論文の内容の要旨

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論文題目

Study on heat shock protein 90 complex formation with SCAP/SREBP and regulation of SREBP function
（熱ショックタンパク質 90 による SCAP/SREBP 複合体形成と SREBP 活性調節に関する研究）

Chapter 1: *Introduction*

Sterol regulatory element-binding proteins (SREBPs) are the master transcription factors that control the expression of genes involved in fatty acids and cholesterol biosynthesis. SREBPs are synthesized on the endoplasmic reticulum (ER) membrane as inactive precursors. Upon synthesis, SREBP binds to its membrane-bound chaperone, SREBP cleavage-activating protein (SCAP). SCAP-binding protects SREBP from rapid degradation and promotes the transport of SREBP to the Golgi apparatus, in which SREBP is cleaved by site-1 and site-2 proteases. The N-terminal transactivation domain of SREBP is released by the proteolytic cleavage and translocates to the nucleus. Hence, SCAP/SREBP-binding is critical to SREBP function and lipid metabolism. It was demonstrated in 1997 that the C-terminal of SREBP interacted with SCAP C-terminus, which contains at least four WD40 repeats. However, up to present there is limited advance in the knowledge regarding the molecular detail of SCAP/SREBP-interaction. In addition, SREBP also plays essential roles in tumorigenesis. Dysregulations of SREBP and lipogenesis is a common strategy of cancer cells to manipulate energy metabolism. Intriguingly, heat shock protein 90 (Hsp90), a conserved chaperone that regulates most of the important cellular processes, is also highly expressed in most cancer cells, chaperoning essential proteins for cancer survival and progression. Recent studies also revealed the involvement of Hsp90 in adipogenesis and fatty liver diseases. Nonetheless, how Hsp90 manipulates lipid metabolism and interplays with SREBP are largely unknown. In this study, we aimed to uncover the molecular detail in the interaction between SCAP and SREBP. In addition, with the attempt to discover new SCAP-binding proteins that regulate SCAP/SREBP, we identified Hsp90 as a novel SREBP regulator. We performed a series of experiments to investigate the interaction between Hsp90 and SCAP/SREBP protein complex and to elucidate the regulatory effect of Hsp90 on SREBP and lipid biogenesis. We demonstrate here that Hsp90 regulates lipid homeostasis through directly controlling the protein level of SCAP and SREBP.

Chapter 2: *Hsp90 physically interacts with the C-termini of SCAP and SREBP*

The C-terminal of SCAP contains a WD40 domain, which is featured by the ability to interact with a wide array of proteins. In this part of the study, we aimed to find new SCAP-binding proteins by a mass spectrum-based proteomic approach. We overexpressed the C-terminus of human SCAP (C-SCAP) in human embryonic kidney 293 cells. C-SCAP-binding proteins were immunoprecipitated and subjected to electrospray ionization quadrupole time-of-flight mass spectrum analysis. More than 40 potential C-SCAP-binding proteins were identified, in which Hsp90 had relatively high sequence coverage (>30%). Since Hsp90 played critical roles in both cancer and lipid metabolism, we were particularly interested in the link between Hsp90 and SCAP. We performed immunoprecipitation experiments using FLAG-SCAP, FLAG-SREBP, and Myc-Hsp90 overexpressed proteins. We showed that Hsp90 interacted with the C-termini of SCAP and SREBP, possibly forming an Hsp90/SCAP/SREBP complex. In agreement with the overexpressed protein experimental result, we were able to co-immunoprecipitate endogenous SCAP and SREBP with Hsp90. Taken together, we demonstrated that Hsp90 formed a protein complex with SCAP and SREBP by interacting with their C-termini. This finding also suggests the possibility that Hsp90 might mediate lipid metabolism by direct regulation of SCAP and SREBP proteins.

Chapter 3: *Hsp90 modulates lipid homeostasis by regulating the stability of SCAP and SREBP*

Hsp90 and SREBP play indispensable roles in sustaining cancer cells survival and coordinating lipid homeostasis. However, the link between Hsp90 and SREBP is elusive. To study the effect of Hsp90 on SCAP/SREBP, we treated human hepatoma HepG2 cells with an Hsp90 inhibitor 17-(Allylamino)-17-demethoxygeldanamycin (17-AAG). 17-AAG decreased SCAP/SREBP protein level, downregulated SREBP-target gene expression, and reduced intracellular triglycerides and total cholesterol levels. This result implies that Hsp90 positively regulates SREBP via controlling SCAP/SREBP protein level. To test this inference, we knockdowned or overexpressed Hsp90 in HepG2 cells by small interfering RNA (siRNA) or lentivirus infection, respectively. Knockdown of Hsp90 substantially reduced the protein level of SCAP/SREBP, causing decreases in triglycerides and total cholesterol levels, whereas Hsp90 overexpression had the opposite effect. These results clearly demonstrate that Hsp90 regulates lipids biogenesis by controlling SCAP/SREBP proteins. We further investigated Hsp90-induced decrease of SCAP/SREBP using protein translation inhibitor cycloheximide (CHX). CHX-chase assay showed that 17-AAG accelerated the degradation of SCAP/SREBP proteins. Moreover, co-treatment of proteasome inhibitor MG132 cancelled the effect of 17-AAG on SCAP/SREBP degradation. These results indicate that Hsp90 stabilizes SCAP/SREBP proteins by protecting them from proteasomal degradation.

Since Hsp90 is a cytoplasmic chaperone and SCAP and SREBP are membrane-bound, we investigated if Hsp90 is indeed associated with SCAP/SREBP on the ER and Golgi membrane. By cell fractionation experiments, we showed that Hsp90 co-existed with SCAP/SREBP and co-immunoprecipitated with

SCAP in both the ER and Golgi fractions. Moreover, we also found that the interaction between Hsp90 and SCAP/SREBP was independent to intracellular sterol levels. Application of cholesterol and 25-hydroxylxholesterol (25-HC) had negligible effect on Hsp90-SCAP/SREBP interaction. In contrast, 17-AAG dramatically inhibited Hsp90-SCAP/SREBP binding. Furthermore, reduction of SCAP/SREBP induced by 17-AAG was unaffected by 25-HC treatment. Altogether, these data suggests that binding to Hsp90 is crucial for the stabilization of SCAP/SREBP complex; however, the binding and stabilizing effect of Hsp90 on SCAP/SREBP is independent to intracellular sterol levels.

Finally, to test our findings *in vivo*, we treated C57/BL6NCrl mice with a more potent 17-AAG analog, 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) by intraperitoneal injections. In agreement with the *in vitro* data, 17-DMAG treatment substantially decreased the protein level of SCAP/SREBP in mouse livers. Moreover, 17-DMAG downregulated hepatic expressions of SREBP-target genes and reduced triglycerides and total cholesterol levels in mouse levers. Taken together, we demonstrated that Hsp90 interacted with SCAP/SREBP on both the ER and Golgi membrane, keeping SCAP and SREBP proteins at steady levels through preventing their proteasomal degradation, thereby maintaining SREBP-target gene expressions and lipid homeostasis in hepatocytes and in mouse livers.

Chapter 4: *SREBP-binding sites in SCAP WD40 domain*

The binding between SCAP and SREBP C-termini has been revealed for nearly 2 decades, however, little is known about the detail of human SCAP/SREBP interaction. To elucidate SREBP-binding motif in SCAP, we developed two strategies based on current knowledge of the WD40 domain structure of SCAP C-terminus. First, using the 7 WD40 annotations of the UniProt database, we constructed SCAP truncated mutants lacking WD40 repeat(s) on its N-terminal, C-terminal, or both ends. Second, taking advantage of a WD40 protein database (WDSPdb) and the crystal structure of yeast SCAP, we deduced more than 30 potential SREBP-binding residues and constructed SCAP mutants carrying corresponding point mutations. SREBP-binding ability of the truncated and point mutated SCAP mutants were tested by co-immunoprecipitation assays. Truncation from N-terminus to WD2–WD3 or from C-terminus to WD7–WD5 dramatically reduced SREBP-binding ability of SCAP. However, simultaneous truncation from both termini showed no incremental effect, in which SCAP mutant having only WD3–WD5 still possessed SREBP-binding ability. This data implies that all WD40 repeats in SCAP contribute partially to SREBP-binding and prompt us to search for the amino acids residues crucial for SREBP-binding.

Based on the information retrieved from the WDSPdb, we constructed 7 SCAP mutant plasmids each carried a point mutation at a potential protein-protein interaction site. Immunoprecipitation experiments revealed that point mutations at leucine 1178 (L1178) and arginine 1248 (R1248) substantially reduced SREBP-binding ability. In addition, structure of yeast SCAP/SREBP1 complex unveiled a positively charged, arginine and lysine-rich region (R/K-patch) in the WD40 domain of SCAP that is essential to

SREBP-binding. Hence, we constructed over 20 plasmids each carried a point mutation at a charged residue, arginine (R), lysine (L), histidine (H), or aspartic acid (D), and tested the effect on SREBP-binding. Immunoprecipitation assays showed that mutations at R984, K1018, R1019, H1118, D1162, and R1187 substantially decreased SREBP-binding ability of SCAP.

In summary, we identified R984, K1018, R1019, H1118, D1162, L1178, R1187, and R1248 as the potential SREBP-binding residues. Interestingly, all these residues reside within WD2–WD7 (aa984–1248) of SCAP, explaining that SREBP-binding ability was uncompromised in WD1-deleted SCAP (aa832–1279) and that SCAP mutant with only WD3–WD5 (aa1005–1195) was still bound to SREBP.

Chapter 5: *Comprehensive discussion*

SREBP plays crucial roles in lipid metabolism and is hijacked by cancer cells to manipulate energy metabolism to favor survival and progression of tumors. Of equal importance, Hsp90 is one of the most abundant and perhaps the most important chaperone in cells. It regulates most of the cellular processes such as signal transduction and cell proliferation and is highly expressed in cancer cells to maintain key proteins required for the malignancies of tumors. However, although extensive studies have advanced greatly the fields of both SREBP and Hsp90 researches, the connection between Hsp90 and SREBP is largely unknown. In this study, with the attempt to identify new SCAP-binding proteins, we identified Hsp90 as a new SREBP regulator that forms complex with SCAP and SREBP. We demonstrated a clear positive regulatory effect of Hsp90 on the protein stability of SCAP/SREBP and on lipid homeostasis. This finding explains at least partially the concurrent increase of Hsp90 and SREBP expressions and lipogenesis in cancer cells and fatty livers. The direct and causal relation between Hsp90 and SREBP in maintenance of lipid homeostasis open new avenue as to the development of strategies against both cancer and metabolic diseases. In addition, we discovered a core surface consisted of at least 8 amino acid residues that is potentially the SREBP-interacting region of SCAP. This finding provides a more in-depth knowledge as to the molecular detail in the interaction between human SCAP and SREBP.

Publication:

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