Identification of the Thyroid Transcription Factor-1 as a Target for Rat MST2 Kinase*

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Thyroid transcription factor-1 (TTF-1) is a homeodomain-containing transcription factor that is required for thyroid-specific expression of the thyroglobulin and thyroperoxidase genes as well as for lung-specific expression of the surfactant protein A, B, and C and the CC10 and the HNF-3 genes. TTF-1 is a phosphoprotein, and the phosphorylation of TTF-1 has been studied already. However, the kinase(s) that could be responsible for this phosphorylation have not been known. In this paper we report the identification by in-gel kinase assay of a 56-kDa serine/threonine kinase that is able to phosphorylate TTF-1 in thyroid cells. The cloning of this kinase revealed that we had identified the rat homolog of the human MST2 kinase. The pathway in which human MST2 functions is not known; however, it does not appear to involve either mitogen-activated protein kinases such as Erk1 and Erk2 nor the stress-activated protein kinases such as JNK and p38. We show that the activity responsible for TTF-1 phosphorylation is constitutive in thyroid cells. Furthermore, we demonstrate that TTF-1 is phosphorylated in vivo by rMST2 at the same residues that had been identified previously as the major phosphorylation sites. Thus, TTF-1 represents the first identified target of this class of protein kinases.

TTF-1\(^1\) is a homeodomain-containing transcription factor originally identified as an important transcriptional activator of thyroglobulin and thyroperoxidase, two genes expressed exclusively in the thyroid (1, 2). TTF-1 protein is present also in lung where it has been demonstrated to be involved in the expression of several genes such as those encoding surfactant protein A, B, and C (3–5), the CC10 gene (6), and the HNF-3\(\alpha\) gene (7). The ability of TTF-1 to activate transcription can be down-regulated, as shown by at least two instances in which the TTF-1 protein can be detected and shown to be able to bind DNA, yet no transcription from known TTF-1-dependent promoters can be observed (8, 9). Many transcription factors are regulated by phosphorylation through several distinct mechanisms that can affect their DNA binding activity, their transcriptional activity, or their ability to interact with other proteins such as cofactors (10–13). In the rat thyroid cell line FRTL-5, TTF-1 is phosphorylated (8), and the phosphorylation has been studied in detail. TTF-1 is phosphorylated exclusively on serine residues, the main phosphorylation sites have been mapped, and their possible functional relevance was studied (14). In this paper we identify the rat counterpart of human MST2 as the activity capable of phosphorylating TTF-1 in thyroid cell extracts. Human MST1 and MST2 have been isolated by polymerase chain reaction screen with primers designed to amplify the catalytic domains of serine/threonine kinases. Both MST1 and 2 have been shown to be quite homologous to the yeast Ste20 and Pak enzymes throughout the kinase domain even though MST1 and 2 do not contain the p21 GTPase-binding domains, nor do they share any significant homology with Ste20 or Pak outside the kinase domain (15, 16). Ste20 is a component of the pheromone response pathway in budding yeast and represents the first identified member of a new family of serine/threonine protein kinases (17, 18). Up until now, several mammalian and yeast homologs to Ste20 have been identified which fall into two classes: those that bind Cdc42 and/or Rac1 and those that do not appear to be regulated in this manner (15, 16, 19–26). MST1 and MST2 are members of the latter class, together with Sps1 and human GC kinase (15, 16, 24, 25). Spas1 in yeast is involved in the activation of a mitogen-activated protein kinase pathway that regulates spore formation (24), and GC kinase has been demonstrated to activate a stress-activated protein kinase pathway (27). The pathway in which MST1 and MST2 function is yet unknown; however, it does not appear to involve either mitogen-activated protein kinases such as Erk1 and Erk2 nor the stress-activated protein kinases such as JNK and p38 (16). We show in this paper that TTF-1 is phosphorylated in vivo by rMST2, thus identifying the first target for this family of kinases.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Transfections, and Preparation of Cell Extracts—**

FRTL-5 and FRTL-5 \(\text{Ha-ras}\) cell lines have been described previously (28). Briefly, these cells were grown in Coon's modified F-12 medium (Seromed) supplemented with 5% calf serum (Life Technologies, Inc.) and six growth factors as described by Ambesi-Impiombato and Coon (28). NIH 3T3 and NIH 3T3 Ha-ras (the 44-9-1-1 cell line kindly provided by M. Barbacid) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum. H441 cells were cultured in RPMI (Irvine Scientific) supplemented with 10% fetal calf serum (Life Technologies, Inc.).

For transient expression, HeLa cells were plated at \(8 \times 10^6\)/100-mm diameter tissue culture dish 24 h before transfection. Cells were harvested 48 h after transfection.

For stable transfection, FRTL-5 cells were plated at \(8 \times 10^5\)/100-mm dish 48 h before transfection. 3 h before transfection, the medium was changed to Dulbecco’s modified Eagle’s medium containing 5% calf serum and growth factors. Selection of positive clones was started 2
days after transfection by adding 300 µg/ml G418 to the medium. Transfections were carried out by calcium phosphate coprecipitation as described elsewhere (8).

To prepare whole cell extract, cells were grown to confluence and washed twice with cold phosphate-buffered saline. Then, the harvested cells were resuspended in whole cell extract buffer (25 mM Hepes at pH 7.7, 0.5 mM MgCl₂, 0.2 mM EDTA, 1% Triton X-100, 0.5 mM dithiothreitol, 20 mM β-glycerophosphate, 0.1 mM Na₃VO₄, 2 µg/ml leupeptin, 100 µg/ml phenylmethylsulfonyl fluoride) and rotated for 30 min at 4°C. The cell suspension was cleared by centrifugation at 10,000 × g for 10 min. Protein concentration was estimated by Bio-Rad protein assay.

*Protein Expression in E. coli*—For the synthesis of wild-type and mutated TTF-1 proteins, *Escherichia coli* M15 strain was transformed with pQE12-TTF-1 or pQE12-S80, two vectors encoding, respectively, wild-type or mutated TTF-1 fused to a His₆ tag at the COOH terminus. For c-Jun synthesis, *E. coli* BL21(D3) cells were transformed with pMT21, a vector encoding human c-Jun fused to a His₆ tag at the COOH terminus, kindly provided by D. Bohmann.

Bacteria harboring the recombinant plasmids were grown at 37°C in LB medium containing 100 µg/ml ampicillin. At A₆₀₀ = 1.0, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM and the incubation continued for 2 h. The cells were collected by centrifugation for 15 min at 6,000 rpm in JA-14 rotor (Beckman), then resuspended and lysed by 40 ml of 0.5 M guanidine HCl. The suspension was sonicated (Branson sonifier macrotip) at setting 6 for 6 × 10 s and centrifuged for 15 min at 10,000 rpm in JA-20 rotor. The pellet was discarded, and 1 ml of Ni²⁺-nitrilotriacetic acid agarose resin (Qiagen) was added to the supernatant. After an overnight incubation on a rotating wheel at room temperature, a column was filled with the slurry. Columns were washed, and bound proteins were eluted with 200 mM imidazole according to instructions supplied with the resin. Purified TTF-1, S80, or c-Jun was dialyzed overnight against 10 mM Hepes, pH 7.5, 50% glycerol, 20 mM KCl, 2 mM MgCl₂, 0.2 mM EDTA, 1 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, and concentrated with Centricon 30 (Amicon).

*In-gel Kinase Assay*—In-gel kinase assays were performed as described by Hibi et al. (29) with slight modifications. 25–250 µg of whole cell extracts from various cell lines was resolved on a 10% SDS-polyacrylamide gel, which was polymerized in the absence of β-mercaptoethanol. In the presence of bacterially expressed TTF-1 (25–40 µg/ml), S80 (25–40 µg/ml), c-Jun (40 µg/ml), or myelin basic protein (Sigma, 100 µg/ml). At the end of the procedure, the gel was dried and then subjected to autoradiography or analyzed by an Instant Image (Hewlett Packard).

cDNA Cloning and Sequencing—Degenerate oligonucleotide primers STRK-1 (5'-CCGGATCCGCGGARHTATGCGCNGTAAARCCT-3') and STRK-2 (5'-CCGGATATCTVTYACNAYTCNGNGSYCATCCARAA-3'), encoding the most conserved and antisense strands of amino acids GE(I/L/V)CGN and CCGGCGAAT(T/C)GCTGAGGTG(T/C)CTGCTGTTCTCTCTCTCTCT-3', were used to amplify a rat thyroid cDNA library. The amplified fragment was ligated into plasmid pBluescript II, then sequenced using T7 DNA polymerase (Pharmacia). The sequences were analyzed using the University of Wisconsin Genetics Computer Groups programs (FastA and Gap). A fragment with high homology with human MST2 was used to screen 1 × 10⁶ plaques from a rat thyroid cDNA library and two clones encoding a protein kinase were obtained, subcloned in pBluescript II, and sequenced entirely.

*Northern Blot Analysis*—Total RNA was isolated from confluent cell culture dishes by the guanidium thiocyanate-phenol-chloroform method (30). 30 µg of total RNA from the different cell lines was size fractionated on a 1% formaldehyde-agarose gel and transferred to Hybond N-plus (Amersham) membrane. The blot was hybridized with a fragment that comprised the entire coding region of MST2 (nucleotides 81–1574 of the cDNA). This probe was labeled with [α-³²P]dATP and [α-³²P]dCTP by random priming. Hybridization was carried out for 16 h at 58°C in hybridization buffer (5 × Denhardt's solution, 6 × SSC, 0.1% SDS, 100 µg/ml denatured salmon sperm DNA). The membrane was washed twice for 30 min with 2 × SSC, 0.1% SDS and twice for 30 min with 0.2 × SSC, 0.1% SDS at 58°C. The blot was normalized by hybridization with a random primer KpnI/SalI 550-bp fragment of the plasmid pGAPDH1 containing the coding region of glyceraldehyde-3-phosphate dehydrogenase. The membrane was exposed overnight at −80°C with an intensifying screen (Kodak Biomax).

*In Vitro Transcription and Translation*—1 µg of pBluescript II containing the cDNA of MST2 (pBS-MST2) was transcribed by T3 RNA polymerase and translated using the tropinin T-coupled reticulocyte lysate system (Promega) according to the manufacturer's instructions. The reaction was incubated for 2 h at 30°C, and an aliquot was run on SDS-polyacrylamide gel electrophoresis. After fixation for 20 min with 10% acetic acid, 10% methanol, the gel was enhanced with an enlightening solution (NEN Life Science Products), dried, and exposed for autoradiography.

*Plasmids*—To construct pHA-rMST2 oligonucleotide primers SHA-1 5'-CCCGGGAAT(T/C)CGCACG(C/G)CCGCGC-3' and SHA-2 5'-CCCGGGAGCCCTCACAGGAAATCTCAGTCC-3' were used to amplify the rat MST2 cDNA from the plasmid pBS-rMST2. The amplified fragment was digested with EcoRI and subcloned into the pECE/HA vector (31), an SV40-based vector containing a hemagglutinin epitope tag (HA). pHA-rMST2 was used both for HeLa and FRTL-5 transfections. The amplified fragment was ligated into plasmid pBluescript II, then sequenced using T7 DNA polymerase (Pharmacia). The sequences were analyzed using the University of Wisconsin Genetics Computer Groups programs (FastA and Gap). A fragment with high homology with human MST2 was used to screen 1 × 10⁶ plaques from a rat thyroid cDNA library and two clones encoding a protein kinase were obtained, subcloned in pBluescript II, and sequenced entirely.

*RESULTS*—The kinase(s) responsible for TTF-1 phosphorylation, we have performed an in-gel kinase assay using as a substrate bacterially expressed TTF-1 polymerized in an SDS-polyacrylamide gel. Extracts from various cell lines were resolved on gels that were polymerized in the presence of either TTF-1 or c-Jun (+TTF-1 and +c-Jun in Fig. 1, respectively). As a control for bands that
could result from autophosphorylation, gels polymerized in the absence of added protein were also used (control in Fig. 1). After electrophoresis, the proteins were renatured in the gel and tested by a kinase assay. The experiment shown in Fig. 1 revealed that a protein kinase, with the apparent molecular mass of 56 kDa, was detected with all extracts used, albeit in variable amounts, in the TTF-1 containing gel (+TTF-1 panel in Fig. 1). The kinase activity was very abundant in FRTL-5 extracts, whereas a little reduction was shown in extracts prepared from FRTL-5 cells transformed by the Ha-ras oncogene. Furthermore, a strong phosphorylation of TTF-1 by extracts from murine fibroblasts NIH 3T3 and NIH 3T3 Ha-ras could also be observed, although it was very much reduced in human lung cells (H441) and in HeLa cells. No band of similar size was obtained in the control gel, demonstrating that the 56-kDa band was not caused by autophosphorylation of a protein kinase present in the extracts.

JNK2 is a well known kinase of 46 kDa which is able to phosphorylate the transcription factor c-Jun and which was first identified by an in-gel kinase assay (29). To determine if the TTF-1 kinase could be JNK2, we performed an in-gel kinase assay using bacterially expressed human c-Jun as substrate (+c-Jun panel in Fig. 1). The experiment revealed that no c-Jun phosphorylation was observed in rat thyroid cells, whereas it was clearly detected in NIH 3T3 and enhanced in NIH 3T3 Ha-ras as expected (29). Taken together, these experiments indicate that the kinase able to phosphorylate TTF-1 in thyroid cells is not JNK2.

The Activity Responsible for TTF-1 Phosphorylation Is Constitutive in Thyroid Cells—To assess if the activity of the identified TTF-1 kinase could be under hormonal control and to get insights on the pathway from the cell surface to the nucleus which could modulate TTF-1 phosphorylation and, perhaps, its activity in FRTL-5 cells, we starved the cells from serum and/or hormones (thyrotropin and/or insulin) and stimulated them with different treatments capable of activating different signal transduction pathways. We have used okadaic acid, which has been described as activating JNK/SAPKs but not ERKs, and conversely, phorbol 12-myristate 13-acetate, which activates ERKs but not JNK/SAPKs (32). Moreover, we heat shocked the cells at 42 °C to activate JNK/SAPKs (33) and alternatively cultured them in high salt conditions to activate the p38 osmosensing pathway (34). Hence, we performed an in-gel kinase assay using whole extracts from the differently treated FRTL-5 cells and quantified the bands by an Instant Imager acquisition and analysis (Fig. 2). In all conditions tested we did not observe great changes in TTF-1 phosphorylation. The TTF-1 kinase also appeared to be insensitive to treatments known to induce JNK/SAPK family members, such as heat-shock and anysomycin (data not shown). The extracts were normalized by Coomassie Blue staining (data not shown).

cDNA Cloning and Comparison with Known Kinases—We have shown previously that TTF-1 protein is phosphorylated exclusively on serine residues (14). For this reason, to clone the cDNA encoding for the kinase responsible for TTF-1 phosphorylation, degenerate oligonucleotide primers designed from highly conserved regions present in the catalytic domains of serine/threonine kinases were used to perform a reverse transcriptase-polymerase chain reaction starting from FRTL-5 poly(A) RNA. Three of the amplified fragments appeared to encode a kinase, and their sequence comparison with the data base revealed a high homology to the human MST2 gene (15). One of these fragments was then used to probe a rat thyroid cDNA library. Two phages containing an identical fragment of 2.6 kilobases were isolated, and the insert was subcloned and sequenced. The primary sequence (Fig. 3A) showed 79% similarity with the cDNA encoding for human MST2. The cDNA contains 1,473 bp of open reading frame encoding a 491-amino acid protein kinase with a predicted molecular mass of ~56 kDa, which showed 96% identity with the human MST2 protein (Fig. 3B). A strong Kozak sequence (gggcccagctg) could be identified at the potential initiation codon (35), and three polyadenylation signals are present in the 3′-untranslated region.

Expression of rMST2 in the Cell Lines—The expression of rMST2 was studied in the same cell lines used to identify the kinase activity. Total RNA was prepared and analyzed by Northern blot hybridization using a probe specific for the entire coding region (nucleotides 81–1574 of the cDNA). A transcript of approximately 3.4 kilobases was observed in the RNA of all the cell lines tested (Fig. 4). In particular, the expression was abundant in the RNAs prepared from FRTL-5 and in NIH 3T3 cells, whereas a reduced expression could be observed in the RNA from the lung cell line H441, and the transcript was detected at only very low levels in HeLa cells. The amount of RNA on the blot was normalized by hybridization with a probe specific for the ubiquitously expressed mRNA of glyceraldehyde-3-phosphate dehydrogenase. Thus, we conclude that the pattern of expression observed for rMST2 correlates well with the presence of the kinase activity identified by in-gel kinase assay.

Generation of rMST2 Stable Transfectants—To verify if rMST2 was really the kinase responsible for TTF-1 phosphorylation, we generated a vector encoding an HA-epitope tagged rMST2 (HA-rMST2) which was transfected transiently in HeLa cells, where the endogenous TTF-1 kinase activity is barely detectable. We also generated FRTL-5 clones stably transfected with the same expression vector. The expression of HA-rMST2 was detected by Western blot using a monoclonal antibody against the HA-epitope (Fig. 5A). Among the few bands de-
ected by the anti-HA antibody we could clearly identify that corresponding to HA-rMST2 by its apparent molecular mass and by its absence in cells transfected with an empty expression vector (Fig. 5A, in FRTL-5 cells, compare clones 3-6, 3-2, and 3-3 with Neo; in HeLa cells compare Trans. with Mock). We next performed an in-gel kinase assay using whole extracts from these transfected cells. It was possible to detect, in addition to the previously observed 56-kDa band, a slightly slower migrating band in all cells shown by Western to express HA-rMST2 (Fig. 5B). This increase in molecular mass is consistent with the size of the HA epitope.

To provide additional evidence for the identity of the 56-kDa protein detected in HeLa cells, rMST2, without HA-tag, was synthesized in a cell-free system and run on a TTF-1 containing gel alongside extracts of HeLa cells either mock transfected or transfected with the HA-rMST2 expression vector. As predicted, in vitro translated rMST2 shows the same mobility as the endogenous 56-kDa band (Fig. 5C).

rMST2 Phosphorylates TTF-1 in the Serine Residues Mapped Previously in Vivo—TTF-1 is phosphorylated exclusively on serine residues and in a previous study we have mapped its phosphorylation sites (14). To determine if rMST2 was indeed able to phosphorylate TTF-1 at the same sites, an in-gel kinase assay was performed. We polymerized within the gel either TTF-1 or S80, a mutated version of TTF-1 protein in which the serines in the phosphorylation sites have been mutated to alanines (Fig. 6). This mutant has already been shown to be unable to incorporate inorganic P-32 in in vivo labeling experiments (14). Equal amounts of extracts from FRTL-5 cells (panel A, lanes 1), FRTL-5 cells stable transfected with HA-rMST2 (lanes 2), HeLa cells mock transfected (lanes 3), and HeLa cells transfected with HA-rMST2 (lanes 4) were analyzed by Western blotting to confirm the expression levels of TTF-1 and S80.
HeLa cells (lanes 2), HeLa cells transiently transfected with HA-rMST2 (lanes 4) were resolved on the two gels. After electrophoresis, the proteins were renatured in the gels and tested by a kinase assay. The experiment revealed that in the gel where wild-type TTF-1 has been polymerized it was possible to observe rMST2 kinase activity in all the extracts, and in particular it was possible to see the activity of both the endogenous kinase as well as that of the HA-tagged version. On the contrary, in the gel where the S80 protein has been polymerized no kinase activity could be detected. This result strongly suggests that both our cloned cDNA and endogenous rMST2 are able to phosphorylate TTF-1 at the same sites that had been mapped previously in vivo.

**DISCUSSION**

We report in this paper the cloning of a rat serine/threonine protein kinase that was named rMST2, since sequence comparison demonstrates it to be the rat counterpart of the human MST2 kinase. The human protein kinases MST1 and MST2 share a considerable homology to Ste20 and Pak kinases throughout their catalytic domain. However, the homology is completely lost outside the catalytic domain, and the pathway in which this subfamily of kinases functions is not known. The inability of growth factors, heat shock, or high osmolarity to increase MST2 activity indicates that this kinase does not belong to the signal transduction cascades known to respond to these stimuli, such as mitogen-activated protein kinase or the HOG kinases (16). Our data also indicate that rMST2 kinase is insensitive to okadaic acid, phorbol 12-myristate 13-acetate, heat shock, and hormone-induced signaling. Thus its activity seems to be constitutive in thyroid cells.

We show, by an in-gel kinase assay, that the transcription factor TTF-1 is in vivo a target for rMST2, thus identifying the first target of MST2 catalytic activity. Several lines of evidence support the hypothesis that rMST2 is indeed the kinase responsible for TTF-1 phosphorylation. First, there is a correlation between the abundance of rMST2 mRNA and the amount of kinase activity capable of phosphorylating TTF-1 in...
The analyzed cell lines. Second, there is correspondence between the molecular weight of TTF-1 kinase activity and the deduced molecular mass of rMST2. Furthermore, we demonstrated, using an HA-tagged rMST2, that a new kinase activity of correspondingly increased molecular mass appeared only in the cells expressing the tagged rMST2. In addition, we were able to show that the cDNA that we have cloned as well as endogenous rMST2 are indeed able to phosphorylate TTF-1 at the same serine residues that had been mapped previously. In fact, when the kinase assay was performed using as a substrate the S80 protein, a mutated version of TTF-1 in which all of the phosphorylated serines had been changed in alanines, we did not observe any kinase activity.

The role of phosphorylation in the regulation of TTF-1 properties is still unclear, and previous studies suggested that phosphorylation of TTF-1 may have an important role only in specific cell types (14). rMST2 mRNA and activity were detected in all cell lines analyzed, although at different extents. In this respect it is of interest that the lung cell line H441, when compared for levels of rMST2 with FRTL-5 cells, shows much less activity and lower mRNA concentration. It is stimulating to think that such a reduced level of a protein kinase capable of phosphorylating TTF-1 could be correlated with the ability of this transcription factor to activate different promoters in lung and thyroid.

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