

# Novel human monoclonal antibodies to insulin-like growth factor (IGF)-II that potently inhibit the IGF receptor type I signal transduction function

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## Abstract

The insulin-like growth factor (IGF) system plays an important role in a variety of physiologic processes and in diseases such as cancer. Although the role of the IGF system in cancer has been recognized many years ago, components of the system have only recently been targeted and shown to affect cell transformation, proliferation, survival, motility, and migration in tissue cultures and in mouse models of cancer. We have been hypothesizing that targeting IGF-II in addition to blocking its interaction with the IGF receptor type I (IGF-IR) would also allow to block that portion of the signal transduction through the insulin receptor that is due to its interaction with IGF-II. Lowering its level may also not induce up-regulation of its production as for IGF-I. Finally, targeting a diffusible ligand as IGF-II may not require penetration of the antibody inside tumors but could shift the equilibrium to IGF-II complexed with antibody so the ligand concentration would decrease in the tumor environment without the need for the antibody to penetrate the tumor. Here, we describe the identification and characterization of three novel anti-IGF-II fully human monoclonal antibodies. They bound with high (subnanomolar) affinity to IGF-II, did not cross-react with IGF-I and insulin, and potently inhibited

signal transduction mediated by the IGF-IR interaction with IGF-II. The most potent neutralizer, IgG1 m610, inhibited phosphorylation of the IGF-IR and the insulin receptor, as well as phosphorylation of the downstream kinases Akt and mitogen-activated protein kinase with an IC<sub>50</sub> of the order of 1 nmol/L at IGF-II concentration of 10 nmol/L. It also inhibited growth of the prostate cancer cell line DU145 and migration of the breast cancer line cells MCF-7. These results indicate an immunotherapeutic potential of IgG1 m610 likely in combination with other antibodies and anticancer drugs but only further experiments in mouse models of cancer and human clinical trials could evaluate this possibility. [Mol Cancer Ther 2006;5(1):114–20]

## Introduction

The insulin-like growth factor (IGF) system plays an important role in a variety of physiologic processes (1, 2) and in pathologic conditions such as cancer (3, 4). Although the role of the IGF system in cancer has been recognized many years ago, components of the system have only recently been targeted and shown to affect cell transformation, proliferation, survival, motility, and migration in tissue cultures and in mouse models of cancer (5–7). Various approaches, including lowering the receptor surface concentration, inhibiting its kinase activity, and disrupting the interaction by antibodies against the receptor or the ligands, have been used to modulate the function of the pathway mediated by the interaction of the IGF receptor type I (IGF-IR) with its ligands IGF-I, IGF-II, and, albeit at a much lower affinity (500- to 1,000-fold less), insulin (6, 8). It has been recently confirmed that IGF-IR is expressed in a broad panel of tumor types, that multiple strategies for inhibition of its function have potent antitumor effects *in vitro*, and that a therapy with a kinase inhibitor had a significant antitumor activity in an orthotopic xenograft model of multiple myeloma (9). Similar results were obtained in another study, where the kinase inhibitor inhibited *in vivo* the IGF-IR signaling in tumor xenografts and significantly reduced the growth of IGF-IR-driven fibrosarcomas (ref. 10; see also the preview article, ref. 8). Thus, it seems that modulation of the activity of the IGF system could add to the arsenal of anticancer therapeutic approaches. However, the complexity of the responses of the IGF system to signals and their dependence on cell type and environment (2) complicates the selection of appropriate strategies for modulation of the IGF system function.

Antibodies have been used for treatment of cancer with varying success throughout the years but only recently showed reproducible success against several cancers, e.g.,

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Herceptin (trastuzumab), which is therapeutically active in HER2-positive breast carcinomas (11). IGF-IR-specific antibodies, similar to other antibodies to growth factor receptors, e.g., trastuzumab, can interfere with cell growth and proliferation (12–14). More recently, several monoclonal antibodies (mAbs) against the IGF-IR have been identified with cancer cell growth inhibitory activity (15–18). Unlike the Herceptin target, HER2, however, the IGF-IR is not very significantly overexpressed in tumors and the antibodies could bind to normal cells expressing the receptor with possible toxic effects. Antibodies against the ligands IGF-I and IGF-II that inhibit the interaction with IGF-IR may not have this type of effects although obviously toxic effects could not be predicted. In addition, one could speculate that binding to the ligand, which is not cell-associated but is diffusible, could shift the equilibrium so the ligand would diffuse out of the tumor environment, thus avoiding problems associated with penetration. Another consideration is that, in addition to IGF-IR, IGF-II also binds to the insulin receptor and can trigger the transmission of a growth signal, which is not affected by anti-IGF-IR antibodies. Because the insulin receptor cannot be used as a target, IGF-II seems to be a potential target for blocking portion of the signal transduction activity of the insulin receptor due to its interaction with IGF-II. Yet, another important consideration is that in many cancer patients, the levels of both mature and premature forms of IGF-II are elevated in their serum.

As a part of our program for developing a panel of human mAbs against components of the IGF system, we, therefore, started with the identification of potent binders to IGF-II. We also chose IGF-II to begin with because lowering the IGF-I concentration may trigger feedback up-regulation by the human growth hormone, whereas such feedback is not known for IGF-II. Recently, a similar strategy was tested by using a mouse mAb, KM1468, which is, however, cross-reactive for IGF-I and IGF-II (19). KM1468 markedly and dose-dependently suppressed the development of new bone tumors and the progression of established tumor foci in nonobese diabetic/severe combined immunodeficient mice implanted with human adult bone as determined by histomorphometry, and it also decreased serum prostate-specific antigen levels compared with the control. Very recently, the same group found that KM1468 inhibits tumor growth and increases survival in a mouse model of hepatic metastasis induced by intrasplenic injection of human colorectal cancer cell line, HT29 (20). These results suggested that targeting the ligands IGF-I and IGF-II can affect tumor growth in mouse models of cancer.

Here, we describe the identification of three novel fully human mAbs that are specific for human IGF-II and do not cross-react with IGF-I and insulin but potently inhibited signal transduction mediated by the IGF-IR interaction with IGF-II. The most potent neutralizer, IgG1 m610, inhibited IGF-IR phosphorylation and phosphorylation of the downstream kinases Akt and mitogen-activated protein kinase (MAPK) with an  $IC_{50}$  of the order

of 1 nmol/L. It also inhibited growth of the prostate cancer cell line DU145 and the migration of the breast cancer line cells MCF-7. These results indicate an immunotherapeutic potential of IgG1 m610, and further experiments in mouse models of cancer are ongoing to evaluate this possibility.

## Materials and Methods

### Expression of Pro-IGF-II (Long IGF-II)

Long IGF-II DNA sequence was cloned from plasmid pRc/CMV-Ligf2 (a gift from Peter Nissley) into an *Escherichia coli* expression vector pRSET, pRSET-Ligf. BL21Lys cells carrying pRSET-Ligf were grown in Luria-Bertani broth at 37°C to  $A_{600} = 0.6$ , then induced with isopropyl-L-thio- $\beta$ -D-galactopyranoside at 1 mmol/L for 3 hours. His-tagged IGF-II was solubilized in PBS + 0.5 mol/L NaCl and purified on HiTrap nickel-chelating column. Purified long IGF-II-His was dialyzed to PBS buffer and purity was examined on SDS-PAGE.

### Phage Display Screening

Recombinant long IGF-II-His was used to screen a human naïve Fab phage library containing  $10^{10}$  unique clones<sup>4</sup> using protocols and reagents similar to those we used previously (21). Long IGF-II-His was coated on a 96-well MaxiSorb plate and blocked with 3% filtered milk/PBS. Purified phage ( $10^{12}$  plaque-forming unit) was allowed to bind to the plate for 2 hours at room temperature. The wells were washed 10 times with PBS + 0.05% Tween 20. Bound phage was eluted with freshly made 100 mmol/L triethylamine and neutralized with 1 mol/L Tris (pH 8.0). Phage was used to infect exponentially growing TG1 cells and rescued by M13KO7 helper phage. Panning was repeated four times with 2  $\mu$ g antigen and 10 times wash after each round.

Two hundred individual colonies after the third round and another 200 colonies after the fourth round were picked and inoculated into 2YT medium in 96-well plate for soluble Fab expression. After 5 hours of growth at 37°C, isopropyl-L-thio- $\beta$ -D-galactopyranoside was added to induce Fab expression.

### Conversion from Fab to IgG1

Fabs in pComb3H were cloned into pDRI2, kindly provided by Dennis Burton (The Scripps Research Institute, La Jolla, CA), which allows simultaneous expression of the heavy chain and light chains (22). Briefly, the heavy chain variable region was first cloned into pDRI2 via *Xba*I and *Sac*I sites. The light chain sequence (VL + CL) was then cloned into pDRI2 via *Hind*III and *Eco*RI sites.

### Expression of Fab and IgG1

HB2151 cells were transformed with pCombIII plasmid containing Fab sequences. Single fresh colonies were inoculated into 2YT medium + 100  $\mu$ g/mL ampicillin + 0.2% glucose. The culture was shaken at 250 rpm at 37°C until  $A_{600} = 0.5$ . Isopropyl-L-thio- $\beta$ -D-galactopyranoside

<sup>4</sup> Z. Zhu and D.S. Dimitrov, manuscript in preparation.

(1 mmol/L) was added to induce expression. After overnight growth at 30°C, the culture was harvested. Bacteria were centrifuged at  $5,000 \times g$  for 15 minutes. The pellet was resuspended in PBS with polymycin B (10,000 units/mL). Soluble Fab was released from periplasm by incubating at room temperature for 45 minutes. The extract was clarified at  $15,000 \times g$  for 30 minutes. The clear supernatant was recovered for purification on protein G column.

IgG1 was expressed in 293 free style cells. CellFectin was used to transfect 293 free style cells according to the instructions of the manufacturer (Invitrogen). Four days posttransfection, the culture supernatant was harvested. IgG was purified on protein G column.

#### ELISA Binding Assay

Antigen was coated on narrow-well, 96-well plate at 50 ng/well overnight at 4°C. For phage ELISA,  $\sim 1 \times 10^{10}$  phage from each round of panning was incubated with antigen. Bound phage was detected with anti-M13-HRP polyclonal antibody (Pharmacia, Piscataway, NJ). For colony screening, 50  $\mu$ L of overnight expression supernatant was incubated with antigen. For Fab-binding kinetics, Fab was titrated from 4,000 to 0.00128 nmol/L. Bound Fab was detected with anti-FLAG-HRP mAb (1:1,000; Sigma). The 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) substrate was added and the reaction was read at  $A_{405}$  nm.

#### Determination of Kinetics Rate Constants and Affinity by Surface Plasmon Resonance

Interactions between various Fabs and IGF-II were analyzed by surface plasmon resonance technology using a Biacore 1000 instrument (Pharmacia). IGF-II was covalently immobilized onto a sensor chip (CM5) using carbodiimide coupling chemistry. A control reference surface was prepared for nonspecific binding and refractive index changes. For analysis of the kinetics of interactions, varying concentrations of Fabs were injected at flow rate of 30  $\mu$ L/min using running buffer containing 150 mmol/L NaCl, 3 mmol/L EDTA, and 0.005% P-20 (pH 7.4). The association and dissociation phase data were fitted simultaneously to a 1:1 Langmuir global model by using the nonlinear data analysis program BIAevaluation 3.2. All the experiments were done at 25°C.

#### Phosphorylation Assay

MCF-7 cells were seeded in a six-well plate at  $1.0 \times 10^6$  per well in complete growth medium. After overnight culture, cells were rinsed with serum-free DMEM and then cultured in serum-free DMEM for 6 hours. Cells were incubated with various concentrations of recombinant Fab or IgG for 30 minutes, and then IGF-II was added to a final concentration of 10 nmol/L. In some cases, antibody and IGF-II were added to the cultures at the same time but were not premixed. Twenty minutes after addition of IGF II, cells were chilled on ice, rinsed in cold PBS, and lysed in 1 mL of lysis buffer [50 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 10% glycerol, 1% Triton X-100, 1.5 mmol/L  $MgCl_2$ , 2 mmol/L sodium vanadate, and protease inhibitors]. Lysates were kept on

ice for 30 minutes, followed by centrifugation at  $17,000 \times g$  for 30 minutes. The supernatant was used for immunoprecipitation: 20  $\mu$ L of protein G Sepharose 4B and 2  $\mu$ g of rabbit anti-IGF-IR  $\beta$  (C-20, Santa Cruz Biotechnologies, Santa Cruz, CA). After extensive wash, the immunoprecipitates were run on 4% to 12% NUPAGE, transferred to polyvinylidene difluoride membrane, and blotted with antiphosphotyrosine mAb 4G10. The membrane was stripped and reprobed with C-20 polyclonal antibody to detect total IGF-IR or C-19 to detect total insulin receptor in the immunoprecipitates. A similar procedure was used for Akt and MAPK but Western blots were done with antibodies recognizing phospho-Akt and phospho-MAPK.

#### Cell Growth Assay

Cells were seeded in 96-well plates at 10,000 per well in complete growth medium. After overnight incubation, they were rinsed gently with serum-free medium twice, and 100  $\mu$ L of serum-free medium containing 10 nmol/L IGF-II and various concentrations of antibodies was added. At day 1, 2, or 3, 20  $\mu$ L of MTS (Promega, Madison, WI) was added to each well and plates were incubated at 37°C for 2 to 4 hours. The reaction was monitored at  $A_{490}$  nm.

#### Cell Migration Assay

Transwell culture plates with 8  $\mu$ m pore size polycarbonate membrane were used according to the instructions of the manufacturer (Corning Life Sciences, Corning, NY). Briefly, the bottom wells contained 2.6 mL DMEM with 5% fetal bovine serum and various concentrations of antibodies. DMEM with 5% fetal bovine serum and serum-free DMEM were used as positive and negative controls. The top inserts contained 1.5 mL of  $0.5 \times 10^6$  MCF-7 single-cell suspension in serum-free DMEM. Cells were incubated in 37°C incubator. Four hours later, cells attached to the upper side of membrane were cleaned off with cotton-tipped applicator. Cells on the downside of membrane were stained with Hema3 kit (Fischer, Middletown, VA). The membranes were removed from the transwell, mounted onto microscopic slides, and cells were counted under a microscope.

## Results

### Selection of Phage-Displayed Fabs Specific for Pro-IGF-II

Most, if not all, of the currently available antibodies to IGF-II are not human but are typically of mouse origin. To develop human mAbs against IGF-II, we used a large naïve human Fab library containing  $\sim 10^{10}$  different phage-displayed Fabs, which was recently developed.<sup>4</sup> A precursor form of IGF-II, pro-IGF-II [or long (big) IGF-II], was expressed and purified as described in Materials and Methods and used as a selecting antigen for screening of the antibody library. After four rounds of panning, screening of 200 individual phage clones was done in phage ELISA with pro-IGF-II as described in Materials and Methods. Of clones that exhibited significant binding to pro-IGF-II and were sequenced, 10 Fabs

had unique sequences (Table 1); they were expressed in bacteria as soluble Fabs, purified, and tested for binding activity. Three Fabs, designated m606, m610, and m616, exhibited significant levels of expression and binding ( $A_{450} > 0.5$ ) to pro-IGF-II in ELISA and were selected for further characterization; the other clones were either not expressed or did not bind to any measurable degree (Table 1; Fig. 1).

#### Specific and High-Affinity Binding of m606, m610, and m616 to IGF-II and pro-IGF-II

The binding of the Fabs was measured by using ELISA and Biacore surface plasmon resonance technology. In an ELISA assay, the  $EC_{50}$  for these antibodies was  $\sim 4$  nmol/L (Fig. 1A; data not shown). The three Fabs bound to pro-IGF-II and to the mature form of IGF-II but not to insulin and IGF-I (Fig. 1B). The binding to pro-IGF-II was slightly better than to IGF-II (Fig. 1B; data not shown), perhaps reflecting the use of pro-IGF-II as selecting antigen. Because of difficulties in production of m616 and similarity of its binding profile with the other two antibodies, we have further focused on m606 and m610. The binding kinetics and affinities of these Fabs to pro-IGF-II were measured by Biacore (Fig. 1C):  $K_{on} = 3.5$  and  $2.9 \times 10^5$ /mol/L/s;  $K_{off} = 2.5$  and  $2.6 \times 10^{-4}$ /s, and  $K_D = 0.7$  and  $0.9$  nmol/L. Further experiments by using Biacore showed that the two Fabs compete between each other for binding to pro-IGF-II, suggesting that they may have overlapping epitopes (data not shown).

Fab m606 and m610 were converted to IgG1, expressed, and purified. The two IgG showed similar binding affinities to long and mature forms of IGF-II, with  $EC_{50}$  of  $\sim 1$  nmol/L (Fig. 2).

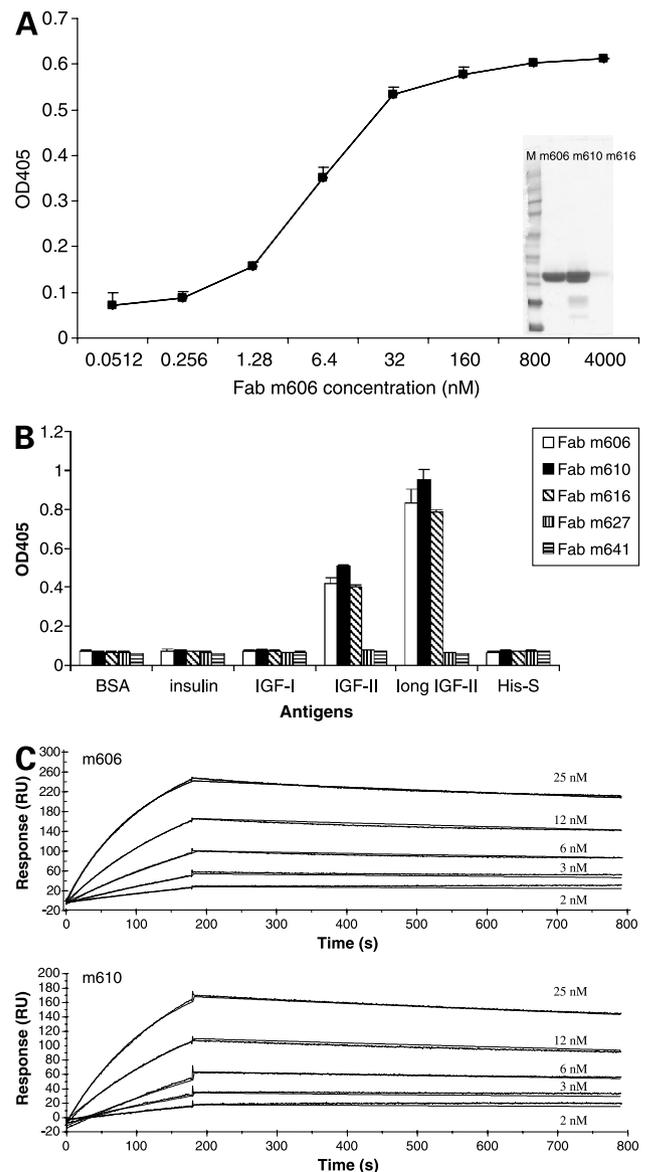
#### Inhibition of IGF-IR and Insulin Receptor Phosphorylation

Upon ligand binding, IGF-IR undergoes autophosphorylation on tyrosine residues of the two  $\beta$ -subunits. To find whether m606 and m610 can inhibit the transmembrane signaling mediated by the IGF-IR interaction with IGF-II, we measured the receptor phosphorylation in MCF-7 cells,

**Table 1. Expression and binding of soluble Fabs selected as phage-displayed Fabs for their strong binding to precursor IGF-II**

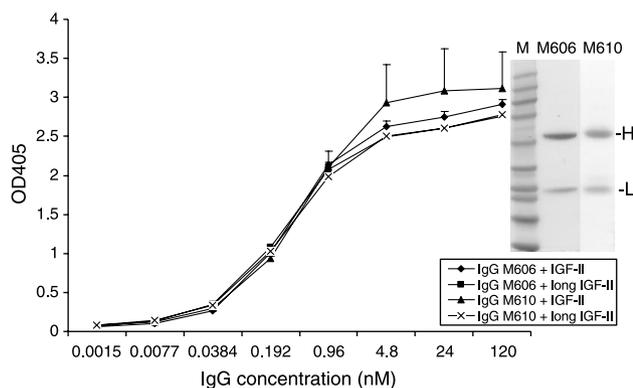
Clone	Expression	Binding	H3 sequence
m606	++++	++++	DRSIAAMGWFDH
m610	++++	++++	DVQWLAYGMDV
m616	++	+++	EKGIGRGITGTTIPYNWFDP
m618	–	–	–
m622	–	–	–
m624	–	–	–
m626	–	–	–
m627	+	–	–
m635	–	–	–
m641	++	–	–

NOTE: Ten clones with unique sequences, which significantly bound to a precursor IGF-II form as phage-displayed Fabs, were expressed as free Fabs, purified and tested for binding to IGF-II in ELISA. The number of + symbols is a measure of their expression and binding activity.



**Figure 1.** Binding affinity, specificity, and binding kinetics of anti-IGF-II Fabs. **A**, ELISA binding of Fab M606 on immobilized long IGF-II. Fab m610 and m616 have similar binding kinetics. *Inset*, SDS gel with purified Fabs. **B**, Fabs were tested for specificity by ELISA with different proteins immobilized on plates. **C**, binding kinetics of Fab m606 and m610 as measured by Biacore for different concentrations of the antibodies represented with different lines. Details of the experiments are provided in Materials and Methods.

which are known to express high levels of IGF-IR. MCF-7 cells kept in serum-free DMEM displayed nondetectable phosphorylation of IGF-IR (Fig. 3). After their incubation with 10 nmol/L IGF-II for 20 minutes, the phosphorylation of IGF-IR was readily detected (Fig. 3A). The phosphorylation was greatly reduced by the antibodies especially by m610; note that to avoid effects of autocrine IGF-II, cells were preincubated with the antibody—the neutralizing effect would be even higher if the antibody is



**Figure 2.** *In vitro* binding of IgG1 m606 and m610 to mature and precursor forms of IGF-II immobilized on ELISA plate. Binding of the antibodies was measured by ELISA with 50 ng of either mature or precursor IGF-II immobilized on plates. OD405, absorbance at 405 nm.

preincubated with IGF-II. The inhibitory effect was concentration dependent with an effective  $IC_{50}$  of 3 nmol/L for Fab m610 at 10 nmol/L IGF-II (Fig. 3A). Similar inhibitory activity was also observed with IgG1 m610, which showed an  $IC_{50}$  of 1 nmol/L at the same IGF-II concentration (Fig. 3B).

In addition to IGF-IR, IGF-II also binds to and activates the insulin receptor. As shown in Fig. 3C, m610 inhibited the phosphorylation of insulin receptor and IGF-IR induced by IGF-II with about the same efficiency in two different protocols—preincubation of the cells with the antibody (lanes 3 and 4) and simultaneous addition of IGF-II and the antibody (lanes 5 and 6). Premixing of the antibody with

IGF-II would lead to even higher neutralizing activity. Note that the total amount of receptors decreases with an increase of free IGF-II reflecting the receptor down-modulation by its ligand (equal amount of cell lysates were used for all immunoprecipitations in Fig. 3C).

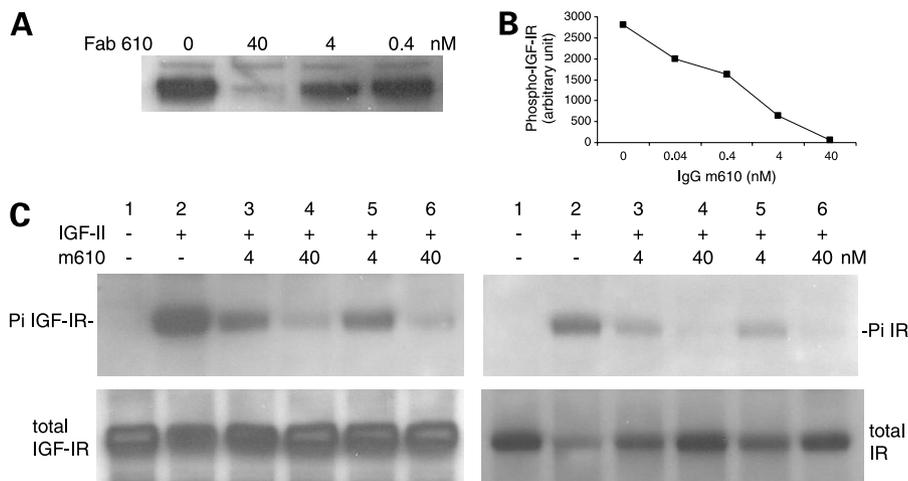
MCF-7 cells have higher levels of IGF-IR than all cell lines we tested (data not shown). To find whether our antibodies are as effective for such cells expressing relatively low levels of IGF-IR as for MCF-7, we also tested the prostate cancer cell line DU145 and the leukemia cell line U937. IgG m610 was capable of inhibiting IGF-II-induced phosphorylation of IGF-IR in both cells, although the  $IC_{50}$  was slightly higher (Fig. 4).

#### Inhibition of Phosphorylation Downstream of IGF-IR

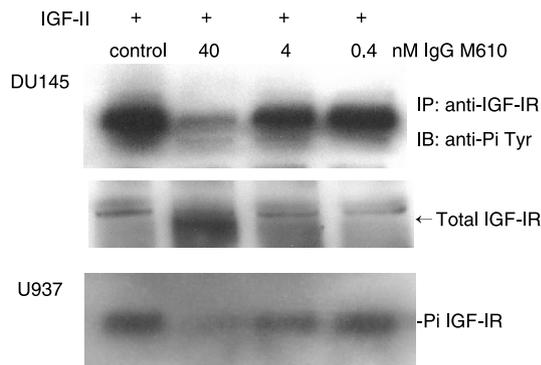
Tyrosine phosphorylation of IGF-IR leads to activation of adaptor proteins insulin response substrate 1 and 2 (IRS-1,2). Activation of IRS-1,2 further causes a phosphorylation cascade, which includes downstream kinases, phosphatidylinositol 3' kinase/Akt and MAPK (or extracellular signal-regulated kinase). To determine whether inhibition of IGF-IR phosphorylation by IgG m610 was capable of preventing signals transduced from IGF-II through its receptor to Akt and MAPK, MCF-7 cells were treated with IGF-II and different doses of IgG m610. Western analysis of phosphorylation of Akt and MAPK indicated that IGF-II-induced phosphorylation of these downstream kinases was also inhibited by IgG m610 in a concentration-dependent fashion. The total amount of MAPK did not change during the duration of the experiment (Fig. 5).

#### Inhibition of Cell Growth and Migration by IgG m610

Components of the IGF system can exert pleiotropic effects on normal and cancer cells. There are a number of



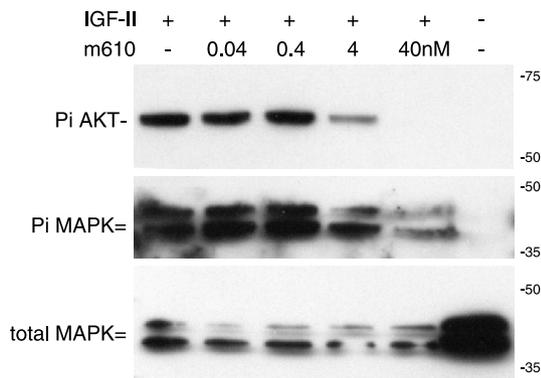
**Figure 3.** IGF-II antibodies inhibited IGF-II-induced phosphorylation of IGF-IR and insulin receptor (IR) in the breast cancer MCF-7 cells. **A**, MCF-7 cells in serum-free medium were preincubated with indicated concentrations of Fab m610 for 30 min. Ten nanomolars of IGF-II were added, and 20 min later cells were collected. Equal amounts of cell lysates were used for immunoprecipitation with anti-IGF-IR $\beta$  antibody (Santa Cruz Biotechnologies). Phosphorylated IGF-IR was detected with mAb 4G10 specific to phosphotyrosine. **B**, a similar test with IgG m610 was done with the same procedure in **A**. The intensity of phospho-IGF-IR was quantified by phosphorimager and was plotted. **C**, phosphorylation of IGF-IR and insulin receptor was monitored in MCF-7 cells treated with IGF-II alone (lane 2) or with indicated concentrations of IgG m610. Lanes 3 and 4, cells were preincubated with the antibody for 30 min before addition of IGF-II. Lanes 5 and 6, cells were treated with the antibody and IGF-II at the same time. *Bottom*, total amount of receptors in the immunoprecipitates.



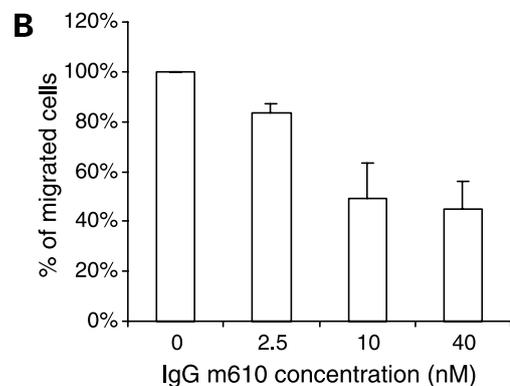
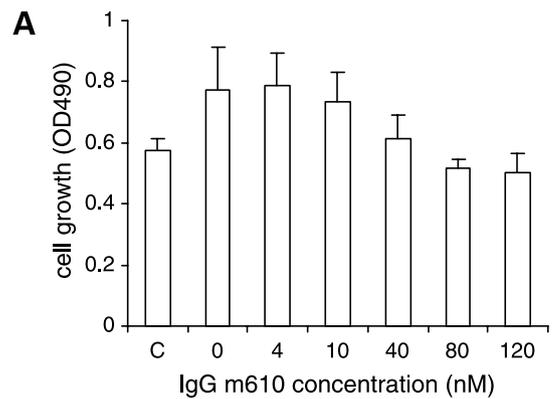
**Figure 4.** IGF-II antibodies (IgG m610) also inhibited phosphorylation of IGF-IR in prostate cancer cell line DU145 and leukemia U937 cells. The experimental procedure was the same as in Fig. 3A.

neutralizing antibodies targeting at IGF-IR and most of these antibodies inhibit cell proliferation. However, a dominant negative form of IGF-IR has been reported to inhibit motility of cells without affecting cell growth (23). We tested the activity of m610 in a cell growth assay using MCF-7, DU145, and U937 cells. A typical result is shown in Fig. 6A for DU145 cells. The antibody concentration required to inhibit cell growth was >10-fold higher ( $IC_{50}$  higher than 10 nmol/L) than the concentration required for signal transduction inhibition. MCF-7 cells did not respond as well as DU145 cells to IGF-II treatment (data not shown).

Motility of cancer cells is essential for tumor metastases. A cell migration assay was therefore done to test whether IgG m610 can inhibit migration of MCF-7 cells through 8  $\mu$ m membrane pores. IgG m610 at 10 nmol/L concentration inhibited 40% of cell migration in medium containing 5% fetal bovine serum (Fig. 6B). DU145 cells showed even larger reduction in motility than MCF-7 cells (data not shown). IGF-II alone in serum-free medium did not induce cell migration, indicating that the migration process requires additional factors.



**Figure 5.** IgG m610 prevented activation of signals downstream of IGF-IR induced by IGF-II. MCF-7 cells were incubated with different doses of IgG m610 and incubated with 10 nmol/L IGF-II. Cell lysates were made 20 min after addition of IGF-II. Western blots were done with antibodies recognizing phospho-Akt, phospho-MAPK, and total MAPK.



**Figure 6.** IgG m610 inhibited DU145 cell growth and reduced motility of MCF-7 cells in medium containing 5% fetal bovine serum. **A**, DU145 cells were starved in serum-free medium for 6 h followed by incubation with IGF-II at 10 nmol/L final concentration and IgG m610 at the indicated final concentrations. Two days later, MTS was added to quantify viable cells. The reaction was monitored by measuring the absorbance at 490 nm ( $OD_{490}$ ). The cells in the control sample (C) were incubated with the same volumes of serum-free medium but in the absence of IGF-II and antibody. **B**, MCF-7 cells were cultured in serum-free medium in transwells with 8  $\mu$ m pores. The bottom wells contained 5% fetal bovine serum and IgG m610 at the indicated concentrations. Cells migrated through the pores after 4 h were stained and counted. Columns, percentage of the number of migrated cells (100% in the absence of antibody).

## Discussion

The major finding of this study is the identification and characterization of three novel fully human mAbs specific for pro-IGF-II and matured IGF-II. These antibodies bound with high affinity to both forms of IGF-II but not to IGF-I and insulin. One of them, m610, was selected for more extensive characterization based on its superior inhibitory activity. This antibody potently inhibited phosphorylation of IGF-IR and the insulin receptor, phosphorylation of downstream kinases, and less potently, cell growth and migration of cells expressing IGF-IR. The relatively high concentrations required for growth inhibition are in agreement with recent reports (e.g., JBC 279, 5017) that in breast cancer cells, such as MDA-MB-435, a dominant negative form of IGF-IR does not inhibit cell proliferation, although it does inhibit IGF-I-stimulated phosphorylation of IGF-IR, IRS-1,2, and Akt. However, it inhibits motility of these cells in culture and metastasis in the mouse model.

The inhibitory activity of the antibodies was cell type dependent with a likely major determinant the surface concentration of the IGF-IR and the insulin receptor. Another major factor is the secretion of IGF-II. For example, we found that MCF-7 cells secrete IGF-II in serum-free medium, resulting in an accumulation of a free ligand at concentrations up to 35 nmol/L after 3 days in culture (data not shown). The autocrine IGF-II would bind the antibody in any cell growth assay that requires more than few days.

Based on these *in vitro* results and recent data based on the use of a mouse mAb cross-reactive to IGF-I and IGF-II (19, 20), we believe that m610, and perhaps the other two antibodies, would also exhibit inhibitory activity *in vivo*. Experiments to test this possibility with mouse models of cancer dependent on IGF-II will definitely define the limits of an approach for cancer treatment based on m610; such experiments will be done after sufficient amounts of this antibody are produced. The finding that IgG1 m610 has approximately the same or even higher activity than Fab m610 is important because it would allow to use the IgG1 antibody format, which is most stable and has longest half-life *in vivo*; the Ig format may also confer certain effector functions *in vivo*.

These antibodies could be also used as research reagents to help dissect the effects of IGF-I from IGF-II because they do not bind to IGF-I and insulin, whereas many of the known antibodies are cross-reactive. Finally, because they are fully human antibodies, one can expect that they would not induce immune response if used in humans. If these antibodies are successful in animal models, they do not need humanization before their use in clinical trials.

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