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Planarian Cytochrome b_{561}: Conservation of a Six Transmembrane Structure and Localization along the Central and Peripheral Nervous System[1]

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Cytochrome b_{561} is a major transmembrane protein of catecholamine and neuropeptide secretory vesicles in the central and peripheral nervous systems of higher animals. We succeeded in cloning a full-length cDNA encoding planarian cytochrome b_{561}. The deduced amino acid sequence shows a very similar six transmembrane topology to those of cytochromes b_{561} of higher vertebrates and contains both putative ascorbate- and monodehydro ascorbate-binding sites. Among the six totally-conserved His residues of cytochrome b_{561} in higher vertebrates, one is substituted with an Asn residue, indicating that His88 and His161 of bovine cytochrome b_{561} play roles as heme *b* ligands at the extravesicular side. Northern- and Western-blot analyses confirmed the expression of the mRNA and protein with the expected sizes in planarians. The distributions of the mRNA and apoprotein were analyzed by *in situ* hybridization and immunohistochemical staining, respectively, showing two morphologically distinct structures, a pair of ventral nerve cords and the cephalic ganglion cluster in the head region. The present results suggest that the usage of ascorbate to supply electron equivalents to neuroendocrine-specific copper-containing monooxygenases is likely to have originated in organisms with a very simple nervous system.

Key words: amidated neuropeptide, ascorbic acid, cytochrome b_{561}, planarian, transmembrane electron transfer.

Most secreted neuropeptides are synthesized as larger precursor proteins and subsequently modified into biologically active peptides by a variety of post-translational processing steps. These post-translational events consist of specific proteolytic cleavages of the precursor proteins as well as other enzymatic events and are highly organized within specific intracellular neurosecretory vesicles. Many of the most prevalent neuropeptides in higher animals (*e.g.* substance P, cholecystokinin, vasopressin, oxytocin, α-MSH, γ-MSH, neuropeptide Y) are α-amidated (*1*), and are inactive (or weakly active) without the α-amide moiety. Another type of neurotransmitter, noradrenaline and adrenaline, is also synthesized and stored in specific secretory vesicles in higher animals (*2*).

Each of the two types of vesicles contains a copper-containing monooxygenase enzyme, peptidylglycine α-amidating monooxygenase (PAM; EC 1.14.17.3), and dopamine β-monooxygenase (DβM; EC 1.14.17.1) for the biosynthesis of these neurotransmitters. PAM catalyzes the COOH-terminal amidation of neuropeptides. The peptide α-amidation is actually a two-step reaction that requires the sequential action of two independent enzyme activities residing in PAM in higher animals (*3*). The first enzyme, peptidylglycine α-hydroxylating monooxygenase (PHM), hydroxylates glycine-extended peptides using molecular dioxygen and electron equivalents (*4*). The second enzyme, peptidyl-α-hydroxylglycine α-amidating lyase (PAL), catalyzes the conversion of peptidyl-α-hydroxyglycine intermediates into α-amidated peptides (*5, 6*). In insects and cnidarians, however, PHM and PAL seem to be coded for by different genes (*7, 8*). On the other hand, DβM catalyzes the hydroxylation of dopamine to form noradrenaline, like PHM, employing molecular dioxygen and electron equivalents (*9*).

The physiological electron donor for these two hydroxylating reactions are believed to be ascorbic acid (AsA^-), which accumulates inside the secretory vesicles (*9*). Since there is no transmembrane transport of AsA^- in the vesicles (*10*), it has been postulated that a common transmembrane electron carrier, cytochrome b_{561}, conveys reducing equivalents from the extravesicular AsA^- to the intravesicular monodehydroascorbic acid (MDA) radical (*10–16*). Cytochrome b_{561}, first observed in chromaffin vesicles of

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Abbreviations: PAM, peptidylglycine α-amidating monooxygenase; DβM, dopamine β-monooxygenase; PAL, peptidyl-α-hydroxylglycine α-amidating lyase; AsA^-, ascorbic acid; MDA, monodehydroascorbic acid.

bovine adrenal medullae (*17, 18*) and later found to distribute in many neuroendocrine tissues (*19–23*), is structurally and functionally very different from other cytochromes (Fig. 1) (*24*). It spans the vesicle membranes (*25*), and has a favorable midpoint potential for interaction with AsA^- and the MDA radical (*26, 27*). Indeed, the purified cytochrome b_{561} reconstituted in artificial vesicle membranes catalyzes transmembrane electron transfer (*28, 29*).

Recently a new member of the cytochrome b_{561} family was identified in plasma membranes of duodenal enterocytes (*30*). Dcytb (for duodenal cytochrome b_{561}) was reported to be responsible for the physiological ferric reductase activity in the duodenal mucosa and an important element in the iron absorption pathway (*30*). Cytochrome b_{561} and Dcytb share a similar membrane topology, potential histidyl heme ligands, and putative binding sites for AsA^- and the MDA radical (*24*), suggesting that Dcytb may react with one or more of these compounds (*30*). Other members of the cytochrome b_{561} family are also known to be present in various plasma membranes such as rabbit neutrophils (*31*) and plant cells (*32, 33*). Those new members may play a role in membrane-spanning electron transfer, but their exact physiological roles may differ and should be elucidated.

Planarians, nonparasitic free-living flatworms of the class Turbellaria, are phylogenetically very distant from vertebrates but already have acquired a central nervous system, mesodermal tissues, and bilateral structure during evolution (*34*). Planarians use both neuropeptides (*35, 36*) and catecholamines (*37*) as neurotransmitters. Planarian neuropeptides (substance P-, FMRFamide-related peptides) are extensively α-amidated (*35, 36, 38, 39*), as observed for other invertebrates. Therefore, it is very interesting to clarify whether cytochrome b_{561} exists or not in planarians. If it does, how much does the protein differ from those of higher animals? Does it catalyze the electron transfer reaction across the neuroendocrine vesicular membranes? Such information will be very important for analyses of both evolution and the mechanism of the electron transfer reaction in the neuroendocrine vesicles. In the present study, we have conducted the molecular cloning of a planarian cytochrome b_{561} cDNA. We analyzed further the expressions of both the cytochrome b_{561} mRNA and protein.

MATERIALS AND METHODS

Organisms—All planarians in this study were derived from a single worm of *Dugesia japonica* collected in the Irima River in Gifu, Japan, and maintained as described (clonal strain: GI) (*40*).

Partial Sequencing of Planarian Cytochrome b_{561} cDNA—A cDNA library of the planarian was made in λ ZAP II (Stratagene, La Jolla, CA) and stocked as 72 groups of 5×10^4 independent phage clones, as previously described (*34*). For the first PCR, two degenerate primers, "S1" (5′-TTYAAYKHICAYCCIMYITKYATG-3′) [coding for the conserved amino acid sequence FN(V/A/Y)HP(L/T)(C/F)M; positions 73–80 of bovine cytochrome b_{561} in Fig. 4 (*24*)] and "A4" (5′-RTGRTRITSRAAIACNGC-3′) [coding for the conserved amino acid sequence AVF(D/E/Q)(Y/H)H; positions 126–131 of bovine cytochrome b_{561} in Fig. 4] were used. For the second, nested PCR, two degenerate primers, "NS1" (5′-AAAAATTYAAYKHICAYCCIMYITKYATG-3′), and "NA2" (5′-AAAGGATCCTCNYKNCKRAANACNCGRTANAC-3′) were used. The former has an adapter sequence carrying a *Hin*dIII site at the 5′ end of "S1" primer. The latter also has an adapter sequence carrying a *Bam*HI site at the 5′ end of a degenerate primer [coding for another conserved amino acid sequence VYRVFR(N/H/R)E; positions 93–100 of bovine cytochrome b_{561} in Fig. 4]. The reaction mixture consisted of 1 μl each of 10× Taq buffer, 2.5 mM dNTPs, 10 μM forward primer, 10 μM reverse primer, cDNA library mixture, and 5 μl of autoclaved H_2O, and 0.05 μl of Taq DNA polymerase (Life Technologies) in a final volume of 10 μl. Thermal cycling was performed for 40 cycles of the following step program: 94°C for 1 min, 40°C for 1 min (primer annealing), 72°C for 1 min (primer extension). Final primer extension was at 72°C for 5 min.

The PCR products were separated in an 8.0% polyacrylamide gel, isolated, and subcloned into the *Bam*HI and *Hin*dIII sites of pBluescript II SK+ (Stratagene). The nucleotide sequences were analyzed with a DNA sequencer (Model DSQ-1000L, Shimadzu, Kyoto). Based on the nucleotide sequence obtained, two specific primers "Kt" (5′-CCT-

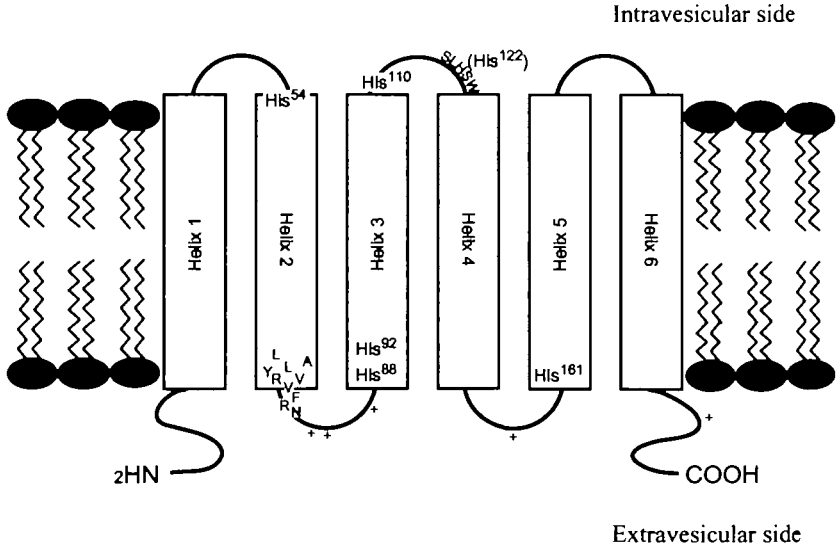

Fig. 1. **Transmembrane structural model of bovine cytochrome b_{561}.** Two fully conserved sequences (^{69}ALLVYRVFR^{77} and ^{120}SLHSW^{124}) and six conserved histidyl residues (His54, His88, His92, His110, His122, and His161) are indicated. His54 and His122 are likely the heme axial ligands at the intravesicular side. His161 and either His92 or His88 are considered to be the heme axial ligands at the extravesicular side. We postulated previously that the two conserved sequences are part of the extravesicular AsA^- and intravesicular MDA-binding sites, respectively (*24*). Other well-conserved basic amino acid residues are indicated by +.

TACTAAAAATAAATACCTTTA-3′) and "asada2" (5′-AACACGCGATAAACCATAATAGC-3′) were synthesized. Nested PCR was performed similarly on the mixture of a planarian cDNA library using "asada2" and M13 reverse primer (PO8; 5′-AGCGGATAACAATTTCACACAGGAAAC-3′) for the first PCR and "Kt" and T3 primer (20-mer) for the second PCR. The PCR products were separated in an 8.0% polyacrylamide gel, isolated, subcloned into pT7Blue T vector (Novagen) with TA cloning, and sequenced.

Stepwise Dilution Screening of the Planarian cDNA Library—A new specific primer "asada3" (5′-TAGTTCAAAATCTTAATGGATTTTTACC-3′) was synthesized based on the nucleotide sequence obtained. Stepwise dilution screening of the planarian cDNA library was performed with PCR using primers "asada3" and "Kt," based on the procedure previously described (*41*). After single plaques carrying the phage-containing cytochrome b_{561} gene were obtained, the single phages were *in vivo*–excised and sequenced as described above.

5′-RACE—The sequence of this clone (cyb561) encoding about 95% of the whole cytochrome b_{561} gene was used to design primers for 5′-rapid amplification of cDNA ends (5′-RACE). By utilizing a Marathon™ cDNA Amplification Kit (CLONTECH), the nucleotide sequence of a whole cytochrome b_{561} cDNA gene was determined and analyzed as described above.

Phylogenetic Analysis—The deduced amino acid sequences of cytochrome b_{561} from various animals and plants (including Dcytbs from human and mouse) were multiply aligned using the program Clustal W 1.8 (operated on the DDBJ CLUSTALW system, DNA Data Bank of Japan, Mishima), and the phylogenetic distance between the protein sequences was determined using the Kimura protein distance method. A phylogenetic tree was created from the distance matrix with the program TreeView PPC (v. 1.6.6) using either the neighbor-joining method or alignment guide tree method.

Northern Blotting—Total RNA was prepared by the CsCl cushion method from asexual planarians. The RNA was glyoxylated, separated in a 1% agarose gel and transferred to a membrane (Hybond-N, Amersham Pharmacia Biotech). The membrane was baked at 80°C for 1 h, prehybridized for 1 h under hybridization conditions in the absence of probe, and hybridized with ^{32}P-labeled DNA probe at 65°C for 18 h in 6× SSC, 5× Denhardt's solution, 100 μg/ml salmon testis DNA, 0.5% SDS. The probe was labeled using a *Bca*Best random DNA labeling kit (Takara Shuzo, Kyoto). The membrane was washed twice for 1 h in 2× SSC, 0.1% SDS, at 65°C, and for 1 h in 0.1× SSC, 0.1% SDS at 65°C,

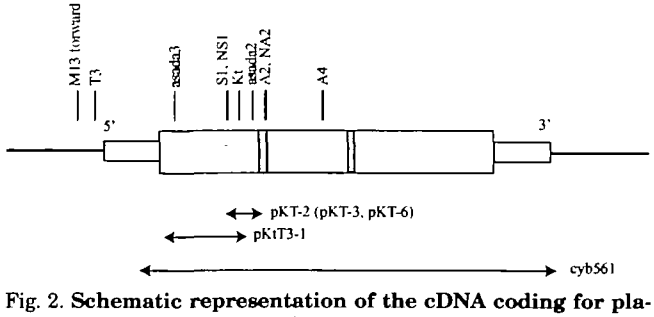

Fig. 2. **Schematic representation of the cDNA coding for planarian cytochrome b_{561} and positions of the degenerate and specific primers used in the present study.** The broad bar and the two flanking small bars represent the coding region and the noncoding regions of the cDNA, whereas the two flanking lines outside indicate the λ ZAP II vector. The two gray boxes represent the conserved sequences (ALLVYRVFR and SLHSW). The degenerate and specific primers are indicated by vertical lines above the figure. The obtained PCR and cDNA clones are indicated below the figure.

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        10         20         30         40         50         60
TTAAAACTTAATATATATTTACATTTTATCTTTACTTATGCATTCCTATGAAGCTGTTGA
                                       M  H  S  Y  E  A  V  D

        70         80         90        100        110        120
TGGAATAAGCAGACTTTCTGTAGTTCAAAATCTTAATGGATTTTTACCATTGATATTTAT
 G  I  S  R  L  S  V  V  Q  N  L  N  G  F  L  P  L  I  F  I

       130        140        150        160        170        180
ATGTGAAGTTTGCGGGCTGGCGATTGTTATAATGACAGCTGTATGGATGGGTGTTCTTCA
 C  E  V  C  G  L  A  I  V  I  M  T  A  V  W  M  G  V  L  Q

       190        200        210        220        230        240
AGATGGGGGATTTGGGTGGACTAAGGAATTGGTTTTTCGCTATCATCCAATGTTCATGAT
 D  G  G  F  G  W  T  K  E  L  V  F  R  Y  H  P  M  F  M  I

       250        260        270        280        290        300
ATTAGGAATGATTTTTATTTATTATGGAAATGCTATTATGGTGTACCGAGTATTTAGAAACAC
 L  G  M  I  F  I  Y  G  N  A  I  M  V  Y  R  V  F  R  N  T

       310        320        330        340        350        360
AAAGAAAATCCGAGCGAAATGGTTGCATGCAGTTCTCAATTTATTAGCTTTAATATTAGG
 K  K  I  R  A  K  W  L  H  A  V  L  N  L  L  A  L  I  L  G

       370        380        390        400        410        420
ATCTGTGGGACTAAAAGCAGTTTTTGATTCTCATAATATGAAAGGAACAGCTAATATGTA
 S  V  G  L  K  A  V  F  D  S  H  N  M  K  G  T  A  N  M  Y

       430        440        450        460        470        480
CAGCTTGCATAGTTGGGTTGGACTTGGATGTGTTATTTTATTCGGTTGTCAGTGGGTACT
 S  L  H  S  W  V  G  L  G  C  V  I  L  F  G  C  Q  W  V  L

       490        500        510        520        530        540
TGGATTTATTTCATTTCTTTTTCCGAAACTACCCGAAACACTGAGATCAGCTATAATGCC
 G  F  I  S  F  L  F  P  K  L  P  E  T  L  R  S  A  I  M  P

       550        560        570        580        590        600
ATTGCATAGAAGTCTAGGTATGATTATTTTGGGTTTGGCCGTAGCTGCTGCTGTTATGGG
 L  H  R  S  L  G  M  I  I  L  G  L  A  V  A  A  A  V  M  G

       610        620        630        640        650        660
AATTACAGAGTACAATAATAATGATAAATCCAAATCACCATCAACTATGCTCGGAAATTT
 I  T  E  Y  N  N  N  D  K  S  K  S  P  S  T  M  L  G  N  F

       670        680        690        700        710        720
TATCGGAATAATTTCATTAATATTTGTAAGTATAGTACTATTTTTGGTTATATGGTCCGA
 I  G  I  I  S  L  I  F  V  S  I  V  L  F  L  V  I  W  S  E

       730        740        750        760        770        780
GTACCGAAGAATTGAACCCGGAACAGAAGAAGAAAGAATTATCCTTAATGATTAATGAAA
 Y  R  R  I  E  P  G  T  E  E  E  R  I  I  L  N  D  *

       790        800        810        820        830        840
CCATTTTGTGGAATTTTTATATGCTTCTAGATTGTCAAATGTAAATTGTCCTGTATTGTG
       850        860        870        880        890        900
ATTGAGCCCCATGACTTTTTTTTATTATTTATTATCATTATTACTATTATTATTATTATTA
       910        920        930        940        950        960
TTATTATTATTTAATATTATCATTATTATTTATGTGATTTCCTCGACTTTAAACTGTTTG
       970        980        990       1000       1010       1020
GTATTTAATGGAATTTTTATTATATGAGTCTTATGTTATTGTGGTTGTCTTGTCATATCG
      1030       1040       1050       1060
TTGTAATAAATTTAATTTTGAAAAAAAAAAAAAAAAAAAA
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Fig. 3. **Nucleotide and deduced amino acid sequences of planarian cytochrome b_{561} cDNA.** The figure consists of the compiled data obtained from sequencing the cDNA clone "cyb561" and the sequence obtained by 5′-RACE. The nucleotides are numbered from the 5′ to the 3′ end, and the amino acid residues are numbered starting with the first ATG in the open reading frame. The translation termination codon is indicated by an asterisk. The sequence obtained by the first degenerate PCR is single underlined, whereas the region used for the production of the polyclonal antibodies is double underlined.

and analyzed using a BAS 2000 image analyzer (Fuji Film).

Whole-Mount In Situ Hybridization—Planarians of 5–7 mm were starved for 7–10 days before use. Whole-mount *in situ* hybridization was performed as described previously (*42*) using a digoxigenin-labeled RNA probe derived from the longest cDNA clone, "cyb561."

Antibody Production—A C-terminal peptide (LFLVIWSEYRRIEPGTEEERIILND) of planarian cytochrome b_{561}, which is considered to be exposed to the extravesicular side of chromaffin vesicles, was overexpressed in *Escherichia coli* as a fusion protein with 6xHis-tagged dihydrofolate reductase protein employing a QIAexpress expression system (QIAexpress Type II Kit, QIAGEN), and purified according to the recommended protocol. The purified fusion protein was emulsified in Freund's complete (initial) or incomplete (booster) adjuvant, and injected subcutaneously into a female Japanese White rabbit. After several booster injections, blood was collected. The experimental protocols were approved by the institutional review committee and meet the guidelines of the governmental agency. Polyclonal antibodies against planarian cytochrome b_{561} were prepared according to the published procedure.

Western Blotting—A single planarian worm was homogenized in 100 μl of 62.5 mM Tris-HCl (pH 6.8) containing 2% SDS, 10% glycerol, and 5% β-mercaptoethanol. Insoluble materials were removed by centrifugation at 15,000 rpm for 5 min. Fifty microliters of the supernatant was separated by electrophoresis in an SDS/12.5% polyacrylamide gel (size: 9 × 8 cm) under reducing condition (*43*). Separated proteins were then transblotted onto a PVDF membrane, and the membrane was separated into 10 sheets with a clean razor. Each membrane sheet was treated with 10% goat serum to block the non-specific binding site. Then the blot was reacted first with rabbit anti-(planarian cytochrome b_{561}) serum (with a dilution of 1/20,000) and detected with an alkaline phosphatase-conjugated secondary

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                  1                                                  49
b561bovine        --------MEGPASPARAPGALPYYVAFSQLLGLIVVAMTGAWLG-MYRGGIAWES--AL
b561sheep         --------MEGPASPAPAPGALPYYVAFSQLLGLTVVAMTGAWLG-MYRGGIAWES--AL
b561pig           --------MESPAGRTPAPGALPYYVAFSQLLGLTVVAVTGAWLG-AYRGGIAWES--AL
b561human         ---------MEGGAAAATPTALPYYVAFSQLLGLTLVAMTGAWLG-LYRGGIAWES--DL
b561mouse         ----------MEHSSASVLLHCRTMWPSPSCLGLTVVAVTGAWLG-LYRGGIAWES--SL
b561Xenopus       ---------MENALSSQNLGFMPYLVAGSQILGIANLAITGAWLA-QLQRRFLWSG--PL
b561elegans       -MSLLFDPGFVILREDQSVKLFNIILVMSQVFGGLAVLLVTIWMS-KFESGFAWNEDPDK
b561planaria      MHSYEAVDGISRLSVVQNLNGFLPLIFICEVCGLAIVIMTAVWMGVLQDGGFGWTK--EL
                                                  *         *         *
                  50                                                 109
b561bovine        QFNVHPLCMIIGLVFLQGDALLVYRVFRNEAKRTTKVLHGLLHVFAFVIALVGLVAVFEH
b561sheep         QFNVHPLCMVIGLVFLQGDALLVYRVFRNEAKRTTKVLHGLLHVFAFVIALVGLVAVFEH
b561pig           QFNVHPLCMIIGLVFLQGDALLVYRVFRNEAKRTTKILHGLLHVLAFVIALVGLVAVFDY
b561human         QFNAHPLCMVIGLIFLQGNALLVYRVFRNEAKRTTKVLHGLLHIFALVIALVGLVAVFDY
b561mouse         QFNVHPLCMVIGMIFLQGDALLVYRVFRREAKRTTKILHGLLHVFAFIIALVGLVAVFDY
b561Xenopus       QFNVHPLCMVLGMVFLCGEALLVYRVFRHETKRSTKILHGVLHIMALVISLVGVIAVFQY
b561elegans       EFNYHPTFMIMGMVFLFGEALLVYRVFRNERKKFSKTLHVILHSCVLVFMLMALKAVFDY
b561planaria      VFRYHPMFMILGMIFIYGNAIMVYRVFRNTKKIRAKWLHAVLNLLALILGSVGLKAVFDS
                   *  **  *  *  *  * *  ******   *   * **  *             ***
                  110                                                161
b561bovine        HRKKG--------YADLYSLHSWCGILVFALFFAQWLVGFSFFLFPGASFSLRSRYRPQH
b561sheep         HRKKG--------YADLYSLHSWCGILVFALFFAQWLVGFSFFLFPGASFSLRSRYRPQH
b561pig           HRKKG--------IADLYSLHSWCGILVFVLFLAQWLVGLGFFLFPGASFSLRSRYRPQH
b561human         HRKKG--------YADLYSLHSWCGILVFVLYFVQWLVGFSFFLFPGASFSLRSRYRPQH
b561mouse         HKKKG--------YADLYSLHSWCGILVFVLYFVQWLVGFSFFLFPGASFSLRSRYRPQH
b561Xenopus       HQANG--------YPDMYSLHSWCGIVTFTLYILQWIIGFSLFFIPGVAFTYRSQFKPLH
b561elegans       HNLHKDPSGNPAPIVNLVSLHSWIGLSVVILYFAQYIVGFITYFFPGMPIPIRQLVMPFH
b561planaria      HNMKG--------TANMYSLHSWVGLGCVILFGCQWVLGFISFLFPKLPETLRSAIMPLH
                  *                  ***** *     *   *   *       *       *    * *
                  162                                                219
b561bovine        VFFGAAIFLLSVATALLGLKEALLFEL--GTKYSMFEPEGVLANVLGLLLATFATVILYI
b561sheep         VFFGAAIFLLSVATALLGLKEALLFEL--GTKYSTFEPEGVLANVLGLLLAAFATVVLYI
b561pig           VFFGAAIFLLSVGTALLGLKEALLFQL--GTKYSAFESEGVLANVLGLLLVAFGAVVLYI
b561human         IFFGATIFLLPVGTALLGLKEALLFNL--GGKYSAFEPEGVLANVLGLLLACFGGAVLYI
b561mouse         IFFGATIFLFSAGHSLLGLKEALLFKL--GSKYSTFEPEGVVANVLGLLLVCFGVVVLYI
b561Xenopus       EFFGRALFLSSIATSLLGLTEKMFS------EYSSHPAEGILVNSLGVLLVVFGAVIAYI
b561elegans       QMFGVLIFIFVSITVAMGISERAAWKHTCWTKEGQMCAQQATSSFVGVFTFLYTVCVLLL
b561planaria      RSLGMIILGLAVAAAVMGITEYNNN-------DKSKSPSTMLGNFIGIISLIFVSIVLFL
                     *              *  *                          *
                  220                          252
b561bovine        LTRADWKRPLQAEEQALSMDFKTLTEGDSPSSQ
b561sheep         LTRADWKRPLQAEEQALSMDFKTLTEGDSPSSQ
b561pig           LTRADWKRPLQAEEQALSMDFKTLTEGDSPSSQ
b561human         LTRADWKRPSQAEEQALSMDFKTLRQGDSPGSQ
b561mouse         LAQAALERGSQAEEQALSMDFKTLTEGDSPTSQ
b561Xenopus       LTREDWRRPPLPEEQALSMDFKTLTEGDSPTDQ
b561elegans       VLNPRWKRQSLPEEEGLHHLTSSHSMSD-----
b561planaria      VIWSEYRRIEPGTEEERIILND-----------
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Fig. 4. **Multiple alignment of cytochrome b_{561} sequences from bovine, human, sheep, pig, mouse, *Xenopus*, *C. elegans*, and planaria.** Amino acid residues common to all sequences are denoted by asterisks below the sequences. The broken underlines indicate the putative membrane spanning segments (helices 1–6). The putative extravesicular AsA^- and intravesicular MDA binding regions are double-underlined. The Asn residue at the 101st position of planarian cytochrome b_{561} is indicated in boldface. The sequences were obtained from bovine (X12783) (*46*), sheep (D88157) (*24*), pig (D88158) (*24*), human (U29462) (*47*), mouse (U16297) (*48*), *Xenopus* (U16364) (*49*), *C. elegans* (Craxton *et al.*, Z27080), and planaria (AB049567) (this study).

anti–rabbit IgG (BioRad) in a mixture containing nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate as substrate.

Immunohistochemistry—After similar processing to the *in situ* hybridization, whole-mount specimens were incubated overnight in a 1/800 dilution of anti-(planarian cytochrome b_{561}). Signals were visualized using an alkaline phosphatase-conjugated secondary anti–rabbit IgG (Bio-Rad) in a mixture containing nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate as substrate.

RESULTS

Using degenerate oligonucleotide primers coding for regions in cytochromes b_{561} conserved among various vertebrates and *C. elegans*, we were able to clone PCR products from a cDNA library of the planarian *Dugesia japonica* (clones pKT-2, pKT-3, pKT-6) (Fig. 2). These three clones showed identical 39-bp long nucleotide sequences. The deduced amino acid sequence (ILGMIFIYGNAIM) is consistent with a partial sequence of cytochrome b_{561}. To obtain longer nucleotide sequences, we carried out nested PCR on the planarian cDNA library using new specific primers ("Kt" and "asada2") synthesized based on the 39-bp sequence and specific primers for the vector sequence (Fig. 2). All the seven cloned PCR products showed nucleotide sequences corresponding to the NH_2-terminal region of cytochrome b_{561}, but lacking a codon for the initial methionine at the 5′ terminal region. Based on this longer nucleotide sequence (pKtT3-1), we synthesized a new specific PCR primer ("asada3") (Fig. 2). Stepwise dilution screening of the planarian cDNA library with PCR (expected PCR product size 187 bp) using the primers, "asada3" and "Kt," resulted in the isolation of five clones containing planarian cytochrome b_{561}-cDNA. The nucleotide sequence of a clone (cyb561) containing the longest insert showed an almost complete (95%) cDNA for the planarian cytochrome b_{561} (Fig. 2). The coding region of this cDNA is 720 bp long and followed by a 3′ untranslated region. The sequence of this clone was used to design primers for 5′-rapid amplification of cDNA end (5′-RACE). Thus, the nucleotide sequence of the whole cytochrome b_{561} cDNA was determined and analyzed. The coding region was 738 nucleotides long preceded by an untranslated 5′ region of 37 bp and followed by a 285-bp untranslated region containing the first 20 nucleotides of the poly A tail (Fig. 3).

The deduced planarian cytochrome b_{561} amino acid sequence gave a length of 245 residues with a theoretical molecular weight of 27,425.45 (Fig. 3). The sequence showed a high degree of similarity to those of cytochromes b_{561} from higher animals. Figure 4 shows a multiple alignment of the deduced amino acid sequences of cytochromes b_{561} from the planarian and *C. elegans* in comparison with other cytochromes b_{561} from various animals. The hydropathy plot (not shown) showed the presence of six membrane-spanning α-helices, as proposed previously based on the sequences of cytochromes b_{561} from higher animals (Fig. 1) (*24*). Of the two highly conserved sequences (*24*), an identical SLHSW sequence at the intravesicular side was observed, whereas an AIMVYRVFR sequence instead of ALLVYRVFR was found at the extravesicular side in planarian cytochrome b_{561}. The two substitutions are, however, very conservative (exchange within the nonpolar side-chain group with similar residue bulk). On the other hand, among the six totally-conserved His residues in higher vertebrates, one His residue (His92, based on numbering of bovine cytochrome b_{561}) is replaced with an Asn residue at the 101st position of planarian cytochrome b_{561}. Positive charges distributing at the cytosolic surface of cytochrome b_{561} of higher vertebrates (*24*) were also well-conserved in planarian cytochrome b_{561}. Phylogenetic analysis of cytochromes b_{561} from various animals (including Dcytbs from human and mouse) and a plant (Fig. 5) showed that planarian cytochrome b_{561} can be classified as a subfamily of the neuroendocrine cytochrome b_{561} rather than the Dcytb subfamily, which is known to be present in the duodenal mucosa of higher animals and is considered to be responsible for physiological ferric reductase activity (*30*). Planarian cytochrome b_{561} was also very distant from plant cytochromes b_{561}.

Northern blot analysis revealed that a cDNA probe (cyb561) coding for a major part of cytochrome b_{561} hybridized with the mRNA giving a single band of approx. 1.1 kb

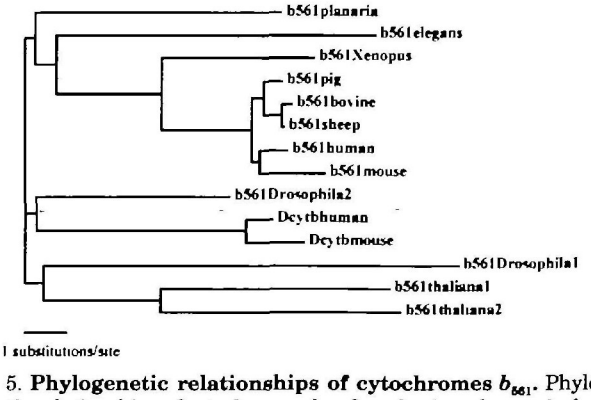

Fig. 5. **Phylogenetic relationships of cytochromes b_{561}.** Phylogenetic relationships of cytochromes b_{561} from bovine, sheep, pig, human, mouse, *Xenopus*, *C. elegans*, planaria (this study), *Drosophila* (Drosophila1, CG8776; Drosophila2, CG1275), *Arabidopsis thaliana* (thaliana1, AB049627; thaliana2, AB049628), and Dcytbs from human (AK027115) and mouse (*30*) were determined as described in the text. The phylogenetic tree was created from the distance matrix using the neighbor-joining method.

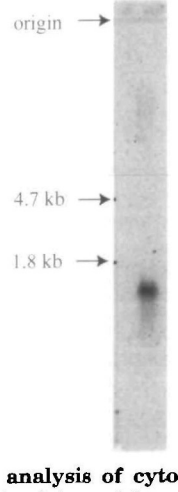

Fig. 6. **Northern blot analysis of cytochrome b_{561}-mRNA in planaria.** Total RNA (10 μg) derived from the total planarian body was probed with cytochrome b_{561} cDNA.

(Fig. 6). Figure 7, A and B, shows dorsal and ventral views, respectively, of the whole-mount *in situ* hybridization using the antisense cyb561 RNA. It was clearly shown that cytochrome b_{561} gene is expressed in two morphologically distinct structures, the pair of ventral nerve cords and the cephalic ganglion cluster in the head region. The distribution of the staining is similar to that of prohormone convertase 2-mRNA (*34*).

Polyclonal antibodies against the C-terminal portion of planarian cytochrome b_{561} were successfully produced in a rabbit. Western blotting analysis of the whole planarian tissues (Fig. 8A) showed a clear, single stained band at 27.5 kDa, almost identical to the theoretical molecular weight of apocytochrome b_{561}. Figure 8, B and C, shows dorsal and ventral views, respectively, of whole-mount immunohistochemical staining using the same polyclonal antibodies used for Western-blotting. The staining shows a much clear view of the central and peripheral nervous system than obtained by *in situ* hybridization. Cephalic ganglions form an inverted U-shaped brain-like structure with branches on each outer side. Two eyes locate on the dorsal side of the third branches of the cephalic ganglions (Fig. 8B). The ventral nerve cords can be seen very clearly and are connected to the cluster of cephalic ganglions in the head region. The left and right sides of the ventral nerve cords are connected by transverse axons between them. These transverse axons form a ladder structure in the whole body (Fig. 8C).

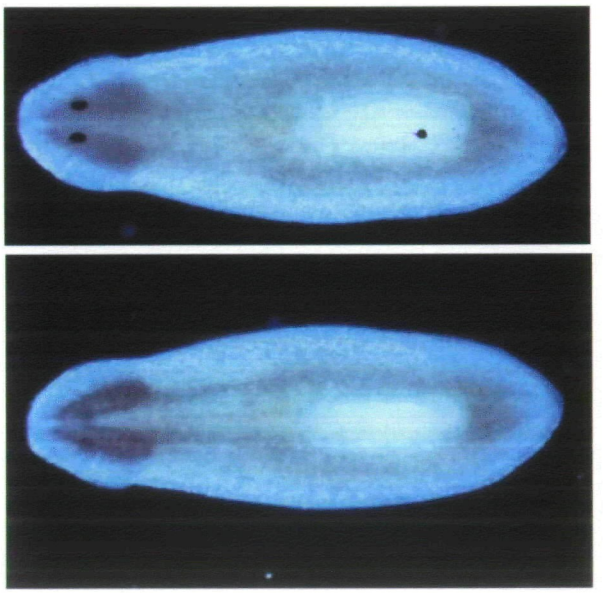

Fig. 7. **Structure of planarian central and peripheral nervous systems visualized by whole mount *in situ* hybridization with the planarian cytochrome b_{561} antisense RNA probe.** (A) Dorsal view. (B) Ventral view. Anterior is left and posterior is right.

DISCUSSION

Flatworms (plathelminthes), including planarians, occupy a very distant position in the phylogenetic tree from that of vertebrates. They are an animal group with the simplest body plan, having a bilateral symmetric nervous system and an archaic brain. The simple nervous system of planarians, however, produces large amounts of a variety of neuropeptides, of which many or perhaps all are α-amidated at their C termini (such as neuropeptide F and FMRFamide-related peptides) (*35, 36*). In addition, both dopamine and noradrenaline have been detected in planarian tissues upon electrochemical detection coupled with liquid chromatography (*37*), suggesting that catecholamines may also be used as neurotransmitters. It has been proposed, however, based on studies of *Cnidarians* which are the most primitive organisms with an organized nervous system, that the use of amidated peptides for intracellular communication preceded the use of catecholamines (*44*).

The deduced amino acid sequence of the cloned cytochrome b_{561} cDNA from planarians suggests that planarian cytochrome b_{561} can be classified as a member of the neuroendocrine subfamily. Clear detection of both the cytochrome b_{561}-mRNA by Northern-blotting and apocytochrome b_{561}-protein by Western-blotting indicate that cytochrome b_{561} is expressed significantly in planarian bodies. The distribution of the mRNA in *in situ* hybridization and the apoprotein in immunohistochemical detection suggests that indeed planarian cytochrome b_{561} is expressed exclusively in the central and peripheral nervous systems. The distribution is similar to that of planarian pro-hormone convertase 2 (PC2) detected by *in situ* hybridization (*34*). PC2 is known to be a protease that processes long precursor proteins to short neuropeptides before α-amidation occurs. This enzyme exists specifically in the neurosecre-

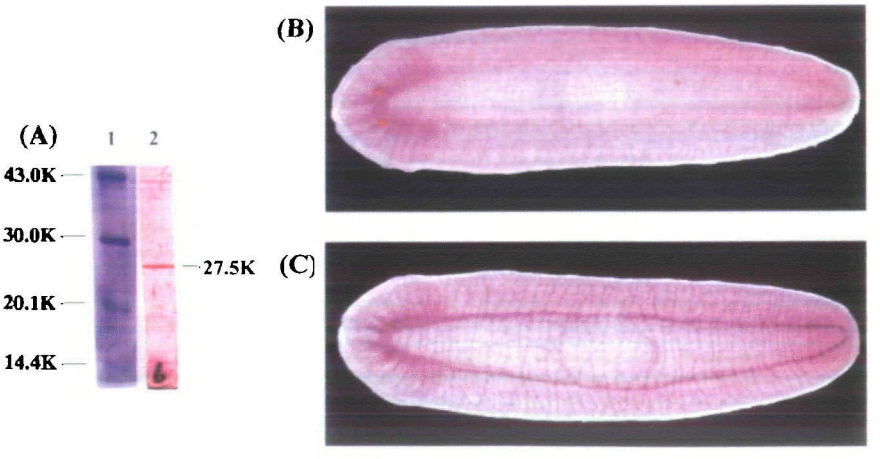

Fig. 8. **Western-blotting analysis of whole planarian tissue (A) and the structure of the planarian central and peripheral nervous systems visualized by whole-mount immunocytochemical staining using anti-planarian cytochrome b_{561} (B, C).** (A) Five microliters of extracted protein from a single planarian worm tissue was separated by SDS-PAGE, transferred to a PVDF membrane, and probed with anti-(planarian cytochrome b_{561}) serum (lane 2). Low molecular mass protein markers are shown in lane 1 stained with Coomassie Brilliant Blue R-250. (B) Dorsal view. (C) Ventral view.

tory vesicles of neurons secreting neuropeptides. Based on these observations, it can be concluded that planarian cytochrome b_{561} exists in the neuroendocrine vesicles of the planarian nervous system and, therefore, most likely plays a role in supplying electron equivalents to the inside of the vesicles, as found in higher animals.

We found recently that purified bovine cytochrome b_{561} contains two hemes B *per* molecule and that each heme *b* center exhibits a distinct EPR signal in the oxidized state (*15*). Comparison of the deduced amino acid sequences of cytochromes b_{561} from various higher animal species showed two conservative sequences at intravesicular and extravesicular sites that we suggested are the putative AsA^- and MDA binding regions, respectively (*24*). Further, we postulated that the two heme B prosthetic groups are located on both sides of the membranes in close contact with the AsA^- and MDA binding regions, to participate in the electron transfer across the vesicular membrane (*24*) (Fig. 1). This proposal was supported by a pulse radiolysis study (*16*) and, more recently, by specific chemical modification studies utilizing a MALDI-TOF-MS technique (*27, 45*).

In the present study, we confirmed the presence of the two conserved sequences (AIMVYRVFR and SLHSW) in planarian cytochrome b_{561} as well (Fig. 4). The former sequence is considered to form a part of the AsA^--binding site at the extravesicular side, whereas the latter is likely to form a part of the MDA-binding site at the intravesicular side in cytochrome b_{561} from higher animals (*24*) (Fig. 1). The present results suggest that the AsA^--related electron transfer system is likely to be operative in planarians as well. The presence of the well-conserved positive charges at the extravesicular side and a relatively compact and conserved tertiary structure of planarian cytochrome b_{561} support this view. We found further that one of the six conserved His residues in higher animal species (His92 of bovine cytochrome b_{561}) is replaced with an Asn residue (Fig. 4). Previously, we suggested that either His88 or His92 and His161 form the heme *b* ligand at the extravesicular side of bovine cytochrome b_{561} (*24*). The present result indicates that His88 and His161 act as the heme *b* ligands at the extravesicular side and His92 does not play an indispensable role in the electron transfer reaction (Fig. 1). This conclusion is consistent with our results from a cDNA cloning study of a cytochrome b_{561} from a plant, *Arabidopsis thaliana*. Of the two forms of cytochrome b_{561} cloned by us from an *A. thaliana* cDNA library, one form was found to have a Gln residue at the positions corresponding to His92 of bovine cytochrome b_{561} (Asada *et al.*, unpublished; the nucleotide sequences were submitted to the DDBJ/EMBL/GenBank databases with accession numbers AB049627 and AB049628). Based on the analysis of the deduced amino acid sequences, the whole tertiary structure of plant cytochrome b_{561} seems very similar to that of animal cytochrome b_{561} (Asada *et al.*, unpublished). Because of a lack of the putative AsA^- binding region at the extravesicular side of the molecule (Asada *et al.*, unpublished), however, the physiological role of plant cytochromes b_{561} (*32, 33*) is not clear at this time. However, the occurrence of the substitution at the position corresponding to His92 of bovine cytochrome b_{561} supports our current assignment of the heme-ligating amino acid residues.

One might suggest that His92 of bovine cytochrome b_{561} (and the corresponding His residues in higher animals) has a specific role in AsA^- binding and, therefore, the occurrence of a substitution of His92 to Asn in planarian cytochrome b_{561} is indicative of other physiological electron donors than AsA^-. The possibility seems, however, unlikely since the location of His92, which is situated much more interiorly than the heme group, is inappropriate for a substrate recognition site. It should be stressed, however, that care must be taken in drawing a final conclusion about the physiological electron donor for planarian cytochrome b_{561} before concrete biochemical evidence is available.

In conclusion, we have succeeded in the molecular cloning of cytochrome b_{561} cDNA from the planarian, *Dugesia japonica*. Analysis of the amino acid sequence suggests that the AsA^--related transmembrane electron transfer *via* cytochrome b_{561} may be operative in planarians also. Significant expression of the cytochrome b_{561} gene product along the planarian nervous system suggests that the use of AsA^- to supply electron equivalents to the neuroendocrine-specific copper-containing monooxygenases is likely to have originated in organisms having a very simple central nervous system, and may be distributed widely among metazoan lineages. Thus, cytochrome b_{561} could be used as an interesting marker for studies on the evolution and the structure of the peptidergic and adrenergic nervous systems.

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