Identification of IGF-II-Binding Site on the Quaternary 3-D Structure of the Insulin Receptor

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The purpose of the project is to obtain the 3D reconstruction structure of the IGF-II/insulin receptor isoform A(IR-A) complex by cryoSTEM to elucidate the IGF-II binding site on IR-A. Gold-labeled IGF-II has been prepared by reacting IGF-II with sulfo-NHS-Nanogold, and purified by size-exclusion HPLC. The purified product, Nanogold-IGF-II, was labeled with gold at its N-terminal alanine. Insulin receptor proteins, IR-A and IR-B have been extracted and purified from transfectant cells. Nanogold-IGF-II, like IGF-II, stimulated the autophosphorylation of IR-A. A complex of Nanogold-IGF-II and IR-A was prepared for cryoSTEM 3D reconstruction. Preliminary STEM of the complex showed images of particles of the expected size with electron-dense gold marker. These results demonstrate that it is possible to prepare a biologically active gold-labeled IGF-II, and that a complex formed between it and the insulin receptor isoform IR-A can be used in cryoSTEM for the 3D reconstruction of the IGF-II/IR-A complex for the purpose of elucidating the binding site of IGF-II in the insulin receptor isoform IR-A.
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**Introduction**

Insulin-like growth factor I and II (IGF-I and IGF-II) are related peptide hormones sharing sequence and structural homology with insulin. While insulin acts mainly to regulate glucose uptake and cellular metabolism, IGFs play a major regulatory role in growth and proliferation of most cells and tissues. IGF-I exerts its action 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. Human IR has two isoforms (IR-A and IR-B) as the result of alternate splicing of a small exon (exon 11) encoding 12 amino acids (residues 718-729) at the carboxyl end of the receptor α-subunit, IR-A being the shorter isoform. It was reported recently that IGF-II binds to IR-A, but not IR-B, with an affinity close to that of insulin (2). It is of particular significance that binding of IGF-II to IR-A leads to predominantly mitogenic responses. Furthermore, significantly IR-A was more abundant in breast and colon cancer tissues than in normal tissues, and receptor autophosphorylation induced by IGF-II reflected the relative abundance of IR-A. Thus as a growth factor IGF-II can interact effectively with IR-A, and could be biologically relevant and significant in cancers where IGF-II is locally produced and IR-A is the predominant form of IR. This research project focused 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 were to obtain the quaternary 3-D structure of the complex of IGF-II and IR-A, to locate the binding site of IGF-II in the structure, and to compare the location with that of the insulin-binding site. We have proposed to use low-dose (6 electrons/Å²), and low-temperature (-150°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. The P.I. and colleagues have successfully used this method to obtain the 3-D structure of the insulin receptor through the use of insulin labeled with Nanogold (3) as an electron-dense ligand to obtain images of the insulin/receptor complex. The 3-D structure thus obtained has made it possible to identify on the IR amino acid residues involved in the binding of insulin (4). The scope of the project included the preparation of biologically active IGF-II labeled with Nanogold, preparation and purification of IR-A and IR-B protein, formation of the Nanogold-IGF-II complex with IR-A, and STEM imaging of the complex for 3-D reconstruction.

**Body**

IGF-II binds to the human insulin receptor isoform A (IR-A) with an affinity equal to that of insulin. While insulin binding to IR-A primarily leads to metabolic responses, IGF-II binding produces mainly mitogenic effects. We have postulated 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. In a recent study, using insulin labeled with Nanogold (Nanogold-insulin) we applied the method of cryoSTEM to reconstruct the 3-D quaternary structure of the insulin/IR complex (3), and were able to pinpoint the insulin-binding site on the 3-D structure of the IR (4). Accordingly, in this research project we have aimed to apply this method to determine the IGF-II-binding site on 3-D structure of
the two IR isoforms (IR-A and IR-B), and to compare with the insulin-binding site. The research project has been planned in a logical sequence: 1) preparation and characterization of Nanogold-IGF-II; 2) preparation and purification of IR-A and IR-B proteins; and 3) determination of the 3-D quaternary structure of each of the ligand-receptor complexes.

In accordance to the Statement of Work, the research project has been scheduled as follows: 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. As a final report, research accomplishments during the 3-year period are described below.

1) Preparation and Characterization of Nanogold-IGF-II

During Year 1 we accomplished the followings using micrograms of IGF-II: i) establishing the optimal conditions (pH, temperature, time, and molar ratios of reactants) to label IGF-II with mono-NHS-Nanogold (Nanoprobes Inc., Stonybrook, NY); ii) separation and purification of the reaction products by size-exclusion HPLC at neutral pH (Figure 1); iii) establishing by peptide sequencing and mass spectrometry (Figure 2) that IGF-II was labeled at its N-terminal alanine with a gold cluster; and iv) showing that the Nanogold-IGF-II obtained was biologically active like native IGF-II when assayed against IR-A (Figure 3). The accomplishments in Year 1 had set the stage for the large-scale preparation of Nanogold-IGF-II at the beginning of Year 2 as planned.

Because we had experienced some lot-to-lot variability in the reactivity of the reagent mono-NHS-Nanogold during Year 1, we thought it prudent to test the reagent in small lots from the supplier (Nanoprobes, Inc.) before ordering a large lot of the reagent for the large-scale reaction. Beginning late April 2001 we were unable to label IGF-II with different lots of the reagent. We spent nearly 3 months rechecking our reaction conditions without success until the supplier, after much probing from us, owed up to us in July 2001 that it had altered the original formulation of the reagent which was now highly positively charged. Because the reagent is proprietary to the supplier, we have been unable to learn exactly what other alterations may have been made to the original reagent such that it no longer reacts with IGF-II. Between July and September 2001 we tried different reaction conditions with the newly formulated reagent in an attempt to label IGF-II without success. After much discussion with the supplier in an attempt to solve the problem, we eventually persuaded the supplier to custom prepare one lot (Lot# JS8061A) of the reagent in the original formulation. A small sample of which was tested and found to be reactive with IGF-II as originally observed. However, the reactivity of this reagent was still less than what we had experienced previously in that the conversion of IGF-II to the gold-labeled derivative was not 100%. Nonetheless, we felt that we should be able to produce a sufficient quantity of gold-labeled IGF-II for use. This unfortunately had set up back several months in our work.

The large-scale preparation of Nanogold-IGF-II using the customized lot of mono-NHS-Nanogold was then carried out in multiple incubations of 30 nmoles of the reagent and 300 μg of IGF-II in 300 μL of sodium phosphate buffer (0.1 M, pH 8.5) at 30°C for 43 hours. The reaction was stopped with 15 μL of ethanolamine, and then lyophilized. An aliquot of the reaction mixture was analyzed by acid-urea polyacrylamide gel
electrophoresis that separates modified IGF-II from native IGF-II by charge. The large-scale reaction produced one gold-labeled product. Figure 4 shows that after 43 hr of incubation the ratio of gold-labeled IGF-II (appeared as a stained band with a slower electrophoretic mobility than native IGF-II) to unreacted IGF-II in the reaction mixture was about 1:1. Aliquots of the reaction mixtures were also separated by electrophoresis in 10-20% Tricine polyacrylamide gel that separates peptides of low molecular weight by size. The gel was stained for the presence of gold with a silver enhancement kit from Nanoprobes, Inc. A stained band with a molecular mass expected of Nanogold-IGF-II was detected, confirming the presence of the gold cluster on the labeled IGF-II. This was additional evidence for the presence of gold as revealed by mass spectrometry.

Each large-scale reaction mixture was separated by HPLC through a size exclusion column (BioSil SEC 1-125-5, 300 mm x 7.8 mm) developed with 50 mM sodium phosphate buffer, pH 7.6, at a flowrate of 0.5 mL/min. Fractions of 0.5 mL were collected. Aliquots of fractions were analyzed by acid-urea polyacrylamide gel electrophoresis. Figure 5 shows the typical separation of Nanogold-IGF-II from unreacted IGF-II and side products of the reactions. Approximately 100 µg of Nanogold-IGF-II were obtained from pooling and re-purification of the fractions. As expected, peptide sequence analysis showed that the N-terminal alanine was derivatized by the Nanogold. The amount of Nanogold-IGF-II obtained was deemed sufficient for the large-scale preparation of Nanogold-IGF-II/IR-A and IR-B complexes for STEM reconstruction.

2) Bio-activity of Nanogold-IGF-II

The bio-activity of the Nanogold-IGF-II obtained from the large scale preparation was assayed using purified IR-A and IR-B receptor proteins purified from fibroblasts from mouse with an IGF-I R knockout and very low numbers of mouse IR. The fibroblasts were transfected with either human IR-A or IR-B. The end-point of the assay was the autophosphorylation of the receptor as stimulated by the gold-IGF-II.

i) Preparation of pure IR-A and IR-B receptors

Cells were grown in 150 cm² flasks until 90% confluent, and then were solubilized in 10 mL 50 mM HEPES, pH 7.6, 150 mM NaCl, 1% Triton-X100, 1 mM PMSF for 1 hour at 4°C. Lysates obtained were purified by affinity chromatography on a MA51 column, an anti-insulin receptor (human) antibody coupled to Affigel 15. The column was equilibrated with 50 mM HEPES, pH 7.6, 150 mM NaCl, 0.1% Triton-X100, 1 mM PMSF (WGA buffer). Lysates from cells were passed over the column and pass-through was collected and was recycled 3 times. The insulin receptors were allowed to bind overnight at 4°C. The column was washed with 50 mL of WGA buffer, followed by 20 mL 50 mM HEPES, pH 7.6, 1 M NaCl, 0.1% Triton-X100, 1 mM PMSF. Insulin receptor protein was eluted with 2.5 M MgCl₂, 120 mM borate, 0.1% Triton-X100, 1 mM PMSF into 180 mL WGA buffer. The eluted insulin receptors were then applied to wheat germ agglutinin agarose column. After washing with WGA buffer, the insulin receptor protein (IR) was eluted with 50 mM HEPES, pH 7.6, 300 mM N-acetyl-D-glucosamine, 0.1% Triton-X100, 1 mM PMSF. IR content was determined by specific IR ELISA. Figure 6 shows the SDS-PAGE of purified IR-A after reduction to its 130-kDa α subunit and 95-kDa β subunit. The differential response of
purified IR-A and IR-B to IGF-II and insulin in receptor autophosphorylation is shown in Figure 7, confirming that, like insulin, IGF-II activates IR-A, but unlike insulin it does not activate IR-B.

**ii) Receptor autophosphorylation ELISA** Purified Nanogold-IGF-II obtained from the large-scale preparation was assayed for its ability to activate receptor autophosphorylation of IR-A in comparison with insulin and IGF-I. In this assay 96-well plates were coated with 2 µg/mL of MA-20, a monoclonal antibody that recognizes and captures both IR-A and IR-B in their native and tyrosine phosphorylated states. After blocking with SuperBlock (Pierce Chemical), cell lysates containing 1 ng IR (IR-A or IR-B) was applied to each well and allowed to bind overnight at 4°C. Next, wells were washed with TBST (20 mM Tris, 150 mM NaCl, 0.05% Tween-20). The plates were then incubated with 2 mM Mn^{2+}, 10 mM Mg^{2+}, insulin or the other ligands for 15 min. at 22°C. Next 10 µM ATP was added and incubation was continued for 1 hour. To read out the tyrosine phosphorylated IR, 100 µL goat anti-PY coupled to horse-radish peroxidase (1:2000) was added for 2 hours at 22°C. After washing with TBST, signal was developed by using TMB substrate. Results shown in Figure 8 confirm that the gold-labeled IGF-II, like insulin, activates IR-A.

### 3) STEM of Nanogold-IGF-II and IR-A Complex

Scanning Transmission Electron Microscopy (STEM) at low temperature of the complex of Nanogold-IGF-II (NG-IGF-II) and IR-A was to be carried out essentially as described for the reconstruction of insulin-IR complex (3). In brief, IR-A was incubated at 4°C overnight in 20 mM HEPES buffer (pH 7.5) with Nanogold-IGF-II (final concentration of ~ 0.5 x 10^{-6} M) at a molar ratio of NG-IGF-II : IR-A of ~ 10:1. Without removing the free NG-IGF-II, the sample (5 µL) was deposited onto a copper grid (300 mesh) coated with a holey plastic film (pore size 5-10 µm) overlaid with a carbon film 23 Å thick. As a step to determine the optimal concentration of the complex for STEM, we have obtained preliminary images of the NG-IGF-II/IR-A complex (Figure 9). Images of particles of the expected size with electron-dense gold cluster can be seen. However, the micrograph indicates that a higher concentration of the complex will be needed for the final STEM. At time of writing, more IR-A at higher concentrations is being purified for the formation of the complex at higher concentrations.

### Key Research Accomplishments

There has been no deviation from the Statement of Work described in the original proposal, except for the delay of several months in the large-scale preparation of Nanogold-IGF-II, a delay caused by the supplier changing the formulation of the reagent mono-NHS-Nanogold as the project progressed into Year 2. Nonetheless, during the entire 3-year funding period, the followings have been accomplished within the Statement of Work:

- Approximately 100 µg of purified Nanogold-IGF-II have been prepared, an amount sufficient for the formation of complex with IR-A and with IR-B for 3-D reconstruction of the complex. The Nanogold-IGF-II with the cluster of gold atoms
attached to its N-terminal alanine is active, like IGF-II and insulin, in stimulating the autophosphorylation of IR-A. Thus a biologically active gold-labeled IGF-II has been prepared.

- Large-scale extraction and purification of IR-A and IR-B from transfectant cells have been carried out to obtain sufficient amount of these receptor proteins for the formation of complex with the Nanogold-IGF-II.
- Preliminary STEM images of the complex of Nanogold-IGF-II and IR-A have been obtained, as a step towards the cryoSTEM 3D reconstruction of the complex.

Reportable Outcomes


Conclusions

The ultimate goal of the work was to obtain the 3D reconstruction of the complex of IGF-II and the insulin receptor isoform IR-A. The body of work carried out during the 3-year funding period has closely adhered to what were proposed in the Statement of Work towards this goal. This includes: 1) the successful preparation of a gold-labeled IGF-II (Nanogold-IGF-II) which is biologically active like IGF-II in activating the insulin receptor isoform IR-A; 2) the preparation and purification of the two insulin receptor isoform IR-A and IR-B for complex formation with the gold-labeled IGF-II.; and 3) preliminary STEM images of the complex of gold-labeled IGF-II and IR-A. Although we have not reached the final goal of obtaining the 3D reconstruction of the IGF-II/IR-A complex within the 3-year time frame, our work has demonstrated that a biologically active gold-labeled IGF-II can be prepared, and that it indeed can form a complex with the insulin receptor isoform IR-A as evident from the preliminary STEM images. The preliminary STEM observation now lays the ground work for the final 3D reconstruction of the complex by cryoSTEM, a goal that we are continuing to pursue.

References

APPENDICES
Figure 1 Acid-urea polyacrylamide gel electrophoresis of fractions from SEC-HPLC separation. Lane 2 reaction mixture obtained from reacting IGF-II with Mono-NHS-Nanogold at 29-30 C for 22 hours. Lane 3 to 14 fractions of SEC-HPLC separation of reaction mixture. Lane 1 and 15 standards: IGF-II, bovine insulin (BI), di-BOC-insulin (DBI)
Figure 2

Mass spectrometry (MALDI) analysis of Fraction 13 (Lane 13 in Figure 1) from SEC-HPLC separation of a reaction mixture as described in Figure 1. The mass number of interest is 12366.41 for the first major peak, and 20087.13 for the second major peak.
Assay of two preparations of NG-IGF-II (IGF-II gold A and IGF-II gold B) in the activation of the tyrosine kinase activity of IR-A \textit{in vitro}. 1 ng of purified IR-A protein was captured overnight at 4\(^\circ\) 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\(^\circ\) C. Next 10 \(\mu\)M ATP was added and incubation was continued for 1 hour. Next 0.3 \(\mu\)g/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.
Figure 4 Acid-urea polyacrylamide gel electrophoresis of reaction mixture obtained from 3 separate reactions, each using 300 μg IGF-II and 30 nmoles of mono- NHS-Nanogold, at 29-30° C.
Lane 1, 3, 5: reaction after 2 min.
Lane 2, 4, 5: reaction after 43 hours; the band migrating ahead of BI but slower than IGF-II is the gold-labeled IGF-II.
Lane 8, 9: mono-NHS-Nanogold without IGF-II under the same reaction conditions.
Std: Standards: DBI (Di-BOC-insulin), BI (bovine insulin) and IGF-II.
Figure 5 Acid-urea polyacrylamide gel electrophoresis of fractions from SEC-HPLC separation.
Lane 1: Reaction mixture from incubation of mono-NHS-Nanogold with IGF-II at 29-30°C for 43 hours.
Lanes 2 – 13: Fractions obtained by size exclusion chromatography. Fraction 6 and 7 were pooled for re-run.
Std: BI (bovine insulin) and IGF-II
Figure 6  Silver-stained reduced SDS-PAGE of purified IR-A showing the 135-KDa α subunit and the 95-KDa β subunit.

IR-A

INSULIN - - +
IGF-II - + -

IR-B

INSULIN - - +
IGF-II - + -

Figure 7  Western blot with anti-PY comparing IR-A and IR-B insulin receptor autophosphorylation, showing IGF-II stimulating IR-A but not IR-B as effectively as insulin.
**Figure 8** ELISA of stimulation of receptor (IR-A) autophosphorylation by insulin, IGF-I, IGF-II (IGF-2), and Nanogold-IGF-II (IGF-2 gold), showing that Nanogold-IGF-II was as active as unmodified IGF-II.
Figure 9

Preliminary STEM results on air-dried specimen of IR labelled with gold-IGF as part of concentration optimization prior to preparation of quick-frozen specimens.

Although air-drying flattens specimen and often results in larger than expected outline, the specimen already provides a check on the success of labelling with the nanogold marker on IGF. Several such IR/IGF complexes are shown in the insets, with the highest densities in the images of the complexes indicating the location of the gold marker. Spots of lesser intensity are due to natural structural densities of within the IR complex.

Unbound nanogold-IGF, which will be separated from the IR/IGF complexes in future preparations, is evident in the large STEM micrograph.