The fusion oncoprotein PML-RARα induces endoplasmic reticulum-associated degradation of N-CoR and ER stress

Md Matiullah Khan*, Teruaki Nomura*, Tomoki Chiba†, Keiji Tanaka†, Hiderou Yoshida‡, Kazutoshi Mori‡ and Shunsuke Ishii*¶

From the *Laboratory of Molecular Genetics, RIKEN Tsukuba Institute, 3-1-1 Koyadai, Tsukuba, Ibaraki 305-0074, Japan, the †Department of Molecular Oncology, The Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8613, Japan, and the ‡Graduate School of Biostudies, Kyoto University, Kyoto 606-8304, Japan

Running title: PML-RARα targets N-CoR to ER

¶To whom correspondence should be addressed. Tel.: +81-29-836-9031; Fax: +81-29-836-9030; E-mail: sishii@rtc.riken.go.jp

1. The abbreviations used are: APL, acute promyelocytic leukemias; ATF6, activating transcription factor 6; ER, endoplasmic reticulum; ERAD, ER-associated degradation; PML, promyelocytic leukemia; RAR-α, retinoic acid receptor-α; NB(s), nuclear body(ies); RA, retinoic acid.
ABSTRACT

PML-RARα, a fusion protein of promyelocytic leukemia (PML) and the retinoic acid receptor-α (RARα), causes acute promyelocytic leukemias (APL). Although the role of nuclear PML-RARα has been extensively studied, a significant amount of PML-RARα is in the cytoplasm. The role cytoplasmic PML-RARα plays in leukemogenesis is unknown. Here we report that PML-RARα induces the N-CoR accumulation in the endoplasmic reticulum (ER), leading to the induction of ER stress and the processing of activating transcription factor 6 (ATF6), the unfolded protein response. PML-RARα stimulates the ubiquitylation of N-CoR via Ubc6 that is involved in the protein quality control. This ER-associated degradation (ERAD) of N-CoR reduces the soluble N-CoR protein levels in the nucleus. The two N-CoR-interacting sites in PML-RARα are required for the ERAD of N-CoR, suggesting the aberrant binding of PML-RARα to N-CoR may induce the ERAD of N-CoR. Over-expression of N-CoR induces the differentiation of APL-derived NB4 cells, suggesting that the low levels of N-CoR in the nucleus may contribute at least partly to PML-RARα-mediated leukemogenesis.
INTRODUCTION

PML-RARα is a fusion protein of PML and the retinoic acid receptor-α (RARα) and causes acute promyelocytic leukemias (APL) (1, 2). The molecular mechanism of leukemogenesis by PML-RARα has been the focus of intensive research in the past decade. PML belongs to a family of Ring finger proteins characterized by the presence of the RBCC motif (3). In PML, this motif consists of a C3HC4-type zinc finger domain known as the Ring finger, two cysteine-rich regions termed B boxes, and a leucine-rich heptad repeat known as the coiled-coil domain. Although exact roles these domains play in PML function are still poorly understood, it is believed that they serve as protein-protein interacting interfaces. PML negatively regulates cellular proliferation (4, 5) and localizes to nuclear dot-like structures known as nuclear bodies (NBs) or the PML oncogenic domain (POD) (6, 7). PML-RARα expression disrupts NB structures in promyelocytic cells. This event is believed to be linked to transformation because reorganization of NBs always occurs when leukemic cells are induced to differentiate by retinoid (RA) treatment.

PML is involved in multiple activities, including Mad- and Rb-mediated transcriptional repression (8, 9), RARα-mediated transcriptional activation (10), and apoptosis (11, 12). PML-RARα blocks all of these functions in a dominant negative manner. For instance, PML binds via its coiled-coil region to multiple corepressors (8), including N-CoR, that mediates the transcriptional repression imposed by unliganded nuclear hormone receptors and Mad (13, 14). The binding of PML to the corepressors
causes the corepressors to be recruited to the NBs (8). In Pml-deficient cells, the transcriptional repression mediated by Mad or Rb is impaired, suggesting that the PML-mediated localization of the corepressors to NBs is an important event needed for the proper function of the corepressors (8, 9). When PML-RARα is expressed, Mad- and Rb-mediated transcriptional repression is inhibited. Mapping studies show that this activity depends on the presence of the two N-CoR-interacting sites of PML-RARα, namely, the coiled-coil domain in the PML part and the CoR box located on the RAR moiety (8, 9). This suggests that PML-RARα may bind aberrantly to the N-CoR molecule via these two sites. Such binding may induce the N-CoR protein to adopt an abnormal conformation. PML is also involved in p53 acetylation (15) and the recruitment of DNA methyltransferase (16). In addition, the oncogenicity of PML-RARα has been found to involve the silencing the genes of the RA signaling pathway by actively recruiting the histone deacetylase complex (17, 18). Thus, the role of nuclear PML-RARα in leukemogenesis has been extensively studied.

Significant levels of PML-RARα are also found in the cytoplasm (6). However, the roles such cytoplasmic PML-RARα molecules may play in leukemogenesis are much less well understood than those played by the nuclear-localized molecules. In this paper, we found that the cytoplasmic PML-RARα molecules may recruit the newly synthesized N-CoR molecules to the endoplasmic reticulum (ER) by directing them into the protein quality control system of the cell. This system is a post-translational process whereby newly synthesized polypeptides that fail to attain the proper structure are either refolded or degraded (19). The mutated proteins or the accidentally generated proteins
by various mechanisms such as chromosome rearrangement are somehow recognized as mis-folded and degraded by the ubiquitin-proteasome system (20). A key site for protein quality control is the ER as it is one of the major sites of protein synthesis and the portal of entry for proteins into the secretory pathway. When mis-folded proteins accumulate in the ER, they are degraded via the proteasome in a process termed ER-associated degradation (ERAD) (21). The accumulation of mis-folded protein in the ER also induces the proteolytic processing of the bZIP transcription factor ATF6 into its active form, which then leads to the transcriptional induction of ER chaperones and folding enzymes (22). The latter process is termed the unfolded protein response (UPR).

Here we report that PML-RARα induces the accumulation of insoluble N-CoR protein primarily in the ER, which leads to decreased levels of soluble N-CoR protein in the nucleus. Since N-CoR protein in the nucleus is vital for the transcriptional repression mediated by the tumour suppressor Mad (14, 23), this reduction of soluble N-CoR protein levels in the nucleus may be at least partly responsible for uncontrolled growth and transformation that is responsible for APL.

MATERIALS AND METHODS

Plasmids and vectors --- The chicken β-actin promoter containing plasmids to express N-CoR, PML-RARα, and PML, and the GST-PML expression vectors encoding various forms of PML were described previously (8). PCR-based method was employed to construct various PML-RARα mutant expression plasmids. In the PML-RARα {RF(Ring finger point mutant)} construct, the cysteine residues at 57 and 60 of Ring
finger domain of PML were substituted with serine. In PML-RARΔC-C, the whole coiled-coil domain (residues 228 to 365) was deleted. Similarly, in the B boxes deletion mutant, the deleted region was the two B-boxes and intervening region, extending from residue 130 to 227. In the PML-RARα (AHT) mutant construct, the A, H and T residues at 223, 224 and 227 sites, which are required for interaction with N-CoR molecule, were replaced by G, G and A residues respectively. The HA-ATF6 expression vector (pCGN-ATF6) was described previously (22). To express Ubc5 or Ubc6 in 293T cells, pcDNA3.1 vector (Invitrogen) was used. In Ubc5(CS) and Ubc6(CS) expression vectors, the active cysteine residue was replaced with serine.

**Immunocytochemistry ---** 293T cells were co-transfected with pact-N-CoR-Flag (1 µg) together with pact-HA- PML-RARα or pact-HA-PML (1 µg), fixed with 4% paraformaldehyde or methanol, and stained with either anti-PML (Santa Cruz), anti-HA (Santa Cruz) and anti-PDI (Stress Gene), Anti-BiP (N-20, Santa Cruz), or anti-Flag (M2, Sigma) antibodies. For visualization of Golgi signals, cells were transfected with Ds-Red-Golgi (0.5 µg) expression plasmid. Staining of NB4 cells was performed under similar conditions using anti-PML (8), anti-N-CoR (C-20, Santa Cruz), anti-Golgin-97 (Molecular Probe) and anti-PDI antibodies and signals were visualized by confocal microscopy or deconvolution. DNA was stained with TO-PRO (Molecular Probe).

**Subcellular fractionation ---** 293T cells co-transfected with N-CoR and PML-RARα plasmids or NB4 cells, were harvested in 2-ml ice-cold homogenizing buffer (10 mM Tris-HCl, pH 7.4, 250 mM sucrose, 5 mM EDTA and protease inhibitor cocktail), homogenized by passing 50 times in a Potter-Teflon homogenizer on ice. Post nuclear
supernatant was obtained (3,000 rpm, 10 min, 4°C) and Nycodenz gradient fractionation was performed essentially as described (24). A step gradient was created in Beckman SW41 centrifuge tubes by loading top to bottom 2.5 ml of 10, 14.66, 19.33, and 24% of Nycodenz solution in saline buffer. The solutions were prepared from 27.6% Nycodenz stock solution and 0.75% NaCl (both in 10 mM Tris-HCl, pH 7.4, 3 mM KCl, 1 mM EDTA, 0.02% NaN₃). The tube was placed vertically for 45 min at room temperature followed by centrifugation (37,000 rpm, 4 h, 15°C, SW41 rotor). 2 ml of the post-nuclear supernatant was layered on top of the gradient and fractionated by centrifugation (37,000 rpm, 1.5 h, 15°C). After centrifugation, fractions (0.8 ml each) were collected from top of the tube using Aliquots (40 µl) of each fraction was resolved by SDS-PAGE and probed with antibodies specific for marker proteins of various subcellular compartments.

Protease sensitivity of N-CoR --- NB4 cells were seeded at 20 x 10⁴ cells/ml and treated with RA (1 µM) for 3 days. Cells were harvested and disrupted with hypotonic buffer(10 mM Tris-HCl, pH 8.0, 10 mM KCl, 1 mM EDTA, 1 mM DTT) and homogenizer. After centrifugation, the supernatant was kept, and nuclei were extracted with NETN buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5% NP40, 10% glycerol, 150 mM NaCl). Nuclei extract and the cytoplasm supernatant were mixed and used as whole cell lysates. Cell lysates were treated with indicated amount of proteinase K or trypsin for 30 min on ice. The degradation products were analyzed with SDS-PAGE, followed by Western blotting with anti-N-CoR antibodies.
ATF6 processing --- NB4 cells were treated with RA (1 μM) or vehicle for 72 hr and lysates were prepared in RIPA buffer. After SDS-PAGE, proteins were transferred to a nitrocellulose membrane and blotted with the anti-ATF6 antibody (rabbit anti-B03N) (22). To detect the processing of exogenous protein, HeLa cells were co-transfected with LipofectAMINE (Invitrogen) with the HA-ATF6 expression vector and the plasmids expressing N-CoR and PML-RARα or PML. Cells were treated with tunicamycin (2.5 μg/ml) or DTT (5 mM) for the indicated time before the preparation of lysates. Twenty-four hours after transfection, lysates were prepared as described above and various forms of HA-ATF6 were detected by using the anti-HA antibody.

Protein solubility, fractionation, and Western blotting --- 293T cells were transfected by LipofectAMINE with the N-CoR expression plasmid pact-N-CoR-Flag (3 μg) and the plasmid expressing various forms of PML-RARα or the control blank DNA (3 μg), and the internal control pact-β-gal (0.5 μg). Forty hours after transfection, cells were passively lysed in NET buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5% NP-40, protease inhibitor cocktail) containing 300 mM NaCl and centrifuged. The soluble fraction was harvested and the insoluble fraction was solubilized by sonication in SDS sample buffer, after which fragmented DNA was removed by sepharose beads. The soluble and insoluble fractions were used for Western blotting with anti-Flag monoclonal antibody and ECL detection reagents (Amersham). The amount of lysate used for Western blotting was normalized on the basis of the β-galactosidase activity. NB4 cells were treated with vehicle, MG132 (5 μM) for 8 hr or with RA (1 μM) for 72 hr. The soluble fraction of NB4 cells was prepared as described above. To determine the
total endogenous N-CoR levels in NB4 cells, lysates were prepared in highly stringent RIPA buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.5% NP-40, 0.5% SDC, 0.5% SDS, and protease inhibitor cocktail) by syringing 10 times with a 21-gauge needle followed by syringing 3 times with a 26-gauge needle. For each lane in Western blotting, 100 µg of protein was used to detect the N-CoR protein with the anti-N-CoR antibody. As a loading control, aliquots of sample were analyzed by SDS-PAGE and stained with Coomassie blue.

**Pulse-chase experiments** --- 293T cells were transfected with the N-CoR-Flag (3 µg) expression vector together with the PML-RARα expression plasmid or the control DNA (3 µg), and pact-β-gal (0.5 µg). Twenty four hours after transfection, cells were labeled with [35S]methionine and [35S]cysteine for 6 hr, and then the radioactivity was chased. At various times, lysates were prepared using the NET buffer containing 300 mM NaCl (to prepare soluble N-CoR) or the lysis buffer (10 mM Tris-HCl, pH 8.0, 500 mM NaCl, 2% SDS) (to prepare both the soluble and insoluble N-CoR), heated for 10 minutes at 95 °C, and immuno-precipitated by anti-Flag antibody after ten-fold dilution and brief sonication. The immuno-complexes were analyzed by SDS-PAGE followed by autoradiography.

**In vivo ubiquitination assays** --- 293T cells were transfected with 2 µg pact-N-CoR-Flag expression vector, 2 µg pcDNA3-Myc-Ub expression vector and 2 µg pact-HA-PML-RARα or empty actin vector. Thirty hours after transfection, the cells were treated with MG132 (10 µM) for 12 hr or with RA (2 µM) for 30 hr. The cells were then scraped into 100 µl lysis buffer (10 mM Tris-HCl, pH 8.0, 500 mM NaCl, 2% SDS),
heated for 10 min at 95 °C, diluted with buffer lacking SDS to reduce the SDS concentration to 0.2%, and then sonicated mildly on ice. After pre-adsorption with Protein G- sepharose, anti-Flag M2 monoclonal antibody was used to precipitate the immuno-complexes, which were then used for Western blotting with anti-Myc antibody (MBL). For the ubiquitination assay of N-CoR in NB4 Cells, NB4 cells (4 x 10^7) were treated with MG132 (5 µM, 12 hr) or RA (1 µM, 3 days), and lysates were prepared as described above, immunoprecipitated with anti-N-CoR antibody, and blotted with anti-ubiquitin antibody (FL-76, Santa Cruz). To examine the effect of the dominant negative form of Ubc6, 293T cells was transfected with 1.5 µg pact-N-CoR-Flag, 1.5 µg pact-HA-PML-RARα and 1 µg pcDNA3-Myc-Ubiquitin with an increasing amount of pcDNA3-Ubc6 (CS mutant) or Ubc5 (CS mutant) expression vectors, and ubiquitination assays were performed as described above.

**GST pull-down assays and co-immunoprecipitation** --- GST pull-down assays were performed essentially as described (8). The GST fusion of full-length PML-RARα or various domains of PML were used with various in vitro-translated Ubc enzymes in a binding buffer containing 10 mM HEPES (pH 6.5), 2.5 mM MgCl_2, 50 µM ZnCl_2, 0.5 mM DTT, and 0.01% BSA. For co-immunoprecipitation of PML-RARα and Ubc6, lysates from 293T cells transfected with 3 µg pact-HA- PML-RARα and 3 µg of FLAG-Ubc6 expression vector were prepared by sonication in LSLD buffer (50 mM Hepes, pH 6.5, 50 mM NaCl, 20 µM NaF, 20% glycerol, and a protease inhibitor cocktail). Immunoprecipitation was performed with anti-Flag antibody, and the anti-HA
antibody was used for Western blotting. For co-immunoprecipitation between endogenous Ubc6 and PML-RARα, lysate of NB4 cells prepared in above mentioned buffer was immunoprecipitated with UbcH6 antibody (Boston Bio-chemicals) and Western was done with anti-RARα antibody to detect endogenous PML-RARα protein.

For co-immunoprecipitation of PML-RARα and PDI, NB4 cells were lysed in PBS-0.1% NP40 by mild sonication, immunoprecipitated with the anti-PDI antibody and blotted with the anti-RAR antibody (Santa Cruz). To detect the association of N-CoR with PDI, a cytosolic fraction of NB4 cells was prepared as described below, immunoprecipitated with anti-PDI antibody, washed with PBS, and N-CoR was detected with goat anti-N-CoR antibody.

*Flow cytometry* --- The retroviral expression plasmid for N-CoR was constructed using the MSCV (murine stem cell virus)-based retroviral vector. The plasmid pMSCV-N-CoR encodes both N-CoR and GFP separated by internal ribosome entry site so that they are expressed together. NB4 cells (10 x 10^6 cells) were transfected with 40 µg of pMSCV-N-CoR or MSCV empty vector using DMRIE-C reagent (Invitrogen) for 24 hr, and then complete medium was added and the cells were cultured for an additional 48 hr. After that, the cells were collected, washed twice with PBS-0.5% BSA, and incubated in 500 µl PBS-0.5% BSA with RPE-conjugated monoclonal mouse anti-human CD14 antibodies (DAKO) for 60 min on ice. The cells were then washed with PBS-0.5% BSA and analyzed on a FACScan flow cytometer (Becton Dickinson). The GFP-positive cells comprised 3-4% of all the NB4 cells. To detect the exogenous N-CoR in NB4 cells, lysate prepared from nuclear fraction of transfected
NB4 cells was subjected to Western blotting using anti-Flag antibody.

To investigate the effect of tunicamycin on RA-mediated differentiation of NB4 cells, NB4 cells were treated with RA (1 µM) alone or with RA and tunicamycin (2.5 µg/ml). Tunicamycin was added in RA-treated cells 24 hours before harvesting. Cells were incubated with RPE-conjugated monoclonal mouse anti-human CD11b antibody (DAKO) and FACS analysis was done as described above.

Luciferase reporter assays --- 293T cells in 6 well plates were co-transfected with the Gal4 site-containing luciferase reporter vector (0.1 µg), the Ubc6(CS) or Ubc5(CS) expression plasmids in increasing amount (0.2 µg increments), PML-RARα (0.2 µg) expression plasmid, and the internal control plasmid pRL-TK (0.01 µg) using Lipofectamin-Plus reagent. Dual luciferase assays were performed as recommended by the manufacturer.

RESULTS

The PML-RARα/N-CoR complex is recruited to the ER --- We previously demonstrated that PML-RARα has two sites that directly bind to N-CoR (8, 9). It is possible that when the N-CoR molecule binds to PML-RARα through these two sites, it adopts an aberrant protein conformation. This may cause N-CoR to become a target of the protein quality control machinery located in the ER. To test this, we assessed whether the PML-RARα/N-CoR complex is bound by protein disulphide isomerase (PDI) and BiP/GRP78 (immunoglobulin binding protein/glucose-regulated protein), two of the ER resident protein, as they have been shown to mediate ER retention and
degradation of poorly assembled and mis-folded proteins (25). 293T cells were cotransfected with the PML-RARα and N-CoR expression vectors and stained with antibodies against either protein together with antibodies that recognize the endogenous PDI and BiP molecule, followed by confocal microscopy analysis. When N-CoR or PML-RARα was expressed alone, the N-CoR signals were localized in the nuclear dot-like structures known as NBs, while PML-RARα was found predominantly in the cytosol like the endogenous PDI and BiP (Fig. 1A). As reported (6), the microspeckled nuclear PML and PML-RARα signals were very weak compared to the cytosolic signals of overexpressed PML-RARα signals. When N-CoR was co-expressed with PML-RARα, both proteins colocalized with the endogenous PDI or BiP molecules in the ER (Fig. 1B). The signal of PDI and BiP proteins in 293T cells co-expressing PML-RARα/N-CoR were much stronger than those of non-transfected cells, suggesting that co-expression of PML-RARα/N-CoR may induce expression of those proteins (Figs. 1A, B). When PML-RARα/N-CoR were co-expressed with Golgi-ds-Red, a vector containing Golgi-targeting sequence, some fraction of the PML-RARα/N-CoR signals colocalized with the Golgi signal (Fig. 1C). RA treatment of transfected cells co-expressing PML-RARα/N-CoR resulted in some degree of nuclear relocation of both PML-RARα and N-CoR, and PML NBs formation (Fig. 1D).

To confirm the localization of the PML-RARα/N-CoR complex in the ER, we performed a Nycodenz gradient fractionation of the cytoplasmic lysates prepared from 293T cells ectopically expressing N-CoR and PML-RARα (Fig. 1E). Subcellular compartments were fractionated by Nycodenz gradient, and each fraction was probed
with antibodies specific to marker protein of the compartment. Fractions 10-16 were
designated ER by their possessing of either PDI or BiP, and fractions 4-8 were
designated Golgi because they contained Golgin-97, the integral membrane protein
localized on the cytoplasmic face of the Golgi apparatus (26). N-CoR was found
predominantly in the ER fraction and to some extent, also in the Golgi fraction. PML-
RARα was known to form a dimer or oligomer (27, 28), and we could detect both
monomer and dimer under standard SDS-PAGE condition, probably due to very strong
capacity to form a dimer. Both the dimeric and monomeric forms of PML-RARα were
found in the ER fraction, while the monomeric PML-RARα was also abundant in the
Golgi fraction. Since it was recently shown that the ER-Golgi traffic is a prerequisite for
efficient ER degradation of mis-folded proteins (29), the localization of N-CoR and
PML-RARα in the ER and Golgi is consistent with our notion that the N-CoR protein
complexed with PML-RARα has an aberrant protein conformation.

When APL-derived NB4 cells were assessed for endogenous PML-RARα and
PDI signals, significant overlapping between them was observed (Fig. 2A, upper panel),
although in the case of NB4 cells a clear judgement of subcellular localization was
slightly difficult due to small cytoplasm of NB4 cells. A considerable amount of the
endogenous N-CoR signals in the cytoplasm overlapped with the PDI signals (Fig. 2A,
middle panel). In addition, overlapping of N-CoR signals with Golgin-97 was observed
(Fig. 2A, lower panel). In NB4 cells treated with RA, the dot-like structure of PML and
N-CoR were observed in the nucleus while most of the PDI signals were in the cytosol
(Fig. 2B).
In the Nycodenz fractionation of the cytoplasmic lysates of NB4 cells, N-CoR was predominantly recovered in the Golgi fraction, though the ER fraction also contained some N-CoR protein (Fig. 2C). The monomeric form PML-RARα was found in the ER fraction, while those of dimeric form were recovered in the Golgi fraction. The difference in the distribution of PML-RARα and N-CoR between transfected 293T cells and NB4 cells may be due to the relatively high amounts of the PML-RARα/N-CoR proteins in the transfected 293T cells. These observations, together with our previous finding that PML-RARα directly associates with N-CoR (8, 9), suggest that N-CoR bound to PML-RARα is recruited to the ER.

*PML-RARα/N-CoR targeted to the ER triggers ER stress*** --- We previously shown that N-CoR directly binds to PML-RARα via the two sites (8). To examine whether the N-CoR protein bound to PML-RARα has the different conformation compared to the unbound form, we investigated the proteases sensitivity of N-CoR. Whole-cell lysates were prepared from the RA-treated and untreated NB4 cells, and digested with different concentration of protease K or trypsin (Fig. 3A). The results of Western blotting indicated that the pattern of N-CoR fragments obtained by both proteases were different between the RA-treated and untreated cell lysates. In the RA-treated lysates, the N-CoR protein appeared to be more sensitive to both proteases. These results suggest that the N-CoR protein conformation changes by binding to PML-RARα.

Since PDI has been shown to mediate ER retention and degradation of poorly
assembled and mis-folded proteins (25), we tried to confirm the association of PDI with PML-RARα and N-CoR by a co-immunoprecipitation assay using lysates prepared from NB4 cells. Significant amounts of PML-RARα and N-CoR proteins, but not another corepressor mSin3A, were co-precipitated by the anti-PDI antibody, confirming that N-CoR bound to PML-RARα forms a complex with PDI in NB4 cells (Fig. 3B). Thus, the PML-RARα/N-CoR complex is recruited to the ER through its interaction with the ER resident protein PDI.

ATF6, a bZIP transcription factor, is synthesized as an ER-localized transmembrane protein with a lumenal-sensing domain and a cytosolic transcription transactivation domain. Upon ER stress, ATF6 translocates from the ER to the Golgi where it is processed to its active form and is then transported to the nucleus, where it binds to the ER stress-inducible elements of a group of chaperone genes (22, 30). To investigate whether the PML-RARα/N-CoR complex induces ER stress and therefore activates the processing of ATF-6, we studied the status of the ATF6 protein in NB4 cells by Western blotting the cell lysates with anti-ATF6 antibody (Fig. 3C). A processed form of ATF6 [pATF6(N)] was detected in NB4 cells, indicating that NB4 cells are in a state of elevated ER stress. When NB4 cells were treated with RA, the amount of processed ATF6 decreased considerably, indicating that RA treatment relieves the stress, possibly through the RA-mediated degradation of the PML-RARα protein (31). In addition, HeLa cells cotransfected with the plasmids expressing N-CoR, PML-RARα and ATF6 also contained the cleavage product and deglycosylated form of ATF6 (Fig. 3D). This form was not detected when ATF6 was expressed on its own.
Localization of high amounts of N-CoR/PML-RARα in the Golgi may disturb the glycosylation of ATF6 in the ER, leading to the accumulation of the deglycosylated form of ATF6. We performed this latter experiment in HeLa cells because they have been frequently used in the study of the ER stress-induced activation of ATF6. In the control experiments, HeLa cells transfected with ATF6 alone were treated with tunicamycin or DTT. As reported previously (30), these treatments also generated the cleavage product and deglycosylated form of ATF6. Thus, the PML-RARα/N-CoR complex is recruited to the ER lumen and induces ER stress and the UPR.

**PML-RARα induces insoluble N-CoR protein accumulation and causes the reduction of soluble N-CoR in the nucleus** --- Recently it was shown that when the misfolded G protein-coupled transmembrane polypeptide Pael-R is overexpressed, it becomes insoluble and ubiquitinated and induces ER stress (32). We therefore investigated whether the N-CoR protein complexes with PML-RARα also results in the insolubility and ubiquitination of N-CoR protein. 293T cells were cotransfected with N-CoR-Flag expression plasmid with the PML-RARα expression vector or empty vector, lysed and the solubility of the ectopically expressed N-CoR protein was examined by immuno-blotting of soluble and insoluble fractions of lysates with the anti-Flag antibody. Compared to N-CoR expressed alone, N-CoR expressed together with PML-RARα was dramatically less soluble in both the lysates of the whole cell (Fig. 4A, left panel) or in the nuclear fraction (Fig. 4A, right panel). In addition, almost all of the N-CoR protein was found as insoluble aggregates in the pellet fraction in the presence of...
PML-RARα (Fig. 4A). The solubility of N-CoR protein was also found to be reduced in NB4 cells, while RA treatment of NB4 cells significantly increased the solubility of N-CoR (Fig. 4B). This indicates a link between N-CoR protein solubility and the differentiation of NB4 cells. Together with the results of immunostaining and the subcellular fractionation described above, these results indicate that the N-CoR in the ER and Golgi becomes insoluble.

We next investigated the half-life of the N-CoR protein in the presence or absence of PML-RARα in transfected 293T cells by pulse-chase labelling. The soluble form of N-CoR protein was stable in the absence of PML-RARα with a half-life of around 16 hr as reported (33), whereas its half-life was reduced to about 1 hr by co-expression of PML-RARα (Fig. 4C). In contrast, the half-life of the mixture of soluble and insoluble forms of N-CoR (in the RIPA buffer lysates) was found to be prolonged about two-fold by the presence of PML-RARα, from 6 hours in the absence of PML-RARα to 12 hours in its presence (Fig. 4D). These findings suggest that PML-RARα causes the N-CoR protein to rapidly accumulate in the ER as the insoluble form, possibly by blocking its physiological turnover and diverting the aggregated N-CoR to the ERAD-mediated degradation pathway.

It has been reported that poorly assembled and mis-folded secretory proteins are ubiquitinated and degraded by the ERAD-mediated protein quality control mechanism (19, 20, 21). To investigate whether PML-RARα-mediated insolubility of N-CoR involves a similar mechanism, we performed in vivo ubiquitination assays using 293T cells. Thus, 293T cells were transfected with N-CoR-Flag and myc-ubiquitin with
or without PML-RARα, treated with the proteasome inhibitor MG132 to block degradation and lysed. Anti-Flag immunoprecipitates were then assessed with the anti-myc antibody. The presence of PML-RARα caused significant levels of ubiquitinated N-CoR protein to accumulate in the MG132-treated 293T cells, which was reduced significantly when cells were treated with RA (Fig. 4E). Similarly, treatment of NB4 cells with MG132 also resulted in the accumulation of ubiquitinated endogenous N-CoR molecules (Fig. 4F). Thus, N-CoR in the presence of PML-RARα becomes heavily ubiquitinated. RA treatment of NB4 cells increased endogenous N-CoR protein levels (Fig. 4F). When N-CoR ubiquitination in NB4 cells treated with RA and MG132 was examined, it was found that the degree of ubiquitination per N-CoR molecule was lower than in RA untreated cells (Fig. 4F). Thus, PML-RARα causes the reduction of soluble form of N-CoR protein in the nucleus and in the cytosol by converting it into an insoluble form.

The ER integral membrane protein Ubc6 mediates PML-RARα-induced ubiquitination of N-CoR --- Ubiquitination is facilitated by specific ubiquitin-conjugating enzymes (Ubc) known as E2 (34). In eukaryotes, multiple Ubc enzymes have been reported so far. To identify the specific Ubc enzyme involved in PML-RARα-mediated ubiquitination of N-CoR, we performed GST-pull down assays using known Ubc enzymes and the GST-PML-RARα protein. Of the multiple in vitro-translated Ubc enzymes tested, only Ubc6 interacted with GST-PML-RARα (Fig. 5A). Ubc6 is an integral membrane protein that is anchored via its hydrophobic C-terminal
tail to the ER and is involved in the ER-mediated protein quality control mechanism (35, 36). The association between Ubc6 and PML-RARα was further confirmed by co-immunoprecipitation and immunocytochemistry assays using 293T cells transfected with the Ubc6 and PML-RARα expression plasmids. The HA-tagged PML-RARα was precipitated along with the Flag-linked Ubc6 protein, indicating that ectopically expressed PML-RARα and Ubc6 form a complex in vivo (Fig. 5B, left panel). Similarly, UbcH6 antibody precipitated endogenous PML-RARα from the lysate of NB4 cells (Fig. 5B, right panel). Expression of UbcH6 protein in NB4 cells was confirmed by Western blotting using the above mentioned anti UbcH6 antibody (data not shown). Moreover, ectopically expressed Ubc6 was found to colocalize with exogenous PML-RARα and endogenous PDI in the cytosol of 293T cells (Fig. 5C). To identify the domain of PML-RARα that interacts with Ubc6, we used the GST fusion proteins containing various portions of PML (Fig. 5D). The GST fusion proteins that contained the Ring finger domain or the B box bound efficiently to the in vitro-translated Ubc6 protein.

We confirmed that Ubc6, in the presence of PML-RARα, specifically mediates N-CoR ubiquitination by using a dominant negative form of Ubc6. All E2 enzymes contain an active cysteine residue that is used for ubiquitin-thiolester formation. In the case of Ubc2 (RAD6) and UbcH10, alteration of this conserved cysteine to serine causes these proteins to form a ubiquitin ester that cannot be transferred to the target proteins, thereby generating the dominant negative forms of these proteins (37, 38). We examined the effect of similar mutants of Ubc6 and Ubc5 (CS mutants) on N-CoR
ubiquitination in MG132-treated 293T cells that ectopically express N-CoR-Flag and myc-ubiquitin with or without PML-RARα. The Ubc6 mutant inhibited the ubiquitination of N-CoR in a dose-dependent manner, whereas the Ubc5 mutant had no effect (Fig. 5E). These findings indicate that Ubc6 is specifically involved in the PML-RARα-mediated ubiquitination of N-CoR and may also play a role in its anchorage to the ER as Ubc6 is an integral membrane protein that is anchored to the ER (37, 38).

The roles played by each PML-RARα domain in targeting N-CoR to the ER ---

To further characterize the roles the individual PML-RARα domains play in the targeting of N-CoR to the ER, we assessed the contribution of the various PML-RARα domains to N-CoR insolubility, ubiquitination, and cytoplasmic localization by transfecting 293T cells with the plasmids to express various PML-RARα mutants (Fig. 6A). PML-RARα contains most of the functional domains of the PML and RARα molecules, including the PML Ring finger, B boxes and coiled-coil domain. As mentioned, the PML-RARα binds to N-CoR via the coiled-coil region of PML and the CoR box in RARα (8). We speculated that this aberrant binding may induce an abnormal conformation of N-CoR, and that this leads to ER stress and ERAD. Consistent with this notion, we found that the deletion of the coiled-coil region (ΔC-C) or point mutations in the CoR box (AHT) abrogated the capacity of PML-RARα to induce N-CoR insolubility (Fig. 6A, left panel). Consistent with these effects on N-CoR insolubility, both of these mutants (ΔC-C and AHT) were also defective in enhancing the ubiquitination of N-CoR (Fig. 6A, right panel). Surprisingly, however, while the
Ring finger mutant (RF) induced N-CoR ubiquitination at the same level as wild-type PML-RARα (Fig. 6A, right panel), it failed to induce any N-CoR protein insolubility (Fig. 6A, left panel). PML did not stimulate insolubility or ubiquitination of N-CoR when over-expressed in 293T cells with N-CoR (Fig. 6A, both panels).

To further characterize the roles the individual PML-RARα domains play in the insolubility of N-CoR, we investigated the subcellular distribution of ectopically expressed N-CoR in 293T cells together with various PML-RARα mutants (Fig. 6B). When expressed alone, N-CoR was uniformly distributed in the nucleus and formed a micro-speckled pattern (Fig. 1A). When N-CoR was co-expressed with PML, N-CoR was recruited into nuclear dot-like structures where it co-localized with PML (Fig. 6B). However, when N-CoR was co-expressed with PML-RARα, only a little nuclear distribution of N-CoR was visible and most of the N-CoR and PML-RARα signals were both observed in the cytosol. Disruption of either of the N-CoR-binding sites in PML-RARα (ΔC-C or AHT) resulted in the failure to retain N-CoR in the cytosol, suggesting that the tight binding of N-CoR via the two sites is required to bring the N-CoR protein to the ER. Interestingly, the Ring finger mutant of PML-RARα (RF) targets the ubiquitinated N-CoR protein to specific sites close to the ER that are quite distinct from the localization pattern mediated by wild-type PML-RARα. A PML-RARα mutant lacking the B box (ΔB box) failed to target N-CoR to the cytosol and to induce a significant level of N-CoR ubiquitination or insolubility. This suggests that the B boxes also participate in generating an aberrant N-CoR protein conformation and possibly also in ubiquitination (Figs. 6A and B). These findings thus reveal the specific role that each
domain of PML-RARα plays in the cytosolic retention and ubiquitination of the N-CoR protein. The coiled-coil domain and N-CoR box of PML-RARα are apparently required in tandem for the cytosolic N-CoR retention, the B boxes participate in the ubiquitination of N-CoR, and the Ring finger domain plays a role in the insolubility of N-CoR protein.

**Down-regulation of N-CoR correlates with PML-RARα-induced leukemogenesis** --- To investigate whether the targeting of the N-CoR protein to the ER by PML-RARα is at least partly contributes to the PML-RARα-induced leukemogenesis, we examined whether the forced expression of N-CoR could similarly promote NB4 differentiation. We found that NB4 cells transiently transfected with the N-CoR expression plasmid indeed underwent differentiation as shown by the expression of the monocytic differentiation marker CD14 (Fig. 7A).

Next we investigated the effect of Ubc6(CS) mutant on PML-RARα mediated abrogation of Mad repression. We reported previously that the nuclear PML-RARα abrogates the Mad-mediated transcriptional repression in a dominant negative fashion (8). As shown above (Fig. 5E), Ubc6 is involved in the PML-RARα-mediated ubiquitination and degradation of N-CoR. If the dominant-negative form of Ubc6 blocks the degradation of mis-folded N-CoR, the accumulated N-CoR that has the aberrant conformation would be transported into the nucleus and may block the Mad-dependent transcriptional repression. When Ubc6(CS) mutant was co-expressed with PML-RARα in 293T cells, it enhanced the dominant negative effect of PML-RARα on Mad
repression as expected (Fig. 7B). The Ubc5(CS) mutant did not affect the Mad-mediated repression. Thus Ubc6 is required to maintain the activity of typical tumor suppressor Mad by reducing the levels of the N-CoR protein complexed with PML-RAR.

We also investigated the possible contributory role of ER stress in the transformation of NB4 cells by examining the effect of tunicamycin on the RA-mediated differentiation of NB4 cells. As shown above (Fig. 3D), tunicamycin induces the ER stress. When it was employed with RA, it blocked the RA-mediated differentiation of NB4 cells significantly as marked by down-regulation of monocytic differentiation marker CD11b (Fig. 7C). In addition, we observed the tunicamycin treatment also blocked the morphological differentiation of NB4 cells into the macrophage-like shapes (data not shown). These results suggest the possible role of ER stress in the PML-RARα-induced leukemogenesis.

DISCUSSION

Based on our findings, we hypothesize that the cytoplasmic PML-RARα molecule engages the N-CoR molecule just after translation in the cytosol by binding to it with its PML-derived coiled-coil domain and its N-CoR-derived CoR motif. The resulting complex probably acquires an abnormal conformation due to this aberrant binding and become a target of the protein quality control machinery located in the ER. We observed that various normal protein, including the transcription factors c-Myb and ATF-2, were not localized in the ER when they were overexpressed (Khan and Ishii, unpublished results), supporting the notion that an abnormal conformation of the protein
is critical for the ER retention. When PML-RARα alone was overexpressed in 293T cells, PML-RARα is located in the ER (Fig. 1A), suggesting that this fusion protein itself has an abnormal conformation. It is also possible that wild-type PML-RARα may simply promote the cytosolic retention of N-CoR by masking its nuclear localization signal (NLS), which the ΔC-C or AHT mutants cannot do. However, the cytosolic retention of the PML-RARα/N-CoR complex is not due to that the PML-RARα molecule lacks the nuclear localization signal (NLS) of PML, which is located in the C terminal region of PML, because an artificial PML-RARα protein containing the full-length PML molecule [PML(full-length)-RARα] was also found to be localized in the cytosol along with N-CoR (data not shown). So far, only secreted and membrane proteins appear to be degraded by the ERAD pathway and thus the postulated movement of a nuclear factor to the ER for degradation may appear to be unusual. However, some transcription factors, including Fos, have also been found in the ER (39). Furthermore, it was recently shown the N-CoR/TAB2 complex is exported to the cytosol in response to IL-1β (40). Thus, our proposal that the N-CoR protein which has the abnormal conformation moves to the ER may not be unusual.

The Ring finger domain serves as a catalytic domain for the ubiquitin ligase activity of many E3 ligases (41). However, in our present study, we found that the in vivo ubiquitination of PML-RARα-induced N-CoR does not require an intact Ring finger of PML-RARα (Fig. 6A). It was recently demonstrated that the Mdm2-mediated nuclear export of the p53 protein requires the presence of an intact Ring finger domain in the Mdm2 protein (42, 43). This suggests that the Ring finger may participate not
only in the recognition of substrates by E3 ligases but may also be involved in other types of protein-protein interactions. Our findings indicate that PML-RARα with an intact Ring finger domain may be involved in transporting the PML-RARα/N-CoR complex across the ER membrane (Fig. 6). Our results also suggest that the Ring finger domain is essential for insoluble protein aggregate formation, possibly because it targets ubiquitinated proteins to ER membrane structures (Fig. 6). Alternatively, the Ring finger may directly induce the insolubility of mis-folded protein by facilitating the formation of tight multi-protein complexes that have the hydrophobic residues on their surfaces (44), whereas a Ring finger mutant may not be able to form such complexes.

The PML-RARα protein did not exhibit any ubiquitination activity when used with recombinant N-CoR and Ubc6 proteins in \textit{in vitro} ubiquitination assays (data not shown). Moreover, the Ring finger mutant of PML-RARα was equally efficient in inducing the ubiquitination of N-CoR in vivo (Fig. 6A). These findings preclude a role for PML-RARα as an E3 ligase in N-CoR ubiquitination. Multiple E3 ligases, including Hrd1p/Der3p, Parkin, and CHIP have been reported to be involved in ERAD (45-47). However, we found that neither Parkin nor CHIP could induce N-CoR ubiquitination in \textit{in vitro} ubiquitination assays (data not shown), suggesting that an unique E3 ligase is involved in the PML-RARα-induced ubiquitination of N-CoR.

Protein insolubility and aggregate formation are the hallmark of the cytopathology observed in many neurodegenerative diseases including Parkinson's disease, Alzheimer's disease and Huntington's disease (48, 49). The common pathological event in all of these diseases is the accumulation of highly ubiquitinated
mis-folded protein as insoluble aggregates and the development of inclusion bodies that are toxic to the cells. Our finding that a fusion protein generated by a chromosomal translocation induces ER stress and UPR represents the first evidence linking the protein quality control mechanism in the ER to uncontrolled growth and transformation. This unique means of oncogenic gain of function may not be restricted to APL. Indeed, it is likely that a broad range of mis-folded proteins arising from mutations, deletions or translocations at the genetic level may derail normal growth control pathways by similar mechanisms. Despite APL being a paradigm of successful differentiation therapy, the frequent development of RA resistance and its toxicity represent a major challenge and highlight the importance of developing new therapeutic approaches. Targeting the quality control mechanism in ER or the isomerase activity of PDI may provide useful alternative approaches in the treatment of APL as well as other forms of human cancers.

Acknowledgements --- We are grateful to A. Kakizuka and R. Evans for the PML and PML-RARα cDNAs, and R. Takahashi for Ds-RED-Golgi. This work was supported in part by Grants-in-Aid for Scientific Research and the Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Science and Technology, and by a grant from the Human Frontier Science Program.
REFERENCES

1. Kakizuka, A., Miller, W.H. Jr., Umesono, K., Warrell, R.P. Jr., Frankel, S.R., Murty, V.V., Dmitrovsky, E. and Evans, R.M. (1991) Cell, 66, 663-674

2. de The, H., Lavau, C., Marchio, A., Chomienne, C., Degos, L. and Dejean, A. (1991) Cell, 66, 675-684

3. Jensen, K., Shiels, C. and Freemont, P.S. (2001) Oncogene, 20, 7223-7233

4. Mu, Z.M., Chin, K.V., Liu, J.H., Lozano, G. and Chang, K.S. (1994) Mol. Cell. Biol. 14, 6858-6867

5. Wang, Z.G., Delva, L., Gaboli, M., Rivi, R., Giorgio, M., Cordon-Cardo, C., Grosveld, F. and Pandolfi, P.P. (1998) Science, 279, 1547-1551

6. Dyck, J.A., Maul, G.G., Miller, W.H. Jr., Chen, J.D., Kakizuka, A. and Evans, R.M. (1994) Cell, 76, 333-343

7. Weis, K., Rambaud, S., Lavau, C., Jansen, J., Carvalho, T., Carmo-Fonseca, M., Lamond, A. and Dejean, A. (1994) Cell, 76, 345-356

8. Khan, M.M., Nomura, T., Kim, H., Kaul, S.C., Wadhwa, R., Shinagawa, T., Ichikawa-Iwata, E., Zhong, S., Pandolfi, P.P. and Ishii, S. (2001) Mol. Cell, 7, 1233-1243

9. Khan, M.M., Nomura, T., Kim, H., Kaul, S.C., Wadhwa, R., Zhong, S., Pandolfi, P.P. and Ishii, S. (2001) J. Biol. Chem., 276, 43491-43494

10. Zhong, S., Delva, L., Rachez, C., Cenciarelli, C., Gandini, D., Zhang, H., kalantry, S., Freedman, L.P. and Pandolfi, P.P. (1999) Nature Genet., 23, 287-295

11. Quignon, F., De Bels, F., Koken, M., Feunteun, J., Ameisen, J.C. and de The, H.
(1998) *Nature Genet.*, **20**, 259-265

12. Wang, Z.G., Ruggero, D., Ronchetti, S., Zhong, S., Gaboli, M., Rivi, R. and Pandolfi, P.P. (1998) *Nature Genet.*, **20**, 266-272

13. Hörlein, A.J., Näär, A.M., Heinzel, T., Torchia, J., Gloss, B., Kurokawa, R., Ryan, A., Kamei, Y., Söderström, M., Glass, C.K., and Rosenfeld, M.G. (1995). Nature **377**, 397-404

14. Heinzel, T., Lavinsky, R.M., Mullen, T.M., Soderstrom, M., Laherty, C.D., Torchia, J., Yang, W.M., Brard, G., Ngo, S.D., Davie, J.R., Seto, E., Eisenman, R.N., Rose, D.W., Glass, C.K. and Rosenfeld, M.G. (1997) *Nature*, **387**, 43-48

15. Pearson, M., Carbone, R., Sebastiani, C., Cioce, M., Fagioli, M., Saito, S., Higashimoto, Y., Appella, E., Minucci, S., Pandolfi, P.P. and Pelicci, P.G. (2000) *Nature* **406**, 207-210

16. Di Croce, L., Raker, V.A., Corsaro, M., Fazi, F., Fanelli, M., Faretta, M., Fuks, F., Lo Coco, F., Kouzarides, T., Nervi, C., Minucci, S. and Pelicci, P.G. (2002) *Science*, **295**, 1079-1082

17. Lin, R.J, Nagy, L., Inoue, S., Shao, W., Miller, W.H.Jr. and Evans, R.M. (1998) *Nature* **391**, 811-814

18. Grignani, F., De Matteis, S., Nervi, C., Tomassoni, L., Gelmetti, V., Cioce, M., Fanelli, M., Ruthardt, M., Ferrara, F.F., Zamir, I., Seiser, C., Grignani, F., Lazar, M.A., Minucci, S. and Pelicci, P.G. (1998) *Nature*, **391**, 815-818

19. Wickner, S., Maurizi, M.R. and Gottesman, S. (1999) *Science*, **286**, 1888-1893

20. Pickart, C.M. (2001) *Annu. Rev. Biochem.*, **70**, 503-533
21. Fewell, S.W., Travers, K.J., Weissman, J.S. and Brodsky, J.L. (2001) Annu. Rev. Genet., 35, 149-191

22. Haze, K., Yoshida, H., Yanagi, H., Yura, T. and Mori, K. (1999) Mol. Biol. Cell, 10, 3787-3799

23. Jepsen, K., Hermanson, O., Onami, T.M., Gleiberman, A.S., Lunyak, V., McEvilly, R.J., Kurokawa, R., Kumar, V., Liu, F., Seto, E., Hedrick, S.M., Mandel, G., Glass, C.K., Rose, D.W. and Rosenfeld, M.G. (2000) Cell, 102, 753-763

24. Tran, K., Thorne-Tjomsland, G., J. DeLong, C., Cui, Z., Shan, J., Burton, L., C. Jamieson, J. and Yao, Z. (2002) J. Biol. Chem., 277, 31187-31200

25. Bottomley, M.J., Batten, M.R., Lumb, R.A. and Bulleid, N.J. (2001) Curr. Biol., 11, 1114-1118

26. Barr, F.A. (1999) Curr. Biol., 9, 381-384

27. Minucci, S, Maccarana, M., Cioce, M., De Luca, P., Gelmetti, V., Segalla, S., Di Croce, L., Giavara, S., Matteucci, C., Gobbi, A., Bianchini, A., Colombo, E., Schiavoni, I., Badaracco, G., Hu, X., Lazar, M.A., Landsberger, N., Nervi, C. and Pelicci, P.G. (2000) Mol. Cell 5, 811-820

28. Lin, R.J. and R.M. Evans, R.M. (2000) Mol. Cell 5, 821-830

29. Taxis, C., Vogel, F. and Wolf, D.H. (2002) Mol. Biol. Cell., 13, 1806-1818

30. Shen, J., Chen, X., Hendershot, L. and Prywes, R. (2002) Dev. Cell 3, 99-111

31. Raelson, J.V., Nervi, C., Rosenauer, A., Benedetti, L., Monczak, Y., Pearson, M., Pelicci, P.G. and Miller, W.H.Jr. (1996) Blood 88, 2826-2832

32. Imai, Y., Soda, M., Inoue, H., Hattori, N., Mizuno, Y., and Takahashi, R. (2001).
Cell 105, 981-902

33. Zhang, J., Guenther, M. G., Carthew, R. W. and Lazar, M.A. (1998) Genes Dev., 12, 1775-1780

34. Hershko, A. and Ciechanover, A. (1998) Annu. Rev. Biochem., 67, 425-479.

35. Tiwari, S. and Weissman, A.M. (2001) J. Biol. Chem., 276, 16193-16200

36. Lenk, U., Yu, H., Walter, J., Gelman, M.S., Hartmann, E., Kopito, R.R. and Sommer, T. (2002) J. Cell. Sci., 115, 3007-3014

37. Sung, P., Prakash, S. and Prakash, L. (1991) J. Mol. Biol., 221, 745-749

38. Townsley, F.M., Aristarkhov, A., Beck, S., Hershko, A. and Ruderman, J.V. (1997) Proc. Natl. Acad. Sci. USA, 94, 2362-2367

39. Bussolino, D.F., Guido, M.E., Gil, G.A., Borioli, G.A., Renner, M.L., Grabois, V.R., Conde, C.B. and Caputto, B.L. (2001) FASEB J., 15, 556-558

40. Baek, S.H., Ohgi, K.A., Rose, D.W., Koo, E.H., Glass, C.K. and Rosenfeld, M.G. (2002) Cell, 110, 55-67

41. Jackson, P.K., Eldridge, A.G., Freed, E., Furstenthal, L., Hsu, J.Y., Kaiser, B.K. and Reimann, J.D. (2000) Trends Cell Biol., 10, 429-439

42. Boyd, S.D., Tsai, K.Y. and Jacks, T. (2000) Nature Cell Biol., 2, 563-568

43. Geyer, R.K., Yu, Z.K. and Maki, C.G. (2000) Nature Cell Biol., 2, 569-573

44. Shiraki, K., Kudou, M., Fujiwara, S., Imanaka, T. and Takagi M. (2002) J. Biochem., 132, 591-595

45. Bays, N.W., Gardner, R.G., Seelig, L.P., Joazeiro, C.A. and Hampton, R.Y. (2001) Nature Cell Biol., 3, 24-29
46. Shimura, H., Hattori, N., Kubo, S., Mizuno, Y., Asakawa, S., Minoshima, S., Shimizu, N., Iwai, K., Chiba, T., Tanaka, K. and Suzuki, T. (2000) *Nature Genet.*, 25, 302-305

47. Murata, S., Minami, Y., Minami, M., Chiba, T. and Tanaka, K. (2001) *EMBO Rep.*, 2, 1133-1138

48. Kopito, R.R. (2000) *Trends Cell Biol.*, 10, 524-530

49. Kopito, R.R. and Ron, D. (2000) *Nature Cell Biol.*, 2, E207-E209
**LEGENDS TO FIGURES**

**FIG. 1.** The PML-RARα/N-CoR complex is recruited to the ER/Golgi. A, Subcellular distribution of N-CoR or PML-RARα. 293T cells were transfected with either the N-CoR or HA-PML-RARα expression vector and visualized by anti-N-CoR or anti-HA antibodies and rhodamine- or FITC-conjugated secondary antibodies. Endogenous PDI or BiP staining were visualized in non-transfected 293T cells by FITC-conjugated secondary antibodies. B, Exogenously expressed PML-RARα and N-CoR colocalize with PDI and BiP. 293T cells were transfected with both the HA-PML-RARα and N-CoR expression plasmids, and immunostained. PML-RARα and N-CoR signals were visualized by rhodamine-conjugated antibodies, while endogenous PDI or BiP staining were performed by FITC-conjugated secondary antibodies. In the right-most panels, nuclear DNA was stained with TO-PRO. In the panels indicated as overlay, all the three signals are superimposed. C, Exogenously expressed PML-RARα and N-CoR are partially targeted to Golgi. 293T cells were co-transfected with the N-CoR and HA-PML-RARα expression vectors together with Ds-Red-Golgi as Golgi marker. N-CoR- or HA-PML-RARα signals were visualized by FITC-conjugated secondary antibodies and Golgi signals were visualized by rhodamine. Nuclear DNA was stained with TO-PRO. All the three signals are superimposed (Overlay). D, Partial nuclear targeting of N-CoR or PML-RARα by RA. Experiments were performed as described in B, except that cells were treated with RA. E, Subcellular fractionation. Cytosolic fractions of 293T cells transfected with N-CoR and PML-RARα expression plasmids were subjected to Nycodenz gradient fractionation analysis. The levels of N-CoR, PDI,
PML-RARα, BiP and Golgin-97 in each fraction were analyzed by immunoblotting. More amount of lysates was used to detect the PML-RARα dimer than that to detect PML-RARα monomer. No signals with anti-lamin B antibody indicates no nuclear cross contamination in the samples used.

FIG. 2. The PML-RARα/N-CoR complex is recruited to the ER/Golgi in NB4 cells. A, Endogenous PML-RARα and N-CoR co-localize with PDI and Golgin-97. (Upper) PML-RARα- and PDI-stainings of NB4 cells were visualized by anti-PML and anti-PDI antibodies and FITC- and rhodamine-conjugated secondary antibodies, respectively. Nuclear DNA was stained with TO-PRO. All the three signals are superimposed (Overlay). (Middle) N-CoR- and PDI-staining visualized by rhodamine- and FITC-conjugated secondary antibodies, respectively. (Lower) N-CoR and Golgin-97 signals were visualized by FITC- and rhodamine-conjugated secondary antibodies, respectively. B, Recovery of nuclear dot-like structure of N-CoR or PML by RA. Experiments were performed as described in A, except that cells were treated with RA for three days. C, Subcellular fractionation. Nycodenz gradient fractionation analysis was performed as described in Fig. 1E by using the cytosolic fractions of NB4 cells.

FIG. 3. The N-CoR protein associated with PML-RARα induces ER stress. A, Protease sensitivity of N-CoR. The whole-cell lysates from RA-treated and untreated NB4 cells were digested with different concentration of protenase K or trypsin. The N-CoR protein fragments were detected by Western blotting. B, The endogenous PML-
RARα/N-CoR complex is co-immunoprecipitated with PDI. The NB4 cell lysates were precipitated with anti-PDI or control IgG, and the immuno-complexes were analyzed by Western blotting using anti-RAR, anti-N-CoR, or anti-mSin3A antibodies. Samples from the lysates were also used directly for Western blotting. C, The ATF6 precursor is cleaved in NB4 cells. Whole cell lysates prepared from RA-treated or untreated NB4 cells were used for Western blotting with the anti-ATF6 antibody. The same amounts of lysates as that used for Western blotting were analyzed by SDS-PAGE followed by Coomassie staining (lower panel). D, ATF6 precursor cleavage is induced when N-CoR is co-expressed with PML-RARα. (Left) HeLa cells were transfected with the HA-ATF6 expression plasmid with the plasmids expressing the proteins indicated above. The ATF6 proteins were detected by Western blotting. (Right) HeLa cells were transfected with the HA-ATF6 expression plasmid alone and treated with tunicamycin (TM) or DTT for the indicated time, and ATF6 was detected. The same amounts of lysates as that used for Western blotting were analyzed by SDS-PAGE followed by Coomassie staining (lower panels).

FIG. 4. PML-RARα induces the insolubility and ubiquitination of N-CoR. A, PML-RARα induces the insolubility of N-CoR protein. 293T cells were transfected with the N-CoR-Flag expression plasmid with the PML-RARα plasmid (+) or the empty vector (-). (Left) Transfected cells were passively lysed, and the soluble and insoluble fractions were separated. The N-CoR protein levels were determined using anti-Flag antibody. The same amounts of lysates as that used for Western blotting were analyzed by SDS-
PAGE followed by Coomassie staining (lower panels). (Right) Transfected cells were lysed and nuclei were isolated, which were then extracted using the same buffer described above. B, RA increases the solubility of N-CoR protein in NB4 cells. NB4 cells were treated with or without RA, and lysates were prepared using either RIPA buffer (RIPA lysate) or buffer containing salt and NP-40 (soluble fraction). The N-CoR levels were examined by Western blotting. C, PML-RARα decreases the half-life of soluble form of N-CoR. 293T cells were transfected with the N-CoR-Flag expression plasmid with or without the PML-RARα expression plasmid, and pulse-chase labeled. The levels of N-CoR in the soluble fraction were examined and plotted. Note that 10-fold more amount of lysate from the PML-RARα-expressing cells were used compared to that of control lysates. D, Effect of PML-RARα on the half-life of mixture of soluble and insoluble N-CoR. Experiment was performed as described in (C) except that the lysates were prepared using the buffer containing SDS. E, PML-RARα induces N-CoR ubiquitination. 293T cells were transfected with the plasmid expressing N-CoR-Flag and myc-ubiquitin with or without the PML-RARα expression plasmid, and treated with MG132. In some case, the cells were treated with RA. N-CoR-Flag was immunoprecipitated and the immuno-complexes were detected with the anti-myc antibody. The filter was reprobed with anti-Flag antibody (lower panel). F, N-CoR ubiquitination is enhanced in NB4 cells. Whole cell lysates were prepared from NB4 cells treated with the drugs indicated above, and immunoprecipitated with the anti-N-CoR antibody. Ubiquitinated N-CoR proteins (upper) and total N-CoR proteins (lower) were detected.
FIG. 5. **Ubc6 associates with PML-RARα and mediates N-CoR ubiquitination.**

A, PML-RARα binds to Ubc6 *in vitro*. (Left) The GST-PML-RARα protein was analyzed by SDS-PAGE followed by Coomassie blue staining. (Right) The binding of various *in vitro*-translated Ubc proteins to GST-PML-RARα was examined. In the input lanes, the amount of Ubc was 10% of that used in the binding assay. 

B, Co-immunoprecipitation of PML-RARα and Ubc6. (Left) Lysates were prepared from 293T cells transfected with the Flag-Ubc6 and HA-PML-RARα expression plasmids and precipitated by anti-Flag or control IgG. The immuno-complexes were analyzed in Western blotting with anti-HA antibodies. Total lysate samples were also used directly in Western blotting. (Right) Similar co-immunoprecipitation experiments were performed using the NB4 cell lysates and anti-UbcH6 antibody.

C, Ubc6 colocalizes with exogenous PML-RARα and endogenous PDI. 293T cells were transfected with the Flag-Ubc6 and HA-PML-RARα expression plasmids and their signals were visualized. Endogenous PDI was also visualized.

D, Identification of the PML domains that bind to Ubc6. The GST-PML constructs used are shown. In the upper panel, the *in vitro*-translated Ubc6 used (input) and the proteins bound to GST-PML were analyzed. In the lower panel, various GST-PML fusions were analyzed by SDS-PAGE followed by Coomassie staining.

E, The dominant negative form of Ubc6 abrogates N-CoR ubiquitination. *In vivo* ubiquitination of N-CoR was examined as described in Fig. 4E, except that increasing amounts of the plasmids that express the dominant negative forms of Ubc6 (left) or Ubc5 (right) were also transfected.
FIG. 6. **Roles played by various PML-RARα domains in N-CoR insolubility, ubiquitination, or ER localization.** A, The effect of various PML-RARα mutants on N-CoR insolubility and ubiquitination. (Upper) The mutants used are shown on the left. The effect of the mutations on the aberrant binding to N-CoR, insolubility and ubiquitination of N-CoR are indicated on the right. (Lower left) To examine the effect on N-CoR insolubility, experiments were performed as described in Fig. 4A using various forms of PML-RARα. (Lower right) To assess the ubiquitination of N-CoR, experiments similar to those described in Fig. 4E were performed. B, Subcellular localization of N-CoR co-expressed with PML-RARα or its various mutants. 293T cells were transfected with the plasmids expressing N-CoR-Flag and the various forms of HA-PML-RARα shown on the left of each row, and analyzed using anti PML, anti-HA and anti-Flag antibodies. In the right-most panel, the signals for both proteins are superimposed. In the case of the RF mutant of PML-RARα, the nuclear DNA was also stained with TO-PRO.

FIG. 7. **Down-regulation of N-CoR correlates with PML-RARα-induced leukemogenesis.** A, Over-expression of N-CoR induces NB4-cell differentiation. (Left) NB4 cells were transfected with the N-CoR and GFP expression plasmid (lower) or control GFP vector (upper) and CD14 expression in GFP-positive cells was examined by flow cytometry. (Right) Levels of exogenous N-CoR in nuclear fraction of transfected NB4 cells was examined by Western blotting. The same amounts of lysates
as that used for Western blotting were analyzed by SDS-PAGE followed by Coomassie staining (lower panel). B, Ubc6(CS) mutant enhances dominant negative effect of PML-RARα. 293T cells were transfected with Gla4-site containing reporter, the Gal4-Mad expression plasmid, the PML-RARα expression plasmid, and the plasmid to express Ubc6(CS) mutant or Ubc5(CS). Luciferase activity was measured and the relative activity (average of three independent experiments) is shown as a bar graph with standard deviations. C, Tunicamycin blocks the RA-mediated differentiation of NB4 cells. NB4 cells were treated with RA alone or with RA and TM, and was subjected to FACS analysis to examine the CD11b expression.
Fig. 1. M. M. Khan et al.
Fig. 2. M. M. Khan et al.
Fig. 3. M. M. Khan et al.
Fig. 4. M. M. Khan et al.
Fig. 5. M. M. Khan et al.
Fig. 6. M. M. Khan et al.
Fig. 7. M. M. Khan et al.
The fusion oncoprotein PML-RAR-α induces endoplasmic reticulum-associated degradation of N-CoR and ER stress
Md Matiullah Khan, Teruaki Nomura, Tomoki Chiba, Keiji Tanaka, Hiderou Yoshida, Kazutoshi Mori and Shunsuke Ishii

J. Biol. Chem. published online December 29, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M312121200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts