

**Original Article****Mechanisms of antibody-mediated insulin-like growth factor I receptor (IGF-IR) down-regulation in MCF-7 breast cancer cells**

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**Summary** The insulin-like growth factor I receptor (IGF-IR) plays a critical role in cell proliferation and survival. We previously reported that a recombinant anti-IGF-IR antibody, scFv-Fc, consisting of 1H7 monoclonal antibody (mAb)-derived single chain antibody (scFv) and human IgG1 Fc, significantly suppressed breast tumor growth, and we proposed IGF-IR down-regulation as a mechanism for tumor growth inhibition (*Horm Metab Res.* 35:836, 2003; *Cancer Res.* 63:627, 2003). This study used MCF-7 breast cancer cells to investigate the effects of anti-IGF-IR mAbs with various epitope specificities on IGF-IR down-regulation and signaling pathways. Despite their differing effects on IGF-IR signaling, all five mAbs used down-regulated IGF-IR. Inhibitor experiments indicated that anti-IGF-IR mAbs induced internalization of IGF-IR from clathrin coated-pits. Pretreatment of MCF-7 cells with methylamine substantially reduced the antibody-mediated IGF-IR down-regulation while MG115 did not. Ubiquitination of IGF-IR did not occur in MCF-7 cells after mAb treatment. These results suggest that anti-IGF-IR antibodies with different epitope-specificities can cause internalization of IGF-IR from clathrin-coated pits and down-regulation *via* a lysosome-dependent pathway in an IGF-IR activation-independent manner.

**Keywords:** Receptor down-regulation, breast cancer, anti-IGF-I receptor antibodies, cancer therapy

**1. Introduction**

Insulin-like growth factors (IGFs) stimulate proliferation, motility, and survival of cells (1). The type I IGF receptor (IGF-IR) mediates the effects of IGF-I and -II. After molecular cloning of human IGF-IR in 1986 (2), the critical roles of IGF-IR signaling were definitively established with experimental systems by manipulating IGF-IR levels in cells and mice (3). Reports indicate that IGF-IR is elevated and thus plays a critical role in several different cancers including: breast, prostate, and liver cancer, glioblastomas, and childhood malignancies (4,5). To suppress IGF-IR signaling, various IGF-IR inhibitors

such as anti-sense DNA, siRNA, antibodies, and small molecular weight competitive or non-competitive inhibitors have been proposed (6). Of those, anti-IGF-IR antibodies have been extensively studied as a seemingly logical strategy to inhibit IGF-IR signaling pathways in cancer (7-10). Also of note is a report that suggested an association between increased blood levels of IGF-I and increased risk of prostate cancer as well as other cancers (11,12). During the last decade IGF-IR signaling has been a subject of major interest in the arena of cancer research.

One of the authors previously reported the production of an anti-IGF-IR monoclonal antibody, 1H7 (13), and of the first recombinant anti-IGF-IR antibody consisting of the 1H7 single chain antibody (scFv) and human IgG1 Fc domain (14). The scFv-Fc significantly suppressed breast tumor growth (14,15). IGF-IR down-regulation was proposed as a possible mechanism for inhibition of breast tumor growth (15,16). Other laboratories and companies have actively participated

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in research and produced anti-IGF-IR antibodies, most of which were also shown to down-regulate IGF-IR (17-20). At least 8 different anti-IGF-IR antibodies that were recently developed are being evaluated in clinical trials (21).

The details of IGF-IR down-regulation mechanisms by anti-IGF-IR antibodies are, however, not completely understood. The aim of this study was to determine mechanisms by which anti-IGF-IR antibodies with apparently distinct epitope specificities cause IGF-IR down-regulation. Effects of various anti-IGF-IR mAbs, 1H7 (13), 2C8 (13), 3B7 (22), 24-57 (23), and  $\alpha$ IR-3 (24) along with scFv-Fc (14), on IGF-IR down-regulation were thus studied using estrogen receptor-positive MCF-7 breast cancer cells.

## 2. Materials and Methods

### 2.1. Materials

IGF-I was purchased from GroPep (Adelaide, Australia). Anti-IGF-IR scFv-Fc was engineered and purified as described previously (14). Anti-IGF-IR mAbs, 2C8 and 3B7, originally produced by the authors (13,22), as well as a polyclonal antibody against ubiquitin, 4PD1, were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Other anti-IGF-IR mAbs such as 24-57 produced by Soos *et al.* (23) and  $\alpha$ IR-3 produced by Kull *et al.* (24) were from BioSource International, Inc (Camarillo, Canada) and Calbiochem (San Diego, CA, USA), respectively. Anti-phosphotyrosine antibody (PY-20) was from BD Transduction Laboratories (Lexington, KY, USA). Antibodies against 44/42 MAPK (phosphor-specific and total), Akt (phosphor-specific and total), and IGF-IR $\beta$  were purchased from Cell Signaling (Beverly, MA, USA). Anti-IGF-IR  $\beta$  mAb (17A3) was kindly provided by Dr. Richard Roth of Stanford University. Anti-rabbit secondary antibody conjugated to alkaline phosphatase (AP) was from Amersham Biosciences (Piscataway, NJ, USA). Protein G-Sepharose was from BIO-RAD Laboratories (Hercules, CA, USA). 4',6-Diamino-2-phenylindole (DAPI) was obtained from Dojindo (Kumamoto, Japan). Cell culture reagents were from Invitrogen/Life Technologies, Inc. (Rockville, MD, USA) unless otherwise stated. All other reagents and chemicals were purchased from Sigma (St. Louis, MO, USA).

### 2.2. Cell lines and culture

MCF-7 cells, obtained from Dr. Douglas Yee of the University of Minnesota Cancer Center (Minneapolis, MN), were routinely maintained in Improved MEM with Zinc Option (Richter's modification) in the presence of 5% fetal bovine serum (FBS), 11.25 nM human insulin (Sigma), 50 units/mL penicillin, and

50  $\mu$ g/mL streptomycin. R<sup>-</sup>IGF-IR mouse fibroblasts (mouse 3T3-like cells derived from animals with a targeted disruption of the IGF-IR gene and transfected with the pECE expression vector containing the cDNA encoding human IGF-IR) were kindly provided by Dr. Giuseppe Pandini (University of Catania, Catania, Italy) and grown in DMEM supplemented with 10% FBS.

### 2.3. Treatment of cells with IGF-I or mAb

MCF-7 cells were grown in 3.5-cm dishes in regular growth media. Confluent cells (70%) were washed twice with PBS and serum deprived for 24 h in regular growth media containing 0.5 mg/mL BSA instead of FBS (SFM). For treatment with IGF-I or various anti-IGF-IR mAbs, media were replaced with SFM containing 1 ng/mL or 100 ng/mL of IGF-I, or 2.5~25 nM of each mAb for 5 min to 24 h as indicated in the figure legends. To determine the effects of various anti-IGF-IR mAbs on signaling pathways, cells were treated for 5 min with mAbs, whereas 24 h incubation was generally used to observe down-regulation by various anti-IGF-IR mAbs.

### 2.4. Down-regulation of IGF-IR in the presence or absence of inhibitors

To address which pathways are responsible for degradation of the internalized IGF-IR-mAb complexes, cells were pretreated with 30  $\mu$ M MG115 (Calbiochem), a proteasome inhibitor, for 2 h, or with 40 mM methylamine, a lysomotropic agent, for 4 h before treatment with various anti-IGF-IR mAbs. MCF-7 cells were then treated without (control), or with either IGF-I (1 or 100 ng/mL), 25 nM scFv-Fc, 2.5 nM 1H7, 5 nM 2C8, 5 nM 3B7, 5 nM 24-57, or 5 nM  $\alpha$ IR-3 for 24 h.

To determine whether IGF-IR is internalized from clathrin-coated vesicles or caveola, MCF-7 cells were preincubated with 2 mM methyl  $\beta$ -cyclodextrin (M $\beta$ ), which causes disassembly of caveola-associated membrane microdomains as a result of cholesterol depletion (25), or 7.5  $\mu$ M chlorpromazine (CP), an inhibitor of clathrin-dependent, receptor-mediated endocytosis (26), for 24 h. The cells were then treated without (control) or with either 25 nM scFv-Fc, 5 nM 2C8, or 5 nM 3B7 for 4 h.

### 2.5. Cell lysis

Cellular proteins prepared as described above were washed three times with ice-cold PBS on ice and lysed with 50  $\mu$ L of TNESV lysis buffer [50 mM Tris-HCl (pH 7.4) containing 1% NP40, 2 mM EDTA (pH 8.0), 100 mM NaCl, 10 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 20  $\mu$ g/mL leupeptin, and 20  $\mu$ g/mL aprotinin]. Lysates were clarified by centrifugation at 12,000  $\times$  g for 20 min at 4°C.

Solubilized cellular proteins were immediately used or stored at  $-20^{\circ}\text{C}$  for experiments. Protein concentrations of the lysates were determined using a Bio-Rad protein assay reagent kit. Lysates (20  $\mu\text{g}/\text{lane}$ ) were subjected to reducing SDS-PAGE followed by immunoblotting with anti-IGF-IR  $\beta$  and anti- $\beta$ -actin as described below.

2.6. Immunoblotting

Cellular fractions were resolved on SDS-polyacrylamide gradient gels (4-20%) and transferred onto Immobilon-P membranes. Nonspecific binding on the membranes was blocked with 5% skim milk in 100 mM Tris-buffered saline, pH 7.4, for 1 h at room temperature. The membranes were incubated with primary antibodies, and then respective proteins were detected using AP-conjugated secondary antibodies and a Vector substrate kit (Vector Laboratories, Inc. Burlingame, CA, USA) as described (27). Primary antibodies used for intracellular signaling were phosphor(p)-IGF-IR, p-IRS-1/2, p-MAPK, p-AKT and total AKT whereas the anti-IGF-IR  $\beta$  antibody was used for immunoblotting the IGF-IR.

2.7. Immunoprecipitations

One mg of total cellular proteins in 200  $\mu\text{L}$  was first incubated with 4  $\mu\text{g}$  of anti-IGF-IR  $\beta$  mAb (17A3) for 2 h in an ice bath. Added to this were 25  $\mu\text{L}$  of 50% Protein G-Sepharose, and the suspension was mixed in a rotator overnight at  $4^{\circ}\text{C}$ . Immune complexes were collected by centrifugation at  $8,000 \times g$  for 2 min. The immunoprecipitates were washed three times by suspending in 200  $\mu\text{L}$  of TNESV followed by centrifugation. After the final wash, immunoprecipitates were suspended in 20  $\mu\text{L}$  of  $1 \times$  SDS-PAGE sample buffer containing 100 mM DTT, boiled for 5 min, and centrifuged. The supernatants were subjected to SDS-PAGE followed by Western blotting with anti-ubiquitin or anti-IGF-IR $\beta$  subunit antibody.

2.8. Immunofluorescence microscopy

Approximately  $1 \times 10^4$  MCF-7 cells were plated on 4-well chamber slides (Nalge Nunc, Naperville, IL, USA) and grown for 24 h in regular growth media. Confluent cells (70%) were washed twice with PBS and serum-deprived for 24 h in SFM. For time-course experiments, cells were treated with either IGF-I (1 ng/mL) or 25 nM scFv-Fc for 30 or 120 min and then subjected to immunofluorescence-staining and microscopy. Alternatively, immunofluorescence-stained images of cells were prepared by preincubation with either methyl-cyclodextrin (2.5 mM) or chlorpromazine (7.5  $\mu\text{M}$ ) for 30 min and then treatment with scFv-Fc (25 nM) for 4 h in the presence of the inhibitors. Cells were fixed, permeabilized, and subjected to immunofluorescence microscopy. Briefly, slides were

rinsed twice with Dulbecco's phosphate-buffered saline (DPBS) and fixed with ice-cold 4% paraformaldehyde in DPBS for 20 min. Cells were permeabilized with DPBS containing 0.25% Triton X-100 for 2 min and washed with DPBS containing 1% BSA. Subsequently, slides were subjected to standard immunofluorescence protocols using rabbit anti-IGF-IR $\beta$  antibody, followed by fluorescein isothiocyanate (FITC)-conjugated anti-rabbit antibody (Invitrogen, Carlsbad, CA, USA). Images were obtained using a Zeiss Axiovert 200M microscope (Carl Zeiss Inc., Oberkochen, Germany).

3. Results

3.1. Characterization of intracellular signaling induced by IGF-I or various anti-IGF-IR antibodies

Cellular proteins prepared from MCF-7 cells that had been treated with IGF-I or antibodies for 5 min were immunoblotted for phosphorylated (p)-IGF-IR, p-IRS-1/2, p-MAPK, p-AKT and Akt. A representative experiment from three independent experiments is shown in Figure 1. Anti-IGF-IR antibodies used were mAbs except for scFv-Fc, which is a recombinant Ab consisting of 1H7 scFv and human IgG1 Fc (14). Characteristics of these antibodies with regard to their epitopes and effects on IGF-I binding are summarized in Table 1. Compared to the control (Figure 1, lane 1), addition of IGF-I, scFv-Fc, 1H7 or 2C8 to MCF-7 cells stimulated phosphorylation of IGF-IR, IRS-1/2 and MAPK within 5 min (lanes 2-5, respectively). In contrast, 3B7, 24-57 or  $\alpha\text{IR}3$  hardly stimulated phosphorylation of IGF-IR, IRS-1/2 or MAPK (lanes 6-8, respectively). IGF-I and scFv-Fc also significantly stimulated Akt phosphorylation. The effects of IGF-IR mAbs on intracellular signaling are summarized in Table 1.

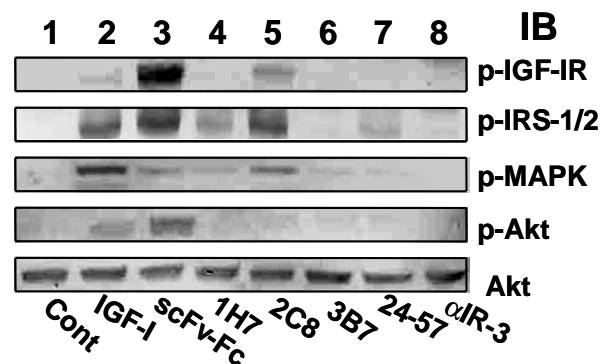
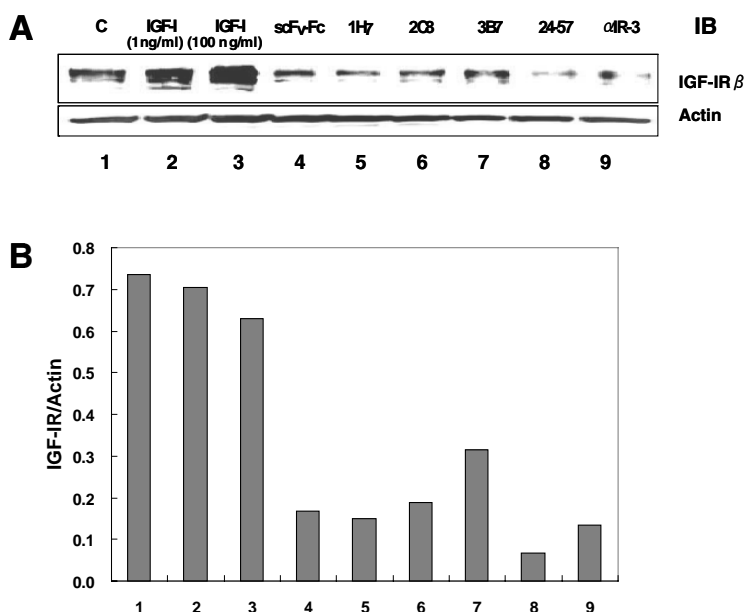


Figure 1. Comparison of intracellular signaling in MCF-7 cells after administration of various anti-IGF-IR antibodies. MCF-7 cells were grown in 3.5 cm dishes in regular growth media. Confluent cells (70%) were washed twice with PBS and serum deprived for 24 h in SFM. Cells were either untreated (lane 1) or treated with IGF-I at 100 ng/mL (lane 2) or antibodies; 25 nM scFv-Fc (lane 3), 2.5 nM 1H7 (lane 4), 5 nM 2C8 (lane 5), 5 nM 2B7 (lane 6), 5 nM 24-57 (lane 7), 5 nM  $\alpha\text{IR}3$  (lane 8), for 5 min. Cellular proteins were prepared for Western blotting with phosphor(p)-IGF-IR, p-IRS-1/2, p-MAPK, p-AKT and total AKT as described in the Methods. All experiments shown were repeated three times with similar results.

**Table 1. Summary of characteristics of anti-IGF-IR mAbs used in this study**

mAb	Effect on IGF-IR signaling (This study)	Effect on IGF-I-binding	Epitope mapping on the $\alpha$ subunit of IGF-IR
1H7	Stimulation	Inhibition (13)	440-514 (29) <sup>a</sup>
1H7 scFv-Fc	Stimulation	ND	440-514 (29) <sup>a</sup>
24-57	No effect	Inhibition (23)	440-514 (30) <sup>a</sup>
$\alpha$ IR-3	No effect	Inhibition (24)	223-274 (31)
3B7	No effect	Stimulation (22)	62-184 (29)
2C8	Stimulation	No effect (13)	ND

ND: Not determined; <sup>a</sup> Although 1H7 and 24-57 binding to the  $\alpha$  subunit were competitive and the 440-514 domain was thus assigned as the epitope for both mAbs (29), this study suggested that their epitopes must differ (see Discussion).



**Figure 2. Anti-IGF-IR antibody induced IGF-IR down-regulation in MCF-7 cells.** MCF-7 cells were either untreated (lane 1) or treated with IGF-I (1 ng/mL, lane 2; 100 ng/mL, lane 3), 25 nM scFv-Fc (lane 4), 2.5 nM 1H7 (lane 5), 5 nM 2C8 (lane 6), 5 nM 3B7 (lane 7), 5 nM 24-57 (lane 8), or 5 nM  $\alpha$ IR-3 (lane 9) for 24 h and solubilized with the TNESV lysis buffer as described in the Methods. Lysates (20  $\mu$ g/lane) were subjected to reducing SDS-PAGE followed by immunoblotting with anti-IGF-IR $\beta$  and  $\beta$ -actin (A). In B, anti-IGF-IR  $\beta$  bands corresponding to lanes 1-9 in A were quantitated.

### 3.2. Anti-IGF-IR antibody-induced IGF-IR down-regulation in MCF-7 cells

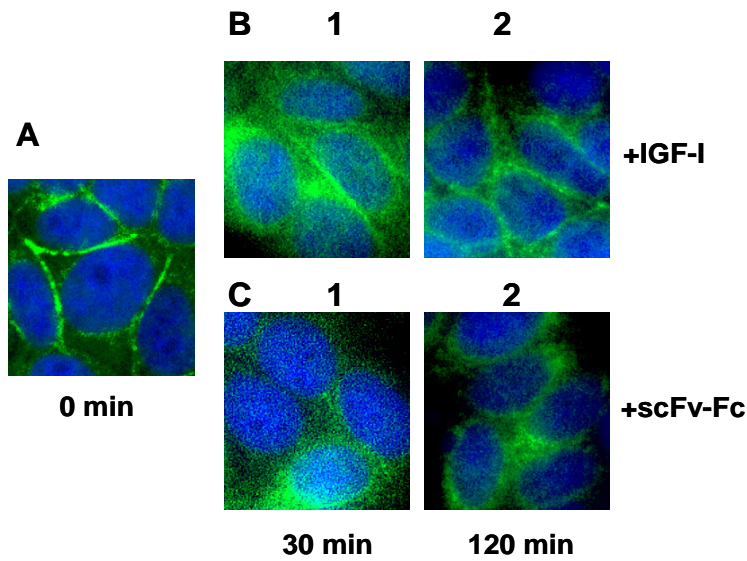
MCF-7 cells, treated with either SFM (control) or SFM containing IGF-I, scFv-Fc, 1H7, 2C8, 3B7, 24-57, or  $\alpha$ IR-3 for 24 h, were solubilized with TNESV lysis buffer. Lysates (20  $\mu$ g/lane) were subjected to reducing SDS-PAGE followed by Western blotting with anti-IGF-IR  $\beta$  subunit or anti- $\beta$  actin (Figure 2A). The amount of the IGF-IR  $\beta$  subunit was quantitated and then normalized against  $\beta$  actin (Figure 2B). The results clearly indicated that, with the exception of the ligand IGF-I, all of the anti-IGF-IR antibodies used induced down-regulation of IGF-IR in MCF-7 cells. Down-regulation is obviously caused by internalization of IGF-IR from the plasma membrane into endosomes followed by eventual degradation of IGF-IR. Treatment of MCF-7 cells with IGF-I did not change the amount of the  $\beta$  subunit on the Western blot, suggesting that IGF-I-bound receptors must be recycled back to the membrane as intact IGF-IR instead of moving to degradation pathways.

IGF-IR recycling after its binding to the ligand was confirmed by immunofluorescence-staining of IGF-IR  $\beta$  subunit in the cells (Figure 3). Before the addition

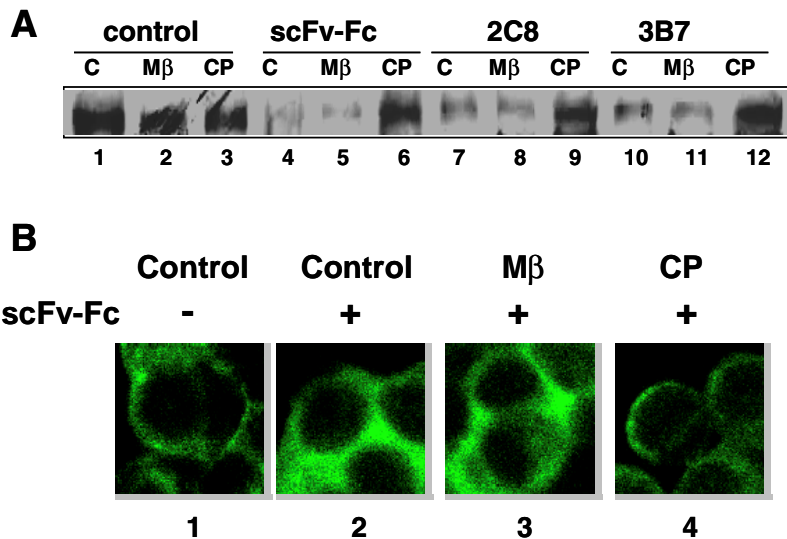
of IGF-I or the antibody, IGF-IR was seen on the cell membrane (Figure 3A), which became diffuse after 30 min of treatment with either IGF-I (Figure 3B1) or scFv-Fc (Figure 3C1). After 120 min of treatment, however, the cell surface intensity clearly increased for the ligand (Figure 3B2) while the diffuse staining was still observed with the scFv-Fc-treated cells (Figure 3C2), indicating that the immunoreactive  $\beta$  subunit epitopes that were most likely to be partially degraded remained inside cells in the latter case.

### 3.3. Internalization of IGF-IR from clathrin-coated vesicles

To determine whether IGF-IR is internalized from clathrin-coated vesicles or caveolae of the plasma membrane, IGF-IR down-regulation by scFv-Fc was measured after MCF-7 cells were preincubated with or without respective inhibitors followed by Western blotting analyses. As shown in Figure 4A, the amount of an intact IGF-IR  $\beta$  subunit did not change after preincubation with methyl  $\beta$ -cyclodextrin (M $\beta$ ) (lane 2) or chlorpromazine (CP) (lane 3). When treated with scFv-Fc, 2C8, or 3B7, IGF-IR  $\beta$  subunit was down-regulated as evidenced by reduced levels of the IGF-



**Figure 3. Internalization of IGF-IR in MCF-7 cells as observed by immunofluorescence microscopy.** Immunofluorescence-stained images of MCF-7 cells were taken before (A) and after treatment with IGF-I (1 ng/mL) for 30 min (B-1) and 120 min (B-2) or 25 nM scFv-Fc for 30 min (C-1) and 120 min (C-2) as described in the Methods, Nuclei were stained with DAPI.



**Figure 4. Internalization of IGF-IR from clathrin-coated vesicles.** In A, MCF-7 cells preincubated with 2 mM methyl-beta-cyclodextrin (Mβ) or 7.5 μM chlorpromazine (CP) were treated without antibodies (control) or with either 25 nM scFv-Fc, 5 nM 2C8, or 5 nM 3B7 for 4 h. Cellular proteins were prepared from MCF-7 cells treated with scFv-Fc, 2C8, or 3B7 in the absence (lanes 1, 4, 7, and 10) or presence of Mβ (lanes 2, 5, 8, and 11) or CP (lanes 3, 6, 9, and 12), followed by Western blotting with IGF-IRβ. In B, shown are immunofluorescence images of MCF-7 cells after scFv-Fc treatment in the absence or presence of inhibitors prepared as described in the Methods. Images 2, 3, and 4 are equivalent to lanes 4, 5, and 6, respectively, whereas image 1 is the control with no antibody (equivalent to lane 1 in A).

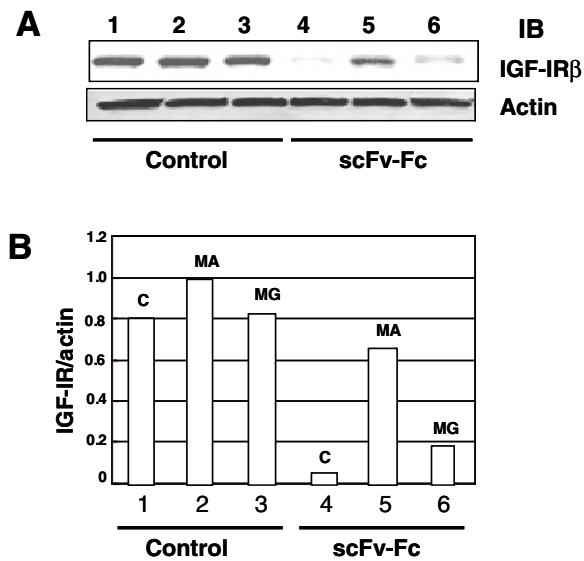
IR β subunit (lane 4, 7, or 10, respectively). This IGF-IR down-regulation induced by scFv-Fc, 2C8, or 3B7 was not affected by preincubation with Mβ (lane 5, 8, or 11, respectively). Antibody-induced IGF-IR down-regulation was, however, prevented when MCF-7 cells were preincubated with CP (lane 6, 9, or 12, respectively). To visualize IGF-IR distribution in MCF-7 cells after antibody treatment, immunofluorescent microscopy was carried out with the scFv-Fc treatment set (equivalent to lanes 4, 5, and 6 in Figure 4A) and no antibody control (equivalent to lane 1 in Figure 4A). Internalization of scFv-Fc and IGF-IR complexes is clearly observed as fluorescence-labeled IGF-IR dispersed in cytosol (Figure 4B2). Preincubation with Mβ did not affect internalization and eventual degradation of scFv-Fc and IGF-IR complexes (Figure 4B3). In contrast, cytosolic staining was markedly reduced by 30 min of preincubation with a relatively low dosage (7.5 μM) of CP (Figure 4B4). These results suggested that IGF-IR is internalized *via*

clathrin-coated vesicles of the plasma membrane after binding with anti-IGF-IR antibodies.

### 3.4. Degradation of IGF-IR

The disappearance of the intact IGF-IR β subunit after antibody treatment as detected by Western blotting indicates that the receptor was readily degraded once it was internalized. Earlier work showed that the lysosomal degradation pathway was responsible for this process (16-18). Previous findings with scFv-Fc were confirmed by the inhibitor experiments shown in Figures 5A and B (Western blot and quantitation of the IGF-IR β subunit, respectively), which show that treatment with methyl amine (MA: lysosomal pathway inhibitor) inhibited IGF-IR down-regulation whereas MG115 (proteasomal pathway inhibitor) did not have much of an effect on the antibody-induced IGF-IR down-regulation in MCF-7 cells.

Next examined was whether IGF-I or scFv-Fc

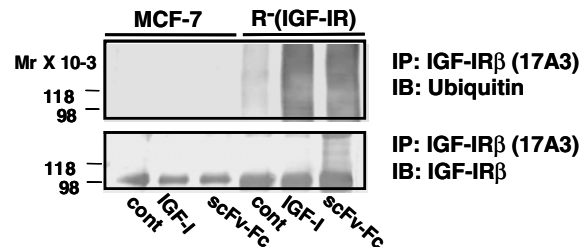


**Figure 5. Potential pathways for IGF-IR down-regulation.** MCF-7 cells were pretreated with 30  $\mu$ M MG115 (MG) for 2 h, or with 40 mM methylamine (MA) for 4 h before treatment with various anti-IGF-IR mAbs as described in the Methods. MCF-7 cells were then treated without (lanes 1, 2, and 3) or with 25 nM scFv-Fc for 24 h. Cellular proteins were prepared for immunoblotting with anti-IGF-IR $\beta$  (A). In B, anti-IGF-IR $\beta$  bands corresponding to lanes 1-6 in A were quantitated.

treatment resulted in ubiquitination of IGF-IR in MCF-7 cells since IGF-I had been known to induce ubiquitination of IGF-IR in mouse embryo fibroblasts overexpressing Grb10 and IGF-IR (p6/Grb10) (28). The results shown in Figure 6 clearly indicate that while both IGF-I and scFv-Fc induced ubiquitination of IGF-IR in fibroblasts overexpressing IGF-IR, so-called R<sup>-</sup> (IGF-IR) cells, neither of them induced ubiquitination of IGF-IR in MCF-7 cells. This result is consistent with the notion that IGF-IR degradation takes place in lysosomes but not in proteasomes in MCF-7 cells. Down-regulation of IGF-IR in MCF-7 cells is thus likely to be the result of IGF-IR-Ab complexes internalized in endosomes readily moving to lysosomes where both IGF-IR and Ab are digested into small peptides.

#### 4. Discussion

The aim of this study was to determine whether or not anti-IGF-IR antibodies, with apparently distinct epitope specificities as summarized in Table 1, cause IGF-IR down-regulation, and if so, to determine the mechanisms by which these antibodies lead to internalization and degradation of IGF-IR. Effects of various anti-IGF-IR mAbs, 1H7, 2C8, 3B7, 24-57, and  $\alpha$ IR-3 along with scFv-Fc, on IGF-IR down-regulation were studied using MCF-7 breast cancer cells in which down-regulation of IGF-IR by scFv-Fc has been previously demonstrated *in vitro* and *in vivo* (15,16). This study not only confirmed the previous finding that IGF-IR was down-regulated by scFv-Fc *via* lysosomal pathways (16) but also further determined IGF-IR



**Figure 6. Ubiquitination of IGF-IR by anti-IGF-IR antibody.** Both MCF-7 and R<sup>-</sup>(IGF-IR) cells were treated without (Cont) or with 100 ng/mL IGF-I or 25 nM scFv-Fc for 15 min. Proteasome inhibitor MG115 (30  $\mu$ M) was added to culture media. After 6 h of incubation, cell lysates were prepared and subjected to immunoprecipitation by anti-IGF-IR $\beta$  (17A3) antibody. Equal amounts of the immunoprecipitates were analyzed using SDS-PAGE followed by immunoblotting with anti-ubiquitin (upper panel) or anti-IGF-IR $\beta$  subunit antibody (lower panel).

internalization/degradation pathways by various anti-IGF-IR mAbs in MCF-7 cells.

As far as the effects of antibodies on IGF-IR signaling are concerned, scFv-Fc, 1H7, and 2C8 were agonistic. Although both scFv-Fc and 1H7 should have the same specificity since scFv-Fc is prepared from 1H7-producing hybridomas, the former had more of an effect on phosphorylation of IGF-IR $\beta$ , IRS-1/2, MAPK, and Akt than the latter. This may be due to the use of concentrations of scFv-Fc that were 10-times higher than those of 1H7, based on the result that scFv-Fc had an affinity constant one-order lower than that of 1H7 (14). Nonetheless, the agonistic nature of 1H7 and 2C8 agrees with a previous report by the authors indicating that 1H7 and 2C8 stimulate autophosphorylation of IGF-IR (13). An interesting point is that 24-57 had little effect on IGF-IR signaling, unlike 1H7. 1H7 binding to IGF-IR was competitively inhibited by 24-57 (29), which has an epitope assigned to the 440-514 domain of the  $\alpha$  subunit (30). This result thus strongly suggests that they do not have the same epitope specificity.

It is clear that in MCF-7 cells, anti-IGF-IR antibody binding to the IGF-IR facilitated degradation of IGF-IR while IGF-I binding did not induce such receptor degradation. After internalization, IGF-IR can be either recycled back to the plasma membrane or processed for degradation into small pieces that can no longer be recognized as an intact  $\beta$  subunit by immunoblotting with anti-IGF-IR $\beta$ . The internalized and degraded  $\beta$  subunit pieces can still be seen by immunostaining of the cells after anti-IGF-IR antibody treatment. Use of various inhibitors demonstrated that the IGF-IR/antibody complex is internalized from clathrin-coated pits and degraded in lysosomes. Furthermore, the present study showed that ubiquitination of IGF-IR did not occur in MCF-7 cells. This result supports that lysosomal pathways play a major role in IGF-IR degradation in MCF-7 cells. In control fibroblasts overexpressing IGF-IR, ubiquitination of the receptor did occur, suggesting that unlike MCF-7 cells, ubiquitination obviously plays an important role in those fibroblast cells (28,32). After this manuscript

was submitted, however, an article similar to ours was published (33). While we compared effects of several different mAbs on cell signaling and down-regulation of IGF-IR, Broussas *et al.* (33) reported that an anti-IGF-IR mAb, h7C10, caused down-regulation of IGF-IR in MCF-7 cells, during which  $\alpha$  and  $\beta$  subunits were degraded using different routes. They showed that ubiquitination of the  $\beta$  subunit occurred when treating with both IGF-I and h7C10, which is contrary to our results described above. Further studies are required to solve this discrepancy.

Internalization and recycling of IGF-IR in relation to sustained Akt activation was recently reported (34), in which IGF-IR was shown to be internalized within 30 min and recycled back to the plasma membrane after 120 min of IGF-I treatment. This time course agrees with the current findings from immunofluorescence microscopy (Figures 3A, B1, and B2). After 120 min, IGF-I treated cells showed IGF-IR on the cell membrane, suggesting recycling of IGF-IR. In contrast, intracellular distribution of IGF-IR was still observed when cells were treated with scFv-Fc for 120 min. Based on the time-course of IGF-IR degradation in MCF-7 cells after treatment with scFv-Fc, less than 10% of the intact  $\beta$  subunit was observed on Western blots after 2 h (16). Thus, intracellular staining of IGF-IR is mostly due to binding of anti-IGF-IR  $\beta$  subunit antibody to the partially degraded  $\beta$  subunit.

With respect to internalization of IGF-IR stimulated by IGF-I, Sehat *et al.* reported involvement of E3 ubiquitin ligases such as c-Cbl and Mdm2, which mediate IGF-IR ubiquitination in osteosarcoma cells and HEK293 cells (35). Mdm2-mediated ubiquitination occurred when cells were stimulated at a low concentration of IGF-I (5 ng/mL) whereas ubiquitination by c-Cbl requires a high concentration (50-100 ng/mL). Mdm2-ubiquitinated IGF-IR was internalized *via* the clathrin endocytic pathway whereas c-Cbl-ubiquitinated receptors were endocytosed *via* the caveolin/lipid raft route. Unlike in the aforementioned cells, IGF-IR ubiquitination did not occur in MCF-7 cells, so IGF-IR internalization and recycling take place. Whether or not sustained Akt activation is required for IGF-IR recycling in MCF-7 cells, which has been proposed by studies using glial progenitor cells (34), is obviously the next question to be answered.

In conclusion, more studies like this and others (33) are required to understand mechanisms of action by therapeutic anti-IGF-IR mAbs because at least 8 different anti-IGF-IR antibodies are now being evaluated in clinical trials (21).

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#### References

1. Valentinis B, Baserga R. IGF-I receptor signalling in transformation and differentiation. *Mol Pathol.* 2001; 54:133-137.
2. Ullrich A, Gray A, Tam AW, Yang-Feng T, Tsubokawa M, Collins C, Henzel W, LeBon T, Kathuria S, Chen E, Jacobs S, Francke U, Ramachandran J, Fujita-Yamaguchi Y. Insulin like growth factor I receptor primary structure: Comparison with insulin receptor suggests structural determinants define functional specificity. *EMBO J.* 1986; 5:2503-2512.
3. Samani AA, Yakar S, LeRoith D, Brodt P. The role of the IGF system in cancer growth and metastasis: overview and recent insights. *Endocr Rev.* 2007; 28:20-47.
4. Larsson O, Girnita A, Girnita L. Role of insulin-like growth factor 1 receptor signalling in cancer. *Br J Cancer.* 2005; 92:2097-2101.
5. Kurmasheva RT, Houghton PJ. IGF-I mediated survival pathways in normal and malignant cells. *Biochim Biophys Acta.* 2006; 66:1-22.
6. LeRoith D, Helman L. The new kid on the block(ade) of the IGF-1 receptor. *Cancer Cell.* 2004; 5:201-202.
7. Bohula EA, Playford MP, Macaulay VM. Targeting the type 1 insulin-like growth factor receptor as anti-cancer treatment. *Anticancer Drugs.* 2003; 14:669-682.
8. Sachdev D, Yee D. Disrupting insulin-like growth factor signaling as a potential cancer therapy. *Mol Cancer Ther.* 2007; 6:1-12.
9. Feng Y, Dimitrov DS. Monoclonal antibodies against components of the IGF system for cancer treatment. *Curr Opin Drug Discov Devel.* 2008; 11:178-185.
10. Ryan PD, Goss PE. The emerging role of the insulin-like growth factor pathway as a therapeutic target in cancer. *Oncologist.* 2008; 13:16-24.
11. Chan JM, Stampfer MJ, Giovannucci E, Gann PH, Ma J, Wilkinson P, Hennekens CH, Pollak M. Plasma insulin-like growth factor-I and prostate cancer risk: a prospective study. *Science.* 1988; 279:563-566.
12. Renehan AG, Zwahlen M, Minder C, O'Dwyer ST, Shalet SM, Egger M. Insulin-like growth factor (IGF)-I, IGF binding protein-3, and cancer risk: systematic review and meta-regression analysis. *Lancet.* 2004; 363:1346-1353.
13. Li SL, Kato J, Paz IB, Kasuya J, Fujita-Yamaguchi Y. Two new monoclonal antibodies against the alpha subunit of the human insulin-like growth factor-I receptor. *Biochem Biophys Res Commun.* 1993; 196:92-98.
14. Li SL, Liang SJ, Guo N, Wu AM, Fujita-Yamaguchi Y. Single chain antibodies against human insulin-like growth factor-I receptor: Expression, purification, and effect on tumor growth. *Cancer Immunol Immunother.* 2000; 49:243-252.
15. Ye JJ, Liang SJ, Guo N, Li SL, Wu AM, Giannini S, Sachdev D, Yee D, Brünner N, Ikle D, Fujita-Yamaguchi Y. Combined effects of tamoxifen and a chimeric humanized single chain antibody against the type I IGF receptor on breast tumor *in vivo*. *Horm Metab Res.* 2003; 35:836-842.

16. Sachdev S, Li SL, Hartell JS, Fujita-Yamaguchi Y, Miller JS, Yee D. A chimeric humanized single chain antibody against the type I insulin-like growth factor receptor renders breast cancer cells refractory to the mitogenic effects of insulin-like growth factor-I. *Cancer Res.* 2003; 63:627-635.
17. Hailey J, Maxwell E, Koukouras K, Bishop WR, Pachter JA, Wang Y. Neutralizing anti-insulin-like growth factor receptor I antibodies inhibit receptor function and induce receptor degradation in tumor cells. *Mol Cancer Ther.* 2002; 1:1349-1353.
18. Jackson-Booth PG, Terry C, Lackey B, Lopaczynska M, Nissley P. Inhibition of the biologic response to insulin-like growth factor I in MCF-7 breast cancer cells by a new monoclonal antibody to the insulin-like growth factor-I receptor. The importance of receptor down-regulation. *Horm Metab Res.* 2003; 35:850-856.
19. Burtrum D, Zhu Z, Lu D, *et al.* A fully human monoclonal antibody to the insulin-like growth factor I receptor blocks ligand-dependent signaling and inhibits human tumor growth *in vivo*. *Cancer Res.* 2003; 63:8912-8921.
20. Wang Y, Hailey J, Williams D, *et al.* Inhibition of insulin-like growth factor-I receptor (IGF-IR) signaling and tumor cell growth by a fully human neutralizing anti-IGF-IR antibody. *Mol Cancer Ther.* 2005; 4:1214-1221.
21. Rodon J, DeSantos V, Ferry RJ Jr, Kurzrock R. Early drug development of inhibitors of the insulin-like growth factor-I receptor pathway: lessons from the first clinical trials. *Mol Cancer Ther.* 2008; 7:2575-2588.
22. Xiong L, Kasuya J, Li SL, Kato J, Fujita-Yamaguchi Y. Growth-stimulatory monoclonal antibodies against human insulin-like growth factor I receptor. *Proc Natl Acad Sci U S A.* 1992; 89:5356-5360.
23. Soos MA, Field CE, Lammers R, Ullrich A, Zhang B, Roth RA, Andersen AS, Kjeldsen T, Siddle K. A panel of monoclonal antibodies for the type I insulin-like growth factor receptor. Epitope mapping, effects on ligand binding, and biological activity. *J Biol Chem.* 1992; 267:12955-12963.
24. Kull FC Jr, Jacobs S, Su YF, Svoboda ME, Van Wyk JJ, Cuatrecasas P. Monoclonal antibodies to receptors for insulin and somatomedin-C. *J Biol Chem.* 1983; 258:6561-6566.
25. Ilangumaran S, Hoessli DC. Effects of cholesterol depletion by cyclodextrin on the sphingolipid microdomains of the plasma membrane. *Biochem J.* 1998; 335:433-440.
26. Orlandi PA, Fishman PH. Filipin-dependent inhibition of cholera toxin: evidence for toxin internalization and activation through caveolae-like domains. *J Cell Biol.* 1998; 141:905-915.
27. Yajima Y, Sato M, Sumida M, Kawashima S. Mechanism of adult primitive mesenchymal ST-13 preadipocyte differentiation. *Endocrinology.* 2003; 144:2559-2565.
28. Vecchione A, Marchese A, Henry P, Rotin D, Morrione A. The Grb10/Nedd4 complex regulates ligand-induced ubiquitination and stability of the insulin-like growth factor I receptor. *Mol Cell Biol.* 2003; 23:3363-3372.
29. Kusada Y, Morizono T, Matsumoto-Takasaki A, Sakai K, Sato S, Asanuma H, Takayanagi A, Fujita-Yamaguchi Y. Construction and characterization of single-chain antibodies against human insulin-like growth factor-I receptor from hybridomas producing 1H7 or 3B7 monoclonal antibody. *J Biochem (Tokyo).* 2008; 143:9-19.
30. Schumacher R, Soos MA, Schlessinger J, Brandenburg D, Siddle K, Ullrich A. Signaling-competent receptor chimeras allow mapping of major insulin receptor binding domain determinants. *J Biol Chem.* 1993; 268:1087-1094.
31. Gustafson TA, Rutter WJ. The cysteine-rich domains of the insulin and insulin-like growth factor I receptors are primary determinants of hormone binding specificity. Evidence from receptor chimeras. *J Biol Chem.* 1990; 265:18663-18667.
32. Monami G, Miliozzi V, Morrione A. Grb10/Nedd4-mediated multiubiquitination of the insulin-like growth factor receptor regulates receptor internalization. *J Cell Physiol.* 2008; 216:426-437.
33. Broussas M, Dupont J, Gonzalez A, Blaecke A, Fournier M, Corvaira N, Goetsch L. Molecular mechanisms involved in activity of h7C10, a humanized monoclonal antibody, to IGF-1 receptor. *Int J Cancer.* 2009; 124:2281-2293.
34. Romanelli RJ, LeBeau AP, Fulmer CG, Lazzarino DA, Hochberg A, Wood TL. Insulin-like growth factor type-I receptor internalization and recycling mediate the sustained phosphorylation of Akt. *J Biol Chem.* 2007; 282:22513-22524.
35. Sehat B, Anderson S, Girmila L, Larsson O. Identification of c-Cbl as a new ligase for insulin-like growth factor-I receptor with distinct roles from Mdm2 in receptor ubiquitination and endocytosis. *Cancer Res.* 2008; 68:5667-5677.

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