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Receptor-mediated activation of a proinsulin-transferrin fusion protein in hepatoma cells

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ABSTRACT

A proinsulin-transferrin (ProINS-Tf) recombinant fusion protein was designed and characterized for the sustained release of an active form of insulin (INS) by hepatoma cells. During incubation with H4IIE hepatoma cells, a gradual decline of ProINS-Tf concentration, with a concomitant generation of the immuno-reactive insulin-transferrin (irINS-Tf), was detected in the culture medium by using INS- or proinsulin (ProINS)-specific radioimmunoassay (RIA) system. Further studies indicated that the conversion of ProINS-Tf to irINS-Tf was a transferrin receptor (TfR) mediated process that was pH-sensitive, and temperature- and microtubule-dependent. These results suggest that the conversion occurred during the slow recycling route of transferrin (Tf)-TfR pathway, possibly processed by proteases in the slow recycling compartments juxtaposed to the trans-Golgi network (TGN). ProINS-Tf exhibited little activity in the short-term promotion of glucose uptake in adipocytes, indicating that it was in an inactive form similar to ProINS. Stimulation of Akt phosphorylation by ProINS-Tf was detected only after prolonged incubation with H4IIE cells. On the other hand, ProINS-Tf pre-incubated with H4IIE cells for 24 h acquired an immediate activity of stimulating Akt phosphorylation. Furthermore, ProINS-Tf elicited a strong activity in the inhibition of glucose production following 24 h incubation with H4IIE cells. Based on these findings, we conclude that the Tf–TfR endocytosis and recycling pathway enables the conversion and release of ProINS-Tf in an active form of irINS-Tf. Results from this study suggest that the Tf–TfR pathway can be exploited for the design of prohormone-Tf fusion proteins as protein prodrugs for their sustained and targeted activation.

1. Introduction

The therapeutic potential of ProINS, the single-chain precursor to INS, has been evaluated in both preclinical and clinical studies [1]. ProINS was marginally hepatospecific, having greater effects on hepatic glucose production than peripheral glucose disposal [2]. Additionally, pharmacokinetic studies demonstrated that both the distribution and the elimination half-life of ProINS were longer than INS [3]. Despite these potential advantages, the application of ProINS faces formidable challenges due to the low potency of ProINS (4 U/mg for ProINS in contrast to 28 U/mg for INS [1]) and the low *in vivo* conversion of ProINS to INS [4,5]. High doses are consequently required for ProINS to achieve *in vivo* pharmacological efficacy. Results from the ProINS clinical trials also showed an increased risk for myocardial infarction, and further clinical studies were subsequently suspended due to safety concerns [1].

The aims of this study were to utilize Tf-fusion protein technology to overcome some of the challenges encountered in the development of ProINS as a hypoglycemic agent. Human Tf is a circulatory serum protein responsible for iron transport, and there are numerous reports on the application of Tf as a fusion protein either to facilitate oral absorption of protein drugs, such as granulocyte-colony stimulating factor-Tf and human growth hormone-Tf fusion proteins [6,7], or to prolong plasma half-life of protein drugs, such as glucagon-like peptide-1-Tf fusion protein [8]. However, another unexploited advantage of Tf-fusion protein technology is the endocytosis and recycling mechanisms of the Tf–TfR pathway. After binding and subsequent receptor-mediated endocytosis of the Tf–TfR complex, Tf unloads iron in the acidic endosomal compartments. Iron-free Tf (apo-Tf) remains bound to TfR intracellularly and is recycled back to the cell surface for release [9,10]. Tf can be delivered to intracellular compartments such as the TGN [11]. Many studies observed the merging of endocytosed Tf with the protein secretory pathway in vesicles located at the TGN [12,13], which conceivably would allow access of endocytosed Tf to secretory proteases that are responsible for the conversion and activation of prohormones. A distinctive feature of Tf is that, unlike most ligands that are sorted to the lysosome for degradation, it is released at the cell surface intact. To our knowledge, this final release step of Tf from TfR following recycling has not been taken advantage of in the development of Tf-fusion proteins.

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In this report, we describe the design and characterization of a ProINS-Tf fusion protein. Our results showed that ProINS-Tf was converted to an active form of INS by hepatoma cells. Furthermore, we demonstrated that the conversion and activation of ProINS-Tf is a TfR-mediated process, occurring inside the recycling compartments along the Tf–TfR pathway. To the best of our knowledge, this is the first report indicating that a ProINS fusion protein can be delivered as a prodrug to be processed and activated by hepatoma cells under the control of the Tf–TfR endocytic and recycling pathway.

2. Materials and methods

2.1. Construction and production of his-tagged ProINS-Tf recombinant fusion protein

A Gly-Gly-Ser-hexa His sequence (–GGSHHHHHH–) was incorporated into the carboxyl-terminal region of the full-length human Tf (residues 1–679) to make a his-tagged Tf (Tf-$GGSH_6$) using PCR-based mutagenesis methods. TFR27 plasmid (ATCC, Manassas, VA) containing the full-length human Tf sequence (NM_001063) was used as PCR templates. The mutagenic forward and reverse primers were designed as 5 -CCGCTCGAGGTCCCTGATAAAACTGTGAGATGGT-3 and 5 -TGCTCTAGACTA**ATGATGATGATGATGATG***GCTGCCCCC*AGGTCTACG-GAAAGTGcaggcttcc-3 (the hexa His sequence is indicated in bold print, and the Gly-Gly-Ser sequence is indicated in italics). The cDNA sequence coding for preproinsulin (NM_000207) was amplified from commercial plasmid (SC120054, Origene, Rockville, MD). Preproinsulin sequence fused upstream in frame with Tf-$GGSH_6$ sequence was subsequently engineered into pcDNA3.1 (+) expression vector (Invitrogen, Carlsbad, CA) through EcoRV, XhoI, and XbaI restriction enzymes sites. A leucyl-glutamyl dipeptide sequence was incorporated between ProINS and Tf due to the XhoI restriction enzyme recognition site.

The plasmids containing preproinsulin-Tf-$GGSH_6$ fusion gene were transiently transfected to HEK293 cells through polyethylenimine-mediated DNA transfection. Conditioned serum-free CD 293 medium (Invitrogen) was collected twice every 4 days, and concentrated using a tangential flow filtration system (Millipore, Billerica, MA). The concentrates were then applied to Ni-NTA column (Qiagen, Valencia, CA) to allow binding of his-tagged ProINS-Tf to Ni-NTA agarose. After washing with 20 mM imidazole to remove the impurities, his-tagged ProINS-Tf was eluted from the column using 250 mM imidazole. The excess imidazole was removed by overnight dialysis using Spectra/Por dialysis membrane (MWCO 12–14 kDa, Spectrum Laboratory, Rancho Dominguez, CA) against phosphate buffer containing 50 mg/mL mannitol and 0.1 mg/mL Tween-20. The purified ProINS-Tf fusion protein was quantified by resolving in 8% non-reducing SDS-PAGE followed by both Coomassie blue staining and Western blot against anti-Tf antibody (T2027, Sigma, St. Louis, MO) and anti-(Pro)INS antibody (INS+ProINS antibody, ab8304, Abcam, Cambridge, MA).

2.2. ProINS- and INS-specific RIA

Cultured H4IIE rat hepatoma cells (ATCC) were treated with ProINS-Tf, in the presence or absence of a 1000-fold excess of apo-Tf (Sigma) or BSA (Sigma). To measure the remaining concentration of ProINS-Tf in the cultured medium, media were collected after different incubation time-points, and subjected to human ProINS-specific RIA (Millipore) according to the manufacturer's instructions. The ProINS-specific RIA has less than 0.1% cross-reactivity with human INS. On the other hand, to quantify the generated irINS-Tf, cell culture media were collected at different incubation time-points, and applied to human INS-specific RIA (Millipore) that has less than 0.2% cross-reactivity with human ProINS. In order to evaluate the cell membrane integrity following the treatment conditions, cells treated with ProINS-Tf, in the presence or absence of excess apo-Tf or BSA for 24 h were analyzed by the lactate dehydrogenase (LDH) release assay (Promega, Madison, WI) according to the manufacturer's instructions.

2.3. 2-Deoxyglucose uptake in cultured adipocytes

3T3-L1 murine fibroblasts (ATCC) were differentiated into adipocytes as previously described [14]. Adipocytes were serum-starved in DMEM containing 0.5% BSA for 16 h before experiments. Cell monolayers were incubated with different concentrations of INS (Sigma), ProINS (R&D System, Minneapolis, MN), or ProINS-Tf in Krebs–Ringer phosphate buffer supplemented with 0.1% BSA for 30 min at 37 °C. After 30 min, 0.5 μCi/mL of 2-deoxy-D-[2, 6-^{3}H] glucose (Perkin Elmer, Waltham, MA) was added to the cells and incubated for 10 min. The reaction was stopped by aspirating the medium, and the cells were washed four times with ice-cold Krebs–Ringer phosphate buffer. Cells were then solubilized with 0.1 M NaOH/0.1% sodium dodecyl sulfate, followed by measurement of the internalized 2-deoxy-D-[2, 6-^{3}H] glucose using a scintillation counter (Perkin Elmer) and protein quantification using the bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Rockford, IL).

2.4. Measurement of Akt phosphorylation

Upon binding of INS to insulin receptor (IR), Akt is phosphorylated following activation of the PI 3-kinase signaling pathway [15]. Quiescent H4IIE cells (~18 h serum-starved) were treated with or without proteins in serum-free conditions. After treatment, cells were immediately lysed with cell extraction buffer (Invitrogen) supplemented with phenylmethanesulfonyl fluoride (Sigma) and protease inhibitor cocktail (Sigma). After protein quantification, an equal amount of cellular proteins (60 μg) was subjected to Western blot analysis by anti-phospho-Akt antibody (Ser 473, 4060, Cell Signaling Technology, Danvers, MA) and anti-beta-actin antibody (AC-15, A5441, Sigma). Immunoreactive bands were detected using enhanced chemiluminescence (GE Health care, Piscataway, NJ), and quantified using Quantity One 1-D Analysis software (Bio-Rad, Hercules, CA).

2.5. Glucose production assay in H4IIE cells

Glucose production was determined using a modified method from previous reports [16]. H4IIE cells were grown in 24-well plates in high-glucose DMEM containing 10% FBS. Serial dilutions of human ProINS, human INS, or ProINS-Tf fusion protein were prepared in serum-free DMEM. Upon confluence, cells were washed with PBS twice to remove excess serum, and treated with various concentrations of proteins for 24 h at 37 °C. Cells were then incubated for 3 h in glucose production medium consisting of serum-, glucose- and phenol red-free DMEM supplemented with 2 mM sodium pyruvate and 40 mM sodium DL-lactate. The medium was harvested and glucose concentrations were measured using the Amplex Red Glucose/Glucose Oxidase Kit (Invitrogen). Cellular protein was quantified using the BCA assay.

2.6. Statistical analysis

The data are presented as average plus standard deviation with $N = 3$ for all experiments. The Student's *t*-test was utilized to compare data sets, where differences with values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Expression and characterization of ProINS-Tf recombinant fusion protein

Human preproinsulin was ligated in frame with carboxyl-terminally his-tagged Tf into pcDNA3.1 vector. Preproinsulin-Tf-$GGSH_6$ fusion gene