Arch. Biol. Sci., Belgrade, 66 (4), 1493-1502, 2014 DOI:10.2298/ABS1404493J

NIR IS DEGRADED BY THE ANAPHASE-PROMOTING COMPLEX PROTEASOME PATHWAY

MYONG HO JEONG[1], MIN KOOK KANG[1], YANG-HEE KIM[2] and SEUNG JIN HAN[1,*]

[1] *Department of Biological Sciences, Inje University, Gimhae,* Gyeongnam 621-749, Republic of Korea
[2] *Department of Molecular Biology, Sejong University,* Seoul 143-747, Republic of Korea

*Corresponding author: hansjin@inje.ac.kr

Abstract - Novel INHAT Repressor (NIR) is a histone acetylation inhibitor that can directly bind histone complexes and the tumor suppressors p53 and p63. Because NIR is mainly localized in the nucleolus and disappears from the nucleolus upon RNase treatment, it is thought to bind RNA or ribonucleoproteins. When NIR moves to the cytoplasm, it is immediately degraded; this degradation was blocked by MG132, a proteasome inhibitor. Furthermore, the central domain of NIR specifically bound APC-C^{Cdh1}. These data show that the stability of NIR is governed by the ubiquitin/proteasome pathway.

Key words: NIR; APC-C; Cdh1; Cdc20; nucleolus,

Abbreviations: NIR – novel inhibitor of histone acetyltransferase (INHAT) repressor; FBS – fetal bovine serum; HEK – human embryonic kidney; PBS – phosphate-buffered saline; rRNA – ribosomal RNA; Mdm2 – murine double minute 2; APC-C – anaphase-promoting complex/cyclosome; HAT – histone acetyltransferase.

INTRODUCTION

More than 50% of human cancers harbor one or more mutations in the gene encoding p53, a critical tumor suppressor (Hollstein et al., 1991). In normal cells, p53 localizes in the nucleus, and its amino-terminal transcriptional activation domain (TAD) is ubiquitinated by the ubiquitin E3 ligase Mdm2 (Xirodimas et al., 2001). Ubiquitinated p53 moves from the nucleus to the cytoplasm, where it is degraded by cytoplasmic proteasomes (Haupt et al., 1997). In stressed cells exhibiting DNA damage, Mdm2 is phosphorylated or sequestered in the nucleolus by association with $p14^{ARF}$, preventing its interaction with p53 and thereby activating p53 (Ivanchuk et al., 2008). When the stress on the cell is transient or mild, p53 arrests cell cycle progression via transcriptional activation of the p53 target p21, a cyclin-dependent kinase inhibitor (el-Deiry et al., 1994). On the other hand, when exposed to sustained or severe stress, cells undergo apoptosis via p53-mediated transcriptional induction of proapoptotic genes, such as those encoding Bax (Miyashita et al., 1995), PUMA (Nakano et al., 2001), and NOXA (O'Prey et al., 2010).

Several histone complex-modifying enzymes are involved in the regulation of transcriptional activation by p53 (Liu et al., 1999). In general, histone acetylation by histone acetyltransferases (HATs) is associated with transcriptional activation and euchromatin formation. Acetylation of lysine neutralizes the positive charge normally present on the histone protein, reducing the affinity between the histone tail and negatively charged DNA and thereby rendering

the DNA more accessible to transcription factors. Via this modification, p53 activity determines whether a candidate gene is expressed or remains in a silenced state (Ogryzko et al., 1996). HAT is also involved in the regulation of p53 itself: following DNA damage, p300 and p/CAF acetylate p53 at several carboxyl-terminal lysine residues (Sakaguchi et al., 1998), leading to modulation of sequence-specific DNA binding by p53 (Gu et al., 1997).

Novel INHAT repressor (NIR), originally isolated as an inhibitor of histone acetyltransferase (INHAT), can directly bind histone complexes and HATs such as p300/CBP or p/CAF (Hublitz et al., 2005). NIR also associates with p53 directly and downregulates the basal activity of p53 in unstressed cells. In this state, NIR is mainly localized in the nucleolus, although a small fraction of NIR is present in the nucleoplasm, where it binds p53 (Hublitz et al., 2005). NIR also binds p63, a p53 superfamily protein, on the promoter of the gene encoding p21, and this interaction inhibits p63-dependent transactivation of p21 (Heyne et al., 2010). When cells are exposed to stressors such as UV irradiation or doxorubicin treatment, p53 and p63 are released from inhibition by Mdm2 and NIR, and can then stimulate the expression of p21. Nucleolar localization of NIR is lost upon RNase treatment, indicating that NIR directly or indirectly binds RNA in the nucleolus (Kang et al., 2013). Expression of NIR is higher in cancer cell lines than in non-cancer cell lines, and decreases in the M phase (Kang et al., 2013). Although NIR might play critical roles in cell cycle regulation, little is known about the mechanisms that regulate NIR itself, especially regarding its degradation.

In addition to control of gene expression, control of protein degradation is also essential for cell cycle regulation. Three major systems govern protein destruction in mammalian cells: the proteasome, lysosome and autophagosome (Clague et al., 2010). In the ubiquitin/proteasome pathway, ubiquitin is conjugated to target proteins by ubiquitin ligases such as F-box proteins and the anaphase-promoting complex (APC) (Stark et al., 2006). F-box proteins are components of the SKP/Cullin/F-box complex (SCF) (Ang et al., 2005). In contrast to SCF, which is activated throughout the cell cycle, APC complexes are activated during specific cell cycle stages to trigger the progression of mitosis: APC-C^{Cdc20} induces degradation of securin to initiate anaphase and also provokes partial degradation of B-type cyclins (Visintin et al., 1997), and APC-C^{Cdh1} causes complete degradation of B-type cyclins to initiate telophase (Stark et al., 2006; Visintin et al., 1997). In this study, we provide evidence that APC is a major ubiquitin ligase involved in regulation of NIR degradation.

MATERIALS AND METHODS

Plasmids construction

To investigate the function of each NIR domain, full-length NIR was divided into NIR-F1, -F2, -F3, -F12, and -F23 fragments, as shown in Fig. 4, by PCR using specific primers. All constructs were subcloned into the *Kpn*I/*Not*I sites of pcDNA3.1 and N-terminally tagged with the HA epitope. To generate GFP-fused NIR fragments, each plasmid was cut with *Kpn*I and *Eco*RV and subcloned into vector pEGFP-C1 treated with *Kpn*I and *Sma*I. Myc- or FLAG-tagged Cdc20, Cdh1, SKP2, and TrCP1 were kindly provided by Dr. D. M. Kang (Ewha Womans University, Republic of Korea) and Dr. J.H. Seol (Seoul National University, Republic of Korea).

Cell fractionation and immunoblotting analysis

Following transfection using the JetPEI transfection reagent (Polyplus Transfection Inc., Illkirch, France), cells were cultured for 24 h, washed with cold phosphate-buffered saline (PBS), and then harvested by centrifugation at 500 g for 10 min at 4°C. To determine the amount of NIR protein, whole-cell extracts were prepared in lysis buffer (25 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% NP-40, 1 mM PMSF, 50 mM NaF, 1 mM sodium orthovanadate, 4 μg/ml aprotinin, and 0.5 μg/ml leupeptin). After rotation at 4°C for 15 min and centrifugation at 16 000 g for 20 min, the supernatant containing soluble proteins was collected (soluble fraction, S). The pellet was resuspended in the same volume of pellet buffer (lysis buffer plus

1% sodium deoxycholate and 0.1% sodium dodecyl sulfate), and was collected as the insoluble fraction after rotation and centrifugation. For cell fractionation, whole-cell extracts were prepared in lysis buffer 1 (25 mM HEPES [pH 7.5], 0.5 mM $MgCl_2$, 5 mM KCl, 1 mM DTT, and 1 mM PMSF) containing 1% NP-40 and rotated at 4°C for 15 min, followed by centrifugation at 500g for 1 min. The supernatant, which contained cytoplasmic proteins, was transferred to a new microtube (cytoplasmic fraction, Cyt), and the pellet was washed three times with lysis buffer 1 containing 0.5% NP-40. After addition of 500 µl of lysis buffer 2 (25 mM HEPES [pH7.9], 350 mM NaCl, 10% sucrose, 0.05% NP-40, 1 mM DTT, and 1 mM PMSF) to the pellet, the sample was rotated for 1 h at 4°C. The supernatant, which contained nuclear proteins, was obtained by centrifugation at 4°C for 10 min (nucleoplasmic fraction, Nuc). To determine the effects of DNase and RNase on the degradation of NIR, the cytoplasmic or nucleoplasmic fraction was treated with 0.1 mg/ml RNase A or with 0.1 mg/ml DNase I and 5 mM $MgCl_2$. Proteins were resolved by SDS-PAGE and transferred to membranes, and the blots were incubated with purified NIR antibody (Kang et al., 2013), FLAG antibody (F1804, Sigma Aldrich, St. Louis, MO), HA antibody (HA 1.1, Sigma Aldrich), or Myc antibody (A7, Abcam, Cambridge, MA). Blots were then incubated with anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA), and proteins were visualized using an enhanced chemiluminescence procedure (GE Healthcare Bio-Sciences, Piscataway, NJ).

Immunocytochemistry

To determine the cellular localization of NIR and the effects of RNase treatment, we modified a previously reported protocol (Galcheva-Gargova et al., 1998). Briefly, HeLa cells grown on coverslips were permeabilized with blocking solution (1% BSA and 0.3% Triton-X 100 in PBS) for 3 min on ice. Cells were washed three times, and then incubated for 60 min at 37°C in PBS with or without 0.1 mg/ml RNase A. Cells were fixed in 4% formaldehyde in PBS for 1 h, and then incubated with NIR antibody for 2 h. The samples were then treated with FITC- or Cy3-conjugated anti-rabbit antibody in blocking solution for 20 min. To detect nuclei, DNA was stained with Hoechst solution (Life Technologies). Mounted coverslips were observed on an Eclipse 2000 microscope (Nikon, Melville, NY).

Immunoprecipitation

To investigate the ability of Cdc20, Cdh1, SKP2, and TrCP1 to bind NIR, one of these genes and NIR were cotransfected in a 1:1 ratio. Twenty-four hours after transfection, the proteasome inhibitor MG132 (final concentration, 10 or 20 nM; Calbiochem, San Diego, CA) was added for 2 or 6 h prior to harvest. Cells were collected in RIPA buffer, and cleared by centrifugation at 13 000 rpm for 20 min at 4°C. Soluble protein (100-400 µg) was incubated with primary antibodies overnight at 4°C, and further incubated for 3 h at 4°C after the addition of 30 µl protein G-Sepharose (50% bead slurry, GE Healthcare Life Sciences, Piscataway, NJ). The beads were washed five times with RIPA buffer and suspended in 10 µl 4× SDS-PAGE sample buffer to elute immunoprecipitated proteins, which were then separated by SDS-PAGE.

RESULTS

RNA is required for NIR stability

NIR was mainly localized in the nucleolus, but disappeared from there following RNase A treatment (Fig. 1A), indicating that nucleolar localization of NIR is dependent on its association with RNA or an RNA-bound protein. Two possible mechanisms might explain this observation: destruction of NIR, or diffusion and dispersion of NIR into the nucleoplasm or cytoplasm. To distinguish between these alternatives, we monitored protein levels by immunoblot analysis following RNase treatment. Cells were fractionated into a soluble fraction, which contained cytosolic and nucleoplasmic proteins, and an insoluble fraction, which contained nucleolar and polymerized insoluble proteins. As shown in Fig. 1B, the amount of NIR was reduced by RNase A treatment, but not by DNase treatment, in both soluble and in-

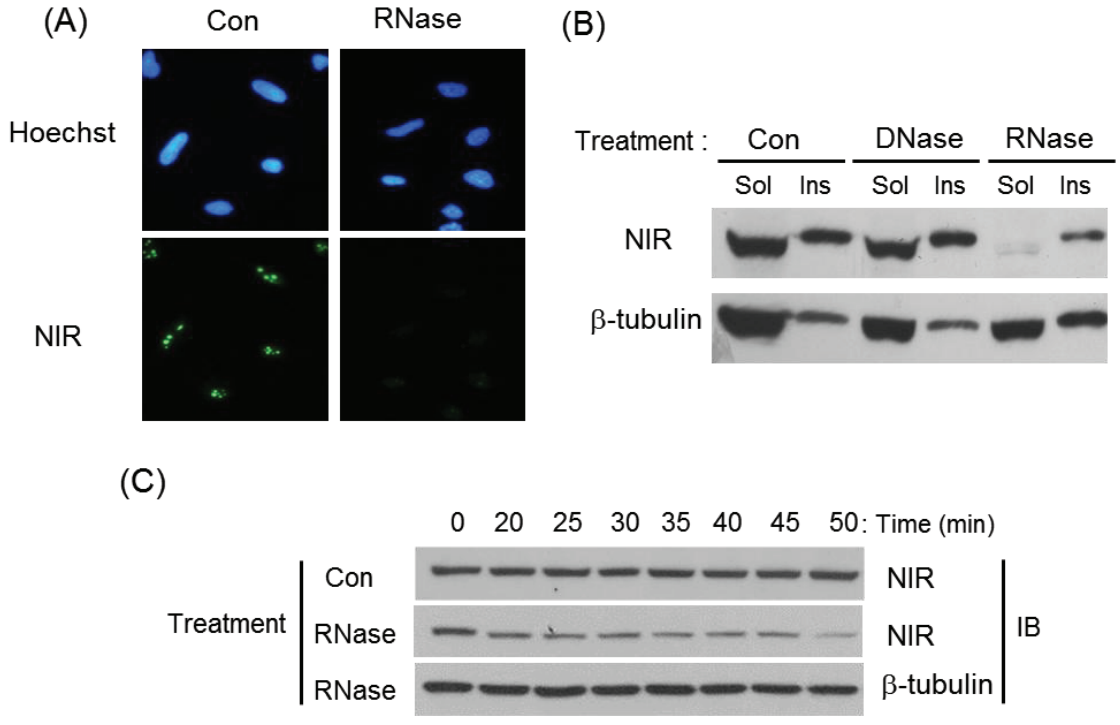

Fig. 1. RNA is required for the nucleolar localization and stability of NIR.
(A) HeLa cell grown on microscope slides were incubated with 0.1 mg/ml RNase A in PBS for 30 min at 37°C. Buffer without enzyme was used as a negative control (Con). Endogenous NIR was detected with NIR antibody and FITC-conjugated anti-rabbit antibodies. Nuclear DNA was stained with Hoechst 33342, and the sample was observed on a florescence microscope. (B) Whole-cell lysates were treated with 4 U DNase I or 0.1 mg RNase A for 1 h at 37°C. After fractionation into soluble (Sol) and insoluble (Ins) fractions, the incubated lysates were separated by SDS-PAGE. NIR was detected with NIR antibody, and β-tubulin was used as a loading control. (C) HEK293T cells were permeabilized and incubated with RNase A for the indicated times. Buffer without enzymes was used as the negative control (Con). After SDS-PAGE, proteins were detected with NIR or β-tubulin antibody.

soluble fractions. This result suggested that the disappearance of NIR from the nucleolus by RNase is due to the destruction of NIR, rather than redistribution within the cell. The degree of diminishment of NIR was greater in the soluble fraction than in the insoluble fraction. The amount of tubulin did not change upon treatment with RNase or DNase, indicating that the disappearance of NIR upon RNase treatment was specific to NIR. Monitoring NIR over a time course following RNase treatment revealed that NIR protein levels decreased gradually after digestion of RNA (Fig. 1C). These results indicate that the NIR protein is unstable, and likely degraded, following release from the nucleolus by RNase treatment.

The ubiquitin/proteasome pathway is involved in NIR degradation

When we overexpressed NIR, we observed a ladder-like pattern of FLAG- or GFP-tagged NIR, as revealed by mobility retardation in SDS-PAGE gels (Fig. 2A). Because the level of NIR protein fluctuates over the course of the cell cycle (Kang et al., 2013), this observation suggested that NIR is degraded as a result of ubiquitination. To test this idea, we transfected HEK293T cells with HA-ubiquitin (HA-Ub) and subjected them to immunoprecipitation with NIR antibody. As shown in Fig. 2B, NIR protein was ubiquitinated, and its mobility was accordingly retarded on SDS-PAGE gels. The attachment of ubiquitin to

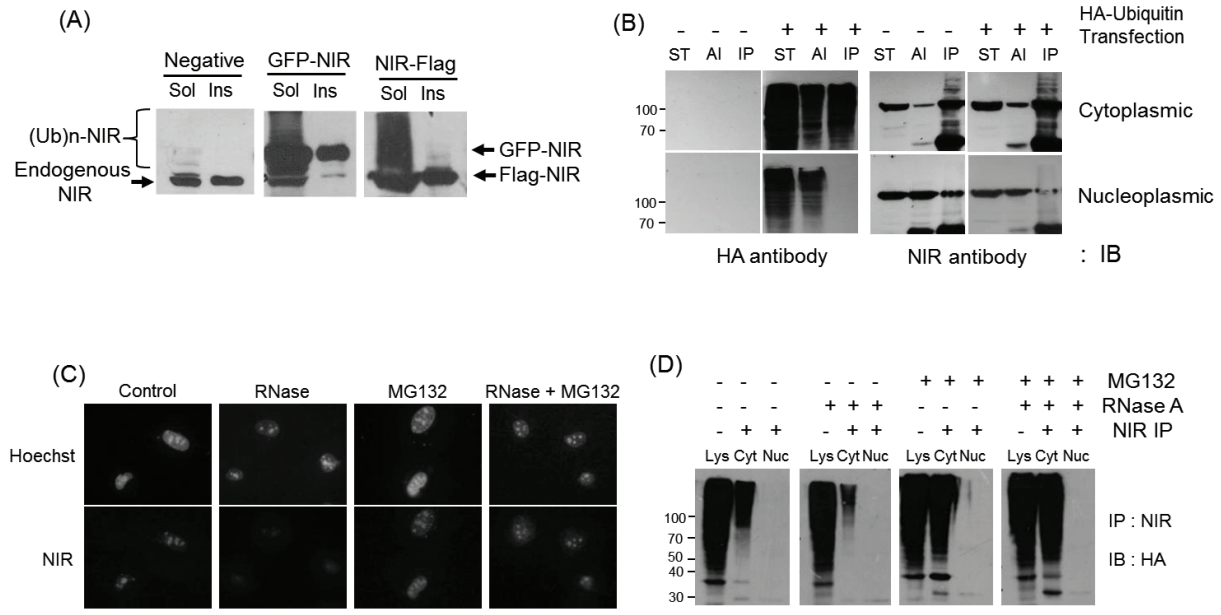

Fig. 2. Ubiquitination is involved in NIR degradation**.**
(A) Gel-mobility retardation was observed by overexpression of FLAG- or GFP-tagged NIR in HEK293T cells. Whole-cell extracts were prepared in cell lysis buffer and fractionated to soluble (Sol) and insoluble (Ins) fractions. Western-blot analysis was performed with NIR antibody after separation of 100 μg protein on an SDS-PAGE gel. (B) NIR is ubiquitinated following overexpression of HA-ubiquitin. HEK293T cells were transfected with or without HA-Ub. Cytoplasmic and nucleoplasmic proteins were immunoprecipitated with NIR antibody; following SDS-PAGE, proteins were detected with HA antibody (for ubiquitin) or NIR antibody. ST: 10% of starting lysate; AI: lysate after immunoprecipitation; IP: immunoprecipitated complex with NIR antibody. The positions of protein standards are indicated on the left (in kDa). (C) MG132 blocks the degradation of NIR induced by RNase A treatment. Immunocytochemistry was performed using NIR antibody after treatment with or without MG132 followed by RNase A addition. (D) Cells were transfected with HA-Ub and incubated with or without 10 nM MG132 for 2 h before each experiment. After fractionation of cytosolic (Cyt) and nuclear (Nuc) proteins, immunoprecipitation was performed with NIR antibody, and protein was detected with HA antibody. Lys: 10 μg lysate.

NIR was detected mainly in the cytoplasmic fraction, whereas ubiquitination of nucleoplasmic NIR was barely detectable (Fig. 2B. lower panel, lane 6). When immunoprecipitated complexes were detected with NIR antibody, consistent results were observed (Fig. 2B, right four panels). These data show that NIR is mainly ubiquitinated in the cytoplasm.

To confirm that the destruction of NIR following RNase A treatment is mediated by ubiquitination followed by proteasomal degradation, we treated HA-Ub–transfected HEK293T cells with MG132, an inhibitor of the proteasome. Whereas RNase A induced the disappearance of NIR from the nucleoli of MG132-untreated cells, NIR protein could still be observed in immunocytochemistry assays after RNase A treatment when cells were incubated with MG132 (Fig. 2C). In addition, the amount of ubiquitinated NIR increased upon the addition of MG132 after RNase A treatment (Fig. 2D; compare the two right panels with the two left panels). These results strongly suggest that the ubiquitin/proteasome pathway is involved in NIR degradation.

NIR is ubiquitinated by an E3 ubiquitin ligase, the APC complex

Because the ubiquitin/proteasome pathway appears to be involved in NIR degradation, we tried to identify the ubiquitin ligase responsible. For this purpose, we cotransfected cells with HA-NIR and either a FLAG-tagged F-box protein (FLAG-TrCP1 or FLAG-Skp2)

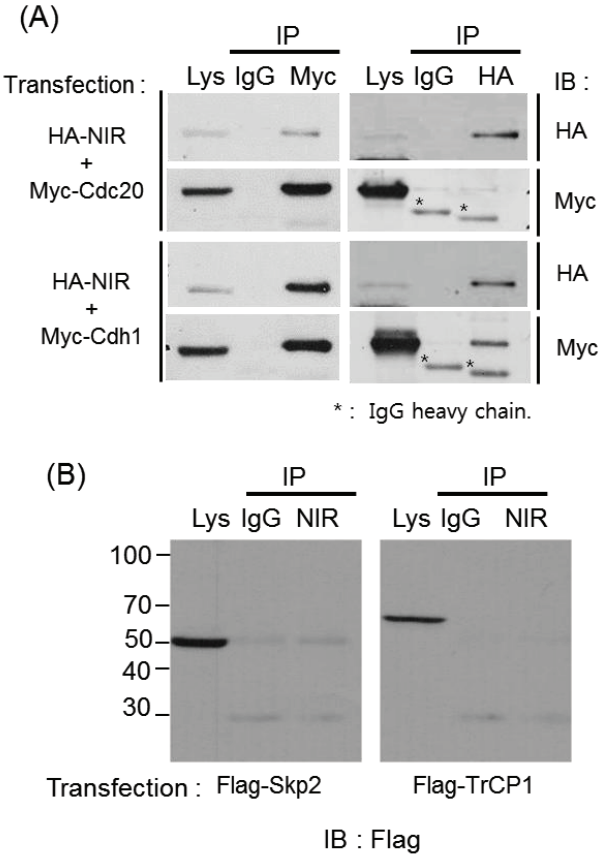

Fig. 3. APC-C is involved in NIR ubiquitination.
(A) Anaphase-promoting complex ligases co-immunoprecipitate with HA-NIR. HEK293T cells were co-transfected with HA-NIR and either Myc-Cdc20 or Myc-Cdh1, components of the APC complex. Cells were cultured for 24 h after transfection, and then treated with 20 nM MG132 for 6 h prior to harvest in RIPA buffer. A portion (10%) of cell lysate used for immunoprecipitation was loaded to show the amount of expressed protein. The cell lysates were immunoprecipitated with IgG, monoclonal Myc antibody, or HA antibody, as indicated. The same blot was stripped and then re-probed with either HA or Myc antibody (right panels). (B) NIR does not bind to Skp2 or TrCP1. HEK293T cells were co-transfected with HA-NIR and either FLAG-Skp2 or FLAG-TrCP1, which are components of the SCF complex. Cell lysates were immunoprecipitated with IgG or monoclonal NIR antibody, and the blot was probed with FLAG antibody. The position of protein standards (in kD) is indicated on the left.

or a Myc-tagged APC ligase (Myc-Cdc20 or Myc-Cdh1). The two APC ligases co-immunoprecipitated with NIR, indicating that they are involved in NIR degradation (Fig. 3), whereas the F-box proteins did not. Both Cdh1 and Cdc20 bound NIR, although Cdh1 bound more strongly. We concluded that NIR degradation is primarily mediated by APC-C.

The central domain of NIR is required for ubiquitination

To determine which domain of the NIR protein is involved in the degradation of NIR following RNase A treatment, we transfected several truncated NIR fragments into HEK293T cells and used immunoblot analysis to monitor the levels of these proteins following RNase A treatment. Levels of full-length NIR and the F12 and F23 fragments were dramatically reduced following RNase A treatment (Fig. 4A). To verify this result, we tagged several forms of NIR with GFP and co-expressed them with Myc-tagged Cdh1. After immunoprecipitation of Cdh1 with Myc antibody, we monitored the binding of truncated forms of NIR. As shown in Figure 4B, only fragments containing the central domain of NIR (F2) co-immunoprecipitated with Cdh1. These data suggest that the central domain of NIR is important for regulation of NIR stability.

DISCUSSION

NIR is an inhibitor of HATs (Hublitz et al., 2005) and a regulator of p53, p63 (Heyne et al., 2010) and the cell cycle (Kang et al., 2013). NIR is mainly localized in the nucleolus, although a small fraction of NIR resides in the nucleoplasm, where it inhibits the transcriptional activity of p53 (Heyne et al., 2010; Hublitz et al., 2005). Because the nucleolus is the cellular 'factory' for rRNA synthesis, rDNA and rRNA are concentrated in this compartment, and many proteins residing in the nucleolus are anchored to RNA or DNA. Nucleophosmin (B23), nucleolin (C23) and Nop2p (the yeast homolog of p120) interact with nucleic acids, and some of them, such as nucleolin, translocate between the nucleolus, nucleoplasm and the cell surface. These proteins are involved in regulation of the cell cycle and proliferation, as well as ribosome biogenesis (Gustafson et al., 1998). Like these proteins, NIR also appears to be at-

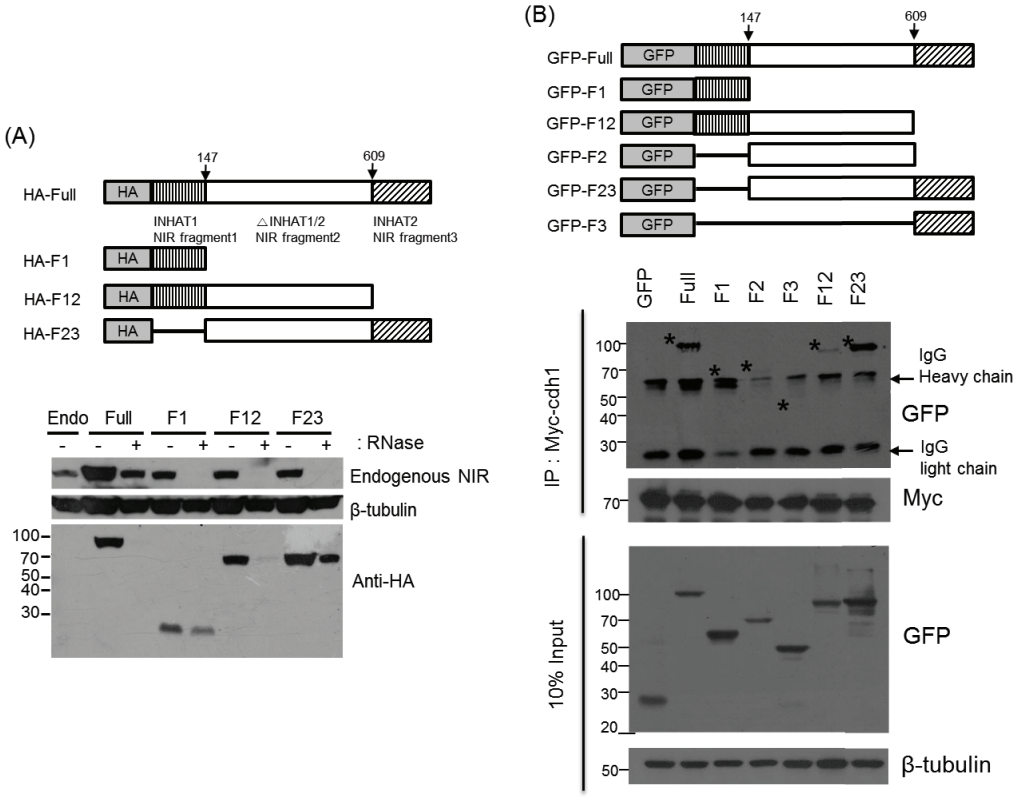

Fig. 4. The central domain of NIR is responsible for its degradation.
(A) The central domain of NIR is the primary target of degradation induced by RNase A treatment. HEK293T cells were transfected with several NIR fragments (upper diagram), and the levels of endogenous and exogenous NIR proteins were determined by immunoblotting after RNase A treatment (lower panels). The levels of F12 and F23 were dramatically decreased by RNase treatment. (B) The central domain of NIR can bind Cdh1. Several truncated forms of GFP-tagged NIR (upper diagram) were co-transfected with Myc-Cdh1, and immunoprecipitation was carried out with Myc antibody. The amount of ubiquitinated NIR fragment co-immunoprecipitated with Myc-Cdh1 was monitored with GFP antibody (top panel). Immunoprecipitated Myc-Cdh1 (second panel from top) and expression of NIR (third panel from top) were monitored with Myc and GFP antibody, respectively. Asterisks indicate the positions of GFP fusion proteins. Numbers at left indicate the migration of protein standards (kD).

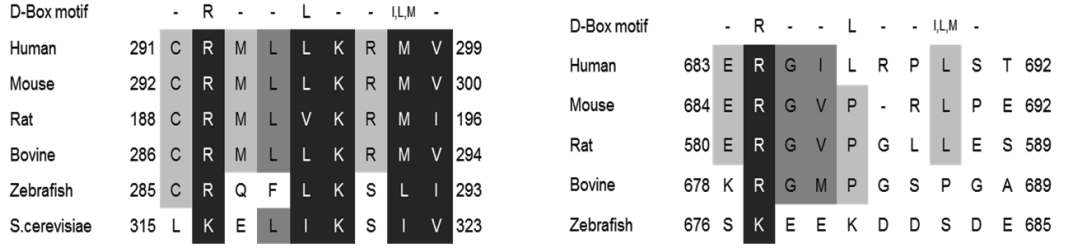

Fig. 5. The D-box of NIR is conserved among several species.
(A) Amino acid alignment of D boxes of NIR family members. Human (NP_056473), mouse (NP_067278.2), bovine (NP_001029498.1), Zebra fish (NP_001003830.1), rat (NP_001029069.1), and *S. cerevisiae* NIR (GenBank Accession Number NP_014849.3) were compared. Sequence alignment was performed using ClustalX 2.1. Residues identical among two or more sequences are boxed in gray. Numbers on the right and the left refer to adjacent amino acids; the initiating methionine (M) was designated as +1. The D boxes were predicted using the Eukaryotic Linear Motif (ELM) website (http://elm.eu.org/search/). The first D-box is highly conserved among several species.

tached to RNA, either directly or indirectly, as demonstrated by the observation that NIR disappeared from the nucleolus upon RNase treatment (Fig. 1). As with other nucleolar proteins, NIR also functions in rRNA biogenesis for both 40S and 60S subunits (Wu et al., 2012). NIR has also been implicated in cell-cycle regulation, although the exact mechanism remains unknown (Kang et al., 2013).

After observing mobility retardation of NIR on SDS-PAGE gels (Fig 1B), we investigated the protein's post-translational modifications. Initially, we tested for phosphorylation of NIR by *in vivo* labeling and phosphoamino acid analysis. NIR is indeed phosphorylated on serine but not tyrosine (data not shown); phosphorylation of threonine was also detectable, but not as clearly as that of serine. However, this phosphorylation of NIR is not the major cause of the electrophoretic shift, which persisted even after lambda phosphatase treatment (data not shown). The physiological role of NIR phosphorylation requires further investigation. Other post-translational modifications such as ubiquitination, acetylation or sumoylation can also cause proteins to migrate differently on SDS-PAGE. These modifications play critical roles in regulating characteristics of proteins, including targeting, stability, and enzyme activity (Cook et al., 2013). Therefore, we asked whether NIR is modulated by one of these modifications. Because the SDS-PAGE retardation pattern of NIR was similar to that of typical ubiquitinated proteins, we predicted that this phenotype might be due to ubiquitination of NIR. Especially in the cytosolic fraction, NIR was extensively ubiquitinated (Fig. 2), and the disappearance of NIR from the nucleolus upon RNase treatment was blocked by treatment with MG132, a proteasome inhibitor (Fig. 2C). In addition, the total amount of NIR protein decreased dramatically upon treatment with RNase (Fig. 1B and 1C), and this decrease was also completely blocked by MG132 treatment. Therefore, the stability of NIR is dependent on its RNA-binding state as well as its localization.

Our effort to identify the ubiquitin ligase of NIR revealed that APC is involved in the NIR degradation pathway (Figs. 3, 4B). In early mitosis, APC-C is activated through binding to Cdc20, whereas in late M phase, Cdc20 is replaced by Cdh1, the second activator of APC-C. After anaphase, APC-C^{Cdh1} is activated by the phosphatase Cdc14 and eliminates B-type cyclin to initiate telophase (Lukas et al., 1999; Mitra et al., 2006; Sorensen et al., 2001). Most NIR protein seems to be recognized and degraded by APC-C^{Cdh1}, which is consistent with the previous finding that NIR expression decreases as the cell passes through M phase (Kang et al., 2013). The simplest possible explanation for NIR degradation is that NIR may be degraded during mitosis because the nucleus and nucleolus disappear during this phase, exposing NIR to the cytoplasm. The exact function of NIR degradation during mitosis should be investigated in future work.

To be recognized by APC-C, a protein must contain specific sequences, e.g., the Destruction-box (D-box) (Glotzer et al., 1991), KEN-box (Pfleger et al., 2000), A-box (Castro et al., 2002; Littlepage et al., 2002), CRY-box (Reis et al., 2006), GXEN motif (Castro et al., 2003), or LXXEXXXN motif (Sullivan et al., 2007). NIR contains two putative D-boxes (Choi et al., 2008) (Fig. 5), and the first one is relatively well conserved among several species. Further work is required to determine which of these motifs, if any, is actually involved in recognition by APC-C. Although our immunoprecipitation data show that both Cdc20 and Cdh1 can bind NIR (Fig. 3), it is possible that another E3 ligase, such as Mdm2, ubiquitinates NIR and promotes its degradation.

Acknowledgments - This work was supported by a 2013 Inje University research grant.

REFERENCES

Ang, X. L. and *J. Wade Harper* (2005). SCF-mediated protein degradation and cell cycle control, *Oncogene*, *24*(17), 2860-2870.

Castro, A., Vigneron, S., Bernis, C., Labbe, J. C. and *T. Lorca* (2003). Xkid is degraded in a D-box, KEN-box and A-box-independent pathway, *Mol Cell Biol*, *23*(12), 4126-4138.

Castro, A., Vigneron, S., Bernis, C., Labbe, J. C., Prigent, C. and *T. Lorca* (2002). The D-Box-activating domain (DAD) is

a new proteolysis signal that stimulates the silent D-Box sequence of Aurora-A, *EMBO Rep*, *3*(12), 1209-1214.

Choi, E., Dial, J. M., Jeong, D. E. and *M. C. Hall* (2008). Unique D box and KEN box sequences limit ubiquitination of Acm1 and promote pseudosubstrate inhibition of the anaphase-promoting complex, *J Biol Chem*, *283*(35), 23701-23710.

Clague, M. J. and *S. Urbe* (2010). Ubiquitin: same molecule, different degradation pathways, *Cell*, *143*(5), 682-685.

Cook, K. M. and *P. J. Hogg* (2013). Post-translational control of protein function by disulfide bond cleavage, *Antioxid Redox Signal*, *18*(15), 1987-2015.

el-Deiry, W. S. et al. (1994). WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis, *Cancer Res*, *54*(5), 1169-1174.

Galcheva-Gargova, Z., Gangwani, L., Konstantinov, K. N., Mikrut, M., Theroux, S. J., Enoch, T. and *R. J. Davis* (1998). The cytoplasmic zinc finger protein ZPR1 accumulates in the nucleolus of proliferating cells, *Mol Biol Cell*, *9*(10), 2963-2971.

Glotzer, M., Murray, A. W. and *M. W. Kirschner* (1991). Cyclin is degraded by the ubiquitin pathway, *Nature*, *349*(6305), 132-138.

Gu, W. and *R. G. Roeder* (1997). Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain, *Cell*, *90*(4), 595-606.

Gustafson, W. C., Taylor, C. W., Valdez, B. C., Henning, D., Phippard, A., Ren, Y., Busch, H. and *E. Durban* (1998). Nucleolar protein p120 contains an arginine-rich domain that binds to ribosomal RNA, *Biochem J*, *331 (Pt 2)*, 387-393.

Haupt, Y., Maya, R., Kazaz, A. and *M. Oren* (1997). Mdm2 promotes the rapid degradation of p53, *Nature*, *387*(6630), 296-299.

Heyne, K., Willnecker, V., Schneider, J., Conrad, M., Raulf, N., Schule, R. and *K. Roemer* (2010). NIR, an inhibitor of histone acetyltransferases, regulates transcription factor TAp63 and is controlled by the cell cycle, *Nucleic Acids Res*.

Hollstein, M., Sidransky, D., Vogelstein, B. and *C. C. Harris* (1991). p53 mutations in human cancers, *Science*, *253*(5015), 49-53.

Hublitz, P. et al. (2005). NIR is a novel INHAT repressor that modulates the transcriptional activity of p53, *Genes Dev*, *19*(23), 2912-2924.

Ivanchuk, S. M., Mondal, S. and *J. T. Rutka* (2008). p14ARF interacts with DAXX: effects on HDM2 and p53, *Cell Cycle*, *7*(12), 1836-1850.

Kang, M. and *S. Han* (2013). NIR is a nucleolar RNA-binding protein that is involved in the cell proliferation, *Arch. Biol. Sci*, *65 (3)*, 929-937.

Littlepage, L. E. and *J. V. Ruderman* (2002). Identification of a new APC/C recognition domain, the A box, which is required for the Cdh1-dependent destruction of the kinase Aurora-A during mitotic exit, *Genes Dev*, *16*(17), 2274-2285.

Liu, L., Scolnick, D. M., Trievel, R. C., Zhang, H. B., Marmorstein, R., Halazonetis, T. D. and *S. L. Berger* (1999). p53 sites acetylated in vitro by PCAF and p300 are acetylated in vivo in response to DNA damage, *Mol Cell Biol*, *19*(2), 1202-1209.

Lukas, C., Sorensen, C. S., Kramer, E., Santoni-Rugiu, E., Lindeneg, C., Peters, J. M., Bartek, J. and *J. Lukas* (1999). Accumulation of cyclin B1 requires E2F and cyclin-A-dependent rearrangement of the anaphase-promoting complex, *Nature*, *401*(6755), 815-818.

Mitra, J., Enders, G. H., Azizkhan-Clifford, J. and *K. L. Lengel* (2006). Dual regulation of the anaphase promoting complex in human cells by cyclin A-Cdk2 and cyclin A-Cdk1 complexes, *Cell Cycle*, *5*(6), 661-666.

Miyashita, T. and *J. C. Reed* (1995). Tumor suppressor p53 is a direct transcriptional activator of the human bax gene, *Cell*, *80*(2), 293-299.

Nakano, K. and *K. H. Vousden* (2001). PUMA, a novel proapoptotic gene, is induced by p53, *Mol Cell*, *7*(3), 683-694.

O'Prey, J., Crighton, D., Martin, A. G., Vousden, K. H., Fearnhead, H. O. and *K. M. Ryan* (2010). p53-mediated induction of Noxa and p53AIP1 requires NFkappaB, *Cell Cycle*, *9*(5), 947-952.

Ogryzko, V. V., Schiltz, R. L., Russanova, V., Howard, B. H. and *Y. Nakatani* (1996). The transcriptional coactivators p300 and CBP are histone acetyltransferases, *Cell*, *87*(5), 953-959.

Pfleger, C. M. and *M. W. Kirschner* (2000). The KEN box: an APC recognition signal distinct from the D box targeted by Cdh1, *Genes Dev*, *14*(6), 655-665.

Reis, A., Levasseur, M., Chang, H. Y., Elliott, D. J. and *K. T. Jones* (2006). The CRY box: a second APCcdh1-dependent degron in mammalian cdc20, *EMBO Rep*, *7*(10), 1040-1045.

Sakaguchi, K., Herrera, J. E., Saito, S., Miki, T., Bustin, M., Vassilev, A. and*erson, C. W.* and *E. Appella* (1998). DNA damage activates p53 through a phosphorylation-acetylation cascade, *Genes Dev*, *12*(18), 2831-2841.

Sorensen, C. S., Lukas, C., Kramer, E. R., Peters, J. M., Bartek, J. and *J. Lukas* (2001). A conserved cyclin-binding domain determines functional interplay between anaphase-promoting complex-Cdh1 and cyclin A-Cdk2 during cell cycle progression, *Mol Cell Biol*, *21*(11), 3692-3703.

Stark, G. R. and *W. R. Taylor* (2006). Control of the G2/M transition, *Mol Biotechnol*, *32*(3), 227-248.

Sullivan, M. and *D. O. Morgan* (2007). A novel destruction sequence targets the meiotic regulator Spo13 for anaphase-promoting complex-dependent degradation in anaphase I, *J Biol Chem*, *282*(27), 19710-19715.

Visintin, R., Prinz, S. and *A. Amon* (1997). CDC20 and CDH1: a family of substrate-specific activators of APC-dependent proteolysis, *Science*, *278*(5337), 460-463.

Wu, J., Zhang, Y., Wang, Y., Kong, R., Hu, L., Schuele, R., Du, X. and *Y. Ke* (2012). Transcriptional repressor NIR functions in the ribosome RNA processing of both 40S and 60S subunits, *PLoS One*, *7*(2), e31692.

Xirodimas, D. P., Stephen, C. W. and *D. P. Lane* (2001). Cocompartmentalization of p53 and Mdm2 is a major determinant for Mdm2-mediated degradation of p53, *Exp Cell Res*, *270*(1), 66-77.