Characterization of the Protein Dimerization Domain Responsible for Assembly of Functional Selenodeiodinases*

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Thyroid hormone metabolism is catalyzed by a small family of selenoenzymes. Type I deiodinase (D1) is the best characterized family member and is an integral membrane protein composed of two 27-kDa subunits that assemble to a functional holoenzyme after translation. To characterize the protein domain(s) responsible for this post-translational assembly event, we used deletion/truncation analysis coupled with immune depletion assays to map the dimerization domain of D1. The results of our studies show that a highly conserved sequence of 16 amino acids in the C-terminal half of the D1 subunit, -D146FL-YL-EAH-DGW163-, serves as the dimerization domain. Based on the high conservation of this domain, we synthesized a novel bait peptide-green fluorescent protein fusion probe (DDDGFP) to examine holoenzyme assembly of other family members. Overexpression of either the DDDGFP or an inert D1 subunit (M4) into SeD2 (accession number U53550)-expressing C6 cells specifically led to the loss of >90% of the catalytic activity. Catalytically inactive D2 heterodimers composed of SeD2: DDDGFP subunits were rescued by specific immune precipitation with anti-SeD2 IgG, suggesting that SeD2 requires two functional subunits to assemble a catalytically active holoenzyme. These findings identify and characterize the essential dimerization domain responsible for post-translational assembly of selenodeiodinases and show that family members can intermingle through this highly conserved protein domain.

Thyroid hormone is essential for the normal growth and development of vertebrates. Thyroxine (3′,5′,3,5-tetraiodothyronine) is the major secretory product of the thyroid gland and must be deiodinated to generate the transcriptionally active metabolite, T₃. Type I iodothyronine deiodinase (D1; EC 3.8.1.4) is the best characterized member of a small family of membrane-bound enzymes that catalyze the production and metabolism of T₃ (1–4). All deiodinase family members are composed of 27–30-kDa polypeptides that contain the novel amino acid, selenocysteine (SeC) that is required for full catalytic activity (3, 5–8). They also share an N-terminal membrane-spanning region and a central core of 14–16 residues surrounding the SeC, but little else is known about the polypeptide. Gel filtration and density gradient centrifugation of detergent-soluble enzyme activity and/or affinity radio-labeled D1 showed that catalytic activity has an Mₜ of ~50,000 and that the ~27-kDa D1 polypeptide co-migrated with a complex, suggesting that D1 is a dimer of ~27-kDa subunits (9–11). The physiochemical properties of the other selenodeiodinase family members remain to be determined, although a recent report using transiently expressed, epitope-tagged D2 and D3 suggested that these family members also formed dimers, albeit at modest levels (12).

Direct analysis of D1 biosynthesis confirmed that the active enzyme was a homodimer formed by the post-translational assembly of two p27 subunits (9). Whether each subunit was catalytically functional or required post-translational assembly to become catalytically active was resolved by the finding that D1 dimers with only one functional subunit had ~50% of the activity of the wild-type enzyme (9, 12). Importantly, these hybrid holoenzymes had a molecular mass of 54 kDa and an S₂₀,ₐ of ~3.5 S, identical to that of the native D1 holoenzyme (9). The ability of each D1 subunit to function as an independent catalytic center was recently confirmed by Curcio-Morelli et al. (12). Interestingly, deletion of the membrane anchor located between residues 9 and 37 of p27 resulted in the formation of catalytically active D1 heterodimers composed of a full-length, membrane-bound subunit and the N-terminal truncated partner lacking a transmembrane domain (9), indicating that insertion of both D1 subunits into the membrane was not required.

Whether holoenzyme assembly required covalent or noncovalent protein-protein interactions was recently examined using transient co-expression of epitope-tagged p27 constructs (12). A minor role for intermolecular disulfide bridging between p27 partners (ranging from 1 to 5% of the total) was recently proposed based on banding pattern(s) of epitope-tagged p27 found after SDS-PAGE analysis. Presumably because of sequence diversity among deiodinase family members, dimer complexes formed between different family members were not observed after SDS-PAGE analysis, suggesting that only like family members could associate (12).

In this study, we mapped the dimerization domain(s) required for deiodinase assembly by exploiting the ability of the native p27 present in LLC-PK1 cells to form dimers with truncation fragments of the immunologically unique, inert rat p27 (M4). We also determined the role of dimerization in the assembly of a catalytically active SeD2 enzyme using C6 cells that constitutively express the 30-kDa SeD2 subunit. The results of our studies show that the highly conserved sequence present in all deiodinase family members from frogs to hu...
mammals, -DFL-YI-EAH-DGW-, serves as the dimerization domain for holoenzyme assembly. For the D1 family member, catalytic activity is preserved when only one subunit is catalytically competent, whereas the D2 isoform required two fully functional subunits to be catalytically active.

**EXPERIMENTAL PROCEDURES**

**Materials**—All reagents were of the highest purity commercially available. Restriction endonucleases and DNA-modifying enzymes were purchased from New England Biolabs (Beverly, MA). L-3,5,3',5'- triiodothyronine (L-3,5,3'-T3) and 125I-labeled rT3 was prepared by radioiodination of 3,3',5-triiodothyronine (T3) using methods described previously (13). Synthetic oligonucleotides were prepared in house or purchased from either Invitrogen or Midland Scientific, Inc. All iodonucleotides were of the L-configuration and were purchased from Henning Berlin GmbH. Dulbecco’s modified Eagle’s medium, antibiotics, Hanks’ buffered salt solution, glucose, trypsin, and G418 were obtained from Invitrogen; supplemented bovine calf serum was from HyClone Laboratories (Boulder, CO); acrylamide and N,N,N’,N’-tetramethylethenediamine were from U.S. Biochemical Corp.; ammonium persulfate and TEMED were from Bio-Rad; and dithiothreitol was from Bio-Rad Laboratories (Burlington, MA; Cat No. 20-390). Acrylamide and N,N,N’,N’-tetramethylethenediamine were from U. S. Biochemical Corp.; ammonium persulfate and TEMED were from Bio-Rad; and dithiothreitol was from Bio-Rad Laboratories (Burlington, MA; Cat No. 20-390). Acrylamide and N,N,N’,N’-tetramethylethenediamine were from U. S. Biochemical Corp.; ammonium persulfate and TEMED were from Bio-Rad; and dithiothreitol was from Bio-Rad Laboratories (Burlington, MA; Cat No. 20-390).

Cell Culture—LLC-PK1 and COS7 cells were grown in 25-cm² flasks in a humidified atmosphere of 5% CO2 and 95% air at 37 °C as described previously (15). Synthetic oligonucleotides were prepared in house or purchased from either Invitrogen or Midland Scientific, Inc. All iodonucleotides were of the L-configuration and were purchased from Henning Berlin GmbH. Dulbecco’s modified Eagle’s medium, antibiotics, Hanks’ buffered salt solution, glucose, trypsin, and G418 were obtained from Invitrogen; supplemented bovine calf serum was from HyClone Laboratories (Boulder, CO; Cat No. 20-390). Acrylamide and N,N,N’,N’-tetramethylethenediamine were from U.S. Biochemical Corp.; ammonium persulfate and TEMED were from Bio-Rad; and dithiothreitol was from Bio-Rad Laboratories (Burlington, MA; Cat No. 20-390).

**Table 1**

| Primer    | Sequence (5’ → 3’) |
|-----------|-------------------|
| X19       | gatctctagggatatgaagctctggagtgcctac |
| X42       | gatctctagggatatgaagctctggagtgcctac |
| X76       | gatctctagggatatgaagctctggagtgcctac |
| X101      | gatctctagggatatgaagctctggagtgcctac |
| X133      | gatctctagggatatgaagctctggagtgcctac |
| C terminus| gcccctcgaggaattttcgggtagtattgg |
| C230      | cctcaattctctagactagacaggtgagagtccggagatctggatcagat |
| C210      | cctcaattctctagactagacaggtgagagtccggagatctggatcagat |
| C190      | cctcaattctctagactagacaggtgagagtccggagatctggatcagat |
| C170      | cctcaattctctagactagacaggtgagagtccggagatctggatcagat |
| C150      | cctcaattctctagactagacaggtgagagtccggagatctggatcagat |
| N terminus| gatctctagggatatgaagctctggagtgcctac |

For X series, the KpnI site is in italic type. For the C series primers, the unique 8-residue-long epitope recognized by the anti-rat p27 antibody (antibody 3050 (9, 14)) (see Table I). A common upstream primer corresponding to nucleotides 7–19 of G21 cDNA and a random primer was used. PCR was done using Vent® DNA polymerase (Stratagene, La Jolla, CA) for a total of 25 cycles according to the following temperature profile: 95 °C, 1.0 min; 50 °C, 1.0 min; 72 °C, 2.0 min, with a final 10-min extended incubation at 72 °C. PCR products were digested with KpnI, gel-isolated on 1.2% low melt agarose gels, and ligated into the KpnI-SmaI sites of pcDNA3 (N-terminal deletions) or into KpnI-HindIII sites of pcDNA3 (C-terminal deletions). All deletion/truncation mutants were confirmed by DNA cycle sequencing.

Complementary synthetic oligonucleotides corresponding to nucleotides 445–496 of G21 cDNA were used to synthesize the D1 dimerization domain-GFP fusion protein (DDDGFP). The 72-mer sense oligonucleotide (5’-TAGACCCACCATGGTCGCTCTTCCTTCTATCATTTTACATGAGAAGCTCAGGGCAGATGATGGGCTGCTG-3’) and its 5’-complementary oligonucleotide (5’-GATTCGACAGGGCATTACCCATCTTTGAGCTGACGCTCTCTCT-3’) were annealed, yielding a double-stranded 72-bp fragment with an N-terminal Met in a Kozak consensus start site, an NheI-compatible 5’ overhang, and a BglII-compatible 3’ overhang. The annealed DDD encoding sequence was appended to the N terminus of GFP by cloning into the NheI BglII sites of peGFP-N1, generating pDDDGFP. Correct insertion was confirmed by DNA cycle sequencing.

**DNA Sequencing**—Double-stranded DNA sequencing was done by the dideoxyxenucleotide method of Sanger et al. (16) using cycle sequencing and iterative primers in the Nucleic Acid Core Facility at the University of Massachusetts Medical School. All sequence information was confirmed by sequencing both strands.

**Antibody Depletion of Deiodinase Dimers**—Anti-epitope antibodies directed against the C-terminal Met of rat p27 or rat SeD2 were used to allow for immune depletion by the species-specific p27 antisera as previously described (antibody 3050 (9, 14)) (see Table I). A common upstream primer corresponding to nucleotides 7–19 of G21 cDNA and a random primer was used. PCR was done using Vent® DNA polymerase (Stratagene, La Jolla, CA) for a total of 25 cycles according to the following temperature profile: 95 °C, 1.0 min; 50 °C, 1.0 min; 72 °C, 2.0 min, with a final 10-min extended incubation at 72 °C. PCR products were digested with KpnI, gel-isolated on 1.2% low melt agarose gels, and ligated into the KpnI-SmaI sites of pcDNA3 (N-terminal deletions) or into KpnI-HindIII sites of pcDNA3 (C-terminal deletions). All deletion/truncation mutants were confirmed by DNA cycle sequencing.

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| C terminus| gcccctcgaggaattttcgggtagtattgg |
| C230      | cctcaattctctagactagacaggtgagagtccggagatctggatcagat |
| C210      | cctcaattctctagactagacaggtgagagtccggagatctggatcagat |
| C190      | cctcaattctctagactagacaggtgagagtccggagatctggatcagat |
| C170      | cctcaattctctagactagacaggtgagagtccggagatctggatcagat |
| C150      | cctcaattctctagactagacaggtgagagtccggagatctggatcagat |
| N terminus| gatctctagggatatgaagctctggagtgcctac |

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Gen, Cambridge, MA) or immobilized rProtein G-Sepharose™ beads (Amersham Biosciences), and 1 μl of either anti-rat p27 IgG (antibody 3050) or anti-SeD2 IgG (antibody 763). Where indicated, excess C-terminal p27 epitope (10 μg/ml, final concentration) was added to block antibody-binding sites prior to the pull-down assay.

Immune complexes were removed by centrifugation, and the deiodinase activity remaining in the clarified supernatant was determined as described below. Expression levels of the exogenous M4 deletion and truncation constructs in LLC-PK1 cells were determined by a competitive binding assay as described previously (9). The minimum detection limit of M4 and its derivatives is ~3 fmol/assay tube (81 pg of p27/tube), and the maximum binding capacity of the anti-rat p27 antiseraum is 22 nmol of M4/mg of antiserum (~600 ng of p27/μl) as determined by Scatchard analysis of the binding data.

SeD2-specific immune complexes were removed by centrifugation and released from the beads by heating at 70 °C for 5 min in Laemml sample buffer. Proteins were separated on 12.5% SDS-polyacrylamide gels, transferred to nitrocellulose, and probed with anti-GFP monoclonal antibodies. Specific immune complexes were identified with horseradish peroxidase-conjugated, anti-mouse IgG (rabbit; Molecular Probes) and visualized by chemiluminescence.

Photomicroscopy—Cells expressing the DDD-GFP fusion protein were seeded onto poly-n-lysine (10 μg/ml)-coated glass coverslips (22 × 22 mm) and grown to ~80% confluence. Cells were then fixed with 4% paraformaldehyde (w/v) in phosphate-buffered saline, and the excess cross-linker was quenched with 100 mM glycine. The distribution of 1 unit equals 1 pmol of 125I-rT3 (100 cpm/fmol), 20 mM dithiothreitol (unless otherwise indicated) in a total volume of 100 μl. D1 reactions were done, in triplicate, in universal deiodinase buffer (100 mM boric acid, 100 mM HEPES, 100 mM glycine) (pH 5.5), 1 mM EDTA, with or without blocking peptide, are summarized in Fig. 2A. More than half of the p27 could be deleted from the N terminus (up to residue 133, including the transmembrane anchor, the subunit D1 Dimerization Domain Mapping by Deletion and Truncation Mutants of M4 Expressed in LLC-PK1 Cells—To define the D1 dimerization domain, we prepared a series of N-terminal deletion (X series) and C-terminal truncation (C series) mutants of M4. X and C series mutants were transfected into LLC-PK1 cells, and constitutively expressing cell lines were isolated by G418 selection. All X-series M4 mutants had a Kozak consensus translation initiation site to ensure adequate expression, and all C-series mutants had the 8-amino acid epitope recognized by the anti-rat p27 IgG at the C terminus. Expression levels of the exogenous X and C series protein(s) in the G418-selected LLC-PK1 cells were determined by competitive displacement analysis and revealed that the cell content of the different truncated/deleted M4s varied from 0.9 ± 0.1 to 1.8 ± 0.3 pmol/mg cell protein, yielding molar ratios of X or C series M4 to p27wt that ranged from ~5 (X133, C230) to 10 (X42, C150), values similar to those reported previously (9).

Cell membranes were then prepared for each X and C series M4 mutant by Percoll gradient centrifugation, and the enzyme activity in detergent extracts of the membrane fractions was measured after immune depletion with anti-rat p27 antisera with or without excess blocking peptide as described under “Experimental Procedures.” As illustrated in Fig. 2A, >95% of the D1 activity in cells expressing the X series of M4 deletion mutants (X19 to X133) was specifically immune-depleted from detergent extracts, just as found previously with the full-length M4 (9). The percentages of D1 heterodimer formation, calculated from the difference in the quantity of residual D1 activity in immune depleted extracts measured in the absence and presence of blocking peptide, are summarized in Fig. 2B. More than half of the p27 could be deleted from the N terminus (up to residue 133, including the transmembrane anchor, the subunit specificity site, and the catalytic center) without altering...
Mean activity is expressed as units/mg protein. Data are reported as the mean ± S.E., n = 3.

| Enzyme source         | D1 dimer | D1 activity |
|-----------------------|----------|-------------|
| Control tDOC extract  | wt:wt    | 100 ± 10 (n = 3) |
| M4-tDOC extract       | M4:p27wt | 48 ± 4 (n = 3) |
| Unbound D1wt          | wt:wt    | 96 ± 10 (n = 3) |
| Unbound M4-D1         | M4-p27wt | 2 ± 4 (n = 3) |
| Immobilized control D1| wt:wt    | 1 ± 4 (n = 3)  |
| Immobilized M4-D1     | M4:p27wt | 45 ± 6 (n = 3) |

**FIG. 2. Mapping of the D1 dimerization domain.** Lysates of LLC-PK1 cells constitutively expressing N-terminal and C-terminal M4 deletion mutants were immune precipitated with anti-rat p27 IgG, and D1 activity was determined in the immune depleted supernatant with or without blocking peptide (A) and in the initial tDOC extracts (B). D1 activity is expressed as units/mg protein. Data are reported as the mean ± S.E. of three separate experiments.

The binding of the truncated M4 to p27wt. These data indicate that the dimerization domain resides in the C-terminal 120 residues of p27.

Direct analysis of progressive truncation mutants from the C terminus of p27 revealed that at least 86 residues could be removed from the C terminus of M4 without altering the interaction between the truncated M4 and p27 (Fig. 2B). However, loss of residues located between positions 150 and 170 completely eliminated the ability of the truncated M4 mutant to interact with p27. These data indicate that the D1 dimerization domain resides in the C terminus of p27 between residues 150 and 170, a region that contains the most highly conserved element of the deiodinase family from frogs to humans.

**Kinetic Analysis of Engineered D1 Heterodimers Lacking a Transmembrane Anchor (X42), a Substrate Specificity Domain (X72), or One Catalytic Center (X133)—** Shown in Fig. 3 are representative initial velocity reaction kinetics for the wild-type D1wt, the full-length M4:p27wt, and the X42:p27wt dimers. All hybrid D1 heterodimers showed ping-pong reaction kinetics. Secondary replots yielded the limiting $K_m$ and $V_{max}$ values (Table III) and revealed that the loss of the N-terminal membrane anchor (X42), the iodothyronine specificity site (X101), and even the catalytic center (X133) resulted in a 2.5-fold decrease in the limiting $K_m$ for the iodothyronine and a 75% fall in the molar activity compared with the native enzyme. Unexpectedly, loss of the membrane anchor increased the reduced thiol cofactor requirement ~4-fold (Table III). These data show that kinetic properties of the D1 reaction are intrinsic to each subunit.

**Characterization of the Interaction(s) between p27wt and the DDDGFP Fusion Protein in LLC-PK1 Cells—** Amino acid residues from 147 to 163 of rat p27 (A147DFLVIVIEAHGW163) are the most highly conserved of all p27 domains across all species and among family members, and our mapping strategy indicated that residues in this region are responsible for assembly of the D1 holoenzyme. To establish that this conserved region was responsible for D1 dimerization, we appended a 19-residue-long peptide containing an initiator Met and residues 147–163 of rat p27 (G21) to the N terminus of the fluorescent reporter protein, GFP, forming a DDDGFP fusion protein. Transient expression of DDDGFP in LLC-PK1 cells resulted in the appearance of multiple punctuate fluorescent signals along the cell periphery and at the borders between adjacent cells (Fig. 4), a pattern identical to that observed previously for the D1 holoenzyme (9, 14). Control cells transfected with the wild type GFP reporter showed no specific membrane localization but abundant reporter protein throughout the cell interior and the nucleus (Fig. 4).

Subcellular fractionation of DDDGFP-expressing cell lysates was on iso-osmotic 16% Percoll gradients as detailed previously (14). Shown in Fig. 5 are the distribution profiles of GFPwt and DDDGFP among the separated cell organelles. In control cells expressing GFP alone, the reporter was found both in the cytosol (top two or three fractions) and in the particulate/nuclear fraction. In cells expressing DDDGFP, little, if any, of the fusion protein was present in the cytosol, and ~70% of the total cell fluorescence co-migrated with the cellular membranes found in fractions 9–11 (22). These data show that exogenous DDDGFP fusion protein was tightly associated with the cellular membrane.

We then examined the functional consequences of the DDDGFP on D1 enzyme activity using the immune depletion approach as detailed under “Experimental Procedures.” D1 activity was measured in cell lysates and in detergent extracts after removing GFP-containing proteins with anti-GFP antibodies. As found with all M4 mutants that formed a heterodimer with p27wt (see Fig. 2), the catalytic activity of the p27wt-DDDGFP heterodimer was ~50% of that present in cells expressing GFP alone, and most, if not all (>95%), of the deiodinating activity present in detergent extracts of DDDGFP, expressing cells was specifically removed by anti-GFP antibodies (Fig. 6). These data confirm that the 17-residue-long DDDGFP peptide contains all of the necessary structural elements required for D1 assembly.

**Analysis of the Role of the DDD Domain in the Assembly of a Functional SeD2 Enzyme—** Although the physicochemical properties of the SeD2 enzyme have not been determined, a comparison of the DDD domain across family members (Table IV) showed a >70% identity for this domain and a fixed positional relationship to the active center SeC, raising the possibility that this domain may also be important for assembly of other family members. To determine the role of the DDD domain in SeD2 assembly, we expressed the DDDGFP fusion protein or a full-length M4 D1 subunit retaining the fixed relationship of elements and among family members, and our mapping strategy indicated that residues in this region are responsible for assembly of the D1 holoenzyme. To establish that this conserved region was responsible for D1 dimerization, we appended a 19-residue-long peptide containing an initiator Met and residues 147–163 of rat p27 (G21) to the N terminus of the fluorescent reporter protein, GFP, forming a DDDGFP fusion protein. Transient expression of DDDGFP in LLC-PK1 cells resulted in the appearance of multiple punctuate fluorescent signals along the cell periphery and at the borders between adjacent cells (Fig. 4), a pattern identical to that observed previously for the D1 holoenzyme (9, 14). Control cells transfected with the wild type GFP reporter showed no specific membrane localization but abundant reporter protein throughout the cell interior and the nucleus (Fig. 4).

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reporter alone had SeD2 activity that did not differ from that in untransfected cells (Fig. 7). These data are similar to the effects of the DDD domain on D1 and suggest that the DDD domain, present in all deiodinase isoforms, is required for the assembly of functional enzymes. Importantly, these data show that different family members will partner with each other through the DDD domain to form mixed isoform heterodimers.

Analysis of the subcellular distribution of DDDGFP in C6 control and SeD2 cells is shown in Fig. 8. C6 cells expressing either GFP or the DDDGFP reporter molecule showed abundant fluorescence through the cell interior and nucleus with no specific labeling of cellular membranes. In contrast, SeD2 cells expressing the DDDGFP reporter molecule showed bright membrane-localized fluorescence with minimal nuclear fluorescence; SeD2 cells expressing GFP alone showed no specific membrane staining and appeared to be identical to the C6 control cells. These data suggest that the DDD domain selectively interacts with a membrane-bound protein, presumably the 30-kDa SeD2 subunit constitutively expressed in SeD2 cells.

Since the presence of DDDGFP in SeD2 cells inactivates enzyme activity, we could not use changes in enzyme activity after antibody “pull-down” to examine the binding of DDD to the SeD2 subunit. Rather, we immune precipitated SeD2 complexes with anti-SeD2 antibodies and determined the presence of DDDGFP in the specific immune precipitates by immunoblot. As shown in Fig. 9, immunoblots of immune precipitated membrane complexes from untransfected SeD2- and GFP-expressing SeD2 cells showed only minor nonspecific bands. In contrast, immunoreactive GFP was present in anti-SeD2 antibody immune precipitates from the DDDGFP-expressing SeD2 cells. These data together with the subcellular distribution of the DDDGFP reporter molecule and the selective loss of SeD2 activity when DDDGFP is present confirm that the DDD serves to assemble a functional SeD2 enzyme similar to its role in D1 assembly.

TABLE III
Limiting kinetic constants for wild type D1, M4-p27wt, and X42, X101, and X133 truncation mutants

| Enzyme source | Limiting $K_m$ (mM) | Molar activity ($\text{mol product min}^{-1} \text{mol D1 dimer}^{-1}$) |
|---------------|---------------------|---------------------------------------------------------|
| Wild type     | 0.5                 | 1780                                                    |
| M4            | 0.2                 | 447                                                    |
| X42           | 0.2                 | 445                                                    |
| X101          | 0.2                 | 390                                                    |
| X133          | 0.2                 | 405                                                    |

DISCUSSION

Thyroid hormone deiodinases are an important class of selenoenzymes that regulate the availability and disposal of thyroid hormone. Assembly of functional enzyme(s) dimers is a key step in the biosynthetic pathway that takes place after the insertion of the selenoprotein monomer into the membrane bilayer (9, 12). In this report, we used deletion/truncation analysis, domain expression, and pull-down assays to identify the protein domain(s) responsible for enzyme assembly and show that a 16-residue-long region found 22 residues downstream of the catalytically essential SeC is required for the holoenzyme assembly of at least two family members.

To eliminate the technical challenges encountered when multiple, catalytically disabled constructs are transiently co-expressed in host cells (12), we used LLC-PK1 cells that constitutively express a full-length D1 selenoprotein and an exogenous, unique inert rat p27 to examine enzyme assembly. Earlier work done with this approach provided direct evidence that the D1 holoenzyme was a homodimer formed from p27 subunits and that the membrane anchor of one the subunits could be removed without altering dimerization (9). Interestingly, at least half of the p27 polypeptide, up to 133 residues, could be removed from the N terminus without altering the
binding of the truncated D1 subunit to the native p27. Progressive truncation from the C terminus of the rat p27 revealed that residues from position 170 to 254 could also be deleted without altering dimerization but that removal of residues between positions 150 and 170 completely eliminated the binding of the truncated subunit to the native p27, suggesting that the domain responsible for enzyme assembly is located between residues 150 and 170.

Comparison of the primary amino acid sequence of this region across family members revealed that a 16-amino acid-long segment located between residues 150 and 170 of p27 is one of the most highly conserved domains in the family. As shown in Table IV, 10 of the 16 residues found between positions 147 and 163 of the rat p27 are identical in all deiodinase family members from fish to humans, and most, if not all, of the variant residues are conservative substitutions. Importantly, the distance between this conserved segment and the active center SeC is fixed at 22 residues in all family members. Because of this high conservation, we generated a domain-specific probe to study enzyme assembly by appending a 17-amino acid-long peptide corresponding to residues 147–163 of p27 to the N terminus of GFP forming the DDDGFP fusion protein.

As expected, overexpression of the soluble DDDGFP fusion protein in LLC-PK1 cells resulted in a ~50% reduction in enzyme activity coincident with specific binding of the fluorescent DDD reporter to the native p27. Immune depletion analysis, subcellular fractionation, and photomicroscopy confirmed that this fusion protein was responsible for the decrease in deiodinating activity and was specifically bound to the full-length p27 in LLC-PK1 cells. These data obtained with the soluble DDDGFP fusion protein are identical to those obtained with the full-length, M4 mutant of rat p27 reported previously (9) and establish that this highly conserved region contains all of the necessary structural requirements for assembly of the D1 holoenzyme.

Based on the finding that DDDGFP fusion protein was a faithful mimic of the protein-protein interaction(s) required for holoenzyme assembly, we turned our attention to the role of this domain in the biosynthesis of catalytically active SeD2. To ensure sufficient quantities of catalytically active SeD2 for study, SeD2 cells (15), C6 astrocytoma cells that constitutively overexpress a functional SeD2, were used as the source of the membrane-bound SeD2 subunits. Unlike our findings for DDD:p27 heterodimers of D1, transient expression of either the DDDGFP fusion or the full-length, M4 mutant of D1 in SeD2 cells completely inactivated SeD2. Since the SeD2:DDDGFP heterodimers were catalytically inactive, we could not evaluate protein-protein interactions by measuring the loss of deiodinating activity by immune depletion. Instead, direct analysis of the binding between the DDDGFP reporter and the 30-kDa SeD2 subunit was done by pull-down assays with anti-SeD2...
antibodies and revealed that the complete loss of SeD2 activity was accompanied by the formation of a DDD:SeD2 heterodimer. The role of the DDD domain in SeD2 assembly was confirmed by subcellular fractionation and by co-localization studies done with the unaltered GFP fluorescent reporter and by radiolabeled wild-type D1; immune precipitates of radiolabeled wild-type D1 failed to show this additional band (12). Since the SeC motif (C-X-X-C motif) in the active center of D1 is required for the formation of disulfide bridges based on SDS-PAGE analysis of incompletely reduced samples. Interestingly, the ~50-kDa D1 band was only found after SDS-PAGE of inactive Cys mutants of D1; immune precipitates of radiolabeled wild-type D1 failed to show this additional band (12).

Using the immune depletion strategy modeled after that developed to study D1 biosynthesis, Curcio-Morelli et al. (12) used transient co-transfection of FLAG-tagged deiodinase constructs to confirm that a D1 dimer containing only one functional subunit was catalytically active (9, 12). Although more than 95% of D1 was immune precipitated by antibodies directed against a single subunit, they estimated that only 1–5% of the holoenzyme was a homodimer formed by intermolecular disulfide bridges based on SDS-PAGE analysis of incompletely reduced samples. Interestingly, the ~50-kDa D1 band was only found after SDS-PAGE of inactive Cys mutants of D1; immune precipitates of radiolabeled wild-type D1 failed to show this additional band (12). Since the SeC motif of the p27 generate a divinylal thiol (-C=X-C= motif) in the active center of the mutant(s), a protein motif shown to have a strong propensity to aggregate during SDS-PAGE (23, 24), it seems likely that the ~50-kDa protein aggregates found after SDS-PAGE resulted from the choice of Cys mutants rather than a specific role for disulfide bridging in the assembly of homodimers.

Whereas the results of our experiments make it unlikely that disulfide bridging participates in the assembly of the D1 homodimer, a role for noncovalent interactions is well documented. First, early work showed that ~95% of detergent-solubilized D1 catalytic activity and the BrAc[125I]thyroxine-labeled p27 co-migrated with a M_w of ~50 kDa on a molecular sieve chromatography (11, 25). Importantly, the choice of detergent has a profound effect on the physicochemical properties that the DDD domain is essential for the post-translational assembly of holoenzymes of at least two deiodinase family members.
FIG. 9. Immune precipitation of SeD2:DDD dimers. Eight 75-
cm² flasks of confluent SeD2 cells were transfected with 20 µg of either pEGFP or pDDDGFP and grown for 2 days at 37 °C. Cell membranes were isolated as detailed under "Experimental Procedures" and solubilized with 0.5% Triton (v/v). Clarified extracts were incubated with anti-SeD2 antiserum (1:100 dilution), immune complexes were isolated with Protein A-agarose beads, and the immune precipitated proteins were separated on a 12.5% SDS-polyacrylamide gel. Separated proteins were electrotransferred to nitrocellulose membranes, and specifically immune precipitated DDDGFP was identified with a monoclonal anti-GFP IgG and visualized using chemiluminescent reagent. Ig HC, rabbit immunoglobulin heavy chain.

of this membrane-bound enzyme and molecular sieve chromatography of D1 activity solubilized with nonionic detergents yielded Mₚ values as high as 400,000 (26, 27), presumably due to the size of the enzyme-detergent micelles. This problem can be overcome by the use of ionic detergents such as taurodeoxycholate at or near their CMD, where they contribute negligibly to the molecular size of the D1 as judged by sucrose density gradient centrifugation (10, 29). Second, that ability of p27 mutants lacking the membrane anchor to bind to the full-length p27 indicates that integration into the bilipid layer is not required for deiodinase assembly (9). Finally, the ability of the DDDGFP fusion protein to bind specifically to the full-length p27 confirms that enzyme assembly is a post-translational event that is independent of membrane association (9).

The participation of selected residues located in the DDD domain in the catalytic reaction was recently proposed by Larsen and co-workers (30) based on hydrophobic cluster analysis coupled with position-specific BLAST protocols. Mutation of any one of three residues, Glu¹⁵⁶, His¹⁵⁸, or Glu¹⁶₃, all located in the DDD domain, impaired catalysis of both D1 and D2 family members in transient expression assays (30). Whether this loss of catalytic function was due to improper enzyme assembly, direct participation in the catalytic reaction, or enhanced enzyme instability could not be evaluated. In light of the role of this domain in enzyme assembly and the improved deiodinase reaction conditions, the role of individual residues in the D1 catalytic reaction, especially in this important functional domain, needs to be re-evaluated.

In summary, assembly of a functional deiodinase holoenzyme requires a small 16-residue-long region that is one of the most highly conserved domains among family members. Identification of the domain responsible for enzyme assembly revealed that deiodinase family members retain the ability to interact with each other to form heterodimers. Whereas the consequence of heterodimer formation between different family members is unknown, it is possible that cells modulate the cellular levels of functional selenodeiodinase enzyme(s) family members by sequestering subunits in a pool of poorly functional heterodimers and thereby regulate production and disposal of bioactive thyroid hormone.

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J. Biol. Chem. 2005, 280:11093-11100.
doi: 10.1074/jbc.M500011200 originally published online January 18, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M500011200

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