Interaction between Liver X Receptor Beta and Estrogen Receptor Alpha Variants *in vitro*

Rosemarie Le, Jacky Wu, Hengameh Rezaei, Arvand Asghari, Michihisa Umetani

Center for Nuclear Receptor and Cell Signaling, University of Houston, Houston, TX 77204, USA

IntroductIon

Liver X Receptors (LXR) are nuclear receptors with ligand-dependent transcription factor function and play an important role in lipid metabolism. LXR has two isoforms, LXRα and LXRβ. These two isoform have similar protein structure and target gene but different tissue distribution patterns. LXRα is primarily expressed in liver, kidney, intestine, adipose tissue, and macrophages, whereas LXRβ is expressed everywhere (Ishikawa et al. 2013). Estrogen Receptors (ER) are also nuclear receptors and function as ligand- inducible transcription factors and signaling molecules. ER regulate gene expression by interacting either in a protein–DNA manner through cognate DNA sequences called responsive elements, or in a protein–protein manner with other signaling molecules (Ishikawa et al. 2013). There are functional overlap between LXR and ER; however, the precise mechanism on the LXR-ER interaction has not been investigated.

Previously, we found that LXRβ and ERα are colocalized and functionally coupled in vascular endothelial cell (EC) plasma membrane caveolae/lipid rafts (Ishikawa et al. 2013). ERα-LXRβ complex plays an important role in vascular NO production and the maintenance of endothelial monolayer integrity in vivo (Figure 1).

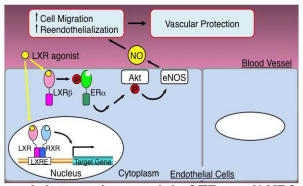


Figure 1. Interaction model of ERα and LXRβ.

We further studied the interaction between ERα and LXRβ. ERα lacking AF-1 (A/B) and DNA binding domain (DBD, C), respectively, showed similar interaction affinity as full length ERα towards LXRβ. ERα lacking ligand binding domain (E) did not show interaction with LXRβ. However, ERα lacking the hinge (D) showed interaction. We concluded that the interaction between ERα and LXRβ involves amino acids 300-330 within the ligand binding domain of ERα and that the interaction is dynamically regulated by ligand binding to either receptor (Ishikawa et al. 2013).

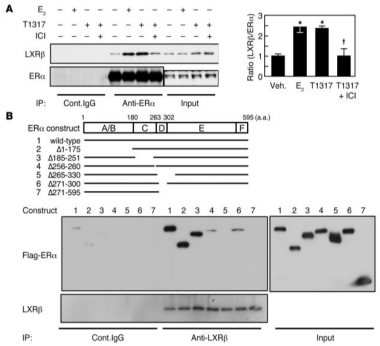


Figure 2. LXRβ and ERα interact directly via the ligand-binding domain of ERα. (A)EA.hy926 cells were treated with different ligands and proceed with co-immunoprecipitation (Co-IP) with IgG or anti-ERα antibody. Co-IP proteins were detected by immunoblotting with anti-LXRβ or anti-ERα antibodies. (B)In vitro Co-IP assay were performed using recombinant wild-type Flag-ERα or mutant ERα and LXRβ protein and pulled down with control IgG or anti-LXRβ antibody.

There are several splicing variants of ERα, including ERα-46, which exist in breast cancer cells (Chantalat et al., 2016). To analyze the interaction between ERα variants and LXRβ, we hypothesized that 1) LXRβ agonist GW3965 (GW) stabilized the complex and 2) there is a protein-protein complex between ERα variants and LXRβ, thus possibly playing a significant role in breast cancer development. We also created a truncated mutation of LXRβ, which lacks DBD, to examine its interaction with ERα.

Model

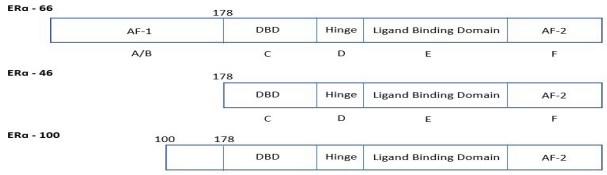


Figure 3. Models of ERα full length and mutated. ERα–66 is the full length protein. ERα-46 has the domain AF-1 (178 amino acids at N-terminus) truncated. ERα-100 has a truncated domain AF-1 (100 amino acids at N-terminus).

results

LXRβ– ERα interaction was increased by their ligands

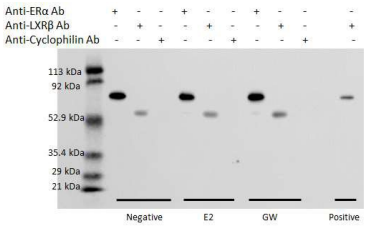
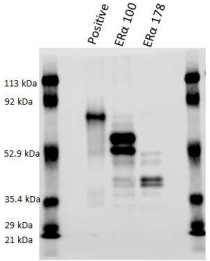


Figure 4. Western blot detecting ERα in the LXRβ – ERα complex. Lane 1, lane 4, and lane 7 were pulled down with ERα Rabbit Antibody in Co-immunoprecipitation Assay (Co-IP). Lane 2, lane 5, and lane 8 were pulled down with LXRβ antibody in Co-IP. Lane 3, lane 6, lane 9 were pulled down with a control antibody in Co-IP, thus, be the negative control. Lane 1, 2, 3 had no ligand. Lane 4, 5, 6 had an ER ligand (E2). Lane 7, 8, 9 had an LXR ligand (GW).

Expression of ERα variant proteins

Figure 5. TNT Expression result of translated proteins. Lane 1 was the full-length ERα. Lane 2 was the ERα-100. Lane 3 was the ERα-46. Multiple bands were observed indicated the presence of other variants.



ERα - 100 interacts with full and truncated LXRβ proteins

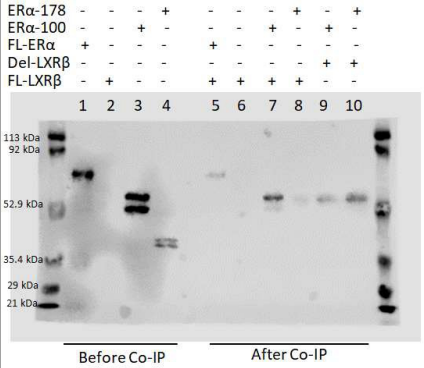


Figure 6. Co-IP result of protein complex between 1) ERα variants and full length LXRβ and 2) ERα-variants and truncated LXRβ. Lane 1, 2, 3, 4 were protein inputs (Before Co-IP). Lane 1 was the full-length ERα. Lane 2 was the negative control. Lane 3 was ERα-100. Lane 4 was ERα-46. Lanes 5-10 were the result from Co-IP. Lane 5 was using full-length ERα and full-length LXRβ. Lane 6 was the negative control. Lane 7 was ERα-100 with full length LXRβ. Lane 8 was ERα-46 with full length LXRβ. Lane 9 was ERα-100 with truncated LXRβ. Lane 10 was ERα-46 with truncated LXRβ.

Methods

Promega TNT Coupled Transcription/Translation system. This system was used to express ERα variants and truncated DBD-LXRβ protein. The reagents were added according to the protocol, and sample solutions were incubated at 30˚C for 90 minutes. The samples were run through a 10% SDS-PAGE, and gel was prepared for western blotting.

Co-immunoprecipitation assays (Co-IP). Co-IP was used to test protein interaction *in vitro*. First, the ERα variant and LXRβ protein from TNT system and GW compound was incubated on a shaker at room temperature for 30 mins. After, 40 µL of ~100% slurry of protein A/G was added to the mixture, and incubated for another hour. The complex was then pulled down with anti-LXRβ antibody. Lastly, the beads were washed with TBS-T, and the samples were prepared for 10% SDS-PAGE, and the Co-IP proteins were detected with western blot using an anti-ERα rabbit antibody.

Western Blot. The Co-IP proteins were denatured at 95˚C in SDSbuffer and separated by 10% SDS-PAGE. Then the proteins were transferred to PVDF membrane at 4˚C at 110V for 1 hour. The membrane was blocked with 5% milk for 1 hour, then incubated with primary anti-ERα rabbit antibody at 4˚C overnight. After that, the membrane was washed and detected with both secondary anti-rabbit antibody and chemiluminescent solution. The membrane was exposed for 2 minutes and analyzed with Li-Cor.

dIscussIon

Although the results were not completely expected, the Co-IP assays displayed interesting results. The Co-IP results (figure 6) have validated that there is no protein-protein complex between ERα-46 and both full length and truncated LXRβ. Rather, full length LXRβ formed a complex with ERα-100, and further validated with truncated LXRβ complex. It is suspected that ERα-100 has higher affinity than other variants for LXRβ. Another unexpected result is the presence of two molecular sizes from ERα variants detected on figure 5. Even though there are two unexpected ERα protein sizes, we predict that the LXRβ interacts with the higher molecular size of the variants. More studies are needed to determine the affinity of LXRβ as well as mutated LXRβ to ERα variants. With these results, we hope to conduct more studies and look into biological significance of ERα-100 and LXRβ complex.

Future studIes

1. "Inverse" Co-immunoprecipitation Assay where ERα Antibody is used to pull down and detect LXRβ.
2. Test for interaction between ERα variants and other LXRβ variants.
3. Determine biological significance of ERα46 and ERα100.
4. Kinetic analysis between the ERα variants and full length LXRβ.

Acknowledgements

Funding supports from Summer Undergraduate Research Fellowship (SURF) to RL and NIH HL127037 to MU.

References

Chantalat, E., Boudou, F., Laurell, H. *et al.* The AF-1-deficient estrogen receptor ERα46 isoform is frequently expressed in human breast tumors. *Breast Cancer Res* **18,** 123 (2016). https://doi.org/10.1186/s13058-016-0780-7

Ishikawa, Tomonori & Yuhanna, Ivan & Umetani, Junko & Lee, Wan-Ru & Korach, Kenneth & Shaul, Philip & Umetani, Michihisa. (2013). LXRβ/estrogen receptor-α signaling in lipid rafts preserves endothelial integrity. *The Journal of clinical investigation*. 123. 10.1172/JCI66533.