Mechanisms of Signal Transduction:

Up-regulation of Acetyl-CoA Carboxylase α and Fatty Acid Synthase by Human Epidermal Growth Factor Receptor 2 at the Translational Level in Breast Cancer Cells

Sarah Yoon, Min-Young Lee, Sahng Wook Park, Jong-Seok Moon, Yoo-Kyung Koh, Yong-Ho Ahn, Byeong-Woo Park and Kyung-Sup Kim

J. Biol. Chem. 2007, 282:26122-26131.
doi: 10.1074/jbc.M702854200 originally published online July 13, 2007

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 36, pp. 26122–26131, September 7, 2007

Up-regulation of Acetyl-CoA Carboxylase α and Fatty Acid Synthase by Human Epidermal Growth Factor Receptor 2 at the Translational Level in Breast Cancer Cells*

Received for publication, April 4, 2007, and in revised form, July 13, 2007 Published, JBC Papers in Press, July 13, 2007, DOI 10.1074/jbc.M702854200

Sarah Yoon[‡1], Min-Young Lee[‡1], Sahng Wook Park[‡], Jong-Seok Moon[‡], Yoo-Kyung Koh[‡], Yong-Ho Ahn[‡], Byeong-Woo Park[§], and Kyung-Sup Kim[‡2]

From the [‡]Department of Biochemistry and Molecular Biology, Brain Korea 21 Project for Medical Science, Institute of Genetic Science, Center for Chronic Metabolic Disease Research and the [§]Department of Surgery, Yonsei University, College of Medicine, 134 Shinchondong, Seodaemungu, Seoul 120-752, Korea

Expression of the *HER2* oncogene is increased in ~30% of human breast carcinomas and is closely correlated with the expression of fatty acid synthase (FASN). In the present study, we determined the mechanism by which FASN and acetyl-CoA carboxylase α (ACCα) could be induced by HER2 overexpression. SK-BR-3 and BT-474 cells, breast cancer cells that overexpress HER2, expressed higher levels of FASN and ACCα compared with MCF-7 and MDA-MB-231 breast cancer cells in which HER2 expression is low. The induction of FASN and ACCα in BT474 cells were not mediated by the activation of SREBP-1. Exogenous HER2 expression in MDA-MB-231 cells induced the expression of FASN and ACCα, and the HER2-mediated increase in ACCα and FASN was inhibited by both LY294002, a phosphatidylinositol 3-kinase inhibitor, and rapamycin, a mammalian target of rapamycin (mTOR) inhibitor. In addition, the activation of mTOR by the overexpression of RHEB in MDA-MB-231 cells increased the synthetic rates of both FASN and ACCα. On the other hand, *FASN* and *ACCα* were reduced in BT-474 cells by a blockade of the mTOR signaling pathway. These changes observed in their protein levels were not accompanied by changes in their mRNA levels. The 5′- and 3′-untranslated regions of both FASN and ACCα mRNAs were involved in selective translational induction that was mediated by mTOR signal transduction. These results strongly suggest that the major mechanism of HER2-mediated induction of FASN and ACCα in the breast cancer cells used in this study is translational regulation primarily through the mTOR signaling pathway.

Because OA-519, a poor prognostic marker found in breast cancer cells, was shown to be a fatty acid synthase (FASN)[3] (1), a number of studies have demonstrated abnormally high levels of FASN in many human epithelial cancers and preneoplastic lesions (2, 3). FASN, a lipogenic enzyme, catalyzes the biosynthesis of palmitic acid that is used for the synthesis of triacylglycerol as a storage fuel molecule as well as membrane lipids including phospholipids and sphingolipids (4). In breast cancer cells, the expression of FASN is closely related to the aggressiveness of cancers as well as to the development, maintenance, and cell cycle progression of human cancers (5–7). Breast cancer cells that overexpress FASN undergo apoptosis when treated with small interfering RNAs (siRNAs) against FASN or FASN inhibitors, such as C75 and cerulenin (8–11). Under physiological conditions, the activities of lipogenic enzymes, including FASN, are tightly regulated by nutritional and hormonal parameters at the transcription level. Sterol regulatory element-binding proteins (SREBP-1a, SREBP-1c, and SREBP-2) are the major transcription factors that mediate this regulation (12). SREBPs reside in endoplasmic reticulum membranes as inactive precursors. To become active, the NH_2-terminal segments of SREBPs are released from the endoplasmic reticulum by proteolytic cleavage and enter the nucleus where they activate their target genes. SREBP-1 preferentially activates the genes involved in fatty acid synthesis, whereas SREBP-2 preferentially activates the genes involved in cholesterol synthesis. Activation of SREBPs has been suggested as one of the major mechanisms by which the induction of FASN in breast cancer cells occurs (13). SREBP-1-mediated induction of the expression of FASN was shown in MCF10a epithelial cells transformed by H-*ras*; SREBP-1 was activated by mitogen-activated protein kinase and phosphatidylinositol 3-kinase (PI3K)/Akt pathways (14, 15) in these cells. Progestin also can induce FASN expression in T47D breast cancer cells through SREBP-1 activation (16).

For the overexpression of FASN in breast cancer cell lines, human epithelial growth factor receptor-2 (HER2) was reported to play an important role (8, 17). HER2 belongs to the type I family of growth factor receptors in which four structurally related proteins are included: HER1 (ErbB1), HER2

* This work was supported by Grant R13-2002-054-01002-0 from the Basic Research Program of the Korea Science and Engineering Foundation and a faculty research grant of Yonsei University College of Medicine for 2006. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Both authors contributed equally to this work.

[2] To whom correspondence should be addressed. Tel.: 822-2228-1676; Fax: 822-312-5041; E-mail: kyungsup59@yumc.yonsei.ac.kr.

[3] The abbreviations used are: FASN, fatty acid synthase; HER2, human epidermal growth factor receptor 2; SREBP-1c, sterol regulatory element-binding protein-1c; ACLY, ATP citrate lyase; ACCα, acetyl-CoA carboxylase α; PI3K, phosphatidylinositol 3-kinase; mTOR, mammalian target of rapamycin; RHEB, *ras* homolog enriched in brain; S6, 40 S ribosomal protein; UTR, untranslated region; RT, real time; TSC, tuberous sclerosis; GAPDH, glyceraldehyde-3-phosphate dehyrogenase; AdGFP, adenoviral vector expressing green fluorescence protein; siRNA, small interfering RNA.

(ErbB2), HER3 (ErbB3), and HER4 (ErbB4). HER2 forms a heterodimer with other HER family members to activate its intrinsic protein-tyrosine kinase activity, triggering a cascade of signal transduction events that result in cellular proliferation (18–20). It was reported that *HER2* gene amplification and/or protein overexpression occurs in 14–30% of all breast cancer cases (21, 22), and its overexpression is associated with rapid tumor growth, poor response to conventional Taxol chemotherapy, and poor prognosis (23, 24). Accordingly, Herceptin, a monoclonal antibody that blocks signals transduced by HER2, has been shown to augment the effects of chemotherapeutic agents in metastatic breast cancers (25).

One of the suggested mechanisms that correlates FASN overexpression with HER2 is that aberrant PI3K transduction cascades by HER2 drive the constitutive activation of SREBP-1c, resulting in transcriptional induction of the *FASN* gene (13, 14). A number of growth factors, hormones, and cytokines activate PI3K, which in turn activates Akt. Activated Akt phosphorylates tuberous sclerosis 2 (TSC2), then represses the GTPase-activating protein activity of TSC1/TSC2 on RHEB, the major regulator of the mammalian target of rapamycin (mTOR). The mTOR protein, one of the downstream effectors of PI3K, is a Ser/Thr kinase that controls cell growth and proliferation by the regulation of protein synthesis (26, 27). In the absence of either TSC1 or TSC2, RHEB-GTP levels are high, which leads to the constitutive activation of the mTOR-raptor signaling pathway. The activation of mTOR leads to the phosphorylation and inactivation of eIF4E-binding protein, resulting in the derepression of translation. Another target of mTOR is the 70-kDa subunit of S6 kinase 1 that controls protein synthesis in response to various hormones, mitogens, and nutrients (28–30). It was reported that mTOR, which is activated by HER2 overexpression, plays a role in the proliferation and survival of breast cancer cells both *in vitro* and *in vivo* (31). Several studies have demonstrated that mTOR inhibition sensitizes certain resistant cancer cells to endocrine therapy with tamoxifen (32, 33) and certain chemotherapeutic drugs, such as paclitaxel, and carboplatin, thus suggesting that the mTOR signaling pathway is a candidate molecular target for breast cancer treatment (34).

Although lipogenesis has proven to be an important process for the survival and proliferation of breast cancer cells, little is known about the mechanisms by which HER2 mediates the regulation of lipogenic enzymes. In the present study, we show that the expression of two lipogenic enzymes, FASN and ACCα, is increased by HER2 overexpression, and that the activation of mTOR by HER2 plays an important role in their overexpression through the selective translational activation of their mRNAs. It is suggested that the 5′- and 3′-untranslated regions (UTRs) of both *FASN* and *ACCα* mRNAs contribute to the increase in the translational efficiency of their mRNAs by mTOR.

EXPERIMENTAL PROCEDURES

Cell Culture—Human breast cancer cell lines, MDA-MB-231, MCF-7, BT-474, and SK-BR-3, were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cells were maintained in the respective media (Invitrogen) as recommended by the ATCC at 37 °C in a humidified atmosphere of 5% CO_2 in air.

Preparation of Recombinant Adenovirus and Viral Transfection—Recombinant adenoviruses expressing HER2 (AdHER2) and RHEB (AdRHEB) were constructed using the AdEasy adenoviral vector system vector (Stratagene, La Jolla, CA) according to the manufacturer's protocol. Briefly, cDNAs encoding HER2 and RHEB were amplified by PCR and cloned into the pShuttle-CMV plasmid. The recombinant shuttle vector constructs and pAdEasy-1 vector were co-transformed into BJ5183 *Escherichia coli* by electroporation. The positive recombinant adenovirus plasmids were identified by restriction digestion. Five micrograms of the recombinant adenovirus plasmids were linearized by digestion with PacI, then transfected into HEK-293A cells using Lipofectamine and the PLUS reagent (Invitrogen). The adenoviruses were amplified in 293A cells, and infective viruses were isolated using $CsCl_2$ ultracentrifugation. The infective recombinant adenoviruses were desalted and the viral titers were determined by plaque-forming assay using an agarose overlay in HEK-293A cells. MDA-MB-231 and BT-474 cells were transduced with recombinant adenovirus at a multiplicity of infection of 100 in serum-free media at 37 °C. Culture medium was replaced with fresh medium containing 10% fetal bovine serum and antibiotics 2 h after transduction. After an overnight incubation, cells were treated with LY294002 (Alexis, San Diego, CA), Herceptin (a generous gift from Genentech, South San Francisco, CA), or rapamycin (Cell Signaling Technology, Danvers, MA) for 24 h. The cells were harvested at 48 h after viral transduction and processed for either immunoblot analysis or RNA isolation. A replicon-defective adenoviral vector expressing green fluorescence protein (AdGFP) was used as a control.

Immunoblot Analyses—The cells treated as indicated in the figure legends were harvested and lysed in 2× SDS loading buffer, then briefly sonicated. Lysates were cleared by centrifugation at 12,000 × *g* for 10 min at 4 °C. Supernatants were collected and protein concentrations were determined by Bradford assay (Bio-Rad). The proteins were subjected to electrophoresis on SDS-polyacrylamide gels and then transferred to Protran nitrocellulose membranes (Schleicher & Schuell). The membranes were blocked in phosphate-buffered saline containing 0.05% (v/v) Tween 20 (PBST) and 5% (w/v) nonfat dried milk, and probed with primary antibodies as indicated in the figure legends. The following antibodies were used: polyclonal antibodies against rat ACCα from Genepia (Seoul, Korea); polyclonal antibodies against S6 protein, phospho-S6 protein, mTOR, phospho-mTOR, and RHEB from Cell Signaling Technology; polyclonal antibodies against HER2 from Oncogene (San Diego, CA); and monoclonal antibodies against α-tubulin from Calbiochem. Polyclonal antibodies against FASN and SREBP-1 were described previously (35, 36). Antibodies against human SREBP-2, and bovine low density lipoprotein receptor were kindly provided by Jay Horton (University of Texas Southwestern Medical Center). Immunoreactive bands were visualized by horseradish peroxidase-conjugated secondary antibodies (Pierce) using the SuperSignal West Pico Chemiluminescent Substrate System (Pierce).

siRNA Experiment—siRNA oligonucleotides for *mTOR* were purchased from Ambion (Austin, TX). A scrambled siRNA (Dharmacon, Chicago, IL) was used as a control. Transfection was performed using FuGENE 6 (Roche Applied Sciences) according to the manufacturer's protocol. Briefly, 100 pmol of siRNA and 6 μl of FuGENE 6 were mixed in 100 μl of serum-free media. After 30 min of incubation, the siRNA-FuGENE 6 mixture was added to the cultured cells. Forty-eight hours after transfection, lysates were prepared and immunoblot analyses were performed.

Cell Viability Assay—Cells (3×10^3 cells/well) were placed in a 96-well culture plate on day 0. On day 1, the medium was replaced with fresh medium containing 10 μg/ml cerulenin (Sigma) solubilized in dimethyl sulfoxide. On day 2, cell viability was determined using the CellTiter 96 Aqueous Non-radioactive Cell Proliferation Assay kit (Promega) according to the manufacturer's protocol.

Northern Blot Analysis and Real-time (RT)-PCR—Total RNA was isolated from cultured cells using the TRIzol reagent (Invitrogen) according to the manufacturer's protocol. For Northern blot hybridization, 20 μg of total RNA were denatured and subjected to electrophoresis on denaturing agarose gels containing formaldehyde. RNA was then transferred to nylon membranes (Schleicher & Schuell), UV cross-linked, and hybridized with *SREBP-1*, *ACLY*, *ACCα*, *FASN*, and *β-actin* cDNAs as probes. The probes were labeled using Rediprime II random primer labeling system (Amersham Biosciences) and [α-^{32}P]dCTP (PerkinElmer Life Sciences). For quantitative RT-PCR, cDNAs were synthesized from 5 μg of total RNA using oligo(dT) and SuperScript reverse transcriptase II (Invitrogen) according to the manufacturer's protocol. The resulting cDNA was subsequently mixed with 2× SYBR Green PCR Master Mix (Applied Biosystems) and various sets of gene-specific primers and then subjected to RT-PCR quantification using the ABI PR PRISM 7300 Real Time PCR System (Applied Biosystems). The sequences of the primers used were as follows: *FASN*, 5′ primer, 5′-TCGTGGGCTACAGCATGGT, and 3′ primer, 5′-GCCCTCTGAAGTCGAAGAAGAA-3′; *ACCα*, 5′ primer, 5′-CTGTAGAAACCCGGACAGTAGAAC-3′, and 3′ primer, 5′-GGTCAGCATACATCTCCATGTG-3′; *ACLY*, 5′ primer, 5′-TGCTCGATTATGCACTGGAAGT-3′, and 3′ primer, 5′-ATGAACCCCATACTCCTTCCCAG-3′; glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*), 5′ primer, 5′-CCCCTTCATTGACCTCAACTAC-3′, and 3′ primer, 5′-GAGTCCTTCCACGATACCAAAG-3′. All reactions were performed in triplicate. The relative amounts of the RNAs were calculated by using the comparative C_T method. As an invariant control, GAPDH mRNA was used.

Measurement of RHEB-induced Translational Activation of FASN and ACCα—MDA-MB-231 cells (8×10^5 cells/10-cm dish) were plated on day 0. On day 1, the cells were transduced with either AdGFP or AdRHEB at a multiplicity of infection of 100 as described above. Twenty-four hours after transduction, the medium was replaced with methionine/cysteine-free Dulbecco's modified Eagle's medium (Invitrogen) for 1 h. Rates of protein synthesis were measured by a pulse labeling experiment; [^{35}S]Met-Cys (PerkinElmer Life Sciences) was added to the medium at a final concentration of 0.3 mCi/ml. Cells were incubated for 0, 1, 2, and 4 h. After incubation, the cells were washed with ice-cold phosphate-buffered saline and harvested with 500 μl of lysis buffer (100 mM Tris-HCl, pH 7.5, 300 mM NaCl, 2% (v/v) Nonidet P-40) on ice. Protein degradation was measured by pulse-chase assay; the cells were rinsed after a 4-h pulse labeling, incubated in medium containing 2 mM L-methionine and 20 μg/ml cycloheximide for 0, 1, 2, and 4 h, and then harvested in lysis buffer.

The cell lysates were collected by centrifugation at 12,000 × *g* for 10 min at 4 °C and precleared by mixing with 50 μl of protein A-agarose (50% slurry), followed by incubation for 2 h on a rotating device at 4 °C. After removal of protein A-agarose by centrifugation, the indicated antibodies were added to the cleared cell lysates and incubated overnight on a rotating device at 4 °C. After the addition of 50 μl of protein A-agarose, the mixture was incubated for 2 h. The protein A-agarose beads were collected by centrifugation, washed twice with 50 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.1% (v/v) Nonidet P-40, and 0.05% (w/v) deoxycholate, and washed once with 10 mM Tris-HCl, pH 7.5, 0.1% (v/v) Nonidet P-40, and 0.05% (w/v) deoxycholate. The immunoprecipitated proteins were eluted from the protein A-agarose by boiling for 5 min with 1× SDS loading buffer, and subjected to electrophoresis on a 6% SDS-polyacrylamide gel. After electrophoresis, the gels were soaked in 30% methanol and 10% acetic acid for 10 min, dried, and exposed to x-ray film.

Generation of Promoter-Reporter Constructs and Transient Transfection Assay—To evaluate the regulatory roles of the UTRs of *ACCα*, *FASN*, and *ACLY* mRNAs on gene expression, two *ACCα* 5′-UTRs, designated Ex1 (599 bp) and Ex2 (161 bp), the 5′-UTR of *FASN* (100 bp), and the 5′-UTR of *ACLY* (101 bp) were amplified from the cDNA prepared using total RNA isolated from BT-474 cells. The amplified PCR fragments were inserted into the HindIII/NcoI sites of the pGL3 promoter vector (Promega), resulting in constructs harboring the amplified 5′-UTR fragments immediately upstream of the open reading frame of the firefly luciferase gene in the pGL3 promoter. The 3′-UTR sequences of *ACCα*, *FASN*, and *ACLY* were obtained by digesting human *ACCα* cDNA image clone 3874305 with XhoI/BamHI, human *FASN* cDNA image clone 6172538 with NcoI/XhoI, and the human *ACLY* cDNA image clone 2959339 with NdeI/XhoI, respectively. The digested fragments with sizes of 2.4, 1, and 1 kb for *ACCα*, *FASN*, and *ACLY*, respectively, were subcloned into the XbaI site of the pGL3 promoter vector, resulting in constructs harboring the 3′-UTR fragments downstream of the firefly luciferase gene. To construct the pGL3 promoter containing both the 5′- and 3′-UTRs, the amplified 5′-UTR fragments were inserted upstream of the firefly luciferase gene in the pGL3 promoter constructs containing the 3′-UTRs of *ACCα*, *FASN*, and *ACLY* downstream of the luciferase gene. After transient transfection of these constructs into MDA-MB-231 cells, both the luciferase assay and PCR were performed to determine the role of the UTR sequences in gene expression under conditions of RHEB overexpression. The cells (5×10^5 cells/well) were plated in 6-well plates on day 0. On day 1, the cells were transfected with the indicated plasmids using Lipofectamine and the PLUS reagent (Invitrogen) according to the manufacturer's protocol with a minor modifi-

cation for the simultaneous transduction with AdRHEB. Plasmid DNA (0.6 μg) was mixed with Lipofectamine and the PLUS reagent in serum-free media. While the complexes were forming, the cells were washed with phosphate-buffered saline, and then incubated with 1 ml of serum-free media containing a multiplicity of infection of 100 of either AdGFP or AdRHEB. After 15 min, the Lipofectamine-DNA complexes were added to the medium. After a 3-h transfection, the medium was replaced with fresh medium containing 10% fetal bovine serum. For BT-474 cells, the same transfection procedures were performed except for transduction with adenoviruses. On day 2, the cells were treated with rapamycin for 24 h. On day 3, the cells were harvested and lysed with 200 μl of reporter lysis buffer (Promega). The luciferase activity assay was performed using 5 μl of the cell extract and 45 μl of luciferase assay reagent (Promega). Each transfection experiment was performed in triplicate, and mean ± S.E. of three independent experiments were calculated. For quantitative analysis of the luciferase mRNA in the transfected cells, poly(A)-rich RNAs were isolated using the PolyATtract mRNA isolation system IV (Promega) from total RNA, and treated with RNase-free DNase (Ambion) to remove the transfected DNA. PCR for the luciferase mRNA was performed using the luciferase primer set: 5′ primer, 5′-AGCGACCAACGCCTTGATT-3′, and 3′ primer, 5′-ACTTCAGGCGGTCAACGATG-3′. To verify the complete removal of transfected DNA, amplification of the SV40 promoter region of the reporter constructs was performed as a negative control with the following primers: 5′ primer, 5′-CTAGCAAAATAGGCTGTCCCC-3′, and 3′ primer, 5′-CTTTATGTTTTTGGCGTCTTC C-3′.

RESULTS

FASN Expression Is Closely Linked to HER2 Expression in Breast Cancer Cell Lines—Increased expression of FASN has previously been demonstrated in breast and ovarian cancer cells (5, 6, 37) and was recently reported to be related to HER2 overexpression (8, 11, 38). FASN and HER2 expression levels were evaluated by immunoblot analysis in four human breast cancer cell lines. HER2 was not detected in MDA-MB-231 and MCF-7 cells, whereas overexpression of HER2 was shown in BT-474 and SK-BR-3 cells (Fig. 1*A*). Consistent with previous reports, cells that overexpress HER2 showed higher expression levels of FASN (Fig. 1*A*, *lanes 3* and *4*) than cells lacking detectable levels of HER2. To determine whether expression of FASN is important for cell growth and survival in these cells, cells were treated with cerulenin (10 μg/ml) for 24 h and viable cells were assayed. Cerulenin, a natural product derived from *Cephalosporium caerulens*, is a suicide inhibitor that binds irreversibly to the catalytic site of the β-ketoacyl synthase domain of multifunctional FASN (39, 40). BT-474 and SK-BR-3 cells that overexpress FASN and HER2 showed higher susceptibility to cerulenin than did MDA-MB-231 or MCF-7 cells, resulting in lower cell viability (Fig. 1*B*). These results suggest a strong association between FASN expression and HER2; the increased expression of FASN might be an important factor for cell growth and survival in breast cancer cells that overexpress HER2.

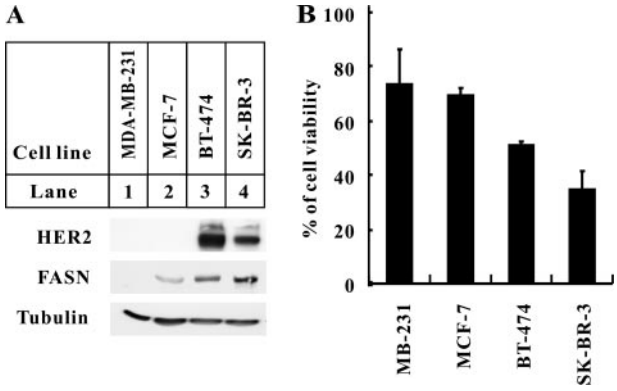

FIGURE 1. **FASN is important for cell survival in HER2-overexpressing breast cancer cells.** *A*, MDA-MB-231, MCF-7, BT-474, and SK-BR-3 cells were cultured in medium containing 10% fetal bovine serum and cell lysates were prepared as described under "Experimental Procedures." Lysates were subjected to SDS-PAGE for immunoblot analysis using polyclonal antibodies against HER2 and FASN. Tubulin was used as a loading control. *B*, the same cell lines shown in *A* were incubated in the presence or absence of 10 μg/ml cerulenin for 24 h. Cell viability was measured as described under "Experimental Procedures," and shown as % of viability of cerulenin-treated cells compared with that of vehicle-treated cells. The data are represented as the mean ± S.D. of triplicate incubations. Similar results were obtained in three independent experiments.

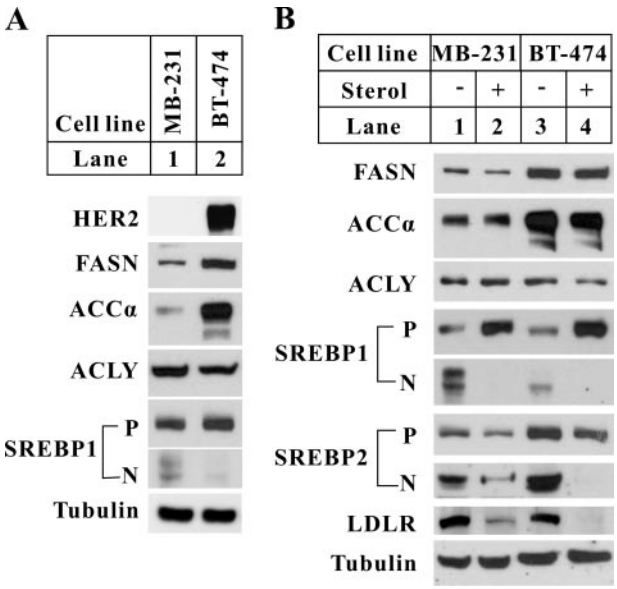

FIGURE 2. **SREBP-1 is not an essential factor for the induction of ACCα or FASN in HER2-overexpressing breast cancer cells.** Cell lysates of MDA-MB-231 and BT-474 cells were prepared as described under "Experimental Procedures." *A*, cells were grown in medium supplemented with 10% fetal bovine serum. *B*, cells were plated in medium supplemented with 10% fetal bovine serum on day 0. On day 2, medium was replaced with the fresh medium supplemented with 10% delipidated serum. Cells were incubated overnight in the absence (*sterol* −) or presence (*sterol* +) of cholesterol (26 μM) and 25-hydroxycholesterol (2.5 μM). The cell lysates were subjected to SDS-PAGE and immunoblot analyses were carried out with antibodies against SREBP-1, SREBP-2, ACLY, FASN, ACCα, LDLR, HER2, and tubulin. *P* and *N* denote the precursor and nuclear forms, respectively, of SREBP-1 or SREBP-2. Similar results were obtained in three independent experiments.

SREBP-1 Is Not the Major Regulator for the Expression of Lipogenic Enzymes in Breast Cancer Cells—To study the role of SREBP-1 on HER2-related increases in FASN, we compared the relative amounts of FASN, ACCα, ACLY, and SREBP-1 in BT-474 and MDA-MB-231 cells (Fig. 2*A*). Whereas certain studies have reported that SREBP-1c is the mediator that

induces the transcription of *FASN* in cancer cells (3, 41), the amounts of the precursor form of SREBP-1 were very similar in both cell lines. The amount of the nuclear form of SREBP-1, which has transcriptional activity, was significantly lower in BT-474 compared with MDA-MB-231 cells. However, the expression levels of ACCα and FASN were significantly increased in BT-474 cells compared with MDA-MB-231 cells. ACLY, which is one of lipogenic enzymes that is regulated by SREBP-1, shows no significant differences between these two cell lines. This result suggests that the overexpression of FASN and ACCα, shown in BT-474 cells, is not mediated by the activation of SREBP-1.

To further study whether the expression of FASN, ACCα, and ACLY is affected by the sterol-dependent control of SREBP processing, immunoblot analyses were performed in cells grown in either the presence or absence of cholesterol/25-hydroxycholesterol supplemented with 10% delipidated serum (Fig. 2*B*). Deprivation of sterol significantly increased the levels of the nuclear forms of SREBP-1 and SREBP-2 in both cell lines (compare Fig. 2*B*, *lanes 1* and *3*, with *A*, *lanes 1* and *2*), whereas the addition of sterol almost completely blocked the processing of SREBP-1 and SREBP-2, resulting in the depletion of the nuclear forms of SREBPs (Fig. 2*B*, *lanes 2* and *4*). As expected, sterols significantly down-regulated the amount of low density lipoprotein receptor, one of the target genes of SREBP-2 in both cell lines, as the nuclear form of SREBP-2 disappeared (Fig. 2*B*, *lanes 2* and *4*). However, the expression of FASN, ACCα, and ACLY was not significantly changed by the presence of sterols. These results strongly suggest that neither SREBP-1 nor -2 is the major effector that increases the expression of FASN and ACCα in BT-474 cells that overexpress HER2.

The PI3K/Akt/mTOR Signaling Pathway Is Involved in the Induction of FASN and ACCα by HER2—To examine whether HER2 overexpression changes the rate of transcription and/or translation of *FASN*, *ACCα*, and *ACLY*, their protein and mRNA levels were determined in MDA-MB-231 cells that were transduced with adenoviruses expressing HER2 (AdHER2). Overexpression of HER2 markedly increased the protein levels of ACCα and FASN, whereas that of ACLY remained unchanged (Fig. 3*A*, *lanes 1* and *2*). However, the mRNA levels for *FASN*, *ACCα*, and *ACLY* were not significantly changed by HER2 overexpression (Fig. 3*B*). When HER2 signaling was blocked by Herceptin, a humanized monoclonal antibody against HER2, the HER2-mediated induction of FASN and ACCα was completely abolished (Fig. 3*A*, *lane 4*) as expected. These results suggest that the induction of FASN and ACCα by HER2 overexpression take place at a post-transcriptional level. To determine whether HER2-mediated induction of ACCα and FASN proteins is mediated through the PI3K/Akt pathway, MDA-MB-231 cells infected with AdHER2 were treated with LY294002, a PI3K inhibitor. The induction of FASN and ACCα transduced by AdHER2 was completely blocked by LY294002 treatment (Fig. 3*A*, *lane 3*), implying that PI3K signaling is involved in the HER2-mediated induction of FASN and ACCα.

The PKI/Akt signals that are triggered by HER2 are able to activate the mTOR signaling pathway, which can regulate the translation of several genes (42–44). To determine whether the induction of FASN and ACCα by HER2 results from the activation of the PI3K/Akt/mTOR pathway, cells were treated with rapamycin. The induction of ACCα and FASN by HER2 was blocked by treatment with rapamycin as well as Herceptin (Fig. 4*A*). Treatment of BT-474 cells with LY294002, rapamycin, or Herceptin also resulted in the decreased expression of FASN and ACCα without a concomitant change in the levels of ACLY (Fig. 4*B*), suggesting that PI3K/Akt and mTOR mediate increased FASN and ACCα levels by endogenous HER2 overexpression. Phosphorylation of the S6 protein, one of the targets of mTOR (Fig. 4*B*), was blocked by these treatments as well. However, the mRNA levels of *FASN* and *ACCα* were not altered by either Ly294002 or rapamycin (Fig. 4*C*). Silencing of *mTOR* by siRNA also led to a marked suppression of FASN and ACCα, in contrast to a slight reduction in ACLY expression (Fig. 4*D*).

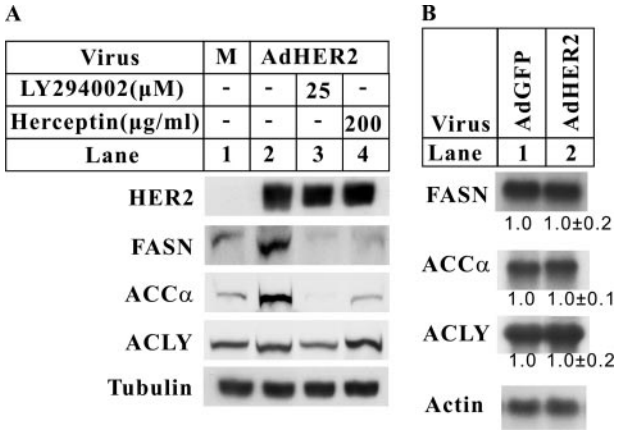

FIGURE 3. **The effects of PI3K inhibitors on the induction of ACCα and FASN by HER2.** *A*, MDA-MB-231 cells, transduced with AdGFP or AdHER2 for 24 h, were treated with 25 μM LY294002 or 200 μg/ml Herceptin for 24 h. The cell lysates were prepared and immunoblot analyses were carried out with antibodies against ACLY, ACCα, FASN, HER2, or tubulin. *B*, 24 h after transduction of MDA-MB-231 cells with AdGFP or AdHER-2, total RNA was isolated and subjected to RT-PCR and Northern blot analysis using ^{32}P-labeled cDNA probes for human *ACLY*, *ACCα*, *FASN*, or *β-actin*. The *numbers below* the figure denote the relative amounts of mRNAs calculated using the comparative C_t method. *β-Actin* mRNA was used as an invariant control.

To determine whether the expression of FASN and ACCα can be induced by mTOR activation in the absence of HER2/PI3K/Akt signals, changes in both FASN and ACCα were analyzed in MDA-MB-231 cells that were transduced with adenovirus that overexpressed RHEB. RHEB, a small GTP-binding protein, is directly upstream of mTOR and its induced expression activates mTOR (45, 46). Overexpression of RHEB in MDA-MB-231 cells increased total mTOR levels as well as the phosphorylated form of mTOR (Fig. 5*A*). RHEB-mediated mTOR activation is accompanied by increases in the levels of both FASN and ACCα, whereas no significant changes in ACLY expression were observed (Fig. 5*A*). The induction of FASN and ACCα is thought to be regulated at a post-transcriptional level because their mRNA levels were unchanged (Fig. 5*B*).

To determine whether the mTOR-mediated increase in FASN and ACCα is due to an increase in translation and/or a decrease in the degradation of FASN and ACCα proteins, metabolic labeling assays were performed in MDA-MB-231 cells transduced with AdRHEB. The overexpression of RHEB significantly increased the rate of protein synthesis of FASN and

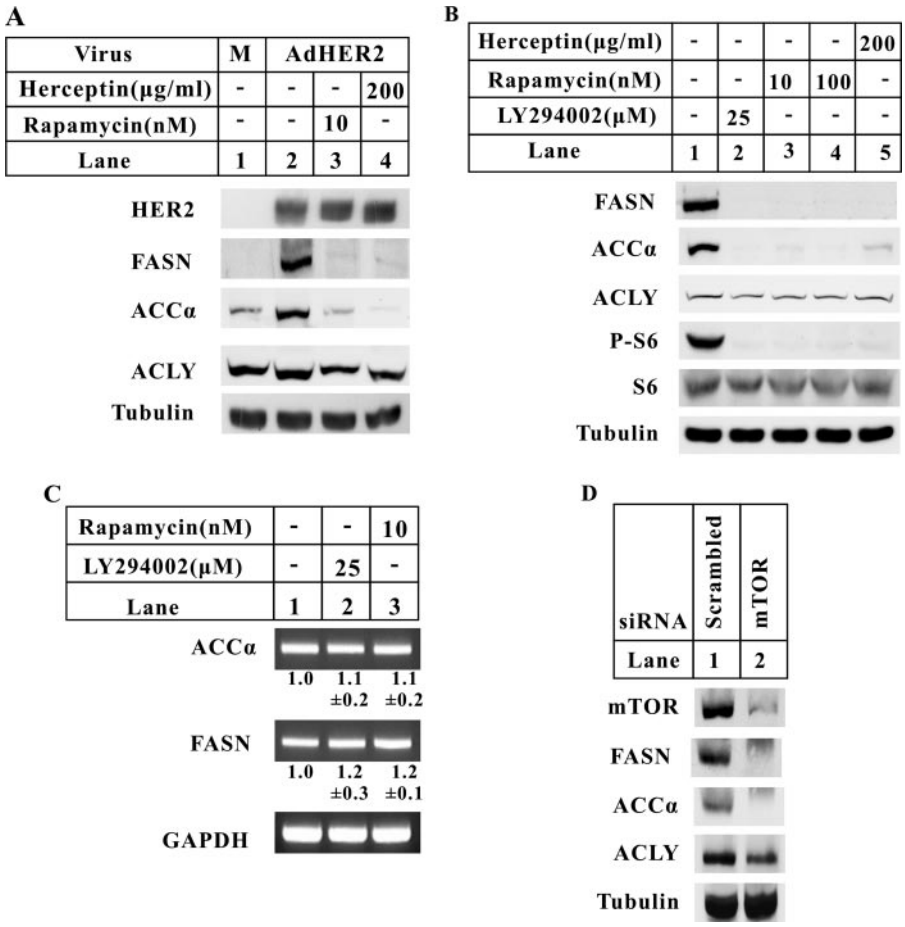

FIGURE 4. **The effects of rapamycin and mTOR siRNA on the induction of ACCα and FASN.** *A*, AdHER2-transduced MDA-MB-231 cells were treated with 10 nM rapamycin or 200 μg/ml Herceptin for 24 h. *B*, BT-474 cells that overexpress HER2 endogenously were treated with 25 μM LY294002, 10 or 100 nM rapamycin, or 200 μg/ml Herceptin for 24 h. The cell lysates were prepared and immunoblot analyses were carried out with antibodies against ACLY, ACCα, FASN, HER2, S6 protein, phospho-S6 protein, or tubulin. *C*, total RNA was prepared from BT-474 cells treated with 25 μM LY294002 or 10 nM rapamycin and RT-PCR was carried out as described under "Experimental Procedures." The *numbers below* the figure denote the relative amounts of mRNAs calculated using the comparative C_t method. *GAPDH* was used as an invariant control. *D*, BT-474 cells were transfected with 100 pmol of scrambled siRNA or mTOR siRNA as described under "Experimental Procedures." Forty-eight hours after transfection, cell lysates were prepared and immunoblot analyses were carried out with antibodies against mTOR, ACCα, FASN, ACLY, or tubulin. Similar results were obtained from two independent experiments.

ACCα, but no significant differences in the levels of ACLY synthesized were found between the cells expressing either GFP or RHEB (Fig. 6*A*). The degradation of ACCα and ACLY were not significantly changed by RHEB overexpression, and the degradation of FASN was increased 4 h after radioisotope labeling (Fig. 6*B*). These results suggest that translational activation via mTOR is the primary mechanism by which the expression of FASN and ACCα is increased in breast cancer cells that overexpress HER2.

The 5′- and 3′-UTRs of ACCα and FASN mRNAs Are Involved in Their Translational Activation by mTOR—To elucidate the mechanism by which FASN and ACCα are activated by mTOR signaling, we hypothesized that either the 5′- or 3′-UTRs of *ACCα* and *FASN* mRNAs might play putative roles in the regulation of their expression. The 5′- and/or 3′-UTR sequences were inserted either upstream or downstream of the luciferase gene in a SV40 promoter-driven reporter construct (pGL3 promoter) as shown in Fig. 7*A*. Insertion of the 5′-UTRs of either *FASN* (100 bp) or *ACLY* (101 bp) markedly increased luciferase activity (Fig. 7, *B* and *D*, *white bars*). The transcription of *ACCα* can be driven by two promoters, P-I and P-II, which generate two different transcripts containing either exon 1 or exon 2 sequences (47, 48). Because most breast cancer cell lines generate both transcripts (data not shown), both 5′-UTRs, designated Ex1 and Ex2 in the present study, were tested. The constructs for Ex1 and Ex2 contained exon 1 and 2 sequences, respectively, followed by exon 4 and a part of exon 5 of *ACCα* mRNA. Exon 1 in Ex1 is relatively long (~550 bp) and has a high GC content and multiple ATG codons, whereas exon 2 in Ex2 is short (~120 bp) and lacks ATG codons. The 5′-UTR in Ex1 strongly inhibited luciferase activity, whereas the 5′-UTR in Ex2 showed an approximate 6.4-fold stimulation of luciferase activity compared with pGL3p (Fig. 7*C*, *white bars*). Overexpression of RHEB further increased the luciferase activities of the constructs with the 5′-UTR of FASN and Ex2 of *ACCα* by 2.3- and 1.7-fold, respectively. However, the 5′-UTR of *ACLY* or Ex1 of *ACCα* did not respond to RHEB overexpression (Fig. 7, *B–D*, *black bars*). The 3′-UTRs of *FASN* and *ACCα* slightly reduced basal luciferase activities (Fig. 7, *B–D*, *white bars*) compared with the luciferase activity of pGL3p; however, they significantly increased luciferase activities by 1.7- and 2.0-fold, respectively, in response to RHEB overexpression (Fig. 7, *B* and *C*, *black bars*). The 3′-UTR of *ACLY* also reduced basal luciferase activity slightly; however, RHEB overexpression did not affect luciferase activity (Fig. 7*D*). The combination of the 5′- and 3′-UTRs of *FASN* attenuated the increase in the basal luciferase activity driven by the 5′-UTR; however, RHEB overexpression increased luciferase activity 3.7-fold (Fig. 7*B*, *5′* and *3′* columns). The presence of Ex2 and the 3′-UTR of *ACCα* showed additive increases in luciferase activity by 3.9-fold in response to RHEB overexpression (Fig. 7*C*, *Ex2* and *-3′* columns, *black bar*). The mRNA levels of the luciferase gene remained unchanged between the constructs containing different UTRs or when RHEB was overexpressed (Fig. 7*E*). The possibility of amplification from transfected plasmid DNA during PCR was excluded by confirming that the

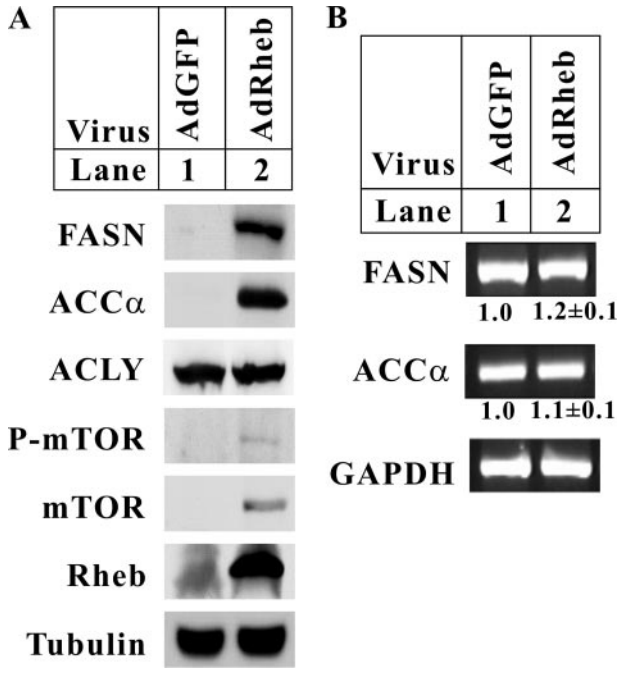

FIGURE 5. **Induction of ACCα and FASN by RHEB overexpression in MDA-MB-231 cells.** *A*, MDA-MB-231 cells were transduced with AdGFP or AdRHEB for 48 h and cell lysates were prepared as described under "Experimental Procedures." Immunoblot analyses were carried out with antibodies against ACLY, ACCα, FASN, mTOR, phospho-mTOR, RHEB, or tubulin. *B*, total RNAs from the cells were prepared 48 h after transfection and RT-PCR was performed as described under "Experimental Procedures." The *numbers below* the figure denote the relative amounts of mRNAs calculated using the comparative C_t method. *GAPDH* was used as an invariant control.

SV40 promoter sequence was not amplified. These results suggest that the UTRs of *FASN* and *ACCα* play important roles in the regulation of gene expression at a translational level, and that this translational activation renders cells responsive to RHEB overexpression.

To further study whether the UTRs of *FASN* and *ACCα* could induce gene expression in breast cancer cells that overexpress HER2, transient transfection in BT474 cells was performed with the reporter constructs bearing both the 5′- and 3′-UTR sequences of *FASN*, *ACCα*, and *ACLY*. The luciferase activities of pGL3p and the construct containing the UTRs of *ACLY* were not significantly affected by rapamycin. However, the increase in the luciferase activities driven by the UTRs of *FASN* and *ACCα* Ex2 were significantly repressed by rapamycin (Fig. 8). These results suggest that the UTRs of *FASN* and *ACCα* mRNA contribute to the mTOR-dependent translational activation of both *ACCα* and *FASN* mRNAs.

DISCUSSION

Mammalian FASN is a multifunctional enzyme that catalyzes *de novo* biosynthesis of palmitate (49). The substrates for this anabolic enzyme, such as acetyl-CoA and malonyl-CoA, are supplied by the serial reactions catalyzed by ACLY and ACCα. Many studies have shown that the levels of these lipogenic enzymes are dramatically but tightly regulated in liver and adipose tissues at the transcriptional level, and that SREBP-1 is reported to be indispensable for the nutritional regulation of these lipogenic enzymes (12). Although the transduction pathways that regulate the expression of FASN in normal and cancer cells appear to share identical downstream elements, the

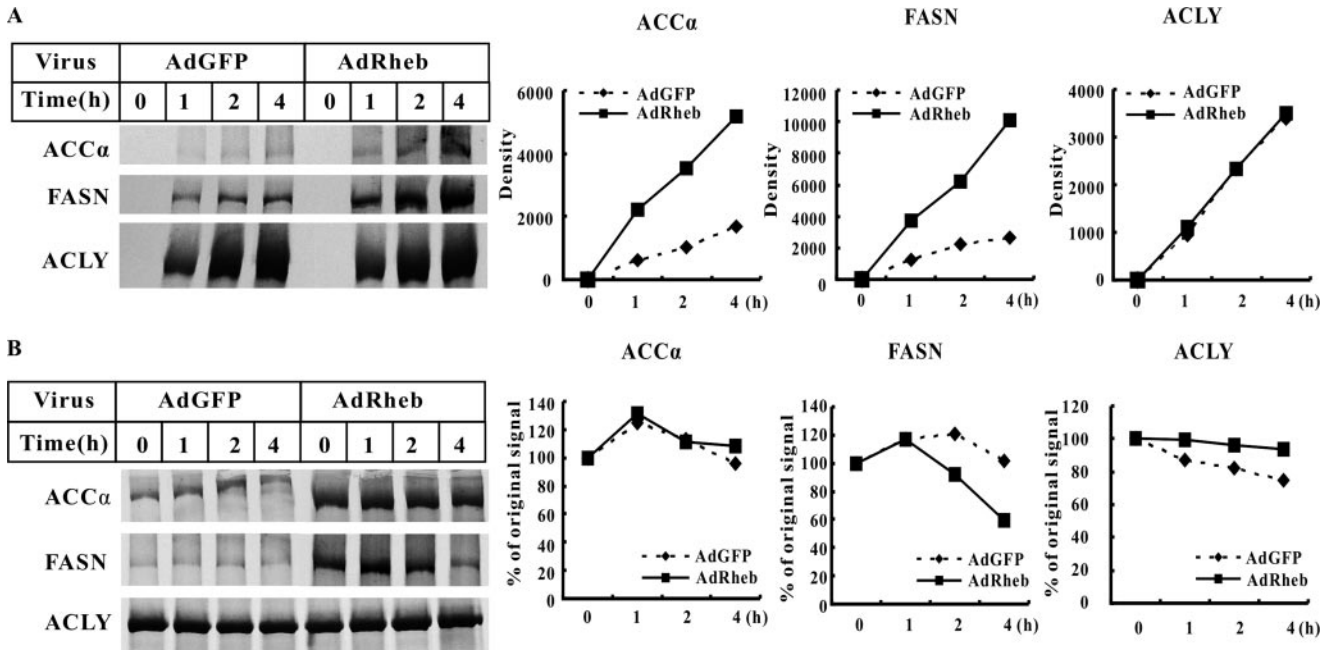

FIGURE 6. **Synthesis and degradation of ACLY, ACCα, and FASN in MDA-MB-231 cells transduced with AdRHEB.** *A*, MDA-MB-231 cells were transduced with AdGFP or AdRHEB and preincubated in Met/Cys-free Dulbecco's modified Eagle's medium for 1 h. After preincubation, [^{35}S]Met-Cys was added and incubated for the indicated times. After labeling, aliquots of the cell lysates were used for immunoprecipitation with antibodies against ACLY, ACCα, or FASN as described under "Experimental Procedures." *B*, AdGFP- and AdRHEB-transduced MDA-MB-231 cells were pretreated with [^{35}S]Met-Cys for 4 h and then the cells were rinsed and incubated in medium containing 2 mM L-methionine and 20 μg/ml cycloheximide for the indicated times prior to preparation of the cell lysates. Immunoprecipitation was performed with antibodies against ACLY, ACCα, or FASN. The protein-antibody complexes were precipitated using protein A-agarose and subjected to 6% SDS-PAGE. The gel was dried and autoradiography was performed. Similar results were obtained in three independent experiments.

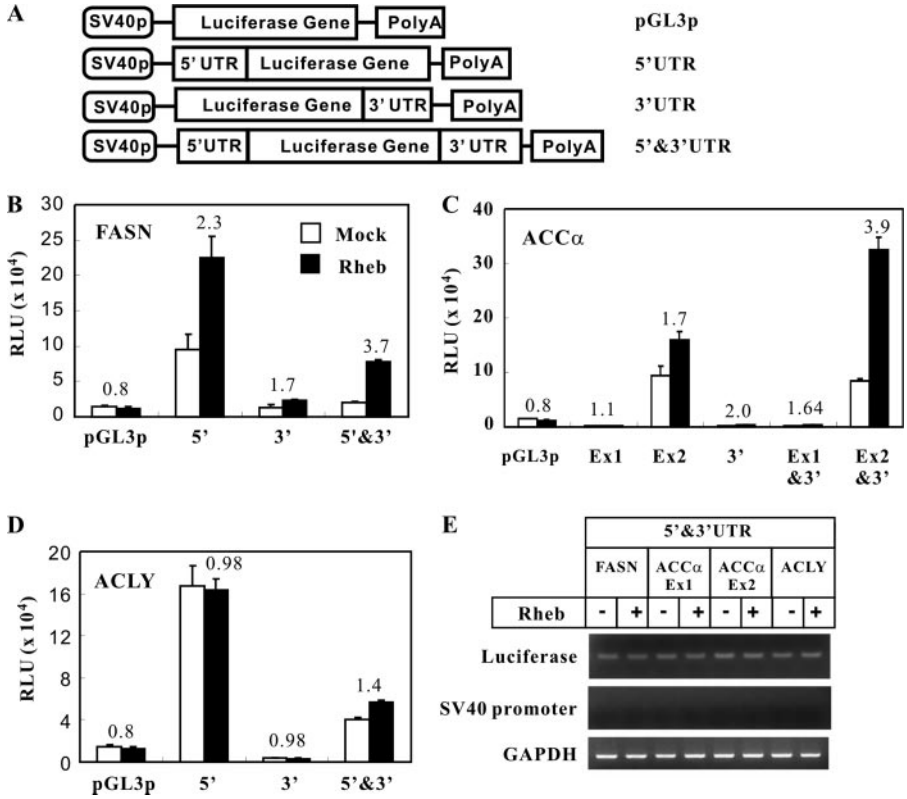

FIGURE 7. **Translational activation of reporter expression by 5′- and/or 3′-UTRs of *FASN* or *ACCα* mRNAs in MDA-MB-231 cells.** *A*, a diagrammatic representation of the pGL3 promoter (*pGL3p*) and its derivative constructs containing the 5′-UTR, 3′-UTR, or both. The pGL3p plasmid contains the SV40 promoter upstream of the luciferase gene and was used as a negative control. The DNA fragments for the 5′-UTR, 3′-UTR, or both (*5′&3′ UTR*) sequences were inserted immediately upstream or downstream of the luciferase gene in pGL3p as described under "Experimental Procedures." *B–D*, MDA-MB-231 cells transduced with either AdGFP or AdRHEB were transiently transfected with pGL3p, 5′-UTR, 3′-UTR, or 5′ and 3′-UTR constructs of *FASN*, *ACCα*, or *ACLY* as indicated in the figure. In *C*, Ex1 and Ex2 represent the two 5′-UTRs of *ACCα*, which contain either exon 1 or exon 2 sequences, respectively. Aliquots of the cell lysates were analyzed 48 h after transfection for luciferase activity. Each result represents the mean ± S.D. of triplicate incubations. The *numbers above* the *black bars* express the -fold increase induced by RHEB overexpression. *E*, the mRNA levels of luciferase transcripts were measured by RT-PCR using poly(A)-rich RNA isolated from the cells transfected with 5′ and 3′-UTR constructs as indicated in the figure. *GAPDH* cDNA was also amplified as a control to demonstrate that equal amounts of total RNA were used for RT-PCR. To show that the luciferase cDNA amplified by RT-PCR was not derived from the transfected plasmid, the primers for the SV40 promoter region were used for PCR.

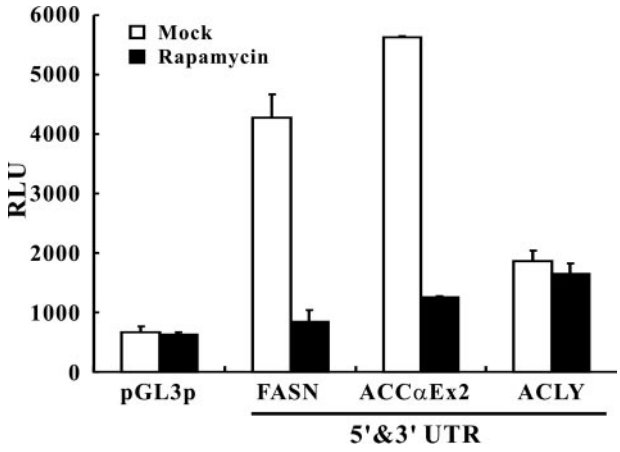

FIGURE 8. **The effects of rapamycin on the translational activation of the reporter gene by the UTRs of FASN and ACCα.** BT-474 cells were transfected with reporter constructs containing both 5′- and 3′-UTRs of *FASN*, *ACCα* (Ex2), and *ACLY* cDNAs with pCMV-β-galactosidase as an internal control; cells were then treated with Me_2SO (*blank bars*) or 100 nM rapamycin (*filled bars*) for 24 h. The cell lysates were analyzed by luciferase assay. Relative luciferase units (*RLU*), normalized to β-galactosidase activity, represent the mean ± S.E. from three independent experiments.

upstream mechanisms that control the expression of FASN in cancer cells, however, must be different from those in normal tissues because cancer-associated FASN expression appears to be insensitive to nutritional signals (3). In prostate cancer cells, the expression of FASN was increased by both androgens and growth factors, and the mechanism for this induction was demonstrated to be the transcriptional regulation mediated by SREBP-1 activation (50, 51). In breast cancer cells, the overexpression of FASN is associated with HER2 amplification and/or overexpression (8, 17), which is considered to be a poor prognostic marker. Kumar-Sinha *et al.* (17) reported that HER2 overexpression increased the transcription of FASN via the PI3K signal pathway. Taken together, transcriptional control has been hypothesized to be the primary mechanism by which FASN is induced in breast cancer cells that overexpress HER2 and SREBP-1 is thought to be a key regulator in this process (3). In the present study, we demonstrated that the HER2-associated induction of FASN and ACCα was achieved through the translational activation mediated by mTOR via the PI3K/Akt pathway in breast cancer cells and not by the transcriptional activation through SREBPs. This translational activation was confined to FASN and ACCα because the other lipogenic enzymes, such as ACLY, malic enzyme, or glucose-6-phosphate dehydrogenase, showed no association with HER2 overexpression (data not shown).

The activation of the Akt/mTOR pathway has been found in breast cancers with high expression levels of HER2, suggesting possible roles of the Akt/mTOR pathway in HER2-mediated tumor progression (31). PKB/Akt was previously demonstrated to protect cancer cells from tamoxifen-induced apoptosis (52). Recently, the activation of mTOR, a downstream effector of the PKB/Akt signal, was reported to play a critical role in the development of hormone-refractory phenotypes in certain breast cancer cells. Accordingly, the inhibition of mTOR by rapamycin resulted in the restoration of sensitivity to tamoxifen (32). The induction of FASN and ACCα by the mTOR signaling pathway shown in the present study suggests that FASN and ACCα are the important target molecules in the HER2/PI3K signaling pathway in breast cancer. It was also reported that the activation of mTOR increased overall translation rates by inactivating eIF4E-binding protein, which in turn activated eIF-4E,

and by activating the 70-kDa subunit of S6 kinase 1, resulting in increasing the synthesis of ribosomal proteins. In the present study, we demonstrated that the activation of mTOR induced by HER2-overexpression led to a selective increase in the translation of FASN and ACCα and not an overall increase in the rate of protein synthesis.

The selective translational controls for CXCR4 by mTOR were previously reported in breast cancer cells (53), which are implicated in tumor metastasis (54). Src and c-myc could be increased by translational controls via the activated Akt/mTOR pathway (44). The 5′-UTR derived from the major promoter of the c-myc gene, P2, mediated translational activation by HER2 signal (42). However, the precise mechanisms by which these selective translational controls are achieved by the mTOR signal require elucidation (42, 55). We, in this study, have found that the 5′-UTR of *FASN* significantly activated gene expression at the translational level, and mTOR activation by RHEB overexpression further augmented this effect. The *ACCα* gene has multiple promoters, including P-I, P-II, and P-III (47, 48). The P-I promoter is unique to humans and produces relatively long 5′-UTRs with multiple ATG codons in its transcript (48). The P-I promoter was shown to be constitutively active in breast cancer cell lines, including MDA-MB-231 and BT-474 (data not shown). The P-II promoter is reported to be regulated by various hormones, including insulin and thyroid hormone, and is highly conserved between species (42). In this study, it was shown that the 5′-UTR (Ex2) derived from *ACCα* P-II strongly activated reporter expression, whereas the 5′-UTR (Ex1) derived from the P-I promoter showed significant suppression of reporter expression as well as non-responsiveness to RHEB overexpression. The 5′-UTR derived from P-III was not evaluated for its transcriptional activation in this study because the anti-ACCα antibody used in this study was raised against 74 amino acids at the NH_2 terminus of ACCα, which is not encoded by transcripts derived from the P-III promoter. The 5′-UTRs derived from P-II also increased reporter expression when mTOR was activated by RHEB overexpression. All 3′-UTRs of *FASN*, *ACCα*, and *ACLY* decreased luciferase activity to a slight degree. However, the 3′-UTRs of *FASN* and *ACCα* may play roles in mTOR-mediated translational activation, but not the 3′-UTR of *ACLY*. These UTR-mediated changes in luciferase activity were shown to result from translational control rather than transcriptional control because the mRNA levels of luciferase were almost identical between the constructs harboring different UTRs.

At present, most of the studies on the regulation of lipogenic enzymes by hormones in lipogenic organs have been focused primarily on the mechanism at the level of transcription. In this study, we demonstrated that ACCα and FASN could be selectively up-regulated at the translational level by the HER2/PI3K/Akt pathway via mTOR activation in breast cancer cells, and that the 5′- and 3′-UTRs of their transcripts play important roles in this regulation. These results suggest that the expression of ACCα and FASN might be regulated at translational levels as well as at transcriptional or post-translational levels in normal lipogenic organs. It also suggests that aberrant translational regulation might contribute to pathological conditions, that is, the constitutive overexpression of lipogenic enzymes as in insulin-resistant diabetes.

REFERENCES

1. Kuhajda, F. P., Jenner, K., Wood, F. D., Hennigar, R. A., Jacobs, L. B., Dick, J. D., and Pasternack, G. R. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 6379–6383
2. Kuhajda, F. P. (2000) *Nutrition* **16,** 202–208
3. Menendez, J. A., and Lupu, R. (2006) *Curr. Opin. Clin. Nutr. Metab. Care* **9,** 346–357
4. Wakil, S. J., Stoops, J. K., and Joshi, V. C. (1983) *Annu. Rev. Biochemistry* **52,** 537–579
5. Jensen, V., Ladekarl, M., Holm-Nielsen, P., Melsen, F., and Soerensen, F. B. (1995) *J. Pathol.* **176,** 343–352
6. Kuhajda, F. P., Piantadosi, S., and Pasternack, G. R. (1989) *N. Engl. J. Med.* **321,** 636–641
7. Milgraum, L. Z., Witters, L. A., Pasternack, G. R., and Kuhajda, F. P. (1997) *Clin. Cancer Res.* **3,** 2115–2120
8. Menendez, J. A., Mehmi, I., Atlas, E., Colomer, R., and Lupu, R. (2004) *Int. J. Oncol.* **24,** 591–608
9. Menendez, J. A., Mehmi, I., Verma, V. A., Teng, P. K., and Lupu, R. (2004) *Mol. Carcinog.* **41,** 164–178
10. Menendez, J. A., Vellon, L., Colomer, R., and Lupu, R. (2005) *Int. J. Cancer* **115,** 19–35
11. Menendez, J. A., Vellon, L., Mehmi, I., Oza, B. P., Ropero, S., Colomer, R., and Lupu, R. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 10715–10720
12. Horton, J. D., Goldstein, J. L., and Brown, M. S. (2002) *J. Clin. Investig.* **109,** 1125–1131
13. Menendez, J. A., Colomer, R., and Lupu, R. (2005) *Med. Hypotheses* **64,** 342–349
14. Yang, Y. A., Morin, P. J., Han, W. F., Chen, T., Bornman, D. M., Gabrielson, E. W., and Pizer, E. S. (2003) *Exp. Cell Res.* **282,** 132–137
15. Yang, Y. A., Han, W. F., Morin, P. J., Chrest, F. J., and Pizer, E. S. (2002) *Exp. Cell Res.* **279,** 80–90
16. Martel, P. M., Bingham, C. M., McGraw, C. J., Baker, C. L., Morganelli, P. M., Meng, M. L., Armstrong, J. M., Moncur, J. T., and Kinlaw, W. B. (2006) *Exp. Cell Res.* **312,** 278–288
17. Kumar-Sinha, C., Ignatoski, K. W., Lippman, M. E., Ethier, S. P., and Chinnaiyan, A. M. (2003) *Cancer Res.* **63,** 132–139
18. Alimandi, M., Romano, A., Curia, M. C., Muraro, R., Fedi, P., Aaronson, S. A., Di Fiore, P. P., and Kraus, M. H. (1995) *Oncogene* **10,** 1813–1821
19. Gullick, W. J., and Srinivasan, R. (1998) *Breast Cancer Res. Treat.* **52,** 43–53
20. Toikkanen, S., and Joensuu, H. (1992) *J. Clin. Oncol.* **10,** 1817
21. Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A., and McGuire, W. L. (1987) *Science* **235,** 177–182
22. Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ullrich, A., and Press, M. F. (1989) *Science* **244,** 707–712
23. Dendukuri, N., Khetani, K., McIsaac, M., and Brophy, J. (2007) *Can. Med. Assoc. J.* **176,** 1429–1434
24. Yu, D., Jing, T., Liu, B., Yao, J., Tan, M., McDonnell, T. J., and Hung, M. C. (1998) *Mol. Cell* **2,** 581–591
25. Slamon, D. J., Leyland-Jones, B., Shak, S., Fuchs, H., Paton, V., Bajamonde, A., Fleming, T., Eiermann, W., Wolter, J., Pegram, M., Baselga, J., and Norton, L. (2001) *N. Engl. J. Med.* **344,** 783–792
26. Hay, N., and Sonenberg, N. (2004) *Genes Dev.* **18,** 1926–1945
27. Harris, T. E., and Lawrence, J. C., Jr. (2003) *Sci. STKE* 2003, re15
28. Dufner, A., and Thomas, G. (1999) *Exp. Cell Res.* **253,** 100–109
29. Proud, C. G., Wang, X., Patel, J. V., Campbell, L. E., Kleijn, M., Li, W., and Browne, G. J. (2001) *Biochem. Soc. Trans.* **29,** 541–547
30. Proud, C. G. (2004) *Biochem. Biophys. Res. Commun.* **313,** 429–436
31. Zhou, X., Tan, M., Stone Hawthorne, V., Klos, K. S., Lan, K. H., Yang, Y., Yang, W., Smith, T. L., Shi, D., and Yu, D. (2004) *Clin. Cancer Res.* **10,** 6779–6788
32. deGraffenried, L. A., Friedrichs, W. E., Russell, D. H., Donzis, E. J., Middle-

ton, A. K., Silva, J. M., Roth, R. A., and Hidalgo, M. (2004) *Clin. Cancer Res.* **10,** 8059–8067
33. Johnston, S. R. (2005) *Clin. Cancer Res.* **11,** 889s–899s
34. Mondesire, W. H., Jian, W., Zhang, H., Ensor, J., Hung, M. C., Mills, G. B., and Meric-Bernstam, F. (2004) *Clin. Cancer Res.* **10,** 7031–7042
35. Kim, K. S., Park, S. W., and Kim, Y. S. (1992) *Yonsei Med. J.* **33,** 199–208
36. Oh, S. Y., Park, S. K., Kim, J. W., Ahn, Y. H., Park, S. W., and Kim, K. S. (2003) *J. Biol. Chem.* **278,** 28410–28417
37. Gansler, T. S., Hardman, W., 3rd, Hunt, D. A., Schaffel, S., and Hennigar, R. A. (1997) *Hum. Pathol.* **28,** 686–692
38. Menendez, J. A., Lupu, R., and Colomer, R. (2004) *Breast Cancer Res. Treat.* **84,** 183–195
39. D'Agnolo, G., Rosenfeld, I. S., Awaya, J., Omura, S., and Vagelos, P. R. (1973) *Biochim. Biophys. Acta* **326,** 155–156
40. Vance, D., Goldberg, I., Mitsuhashi, O., and Bloch, K. (1972) *Biochem. Biophys. Res. Commun.* **48,** 649–656
41. Swinnen, J. V., Brusselmans, K., and Verhoeven, G. (2006) *Curr. Opin. Clin. Nutr. Metab. Care* **9,** 358–365
42. Galmozzi, E., Casalini, P., Iorio, M. V., Casati, B., Olgiati, C., and Menard, S. (2004) *J. Cell. Physiol.* **200,** 82–88
43. Koziczak, M., and Hynes, N. E. (2004) *J. Biol. Chem.* **279,** 50004–50011
44. Tan, M., Li, P., Klos, K. S., Lu, J., Lan, K. H., Nagata, Y., Fang, D., Jing, T., and Yu, D. (2005) *Cancer Res.* **65,** 1858–1867
45. Castro, A. F., Rebhun, J. F., Clark, G. J., and Quilliam, L. A. (2003) *J. Biol. Chem.* **278,** 32493–32496
46. Inoki, K., Li, Y., Xu, T., and Guan, K. L. (2003) *Genes Dev.* **17,** 1829–1834
47. Lopez-Casillas, F., and Kim, K. H. (1989) *J. Biol. Chem.* **264,** 7176–7184
48. Mao, J., Chirala, S. S., and Wakil, S. J. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 7515–7520
49. Wakil, S. J. (1989) *Biochemistry* **28,** 4523–4530
50. Heemers, H., Maes, B., Foufelle, F., Heyns, W., Verhoeven, G., and Swinnen, J. V. (2001) *Mol. Endocrinol.* **15,** 1817–1828
51. Swinnen, J. V., Heemers, H., Deboel, L., Foufelle, F., Heyns, W., and Verhoeven, G. (2000) *Oncogene* **19,** 5173–5181
52. Jordan, N. J., Gee, J. M., Barrow, D., Wakeling, A. E., and Nicholson, R. I. (2004) *Breast Cancer Res. Treat.* **87,** 167–180
53. Li, Y. M., Pan, Y., Wei, Y., Cheng, X., Zhou, B. P., Tan, M., Zhou, X., Xia, W., Hortobagyi, G. N., Yu, D., and Hung, M. C. (2004) *Cancer Cell* **6,** 459–469
54. Muller, A., Homey, B., Soto, H., Ge, N., Catron, D., Buchanan, M. E., McClanahan, T., Murphy, E., Yuan, W., Wagner, S. N., Barrera, J. L., Mohar, A., Verastegui, E., and Zlotnik, A. (2001) *Nature* **410,** 50–56
55. West, M. J., Stoneley, M., and Willis, A. E. (1998) *Oncogene* **17,** 769–780