Identification of the Key Residues Responsible for the Assembly of Selenodeiodinases*

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Type I deiodinase is the best characterized member of a small family of selenoenzymes catalyzing the bioactivation and disposal of thyroid hormone. This enzyme is an integral membrane protein composed of two 27-kDa subunits that assemble into a functional enzyme after translation using a highly conserved sequence of 16 amino acids in the C-terminal half of the polypeptide, \textsuperscript{149}DFLXXX[EXAHXXDWG\textsuperscript{163}]. In this study, we used alanine scanning mutagenesis to identify the key residues in this domain required for holoenzyme assembly. Overexpression of sequential alanine-substituted mutants of a dimerization domain–green fluorescent protein fusion showed that sequence \textsuperscript{152}IYI\textsuperscript{154} was required for type I enzyme assembly and that a catalytically active monomer was generated by a single I152A substitution. Overexpression of the sequential alanine-substituted dimerization domain mutants in type II selenodeiodinase-expressing cells showed that five residues (\textsuperscript{153}FLIVY\textsuperscript{157}) at the beginning and three residues (\textsuperscript{164}SDG\textsuperscript{166}) at the end of this region were required for the assembly of the type II enzyme. In vitro binding analysis revealed a free energy of association of \(-60 \pm 5\) kJ/mol for the noncovalent interaction between dimerization domain monomers. These data identify and characterize the essential residues in the dimerization domain that are responsible for the post-translation assembly of selenodeiodinases.

Thyroid hormone deiodination is catalyzed by a small family of membrane-anchored selenoenzymes. The mRNAs of all deiodinase family members encode 27–30-kDa polypeptides containing the novel amino acid selenocysteine (Sec)\textsuperscript{2} (1–3), and catalytically active enzymes are generated by the post-translational assembly of two enzyme subunits (4–6). The dimerization domain responsible for enzyme assembly is located between residues 148 and 163 of the 27-kDa type I deiodinase (D1) subunit, and it is highly conserved across species and among family members (5). Different family member subunits interact with each other through this domain to assemble hybrid enzymes. Interestingly, the hybrid enzyme formed from the combination of a catalytically inert D1 subunit and a functional type II selenodeiodinase (SeD2) subunit is inactive, whereas hybrid enzyme dimers formed between a catalytically active D1 subunit and an inert D1 subunit or a binding surrogate retain catalytic activity, albeit at \(-50\%\) of that of a wild-type homodimer (5).

On the basis of the high conservation of this 16-residue-long deiodinase dimerization domain (DDD), we generated a DDD–green fluorescent protein (GFP) fusion that substitutes for one of the binding partners in the assembly process (5). This soluble non-catalytic surrogate of the deiodinase subunit was found (i) to co-localize with both the D1 and SeD2 subunits, (ii) to form heterodimers with both the D1 and SeD2 subunits that could be isolated by immunoprecipitation, and (iii) to impair the catalytic activity of the assembled D1 and SeD2 heterodimers just like the heterodimers containing a catalytically inactivated full-length deiodinase subunit (6). Notably, the soluble DDD–GFP deiodinase subunit surrogate eliminates the potential untoward effects resulting from mutagenesis-induced conformation changes in the polypeptide regions flanking the DDD in the native deiodinase subunit.

In this study, we used alanine scanning mutagenesis to identify key residues in the DDD that are required for holoenzyme assembly. The results show that three residues (Ile\textsuperscript{152}, Tyr\textsuperscript{153}, and Ile\textsuperscript{154}) are responsible for the assembly of a D1 homodimer and that tight binding of the D1 dimerization domain (\(\Delta G = -60 \pm 5\) kJ/mol for the isolated DDD) prevents holoenzyme disassembly and/or the dynamic exchange of binding partners. Site-directed mutagenesis was then used to generate a dimer-deficient full-length D1 subunit. This dimer-deficient D1 subunit was catalytically active and did not form dimers. For SeD2, the assembly of the type II deiodinase homodimer required a broader region of the DDD and utilized residues at both ends of this domain. These data illustrate that the deiodinase family utilizes a common dimerization domain for the assembly of a fully functional enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials**—All reagents used were of the highest purity commercially available. Restriction endonucleases and DNA-modifying enzymes were purchased from New England Biolabs (Beverly, MA). 3',5',3'-Triiodothyronine was prepared by radioiodination of 3,3'-diiodothyronine using methods described previously (7). Synthetic oligonucleotides were purchased from Invitrogen or Midland Scientific, Inc. (Omaha, NE). Synthetic peptides were prepared by Genemed Synthesis, Inc. (South San Francisco, CA). All iodothyronines were of the L-configuration and were purchased from Henning Berlin GmbH & Co. (Berlin, Germany). Dulbecco’s modified Eagle’s medium, antibiotics, Hanks’ buffered salt solution, glucose, trypsin, and Geneticin® (G418) were obtained from Invitrogen. Supplemented bovine calf serum was from Hyclone (Boulder, CO). Acrylamide and N,N’-methylenebisacrylamide were from National Diagnostics (Atlanta, GA). Ammonium persulfate and TEMED were from Bio-Rad.

**Cell Culture**—LLC-PK1 and SeD2 cells (C6 cells constitutively expressing exogenous rat SeD2) (8) were grown in 25-cm\(^2\) flasks, unless stated otherwise, in a humidified atmosphere of 5% CO\(_2\) and 95% air at 37 °C as described previously (6, 8). The growth medium was composed of Dulbecco’s modified Eagle’s medium containing 15 mM sodium bicarbonate, 25 mM HEPES (pH 7.2), 25 mM glucose, 1 mM sodium pyruvate, 10% (v/v) bovine calf serum, 50 milliliters/ml penicillin, and 90 µg/ml strep-
tomycin. Constitutive expression of SeD2 in C6 cells was maintained by adding 200 μg/mg G418 to the growth medium (8). HEK293 cells were grown as described above with 10% (v/v) fetal bovine calf serum substituted for bovine calf serum. All cell types were subcultured every 3–5 days by seeding 50,000 cells/cm² into 25-cm² flasks. The culture medium was changed three times weekly.

**Alanine Scanning Mutagenesis of the p27 Dimerization Domain**—Complementary synthetic oligonucleotides corresponding to nucleotides 445–496 of rat p27 (GenBank® accession number X57999) were used to create a series of DDD constructs with serial alanine substitutions along the D1 dimerization domain. 72-mer complementary oligonucleotides based on the native DDD sequence (5'- TAGACCCATCTGGTCGCTAGTTCTCTATATAAGGGAGCTTCTTCAATGTAAATGATGAGGAAGTCAGCACCAGGGCTCTG-3') and 5'-GATCCAGAGCCATCCATCTGTGGC-GTGAGCTTCTTCAATGTAAATGATGAGGAAGTCAGCACCAGGGCTCTG-3') were annealed, yielding a double-stranded 72-bp fragment (see Ref. 5) with an N-terminal Met residue in a Kozak consensus start site and NheI and BglII overhangs, and ligated in-frame into the pADD-GFP plasmid (Clontech), generating sequential alanine-substituted dimerization domain (ADD)-GFP fusion proteins(s). Correct insertion was confirmed by DNA cycle sequencing.

**Generation of ADD-GFP-expressing LLC-PK1 and SeD2 Cells**—LLC-PK1 or SeD2 cells were grown in 25-cm² flasks to 60% confluence and transfected with 5–10 μg of pEGFP-N1 (Clontech), generating serial alanine-substituted dimerization domain (ADD)-GFP fusion protein(s). Correct insertion was confirmed by DNA cycle sequencing.

**Immunoprecipitation of D1 Monomers and Heterodimers**—1 μl of anti-p27 antisera (R3050) (6) and 10 μl of protein A beads (Repligen Corp., Waltham, MA) were added to individual sucrose gradient fractions and incubated for 90 min at 4 °C. Immune complexes were collected by centrifugation, and the protein A beads were washed with 10 volumes (v/v) of lysis buffer (20 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, and 5 mM taurodeoxycholate). Gradients were centrifuged for 24 h at 3.9 × 10⁸ gmin in the SW 60Ti rotor, and 100-μl fractions were collected from the top of the sucrose gradient.

**DDD Peptide Competitive Inhibition Assays**—The binding properties of selected synthetic alanine-substituted DDD mutant proteins and the DDD were determined by a fluorescence-based competitive binding assay. Reacti-Bind™ streptavidin-coated polystyrene plates (96-well; Pierce) were incubated with a 3-fold molar excess (360 pmol) of the C-terminal biotinylated DDD peptide (ADFLVIYIEEAHATDGW/LAILK-biotin; DDD-biotin) at room temperature for 1 h. Unbound peptide was removed by washing three times with 200 μl of phosphate-buffered saline (pH 7.2) at 70 °C, and the plate was kept at 4 °C until used.

Fluorescein isothiocyanate (FITC)-labeled DDD (ASADFLVIYEEAHATDGW/LAILK-biotin; DDD-FITC) was dissolved in phosphate-buffered saline (final concentration of 154 mM at pH 7.2) and used as the reporter molecule. In brief, 100 μl of DDD-FITC was mixed with 100 μl of DDD or ADD peptide (A152DD, A133DD, or A154DD) at 0–300 μM; preformed DDD dimers were destabilized by heating to 70 °C (see “Results”); and 100-μl aliquots of each probe/peptide mixture were added to individual wells of the DDD-coated plate. Binding reactions were performed for 30 min at 4 °C. Unbound peptide was removed by aspiration; the wells were rinsed three times with 200-μl aliquots of phosphate-buffered saline heated to 60 °C; and 100 μl of phosphate-buffered saline at room temperature was then added to each well. Fluorescence was determined on a SpectraMax Gemini XS fluorometer (Molecular Devices Corp.) at wavelengths of 492 nm (excitation) and 520 nm (emission) and processed using SoftMax Pro Version 4 software.

**Analytical Methods**—D1 activity was determined in cell lysates by measuring the release of radioactive iodide from 10 μM 3',5',3'-[125I]triiodothyronine (100 cpm/ml) in 20 mM dithiothreitol (unless indi-
RESULTS

At least two members of the selenodeiodinase family assemble into catalytically functional homodimers through a highly conserved DDD. Interestingly, a soluble DDD-GFP fusion protein substitution (schematically represented in Fig. 1A) also specifically partners with both D1 and SeD2 subunits to form stable heterodimers (5, 6), indicating that the isolated 16-residue-long domain contains all of the elements required for dimerization. To determine the role of individual residues in enzyme assembly, we used alanine scanning mutagenesis and our DDD-GFP fusion protein to measure the influence of the alanine-substituted DDD on catalytic activity.

We then examined the subcellular distribution of the individual ADD-GFP mutants in LLC-PK1 cells to confirm that the altered D1 activity in cells expressing the ADD-GFP fusion proteins reflected the formation of heterodimer(s) between the fusion protein and native p27. The photomicrographs in Fig. 2 show examples of the subcellular distribution of representative ADD-GFP fusion proteins in LLC-PK1 cells. As observed previously for cells expressing GFP (5), fluorescence was found throughout the cell interior. On the other hand, expression of the ADD-GFP series, or GFP alone, and cells constitutively expressing the individual DDD-GFP fusion protein and measured the influence of the alanine series in LLC-PK1 cells to confirm that the altered D1 catalytic activity reflected the distribution of representative ADD-GFP fusion proteins in LLC-PK1 cells to confirm that the altered D1 catalytic activity reflected the formation of heterodimer(s) between the fusion protein and native p27. The photomicrographs in Fig. 2 show examples of the subcellular distribution of representative ADD-GFP fusion proteins in LLC-PK1 cells. As observed previously for cells expressing GFP (5), fluorescence was found throughout the cell interior. On the other hand, expression of the ADD-GFP series, or GFP alone, and cells constitutively expressing the individual DDD-GFP fusion protein and measured the influence of the alanine series fusion proteins in LLC-PK1 cells.

FIGURE 1. A, schematic representation of the DDD-GFP fusion protein; B, analysis of D1 activity in LLC-PK1 cells constitutively expressing the ADD-GFP series of DDD mutants. LLC-PK1 cells were transfected with 10 μg of pEGFP-N1 (GFP control), pDDD-GFP, or individual ADD-GFP plasmids to produce constitutively expressing mutant cell lines. D1 catalytic activity was determined as described under “Experimental Procedures.” Data are representative of the mean ± S.E. of at least three individual assays.

FIGURE 2. Representative photomicrographs of LLC-PK1 cells transiently expressing the alanine series fusion proteins. Cells on coverslips were transfected with pEGFP-N1, pA152DD-GFP, or pA160DD-GFP and fixed after 24 h. Fluorescence microscopy was performed as described under “Experimental Procedures.” Photomicrographs are representative of 20–30 individual cells.
Alanine Scanning Mutagenesis of the DDD

Characterization of the Binding Properties of DDD Peptides—Based on the functional consequences of alanine mutants of DDD-GFP in situ, individual 26-residue-long DDD peptides were synthesized to examine the role of individual residues in dimerization without the constraints imposed by the flanking regions of its membrane-bound binding partner or the GFP reporter. The 17-residue-long DDD with a random leashing of seven residues was synthesized, and a C-terminal biotin (DDD-biotin) or fluorescein (DDD-FITC) tag was added. Streptavidin microtiter plates were coated with the biotinylated DDD peptide, and the wells were washed free of unbound DDD-biotin. Approximately 20 pmol of DDD-biotin was immobilized as judged by UV spectrophotometry. Because the DDD is necessary and sufficient for deiodinase dimerization, we expected the synthetic DDD peptides to dimerize in solution and the DDD dimers to be immobilized on the streptavidin-coated wells. Consistent with this supposition, little, if any, DDD-FITC reporter bound to the DDD-coated wells when binding assays were performed at 37 °C. To destabilize the DDD dimers sufficiently to allow dynamic interactions of DDD monomers both in solution and bound to the wells, the plates were incubated at increasing temperatures from 55 to 70 °C with the DDD-FITC reporter in the absence and presence of a 20-fold molar excess of the DDD. There was a progressive increase in the quantity of DDD-FITC bound to the immobilized DDD from 11.8 ± 0.5 to 46.5 ± 2.8% of the total DDD-FITC as the temperature was increased from 55 to 70 °C, and the quantity of exchangeable binding peaked at ∼70 °C. At all temperatures, the addition of a 20-fold molar excess of the DDD decreased the quantity of DDD-FITC bound to ∼9–14% of the total, indicating that nonspecific binding of DDD-FITC was ∼12% of the total fluorescent reporter. The relationship between temperature and specific binding of DDD-FITC to the immobilized DDD is shown in Fig. 3. Regression analysis of the Arrhenius plot yielded a free energy of association of −60 ± 5 kJ/mol for the noncovalent dimerization reaction. Based on these findings, all subsequent competitive binding studies were performed at 70 °C.

The dimeric binding of DDD-FITC to the immobilized DDD. With increasing concentrations of DDD-FITC, there was a progressive increase in the quantity of DDD-FITC specifically bound that approached a plateau of ∼8 pmol of DDD-FITC/well when the concentrations exceeded 300 μM. Competition between DDD-FITC and three 26-residue-long ADD peptides with alanine substitutions at Ile152 (A152DD), Tyr153 (A153DD), and Ile154 (A154DD) was then evaluated. Compared with the DDD, which decreased DDD-FITC binding by >95%, the addition of a 20-fold molar excess of the individual ADD peptides decreased DDD-FITC binding by 50–65%, confirming that these individual residues (Ile152, Tyr153, and Ile154) contribute partially to the dimerization reaction (Fig. 4B).

Identification of Key Residues Required for SeD2-DDD-GFP Complex Assembly in SeD2-expressing C6 Astrocytoma Cells—Unlike the catalytically active p27/DDD-GFP heterodimer in LLC-PK1 cells, heterodimers formed between the DDD-GFP fusion probe and the SeD2 subunit are catalytically inactive (5). This provides a straightforward assay to examine the potential interaction(s) between ADD-GFP proteins and the 29-kDa SeD2 subunit. Constitutive expression of individual members of the ADD-GFP series of mutants ranged from a low of 200 ± 30 RFU/mg of cell protein in A160DD-GFP cells to a high of 670 ± 80 RFU/mg of cell protein in A152DD-GFP cells. The level of fusion protein found in DDD-GFP cells was 175 ± 22 RFU/mg of cell protein. Unexpectedly, the residues responsible for binding of ADD-GFP to SeD2 were more extensive than those involved in D1 assembly. Significant SeD2 activity ranging from 10 to 54% of the wild-type control was observed when alanine substitutions were made at positions equivalent to Phe153, Leu154, Leu155, Val156, Tyr157, Ser164, Asn165, and Gln166 in the DDD of the SeD2 subunit (Fig. 5). These data suggest that two subdomains in the DDD are responsible for SeD2 dimerization, with the N-terminal subdomain including two of the three residues responsible for D1 assembly.

Characterization of the Dimer-deficient D1 Subunit p27A152—On the basis of the finding that substituting Ile152 with alanine prevents the
BrAc[125I]T4; cell membranes were solubilized with taurodeoxycholate; p27A152 polypeptide was then identified by immunoprecipitation using and the membrane proteins were separated by sucrose density gradient and without 1

Procedures." Taurodeoxycholate-solubilized membrane proteins were incubated with 10

A

membranes of LLC-PK1 cells constitutively expressing p27 and dimer-deficient p27A152

was located at the top of the gradient between fractions 2 and 5 with an

SeD2 catalytic activity was determined as described under "Experimental Proce-

D1 subunit BrAc[125I]T4-labeled D1 in HEK293 cells constitutively expressing p27 or dimer-deficient p27A152

the ADD-GFP series of DDD mutants. BrAc[125I]T4-labeled cell membrane proteins were separated on 4 –12% linear sucrose density gradients as described under "Experimental Procedures." Each frac-

FIGURE 7. A, analysis of D1 activity in HEK293 cells expressing p27 or dimer-deficient p27A152. HEK293 cells were transfected with rat p27 or dimer-deficient p27A152 and grown for 24 h Cell lysates were prepared, and D1 activity was determined as described under "Experimental Procedures." 1 unit equals 1 pmol of iodide released per min. Data are reported as the means ± S.E. of three separate experiments. B, distribution of immuno-

labeled with BrAc[125I]T4, and membranes isolated as described under "Experimental

estimated molecular mass of 25–30 kDa, and little, if any, p27A152 was

To exclude the possibility that the lack of a p27/p27A152 dimer in the LLC-PK1 cells was due to overexpression of the "dimer-deficient" mutant relative to native p27, we exploited the finding that non-Sec mutants of selenodeiodinases are expressed in large excess over the wild-type selenodeiodinase subunit: typical expression ratios of p27Sec to native p27Sec are >10-fold (6, 13). HEK293 cells, which lack all sel-

FIGURE 5. Analysis of type II deiodinase activity in SeD2 cells constitutively expressing the ADD-GFP series of DDD mutants. SeD2-expressing C6 astrocytoma cells were grown to 60% confluence in growth medium containing 200 μg/ml G418 and transfect-

Sucrose density gradient centrifugation of BrAc[125I]T4-labeled detergent-solubilized BrAc[125I]T4-labeled D1 in HEK293 cells constitutively expressing p27 (■) and dimer-deficient p27A152 (○) constructs. BrAc[125I]T4-labeled cell membranes were isolated on 16% Percoll gradients and solubilized with 5 mm taurodeoxycholate. Clarified detergent extracts were loaded onto 4–12% linear sucrose density gradients and centrifuged at 3.9 × 104 g

FIGURE 6. A, distribution of immunoprecipitated (Iptted) BrAc[125I]T4-labeled D1 in membranes of LLC-PK1 cells constitutively expressing p27 and dimer-deficient p27A152 constructs. BrAc[125I]T4-labeled cell membrane proteins were separated on 4–12% linear sucrose density gradients as described under "Experimental Procedures." Each fraction was incubated with 10 μl of protein A beads and 1 μl of anti-p27 antiserum (R3050) for 90 min, and immune complexes were isolated by centrifugation. Data are representative of three separate experiments. The stippled bar identifies the expected position of the D1 homodimer. B, immunoprecipitation of BrAc[125I]T4-labeled M4-GFP fusion protein in HEK293 cells coexpressing M4-GFP and dimer-deficient p27A152 constructs. HEK293 cells were cotransfected with equal amounts (5 μg/cm2 flask) of dimer-deficient p27A152 and the GFP-tagged cysteine mutant of p27 (M4-GFP) (5). Cells were affinity-labeled with BrAc[125I]T4, and membranes isolated as described under "Experimental Procedures." Taurodeoxycholate-solubilized membrane proteins were incubated with and without 1 μl of anti-GFP IgG for 90 min, and immune complexes were isolated as described for A Proteins specifically bound to the protein A beads were resolved on 12.5% SDS-polyacrylamide gel, and the positions of affinity-labeled proteins were identified by autoradiography.

DDD-GFP fusion protein from partnering with native p27, we generated a full-length D1 subunit with the I152A substitution (p27A152) and examined the consequences of this single residue exchange on its ability to dimerize with the native p27 selenoprotein in LLC-PK1 cells. D1 subunits in p27A152-expressing LLC-PK1 cells were affinity-labeled with BrAc[125I]T4; cell membranes were solubilized with taurodeoxycholate; and the membrane proteins were separated by sucrose density gradient centrifugation as described previously (6). The distribution of the p27A152 polypeptide was then identified by immunoprecipitation using antibodies directed against its C terminus. As shown in Fig. 6A, p27A152 was located at the top of the gradient between fractions 2 and 5 with an

We then examined the catalytic potential of dimer-deficient p27A152. As illustrated in Fig. 7A, HEK293 cells transfected with the p27A152 construct showed deiodinating activity (14.9 ± 0.3 units/mg of protein) that was 50–60% lower than that observed in cells transfected with equal amounts of the p27 plasmid (33.5 ± 2.3 units/mg of protein). Thus, the p27A152 mutant retained ~50% of the catalytic activity of its wild-type parent.

Sucrose density gradient centrifugation of BrAc[125I]T4-labeled solubilized HEK293 cell membranes showed that immunoreactive p27A152 sedimented between fractions 2 and 7, whereas the immunoreactive p27
homoenzyme sedimented between fractions 12 and 18 (Fig. 7B), characteristic of the D1 homodimer (4). Taken together, these data confirm that (i) dimer-deficient p27<sup>A152</sup> is catalytically active; (ii) p27<sup>A152</sup> is selectively affinity-labeled with the alkylating D1 substrate analog BrAcT<sub>4</sub> and (iii) p27<sup>A152</sup> does not partner with the native p27 subunit or with itself.

**DISCUSSION**

Thyroid hormone deiodinases compose a small family of selenoenzymes that regulate the availability and disposal of thyroid hormone. A key step in their biosynthetic pathway is the assembly of a functional homodimer. This assembly process takes place after the insertion of the selenoprotein monomers into the membrane bilayer and requires a short 16-residue-long region that is one of the most highly conserved domains among family members (5, 6, 14). Notably, the conservation of this dimerization domain across family members allows different family members to partner, and the resulting heterodimers have catalytic properties that differ from those of the two parent homoenzymes (5, 6). To identify the individual residues responsible for the assembly of deiodinase dimers, we used alanine scanning mutagenesis coupled with functional analysis, affinity labeling, and density gradient centrifugation. We then determined the binding properties of isolated DDD peptides by competitive binding analysis. The data show that the D1 and SeD2 family members utilize different segments of the DDD for homoenzyme assembly and that a catalytically active monomeric deiodinase is generated by a single I152A substitution in the p27 dimerization domain.

Alanine scanning mutagenesis is a powerful tool used to explore the role of individual residues in the function of a protein, and this tool was exploited to identify the residue(s) in the 16-residue-long DDD that contribute to the assembly of the deiodinase homoenzyme. To eliminate the contribution of structural elements that flank the DDD in the full-length enzyme subunit, we made a DDD-GFP fusion protein that would serve as a surrogate for one of the binding partners. Because the expression of this soluble non-Sec-containing reporter is routinely 5- to 10-fold greater than that of the rare native Sec-containing deiodinase subunit (5), the dimerization reaction is driven toward heterodimer formation. By also limiting the functional component of the DDD to its core element (5) and using the altered catalytic activity of its native binding partner, changes in the binding of sequential alanine-substituted DDD-GFP fusion probes to full-length p27 are less influenced by misfolding artifacts that often occur in the mutant protein(s). For example, amino acid substitution for the acidic Glu<sup>136</sup>, the basic His<sup>154</sup>, and the non-polar Trp<sup>163</sup> residues leads to the loss of catalytic activity (15, 16), but does not prevent heterodimer assembly, thereby eliminating the possibility that altered enzyme assembly is responsible for the loss of catalytic activity by mutation of these residues (15, 17).

Three residues (Ile<sup>152</sup>, Tyr<sup>153</sup>, and Ile<sup>154</sup>) are essential for D1 assembly as judged by the inability of their alanine-substituted mutants to impair deiodination or to bind to membrane-bound p27 in LLC-PK1 cells. To characterize the participation of individual residues in the interaction(s) between DDD monomers (interactions that are based on noncovalent bond formation), we synthesized peptides composed of the 17-residue-long DDD and developed a competitive binding assay. Because the DDD peptide(s) were expected to self-associate, it was not surprising to find that dynamic interactions between the immobilized DDD “acceptor” and the DDD-FITC ligand required an elevated temperature. This finding is consistent with the strength of the binding between DDD-GFP and its membrane-bound binding partner, which allowed us to map this domain in cells. Melting curves generated by measuring the quantity of free DDD acceptor monomers yielded a free energy of activation for dimerization of $-60 \pm 5$ kJ/mol for these “out-of-context” peptides and revealed the strong propensity for DDD peptides to self-associate. Although competition for the immobilized DDD monomers between the DDD-FITC reporter and a 20-fold molar excess of the DDD or individual ADD peptides confirmed the individual contributions of Ile<sup>152</sup>, Tyr<sup>153</sup>, and Ile<sup>154</sup> to the dimerization reaction, each residue contributed only partially to the dimerization reaction. Unlike the inability of the dimer-deficient p27<sup>A152</sup> point mutant to self-assemble and the failure of a subset of ADD-GFP fusion proteins to partner with native p27, the competition between individual alanine-substituted peptides revealed the shared contribution of these three residues to the overall dimerization reaction. These context-dependent differences in the behavior of the DDD raise the possibility that the flanking regions exert sufficient strain on the DDD to prevent homodimer assembly. Alternatively, cooperation between the responsible residues in the dimerization reaction may differ when dynamic binding is assessed because the ability of single residue(s) to completely prevent dimerization is dependent on the ratio of the soluble DDD surrogate to the local concentrations of full-length p27 in the endoplasmic reticulum. More important, once a D1 homodimer forms, it is highly unlikely to exchange binding partners.

Similarly, analysis of SeD2 assembly showed that expression of the DDD-GFP fusion probe in SeD2-expressing C6 cells completely inactivates deiodination (5), indicating that both SeD2 subunits are required for catalysis. Expression of the ADD series in SeD2 cells revealed that the residues required for dimerization were more extensive that those used for D1 assembly and were found at both ends of the DDD. Single alanine substitutions at any one of a five-residue stretch from positions 153 to 157 at the beginning of the DDD, including two of the three residues required for D1 assembly, or in a three-residue region located at its C-terminal end (positions 160-162) eliminated the ability of DDD to impair SeD2 catalysis. These differences between the D1 and SeD2 subunits in the residues required for dimerization were unexpected, but may contribute to the unique catalytic properties of these two isoenzymes. Interestingly, the three amino acids that differ between the D1 and SeD2 dimerization domains, Ile<sup>151</sup> and Leu<sup>155</sup>, Glu<sup>155</sup> and Asp<sup>159</sup>, and the structurally more significant Ala<sup>160</sup> and Pro<sup>163</sup>, do not appear to contribute to SeD2 homoenzyme assembly because alanine substitution at any of these positions did not affect DDD binding to the full-length SeD2 subunit.

The difference(s) in the functional outcome of impaired homoenzyme assembly between D1 and SeD2 appear to reside in elements outside of the DDD. Rat D1 and SeD2 share ~31.5% amino acid identity, and hydrophobic cluster analysis suggests that the substrate-binding sites/active centers of D1 and SeD2 are similarly organized (16). However, the inability of the SeD2 subunit to catalyze deiodination by itself indicates that protein-protein interactions in the SeD2 homodimer are necessary to form a catalytically active enzyme. Although the responsible protein domains are unknown, three unique segment insertions are found in the SeD2 subunit. Segment A (residues 88–112; located 17 residues upstream of the active center Sec residue, segment B (residues 168–181; located adjacent to the DDD, and segment C (residues 107–205; located in the C terminus) are likely candidates for study.

On the basis of the ability of a single point mutation to prevent the DDD-GFP fusion protein from partnering with native p27, we constructed a “dimerization-deficient” D1 subunit by substituting alanine for Ile<sup>152</sup> in full-length p27 (p27<sup>A152</sup>). Transient expression of p27<sup>A152</sup> in LLC-PK1 cells revealed that the substrate-binding site of exogenous p27<sup>A152</sup> was intact as judged by its interaction with the affinity label BrAcT<sub>4</sub>, but the point mutant did not partner with native p27 or an
overexpressed Cys mutant of p27 (M4-GFP) and remained as a membrane-bound monomer. Because the abundant activity of native D1 in these cells overwhelmed that contributed by p27A152, we turned to transient expression of p27A152 in HEK293 cells, which lack native D1. Coexpression of M4-GFP and p27A152 in HEK293 cells confirmed that p27A152 does not partner with native p27. Interestingly, expression of p27A152 yielded a catalytically functional membrane-bound deiodinase that did not self-associate to form a homodimer, consistent with the findings in LLC-PK1 cells expressing this point mutant. Thus, the catalytic potential of the D1 enzyme is an intrinsic property of individual subunits that constitute the holoenzyme, and the assembly of a homodimer is not obligatory to generate a functional enzyme.

The generation of catalytically active monomeric D1 by the mutation of Ile152 in the DDD is inconsistent with the proposed role of intermolecular disulfide bridging in holoenzyme assembly (2, 14). Because the isolated DDD forms stable heterodimers with both the D1 and SeD2 family members (5) and because cysteine is not one of the residues required for holoenzyme assembly, it is unlikely that intermolecular disulfide bridging contributes to enzyme assembly. Nonetheless, it is clear that intramolecular disulfide bridging is essential for the proper folding of individual deiodinase subunits.

In summary, a small non-polar patch (I152YI154) located at the beginning of the 16-residue-long dimerization domain is required for the assembly of the D1 enzyme. For its family member SeD2, two subdomains located at the ends of this region are required for the assembly of a catalytically active holoenzyme. Family members also differ in the catalytic potential of individual subunits; D1 subunits are fully capable of catalyzing deiodination alone, whereas the SeD2 isoenzyme appears to require two functional subunits for catalytic activity. The strength of binding through noncovalent interactions between the DDD of each binding partner suggests that, once two enzyme subunits come in contact with each other, they assemble into a highly stable dimeric complex. Thus, formation of hybrid deiodinase dimers composed of different family member subunits is driven by the relative concentrations of each subunit; and once formed, heterodimers have little, if any, chance to reassemble into a homodimer with its cognate partner.

REFERENCES
1. Berry, M. J., Banu, L., and Larsen, P. R. (1991) Nature 349, 438–440
2. Bianco, A. C., and Larsen, P. R. (2005) Thyroid 15, 777–786
3. Kohrle, J. (2000) CMLS Cell. Mol. Life Sci. 57, 1853–1863
4. Safran, M., and Leonard, J. L. (1991) J. Biol. Chem. 266, 3233–3238
5. Leonard, J. L., Simpson, G., and Leonard, D. M. (2005) J. Biol. Chem. 280, 11093–11100
6. Leonard, J. L., Visser, T. J., and Leonard, D. M. (2001) J. Biol. Chem. 276, 2600–2607
7. Weekes, J., and Orskov, H. (1973) Scand. J. Clin. Lab. Invest. 32, 357–360
8. Leonard, J. L., Leonard, D. M., Safran, M., Wu, R., Zapp, M. L., and Farwell, A. P. (1999) Endocrinology 140, 2206–2215
9. Sanger, F., Nicklen, S., and Coulston, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467
10. Leonard, J. L., Ekenbarger, D. M., Frank, S. J., Farwell, A. P., and Koehrle, J. (1991) J. Biol. Chem. 266, 11262–11269
11. Farwell, A. P., Safran, M., Duhord, S., and Leonard, J. L. (1996) J. Biol. Chem. 271, 16369–16374
12. Visser, T. J., Leonard, J. L., Kaplan, M. M., and Larsen, P. R. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 5080–5084
13. Berry, M. J., Maia, A. L., Kieffer, J. D., Harney, J. W., and Larsen, P. R. (1992) Endocrinology 131, 1848–1852
14. Curcio-Morelli, C., Gereben, B., Zavacki, A. M., Kim, B. W., Huang, S., Harney, J. W., Larsen, P. R., and Bianco, A. C. (2003) Endocrinology 144, 937–946
15. Berry, M. J. (1992) J. Biol. Chem. 267, 18055–18059
16. Callebaut, I., Curcio-Morelli, C., Morrow, J. P., Gereben, B., Buettner, C., Huang, S., Castro, B., Fonseca, T. L., Harney, J. W., Larsen, P. R., and Bianco, A. C. (2003) J. Biol. Chem. 278, 36887–36896
17. Mol, J. A., Docter, R., Hennemann, G., and Visser, T. J. (1984) Biochim. Biophys. Res. Commun. 120, 28–36