Involvement of Heat Shock Protein 90 in the Degradation of Mutant Insulin Receptors by the Proteasome*

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Takeshi Imamura, Tetsuro Haruta, Yasumitsu Takata, Isao Usui, Minoru Iwata, Hajime Ishihara, Manabu Ishiki, Osamu Ishibashi, Eiichi Ueno, Toshiyasu Sasaoka, and Masashi Kobayashi‡

From the First Department of Medicine, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-01, Japan

We previously reported three families with type A insulin-resistant syndrome who had mutations, either Asp1179 or Leu1193, in the kinase domain of the insulin receptor. The extreme insulin resistance of these patients was found to be caused by the decreased number of insulin receptors on the cell surface, due to the intracellular rapid degradation (Imamura, T., Takata, Y., Sasaoka, T., Takada, Y., Moriga, H., Haruta, T., Sawada, T., Iwanishi, M., Yang, G. H., Suzuki, Y., Hamada, J., and Kobayashi, M. (1994) J. Biol. Chem. 269, 31019–31027). In the present study, we first examined whether these mutations caused rapid degradation of unprocessed proreceptors, using the exon 13 deleted mutant insulin receptors (ΔEx13-IR), which were accumulated in the endoplasmic reticulum as unprocessed proreceptors. The addition of Asp1179 or Leu1193 mutation to ΔEx13-IR caused accelerated degradation of the unprocessed ΔEx13-IR in the transfected COS-7 cells. Next, we tested whether these mutant receptors were degraded by the proteasome. Treatment with proteasome inhibitors Z-D-FK and MG-132 prevented the accelerated degradation of both mutant receptors, resulting in increased amounts of the mutant receptors in the COS-7 cells. Essentially the same results were obtained in the patient’s transformed lymphocytes. Finally, we found that these mutant receptors bound to heat shock protein 90 (Hsp90). To determine whether Hsp90 played an important role in the accelerated receptor degradation, we examined the effect of anti-Hsp90 antibody on the mutant receptor degradation. The microinjection of anti-Hsp90 antibody into cells prevented the accelerated degradation of both Asp1179 and Leu1193 mutant insulin receptors. Taken together, these results suggest that Hsp90 is involved in dislocation of the mutant insulin receptors out of the endoplasmic reticulum into the cytosol, where the mutant receptors are degraded by the proteasome.

Various mutations in the insulin receptor gene have been reported in patients with severe insulin resistance (1). Analysis of the mutated insulin receptors in the cells gave us the opportunity to understand the function and processing of insulin receptor protein. Among the various mechanisms for the insulin resistance in these patients, certain patients with the insulin receptor mutations showed a reduced number of insulin receptors on the cell surface although the mRNA of insulin receptor was normally expressed (1). Two major causes for this phenomenon have been described: the first is the impaired protein processing of mutated insulin receptors and accumulation in the endoplasmic reticulum (ER)1 (3–13), and the second is the accelerated intracellular degradation of mutant receptor proteins (14, 15). We previously reported the three families of type A insulin-resistant syndrome who had a mutation (Asp1179 or Leu1193)2 in the kinase domain of the insulin receptor leading to the accelerated intracellular degradation of the unprocessed proreceptors (15).

Interestingly, Arg209 and Val382 mutant insulin receptors, which were accumulated in the ER, were associated with a molecular chaperone, immunoglobulin heavy chain binding protein (BiP), one of the heat shock proteins in the ER (16). Thus, it was likely that the difference in the molecular chaperones to which the mutant insulin receptor tightly bound determined the fate of mutant insulin receptors. Although the association of these specific chaperons with some of mutant insulin receptors has been described, the role of molecular chaperones and the site where these mutants degraded have not been well characterized.

The mechanism of intracellular degradation of abnormal proteins in the secretory pathway has not been clearly understood. However, recent observations suggest that unfolded or unassembled proteins are retained in the ER by chaperones, transported back into the cytosol and degraded by the proteasome (17–20). In the present study, to investigate the mechanism of the accelerated degradation of both Asp1179 and Leu1193 mutant insulin receptors, we examined whether these mutations caused accelerated degradation of unprocessed proreceptors retained in the ER and whether these mutant receptors were degraded by the proteasome, and finally which molecular chaperone was involved in degradation. We now report that the mutant insulin proreceptors in the ER are rapidly transported to the proteasome for degradation through the binding to Hsp90.

EXPERIMENTAL PROCEDURES

Cell Lines and Materials—COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum as described (21). GST gene fusion system was purchased from Amer-
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Sham Pharmacia Biotech. Proteasome inhibitors MG-115 and MG-132 and protease inhibitor E-64-d were from Peptide Institute Inc. (Osaka, Japan). Enhanced chemiluminescence reagents were from Amersham Pharmacia Biotech. Hsp90 and Hsc70 protein, and antibodies against Hsp90, Hsc70, Hsp60, BiP, and ubiquitin were purchased from Stressed Protein Science (Osaka, Japan). Anti-calnexin antibody was provided by Dr. I. Wada (Sapporo University of Medical Science, Sapporo, Japan), and polyclonal anti-insulin receptor C-terminal antibody was provided by Dr. J. M. Olefsky. Mouse monoclonal anti-insulin receptor α-subunit antibody was purchased from Cosmo Bio Co. Ltd. (Tokyo, Japan). Mouse immunoglobulin G (IgG) and fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit and anti-mouse IgG antibodies were from Jackson Laboratories (West Grove, NY). Electrophoresis reagents were from Bio-Rad. All other reagents were of analytical grade and were purchased from Sigma or Wako Pure Chemical Industries (Osaka, Japan).

Construction and Expression of Wild-type and Mutant Insulin Receptors—Expression plasmids (pGEMSVHHR) of wild-type and mutant (exon 13 deletion (ΔEx13), Asp1179 Leu1193 ΔEx13 + Asp1179, and ΔEx13 + Leu1193) human insulin receptor were constructed as described previously (15, 22). COS-7 cells were transfected with these vectors by electroporation, using a Gene Pulser (Bio-Rad). The cells were electroporated with a total of 40 µg of DNA at 340 V and 960 microfarads to obtain a high degree of transfection efficiency, according to Yamauchi et al. (23). Briefer—COS-7 cells were transfected for steady-state labeling experiments at 60 h after transfection. The transfected cells were incubated in methionine-free DMEM containing 10% dialyzed fetal bovine serum and Tran35S-label (0.1 mCi/ml; 1022 Ci/mmol; ICN Biochemicals Inc.) at 37 °C for 24 h. Treated precipitates were separated by SDS-PAGE and autoradiography (15).

Expression of Glutathione S-Transferase-Insulin Receptor Cytoplasmic Domain Fusion Protein—The cytoplasmic domain of insulin receptor (IRc) was inserted into the baculovirus expression vector pICG (codon 958) and Spel (nucleotide 13100 of 3’ intron) (24). The fragments containing the cytoplasmic domain of insulin receptor (IRc) were inserted into blunt end by use of T4 DNA polymerase. The GST fusion protein expression vector pGEX-5X-2 was digested with SmaI, inserted into blunt end similarly, and treated with calf intestine alkaline phosphatase. Two fragments were ligated, and then the plasmids were transferred into E. coli BL21 competent cells (Takara, Tokyo, Japan). The plasmids containing the cytoplasmic insulin receptor cDNA were selected by enzyme restriction and confirmed by sequencing. After transfection of the plasmids containing GST-IRc cDNA into E. coli BL21 strain by electroporation, GST-IRc fusion proteins were purified according to the specifications of the manufacturer (Amersham Pharmacia Biotech).

Assocation of GST-IRc Fusion Proteins with Heat Shock Proteins—After purification through glutathione-Sepharose 4B, 3 µg each of the fusion proteins were incubated with 9.9 µg of Hsp90 and/or Hsc70 in 1 ml of phosphate-buffered saline (PBS) for 1 h at 4 °C. After washing three times with PBS, the glutathione-Sepharose 4B-coupled protein complex was centrifuged at 500 x g and was dissolved in Laemmli sample buffer (25) to be analyzed by Western blotting.

Western Blotting Analysis—Cells were washed three times with PBS and solubilized in boiled Laemmli sample buffer for experiments of the proteasome inhibitors. For the immunoprecipitation assay, cells were lysed in a pH 7.4 buffer, containing 30 mM Tris, 150 mM NaCl, 10 mM EDTA, 0.5% sodium deoxycholate, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, and 10 µg/ml leupeptin. The cell lysates were centrifuged to remove insoluble materials. The supernatants were used for immunoprecipitation with the indicated antibodies for 4 h at 4 °C. The precipitates were separated by SDS-PAGE and transferred to Immobilon-P using a Bio-Rad Transblot apparatus. The membranes were blocked in a pH 7.5 buffer, containing 50 mM Tris, 150 mM NaCl, 0.1% Tween 20, and 2.5% bovine serum albumin, for 2 h at 20 °C and were then probed with the antibodies for 2 h at 20 °C. After washing the membranes in a pH 7.5 buffer, the individual filters were incubated 50 mM Tris, 150 mM NaCl, and 0.1% Tween 20, blots were incubated with horseradish peroxidase-linked second antibody followed by enhanced chemiluminescence detection using the ECL reagent according to the manufacturer’s instructions (Amersham Pharmacia Biotech) (15).

Treatment with Proteasome Inhibitors—Transfected COS-7 cells expressing mutant or wild-type insulin receptors were incubated in fetal calf serum-free DMEM with 0.3% MeSO, 1% bovine serum albumin, and with 50 µM proteasome inhibitors, Z-Leu-Leu-Val-A (MG-115), or Z-Leu-Leu-Leu-H (MG-132), or with 50 µM cysteine protease inhibitor E-64-d in CO2 incubator at 37 °C. After 2 h of incubation, the cells were washed twice with PBS and then dissolved in Laemmli sample buffer to be analyzed by Western blotting, as described above.

Microinjection Assay—Cells were grown on glass coverslips and rendered quiescent by starvation for 24 h in serum-free DMEM. Antibodies in a buffer containing 5 mM NaPO4 and 100 mM KCl, pH 7.4, were microinjected into cells using a glass capillary needle. Approximately 1 x 10-11 liter of the buffer was injected into each cell. The injection in microinjected COS-7 cells was visualized by confocal fluorescence microscopy (Olympus, Japan).

RESULTS

Accelerated Degradation of Mutant Insulin Proreceptor—We previously suggested that the accelerated degradation of both Asp1179 and Leu1193 mutant insulin receptors occurred in the stage of proreceptor (15). To examine whether Asp1179 or Leu1193 mutation may cause the rapid degradation of the proreceptors retained in the ER, we used the exon 13 deletion mutant (ΔEx13) of insulin receptor, which was accumulated in the ER as the uncleaved proreceptor and could not undergo further processing (22). We introduced Asp1179 or Leu1193 mutation into the ΔEx13 mutant insulin receptor cDNAs, i.e. ΔEx13 + Asp1179 or ΔEx13 + Leu1193. After transfection of these cDNAs into COS-7 cells, we examined the synthetic and processing time course of these mutant receptors. These double mutant insulin receptors produce only a single 190-kDa protein corresponding to the ΔEx13 proreceptor form (Fig. 1A). Insulin binding to the COS-7 cells (1 x 106 cells) transfected with wild-type, mock, ΔEx13, ΔEx13 + Asp1179, and ΔEx13 + Leu1193 cDNAs were 15 ± 0.9, 9 ± 0.3, 0.9, 0.8, and 0.9% of total, respectively. Therefore, both double mutant receptors as well as the ΔEx13 mutant receptor were not transported to the cell surface. To investigate the degradation of these mutant insulin proreceptors, steady-state labeling studies were carried out in the transfected COS-7 cells. As shown in Fig. 1 (A and B), the labeled ΔEx13 proreceptor slowly degraded, and t1/2 for the ΔEx13 proreceptor was 15 h (Fig. 1B). In contrast, both the ΔEx13 + Asp1179 and ΔEx13 + Leu1193 mutant proreceptors disappeared rapidly with a half-life of 6 h, which was 2.5 times faster than that for ΔEx13 proreceptors. These results indicated that both Asp1179 and Leu1193 mutations of the insulin receptor caused the accelerated degradation of the unprocessed proreceptor accumulated in the ER.

The Role of Proteasome in Degradation of the Mutant Insulin Receptors Associated with the ER—To investigate whether the proteasome was involved in the mutant receptor degradation, we examined the effect of proteasome inhibitors. The COS-7 cells expressing the wild-type or the mutant insulin receptors were microinjected with MG-115 (Z-Leu-Leu-Val-A), MG-132 (Z-Leu-Leu-Leu-H), or E-64-d. Fig. 2 shows the two major bands of 190 and 95 kDa. The 190-kDa band corresponds to the proreceptor and 95-kDa band to the mature β-subunit. Treatment with E-64-d, which inhibited the calpain and cathepsin protease activities, made no changes in the intensities of the wild-type and mutant receptors. However, in the Leu1193 and

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The study investigates the degradation of mutant insulin proreceptors in transfected COS-7 cells. It was observed that the amount of proreceptor associated with Hsp90 was 6- and 7-fold greater than that bound to the wild-type GST-IRc. The binding to GST-IRc with Asp1179 or Leu1193 mutation was significantly associated with these mutants.

The study also examines whether these phenomena were similarly observed in the cells from these patients with extreme insulin resistance. The transformed lymphocytes from the patient with type A insulin-resistant syndrome having heterozygous Asp1179 mutation in the insulin receptor were similarly treated. As shown in Fig. 3, the intensities of the proreceptor and the β-subunit bands from the patient’s transformed lymphocytes were increased 2.5-fold by treatment with the proteasome inhibitors, but not with E-64-d.
bodies. However, association between Hsp90 and ubiquitin was observed by co-immunoprecipitation with either anti-Hsp90 or anti-ubiquitin antibody in COS-7 cells transfected with the wild-type or mutant insulin receptor cDNAs (data not shown).

**Microinjection of anti-Hsp90 Antibody to Transfected COS-7 Cells**—To investigate whether Hsp90 played an important role in the accelerated receptor degradation, we studied the effect of anti-Hsp90 antibody on the mutant receptor degradation. Insulin receptor was visualized by immunofluorescence microscopy utilizing FITC-labeled anti-rabbit IgG for anti-insulin receptor antibody (Fig. 5, left side), and injected cells were identified by rhodamine-labeled anti-mouse IgG antibody for anti-Hsp90 antibody (Fig. 5, A–D, right side), or rhodamine-labeled anti-rat IgG for anti-Hsc70 antibody (Fig. 5, E–H, right side). Microinjection of either anti-Hsp90 antibody or anti-Hsc70 antibody into COS-7 cells transfected with the wild-type insulin receptor cDNA (Fig. 5A) did not change the amount of insulin receptors. COS-7 cells, which were transfected with Asp1179 or Leu1193 mutant receptor cDNA, had a small amount of receptors compared with the cells transfected with wild-type cDNA (Fig. 5, B, C, F, and G; un.injected cells). However, microinjection of anti-Hsp90 antibody led to the clearly increased amount of the Asp1179 or Leu1193 mutant insulin receptors at 12 h after microinjection (Fig. 5, B and C; injected cells). On the other hand, microinjection of anti-Hsc70 (Fig. 5, F and G; injected cells) or anti-BiP antibody (data not shown) did not change the amount of the receptors.

To directly prove that Hsp90 is involved in the degradation of the mutant receptors, we injected anti-Hsp90 antibody into 1000 transfected COS-7 cells and subjected them to immunoblot analysis (Fig. 6). In the cells expressing Asp1179 or Leu1193 mutant insulin receptor, the amount of receptors was increased to 2.5- or 3.3-fold, respectively, by injection with anti-Hsp90 antibody. However, in the cells expressing the wild-type receptors, there was no change in the amount of receptors by the antibody microinjection. Thus, the antibody binding to Hsp90 suppressed the accelerated mutant receptor degradation, most likely by preventing mutant receptors from association with Hsp90 and from subsequent degradation by the proteasome.

**DISCUSSION**

Most of the naturally occurring mutations in the \( \beta \)-subunit of the insulin receptor interfere with processing of proreceptors to the subunits, leading to accumulation of proreceptors in the ER. As reported previously, these mutant insulin receptors were tightly associated with BiP, an intra-ER chaperone, and remained in the ER (16). Similarly, the mutant insulin receptor lacking the coding region of exon 13 was not processed to the subunits and was also associated with BiP (22, 29). Since this \( \Delta \text{Ex}13 \) insulin receptor remained unprocessed and was easily distinguished from normal matured insulin receptors, we created double mutant insulin receptors that had both \( \Delta \text{Ex}13 \) and either Asp1179 or Leu1193 mutations in the tyrosine kinase domain, and monitored the processing and degradation of this mutant insulin receptors. Addition of either Asp1179 or Leu1193 mutations to \( \Delta \text{Ex}13 \) insulin receptor led to rapid receptor degradation (15), suggesting that these point mutations were specific for the cause of accelerated degradation of unprocessed insulin receptors that existed in the ER.

The ER-associated proteins destined for degradation are transported to the cytosol, where proteolysis is catalyzed by the proteasome (17, 18). By using two proteasome inhibitors, we clearly demonstrated that proteasome played a major role in degradation of the mutant receptors. Furthermore, the inhibitors increased the amount of insulin receptor protein in the transformed lymphocyte derived from the patient with Asp1179 mutation. Thus, the proteasome inhibitors rescued these mutant receptors, which could be transported to the cell surface. These results suggest that both Asp1179 and Leu1193 mutant insulin proreceptors are degraded by the proteasome in the cytosol before their transport from the ER to the Golgi apparatus, because the degradation occurs before the processing from the proreceptor to the \( \alpha \)- and \( \beta \)-subunits takes place at the Golgi apparatus.

The degradation by the proteasome usually requires polyubiquitination of the targeted proteins (18, 30–32). However, we could not detect any specific association of ubiquitin with these mutant insulin receptors. Therefore, Asp1179 and Leu1193 mutant insulin receptor proteins were degraded by the proteasome without direct ubiquitination in the transfected COS-7 cells and the patient's cells. In fact, the proteasome degradation of certain proteins does not require ubiquitination (19, 33, 34).

To determine the mechanism by which these mutant insulin proreceptors were transported out of the ER and then presented to the proteasome, we tested the possibility that specific molecular chaperones might bind to the mutant proteins and present them to the proteasome. We demonstrated that Hsp90 was tightly associated with these mutant receptors, both in the cells and in the cell-free system, using GST fusion proteins. Furthermore, the degradation of the mutant insulin receptor protein was partially inhibited by microinjection of the anti-Hsp90 antibody, leading to the increased amount of these mutant insulin receptors in the cells. These experiments sug-
gested that Hsp90 played a key role in transport of these mutant receptors out of the ER and in the subsequent degradation by the proteasome in the cytosol.

Hsp90, which exists in the cytosol, functions as a molecular chaperon, by the complex formation with steroid receptor (35, 36), pp60v-src (37, 38), Raf-1 (39), or casein kinase II (40). However, it has not been reported that Hsp90 participates in the degradation of ER-associated misfolded proteins. Hsp90 functions in co-operation with several kinds of molecular chaperones, such as Hsc70, as we demonstrated in GST-IRc-Hsp90 binding studies in the presence of Hsc70. Furthermore, we observed that Hsp90 associated with ubiquitin in the COS-7 cells expressing Asp1179 or Leu1193 mutant type or wild-type insulin receptors. These results suggest that the association of the mutant insulin receptors with Hsp90 might be followed by further complex formation with ubiquitin and Hsc70. It is also possible that other chaperons, which we could not identify, might participate in the ER-associated protein degradation.

The mechanism whereby misfolded proteins are dislocated from the ER is not clearly understood. Recent observations by Weirtz et al. (33) suggest that the Sec61 complex is thought to have a role in the dislocation of proteins from the ER to the cytosol, and that the misfolded proteins could be caught in transit through the channel. It should be further investigated whether the mutant insulin receptors such as Asp1179 or Leu1193 mutant receptor are also dislocated from the ER to the cytosol in the similar fashion.

Asp1179 and Leu1193 mutations may cause a significant change in tertiary structure of the receptor that can be specifically recognized by Hsp90. The close location and surface exposure of Glu1179 and Trp1193 in the tertiary structure of insulin receptor β-subunits (41) would support the importance of these amino acids for Hsp90 binding. Thus, it is likely that other mutations in the kinase domain may cause similar phenomena as shown in Asp1179 and Leu1193 mutations. Another mutation, Asp1048 in the kinase domain (42), however, did not lead to accelerated degradation, probably due to a relatively
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normally folded structure of the receptor protein. Although there are some other mutants of the kinase domain that show the decreased insulin binding (43, 44), it remains to be determined whether they are also degraded in the same manner as described above. On the other hand, in the α-subunit of the insulin receptor, only the Glu460 mutation showed the accelerated receptor degradation (14). Since it was reported that this degradation was different from that of Asp1179 and Leu1193 mutant receptors.

In conclusion, we have clarified a novel mechanism for degradation of the mutant insulin receptors by proteasome, in which Hsp90 plays a key role.

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