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Therapeutic peptide production in *Drosophila*

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Abstract

Bioactive peptides are important therapeutic drugs, yet conventional methods of peptide synthesis are challenged to meet increasing demand. We developed a novel and efficient means of metabolic engineering: therapeutic peptide production in *Drosophila* and as a proof of concept, we demonstrate production of fully-matured human insulin. This *in vivo* system offers an innovative means to produce valuable bioactive peptides for therapies, its inherent flexibility facilitates drug development, and its ease of producing fully processed peptides simplifies metabolic engineering of new peptide products.

Keywords

Drosophila; insulin; therapeutic peptides; DIMM

1. Introduction

Bioactive peptides are receiving more attention as potential therapeutics because they possess high target specificity, low toxicity and a low incidence of side effects [19]. The choice of methods with which to synthesize bioactive peptides is based on peptide size and complexity. It falls within three main technologies: (i) Chemical synthesis, (ii) biosynthesis in microbes and (iii) biosynthesis in transgenic organisms. Chemical peptide synthesis is well suited to produce peptides of small size (< 30 amino acid (AA)) in large amounts. Yet it is expensive for peptides that are longer than 30 AA, or which possess more complex structures (i.e., display multiple chemical modifications) [2]. Microbial peptide synthesis (using yeast and bacteria) can produce longer peptides, but only at considerable cost [19]. To address the increasing clinical need for longer and more complex peptides, we have designed a novel form of metabolic engineering which seeks to capitalize on two factors: the conserved nature of peptide processing across Metazoa, and the flexibility and scope of *Drosophila* genetics.

Here we use human insulin as our model peptide. The hormone insulin controls glucose homeostasis and comprises two separate peptides (a 21 AA “A” chain and a 30 AA “B” chain) produced from a single precursor. The insulin precursor (110 AA) is processed by

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serial enzymatic reactions to produce the distinctive conformation of the active form, which contains three specific disulfide bridges between A and B chains [4]. Insulin is administered therapeutically to the majority of type I and some type II diabetics. According to the recent World Health Organization report (2011) [5], it is estimated that roughly 346 million people worldwide suffer from diabetes and that in 2004, 3.4 million died from the high glucose-related diseases each year. It is further estimated that the number of diabetes-related deaths will double by 2030.

While projections for increasing demand for insulin are thus widely accepted, there remain many limitations to increasing commercial insulin production, due to the complexity of its final bioactive structure. Two conventional methods to produce therapeutic insulin (de novo chemical synthesis, and the purification of authentic peptide from pancreas) have proven to be not economically feasible. Instead, the bulk of therapeutic insulin is produced by metabolic engineering in microbes. While microbial DNA technology has been thus adapted to produce synthetic human insulin in large quantities, it remains twice as expensive to produce as animal-derived insulin [10]. Here, we describe novel technology to produce long and complex bioactive peptides efficiently *in vivo* in *Drosophila* by harnessing the power of a dedicated secretory cell master regulator, the bHLH transcription factor DIMMED [1].

In *Drosophila*, DIMM is dedicated to organizing the molecular and cellular properties of peptidergic neurosecretory neurons and endocrine cells [7,9, 13,14]. It promotes the high-level production and release of secretory peptides and peptide hormones [15]. Importantly, DIMM can confer the authentic properties of high secretory production and capacity onto cells that do not normally display these properties [7]. It does so by promoting a comprehensive secretory phenotype at the transcriptional level. The DIMM-dependent secretory pathway causes novel neuropeptide precursors to be processed to completion; it also permits them to be packaged within ultrastructurally normal large dense core vesicles [7]. Significantly, DIMM is normally expressed in insulin-expressing neurons of the fly, suggesting that the biology of DIMM regulation and of insulin production are evolutionarily-linked [15]. We hypothesized that the co-mis-expression of DIMM and human pro-insulin in non-peptidergic *Drosophila* photoreceptors would efficiently generate fully-processed and bioactive hINS. If this hypothesis were valid, then active/mature insulin could be produced directly in a eukaryotic system, without recourse to the expense of subsequent *ex vivo* enzymatic/chemical reactions to generate proper secondary and tertiary structure.

2. MATERIALS AND ETHODS

2.1. *Drosophila* Strains

Two gal4 lines, *GMR-gal4* (II) [8] and *PHM-gal4* (III) [14] were used to drive human insulin and dimm in the eye, and in subsets of brain neurons, respectively. We generated several transgenic UAS-*human insulin* lines, and here used two independent lines, *UH-2* and *UH-17*, to assure that no effects were position-specific. To increase insulin production beyond levels found with a single gal4 transgene, we combined the *GMR-gal4* and *Phm-gal4* elements as follows: we crossed *w*; *GMR-gal4*, UAS-*dimm*; UAS-*hINS* with *PHM-gal4* and maintained them at ambient temperature (~25°C).

2.2. Immunohistochemistry

We used affinity-purified guinea pig anti-Dimm (1:250) [1], and mouse monoclonal anti-insulin (1:500, Sigma-Aldrich, St. Louis MO) as primary antibodies, and Cy3-conjugated or Alex-488-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) for immunocytochemistry. Immunostaining methods were as previously described [9]. Images

were acquired on an Olympus FV500 laser scanning confocal microscope and manipulated by Image J and Adobe Photoshop software to adjust contrast and/or levels.

2.3. ELISA assay (Insulin and its receptor)

Frozen adult fly heads (10 fly head/ 200 μ l buffer) were homogenized in buffer (1.5% HCl + 70% ethanol) and sonicated with a microtip (5 second, 40% power) (Branson Sonifier cell disruptor, Danbury, CT). After centrifugation at 13,000 rpm for 20 min, the supernatant was transferred to a new tube and solution evaporated by speed vacuum for 1 hour. For insulin ELISA assay, the samples were resuspended in 1 $\times$ PBS. We measured insulin with an ELISA according to the manufacturer's protocol, with minor modifications (Calbiotech Inc. #IS130D; Spring Valley, CA). For insulin receptor ELISA assay, the samples were dissolved in 5 ml of 5% acetonitrile (ACN)/ 0.1% trifluoroacetic acid (TFA) solution. A C18 column (Varian Inc., Palo Alto, CA) was pre-equilibrated with 5 ml of 0.1% TFA/5% ACN, 1 ml of 0.1% TFA/5% ACN/100 μ g BSA, and 5 ml of 0.1% TFA/5% ACN. Samples were resuspended in 5ml of 0.1% TFA + 5% ACN, then loaded onto a C18 column; the flow-through was re-loaded onto the same column. Without further washes, material was then eluted with 5 ml of 0.1% TFA/60% ACN/BSA (100 μ g/ml). The eluents were evaporated with a speed vacuum for 2 hr. The fractions were then lyophilized overnight and stored at -20°C until use. To perform receptor auto-phosphorylation assays, we used a CHO-hIR stable cell line that expresses a human insulin receptor [20]. Approximately one million cells in each of six well plates were incubated for a day and the medium changed to a serum-free one overnight. Partially purified C-18 samples were re-dissolved in 1 $\times$ PBS (10 head extract/100 μ l 1 $\times$ PBS) and standard controls (0, 10, 100, 1000nM Insulin) were added to the cells and incubated for 10 min in the CO_2 incubator (37°C), then washed with 1 $\times$ PBS, twice. 150 μ l of 1 $\times$ RIPA buffer (50 mM Tris-HCl, pH 7.4; 1% NP-40; 0.25% Na-deoxycholate, 50 mM NaCl) + 1 $\times$ Complete Protease inhibitor, 0.5 $\times$ Phosphatase inhibitor (pSTOP) (Roche, Indianapolis, IN)) were added and incubated for 20 min. on ice. The collected lysates were centrifuged at high speed for 10 min. The supernatants were diluted 5 times (20 μ l lysates + 80 μ l ELISA diluent) and the ELISA assay was performed following the manufacturer's instructions (Star phospho-IR (Tyr 1162/1163) ELISA kit, Millipore, Temecula, CA). Standard Insulin controls produced results that were highly reproducible.

2.4. Mass spectrometric measurements

To determine the chemical form of the r-hINS, we used two different LC-MS platforms to detect and sequence the peptides, one with higher sensitivity and one that provided tandem MS capabilities.

2.4.1. Sample preparation—Frozen fly heads (~60 mg) expressing r-INS were thoroughly homogenized in 4ml acidified acetone (40:6:1 acetone/water/12M HCl, by volume), followed by sonication, vortexing and centrifugation. The supernatant was collected and dried in a Savant Speed Vac concentrator (Thermo Fisher Scientific, Waltham, MA), which was then reconstituted in 15 μ l solvent A (95% water/5% acetonitrile (ACN)/ 0.1% formic acid (FA)/0.01% Trifluoroacetic acid (TFA)). The sample was split with 3 μ l of the homogenate used for intact insulin detection, while the remaining 12 μ l used for disulfide bond reduction and alkylation before LC-MS analysis.

After adjusting the pH of fly head extract to basic with 1 M NH_4HCO_3 , 8 μ l of 200 mM 1,4-Dithio-DL-threitol (DTT) in 100 mM NH_4HCO_3 was added to reduce disulfide bonds. The sample was then immersed in a boiling water bath for 10 min, followed by vortexing, centrifugation, and incubation at the room temperature for an hour. Alkylation was performed by adding 6.4 μ l of 1 M iodoacetamide in 100 mM NH_4HCO_3 into the reduced solution, and allowing one and a half hours for a complete reaction. The last step was to

remove unreacted iodoacetamide by adding 32 μ l of DTT into the solution. Upon completion of the reaction, samples were desalted using PepClean C18 spin columns (Pierce, Rockford, IL) and its standard protocols, and the eluent was dried in a Savant Speed Vac concentrator (Thermo Fisher Scientific, Waltham, MA) and reconstituted in 8 μ l solvent A. The sample was ready for LC-MS analysis.

2.4.2. Peptide separation and MS measurement—After reduction/alkylation procedures, the sample was fractionated using a UltiMate/Switchos/Famos capillary LC system (Dionex Co., Sunnyvale, CA) with a C18 reverse phase column (Dionex Acclaim, 300 μ m i.d. $\times$ 15cm, particle size 3 μ m, and pore size 100 Å) at a 7 μ L/min flow rate over a 70 min run. A five-step linear solvent gradient was created by mixing solvent A (95% water and 5% ACN, 0.1% FA and 0.01% TFA), and B (95% ACN, 5% water, 0.1% FA, and 0.01% TFA) as follows: 4%–10% B in 10 min, 10%–50% B in next 40 min, 50%–90% B in next 3 min, isocratic 90% B for 4 min, 90%–4% B in 3 min, and isocratic 4% B for 10 min. Fractions were manually collected, and subjected to a second stage LC separation and MS studies.

To detect both intact and reduced/alkylated insulin with high MS resolution and accuracy, we employed a maXis 4GTM quadrupole time-of-flight (Q-TOF) mass spectrometer with an electrospray ionization (ESI) source (Bruker Daltonics, Bremen, Germany). The Dionex UltiMate 3000 nanoLC system (Dionex Co., Sunnyvale, CA) was utilized before MS to reduce sample complexity. It was equipped with a C18 reverse phase column (Dionex Acclaim, 75 μ m i.d. $\times$ 15 cm, particle size 3 μ m, and pore size 100 Å), and used the same water/ACN solvent system with 0.1% FA and 0.01% TFA at a flow rate of 300 nL/min. Second stage separation parameters were optimized individually for each fraction, while the separation of non-reduced sample used the same gradient as described above. The scan mass-to-charge (m/z) ranges for MS were 300–2000, and the data were analyzed using the DataAnalysis software (Bruker Daltonics, Bremen, Germany).

2.4.3. Peptide sequencing—To further sequence peptide by tandem MS, a NanoAcquity Ultra Performance LC (UPLC) system connected to ESI-Q-TOF (Waters Premier, Milford, MA) was utilized with a C18 reverse phase column (Waters Atlantis dC18, 75 μ m i.d. $\times$ 15 cm, particle size 3 μ m, and pore size 100 Å). The solvent gradient over a 90 min run was generated by the same water/ACN solvent system with 0.1% FA at a flow rate of 400 nL/min, and was optimized for each individual fraction. MS/MS fragmentation of eluting peptides was controlled by MassLynx 4.1 software (Waters, Milford, MA) in a data-dependent manner. The precursor ion selection for tandem MS was limited to four ions per MS scan sorted by intensity. Two peptides were included for fragmentation, m/z 1306.1 $\pm$ 0.3 and 871.1 $\pm$ 0.3 for +2 and +3 charged insulin chain A, and 1181.6 $\pm$ 0.3, 886.4 $\pm$ 0.3 and 709.4 $\pm$ 0.3 for +3, +4 and +5 charged insulin chain B. The scan m/z ranges for the MS and MS/MS were 200–2000 and 50–2000 Da, respectively. After MS/MS data collection and conversion to .pkl file format, the PEAKS Studio 5.2 software (Bioinformatics Solutions, Inc., Waterloo, CA) was performed for peptide identification, and the criteria for confident peptide assignments, including the PEAKS score, post-translational modification (PTM) match, mass error, and manual verification, were described previously [3].

3. RESULTS

3.1. The accumulation of recombinant human insulin is DIMM-dependent

To produce the *hINS* gene ectopically, we generated p{UAS-*hINS*} transgenic flies and tested whether hINS protein expression is dependent on DIMM using the *GMR*-gal4 driver which is expressed specifically in photoreceptors (summarized in Figure 1A). For initial

evaluation of ectopic hINS expression, we performed immunocytochemistry with anti-(h)insulin antibodies in flies overexpressing DIMM. Immunoreactivity for hINS was present in transformed photoreceptors and fully-dependent on DIMM (Figure 1B); in flies lacking the UAS-*dim* transgene, we did not detect hINS expression (not shown). These results strongly suggested that hINS is produced and will accumulate in *Drosophila* photoreceptors, within the permissive cellular environment fostered by DIMM co-expression.

3.2. DIMM directs full maturation of human insulin in *Drosophila*

Next, to examine whether hINS biosynthesis proceeds to completion in *Drosophila* neurons co-expressing DIMM, we performed an ELISA assay that is sensitive to fully-processed hINS, but not to pro-insulin or any intermediate form or to *Drosophila* insulin-like peptides. We found that *Drosophila* of the genotype *GMR> hINS, dim* could produce detectable amount of hINS-like immunoreactivity, while control flies did not (Figure 1C). The average yield of insulin from this measurement was 7.86 ± 0.62 ng, while the control was 0.38 ± 0.03 ng (per 10 adult heads, N=4). To maximize the yield of hINS, we changed the ambient temperature from 25°C to 29 °C, after adult fly eclosion, (29 °C is the optimal temperature for yeast GAL4 activity [12] and found a ~50 % increase in hINS yield - 11.63 ± 3.88 ng per 10 adult fly heads. Next, we tested whether adding a driver to recruit additional cells to express DIMM and hINS would increase the hINS yield: we combined *GMR-gal4* and *PHM-gal4* with UAS-*dim* and UAS-*hINS* together and incubated the resultant flies for 5–7 days at 29 °C. The yield of hINS from the combined *GMR-PHM gal4* lines (20.53 ± 3.88 ng/10 adult fly heads, N=4) was increased up to 75%, compared to that derived from flies expressing the single *GMR-gal4* driver (11.63 ± 3.88 ng/10 adult fly heads, N=4). At 25°C, the combined GAL4 lines did not increase the yield. Thus the addition of a second GAL4 driver and the increase in ambient temperature (to 29 °C) significantly increased the yield of insulin by ~2.6-fold.

3.3. Recombinant human insulin is fully processed

Next, recombinant human insulin, extracted from adult fly heads, was evaluated by mass spectrometry to confirm that it undergoes precise and complete post-translational processing [10, 11, 17]. If r-hINS has the same sequence as hINS, we would expect to detect a peptide with isotopic molecular weight 5803.66, assuming one intra-chain and two inter-chain disulfide bonds. After reduction and alkylation, two alkylated peptides, r-hINS chain A and chain B, are expected at isotopic molecular weights 2610.19 and 3541.77. After peptide extraction and liquid chromatography separation, the samples were analyzed using electrospray ionization (ESI) coupled to quadrupole time-of-flight (Q-TOF). The mass of the predicted intact r-hINS (Mass=5804.80, z=+5) was observed (Figure 2A). To verify the assignment of this peak, we reduced the disulfide bonds with 1,4-Dithio-DL-threitol (DTT), followed by alkylating the thiols with iodoacetamide [15]. The masses of the expected r-hINS chain A (Mass=2609.66, z=+2) and chain B (Mass=3541.15, z=+5 and +4) were detected (Figure 2B–D). In addition, the retention times of both intact and reduced r-hINS extracted from *Drosophila* heads are consistent with those of authentic hINS standards. We further confirmed the sequence of r-hINS chain B via tandem MS using another mass spectrometer, an ESI-Q-TOF (Figure 2E). Together, these data strongly support that r-hINS in *Drosophila* heads is expressed and processed into authentic hINS.

3.4. The r-hINS is functional

The insulin receptor (IR) forms a heterotetramer, composed of two α subunits and two β subunits. While α subunits are exposed extracellularly and connect with β subunits by disulfide bonds, the single β subunit forms the extracellular, transmembrane and cytoplasmic tyrosine kinase domains. With insulin binding, the IR β subunits are autophosphorylated at the three tyrosine residues, which are necessary for further signal

transduction. To monitor the biological activity of the recombinant hINS (r-hINS), we used a stable CHO-IR cell lines that express insulin receptor and measured the auto-phosphorylation of IR using ELISA assay. We found that partially purified r-hINS could activate the IR to significant levels in each of three independent experiments, compared to the commercial insulin treatment (Figure 3).

4. DISCUSSION

Peptide-based drugs now receive increased attention because of several advantageous properties, including high potency, high specificity and low toxicity. Although chemical synthesis could be considered as a major route for industrial production, recombinant biotechnology remains a valuable alternative. As a proof of concept, we have successfully produced bioactive recombinant human insulin in *Drosophila* neurons. This novel technology can be easily and directly applied for production of numerous, potentially-therapeutic peptides.

This peptide production platform has several advantages to complement those of existing methods. **First**, bioactive peptide synthesis is derived in its entirety from *in vivo* biological processes, and therefore does not resort to costly chemical treatment *ex vivo*. This is significant because the expense of chemical processing represents as much as half the cost of microbial derived human insulin production [16]. **Second**, the yield of bioactive peptide could be increased considerably because mature peptides will be accumulated in DIMM-transformed *Drosophila* neurons without apparent release [7]. **Third**, the desired peptide products can be readily modified by standard molecular genetic designs, thus permitting facile consideration of peptide structures that could increase potency, stability and/or bioavailability. For example, post-translational modifications exhibited by some N- or C-termini of bioactive peptide are produced by specific enzymes such as N-acetyl transferase (acetylation) and peptidylglycine alpha-amidating mono-oxygenase (amidation) respectively. Thus the *Drosophila* peptide biosynthetic system provides exceptional flexibility to make and test more modified forms. **Fourth**, bioactive peptides can be easily purified because they are produced in tissues that are highly enriched in peptide. *Drosophila* photoreceptors make up a large fraction of all cells in the *Drosophila* head, and heads can be isolated easily (and in an automated fashion) from bodies. Furthermore, h-INS extraction and purification could utilize well-established methods for peptides, as the material would reside within normal sub-cellular compartments. Finally, how large scale a production of *Drosophila* would be needed to generate sufficient yield of r-hINS? We cannot say with certainty at this point, but submit there are well-established mass production methods available for fly husbandry. For example, efforts to mass-produce medfly pupae succeeded in producing at least 500 million per week [18]. This novel technology – here shown suitable to production of human insulin – has broader application for the efficient generation of medium-sized therapeutic bioactive peptides (both native and modified) for which chemical synthesis is not cost-effective. These include ghrelin, calcitonin and leptin, among many others. The innovative features of this approach may therefore contribute to future designs of drug production and in so doing provide substantial benefit to public health.

In spite of its positive attributes, this proof of concept technology needs obvious improvements. First, it would be useful to find a means to rationally incorporate unnatural D-amino acids to increase the resistance against peptidases and potentially increase ligand affinity. Second, we anticipate a need to devise protection from endopeptidases due to the existence of cryptic cleavage sites within some peptide precursors. Third, and most importantly, production yield will require critical appraisal and improvement. We anticipate that several adjustments will be immediately helpful, based on standard *Drosophila* husbandry (mass culture systems) and based on specific genetics methods that could make

immediate improvements. A similar rational strategy has been employed to improve the yield of plant-derived insulin [18]. In spite of these issues, the specificity of eukaryotic peptide biosynthesis and the power of *Drosophila* genetics argue that this new form of metabolic engineering is innovative and could be a useful adjunct to the diverse mix of methods currently used to meet the increasing demand for therapeutic bioactive peptides. It will provide exceptional opportunities to circumvent current obstacles to produce long and complex peptide efficiently, and its ease of implementation makes it promising for rapid production. In so doing it could contribute to developing newly-modified forms of therapeutic peptide drugs at considerably lowered cost.

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HIGHLIGHTS

We developed a novel platform to produce biologically-active peptides in *Drosophila*.

As a proof-of-concept, biologically active human insulin was successfully produced.

This method could provide a useful alternative to commercial peptide production.

This method also presents great potential for peptide drug development.

Figure 1. Human insulin production in *Drosophila*

(A) A schematic drawing of this experiment.

(B) The accumulated human insulin protein found within eye imaginal discs is dependent on DIMM. (*GMR>dimm; hINS*). DIMM (Red); Insulin (Green).

(C) Measurement of recombinant human insulin (r-hINS) per 10 adult fly heads estimated (10 HE). A one-tailed Student's t-Test was used for the statistical analysis. (* : $P < 0.05$; ** : $P < 0.01$; NS : Not significant.)

Figure 2. Mass spectra confirm that the chemical form of the recombinant human insulin from *Drosophila* is authentic

(A) Intact r-hINS ($z=5$) before reduction/alkylation. (B) r-hINS chain A ($z=2$) after reduction/alkylation (C, D) r-hINS chain B ($z=5$ and 4) after reduction/alkylation. The naturally occurring isotopic distributions found in these peptides results in multiple MS peaks for each peptide with an interval of $1/z$ (charge) along the m/z axis. The measured peptide masses match expected masses at 200 ppm mass accuracy. (E) Tandem MS spectra of r-hINS chain B after reduction/alkylation (m/z 709.27, $z=5$), with the precursor ion mass-matching the expected peptide within 110 ppm, and assigned ions matching expected fragments within 0.3 Da. X=CH₂CONH₂. Here, peptide is mainly cleaved at peptide bonds (C-N bonds) with the formation of b and y ions, and the positions of cleavage have been indicated in the peptide sequence.

Figure 3. Recombinant human insulin is functional

Recombinant human insulin (rhINS) activates its receptor in CHO-hIR stable cell lines. The data represents an ELISA assay for the autophosphorylation of human Insulin receptor following stimulation by commercial human insulin (left) or by head extracts (5 HE) of a wild type control (Canton S) and experimental genotypes (GAL4>UAS). A one-tailed Student's t-Test was used for the statistical analysis. (* : $P < 0.05$)