sequence near the NH\(_{2}\) terminus of 422(aP2) protein (23). Nevertheless, it was important to verify the identity of pp15 as the phosphorylation product of 422(aP2) protein by independent means. This was accomplished by two approaches.

First, as shown in Fig. 4 \(^{32}\)Ppp15 in the cytosol from \(^{32}\)P-labeled 3T3-L1 adipocytes (treated with insulin, PAO, and vanadate to the accumulation of pp15) was specifically immunoprecipitated almost quantitatively (86%) by an affinity purified antibody directed against a synthetic peptide corresponding to the COOH-terminal sequence of 422(aP2) protein

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\(^{3}\) Portions of this paper (including "Experimental Procedures," Figs. 1–7, and Tables I–III) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
protein. Immunoprecipitation of [32P]pp15 was almost completely blocked by the synthetic peptide antigen (Fig. 4). The fact that this antibody, which is directed against an epitope different from the phosphorylation site on 422(aP2) protein, efficiently precipitates pp15 supports the view that this protein is a cellular substrate of the insulin receptor tyrosine kinase. The two minor phosphoproteins that were also specifically immunoprecipitated (Fig. 4) are probably phosphorylated isoforms of 422(aP2) protein.

Second, purified 422(aP2) protein was phosphorylated in vitro by the tyrosine kinase of the insulin receptor isolated and purified from membranes of 3T3-L1 adipocytes. The receptor was first activated by autophosphorylation for 5 min in the presence of insulin and [γ-32P]ATP as previously described (6) after which purified 422(aP2) protein was added and its phosphorylation monitored. After quenching the reaction, the proteins were separated by two-dimensional gel electrophoresis and 32P-labeled proteins were visualized by autoradiography (Fig. 5A). The migration of [32P]pp15 isolated from 3T3-L1 adipocytes is shown for comparison (Fig. 5B). A mixture containing equal amounts of 32P from both [32P]pp15 and phosphorylated [32P]422(aP2) protein was also analyzed on a companion gel (Fig. 5C). Since pp15 and phosphorylated 422(aP2) protein migrate identically, it is evident that they possess similar pI values and subunit molecular weights. Interestingly, although the 422(aP2) protein used as substrate for the receptor kinase was apparently homogeneous by staining with Coomassie Blue, a small satellite phosphoprotein (in addition to pp15) appeared upon autoradiography (Fig. 5A). This satellite phosphoprotein spot (which was removed by the purification procedure) is found when extracts of intact 3T3-L1 adipocytes labeled with 32Pi are subjected to immunoprecipitation using anti-422(aP2) protein antibodies (Fig. 4).

Phosphoamino acid analysis of [32P]422(aP2) protein purified by two-dimensional polyacrylamide gel electrophoresis revealed that 422(aP2) protein, phosphorylated by the insulin receptor in vitro, occurs exclusively on tyrosine (Fig. 6A) as is the case for [32P]pp15 isolated from intact cells (Fig. 6B). The 32P-labeled tryptic phosphopeptides generated from pp15 and phosphorylated 422(aP2) protein were compared by reverse-phase HPLC. [32P]pp15 and 32P-phosphorylated 422(aP2) protein were first isolated from two-dimensional polyacrylamide gels and then exhaustively digested with trypsin. As illustrated in Fig. 7, the HPLC elution profiles of the [32P]phosphopeptides were virtually identical. Although pp15 is phosphorylated on a single tyrosine, cleavage by trypsin is incomplete, yielding three phosphopeptides (Fig. 7 and Ref. 23). The three negatively charged amino acids, i.e. phospho-tyrosine and 2 aspartic acid residues, adjacent to Lys21 in pp15 and phosphorylated 422(aP2) protein (See Table III, peptide C), appear to inhibit cleavage by trypsin. The same tryptic site is readily cleaved, however, in unphosphorylated 422(aP2) protein (see Table III, peaks C and A). Sequence analysis of the three phosphopeptides (23) revealed that the first peak is a limit digestion while peaks 2 and 3 are derived from incomplete digestion.

Activation by Long-chain Fatty Acid of Insulin Receptor Tyrosine Kinase-catalyzed Phosphorylation of 422(aP2) Protein—As demonstrated above the insulin receptor tyrosine kinase catalyzes the phosphorylation (on tyrosine) of purified 422(aP2) protein in vitro. The rate of phosphorylation of this protein by the isolated receptor was, however, relatively slow. Since 422(aP2) protein is structurally similar to a family of fatty acid-binding proteins (25–27) and is itself known to bind fatty acid (25, 44–46), the possibility was considered that this interaction might alter its properties as a substrate. Moreover, since pp15 accumulates in insulin-treated 3T3-L1 adipocytes only when PAO is present, it was also important to determine whether PAO per se interacts directly with 422(aP2) protein to render it a better substrate for the insulin receptor tyrosine kinase. PAO is an agent that forms relatively stable complexes with vicinal or neighboring thiols (47).

To assess the effect of fatty acid on the properties of 422(aP2) protein as substrate of the insulin receptor tyrosine kinase, 422(aP2) protein was first incubated with oleic acid (10:1 molar ratio of fatty acid to protein) to allow the fatty acid to bind. Then insulin receptor, previously autophosphorylated for 5 min either in the presence or absence of insulin, was added to the 422(aP2) protein-oleic acid complex, and protein substrate phosphorylation was followed for 5 min. As shown in Fig. 8A, oleic acid introduced into the reaction mixture with 422(aP2) protein at the time substrate phosphorylation was initiated had no effect on the extent of basal or insulin-stimulated autophosphorylation of the receptor’s β-subunit. In other experiments not shown, it was also demonstrated that oleic acid had no effect on receptor autophosphorylation even when the receptor had been preincubated with fatty acid prior to initiating autophosphorylation. Oleic acid did, however, dramatically increase the rate of phosphorylation of 422(aP2) protein by receptor autophosphorylated both in the presence and absence of insulin (Fig. 8B).

The effect of oleic acid is entirely dependent upon insulin receptor since, in the absence of receptor, phosphorylation of 422(aP2) protein did not occur (results not shown). It was also shown (Fig. 8, A and B) that neither PAO nor the solvents (ethanol and dimethyl sulfoxide), in which the fatty acid and PAO are introduced into the reaction mixture, affect autophosphorylation of the receptor or receptor-catalyzed substrate phosphorylation. Finally, it was determined (results not shown) that the sodium salt of oleic acid can replace oleic acid as substrate of the insulin receptor tyrosine kinase. 422(aP2) protein was treated sequentially with buffer alone (open bar) or ethanol (1% final concentration, closed bar) or oleic acid in ethanol (10:1 mol ratio, oleic acid to 422(aP2) protein; hatched bar) for 90 min at 37 °C then with buffer alone or dimethyl sulfoxide (DMSO) (0.035% final concentration) without or containing PAO (95 μM final concentration) for 5 min at room temperature. Insulin receptor was autophosphorylated for 5 min at 23 °C with [γ-32P]ATP either in the absence or presence of 1 μM insulin. The 422(aP2) protein substrate (20 μM final concentration) was then added to the activated receptor and incubated an additional 5 min before the reaction was terminated. Samples were then analyzed by one-dimensional SDS-PAGE and the 32P activity incorporated into the β-subunit of the insulin receptor (panel A) and 422(aP2) protein (panel B) was quantitated by scintillation counting.

Fig. 8. Effect of insulin (INS), oleic acid, and PAO on autophosphorylation of the β-subunit of the insulin receptor and on the phosphorylation of 422(aP2) protein catalyzed by the insulin receptor tyrosine kinase. 422(aP2) protein was treated sequentially with buffer alone (open bar) or ethanol (1% final concentration, closed bar) or oleic acid in ethanol (10:1 mol ratio, oleic acid to 422(aP2) protein; hatched bar) for 90 min at 37 °C then with buffer alone or dimethyl sulfoxide (DMSO) (0.035% final concentration) without or containing PAO (95 μM final concentration) for 5 min at room temperature. Insulin receptor was autophosphorylated for 5 min at 23 °C with [γ-32P]ATP either in the absence or presence of 1 μM insulin. The 422(aP2) protein substrate (20 μM final concentration) was then added to the activated receptor and incubated an additional 5 min before the reaction was terminated. Samples were then analyzed by one-dimensional SDS-PAGE and the 32P activity incorporated into the β-subunit of the insulin receptor (panel A) and 422(aP2) protein (panel B) was quantitated by scintillation counting.
Fig. 9. Binding of oleic acid to 422(aP2) protein determined by the liposome transfer or hydroxalkoxypropyl-dextran (HAP-dextran) methods. A, liposomes containing variable amounts of [3H]oleic acid were incubated with 10 μM 422(aP2) protein for 1.5 h at 37°C. The amount of oleate bound to 422(aP2) protein was then determined as described under “Experimental Procedures.” *Inset,* Scatchard analysis of binding isotherm. B, [3H]oleic acid was added to 422(aP2) protein in ethanol and incubated 1.5 h at 37°C. Free fatty acid was then removed with ice-cold HAP-dextran and the labeled oleate in the supernate determined as described under “Experimental Procedures.” *Inset,* Scatchard analysis of binding isotherm.

Since oleic acid markedly alters the properties of 422(aP2) protein as substrate for the receptor kinase, it was of interest to determine 1) whether purified 422(aP2) protein contained bound fatty acid, and 2) the affinity with which exogeneous fatty acid binds to the protein. To determine whether the 422(aP2) protein preparation contained fatty acid, the purified protein was extracted with a chloroform-methanol mixture and any fatty acids in the extract were converted to their methyl esters and analyzed by gas chromatography (GC). GC analysis revealed that the purified 422(aP2) protein preparation was devoid of detectable fatty acids. Control experiments in which oleic acid was added exogenously to purified 422(aP2) (at a 1:1 molar ratio of oleic acid to protein), followed by incubation at 37°C for 1.5 h, extraction and methylation of the fatty acid and analysis by GC, demonstrated that the fatty acid could be quantitatively extracted, methylated, and analyzed by GC. By this method myristic, palmitic, stearic, oleic, linoleic, linolenic, and arachidonic acids could have been detected at a fatty acid to protein molar ratio of <0.003:1.0. We conclude that our purified 422(aP2) protein preparation is devoid of bound fatty acids.

The capacity of purified 422(aP2) protein to bind [3H]oleic acid was investigated using two different methods (the liposome delivery and hydrophobic affinity matrix methods) for delivering labeled fatty acid to the protein and for removing unbound free fatty acid following its equilibration with the protein (28, 42). For the liposome delivery method, liposomes containing egg lecithin and cholesterol in a 3:1 molar ratio and 5–200 μM [3H]oleic acid were incubated with 422(aP2) protein for 1.5 h at 37°C to allow transfer of the fatty acid from the vesicles to the protein. Liposome-bound oleic acid was then separated from that bound to the protein by centrifugation. For the hydrophobic affinity matrix method, [3H]oleic acid in ethanol was incubated with 422(aP2) for 1.5 h at 37°C after which fatty acid not tightly bound to 422(aP2) protein was removed with ice-cold hydroxalkoxypropyl-dextran (HAP-dextran). Scatchard analyses of the results of the binding experiments using both techniques showed (insets, Fig. 9, A and B) that 422(aP2) protein possesses a single high affinity (or slowly dissociating) fatty acid-binding site with an apparent Kd of either 22 μM (liposome method, Fig. 9A) or 5 μM (HAP-dextran, Fig. 9B). The Kd values determined by these methods overestimate the true Kd, since liposomes or the hydrophobic affinity matrix bind fatty acid and thus establish a competitive equilibrium. The fatty acid binding properties of 422(aP2) protein described above are in agreement with those reported by Matarese and Bernlohr (25).

The time course (not shown) of phosphorylation of 422(aP2) protein by the insulin receptor tyrosine kinase in the absence or presence of oleic acid (101, oleic acid/422(aP2) protein molar ratio) revealed that substrate phosphorylation was linear for 5 min. Thus, all substrate phosphorylation reactions were carried out for 5 min unless otherwise stated.

The dependence of phosphorylation rate on concentration of 422(aP2) protein was investigated in the absence and presence of oleic acid (Fig. 10). Lineweaver-Burk analysis of these data (Fig. 10, inset) indicates that the addition of oleic acid, and presumably its binding to the protein, caused a decrease of the Km for 422(aP2) protein in the phosphorylation reaction from 170 to 3 μM, but had little effect on the Vmax. Insulin had no effect on the Km for 422(aP2) protein but increased Vmax, 3–4-fold (result not shown).

The dependence of the rate of phosphorylation of 422(aP2) protein on oleic acid concentration was also investigated. 422(aP2) protein was incubated at 37°C with the fatty acid for 1.5 h prior to initiating the phosphorylation reaction. The rate of 422(aP2) protein phosphorylation increased markedly with oleic acid concentration before reaching a maximal rate near a 6:1 molar ratio of fatty acid to 422(aP2) protein (Fig. 11).

Homologous series of long-chain saturated and unsaturated fatty acids were tested for their ability to activate the phosphorylation of 422(aP2) protein. Fatty acids were added to 422(aP2) protein at a 10:1 molar ratio of fatty acid to protein, incubated 1.5 h at 37°C, and then added to preactivated receptor for 5 min. All fatty acids tested dramatically increased 422(aP2) phosphorylation (Fig. 12), although unsaturated fatty acids generally had a greater activating effect than saturated fatty acids.

Effect of Oleic Acid on the Phosphorylation of 422(aP2) Protein Catalyzed by the Cyttoplasmic Domain of the Insulin Receptor—The insulin receptor preparation used in the substrate phosphorylation studies described above was extracted from membranes, purified, and assayed in the presence of the non-ionic detergent, Triton X-100. Detergent was necessary, since in its absence, the receptor lost its capacity for allosteric activation by insulin and, thus, lost its ability to undergo autophosphorylation and to catalyze substrate phosphorylation at significant rates. Detergent micelles, present during the phosphorylation of 422(aP2) protein, might serve as a "sink" to sequester free fatty acid thereby reducing the sto-
Fatty Acids Activate Phosphorylation of 422(aP2) Protein

FIG. 10. Effect of oleic acid on the 422(aP2) protein concentration dependence of phosphorylation of the protein substrate catalyzed by the insulin receptor tyrosine kinase. Ethanol (○) or oleic acid in ethanol (●) was added to 422(aP2) protein (10:1 molar ratio, fatty acid to protein) and the mixtures were incubated 1.5 h at 37 °C. Various sample dilutions were prepared such that the final 422(aP2) protein concentration in the reaction mixture ranged from 1 to 200 μM. Phosphorylation catalyzed by insulin receptor purified from 3T3-L1 adipocytes was conducted as described under "Experimental Procedures." Samples were analyzed by one-dimensional SDS-PAGE and the radioactivity incorporated into 422(aP2) protein was measured. Insert, Lineweaver-Burk analysis.

FIG. 11. Oleic acid concentration dependence of phosphorylation of 422(aP2) protein catalyzed by the insulin receptor. Varying amounts of oleic acid were added in ethanol (1% final concentration) to 422(aP2) protein (20 μM final concentration) and the mixtures incubated for 1.5 h at 37 °C. Samples were subjected to phosphorylation catalyzed by the insulin receptor from 3T3-L1 adipocytes previously autophosphorylated with (○) or without (●) insulin as described under "Experimental Procedures" and then analyzed by SDS-PAGE and the ^32P radioactivity incorporated into 422(aP2) protein measured.

FIG. 12. Specificity of the activation of 422(aP2) protein substrate phosphorylation by different saturated and unsaturated fatty acids. Fatty acids in ethanol were added to 422(aP2) protein (10:1 molar ratio, fatty acid to protein) and the mixtures incubated 1.5 h at 37 °C. 422(aP2) protein (10 μM final concentration) was added to 3T3-L1 adipocyte insulin receptor previously autophosphorylated with open bars or with hatched bars insulin, and mixtures were then further incubated for 5 min at 23 °C before the reaction was quenched. Samples were analyzed by two-dimensional gel electrophoresis and the ^32P radioactivity incorporated into 422(aP2) protein quantitated.

Effect of Bound Fatty Acid on the Accessibility to Iodination of Tyrlg and Tyr^142

The foregoing results...
together with recent crystallographic data on a structurally similar protein, myelin P2 protein (27), strongly suggest that Tyr\textsuperscript{19} in native 422(aP2) protein without bound fatty acid is inaccessible to the active site of the insulin receptor tyrosine kinase. Moreover, it appears that upon binding fatty acid, a conformational change occurs whereby Tyr\textsuperscript{19} becomes more readily accessible to the kinase active site. To test this hypothesis by an independent method, the accessibility of the 2 tyrosine residues, Tyr\textsuperscript{19} and Tyr\textsuperscript{19\#}, in 422(aP2) protein to Tyr\textsuperscript{19} is iodinated at a similar rate, but the reaction continues for at least 20 min, resulting in a greater total incorporation of fatty acid was attributed to Tyr\textsuperscript{19}. When fatty acid is included at a 10-fold molar excess over 422(aP2) protein, 422(aP2) protein (final concentration 10\) \(\mu\)M was treated with buffer alone (0) or with ethanol (final concentration 0.4%) with (0) or without (0) a 10-fold molar excess of oleic acid for 90 min at 37 \(^\circ\)C. The samples were iodinated for the indicated times using lactoperoxidase as described under “Experimental Procedures.” After one-dimensional SDS-PAGE labeled 422(aP2) protein was excised from each gel and radioactivity was measured. The gel segments were then subjected to trypsinization and the resulting peptides were analyzed by reverse-phase HPLC (see “Experimental Procedures”) to distinguish between label incorporated at Tyr\textsuperscript{19} and Tyr\textsuperscript{19\#}. A, radioactivity incorporated into 422(aP2) protein, B, radioactivity incorporated into Tyr\textsuperscript{19} (dashed lines) and Tyr\textsuperscript{19\#} (dotted lines) in the presence (0) or absence (0) of oleic acid.

**Fig. 14. Effect of oleic acid on the iodination of 422(aP2) protein.** 422(aP2) protein (final concentration 10 \(\mu\)M) was treated with buffer alone (0) or with ethanol (final concentration 0.4%) with (0) or without (0) a 10-fold molar excess of oleic acid for 90 min at 37 \(^\circ\)C. The samples were iodinated for the indicated times using lactoperoxidase-catalyzed iodination of Tyr\textsuperscript{19} and low lactoperoxidase-catalyzed iodination of Tyr\textsuperscript{19\#}.

**DISCUSSION**

There is compelling evidence that the tyrosine kinase activity of the insulin receptor is important in signal transmission initiated by the binding of insulin (1). Previously, we reported (17, 21) the accumulation of a 15-kDa protein (pp15) phosphorylated on tyrosine in 3T3-L1 adipocytes treated with insulin and PAO. Insulin appeared to activate receptor catalyzed phosphorylation of p15 while PAO blocked the turnover of the pp15 tyrosine phosphoryl group (17, 20–22), thereby leading to its accumulation. Our recent discovery (23) that pp15 is \(O\)-phospho-Tyr\textsuperscript{19}\textsuperscript{22}-422(aP2) protein initiated our attempts to reconstitute the phosphorylation reaction in \textit{vitro} using purified proteins. Although there is no known catalytic activity for any fatty acid-binding protein, our strategy for maintaining the native state of 422(aP2) protein was to employ purification methods which are generally regarded as mild with respect to maintenance of enzyme activities. Our procedure differs from that of Matarese and Bernlohr (25) in that we used ammonium sulfate fractionation instead of gel filtration, delipidation with Lipidex-1000, and thiol affinity chromatography. We obtained purified protein free of endogenous fatty acid in a yield (38%) probably similar to that reported by these authors.

**Quantitative analysis of 3T3-L1 adipocytes with immunochemical methods showed that 422(aP2) protein is abundant, comprising about 3% of cellular protein. By far the vast majority of this protein is accounted for by the form we have purified. Interestingly, two minor 15-kDa phosphoproteins were identified (Fig 4) on two-dimensional gels of immunoprecipitates from \(3^P\)-labeled whole cell extracts with anti-COOH-terminal (422(aP2) protein) antibody. Isoforms have been reported for other fatty acid-binding proteins. Rat liver FABP can be separated by either isoelectric focusing (49) or...
Fatty Acids Activate Phosphorylation of 422(aP2) Protein

In the absence of fatty acid, Tyr^{19} is sequestered from the insulin receptor in the ligand (fatty acid)-binding cavity of the β-sheet domain. In this state, phosphorylation occurs exclusively, but to a low extent, on Tyr^{28}. This drawing is adapted from the crystal structure of bovine myelin P2, a closely related homologue of 422(aP2) protein, reported by Jones et al. (27).

In the presence of fatty acid, Tyr^{19} becomes accessible to the insulin receptor protein tyrosine kinase by an unknown mechanism, here represented through a hinge motion of the α-helical (bold lines) domain with respect to the β-sheet domain. In this state, phosphorylation of both Tyr^{19} and Tyr^{28} occurs.

Fig. 15. Proposed model for fatty acid-induced activation of 422(aP2) protein. In the absence of fatty acid, Tyr^{19} is sequestered from the insulin receptor in the ligand (fatty acid)-binding cavity of the β-sheet domain. In this state, phosphorylation occurs exclusively, but to a low extent, on Tyr^{28}. This drawing is adapted from the crystal structure of bovine myelin P2, a closely related homologue of 422(aP2) protein, reported by Jones et al. (27). In the presence of fatty acid, Tyr^{19} becomes accessible to the insulin receptor protein tyrosine kinase by an unknown mechanism, here represented through a hinge motion of the α-helical (bold lines) domain with respect to the β-sheet domain. In this state, phosphorylation of both Tyr^{19} and Tyr^{28} occurs.

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Phosphorylation of purified 422(aP2) protein in vitro by the 3T3-L1 insulin receptor tyrosine kinase was dependent on the addition of fatty acid. Kinetic analysis showed that this effect was largely explained by a reduction in the apparent $K_m$ for 422(aP2) protein from 170 to 3 μM with only an insignificant change in $V_{max}$ (Fig. 10). Fatty acid did not affect the autophosphorylation of the insulin receptor. Although all fatty acids tested gave some stimulation of phosphorylation, there was a slight preference for unsaturated fatty acids (Fig. 14). The plateau that follows the linear phase suggests that the rate of spontaneous transition from the active state to the inactive state is slow. When fatty acid is present, the linear phase of iodination persists much longer indicating an increased fraction of the protein substrate in the active state.

Our work is the first reported instance of an ligand-induced conformational change in a fatty acid-binding protein leading to an altered function. No other fatty acid-binding protein is known to undergo tyrosine phosphorylation. Despite these considerations, several other fatty acid-binding proteins show extensive primary sequence homology and presumably also have tertiary structures similar to 422(aP2) protein. Four of these proteins, bovine myelin P2 (52), rat heart FABP (53), rat cellular retinoid-binding protein (54, 55), and bovine mammary-derived growth inhibitor protein (26) possess a conserved tyrosine residue analogous to Tyr^{28} of 422(aP2) protein in the context of a consensus protein tyrosine kinase recognition motif (56). The potential for these related proteins to undergo ligand-induced conformational changes or substrate activation for tyrosine phosphorylation has not been explored.

The significance of fatty acid-induced substrate activation of 422(aP2) protein in the 3T3-L1 adipocyte may relate to the anti-lipolytic function of insulin. It has been known (57, 58) that insulin inhibits lipolysis in adipocytes stimulated by β-adrenergic and other lipolytic hormones. These hormones activate the triacylglycerol lipase of adipocytes through phosphorylation of the enzyme by the cAMP-dependent protein kinase (58, 59). Lipolysis is also subject to feedback inhibition by free fatty acids which accumulate in adipocytes under various physiological circumstances (60). Recent evidence from Bernlohr's laboratory (25, 44–46) has shown convincingly that 422(aP2) protein mediates intracellular fatty acid transport in 3T3-L1 adipocytes. In this connection our results reveal that loading 422(aP2) protein with fatty acid renders the protein susceptible to phosphorylation by the insulin receptor tyrosine kinase. Conceivably, phosphorylation of 422(aP2) protein might decrease its capacity to bind/transport fatty acids and lead to their accumulation, thereby inhibiting lipolysis. Thus, insulin might serve its anti-lipolytic role both by altering the phosphorylation state of the lipase and by causing the accumulation of free fatty acids. The possible role of phosphorylation of 422(aP2) protein by these mechanisms is currently under investigation with intact 3T3-L1 adipocytes.

The role of fatty acid binding proteins in biology has long been debated. The putative consequences of fatty acid binding have been limited to protection of cells from toxic high local concentrations of fatty acid. Our observations suggest the existence of dynamic conformational changes that may produce intercellular signaling in a wide range of biologic systems.

A R. C. Hresko, unpublished results.

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EXPERIMENTAL PROCEDURES

Materials. PAG was obtained from Aldrich. Digitonin, Staphylococcal Protein A-Sepharose 4 Fast Flow, aspirin, and fatty acid free bovine serum albumin (Type V) were purchased from Sigma Chemical Co. HPLC grade methanol and acetone were obtained from Fisher Scientific. 5 M Mops buffer was obtained from Fisher Scientific. 5 M Tris buffer was obtained from Bio-Rad Laboratories Inc. Polyethylene glycol 4000, 8000, and 20,000 were obtained from Sigma Chemical Co. 1 M sodium acetate buffer, pH 5.0, was obtained from Sigma Chemical Co. All other chemicals were of analytical grade.

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PAG binding experiments were conducted using 1 μg of PAG in 1 ml of assay buffer. The assay buffer contained 50 mM Tris, 150 mM NaCl, 15 μg/ml of BSA, and 1 μg of digitonin. The 2.5 mg/ml of PAG was added to the assay buffer. The reaction was incubated at 4°C for 1 hour. The reaction was then terminated by adding 1 ml of 1 M sodium acetate buffer, pH 5.0. After centrifugation, the dialyzed solution was concentrated using a Centricon-10 concentrator (Amicon, Beverly, MA). The sample was then injected at a linear flow of 0.5 ml/min through the column. All samples were run in triplicate. The eluted protein was then quantified by spectrophotometry.

Bacterial growth. Staphylococcal Protein A-Sepharose 4 Fast Flow was prepared by mixing 10 ml of the protein solution with 100 ml of 5 M Mops buffer, pH 7.0. The mixture was then incubated at 4°C for 1 hour. The mixture was then centrifuged at 10,000 g for 10 minutes. The supernatant was discarded and the precipitate was washed with 10 ml of 5 M Mops buffer, pH 7.0. The procedure was repeated three times. The precipitate was then dissolved in 5 M Mops buffer, pH 7.0. The mixture was then incubated at 4°C for 1 hour. The mixture was then centrifuged at 10,000 g for 10 minutes. The supernatant was discarded and the precipitate was washed with 10 ml of 5 M Mops buffer, pH 7.0. The procedure was repeated three times. The precipitate was then dissolved in 5 M Mops buffer, pH 7.0.

Bioassay determination of 422(aP2) protein. A rabbit was immunized with 2 ml of complete Freund's adjuvant containing 1 mg of synthetic 422(aP2) peptide conjugated to bovine serum albumin. The booster was given at monthly intervals on four occasions with 2 ml of incomplete Freund's adjuvant containing 1 mg of synthetic 422(aP2) peptide conjugated to bovine serum albumin. The antibody was then purified by affinity chromatography on a Staphylococcal Protein A-Sepharose column. The antibody was then used to precipitate 422(aP2) labeled C-terminal synthetic peptide in the presence of Staphylococcal Protein A-Sepharose 4 Fast Flow. The 422(aP2) peptide was then quantified by spectrophotometry.

Phosphoamidase. A phosphoamidase was purified from Staphylococcus aureus and used to hydrolyze the C-terminal synthetic peptide. The phosphoamidase was then used to hydrolyze the C-terminal synthetic peptide in the presence of Staphylococcal Protein A-Sepharose 4 Fast Flow. The 422(aP2) peptide was then quantified by spectrophotometry.

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Fatty Acids Activate Phosphorylation of 422(P2) Protein

Immunoprecipitation of 422(P2) protein with anti-422(P2) mAB serum. Forty-five μl of 422(P2)-containing Triton X-100 was added to each well, followed by washes as indicated by adding 5 μl of 1 M SDS and heating to 100°C for 10 min. An aliquot (5 μl) of the clarified supernatant was analyzed by 2-dimensional gel electrophoresis. The gel was stained with 0.1 μl of protein A-Sepharose (10 mg/ml) in SDS-PAGE buffer is added to each well. The mixture were incubated at room temperature for 2 h on a rotating wheel before centrifugation. The pellets were washed three times in 450 μl of PBS, then washed with 10 μl of PBS buffer. The entire samples were analyzed by 2-dimensional gel electrophoresis. The 2-dimensional gels were fixed, stained and dried before autoradiography. Equal areas of the 2-subunit molecular weight band was excised and the radioactive composition of the 2-gel fragment determined.

Analysis of phosphorylation of 422(P2) protein in the presence of or absence of serum at acidic pH was performed under conditions designed to mimic those used for phosphorylation experiments. Three milligrams of 422(P2) protein in 15µl of 50mM Na acetate pH 5.8 were added to 500µl of BSA or a 1:1 ratio of BSA and 500µl of FPLC column eluate were added to each well. The mixtures were incubated at room temperature for 2 h on a rotating wheel before centrifugation. The pellets were washed three times in 450 µl of PBS, then washed with 10 µl of PBS buffer. The entire samples were analyzed by 2-dimensional gel electrophoresis. The 2-dimensional gels were fixed, stained and dried before autoradiography. Equal areas of the 2-subunit molecular weight band was excised and the radioactive composition of the 2-gel fragment determined.

Table I. Purification of 422(P2) Protein from 375-L1 Adipocytes

| Purification Step | Total Protein | Yield | Specific Activity | Fold |
|-------------------|---------------|-------|------------------|------|
| Whole Cells       | 115           | 100   | 1                | 1    |
| Diphosphatase     | 112           | 18.1  | 5.4              | 5.4  |
| Trisphosphatase   | 25            | 69.7  | 1.0              | 1.0  |
| Sulfate Dehydrogenase | 83       | 64.1  | 2.0              | 2.0  |
| Alkaline Phosphatase | 23         | 95.5  | 0.9              | 0.9  |
| Mono C 3 Column   | 73            | 58.2  | 0.9              | 0.9  |

The amount of 422(P2) protein in whole cells was determined in a separate set of experiments otherwise identical to that used in the purification of 422(P2) protein.

Table II. Comparison of the amino acid composition of 375-L1 protein purified using the 422(P2) mAB or 12CA5 mAB

| Amino Acid | Composition of purified 153Na mAB protein a | Composition based on the purified 153Na mAB (n mole/mg protein) b | Composition based on the purified 153Na mAB (n mole/mg protein) c |
|------------|---------------------------------------------|---------------------------------------------------|---------------------------------------------------|
| Ala        | 7.3                                        | 7.3                                               | 7.3                                               |
| Val        | 7.5                                        | 7.5                                               | 7.5                                               |
| Ile        | 11.8                                       | 11.8                                              | 11.8                                              |
| Leu        | 10.4                                       | 10.4                                              | 10.4                                              |
| Thr        | 0.7                                        | 0.7                                               | 0.7                                               |
| Asp         | 1.8                                        | 1.8                                               | 1.8                                               |
| Glu         | 2.8                                        | 2.8                                               | 2.8                                               |
| Pro         | 1.2                                        | 1.2                                               | 1.2                                               |
| Gln         | 2.0                                        | 2.0                                               | 2.0                                               |
| Gly         | 2.0                                        | 2.0                                               | 2.0                                               |
| Asn         | 0.6                                        | 0.6                                               | 0.6                                               |
| Cys         | 1.5                                        | 1.5                                               | 1.5                                               |
| Ser         | 0.7                                        | 0.7                                               | 0.7                                               |
| His         | 0.2                                        | 0.2                                               | 0.2                                               |
| Arg         | 3.5                                        | 3.5                                               | 3.5                                               |
| Lys         | 2.2                                        | 2.2                                               | 2.2                                               |
| Tyr         | 0.9                                        | 0.9                                               | 0.9                                               |
| Phe         | 0.6                                        | 0.6                                               | 0.6                                               |
| Trp         | 0.1                                        | 0.1                                               | 0.1                                               |
| Met         | 0.1                                        | 0.1                                               | 0.1                                               |
| Cys (DM)    | 0.1                                        | 0.1                                               | 0.1                                               |
| Met (DM)    | 0.1                                        | 0.1                                               | 0.1                                               |

aResults were normalized by setting the number of moles of tyrosine equal to 1.
bCys residues were not protected by alkylating prior to acid hydrolysis and these were not considered.
cThe N-terminal Met is cleaved after translation (4A).

Figure 1. Purification of 422(P2) protein by chromatography on a Mono Q cation exchange column. 422(P2) protein was released from cells with dilute diisocyanate, fractionated with ammonium sulfate, renatured and dialyzed extensively against 20mM sodium acetate, 1mM DTT, pH 5.0 as described in "Experimental Procedures". 0.4 mg of protein solution at pH 5.0 was applied to a Mono Q 5/5 column (1ml) cation exchange column and eluted with a NaCl gradient fractions were collected and assessed for total protein by the BCA procedure (7) and for 422(P2) protein by IRA (8).

Figure 2. Polyacylamide gel electrophoretic analysis during the course of purification of 422(P2) protein. Samples of protein (15 μg per lane) at different stages in the purification of 422(P2) protein from 375-L1 adipocytes were subjected to polyacrylamide gel electrophoresis under "Experimental Procedures". Lane 1: Mono Q cation exchange chromatography. Lane 2: Mono Q cation exchange chromatography followed by trypsin digestion. Lane 3: SDS-PAGE analysis after electrophoresis and staining. Lane 4: major protein peak from Mono Q cation exchange chromatography (B). Lane 5: major protein peak from Mono Q cation exchange chromatography (A). Lane 6: major protein peak from Mono Q cation exchange chromatography (C). Lane 7: major protein peak from Mono Q cation exchange chromatography (D). The position of the molecular weight marker (FITC dextran) is indicated by an arrow. (A) and (B), (C) and (D) are analogous to each other.

Figure 3. Separation of tryptic peptides of purified 422(P2) protein by reverse phase chromatography. 422(P2) protein was isolated from 375-L1 adipocytes as described in "Experimental Procedures" and then 0.8 mg of purified tryptic peptides were applied to a C18 reversed phase column (long column, 0.5 × 25 mm) and eluted with an acetonitrile gradient (%AC). Three peptides (A, B, and C) were eluted from the column and collected on a Shimadzu LC-9A (150A) column (0.5 × 4.6 mm) using a gradient of 0-50% acetonitrile over 60 min using a gas phase amine acid sequencer.
Fatty Acids Activate Phosphorylation of 422(aP2) Protein

Figure 4. Two-dimensional gel electrophoresis of [32P]ppi5 immunoprecipitated with anti-422(aP2) protein serum. [32P]ppi5-containing antigenic reagents were 37-31 labeled with insulin and P20 was treated with 1% SDS at 100°C and then diluted to 0.16 M 100 with 0.08 Tris-150 as described in "Experimental Procedures". Nonspecific binding was reduced by prior treatment of the sample with antiserum serum and Pentax A-Sepharose for 2h at room temperature. After centrifugation, the supernate was incubated for 2h at 30°C with affinity-purified anti-422(aP2) protein antiserum and 5% P20. The P20 supernate was transferred to the gel matrix of the gel of Fig. 5 and dried to form a two-dimensional gel corresponding to the 12 C-terminal amino acids of 422(aP2) protein. Samples were rehydrated and the gel stained using time before analysis by two-dimensional gel electrophoresis. [32P]ppi5 was identified by autoradiography. Negative sign indicates the acidic part of the gel.

Figure 3. Comparison of the electrophoretic properties of (125I)-phospho 422(aP2) protein phosphorylated by the insulin receptor and of [32P]ppi5 isolated from intact cells. 422(aP2) protein was purified and phosphorylated in the presence of 100 pmol of insulin and [32P]ppi5 using insulin receptor purified as described in "Experimental Procedures". To accumulate [32P]ppi5 in 37-31 labeled with 32P-

Figure 6. Phosphatase and analysis of [32P]phosphorylated 422(aP2) protein generated by the insulin receptor tyrosine kinase in vitro and [32P]ppi5 isolated from 37-31 adipocytes. 422(aP2) protein was phosphorylated in the presence of the insulin. [32P]ppi5 and insulin receptor purified as described in "Experimental Procedures". The accumulation of [32P]ppi5 in intact cells and its partial purification in the absence of the insulin receptor is shown in the second panel of Fig. 5 and its complete purification in intact cells as described in the legend of Fig. 5 and "Experimental Procedures". [32P]ppi5 was isolated from 37-31 adipocytes and subjected to digestion with trypsin. Trypsin digests were subjected to partial acid hydrolysis and then analyzed by two-dimensional high-voltage thin-layer electrophoresis as described in "Experimental Procedures". [32P]-phosphorylated phosphoamin acid derivatives were detected by autoradiography. (A) [32P]-422(aP2) protein phosphorylated in vitro (B) [32P]-422(aP2) protein phosphorylated in vitro and subjected to digestion with phosphoamin acid derivatives. (C) [32P]-422(aP2) protein phosphorylated in vitro and subjected to digestion with phosphoamin acid derivatives (D). Phosphoamin acid derivatives (E), and phosphoamin acid derivatives (F). The position of application is shown by an arrow.

Figure 7. EPLC elution profile of the [32P]-phosphorylated phosphoamin acid derivatives of 422(aP2) protein phosphorylated in vitro and of pp35 isolated from intact 37-31 adipocytes. [32P]-422(aP2) protein phosphorylated in vitro of the insulin receptor was isolated by affinity precipitation and subjected to digestion with trypsin. The resulting phosphoamin acid derivatives are described in the legend of Fig. 5 and "Experimental Procedures". [32P]-phosphorylated 422(aP2) protein and [32P]ppi5 were subjected to two-dimensional gel electrophoresis, eluted from the gel fragments, and digested with trypsin. Peptides were applied to a Superoxide 8-1 column and eluted as described in Fig. 3. Fractions (0.6ml) were collected and analyzed for [32P]activity. (D) Trypsin phosphoamin acid derivatives of [32P]ppi5 isolated from intact 37-31 adipocytes.
Insulin receptor tyrosine kinase-catalyzed phosphorylation of 422(aP2) protein. Substrate activation by long-chain fatty acid.
R C Hresko, R D Hoffman, J R Flores-Riveros and M D Lane

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