Activation of TRAP/Mediator Subunit TRAP220/Med1 Is Regulated by Mitogen-Activated Protein Kinase-Dependent Phosphorylation

Pradeep K. Pandey,¹ T. S. Udayakumar,¹ Xinjie Lin,¹ Dipali Sharma,^{2†} Paul S. Shapiro,³
and Joseph D. Fondell^{1*}

*Department of Physiology and Biophysics, Robert Wood Johnson Medical School, UMDNJ, Piscataway, New Jersey 08854¹;
Department of Physiology, University of Maryland School of Medicine, Baltimore, Maryland 21201²; and Department of
Pharmaceutical Sciences, University of Maryland School of Pharmacy, Baltimore, Maryland 21201³*

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The TRAP/Mediator coactivator complex serves as a molecular bridge between gene-specific activators and RNA polymerase II. TRAP220/Med1 is a key component of TRAP/Mediator that targets the complex to nuclear hormone receptors and other types of activators. We show here that human TRAP220/Med1 is a specific substrate for extracellular signal-regulated kinase (ERK) of the mitogen-activated protein kinase (MAPK) family. We demonstrate that ERK phosphorylates TRAP220/Med1 in vivo at two specific sites: threonine 1032 and threonine 1457. Importantly, we found that ERK phosphorylation significantly increases the stability and half-life of TRAP220/Med1 in vivo and correlates with increased thyroid hormone receptor-dependent transcription. Furthermore, ERK phosphorylates TRAP220/Med1 in a cell cycle-dependent manner, resulting in peak levels of expression during the G₂/M phase of the cell cycle. ERK phosphorylation of ectopic TRAP220/Med1 also triggered shuttling into the nucleolus, thus suggesting that ERK may regulate TRAP220/Med1 subnuclear localization. Finally, we observed that ERK phosphorylation of TRAP220/Med1 stimulates its intrinsic transcriptional coactivation activity. We propose that ERK-mediated phosphorylation is a regulatory mechanism that controls TRAP220/Med1 expression levels and modulates its functional activity.

Mediator is a multisubunit transcriptional coactivator complex evolutionarily conserved from yeasts to mammals that serves as a functional interface between DNA-bound transcription factors and RNA polymerase II (pol II) (5, 32, 39). In humans, the complex was originally isolated as a thyroid hormone receptor (TR)-associated protein (TRAP) complex that facilitates ligand-dependent gene expression by TR in vitro (12) and was thus termed TRAP/Mediator. More recent studies have established TRAP/Mediator and other highly related human Mediator complexes (ARC, CRSP, PC2, and DRIP) as essential coactivators for a broad range of nuclear hormone receptors (NRs) and other types of transcriptional activators, including SREBP, p53, NF- κ B, VP16, and E1A (reviewed in references 28, 32, 44, and 53). In contrast to coactivators that function by covalently modifying histones (56) or rearranging higher-ordered chromatin (3), TRAP/Mediator appears to function by facilitating the recruitment of RNA pol II and by subsequently activating the basal transcription apparatus (4, 60).

TRAP220/Med1 (also termed PBP, ARC/DRIP205, or Med220) is a peripherally associated subunit of the human TRAP/Mediator complex (31, 54, 55) that facilitates direct ligand-dependent interactions with NRs by virtue of its two signature LXXLL motifs (45, 46, 67, 71) shown previously in other types of coactivators to mediate NR binding (16). Indeed, ligand-dependent recruitment of TRAP/Mediator to NR target genes via TRAP220/Med1 is believed to facilitate an essential activation step in NR-regulated gene expression (19, 34). TRAP220/Med1

gene knockout in mice is embryonic lethal (21, 72), and primary embryonic fibroblasts derived from *TRAP220/Med1* null embryos exhibit retarded cell cycle progression in growth and mitogenicity assays (21). Thus, in addition to NR coactivation, TRAP220/Med1 appears to play a pivotal coregulatory role in cellular growth and cell cycle control. Consistent with this notion, recent studies suggest that TRAP220/Med1 can also interact with a number of other transcription factors essential for cell growth and development, including the GATA family of proteins (8), BRCA-1 (58), and p53 (14, 20).

Intriguingly, several lines of evidence show that cytokines, growth factors, and intracellular cell cycle signaling can modulate the functional activity of NR coregulatory factors via posttranslational phosphorylation events (reviewed in references 17, 51, and 66). For example, phosphorylation of specific members of the steroid receptor coactivator (SRC)/p160 family of proteins via different signal transduction pathways can enhance nuclear localization (62, 64), inhibit interactions with non-NR activators (27), or stimulate intrinsic SRC/p160 coactivator activity (13, 48, 65). Similarly, phosphorylation of the NR coactivator PGC-1 via the mitogen-activated protein kinase (MAPK) p38 significantly stabilizes protein expression, leading to increased levels of coactivator activity (43). Misra et al. (37) recently showed that recombinant mouse TRAP220/Med1 is an in vitro substrate for protein kinase A (PKA), protein kinase C (PKC), and MAPKs. Nonetheless, the underlying molecular mechanisms by which phosphorylation might influence TRAP220/Med1 functional activity remain unknown.

We report here that human TRAP220/Med1 is an in vivo substrate for activated extracellular signal-regulated kinase (ERK) of the MAPK family and further show that ERK phosphorylates TRAP220/Med1 in a cell cycle-dependent manner.

* Corresponding author. Mailing address: Robert Wood Johnson Medical School, UMDNJ, 683 Hoes Lane, Piscataway, NJ 08854. Phone: (732) 235-3348. Fax: (732) 235-5823. E-mail: fondelljd@umdnj.edu.

† Present address: Winship Cancer Institute, Emory University School of Medicine, Atlanta, GA 30322.

Using deletion and point mutagenesis, we mapped the ERK-specific phosphorylation sites on the TRAP220/Med1 protein to two distinct amino acid residues, threonine 1032 and threonine 1457, both of which exist in the context of a consensus MAPK phosphorylation site, P(X)_{1/2}S/TP. Relevant to the functional and physiological consequences of this posttranslational modification, we show that phosphorylation of TRAP220/Med1 by ERK significantly increases the stability and half-life of the TRAP220/Med1 protein in vivo and correlates with increased TR-dependent transcription. Importantly, we further show that ERK phosphorylation stimulates intrinsic TRAP220/Med1 transcriptional activity. Our findings thus suggest that activated MAPK-ERK signaling pathways trigger regulatory phosphorylation events that control TRAP220/Med1 expression levels and modulate its functional activity.

MATERIALS AND METHODS

Antibodies. Rabbit polyclonal antibodies against TRAP220/Med1 were described previously (68). Mouse monoclonal antibodies against TR, α -tubulin, and the hemagglutinin (HA) epitope were obtained from Santa Cruz Biotechnology. Mouse monoclonal antibodies against β -actin and the FLAG epitope were from Sigma.

Plasmids. The mammalian expression vectors pBK-CMV-FLAG-TR α and pSG5-HA-TRAP220, and the luciferase reporter genes 2xT₃-RE-tk-Luc and p5xGal4-Luc were described previously (68). The enhanced green fluorescent protein (EGFP)-TRAP220/Med1 fusion construct pEGFP-TRAP220/Med1 was generated by subcloning a TRAP220/Med1 fragment containing amino acids 314 to 1581 into the XbaI site of pEGFP (Clontech). The Gal4-TRAP220/Med1 fusion construct pCMX-Gal4-TRAP220 was generated by subcloning a TRAP220/Med1 fragment containing amino acids 326 to 1581 into the EcoRI/BamHI sites of pCMX-Gal4 (57). The MKK1 expression vectors pMCL-HA-MKK1wt and pMCL-HA-MKK1(N44) (33) and ERK2 expression vectors pCMV5-ERK2-dead and pCMV5-ERK2 -constitutively active (11) were kindly provided by Natalie Ahn (University of Colorado).

Site-directed mutagenesis. MAPK-ERK phosphorylation site point mutants (see Fig. 3G) were introduced into the pSG5-HA-TRAP220, pEGFP-TRAP220/Med1 and pCMX-Gal4-TRAP220 plasmids using specific mutant oligonucleotides and the Quick Change site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. For the C-terminal TRAP220/Med1 deletion mutants (Fig. 3D), stop codons were introduced into the open reading frame of pSG5-HA-TRAP220 at the residues indicated. For the N-terminal deletion mutant Δ 1233 (Fig. 3D), a C-terminal TRAP220/Med1 PCR fragment containing amino acid residues 1233 to 1581 was subcloned in frame into the pSG5-HA vector (68). All mutant constructs were confirmed by DNA sequencing.

Cell culture and synchronization. HeLa and COS cells and the α -2 cell line that stably expresses FLAG-TR α (12) were all routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and 20 mM HEPES (all media and supplements were from Invitrogen). HeLa cells were arrested at the border of the G₁/S phase of the cell cycle using the double-thymidine-block approach as described previously (63). Upon addition of serum, cells were released into different cell cycle stages (0 h for G₁/S phase, 3 h for S phase, 8 h for G₂/M, and 15 h for G₁). Synchrony was monitored by fixing the cells in 70% ethanol (EtOH), staining with propidium iodide (20 μ g/ml), and analyzing using a Cytomics FC 500 flow cytometer (Beckman Coulter Inc.).

Growth factors and inhibitors. Epidermal growth factor (EGF) was purchased from Invitrogen. The protein synthesis inhibitor cycloheximide was from Sigma. The kinase inhibitors U0126, SB202190, roscovitine, 4-cyano-3-methylisouquinoline and bisindolylmaleimide were all from Calbiochem. L-JNK1 was from Alexis Biochemicals. Butyrolactone I was from BIOMOL.

Western blotting. Immunoprecipitated TRAP220/Med1 protein, or equal amounts of cellular or nuclear extracts, were resolved on sodium dodecyl sulfate (SDS)-polyacrylamide gels and subsequently transferred to nitrocellulose membranes and probed with specific antibodies described in the figure legends. Immunodetection was performed using the enhanced chemiluminescence (ECL) system, Amersham Pharmacia Biotech Inc.). Nuclear extracts were generated as previously described (68) and, when indicated, pretreated with calf intestinal phosphatase (CIP) (New England Biolabs) (1 U per 5 μ g of nuclear extract). Western blot signals were quantified by densitometry using SCION image analysis software (an NIH shared software).

Immunoprecipitation of native phosphorylated TRAP220/Med1. For non-synchronized experiments (see Fig. 1A), HeLa cells (2×10^7) were grown in serum- and phosphate-free DMEM (Invitrogen) for 4 h and then incubated with ^{32} P_i (0.1 mCi/ml) for an additional 6 h. For synchronized experiments (see Fig. 7), HeLa cells (2×10^6) were synchronized into different cell cycle phases (per above) and then incubated in 2 ml serum- and phosphate-free media containing ^{32} P_i (0.125 mCi/ml) for 10 min. Control experiments lacking ^{32} P_i were performed in parallel. The cells were lysed in lysis buffer (50 mM Tris-Cl [pH 7.4], 150 mM NaCl, 5 mM EDTA, and 0.5% NP-40 [500 μ l per 2×10^6 cells]) for 1 h at 4°C. 1 mg of protein from each sample was combined with 5 μ l of anti-TRAP220 antibodies and 10 μ l (packed) protein A-agarose beads (Roche) overnight at 4°C. The beads were then washed three times in lysis buffer, and the precipitated immune complexes were resolved by SDS-polyacrylamide gel electrophoresis (PAGE). The gel was either dried for autoradiography or processed for Western blotting analyses.

In vitro kinase assay. Expression and purification of HA-TRAP220/Med1 from insect Sf9 cells using recombinant baculovirus were carried out exactly as previously described (61). Purified HA-TRAP220/Med1 protein (50 ng) was incubated in kinase buffer (20 mM MOPS [morpholinopropanesulfonic acid; pH 7.2], 25 mM β -glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 15 mM magnesium chloride, 100 μ M cold ATP) together with 2 μ Ci [γ - ^{32} P]ATP plus 1 ng of purified MAPK-ERK1 (Upstate) for 10 min at room temperature. The reaction was terminated by adding 2 $\times$ SDS loading buffer, and the samples were resolved on an 8% SDS-PAGE. The gel was then dried and exposed by autoradiography.

TRAP220/Med1 RNA interference. Small interfering RNA (siRNA) specific for TRAP220/Med1 was purchased from Dharmacon RNA Technology, Inc. The 21-nucleotide MED1 siRNA is specific for the 3'-untranslated region of the native TRAP220/Med1 mRNA (NCBI accession no. NM_004774). A nonspecific (scrambled) siRNA (Dharmacon) was used as a control. HeLa cells were transfected with TRAP220/Med1 siRNA or control siRNA at a final concentration of 100 nM using Lipofectamine with Plus reagent (Invitrogen).

Transient transfections, immunoprecipitation of ectopic TRAP220/Med1, and luciferase assays. Transient transfections using HeLa or α -2 cells were performed in 6- or 12-well plates using Lipofectamine 2000 or Lipofectamine in combination with the Plus reagent (Invitrogen) according to the manufacturer's instructions. The addition of ^{32} P_i (0.2 mCi/ml), EGF (100 ng/ml), or specific kinase inhibitors was carried out as specified in the figure legends. In experiments involving T3-dependent transcription, the transfected cells were subsequently cultured in DMEM containing dialyzed FBS (Invitrogen) for 24 h in the presence or absence of T3 (10^{-7} M final concentration). Cells were then harvested, and luciferase activity was determined using the Promega luciferase assay system. Luciferase values were normalized by using either a *Renilla* luciferase (pRL-TK) or a β -galactosidase (pSV- β gal) (Promega) expression vector internal control. For experiments involving immunoprecipitation of ^{32} P-labeled or unlabeled ectopically expressed wild-type and mutated HA-TRAP220/Med1 proteins, cell lysates were prepared in lysis buffer (50 mM Tris HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, 0.2 mM phenylmethylsulfonyl fluoride [10^6 cells/250 μ l lysis buffer]). Protein concentrations were determined by Bradford assay. Equal amounts of protein lysate were then incubated with 5 μ l packed anti-HA agarose beads (Sigma) at 4°C overnight and then washed three times with lysis buffer. The immunoprecipitated protein was then fractionated by SDS-PAGE and analyzed by either autoradiography or Western blotting as indicated in the text.

Phosphoamino acid analysis. HeLa cells (8×10^5) were transfected with 4 μ g of pSG5-HA-TRAP220 as described above. Twelve hours posttransfection, cells were serum starved for 24 h and then incubated in phosphate-free medium containing ^{32} P_i (0.2 mCi/ml) with or without EGF (100 ng/ml) for an additional 20 min. Cells were lysed in lysis buffer and subsequently incubated with anti-HA agarose beads as described above. Immunoprecipitated HA-TRAP220/Med1 was resolved by SDS-PAGE (8% polyacrylamide), transferred to an Immobilon-P membrane (Millipore), and then exposed by autoradiography. The predicted TRAP220/Med1 protein bands were excised from the membrane and treated with 200 μ l of 6 M HCl at 100°C for 90 min. The pellets were washed with deionized water. The supernatants were pooled and dried by vacuum. The final pellets were resuspended in 5 μ l of water and 1 μ l of phosphoamino acid standard containing 10 mg/ml phosphoserine, phosphothreonine, and phosphotyrosine. A thin-layer chromatograph plate was spotted with the resulting sample, which was resolved by ascending chromatography with 70 ml isopropanol, 15 ml of 10 M HCl, and 15 ml of deionized water. When the chromatography was finished, the plate was dried and developed with a solution of ethanol containing 0.2% ninhydrin (Sigma). The plate was then heated at 100°C for 25 to 30 min to develop the standards and then exposed for autoradiography.

Pulse-chase analysis. COS cells (5×10^5) were transfected with pSG5-HA-TRAP220 (5 μ g) alone or together with pMCL-HA-MKK(Δ 4) (1 μ g). Twenty-four hours posttransfection, the cells were serum starved for 24 h, washed twice with phosphate-buffered saline (PBS), and then incubated with methionine/cysteine-free DMEM (Invitrogen) for 30 min. The cells were then pulsed with [^{35} S]methionine/cysteine (100 μ Ci/ml) for 1 h in the presence or absence of EGF (100 ng/ml) as indicated in the Fig. 5 legend. The medium was then removed, and the cells were washed twice with PBS. DMEM chase medium containing 10 mM cold methionine/cysteine (and in some cases) supplemented with EGF (100 ng/ml) was then added for the lengths of time indicated. At each chase time point, the cells were washed with PBS twice, lysed in lysis buffer for 1 h, and subsequently incubated with anti-HA beads (5 μ l packed) as described above. The immunoprecipitates were washed three times in ice-cold lysis buffer, resolved by SDS-PAGE, and visualized by autoradiography. The radioactive bands were quantified using a Storm PhosphorImager (Molecular Dynamics) and via densitometry using the SCION image analysis software. The resulting data were analyzed using Sigma plot software.

RT-PCR analysis. For reverse transcription-PCR (RT-PCR), total RNA was prepared from HeLa cells using the RNeasy mini kit (Qiagen). First-strand cDNA synthesis was generated by incubating 1 μ g of total RNA with 200 mM (final concentration) deoxynucleoside triphosphates (dNTPs) $1 \times$ reverse transcriptase buffer, and 1 U of reverse transcriptase (New England Biolabs) in a total volume of 10 μ l at 42°C for 90 min. First-strand cDNA was PCR amplified using *Taq* DNA polymerase (Takara) together with specific primers for either human TRAP220/Med1 or β -actin for 25 cycles. The PCR product was analyzed on a 1.5% ethidium bromide stained agarose gel. Real-time PCR reactions were performed using an Opticon thermocycler (MJ Research) according to manufacturer instructions using the SYBR Green I real-time PCR kit (Invitrogen). The resulting data was analyzed using Opticon software.

Immunocytochemistry. For immunofluorescence microscopy, COS cells were seeded on coverslips in 12-well plates (10^5 cells/well) and transfected as described above with either the wild-type or mutant pEGFP-TRAP220/Med1 construct alone or together with the pCMV5-ERK2 (constitutively active) construct. EGF (100 ng/ml) and U0126 (50 μ M) were added as indicated in the Fig. 8 legend. The subcellular locations of the GFP-TRAP220/Med1 proteins were visualized using a Nikon E1000 microscope and analyzed using IMAGEPRO software.

RESULTS

TRAP220/Med1 is phosphorylated by activated MAPK-ERK *in vivo*. Given that transcriptional coactivators are key regulatory targets for signal transduction pathways (see references 17, 27, 37, 43, 48, 51, 62, and 64–66 and references therein), we asked whether human TRAP220/Med1 might also be a substrate for kinase-dependent signaling. Accordingly, the phosphorylation status of the native TRAP220/Med1 protein was examined by immunoprecipitating the protein from ^{32} P_i-labeled HeLa cells with TRAP220/Med1 antibodies followed by SDS-PAGE fractionation and autoradiography. Notably, a single labeled protein migrating with a relative molecular mass of 220 kDa was detected (Fig. 1A). Furthermore, when nuclear extract from HeLa cells or the HeLa-derived α -2 cell line was pretreated with CIP, a faster-migrating (dephosphorylated) TRAP220/Med1 protein was observed by Western blotting (Fig. 1B). These findings demonstrate that endogenously expressed human TRAP220/Med1 is a phosphoprotein *in vivo*.

The family of MAPKs are critical mediators of intracellular signaling pathways that translate autonomous or extracellular stimuli (e.g., growth factors or stress) into cellular responses (29, 41). By phosphorylating specific serines and threonines of target protein substrates, MAPKs regulate a broad range of cellular activity, including gene expression, mitosis, movement, metabolism, and programmed death (29). Metazoans express three well-characterized MAPK subfamilies: ERK1/2, the c-Jun NH₂-terminal kinases (JNKs), and the p38 enzymes (41). To investigate a potential role for MAPKs in TRAP220/Med1

phosphorylation, HeLa cells were treated with EGF, a potent activator of ERK signaling pathways (29, 41). Subsequently, the TRAP220/Med1 protein was assayed by SDS-PAGE electromobility for gain of phosphorylation. Remarkably, TRAP220/Med1 in EGF-stimulated cells became markedly hyperphosphorylated (Fig. 1C) and remained so up to 6 h post-EGF treatment (see below). These findings show that TRAP220/Med1 exists as a basally phosphorylated protein and becomes hyperphosphorylated upon growth factor stimulation.

To gain a better understanding of which MAPK subfamilies are involved in TRAP220/Med1 phosphorylation, HeLa cells were transiently transfected with an expression vector for MAPK kinase 1 (MKK1), the immediate upstream activator of ERK1/2 (29, 41). Two forms of MKK1 were transfected: an HA-tagged wild-type construct (HA-MKK1 wt) and a constitutively active mutant [HA-MKK1(N Δ 4)] possessing a basal activity 45 times that of the wild-type enzyme (33). Significantly, whereas MKK1wt induced a noticeable increase in TRAP220/Med1 phosphorylation, MKK1(N Δ 4) triggered an even more pronounced increase in phosphorylation (Fig. 1D). To more directly implicate ERK in TRAP220/Med1 phosphorylation, HeLa cells were next transfected with a constitutively active ERK2 (L73P/S151D) mutant construct (11). As expected, transfection of the constitutively active construct robustly induced TRAP220/Med1 phosphorylation whereas transfection of a kinase-“dead” ERK2 mutant (K52R) had no effect (Fig. 1E).

To demonstrate that ERK phosphorylation of TRAP220/Med1 was not just restricted to the endogenous protein, HeLa cells were transiently transfected with an HA-TRAP220/Med1 expression vector in the presence of ^{32} P_i and then subjected to anti-HA immunoprecipitation (Fig. 2A and B). Importantly, stimulation of the cells with EGF (Fig. 2A) or cotransfection with the MKK1(N Δ 4) or ERK2(L73P/S151D) expression constructs (Fig. 2B) markedly enhanced the phosphorylation of ectopic TRAP220/Med1. In addition to ERK1/2, EGF is capable of activating other subtypes of MAPKs (p38 and JNK) and other non-MAPK phosphokinases (PKA and PKC) (reviewed in references 38 and 42). To investigate whether other phosphokinases are involved in EGF-induced TRAP220/Med1 phosphorylation, HeLa cells were again transiently transfected with HA-TRAP220/Med1, but this time in the presence of specific chemical inhibitors for MKK1/2 (U0126), p38 (SB202190), JNK, (L-JNK-1), PKA (4-cyano-3-methylisoquinoline), and PKC (bisindolylmaleimide I). The cells were then cultured in the presence of ^{32} P_i, with or without EGF stimulation, and then subjected to anti-HA immunoprecipitation. Importantly, only the addition of the U0126, a specific inhibitor of MKK1/2, could abolish EGF-induced phosphorylation of TRAP220/Med1 (Fig. 2A and D). Given that MKK1/2 is the immediate upstream activator of ERK1/2, these results indicate that EGF-induced phosphorylation of TRAP220/Med1 occurs almost exclusively through the activated MAPK-ERK signaling pathway.

Although the data in Fig. 1 and 2 clearly show that TRAP220/Med1 phosphorylation depends on ERK, there remained the remote possibility that the actual phosphorylation is performed by some other kinase that is itself dependent on ERK. To rule out this possibility, we tested whether purified recombinant full-length TRAP220/Med1 is a substrate for purified ERK1 *in vitro*. As shown in Fig. 2E, the purified TRAP220/Med1 protein was efficiently phosphorylated *in vitro*

FIG. 1. TRAP220/Med1 is phosphorylated in vivo by MAPK-ERK. (A) TRAP220/Med1 is a phosphoprotein in vivo. HeLa cells were cultured in serum- and phosphate-free medium for 4 h and subsequently in $^{32}\text{P}_i$ (0.1 mCi/ml) for 6 h. ^{32}P -labeled TRAP220/Med1 was immunoprecipitated using anti-TRAP220 antibodies, resolved by SDS-PAGE, and detected by autoradiography. (B) TRAP220/Med1 phosphorylation detected by electromobility shift. Nuclear extracts (5 μg) prepared from α -2 or HeLa cells were treated (or not) with CIP, fractionated by 5% SDS-PAGE for 48 h at 4°C , and then probed by immunoblotting with anti-TRAP220 antibodies (α -TRAP220). (C) EGF induces TRAP220/Med1 hyperphosphorylation (Hyper-Phos.). HeLa cells were grown in 10% serum (lanes 1 and 2) or serum starved for 12 h and then treated (or not) with EGF (100 ng/ml) for 30 min (lanes 3 and 4). Nuclear extract was then prepared and probed by immunoblotting with anti-TRAP220 antibodies. (D) MKK1 overexpression induces TRAP220/Med1 phosphorylation. HeLa cells were transiently transfected with mammalian expression vectors for either wild-type (wt) HA-MKK1 or a constitutively active MKK1 mutant (N Δ 4). Whole-cell extracts were then prepared and probed with antibodies specific for TRAP220/Med1 or the HA tag (α -HA). (E) ERK2 overexpression induces TRAP220/Med1 phosphorylation. HeLa cells were transiently transfected with mammalian expression vectors for either constitutively (constit.) active ERK2 (L73P/S151P) or a kinase-dead mutant (K52R) and then probed by immunoblotting with antibodies specific for TRAP220/Med1.

by ERK1. This finding strengthens the notion that TRAP220/Med1 is indeed a direct substrate for ERK in vivo.

Identification of MAPK-ERK-mediated phosphorylation sites on TRAP220/Med1. The human TRAP220/Med1 protein sequence contains seven consensus MAPK phosphorylation sites [P(X) $_{1/2}$ S/TP] (41) within its open reading frame (Fig. 3A). In order to identify the specific type of TRAP220/Med1 amino acids that are phosphorylated by ERK in vivo, HeLa cells were transiently transfected with HA-TRAP220 and then were stimulated (or not) with EGF in the presence of $^{32}\text{P}_i$. The immunopurified radiolabeled TRAP220/Med1 protein (Fig. 3B) was then excised and subjected to phosphoamino acid analysis (Fig. 3C; see Materials and Methods for details). Our results clearly indicate that TRAP220/Med1 phosphorylation by ERK occurs predominantly on threonine residues but not on serine or tyrosine residues.

In an effort to determine more precisely the specific ERK-mediated phosphorylation sites on the TRAP220/Med1 protein in vivo, a series of deletion mutants were generated (Fig. 3D and E). Each mutant was transiently transfected into

HeLa cells in the presence of EGF and $^{32}\text{P}_i$ and tested for phosphorylation. Figure 3F shows that ERK-mediated phosphorylation of TRAP220/Med1 only occurs on the C-terminal end of the protein between residues 918 and 1581. Given that phosphorylation is not observed at serine residues (Fig. 3C), these findings strongly implicate threonine 1032, threonine 1215, or threonine 1457 as the most likely target residues for ERK phosphorylation.

To establish the relative importance of each threonine residue as a potential substrate for ERK in vivo, we mutated each site (threonine to alanine) within the full-length HA-TRAP220 expression vector and then tested each point mutant for EGF-induced phosphorylation (Fig. 3G). Mutation of threonine 1215 had no significant effect on EGF-induced phosphorylation (lane 8), whereas mutation of either threonine 1032 or threonine 1457 significantly reduced the phosphorylation level (compare lanes 2, 5, and 9). Importantly, a double mutation of both threonine 1032 and threonine 1457 completely abolished EGF-induced phosphorylation (lane 10). In contrast, mutation of any of the other four potential MAPK phosphorylation sites

FIG. 2. Ectopically expressed recombinant TRAP220/Med1 is phosphorylated in vivo and in vitro by ERK. (A and B) HeLa cells (8×10^5) were transiently transfected with pSG5-HA-TRAP220 (4 μ g) alone (A) or together with either HA-MKK1(N Δ 4) (4 μ g) or constitutively (constit.) active ERK2 (4 μ g) (B). Twelve hours posttransfection, cells were serum starved for 24 h and then incubated in phosphate-free media containing ^{32} P_i (0.2 mCi/ml) with or without EGF (100 ng/ml) or U0126 (30 μ M) for 30 min. Anti-HA immunoprecipitates were resolved by 8% SDS-PAGE and exposed for autoradiography. (C) As a control for expression, HeLa cells were transfected with pSG5-HA-TRAP220 in the absence of ^{32} P and probed by immunoblotting with anti-HA antibodies. (D) HeLa cells were transiently transfected with pSG5-HA-TRAP220 as in panel A. Twelve hours posttransfection, cells were serum starved for 24 h and then pretreated with the kinase inhibitors U0126 (30 μ M), SB202190 (2.5 μ M), L-JNK-1 (1 μ M), 4-cyano-3-methylisoquinoline (300 nM), and bisindolylmaleimide I (Bisindolyl.; 100 nM) for 90 min as indicated. The cells were then incubated in fresh phosphate-free media containing ^{32} P_i (0.2 mCi/ml) in the presence or absence of EGF (100 ng/ml) for 20 min. Anti-HA immunoprecipitates were resolved by SDS-PAGE and exposed for autoradiography. (E) MAPK-ERK phosphorylates TRAP220/Med1 in vitro. Purified baculovirally expressed full-length HA-TRAP220/Med1 (50 ng) was incubated with [γ - ^{32} P]ATP in the presence or absence of purified ERK1 (1 ng) as indicated. Reactions were resolved by SDS-PAGE and exposed for autoradiography (top panel). As a loading control, parallel experiments were performed in the absence of [^{32} P]ATP, resolved by 8% SDS-PAGE, and probed by immunoblotting with anti-HA antibody (bottom panel).

(residues T628, T739, S1156, or S1192) had no significant effect on EGF-induced phosphorylation. We therefore conclude that threonine 1032 and threonine 1457 are bona fide ERK phosphorylation sites on the human TRAP220/Med1 protein in vivo.

ERK phosphorylation of TRAP220/Med1 stabilizes protein expression. We observed earlier that hyperphosphorylation of TRAP220/Med1 by ERK appeared to be correlated with increased protein levels (Fig. 1D). We therefore hypothesized that ERK-dependent phosphorylation of TRAP220/Med1 might be a regulatory mechanism that modulates TRAP220/Med1 cellular concentration. To address this issue more thoroughly, extracts were prepared from EGF-treated HeLa cells and then probed by Western blotting with TRAP220/Med1 antibodies (Fig. 4A and C). Remarkably, we found that EGF treatment nearly doubled the amount of TRAP220/Med1. The increase was clearly mediated by activated ERK since transfection of HeLa cells with constitutively active MKK1(N Δ 4) similarly increased TRAP220/Med1 protein levels (Fig. 4D) and treatment with U0126 abrogated the effect (Fig. 4A). Addition of the protein synthesis inhibitor cycloheximide prior to EGF treatment had no noticeable inhibitory effect (Fig. 4A), thus indicating that MAPK-ERK regulation of TRAP220/Med1 protein levels does not require de novo protein synthe-

sis. Furthermore, activation of ERK by EGF had no significant effect on TRAP220/Med1 mRNA expression as determined using semiquantitative and real-time RT-PCR (Fig. 4B). These findings are thus consistent with the notion that direct post-translational phosphorylation of TRAP220/Med1 by ERK stabilizes TRAP220/Med1 protein expression.

Elevated levels of TRAP220/Med1 could be detected at least 6 h post-EGF stimulation, thus suggesting that the duration of ERK-dependent protein stabilization is relatively protracted (Fig. 4C). Moreover, activation of ERK in HeLa cells resulted in increased expression of ectopic HA-TRAP220/Med1, yet had no apparent stabilizing effect on ectopic FLAG-TRAP80/Med17 protein expression (Fig. 4E). Surprisingly, the TRAP220/Med1 double mutant (T1032A T1457A) that was resistant to ERK phosphorylation (Fig. 3G) had an elevated level of expression in the control treatment that was insensitive to further stimulation by EGF or MKK1(N Δ 4) (Fig. 4E). While this result might at first appear paradoxical, it is important to note that phosphorylation has been shown to stabilize the protein expression of other transcription factors such as p53 and PGC-1, yet mutation of their respective serine/threonine phosphorylation sites to alanine similarly resulted in increased protein expression (43, 49, 50). Thus, in these cases, alanine

FIG. 3. Identification of ERK phosphorylation sites on TRAP220/Med1. (A) Schematic representation of TRAP220/Med1 showing the location of consensus MAPK phosphorylation sites [P(X)_{1/2} S/TP] with threonine (T) or serine (S). (B and C) ERK phosphorylates TRAP220/Med1 at threonine residues. HeLa cells (8×10^5) were transiently transfected with pSG5-HA-TRAP220 (4 μ g). Twelve hours posttransfection, cells were serum starved for 24 h and then incubated in phosphate-free media containing ^{32} P_i (0.2 mCi/ml) with or without EGF (100 ng/ml) for 20 min. The anti-HA-immunoprecipitated TRAP220/Med1 protein was resolved by SDS-PAGE, transferred to a nylon membrane, and exposed for autoradiography (B). The radiolabeled TRAP220/Med1 protein was excised from the membrane, hydrolyzed in HCl, analyzed together with phosphoamino acid markers by thin-layer chromatography, developed with ninhydrin to reveal mobility of nonlabeled markers (indicated by dashed circles), and subsequently exposed for autoradiography. (D) Schematic representation of TRAP220/Med1 N- and C-terminal deletion mutants indicating location of consensus MAPK phosphorylation sites. (E) Expression of TRAP220/Med1 deletion mutants. HeLa cells (8×10^5) were transfected with 4 μ g of full-length (FL) pSG5-HA-TRAP220 or each deletion mutant (panel D). Twenty-four hours posttransfection, whole-cell lysate was prepared, resolved by SDS-PAGE, and probed with anti-HA antibodies. The N Δ 1233 deletion mutant (~ 38 kDa) was resolved on a separate gel. The expressed HA-tagged proteins are indicated by open arrows. (F) ERK phosphorylation of TRAP220/Med1 deletion mutants. HeLa cells were transfected exactly as in panel B, except that only 0.5 μ g of the N Δ 1233 construct was transfected due to high expression levels. Twelve hours posttransfection, the cells were serum starved for 24 h and then incubated in phosphate-free media containing ^{32} P_i (0.2 mCi/ml) with or without EGF (100 ng/ml) for 20 min. Anti-HA immunoprecipitates were resolved by SDS-PAGE and exposed by autoradiography. (G) ERK phosphorylation of TRAP220/Med1 point mutants. Consensus serine (S) or threonine (T) MAPK-ERK phosphorylation sites in TRAP220/Med1 (shown schematically in the upper panel) were mutated to alanine within the full-length pSG5-HA-TRAP220 vector. In the bottom panel, each mutant was transfected into HeLa cells and assayed for EGF-induced phosphorylation as described for panel F.

FIG. 4. ERK phosphorylation of TRAP220/Med1 stabilizes protein expression. (A and B) HeLa cells (5×10^6) were serum starved for 24 h and then treated (or not) with EGF (100 ng/ml), U0126 (30 μ M), or cycloheximide (CHX) (30 μ g/ml) for 1 h as indicated. Nuclear extract and total RNA were then prepared from equal numbers of cells. The nuclear extract (50 μ g) was probed by immunoblotting with anti-TRAP220 antibodies (A). The same blot was then stripped and reprobed with antibodies against β -actin. The total RNA (1 μ g) was analyzed by semiquantitative RT-PCR using primers specific for TRAP220 and β -actin (B, top) and by SYBR green real-time PCR (B, bottom). (C) HeLa cells (8×10^5) were serum starved for 24 h and then treated (or not) with EGF (100 ng/ml) for different lengths of time (shown on the x axis as 2-h intervals from 0 to 12 h). Equivalent amounts of whole-cell lysate were probed by immunoblotting with anti-TRAP220 antibodies (C, top). Each band was quantitated densitometrically using the SCION image software and normalized against α -tubulin immunoblot signals from the same blot (data not shown). The data are presented graphically using Microsoft Excel (C, bottom). The y axis shows 10-point increments running from 0 to 70. (D) HeLa cells (8×10^5) were transiently transfected with HA-MKK1(NΔ4) (2 μ g) or an empty vector control. Starting at 24 h posttransfection, whole-cell lysate was prepared at different time points (indicated) and probed by immunoblotting using anti-TRAP220 antibodies. (E) HeLa cells (8×10^5) were transfected with either the pSG5-HA-TRAP220 wild type (2 μ g) or the pSG5-HA-TRAP220 double mutant (T1032A/T1457A) (2 μ g) alone or together with HA-MKK1(NΔ4) (1 μ g) (lanes 3 and 6). pCIN4-FLAG-TRAP80 (1 μ g) was transfected in each reaction as an internal control. Twenty-four hours posttransfection, cells were serum starved for 24 h and then treated (or not) with EGF (100 ng/ml) for 30 min. Whole-cell lysates were then prepared, and equal amounts of protein were probed by immunoblotting with anti-HA or anti-FLAG antibodies.

substitutions presumably alter the association of regulatory machinery involved in protein turnover (43).

The experiments performed in Fig. 4A to E were also carried out using COS cells with very similar results (data not shown). To clearly establish whether or not ERK-dependent protein stabilization of TRAP220/Med1 occurs at the level of reduced protein turnover, pulse-chase experiments were performed using COS cells with [^{35} S]methionine/cysteine in the presence or absence of EGF (Fig. 5A) or in the presence of cotransfected MKK1(NΔ4) (Fig. 5B). As shown clearly in Fig. 5, either EGF- or MKK-stimulated phosphorylation of TRAP220/Med1 via ERK significantly increases the half-life of

TRAP220/Med1 from approximately 2 to 3 h to 6 to 7 h. Consistent with these findings, we found that protein expression from a transiently transfected GFP-TRAP220/Med1 fusion vector persisted significantly longer in cells additionally treated with EGF or cotransfected with MKK1(NΔ4) than in control cells (Table 1). Addition of the ERK inhibitor U0126 nullified this effect.

One functional consequence of ERK-induced stabilization of TRAP220/Med1 expression might be to quantitatively increase its availability to gene-specific activators that require TRAP220/Med1 for transcriptional coactivation. Given that TRAP220/Med1 is an essential coactivator for TR (12, 19, 21,

FIG. 5. ERK phosphorylation increases the half-life of TRAP220/Med1. COS cells (5×10^5) were transfected with pSG5-HA-TRAP220 (4 μ g) alone or together with HA-MKK1(N Δ 4) (1.5 μ g). Twenty-four hours posttransfection, cells were serum starved for 24 h and then incubated in methionine/cysteine-free media for 30 min. The cells were then pulse-labeled in [^{35} S]methionine/cysteine (100 μ Ci/ml) with or without EGF (100 ng/ml) for 1 h and then incubated in chase medium for different lengths of time (indicated). At each chase time point, equivalent amounts of cell lysate were subjected to anti-HA immunoprecipitation and then resolved by SDS-PAGE. The radioactive bands were then exposed and quantitated by autoradiography and phosphorimaging.

34), we asked whether ERK phosphorylation of TRAP220/Med1 affects thyroid hormone (T3)-dependent transcription by TR. Accordingly, HeLa cells were transfected with a TR α expression vector and a T3-responsive reporter gene and then cultured in the presence or absence of T3 and/or EGF (Fig. 6A). Interestingly, T3-dependent transcription from the reporter gene was stimulated in the presence of EGF and concomitantly correlated with an increase in native TRAP220/Med1 expression. In contrast, we observed no EGF-induced increase in ectopic TR α protein expression.

As shown previously, overexpression of recombinant TRAP220/Med1 can also coactivate T3-dependent transcription (31, 67) (Fig. 6B). Consistent with the findings in Fig. 6A, when ectopic HA-TRAP220/Med1 was expressed along with constitutively

active MKK(N Δ 4), T3-dependent transcription was further stimulated and again correlated with elevated levels of the HA-tagged TRAP220/Med1 protein (Fig. 6B). Taken together, our results show that ERK directly phosphorylates TRAP220/Med1 at two threonine residues thereby increasing its half-life and that ERK-induced stabilization of TRAP220/Med1 expression directly correlates with increased TR-dependent transcription.

ERK phosphorylates TRAP220/Med1 in a cell cycle-dependent manner. MAPK-ERK signaling pathways are essential for mammalian cell cycle progression (reviewed in reference 23), being required for both entry and progression through the G₁/S phase (29, 41) and, as recently demonstrated using synchronized HeLa cells, for entry and progression through mi-

TABLE 1. MAPK-ERK phosphorylation of transiently transfected GFP-TRAP220/Med1 stabilizes expression^a

Treatment	% of transfected cells at:				
	0 h	4 h	12 h	18 h	24 h
- EGF	31.8 $\pm$ 7.2	31.2 $\pm$ 3.4	4.9 $\pm$ 2.3	1.1 $\pm$ 0.9	0
+ EGF	31.3 $\pm$ 6.6	35.3 $\pm$ 9.1	33.7 $\pm$ 3.2	32.5 $\pm$ 4.2	21.0 $\pm$ 4.4
+ MKK(N Δ 4)	36.1 $\pm$ 4.7	33.4 $\pm$ 6.5	33.1 $\pm$ 4.6	30.0 $\pm$ 1.9	25.9 $\pm$ 4.3
+ EGF + UO126	27.4 $\pm$ 8.7	15.8 $\pm$ 3.4	7.0 $\pm$ 3.02	4.5 $\pm$ 1.1	0

^a COS cells were transfected with pEGFP-TRAP220/Med1 alone or together with HA-MKK1(N Δ 4). Twenty-four hours posttransfection, cells were serum starved for 24 h and then treated (or not) with EGF (100 ng/ml) or with UO126 (25 μ M) for the times indicated. The percentage of transfected cells at each time point is shown. In each experiment (n), 200 cells were examined. Values are presented as the mean $\pm$ standard error ($n = 3$).

FIG. 6. ERK stabilization of TRAP220/Med1 protein expression correlates with increased TR-dependent transcription. (A) HeLa cells (10^5) were transfected with p2xT₃RE-tk-Luc (0.3 μ g) and pBK-CMV-FLAG-TR α (1 μ g). Twelve hours posttransfection, the cells were serum starved for 18 h and then treated (or not) with T3 (10^{-7} M) for an additional 12 h. EGF (100 ng/ml) was added as indicated 6 h prior to the analysis of luciferase activity. Whole-cell lysate was then prepared and analyzed for luciferase reporter activity and for protein expression by immunoblotting, first using anti-TRAP220 antibodies and subsequently using anti-TR antibodies (shown at the bottom). Luciferase activity results are presented as the mean $\pm$ standard error of triplicate transfections. (B) HeLa cells (10^5) were transfected with p2xT₃RE-tk-Luc (0.3 μ g) and pBK-CMV-FLAG-TR α (1 μ g) together with pSG5-HA-TRAP220 (0.25 μ g) and HA-MKK1(N Δ 4) (0.25 μ g) as indicated. Twelve hours posttransfection, cells were treated (or not) with T3 (10^{-7} M) for an additional 24 h. Immunoblotting with anti-HA antibodies and luciferase assays were carried out as in panel A.

tosis (30, 47). Given our results showing that TRAP220/Med1 is an *in vivo* substrate for activated ERK, we asked whether TRAP220/Med1 might also exhibit cell cycle-dependent phosphorylation. Accordingly, HeLa cells were growth arrested at the G₁/S-phase boundary by double-thymidine block and then analyzed as the cells progressed through S phase, G₂/M phase, and back into G₁ phase (see Materials and Methods). Synchronization in each cell cycle phase was verified by flow cytometry (Fig. 7A). To assay for cell cycle-dependent phosphorylation, the synchronized cells were exposed to a short pulse of ^{32} P_i followed by anti-TRAP220/Med1 immunoprecipitation. Strikingly, we found that TRAP220/Med1 undergoes marked phosphorylation during the G₂/M phase of the cell cycle (Fig. 7B). Consistent with our earlier findings correlating TRAP220/Med1 phosphorylation with increased protein levels, we observed that TRAP220/Med1 protein levels were approximately twofold higher during G₂/M than in other phases of the cell cycle (Fig. 7C). In contrast, TRAP220/Med1 mRNA levels remained relatively constant throughout the cell cycle (Fig. 7D), thus suggesting that posttranslational phosphorylation serves to stabilize TRAP220/Med1 expression at G₂/M.

To verify that the observed cell cycle-dependent phosphoryla-

tion of TRAP220/Med1 was mediated by ERK, and to further investigate the possible involvement of other cell cycle-dependent kinases (cdk's), we repeated the cell cycle phosphorylation assay in the presence of specific inhibitors (Fig. 7E). Given the observed phosphorylation of TRAP220/Med1 at G₂/M (Fig. 7B), we specifically used butyrolactone I, a potent inhibitor of cyclin B/cdc2, as well as roscovitine, a common inhibitor of all cdk's (9, 35). In these experiments, HeLa cells were synchronized in G₂/M, treated with the various kinase inhibitors for 1 h, and then exposed to a short pulse of ^{32} P_i followed by anti-TRAP220/Med1 immunoprecipitation. As shown in Fig. 7E, neither butyrolactone I nor roscovitine inhibited TRAP220/Med1 phosphorylation in G₂/M-synchronized HeLa cells. However, and consistent with our earlier findings, addition of the MKK inhibitor U0126 significantly decreased phosphorylation (Fig. 7E).

In agreement with our earlier results, we also found that inhibition of TRAP220/Med1 phosphorylation at G₂/M by U0126 was concomitantly associated with a decrease in protein expression (Fig. 7F, compare lanes 1 and 4). These findings thus demonstrate that ERK can phosphorylate TRAP220/Med1 in a cell cycle-dependent manner, resulting in peak levels of expression during the G₂/M phase of the cell cycle. Given

FIG. 7. ERK phosphorylates TRAP220/Med1 in a cell cycle-dependent manner. (A) HeLa cells were synchronized in G_1/S , S, G_2/M , and G_1 by double-thymidine block and release. Synchronization was verified by flow cytometry. For each panel, the x axis runs from 0 to 1,023. (B) Synchronized HeLa cells were pulsed with $^{32}\text{P}_i$ (0.125 mCi/ml) for 10 min. Parallel reactions lacking $^{32}\text{P}_i$ were performed side-by-side. TRAP220/Med1 was isolated by anti-TRAP220 immunoprecipitation (IP) and resolved by SDS-PAGE. Reactions containing $^{32}\text{P}_i$ were detected by autoradiography (upper panel), while reactions lacking ^{32}P were detected by anti-TRAP220 immunoblotting (middle panel). In each synchronized experiment, 50 μg of protein was taken from each sample (before immunoprecipitation) and probed by anti- α -tubulin immunoblotting (bottom panel). WB, Western blot. (C and D) HeLa cells were synchronized in G_1/S , S, G_2/M , and G_1 . Equal amounts of whole-cell lysate (100 μg) were probed by anti-TRAP220 and anti- α -tubulin immunoblotting (C). Equal amounts of total RNA (1 μg) were analyzed by RT-PCR using primers specific for TRAP220 and β -actin (D). Immunoblot and RT-PCR signals were quantified by densitometry. In panel C, the y axis runs in increments of 50 from 0 to 300. In panel D, the y axis runs in increments of 20 from 0 to 140. (E) HeLa cells were first synchronized in G_2/M ; treated (or not) with the kinase inhibitor butyrolactone (50 μM , lane 2), U0126 (50 μM , lane 4), or roscovitine (1 μM) for 90 min; and then pulsed with $^{32}\text{P}_i$ (0.125 mCi/ml) for 10 min. TRAP220/Med1 was then isolated by anti-TRAP220 immunoprecipitation, resolved by SDS-PAGE, and detected by autoradiography (upper panel). As a loading control, 50 μg of protein from each sample taken before immunoprecipitation was probed by anti- α -tubulin immunoblotting (lower panel). (F) HeLa cells synchronized in G_2/M were treated with the various kinase inhibitors for 90 min exactly as outlined in panel E. Equal amounts of whole-cell lysate (100 μg) were probed by anti-TRAP220 and anti- α -tubulin immunoblotting.

FIG. 8. ERK-induced shuttling of TRAP220/Med1 into the nucleolus. COS cells were transfected on coverslips with expression vectors for pEGFP (A) or the pEGFP-TRAP220/Med1 wild-type (GFP220) (B to E), pEGFP-TRAP220/Med1 double-mutant T1032A/T1457A (GFP220-DM) (F), or constitutively active ERK2 (L73P/S151P) (C) expression vectors. Twenty-four hours posttransfection, cells were serum starved for 24 h and then treated (or not) with EGF (100 ng/ml for 30 min) or U0126 (25 μ M for 90 min) as indicated. The cells were then fixed and examined by fluorescent microscopy.

that ERK activity is required for mitotic entry, these data may implicate TRAP220/Med1 as a specific regulatory target for ERK signaling at G₂/M. Moreover, these findings may have implications for the role TRAP220/Med1 in transcriptional regulation of cell cycle progression (see Discussion).

ERK-dependent subnuclear shuttling of TRAP220/Med1. Several recent reports show that phosphokinase signaling pathways can influence the import and export of NR coactivators and corepressors in and out of the nucleus (17, 18, 62, 64). We therefore investigated whether ERK phosphorylation affects TRAP220/Med1 nuclear localization. Using anti-TRAP220/Med1 immunocytochemistry and immunoblotting of cytoplasmic versus nuclear fractions, we observed that TRAP220/Med1 resides predominantly in the nucleus of both HeLa and COS cells and that the ratio of cytoplasmic to nuclear protein was relatively unaffected by the presence or absence of activated ERK (data not shown).

To begin to explore whether TRAP220/Med1 phosphorylation affects subnuclear localization, we transiently transfected COS cells with the pGFP-TRAP220/Med1 fusion construct and visualized the expressed protein using fluorescent microscopy. Consistent with our earlier findings, we found that TRAP220/Med1 resides predominantly in the nucleus (Fig. 8). Curiously, we found that when the cells were either treated with EGF or cotransfected with constitutively active ERK2, a subset of the

TRAP220/Med1 protein reproducibly translocated into the nucleolus (compare Fig. 8B with 8C and D). The observed nucleolar shuttling was clearly mediated through activated ERK since treatment with the MKK1/2 inhibitor U0126 blocked this effect (Fig. 8E). Furthermore, when a pGFP-TRAP220/Med1 mutant construct lacking ERK phosphorylation sites (T1032A, T1457A) was used, TRAP220/Med1 failed to translocate into the nucleolus in the presence of EGF (Fig. 8F). While the physiological significance of these data remains unclear, the findings raise the possibility that phosphorylated TRAP220/Med1 (and possibly other components of the TRAP/Mediator complex) plays a novel coregulatory role in RNA polymerase I (RNA pol I) transcription. Alternatively, these data may reveal a novel regulatory mechanism in which activated ERK sequesters a subset of TRAP220/Med1 proteins into specific nuclear compartments.

TRAP220/Med1 intrinsic coactivator activity is stimulated by ERK. Our findings thus far suggest that ERK-induced stabilization of TRAP220/Med1 protein expression increases its availability as a coactivator for gene-specific activators like TR. We extended this analysis and further asked whether activated ERK might directly potentiate intrinsic TRAP220/Med1 transcriptional activity. For this purpose, we fused TRAP220/Med1 to a GAL4 DNA binding domain (DBD) and assayed the construct for transcriptional activation in HeLa cells cotrans-

FIG. 9. MAPK-ERK stimulates intrinsic TRAP220/Med1 transcriptional activity. (A) HeLa cells (1×10^5) were transfected with 0.5 μ g of pCMX-Gal4, pCMX-Gal4-TRAP220 wild-type (wt) or pCMX-Gal4-TRAP220 (T1032A/T1457A) double-mutant (DM) expression vectors together with 0.5 μ g of the p5xGal4-Luc reporter gene and 200 ng of the pSV- β gal internal control vector. One day after transfection, cells were serum starved for 24 h and then treated with EGF (100 ng/ml) or EGF plus UO126 (25 mM) for 6 h. Whole-cell lysate was then prepared and assayed for luciferase reporter activity and for GAL4-TRAP220 protein expression by immunoblotting (shown at the bottom). (B) TRAP220/Med1 mutation blocking ERK phosphorylation impairs TR-dependent transcription. HeLa cells (10^5) were transfected with 100 pmol of either TRAP220/Med1 siRNA or a nonspecific control together with 1 μ g of pBK-CMV-FLAG-TR α and 0.3 μ g of the 2xT₃RE-tk-luciferase reporter gene. As indicated, selected reaction mixtures were also cotransfected with 0.2 μ g of the pSG5-HA-TRAP220 wild type or pSG5-HA-TRAP220

fectected with a reporter gene containing GAL4 upstream activation sites (UAS). As shown in Fig. 9A, fusion of TRAP220/Med1 to GAL4 increased transcription greater than ninefold. Importantly, addition of EGF nearly doubled the intrinsic TRAP220/Med1 transcriptional activity.

The EGF-induced stimulation of intrinsic TRAP220/Med1 transcriptional activity is clearly mediated via activated ERK since treatment with U0126 markedly abrogated the increase. Moreover, transfection of a GAL4-TRAP220/Med1 construct containing the ERK-phosphorylation-resistant double mutation (T1032A T1457A) was insensitive to stimulation by EGF (Fig. 9A). These data cannot be accounted for by an EGF-induced increase in TRAP220/Med1 protein stability, since expression levels of the double-mutant protein are comparable to (or greater than) wild-type levels in the presence of EGF (Fig. 4E and Fig. 9A, bottom). These findings thus suggest that in addition to regulating protein stability and possibly subnuclear location, activated ERK can also modulate the intrinsic transcriptional coactivator activity of TRAP220/Med1.

We further examined TRAP220/Med1 coactivator function within the context of T3-dependent gene activation by TR. In these experiments, siRNA duplexes were generated against the 3' untranslated region of the native TRAP220/Med1 mRNA (see Materials and Methods) and used to knock down TRAP220/Med1 expression in HeLa cells via RNA interference. To verify that loss of endogenous TRAP220/Med1 expression impairs TR signaling, HeLa cells transfected with TRAP220/Med1 siRNA were subsequently transfected with a TR α expression vector and a T3-responsive reporter gene. The cells were then cultured in the presence or absence of T3, and transcription from the reporter gene was measured (Fig. 9B). T3-dependent transcription was observed in the presence of control siRNA, but as expected, the T3 response was significantly impaired in the presence of TRAP220/Med1 siRNA (Fig. 9B).

Since the TRAP220/Med1 siRNA is specific for the 3' untranslated region of the native mRNA, we were able to test whether ectopic HA-TRAP220/Med1 expression (lacking the 3' untranslated region) could rescue the T3 response. As shown in Fig. 9B, transfection of the wild-type TRAP220/Med1 construct restored T3-dependent transactivation to near normal levels. However, transfection of the TRAP220/Med1 construct containing the ERK-phosphorylation-resistant double mutation (T1032A T1457A) failed to restore T3-dependent activation (Fig. 9B). Once again, both the wild-type and double-mutant HA-tagged TRAP220/Med1 proteins were expressed with equal efficiency, presumably ruling out ERK-induced protein stabilization as a mechanism for coactivation by the wild-type protein. We thus conclude that in addition to conferring protein stability, ERK signaling pathways can also stimulate the intrinsic coactivation function of TRAP220/Med1. The molecular mechanisms underlying this stimulation by ERK are under investigation.

DISCUSSION

The TRAP/Mediator complex plays a central role in regulating the expression of protein-encoding genes by virtue of its ability to functionally interact with both gene-specific transcription factors and the RNA pol II-associated basal transcription machinery (32, 44, 53). TRAP220/Med1 is a key component of TRAP/Mediator in that it targets and anchors the complex to a broad range of NRs as well as other types of gene-specific activators. Accordingly, TRAP220/Med1 ablation in mice is embryonic lethal (21, 72) and null embryonic fibroblasts (isolated prior to embryonic death) display impaired cell cycle progression (21). Despite its pivotal role in cellular growth and development, however, very little is known about basic cellular signaling mechanisms that regulate the functional activity of TRAP220/Med1 and that of the TRAP/Mediator holocomplex. Furthermore, the underlying regulatory mechanisms by which TRAP220/Med1 might be involved in cell cycle progression remain unclear.

It was recently reported that activated MAPK signaling can lead to phosphorylation of gene-specific transcription factors which, in turn, stimulate their binding to the TRAP/Mediator complex (52). Here we investigated whether the TRAP/Mediator complex itself is a regulatory target for MAPK signaling. We have shown that the TRAP220/Med1 subunit is a direct substrate for activated MAPK-ERK *in vivo* and precisely identify the phosphorylation sites. With regard to the physiological and functional relevance of this posttranslational event, we have demonstrated that ERK phosphorylation significantly stabilizes TRAP220/Med1 protein expression and occurs in a cell cycle-dependent manner, resulting in peak TRAP220/Med1 expression levels during the G₂/M phase. Furthermore, we observed that ERK-mediated phosphorylation stimulates the intrinsic coactivation function of TRAP220/Med1 and appears to influence its subnuclear localization.

ERK phosphorylates TRAP220/Med1 *in vivo* at two specific amino acid residues, threonine 1032 and threonine 1457 (Fig. 3). Both sites are located in the C-terminal portion of TRAP220/Med1, the function of which remains poorly defined but apparently does not directly interact with the core TRAP/Mediator complex (31). The same relative residues were identified in the mouse TRAP220/Med1 protein as *in vitro* MAPK phosphorylation sites (37). The same study also reported that mouse TRAP220/Med1 is an *in vitro* substrate for PKA and PKC, possibly accounting for the basal phosphorylation observed here with the human protein *in vivo* (Fig. 1). We found that ERK hyperphosphorylation of TRAP220/Med1 markedly inhibits protein degradation (Fig. 4 and 5), presumably by inhibiting TRAP220/Med1 interaction with the protein degradation machinery. Interestingly, mutation of the two ERK phosphorylation sites into alanine also inhibited TRAP220/Med1 turnover (Fig. 4E). Analogous observations were re-

(T1032A/T1457A) double mutant. Twenty-four hours posttransfection, cells were incubated for an additional 24 h in the presence or absence of T3 (10^{-7} M) and then harvested for luciferase activity measurement. Luciferase values were normalized against an internal control. Equivalent amounts of whole-cell extract prepared from the transfected cells were also probed by immunoblotting with anti-TRAP220 and anti-HA antibodies (shown in the inset). In both panels A and B, luciferase results are presented as the mean $\pm$ standard error of triplicate transfections.

ported for the transcription factors PGC-1 and p53 (43, 49, 50), thus suggesting that alanine substitutions affect the association of regulatory machinery involved in protein turnover (43). The molecular mechanisms and events underlying this stabilization are currently under investigation.

An important mechanism of regulated gene expression in higher eukaryotes involves the quantitative modulation of distinct coactivator and corepressor expression levels in different tissues and in response to various cellular signals (17, 51). Therefore, and relevant to the findings reported here, stabilization of TRAP220/Med1 protein expression via activated ERK may enhance or promote transcription from specific genes that are regulated by factors which recruit TRAP/Mediator via the TRAP220/Med1 subunit. Indeed, we found that ERK-induced stabilization of TRAP220/Med1 expression can be directly correlated with increased TR-dependent transcription (Fig. 6). It's interesting to note in this regard that TRAP220/Med1 is overexpressed in estrogen receptor-positive primary breast cancers and breast cancer cell lines (70) that concomitantly express amplified or hyperactivated MAPKs (2, 10, 22, 26). Given that TRAP220/Med1 plays a key mediator role in estrogen receptor-dependent transcription (1, 6, 24, 36, 70), our findings are thus consistent with the notion that TRAP220/Med1 may be a regulatory target for activated MAPKs that ultimately affect the growth, proliferation, and survival of breast cancer cells. Similarly, other specific components of the TRAP/Mediator complex have been shown to be required for transforming growth factor β signaling pathways that have essential roles in tumorigenesis (25).

MAPK-ERK signaling pathways are essential for mammalian cell cycle progression (23). Although the proliferative role of ERK is best defined at G₀/G₁ (29), recent studies using human HeLa cells additionally show that ERK becomes activated late in S phase, remains active through the end of mitosis, and is functionally required for mitotic entry and progression through mitosis (30, 47). Despite this work, little is known about the specific molecular targets of ERK during mitotic progression. Importantly, we show here that TRAP220/Med1 is a specific target for ERK phosphorylation during the G₂/M phase of the cell cycle (Fig. 7). These findings thus raise the intriguing possibility that TRAP220/Med1, likely in association with the TRAP/Mediator complex, plays a regulatory role in cell cycle progression, presumably by modulating the expression of genes important for facilitating the G₂/M transition. Two lines of evidence are consistent with a possible TRAP220/Med1 regulatory role in mitotic cell division *in vivo*. First, *TRAP220/Med1* null mouse embryos are unusually small and exhibit retarded cell growth (21, 72). Indeed, the cause of death in a subset of the mutants is believed to be directly attributed to retarded cell growth at very early stages of embryogenesis (21). Secondly, *TRAP220/Med1* null mouse embryonic fibroblasts (isolated prior to embryonic death) display impaired cell cycle progression in growth and mitogenicity assays (21). Future studies will be directed toward determining the specific genes regulated by TRAP/Mediator at G₂/M and understanding how their expression is affected by activated MAPK-ERK signaling.

Although previous reports showed that phosphokinases can influence the import and export of distinct coregulatory factors to and from the nucleus (17, 18, 62, 64), we found that

TRAP220/Med1 resides predominantly inside the nucleus in both the presence and absence of activated ERK. Surprisingly, however, we found that activated ERK triggered the translocation of a subset ectopically expressed TRAP220/Med1 protein into the nucleolus (Fig. 8). While the physiological implications of these data remain unclear, our findings are suggestive of a possible novel role for TRAP220/Med1 (and possibly other TRAP/Mediator subunits) in RNA pol I-mediated transcription of the ribosomal DNA gene. Consistent with this view, other transcription factors generally associated with RNA pol II transcription (e.g., TFIIF and TAF1) were recently shown to also mediate RNA pol I transcription (reviewed in reference 15). Moreover, and consistent with a specific role for phosphorylated TRAP220/Med1, nucleolar ribosomal gene expression is markedly activated by ERK (69). On the other hand, activated ERK might sequester TRAP220/Med1 (and possibly other TRAP/Mediator subunits) in the nucleolus as part of a novel regulatory mechanism. Indeed, numerous other nuclear cofactors and enzymes (e.g., ARF, MDM2, telomerase, and cdc14) are functionally regulated via MAPK-dependent sequestration into the nucleolus (reviewed in reference 7). Clearly more experimentation will need to be carried out in order to investigate these possibilities.

While increased protein stability likely plays an important role in the activation of TRAP220/Med1, our findings suggest that MAPK-ERK activates the TRAP220/Med1 protein via additional mechanisms. For example, the phosphorylation-deficient TRAP220/Med1 double-mutant allele had similar stability and accumulated to comparably high levels as the wild-type phosphorylated protein, yet was not nearly as potent in activating transcription (Fig. 9). These results suggest ERK-mediated phosphorylation activates intrinsic TRAP220/Med1 transcriptional activity, possibly by enhancing direct TRAP220/Med1 interactions with other gene-specific activators or other TRAP/Mediator subunits, or possibly by promoting interactions with other novel gene-specific cofactors (e.g., PGC-1 and PARP-1) not stably associated with the TRAP/Mediator complex (40, 59). Alternatively, it is conceivable that phosphorylation might activate an as-of-yet uncharacterized functional activity of TRAP220/Med1 protein that serves to stimulate transcription. In sum, our findings suggest that activated MAPK-ERK signaling pathways trigger regulatory phosphorylation events that regulate the functional activity of TRAP220/Med1 at multiple levels.

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