W276 Mutation in the Endothelin Receptor Subtype B Impairs G_q Coupling but Not G_i or G_o Coupling†

Fumiaki Imamura,‡,§ Ikuyo Arimoto,‡,|| Yoshinori Fujiyoshi,‡,⊥ and Tomoko Doi*,‡,#

Department of Biophysics, Graduate School of Science, Kyoto University, Oiwake, Kitashirakawa, Sakyo-ku, Kyoto 606-8502, and CREST, Japan Science and Technology Corporation

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ABSTRACT: The mutation of W276 to cysteine within the human endothelin receptor subtype B (ET_BR) is associated with Hirschsprung's disease, a congenital intestinal disease. The sequence surrounding W276 is highly conserved between the endothelin receptor subtypes A and B. We have introduced sets of mutations into W275 and W276 of the ET_BR gene, and the corresponding W257 and W258 of the ET_AR gene, and studied their coupling properties with G_i, G_o, and G_q in reconstituted phospholipid vesicles. The prepared mutants all showed a similar affinity for endothelin-1. The W276C/ET_BR and W276A/ET_BR mutants had reduced activities in G_q coupling but not in G_i/G_o coupling, while the W275A/ET_BR displayed reduced activities in G_i/G_q coupling, with normal G_o coupling. On the other hand, W257A/ET_AR and W258A/ET_AR exhibited wild-type activities in all examined G protein couplings. These results suggest that the defects in the G_q signaling pathway by the ET_BR are connected with Hirschsprung's disease and that the two conserved tryptophans play distinct roles in signal transduction by the two receptor subtypes. In addition, W275 and W276, which are thought to be located near the extracellular side of the transmembrane helix 5, play important roles in forming the active structure of ET_BR.

Endothelins (ETs),[1] consisting of ET-1, ET-2, and ET-3, are 21 amino acid vasoconstrictive peptides that transmit a number of signals via the ET receptor subtypes A (ET_AR) and B (ET_BR) (*1*). ET_AR and ET_BR are G protein-coupled receptors, distributed throughout the body, that couple to G_i, G_o, G_s, and G_q, depending upon the tissue or cell type (*2*). ET_AR is selective to ET-1 and ET-2, whereas ET_BR binds three isopeptides with similar affinities. While ETs send regulatory signals to maintain homeostasis in the circulatory and endocrine systems (*3*−*5*), they also play noticeable roles as mitogenic signals in many cell types (*6*, *7*).

Hirschsprung's disease (HSCR) is a hereditary disease, characterized by the absence of intrinsic ganglion cells in the submucosal and myenteric plexuses of the distal gastrointestinal tract. HSCR is considered to be a multigenic disorder and a developmental defect resulting from abnormal neural crest cell migration (*8*, *9*). The ET_BR gene has been identified as one of the susceptibility genes in HSCR along with the RET proto-oncogene (receptor tyrosine kinase) and the ET-3 gene (*10*, *11*). Various mutations in the three genes were identified from naturally occurring HSCR patients (*10*, *12*−*19*). Puffenberger and co-workers were the first to show that the W276C mutation in the ET_BR was associated with HSCR, resulting in an impaired ligand-dependent [Ca^{2+}] increase in transfected cells (*12*).

To dissect the complex signal transduction by the ET_AR and the ET_BR, we have been studying the biochemical properties of ET_AR and ET_BR in a purified and reconstituted system using Sf9-expressed protein. We have shown that the ET-1-bound ET_AR and ET_BR activate G_q to different extents and exhibit different affinities to G_i; nevertheless, they are highly homologous to each other (*20*). In the secondary structure model, W276 is located in the putative transmembrane helix 5 near the extracellular side, and the surrounding sequences in the ET_BR and those in the ET_AR are highly conserved. To reveal the structural properties of the subtypes in the activated form, we have introduced a series of mutations into W275 and W276 in ET_BR and into W257 and W258 in ET_AR and have studied their coupling efficiencies with G_i, G_o, and G_q in the reconstituted system after expression in Sf9 cells and purification. The W276C/ET_BR and W276A/ET_BR mutants exhibited about 50% reduction in G_q activation, with wild-type activation of G_i and G_o, suggesting the involvement of W276 not in the direct ET-1 binding, but in the conformational change of ET_BR following ligand binding. These results suggest the importance of signaling via the G_q pathway in neural crest cell development.

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* To whom correspondence should be addressed. Phone: 81-75-753-4216. Fax: 81-75-753-4218. E-mail: doi@nb.biophys.kyoto-u.ac.jp.

‡ Kyoto University.

§ Phone: 81-75-753-4216. Fax: 81-75-753-4218. E-mail: fumiaki@em.biophys.kyoto-u.ac.jp.

|| Phone: 81-75-753-4216. Fax: 81-75-753-4218.

⊥ Phone: 81-75-753-4215. Fax: 81-75-753-4218. E-mail: yoshi@em.biophys.kyoto-u.ac.jp.

CREST.

[1] Abbreviations: ETs, endothelins; ETRs, endothelin receptors; ET_AR and ET_BR, endothelin receptor subtypes A and B; G protein, heterotrimeric guanine nucleotide-binding protein; GPCR, G protein-coupled seven transmembrane receptor; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); HSCR, Hirschsprung's disease; PTX, pertussis toxin; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfate; DTT, dithiothreitol.

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EXPERIMENTAL PROCEDURES

Materials. The cyclic-peptide antagonist for ET_BR, RES701-1, was generously provided by Dr. M. Yoshida (Kyowa Hakko Kogyo Co. Ltd.). The cDNAs encoding human ET_AR and ET_BR were kindly provided by Dr. T. Masaki (National Cardiovascular Research Institute) and Dr. Y. Furuichi (AGENE Research Institute), respectively. The cDNAs for α_q and γ_2 were generously supplied by Drs. V. Mancino and M. Simon (California Institute of Technology). The pQE60-based expression plasmids for rat α_{i1} in *Escherichia coli* strain BL21, and the recombinant virus of rat α_{i1} for expression in Sf9 cells, were generously provided by Drs. H. Itoh and Y. Kaziro (Tokyo Institute of Technology). The hybridoma producing the anti-bovine rhodopsin monoclonal antibody 1D4 was from Dr. R. Molday (University of British Columbia).

Construction of the Mutant ETR Gene in the Transfer Plasmids. To achieve a simple purification, the tag-modified ET_AR was used as the wild-type receptor, with six histidines in the N-terminal tail and with the epitope sequence, KTETSQVAPA, for the anti-rhodopsin monoclonal antibody 1D4 at the C-terminus, as described (*20*). The modified ET_AR mimicked the wild-type in its ligand binding and G protein coupling activities. The wild-type ET_BR gene and the modified wild-type ET_AR gene were introduced into the transfer vector pVL1393 (Invitrogen) and were replaced with mutated fragments produced by PCR as described.

Purification of ET_AR and ET_BR. Cell culture, expression of ET_ARs and ET_BRs in Sf9 cells, preparation of Sf9 cell membranes, and purification of the ET_ARs by 1D4-immunoaffinity chromatography were carried out as described (*20*). After the antibody-affinity chromatography, nickel affinity column chromatography was performed for the ET_ARs. For ET_BR, ligand-affinity purification using biotinylated ET-1 (21) gives better purity than 2A5-immunoaffinity purification (unpublished work), while it requires the wild-type stability in a detergent-solubilized solution because of using NaSCN. The wild-type ET_BRs purified either by ligand-affinity or by immunoaffinity chromatography showed similar efficiency to each other in G protein coupling (*21*). Since W275F/ET_BR was expressed well and stable enough, it was purified by ligand-affinity chromatography. Other mutant receptors were purified by immunoaffinity chromatography.

Expression and Purification of G Proteins. The rat α_{i1} subunit expressed in *E. coli* strain BL21, the bovine α_o subunit from bovine brain, and the $\beta_1\gamma_2$ and the $\alpha_q\beta_1\gamma_2$ subunits coexpressed in Sf9 cells were prepared as described (*20*). The concentrations of the $\beta_1\gamma_2$ and $\alpha_q\beta_1\gamma_2$ subunits were calculated from the protein concentrations after purification. The α_{i1} subunit was stored in 50 mM potassium phosphate (pH 8.0) with 10 μM GDP. The α_o, $\beta_1\gamma_2$, and $\alpha_q\beta_1\gamma_2$ subunits were stored in a 0.6% (w/v) Chaps solution [20 mM Tris-HCl, 1 mM DTT, 120 mM potassium phosphate (pH 8.0), and 0.6% Chaps].

Reconstitution of Receptors and G Proteins. Purified receptors (10−1000 pmol), in 100−350 μL of 20 mM Tris-HCl (pH 7.5), 0.1 M NaCl, and 0.1% digitonin, were reconstituted into phospholipid vesicles as described previously (*21*), using an Ultrogel AcA34 gel filtration column. The void fractions without detergent were collected, divided into aliquots, and stored at −70 °C. G proteins, at a 50−100-fold molar excess, were coreconstituted into the isolated receptor vesicles, as described previously (*20*). Then, the phospholipid vesicles were used for ligand-dependent [γ-^{35}S]-GTPγS binding assays. The [γ-^{35}S]GTPγS binding rates in the presence of ET-1 were analyzed according to the apparent first-order kinetics.

Radioligand Binding Assays. Saturation binding assays, competition binding assays, and determinations of the dissociation constant (K_D), the maximum ligand binding sites (B_{max}), and the K_i, were performed using the LIGAND program (Biosoft). The amounts of ETRs contained in the reconstitution mixtures of [γ-^{35}S]GTPγS binding assays were measured by the [^{125}I]ET-1 binding assays as described (*21*).

RESULTS

Construction of the Mutant ETRs. Figure 1 shows the secondary structure models of the human ET_AR and ET_BR. The W257 and W258 residues of ET_AR and the corresponding W275 and W276 residues of ET_BR are located near the extracellular side in the putative transmembrane helix 5, in which the amino acid sequence KDWWLF is well conserved between the subtypes and among species. The two conserved tryptophans were mutated to alanine, phenylalanine, and cysteine (only for W276), as listed in Table 1.

Expression in Sf9 Cells and Ligand-Binding Properties of the Mutant ETRs. The constructed mutants were expressed in Sf9 cells by means of a baculovirus-based system. The cell membranes were examined for their ligand-binding properties. Table 1 summarizes the apparent dissociation constants (K_D) and the maximum binding (B_{max}), calculated from the Scatchard analysis. All of the mutants showed affinities to ET-1 (70−150 pM) similar to those of the wild-type receptors. However, the numbers of binding sites differed. In particular, the binding sites of mutants substituted with alanine or cysteine were largely reduced (mostly less than one-fifth of those of the wild-type). The apparent K_i values for the various ligands are summarized in Table 2. While the W276A/ET_BR and W275A/ET_BR mutants showed wild-type affinity to ET-3, the affinities of the W257A/ET_AR and W258A/ET_AR mutants to ET-3 were reduced to less than one-tenth. The affinity of ET_BR to the agonist BQ788 was affected by the mutation of either W275 or W276, whereas its affinity to the antagonist RES701-1 was affected only by the mutation of W276.

The expressed mutant receptors were analyzed by immunoblotting, as shown in Figure 2. All of the ETRs showed major bands at 40−44 kDa. The ET_ARs displayed smeared multiple bands around 40 kDa, due to glycosylation.

Purification and Reconstitution of ETRs into Phospholipid Vesicles. The ETRs were purified by antibody-affinity column chromatography as described previously, except for W275F/ET_BR, which was purified by ligand-affinity column chromatography. The ET_ARs were further purified by nickel-affinity column chromatography. The purified ETRs were reconstituted into phospholipid vesicles as described in the Experimental Procedures. A silver-stained analysis of the isolated vesicles shows that the ETRs were purified to the majority (Figure 3). However, the purities of the receptors differed, depending on the amounts of expressed proteins and the stabilities of mutant receptors in the solubilized solutions.

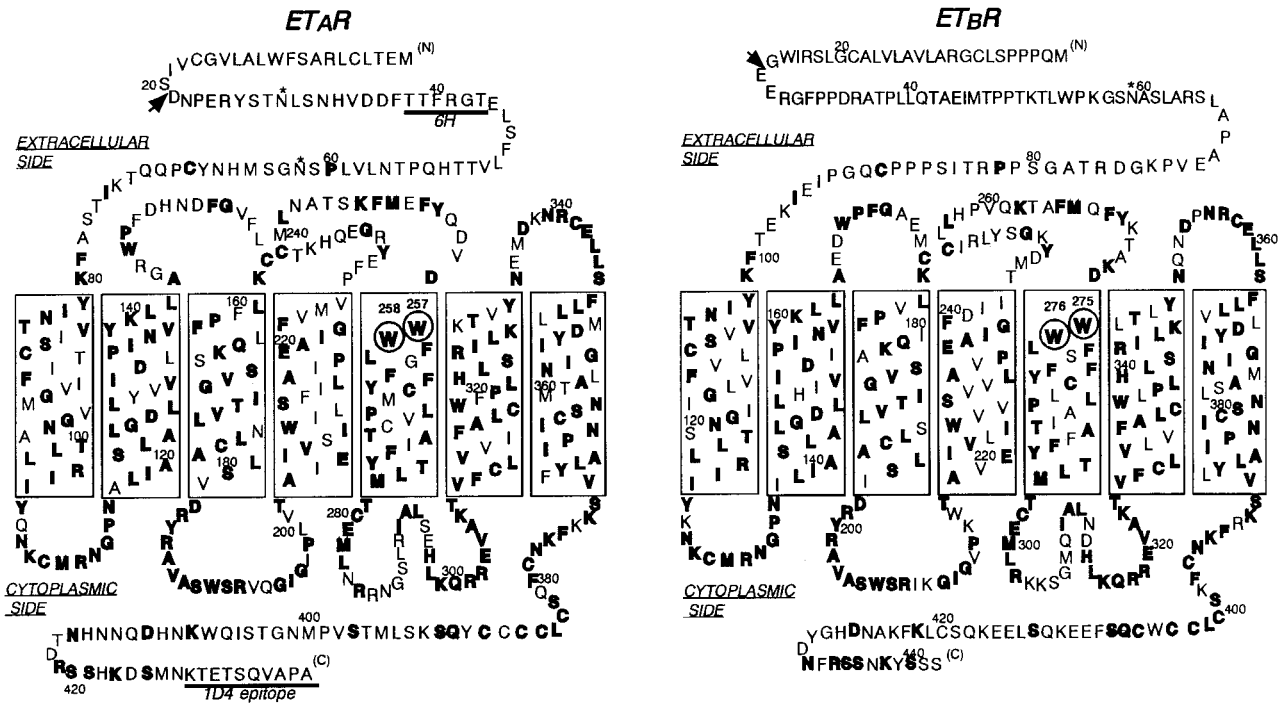

FIGURE 1: (A) Secondary structure models of human ET_AR and ET_BR. The putative seven α helices are boxed. To facilitate the purification, residues 38−43 of ET_AR were replaced with six histidines, and the 1D4 epitope sequence was attached to the C-terminus of ET_AR. Conserved residues in subtypes are shown in bold. The mutated tryptophan residues are marked with circles. The asterisks indicate the potential N-linked glycosylation sites, and the arrows indicate the signal peptidase cleavage sites.

Table 1: Densities and Affinities of the Wild-Type and the Mutant ET_ARs and ET_BRs Expressed in Sf9 Cells[a]

receptor	K_D (pM)	B_{max} (pmol/mg protein)
ET_AR	78 ± 8	25.0 ± 1.4
W257A/ET_AR	138 ± 18	3.4 ± 0.3
W257F/ET_AR	97 ± 11	11.1 ± 0.8
W258A/ET_AR	80 ± 9	5.6 ± 0.4
ET_BR	149 ± 23 (23 ± 2)[b]	86.5 ± 9.1
W275A/ET_BR	97 ± 14	6.6 ± 0.6
W275F/ET_BR	159 ± 14	43.5 ± 2.7
W276C/ET_BR	85 ± 14	11.7 ± 1.1
W276A/ET_BR	98 ± 11	6.4 ± 0.4
W276F/ET_BR	(23 ± 1)[b]	9.7 ± 0.5

[a] Sf9 cell membranes, containing about 5 fmol of ETRs, were used for the examination of binding parameters by a saturation isotherm of [^{125}I]ET-1 binding. Each experiment was done at least two times. [b] Experiments were performed at another period of time.

Table 2: Ligand Affinities for Wild-Type and Mutant ETRs[a]

	agonists K_i (nM)			antagonists K_i (nM)		
receptor	ET-1	ET-3	IRL1620	BQ123	BQ788	RES701-1
ET_AR	0.28	11	nd	16	nd	nd
W257A/ET_AR	0.25	263	nd	21	nd	nd
W258A/ET_AR	0.16	106	nd	25	nd	nd
ET_BR	0.20	0.34	1.7	nd	9.4	53
W275A/ET_BR	0.16	0.18	4.6	nd	152	27
W276A/ET_BR	0.16	0.24	3.4	nd	89	799

[a] Competitive binding experiments between [^{125}I]ET-1 (50 pM) and unlabeled ligands were performed as described in the Experimental Procedures. Sf9 cell membranes containing 15−20 fmol of each receptor were assayed. The K_i values were calculated from the IC_{50} values determined from a nonlinear regression of the competition experiments and are the averages of two to three experiments. The maximum range of the K_i value was ±19% of the mean values. nd, not determined.

Coreconstitution of ETRs with G_i, G_q, or G_o Proteins. The ETRs in the phospholipid vesicles were coreconstituted with purified G proteins to test the coupling efficiencies. Figure 4 shows the ET-1-dependent stimulation of [^{35}S]GTPγS binding of G_i ($\alpha_{i1}\beta_1\gamma_2$), G_q ($\alpha_q\beta_1\gamma_2$), and G_o ($\alpha_o\beta_1\gamma_2$) by the ETRs. The assays in each panel were performed at similar receptor concentrations, indicated in the insets, with identical amounts of G proteins.

The W275F/ET_BR always displayed slightly higher G protein stimulation in the presence of the antagonist, RES701-1, than the wild-type receptor. We observed that the ligand-affinity-purified ET_BR tended to give higher ligand-independent G protein activation. We assume that an artificial receptor-activation occurred in the course of ligand-affinity purification by the use of the chaotropic reagent, NaSCN, as discussed previously (*20*). The W258A/ET_AR mutant exhibited a wild-type ligand-dependent activation with a high background in coupling with G_o, presumably due to the higher content of impurities in the vesicles. Considering these points, a comparison of the coupling efficiencies of the mutant receptors to those of the wild-type receptors is summarized in Table 3.

Mutant ET_ARs. None of the mutant ET_ARs showed significantly different G protein activations as compared to the wild-type; namely, they displayed 0.7−1.7-fold activities of those of the wild-type receptor in the stimulation of [^{35}S]-GTPγS binding of G proteins.

Mutant ET_BRs. Both W276C/ET_BR and W276A/ET_BR showed G_q stimulation reduced to 60 and 30% of the wild-type activity, respectively, whereas they displayed G_i and G_o activations similar to those of the wild-type ET_BR. The W275A/ET_BR exhibited more severe defects, with G_q and G_i activations decreased to about 20 and 60% of the wild-type, respectively. On the other hand, the replacements of W275 and W276 with phenylalanine had no significant effects on G protein coupling. These mutants displayed 0.8−1.4-fold activities as compared with the wild-type.

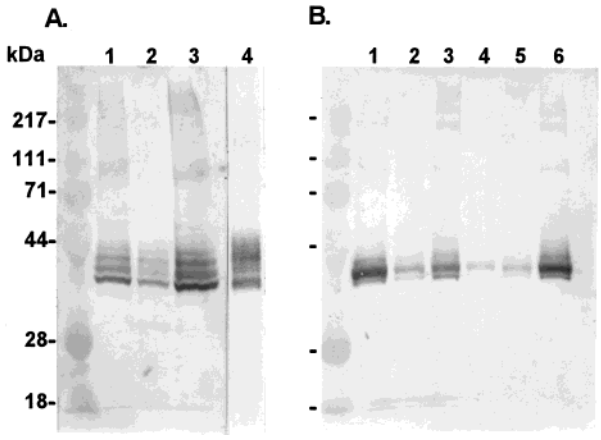

FIGURE 2: Immunoblotting analysis of ET_AR (A) and ET_BR (B) expressed in Sf9 cells. Membranes prepared from Sf9 cells expressing each receptor and containing 30 μg of protein, were resolved by 12.5% SDS−PAGE, transferred to a nitrocellulose membrane, and probed with the 1D4 mAb for the ET_ARs and the 2A5 mAb for the ET_BRs. (A) Lane 1, W258A/ET_AR; lane 2, W257A/ET_AR; lane 3, W257F/ET_AR; lane 4, ET_AR. (B) Lane 1, ET_BR; lane 2, W276C/ET_BR; lane 3, W276A/ET_BR; lane 4, W276F/ET_BR; lane 5, W275A/ET_BR; lane 6, W275F/ET_BR.

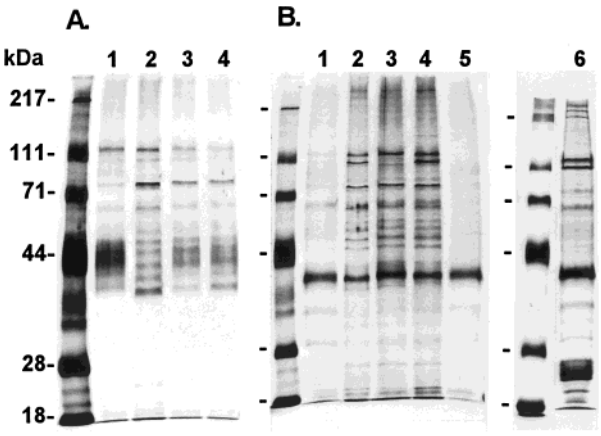

FIGURE 3: The purified ET_ARs (A) and ET_BRs (B) were analyzed by 12.5% SDS−PAGE visualized by silver staining. (A) Lane 1, ET_AR; lane 2, W258A/ET_AR; lane 3, W257A/ET_AR; lane 4, W257F/ET_AR. (B) Lane 1, ET_BR; lane 2, W276C/ET_BR; lane 3, W276A/ET_BR; lane 4, W275A/ET_BR; lane 5, W275F/ET_BR; lane 6, W276F/ET_BR.

The defective mutants, W275A/ET_BR and W276A/ET_BR, were further examined with respect to their efficiencies of G protein activation. Figure 5A shows the effect of the ET-1 concentration on the [^{35}S]GTPγS binding of G_i and G_q reconstituted with the ET_BRs. The K_D values for ET-1 of the wild-type and mutant ET_BRs are similar to each other and are not affected by the presence of G proteins. Therefore, the EC_{50} values of ET-1 in the [^{35}S]GTPγS binding of the G protein reflect the affinities of the ligand-bound forms of the ET_BRs to the G proteins. For G_i activation, W275A/ET_BR, W276A/ET_BR and the wild-type ET_BR exhibited EC_{50} values of 0.8 ± 0.2, 1.2 ± 0.1, and 0.6 ± 0.1 nM, respectively. For G_q activation, they also showed values similar to each other, namely 0.7 ± 0.1, 0.8 ± 0.1, and 0.66 ± 0.04 nM, respectively. Figure 5B shows the effect of the GDP concentration on the [^{35}S]GTPγS binding of G_q. The W276A/ET_BR mutant displayed an IC_{50} value similar to that of the wild-type ET_BR (0.54 ± 0.03 and 0.77 ± 0.05 μM, respectively). Thus, the defective mutant receptors retain the wild-type affinity for G_q and G_i, and W276A/ET_BR is able to form activated G_q, which has a wild-type affinity for GTP.

DISCUSSION

Human ET_AR and ET_BR share approximately 76% homology in the transmembrane and cytoplasmic domains, except for the C-terminal 35 residues. We previously observed subtle differences in the signal transduction of these ETR subtypes, such that ET_AR activated G_q 2−3-fold more efficiently than ET_BR, and ET_BR had a higher affinity for G_i than ET_AR, in the reconstituted system using Sf9-expressed proteins. The purified and reconstituted system allows analyses of the direct interactions of the ETRs and G proteins. Using this system, we studied which parts of the receptor molecule are involved in the conformational change accompanying the binding of a peptide ligand, ET-1, and how such homologous receptors send refined signals.

Many mutations in the ET_BR genes were found to associate with HSCR, a hereditary developmental disorder of the autonomic innervation of the gut, such as C109R, which leads to a translocation defective mutant, W276C and S390R, which lead to mutants defective in intracellular signaling, as well as the premature chain termination mutations at W275, Y293L, and N378I, and so on (*12*−*19*). To obtain insight from these natural mutations into the structural changes that occur in the receptor molecule after binding to ET, we focused on W276 and the neighboring W275 mutations and compared their properties in interactions with G proteins with the corresponding ET_AR mutant receptors, because W276C neither affects the affinity of ET_BR to ET-1 and ET-3 nor is directly involved in G protein interactions (*12*).

The substitution of alanine for W276 and W275 did not affect the affinities of ET_BR for ET-1, ET-3, and IRL1620, whereas the corresponding substitution of alanine for W257 or W258 of the ET_AR reduced the affinity for ET-3, while retaining the wild-type affinity for ET-1. This suggests that the binding pockets for ET-1 and ET-3 in the ET_AR are not identical. Likewise, the affinities for BQ788 of W275A/ET_BR and W276A/ET_BR are decreased to less than one-tenth of the wild-type value, and the affinity for the antagonist, RES701-1, is only affected by the W276A mutation, and not by the W275A mutation. These data suggest that the binding pockets in ET_BR for BQ788, RES701-1, and ETs are different. The presence of two ligand-binding subdomains (one consisting of transmembrane helices 1−3 and 7, and the other consisting of helices 4−6) has been shown within the ETR molecules by using receptor subtype chimeras, and each subdomain displays specificities for the carboxy-terminal message domain and for the amino-terminal address domain of ET, respectively (*22*). The results suggest that the side chains of the two conserved tryptophans in the transmembrane helix 5 of the ETRs are not involved in the direct binding to the address domain of ET-1.

The observation that W276C/ET_BR and W276A/ET_BR are defective only in G_q coupling, but not in G_i or G_o coupling, suggests impaired signal transduction via the G_q pathway. When W276C/ET_BR was expressed in CHO cells, reduced Ca^{2+} responses were observed (*12*). It is not clear from our studies whether the impaired G_q activation or the low level

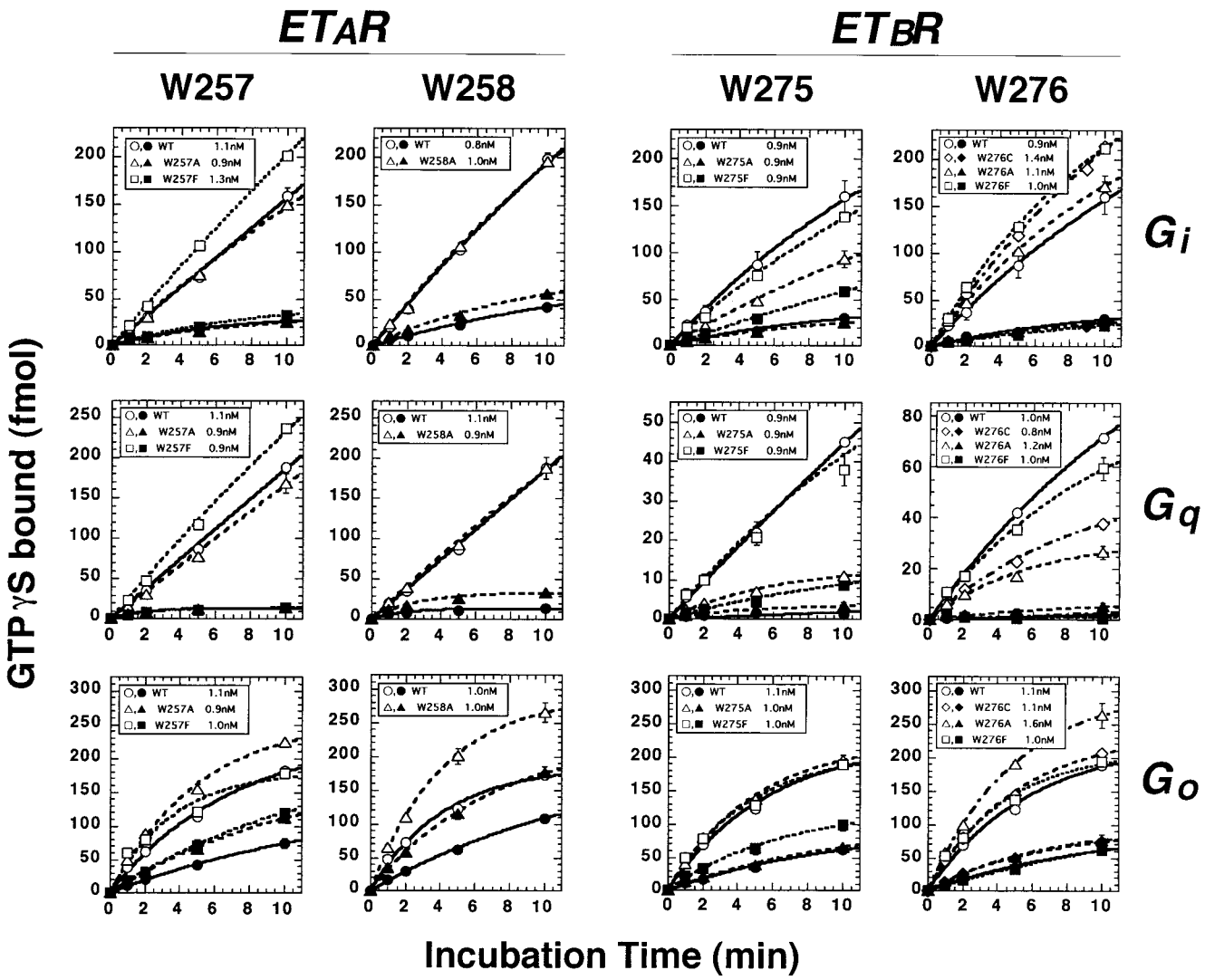

FIGURE 4: ET-1-dependent stimulation of [^{35}S]GTPγS binding to G_i, G_q, and G_o by the wild-type and mutant ETRs. The receptors and the G proteins in the reconstituted vesicles, prepared as described in the Experimental Procedures, were incubated with 5 nM [^{35}S]GTPγS at 30 °C for G_i and G_q, and at 25 °C for G_o. The open symbols represent assays in the presence of 1.0 μM ET-1, and the closed symbols represent assays in the absence of ET-1 (for ET_AR) or in the presence of 10 μM RES701-1 (for ET_BR). GDP, at a concentration of 1.0 μM, was added to the assays for G_i and G_o. The amounts of the receptors in the isolated phospholipid vesicles were measured by [^{125}I]ET-1 binding, and the amounts of the α subunit added to the coreconstitution mixtures were measured by [^{35}S]GTPγS binding as described (*21*). The concentrations of receptors in the vesicles are shown in the table of each panel. The following amounts of G proteins were contained in the 20 μL aliquots of the respective coreconstitution mixtures: in the assays with G_i, 1900 fmol of α_{i1} (95 nM); in the assays with G_o, 2100 fmol of α_o (105 nM). Together with each α subunit, 1600 fmol of $\beta\gamma$ subunits was added. In assays of the ET_AR mutants and the W275-ET_BR mutants with G_q, approximately 740 fmol of G_q ($\alpha_q\beta_1\gamma_2$) (~37 nM) was contained, as calculated from the amounts of protein added. For the assays of the W276-ET_BR mutants, approximately 1260 fmol of G_q (~63 nM) was used. Data are the means of duplicate or triplicate determinations.

of expression of the mutant receptor is related to HSCR. However, considering the expression of W276C/ET_BR at the wild-type level in CHO cells (*12*), the impaired signaling via the G_q pathway may be more significantly related to HSCR.

The other HSCR susceptibility genes are the ET-3 gene and the RET proto-oncogene, which encodes a growth factor receptor tyrosine kinase that is expressed mainly in neural crest-derived cells and is known to activate mitogen-activating protein (MAP) kinase cascades (*23*−*26*). The knockout of the ET-3 gene in mice or mutations in the RET gene also lead to defects similar to HSCR, such as aganglionic megacolon or pigmentary disorder (*10*, *11*). The ET_BR mediates mitogenic signals in part via MAP kinase activation (*6*, *7*). Although the mechanisms of MAP kinase activation by G protein-coupled receptors (GPCRs) are not clear, GPCRs and protein tyrosine kinases appear to use many of the same signal transduction proteins to activate MAP kinase cascades (*27*, *28*). At the developmental stage of neural crest-cell migration, the ET_BR-mediated G_q activation may converge with a pathway mediated by the tyrosine-phosphorylated RET protein.

The W276A, W276C/ET_BR, and W275A/ET_BR mutants retain the wild-type affinity for ET-1 and ET-3, whereas they fail to activate either G_q or G_i with wild-type efficiency. These results suggest that W275 and W276 play important roles in the formation of the activated structure of the ET_BR, and that the large aromatic side chains are required to interact properly with the neighboring residues. However, these residues are not essential for activating G proteins. In the cases of bovine rhodopsin and the β_2-adrenergic receptor, movements of the transmembrane helices 3 and 6 during

Table 3: GTPγS Binding of G Proteins Stimulated by the Mutant ETRs, as Compared to Those of the Wild-Type ETRs[a]

receptor	G_i	G_q	G_o
ET_AR	1.0	1.0	1.0
W257A/ET_AR	1.3	1.1	1.5
W257F/ET_AR	1.2	1.7	0.9
W258A/ET_AR	0.7	1.1	1.3
ET_BR	1.0	1.0	1.0
W275A/ET_BR	**0.4 (↓)**	**0.2 (↓)**	1.2
W275F/ET_BR	0.8[b]	0.9[b]	1.1[b]
W276C/ET_BR	0.9	**0.6 (↓)**	1.1
W276A/ET_BR	1.0	**0.3 (↓)**	1.0
W276F/ET_BR	1.4	0.9	1.3

[a] The ETR-stimulated GTPγS binding values at 5 min presented in Figure 4 were calculated by nonlinear least-squares fitting to the first-order rate equation and were normalized by dividing by the receptor concentrations. The values obtained in the absence of ET-1 or the presence of RES701-1 were subtracted as backgrounds from the values obtained in the presence of ET-1. The subtracted values are expressed relative to that of the wild-type as 1.0. Downward arrows indicate significantly lowered coupling efficiencies by the mutant receptors, as compared to those of the wild-type. [b] Because of the artificial activation, each value obtained from the assays of ET_BR in the presence of RES701-1 was subtracted as background.

receptor activation have been reported using spectroscopic techniques (*29*−*33*). In addition, studies of chimeric luteinizing hormone/follicle-stimulating hormone receptors suggested the importance of the interaction of transmembrane helices 5 and 6 in stabilizing the inactive structure (*34*). Although we do not know the structural movements of ET_BR during the course of ligand binding, by analogy, transmembrane helix 5, particularly the region near the extracellular side including W275 and W276, may play roles in supporting the movements of transmembrane helices 3 and 6 or in stabilizing the activated structure of ET_BR. We previously reported that RES701-1, an antagonist for ET_BR, functioned as an inverse agonist to stabilize ET_BR, activated by a denaturing reagent, in an inactive form (*20*). The result that the W276A/ET_BR mutant showed only one-tenth of the affinity for RES701-1 also suggests that W276 has a structural role in the course of ET_BR activation.

However, it is not clear why only G_q coupling not G_i/G_o coupling is affected by the W276 mutation. It may suggest that the W276 mutation causes a local structural defect which only affects interaction with G_q or that more strict receptor structure is required for optimum G_q activation. The D79N mutation of α_{2A}-adrenergic receptor has been reported to exhibit a selective defect in coupling to potassium current but not in coupling to calcium current in AtT20 mouse pituitary cells (*35*). The R332Q mutation of prostaglandin E receptor EP3D also caused a selective defect in G_s coupling but not in G_i coupling in CHO cells (*36*). Compared to these mutated residues, which are very critical for ligand binding, located in the middle of the transmembrane helices, it is of interest that the W276 residue of ET_BR located in the transmembrane region very near the extracellular side in the secondary structure model affects the structure of the cytoplasmic region that interacts with G proteins. At least, these results suggest that the required receptor structure to perform optimum activation is different for respective G proteins. Elucidation of the three-dimensional structure of ET_BR should facilitate further understanding of receptor-G protein interaction.

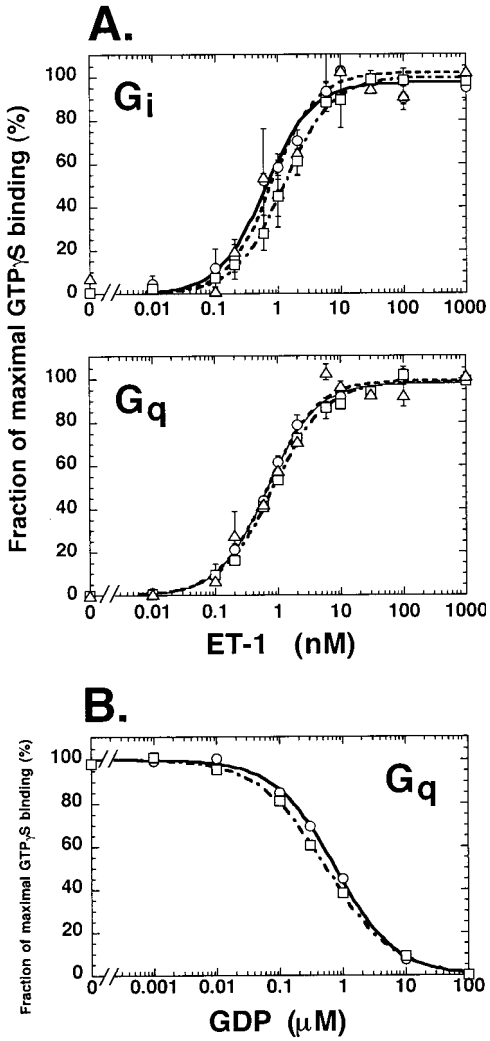

FIGURE 5: Effect of ET-1 (A) and GDP (B) concentrations on the [^{35}S]GTPγS binding of G_i and G_q to the ET_BRs. (A) Each sample was incubated for 10 min at 30 °C. Data are the means of duplicate determinations. Each 20 μL aliquot contained 1600 fmol of α_{i1}, 1600 fmol of βγ subunits, and either 9.3 fmol of ET_BR (○), 13.7 fmol of W275A/ET_BR (△), or 14.4 fmol of W276A/ET_BR (□) in the upper panel, and 1184 fmol of G_q and either 18.8 fmol of ET_BR (○), 18.2 fmol of W275A/ET_BR (△), or 24.0 fmol of W276A/ET_BR (□) in the lower panel. (B) Each sample was incubated for 10 min at 30 °C. Data are the means of duplicate determinations. Each 20 μL aliquot contained 1600 fmol of G_q and either 11.8 fmol of ET_BR (○) or 15.9 fmol of W276A/ET_BR (□).

While the W276A, W276C/ET_BR, and W275A/ET_BR mutants are defective in coupling with G_q or G_i, they showed wild-type affinity for G_q and G_i (Figure 5A), and the W276A/ET_BR-activated G_q exhibited wild-type affinity for GTP as well (Figure 5B). Activation of G proteins by ligand-activated GPCRs involves the following four steps: (i) binding of a G protein (αβγ subunits) to an activated receptor, (ii) structural changes in the G protein (mainly in the α subunit) leading to the release of GDP from the α subunit, (iii) binding of GTP to the α subunit, and (iv) release of the α subunit and the βγ subunits from the receptor. The results suggest that the defects observed in G protein coupling of mutant receptors are due to inefficiencies in either step ii or iv.

In contrast with the ET_BR mutants, the corresponding ET_AR mutants, W257A/ET_AR and W258A/ET_AR, displayed wild-type properties in ET-1 binding and in G protein coupling. Since ET_AR displays a clear specificity for the

address domain of ET, the binding subdomain formed by transmembrane helices 4, 5, and 6 of ET_AR may have a more stable activated structure, which might be a reason for the wild-type efficiency in G_q coupling of the ET_AR mutants. This property could also be related to the higher efficiency of G_q activation by ET_AR as compared to ET_BR. Thus, although ET_AR and ET_BR are highly homologous to each other, and W275, W276 (W257 and W258 in ET_AR), and the surrounding sequences are conserved, these residues are endowed with different roles in the conformational change process and, therefore, in G protein activation as well. Since the binding pockets for ET-1 are formed differently in ET_AR and in ET_BR, the structures of the ET-1-bound receptors could differ. Such variety may contribute further to the different recognition of G proteins by the receptors.

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