Sp1 is a basic transcriptional factor that binds to the GC-rich region in the promoter of the target gene. It is involved in transcription of numerous genes by recruiting transcriptional factors to the promoters of target genes. In this study, we found in vivo and in vitro that Hsp90α was recruited to the GC-rich region of the 12(S)-lipoygenase promoter through interaction with Sp1 in A431 cells by employing DNA affinity immunoprecipitation assay and chromatin immunoprecipitation assay. When Hsp90α was inhibited by geldanamycin (GA, a specific inhibitor of the Hsp90 family) or by siRNA of Hsp90α to block its activity or to knockdown protein levels, respectively, luciferase activity (driven by the 12(S)-lipoygenase promoter) and both mRNA and protein levels of 12(S)-lipoygenase were reduced significantly in cells. In addition, the effect of GA was abolished when the Sp1 binding sites of 12(S)-lipoygenase were mutated in A431 cells. Interestingly, binding of Sp1 to the 12(S)-lipoygenase promoter was also decreased upon GA treatment in cells. In conclusion, our results indicate that Sp1 interacts with Hsp90α to recruit it to the promoter of 12(S)-lipoygenase and then to regulate gene transcription by modulating the binding ability of Sp1 to promoters.

Sp1 is one of the first transcription factors purified and cloned from mammalian cells (1, 2). It can recognize and specifically bind to GC-rich sites within the simian virus 40 promoter via three Cys2His2 zinc finger motifs localized at its C-terminal region to regulate the transcription of the target genes (3, 4). In addition to the zinc finger domain of the C-terminal region, the N-terminal regions of the Sp1 are much more variable and contain transcriptional activation or repression domains (5, 6). Sp1 is generally considered as a factor that primarily determines the core activity of the promoter by direct interaction with other factors of the basal transcriptional machinery and by cooperation with several transcriptional activators such as CRSP, p300/CBP, steroidogenic factor-1 (SF-1), vitamin D3 receptor, and TAFII130 (7–12). Recent studies reveal that both DNA binding activity and transactivational activity of Sp1 may be influenced by the post-translational modification of Sp1 such as phosphorylation, glycosylation, and acetylation (13, 14). Previous studies indicate that Sp1 is phosphorylated by casein kinase II, which has been reported to repress Sp1 activity, and by DNA-dependent protein kinase and CDKII, to positively regulate the transactivity and DNA binding affinity of Sp1 (15–18). In addition, glycosylation of Sp1 was found to regulate the proteasome-dependent degradation, and it is acetylated to regulate the DNA binding affinity or transactivation (19–24). Therefore, post-translational modification on Sp1, because of interaction with other factors, may play an important role in the regulation of Sp1 activity.

Hsp90, a constitutent molecular chaperone, is an abundant protein, comprising 2% of total cellular proteins under non-stress conditions. It is essential for numerous cellular proteins that regulate signal transduction such as transcription factors, protein kinases, and nitric-oxide synthase, and involved in various cellular processes, such as cell proliferation, differentiation, and apoptosis (25–29). Unlike Hsp70, Hsp90 does not act generally in nascent protein folding, and instead, binds to the client proteins to stabilize the folding of proteins (30, 31). The crystal structure of Hsp90 reveals that the N-terminal domain of Hsp90 binds ATP, which is consistent with the observation that ATP hydrolysis is required for conformational changes involved in refolding protein substrates or client proteins of Hsp90. Geldanamycin (GA), a benzoquinone ansamycin, and radicicol, a macrocyclic anti-fungal antibiotic, can compete the ATP binding site on Hsp90 to inhibit its activity (32, 33). Hsp90 resides mostly in the cytoplasm to form the main functional component of an important cytoplasmic chaperone complex (34). However, it has been also found inside the nucleus and outside the cells in unstressed or stressed cells and cancer cells (35–38). Most studies about nuclear Hsp90 focus on how the Hsp90 shifts glucocorticoid receptor (GR) into the nucleus and regulates nuclear retention (38, 39). However, whether the Hsp90 has additional functions within the nucleus is not very clear.

Arachidonate 12(S)-lipoygenase (arachidonate: oxygen 12-oxidoreductase; EC 1.13.11.31) in the platelet was the first mammalian lipoygenase discovered (40). It catalyzes the transformation of arachidonic acid into 12(S)-hydroperoxycisatetraenoic acid, followed by conversion to 12(S)-HETE. 12(S)-HETE plays a significant role in the pathogenesis of some epidermal and epithelial inflammation. A markedly elevated 12(S)-HETE level was found in psoriatic plaque, whereas the level of prostaglandins E2 and F2α was only minimally elevated (41, 42). Therefore, high levels of 12(S)-HETE may contribute to the inflammatory changes and the abnormal epidermal hyperproliferation in the development of a psoriatic plaque. In terms of promoter activity, the two simian virus 40 promoter factor 1 (Sp1) binding sites residing at −158 to −150 bp and −123 to −114 bp were found to be required for the transcription of the 12(S)-lipoygenase (43). Under normal conditions, Sp1 recruited to the promoter of 12(S)-lipoygenase is responsible for...
its basal transcription. Upon induction with phorbol 12-myristate 13-acetate (PMA) or epidermal growth factor (EGF), c-Jun can be recruited to the promoter through Sp1 to regulate transcription of 12(S)-lipoxygenase (43, 44).

In this study, we first found that Sp1 could recruit Hsp90α to the GC-rich region of 12(S)-lipoxygenase promoter in A431 cells. Interestingly, a specific inhibition of Hsp90 by GA blocked Sp1 activity and siRNA of Hsp90α resulted in knockdown of the Hsp90 level, inhibiting the transcriptional activity of 12(S)-lipoxygenase in A431 cells. Furthermore, the DNA binding affinity of Sp1 was inhibited in the presence of GA.

**EXPERIMENTAL PROCEDURES**

**Materials**—Polyclonal antibodies against Sp1 and Hsp90 were from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibodies against 12(S)-lipoxygenase were purchased from Oxford Medical Research Inc (Oxford, MI). Monoclonal antibodies against acetyl-H3, tubulin α, and TFIIID (TPB) were purchased from Upstate Biotechnology (Lake Placid, NY). FITC-conjugated donkey anti-mouse and Cy5-conjugated donkey anti-rabbit antibodies were purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). GA was purchased from Sigma. Biotinylated oligonucleotides were obtained from MD Bio, Inc. (Taipei, Taiwan). The luciferase assay system was from Promega. Luciferase plasmid pXP-1 was a gift from Dr. T. Sakai of Kyoto Prefecture University of Medicine. TRIzol RNA extraction kit, SuperScript™II, Lipofectamine 2000, RNase inhibitor, Dulbecco’s modified Eagle’s medium, and Opti-MEM medium were obtained from Invitrogen Life Technologies (Grand Island, NY). [α-32P]UTP (3000 Ci/mmole), [γ-32P]dATP (6000 Ci/mmole), and [35S]methionine (1000 Ci/mmole) were purchased from Amersham Biosciences. The plasmids, pSM2 and pSM2-shHsp90 were purchased from Gene-Discovery Inc (Taipei, Taiwan). Fetal bovine serum was from HyClone Laboratories (Logan, UT).

All other chemical reagents used were of the highest purity obtainable.

**Cell Culture**—Human epidermoid carcinoma A431 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100 μg/ml streptomycin sulfate, and 100 units/ml penicillin G sodium under 37 °C, 5% CO2 incubator. The 90% confluent cells were then resuspended in 400 μl of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM DTT, 2 μg/ml leupeptin, and 2 μg/ml pepstatin). After staying on ice for 10 min, samples were vortexed for 10 s and sucked back and forth six times using 25-gauge needles. Nuclei were pelleted by centrifugation at 12,000 × g for 30 s. The pellets were resuspended in 100 μl of buffer C (20 mM HEPES, pH 7.9, 420 mM NaCl, 0.2 mM EDTA, 25% glycerol, 1.5 mM MgCl2, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM DTT, 2 μg/ml leupeptin, and 2 μg/ml pepstatin) and put on ice for 20 min. Finally, nuclear extracts were prepared by centrifugation at 12,000 × g for 3 min to remove the pellet and stored at −80 °C until use.

**Western Blotting**—Whole cell extracts were fractionated by SDS-PAGE and transferred to a nitrocellulose membrane using a transfer apparatus according to the manufacturer’s protocols (Bio-Rad). After incubation with 3% nonfat milk in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Tween 20) for 60 min, the membrane was washed once with TBST and incubated with antibodies against Sp1 (1:1000), Hsp90 (1:2000), 12(S)-lipoxygenase (1:500), acetyl-lysine (1:1000), tubulin α (1:3000), or actin (1:5000) at room temperature for 12 h. Membranes were washed three times for 10 min and incubated with a 1:3000 dilution of horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies for 120 min. Blots were washed with TBST three times and developed with the ECL system (Amersham Biosciences) according to the manufacturer’s protocols.

**Immunoprecipitation**—Cell nuclear extracts were prepared, and the protein concentration was determined using a bichinchoninic acid protein assay kit (Pierce), and an equal amount of protein was used in each experiment. Nuclear extracts were preincubated with protein A/G-Sepharose for 1 h at 4 °C and centrifuged to remove the pellets. The supernatants were transferred to new tubes and incubated with either anti-Hsp90 or anti-Sp1 antibodies at a dilution of 1:200 for 12 h at 4 °C. The immunoprecipitated pellets were subsequently incubated with protein A/G-Sepharose, washed five times with lysis buffer, and separated on an SDS-7% polyacrylamide gel. After electrophoresis, the gels were processed for immunoblot analysis.

**Transfection and Reporter Gene Assay**—A431 cells (2.5 × 105) were seeded on 3.5-cm dishes and reached 40–50% confluence on the day of transfection. Cells were transfected with plasmids by lipofection using Lipofectamine 2000 according to the manufacturer’s instruction with a slight modification. For use in transfection, 1 μg of pXP-1 or pXLO-7 luciferase plasmids was combined with 1 μl of Lipofectamine 2000 in 200 μl of Opti-MEM medium without serum, and incubated at room temperature for 30 min. Cells were transfected by changing the medium with 2 ml of Opti-MEM medium containing the plasmids and Lipofectamine 2000, and then incubating at 37 °C in 5% CO2 for 6 h. After change of Opti-MEM medium to 2 ml of fresh medium containing 10% fetal bovine serum, cells were incubated for an additional 18 h. The luciferase activity in the cell lysate was determined as described previously (44).

**DNA Affinity Precipitation Assay (DAPA)**—The oligonucleotide, 5′-TTTGGGCTAGTCTGGGGCGGGG-3′, localized −171 to −150 bp within the promoter of 12(S)-lipoxygenase and its mutant, 5′-TTTGGGCTAGTCTAAAAAAA-3′, were biotinylated at the 5′-termini, and then annealed with their complementary strands, respectively. The DNA affinity precipitation assay was performed by incubating 2 μg of biotinylated DNA probe with 300 μg of nuclear extract that was precleared with 10 μl of streptavidin-agarose beads and 1 μg of poly(dl-dC) for 1 h, and then incubated with 20 μl of streptavidin-agarose in binding buffer (1 μg of poly(dl-dC), 20 mM HEPES, pH 7.9, 0.1 mM KCl, 2 mM MgCl2, 15 mM NaCl, 0.2 mM EDTA, 1 mM DTT, and 10% (v/v) glycerol) for 1 h. Beads were collected and washed with the binding buffer containing 0.5% Nonidet P-40 three times. Proteins bound to the beads were eluted with 2× sample buffer and separated by 7% SDS-PAGE for immunoblot analysis, and by 8–20% gradient SDS-PAGE for silver staining.

**Chromatin Immunoprecipitation (ChIP)**—Assays were performed as described previously (45). Cells (1 × 106) were cross-linked using 0.5% formaldehyde in PBS for 15 min at room temperature. The cells were washed three times with PBS, and the cell lysate was then collected with lysis buffer. The chromatin was fragmented by sonication to an average size of 500 bp. The samples were precleared with 1 μg of poly(dl-dC) and 10 μl of protein A/G-agarose for 1 h, and then immunoprecipitated with 5 μg of IgG, anti-Sp1, anti-Hsp90, anti-TFIIID (TBP), anti-acetyl tubulin, anti-lysine, and anti-Hsp90.
RESULTS

Hsp90α Was Recruited by Sp1 to the Promoter of 12(S)-Lipoxygenase in A431 Cells—Sp1 acts as an anchor protein to recruit other transcriptional factors to carry out the transcriptional mechanism (43). Therefore, a biotinylated DNA fragment localized at the −171 to −150 bp region of the 12(S)-lipoxygenase promoter containing Sp1 binding sites was used as a probe to study the factors that could be recruited to the promoter by Sp1. The samples were analyzed by immunoblotting with anti-Sp1 antibodies and shown in Fig. 1A. This indicated that Sp1 could bind to the GC-rich region of 12(S)-lipoxygenase, but not to the mGC-rich region in which the sequence was modified from 5′-TTGGGCT-AGCTGGGCGGGGGG-3′ to 5′-TTGGGCTAGCTAAGAACAAC-3′ (Fig. 1A). These samples were subsequently analyzed by two-dimensional SDS-PAGE, revealing that more proteins were recruited to the GC-rich probe, but not to the mGC-rich probe (Fig. 1B). The proteins on the gel were collected and analyzed employing MALDI-TOF MS to verify their identities. Among those, Hsp90α was clearly identified (Fig. 1C). ChiP assay was carried out to mimic the in vivo recruitment of Hsp90α to the promoter of 12(S)-lipoxygenase. The results revealed that GC-rich region of 12(S)-lipoxygenase promoter could be pulled down by anti-Sp1 and anti-Hsp90α antibodies (Fig. 1D). These results indicated that the Hsp90α could be recruited to the promoter of 12(S)-lipoxygenase by Sp1 in A431 cells. Further, immuno precipitation with anti-Sp1 antibodies followed by immunoblotting with anti-Hsp90α and anti-Sp1 antibodies was performed. As shown in Fig. 2A, Hsp90α could be immunoprecipitated by anti-Sp1 antibodies. In addition, we also expressed and purified the GST-Sp1 protein from Escherichia coli and used it to pull-down interacting proteins residing in the nuclear extract. Immunoblots with anti-Hsp90α and anti-GST antibodies revealed that GST-Sp1 could pull-down the Hsp90α, but the GST protein alone could not (Fig. 2B). To investigate whether Sp1 and Hsp90α interacted directly, both Hsp90α and GST-Sp1 were expressed and purified for in vitro interaction assay. These results revealed that no direct interaction between Sp1 and Hsp90α occurred (Fig. 2C). In addition, cells were also observed with confocal microscopy to study the co-localization between the Sp1 and Hsp90α (Fig. 2D). The result revealed that a little Hsp90α was inside the nucleus, and it was co-localized with the Sp1. In contrast, tubulin α, which has been reported to be absent in the nucleus, was used as a negative control. Taken together, these results indicated that Hsp90α interacted indirectly with Sp1, and these two proteins were recruited together to the nucleus to bind to the promoter of 12(S)-lipoxygenase in A431 cells.

GA Inhibited the Transcriptional Activity of 12(S)-Lipoxygenase—To investigate the effect of the Hsp90α on the transcription of the target gene, A431 cells were treated with GA to inhibit the function of Hsp90α by competing with ATP binding at the ATP binding site. The protein synthesis rate, mRNA level, and luciferase activity driven by 12(S)-lipoxygenase promoter (Fig. 3C) in cells were then analyzed. A431 cells were treated with various concentration of GA for 18 h and then labeled with [35S]methionine for 1 h. IKKα, which can be inhibited by GA treat-
ment was used as a positive control, whereas Sp1 was used as a negative control. The 12(S)-lipoxygenase, IKKα, and Sp1 levels were then analyzed with immunoprecipitation by anti-12(S)-lipoxygenase, anti-IKKα, and anti-Sp1 antibodies, respectively (48, 49). After autoradiography, the results revealed that synthesis rate of 12(S)-lipoxygenase in A431 cells (Fig. 3A). To find out if the inhibition was in the transcriptional step or the translational step, cells were treated with 2 μM GA for 9 h, and RNA was then extracted and examined by RT-PCR. This result indicated that the mRNA level of 12(S)-lipoxygenase and IKKα were reduced upon GA treatment in A431 cells, but no difference was observed in that of GAPDH with or without GA treatment (Fig. 3B). Furthermore, luciferase activity assay revealed that when the plasmid, pXLO7-1, containing the key promoter region of 12(S)-lipoxygenase, was transfected into A431 cells, the promoter activity was inhibited to 49, 42, and 28.5% upon treatment with 0.5, 1, and 2 μM GA, respectively (Fig. 3C). Taken together, inhibition of Hsp90α by GA reduced the transcription of 12(S)-lipoxygenase in A431 cells.

Knockdown of Hsp90α Reduced the Expression of 12(S)-Lipoxygenase—GA can inhibit the activity of the Hsp90 family, including Hsp90α, Hsp90β, and Grp96 (32). To more specifically elucidate the role of Hsp90α in the transcription of 12(S)-lipoxygenase, siRNA was designed to knockdown the level of Hsp90α in cells. Plasmids, pSM2 and pSM2-shHsp90a were transfected into the AmphoPack-293 cells, respectively. After 72 h of incubation, viral particles of retrovirus-shHsp90a and retrovirus were collected in the supernatant. A431 cells were infected with the retrovirus or retrovirus-shHsp90a for 48 h, and the cell lysates were then collected for immunoblotting analysis. The results revealed that there was no significant change in Sp1 level, which served as a negative control, and both Hsp90α and 12(S)-lipoxygenase levels were reduced upon infection with the retrovirus-shHsp90α at a m.o.i. of 5 in A431 cells. Little change was observed when the cells were infected by the retrovirus (Fig. 4A). The levels of Hsp90α and 12(S)-lipoxygenase were reduced to 24 and 50%, respectively after retrovirus-shHsp90a infection for 48 h, but there was no significant difference upon retrovirus infection (Fig. 4B). In addition, cells were infected with retrovirus at an m.o.i. of 10 or retrovirus-shHsp90α at an m.o.i. of 1, 5, and 10, and then co-transfected with 1 μg of pXLO7-1. After 18 h of incubation, cells lysates were collected for the luciferase activity assay. These results revealed that the luciferase activity driven by the promoter of 12(S)-lipoxygenase was reduced to 72, 67, and 53% in cells treated with 0.5, 1, and 2 μM GA, respectively, after normalization with that of vector pXP-1 luciferase activity driven by the wild-type promoter of 12(S)-lipoxygenase (Fig. 4C). Moreover, the RNA level of 12(S)-lipoxygenase decreased significantly after knockdown of Hsp90α by retrovirus-SM2-shHsp90α, when no change was observed in GAPDH levels with or without GA treatment (Fig. 4D).

Effect of GA on Transcriptional Activity of 12(S)-Lipoxygenase Was Abolished with the Mutant 12(S)-Lipoxygenase Promoter at the Sp1 Binding Sites—To examine whether the effect of Hsp90α on the transcriptional activity of 12(S)-lipoxygenase was Sp1-dependent, three Sp1 binding sites (43), Sp1–1, Sp1–2, and Sp1–3, localized within 150 bp of the 12(S)-lipoxygenase promoter were mutated individually or together to study the relationship between Hsp90α and transcriptional activity of 12(S)-lipoxygenase (Fig. 5). The results show that the luciferase activity driven by the wild-type promoter of 12(S)-lipoxygenase was reduced to 26.5% (normalized with that driven by vector pXP-1 under GA treatment). In addition, the activity driven by the mutated 12(S)-lipoxygenase that single mutation on Sp1–1, Sp1–2, or Sp1–3 of the promoter were recovered to 60, 86, and 87.5%, respectively upon the
treatment with GA. Interestingly, the level of activity was completely recovered when all three Sp1 binding sites were mutated with GA treatment. These results clearly indicated that Hsp90 was involved in the transcription of 12(S)-lipoxygenase in an Sp1-dependent fashion in A431 cells.

Nuclear Hsp90 Was Involved in the Transcription of 12(S)-Lipoxygenase—To study the importance of nuclear Hsp90 on the transcription of 12(S)-lipoxygenase, the DNA fragment of 12(S)-lipoxygenase localized within −220 to +500 bp (T1, containing the promoter and partial coding region), −220 to +1 bp (T2, containing the promoter only), and +1 to +500 bp (T3, containing the partial coding region) were used for the in vitro transcription assay (Fig. 6A). The DNA fragments were incubated with nuclear extracts in cells treated with or without GA in the presence of [32P]UTP. RNA synthesis was subsequently analyzed with 6% acrylamide gel containing urea (Fig. 6B). No signal was detected when either the promoter region (T2) or the coding region (T3) was used (Fig. 6B, lanes 1 and 2). However, a signal was observed at 500 bp in the absence or presence of RNase i, when the template containing both promoter and coding regions (T1) was used (Fig. 6B, lanes 3 and 4). When the nuclear extracts were treated with 1 or 5 μM GA to block the Hsp90α, the signals were decreased (Fig. 6B, lanes 5 and 6). The results revealed that RNA synthesis of 12(S)-lipoxygenase was inhibited under GA treatment.

Sp1 Binding to Promoter of 12(S)-Lipoxygenase Was Inhibited by GA—To study the mechanism of the Hsp90α effect on the target gene, A431 cells were treated with or without GA and fixed with formaldehyde. The nuclear extracts were prepared, and a ChIP assay was undertaken. These results revealed that Sp1 bound quite strongly to the pro-

FIGURE 2. Hsp90α interacts with Sp1 in cells. A, nuclear extracts were extracted from A431 cells, and then incubated with anti-Sp1 and anti-IgG antibodies. The samples were analyzed with immunoblotting for Hsp90α and Sp1. B, GST-Sp1 was expressed in E. coli and purified by glutathione beads. 1 μg of GST-Sp1 or GST was incubated with nuclear extracts of A431 cells at 4 °C. The samples were separated with 7% SDS-PAGE and analyzed by immunoblotting using anti-Sp1 and anti-Hsp90α antibodies. The GST-beads were used as a negative control. C, GST-Sp1 and Hsp90α were expressed in E. coli, 1 μg of GST-Sp1 and Hsp90α were used to do the in vitro direct interaction assay. D, cells were seeded and fixed, and the colocalization was then analyzed with anti-Hsp90α, anti-tubulin α, and anti-Sp1 antibodies. After washing with PBS, samples were stained with FITC-anti-mouse antibodies for Hsp90α and tubulin α, and Cy5-anti-rabbit antibodies for Sp1.
moter of 12(S)-lipoxygenase (Fig. 7A, lane 1). When the cells were treated with 0.5, 1, and 2 μM GA, the Sp1 binding signals were decreased gradually (Fig. 7A, lanes 2–4). Consistent with the above results, the recruitment of Hsp90α was also decreased significantly upon GA in a dose-dependent manner (Fig. 7A, lanes 6–9). In addition, no signal shown in lanes 5 and 10 of Fig. 7A was observed, indicating that the specific interaction between Sp1 and promoter was required. Furthermore, we also investigated the binding conditions of other transcription factors to the promoter of 12(S)-lipoxygenase with or without GA treatment by ChIP assay (Fig. 7B). The quantified data from several independent experiments indicated that in the presence of GA, recruitment of Sp1, Hsp90α, TFIID (TBP), and acetyl-H3 to the promoter of 12(S)-lipoxygenase was reduced to about 23, 65, 35, and 34%, respectively, but no obvious signal variation was detected when the actin or IgG was tested (Fig. 7C). These results demonstrated that Sp1 binding to the promoter of 12(S)-lipoxygenase was influenced by the Hsp90α in A431 cells.
FIGURE 4. Expression of 12(S)-lipoxygenase was inhibited by siRNA of Hsp90α in cells. A, pSM2-shHsp90α or pSM2 was transfected to the Amphotopack-293 cells, and cells were then incubated for 3 days. The recombinant retrovirus particles containing siRNA of Hsp90α or SM2 were produced. A431 cells were infected with the recombinant virus at an m.o.i. of 5, and then incubated for 3 days. After labeling with [35S]methionine for 1 h, the cell lysates were collected, and the immunoprecipitation with anti-12(S)-lipoxygenase, anti-Hsp90, and anti-Sp1 antibodies were performed. The samples were then analyzed by SDS-PAGE, and the expression of actin was used as an internal control. B, signals shown in A were quantified from three independent experiments. C, plasmid, pXLO7-1, was co-transfected into the retrovirus-infected A431 cells, and cells were then incubated for 1 day. The cell lysates were collected, and the transcriptional activity of 12(S)-lipoxygenase was analyzed by the luciferase assay. All of the experiments were performed three times at least independently, and statistical analysis was performed by Student’s t test. D, both retrovirus-SM2- and retrovirus-SM2-siHsp90α-infected A431 cells, and untreated cells were then incubated for 1 day. RNA was isolated, and the RNA levels of Hsp90α, 12(S)-lipoxygenase, and GAPDH were studied by RT-PCR.
DISCUSSION

Sp1 exerts its role in the regulation of target genes by binding to the GC-rich promoter region of the target gene. Numerous transcription factors cannot bind to DNA directly, but rather are recruited to the promoter through other transcription factors that directly bind to promoter. Therefore, Sp1 can be considered an important anchor protein that is able to recruit many factors to the promoter region of the target genes to carry out the transcription mechanism; such as recruiting c-Jun to enhance the transcriptional activity of the target gene(s) and recruiting p300 to promoter to acetylate the other transcription factors to regulate the transcription (44, 50–52). In this study, we used the biotinylated GC-rich promoter region of 12(S)-lipoxygenase as a probe to detect the proteins recruited through Sp1 prior to two-dimensional SDS-PAGE analysis. Interestingly, after ruling out nonspecific binding by the mutated probe as shown in Fig. 1, many spots appeared in the two-dimensional gel. We identified the specific binding proteins by MALDI-TOF MS and observed that Hsp90/H9251 could be recruited to the promoter region of 12(S)-lipoxygenase through Sp1 in A431 cells. Other co-chaperones such as TCP-1, Cdc37, and Hsp70 were also found in the recruited complex (data not shown). Therefore, recruitment of the whole chaperone complex to the promoter of 12(S)-lipoxygenase occurred. Although Hsp90 mainly stays and functions in the cytosol, several reports showed that Hsp90 can translocate into the nucleus to regulate the nuclear retention of GR (53–55). In addition, several Hsp90 client proteins such as p53 involved in signal transduction pathway have been shown to enter the nucleus to execute their functions (56, 57). In this study, two pieces of evidence supported that Hsp90/H9251 could be recruited to promoter of 12(S)-lipoxygenase in A431 cells. First, the results of DAPA and ChIP assay (Fig. 1) showed that Hsp90/H9251 could be recruited to the 12(S)-lipoxygenase promoter. Second, the immunoprecipitation and pull-down assays using the nuclear extracts (Fig. 2, A and B), and immunofluorescence microscopic analysis to study the co-localization between Sp1 and Hsp90 in vivo also revealed that Hsp90/H9251 could be recruited to the nucleus (Fig. 2D).

Next, the Hsp90 inhibitor, GA, which binds to the N terminus of...
Hsp90α was used to block the activity of the Hsp90α, and siRNA of Hsp90α was further used to reduce the level of Hsp90α. Both experiments revealed that the expression of 12(S)-lipoxygenase was inhibited in the presence of inactive Hsp90 or Hsp90 knockdown conditions (Figs. 3 and 4). However, previous studies indicated that Hsp90 interacts with P52 (rIPK) to inhibit the P58 (IPK) to mediate downstream control of PKR activity and eIF2β phosphorylation. The dephosphorylated eIF2β cannot inhibit the translational mechanism (58), and therefore, Hsp90α enhances translation efficiency. In our present study, we still could not rule out the possibility that the translational activity of the 12(S)-lipoxygenase was affected under GA treatment or Hsp90α siRNA treatment in A431 cells. However, our results show that the transcriptional activity was affected under GA treatment in A431 cells. First, the results shown in Fig. 3 revealed that the mRNA level was reduced under GA treatment. Second, the reporter assay indicated that the promoter activity of 12(S)-lipoxygenase was reduced to about 25% under GA treatment. Third, the in vitro transcription assay shown in Fig. 6 revealed that the RNA synthesis level was reduced under GA treatment. According to the previous studies, Hsp90 can affect many proteins in cell cycle and signal transduction (59–62). Indeed, in our experiments, there is no doubt that Hsp90α, recruited by Sp1 to the nucleus, was important for the transcription of 12(S)-lipoxygenase in A431 cells. The reporter assay shown in Fig. 5 revealed that mutation at three Sp1 binding sites localized on the promoter of 12(S)-lipoxygenase resulted in reduction of the inhibition level because of GA treatment in A431 cells. These results revealed that interaction between Sp1 and Hsp90α was very important for the transcription of 12(S)-lipoxygenase. Recently, many kinases have been shown to translocate to the nucleus with their substrate to carry out their functional biological roles within the nuclei (63). Hsp90α has large conformational flexibility to form a multitude of dynamic co-chaperone complexes, which contribute to functional diversity (64). This assists a wide range of the client proteins, to stabilize

**FIGURE 7.** Sp1 binding to promoter of 12(S)-lipoxygenase was decreased by GA treatment in cells. A, cells were treated with 0.5, 1, and 2 μM GA. After fixation with 0.5% formaldehyde, nuclear extract was extracted and incubated with anti-Sp1 antibodies and anti-Hsp90α. The samples were then analyzed by Southern blotting. B, the other binding factors, TFIID, histone 3, Hsp90α, and actin were also studied by using anti-TFIID (TBP), anti-acetyl-H3, and anti-Hsp90 antibodies. The samples were then analyzed by Southern blotting. C, the signals shown in B from several independent experiments were quantified, and the actin and IgG were used as negative controls. The letter n represents the number of independent experiments.
DNA Binding Affinity of Sp1 under GA Treatment

their structures. A large number of the client proteins have a consensus sequence TPR (65–67). A remarkable proportion of its substrates are proteins related to the protein kinases, cell cycle control, and signal transduction (68, 69). There is no TPR sequence inside the Sp1, and hence no direct interaction between Sp1 and Hsp90α occurred. Rather, this recruitment by Sp1 is indirect. The role of Hsp90α recruited to Sp1 might affect other factor(s) recruited by Sp1 to regulate the transcriptional activity of 12(S)-lipoxygenase. In summary, in this study, we demonstrated that Hsp90α could be recruited by Sp1 to the promoter of 12(S)-lipoxygenase to modulate its transcriptional activity, by affecting the binding of Sp1 to the gene promoter in A431 cells.

Acknowledgments—We thank National Cheng-Kung University Proteomics Core Laboratory for assistance in two-dimensional gel electrophoresis and mass spectrometry analysis. Thanks are also due to Dr. Wai-Ming Kan and Dr. Margaret Dah-Tsyu Chang for their critical reviewing of the manuscript.

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Hsp90α Recruited by Sp1 Is Important for Transcription of 12(S)-Lipoxygenase in A431 Cells
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J. Biol. Chem. 2005, 280:36283-36292.
doi: 10.1074/jbc.M504904200 originally published online August 23, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M504904200

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