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Regulation of Nur77 protein turnover through acetylation and deacetylation induced by p300 and HDAC1

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ABSTRACT

Although the roles of Nur77, an orphan member of the nuclear hormone receptor superfamily, in the control of cellular proliferation, apoptosis, inflammation, and glucose metabolism, are well recognized, the molecular mechanism regulating the activity and expression of Nur77 is not fully understood. Acetylation of transcription factors has emerged recently as a major post-translational modification that regulates protein stability and transcriptional activity. Here, we examined whether Nur77 is acetylated, and we characterized potential associated factors. First, Nur77 was found to be an acetylated protein when examined by immunoprecipitation and western blotting using acetyl protein-specific antibodies. Second, expression of p300, which possesses histone acetyltransferase activity, enhanced the acetylation and protein stability of Nur77. Treatment with a histone deacetylase (HDAC) inhibitor, trichostatin A, also increased Nur77 acetylation. Among the several types of HDACs, HDAC1 was found as the major enzyme affecting protein level of Nur77. HDAC1 decreased the acetylation level, protein level, and transcriptional activity of Nur77. Interestingly, overexpression of Nur77 induced expression of both p300 and HDAC1. Finally, the expression of Nur77 increased along with that of p300, but decreased with induction of HDAC1 after treatment with epithelial growth factor, nerve growth factor, or 6-mercaptopurine, suggesting that the self-control of the acetylation status contributes to the transient induction of Nur77 protein. Taken together, these results demonstrate that acetylation of Nur77 is modulated by p300 and HDAC1, and suggest that acetylation is an important post-translational modification for the rapid turnover of Nur77 protein.

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1. Introduction

Nur77 (also called NR4A1, TR3, NAK-1, and NGFI-B) plays important roles in the regulation of cellular proliferation, apoptosis, and energy metabolism. It belongs to the NR4A (nuclear receptor group 4A) subfamily of nuclear hormone receptors and its DNA-binding domain (DBD) shows 90% homology with other subfamily members, Nurr1 and Nor-1 [1,2]. All of NR4A subfamilies are encoded by immediate early genes whose expression is induced in response to a variety of signals including mitogens and cellular stress [3–5]. Interestingly, Nur77 is activated by ligand independently, which may be explained by conserved, tightly packed, and bulky hydrophobic residues occupying the ligand-binding domain (LBD) of NR4A groups [6,7]. Both Nur77 mRNA and protein appear to be unstable *in vivo*, and thereby their expression is transiently induced by stimulation [3,5,8]. Because Nur77 acts independently to a ligand and is unstable *in vivo*, the regulation of its expression and post-translational modification are thought to be important to its function.

Post-translational modification is an important process in the regulation of nuclear hormone receptors. Phosphorylation, methylation, acetylation, ADP ribosylation, glycosylation, ubiquitination, sumoylation, and neddylation affect a broad spectrum of protein structures and functions [9]. Acetylation has emerged recently as a central step in the regulatory modification processes, and more than 100 proteins have been found to be acetylated. Acetylation commonly occurs at a lysine residue, and the functional consequences of acetylation are diverse. Acetylation affects the nuclear localization, stability, transcriptional activity, DNA binding, and interactions with other cofactors and proteins [10,11]. Protein acetylation is regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs), which have opposite roles [12–14]. p300/CBP possess strong acetylase function on both histone and non-histone proteins. HDACs are classified

Abbreviations: HAT, histone acetyltransferase; HDAC, histone deacetylase; IP, immunoprecipitation; TSA, trichostatin A; DBD, DNA-binding domain; LBD, ligand-binding domain; NGF, nerve growth factor; EGF, epithelial growth factor; PMA, phorbol myristate acetate; CHX, cycloheximide; 6-MP, 6-mercaptopurin.

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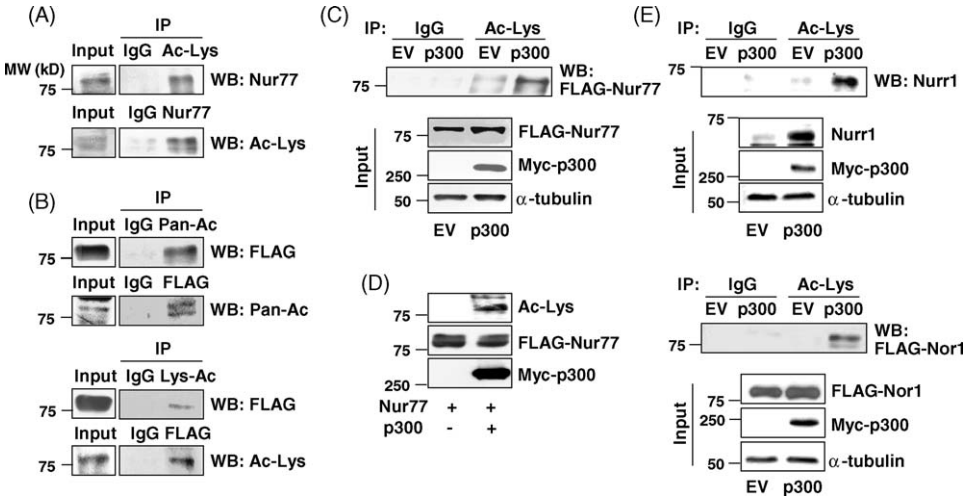

Fig. 1. Nur77 is acetylated in the presence of p300. (A) 2 mg of HepG2 cell lysates was immunoprecipitated (IP) using anti-Ac-lysine (Lys) or anti-Nur77 antibody, and probed by western blotting (WB) using anti-Nur77 or anti-Ac-lysine antibody, respectively. Immunoprecipitation with normal IgG was used as control. The expression of Nur77 in cell lysates was analyzed by western blotting as input. (B) NIH3T3 cells were transfected with FLAG-Nur77. 700 μg of whole-cell lysates were immunoprecipitated using anti-pan-Ac, anti-Ac-lysine, or anti-FLAG antibody and then probed by the indicated antibodies. (C) HEK293 cells were transfected with FLAG-Nur77 together with Myc-p300 or empty vector (EV). 500 μg of whole-cell lysates were immunoprecipitated using anti-Ac-lysine or normal IgG antibodies, and then probed by anti-FLAG antibody. (D) *In vitro* acetylation assay was performed as described in Section 2. Purified FLAG-Nur77 and Myc-p300 were incubated in the presence of acetyl-CoA for 1 h at 37 °C. The levels of acetylated and total recombinant Nur77 proteins and p300 were shown by western blot analysis. (E) HEK293 cells were transfected with pCMX-Nurr1 (upper) or FLAG-Nor-1 (lower) together with Myc-p300 or empty vector (EV) as indicated. 500 μg of whole-cell lysates were immunoprecipitated using anti-Ac-lysine or normal IgG antibodies, and then probed by anti-Nurr1 or anti-FLAG antibody.

into four classes and two families: classical (classes I, II, and IV) and silent information regulator 2 (Sir2)-related protein (sirtuin) families (class III) [14]. Because HATs and HDACs have been linked to diverse cellular factors of biological importance, many strategies have been used in therapeutic intervention.

Nur77 is also a target of post-translational modification [15]. Phosphorylation of Nur77 is induced by natural growth factors such as nerve growth factor (NGF) and epithelial growth factor (EGF), and chemicals such as phorbol myristate acetate (PMA) [16–19]. Several kinases, such as Jun N-terminal kinase, p90 ribosomal S6 kinase, and protein kinase B are known to phosphorylate Nur77 [18–20]. The fact that Nur77 interacts directly with coactivators that have HAT activity [21–23], suggests that Nur77 is acetylated by these cofactors. Here we examined whether Nur77 is an acetylated protein and we characterized potential associated coregulators. We also aimed to identify the role of acetylation in the regulation of Nur77 function.

2. Materials and methods

2.1. Cell and cell culture

Human hepatocellular carcinoma cell line, HepG2 (ATCC HB-8065), human cervical carcinoma cell line, HeLa (ATCC CCL-2) and mouse fibroblast cell line, NIH3T3 (ATCC CRL-1658) were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS). Human embryonic kidney cell line, HEK293 (ATCC CRL-1573) was maintained in Iscove's Modified Dulbecco's Medium containing 10% FBS. Rat pheochromocytoma cell line PC12 (ATCC CRL-1721) was maintained in Dulbecco's modified Eagle's medium containing 10% horse serum and 5% FBS. Cells were incubated at 37 °C in 5% CO_2/95% air. HeLa cells were subjected to serum deprivation for 20 h and PC12 cells had serum starvation for 4 h before treating growth factors. EGF and NGF were purchased from Sigma (St. Louis, MO, USA) and Invitrogen Corporation (Carlsbad, CA, USA), respectively. Cycloheximide (CHX), MG132, and 6-mercaptopurin (6-MP) were purchased from Sigma, Calbiochem (Darmstadt, Germany), and Fluka Ltd. (Buchs, Switzerland), respectively.

2.2. Plasmids, siRNA and transient transfection

The reporter NurRE-pomc-Luc and eukaryotic expression vectors encoding FLAG-Nur77, pCMX-Nurr1, FLAG-HDAC1, -2, -4, and -7, and Myc-p300 were described previously [23–26]. FLAG-Nor-1 was constructed by inserting a PCR-amplified full-length

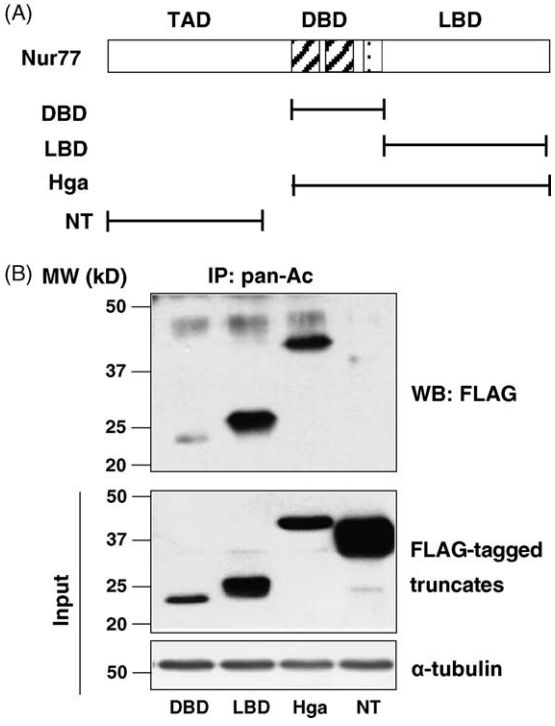

Fig. 2. Identification of acetylated domains of Nur77. (A) Schematic representation of the truncated mutants of Nur77. (B) Plasmids encoding the FLAG-tagged Nur77 truncates were transfected into NIH3T3 cells. 24 h after transfection, cell lysates were obtained, immunoprecipitated by anti-pan-Ac antibody and analyzed by western blotting with anti-FLAG antibody. The expression of FLAG-tagged Nur77 truncates was analyzed by western blotting as input.

mouse Nor-1 fragment into the EcoRI/BamHI site of the 3XFLAG-CMV-10 vector (Sigma). Transient expression of proteins and reporter gene analysis were as previously described [27]. The siRNA duplexes targeting p300, HDAC1, and nonspecific siRNA (si-GFP) were transfected into cells as previously described [26,28].

2.3. Western blotting and immunoprecipitation

Western blotting and immunoprecipitation were performed as previously described using specific antibodies against Nur77 (BD Bioscience Pharmingen, San Diego, CA, USA), FLAG (Sigma), Nurr1, p300, HDAC1, Myc, normal IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and α-tubulin (Calbiochem) [26,27]. To detect acetylated proteins, 500 μg whole-cell lysates were incubated with 1 μg anti-pan-acetyl (Ac) antibody (Santa Cruz Biotechnology), or anti-Ac-lysine antibody (Cell Signaling Technology, Inc., Danvers, MA, USA), precipitated by adding 30 μl protein-A or G agarose slurry (Upstate, Billerica, MA, USA), and then probed with anti-FLAG, anti-Nur77 antibody, or normal IgG. To detect ubiquitinated proteins, whole-cell lysates were immunoprecipitated by 1 μg anti-FLAG antibody, and probed using anti-ubiquitin antibody (Santa Cruz Biotechnology).

2.4. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was prepared using the Easy-Blue™ total RNA extraction kit (iNtRON Biotechnology, Korea) according to the manufacturer's instructions. RT-PCR was performed as described

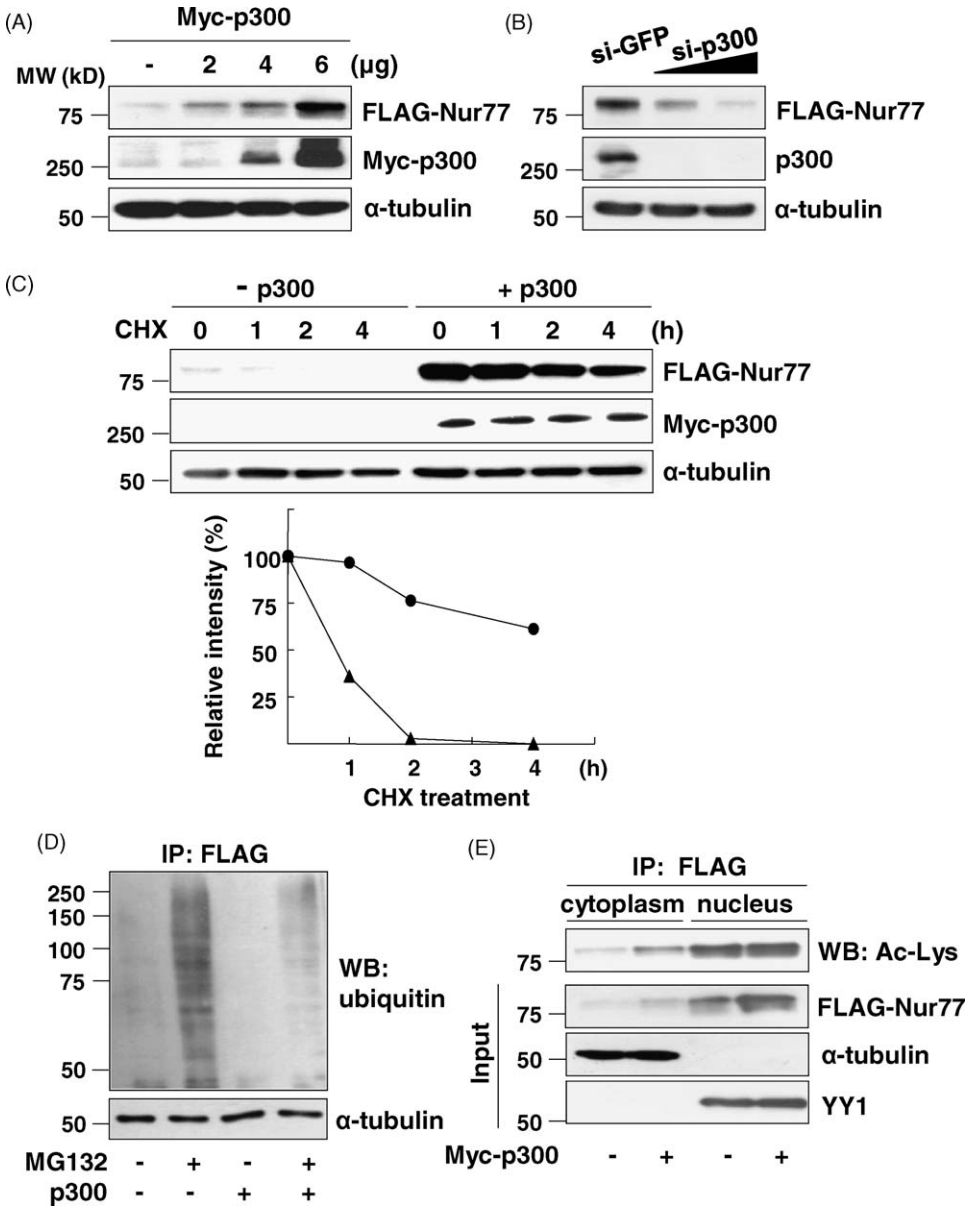

Fig. 3. p300 increases stability of Nur77 protein. (A) HeLa cells were transfected with FLAG-Nur77 and Myc-p300. The expression of proteins was analyzed by western blotting using anti-FLAG or anti-Myc antibody. (B) HeLa cells were transfected with FLAG-Nur77, si-p300 or nonspecific siRNA (si-GFP). The expression of proteins was analyzed by western blotting. (C) HeLa cells were transfected with FLAG-Nur77 together with Myc-p300 (●) or empty vector (▲). 24 h after transfection, cells were treated with 10 μM cycloheximide (CHX) for the indicated time periods. The expression of protein was analyzed by western blotting (upper). The density of FLAG-Nur77 protein band was determined using image analysis system. The values were normalized to that of α-tubulin and expressed as percent of the CHX-untreated control (lower). (D) HeLa cells were transfected with FLAG-Nur77 together with Myc-p300 or empty vector. 24 h after transfection, cells were treated with 10 μM MG132 for 2 h. 500 μg of whole-cell lysates were immunoprecipitated (IP) using anti-FLAG antibody, and probed by anti-ubiquitin antibody. (E) Plasmid encoding the FLAG-Nur77 was transfected into HEK293 cells together with or without p300. 24 h after transfection, the nucleus and the cytoplasmic fractions were prepared, immunoprecipitated (IP) using anti-FLAG antibody and probed by western blotting (WB) using anti-Ac-lysine antibody. The expression of Nur77 in the fractions was analyzed by western blotting as input.

previously using specific primers for p300 and HDAC1 [27,28]. The expression of β-actin was monitored as a control.

2.5. In vitro acetylation assay

Briefly, HEK293 cells were transfected with expression vector for FLAG-Nur77 or Myc-p300 [29]. FLAG-Nur77 and Myc-p300 proteins in the transfected HEK293 cells were immunoprecipitated using anti-FLAG or anti-Myc antibody. 1 mg each purified immunoprecipitated proteins were incubated in 20 μl reaction buffer (500 μM Tris pH 7.9, 10% glycerol, 1 μM DTT, 10 mM sodium butyrate, and 20 μM acetyl-CoA) for 1 h at 37 °C. Acetylation of Nur77 was analyzed by SDS-PAGE followed by western blotting with anti-Ac-lysine antibody.

3. Results

3.1. Nur77 is acetylated by p300

Acetylation has emerged as a major post-translational modification of various transcription factors, which changes biochemical functions such as transcriptional activity and subcellular localization [9]. Because acetylation modification of Nur77 has not been studied for Nur77, we first examined whether Nur77 protein is acetylated by immunoprecipitation and subsequent western blotting using specific antibodies against acetylated proteins such as anti-pan acetyl and anti-Ac-lysine antibodies. Endogenously present Nur77 in HepG2 immunoprecipitated by anti-Nur77 antibody and probed by anti-Ac-lysine antibody, showed clear evidence of the acetylation of Nur77 protein. The acetylation of Nur77 was confirmed by reciprocal immunoprecipitation and western blotting (Fig. 1A). Acetylation of exogenously introduced FLAG-tagged Nur77 was also detected by anti-FLAG and anti-acetyl protein antibodies (Fig. 1B). Next, we explored the possibility that Nur77 is acetylated by p300, which interacts with Nur77 *in vivo* [21–23]. As shown in Fig. 1C, overexpression of p300 in HeLa cells induced the acetylation of Nur77 *in vivo*. An *in vitro* acetylation assay employing Nur77 and p300 purified by immunoprecipitation with corresponding antibodies showed that the amount of acetylated Nur77 increased in the presence of p300 protein (Fig. 1D). Taken together, these results indicate that Nur77 is present in the acetylated form *in vivo* and the acetylation is

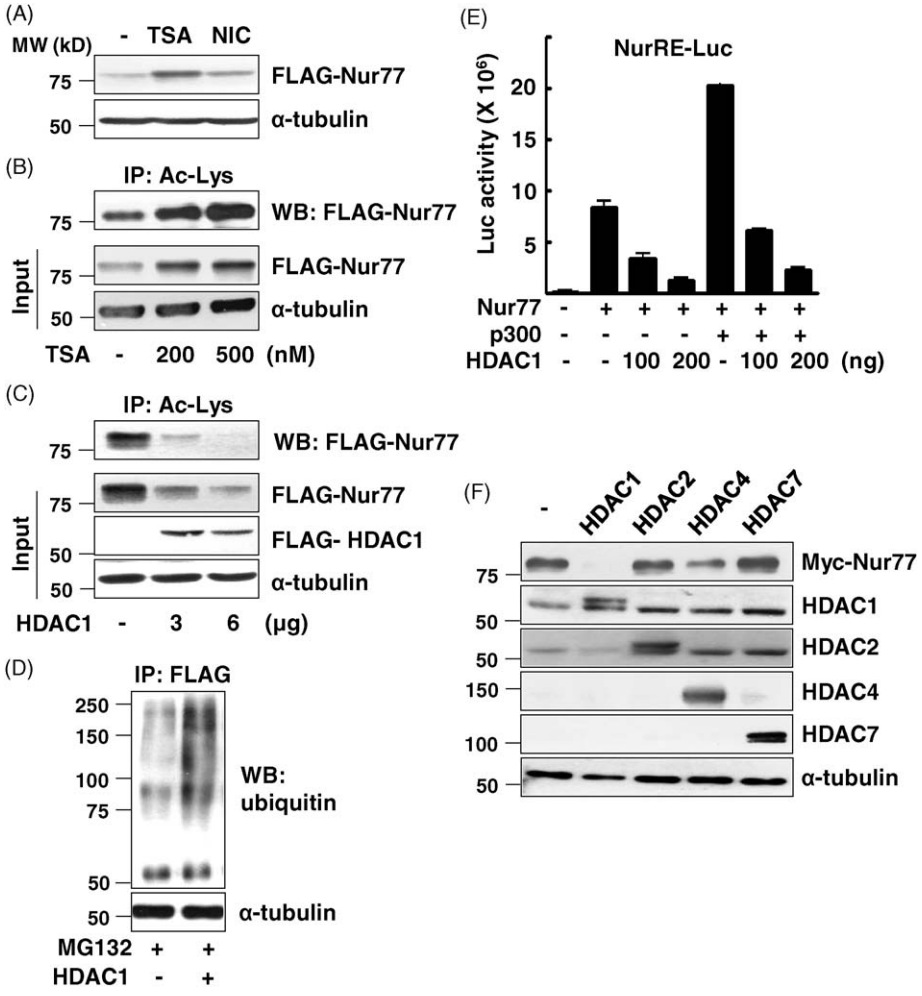

Fig. 4. Nur77 is deacetylated in the presence of HDAC1. (A) HeLa cells were transfected with FLAG-Nur77 and then treated with 500 nM TSA or 10 nM nicotinamide (NIC) for 16 h. The expression of proteins was analyzed by western blotting. (B) HeLa cells were transfected with FLAG-Nur77 and then treated with or without TSA for 24 h. 500 μg of whole-cell lysates were immunoprecipitated (IP) using anti-Ac-lysine antibody, and then probed by anti-FLAG antibody. The expression of proteins was analyzed by western blotting as input. (C) HeLa cells were transfected with FLAG-Nur77 and the indicated amount of FLAG-HDAC1. 500 μg of whole-cell lysates were immunoprecipitated using anti-Ac-lysine antibody, and then probed by anti-FLAG antibody. The expression of proteins was analyzed by western blotting as input. (D) HeLa cells were transfected with FLAG-Nur77 together with Myc-HDAC1. 24 h after transfection, cells were treated with 10 μM MG132 for 2 h. 1000 μg of whole-cell lysates were immunoprecipitated (IP) using anti-FLAG antibody, and probed by anti-ubiquitin antibody. (E) HEK293 cells were transfected with NurRE-Luc reporter and expression vector for Nur77, p300 or HDAC1 as indicated. Luciferase activity was normalized for transfection efficiency by corresponding β-galactosidase activity. The bars represent the mean ± S.D. of three independent experiments. (F) HeLa cells were transfected with Myc-Nur77 and the indicated HDACs. The expression of proteins was analyzed by western blotting.

catalyzed by p300. Similarly, we observed that Nurr1 and Nor-1 were also acetylated in the presence of p300 (Fig. 1E). Immunoprecipitation of the FLAG-tagged Nur77 domains using anti-acetyl protein antibodies revealed that Nur77 was acetylated mainly in the LBD (23) (Fig. 2).

3.2. p300 increases the protein stability of Nur77

p300 induces acetylation of non-histone proteins such as c-Myc and ATF-4, and thereby enhances the stability of these proteins [30,31]. Therefore, we tested whether p300 alters the protein level of Nur77. Exogenously introduced p300 increased the protein level of Nur77 in a dose-dependent manner (Fig. 3A). Knock down of p300 by transfection with siRNA markedly decreased in the expression of Nur77. These results indicate that p300 plays an important role in the control of Nur77 protein level (Fig. 3B). The kinetics of Nur77 degradation after cycloheximide treatment showed that about 60% of Nur77 protein remained in the presence of p300, whereas Nur77 protein degraded completely in the absence of p300 after 4 h of cycloheximide treatment (Fig. 3C). Although Nur77 was ubiquitinated in the presence of MG132, ubiquitination was barely detected in the presence of p300 (Fig. 3D). To examine whether subcellular localization of Nur77 is altered after acetylation, the amount of acetylated Nur77 was measured in the subcellular fractions. As shown in Fig. 3E, most of the acetylated Nur77 was present in the nucleus in the presence or absence of p300, indicating that acetylation does not affect the subcellular localization of Nur77. Together, these data indicate that p300 enhances the protein stability of Nur77, by inhibiting ubiquitin/proteasome-mediated degradation of Nur77.

3.3. HDAC1 induces the deacetylation of Nur77

Next, we examined whether HDACs are involved in the deacetylation of Nur77. Trichostatin A (TSA), a classical HDAC inhibitor, strongly increased the protein level of Nur77, whereas nicotinamide, an inhibitor of SIRT, did not, suggesting that HDACs are involved in the deacetylation of Nur77 (Fig. 4A). TSA treatment increased the acetylation level of Nur77 (Fig. 4B). Further overexpression of HDAC1 strongly decreased the acetylation level of Nur77 (Fig. 4C). Ubiquitination of Nur77 was further enhanced in the presence of HDAC1, indicating that deacetylation of Nur77 is associated with ubiquitin-mediated degradation (Fig. 4D). HDAC1 decreased the p300-induced transcriptional activity of Nur77 when measured by a reporter encoding NurRE (Fig. 4E). Other HDACs, such as HDAC2 and HDAC7, did not alter the Nur77 protein level, and HDAC4 induced a mild decrease (Fig. 4F).

3.4. Turnover of Nur77 protein is regulated by p300 and HDAC1

Interestingly, we observed that overexpression of Nur77 increased both the transcript and protein levels of p300 (Fig. 5A). This result suggests that a positive feedback mechanism exists for the activation of Nur77. Similar to p300, HDAC1 expression was elevated at both the transcript and protein levels in the presence of Nur77, indicating that the deacetylation of Nur77 is also controlled by Nur77 by inducing HDAC1 (Fig. 5B). Together these results suggest that the induction of p300 and HDAC1 by Nur77 plays a role in the turnover of Nur77 protein itself.

Nur77 protein is transiently induced by growth factors such as EGF and NGF [5]. 6-Mercaptopurine (6-MP) increases the protein level and the transactivation function of Nur77 [27,32]. We hypothesized that autoregulation of Nur77 at the protein level by acetylation and deacetylation induced by p300 and HDAC1 contributes to the transient induction of Nur77 protein. To examine this possibility, we monitored the expression pattern of

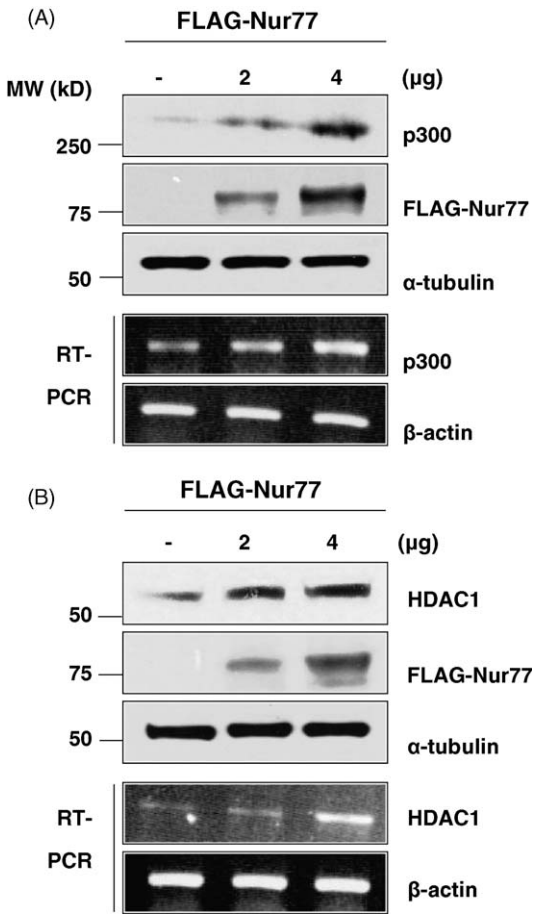

Fig. 5. Nur77 increases expression of Nur77 and HDAC1 at transcription-level. (A) HeLa cells were transfected with the indicated amount of FLAG-Nur77. The protein and mRNA expression of FLAG-Nur77 and p300 were analyzed by western blotting (WB) and RT-PCR, respectively. (B) HeLa cells were transfected with the indicated amount of FLAG-Nur77. The protein and mRNA expressions of FLAG-Nur77 and HDAC1 were analyzed by western blotting and RT-PCR, respectively.

p300 and HDAC1 along with Nur77 after treatment with various activators. As expected EGF, NGF, and 6-MP induced the transient expression of Nur77 protein, although the time course differed between the stimuli. Interestingly, the induction profile of p300 was similar to that of Nur77, whereas expression of HDAC1 increased after the peak in Nur77 expression (Fig. 6A). The expression profiles of Nur77, p300, and HDAC1 may provide evidence for the autoregulated turnover of Nur77 after activation by various activators (Fig. 6B).

4. Discussion

Increasing evidence indicates that protein is post-translationally regulated by acetylation [10]. Our study is the first to show that Nur77 is acetylated *in vivo* and *in vitro* by p300 using acetylated specific antibodies, *i.e.*, anti-Pan-acetyl and anti-Lys-Ac antibodies (Fig. 1). Previously, Li et al. reported that Nur77 is not acetylated by p300, although two regions of Nur77 protein, the DBD and aa 439–521 in the LBD, are capable of binding to p300 [22]. Wang et al. also showed that NR4A and NR2B lack the acetylation motif KXKK/RXKK, which is conserved between species and the phylogenetically related nuclear receptors of estrogen receptor α [33]. In contrast to these findings, our domain-mapping study showed that Nur77 is acetylated mainly in the LBD (Fig. 2). The reason for this contradictory result is not understood at present, and the identification of the acetylation motif is required for further investigation.

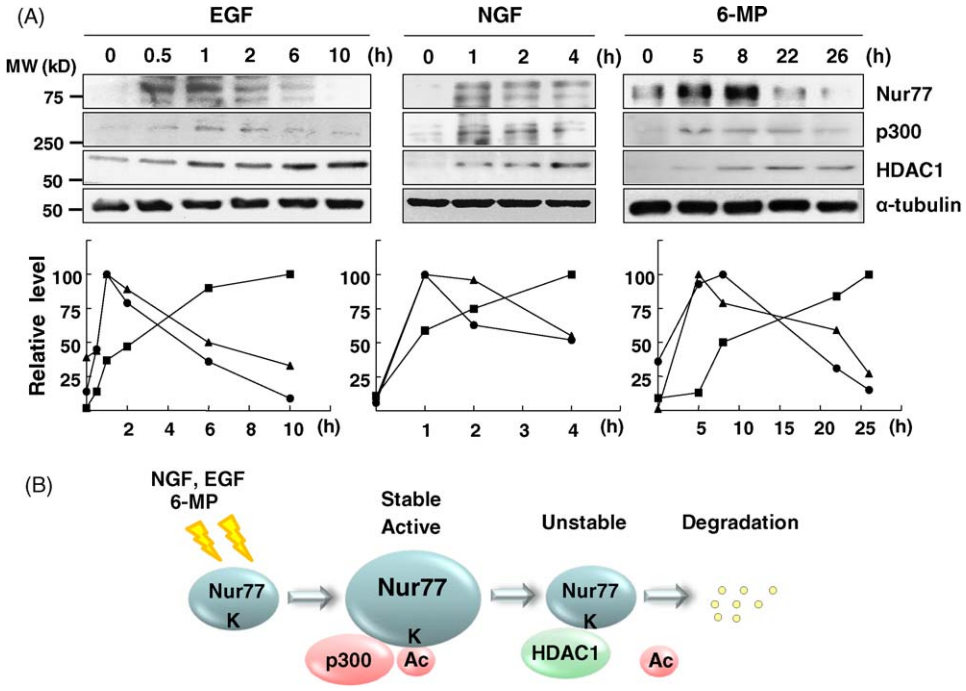

Fig. 6. Kinetics of Nur77 expression along with p300 and HDAC1 expression after treatment of Nur77 activator. (A) HeLa, PC12 or HepG2 cells were treated with 200 ng/ml EGF, 100 ng/ml NGF, 50 μM 6-MP, respectively. The expression of Nur77, p300, HDAC1 and α-tubulin was analyzed by western blotting (upper). The intensity of Nur77 (●), p300 (▲) and HDAC1 (■) was determined using an image analysis system (lower). The values were normalized to that of α-tubulin. (B) Schematic model for regulation of Nur77 protein turnover by p300 and HDAC1.

We found that the protein stability of Nur77 was enhanced when Nur77 was acetylated by p300 (Fig. 3). This observation is similar to the p300-induced acetylation of p53, Smad7, and ATF4, which blocks the ubiquitination and degradation of these proteins [24,31,34,35]. To our knowledge, the nuclear receptors such as estrogen receptor α, androgen receptor and liver X receptor α, are present as acetylated forms [13,33,36,37]. Acetylation modification of these receptors is associated with the subcellular localization, transactivation, or other post-translational modifications such as ubiquitination. The subcellular localization of Nur77 is one of critical factors that determine Nur77 function [38]. Although most Nur77 is present in the nucleus, apoptotic stimuli induce the translocation of Nur77 from the nucleus to the mitochondria, which leads to cytochrome c release into the cytoplasm. Acetylation modification did not alter the subcellular localization of Nur77 in our study (Fig. 3E). Although acetylated Nur77 was present in the nucleus and cytoplasm, most Nur77 acetylation is likely to occur in the nucleus where both p300 and Nur77 are predominantly located. How the acetylated Nur77 moves to cytoplasm and its role in the cytoplasm should be investigated. Previously, we and others showed that physical interaction of Nur77 with p300 results in transcriptional activation, and that the interaction of Nur77 with the corepressor complex containing HDACs is associated with transcription repression [21,22,39]. Therefore, p300 and HDAC1 play a dual function in regulating Nur77 through the acetylation and deacetylation of Nur77 itself and the histones located near the target genes of Nur77, *i.e.*, regulation of protein stability and transactivation function.

In many cases, p300 induces protein stabilization by competing with ubiquitination on the same residue, and acetyltransferases and deacetylases are important for the regulation of protein turnover [40]. For example, p300 induces the acetylation and subsequent stabilization of p53 protein, which is negatively regulated by MDM2-HDAC1-mediated deacetylation and further ubiquitination-mediated degradation [24,34]. Other examples might be Smad7 and ATF4, which are acetylated by p300 and thereby stabilized by inhibiting its ubiquitination [31,35]. Nur77 is known as an early response gene, whose expression is transiently induced by growth factors [5,19]. In our study, the expression of Nur77 increased along with that of p300, but decreased with induction of HDAC1, suggesting that the control of the acetylation status by p300 and HDAC1, contributes to the transient induction of Nur77 protein (Fig. 6). It is well known that Nur77 is induced at the transcriptional level after physiological stimulation, and the regulation of its protein stability may contribute partly to the fine tuning of Nur77 turnover. Interestingly, transcripts of p300 and HDAC1 are induced by Nur77 itself, indicating that the p300/HDAC1-mediated regulation of Nur77 protein stability is controlled automatically (Fig. 5). In addition, it was reported that HDAC1 repressed HAT activity of p300 by deacetylating p300 [41]. Nur77 also negatively regulates the HAT activity of p300 by blocking a conserved transcriptional adapter motif [22]. These observations together with ours suggest that a complicated network of mechanisms is associated with turnover of Nur77 function and that p300/HDAC1-induced dynamic changes in the acetylation of Nur77 protein play an important role in the control of Nur77 protein turnover.

Acknowledgments

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