Vol. 196, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1993 Pages 92-98

TWO NEW MONOCLONAL ANTIBODIES AGAINST THE α SUBUNIT OF THE HUMAN INSULIN-LIKE GROWTH FACTOR-I RECEPTOR

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Received August 13, 1993

Summary: Recently, we have reported three monoclonal antibodies (mAbs) against purified human placental insulin-like growth factor (IGF)-I receptors. These antibodies, in contrast to the well-studied mAb αIR-3, stimulate binding of IGF-I and IGF-II to the receptor and DNA synthesis as well [Xiong, *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 1992(89), 5356]. Here we describe two additional mAbs, 1H7 and 2C8, against the IGF-I receptor that have characteristics different from either αIR-3 or our previously reported mAbs. Both 1H7 and 2C8 bind to the α subunit of the IGF-I receptor as determined by immunoblotting. MAb 1H7 inhibited the binding of IGF-I and IGF-II to the IGF-I receptor while 2C8 had no effect on the binding of either ligand to the receptor. When their effects on DNA synthesis were examined using NIH 3T3 cells expressing human IGF-I receptors, 1H7 inhibited basal and IGF-I- or IGF-II-stimulated DNA synthesis whereas 2C8 stimulated basal DNA synthesis but provided no synergism in the presence of IGF-I or IGF-II.

The IGF-I receptor which is responsible for the growth effects of both IGF-I and IGF-II, is structurally and functionally similar to the insulin receptor (1). Both receptors are glycoproteins of Mr=~350,000 composed of two extracellular α subunits and two transmembrane β subunits. The cytoplasmic protein tyrosine-kinases are activated by ligand binding to the extracellular domain of the receptors. Kinase activation in turn stimulates an intracellular cascade of molecular interactions involving multiple signaling pathways, leading to the growth and metabolic effects of IGFs and insulin (2,3).

As usually defined, insulin elicits metabolic effects such as increases in glucose uptake and glycogen synthesis whereas IGFs are more potent in mitogenic activity (4,5). However, an overlap in the biological activities of insulin and IGFs can be observed in cells that express both insulin and IGF-I receptors (6). Thus, mAbs that block or inhibit the biological activity of either receptor would help us delineate signal

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Abbreviations used: IGF, insulin-like growth factor; mAb, monoclonal antibody; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide electrophoresis.

0006-291X/93 $4.00

transduction pathways specific for insulin or IGFs. While a number of mAbs against the insulin receptor have been reported (7-11), to date the availability of mAbs against the IGF-I receptor has been limited. A mAb called αIR-3 is the only one that has been commonly used among investigators in the field (12-16). This mAb inhibits IGF-I- and IGF-II-stimulated DNA synthesis at the same time it inhibits the binding of IGF-I to its receptor more potently than IGF-II (16,17). Recently, we reported three IGF-I receptor mAbs which stimulated binding of IGF-I and IGF-II to the receptor as well as basal and IGF-stimulated DNA synthesis (18). Soos *et al.* also reported a panel of mAbs against the recombinantly expressed IGF-I receptor (19). In this report, we describe new mAbs against the IGF-I receptor which appear to have characteristics different from the mAbs previously reported.

Materials and Methods

Materials: IGF-I and insulin receptors were purified from human placental membranes as described (15,20). αIR-3 and 4B1 were gifts from Dr. S. Jacobs (The Wellcome Research Laboratories, Research Triangle Park, NC) and Dr. P. Salvaterra (Beckman Research Institute of the City of Hope), respectively. NIH3T3 cells expressing ~10^6 human IGF-I receptors per cell (2) were provided by Dr. U. Ullrich (Max Plank Institute, Martinsried, Germany). Mouse monoclonal isotyping reagents and rabbit anti-mouse IgG were purchased from Sigma. Goat anti-mouse Ig's conjugate with alkaline phosphatase was purchased from Tago, Inc., (Burington, CA). Recombinant IGF-I was purchased from Mallinckrodt. Recombinant IGF-II was a gift from Dr. Y. Sato (Daiichi Pharmaceutical, Tokyo). [γ-^{32}P]ATP(6000Ci/mmol), ^{125}I-labeled IGF-I (^{125}I-IGF-I; 2000Ci/mmol) and [^{3}H]thymidine (6.7 Ci/mmol) were purchased from New England Nuclear, Amersham and ICN, respectively. ^{125}I-IGF-II was a gift from Dr. J.F. Perdue (American Red Cross, Rockville, MD (21).

Immunization, hybridoma production, and screening: BALB/c female mice were immunized with the purified human placental IGF-I receptor by standard procedures as described (18). Hybridomas were produced as previously described (18). Screening of hybridomas producing mAbs against the IGF-I receptor was carried out by ELISA and dot-blot assays using purified IGF-I receptor as described (18).

Immunoblotting: Purified IGF-I and insulin receptors were subjected to SDS-PAGE (7.5% gel). Proteins were electrophoretically transferred to a nitrocellulose membrane (22). The membranes were incubated with either 1H7 or 2C8 at 4°C for 16 h, washed three times with PBS, and incubated with goat anti mouse Ig's conjugated with alkaline phosphatase (1:3500 dilution) at 25°C for 3 h. The membrane was washed once with 0.1 M Tris-HCl buffer, pH 9.5, containing 0.1 M NaCl and 5 mM $MgCl_2$, then incubated with bromochloroindol phosphate-nitro blue tetrazolium substrate solution as previously described (23). The reaction was stopped with PBS containing 20 mM EDTA.

IGF Binding assays: ^{125}I-IGF-I or ^{125}I-IGF-II binding to the purified IGF-I receptor in the presence of different concentrations of mAbs was measured as described (18).

Phosphorylation of the IGF-I receptor: Purified IGF-I receptor was incubated with buffer, IGFs, or mAbs at 25°C for 40 min in 12.5 μl of 50 mM Tris-HCl buffer, pH 7.4, containing 0.1% Triton X-100. Autophosphorylation was initiated by adding 2.5 μl of [γ-^{32}P]ATP/metal mixtures to give final concentrations of 0.1% Triton X-100, 2 mM

$MnCl_2$, 15 mM $MgCl_2$ and 40 μM [γ-^{32}P]ATP (~12,000 cpm/pmol). After 40 min, the reaction was terminated by the addition of 7.5 μl of 3x SDS-PAGE sample buffer. The samples were boiled for 5 min and analyzed by mini SDS-PAGE under reducing conditions. The gel was stained with silver, and radioactive bands were quantitated by the AMBIS Radioanalytic Imaging System (AMBIS System, Inc., San Diego, CA).

[^{3}H]Thymidine incorporation into DNA: NIH3T3 cells expressing human IGF-I receptors were grown to subconfluency in DMEM containing 10% FBS in 24-well plates (~2x10^4 cells/well). The cells were cultured in 1 ml of DMEM containing 0.25% BSA for 24 h and then incubated with different concentrations of mAbs in the absence or presence of IGFs for 16 h. The cells were subsequently pulsed with 0.5 μCi of [^{3}H]thymidine for 5 h. The magnitude of [^{3}H]thymidine incorporated into trichloroacetic acid-insoluble DNA was quantified by liquid scintillation counting.

Results and Discussion

Specificity and Crossreactivity: Two hybridomas producing mAbs 1H7 and 2C8 were cloned by reactivity with purified IGF-receptor. Both mAbs were determined to be IgG1. The specificity of two new mAbs was examined by immunoblotting using purified human placental IGF-I receptor and insulin receptor. Both 1H7 and 2C8 reacted with the α subunit of the IGF-I receptor (Fig. 1), but did not recognize the insulin receptor (data not shown).

Effects of mAbs on Binding Activity of Purified IGF-I Receptors: Effects of 1H7 and 2C8 on IGF-I and IGF-II binding to purified IGF-I receptors were analyzed together with αIR-3, which is known to inhibit IGF-I binding more potently than IGF-II.

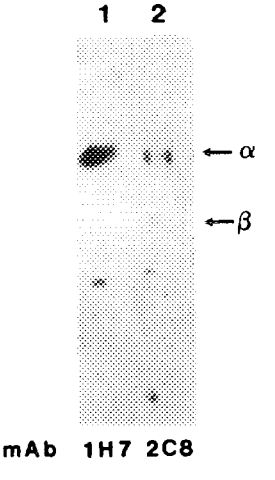

Figure 1. Immunoblotting of the purified IGF-I receptor by mAbs, 1H7 and 2C8. Purified human placental IGF-I receptor (~100 ng) was applied to a SDS-PAGE (7.5%) gel and blotted onto a nitrocellulose membrane. Culture supernatants from the 1H7 and 2C8 hybridomas provided activities that reacted with the α subunit of IGF-I receptor; lane 1, 1H7; lane 2, 2C8.

1H7 inhibited IGF-II binding more potently than IGF-I binding to the IGF-I receptor (Fig. 2). When compared to the binding inhibition of IGF-I and IGF-II to the receptor by αIR-3, 1H7 was more potent in inhibiting IGF-II binding but less effective in inhibiting IGF-I binding (Fig. 2). In contrast, 1C8 had neither stimulatory nor inhibitory effect on the binding of IGF-I or IGF-II to the IGF-I receptor (Fig. 2).

Effects of mAbs on Autophosphorylation of the IGF-I Receptor β Subunit: The effects of1H7 and 2C8 on autophosphorylation of the purified IGF-I receptor were examined and compared with those of IGF-I, IGF-II, αIR-3, and 3B7, our previously reported mAb (18). As summarized in Table I, 1H7 and 2C8 stimulated autophosphorylation of the β subunit by ~1.6- and 1.3-fold, respectively, over the basal activity. The magnitude of the stimulation, however, significantly smaller than that of αIR-3, 3B7, and IGFs.

Effects of mAbs on DNA Synthesis: Effects of mAbs on [^{3}H]thymidine incorporation into NIH3T3 cells expressing human IGF-I receptors in the absence or presence of IGF-I or IGF-II were measured. As controls, growth-stimulatory mAb, 3B7, and growth-inhibitory mAb, αIR-3, were included. The results are shown in Fig. 3. 2C8 markedly stimulated basal DNA synthesis without significant effects on IGF-I- or IGF-II-stimulated DNA synthesis while 3B7 stimulated DNA synthesis both in the absence and presence of IGF-I or IGF-II. 1H7 had an inhibitory effect of ~18% on basal DNA synthesis while it inhibited both IGF-I and IGF-II stimulated DNA synthesis by ~55%.

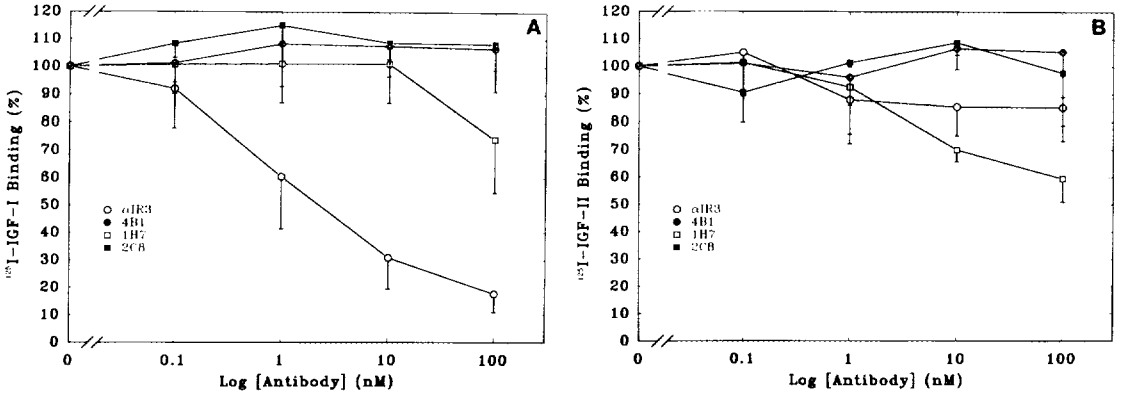

Figure 2. Effects of mAbs on ^{125}I-IGF-I and ^{125}I-IGF-II binding to the purified human placental IGF-I receptors. The receptors (~10 ng) were incubated with ^{125}I-IGF-I (**A**) or ^{125}I-IGF-II (**B**) in the presence of indicated concentrations of mAbs, and radioactivity bound to the receptors was determined. The binding activity is expressed as the per cent of binding in the absence of mAbs. mAbs used are 2C8 (■), 1H7 (❑), αIR-3 (○), and 4B1 (●, anti-cholin acetyltransferase; a negative control). Data points are average ± SD of four experiments.

Table I. Effects of mAbs and IGFs on autophosphorylation of the purified IGF-I receptor β subunit

Addition	Fold stimulation	
	Experiment 1	Experiment 2
None (basal)	1	1
Ligand		
130 nM IGF-I		1.9
130 nM IGF-II		3.2
mAb		
200 nM 1H7	1.6	1.7
200 nM 2C8	1.5	1.3
200 nM 3B7	3.1	2.7
200 nM αIR-3	2.7	2.9

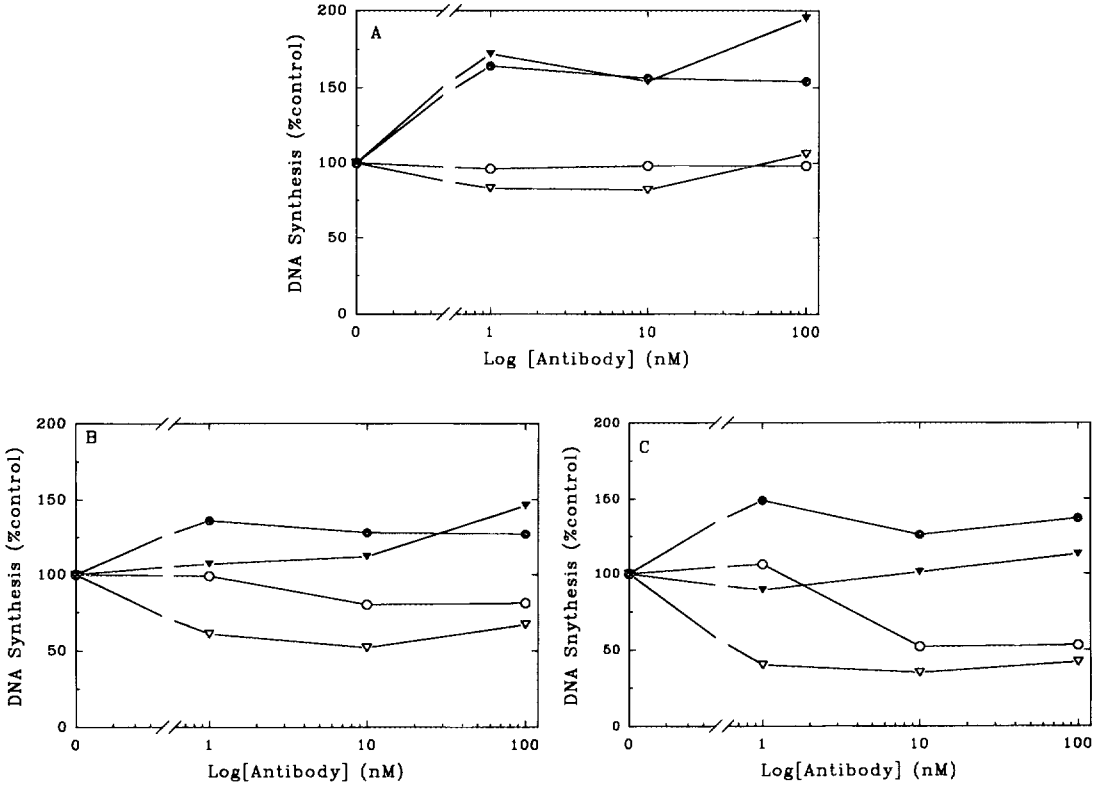

Figure 3. Effects of mAbs on the stimulation of DNA synthesis. NIH3T3 cells expressing human IGF-I receptors plated on 24-well plates were serum-depleted and incubated with different concentrations of mAbs in the absence (**A**) or presence of IGF-I at 0.1 μg/ml (**B**) or IGF-II at 1 μg/ml (**C**). [^{3}H]Thymidine incorporation was then measured as described in the Methods. mAbs used were 2C8 (▼), 1H7 (▽), 3B7 (●), and αIR-3 (○). Shown is one of two similar results.

Newly characterized mAbs, 1H7 and 2C8, are shown to be different from previously reported mAbs (8,18,19). First, 1H7 and 2C8 reacted well with the IGF-I receptor α subunit on immunoblots whereas αIR-3, our previously reported mAbs, and other mAbs prepared by Soos *et al.* did not give strong reactions on immunoblots (19). Second, 1H7 inhibited IGF-II binding to the IGF-I receptor more potently than IGF-I, which is opposite of αIR-3. Therefore, 1H7 and αIR-3 are complementary to each other with respect their effects on binding activity, although both mAbs inhibited IGF-I- and IGF-II-stimulated DNA synthesis. Third, 2C8 stimulated only the basal DNA synthesis without any effects on the binding of IGFs whereas our previously reported mAbs stimulated both IGF-I and IGF-II binding to the receptor as well as basal and ligand-stimulated DNA syntheses. Epitope analysis of these new mAbs is in progress in our laboratory. The new mAbs will be thus useful in studying IGF-I receptor-mediated signal transductions.

Acknowledgments

We thank Faith Sorensen for secretarial assistance. This work was in part supported by National Institute of Health Grant DK34427.

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