Tip60 and HDAC7 Interact with the Endothelin Receptor A and May Be Involved in Downstream Signaling*

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He-Jin Lee‡, Miyoung Chun§, and Konstantin V. Kandror¶

From the Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts 02118

Endothelins exert their biological effects through G protein-coupled receptors. However, the precise mechanism of downstream signaling and trafficking of the receptors is largely unknown. Here we report that the histone acetyltransferase Tip60 and the histone deacetylase HDAC7 interact with one of the ET receptors, ETA, as determined by yeast two-hybrid analysis, glutathione S-transferase pull-down assays, and co-immunoprecipitation from transfected COS-7 cells. In the absence of ET-1, Tip60 and HDAC7 were localized mainly in the cell nucleus while ETA was predominantly confined to the plasma membrane. Stimulation with ET-1 resulted in the internalization of ETA to the perinuclear compartment and simultaneously in the efflux of Tip60 and HDAC7 from the nucleus to the same perinuclear compartment where each protein co-localized with the receptor. Upon co-transfection with ETA into COS-7 cells, Tip60 strongly increased ET-1-induced ERK1/2 phosphorylation, whereas HDAC7 had no significant effect. We thus suggest that protein acetylase and deacetylase interact with ETA in a ligand-dependent fashion and may participate in ET signal transduction.

The endothelin family consists of three known members, ET-1, ET-2, and ET-3, all of which have potent vasoconstrictive activity (1, 2). In addition, endothelins control many other cellular processes including gene expression, cytoskeletal reorganization, differentiation, and cell growth (3–9). They can also stimulate secretion of neuropeptides, pituitary hormones, and atrial natriuretic peptide from neural and neuroendocrine cells (10–12) and induce translocation of Glut4-containing vesicles in 3T3-L1 adipocytes (13, 14).

Endothelins interact with the specific G protein-coupled receptors A (ETA) and B (ETB). The extracellular N terminus of these receptors is involved in ligand binding, whereas the intracellular C-terminal region is implicated in downstream signaling, receptor internalization, and desensitization (15–19). ET-1 interacts mainly with ETA, whereas the affinity of ET-2 and ET-3 to this receptor is much lower (20). In contrast, ETB has similar specificity for all three endothelin subtypes (21).

The diversity of endothelin action may be explained not only by the multiplicity of ligands and receptor heterogeneity but also by the ability of the receptor to activate different signaling pathways. Binding of ET-1 to ETA leads to the increase in intracellular calcium levels via activation of phospholipases A2, C, and D and Ca2+ channels (22–26). It has also been documented that ET-1 activates the MAP kinase pathway (27–29). The sequence of biochemical events in which ETA and other heptahelical receptors activate this pathway is not clear and may depend on the cell type. Several possible mechanisms have been proposed. For example, Src and/or other upstream signal-transducing proteins may interact with these receptors directly or through arrestin (30–32). Also, ET-1 and several other ligands may turn on ERK1/2 by “transactivation” of the receptor-tyrosine kinase; in particular, the epidermal growth factor receptor (33–35). Yet another mechanism involves calcium and tyrosine phosphorylation of PYK2 (36, 37). It has also been shown that internalization of G protein-coupled receptors and/or transactivation of receptor-tyrosine kinases is crucial for the activation of the MAP kinase pathway (38, 39).

Binding of ET-1 to ETA causes rapid receptor internalization via clathrin-coated pits and/or caveolae (40, 41). Internalized ETA then traffics through the endosomal pathway to the pericentrilobular recycling compartment that can also be marked by fluorescent-labeled transferrin (42). Eventually, a significant fraction of ETA recycles back to the plasma membrane (42, 43).

To identify novel proteins that interact with ETA and may affect its biological functions, we have utilized the yeast two-hybrid system using the ETA C-terminal region as bait. Two ETA-interacting proteins were identified: a histone acetyltransferase (HAT) Tip60 (44) and a human homolog of mouse HDAC7 (45) that represents a new member of the histone deacetylase (HDAC) family. We have further shown that HAT and HDAC7 proteins undergo ET-1-dependent translocation from nucleus to cytoplasm, where they co-localize and interact with ETA. Moreover, co-expression of Tip60 with ETA significantly potentiated phosphorylation of ERK1/2 in response to ET-1 stimulation. We propose that Tip60 and HDAC7 act as novel components of ETA-mediated signal transduction.

EXPERIMENTAL PROCEDURES

Materials—The following antibodies were used for this work: monochonal anti-Myc antibody (InVitrogen), monoclonal anti-FLAG antibody (Sigma), monoclonal anti-p44/42 MAP kinase (Erk1/2) antibody (New England Biolabs), monoclonal anti-phospho-p44/42 MAP kinase antibody (New England Biolabs), polyclonal anti-ETA antibody (Upstate Biotechnology), Cy3-conjugated rabbit anti-rabbit IgG (Jackson Immuno Research Laboratory), horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG (Sigma) and affinity-purified nonspecific mouse IgG (Sigma). Other reagent grade chemicals were from Sigma.

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‡ Present Address: Parkinson’s Inst., Sunnyvale, CA 94089.
§ Present Address: Millennium Pharmaceuticals, Cambridge, MA 02139.
¶ To whom correspondence should be addressed: 715 Albany St., Boston, MA 02118. Tel.: 617-638-5049; Fax: 617-638-5339; E-mail: kandror@biochem.bumc.edu.

1 The abbreviations used are: ET, endothelin; HAT, histone acetyltransferase; HDAC, histone deacetylase; Tip60, TAT-interactive protein of 60 kDa; MAP, mitogen-activated protein; FITC, fluorescein isothiocyanate; DMEM, Dulbecco’s modified Eagle’s medium; PCR, polymerase chain reaction; GST, glutathione S-transferase; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; RT, room temperature.
Cell Culture—Transformed African monkey kidney cell line COS-7 and rat smooth muscle cell line A10 were maintained in high glucose Dulbecco’s modified Eagle’s medium (MEM, Life Technologies, Inc.) with 10% fetal bovine serum (Hyclone) in a 37 °C, 5% CO2 incubator.

Plasmids—Plasmids (4 μg each) were transfected into COS-7 cells using LipofectAMINE (Life Technologies, Inc.) and cells were harvested or stained after 72 h of transfection. Cells were washed twice with cold PBS and harvested in the extraction buffer (25 mM Tris, pH 7.4, 1% Triton X-100, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 mM PMSF). For the analysis of ERK1/2 phosphorylation, cells were harvested for 10 min. The eluates were analyzed by Western blot.

RESULTS

To identify proteins interacting with ETA, the human brain cDNA library (~8 × 10^6 clones) was screened with the C-terminal region of ETA (amino acids 365–420) as a bait using yeast two-hybrid system. The full selection process yielded two strong positive clones. A BLAST search revealed that the first clone was identical to Tat-interactive protein 60 or Tip60 (44), which belongs to the subfamily of histone acetyltransferases called MYST (49). The second clone encoded a novel protein with a region of strong homology to the conserved catalytic domain of the class II histone deacetylases, which includes HDAC4, HDAC5, and HDAC6 (50). According to a recent report (45), this protein represents a human homolog of mouse HDAC7.

To confirm interaction of Tip60 and HDAC7 with ETA, the yeast two-hybrid system was used. The interactions were further verified by co-immunoprecipitation experiments using transfected COS-7 cells. ETA with Myc epitope and polyHis tag at the C terminal (ETA-Myc) was expressed in COS-7 cells alone or co-expressed with either Tip60 or cHDAC7, both in the pFLAG-CMV2 vector. Cells were homogenized, and ETA-Myc was immunoprecipitated from cell lysates (50 μg) with anti-Myc antibody (1 μg) and protein G-Sepharose (25 μl of packed beads). Immunopurified proteins were analyzed by Western blot with anti-FLAG antibody. A representative result from at least three independent experiments is shown.

To study the intracellular localization of Tip60, HDAC7, and...
This pathway in COS-7 cells transfected with ETAmychis. How-
in Fig. 4, stimulation with ET-1 causes transient activation of
stimulation. As described previously (52, 53) and is also shown
activation of p44/42 MAP kinase (Erk1/2) in response to ET-1
volved in the downstream signaling of ETA, we analyzed the
cytosol upon ET-1 stimulation.

The nucleus of basal cells and is partially relocated to the
presence of endogenous Tip60 in these samples was analyzed
separated into the nuclear and the cytosolic fractions, and the


cells. A10 cells treated and not treated with ET-1 were
in biochemical experiments with non-transfected smooth mus-

acetylation/deacetylation of histones is crucial for the regu-
lation of transcription of many genes. Known substrates for
HATs, however, are not limited to histones and include several
transcription factors, importin, and c-tubulin (54–59). Acety-

Our studies on the localization of Tip60 and HDAC7 revealed
that under basal conditions the major pools of Tip60 and
HDAC7, like other HATs and HDACs, reside in the cell nu-
ucleus. ET-1 stimulation led to the redistribution of Tip60 and
HDAC7 from the nucleus into the perinuclear region where
each protein co-localized with the internalized ETA. Efflux of
Tip60 and HDAC7 from the nucleus may have dual effects.
First, it may result in changes in the transcription of several
ET-1 responsive genes, such as c-myc, c-jun, c-fos, etc. Second,
interaction of Tip60 and HDAC7 with ETA or ETA-associated
proteins may affect cytoplasmic signaling pathways, such as
phosphorylation of ERK.

It was recently shown that other class II histone deacety-

FIG. 2. Co-localization of Tip60 and HDAC7 with ETA by con-
focal laser-scanning microscopy. COS-7 cells expressing ETA and
Tip60 (a) or ETA and HDAC (b) were incubated in the absence or
presence of ET-1 (10 nM) for 10 min at 37 °C. Fixed cells were stained
with rabbit polyclonal anti-ETA antibody and mouse monoclonal anti-
FLAG antibody followed by Cy3-conjugated anti-rabbit IgG for the
detection of Tip60 and HDAC7 (green). A representative result from at least three independent experiments is shown.

ETA, transfected COS-7 cells were incubated in the presence or
in the absence of ET-1. These cells were immunostained with
rabbit polyclonal anti-ETA antibody, which specifically recog-
nizes the N terminus of ETA (46) and with monoclonal anti-
FLAG antibody for the detection of Tip60 and HDAC7. Confocal
images showed that in the absence of ET-1 ETA was
expressed mainly on the cell surface (Fig. 2). Tip60 (Fig. 2a)
and HDAC7 (Fig. 2b) were detected mainly in the cell nucleus
similar to all other known histone acetyltransferases and
deacetylases (50, 51).

Treatment of cells with ET-1 changed the intracellular local-
ization of all three proteins. As expected, ETA was internalized
from the cell surface into the perinuclear region (Fig. 2, a and
b). ET-1 stimulation also caused a dramatic redistribution of
both Tip60 and HDAC7 from the nucleus into the same perinu-
clear region where these proteins co-localized with ETA. Note,
that Fig. 2b shows two cells (bottom middle panel). One cell
(transfected with both HDAC7 and ETA) demonstrates the
redistribution of HDAC7 (green) from the nucleus to the ETA-
containing perinuclear region upon ET-1 administration. An-
other cell (transfected with HDAC7 only) does not show this
effect. Thus, expression of ETA is required for the ET-1-dep-

ET-1-induced efflux of Tip60 from the nucleus was confirmed
in biochemical experiments with non-transfected smooth mus-
cle cells. A10 cells treated and not treated with ET-1 were
separated into the nuclear and the cytosolic fractions, and the
presence of endogenous Tip60 in these samples was analyzed
by Western blot. It is evident in Fig. 3 that Tip60 is localized in
the nucleus of basal cells and is partially relocated to the
cytosol upon ET-1 stimulation.

To determine whether or not Tip60 and/or HDAC7 are in-
volved in the downstream signaling of ETA, we analyzed the
activation of p44/42 MAP kinase (Erk1/2) in response to ET-1
stimulation. As described previously (52, 53) and is also shown
in Fig. 4, stimulation with ET-1 causes transient activation of
this pathway in COS-7 cells transfected with ETAmychis. How-

however, in cells transfected with ETAmychis together with Tip60,
the level of ET-1-induced ERK1/2 phosphorylation is dramati-
cally increased, which suggests that Tip60 may be directly
involved in the acute downstream signaling of ETA. Deletion of
the C-terminal region of ETA abolishes the effect of Tip60,
directing that direct interaction is required for the elevation of
ERK1/2 phosphorylation (data not shown).

The effect of exogenously expressed HDAC7 on the ERK1/2
pathway was inconsistent. In some cases, ERK1/2 phosphoryl-
ation was attenuated, whereas other experiments showed no
effect (data not shown). However, we were never able to detect
an increase in ERK1/2 phosphorylation in ETAmychis- and
HDAC7-transfected cells.

DISCUSSION

Acetylation/deacetylation of histones is crucial for the regu-
lation of transcription of many genes. Known substrates for
HATs, however, are not limited to histones and include several
transcription factors, importin, and c-tubulin (54–59). Acety-
lation affects DNA-binding activity, protein stability, and pro-
tein-protein interaction (51). Therefore, acetylation and
deacetylation may represent general post-translational modi-
fications that take place in both nuclear and cytosolic compart-
ments of the cell (reviewed in Ref. 51). In fact, we have shown
that Tip60, a HAT family protein, can potentiate the effect of
ET-1 on ERK1/2 phosphorylation, which suggests that the
acetylation event may also regulate receptor-mediated signal-
pathways.

Our studies on the localization of Tip60 and HDAC7 revealed
that under basal conditions the major pools of Tip60 and
HDAC7, like other HATs and HDACs, reside in the cell nu-
ucleus. ET-1 stimulation led to the redistribution of Tip60 and
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interaction of Tip60 and HDAC7 with ETA or ETA-associated
proteins may affect cytoplasmic signaling pathways, such as
phosphorylation of ERK.

It was recently shown that other class II histone deacetyl-
lases, HDAC4 and 5, shuttle between the nucleus and the cytosol (60, 61). The physiological importance of the nucleo-cytoplasmic distribution of HDAC4 and 5 was emphasized in a recent report demonstrating that muscle differentiation can be controlled by nuclear export of HDAC4 and 5 in response to calcium/calcmodulin-dependent protein kinase signaling (62). On the other hand, constitutive activation of the MAP kinase pathway in Ras- or MEK1-transfected cells results in the increased nuclear localization of HDAC4 (63). ERK1/2 was shown to be involved in other regulatory events. For example, we suggest that Tip60 may protect ETA from degradation by phosphorylation of HDAC4 (63). With the experimental system established in our laboratory, future work should be able to ascertain the role of HDAC4 in the cytosolic compartment and induce their efflux from the nucleus. There is evidence that protein 14-3-3 may play an important role in cytoplasmic retention of HDAC4 and 5 (61, 64). With the experimental system established in our laboratory, future work should be able to ascertain the role of 14-3-3 or other candidate proteins in ET-1-induced nucleo-cytoplasmic transport of Tip60 and HDAC7.

In addition to the MAP kinase pathway, Tip60 and HDAC7 may be involved in other regulatory events. For example, we have noticed that transfection of cells with Tip60 increases (or with HDAC7 decreases) the level of ETA (data not shown). This suggests that Tip60 may protect ETA from degradation by re-routing the receptor from lysosomes to the recycling pathway. We are now trying to follow up on these experiments to uncover the potential role of acetylation/deacetylation in the regulation of receptor trafficking and stability.

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