Dual Specificity Mitogen-activated Protein (MAP) Kinase Phosphatase-4 Plays a Potential Role in Insulin Resistance*

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Insulin is the key hormone that controls glucose homeostasis. Dysregulation of insulin function causes diabetes mellitus. Among the two major forms of diabetes, type 2 diabetes accounts for over 90% of the affected population. The incidence of type 2 diabetes is highly related to obesity. To find novel proteins potentially involved in obesity-related insulin resistance and type 2 diabetes, a functional expression screen was performed to search for genes that negatively regulate insulin signaling. Specifically, a reporter system comprised of the PEPCK promoter upstream of alkaline phosphatase was used in a hepatocyte cell-based assay to screen an expression cDNA library for genes that reverse insulin-induced repression of PEPCK transcription. The cDNA library used in this study was derived from the white adipose tissue of ob/ob mice, which are highly insulin-resistant. The mitogen-activated dual specificity protein kinase phosphatase 4 (MKP-4) was identified as a candidate gene in this screen. Here we show that MKP-4 is expressed in insulin-responsive tissues and that the expression levels are up-regulated in obese insulin-resistant rodent models. Heterologous expression of MKP-4 in preadipocytes significantly blocked insulin-induced adipogenesis, and overexpression of MKP-4 in adipocytes inhibited insulin-stimulated glucose uptake. Our data suggest that MKP-4 negatively regulates insulin signaling and, consequently, may contribute to the pathogenesis of insulin resistance.

Type 2 diabetes mellitus is growing as a major public health problem in the United States and other industrialized countries (1). The rapid increase in the number of people diagnosed with the disease has triggered more interest in finding novel and better therapeutic agents (2). Type 2 diabetes is a polygenic disease with complicated etiology. Although significant progress has been made in the identification of genes that contribute to the disease, many pieces of the puzzle are still missing. Insulin resistance, characterized by elevated circulating insulin levels and impaired glucose tolerance, usually precedes Type 2 diabetes. Whether an individual with insulin resistance ultimately develops diabetes depends on the secretion capacity of pancreatic β cells (3). Drugs aiming to stimulate insulin secretion can improve hyperglycemia but only work on patients with relatively mild dysfunction (4). Improving insulin sensitivity promises a preferable approach to treat diabetes.

Insulin is an anabolic hormone that controls many aspects of metabolism. The MAP kinase cascade and the PI3-kinase pathway are two of the signaling networks activated by insulin upon binding to its receptor. The activation of ERK by insulin seems to play a major role in insulin-mediated mitogenesis, whereas PI3-kinase has been implicated as a major key player of the metabolic arm. Defects in the insulin-stimulated metabolic cascade lead to insulin resistance. Therefore, to identify additional genes involved in negatively regulating insulin-stimulated metabolic pathway(s), we employed an expression cloning approach using the promoter of the phosphoenolpyruvate carboxykinase gene (PEPCK) placed upstream of secreted alkaline phosphatase as a surrogate reporter system for the metabolic effects of insulin.

The action of insulin modifies the activity of a multitude of proteins within minutes and regulates expression of about 100 genes in a matter of hours (5). Among the genes regulated by insulin, the expression of PEPCK, a key enzyme in hepatic gluconeogenesis, has been studied most extensively (6, 7). There has been no evidence to support post-translational modification or allosteric activation of PEPCK by insulin, and transcriptional regulation is thought to be the only way by which insulin regulates PEPCK levels (5). Therefore, the PEPCK promoter is an ideal tool in a functional screening to reflect transcriptional changes in response to insulin stimulation. Furthermore, it has been shown that insulin-mediated repression of PEPCK transcription can be completely blocked by wortmannin (a PI3-kinase inhibitor), indicating the essential role of PI3-kinase in this pathway (8). On the other hand, kinase inhibitors and dominant-negative mutants of ERK pathway, which block insulin mitogenic signaling, had no effect on insulin-mediated PEPCK promoter repression (9, 10). Therefore, regulation of PEPCK transcription by insulin seems to use the same signaling pathways involved in insulin-mediated metabolic effects. Our hypothesis is that genes blocking the inhibitory effect of insulin on PEPCK transcription are also likely to attenuate the metabolic signaling pathway(s) elicited by insulin. Using this approach, we identified mitogen-activated protein kinase phosphatase 4 (MKP-4), a dual specificity protein phosphatase (11), as a candidate gene involved in the negative regulation of insulin signaling. Further experiments...

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† The abbreviations used are: MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; MKP, mitogen-activated dual specificity protein kinase phosphatase; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH2-terminal kinase; P38-kinase, phosphatidylinositol 3-kinase; AP, alkaline phosphatase; GFP, green fluorescent protein; DMEM, Dulbecco’s modified Eagle’s medium; PEPCK, phosphoenolpyruvate carboxykinase; PTP-1B, protein-tyrosine phosphatase 1B.
in this study provide supporting evidence for a role of MKP-4 in the regulation of other insulin-mediated metabolic effects such as adipocyte differentiation and glucose uptake. This is the first time that a dual specificity protein phosphatase has been implicated in insulin resistance.

MATERIALS AND METHODS

Cells and Reagents—The rat hepatoma cell line H4IIE was cultured in DMEM supplemented with 5% fetal bovine serum (Invitrogen). For the PEPCK promoter assay, H4IIE cells were cultured in DMEM supplemented with 0.1% fetal bovine serum as well as a mixture of 100 μM cAMP, 1 μM dexamethasone, and/or 10 μM insulin, as indicated in the figures. The 3T3-F442A cell line was cultured in DMEM supplemented with 10% bovine calf serum (Hyclone). For differentiation, 3T3-F442A cells were switched to DMEM supplemented with 10% cosmic calf serum (Hyclone). Cells were first induced with a mixture of 5 μg/ml insulin, 1 μM dexamethasone, and 0.5 mM isobutyl-methylxanthine for 3 days and then maintained in 5 μg/ml insulin until they fully differentiated into adipocytes.

Construction of the Reporter Construct and cDNA Library—The PEPCK promoter (~548 to +73) (12) was amplified from rat genomic DNA and cloned upstream of the placent al alkaline phosphatase coding sequence (13) in the pM67 vector. For the clDNA library construction, RNA was extracted from the white adipose tissue of ob/ob mice and reverse-transcribed using oligo(dT). The cDNAs were then cloned into the pM67 expression vector.

Transfection of H4IIE Cells—Cells were seeded into 96-well plates (1.5 dilution from a confluent 10-cm dish) 1 day prior to transfection. Fresh medium (DMEM/5% fetal calf serum) was added 5 h prior to transfection. The following day, the PEPCK reporter construct (0.156 μg/well) was co-transfected with dominant-negative p85 (DNp85) or single cDNA clones from the white adipose tissue (0.125 μg/well) into H4IIE cells using FuGene 6 (0.38 μl/well) (Roche Applied Science). Medium was then exchanged, the following morning, to DMEM/0.5% fetal calf serum. Stimulants and/or inhibitors were added 5 h later, as indicated in the figures, and incubated for 16 h. Then, medium was collected on the following day and assayed for alkaline phosphatase activity.

Alkaline Phosphatase Assay—The medium was collected from transfected H4IIE cells and spun down to remove floating cells. The samples were heat-inactivated at 65 °C for 30 min. The Great EscAPE kit (Clontech) was used to detect the alkaline phosphatase activity in conditioned medium. The samples were read on the Wallace Luminometer (Dy nex Tech).

Construction of Expression Vectors and Establishment of Cell Lines Stably Expressing MKP-4—The full-length cDNA of mouse MKP-4 was cloned into a retroviral vector expressing the puromycin resistance gene. The retroviral expression vectors were transferred into a packaging cell line Bosc23 (14) by the LipofectAMINE method (Invitrogen). Cloned into a retroviral vector expressing the puromycin resistance (Clontech) was used to detect the alkaline phosphatase activity in conditioned medium. The samples were read on the Wallace Luminometer (DyneX Tech).

RESULTS

Identification of MKP-4—As a Negative Regulator of Insulin Signaling—Individual clones of a cDNA expression library derived from the white adipose tissue of ob/ob mice were co-transfected with a PEPCK promoter (~600 bp) construct into H4IIE cells, an insulin-responsive rat hepatoma cell line (17). The PEPCK promoter was placed upstream of the secreted alkaline phosphatase (PEPCKp600-SEAP). It is well known that dexamethasone and cAMP strongly stimulate transcription of the PEPCK gene, whereas insulin shuts down the PEPCK gene expression exclusively at transcription level (6, 7). The 600-bp PEPCK promoter used in this study contains an insulin response unit, a glucocorticoid response unit, and a cAMP response unit (7). Using the secreted placental alkaline phosphatase (AP) as a reporter gene, the rate of transcription is reflected by the activity of AP in the conditioned media. Complementary DNA clones that resulted in high AP activity even in the presence of insulin were selected and confirmed in secondary assays. As a positive control, a dominant-negative form of the regulatory subunit of PI3-kinase, p85 (DNp85) (18), was cotransfected with PEPCKp600-SEAP. The mutant p85 rescued AP activity to ~50% of the control level in the presence of insulin (Fig. 1). The control level was defined as AP activity stimulated by dexamethasone plus cAMP in the absence of insulin. About 10,000 clones were screened using this system. This study describes the identification of one of the cDNA clones, which reproducibly restored AP activity to a similar extent when compared with the DNp85 (Fig. 1). Follow-up sequence analysis revealed that this clone is the mouse orthologue. Since the 67-residue repetitive sequence is also present in the N terminus of the mouse orthologue. This is the first time that a dual specificity protein phosphatase has been implicated in insulin resistance.
The expression of MKP-4 was examined in various murine tissues including the white and brown adipose tissues. Mouse MKP-4 was only detectable in white adipose tissue, kidney, and testis (Fig. 3A), suggesting that the expression of MKP-4 is specific to insulin-responsive tissues. Since liver, muscle, white adipose tissue, and brown adipose tissue express high levels of insulin receptor and play important roles in insulin sensitivity, the up-regulation of MKP-4 in insulin-responsive tissues indicates a potential role for MKP-4 in insulin signaling and in obesity-related insulin resistance.

**MKP-4 in Adipocyte Function**—To further understand the role of MKP-4 in adipocyte biology, MKP-4 was stably expressed in 3T3-F442A preadipocytes by a retroviral approach. Then, the cells were induced to differentiate by either insulin or a mixture of insulin, dexamethasone, and isobutylmethylxanthine. The experiment was terminated when more than 90% of control vector-expressing cells induced by the mixture fully differentiated into adipocytes, which is 7 days after induction. Cells stably expressing MKP-4 differentiated poorly under both induction conditions as shown by oil red O staining (Fig. 5A). This experiment indicates that MKP-4 has an inhibitory effect on adipogenesis under both conditions. The expression level of MKP-4 was significantly increased in the white adipose tissue of ob/ob, db/db, and diet-induced rodent obesity models (Fig. 4, top panels, lanes 1–8). Although MKP-4 was also expressed in kidney and testes, no regulation was observed in the above rodent obesity models (Fig. 4, bottom panels, lanes 9–16), suggesting that the up-regulation of MKP-4 is specific to insulin-responsive tissues. Since liver, muscle, white adipose tissue, and brown adipose tissue express high levels of insulin receptor and play important roles in insulin sensitivity, the up-regulation of MKP-4 in insulin-responsive tissues indicates a potential role for MKP-4 in insulin signaling and in obesity-related insulin resistance.
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MKP-4 has been reported to inactivate both ERK and P38, whereas its effect on JNK remains controversial (11, 19). Since both P38 and MKP-4 are capable of enhancing PEPCK transcription, it is unlikely that MKP-4 blocks insulin-mediated repression on PEPCK promoter by inactivating P38. It is well known that a PI3-kinase-dependent pathway is required for repression of PEPCK transcription by insulin. These data suggest that MKP-4 is involved in dephosphorylating kinase components in the insulin-induced metabolic cascade. This piece of evidence demonstrates that the MAP kinase pathway can cross-talk with the metabolic pathway. Further studies are necessary to identify the substrates of MKP-4 in insulin-mediated metabolic responses.

To our knowledge, this is the first time that a dual specificity

DISCUSSION

A functional expression cloning approach was applied to identify genes that may play a role in insulin resistance in this specific study. Complementary DNA clones from the insulin-resistant adipose tissue of ob/ob mice were screened. As a result of this screen, MKP-4, a dual specificity protein phosphatase, was found to block insulin signaling in this system. This phosphatase belongs to the MKP family, which leads to the downregulation of MAPK signaling cascades by dephosphorylating critical tyrosine and serine/threonine residues on MAPKs (20). It is interesting to note that a member of the MKP family inhibits insulin-mediated repression of the PEPCK promoter since complete blockage of ERK-mediated signaling, with kinase inhibitors and dominant negatives, has no effect on PEPCK gene expression (9, 10). In addition to the ERK pathway, recent studies showed that the stress-induced P38 pathway can also be activated by insulin (21). Interestingly, P38 is involved in ATF-2-mediated induction of PEPCK transcription (22). MKP-4 has been reported to inactivate both ERK and P38, whereas its effect on JNK remains controversial (11, 19). Since both P38 and MKP-4 are capable of enhancing PEPCK transcription, it is unlikely that MKP-4 blocks insulin-mediated repression on PEPCK promoter by inactivating P38. It is well known that a PI3-kinase-dependent pathway is required for repression of PEPCK transcription by insulin. These data suggest that MKP-4 is involved in dephosphorylating kinase components in the insulin-induced metabolic cascade. This piece of evidence demonstrates that the MAP kinase pathway can cross-talk with the metabolic pathway. Further studies are necessary to identify the substrates of MKP-4 in insulin-mediated metabolic responses.

To our knowledge, this is the first time that a dual specificity
phosphatase has been implicated in insulin resistance. Previous work in the field was focused extensively on protein-tyrosine phosphatases. Among the protein-tyrosine phosphatases that attenuate the insulin signaling pathway, PTP-1B is the most extensively characterized and well validated, whereas the role of leukocyte antigen-related protein-tyrosine phosphatase (LAR) remains controversial (23, 24). The major impact site of PTP-1B is known to be in liver, and overexpression of PTP-1B in adipocytes did not affect glucose uptake (25). Our results suggest that MKP-4 might be a major phosphatase involved in...
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adipose insulin resistance since it is expressed and up-regulated in adipose tissue of several rodent obesity models. Furthermore, MKP-4 can blunt insulin-stimulated glucose uptake in cultured adipocytes, which supports our hypothesis. In addition, MKP-4 is undetectable in other insulin-responsive tissues under normal circumstances but becomes detectable in brown adipose tissue, liver, and muscle of obese mice, indicating that MKP-4 might also attenuate insulin signaling in other insulin-responsive tissues in the obese state.

The mitogen activated dual specificity MAP kinase phosphatase family has nine members (20). These phosphatases have distinct tissue distribution patterns and different substrate preferences. The closest homologues of MKP-4 are MKP-3 and MKP-X, sharing sequence identity of 56 and 61%, respectively, at the amino acid level. In fact, MKP-3 was also identified as a weaker candidate gene during the functional expression screen. The appearance of two dual specificity phosphatases in our screen indicates the involvement of a new class of phosphatases in insulin action and insulin resistance. When compared with tyrosine phosphatases, which only dephosphorylate tyrosine residues, dual specificity phosphatases also dephosphorylate serine/threonine residues. One feature of this class of protein phosphatases is that they contain both a regulatory domain and a catalytic domain, whereas PTP-1B is comprised only of a catalytic domain, and contains both a regulatory and a catalytic domain, which is constitutively active. Phosphorylation of IRS-1 on serine residues has been proposed as a mechanism for inducing IRS1 degradation as well as reducing the tyrosine kinase activity of insulin receptor (26, 27). It remains to be studied whether dual specificity phosphatases such as MKP-4 have any effect on IRS1 serine/threonine phosphorylation. Future loss-of-function studies may further define the role of MKP-4 in insulin resistance in vivo. If confirmed to be an important regulator in obesity-related insulin resistance, MKP-4 presents a new molecular target for pharmaceutical intervention to improve systemic insulin sensitivity.

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