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Gold-labeled IGF-II has been prepared in micro-scale by reacting IGF-II with sulfo-NHS-Nanogold, and purified by size-exclusion HPLC. Two gold-labeled IGF-II products, differing in electrophoretic mobility under acidic condition, were obtained. Both products were derivatized with the Nanogold at their N-terminal alanine. Insulin receptor proteins, IR-A and IR-B have been extracted and purified from transfectant cells. As expected from previous studies in the transfectant cells, IR-A in contrast to IR-B was activated by IGF-II comparable to that by insulin. The two gold-labeled IGF-II derivatives when tested in a preliminary study for their activity on IR-A were found to have a good level of activity in stimulating the autophosphorylation of IR-A. In summary, methods have been established to prepare and purify the reagents, namely the gold-labeled IGF-II and the insulin receptor proteins, that are needed to form the ligand-receptor complex for the identification of the IGF-II-binding site of the insulin receptor by STEM reconstruction of the 3-D quaternary structure of the complex.

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Introduction

Insulin-like growth factor I and II (IGF-I and IGF-II) are related peptides sharing homology with insulin. While insulin acts mainly to regulate glucose uptake and cellular metabolism, in most cells and tissues IGFs play a major regulatory role in growth and proliferation. IGF-I and IGF-II are believed to exert their actions through the IGF-I receptor (IGF-I-R) which is highly homologous to the transmembrane insulin receptor (IR) (1). However, there is experimental evidence indicating that IGF-II may signal via IR. The human IR exists in two isoforms (IR-A and IR-B) as the result of alternate splicing of a small exon (exon 11) encoding 12 aminoacids (residues 718-729) at the carboxyl end of the receptor α-subunit. It was reported recently that IGF-II binds to IR-A, but not IR-B, with an affinity close to that of insulin (2) significantly resulting in predominantly mitogenic responses. It was further observed that in breast and colon cancer tissues the relative abundance of IR-A was significantly higher than in normal tissues, and that receptor autophosphorylation induced by IGF-II reflected the relative abundance of IR-A. These novel observations show that IGF-II can interact effectively with IR-A as a growth factor, and may acquire biological relevance and significance in cancers where IGF-II is locally produced and IR-A is the predominant isoform of IR. The present research focuses on the question of what may be the structural basis for the preferential binding of IGF-II to IR-A over IR-B. The aims of the research are to obtain the quaternary 3-D structure of the complex of IGF-II and IR-A, to locate structurally the binding site of IGF-II, and to compare its location with that of the insulin-binding site. The research applies the method of low-dose (6 electrons/Å^2), and low-temperature (-150^{0}C) dark field scanning transmission electron microscopy (STEM) to obtain the quaternary 3-D structure of the complex formed between IGF-II and IR-A. This method has been successfully carried out by the P.I. and colleagues to elucidate the 3-D structure of the insulin/receptor complex through the use of insulin labeled with Nanogold (3). The scope of the research includes the preparation of biologically active IGF-II labeled with Nanogold, preparation and purification of IR-A and IR-B, formation of the Nanogold-IGF-II complex with IR-A, and STEM of the complex for 3-D reconstruction.

Body

Insulin-like growth factor II (IGF-II) binds with an affinity equal to that of insulin to the human insulin receptor isoform A (IR-A). While insulin binding to IR-A leads to primarily metabolic responses, IGF-II binding produces mainly mitogenic effects. We hypothesize that the differential response is a result of IGF-II binding to a site different from but shared with the insulin-binding site on IR-A. Using Nanogold-insulin and applying the method of low-dose electron cryomicroscopy we have recently succeeded in pinpointing the insulin-binding site on the quaternary 3D structure of the insulin receptor. Accordingly, we propose to apply this method to determine the IGF-II-binding site on the quaternary 3D structure of the two isoforms (IR-A and IR-B) of the insulin receptor and to compare with the insulin-binding site. We have proposed to carry out the research in a

logical sequence: 1) preparation and characterization of Nanogold-IGF-II; 2) preparation and purification of IR-A and IR-B; and 3) determination of the quaternary 3D structure of each of the ligand-receptor complex.

The research has been scheduled as follows in accordance to the approved Statement of Work: **Year 1-2:** preparation and characterization of Nanogold IGF-II, and preparation and purification of IR-A and IR-B; **Year 2-3:** determination of the quaternary 3-D structure of the IGF-II-IR-A and IGF-II-IR-B complex. Research accomplishments during **Year 1** are described below.

1) Preparation and Characterization of Nanogold IGF-II

Much of the effort during this first year has been devoted to first working out the optimal conditions for the preparation and purification of the Nanogold-IGF-II at micro-scales. Once the optimal conditions are determined, the preparation will be scaled up.

IGF-II was derivatized with mono-NHS-Nanogold (Nanoprobes Inc., Stoney-brook, NY) based on the method we have previously used to prepare Nanogold-insulin (3). We carried out small-scale reactions to optimize the reaction conditions in respect to time, pH, molar ratios, and temperature. The reaction was monitored by 10-20% Tricine PAGE (to detect the reaction products by their increase in size over IGF-II) and acid-urea 18.75% PAGE (to detect a change in charge due to the derivatization of reactive amino groups in IGF-II).

The N-terminal alanine and Lys65 of IGF-II are amenable to derivatization with mono-NHS-Nanogold to give rise potentially to three Nanogold-IGF-II products (two mono-substituted and one di-substituted IGF-II). **Figure 1** shows that when IGF-II (25 μg) was derivatized with 6 nmoles of mono-NHS-Nanogold at pH 7.5, for 22 hr. at 37^0 C two major products appeared with electrophoretic mobility slower than IGF-II in acid-urea PAGE, indicating that the reactive amino groups (one or both) have been derivatized. Mass spectrometry analysis of the reaction mixture by MALDI showed the presence of a component with a mass of approximately 20,000 Da (**Figure 2**), equivalent to the addition of one cluster of Nanogold to one molecule of IGF-II. No higher molecular weight component was detected. We therefore concluded that the reaction mixture contained mono-substituted Nanogold-IGF-II as the product.

We then proceeded to purify the reaction products by RP-HPLC on a μBonda-pak C-18 column (300 mm x 3.9 mm) developed with a gradient of 25-45% acetonitrile in water containing 0.1% TFA. Peak fractions were analyzed by acid-urea PAGE. **Figure 3** shows the two reaction products in the reaction mixture were clearly separated by RP-HPLC. Amino acid sequencing of the two peak fractions showed that the N-terminus of each was blocked. However, mass spectrometry analysis of the two peak fractions after concentration by lyophilization (data not shown) did not detect a large molecular mass component originally present in the reaction mixture as mentioned above. We concluded that the acidic (TFA) condition of separation had likely destroyed the integrity of the gold cluster of the Nanogold-IGF-II, releasing the 70 atoms of gold from the Nanogold-IGF-II, but leaving its N-terminal blocked. Therefore, RP-HPLC in the presence of TFA is not suitable for the purification of Nanogold-IGF-II.

In order to avoid the disruption of the gold cluster, we decided to separate the reaction mixture by size-exclusion chromatogaphy (SEC) using a column (300 mm x

7.8 mm) of Bio-Sil SEC I-125-5 (Bio-Rad) which has an effective range of protein separation of 5000-100,000 kDa. The column was developed with 50 mM phosphate buffer, pH 7.6, containing 150 mM NaCl at a flow rate of 0.5 mL/min. Fractions of 0.5 mL were collected for analysis by electrophoresis. **Figure 4** shows the acid-urea PAGE analysis of the fractions obtained from the SEC separation of the reaction mixture from the derivatiz-ation of IGF-II with Nanogold at room temperature for 22 hr. It can be seen that the product, Nanogold-IGF-II A (**NGIGF-IIA**), appeared as a band with an electrophoretic mobility much slower than IGF-II and migrating just behind insulin, was eluted in fractions 12 and 13. Mass spectrometry analysis of fraction 14 showed the presence of a component with a mass of about 20,000 Da (**Figure 5**). When IGF-II was derivatized with the Nanogold at 37 ^{0}C a product appeared as a band migrating between insulin and IGF-II was obtained (**Figure 6**). We identified this product as Nanogold-IGF-II B (**NGIGF-IIB**). Peptide sequence showed the N-terminus of both of the products was blocked. We therefore conclude that it is possible to separate and purify the Nanogold-IGF-II from the reaction mixture by SEC under neutral conditions without destroying the gold cluster.

2) <u>Preparation, Purification of IR-A and IR-B, and the Assay of the Bioactivity of Nanogold-IGF-II</u>

Receptor proteins were extracted from R^- mouse fibroblasts expressing respect-ively IR-A and IR-B. The proteins were purified by sequential chromatography on WGA-agarose and on agarose coupled with the monoclonal anti-IR antibody MA-20, The purified IR-B had a high affinity for insulin (1nM), but not for IGF-1 or IGF-II. In contrast the purified IR-A bound IGF-II with an affinity that was nearly one half of that of insulin (2 nM). This affinity of the IR-A for IGF-II was similar to the affinity of the IGF-I receptor for this ligand. The purified IR-A was tested for its response to insulin, IGF-I and IGF-II in autophosphorylation. **Figure 7** shows that IR-A responded equally well to either insulin or IGF-II in the autophosphorylation of its β subunit, whereas IGF-I was only about one-tenth as effective as either insulin or IGF-II in stimulating the auto-phosphorylation of IR-A.

We have begun the preliminary assay of the activity of the Nanogold-derivatized IGF-II in the stimulation of the receptor tyrosine kinase (TK) activity of IR-A *in vitro*. It can be seen from **Figure 8** that the two Nanogold-IGF-II products (**NGIGF-IIA** and **NGIGF-IIB**) obtained as described above were active in stimulating the TK activity of IR-A in a dose-dependent manner.

Key Research Accomplishments

- We have successfully derivatized IGF-II with Nanogold.
- Two reaction products, one obtained at room temperature and one at 37° C, showed a difference in electrophoretic mobility under acidic condition.

- The amino termini of both Nanogold-IGF-II products were derivatized with the Nanogold cluster.
- We have extracted and purified the two insulin receptor proteins, IR-A and IR-B. As expected, IR-B showed a low affinity for either IGF-I or IGF-II when compared to insulin, whereas IR-A bound IGF-II with a high affinity (2 nM compared to 1 nM for insulin).
- IGF-II was equally as effective as insulin in stimulating the autophosphorylation of IR-A.
- Both Nanogold derivatives of IGF-II (NGIGF-IIA and NGIGF-IIB) were active in stimulating the tyrosine kinase activity of IR-A.

Reportable Outcomes

1. An abstract entitled "Studies of the purified fetal insulin receptor (IR-A) reveal that it is an IGF-II receptor" by B. Maddux, Ira D. Goldfine, C.C. Yip, R. Sportsman, and R. Vigneri, was presented at the annual meeting of the American Diabetes Association, 2001.

Conclusions

In this first year of the IDEA grant period we have achieved the synthesis of two Nanaogold derivatives of IGF-II (NGIGF-IIA and NGIGF-IIB) with differing electrophoretic mobility in acidic urea PAGE, indicating a difference in charge. A method of purification has been established to purify these two products. Both Nanogold derivatives of IGF-II were derivatized at their N-terminal alanine. The formation of the two Nanogold derivatives of IGF-II may be related to the detection of two stained bands in IGF-II when electrophoresed in acidic condition (see **Figure 1**, Lane 1 and 10).

Insulin receptor protein, IR-A and IR-B, have been extracted and purified from transfectant cells. As expected from the original observation in intact transfectant cells (2), IGF-II binds to and activates IR-A *in vitro* with an avidity similar to that of insulin, whereas with IR-B it is about one-tenth as active as insulin.

Preliminary assay of NGIGF-IIA and NGIGF-IIB for tyrosine kinase activation of IR-A indicates both Nanogold derivatives were active.

In summary, we have established the conditions for the synthesis and purification of Nanogold IGF-II in microscale (μg). In the coming next year, the method of synthesis and purification will be scaled up to the mg level. The chemical nature of the two derivatives, both being active towards the insulin receptor protein IR-A, will be further characterized by tryptic fragmentation and mass spectrometry analysis. Production of IR-A and IR-B will also be scaled up to the mg level.

In conclusion, the research project is on target. The research project is moving towards the production of the reagents in mg quantities needed (i.e. the Nanogold labeled IGF-II, and the insulin receptor proteins IR-A and IR-B) for the STEM determination of the IGF-II-binding site of IR-A, the objective of the research.

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1. Ullrich, A. *et al.*, EMBO J. **5:** 2503-2512 (1986).
2. Frasca, F., *et al.,* Mol. Cell. Biol. **19:** 3278-3288 (1999).
3. Luo, R. Z-T., Beniac, D. R., Fernandes, A., Yip, C. C., and Ottensmeyer, F. P., Science **285:** 1077-1080 (1999).

APPENDICES

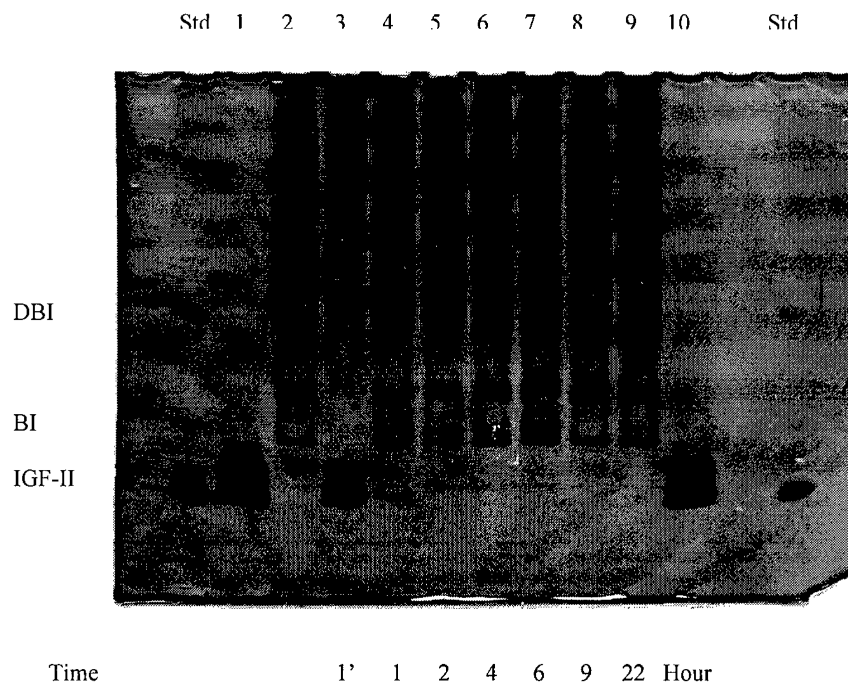

Figure 1

Acid-urea PAGE of reaction mixture obtained from reacting 25 μg of IGF-II with 6 nmoles of mono-NHS-Nanogold at 37° C in 20 μL of 0.1 M sodium phosphate buffer, pH 7.5, in a time study from 1 min. to 22 hours (Lanes 3-9). Lane 2 shows reaction mixture obtained after 1 hour at room temperature and 21 hours at 4° C. Lanes 1 and 10 were IGF-II. Standards (Std.) used as markers are: IGF-II, BI (bovine insulin), and DBI (di-BOC-insulin.

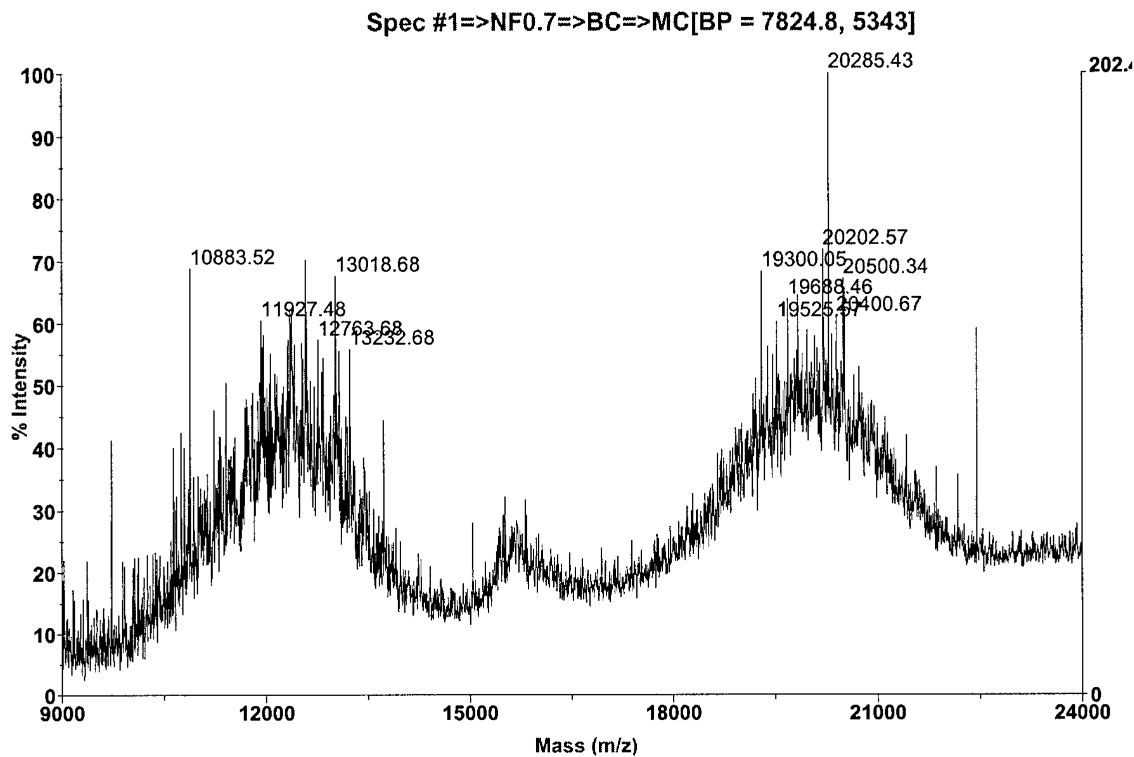

Figure 2

Mass spectrometry (MALDI) analysis of an aliquote of the reaction mixture obtained as described in **Figure 1** showing the presence of a high molecular mass component of 20,000 Da.

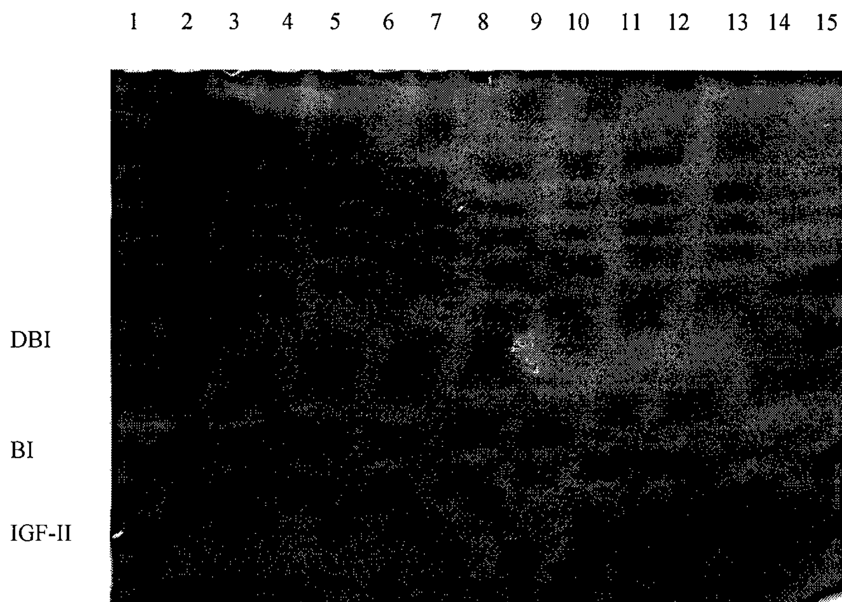

Figure 3

Acid-urea PAGE of peaks from RP-HPLC separation of a reaction mixture obtained as described in **Figure 1**, except only 10 µg, instead of 20 µg, of IGF-II were used. Lane 2: reaction mixture; Lanes 3-14: peaks from the RP-HPLC separation; Lane 1: standard markers of IGF-II, BI (bovine insulin), DBI (di-BOC-insulin).

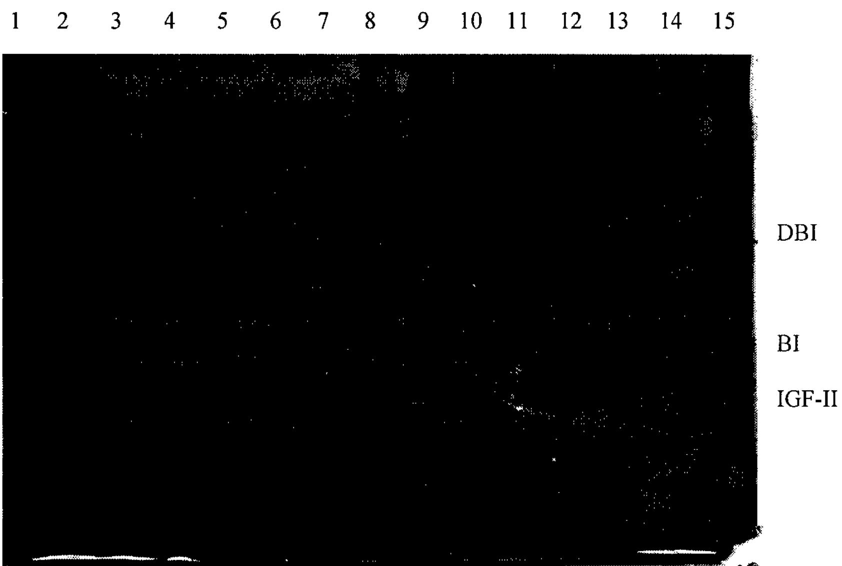

Figure 4

Acid-urea PAGE of fractions from SEC-HPLC separation of a reaction mixture obtained as described in **Figure 1** at room temperature. Lane 2: reaction mixture; Lanes 3-14: fractions from the SEC-HPLC separation. Standard markers (Lane 1 and 15): IGF-II, BI (bovine insulin), DBI (di-BOC-insulin).

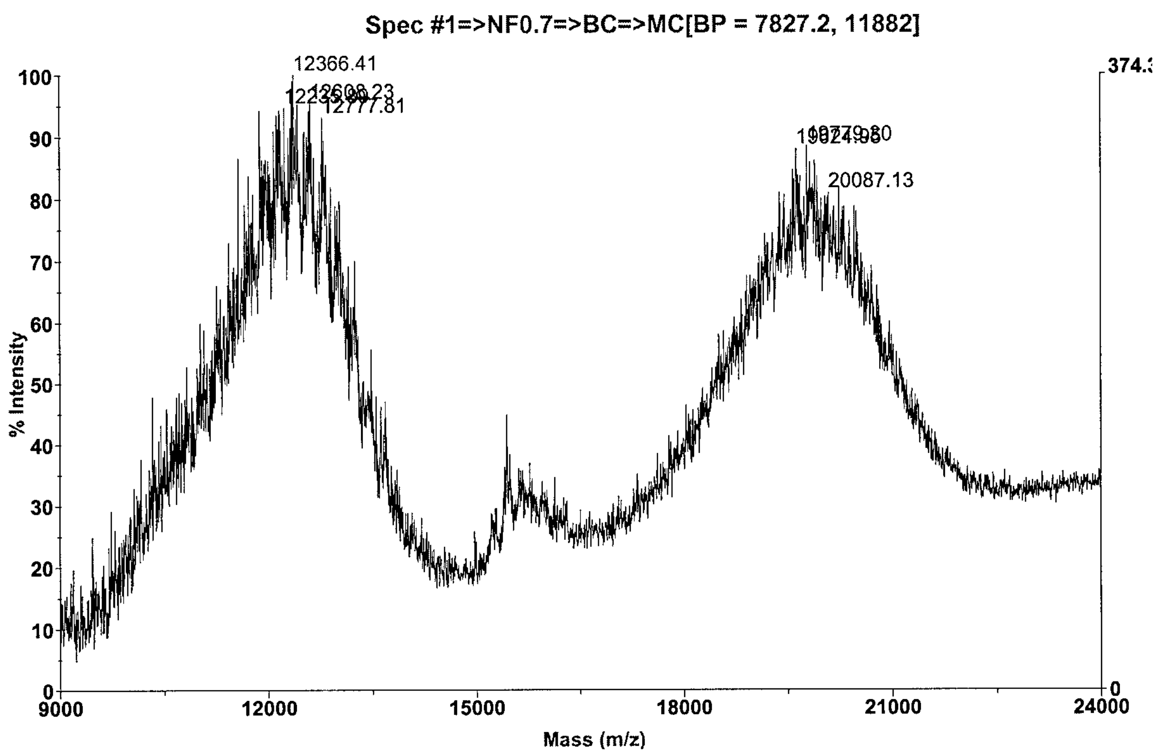

Figure 5

Mass spectrometry (MALDI) analysis of Fraction 13 (Lane 13 in **Figure 4**) from SEC-HPLC separation of a reaction mixture as described in **Figure 4**.

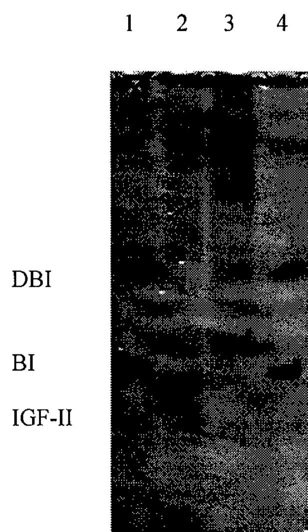

Figure 6

Acid-urea PAGE of fractions from SEC-HPLC separation. Lane 2: a fraction from SEC-HPLC separation of a reaction mixture obtained at 37° C. This fraction contained NGIGF-IIB but was not clearly separated from unreacted IGF-II. Lane 3: a SEC-HPLC fraction from a reaction mixture obtained at room temperature, showing the presence of NGIGF-IIA. Lanes 1 and 3: standard markers of IGF-II, BI (bovine insulin), DBI (di-BOC-insulin).

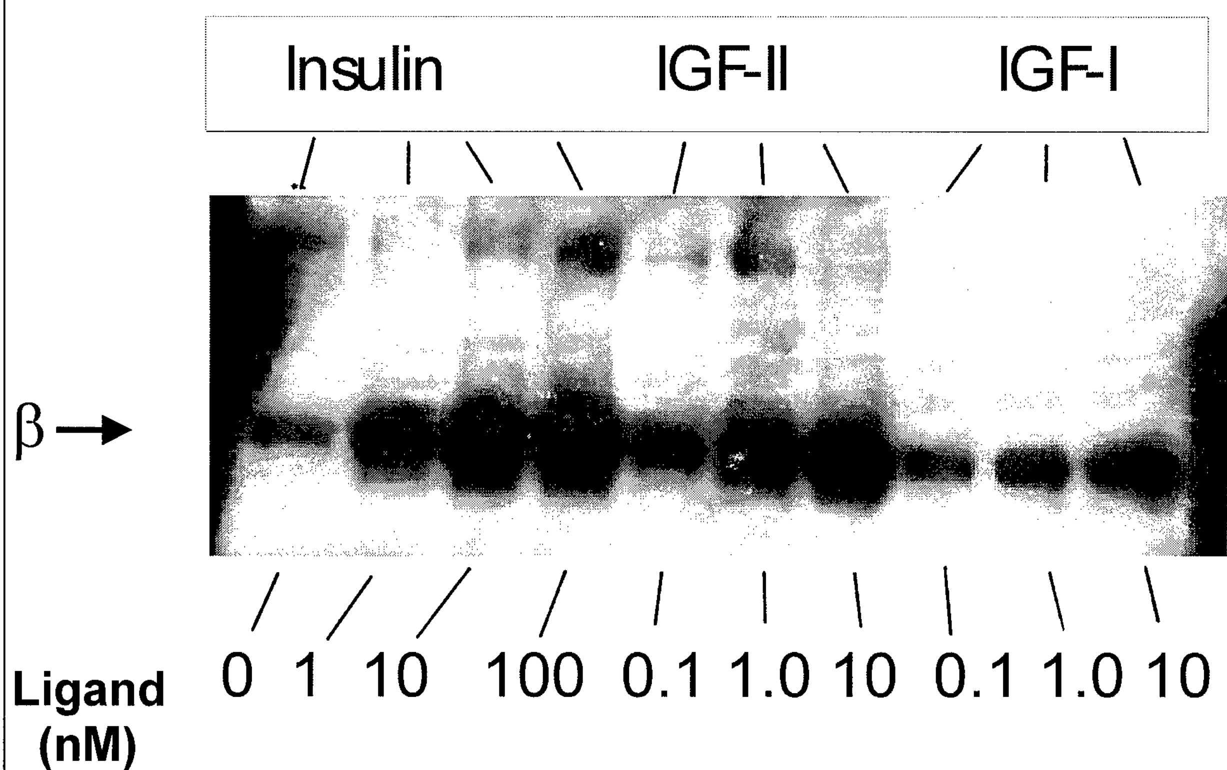

Figure 7

Activation of IR-A autophosphorylation *in vitro* by insulin, IGF-II, and IGF-I as shown by the phosphorylation of the β subunit.

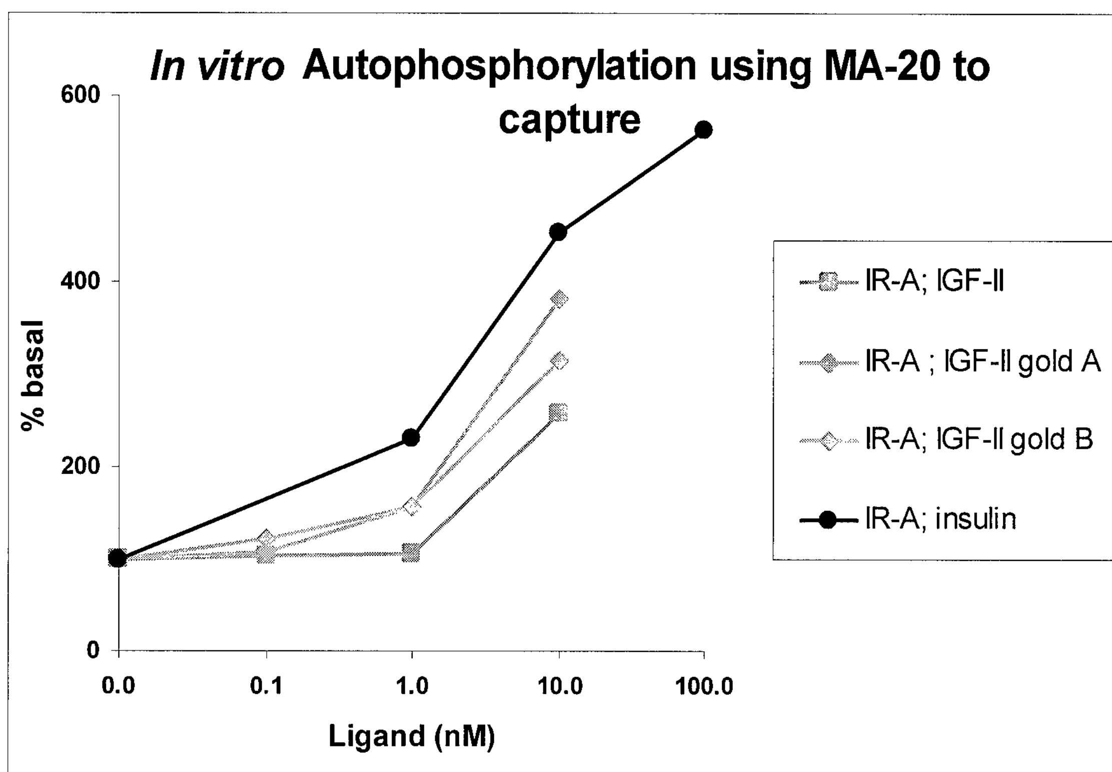

Figure 8

Assay of NGIGF-IIA (IGF-II gold B) and NGIGF-IIB (IGF-II gold A) in the activation of the tyrosine kinase activity of IR-A *in vitro*. 1 ng of purified IR-A protein was captured overnight at 4° C on plates pre-coated with the anti-IR monoclonal antibody MA-20. The plates were washed, then incubated with 2 mM Mn^{++}, 10 mM Mg^{++}, insulin or the other ligands for 15 min. at 22° C. Next 10 µM ATP was added and incubation was continued for 1 hour. Next 0.3 µg/mL anti-PY biotin was added to the plate for 2 hours. The plates were washed, then incubated with SAHRP for 30 minutes. Next signal was developed using TMB reagent, development time: 12 minutes.