Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 332 (2005) 33–36
BBRC
www.elsevier.com/locate/ybbrc

The conserved WRPW motif of Hes6 mediates proteasomal degradation ☆

Seon Ah Kang [a], Jae Hong Seol [b], Jaesang Kim [a,*]

[a] *Division of Molecular Life Sciences and Center for Cell Signaling Research, Ewha Womans University, 11-1 Daehyun-dong, Seodaemun-gu, Seoul 120-750, Korea*
[b] *Seoul National University School of Biological Sciences, San 56-1, Shillim-dong, Kwanak-gu, Seoul 151-742, Korea*

Received 14 April 2005
Available online 27 April 2005

Abstract

Hes6 belongs to a subfamily of basic helix–loop–helix transcription factors that includes Drosophila *Hairy* and *Enhancer of split* genes. Like other members of the family, Hes6 features the WRPW motif which is consisted just of four amino acids at its C-terminus. Here, we show that WRPW motif deletion mutant protein is substantially stabilized in comparison to the full length protein and that the enhanced stability is due to its resistance to proteasomal degradation. The WRPW motif also appears to be sufficient for acceleration of proteolysis as its fusion to two heterologous proteins, the green fluorescent protein (GFP) of *Aequoria victoria* and Gal4 DNA binding domain of *Saccharomyces cerevisiae*, significantly destabilized the proteins. These findings demonstrate a novel function of this conserved motif as a degradation signal and raise the possibility of utilizing it for controlling the level of ectopically expressed gene products.

Keywords: Proteolysis; Proteasome; Ubiquitination; Hes6; WRPW

Hes genes constitute a conserved basic helix–loop–helix transcription factor subfamily with a unique combination of molecular characteristics [1–3]. They have a highly conserved basic helix–loop–helix domain, a Hes gene family unique domain known as the orange domain, and the WRPW motif at the C-terminus. Hes genes are known to bind to the so-called N-box in a dimeric form and interact with various co-factors including Groucho/TLE co-repressors to regulate specific target genes. WRPW motif in fact is responsible for direct interaction with Groucho/TLE proteins and thereby functions as the de facto repressor domain of this family of transcription factors [4–6].

Hes genes function in a variety of developmental programs often as a downstream effector of Delta-Notch signaling [2,7]. One of their more recently defined roles is in the somite formation in which at least two Hes genes, Hes1 and Hes7, show a cyclical pattern of expression coinciding with the formation of individual somites [8–10]. Importantly, Hes1 has been shown to be degraded in a ubiquitin–proteasome dependent manner [9]. Furthermore, derivatives of Hes7 whose potential ubiquitination target lysine residues are mutated show deregulated somite formation [10]. The implication of such observations is that Hes1 and Hes7 proteins undergo a rapid degradation in a tightly regulated manner and that such degradation is an integral part of the somitogenesis.

Hes6, a member of the Hes gene family, contains all of the essential molecular features of this family [11,12]. It is expressed in a variety of precursor cells

☆ *Abbreviations:* Hes, *Hairy* and *Enhancer of split*; TLE, transducin-like element; IRES, internal ribosome entry site; EGFP, enhanced green fluorescent protein.

* Corresponding author. Fax: +82 2 3277 3760.
E-mail address: jkim1964@ewha.ac.kr (J. Kim).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.04.089

including myoblasts and neuroblasts. It is known to promote differentiation of these precursor cells in opposition to other Hes genes including Hes1 [11–14]. One of the recombinant derivatives of Hes6, the WRPW motif deletion mutant, which had been expected to function as a dominant negative variant to Hes6, showed a much higher level of protein than the wild type. Here, we show that this difference is due to a significantly lowered level of protein turnover by 26S proteasome. We further show that WRPW-motif can function as a degradation signal when appended to heterologous proteins. These observations raise the possibility of utilizing this simple motif and its variants to regulate the turnover rate and thus the steady state level of exogenous proteins.

Materials and methods

Plasmids. Construction of expression vector plasmids was performed using standard molecular cloning techniques. The full length sequence of Hes6 was amplified from a cDNA plasmid and inserted upstream of IRES-GFP cassette in a shuttle vector. The DNA fragment containing the wild type Hes6 sequence and IRES-GFP (Hes6WT-IRES-GFP) was then cloned into pcDNA3.1. To generate the WRPW motif deletion mutant of Hes6 with IRES-GFP (Hes6MT-IRES-GFP), a downstream PCR primer bearing a single nucleotide substitution that turns W221 codon (TGG) into a stop codon (TGA) was used. To generate FLAG-Hes6WT and FLAG-Hes6MT, Hes6WT and Hes6MT fragments were PCR amplified and cloned in-frame into a FLAG epitope containing expression vector, pFLAG-CMV2 (gift of S.Y. Lee). EGFP and EGFP-WRPW fragments were PCR amplified from a cDNA plasmid using a common upstream primer and a pair of downstream primers with single nucleotide difference resulting in either the full length EGFP or mutant EGFP with the WRPW motif appended. The PCR products were used to generate expression plasmids in pcDNA3.1. HA-Gal4DB and HA-Gal4DB-WRPW were generated using a similar strategy with the HA-epitope tag in the N-terminus in pcDNA3.1. HA-ubiquitin expression plasmid was a gift from J.B. Yoon. Details of cloning procedures and the sequence of PCR primers can be provided upon request.

Antibodies. The following primary antibodies were used for immunoblotting and immunoprecipitation: β-actin (mouse monoclonal; Sigma), GFP (rabbit IgG fraction; Molecular Probes), GFP (mouse monoclonal; BD Sciences), FLAG-epitope (mouse monoclonal; Sigma), and HA-epitope (rat monoclonal; Roche). In order to generate rabbit polyclonal antibody to Hes6, the peptide DDLCSDLEEIPEAELNRVC was synthesized and coupled to keyhole limpet hemacyanin prior to injection. Antibody production was carried out by Covance Research Products (Richmond, CA, USA).

Cell culture, transfection, and immunoblotting. HeLa and 293T cells were cultured in DMEM supplemented with 10% FBS, 1% penicillin, and 1% streptomycin. The cells were seeded 1 day prior to transfection at the density of 5×10^5 cells per 60 mm dish. The transient transfection using Effectene (Qiagen) was carried out with 1 μg of expression vector plasmids described above except for the experiment described in Fig. 2B in which a total of 2 μg was used. For immunoblotting assays, cells were scraped and lysed in SDS gel loading buffer 24 h after transfection. Where indicated, MG132 (A.G. Scientific) was added to 10 μM during the last 8 h of the culture. Proteins were resolved by 12% SDS–PAGE and analyzed with indicated primary antibodies. Membranes were developed using the enhanced chemiluminescence system (Amersham Biosciences) except for detecting GFP in experiments described in Figs. 1A and B in which alkaline phosphatase coupled anti-mouse antibody (Promega) was used as the secondary antibody.

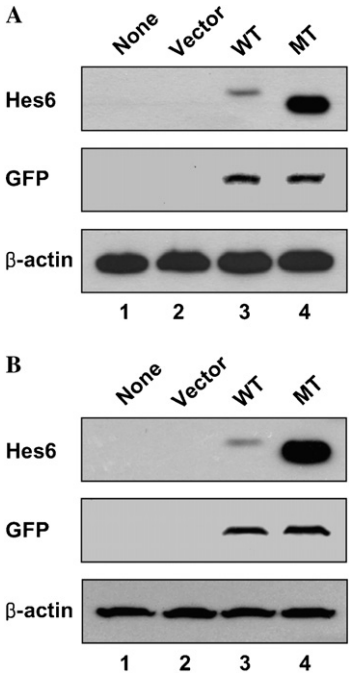

Fig. 1. Expression of Hes6WT and Hes6MT. HeLa (A) and 293T cells (B) were transfected with Hes6WT-IRES-GFP (lane 3) and Hes6MT-IRES-GFP (lane 4). GFP functions as an internal transfection and transcription level control. Hes6 and GFP are not seen in no transfection control (lane 1) or the vector (pcDNA3.1) only control (lane 2). β-Actin is used as a gel-loading control. Antibodies to Hes6, GFP, and β-Actin are used as indicated. Hes6WT protein is seen at a significantly lower level than Hes6MT protein.

Ubiquitination and immunoprecipitation. Cells were cultured and transfected with the indicated combinations of expression plasmids as shown in Fig. 3B. Cells were lysed in Nonidet P-40 containing lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 1% deoxycholate, 0.025% SDS, and protease inhibitors) 24 h afterwards. Cell lysates were incubated with anti-FLAG antibody overnight at 4 °C, and immune complexes were collected by incubation (2 h, 4 °C) with protein G–Sepharose (Sigma). After resolution by 12% SDS–PAGE, ubiquitinated proteins were detected with anti-HA antibody.

Results and discussion

In the course of generating a WRPW motif deletion mutant as a potential dominant negative derivative of Hes6, we noticed that the protein level was much higher for the deletion mutant than for the full length protein. To examine the phenomenon more precisely, we generated a point mutant of Hes6 that contains a stop codon in place of W221 (TGG → TGA). The full length gene (Hes6WT) and the WRPW deletion mutant (Hes6MT) were inserted upstream of IRES-GFP cassette which would function as an internal transcription and transfection control. When expressed in HeLa cells, the mutant product, with a slightly higher mobility in SDS–PAGE,

was seen at a much higher level than the wild type product (Fig. 1A). Consistent with the equal levels of transfection and transcription, GFP showed equal levels of expression. β-Actin, which functions as a loading control, was also seen in equal levels in all lanes (Fig. 1A). Qualitatively and quantitatively similar results were obtained using 293T cells (Fig. 1B). The same can be said of all subsequent experiments, and all results presented are highly replicable. Given the unlikelihood that transcriptional levels were different between the two expression vectors, we hypothesized that the protein turnover rate was the reason behind the difference. The principal mechanism of protein turnover is 26S proteasome mediated proteolysis [15–17]. We tested the involvement of 26S proteasome in Hes6 turnover by treating cells with the proteasome inhibitor MG132. An elevated level of Hes6WT was readily noticed after 8 h of treatment with 10 μM MG132 while the level of Hes6MT showed only a slight increase (Fig. 2A). We could not detect the appearance of higher molecular weight species upon MG132 treatment which would potentially represent the ubiquitinated Hes6. To test whether Hes6 is indeed ubiquitinated, we resorted to a more sensitive experiment. We designed a FLAG-epitope-tagged Hes6WT, and Hes6MT and co-transfected them with a HA-epitope-tagged ubiquitin expression vector plasmid. Immunoprecipitation with anti-FLAG antibody followed by immunoblotting with anti-HA antibody revealed that Hes6WT but not Hes6MT was efficiently ubiquitinated (Fig. 2B). When MG132 was applied to the transfected cells, we saw a substantial increase in the amount of ubiquitinated Hes6WT. Hes6MT also showed ubiquitination although to a significantly less extent consistent with the slight increase upon MG132 treatment (Figs. 2A and B).

Lastly, we asked if WRPW motif is sufficient for promoting the protein turnover by 26S proteasome. To this end, WRPW was appended to EGFP and the DNA binding domain of Gal4 (Gal4DB). A fusion protein with EGFP had been used to demonstrate the degradation signaling role of C-terminus domain of mouse ornithine decarboxylase [18]. When EGFP and EGFP-WRPW expression plasmids were transfected to cells and the protein levels were compared, the steady state EGFP-WRPW expression level was significantly less than that of EGFP (Fig. 3A). Consistent with a more rapid turnover of EGFP-WRPW by 26S proteasome, MG132 treatment substantially enhanced the level of EGFP-WRPW protein. A similar approach was taken with Gal4DB and Gal4DB-WRPW, and we obtained a similar result to what was seen with EGFP (Fig. 3B). Taken together, these data demonstrate that WRPW motif is sufficient for promotion of 26S proteasome mediated protein degradation.

Our data reveal a novel role of the WRPW motif apart from its role in mediating interaction with Groucho/TLE co-repressor proteins [4,5]. Specifically, WRPW domain

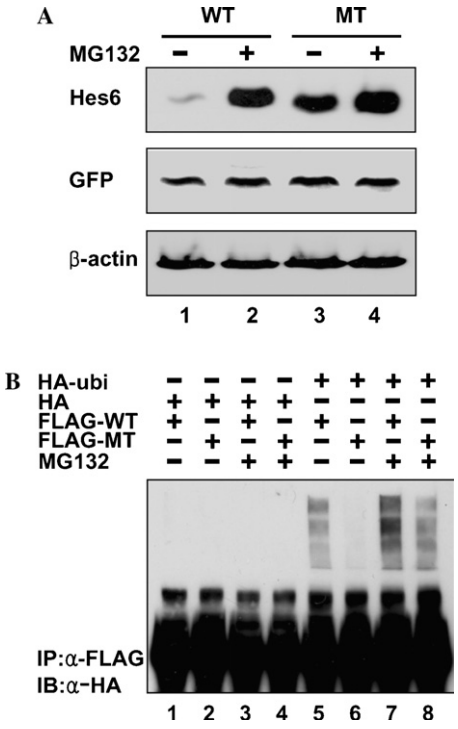

Fig. 2. Hes6 is degraded by ubiquitin–proteasome system. (A) Hes6WT-IRES-GFP and Hes6MT-IRES-GFP were expressed in HeLa cells, and cells were subsequently either untreated (lanes 1 and 3) or treated (lanes 2 and 4) with 10 μM MG132, an inhibitor of 26S proteasome. A dramatic increase in the protein level of Hes6WT is seen with the addition of MG132. A much smaller but detectable increase is also seen with Hes6MT upon treatment with MG132. (B) HeLa cells were transfected with FLAG-epitope-tagged Hes6WT (FLAG-WT; odd number lanes), Hes6MT (FLAG-MT; even number lanes), and HA epitope tagged ubiquitin (lanes 5–8) in the indicated combinations. In lanes 1–4, cells were transfected with pcDNA-HA which is essentially the vector for HA-ubiquitin construct. After lysis and immunoprecipitation with anti-FLAG antibody, the ubiquitinated Hes6 derivatives were detected by immunoblotting with anti-HA antibody. High molecular weight species representing ubiquitinated Hes6 were seen only if HA-ubiquitin expression construct was included in the transfection (lanes 5–8). More ubiquitinated Hes6 is seen with Hes6WT than with Hes6MT (lanes 5 and 6), and the levels of ubiquitinated Hes6WT and Hes6MT increased with MG132 treatment (lanes 7 and 8).

is shown to be a "degradation signal" of Hes6. Other members of Hes gene family have been shown to be ubiquitinated and degraded in a regulated fashion [9,10]. Most likely, WRPW motif is involved in the turnover process of these proteins as well. It should also be noted that even Hes6MT showed a slight increase in protein level and a significant level of ubiquitination upon treatment with MG132. These observations indicate that WRPW motif is not the sole signal for Hes6 degradation. There are precedents where multiple motifs control ubiquitination and degradation of a given protein [19,20].

WRPW is a well-defined simple motif and easy to append to the C-terminus. Naturally occurring and recombinant variants of the WRPW motif may mediate variable degrees of degradation upon fusion to heterologous proteins. The WRPW may thus represent a useful

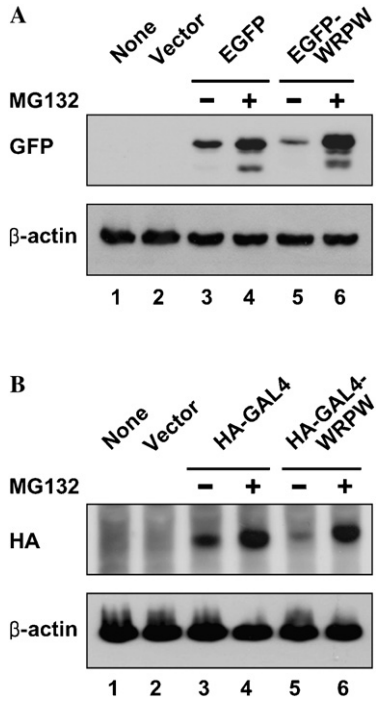

Fig. 3. WRPW is sufficient for promoting the proteasome mediated degradation. (A) WRPW motif appended EGFP (EGFP-WRPW; lane 5) shows a significantly lower protein level compared to EGFP (lane 3). The protein levels were increased and equalized upon treatment with MG132 (lanes 4 and 6). (B) WRPW motif appended Gal4 DNA binding domain (Gal4DB-WRPW; lane 5) shows a significantly lower protein level compared to Gal4DB (lane 3). The protein levels were increased and equalized upon treatment with MG132 (lanes 4 and 6).

tool in adjusting the level of ectopically expressed proteins. In other words, appending WRPW variants to EGFP could result in several derivatives with variable half-lives. If so, in building a reporter construct, investigators can choose a recombinant derivative with a half-life that closely matches the half-life of the protein coded by the gene under investigation. The resulting reporter system would represent a closer approximation of the gene expression.

Acknowledgments

We thank S.Y. Lee for his critical comments on the manuscript. We thank D.J. Anderson for permitting the use of anti-Hes6 antiserum. This research was supported by the Ewha Womans University Research Grant of 2003, by the Korea Science and Engineering Foundation through the Center for Cell Signaling Research at Ewha Womans University, and by a grant (M103KV010010 04K2201 01020; to J. Kim) from Brain Research Center of the 21st Century Frontier Research Program funded by the Ministry of Science and Technology, the Republic of Korea.

References

[1] R. Kageyama, S. Nakanishi, Helix–loop–helix factors in growth and differentiation of the vertebrate nervous system, Curr. Opin. Genet. Dev. 7 (1997) 659–665.
[2] T. Iso, L. Kedes, Y. Hamamori, HES and HERP families: multiple effectors of the Notch signaling pathway, J. Cell. Physiol. 194 (2003) 237–255.
[3] J. Lewis, Neurogenic genes and vertebrate neurogenesis, Curr. Opin. Neurobiol. 6 (1996) 3–10.
[4] A.L. Fisher, S. Ohsako, M. Caudy, The WRPW motif of the hairy-related basic helix–loop–helix repressor proteins acts as a 4-amino-acid transcription repression and protein–protein interaction domain, Mol. Cell. Biol. 16 (1996) 2670–2677.
[5] D. Grbavec, S. Stifani, Molecular interaction between TLE1 and the carboxyl-terminal domain of HES-1 containing the WRPW motif, Biochem. Biophys. Res. Commun. 223 (1996) 701–705.
[6] A.L. Fisher, M. Caudy, Groucho proteins: transcriptional corepressors for specific subsets of DNA-binding transcription factors in vertebrates and invertebrates, Genes Dev. 12 (1998) 1931–1940.
[7] S. Bray, A Notch affair, Cell 93 (1998) 499–503.
[8] Y. Bessho, H. Hirata, Y. Masamizu, R. Kageyama, Periodic repression by the bHLH factor Hes7 is an essential mechanism for the somite segmentation clock, Genes Dev. 17 (2003) 1451–1456.
[9] H. Hirata, S. Yoshiura, T. Ohtsuka, Y. Bessho, T. Harada, K. Yoshikawa, R. Kageyama, Oscillatory expression of the bHLH factor Hes1 regulated by a negative feedback loop, Science 298 (2002) 840–843.
[10] H. Hirata, Y. Bessho, H. Kokubu, Y. Masamizu, S. Yamada, J. Lewis, R. Kageyama, Instability of Hes7 protein is crucial for the somite segmentation clock, Nat. Genet. 36 (2004) 750–754.
[11] S. Bae, Y. Bessho, M. Hojo, R. Kageyama, The bHLH gene Hes6, an inhibitor of Hes1, promotes neuronal differentiation, Development 127 (2000) 2933–2943.
[12] N. Koyano-Nakagawa, J. Kim, D. Anderson, C. Kintner, Hes6 acts in a positive feedback loop with the neurogenins to promote neuronal differentiation, Development 127 (2000) 4203–4216.
[13] M.O. Gratton, E. Torban, S.B. Jasmin, F.M. Theriault, M.S. German, S. Stifani, Hes6 promotes cortical neurogenesis and inhibits Hes1 transcription repression activity by multiple mechanisms, Mol. Cell. Biol. 23 (2003) 6922–6935.
[14] X. Gao, T. Chandra, M.O. Gratton, I. Quelo, J. Prud'homme, S. Stifani, R. St-Arnaud, HES6 acts as a transcriptional repressor in myoblasts and can induce the myogenic differentiation program, J. Cell Biol. 154 (2001) 1161–1171.
[15] M. Hochstrasser, Ubiquitin-dependent protein degradation, Annu. Rev. Genet. 30 (1996) 405–439.
[16] P. Roos-Mattjus, L. Sistonen, The ubiquitin–proteasome pathway, Ann. Med. 36 (2004) 285–295.
[17] A. Hershko, A. Ciechanover, The ubiquitin system, Annu. Rev. Biochem. 67 (1998) 425–479.
[18] X. Li, X. Zhao, Y. Fang, X. Jiang, T. Duong, C. Fan, C.C. Huang, S.R. Kain, Generation of destabilized green fluorescent protein as a transcription reporter, J. Biol. Chem. 273 (1998) 34970–34975.
[19] A. Herbst, S.E. Salghetti, S.Y. Kim, W.P. Tansey, Multiple cell-type-specific elements regulate Myc protein stability, Oncogene 23 (2004) 3863–3871.
[20] M. McMahon, N. Thomas, K. Itoh, M. Yamamoto, J.D. Hayes, Redox-regulated turnover of Nrf2 is determined by at least two separate protein domains, the redox-sensitive Neh2 degron and the redox-insensitive Neh6 degron, J. Biol. Chem. 279 (2004) 31556–31567.