The **PTC1** chimeric oncogene is generated by the fusion of the tyrosine kinase domain of the **RET** proto-oncogene to the 5′-terminal region of another gene named **H4** (D10S170). This oncogene has been detected only in human papillary thyroid carcinomas. We have previously demonstrated that the putative leucine zipper in the N-terminal region of **H4** can mediate oligomerization of the **PTC1** oncoprotein in vitro. In this study, we further demonstrated that the **PTC1** oncoprotein forms a dimer in vivo, and the leucine zipper is responsible for this dimerization. The **H4** leucine zipper-mediated dimerization is essential for transforming activity of the **PTC1** oncoprotein. Introducing a loss-of-function **PTC1** mutant into **PTC1**-transformed NIH3T3 cells suppressed the transforming activity of **PTC1** and reversed the transformed phenotype of these cells, presumably by forming inactive heterodimers between the two forms of **PTC1**. Taken together, these data indicate that constitutive dimerization of the **PTC1** oncoprotein is essential for **PTC1** transforming activity and suggest that constitutive oligomerization acquired by rearrangement or by point mutations may be a general mechanism for the activation of receptor tyrosine kinase oncogenes.

The **PTC1** chimeric oncogene, formed by intrachromosomal rearrangement between **H4** (D10S170) and the **RET** proto-oncogene, has been detected in 2.5–30% of papillary thyroid carcinomas (1–3). The product of the **PTC1** oncogene is a fusion protein containing the N terminus of **H4** fused to the tyrosine kinase domain of c-**RET** (1). The **H4** gene shows no significant homology to known genes, and the function of **H4** protein is unknown (4). The **RET** proto-oncogene encodes a receptor-type tyrosine kinase (5, 6), whose ligand has been identified as glial cell line-derived neurotrophic factor (7–9). A variety of mutations involving the **RET** proto-oncogene have been found to be associated with a number of human neuroendocrine diseases, such as multiple endocrine neoplasia type 2 inherited cancer syndromes (10–12), the congenital developmental defect **Hirschsprung’s disease** (13), and some sporadic medullary thyroid carcinomas (14).

The **PTC1** oncoprotein has been demonstrated to be hyperphosphorylated (15) and to exert transforming activity in NIH3T3 cells (16). The causative role of the **PTC1** oncogene in human papillary thyroid carcinoma is further supported by the fact that our transgenic mouse model with targeted expression of the **PTC1** oncogene in the thyroid gland develops papillary thyroid carcinomas (17). However, the molecular mechanism of **PTC1** activation in the development of papillary thyroid tumors has yet to be elucidated. We have previously demonstrated that the **PTC1** chimeric oncogene shows unscheduled expression in the thyroid follicular cells and that recombinant proteins containing the putative leucine zipper domain of **H4** form oligomeric complexes in vitro (18). As dimerization is considered to be a crucial step for receptor tyrosine kinase activation (19, 20), we hypothesized that both unscheduled expression of **RET** tyrosine kinase and constitutive oligomerization of **PTC1** proteins are responsible for **PTC1**-transforming activity in the thyroid.

In this study, we further demonstrated that the leucine zipper region of **H4** is responsible for the dimerization of the **PTC1** oncoprotein in vivo. Our data also indicated that the leucine zipper-mediated dimerization is essential for tyrosine hyperphosphorylation and the transforming activity of **PTC1**. Furthermore, the transforming activity of **PTC1** can be suppressed by introducing a loss-of-function mutant of **PTC1** into **PTC1**-transformed NIH3T3 cells.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Antibodies**—The African green monkey kidney cell line, COS-7 cells (ATCC 1651), was maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. Both NIH3T3 and NIH3T3/PTC1 were maintained in Dulbecco’s modified Eagle’s medium with 10% donor calf serum. All media contain 100 units/ml penicillin and 100 μg/ml streptomycin. The monoclonal antibody **MSJ** was generated in our laboratory. Its epitope was tentatively mapped to amino acid residues 824–828 of **RET**. The polyclonal antibody **C17**, was raised against a synthetic peptide (CKRRDYLDLAASTPSDSL) located at amino acid residues 1011–1027 of the C terminus of the **RET** tyrosine kinase (Immu-Dynamics, Inc.). The **C17** antibody was purified through an affinity column covalently bound with the synthetic peptide. The monoclonal antibody against phosphotyrosine (4G10) was obtained from Upstate Biotechnology Inc.

**Plasmid Constructs**—The **PTC1/CMV** was constructed by excising the **PTC1** cDNA from the plasmid **TPC-1** (15) and inserting it into the **XhoI** and **Apol** sites of p**Rc/CMV** (Invitrogen). The **PTC1/LNCX** was obtained by excising the **PTC1** fragment from **PTC1/CMV** with HindIII and inserting it into the **HindIII** site of p**LNCX**. To clone **PTC1**Δ**z**/CMV and **PTC1**Δ**z**/LNCX, the **T7** primer and another primer (**5′-AAGGATCCCGGAACGGCGAGATGA-3′**), which is located to a region just ahead of the leucine zipper, were used to perform polymerase chain reaction using **PTC1/CMV** as DNA template. The 250-base pair polymerase chain reaction product was digested with **BamHI** and used to replace the 400-base pair **BamHI** fragment of **H4** in **PTC1/CMV** and **PTC1/LNCX**. To clone **PTC1**Δ**n**/CMV and **PTC1**Δ**n**/LNCX, a sense primer (**5′-AGTGGATCCGCCATGCGGCGCGGCTCCGCTCGGG-3′**), which is located at the beginning of the leucine zipper region, was paired with an antisense primer (**5′-AGTTTCTCGAGGAAATCC-3′**), which is located at the beginning of the **RET** tyrosine kinase domain, to perform

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‡ To whom correspondence should be addressed: 302 Hamilton Hall, 1645 Neil Ave., Columbus, OH 43210. Tel.: 614-292-4312; Fax: 614-292-4888.

1 Q. Tong, S. Xing, and S. M. Jhiang, unpublished data.
polymerase chain reaction on the PTC1/CMV template. A 200-base pair BamHI fragment was excised from the polymerase chain reaction product and used to replace the 400-base pair H4 fragment of PTC1/CMV and PTC1/LNCX. The PTC1ΔC/CMV was generated by unidirectional deletion of the PTC1 insert, starting from the C terminus of PTC1. A mutation \((\text{Arg}^\text{897} \rightarrow \text{Glu})\), found in some patients with Hirschsprung’s disease (13), was first introduced into c-RET cDNA by site-directed mutagenesis (Muta-gene M13 in vitro mutagenesis kit, Bio-Rad) and later subcloned into PTC1/CMV and PTC1Δzip/CMV to obtain PTC1HS/CMV and PTC1ΔzipHS/CMV.

**DNA Transfection**—For transient transfection, \(5 \times 10^5\) COS-7 cells were transfected with 10–20 μg of various DNA constructs using the calcium phosphate transfection kit (Life Technologies, Inc.). For the focus formation assay, \(1.5 \times 10^5\) NIH3T3 cells were transfected with 0.3 μg of various DNA constructs. Three weeks later, the foci were stained with Giemsa and counted. For NIH3T3 stable transfectants, 4 \(\times 10^4\) NIH3T3 cells were transfected with 1 μg of various DNA constructs, and the G418-resistant colonies were screened for PTC1 expression by immunoprecipitating the cell lysates with the antibody C17, followed by immunoblot analysis with the antibody MSJ.

**Immunoprecipitation and Immunoblot Analysis**—Cells were lysed in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, and 0.5 mM NaOVO₄. For immunoprecipitation, cell lysates were incubated at 4°C overnight with C17 polyclonal antibody that was covalently conjugated to protein A beads (21). For the immunoblot assay, proteins were separated by SDS-PAGE² and then transferred to a nitrocellulose membrane. The membrane was blocked in TBST buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.1% Tween 20) containing 5% nonfat dry milk at 4°C overnight. After incubation with the primary antibody for 1 h and horseradish peroxidase-conjugated anti-mouse IgG (Transduction Laboratories) for an additional hour, the membrane was treated with ECL reagent (Amersham Corp.) and exposed to x-ray film.

**Soft Agar Assay**—The soft agar assay was carried out as follows. A bottom layer of agar was prepared in 60-mm Petri dishes using 3 ml of 0.5% noble agar (Difco) in normal growth medium. Next, 3 ml of 0.35% noble agar in normal growth medium containing 9 \(\times 10^5\) cells were added on top of the solidified bottom layer. Colonies with anchorage-independent growth were counted 2 weeks later.

### RESULTS

**PTC1 Oncoprotein Forms Dimers in Vivo, and Its Dimerization Is Mediated by the Leucine Zipper of H4**—To investigate the oligomerization status of PTC1 oncoprotein in eukaryotic cells, the PTC1 oncoprotein was expressed in COS-7 cells by transient transfection with a DNA construct PTC1/CMV. The lysates of COS-7 transfected cells expressing PTC1 were treated with glutaraldehyde to stabilize the oligomeric protein complexes (Fig. 1b). Without cross-linking treatment, PTC1 oncoprotein can be detected at the size of 53 kDa, since the PTC1 used in our experiment is the alternative spliced isoform with a shorter C terminus (6). After 1 min of treatment with glutaraldehyde, a 105-kDa protein complex can be readily detected. By 16 min, the PTC1 monomer can no longer be detected. We consistently observed that the PTC1 protein complexes had a stronger signal intensity than the PTC1 monomers in immunoblot detection. One possible explanation is that the formation of protein complexes may alter the conformation of the RET tyrosine kinase for better binding of the MSJ antibody to the epitope site.

To investigate the role of the leucine zipper of H4 in the formation of PTC1 protein complexes, two forms of PTC1 mutant, PTC1Δzip and PTC1ΔN, were constructed. PTC1Δzip had the leucine zipper region of H4 (amino acid residues 56–102) deleted. PTC1ΔN had the N terminus of H4 (amino acid residues 3–52) deleted but retained the leucine zipper (Fig. 1a). As shown in Fig. 1b, the PTC1Δzip (46 kDa) fails to form protein complexes even after a 16-min treatment with glutaraldehyde. In contrast, PTC1ΔN (49 kDa) retains the ability to form protein complexes. These data indicate that the formation of PTC1 protein complex is mediated by the leucine zipper of H4.

A co-immunoprecipitation assay was performed to further investigate whether the PTC1 forms homodimers. A mutated form of PTC1, PTC1ΔC, had the extreme C terminus of PTC1 (amino acid residues 345–461) deleted. Therefore, PTC1ΔC is no longer recognized by the polyclonal antibody C17 (Fig. 2a). When the PTC1ΔC was co-expressed in COS-7 cells with either PTC1, PTC1Δzip, or PTC1ΔN, the PTC1ΔC-encoded protein cannot be immunoprecipitated by C17 unless it forms protein complexes with other forms of PTC1 containing an intact C terminus. The expression of PTC1 and its mutants in various cell lysates is detected by the MSJ monoclonal antibody, which can react with all forms of PTC1 protein including PTC1ΔC (Fig. 2b). The PTC1ΔC product (38 kDa) cannot be immunoprecipitated by C17 when it was expressed alone or co-expressed with PTC1Δzip (Fig. 2c, lanes 2 and 4), but can be co-immunoprecipitated when it was co-expressed with PTC1 or PTC1ΔN (Fig. 2c, lanes 3 and 5). From the results of the cross-linking experiment and the co-immunoprecipitation assay, we conclude that PTC1 oncoprotein forms a homodimer in vivo, and the dimerization is mediated by the leucine zipper domain of H4.

**The H4 Leucine Zipper-mediated Dimerization Is Essential for Tyrosine Hyperphosphorylation of the PTC1 Oncoprotein**—It has been shown that dimerization of receptor tyrosine kinases promotes the transphosphorylation of tyrosine residues on intracellular domains (20). To investigate whether there is a correlation between dimerization and transphosphorylation, the tyrosine phosphorylation levels of PTC1, PTC1Δzip, and PTC1ΔN proteins expressed in COS-7 cells were examined (Fig. 3). The equivalent amount of PTC1,
PTC1∆zip, and PTC1∆N proteins used in the immunoblot analysis was also shown. The PTC1 and PTC1∆N oncoprotein are hyperphosphorylated on tyrosine residues. However, deletion of the leucine zipper region of PTC1 dramatically reduced the tyrosine phosphorylation level of the PTC1∆zip-encoded protein.

The Leucine Zipper-mediated Dimerization Is Required for the PTC1 Transforming Activity—To correlate the leucine zipper-mediated dimerization and tyrosine hyperphosphorylation with the transforming activity of PTC1, both focus formation assay and soft agar assay were performed to evaluate the transforming activity of PTC1, PTC1∆zip, or PTC1∆N. The expression of PTC1, PTC1∆zip, or PTC1∆N proteins encoded by the corresponding DNA constructs was initially confirmed by immunoblot with anti-phosphotyrosine antibody 4G10. In the experiment is demonstrated by immunoblot with the MSJ antibody.

TABLE I

| DNA construct | Foci/µmol | G418<sup>+</sup> colonies/µmol |
|---------------|-----------|---------------------------------|
| pLNCX         | <1        | 5.7 × 10<sup>2</sup>             |
| PTC1          | 5.4 × 10<sup>2</sup> | 6.0 × 10<sup>2</sup>             |
| PTC1∆zip      | <1        | 5.7 × 10<sup>2</sup>             |
| PTC1∆N        | 4.0 × 10<sup>2</sup> | 5.5 × 10<sup>2</sup>             |

For soft agar assay, NIH3T3 stable transfectants expressing PTC1, PTC1∆zip, or PTC1∆N were established. We consistently observed that the protein expression levels of PTC1 or PTC1∆N were higher than those of PTC1∆zip in corresponding NIH3T3 stable transfectants (Fig. 4). The clones expressing the lowest amount of PTC1 (PTC1–5 and PTC1–10), a clone expressing the highest amount of PTC1∆zip (PTC1∆zip-3), and clones expressing both high (PTC1∆N-2) and low (PTC1∆N-1) amounts of PTC1∆N were selected for soft agar assay. The result of soft agar assay demonstrated that NIH3T3 cells expressing PTC1 or PTC1∆N, but not PTC1∆zip, acquired anchorage-independent growth in soft agar (Table II).

Although no difference in the ability to form foci was observed between cells expressing PTC1 and cells expressing PTC1∆N (Table I), fewer colonies were formed in soft agar for PTC1∆N-expressing cells compared with PTC1-expressing cells (Table II). Even though two different clones of both PTC1 and PTC1∆N were tested by soft agar assay, the clonal effect cannot be completely excluded. Alternatively, the N-terminal region of H4 may contain some elements contributing to the anchorage-independent growth of PTC1 but not to the loss of contact inhibition. Taken together, the results of focus formation assay and soft agar assay indicate that the leucine zipper-mediated dimerization is required for the PTC1 transforming activity.

The PTC1 Transforming Activity Can Be Suppressed by a Loss-of-function PTC1 Mutant through the Formation of Heterodimers—Since intermolecular phosphorylation of dimerized receptor tyrosine kinases is crucial for the PTC1 activation, it is possible to suppress the PTC1 transforming activity by introducing a loss-of-function PTC1 mutant to form inactive heterodimers with PTC1. A mutation at codon 897 of c-RET, changing arginine to glutamine, was identified in some pa-
FIG. 4. The expression of PTC1, PTC1Δzip, or PTC1ΔN proteins in the corresponding NIH3T3 stable transfectants. Lysates of various NIH3T3 stable transfectants (1 × 10⁶ cells) were immunoprecipitated with the C17 anti-RET polyclonal antibody, followed by immunoblotting with the MSJ anti-RET monoclonal antibody. Three stable transfectants of each DNA construct (PTC1, clones 5, 7, and 10; PTC1Δzip clones 2, 3, and 11; or PTC1ΔN, clones 1, 2, and 3) are shown.

Table II

| Cell line | Number of colonies |
|-----------|-------------------|
| NIH3T3    | 0                 |
| PTC1-5    | 255 ± 30          |
| PTC1-10   | 228 ± 6           |
| PTC1Δzip-3| 0                 |
| PTC1ΔN-1  | 44 ± 6            |
| PTC1ΔN-2  | 50 ± 16           |

DISCUSSION

In this study, we demonstrated that the PTC1 oncprotein forms dimers in vivo and the dimerization of PTC1 is mediated by the leucine zipper in the H4 portion of PTC1. Our data also indicated that the leucine zipper-mediated dimerization was essential for the constitutive phosphorylation and the transforming activity of the PTC1 oncprotein. Furthermore, a loss-of-function PTC1 mutant can reverse the transformed phenotype of NIH3T3/PTC1 cells maintained transformed phenotype, which was significantly different from that of NIH3T3/PTC1 cells transfected with pRC/CMV vector (p < 0.001) or cells transfected with PTC1ΔzipHS/CMV (p < 0.05).

The leucine zipper-mediated dimerization is required for the activation of PTC1 tyrosine kinase.

In human papillary thyroid carcinomas, three different forms of the PTC oncogenes have been identified in which the RET tyrosine kinase domain becomes fused with the N-terminal sequences of three different genes (1, 24–26). In addition to PTC1, the PTC2 oncprotein has also been shown to form dimers in vivo (24), and the dimerization is required for the mitogenic activity of PTC2 (27). Although PTC3 has not been shown to form oligomers experimentally, a potential coiled-coil motif was identified in the ELE1 sequence of PTC3.1 In addition to PTC oncogenes, RETI and RETII transforming genes, which were formed by rearrangements during in vitro transfection (28, 29), also form dimers in vivo (30). Therefore, constitutive oligomerization of RET appears to be a common mechanism for the activation of rearranged RET oncproteins. In fact, constitutive oligomerization may serve as a common mechanism for oncogenic activation of other receptor tyrosine kinases in thyroid tumors. The TPR protein, which is involved in a rearrangement with the tyrosine kinase domain of the TRK nerve growth factor receptor in human papillary thyroid carcinoma (31), contains a leucine zipper domain and forms dimers in vivo (32). All of the tyrosine kinase oncogenes formed by rearrangement in human papillary carcinomas seem to be activated by constitutive oligomerization.

Rodrigues and Park (32) have proposed that oligomerization may serve as a general mechanism for oncogenic activation of receptor tyrosine kinases in many types of tumors. They demonstrated that the leucine zipper domain in TPR mediates dimerization of the TPR-MET oncprotein, and this constitutive dimerization is essential for its transforming activity. Not only can rearrangement cause oligomerization to activate receptor tyrosine kinases, but point mutations have also been shown to promote constitutive dimerization of receptor tyrosine kinases. Point mutations of RET found in MEN2A patients, resulting in the substitution of one of five Cys residues in the
extracellular domain, caused constitutive dimerization and activation of RET tyrosine kinase (33, 34). An amino acid substitution of the NEU oncogene has also been shown to induce oncoprotein aggregation, and increase tyrosine kinase activity and transforming activity (35). Therefore, the approach of using a loss-of-function mutant to suppress the transforming activity of the corresponding oncogene provides a new strategy to specifically intervene with the oncogenic activity of various receptor tyrosine kinase oncoproteins.

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