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Cloning and Expression of a Novel Nuclear Matrix-associated Protein That Is Regulated during the Retinoic Acid-induced Neuronal Differentiation*

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Retinoic acid (RA), a derivative of vitamin A, is essential for the normal patterning and neurogenesis during development. RA treatment induces growth arrest and terminal differentiation of a human embryonal carcinoma cell line (NT2) into postmitotic central nervous system neurons. Using RNA fingerprinting by arbitrarily primed polymerase chain reaction, we identified a novel serine/threonine-rich protein, RA-regulated nuclear matrix-associated protein (Ramp), that was down-regulated during the RA-induced differentiation of NT2 cells. Prominent mRNA expression of ramp could be detected in adult placenta and testis as well as in all human fetal tissues examined. The genomic clone of ramp has been mapped to the telomere of chromosome arm 1q, corresponding to band 1q32.1-32.2. Associated with the nuclear matrix of NT2 cells, Ramp translocates from the interphase nucleus to the metaphase cytoplasm during mitosis. During the late stage of cytokinesis, Ramp concentrates at the midzone of the dividing daughter cells. The transcript expression of ramp is closely correlated with the cell proliferation rate of NT2 cells. Moreover, overexpression of Ramp induces a transient increase in the proliferation rate of NT2 cells. Taken together, our data suggest that Ramp plays a role in the proliferation of the human embryonal carcinoma cells.

Retinoic acid (RA), a derivative of vitamin A, serves as an important soluble factor that mediates the differentiation of both the neuronal and hematopoietic precursor cells. As a natural morphogen, RA specifies the axial patterning during the embryonic development and affects neurogenesis (1, 2). RA is a powerful differentiating agent and induces the differentiation of many cell types such as epithelial cells and mesenchyme cells (3, 4). It also induces the differentiation of cancer cells such as teratocarcinoma and leukemia (5) and many immortalized cell lines. Thus, RA has been used as an effective therapeutic agent for the treatment of acute promyelocytic leukemia (6).

The human embryonal carcinoma cell line, NTera2 cl. D1 (NT2), has been extensively used as a model system to study growth and differentiation as well as cancer therapy. RA treatment induces growth arrest and terminal differentiation of NT2 cells into postmitotic central nervous system neurons (7, 8). The RA-induced neuronal differentiation of NT2 cells is concomitant with the up-regulation of the homeotic genes, such as Hox 2.1 and Hox 2.2. On the contrary, the expression of growth factors, including transforming growth factor-α and basic fibroblast growth factor, and proto-oncogenes, such as c-myc and N-myc, are down-regulated during the neuronal differentiation (9). These responses are mediated through two classes of nuclear receptors, the RA receptors and retinoid X receptors (10–12). Although it is well-documented that RA receptors and retinoid X receptors play essential roles in RA-mediated events, including primitive endodermal differentiation, cell proliferation, and apoptosis, the molecular mechanisms underlying these diverse physiological actions have only recently been explored (13–15). Even less is known about how different genes are regulated by RA during these processes. The involvement of other signaling molecules, such as the coordinated regulation of the neurotrophin receptors, may mediate RA-induced central nervous system neuronal differentiation (17). Thus, one major thrust has been the identification of genes that are regulated during the RA-mediated cellular events (18, 19).

We have employed the RNA fingerprinting by arbitrarily primed PCR to identify candidate genes that are differentially regulated during the RA-induced neuronal differentiation of the NT2 cells (20). Among the candidate genes identified, clone 8.31 encoded a novel gene that was down-regulated during the RA-induced neuronal differentiation of NT2 cells. Here we report the cloning and characterization of this novel gene, designated RA-regulated nuclear matrix associated protein (Ramp). Ramp is a serine/threonine-rich protein that is associated with the nuclear matrix protein of the NT2 cells, and translocates from the nucleus to the cytoplasm during mitosis and cytokinesis. Our data suggest that Ramp plays a role in the cell proliferation of these human embryonal carcinoma cells.
**EXPERIMENTAL PROCEDURES**

**Cloning of Full-length cDNA of Ramp**—Full-length cDNA of clone 8.31 (ramp) was obtained by screening an expression cDNA library prepared from undifferentiated NT2 cells (Stratagene) using the partial 8.31 cDNA fragment obtained by RNA fingerprinting by arbitrarily primed PCR as probes. The cDNA sequence encoding ramp has been submitted to GenBank™ (accession number AF345896). Radioactive cDNA probes were prepared using M13 template DNA labeled with 

1. Biotin (Amersham Pharmacia Biotech) and hybridization was performed at 60 °C. Single phages were obtained and transformed into XLOLR bacterial cells (Stratagene) and the cDNA fragment was cloned into pBK-CMV mammalian expression vector by in vivo excision.

2. **Cell Culture and Cell Proliferation Assays**—NT2 cells were cultured as previously described (21). Cells were maintained in Opti-MEM I reduced serum medium (Life Technologies, Inc.) supplemented with 5% fetal bovine serum (fetal bovine serum, Life Technologies, Inc.). NT2 cells were differentiated with 10 μμ of all-trans RA (t-RA; Sigma Chemical Co.) in Dulbecco's modified Eagle's medium (high glucose formulation) containing 10% fetal bovine serum. Cell synchronization was performed by treating NT2 cells with nocodazole (1 μμ) for 12 h, which arrested the cells at G2-M. Transfection of NT2 cells was performed using FuGENE reagent (Roche Molecular Biochemicals) in 96-well plates. Expression vectors (0.15 μμ) plus 0.05 μμ of the reference plasmid pCMV-βgal, expressing β-galactosidase as internal control for normalization, were used. For cell proliferation assays, NT2 cells were transiently transfected with expression vectors containing wild type ramp, 6X histidine-, or EGF-tagged ramp. Reference plasmid pCMV-βgal was co-transfected to serve as internal control for normalizing the transfection. Mixtures of recombinant alkaline phosphatase and β-galactosidase assay were performed as previously described (Roche Molecular Biochemicals) (22). BrdUrd incorporation assays were performed using BrdUrd-based cell proliferation enzyme-linked immunosorbent assay (Roche Molecular Biochemicals). Briefly, cells were labeled with BrdUrd solution for 2 h at 37 °C. The cells were then fixed and denatured using FixDenat solution. After incubation with horseradish peroxidase-conjugated anti-BrdUrd, the cells were incubated with substrate solution. Absorbance was measured at 450 nm. All experiments were repeated at least three times.

**Promoter Constructs and Alkaline Phosphatase Assays**—Genomic DNA fragment (−2396 bp) containing the putative ramp promoter and the first exon (2304–2354 bp) of ramp cDNA (data not shown). The genomic DNA fragment was then fused to the 5′-end of the promoterless reporter secretory form of human placental alkaline phosphatase (SEAP, CLONTECH). Deletion mutant (RampΔ, ~311 bp) was constructed by deleting a −2080-bp DNA fragment from the 5′-end of the putative promoter. For promoter analysis, a putative ramp promoter was subcloned to the 5′-region of the reporter gene SEAP. SV40 early and acetylcholine receptor ε subunit promoters (23) were used as control in the experiment. NT2 cells transfected with promoter constructs were treated with 1 mM sodium orthovanadate and protease inhibitor mixture (Complete, Roche Molecular Biochemicals). Cells were then lysed with lysis buffer (50 mM Tris-Cl at pH 8, 150 mM NaCl, 1% Triton-X, 1× aprotinin; Sigma, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 1× Complete) at 4 °C for 10 min. Lysates were collected and centrifuged to remove cell debris. Protein assays were performed with the Bio-Rad protein assay kit based on the Bradford dye-binding procedure (Bio-Rad). Protein samples (typically 40 μg) were separated by SDS-polyacrylamide gel electrophoresis in 10% resolving gel using a Hoeffer minigel apparatus. Proteins were then electrotransferred to nitrocellulose membrane (Amersham Pharmacia Biotech). After blocking at room temperature for 1 h using TBS-Tween containing 5% non-fat milk, membranes were incubated overnight at 4 °C with primary antibodies in TBS-Tween containing 5% non-fat milk. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (1:2500, Zymed Laboratories Inc.) for 1 h at room temperature. After three washes with TBS-Tween, immunoreactive bands were detected using an ECL kit (Amersham Pharmacia Biotech) and visualized by x-ray film (Fuji).

**RESULTS**

**Cloning of the Full-length Coding Sequence of Ramp**—The cDNA encoding full-length 8.31 (ramp) was obtained by screening a cDNA library prepared from the undifferentiated NT2 cells. Double-stranded sequencing by T7 DNA polymerase revealed that the cDNA (−2831 bp) is novel in its gene identity. The full DNA sequence and its deduced amino acid sequence are shown in Fig. 1A. The cDNA contains a single open reading frame corresponding to a protein of 730 amino acids (−79.4 kDa). The ATG codon preceded by a consensus Kozak sequence, P<sub>2</sub>P<sub>1</sub>AP, at residues 637–641. Moreover, there is a hydrophilic residue (Fig. 1B). Amino acid identity to Human and Mouse proteins begins at residues 13.04% arginines, lysines, and histidines. There are five potential glycosylation sites at residues 190, 248, 289, 299, and 316, and three putative cAMP- and cGMP-dependent kinase phosphorylation sites at residues 413–416, 482–485, and 688–691. Another interesting feature is the presence of a proline-rich motif that shares high homology with the SH3-binding consensus, PXXP, at residues 637–641. Moreover, there is a leucine zipper located at 577 and two WD repeats at 113 and 231 of the cloned ramp cDNA.
Ramp protein contains several putative N-glycosylation sites, it is possible that the ~180-kDa immunoreactive represents the glycosylated protein.

**Ramp Is Expressed in the Placenta and Testis**—To obtain clues on the potential function(s) of ramp, we have examined the expression profile of ramp in both adult and fetal human tissues. Among the tissues examined, including brain, colon, heart, kidney, liver, lung, skeletal muscle, placenta, small intestine, spleen, stomach, and testis, two transcripts (~5 and ~3 kb) of ramp were predominantly expressed in the placenta and testis (Fig. 2A). Interestingly, skeletal muscles also expressed low level of ramp (upon long exposure of the autoradiogram).

The expression of ramp was observed in all the human fetal tissues examined, which included brain, lung, liver, and kidney. All fetal tissues expressed the ~5- and ~3-kb ramp transcripts. Two extra transcripts of ~4 and ~2 kb were observed in the messenger RNA prepared from the fetal lung (Fig. 2B). However, similar prominent expression of ramp was not observed in the corresponding adult tissues.

Based on abundant level of expression of ramp in fetal liver, dot blot analysis was performed to examine the mRNA level in hematopoietic tissues (Fig. 2C). Although ramp was detected in all hematopoietic tissues examined, mRNA level was highest in thymus and bone marrow. A low level of ramp transcript was detected in the spleen and lymph node, and only a barely detectable level was observed in the peripheral leukocytes.

**Chromosomal Localization of Ramp by Fluorescence in Situ Hybridization**—DNA from a genomic clone of ramp was labeled with digoxigenin-dUTP by nick translation. Labeled probe was combined with sheared human DNA and hybridized to normal metaphase chromosomes derived from PHA-stimulated peripheral blood lymphocytes. The initial experiment resulted in the specific labeling of the long arm of a group A chromosome, which was believed to be chromosome 1 on the basis of size, morphology, and banding pattern (Fig. 3A). Thus, a biotin-labeled probe, which was specific for the heterochromatic region of chromosome 1, was co-hybridized with the genomic clone of ramp. The specific labeling of genomic fragment containing ramp was labeled green (indicated by white arrows), and the heterochromatin of the chromosome 1 labeled red (indicated by gray arrows) (Fig. 3B). A total of 80 metaphase cells were analyzed with 76 exhibiting specific labeling. Measurements of 10 specifically labeled chromosomes 1 demonstrated that the genomic clone of ramp is located at a position that is 62% of the distance from the heterochromatin-euchromatin boundary of the telomere of chromosome arm 1q, an area that corresponds to band 1q32.1–32.2. The result is schematized in Fig. 3C.

**RA Treatment Down-regulated the Expression of Ramp in NT2 Cells**—To examine the regulation of ramp expression by RA, the full-length ramp cDNA was used as a probe to examine its change in expression profile upon treatment with RA for 0–28 days (Fig. 4A). Two transcripts (~4.5 and ~3.5 kb) of ramp were detected in NT2 cells. The transcript expression of ramp was transiently up-regulated after 1 day of RA treatment, followed by a decrease, reaching a low level of expression at day 28. Moreover, total proteins were obtained from NT2 cells treated with t-RA for 6 or 12 days. An immunoprecipitation assay was performed using anti-Ramp (C terminus) antibodies. The resulted immunoprecipitates were electrophoresed and immunoblotted with anti-Ramp antibodies. As depicted in Fig. 4B, undifferentiated NT2 cells expressed abundant level of Ramp proteins, which was repressed after 6 days of RA treatment. Interestingly, additional immunoreactive bands (~175 and ~180 kDa) were also detected by the anti-Ramp antibodies employed in the study.

To confirm that the full-length cDNA encoded a functional protein, coupled in vitro transcription and translation was performed to estimate the molecular weight of the encoded protein. It was demonstrated that the cloned full-length ramp cDNA could be translated into a protein of ~85 kDa (Fig. 1C). In addition, a chimeric enhanced green fluorescence protein (EGFP)-tagged Ramp protein (E-Ramp) was constructed by fusing the EGFP-encoding cDNA to the N terminus of the full-length ramp cDNA. The chimeric cDNA construct was transiently expressed in CHO cells and total proteins were collected. Immunoblotting using the anti-Ramp polyclonal antibodies revealed the expression of the EGFP-tagged Ramp proteins (Fig. 1D). Two immunoreactive bands (~110 and ~180 kDa) were observed, the identity of which was confirmed by immunoblotting with anti-EGFP antibodies. Because the
**Fig. 2.** Expression profile of *ramp* in human tissues. Northern blots of multiple tissues (A, adult tissues; B, fetal tissues; CLONTECH) were hybridized using the full-length *ramp* cDNA as a probe. Size markers in kilobases are as indicated. C, 2 μg of poly(A)*⁺* RNAs from human normal tissues (CLONTECH) was used in the dot blot to examine the expression of *ramp* in various adult tissues of hematopoietic origin, as well as fetal tissues.

**Fig. 3.** Chromosomal localization of *ramp* by FISH. A, *ramp* genomic DNA probes labeled with digoxigenin dUTP were hybridized to normal metaphase chromosomes derived from PHA-stimulated peripheral blood lymphocytes. Positive signals were detected by incubation with fluorescent anti-digoxigenin antibodies and counterstained with DAPI. Specific labeling of the long arm of chromosome 1 was observed based on the size, morphology, and banding pattern. B, biotin-labeled probe specific for the heterochromatic region of chromosome 1 was co-hybridized with *ramp*. Heterochromatin was labeled with Texas red avidin in red (indicated in the black and white figure by gray arrows) and *ramp* in green (indicated by white arrows). Measurements of 10 specifically labeled chromosomes 1 demonstrated that *ramp* is located at a position, which is 62% of the distance from the heterochromatic-euchromatic boundary to the telomere of chromosome arm 1q, an area that corresponds to band 1q32.1–32.2. A total of 80 metaphase cells were analyzed with 76 exhibiting specific labeling. C, two ideograms illustrating the chromosomal position of *ramp* at 1q32.1–32.2. Both ideograms are from the International System for Human Cytogenetic Nomenclature 1995.
Ramp Is Localized to the Nucleus and Is a Nuclear Matrix-associated Protein—To define the subcellular localization of Ramp protein, we transiently transfected the ramp expression vector into CHO cells. Transfected CHO cells were immunostained with anti-Ramp polyclonal antibodies and counterstained with DAPI. It was observed that the Ramp protein was localized to the nucleus (Fig. 5A). A chimeric Ramp was constructed by fusing a 6X histidine tail to the C terminus of the Ramp. After transient transfection into CHO cells, the expression of Ramp was detected by double staining the transfected cells using the anti-6X histidine antibodies and anti-Ramp antibodies. Ramp protein was localized to the nuclear region of the transfected CHO cells, although some expression of Ramp could also be detected in the cytoplasm (Fig. 5B). The nuclear-cytoplasmic trafficking of the transfected Ramp observed in CHO cells raised the possibility that Ramp is associated with the nuclear matrix.

To confirm that Ramp is a nuclear matrix-associated protein, we examined whether Ramp was localized to the nuclear matrix proteins of the NT2 cells. Nuclei were digested with DNase I and extracted with 2 M NaCl to enrich for the nuclear matrices and its associated proteins. The nuclear matrix preparations were then electrophoresed and immunoblotted with anti-Ramp antibodies. Ramp proteins were detected in the crude nuclear preparation as well as the DNase I-treated and NaCl-washed purified nuclear matrix proteins (Fig. 5C).

Ramp Redistributes during the Cell Cycle of the NT2 Cells—The subcellular distribution of Ramp was then examined throughout the cell cycle of the unsynchronized NT2 cells. During the interphase, strong Ramp immunoreactivity was detected mainly in the nuclei of the NT2 cells (Fig. 6A). Positive signal, although less concentrated, could also be observed in the cytoplasm. At pro-metaphase and metaphase, Ramp was excluded from the condensed chromosomes, with the staining localized to the cytoplasmic region. In telophase, the Ramp staining remained in the cytoplasmic region of the daughter cells. It is interesting to observe that Ramp became concentrated at the midzone as the dividing NT2 cells progressed to late anaphase in cytokinesis. The subcellular localization of Ramp during the cell cycle raises the possibility that Ramp is a nuclear matrix-associated protein and actively participates in the cell division of NT2 cells.

NT2 cells were then synchronized at G1-M phase by treatment with nocodazole. At pro-metaphase, NT2 cells revealed a strong DNA (DAPI) staining of the condensing chromosomes. Immunofluorescent staining using specific Ramp antibodies confirmed our previous observations with unsynchronized NT2 cells. Ramp proteins were localized to the cytoplasm and excluded from the chromatins (Fig. 6B). The G1-M-arrested NT2 cells were then immunostained with control antibodies that detected the tyrosine-phosphorylated cdc2 (Phospho-cdc2 Y15). Interestingly, the subcellular distribution of phospho-cdc2 Y15 was similar to that of the Ramp proteins. Thus, the localization of the Ramp proteins is consistent with our hypothesis that Ramp actively participates in the process of cell division.

RA Could Up-regulate the Transcriptional Expression of Ramp in NT2 Cells—To evaluate the effect of RA on the expression of the ramp gene, we cloned and sequenced a ~2639-bp genomic DNA fragment containing the putative ramp promoter and the first exon (2304–2354 bp) of ramp cDNA (data not shown). The genomic DNA fragment was then fused to the 5′-end of the promoterless reporter SEAP. The SV40 early and the acetylcholine receptor e promoters (ACHRe) (23) were used as control promoter reporter plasmids in the study. Transient transfection into NT2 cells resulted in the constitutive expression of SEAP, demonstrating that the putative ramp promoter was transcriptionally active in undifferentiated NT2 cells. The transfected NT2 cells were treated with 10 μM t-RA for 24 h, and the SEAP expression was monitored. RA up-regulated the relative expression of SEAP by ~40% (p < 0.005; Fig. 7A). This is consistent with our previous observations that the expression of ramp was transiently up-regulated during the early stage of neuronal differentiation. Deletion of the 2080-bp DNA fragment from the putative ramp promoter (RampΔ) reduced the expression level of the reporter SEAP as well as the induction by RA. Similarly, RA treatment did not significantly affect the expression level of the control SV40 early and AChRe promoters.

As the first step to explore the possibility that Ramp might play a role in the cell proliferation of the NT2 cells, we examined the effect of short term RA treatment on the proliferation of NT2 cells. NT2 cells were treated with t-RA (10 μM) for 1–7 days. The cell proliferation was quantitated by measuring the metabolic rate of viable cells and by determining the total cell count of NT2 cells. When NT2 cells were treated with t-RA for 1 day, there was a significant increase in the proliferation of NT2 cells, both in terms of metabolic activities (12%, Fig. 7B) as well as in total cell number (~18.9%, Fig. 7C). At day 2, significant difference between the RA treatment or the control solvent treatment was not observed. This was followed by a sharp decrease in the total metabolic rate at day 4 of RA treatment. To further confirm the effect of RA on the proliferation of NT2 cells, we examined the rate of cell proliferation by monitoring the BrdUrd incorporation after 1–3 days of RA treatment. As shown in Fig. 7D, the rate of BrdUrd incorporation was increased by more than 10% of the control after 1 day of RA treatment. Similar down-regulation was observed after day 2 of RA treatment. Thus, our data demonstrated a good correlation between the expression of ramp and the cell proliferation rate of NT2 cells.

**Fig. 4.** RA treatment down-regulated the expression of Ramp in NT2 cells. A, Northern blot analysis of ramp expression during the RA-induced neuronal differentiation of NT2 cells. Total RNA (10 μg) prepared from NT2 cells treated with t-RA for 0–28 days was hybridized with the full-length ramp cDNA at high stringency. The position of the 28S ribosomal RNAs is indicated, whereas arrowheads depict the positions of the ramp transcripts. B, total protein lysates were obtained from NT2 cells either treated with t-RA or control solvent for 6 or 12 days. Antibodies specific to the N terminus (N) were added, and the immunoprecipitates were electrophoresed. Immunoblotting was performed using anti-Ramp (C terminus specific; C) antibodies.
Cloning and Expression of Ramp

Overexpression of Ramp Enhanced the Cell Proliferation of NT2 Cells—To further evaluate the effect of ramp on the cell proliferation, we transiently transfected the ramp expression vector into the NT2 cells. Control β-galactosidase expression vector driven by the constitutively expressed CMV promoter was used for normalizing the transfection efficiency. Rate of cell proliferation was monitored by MTT assays 48 h after transfection. Overexpression of ramp significantly up-regulated the proliferation rate of the NT2 cells by ~10% (Fig. 8A). We could observe similar up-regulation of cell proliferation rate (~14%) when the human neuroblastoma SY5Y cells were transfected transiently with the wild type ramp (Fig. 8B).

The effect of ramp on the proliferation of NT2 cells was also monitored by the BrdUrd incorporation assays. We transiently transfected the EGFP-tagged ramp (E-Ramp) expression vector into the NT2 cells and examine the rate of BrdUrd incorporation. Because the expression of retinoid receptor (RXRα) was not affected during the early stage of RA treatment (13), EGFP-tagged RXRα (E-RXR) was used as negative control in the experiment. Overexpression of E-Ramp significantly up-regulated the NT2 cell proliferation rate by ~30% whereas transient transfection of E-RXR did not significantly affect the NT2 cell proliferation rate (Fig. 8C).

DISCUSSION

In this paper, we report the identification and characterization of a novel gene, designated ramp. This gene was initially identified as a candidate gene that was down-regulated during the RA-induced neuronal differentiation of human NT2 cells. Cloning of the full-length cDNA reveals that Ramp does not share any homology with known proteins, and that it is a serine/threonine-rich protein expressed predominantly in adult placenta, testis, and hematopoietic tissues such as thymus and bone marrow. We demonstrate that Ramp associates with the nuclear matrix in the undifferentiated NT2 cells. Furthermore, the distinct subcellular localization of Ramp during the cell cycle, together with its capabilities to enhance the cell proliferation, suggests a role for Ramp in the mitosis and cytokinesis of the NT2 cells.

The deduced amino acid sequence of Ramp exhibits a number of interesting features. Although Ramp contains a putative nuclear localization signal, prediction based upon the amino acid composition using PSORT server reveals that Ramp can both be a cytoplasmic and nuclear protein. Another interesting feature is the proline-rich motif, conforming to the SH3-binding consensus PXXP (27). The sequence TLPLPLRP shares high homology with the proline-rich domain of yet another serine/threonine-rich protein, Sirm (28). In addition, Ramp contains an LXXLL motif. The LXXLL motif is demonstrated to be essential for the association of thyroid hormone receptor-binding protein to the liganded thyroid hormone receptor and retinoic acid receptor (29). It is well known that cellular communication is made possible in part by the post-translational modification of the signaling proteins. With serine and threonine residues constituting more than 20% of the Ramp protein, Ramp may serve different functions depending on the phosphorylation status. We have observed that Ramp proteins are phosphorylated at serine in undifferentiated NT2 cells (data not shown). This suggests that phosphorylation of Ramp may involve protein kinases, such as Akt, RSK1, and p70S6K (30). However, its potential signaling kinases and phosphatases remain to be identified. The presence of two WD repeats in Ramp is also of interest. WD repeats were first identified in the β-subunit of heterotrimeric GTP-binding proteins (G proteins) (31). The role of WD repeats in facilitating protein-protein interaction has been proposed (32). It is noteworthy that many of these mammalian WD repeat-containing proteins, such as chromatin assembly factor I, retinoblastoma-binding proteins, and Cdc20/fizzy, are involved in cell cycle regulation (32–34). Our preliminary data on the analysis of ramp promoter suggest that the transcriptional expression of ramp in NT2 cells is up-regulated during the early phase of RA treatment. Moreover, our data further demonstrate that the essential regulatory elements are located in the 2102-bp DNA fragment of the putative ramp promoter. However, the detailed regulatory mechanism of the ramp expression still remains unknown and awaits to be determined.

Subcellular localization of Ramp provides insight into the potential functions of Ramp. Although low expression of Ramp is detected in the cytoplasm, it is more condensed to the nuclei of the undifferentiated NT2 cells. In actively dividing cells, as
revealed by the DAPI counterstain, higher expression of Ramp is observed and localized in the cytoplasmic region of the NT2 cells. The subcellular distribution of Ramp suggests its possible role as a nuclear matrix-associated protein, which is the set of proteins that resists the nuclease treatment, as well as the high salt treatment (26). In light of the fact that the nuclear matrix-associated proteins normally redistribute during the mitosis and cytokinesis (35), we have observed that Ramp is concentrated at the midzone of the late telophase NT2 cells, consistent with a potential role of Ramp in cytokinesis. During cytokinesis, daughter cells are cleaved in two by the constriction of an actin-rich contractile ring, which encircles the equator of the dividing cells. Myosin II present in the contractile ring is essential for the constriction of the furrow (36). In this midzone, the microtubule bundle between the chromosomes of the daughter cells and assemble with other midzone-associated proteins, such as kinesin, and inner centromere protein, to form the midbody (37). These structures strongly affect the proper completion of cytokinesis, thus requiring an accurate assembly of the midzone proteins. When NT2 cells are arrested at the pro-metaphase (G2-M) by nocodazole, Ramp becomes

**Fig. 6.** Cell cycle redistribution of Ramp in NT2 cells. A, undifferentiated NT2 cells were fixed and double-stained using specific anti-Ramp (Ramp) and anti-α-tubulin antibodies (Tubulin). Nuclei are visualized by counterstaining with DAPI. Results of the Ramp staining are indicated in the first column, α-tubulin in the second column, and DAPI in the third column. Results obtained by superimposing the first and second columns are indicated in the fourth column (Combine) on the right. B, NT2 cells were synchronized using nocodazole (1 μM), fixed, and double-stained with anti-Ramp and anti-phospho-cdc2 Y15 antibodies as indicated. Results obtained by superimposing the first and second columns are indicated in the fourth column on the right. Nuclei were visualized by counterstaining with DAPI. The scale bar represents 10 μm.

**Fig. 7.** RA transiently up-regulated the transcriptional expression of ramp promoter and the cell proliferation of NT2 cells. A, Ramp promoter vectors were constructed by subcloning the upstream genomic DNA fragment of ramp to the 5′-end of the SEAP reporter gene. Ramp deletion promoter vector (RampΔ) was constructed by deleting a 2080-bp DNA fragment from the 5′-end of the putative ramp promoter. SV40 early and AChR promoters were used as control in the study. Promoter vectors were transiently transfected into the NT2 cells. The efficiency of transfection was normalized using a β-galactosidase expression vector in each transfection. The cells were lysed after 24-h treatment with t-RA (10 μM). Alkaline phosphatase and β-galactosidase assays were performed. Bars show mean ± S.E.; n = 5. B, NT2 cells were treated with t-RA (10 μM) for 1–7 days. MTT assays were performed on days 1, 2, 4, and 7 as described. C, NT2 cells were trypsinized and counted using a Coulter cell counter on the days as indicated. D, NT2 cells were treated with t-RA (10 μM) for 1–3 days. BrdUrd assays were performed as described. For B to D, the mean absorbance obtained using solvent control was designated as 100%. Results shown represent the mean ± S.E. of a typical experiment; n = 6. The mean is shown as: *, p < 0.005, unpaired Student’s t test, compared with the control.
totally excluded from the condensed chromosomal DNA. During the progression into mitosis, the phospho-cdc2 Y15 is activated by the cdc25 phosphatase by dephosphorylating the cdc2 (38). Interestingly, we have demonstrated that the subcellular distribution of Ramp is in close association with the phospho-cdc2, which signifies the progression of the NT2 cells into mitosis. The precise interplay between the cyclins, Ramp, and cdc2, which signifies the progression of the NT2 cells into mitosis, is explained by the up-regulation of cell proliferation rate during the RA-induced neuronal differentiation of NT2 cells. Ramp is a serine/threonine-rich protein associated with the nuclear matrix of NT2 cells. While the immunocytochemical study suggests its role in the cell cycle, promoter analysis, overexpression studies and cell proliferation assays support its role in the cell proliferation of NT2 cells. The mapping of ramp to the chromosome 1q32.1–2 also suggests that it may potentially be involved in genetic diseases such as the complement malfunctioning and Usher disease.

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