Sterol Regulatory Element-binding Protein Negatively Regulates Microsomal Triglyceride Transfer Protein Gene Transcription*

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We herein report that mRNA expression of microsomal triglyceride transfer protein (MTP) and its protein synthesis decline in response to sterol depletion in HepG2 cells, and we functionally characterized the MTP gene promoter in an effort to investigate the molecular mechanisms by which MTP gene transcription is regulated. Luciferase assays using truncated versions of the reporter gene revealed that the region at −124 to +33 base pairs of the human promoter contains the elements required for the suppression of transcription by sterol depletion. Enforced expression of an active form of sterol regulatory element-binding protein (SREBP)-1 (amino acids 1–487) or -2 (amino acids 1–481), both of which are activated under sterol-depleted conditions, is able to mimic sterol-mediated down-regulation. Either further truncation of the promoter region or mutation of the putative SREBP-binding sequence (5′-GCAGCCCA3′, −124 to −116 base pairs) abolishes the sterol- and SREBP-dependent transcriptional regulation. Gel mobility shift assay showed that recombinant SREBP-2-(1–481) is able to bind the sequence. Enforced expression of a truncated form of SREBP-2 (amino acids 31–481), which acts as an inhibitor of transcription of the low density lipoprotein receptor gene because it lacks the transcriptional activation domain, also diminishes the luciferase activity, suggesting that direct binding to the promoter region might be sufficient and that the mechanism by which SREBPs inhibit MTP gene expression is distinct from that for the transcriptional stimulation of sterol-regulated genes. Although the SREBP-binding site overlaps a negative insulin-responsive element, insulin negatively regulates MTP gene expression even when the amount of the active form of SREBPs is quite low under the sterol-loaded conditions, indicating that SREBPs only slightly mediate, if at all, the insulin effects. Overall, we conclude that SREBPs are responsible for regulation of lipoprotein secretion via their control of MTP gene expression. Moreover, our results describe for the first time a novel mechanism by which SREBPs negatively regulate expression of the gene encoding the protein involved in lipid metabolism.

Microsomal triglyceride transfer protein (MTP)1 plays a critical role in the assembly and secretion of very low density lipoproteins in the liver and chylomicrons in the intestine. MTP exists in the lumen of the endoplasmic reticulum as a heterodimer with protein-disulfide isomerase and is involved in the transfer of triglycerides, cholesterol esters, and phospholipids to newly synthesized apoB (1, 2). In human patients with abetalipoproteinemia, the absence of functional MTP results in a defect in the assembly and secretion of plasma lipoproteins containing apoB (3, 4). In the absence of either MTP lipid transfer activity or sufficient lipid, apoB translocation and lipoprotein assembly are blocked, and apoB is rapidly degraded by a ubiquitin-dependent proteasome process. Under physiological conditions, only a portion of de novo synthesized apoB is secreted; the remaining portion is degraded (5–7). These findings raise the possibility that changes in MTP activities under various physiological conditions may modulate lipoprotein production and secretion in the liver and intestine.

Recent studies have demonstrated that a high-fat diet fed to hamsters causes an increase in the hepatic MTP mRNA levels (8, 9) and that insulin or high concentrations of glucose decrease MTP mRNA levels in HepG2 cells (10). Insulin treatment has also been reported to decrease very low density lipoprotein secretion from hepatocytes (11). On the other hand, oleate stimulates apoB-containing lipoprotein secretion by preventing the intracellular degradation of apoB (12, 13), not by altering MTP mRNA levels (10). It has recently been shown that oxysterols regulate the production of lipoproteins by modulating the ubiquitin conjugation of apoB and its subsequent degradation by the proteasome (14). However, little is known about the effect of sterols on MTP mRNA and protein levels.

It has been reported that sterols affect the transcription of a number of genes encoding enzymes and proteins involved in cholesterol and fatty acid metabolism through the actions of sterol regulatory element-binding proteins (SREBPs) (15). SREBPs activate the transcription of genes encoding enzymes involved in cholesterol synthesis, including 3-hydroxy-3-methylglutaryl (HMG)-CoA synthase, HMG-CoA reductase, farnesyl-diphosphate synthase, and squalene synthase, and the low density lipoprotein (LDL) receptor. SREBPs also stimulate the transcription of genes encoding fatty acid synthesis enzymes (acetyl-CoA carboxylase, fatty-acid synthase, glycerol-3-phosphate acyltransferase, and stearoyl-CoA desaturase).

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1 The abbreviations used are: MTP, microsomal triglyceride transfer protein; SRE, sterol regulatory element; SREBP, sterol regulatory element-binding protein; LDL, low density lipoprotein; HMG, 3-hydroxy-3-methylglutaryl; bp, base pair(s); FCS, fetal calf serum; PBS, phosphate-buffered saline; LPDS, lipoprotein-deficient serum; HNF, hepatocyte nuclear factor; USF, upstream stimulatory factor.
FIG. 1. Human MTP promoter sequence (10, 22). The transcription start site is position +1. The TATA-like sequence is underlined. The functional elements (insulin-responsive element (IRE) and AP-1-binding site (AP-1)) are overlined. The SRE site is boxed. The sites used for preparation of truncated reporter gene constructs are indicated by arrows. The mutant sequence in the SRE site is shown by italic letters under the individual original sequence.

Unlike other members of the basic helix-loop-helix leucine zipper family, SREBPs are synthesized as precursors bound to the endoplasmic reticulum membrane and nuclear envelope (16, 17). The transcriptionally active N-terminal portion including the basic helix-loop-helix leucine zipper domain is released from the membrane by two-step proteolysis (18). In sterol-depleted cells, SREBPs are cleaved at site 1 in the endoplasmic reticulum luminal loop by site-1 protease, which accomplishes the basic helix-loop-helix leucine zipper domain is re-leased from the membrane by two-step proteolysis (18). In sterol-depleted cells, SREBPs are cleaved at site 1 in the endoplasmic reticulum luminal loop by site-1 protease, which allows site-2 protease to cleave at site 2 within the first membrane-spanning region. The activity of site-1 protease is subject to negative feedback regulation by cholesterol. When cells are overloaded with sterols, the proteolytic process is blocked; the NH$_2$-terminal domains are not released; and transcription of the target genes declines.

In this report, we examine whether MTP mRNA and protein levels are affected by sterols and SREBPs, testing our hypothesis that SREBPs might control lipoprotein production and secretion by regulating MTP gene expression. We also characterize the human MTP promoter and define the region responsible for the sterol-dependent regulation. Furthermore, we investigate how SREBPs regulate MTP gene expression, compared with insulin-negative effects.

MATERIALS AND METHODS

Construction of Reporter Genes for Luciferase Assay—The luciferase reporter plasmids were constructed by cloning the BglII-HindIII polymerase chain reaction fragments coding the 5'-untranslated region of the human MTP gene into the same restriction sites of a pGL3 basic vector (Promega). To generate pMTP-204, pMTP-124, pMTP-109, and pMTP-100, polymerase chain reaction primers were designed to hybridize at the corresponding position (19) and were coupled with the pMTP-33 reporter plasmids were constructed by cloning the 5'- flanking region corresponding to amino acid 31 as an initiator codon.

SREBP Regulates MTP Gene Expression

Northern Blot Analysis—HepG2 cells were set up on day 0 (1.5 x 10$^5$ cells/100-mm dish) in medium A supplemented with 7% FCS. On day 1, the medium was removed, and the cells were then washed with PBS and refed with medium A containing 5% LPDS supplemented with either sterols or the inhibitor, as described above. After 48 h of culture, total RNA was extracted and fractionated on formaldehyde-agarose gels and then transferred to nylon membranes (Roche Molecular Biochemicals). Riboprobes were prepared using human MTP cDNA (890–1560 bp downstream of the initiator codon) and human gyceraldehyde-3-phosphate dehydrogenase cDNA (963–1245 bp) with a DIG RNA labeling kit (Roche Molecular Biochemicals). Hybridization signals were quantified with an Autoradiography (Amersham Pharmacia Biotech) using a FluorImage 595 (Molecular Dynamics, Inc.).

Western Blot Analysis—HepG2 cells were set up on day 0 (5 x 10$^5$ cells/100-mm dish) in medium A supplemented with 7% FCS. On day 1, the medium was removed, and the cells were then washed with PBS and refed with medium A containing 5% LPDS with or without 5 μg/ml cholesterol. After 14 days of culture, the cells were refed with medium A containing 5% LPDS supplemented with either sterols or the inhibitor, as described above. After 2 days of culture, the cells were harvested, and Western blot analysis was carried out using a polyclonal antibody against human MTP with the Vistra fluorescence Western blotting kit (Amersham Pharmacia Biotech). Signals for 97-kDa MTP were quantified using a FluorImage 595.

Gel Mobility Shift Assay—A double-stranded DNA fragment corresponding to nucleotides −129 to −112 was 3' end-labeled with digoxigenin-11-ddUTP using a DIG gel shift kit (Roche Molecular Biochemicals). The reaction mixture (20 μl) contained 100 ng of recombinant SREBP-2 (1–481), 30 fmol of the end-labeled probe, 20 μg Hepes-KOH (pH 7.6), 1 μm EDTA, 10 mM (NH$_4$)$_2$SO$_4$, 1 mM dithiothreitol, 0.2% (w/v) Tween 20, and 30 mM KCl. Each reaction mixture was incubated at room temperature for 20 min. Following the addition of 0.5 μg of antibodies, the reaction mixture was placed on ice for 30 min and then loaded directly onto a 6% polyacrylamide gel in 0.5× buffer containing 45 mM Tris borate and 1 mM EDTA. In competition assays, an excess amount of an unlabeled 17- bp fragment was added prior to addition of the labeled probe. The bands were detected by an anti-digoxigenin antibody (Roche Molecular Biochemicals).

Insulin Effect—HepG2 cells were set up on day 0 in medium A supplemented with 2.5% FCS. On day 1, after 4 h of transfection, the
medium was removed, and the cells were then washed with PBS and refed with medium A containing 2.5% LPDS supplemented with the indicated concentration of insulin in the presence of either sterols or the inhibitor, as described above. After 48 h of culture, the cells were harvested, and luciferase assays were carried out. For Western blot analysis of SREBP-2, the nuclear extracts of HepG2 cells without transfection were prepared as described previously (16).

Antibodies—Polyclonal antibodies (RS001 against human MTP and RS004 against human SREBP-2) were produced by immunizing rabbits with a fusion protein encoding six consecutive histidines followed by amino acids 300–507 of human MTP and amino acids 1–481 of human SREBP-2, respectively. The fusion protein constructs were cloned into a pET28(a) vector (Novagen), expressed in E. coli, and purified by Ni²⁺-Sepharose affinity chromatography.

RESULTS

Regulation of MTP mRNA and Protein Levels—HepG2 cells were cultured with LPDS in the presence of either sterols (sterol-loaded conditions) or a HMG-CoA reductase inhibitor (pravastatin) plus mevalonic acid (sterol-depleted conditions), and their total RNA was prepared. In the absence of sterols, the MTP mRNA level was reduced by 53% (Fig. 2A). To see if such reduced mRNA levels lead to an decrease in MTP protein levels, Western blot analysis was carried out. Because the half-life of the MTP protein has been reported to be relatively long (4.4 days), HepG2 cells were cultured for a longer than 124 h. Fifty-μg total RNA samples were fractionated on 7% polyacrylamide gel and blotted with antibodies against human MTP. In both Northern and Western blots were quantified with a FluorImager 595.

MTP Gene Expression Is Regulated by Sterols—It is of interest to determine whether the above phenomenon is due to transcriptional regulation of the MTP gene. Thus, we isolated the 5′-flanking region of the human MTP gene and searched for the sequence motifs potentially responsible for such regulation. It has been demonstrated using luciferase assays that the MTP promoter activity is positively regulated by cholesterol (22). We constructed various deletion versions of reporter genes (Fig. 1) and carried out luciferase assays. HepG2 cells were transfected with these reporter genes and cultured under sterol-loaded or -depleted conditions for 2 days. Fig. 3 shows that a significant decrease in luciferase activity was observed under the sterol-depleted conditions with the −204 and −124 bp reporter genes (pMTP−204 and pMTP−124, respectively), but there was no alteration with the −109 and −100 bp reporter genes (pMTP−109 and pMTP−100, respectively). The magnitude of suppression of luciferase activity by sterol depletion (40–50%) was similar to that of endogenous MTP mRNA (Fig. 2). Deletion of the region between −109 and −100 bp containing a putative AP-1-binding site (Fig. 1) reduced luciferase activity dramatically, suggesting that this region is critical for transcription.

SREBP Can Regulate MTP Gene Expression—To determine whether the above phenomenon is due to effects of SREBPs that are activated by proteolysis under sterol-depleted conditions, HepG2 cells were cotransfected with one of the reporter genes and an expression construct of an active form of human SREBP-2. The magnitude of suppression of luciferase activity by sterol depletion (40–50%) was similar to that of endogenous MTP mRNA (Fig. 2). Deletion of the region between −109 and −100 bp containing a putative AP-1-binding site (Fig. 1) reduced luciferase activity dramatically, suggesting that this region is critical for transcription.

SREBP-2 Can Bind the Sterol Regulatory Element—The region between −124 and −109 bp contains a GCAGCCCAC sequence (−124 to −116 bp), resembling the SRE sequence in the LDL receptor gene (TCACCCCAC). To determine whether the putative SRE is able to bind SREBP-2, we performed gel mobility shift assays with recombinant human SREBP-2 (−116 to −109 bp) and a digoxigenin-labeled DNA fragment. As shown in Fig. 4, a single-shifted DNA-protein complex was observed in the presence of recombinant SREBP-2 (lane 2). The band almost completely disappeared in the presence of an excess amount of an unlabeled wild-type probe, but not a mutant probe (lanes 4 and 5), and was supershifted by antibodies.
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HepG2 cells were transfected with one of the reporter gene constructs, an expression construct for β-galactosidase, and an expression plasmid encoding the active form of either SREBP-1 (amino acids 1–487) or SREBP-2 (amino acids 1–481). The cells were cultured with medium containing 7% FCS for 48 h. Luciferase values were normalized to β-galactosidase activity. Relative activities of the respective constructs without expression of SREBP-2 are considered as 100%. The values given are the average of data from more than three experiments. Data are expressed as means ± S.D.

| Reporter gene construct       | Activity     |
|------------------------------|--------------|
|                             | SREBP-2-(1–481) | SREBP-1-(1–487) |
| pMTP–204                    | 51 ± 3       | —             |
| pMTP–124                    | 60 ± 6       | 48 ± 5        |
| pMTP–109                    | 106 ± 12     | 102 ± 8       |
| pMTP–100                    | 126 ± 15     | —             |

—, not determined.

Competitor: — — — wt Mut
Anti-SREBP2: — — — —
SREBP2(1–481): — + + + +
Supershifted Bound: 1 2 3 4 5

Insulin and SREBPs Negatively Regulate MTP Gene Expression Independently—It has been reported that insulin negatively regulates MTP gene expression and that the region between −123 and −112 bp is essential for the insulin response (22). The fact that the SRE in the MTP promoter is found in this region raises the possibility that SREBP-2 might mediate certain gene regulatory effects of insulin or that SREBP-2 might simply mimic the functions of an unknown DNA-binding protein activated by insulin. To see if the insulin effect is dependent on SREBP-2, luciferase assays using various reporter genes in the presence or absence of insulin were performed. HepG2 cells were transfected with one of the reporter genes and cultured under either sterol-loaded or -depleted conditions in the presence of the indicated concentration of insulin for 2 days. When cells were transfected with the −124 bp reporter gene, MTP gene expression was suppressed under sterol-depleted conditions (Fig. 7A, white bars) by 38 and 53% at insulin concentrations of 0.1 and 1.0 μM, respectively. However, insulin did not affect the reduced luciferase activity that occurred under sterol-depleted conditions (Fig. 7A, shaded bars). Luciferase activity with the LDL receptor reporter gene was increased under sterol-depleted conditions in the presence or absence of insulin (Fig. 7B). Fig. 7C shows that the amounts of active forms of nuclear SREBP-2 under sterol-depleted conditions were significantly low in the presence or absence of insulin as determined by Western blotting. Therefore, the suppression of MTP gene expression by insulin under sterol-depleted conditions might imply that SREBP-2 only slightly mediate, if at all, the insulin-negative effect. When cells were transfected with the −109 bp reporter gene without both the SRE and the putative insulin-responsive element, luciferase activity was not changed by the addition of sterols or insulin to the medium, in accordance with the results in Fig. 3 as well as observations in a previous report (22) (data not shown).

DISCUSSION

The purpose of this study was to gain insight into the effects of sterols on the MTP activity that controls the secretion of apoB-containing lipoproteins, chylomicrons, and very low density lipoproteins from the small intestine and liver. We have demonstrated that sterols regulate MTP gene expression and the amount of the protein in HepG2 cells (Fig. 2). We performed a series of luciferase assays to identify the sequence motifs responsible for transcriptional regulation of the MTP gene. Since it has been reported that all the putative positive and negative response elements for the liver-specific MTP gene expression are localized within the human MTP promoter (−142 bp region (22)), we focused on the promoter activity of the first 200 bp 5' to the transcription start site. The following line of evidence indicates that SREBP-2 can negatively regulate MTP gene expression through binding to the SRE site in the promoter. First, the reporter gene containing at least the −124...
bp region was positively regulated by sterols, i.e. was negatively regulated by sterol deprivation (Fig. 3). When either human embryonic kidney 293 or HeLa cells were transfected with the reporter genes plus an expression plasmid for β-galactosidase, and either an expression plasmid (pSREBP2(1–481)) or the vector without the insert (10 ng). The cells were incubated with medium containing 7% FCS for 48 h. Luciferase values were normalized to β-galactosidase activity. Promoter activities of the pMTP−124 construct either under sterol-loaded conditions (1500–2200 relative light units/unit) or in the absence of an active form of SREBP-2 (1200–2000 relative light units/unit) are considered as 100%. The values given are the average of data from more than three experiments. Data are expressed as means ± S.D. In each experiment, both sterol depletion and SREBP-2 significantly suppressed the pMTP−124 promoter activities.

Fig. 5. Effect of disruption of the SRE on the expression of reporter genes. A, HepG2 cells were transfected with the indicated reporter plasmid and an expression plasmid for β-galactosidase. In pMTP−124SREKO, the SRE of pMTP−124 is replaced. The cells were cultured under either sterol-loaded (+) or -depleted (−) conditions for 48 h. B, HepG2 cells were transfected with the indicated reporter plasmid, an expression plasmid for β-galactosidase, and either an expression plasmid (pSREBP2(1–481)) or the vector without the insert (10 ng). The cells were incubated with medium containing 7% FCS for 48 h. Luciferase values were normalized to β-galactosidase activity. Promoter activities of the pMTP−124 construct either under sterol-loaded conditions (1500–2200 relative light units/unit) or in the absence of an active form of SREBP-2 (1200–2000 relative light units/unit) are considered as 100%. The values given are the average of data from more than three experiments. Data are expressed as means ± S.D. In each experiment, both sterol depletion and SREBP-2 significantly suppressed the pMTP−124 promoter activities.

Fig. 6. Effect of deletion of the NH2-terminal transactivation domain in SREBP-2 on the expression of reporter genes. A and B, HepG2 cells were transfected with the indicated reporter and expression plasmids (10 ng). An expression plasmid (pSREBP2(31–481)) encodes amino acids 31–481 of SREBP-2 lacking most of the transactivation domain. The cells were incubated with medium containing 7% FCS for 48 h. Luciferase values were normalized to β-galactosidase activity. Promoter activities of the pMTP−124 construct in the absence of SREBP-2 (1200–1800 relative light units/unit) are considered as 100%. The values given are the average of data from four experiments. Data are expressed as means ± S.D.

It has been demonstrated that SREBP's stimulate the expression of target genes in cooperation with either of the general transcription factors Sp1 (24) and NF-Y (21). Recent studies show that CAMP response element-binding protein-binding protein is capable of binding the NH2-terminal transactivation domain of SREBP's and is required for SREBP-regulated tran-
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SREBP-2 was quite low (Fig. 7, A and C). Second, increased amounts of active forms of nuclear SREBPs under sterol-depleted conditions suppressed the transcription of the MTP gene even at lower concentrations of insulin, suggesting that the inhibitory effects of SREBPs do not require the signaling induced by insulin (Fig. 7A). Although a previous report (27) demonstrated that SREBP-1 mediates activation of the LDL receptor by insulin and that the effect might be linked to the mitogen-activated protein kinase cascade, in the current study, the sterol-mediated transcriptional regulation of the LDL receptor was not significantly altered in the presence or absence of insulin (Fig. 7B). However, we cannot rule out the possibility that a trace amount of the active form of nuclear SREBPs under sterol-loaded conditions mediates the insulin effect.

It has been demonstrated that the insulin response sequence on the fatty-acid synthase promoter contains an E-box CANNTG sequence that is able to interact with the ubiquitous basic helix-loop-helix leucine zipper transcription factors, upstream stimulatory factor (USF)-1 and USF2 (28). Two adjacent SRE sequences on the fatty-acid synthase promoter flank the E-box. Unlike MTP gene expression, expression of the fatty-acid synthase gene is stimulated by insulin and SREBPs (28, 29). Analysis of endogenous fatty-acid synthase mRNA expression in USF1 and USF2 knockout mice revealed that USFs and SREBPs independently activate fatty-acid synthase gene expression (30). It is therefore possible that SREBPs might be able to substitute for the unidentified factor(s) bound to the MTP insulin-responsive element by binding an overlapping site, thereby down-regulating MTP gene expression. In
our experiments, we found that further deletion of the NH2-terminal portion of SREBP-2 from amino acids 32 to 180 abolished the inhibitory effect (data not shown). Because only 29% of the amino acid sequence for the NH2-terminal region (amino acids 32–180) of SREBP-1 and -2 is identical, it appears that the entire region might be required for sufficient inhibition of the transcriptional activation of the MTP gene rather than in association with unidentified corepressor. As shown in Fig. 2, deletion of the region between –109 and –100 bp of the MTP promoter, which is located close to the SRE and contains a putative AP-1-binding site (19, 22), diminished the luciferase activity dramatically. It is possible that insulin and SREBPs may disrupt the interaction between AP-1 and this region, thereby diminishing MTP gene expression. Further studies will be required to elucidate the precise mechanism.

Horton and co-workers (31–33) reported that MTP mRNA was elevated 3–6-fold in the livers of transgenic mice overexpressing SREBP-2, but was not significantly increased in mice either overexpressing mutant SREBP cleavage-activating protein, which stimulates the proteolytic processing of endogenous SREBPs in a sterol-independent manner (1.7-fold), or overexpressing SREBP-1a (1.2-fold). We are aware that these findings are apparently not in accordance with our results, but as the authors themselves stated in these reports, it remains to be elucidated whether the elevation of MTP mRNA in these animals is a direct effect of altered SREBP activity or whether it is secondary to increased lipid content in the liver. It is possible that overexpression of SREBPs in these transgenic mice might activate certain crucial genes such as AP-1 and/or HNF-4, which might be involved in MTP gene expression (22). Furthermore, a profound elevation of fatty acid synthesis in these mice might modulate the level of HNF-4 transcription by the production of an increased amount of fatty acyl-CoA ligands for HNF-4 (34). In the absence of resolution of these competing explanations, the results obtained from these transgenic mice studies cannot therefore be taken to be in clear contradiction to the findings that we report. Further investigation will be necessary to determine the source of the ambiguity.

In summary, sterols positively regulate MTP gene expression and protein synthesis in HepG2 cells. Unlike acute insulin effects, stimulated transcription of the MTP gene by higher intracellular sterol levels over the longer term is able to bring about an increase in MTP protein levels, despite the slow turnover rate of the MTP protein. It is likely that elevated MTP activity augments lipoprotein production and secretion by facilitating the assembly of apoB and lipids, preventing the intracellular degradation of apoB. It is noteworthy that cholesterol is one of the determinants of lipoprotein production and secretion through its effect on MTP activities. The sterol-dependent transcriptional regulation of the MTP gene is mediated by SREBPs through binding to the SRE in the MTP promoter. Here we report for the first time a novel mechanism by which SREBPs are able to inhibit MTP gene expression, a mechanism distinct from that of the transcriptional stimulation of the sterol-regulated genes related to cholesterol and fatty acid metabolism. In this regard, it is important to note that SREBPs play a central role in lipid metabolism, regulating not only the synthesis and uptake of cholesterol and fatty acids, but also lipoprotein production and secretion.

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