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Insulin-sensitive Myelin Basic Protein Phosphorylation on Tyrosine Residues

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Rat brain plasma membranes were solubilized in detergent and a glycoprotein-enriched fraction was obtained by lectin affinity chromatography. This glycoprotein fraction contained insulin receptors, as well as protein kinases capable of phosphorylating some exogenously added substrates such as MAP2 (microtubule associated protein 2) and MBP (myelin basic protein), but not ribosomal protein S6. Phosphoamino acid analysis of MAP2 and MBP showed that phosphotyrosine residues, as well as phosphoserine/phosphotheronine residues, were present in both proteins under basal conditions. Whereas the addition of insulin to the rat brain membrane glycoprotein fraction in vitro had no effect on MAP2 phosphorylation, MBP phosphorylation was stimulated 2.7-fold in response to insulin. This phenomenon was dose-dependent, with half-maximal stimulation of MBP phosphorylation observed with 2 nM insulin. Phosphoamino acid analysis of MBP indicated that insulin stimulated the phosphorylation of tyrosine residues nearly three-fold, whereas the phosphorylation of serine or threonine residues was not increased. These results identify MBP as a substrate for the rat brain insulin receptor tyrosine-specific protein kinase in vitro.

The mechanism of insulin action has not yet been clearly characterized. One proposed mechanism of insulin signal transduction involves the activation of the insulin receptor tyrosine-specific kinase, which may subsequently phosphorylate as yet unidentified target proteins on tyrosine residues. Among the candidate substrates for insulin receptor tyrosine kinase is a cytosolic enzyme known as MAP (microtubule-associated protein or mitogen-activated protein) kinase (1); this kinase is thought to be activated by phosphorylation on tyrosine residues and subsequently to phosphorylate its respective substrates,which may include S6 kinase and MAP2, on

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The abbreviations used are: MBP, myelin basic protein; MAP2, microtubule-associated protein 2; SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis; CNS, central nervous system.

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serine residues (2,3). Evidence that insulin action in vivo leads to an increase in MAP2 or S6 phosphorylation on serine residues is consistent with this hypothesis, but it has not been possible to isolate and reconstruct such a hormone-sensitive phosphorylation cascade in vitro. In a recent report, this laboratory identified a subfraction of rat brain plasma membranes which could be solubilized in detergent and retained on a wheat germ agglutinin-Sepharose column (4). This fraction contained several types of protein kinases, including cyclic AMP-dependent protein kinase, which could phosphorylate endogenous brain MAP2 on serine residues (4). Additionally, this fraction contained insulin receptor tyrosine kinase activity; immunodepletion studies indicated that this latter enzyme could phosphorylate MAP2 directly on tyrosine residues in vitro (5). However, phosphorylation of MAP2 by this fraction could not be stimulated by the addition of insulin in vitro.

Myelin basic protein (MBP) is the major basic protein of the CNS myelin; MBP is heterogenous in size (21.5, 18.5, 17.2 and 14.2 kDa) and it is a linear basic protein with no disulfide bonds (6). MBP has been used as a model substrate for both cAMP-dependent protein kinase and Ca^{2+}/phospholipid dependent protein kinase (7,8). In all of these reactions, MBP phosphorylation occurs on serine/threonine residues. In this communication, we describe for the first time insulin-sensitive MBP phosphorylation on tyrosine residues.

Methods

Rat brain plasma membranes were isolated by differential centrifugation as described by Haskell et al. (9). Rat brain plasma membranes were solubilized in 25 mM Hepes, pH 7.6, containing 1% Triton X-100 and loaded onto a column of wheat germ agglutinin-Sepharose (Pharmacia LKB, Uppsala, Sweden). The column was washed extensively with buffer containing 100 mM NaCl, 0.05% Triton X-100, 2.5 mM KCl, 1 mM $CaCl_2$ and 25 mM HEPES, pH 7.6; the glycoprotein fraction bound to the column was eluted with the same buffer containing 0.3 M N-acetyl-D-glucosamine. The eluted glycoproteins were dialyzed and concentrated to their original volume using an Amicon PM 30 ultrafiltration unit. Aliquots of the glycoprotein fraction of rat brain plasma membranes, isolated as described above, were pre-incubated with porcine insulin (10 µg/ml) at 23°C for 30 min. The phosphorylation reaction was initiated by the addition of a solution containing (final concentrations) 50 µM [γ-^{32}P) ATP (14 µCi/sample), 5 mM $MnCl_2$, 12 mM $MgCl_2$, 100 µM Na_3VO_4, 100 µM NaF and 25 mM Hepes (pH 7.4) and allowed to incubate for various times, as described by Pillion et al. (10). The reaction was terminated by heating to 100°C for 3 min in Laemmli (11) sample buffer with dithiothreitol. The phosphoproteins were separated by SDS-PAGE on a 4-16% gradient gel and identified by autoradiography. MAP2 was purified from bovine brain as described by Kim et al (12). S6 was kindly provided by Dr. Natalie Ahn, Department of Pharmacology, University of Washington at Seattle. Phosphoamino acids were analyzed by the procedure described by Huganir et al. (13).

Results

Rat brain plasma membranes were isolated by differential centrifugation and solubilized in the presence of 1% Triton X-100. A glycoprotein-enriched fraction was then purified by passing the detergent-soluble membrane material over a wheat germ agglutinin-Sepharose column. Rat brain plasma membrane glycoproteins were eluted from the column and added to a phosphorylation assay in the absence or presence of insulin or EGF with the following exogenous substrates: bovine brain MAP2, ribosomal protein S6 or MBP in the absence or presence of insulin or EGF. Subsequently, the assay mixture was transferred to SDS-polyacrylamide gels and, following electrophoresis, evaluated by autoradiography. Fig. 1 is an autoradiogram showing ^{32}P-incorporation into MAP2, S6 and MBP following an incubation with rat brain plasma membrane glycoproteins. The addition of insulin to the glycoprotein fraction in vitro stimulated the autophosphorylation of the insulin receptor ß-subunit (Mr 95,000) (lanes 2, 5 and 8); the addition of epidermal growth factor (EGF) had no effect on the autophosphorylation of the EGF receptor (Mr 170,000) (lanes 3, 6 and 9). These data confirm that functional insulin receptors, but not EGF receptors, are present in the rat brain plasma membrane glycoprotein fraction. Exogenous bovine brain MAP2 (lanes 1-3) was phosphorylated by the rat brain plasma membrane

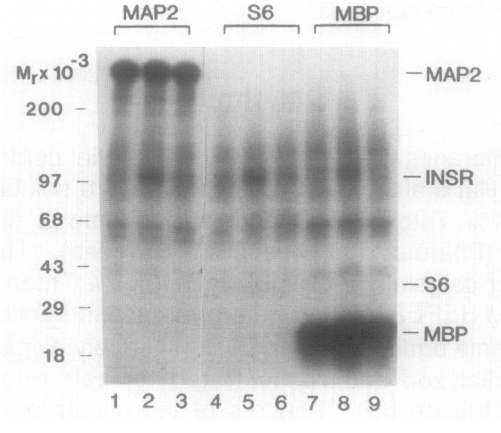

Fig. 1. Phosphorylation of MAP2, S6, and MBP by the lectin-purified plasma membrane glycoprotein fraction from rat brain.
Rat brain plasma membranes were isolated, solubilized in 1% Triton X-100 and fractionated on a wheat germ agglutinin-Sepharose column. Aliquots of this fraction were pre-incubated without (lanes 1,4 and 7) or with porcine insulin (10 µg/ml) (lanes 2, 5, and 8) or EGF (1.2 µg/ml) (lanes 3, 6, and 9) at 23°C for 30 min. A phosphorylation reaction was conducted for 15 min at 23°C. The phosphoproteins were separated by SDS-PAGE on a 4-16% gradient gel and identified by autoradiography. The positions of MAPs, S6 protein and myelin basic protein (MBP) were determined by staining the gel with Coomassie Brilliant Blue and the position of the Mr 95,000 insulin receptor beta-subunit (INSR) was confirmed by immunoblot analysis.

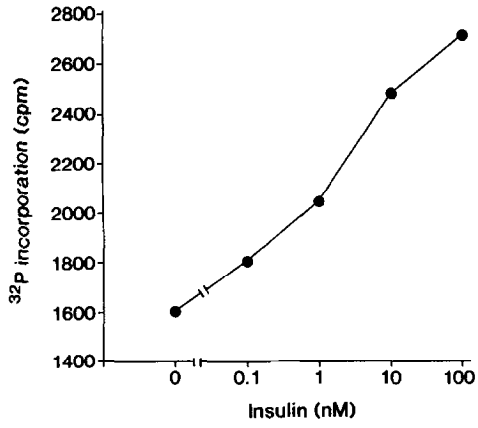

Fig. 2. Effect of insulin concentration on MBP phosphorylation by the rat brain plasma membrane glycoprotein fraction.
Aliquots of the rat brain plasma membrane glycoprotein fraction were pre-incubated with different concentrations of porcine insulin (0-100 nM) at 23°C for 30 min. MBP (5 μg) was added and a phosphorylation reaction was conducted as described in Fig. 1. The phosphoproteins were separated by SDS-PAGE on a 4-16% gradient gel and identified by autoradiography. The MBP bands were cut from the dried gel and ^{32}P incorporated into the bands was measured by counting Cerenkov radiation.

glycoprotein fraction, but neither insulin (lane 2) nor EGF (lane 3) had any effect on MAP2 phosphorylation. In contrast to the results obtained with exogenous MAP2, there was no phosphorylation of exogenous ribosomal protein S6 by the rat brain plasma membrane glycoprotein fraction, either in the presence or absence of insulin or EGF (lanes 4-6). Myelin basic protein (MBP) behaved in a different manner from either MAP2 or S6 when added to the reaction mixture. MBP was phosphorylated by the rat brain plasma membrane glycoprotein fraction in the absence of insulin (lane 7); however, unlike the situation described above with MAP2, addition of 10 μg/ml insulin stimulated MBP phosphorylation (lane 8 vs lane 7). EGF had no stimulatory effect on MBP phosphorylation (lane 9).

The effect of insulin concentration on the phosphorylation of MBP by the rat brain plasma membrane glycoprotein fraction was determined (Fig. 2). MBP phosphorylation by rat brain plasma membrane glycoproteins was increased by insulin in a dose-dependent manner and reached a maximum at an insulin concentration of 100 nM (Fig. 2); the addition of 1 μM insulin had no further stimulatory effect. The half-maximal insulin concentration was approximately 2 nM (Fig. 2). The results described in Figs. 1 and 2 were obtained from experiments in which the phosphorylation reactions were carried out for 15 min. When MBP phosphorylation reactions were carried out for 0-15 min in the presence and absence of 10 μg/ml

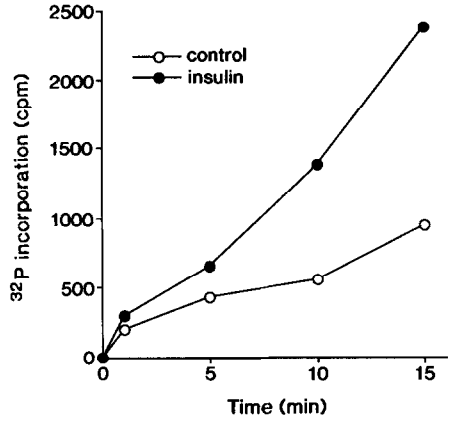

Fig. 3. Time-dependent MBP phosphorylation by the rat brain plasma membrane glycoprotein fraction.
Aliquots of the rat brain plasma membrane glycoprotein fraction were pre-incubated with or without porcine insulin (10 μg/ml) at 23°C for 30 min. MBP (5 μg) was added to the reaction mixture and a phosphorylation reaction was conducted as described in Fig. 1 for different time periods (0-15 min). The phosphoproteins were separated by SDS-PAGE on a 4-16% gradient gel and identified by autoradiography. MBP bands were cut from the dried gel and ^{32}P incorporated into the bands measured by counting Cerenkov radiation.

insulin, MBP phosphorylation increased with time (Fig. 3). The stimulatory effect of insulin on MBP phosphorylation was maximal after 15 min; ^{32}P-incorporation into MBP was enhanced approximately 3-fold in samples incubated with insulin, relative to samples incubated without insulin.

Phosphoamino acid analysis was performed to characterize the amino acid specificity of MBP phosphorylation in the absence or presence of insulin (Fig. 4). In the absence of insulin, MBP was phosphorylated to approximately the same extent on both serine and tyrosine residues; addition of insulin to the reaction mixture, however, stimulated the phosphorylation of MBP on tyrosine residues (Fig. 4A, right panel vs. left panel). The radioactivity incorporated into phosphoamino acids depicted in Fig. 4A was subsequently counted with a liquid scintillation counter (Fig. 4B). The addition of insulin to the reaction mixture had little effect on MBP phosphorylation on either threonine or serine residues, but it increased phosphorylation on tyrosine residues by nearly three-fold (Fig. 4B).

Discussion

Attempts to identify and characterize target substrate proteins for the insulin receptor tyrosine kinase have met with only marginal success. Many proteins have been suggested as potential substrates for insulin receptor tyrosine kinase, including pp240,

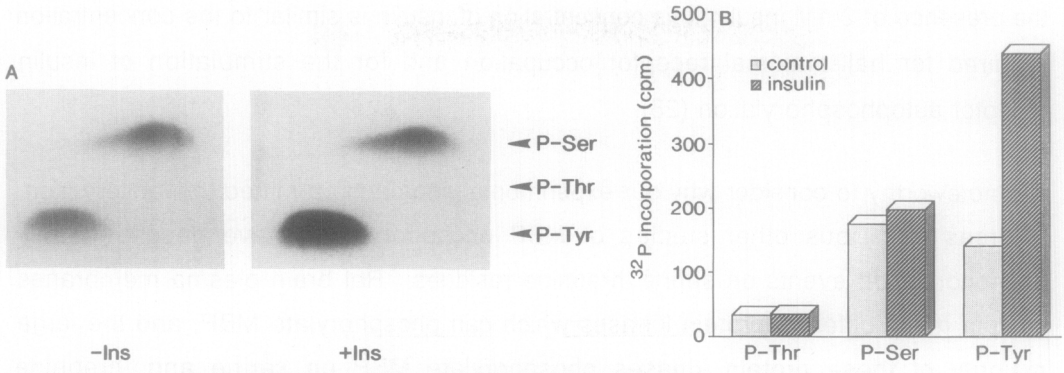

Fig. 4. Phosphoamino acid analysis of MBP phosphorylated by the rat brain plasma membrane glycoprotein fraction.
Aliquots of the rat brain plasma membrane glycoprotein fraction were pre-incubated with or without porcine insulin (10 µg/ml) at 23°C for 30 min. The phosphorylation reaction was conducted for 15 min in the presence of 5 µg MBP as described in Fig. 1. The phosphoproteins were separated by SDS-PAGE on a 4-16% gradient gel and identified by autoradiography. MBP bands were cut from the dried gel and acid-hydrolyzed peptides were subjected to two-dimensional thin-layer electrophoresis. The plates were dried and subjected to autoradiography (A). The positions of phosphoamino acid standards are indicated on the right. The area corresponding to the phosphoamino acids were scraped from the plate and counted in a liquid scintillation spectrophotometer to quantitate ^{32}P (B).

pp185, pp120, pp46, pp19, pp15, calmodulin, Ca++/calmodulin-dependent protein kinase, MAP kinase, tubulin, MAPs, the progesterone receptor and GTP-binding proteins (G-proteins) (14-25). Despite intense efforts to characterize and reconstruct an insulin-responsive intracellular cascade of phosphorylation events, it has not been possible to describe insulin signal transduction in terms of a discrete insulin-sensitive phosphorylation reaction involving phosphorylation of a known protein on tyrosine residues. In this communication, myelin basic protein (MBP) phosphorylation by rat brain plasma membrane glycoproteins was evaluated. MBP has been used by other investigators as a model substrate for serine-specific protein kinases such as cAMP-dependent and Ca++/phospholipid-dependent protein kinases and MAP kinase (7,8,26). Recently, MBP has also been used as a substrate for a cytosolic tyrosine-specific protein kinase from porcine spleen (CPTK-40) (27). Hence, there is a precedent for phosphorylation of MBP on tyrosine residues, although no neural enzyme capable of this activity has been identified previously. Our results indicate that MBP phosphorylation by rat brain plasma membrane glycoproteins occured on both serine and tyrosine residues, but only phosphorylation on tyrosine residues was stimulated by the addition of insulin in vitro. The specificity of this reaction was underscored by the observation that MAP2 phosphorylation in vitro was not increased by the addition of insulin. Half-maximal stimulation of MBP phosphorylation occured in

the presence of 2 nM insulin; this concentration of insulin is similar to the concentration required for half-maximal receptor occupation and for the stimulation of insulin receptor autophosphorylation (28).

It is noteworthy to consider why our experimental approach permitted this observation, whereas numerous other studies of MBP phosphorylation have described only phosphorylation events on serine/threonine residues. Rat brain plasma membranes contain many different protein kinases which can phosphorylate MBP, and the large majority of these protein kinases phosphorylate MBP on serine and threonine residues. The glycoprotein fraction used in this study contains only a small percentage of the total proteins in the cell membrane and we have shown that many types of protein kinase activity which could phosphorylate MBP in vitro are not retained in the glycoprotein fraction (5,29); for example, no S6 kinase activity was identified in the rat brain plasma membrane glycoprotein fraction. Hence, the tyrosine-specific phosphorylation events that are described in these experiments may easily have been overlooked in protocols which utilized plasma membranes or cellular extracts as a source of protein kinase activity.

These results raise the possibility that myelin basic protein (MBP) may represent an endogenous substrate for the insulin receptor tyrosine-specific protein kinase in vivo. This hypothesis raises some important questions about the physiologial role of MBP phosphorylation and the well-documented neuropathy that occurs in patients with long-standing diabetes mellitus. Previous studies have shown that the insulin receptor is widely distributed throughout the mammalian brain (30,31) and, moreover, that diabetic patients tend to have reduced or altered myelination, as well as impaired nerve conductance velocity (32,33). Our observation that the rat brain insulin receptor can directly phosphorylate MBP on tyrosine residues may provide a biochemical explanation for the altered pattern of myelination and nerve conductence velocity observed in diabetics; i.e., altered phosphorylation of MBP on tyrosine residues may represent a previously unrecognized pathophysiological hallmark of diabetic neuropathy. Caution must be exercised, however, since many proteins are phosphorylated by the insulin receptor tyrosine kinase in vitro but fail to undergo phosphorylation on tyrosine residues in vivo.

In conclusion, this investigation has identified a novel substrate for the insulin receptor tyrosine-specific kinase: myelin basic protein. MBP phosphorylation on tyrosine residues can occur in vitro and the addition of insulin to the reaction mixture increases phosphotyrosine formation without affecting the rate of phosphoserine or

phosphothreonine formation. It remains to be determined if MBP phosphorylation on tyrosine residues occurs in vivo in response to insulin

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