Decreased responsiveness of naturally occurring mutants of human estrogen receptor α to estrogens and antiestrogens

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Abstract

Estrogen receptor α (ERα) is a ligand-inducible transcription factor that mediates the biological effects of estrogens and antiestrogens. Many point mutations in the human ERα gene have been reported to be associated with breast cancer, endometrial cancer, and psychiatric diseases. However, functional analyses for most mutants with amino acid changes are still lacking. In the present study, to investigate the effects of point mutations on the function, gel-shift assays and luciferase assays were performed for eight kinds of mutated ERα proteins, including a single nucleotide change of C207G (N69K), G478T (G160C), T887C (L296P), A908G (K303R), C926T (S309F), A1058T (E353V), A1186G (M396V) and G1231deletion (411fsX7). The mutated ERα expression plasmids were constructed by site-directed mutagenesis. With gel-shift assays using *in vitro* translated ERα proteins, binding to the consensus estrogen response element (ERE) was observed for the mutated ERα proteins except ERα (G160C) and ERα (411fsX7), the binding of which was comparable with that of the wild type. Western blot analyses showed that ERα (G160C) could not be efficiently translated with the *in vitro* transcription/translation system and that ERα (411fsX7) produced a truncated protein. To investigate the transactivation potency, wild-type or mutated ERα expression plasmids were cotransfected with pGL3-3EREc38 reporter plasmid into human breast adenocarcinoma MDA-MB-435 cells. The concentration-response curves (10 pM - 100 nM E2) of the mutant ERα proteins except ERα (E353V) and ERα (411fsX7) were similar to that of wild-type ERα. However, at a low level of E2 (100 pM), the mutants ERα (N69K), ERα (L296P), ERα (S309F), and ERα (M396V) showed a significant decrease of transactivation compared with that of the wild-type ERα. The mutants ERα (E353V) and ERα (411fsX7) did not show responsiveness to E2 and antiestrogens, 4-hydroxytamoxifen (4OHT) and ICI 182,780. The mutant ERα (S309F) showed decreased responsiveness for the antiestrogenicity of 4OHT. In conclusion, we found that some of the naturally occurring human ERα mutants with amino acid changes may have an altered responsiveness to estrogen and antiestrogens.

Keywords: Estrogen receptor; mutation; transcriptional activity; estradiol; antiestrogens

1. Introduction

Estrogens play a crucial role in female sexual development and in the regulation of the menstrual cycle, have important effects on prostatic hyperplasia, protective effects on the cardiovascular system, and influence lipid and bone metabolism [1]. In addition, estrogens are involved in the growth and development of both uterine and mammary cancers [2,3]. The effects of estrogens are mediated primarily via specific nuclear receptors, estrogen receptors (ERs), which function as ligand-inducible transcription factors [4]. ER is expressed in two thirds of breast cancers and endometrial cancers, and is used as a guide for hormonal therapy and for the prognosis [5,6].

Two ER subtypes, ERα and ERβ, encoded by different genes, have been isolated in mammals [7,8]. Most of the biological actions exerted by estrogens are mediated by ERα [9]. Upon binding estrogens, the receptor dimerizes and binds to the estrogen response element (ERE) located in the 5'-flanking region of estrogen-responsive genes. The ligand-activated ERα interacts with transcription factors and other components of the transcription complex to modulate gene expression [10]. ERα, like other members of the nuclear receptor superfamily, contains six functional domains designated A to F [11]. The amino-terminal A/B region of the receptor exhibits a hormone-independent transactivation function (Fig. 1). The C region is principally involved in receptor-DNA interaction. The D region contains a part of the ligand-dependent, transactivation domain AF-2a and a portion of the nuclear localization signal. The carboxyl-terminal domains (E/F) are structurally and functionally complex and contain hormone binding, dimerization, and hormone-dependent transactivation functions [12,13].

A number of point mutations of ERα have been identified in patients with a variety of disease states, including breast cancer, endometrial cancer, and psychiatric diseases [14]. Conclusive studies to determine whether they are somatic mutations or genetic polymorphisms have been limited. Furthermore, their correlations with the clinical features have not been completely determined. It should be noted that some of these are silent mutations or polymorphisms that do not affect the protein sequence, despite their association

with disease states. Regarding mutations that cause amino acid changes, a few studies have sought to characterize the ERα mutants [14]. In these studies, the assessed mutations and the experimental methods used were different. Furthermore, functional analyses for most of the ERα mutants still remain to be performed. To address these issues, we sought to examine the effects of the mutations on the function of ERα with a unified method for multiple mutants. The present study focused on eight mutations existing in various domains, including C207G (N69K) [15], G478T (G160C) [16-18], T887C (L296P) [19], A908G (K303R) [18,20], C926T (S309F) [17,18,21], A1058T (E353V) [22], A1186G (M396V) [15] and G1231deletion (411fsX7; frame-shift at codon 411 resulting in the termination at codon 418) [23]. Gel-shift assays and luciferase assays were performed to investigate the binding affinity to DNA, the transcriptional activity and the responsiveness to agonists or antagonists of these ERα mutants.

2. Materials and Methods

2. 1. Chemicals and reagents

Taq DNA polymerase was obtained from Greiner Japan (Tokyo, Japan). Restriction enzymes were purchased from Takara (Kyoto, Japan), Nippon Gene (Tokyo, Japan), or New England Biolabs (Beverly, MA). Primers were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). 17β-Estradiol (E2) and 4-hydroxytamoxifen (4OHT) were obtained from Sigma-Aldrich (St. Louis, MO). 7α-[9 (4,4,5,5,5-Pentafluoropentylsulfonyl)nonyl] estra-1,3,5(10)-triene-3,17β-diol (ICI 182,780) was purchased from Tocris Cookson (Bullsin, MO). phRL-TK plasmid and a dual-luciferase reporter assay system were from Promega (Madison, WI). Lipofectamine transfection reagent was from Invitrogen (Melbourne, Australia). A pGL3 plasmid containing three copies of EREc38 (pGL3-3EREc38) was a generous gift from Dr. Carolyn M. Klinge (University of Louisville School of Medicine, Louisville, KY). The wild-type human ERα expression vector (pSG5-HE0), constructed previously [24,25], was a gift from Dr. Pierre Chambon (Institut de

genetique et de Biologie Moleculaire et Cellulaire, Strasbourg, France). [γ-^{32}P]ATP was from Amersham (Buckinghamshire, United Kingdom). Mouse anti-human ERα monoclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). All of the other chemicals and solvents were of the highest grade commercially available.

2.2. Construction of expression plasmids of mutated ERα by site-directed mutagenesis

To construct the expression vectors of mutated ERα, a single nucleotide change was inserted into the wild-type ERα expression vector by site-directed mutagenesis with a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The forward and reverse mutagenic primers were as follows:

C207G-S: 5'-CCG CCG CCA A**G**G CGC AGG TCT ACG GTC AGA C-3'

C207G-AS: 5'-GTC TGA CCG TAG ACC TGC GC**C** TTG GCG GCG G-3'

G478T-S: 5'-GAT AAT CGA CGC CAG **T**GT GGC AGA GAA AGA TTG GCC-3'

G478T-AS: 5'-GGC CAA TCT TTC TCT GCC AC**A** CTG GCG TCG ATT ATC-3'

T887C-S: 5'-CTT TGG CCA AGC CCG C**C**C ATG ATC AAA CGC TCT AAG-3'

T887C-AS: 5'-CTT AGA GCG TTT GAT CAT G**G**G CGG GCT TGG CCA AAG-3'

A908G-S: 5'-CAA ACG CTC TAA GA**G** GAA CAG CCT GGC CTT GTC CC-3'

A908G-AS: 5'-GGG ACA AGG CCA GGC TGT TC**C** TCT TAG AGC GTT TG-3'

C926T-S: 5'-GAA CAG CCT GGC CTT GT**T** CCT GAC GGC CG-3'

C926T-AS: 5'-CGG CCG TCA GG**A** ACA AGG TTA GGC TGT TC-3'

A1058T-S: 5'-CAA CCT GGC AGA CAG GG**T** GCT GGT TCA CAT G-3'

A1058T-AS: 5'-CAT GTG AAC CAG C**A**C CCT GTC TGC CAG GTT G-3'

A1186G-S: 5'-GTC TGG CGC TCC **G**TG GAG CAC CCA GTG AAG CTA C-3'

A1186G-AS: 5'-GTA GCT TCA CTG GGT GCT CCA **C**GG AGC GCC AGA C-3'

G1231del-S: 5'-GCT CCT AAC TTG CTC TT**G A**CA GGA ACC AGG G-3'

G1231del-AS: 5'-CCC TGG TTC CTG **TC**A AGA GCA AGT TAG GAG C-3'

Mutated sites are indicated by underlined bold letters. Nucleotide sequences were confirmed by DNA sequencing analyses.

2.3. Gel shift assay

The oligonucleotide for the consensus ERE (5'-GTC CAA AGT CAG GTC ACA GTG ACC TGA TCA AAG TT-3') was from the *Xenopus* vitellogenin A2 gene [26]. The oligonucleotide was labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase (Toyobo). The wild-type and mutated ERα proteins were synthesized using TNT quick coupled transcription/translation systems (Promega). The reaction mixture contained 5 μL of the *in vitro* translated ERα protein, 2 μg of poly(dI-dC), 1 μg of salmon sperm DNA, and 30 fmol of the radiolabeled probe (~50,000 cpm) in a final volume of 15 μL of binding buffer [25 mM HEPES-KOH (pH 7.9), 0.5 mM EDTA, 50 mM KCl, 10% glycerol, 0.5 mM DTT, and 0.5 mM (*p*-amidinophenyl) methanesulfonyl fluoride]. Binding reactions were performed on ice for 30 min. To determine the specificity of the binding to the oligonucleotides, competition experiments were conducted by coincubation with 50- and 200-fold excesses of unlabeled competitors. In super-shift assays, 2 μg of anti-ERα monoclonal antibodies were preincubated with the *in vitro* translated ERα protein on ice for 20 min. The DNA-protein complexes were separated under nondenaturing conditions on 4% polyacrylamide gels with 0.5 X Tris-borate EDTA as the running buffer. The gels were dried, and then the DNA-protein complexes were detected and quantified with a Fuji Bio-Imaging Analyzer BAS 1000 (Fuji Film, Tokyo, Japan).

2.4. SDS-PAGE and Western blot analyses of in vitro translated wild-type or mutated ERα proteins

SDS-polyacrylamide gel electrophoresis and Western blot analysis for the *in vitro* translated ERα proteins were performed according to Laemmli [27]. The *in vitro* transcription/translation mixtures including the wild-type or mutated ERα expression plasmids (4 μl) were separated on 15% polyacrylamide gel and transferred electrophoretically to a nitrocellulose membrane. A mixture including an empty pSG5 vector was applied as a negative control. Mouse anti-human ERα antibody was used. Biotinylated anti-mouse IgG

and a VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA) were used for diaminobenzidine staining.

2.5. Cell culture, transfection, and luciferase assay

The human breast adenocarcinoma cell line MDA-MB-435, which is ER-negative, was obtained from American Type Culture Collection (Rockville, MD). The cells were cultured in DMEM (Nissui Pharmaceutical, Ibaraki, Japan) supplemented with 10% (v/v) fetal bovine serum (Invitrogen). These cells were maintained at 37°C under an atmosphere of 5% CO_2-95% air. A transient expression system using the luciferase reporter gene was used to characterize the function of the mutated ERα proteins. The cells (2×10^5) were seeded into 24-well plates and then incubated for 24 hr. The medium was replaced with a phenol red-free DMEM medium (Invitrogen) containing 10% dextran-coated charcoal-treated fetal bovine serum. After 24 hr, the cells were cotransfected with 450 ng of pGL3-3EREc38 plasmid, 50 ng of control reporter plasmid (phRL-TK), and 500 ng of each ERα expression plasmid using Lipofectamine transfection reagent. After 24 h of transfection, the medium was replaced with medium containing 0.1% (v/v) DMSO or various concentrations (10 pM - 100 nM) of E2. For the inhibition studies, the transfected cells were incubated with 100 nM of 4OHT or ICI 182,780 in the absence or presence of 10 nM of E2. After 24 h of treatment with the agonist or antagonist, the cells were resuspended in passive lysis buffer, and then the luciferase activity was measured with a luminometer (ARVO MX, PerkinElmer, Osaka, Japan) using the dual-luciferase reporter assay system. The relative luciferase activities were normalized with the *Renilla* luciferase activities. To confirm the reproducibility of the luciferase assays, three independent experiments were performed for each assay.

2.6. Statistical analyses

Data are expressed as mean ± SD of triplicate determinations. Statistical significance was determined by analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Binding of mutated ERα proteins to consensus ERE

To examine the binding potency of the mutated ERα proteins to the consensus ERE, gel shift assays were performed. *In vitro* translated wild-type or mutated ERα proteins were incubated with a ^{32}P-labeled consensus ERE. Wild-type ERα showed a clear shifted band, which was super-shifted with the anti-ERα antibody (Fig. 2A). No super-shifted band was observed with normal mouse IgG (data not shown). The binding was competed by excess amounts of unlabeled consensus ERE. When the mutants ERα (N69K), ERα (L296P), ERα (K303R), ERα (S309F), ERα (E353V) and ERα (M396V) were added, a shifted band of similar intensity as the wild type was observed. The super-shifted band with the anti-ERα antibody and competition with excess amounts of unlabeled consensus ERE were also similar to those of the wild type. In contrast, the mutant ERα (G160C) faintly bound to ERE, and the intensity of the super-shifted band was weaker than that of the wild type. The mutant ERα (411fsX7) was moderately bound to ERE.

To investigate the cause of the faint bands with ERα (G160C) and ERα (411fsX7), Western blot analyses of the *in vitro* translated ERα proteins were performed. The *in vitro* transcription/translation mixture that included the wild-type ERα showed a distinct band with 66 kDa (Fig. 2B). The band corresponded to the full-length ERα protein. No band was observed with the *in vitro* transcription/translation mixture that included the empty pSG5 vector (mock). The *in vitro* transcription/translation mixtures that included the mutants except ERα (411fsX7) showed a band with the same mobility as that of the wild type. However, the band density for ERα (G160C) was prominently attenuated. The *in vitro* transcription/translation mixture that included ERα (411fsX7) showed a band with 46 kDa, which corresponded to a truncated protein with 417 amino acids.

3.2. E2-dependent transactivation potency of mutated ERα proteins

To investigate the transactivation potency of the mutated ERα proteins, luciferase assays were performed. The responsiveness to E2 was assessed at concentrations ranging from 10 pM to 100 nM. In wild-type ERα, a significant increase in the transactivation was observed at 100 pM E2, with maximal transactivation (ca. 10-fold) at 100 nM E2. The concentration-response curves of the mutant ERα proteins except ERα (E353V) and ERα (411fsX7) were similar to that of wild-type ERα. ERα (E353V) and ERα (411fsX7) did not show transactivation at any concentration of E2. At 100 pM E2, the mutants ERα (N69K), ERα (L296P), ERα (S309F), and ERα (M396V) showed significant decreases of transactivation compared with the wild-type ERα. With RT-PCR analyses, it was confirmed that the expression levels of ERα mRNA in the cells transfected with mutated ERα expression plasmids were almost the same as that of the wild type (data not shown).

3.3. Responsiveness to 4OHT and ICI 182,780 of mutated ERα proteins

We investigated the responsiveness of the mutated ERα proteins to antiestrogens, 4OHT and ICI 182,780. The cells transfected with the wild-type or mutated ERα expression vector were treated with 100 nM of 4OHT or ICI 182,780 in the presence of 10 nM E2. In the wild-type ERα, the E2-dependent transactivation was markedly suppressed by 4OHT and ICI 182,780 (Fig. 4). The responsiveness of the mutants ERα (N69K), ERα (G160C), ERα (L296P), ERα (K303R) and ERα (M396V) was almost the same as that of the wild-type ERα. In contrast, the mutant ERα (S309F) showed significantly ($P < 0.05$) lower antiestrogenicity of 4OHT than did the wild-type ERα. The antiestrogenicity of 4OHT and ICI 182,780 was not determined for the mutants ERα (E353V) and ERα (411fsX7), since these mutants were inactive with E2.

The antiestrogens 4OHT and ICI 182,780 sometimes act as an ER agonist [28]. To examine the responsiveness of the mutated ERα proteins to the estrogenicity of 4OHT and ICI 182,780, the transcriptional activities were assessed in the absence of E2. In the wild-type ERα, the transcriptional activity was about 2-fold increased by 4OHT. A similar activation by 4OHT was observed with the mutated ERα (N69K), ERα (G160C), ERα (L296L), ERα

(K303R), ERα (S309F) and ERα (M396V). However, the transactivation was not observed with ERα (E353V) and ERα (411fsX7). Concerning ICI 182,780, no estrogenicity was observed for the wild-type and mutated ERα proteins.

4. Discussion

We characterized the functional changes in human ERα mutants with amino acid changes of a single nucleotide substitution. In the present study, we investigated eight mutants in which the amino acid changes of N69K and G160C are located in the AF-1 region; L296P, K303R, and S309F are in the region responsible for AF-2a and nuclear localization; E353V, M396A, and 411fsX7 are in the AF-2 region. Our findings provide evidence that some of these mutations decrease the responsiveness to estrogens or antiestrogens.

ERα (G160C) did not obviously bind to ERE by gel-shift analyses, although the mutation is not located in the C region. In contrast, with the luciferase assays, the mutant demonstrated significant transactivation by E2, which was comparable with that of the wild-type ERα. A likely explanation for these observations is that ERα (G160C) protein could not be efficiently translated under the *in vitro* transcription/translation system. Western blot analyses were able to prove this (Fig. 2B). Andersen et al [16] considered that the amino acid substitution of G160C might theoretically change the conformation of the receptor by creating disulfide bridges with other cysteins in the protein. However, no dramatic change in the function of ERα (G160C) was observed in the present study. The ERα (G160C) decreased the responsiveness to E2 only at a low concentration.

Although mutated ERα (E353V) showed DNA-binding activity that was similar to that of the wild-type ERα (Fig. 2), no transactivation by E2 was observed at all (Fig. 3), indicating that glutamic acid at 353 has a critical role in the E2-dependent transcriptional activity. This observation is in accordance with a previous report of Brzozowski et al [29] who determined the structure of the ligand-binding domain and E2 complex and suggested that glutamic acid at 353 makes direct hydrogen bonds with E2. In addition, our study revealed that this mutant

also did not respond to 4OHT.

The G1231deletion causes a frame-shift at codon 411 which results in an early stop codon at 418. Conceivably, an immature ERα protein lacking over half of its ligand-binding domain is produced. The production of the truncated ERα (46 kDa) was confirmed by Western blot analyses of the *in vitro* translated protein. Interestingly, the gel-shift assays showed a shifted band with ERα (411fsX7) with a similar mobility as that of the wild type, indicating that the lack of the carboxy-terminal domain from codon 417 did not affect the DNA binding of ERα. A change in the size or molecular weight in the truncated protein might not be detected with the gel-shift assay. Although mutant ERα (411fsX7) has the DNA binding potency, E2-dependent transactivation was not observed because of the lack of E2 binding.

Fuqua et al [30] reported that mutant ERα (K303R) showed increased sensitivity to estrogen as compared with that of the wild-type ERα in stably transfected MCF-7 cells, which showed increased proliferation at subphysiological levels of estrogen. In contrast, normal responsiveness of the mutant to estrogens was observed with the luciferase assay in MDA-MB-435 cells in the present study. With the luciferase assay, in MCF-7 cells both wild-type and mutant ERα (K303R) enhanced the E2-dependent transactivation (data not shown), but the effects of the mutant were not higher than that of the wild-type ERα, indicating that the discrepancy might not be due to differences in the cell lines. Considering the cell system, it might be difficult to evaluate the ERα responsiveness with the cell proliferation response, because the change of the proliferation might result from the modulation of some factors besides ERα transactivation [31]. Fuqua et al [30] reported that mutant ERα exhibited estrogen binding affinity that was similar to that of wild-type ERα but increased binding to the coactivator transcriptional intermediary factor 2 (TIF2), which may partially explain its increased estrogen responsiveness. It should be noted that the evaluation of the biological effects of ERα mutants might not be easy in MCF-7 cells, since endogenous ERα is simultaneously expressed. In the present study, we directly investigated the binding to DNA and the transcriptional activity of mutant ERα, and found that ERα (K303R) showed normal responsiveness to estrogens and antiestrogens.

4OHT and ICI 182,780 are known as a partial antagonist and a pure antagonist of ERα, respectively [32]. These compounds, especially 4OHT, sometimes work as agonists of ERα [28]. The mechanisms of the antiestrogenic action of 4OHT and ICI 182,780 are different. 4OHT competes with E2 for ERα, whereas ICI 182,780 affects the stability and inhibits the dimerization of ERα [33,34]. In this study, the suppression of E2-induced transcriptional activity by 4OHT and ICI 182,780 was observed in the wild-type ERα and the mutant ERα proteins except ERα (E353V) and ERα (411fsX7). Since there was no significant difference in the antiestrogenicity of ICI 182,780 for the wild-type and mutated ERα, these mutations may not be involved in the effects of ICI 182,780. In ERα (S309F), although the percentage of suppression by ICI 182,780 was similar to that of the wild type, the percentage of suppression by 4OHT was significantly decreased compared to that of the wild-type ERα. However, the transactivation potencies of ERα (S309F) by E2 or 4OHT were almost equal to those of the wild-type ERα. Previously, it was considered that the substitution of an uncharged polar residue serine for a nonpolar residue phenylalanine may change the polarity of the protein and alter its conformation [17]. Our results clarified that the amino acid change of S309F may have an impact only on the antiestrogenicity of 4OHT. This mutant would contribute to the resistance to antiestrogens in hormonal therapy.

Previously, it was reported that some naturally occurring mutations, such as D351Y, K303R, and Y537N, enhance the responsiveness to estrogens or antiestrogens. The D351Y mutation increases the estrogenicity of 4OHT [35-37]. As discussed above, K303R showed increased sensitivity to E2 [30]. In the absence of estrogens, the Y537N mutation exhibited a higher transactivation level compared with the wild type [38]. Since hyper-responsiveness of ER is one of the risk factors for breast cancer, the finding of these mutants in breast cancer tissues is reasonable. In contrast, the mutations investigated in the present study did not reveal hyper-responsiveness to estrogens. However, these mutants may modulate the responsiveness to estrogen and antiestrogens.

In summary, we found that some ERα mutations decrease the responsiveness to estrogens and antiestrogens. The functional analyses of the mutations as in this study will help to

determine whether they indeed play a role in the progression of breast cancer or endometrial cancer and/or resistance to hormonal therapy.

Acknowledgements

This work was supported in part by Research on Advanced Medical Technology, Health and Labor Science Research Grants from the Ministry of Health, Labor and Welfare of Japan. We are grateful to Dr. Pierre Chambon of Institut de Genetique et de Biologie Moleculaire et Cellulaire (Strasbourg, France) for the gift of wild-type ERα expression vectors. We acknowledge Mr. Brent Bell for reviewing the manuscript.

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Figure legends

Fig. 1. Schematic representation of the human ERα cDNA. Exons are numbered in the corresponding blocked region. The ATG start codon and the TAG stop codon are shown above. ERα protein is divided into structural and functional domains A-F. Region A/B is implicated in the transactivating function (AF-1). Region C encodes the DNA-binding domain. Region D contains a part of the ligand-dependent transactivation domain AF-2a and a portion of the nuclear localization signal. Region E/F contains the ligand binding domain and the ligand-dependent transactivation domain (AF-2). The locations of the mutations investigated in this study are indicated with vertical arrows. Numbers indicate the nucleotide position on cDNA when the A in the initiation codon is nucleotide 1. The amino acid changes are represented in parentheses.

Fig. 2. **A.** Gel-shift assays of wild-type or mutated ERα for binding to ERE. Wild-type or mutated ERα was translated *in vitro.* Radiolabeled oligonucleotide of cERE from the *Xenopus* vitellogenin A2 gene was used as a probe. Cold oligonucleotides were used as a competitor at 50- and 200-fold molar excess. For super shift analysis, 2 μg of anti-ERα antibodies were pre-incubated with ERα proteins on ice for 20 min. The lower arrow indicates the position of the ERα dependent band and the upper arrow indicates the super-shifted ERα complex. **B.** Western blot analyses of *in vitro* translated ERα using mouse anti-human ERα antibody. The *in vitro* transcription/translation mixture expressing wild-type or mutated ERα except ERα (G160C) and ERα (411fsX7), showed a distinct band of 66 kDa. ERα (411fsX7) showed a distinct band of 46 kDa.

Fig. 3. E2 concentration-dependent transcriptional activities of wild-type or mutated ERα protein in MDA-MB-435 cells. Wild-type or mutated ERα expression plasmids were transiently co-transfected with PGL3-3EREc38 and phRL-TK plasmids into the cells. After 24 hr, the cells were treated with various concentrations of E2 for 24 hr. The relative

luciferase activity was normalized with the *Renilla* luciferase activity and converted to fold induction above the vehicle control value. Each column represents the mean ± SD of triplicate determinations (* $P < 0.05$ and ** $P < 0.001$, compared with wild-type ERα).

Fig. 4. Antiestrogenicities of 4OHT and ICI 182,780 for wild-type or mutated ERα proteins in MDA-MB-435 cells. Wild-type or mutated ERα expression plasmids were transiently co-transfected with PGL3-3EREc38 and phRL-TK plasmids into the cells. After 24 hr, the cells were treated with 100 nM of 4OHT or 100 nM of ICI 182,780 in the presence of 10 nM of E2 for 24 hr. **A**. The relative luciferase activity was normalized to the *Renilla* luciferase activity. **B**. Percentage of repression of the transcriptional activity by 4OHT or ICI 182,780. Each column represents the mean ± SD of triplicate determinations (* $P < 0.05$, compared with the wild-type ERα). NS: no suppression.

Fig. 5. Estrogenicities of 4OHT and ICI 182,780 for wild-type or mutated ERα protein in MDA-MB-435 cells. Wild-type or mutated ERα expression plasmids were transiently co-transfected with PGL3-3EREc38 and phRL-TK plasmids into the cells. After 24 hr, the cells were treated with 100 nM of 4OHT or 100 nM of ICI 182,780 for 24 hr. The relative luciferase activity was normalized to the *Renilla* luciferase activity. Each column represents the mean ± SD of triplicate determinations (* $P < 0.05$, compared with wild-type ERα).

Fig. 1. Komagata et al.

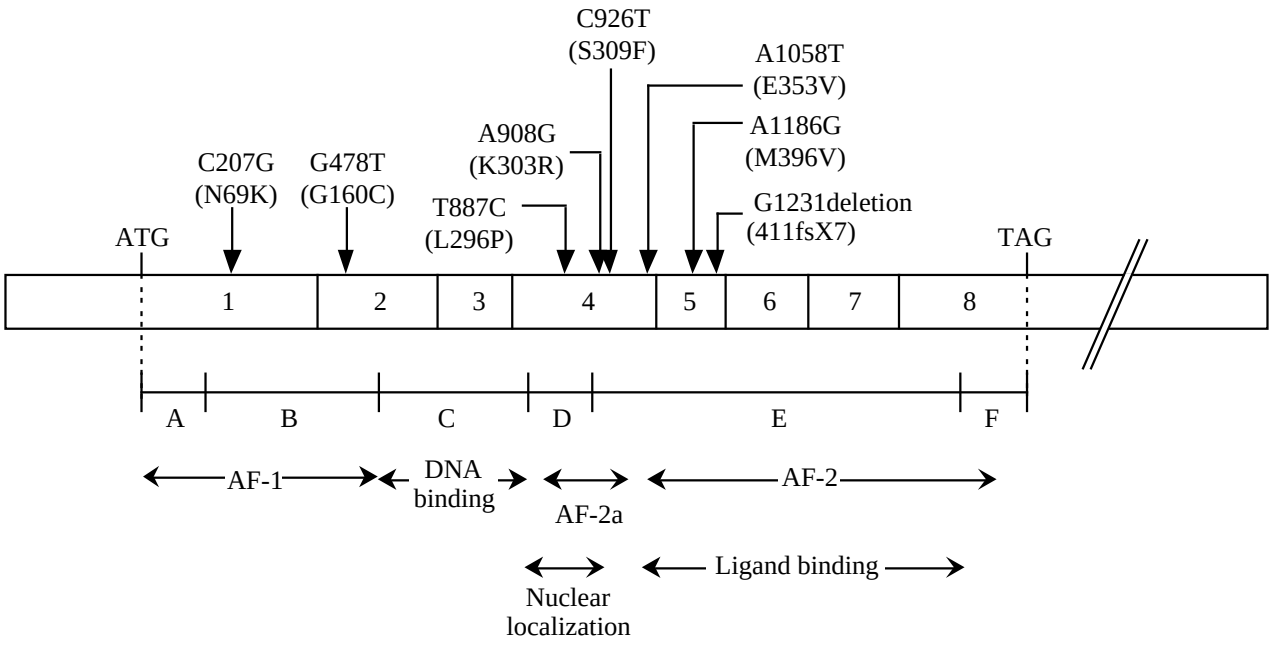
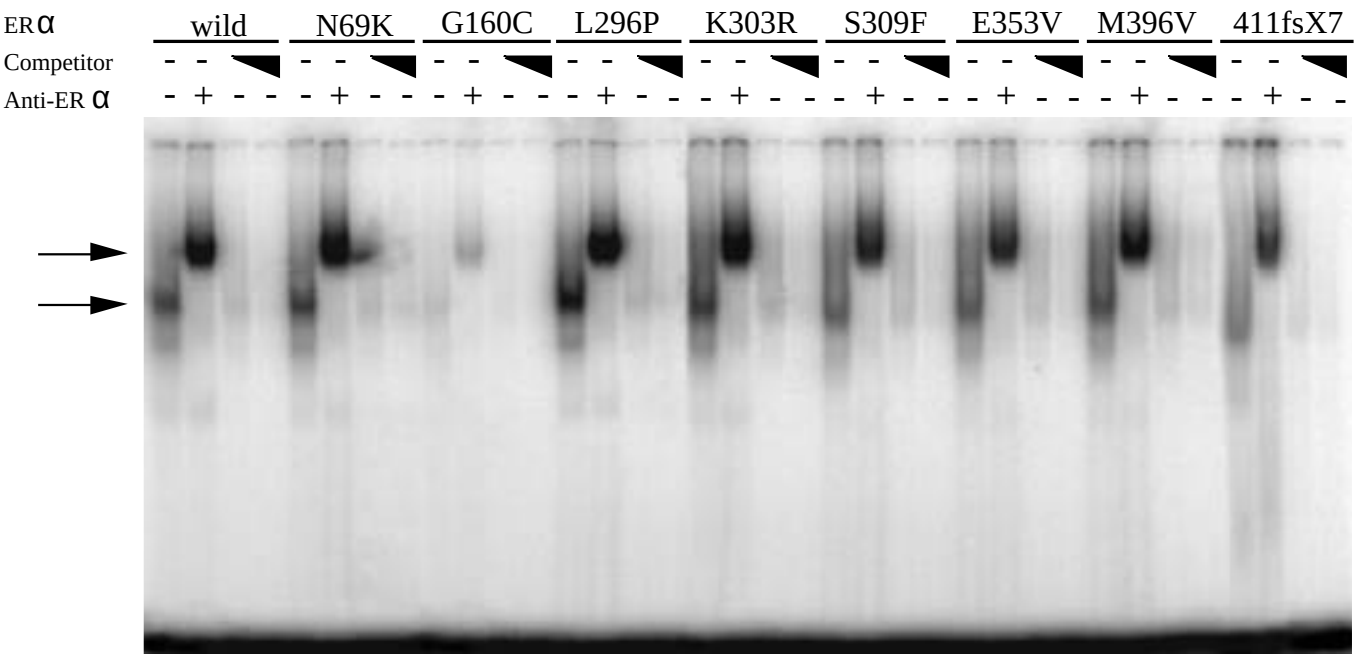


Fig. 2. Komagata et al.

A



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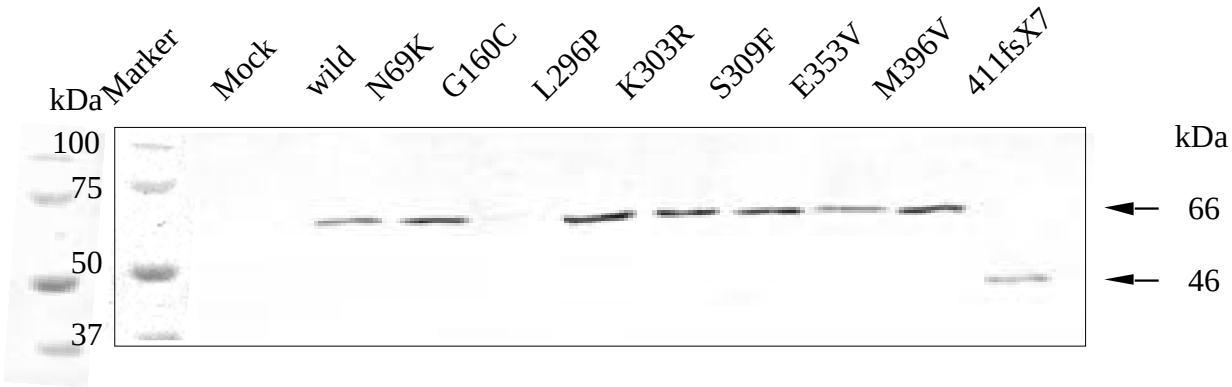

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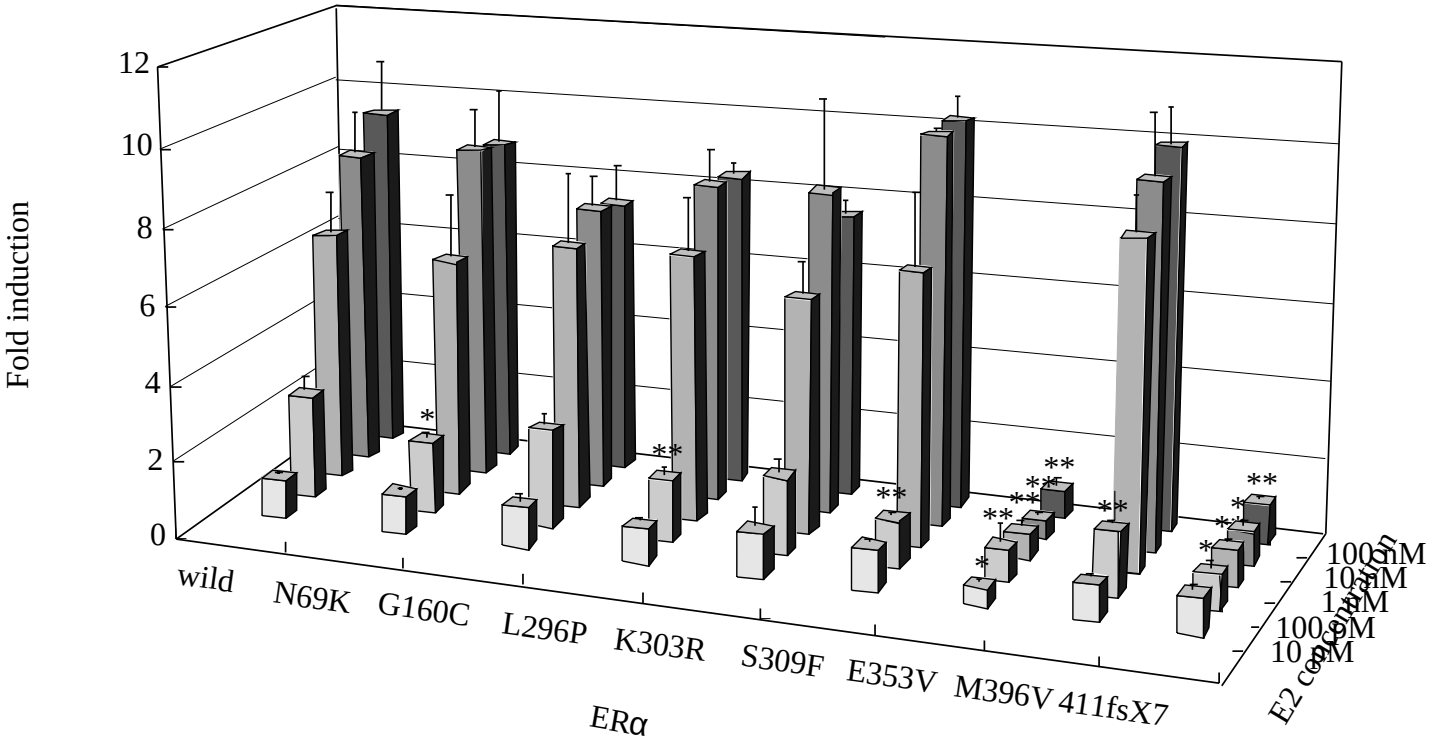

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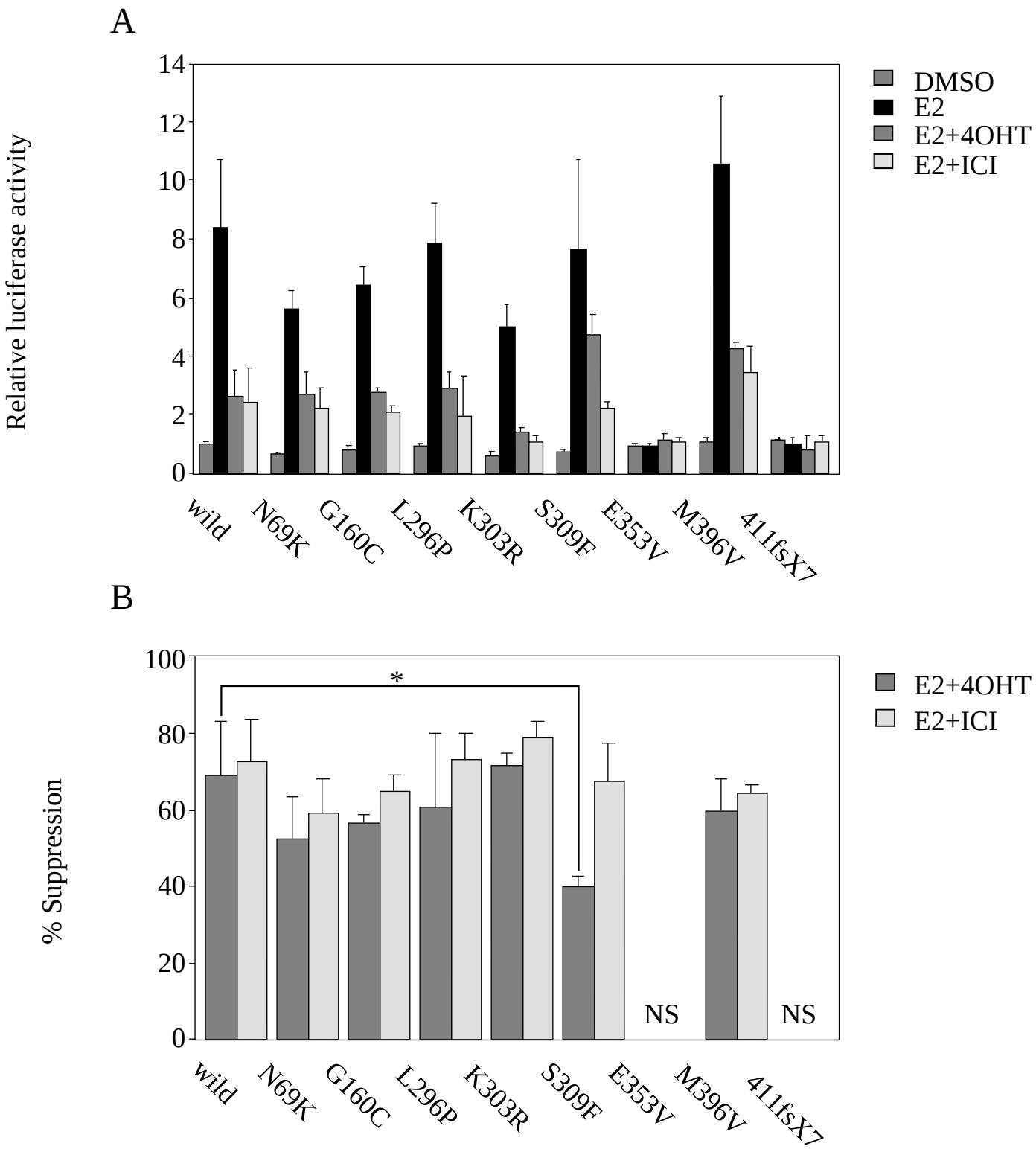

Fig. 5. Komagata et al.