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Down-Regulation of Cell Surface Insulin Receptor and Insulin Receptor Substrate-1 Phosphorylation by Inhibitor of 90-kDa Heat-Shock Protein Family: Endoplasmic Reticulum Retention of Monomeric Insulin Receptor Precursor with Calnexin in Adrenal Chromaffin Cells

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ABSTRACT

Treatment (≥6 h) of cultured bovine adrenal chromaffin cells with geldanamycin (GA) or herbimycin A (HA), an inhibitor of the 90-kDa heat-shock protein (Hsp90) family, decreased cell surface ^{125}I-insulin binding. The effect of GA was concentration (EC_{50} = 84 nM)- and time ($t_{1/2}$ = 8.5 h)-dependent; GA (1 μM for 24 h) lowered the B_{max} value of ^{125}I-insulin binding by 80%, without changing the K_d value. Western blot analysis showed that GA (≥3 h) lowered insulin receptor (IR) level by 83% ($t_{1/2}$ = 7.4 h; EC_{50} = 74 nM), while raising IR precursor level by 100% ($t_{1/2}$ = 7.9 h; EC_{50} = 300 nM). Pulse-label followed by reducing and nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that monomeric IR precursor (~190 kDa) developed into the homodimeric IR precursor (~380 kDa) and the mature $\alpha_2\beta_2$ IR (~410 kDa) in nontreated cells, but not in GA-treated cells; in GA-treated cells, the homodimerization-incompetent form of monomeric IR precursor was degraded via endoplasmic reticulum (ER)-associated protein degradation. Immunoprecipitation followed by immunoblot analysis showed that IR precursor was associated with calnexin (CNX) to a greater extent in GA-treated cells, compared with nontreated cells. GA had no effect on IR mRNA levels and internalization rate of cell surface IRs. In GA-treated cells, insulin-induced tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) was attenuated by 77%, with no change in IRS-1 level. Thus, inhibition of the Hsp90 family by GA or HA interrupts homodimerization of monomeric IR precursor in the ER and increases retention of monomeric IR precursor with CNX; this event retards cell surface expression of IR and attenuates insulin-induced activation of IRS-1.

Insulin receptors (IRs) consist of two extracellular α- and two transmembrane β-subunits (~135 and ~95 kDa, respectively) that are encoded by the same gene and derived from the single-chain IR precursor molecule. IR precursor undergoes cotranslational glycosylation, intrachain disulfide-bond formation/isomerization (rearrangement), and disulfide-linked homodimerization at the endoplasmic reticulum (ER). The homodimeric IR precursor is proteolytically processed at the *trans*-Golgi network (TGN) into the disulfide-linked $\alpha_2\beta_2$ complex, which is transported to plasma membrane via as yet unidentified mechanisms (Ronnett et al., 1984; Arakaki et al., 1987; Olson et al., 1988; Caro et al., 1994; Cheatham and Kahn, 1995; Bass et al., 1998; Elleman et al., 2000). Binding of insulin to the α-subunit causes autophosphorylation of the β-subunit, resulting in the endocytic internalization of IRs via clathrin-coated vesicles. IR internalization may trigger phosphorylation of insulin receptor substrate-1 (IRS-1) at the multiple tyrosine residues, which create binding sites for signal-transducing molecules containing Src homology-2 domain, thus initiating the pleiotropic effects of insulin (Cheatham and Kahn, 1995; Balbis et al., 2000). Little is known, however, about the quality control mechanisms ensuring the conformational maturation of monomeric IR precursor into the $\alpha_2\beta_2$ complex.

As the growing polypeptide of glycoprotein enters the ER lumen via Sec61 translocon (Aridor and Balch, 1999), the

ABBREVIATIONS: IR, insulin receptor; ER, endoplasmic reticulum; TGN, *trans*-Golgi network; IRS-1, insulin receptor substrate-1; CNX, calnexin; CRT, calreticulin; Hsp90, 90-kDa heat-shock protein; Grp94, 94-kDa glucose-regulated protein; CFTR, cystic fibrosis transmembrane conductance regulator; GA, geldanamycin; HA, herbimycin A; Src, SH2 domain of pp60; ErbB2, estrogen receptor β_{B-2}; MG132, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal; MG115, carbobenzoxy-L-leucyl-L-leucyl-L-norvinal; BFA, brefeldin A; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DMSO, dimethyl sulfoxide; KRP, Krebs-Ringer phosphate; BSA, bovine serum albumin; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; SSC, standard saline citrate; ERAD, endoplasmic reticulum-associated protein degradation; CHO, Chinese hamster ovary; kb, kilobase(s).

oligosaccharide core unit, $Glc_3Man_9GlcNAc_2$, is cotranslationally transferred to its *N*-linked glycosylation site by oligosaccharyltransferase and sequentially trimmed by glucosidases I and II (Helenius et al., 1997; Ruddon and Bedows, 1997; Ellgaard and Helenius, 2001; Lehrman, 2001). Calnexin (CNX), a lectin chaperone of the ER transmembrane protein, and calreticulin (CRT), a CNX homolog in the ER lumen, bind to monoglucosylated glycoprotein intermediate bearing $Glc_1Man_{9-5}GlcNAc_2$, thus enhancing correct glycoprotein folding/assembly and tethering incompletely folded/assembled glycoprotein in the ER (Jackson et al., 1994; Rajagopalan et al., 1994). Also, CNX functions as a molecular chaperone by recognizing the exposed hydrophobic polypeptide segments that are normally buried inside the native mature glycoproteins (Danilczyk and Williams, 2001). Glucosidase II-catalyzed trimming of final glucose residue from $Glc_1Man_{9-5}GlcNAc_2$ causes dissociation of glycoprotein from CNX/CRT; only the incompletely folded/assembled glycoprotein is reglucosylated by UDP-glucose/glycoprotein glucosyltransferase, a folding sensor, and reassociates with CNX/CRT, multiple rounds of association-dissociation cycles being postulated to occur (Helenius et al., 1997; Aridor and Balch, 1999; Ellgaard and Helenius, 2001; Lehrman, 2001). When the oligosaccharide chain of finally misfolded glycoprotein is cleaved by the ER mannosidase I, it is inspected by a $Man_8GlcNAc_2$-specific, as-yet-unidentified, lectin chaperone (Fagioli and Sitia, 2001; Lehrman, 2001) and retro-dislocated via Sec61 translocon into cytoplasm, where it is proteolytically degraded by the ubiquitin-proteasome system (Aridor and Balch, 1999).

The 90-kDa heat-shock protein (Hsp90), a molecular chaperone in the cytoplasm, and the 94-kDa glucose-regulated protein (Grp94), an Hsp90 homolog in the ER lumen, ensure correct conformational maturation and translocation of signaling molecules, such as steroid hormone receptors, Src-tyrosine kinase, growth factor receptors, and cystic fibrosis transmembrane conductance regulator (CFTR). These findings were obtained by using the ansamycin derivative geldanamycin (GA) or herbimycin A (HA), an inhibitor of the Hsp90 family (Whitesell et al., 1994; Sepp-Lorenzino et al., 1995; Loo et al., 1998; Buchner, 1999; Xu et al., 2001; Young et al., 2001). GA binds to the adenosine nucleotide binding site of the N-terminal domain of Hsp90 with affinity higher than that of ATP and inhibits the ATPase activity/chaperone function of Hsp90 (Whitesell et al., 1994; Buchner, 1999; Young et al., 2001). GA blocked dissociation from Hsp90 of glucocorticoid receptors (Young and Hartl, 2000) and heat-denatured firefly luciferase (Schneider et al., 1996), while disrupting heteroprotein complex formation of Hsp90 with *v*-Src (Whitesell et al., 1994), CFTR (Loo et al., 1998), or ErbB2 (Xu et al., 2001).

In cultured bovine adrenal chromaffin cells (embryologically derived from the neural crest), IRs play crucial roles, such as up-regulation of cell surface voltage-dependent Na^+ channels (Yamamoto et al., 1996) and enhancement of voltage-dependent Ca^{2+} channel gating and of exocytic secretion of catecholamines (Yamamoto et al., 1996), as well as increased synthesis of various bioactive proteins (Wilson et al., 1985). We previously showed that protein kinase C-α up-regulated cell surface expression of IRs via transcriptional/translational events (Yamamoto et al., 2000). Peptidyl prolyl *cis-/trans*-isomerase activity of cytoplasmic immunophilins (Shiraishi et al., 2000) and Ca^{2+}-ATPase activity of the ER (Shiraishi et al., 2001) accelerated cell surface externalization of IRs from the TGN. Our present study shows that chronic treatment of chromaffin cells with GA or HA interrupted homodimerization of monomeric IR precursor in the ER, increasing its retention with CNX; this event down-regulated cell surface expression of IRs, thus attenuating insulin-induced tyrosine phosphorylation of IRS-1.

Materials and Methods

Materials. Eagle's minimum essential medium was obtained from Nissui Seiyaku (Tokyo, Japan). Dulbecco's methionine- and cysteine-free modified Eagle's medium, and TRIzol reagent were obtained from Invitrogen (Carlsbad, CA). Calf serum, phenylmethylsulfonyl fluoride, leupeptin, aprotinin, Na_3VO_4, NaF, *N*-ethylmaleimide, and Nonidet P-40 were from Nacalai Tesque (Kyoto, Japan). GA, HA, lactacystin, MG132, and MG115 were obtained from Calbiochem-Novabiochem (San Diego, CA). Brefeldin A (BFA) was from Sigma-Aldrich (St. Louis, MO). Rabbit polyclonal IR β-subunit antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal IRS-1 antibody was from Upstate Biotechnology (Lake Placid, NY). Mouse monoclonal phosphotyrosine-specific antibody (PY20) was from Transduction Laboratories (Lexington, KY). Rabbit polyclonal CNX antibody was from Stressgen Biotechnologies (Victoria, BC, Canada). Protein A-agarose, protein G-agarose, and Oligotex-dT30<Super> were from Nippon Roche (Tokyo, Japan). BcaBEST labeling kit and Noninterfering Protein Assay kit were from Takara (Kyoto, Japan). ^{125}I-Labeled anti-rabbit IgG, ^{125}I-anti-mouse IgG, ^{125}I-anti-protein G, ^{125}I-insulin (~2000 Ci/mmol), Redivue Pro-mix L-[^{35}S] in vitro cell labeling mix (containing ~70% L-[^{35}S]methionine and ~30% L-[^{35}S]cysteine) (>1000 Ci/mmol), and [α-^{32}P]dCTP (>4000 Ci/mmol) were obtained from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK). ^{125}I-Insulin was diluted with nonradioactive human insulin, Humulin R (Eli Lilly, Kobe, Japan), and ^{125}I-insulin (3.125 Ci/mmol) was used for the ^{125}I-insulin binding assay. cDNA for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was obtained from BD Biosciences Clontech (Palo Alto, CA). Plasmid containing human IR cDNA (pSelect HIR) was generously donated by Drs. Graeme Bell and Donald F. Steiner, as reported previously (Yamamoto et al., 2000).

Primary Culture of Adrenal Chromaffin Cells and Drug Treatment. Isolated bovine adrenal chromaffin cells were cultured (4×10^6 per dish, 35-mm diameter; Falcon Plastics, Oxnard, CA) in Eagle's minimum essential medium containing 10% calf serum under 5% CO_2/95% air in a CO_2 incubator (Yamamoto et al., 1996, 2000; Shiraishi et al., 2000, 2001). Three days (60–62 h) later, the cells were treated in the fresh medium without or with 0.001 to 10 μM GA or 1 μM HA for up to 96 h in the absence or presence of 10 μM lactacystin, 50 μM MG132, or 50 μM MG115. GA and HA were dissolved in dimethyl sulfoxide (DMSO), with the final concentration of DMSO in the test medium being ~0.2%; treatment of cells with 0.2% DMSO for up to 96 h did not alter ^{125}I-insulin binding and tyrosine phosphorylation levels of IRS-1, compared with nontreated cells. The culture medium contained 3 μM cytosine arabinoside to suppress the proliferation of nonchromaffin cells; when chromaffin cells were further purified by differential plating (Yamamoto et al., 1996, 2000), ^{125}I-insulin binding was similar between purified and conventional chromaffin cells; also, GA (1 μM for 24 h) decreased ^{125}I-insulin binding by 83 and 80% in purified and conventional chromaffin cells, respectively.

^{125}I-Insulin Binding. Cells were washed with ice-cold Krebs-Ringer phosphate (KRP) buffer [154 mM NaCl, 5.6 mM KCl, 1.1 mM $MgSO_4$, 2.2 mM $CaC1_2$, 0.85 mM NaH_2PO_4, 2.15 mM Na_2HPO_4, 5 mM glucose, and 0.5% bovine serum albumin (BSA), pH 7.4] and incubated with 0.025 to 10 nM ^{125}I-insulin in 1 ml of KRP buffer at

4°C for 6 h in the absence (total binding) and presence (nonspecific binding) of 1 μM unlabeled insulin. The cells were immediately washed, solubilized in 0.2 M NaOH, and counted for radioactivity. Specific binding was calculated as the total binding minus nonspecific binding. The B_{max} and K_d values of ^{125}I-insulin binding were almost identical to those of previous studies in bovine adrenal chromaffin cells; these values correspond to the binding of ^{125}I-insulin to IRs (but not insulin-like growth factor I receptors) (Yamamoto et al., 1996, 2000; Shiraishi et al., 2000, 2001). ^{125}I-Insulin binding represents cell surface (but not internalized) IRs, because ^{125}I-insulin associated with chromaffin cells was completely removed by washing the cells with ice-cold acidic, pH 4.0, KRP buffer twice, each for 7 min (Yamamoto et al., 2000).

Internalization Rate of Cell Surface IRS. One strategy to measure internalization rate may be the use of BFA; BFA prevents vesicular exit from the TGN of newly-synthesized proteins by inhibiting guanine nucleotide exchange protein of ADP-ribosylation factor 1, a monomeric GTPase (Moss and Vaughan, 1995). Previous fluorescence study showed that BFA treatment (0.28–2.8 μg/ml for ~2 h) was sufficient to cause disassembly of Golgi membrane in most (>90%) adrenal chromaffin cells (Xu and Tse, 1999). To examine the effect of GA on internalization rate of cell surface IRs, cells were preincubated with 10 μg/ml BFA at 37°C for 30 min, and treated without or with 1 μM GA in the continuous presence of BFA for up to 24 h. The cells were washed, and subjected to ^{125}I-insulin binding assay (Shiraishi et al., 2000, 2001; Yamamoto et al., 2000).

Western Blot Analysis of IR Molecules. Cells were washed with ice-cold Ca^{2+}-free phosphate-buffered saline (PBS) and solubilized in 500 μl of 2× SDS electrophoresis sample buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 10% 2-mercaptoethanol, and 4% SDS) at 98°C for 3 min. Total quantities of cellular proteins, as measured by the Noninterfering Protein Assay kit, were not changed between nontreated and GA-treated cells. The same amounts of proteins (7.0–7.5 μg per lane) were separated by SDS-7.5% polyacrylamide gel electrophoresis (PAGE) and transferred onto a nitrocellulose membrane. The membrane was preincubated with 5% dry milk in PBS and reacted overnight at 4°C with rabbit antibody against the C-terminal amino acid sequence (1365–1382) of IR β-subunit (Cheatham and Kahn, 1995). After repeated washings, the immunoreactive bands were labeled with ^{125}I-anti-rabbit IgG (1:1000) and analyzed by a Bioimage BAS 2000 analyzer (Fuji Film, Tokyo, Japan).

Metabolic Labeling and Analysis of IR Synthesis. Cells were incubated at 37°C for 1 h in the methionine- and cysteine-free culture medium in a CO_2 incubator, then pulse-labeled for 30 min with 100 μCi/ml of [^{35}S]methionine plus [^{35}S]cysteine in the absence or presence of 1 μM GA, and chased for up to 6 h in the continuous absence or presence of GA in the normal culture medium containing 0.1 mM methionine and 0.26 mM cysteine. Cellular uptake of the radioactivity was comparable between nontreated and GA-treated cells.

For analysis of [^{35}S]-labeled IR biosynthesis, cells were washed with ice-cold Ca^{2+}-free PBS twice, solubilized at 4°C for 15 min in 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 mM EDTA, 20 μg/ml aprotinin, and 10 μg/ml leupeptin), and centrifuged at 12,000*g* for 10 min at 4°C. When analyzed by nonreducing SDS-PAGE (see below), the lysis buffer contained 5 mM *N*-ethylmaleimide (Olson et al., 1988). Proteins in the supernatant were immunoprecipitated with IR β-subunit antibody for 2 h at 4°C and reacted with protein A-agarose for 1 h. The immunoprecipitates were washed three times with the lysis buffer by repeated resuspension and centrifugation, finally solubilized at 98°C in Laemmli's sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 0.1% bromphenol blue, and 10% glycerol) in the absence or presence of 5% mercaptoethanol, and centrifuged to remove protein A-agarose. Proteins in the supernatant were size-fractionated by reducing 7.5% SDS-PAGE or nonreducing 3 to 10% linear gradient SDS-PAGE. The gel was fixed in 20% ethanol and 7.5% acetic acid, dried, and exposed to an imaging plate for analysis by a Bioimage BAS 2000 analyzer.

Immunoprecipitation, PAGE, and Immunoblot Analysis of IRS-1, Tyrosine-Phosphorylated IRS-1, CNX, and IR Precursor. Cell lysates in the lysis buffer were subjected to immunoprecipitation with IRS-1 antibody or CNX antibody. The immunoprecipitates were reacted with protein A-agarose or protein G-agarose, washed with lysis buffer, finally suspended in 2× SDS electrophoresis sample buffer at 98°C, and centrifuged; the resultant supernatant was separated by 7.5% SDS-PAGE and transferred to membrane for immunoblot analysis. To measure insulin-induced tyrosine phosphorylation of IRS-1, cells were treated at 37°C without or with 100 nM insulin for 10 min in the KRP buffer, washed, and solubilized in the lysis buffer containing 10 mM Na_3VO_4 and 100 mM NaF; the cell lysates were subjected to immunoprecipitation with IRS-1 antibody.

For immunoblot analysis, the membrane was preincubated with Tween-Tris-buffered solution (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20) containing 1% BSA and 0.05% NaN_3, and reacted overnight at 4°C with the following antibodies against either IRS-1, phosphotyrosine, CNX, or IR β-subunit. The immunoreactive bands were labeled with ^{125}I-anti-rabbit IgG, ^{125}I-anti-mouse IgG, or ^{125}I-anti-protein G, and analyzed by a Bioimage BAS 2000 analyzer.

Northern Blot Analysis of IR mRNA Levels. Total cellular RNA was isolated from cells by acid guanidine thiocyanate-phenol-chloroform extraction using TRIzol reagent. Poly(A)$^+$ RNA was purified by Oligotex-dT30<Super>, electrophoresed on 1% agarose gel containing 6.3% formaldehyde in buffer [40 mM 3-(*N*-morpholino) propanesulfonic acid, pH 7.2, 0.5 mM EDTA, and 5 mM sodium citrate], transferred overnight to a nylon membrane (Hybond-N; Amersham Biosciences) in 20× saline-sodium citrate (SSC; 1× SSC = 0.15 M NaCl and 0.015 M sodium citrate), and cross-linked using a UV cross-linker (Funakoshi, Tokyo, Japan). The IR cDNA fragment (nucleotides 1–4608), obtained by digestion of pSelect HIR with *Sal*I (Yamamoto et al., 2000), and GAPDH cDNA (1.1 kbp) were labeled with [α-^{32}P]dCTP using the BcaBEST labeling kit. The membrane was prehybridized at 42°C in 6× SSC, 10× Denhardt's solution (2% BSA fraction V, 2% polyvinylpyrrolidone, and 2% Ficoll 400), 50% formamide, 0.5% SDS, and 50 μg/ml salmon sperm DNA, and then hybridized with the IR probe under the same condition for 18 h. It was washed at 55°C in 2×, 1×, and 0.2× SSC containing 0.1% SDS, each for 30 min twice, and subjected to autoradiography. The same membrane was hybridized with the GAPDH probe, after it was thoroughly washed in 0.1% SDS at 100°C to remove the IR probe. The autoradiogram was quantified by a Bioimage BAS 2000 analyzer.

Statistical Methods. ^{125}I-Insulin binding was performed in triplicate. All experiments, except for Northern blot analysis, were carried out five times. Data are mean ± S.E.M. values. Significance ($p < 0.05$) was determined by one-way or two-way ANOVA with post hoc mean comparison by the Newman-Keuls multiple range test. Student's *t* test was used when two means of group were compared.

Results

Decrease of Cell Surface ^{125}I-Insulin Binding in GA- or HA-Treated Adrenal Chromaffin Cells. Cells were treated without or with 0.001 to 10 μM GA for 24 h, and ^{125}I-insulin binding was assayed (Fig. 1A). GA treatment decreased ^{125}I-insulin binding by 80% in a concentration-dependent manner (EC_{50} = 84 nM). Treatment with HA (1 μM for 24 h) decreased ^{125}I-insulin binding by 37%, and its extent was comparable with that (39% decrease) of a 10-fold lower concentration of GA treatment (0.1 μM for 24 h) (Fig. 1A). Previous studies showed that *v*-Src-induced, Hsp90-mediated oncogenic morphological transformation of fibroblasts was prevented by HA ~10-fold less effectively, compared with GA (Whitesell et al., 1994). As shown in Fig. 1B, treat-

ment with 1 μM GA did not significantly alter ^{125}I-insulin binding at 3 h but lowered the binding capacity by 26, 64, and 80% at 6, 12, and 24 h, reaching its near-maximum 87% reduction at 72 h with a $t_{1/2}$ of 8.5 h. Scatchard plot analysis (Fig. 1C) revealed that GA treatment (1 μM for 24 h) significantly lowered the B_{max} values from 98.6 ± 4.5 to 20.0 ± 5.3 fmol/4 × 10^6 cells, without changing the K_d values (3.4 ± 0.2 nM, nontreated cells; 3.2 ± 0.2 nM, GA-treated cells; n = 5).

No Change of Immunoreactive IRS-1 Level and Attenuation of Insulin-Induced Tyrosine Phosphorylation of IRS-1 in GA-Treated Cells. We examined whether GA-induced down-regulation of cell surface IRs may decrease the intrinsic tyrosine kinase activity of IR. Cells were treated without or with 1 μM GA for 24 h and incubated without or with 100 nM insulin for 10 min for the measurement of insulin-induced tyrosine phosphorylation of IRS-1. As shown in Fig. 2 (top), IRS-1 level was similar between nontreated (Fig. 2, lanes 1 and 2) and GA-treated cells (Fig. 2, lanes 3 and 4). In contrast (Fig. 2, bottom), insulin-induced tyrosine phosphorylation of IRS-1 was attenuated by 77% in GA-treated cells (Fig. 2, lanes 3 and 4), compared with nontreated cells (Fig. 2, lanes 1 and 2).

Internalization of Cell Surface IRs: No Effect of GA Treatment. We examined whether GA may accelerate constitutive endocytic internalization of cell surface IRs, thus decreasing cell surface density of functional IRs. In various intact cells, BFA treatment (2.5–10 μg/ml for 2–36 h) blocked cell surface vesicular externalization from the TGN of newly synthesized proteins (e.g., renal epithelial Na^+ channels, α_{1B}-adrenoceptors, transferrin receptors, and glucose transporter-4), while having no effect on ADP-ribosylation factor 6-catalyzed internalization of receptors and ion channels (Moss and Vaughan, 1995; Shiraishi et al., 2000, 2001; Yamamoto et al., 2000). Adrenal chromaffin cells were treated without or with 1 μM GA in the presence of 10 μg/ml BFA for up to 24 h, and ^{125}I-insulin binding was assayed at the indicated times (Fig. 3). Cell surface ^{125}I-insulin binding was progressively decreased, but the internalization rate of cell surface IRs was similar between nontreated ($t_{1/2}$ = 8.0 h) and GA-treated ($t_{1/2}$ = 7.7 h) cells.

IR mRNA Levels: No Effect of GA Treatment. We examined whether GA treatment (1 μM for ~24 h) may decrease IR mRNA levels by Northern blot analysis (Fig. 4). IR probe hybridized to two major (~9.4 and ~7.0 kb) and one minor (~5.0 kb) transcripts of IRs, in accordance with the molecular sizes of multiple species of IR mRNAs (Yamamoto et al., 2000); these multiple transcripts encompass, in addition to the coding region, different lengths of 5′- and 3′-untranslated regions (Tewari et al., 1989). The levels of IR mRNAs were normalized against GAPDH mRNA levels; GA treatment did not significantly change 9.4-kb IR mRNA (97.9 ± 7.5, 98.8 ± 8.3, 103.8 ± 10.2, and 104.5 ± 15.0%), 7.0-kb IR mRNA (98.4 ± 5.0, 100.5 ± 8.2, 108.5 ± 10.2, and 109.0 ± 12.3%), and 5.0-kb IR mRNA (98.0 ± 10.0, 102.5 ±

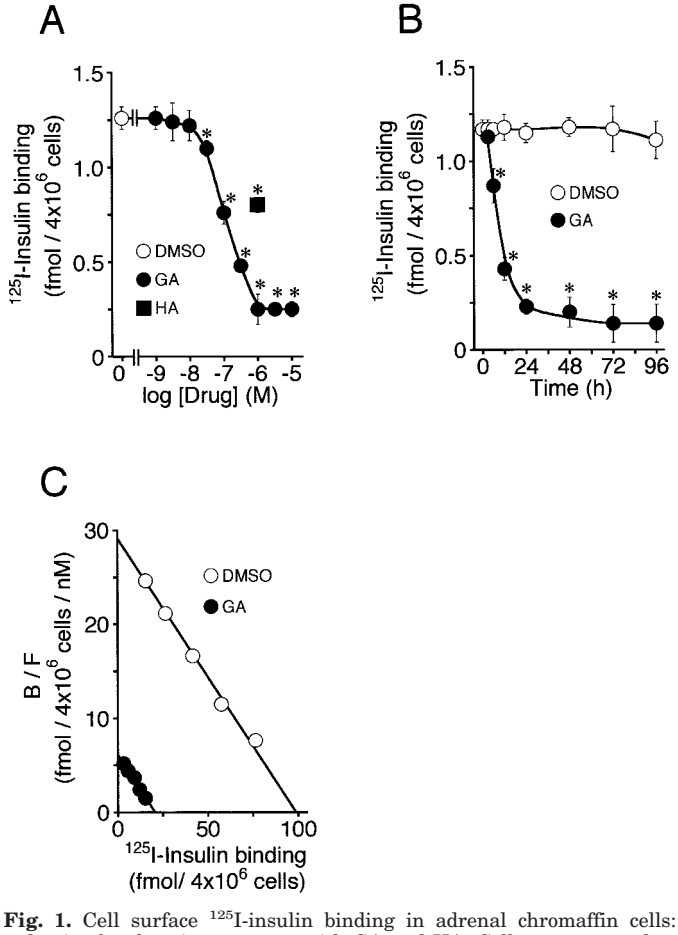

Fig. 1. Cell surface ^{125}I-insulin binding in adrenal chromaffin cells: reduction by chronic treatment with GA and HA. Cells were treated at 37°C without or with 0.001 to 10 μM GA or 1 μM HA for 24 h (A), or 1 μM GA for the indicated periods (B), and binding of ^{125}I-insulin was assayed. Mean ± S.E.M. (n = 5). *, $p < 0.05$, compared with nontreated cells. C, Scatchard plot of ^{125}I-insulin binding to cells treated without or with 1 μM GA for 24 h. Data are typical from five separate experiments with similar results. B/F, bound/free.

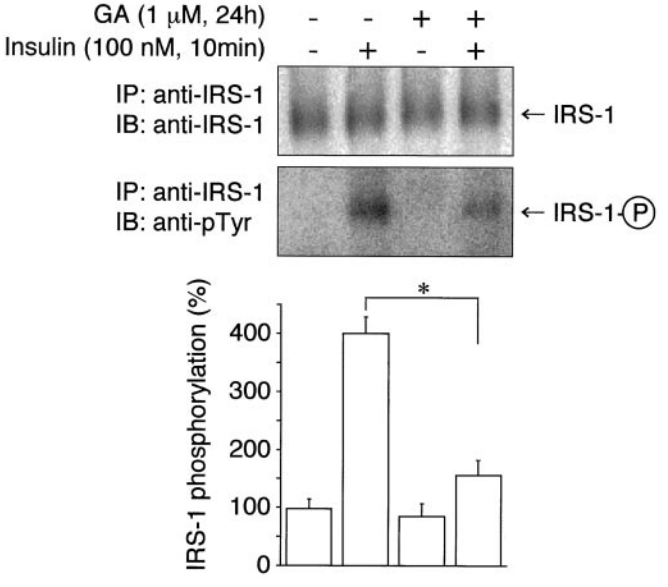

Fig. 2. Attenuation of insulin-induced tyrosine phosphorylation of IRS-1 in GA-treated cells. Cells were treated at 37°C without (−) or with (+) 1 μM GA for 24 h and incubated without (−) or with (+) 100 nM insulin for 10 min. Cell lysates were subjected to immunoprecipitation (IP) with IRS-1 antibody; the immunoprecipitates were separated by SDS-PAGE, transferred to membrane, and subjected to immunoblot (IB) analysis using IRS-1 antibody (anti-IRS-1; top) or phosphotyrosine-specific antibody (anti-pTyr; bottom). These blots were typical from five independent experiments with similar results. IRS-1 tyrosine phosphorylation levels were quantified by a Bioimage analyzer, a value of 100% representing the level obtained in cells that were incubated at 37°C for 24 h in the absence of GA and DMSO. Mean ± S.E.M. (n = 5). *, $p < 0.05$, compared with nontreated cells.

12.3, 98.5 ± 10.2, and 100.0 ± 15.3%) levels of nontreated cells at 3, 6, 12, and 24 h, respectively (n = 3).

Cellular Levels of Immunoreactive IR β-Subunit and IR Precursor Molecule: Opposite Effects of GA Treatment. We examined whether GA treatment may interfere with post-transcriptional events of IR synthesis, thereby causing down-regulation of cell surface IRs. By using IR β-subunit antibody, we measured IR β-subunit and IR precursor levels by Western blot analysis under the reducing condition (Fig. 5A). The antibody recognized a single major band (~95 kDa) and a minor band (~190 kDa), in agreement with the molecular sizes of mature IR β-subunit and monomeric IR precursor molecule, respectively (Ronnett et al., 1984; Arakaki et al., 1987; Olson et al., 1988; Caro et al., 1994; Bass et al., 1998; Elleman et al., 2000; instruction from Santa Cruz Biotechnology, the antibody manufacturer). The antibody, when reacted with the control blocking peptide before the Western blot analysis, did not detect these bands (data not shown). The levels of these immunoreactive bands were quantified by a Bioimage analyzer; GA treatment for 24 h decreased IR β-subunit level by ~83% with an EC_{50} of 74 nM, while increasing IR precursor level by ~100% with an EC_{50} of 300 nM (Fig. 5, A and B).

Cells were treated without or with 1 μM GA for up to 96 h (Fig. 6, A and B); GA decreased IR β-subunit level by 20% as soon as 3 h and further lowered its level by 33, 66. and 80% at 6, 12, and 24 h, causing the 91% fall at 96 h with a $t_{1/2}$ of 7.4 h. In contrast, the same GA treatment increased IR precursor level by 30% at 6 h, causing the maximum plateau increase of 81% between 24 and 96 h (Fig. 6, A and C).

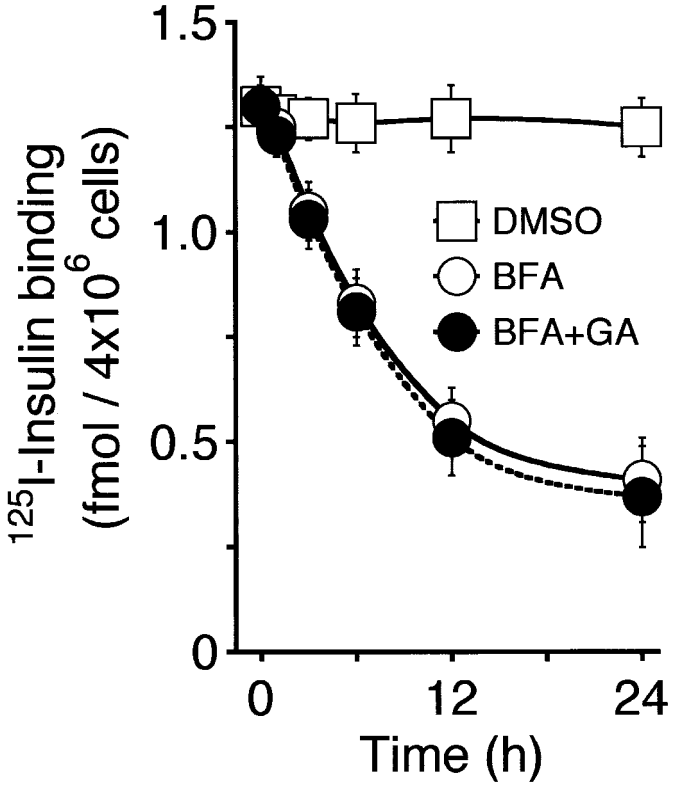

Fig. 3. Internalization of cell surface IRs: no effect of GA treatment. In the absence or presence of 10 μg/ml BFA, cells were treated without or with 1 μM GA for up to 24 h and washed, and the remaining cell surface IR was measured by ^{125}I-insulin binding assay at the indicated times. Mean ± S.E.M. (n = 5).

It has been shown that chronic (~24 h) treatment of various cells with GA or HA decreased cellular levels of insulin-like growth factor I receptor (Sepp-Lorenzino et al., 1995), ErbB2 (Xu et al., 2001), and CFTR (Loo et al., 1998) by accelerating their proteosomal proteolytic degradation, as evidenced by using lactacystin, MG132, or MG115, an inhibitor of proteasome. In our present study (Fig. 7), concurrent treatment of GA with either 10 μM lactacystin, 50 μM MG132, or 50 μM MG115 failed to prevent GA (1 μM for 24 h)-induced reduction of IR β-subunit level, suggesting that proteasomal degradation of mature IRs is not involved in the GA-induced reduction of mature IR level. Thus, GA-induced increase of IR precursor level and decrease of IR β-subunit level suggest that GA may interfere with post-translational processing of monomeric and/or dimeric IR precursor molecule(s) into the tetrameric mature IR.

Conformational Maturation of [^{35}S]Methionine/Cysteine-Labeled IR: Impairment by GA Treatment of Homodimerization of Monomeric IR Precursor. IRs were pulse-labeled with [^{35}S]methionine/cysteine, then immunoprecipitated with IR β-subunit antibody, and separated by SDS-PAGE under the nonreducing condition (Fig. 8, top). In nontreated cells, the monomeric form of IR precursor (~190 kDa) was converted to the dimeric form of IR precursor (~380 kDa) at 0.5 h and further processed into the tetrameric form of mature IR (~410 kDa) between 1 and 3 h, as reported previously (Ronnett et al., 1984; Arakaki et al., 1987; Olson et al., 1988; Caro et al., 1994; Bass et al., 1998). In GA-treated cells, the monomeric form of IR precursor was synthesized to the extent comparable with that in nontreated cells, but it failed to develop into the dimeric and tetrameric forms of IR. As shown in Fig. 8 (bottom), IR species were separated by the reducing SDS-PAGE. In nontreated cells, labeled monomeric IR precursor was processed into the α- and β-subunits at 1 h, and it was completed at ~3 h. In GA-treated cells, however, IR precursor was not processed into the α- and β-subunits. These correlative results suggest that GA impairs homodimerization of monomeric IR precursor in the ER, an event required for the transport of IR precursor from the ER to the Golgi apparatus (Olson et al., 1988). Thus, GA abolished proteolytic processing of dimeric IR precursor into the tetrameric $\alpha_2\beta_2$ IR, which is catalyzed by the endopeptidase furin in the TGN (Cheatham and Kahn, 1995).

Figure 8 also shows that radioactivity of pulse-labeled mo-

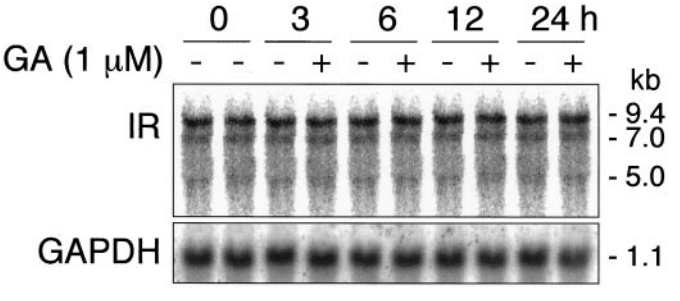

Fig. 4. Northern blot analysis of IR mRNA levels: no effect of GA treatment. Cells were treated at 37°C without (−) or with (+) 1 μM GA for the indicated periods; poly(A)$^+$ RNA was extracted, separated by electrophoresis on agarose gel, and transferred to membrane. The membrane was sequentially hybridized to each ^{32}P-labeled cDNA probe for IRs (top) and GAPDH (bottom) after removal of the former probe. Data are typical from three independent experiments with similar results.

nomeric IR precursor faded away in GA-treated cells after 2 h of the chase period. This result implies that the homodimerization-incompetent form of monomeric IR precursor in GA-treated cells was degraded via ER-associated protein degradation (ERAD). However, the molecular mechanism of ERAD remains largely unknown, in particular, for transmembrane proteins, such as IR precursor; this mechanism may involve cleavage of the transmembrane domain by cysteine and serine proteases, as well as Sec61-mediated retro-translocation of them from the ER to proteasome (Zhang et al., 2001).

Association of IR Precursor with CNX: Enhancement by GA Treatment. IRs are heavily glycosylated (~64 kDa) at, at least, 16 asparagine residues (Elleman et al., 2000), and association of monomeric IR precursor with CNX (Bass et al., 1998) plays crucial roles in ensuring conformational maturation of IR precursor and cell surface targeting of $\alpha_2\beta_2$ IR (Ronnett et al., 1984; Arakaki et al., 1987; Olson et al., 1988; Caro et al., 1994; Bass et al., 1998; Elleman et al., 2000; Hwang et al., 2000). As shown in Fig. 9A (top), the CNX level was comparable between nontreated and GA (1 μM for ~24 h)-treated cells. In contrast, GA treatment increased association of monomeric IR precursor with CNX by 42% at 3 h, causing the plateau 50% increase between 6 and 24 h (Fig. 9, A, bottom, and B).

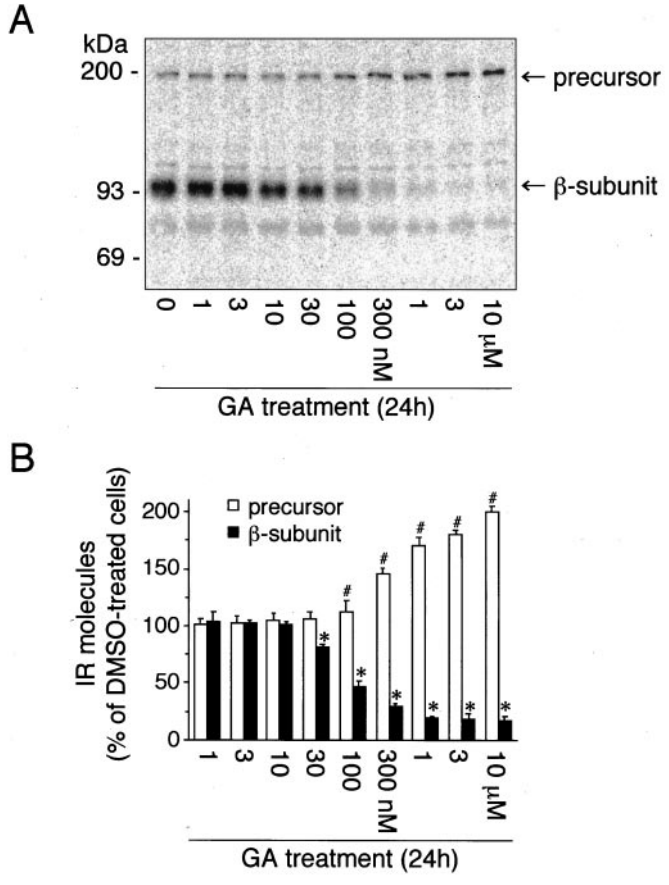

Fig. 5. Western blot analysis: decrease of IR β-subunit level and increase of IR precursor level caused by various concentrations of GA. A, cells were treated at 37°C without or with 0.001 to 10 μM GA for 24 h; cell lysates were separated by SDS-PAGE, transferred to membrane, and subjected to Western blot analysis with IR β-subunit antibody. This blot is typical from five separate experiments with similar results. B, immunoreactivities in A were quantified by a Bioimage analyzer, levels of IR β-subunit and IR precursor in nontreated cells representing 100%. Mean ± S.E.M. (n = 5). *, #, $p < 0.05$, compared with nontreated cells.

Discussion

In various cells including reticulocytes and v-Src-transformed fibroblasts, previous study documented that GA specifically bound to Hsp90 and formed a stable complex with Hsp90 (Whitesell et al., 1994). Previous binding study showed that GA exhibited higher affinity for Hsp90 (IC_{50} = ~0.3 μM) than for Grp94 (IC_{50} = ~1 μM); GA binding to Hsp90 was saturable at 1 μM, whereas GA binding to Grp94 became maximal at 30 μM (Xu et al., 2001). Future availability of WX514, a GA derivative with low affinity for Grp94 (Xu et al., 2001), may allow us to discriminate more readily between Hsp90 and Grp94. In the present study, chronic (≥6 h) treatment of adrenal chromaffin cells with GA decreased cell surface ^{125}I-insulin binding by 87% in a concentration (EC_{50} = 84 nM)- and time ($t_{1/2}$ = 8.5 h)-dependent manner. The almost maximum reduction of ^{125}I-insulin binding was obtained with GA treatment (1 μM for 24 h); the B_{max} value was lowered by 80% with no change in the K_d value. In GA (1 μM for 24 h)-treated cells, insulin-induced tyrosine phosphorylation of IRS-1 was attenuated by 77%, with no change in IRS-1 level. Because the K_d values of ^{125}I-insulin binding were similar between nontreated and GA-treated cells, attenuation of insulin-induced activation of IRS-1 in GA-treated cells is attributed to the down-regulation of cell-surface functional IRs. Internalization rate of cell surface IRs was comparable between nontreated and GA (1 μM)-treated cells for up to 24 h. Western blot analysis showed that chronic treatment with GA decreased the IR β-subunit level by 83% in a concentration (EC_{50} = 74 nM)-dependent manner, being comparable with those (87% decrease; EC_{50} = 84 nM) of

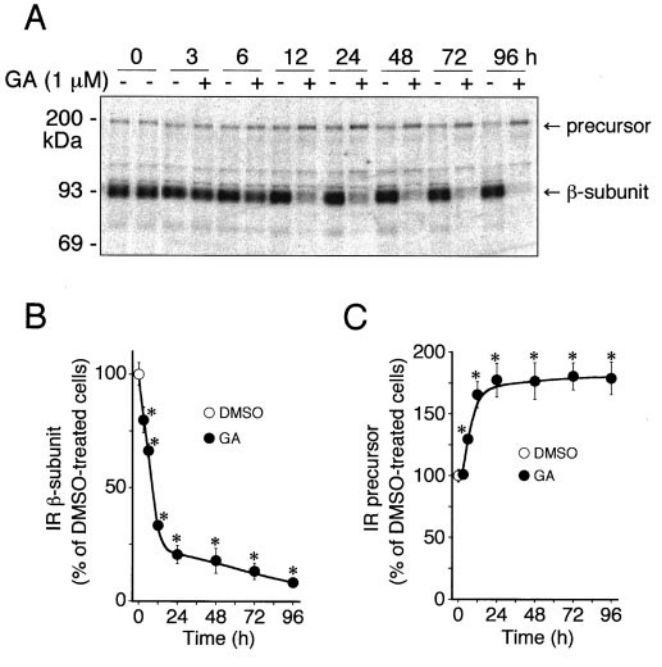

Fig. 6. Western blot analysis: time-dependent effects of GA on IR β-subunit and IR precursor levels. A, cells were treated at 37°C without (−) or with (+) 1 μM GA for the indicated periods, lysed, and subjected to Western blot analysis using IR β-subunit antibody. Data are typical from five separate experiments with similar results. B and C, quantification of immunoreactivities in A by a Bioimage analyzer, a value of 100% representing the immunoreactivity in nontreated cells at the left lane in each incubation time. Mean ± S.E.M. (n = 5). *, $p < 0.05$, compared with nontreated cells.

GA-induced reduction of ^{125}I-insulin binding. GA decreased the IR β-subunit level by 20% as soon as 3 h, when ^{125}I-insulin binding was not yet lowered, and reduction of ^{125}I-insulin binding became evident between 3 and 6 h of GA treatment. These correlative results suggest that GA decreases cellular level of mature $\alpha_2\beta_2$ IRs, thereby causing down-regulation of cell surface IRs. Northern blot analysis, however, showed that steady-state levels of 9.4-, 7.0-, and 5.0-kb IR mRNAs were similar between nontreated and GA (1 μM)-treated cells for up to 24 h. Thus, GA may retard post-transcriptional steps required for the synthesis of mature IRs, thus causing down-regulation of cell surface IRs.

Western blot analysis also showed that GA treatment raised the cellular level of the IR precursor molecule (~190 kDa) by 100% in a concentration-dependent manner between 0.1 and 10 μM, at which GA caused concentration-dependent reductions of ^{125}I-insulin binding and IR β-subunit level. GA (1 μM)-induced increase of IR precursor level became evident between 3 and 6 h, and developed to the maximum at 24 h, when GA decreased ^{125}I-insulin binding and IR β-subunit level in a time-dependent manner. Pulse-label with [^{35}S]methionine/cysteine followed by nonreducing SDS-PAGE documented that monomeric IR precursor (~190 kDa) was processed into the dimeric IR precursor (~380 kDa), and the tetrameric mature IR (~410 kDa) in nontreated cells, whereas this conformational maturation of monomeric IR precursor was almost completely blocked in GA (1 μM)-treated cells. SDS-PAGE under the reducing condition showed that monomeric IR precursor was processed into the mature $\alpha_2\beta_2$ IR in a time-dependent manner in nontreated cells, but not in GA-treated cells. These results suggest that inhibition of the Hsp90 family by GA remarkably inhibits homodimerization of monomeric IR precursor in the ER, thereby causing down-regulation of cell surface IR. Also, pulse-label followed by nonreducing and reducing SDS-PAGE revealed that in GA-treated cells, the homodimerization-incompetent form of monomeric IR precursor was degraded within 2 h via ERAD. However, the monomeric IR precursor began to accumulate between 3 and 6 h after GA treatment, reaching the maximum plateau increase at 24 h.

In the ER, N-linked glycosylation and disulfide-bond formation/isomerization of the immature form of monomeric IR precursor were indispensable to its conformational maturation, such as the acquisition of insulin binding capacity (Olson et al., 1988) and intrinsic tyrosine kinase activity (Hwang et al., 2000). Previous pulse-label studies in hepatoma Fao cells and 3T3-L1 adipocytes documented that the primary translational product of the IR gene was the aglyco form (~180 kDa) of monomeric IR precursor; it rapidly ($t_{1/2}$ = 15 min) developed into the longer-lived mature form (~190 kDa) of monomeric IR precursor (Goldstein and Kahn 1988; Olson et al., 1988). When N-linked glycosylation was blocked by tunicamycin in 3T3-L1 adipocytes, the aglyco form of IR precursor did not develop into the mature form of monomeric IR precursor and did not acquire insulin binding capacity (Ronnett et al., 1984; Olson et al., 1988). Similar results were obtained in 3T3 fibroblasts (Caro et al., 1994) or Chinese hamster ovary (CHO) cells transfected with human IR (Elleman et al., 2000), in which N-linked glycosylation of IR precursor was blocked by the mutation of multiple asparagine residues to glutamine. When glucosidase I- and II-catalyzed sequential glucose trimming of the oligosaccharide core unit of monomeric IR precursor was blocked by castanospermine or 1-deoxynojirimycin in IM-9 lymphocytes, the monomeric IR precursor remained its higher molecular size (~205 kDa), and the inability of monomeric IR precursor to associate with CNX/CRT hampered its processing into the α- and β-subunits (Arakaki et al., 1987). In CHO cells transfected with human IRs, CNX/CRT bound to monomeric (but not dimeric) IR precursor, thus increasing the efficiency of intrachain disulfide-bond formation/isomerization of monomeric

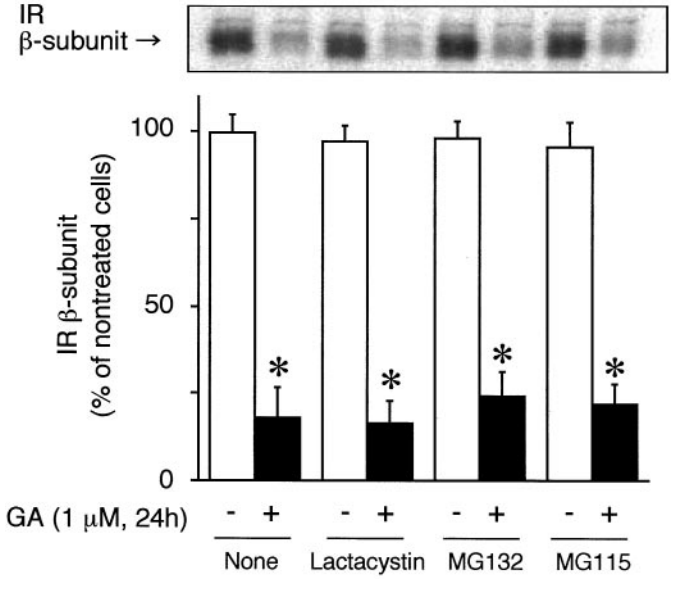

Fig. 7. No prevention by proteasome inhibitors of GA-induced decrease of IR β-subunit level. In the absence (None) or presence (indicated under the horizontal line) of 10 μM lactacystin, 50 μM MG132, or 50 μM MG115, cells were treated without (−) or with (+) 1 μM GA for 24 h and subjected to Western blot analysis with IR β-subunit antibody. This blot is typical from five separate experiments with similar results. Immunoreactivities were quantified by a Bioimage analyzer, a value of 100% representing the level in cells not treated with DMSO and any test drug. Mean ± S.E.M. (n = 5). *, $p < 0.05$, compared with GA-nontreated cells.

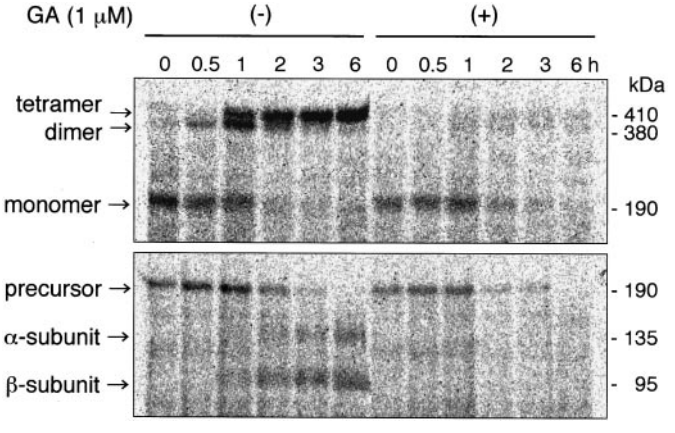

Fig. 8. Time course of [^{35}S]methionine/cysteine-labeled IR synthesis analyzed by reducing and nonreducing SDS-PAGE: interruption by GA of homodimerization of monomeric IR precursor. Cells were pulse-labeled with [^{35}S]methionine/cysteine for 30 min and chased for up to 6 h in the absence (−) or presence (+) of 1 μM GA; cells were washed, solubilized, and centrifuged; and proteins in the supernatant were immunoprecipitated with IR β-subunit antibody. The immunoprecipitates were solubilized and separated by nonreducing 3 to 10% linear gradient SDS-PAGE (top) and reducing 7.5% SDS-PAGE (bottom). Data are typical from five separate experiments with similar results.

IR precursor (Bass et al., 1998). In CHO cells treated with castanospermine, the inability of monomeric IR precursor to associate with CNX/CRT accelerated homodimerization of IR precursor but produced misfolded IR precursor, of which processing was delayed and cell surface expression was decreased (Bass et al., 1998).

In our present study, monomeric IR precursor was associated with CNX to a greater extent (~54%) in GA (1 μM)-treated cells between 3 and 24 h, when monomeric IR precursor level increased in GA-treated cells. A recent study showed that, if nascent growing polypeptides (e.g., IR precursor) (Elleman et al., 2000) have N-linked oligosaccharide chain within the first ~50 amino acid residues from their N terminus, the polypeptides preferentially interact with CNX/CRT without prior association with immunoglobulin heavy-chain binding protein (Bip/Grp78) (Molinari and Helenius, 2000). Molinari and Helenius (1999) provided the first evidence in mammalian living cells that CNX/CRT promotes disulfide-linked protein folding/assembly by acting as a scaffold protein to recruit protein disulfide isomerase and ERp57, a new member of the protein disulfide isomerase family in the ER lumen (Ellgaard and Helenius, 2001). In CHO cells expressing human IRs, previous pulse-label study showed that impairment of disulfide-bond formation by dithiothreitol produced the misfolded immature form of monomeric IR precursor that failed to develop into the mature form of monomeric IR precursor (Bass et al., 1998). In our present study, however, GA treatment did not perturb synthesis of the mature form (~190 kDa) of monomeric IR precursor, implying that the Hsp90 family may not be important for executing the intrachain disulfide-bond formation/isomerization of monomeric IR precursor. Disulfide-bond formation/isomerization-related conformational maturation of proteins in intact cells is a complex and, as yet, not fully defined process (Ruddon and Bedows, 1997; Molinari and Helenius, 1999). There has been no report specifying the mechanism that regulates disulfide-linked homodimerization of monomeric IR precursor (Olson et al., 1988; Bass et al., 1998). Our present study provides the evidence that the Hsp90 family is involved in the homodimerization of monomeric IR precursor. Previous immunoprecipitation studies showed that Hsp90 was associated with the cytoplasmic domain of IR β-subunit (Takata et al., 1997) and with the intrinsic tyrosine kinase motif of the cytoplasmic domain of ErbB2 (Xu et al., 2001). Based on these previous results, it may be intriguing to conjecture that Hsp90 interacts with the cytoplasmic domain of monomeric IR precursor and plays an essential role in accomplishing homodimerization of IR precursor, because the homodimerization process seemingly proceeds within the ER lumen and involves disulfide-bond formation/isomerization at the ER lumenal domain of IR precursor (Olson et al., 1988; Cheatham and Kahn, 1995; Bass et al., 1998).

In rat-1 fibroblasts expressing human IRs, microinjection of Hsp90 antibody abolished insulin-induced mitogenesis, suggesting the essential role of Hsp90 in mediating intracellular signal transduction of cell surface IRs (Takata et al., 1997). In an expression study in COS-7 cells, human IR precursor mutant at the intrinsic tyrosine kinase domain (Glu1179Asp or Trp1193Leu) normally bound to CNX, but was associated with Hsp90 to a greater extent than wild-type IR precursor; the increased association with Hsp90 culminated in the accelerated proteasomal degradation of mutant IR precursor and caused down-regulation of cell surface IRs (Imamura et al., 1998). Conversely, an expression study in thermosensitive mutant ts20 lung cells implied that inhibition of the Hsp90 family by HA rather promoted degradation of human IRs only at the permissive (but not nonpermissive) temperature, at which the ubiquitin-proteasome system could be activated in the mutant ts20 lung cells (Sepp-Lorenzino et al., 1995). Thus, our present study provides the first evidence that in GA-treated cells, monomeric IR precursor was incompetent to undergo homodimerization and retained with CNX in the ER, thus causing down-regulation of cell-surface functional IRs. Chaperone function of the Hsp90 family is indispensable to normal conformational maturation of IRs.

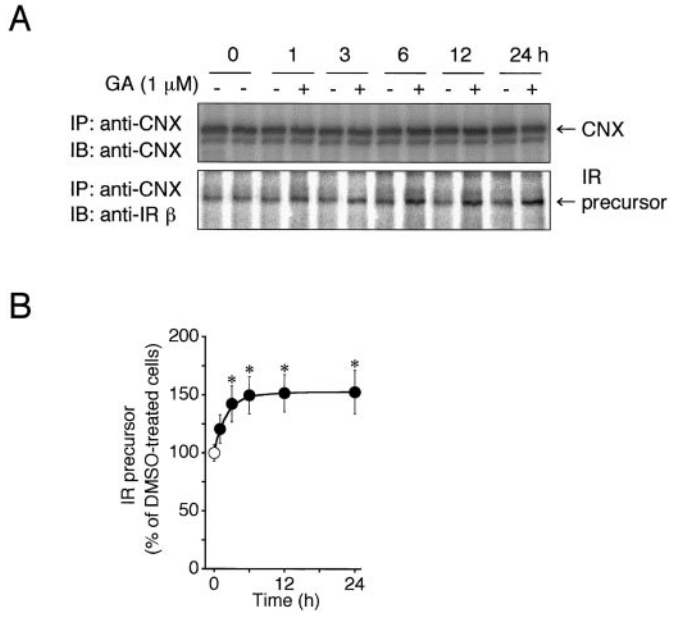

Fig. 9. Increased association of IR precursor with CNX in GA-treated cells. A, cells were treated at 37°C without (−) or with (+) 1 μM GA for the indicated periods, and subjected to immunoprecipitation with CNX antibody and IB analysis with CNX antibody (top) or IR β-subunit antibody (bottom). Data are typical from five separate experiments with similar results. B, immunoreactivities in A were quantified by a Bioimage analyzer, the level of nontreated cells at the left lane in each incubation time being assigned a value of 100%. Mean ± S.E.M. ($n = 5$). *, $p < 0.05$, compared with nontreated cells.

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