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Structural differences between the ligand-binding pockets of estrogen receptors alpha and beta

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Abstract. The estrogen receptor (ER) is a member of the nuclear receptor superfamily and has two subtypes: ERα and ERβ. Inhibition of ERα is an effective therapeutic strategy in breast cancer. In contrast, ERβ is the presumed drug target of various autoimmune diseases. Many experimental structures of ERα/β have been reported; however, their structures vary due to ligand variability. Here, we performed structural bioinformatics studies for three-dimensional structures of ERs retrieved from the protein data bank (PDB) and clarify the detailed structural differences between ERα and ERβ. In total, 48 structures registered in the PDB were analyzed by HBOP and HBSITE, which we developed to identify ligand-binding cavities in proteins. PDB entries were clustered by number, shape, size, and location of the ligand-binding pockets. In addition, C-terminal domain shapes, which are divided into “agonist form” and “antagonist form,” were also used for clustering. We classified 27 entries of ERα into five clusters and 21 entries of ERβ into seven clusters (a total of 12 clusters). Differences in the pockets and hydrogen bonds with ligands were observed between ERα and ERβ and occurred even within the same ER species. Therefore, we conclude that the structure of ERs is diverse and is affected by ligands.

1. Introduction

Estrogen receptors (ERs) are nuclear hormone receptors that mediate the physiological effects of estrogens. Two types of ER exist, ERα and ERβ, whose function and distribution differ from each
other [1–3]. ERα is mainly expressed in the uterus, prostatic stroma, ovarian theca cells, Leydig cells in the testis, epididymis, bone, breast, various regions of the brain, liver, and white adipose tissue. In contrast, ERβ is expressed in the colon, prostatic epithelium, testis, ovarian granulosa cell, bone marrow, salivary gland, vascular endothelium, and certain regions of the brain [3,4]. The transcriptional function of ERα includes involvement in osteoporosis, cardiovascular disease, and breast cancer. On the other hand, ERβ is the presumed drug target for various autoimmune diseases [5]. Therefore, ERβ-selective ligands are predicted to be an effective therapy for autoimmune diseases. Recently, we developed ERβ-selective compounds and clarified the structure–activity relationships between these ligands [6–8]. In these studies, several compounds, including carborane or bis (4-hydroxyphenyl) methane moieties as a hydrophobic core, were found to be selective ERβ ligands. To develop such ligands, it is essential to understand the detailed structural differences between ERα and ERβ. Despite the functional differences, ERα and ERβ share homology, and the degree of amino acid sequence similarity is approximately 97% in the DNA-binding domain and approximately 56% in the ligand-binding domain [9]. Actually, aligned amino acid sequences of ligand-binding domains indicate that those sequences are well conserved (Fig. 1). To date, many experimental structures have been determined; however, ligand-binding pocket structures are diverse because of the wide variety of ligands [10–12]. In addition, the shape of C-terminus is thought to be important for ER activation [13]. Ligand-based selectivity studies have been conducted; however, there is insufficient information to develop ER-selective ligands [14], and structure-based clustering for ER has not been performed. In this study, we classified ERα and ERβ according to number, shape, size, location of the ligand-binding pockets, and shape of the C-terminal domains. Our results indicate that ERs can form diverse structures and the structures are affected by ligands. Furthermore, this structural information could be beneficial for the development of ER subtype-selective ligands.

Figure 1. Alignment of amino acid sequences of ERα and ERβ. The sequences of ligand-binding domain of ERs aligned using BLAST are shown. Black square indicates identity region.
2. Methods
To compare the three-dimensional structures of ERs, we retrieved 48 crystal structures, comprising 27 entries for ERα and 21 entries for ERβ, from the protein data bank (PDB; Tables 1 and 2). The HETATM, such as water molecules and ligands, in these entries were deleted for analysis. Ligand-binding cavities of ERs were identified using HBOP and HBSITE programs [15,16], from which hydrophobic potentials [17] were calculated around the proteins and the hydrophobic areas were detected as tentative binding pockets. The number, shape, size, and location of the identified cavities were compared among the PDB structures, and the structural features of the ligand-binding pockets were used to elucidate the differences between ERα and ERβ. To investigate ligand interactions for ERα and ERβ, we analyzed the amino acid residues forming hydrogen bonds with ligands in the PDB structures. In addition, ER structures were clustered according to the C-terminal structures important for transcriptional activity.

| Table 1. Retrieved ERα structures from the PDB |
|-----------------------------------------------|
| 1A52  | 1GWR  | 1L21  | 1XP9   |
| 1QKT  | 1SJ0  | 1XQC  | 2AYR   |
| 2OUZ  | 2POG  | 2Q70  | 2R6W   |
| 2R6Y  | 3DT3  | 3ERT  | 1G50   |
| 1ERE  | 1GWQ  | 3ERD  | 1PCG   |
| 1QKU  | 1XP1  | 1XP6  | 1XPC   |
| 1ERR  | 1R5K  | 1UOM  |        |

| Table 2. Retrieved ERβ structures from the PDB |
|-----------------------------------------------|
| 1NDE  | 1U3Q  | 1U9E  | 1U3S   |
| 1X7B  | 2JJ3  | 2I7X  | 1U3R   |
| 1QKM  | 1QKN  | 1X7J  | 1X76   |
| 1X78  | 1YY4  | 1YYE  | 2GIU   |
| 2NV7  | 1L2J  | 2I7Y  | 2QTU   |
| 2Z4B  |        |        |        |

3. Results and Discussion
3.1. Pocket number and size of ERs
The ER pockets identified using HBOP and HBSITE are shown in Tables 3 and 4. The hydrophobic pockets detected by HBOP and HBSITE are “HBSx,” in which “x” is the rank of pockets ordered by the degree of hydrophobicity. In the table, the volume of pocket is written in Å³. Some pockets detected by HBOP and HBSITE were connected and formed one pocket; therefore, they were defined as a single pocket and indicated by same mark († or ‡). The under bar indicates the pockets in which ligands were actually bound in the PDB structures. In this study, “ligand-binding pockets” refers to the tentative binding pockets detected by HBOP and HBSITE, and the actual pocket in which the ligand was experimentally observed is defined as the “actual pocket.” In Tables 3 and 4, HBSx is the ligand-binding pocket, and the underlined pocket is the actual pocket. For ERα, 19 entries had three ligand-binding pockets, seven entries had four, and another one had five. The minimum volume of the actual
pocket was 152 Å³, and the maximum volume was 238 Å³ (average = 205 Å³). Similarly, for ERβ, three to five ligand-binding pockets were detected. Twelve entries had three ligand-binding pockets, seven entries had four, and two entries had five. The minimum volume of the actual pocket was 115 Å³ and the maximum volume was 245 Å³ (average = 165.8 Å³). Similarity, for ERβ, three to five ligand-binding pockets were detected. Twelve entries had three ligand-binding pockets, seven entries had four, and two entries had five. The minimum volume of the actual pocket was 115 Å³ and the maximum volume was 245 Å³ (average = 165.8 Å³). The standard deviation of the actual pocket volume of ERα and ERβ was 27.75 and 31.11, respectively. These results indicate that the cavities of ERs can form diverse shape by ligand, and ERs have some cavities besides the actual pocket. Even within the same ER subtype, the number and size of the cavities were different in the PDB structures. Such differences within the same subtypes may be affected by the ligands.

| PDB ID | HBS1 | HBS2 | HBS3 | HBS4 | HBS5 | No. of Pockets |
|--------|------|------|------|------|------|----------------|
| 1A52   | 168  | 210† | 61   | 20†  | —    | 3              |
| 1ERE   | 192  | 179  | 10†  | 13†  | —    | 3              |
| 1ERR   | 128  | 195  | 25   | —    | —    | 3              |
| 1G50   | 178  | 169  | 50   | 11   | 10   | 5              |
| 1GWQ   | 152  | 205  | 25   | —    | —    | 3              |
| 1GWR   | 185  | 149  | 13   | 27   | —    | 4              |
| 1L2I   | 205  | 153  | 14   | —    | —    | 3              |
| 1PCG   | 182  | 156  | 32   | —    | —    | 3              |
| 1QKT   | 138  | 237  | 48   | —    | —    | 3              |
| 1QKU   | 188  | 24†  | 78†  | 29‡  | 13‡  | 3              |
| 1R5K   | 167  | 217  | 48   | 24   | —    | 4              |
| 1SJ0   | 147  | 222  | 39   | —    | —    | 3              |
| 1UOM   | 137  | 201  | 46   | 13   | —    | 4              |
| 1XP1   | 141  | 233  | 42   | 16   | —    | 4              |
| 1XP6   | 145  | 238  | 36   | —    | —    | 3              |
| 1XP9   | 133  | 217  | 23   | 10   | —    | 4              |
| 1XPC   | 137† | 221  | 35   | 10†  | 10   | 4              |
| 1XQC   | 120  | 186  | 56   | —    | —    | 3              |
| 2AYR   | 139  | 226  | 44   | —    | —    | 3              |
| 2OUZ   | 130  | 200  | 44   | 10   | —    | 4              |
| 2POG   | 149  | 199  | 46   | —    | —    | 3              |
| 2Q70   | 140  | 216  | 43   | —    | —    | 3              |
| 2R6W   | 126  | 210† | 14†  | 30†  | 10   | 4              |
| 2R6Y   | 143  | 199  | 47   | —    | —    | 3              |
| 3DT3   | 140  | 237  | 27   | —    | —    | 3              |
| 3ERD   | 163  | 134‡ | 10†  | 10‡  | 26‡  | 3              |
| 3ERT   | 151  | 220  | 50   | —    | —    | 3              |

a The volume of the detected pockets is shown in Å³.

b Pockets with the same marks († or ‡) were defined as a single pocket because they were connected and formed one pocket.

c Underline indicates the actual pocket in the PDB structure.
Table 4. Number and size of pockets in ERβ<sup>a,b,c</sup>

| PDB ID | HBS1 | HBS2 | HBS3 | HBS4 | HBS5 | HBS6 | No. of Pockets |
|--------|------|------|------|------|------|------|---------------|
| 1L2J   | 157  | 237  | 10   | 49†  | 70†  | 22†  | 4             |
| 1NDE   | 156  | 245  | 27   | 16†  | 13   | 14†  | 5             |
| 1QKM   | 134  | 115  | 15   | 12   | —    | —    | 4             |
| 1QKN   | 154  | 188† | 52†  | 23   | —    | —    | 3             |
| 1U3Q   | 135  | 92   | 11   | 10   | —    | —    | 4             |
| 1U3R   | 160  | 111  | 15   | —    | —    | —    | 3             |
| 1U3S   | 160  | 121  | 11   | —    | —    | —    | 3             |
| 1U9E   | 148  | 124  | 23   | —    | —    | —    | 3             |
| 1X7B   | 138  | 139  | 45   | —    | —    | —    | 3             |
| 1X7J   | 148  | 133  | 40   | —    | —    | —    | 3             |
| 1X76   | 145  | 130  | 41   | —    | —    | —    | 3             |
| 1X78   | 144  | 124  | 34   | —    | —    | —    | 3             |
| 1YY4   | 166  | 107  | 25   | 13   | —    | —    | 4             |
| 1YYE   | 142  | 127  | 55   | —    | —    | —    | 3             |
| 2GIU   | 125† | 192  | 17   | 19   | 12†  | —    | 4             |
| 2J7X   | 169  | 153  | 23   | —    | —    | —    | 3             |
| 2J7Y   | 156  | 155  | 18   | —    | —    | —    | 3             |
| 2JJ3   | 168  | 151  | 18†  | 28   | 14†  | —    | 4             |
| 2NV7   | 148  | 130  | 29   | —    | —    | —    | 3             |
| 2Q14    | 163  | 167  | 20†  | 28   | 21†  | —    | 4             |
| 2Z4B   | 139  | 158  | 16   | 25   | 13   | —    | 5             |

<sup>a</sup> The volumes of the detected pockets are shown in Å<sup>3</sup>.  
<sup>b</sup> Pockets with same marks († or ‡) were defined as a single pocket because they were connected and formed one pocket.  
<sup>c</sup> Underline indicates the actual pocket in the PDB structure.

We classified the PDB entries by number, shape, size, and location of the ligand-binding pockets. As shown in Figs. 2 and 3, ERα and ERβ were clustered into five and seven groups, respectively. Therefore, all ER structures were classified into 12 groups. For ERα, the entries included in groups A and B had three ligand-binding pockets, the entries in groups C and D had four, and one entry in group E had five. Furthermore, the volumes of the actual pockets were different; that of group B was larger than that of group A, and that of group C was larger than that of group D. For ERβ, the entries included in groups F and G had three ligand-binding pockets, the entries in groups H, I, and J had four, and the entries in groups K and L had five. The shapes of the ligand-binding pockets were different between groups F and G, and were also different between groups H, I, and J. The volumes of the actual pockets differed between groups K and L (K > L). There were large differences in shape and
number of cavities not only between different subtypes but also between entries within the same subtypes. Although entries with similar ligands tend to have similar structures of actual pockets, some exceptions were found. For example, 1ERE and 1QKT which have same ligand (estrogen) were classified into groups A and B, respectively. These exceptions might be caused by the flexibility of actual pocket, but the structural flexibility cannot be evaluated by X-ray structures. We will investigate the structural flexibility of ERs using molecular dynamics simulations. Although few atypical forms of ligand-binding pockets were observed, the shape and size of cavities for ERs mainly depend on ligands.

| group | (a) | (b) | PDB ID | No. of pockets |
|-------|-----|-----|--------|---------------|
| A     |     |     | 1ERE, 1GWQ, 1L2I, 1PCG, 1QKU, 3ERD | 3             |
| B     |     |     | 1A52, 1ERR, 1QKT, 1SJ0, 1XP6, 1XQC, 2AYR, 2POG, 2Q70, 2R6Y, 3DT3, 3ERT | 3             |
| C     |     |     | 1R5K, 1UOM, 1XP1, 1XP9, 1XPC, 2OUZ, 2R6W | 4             |
| D     |     |     | 1GWR | 4             |
| E     |     |     | 1G50 | 5             |

**Figure 2.** Clusters of ERα classified by their pockets. Both (a) and (b) show grid points with high hydrophobic potentials conducted by HBOP and HBSITE analysis and were identified as ligand-binding pockets. (b) shows the view from underneath (a).

### 3.2. Hydrogen bonding between ERs and ligands

The specific recognition between protein and ligand mainly depends on hydrogen bonds. We investigated hydrogen bonds between ERs and ligands to elucidate ligand-recognition mechanisms for development of subtype-selective ligands. In ERα, Asp351, Glu353, Leu387, Arg394, Gly521, and His524 form hydrogen bonds with ligands (Fig. 4 and Table 5). In particular, Glu353, Arg394, and His524 are considered to be important residues for ligand binding because hydrogen bonds between
**Figure 3.** Clusters of ERβ classified by their pockets. Both (a) and (b) are grid points with high hydrophobic potentials conducted by HBOP and HBSITE and were identified as ligand-binding pockets. (b) shows a view from underneath (a).

These residues and ligands are observed in the majority of ERα structures. These residues were previously suggested to be important for ligand binding [18,19]. Hydrogen bond formation between residues and ligands in ERβ are described in Fig. 5 and Table 6. Asp351, Glu353, Leu387, Arg394,
Gly521 and His524 in ERα correspond to Asp303, Glu305, Leu339, Arg346, Gly472 and His475, respectively. Although no interaction was observed at Leu339, other residues corresponding to ERα residues formed hydrogen bonds with ligands. Hydrogen bond formation at Glu305, Arg346, and His475 was frequently observed as same as corresponding residues of ERα. Since those hydrogen-bonded residues are conserved among ERs, these are considered to be particularly important biologically. However, only half the expected bonds at Arg346 were formed. Furthermore, in ERα, Asp351 formed hydrogen bonds in 15 of the 27 entries, whereas in ERβ, Asp303 formed hydrogen bonds in only two entries. Therefore, the major differences in residues forming hydrogen-bond with ligands are aspartate (351 in ERα and 303 in ERβ) and Arginine (394 in ERα and 346 in ERβ). ERα had more entries forming hydrogen bond in both residues. These differences could be useful for the development of subtype-selective ligands.

**Figure 4.** Example of hydrogen bonding between ERα and ligand (PDB ID: 1SJ0). Red lines represent hydrogen bonds formed between Arg394 and His524 and the ligand. Ligand carbon atoms are shown in gray. Nitrogen and oxygen atoms are shown in blue and red, respectively. Conserved hydrogen-bond forming residues between ERα and ERβ are shown in red, and residues considered to be important for selectivity are indicated in blue.

**Table 5.** Residues of ERα forming hydrogen bonds with ligands

| Residue | No. of entries |
|---------|---------------|
| Asp351<sup>b</sup> | 15 |
| Glu353<sup>b</sup> | 26 |
| Leu387 | 1 |
| Arg394<sup>b</sup> | 25 |
| Gly521 | 6 |
| His524<sup>b</sup> | 20 |

<sup>a</sup> 27 entries in total

<sup>b</sup> Conserved hydrogen-bond forming residues between ERα and ERβ are underlined by solid line, and residues considered to be important for selectivity are underlined by dotted line.
Figure 5. Example of hydrogen bonding between ERβ and ligand (PDB ID: 1QKN). Red lines represent hydrogen bonds formed between Arg346 and His475 and the ligand. Ligand carbon atoms are shown in gray. Nitrogen and oxygen atoms are shown in blue and red, respectively. Conserved hydrogen-bond forming residues between ERα and ERβ are shown in red, and residues considered to be important for selectivity are indicated in blue.

Table 6. Residues of ERβ forming hydrogen bonds with ligands

| Residue  | No. of entries a |
|----------|------------------|
| Asp303b  | 2                |
| Glu305b  | 21               |
| Leu339   | 0                |
| Arg346b  | 10               |
| Gly472   | 1                |
| His475b  | 18               |

a 21 entries in total.

b Conserved hydrogen-bond forming residues between ERα and ERβ are underlined by solid line, and residues considered to be important for selectivity are underlined by dotted line.

3.3. C-terminal structure of ERs
We focused on the C-terminal structure of ERs because the agonist and antagonist forms were observed in the C-termini of the experimental structures [13]. Fig. 6 shows examples of ER C-terminal structures shown as top and side views. The ER C-terminal region formed a helix structure that is exposed to solvent in the inactive form (Fig. 6A). Agonist binding causes C-terminal helix modification and decreases exposure to the solvent (Fig. 6B). One ERα, PDB ID: 1A52, showed a fully-extended form (Fig. 6C), which may have been an artifact. The agonist-binding crystal structures
were in their active forms, and antagonist binding was in its inactive form (Table 7). Partial agonist-binding structures almost establish inactive forms. Whether ERs form active or inactive forms depends on ligands; therefore, structural information is useful for agonist/antagonist design. In addition, because the partial agonists could be candidates for tissue-selective ER modulators, detailed research for inactive forms, such as hydrogen bond analyses in this study, may assist molecular targeted drug design for ERs to reduce side effects.

**Figure 6.** Examples of inactive, active, and fully-extended forms are shown as top and side views. The C-terminal helix is shown in purple. (A) Inactive form (PDB ID: 1ERR), (B) active form (PDB ID: 1ERE), and (C) fully-extended form (PDB ID: 1A52).

**Table 7.** Number of active, inactive, and fully-extended forms

| Subtype | Ligand      | Active form | Inactive form | Extended form |
|---------|-------------|-------------|---------------|---------------|
|         | Agonist     | 7           | 0             | 1             |
| ERα     | Antagonist  | 0           | 6             | 0             |
|         | Partial agonist | 1       | 12            | 0             |
|         | Agonist     | 13          | 2             | 0             |
| ERβ     | Antagonist  | 0           | 2             | 0             |
|         | Partial agonist | 1       | 3             | 0             |
4. Conclusion
In this study, we clarified the structural differences between ERα and ERβ crystal structures registered in the PDB. The ligand-binding pockets of ERs are diverse and can be changed by ligands; therefore, more comprehensive structural analyses such as molecular dynamics simulations are required for structure-based drug design (SBDD). In addition, those ligand-free structures have not been determined. It is essential for investigation of ligand-independent ERs structure and conformation change by ligand binding. We will analyze the conformation change using molecular dynamics simulations in the near future. This analysis may reveal important residue for selectivity. Furthermore, chemical investigations of the influences caused by ligands should be performed to clarify more details of structural differences by ligands. On the other hand, important protein–ligand interactions were observed. Conserved and different hydrogen bonding residues between ERα and ERβ were revealed. In particular, hydrogen bonding between Asp351 of ERα and ligands was frequently found; however, Asp303 of ERβ did not participate greatly in ligand interactions. This difference can be available for the development of ER subtype-selective ligands. In addition to this, the interaction energy of ligands and ERs may be useful for new ligand design. Structural information on active–inactive forms are abundant, and stimulant and inhibitor design may be assisted using this information.
ERs have cavities in addition to actual pockets, making it possible to develop allosteric ligands. We obtained valuable structural information for the development of ER functional modulators by only structural bioinformatics analyses. We intend to pursue more detailed structural bioinformatics studies, for example principle component analysis, to develop ER subtype-selective ligands using SBDD.

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