The Generation and Characterisation of Human Insulin-like Growth Factor Mutants

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Table of Contents

Table of Contents.................................................................i
Abstract....................................................................................iv
Declaration................................................................................vi
Acknowledgments ......................................................................vii
Abbreviations............................................................................viii

Chapter 1  Introduction .............................................................1
  1.1  Thesis overview..............................................................1
  1.2  The Insulin-like Growth Factor axis ....................................2
    1.2.1  IGF evolution...........................................................2
    1.2.2  Gene structure, expression and physiological effects of the IGFs 5
    1.2.3  Regulation of the IGFs................................................8
    1.2.4  Structure of IGF-I and IGF-II .......................................13
  1.3  The function of the Insulin-like Growth Factor I Receptor ..........15
  1.4  The structure of the Insulin-like Growth Factor I Receptor ..........15
  1.5  Insulin receptor isoforms and their functions .........................16
    1.5.1  Distribution and functions of the IR-A ..........................17
    1.5.2  Distribution and functions of the IR-B ..........................17
  1.6  Hybrid receptors and their functions ....................................18
    1.6.1  IR-A/IR-B hybrid receptors..........................................18
    1.6.2  IR/IGFR-IR hybrid receptors ........................................18
  1.7  The structure of the Insulin Receptor ....................................19
  1.8  Characteristics and mechanism of ligand binding to the IR and IGF-IR 21
    1.8.1  IGF receptor binding sites are primarily located in the B and A-domains 25
    1.8.2  Receptor binding specificity is determined by the IGF C and D-domains 25
    1.8.3  IR and IGF-IR signal transduction...................................27
  1.9  The IGF system and cancer ...............................................30
    1.9.1  IGF ligands and the IGF-IR in cancer .............................31
    1.9.2  IGF-II and the IR-A in cancer .....................................31
    1.9.3  Targeting the IGF system for use as potential cancer therapeutics ....32
  1.10 Directed evolution.........................................................37
    1.10.1 Directed evolution requires protein function to be physically achievable and obtainable through evolution ......................38
    1.10.2 An overview of library construction for directed evolution ........43
    1.10.3 An overview of screening and selection for directed evolution ....44
    1.10.4 Directed evolution strategies ........................................46
    1.10.5 Directed evolution in Neurospora ....................................56
  1.11 Aims ...............................................................................65

Chapter 2  Materials and methods .............................................66
  2.1  Materials .......................................................................66
    2.1.1  Chemicals, reagents & enzymes ..................................66
    2.1.2  Neurospora crassa stocks ..........................................66
    2.1.3  Bacterial stocks ......................................................67
    2.1.4  Plasmid stocks .......................................................68
    2.1.5  Mammalian cell lines ................................................69
    2.1.6  Antibodies .............................................................69
Chapter 4

4.4 Characterisation of transplaced IGF-I expression vectors

4.4.1 Targeted transplacement of the IGF-I expression constructs into Neurospora

4.4.2 Western blot characterisation of the IGF-I expression constructs in Neurospora

4.4.3 IGF-IR ligand binding assay of the IGF-I expression constructs in Neurospora

4.4.4 Characterisation of RIP-generated IGF-I mutants

4.4.5 Discussion

4.4.6 Diversifying the human IGF-I coding sequence in Neurospora crassa

4.4.7 IGF-I secretion in Neurospora crassa
Abstract

Human insulin-like growth factor-I and -II (IGF-I and -II) are structurally homologous ligands with differential binding affinities for the insulin/IGF family of cell surface receptors. High affinity binding of IGF-I and IGF-II ligands to the insulin-like growth factor I receptor (IGF-IR), insulin receptor exon 11- (IR-A) and exon 11+ (IR-B) isoforms stimulates receptor tyrosine kinase activity regulating numerous biological responses such as cell cycle progression, proliferation and cell survival. Deregulation of the IGF-IR and IR-A signalling axis is common in cancer. Both IGF-I and IGF-II have been implicated in cancer initiation and progression due to ligand overexpression and autocrine ligand production.

The focus of this study was to diversify human IGF-I as part of the ongoing development of an in vivo gene diversification system associated with the filamentous fungus Neurospora crassa (Catcheside et al., 2003). The aim of this project was to generate, express, secrete and screen novel IGF-I variants to identify IGF-IR antagonists as potential cancer therapeutics. Mutant IGF-I DNA sequences were generated with high efficiency using the Neurospora Repeat-Induced Point Mutation (RIP) & HotSpot-Mediated Recombination (HSMR) gene diversification platforms. Multiple IGF-I expression vectors were constructed and the novel secretion of human IGF-I protein in Neurospora was achieved with the fusion of the IGF-I protein to the endogenous glucoamylase (gla-1) protein. However, the abundance of mature IGF-I protein in culture medium was relatively low due, at least in part, to extracellular proteolytic degradation. In addition, the IGF-IR binding assay was found to be unsuitable for reliable screening of secreted IGF-I protein in Neurospora culture medium. Resolution of these issues were not pursued in this project as the generation of protease deficient Neurospora expression strains and development of a more robust screening methodology were already being undertaken by Neugenesys Corporation and were unlikely to be resolved within the duration of this project. Consequently, the project direction was diverted to the characterisation of IGF residues involved in receptor binding and activation through site-directed mutagenesis.
The IGF-I and IGF-II ligands share a common domain structure and exhibit differential receptor binding and activation which is primarily due to the IGF C-domain. Understanding the role of specific residues within the C-domains could make a significant contribution to our fundamental understanding of ligand-receptor interactions in the insulin/IGF system and enable the rational design of IGF-IR and IR-A antagonists for use as anti-cancer agents. As such, a second aspect of this study aimed to delineate the C-domain residues conferring differential receptor binding affinity and activation by generating and characterising three IGF-II analogues. Mutants C1 and C2 contained partial IGF-I C-domain substitutions (IGF-I residues underlined): GYGSSSRRSR & SRVSRRAPQT, respectively. Mutant C3 contained a truncated IGF-I C-domain (GSSSRRAT) which approximated the size of the IGF-II C-domain. The three IGF-II analogues were characterized by competitive receptor binding assay, receptor phosphorylation assay and cell survival assay using the IR-A, IR-B and IGF-IR receptors.

The C1 analogue bound the IR-A and IGF-IR with high affinity but bound the IR-B with only moderate affinity suggesting a negative interaction between the C1 C-domain and the exon-11 encoded peptide in the IR-B. The IR-A, IR-B and IGF-IR activation and cell survival responses were generally proportional to the relative binding affinity of C1. In contrast, C2 acted as a partial agonist against the IR-A, IR-B and IGF-IR with high receptor binding affinities but substantially lower activation and cell survival responses in all three receptors. These data indicated both flanks of the IGF C-domain play important roles in receptor binding, activation and cell survival. The C3 analogue had generally poor IR-A, IR-B and IGF-IR binding affinities, activation and cell survival responses which indicated this analogue lacked critical C-domain residues required for high affinity receptor binding and activation.
Declaration

I certify that this thesis does not incorporate without acknowledgment any material previous submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

S.T. Henderson
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Abbreviations

%CV  coefficient of variation expressed as a percentage
AA  amino acid
amp  ampicillin
BCA  bicinchoninic acid
Bis-tris  bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane
boil prep  boiled preparation of *Neurospora* macroconidial gDNA for PCR
bp  base pair
BAP  biotinylated alkaline phosphatase
BCIAD  IGF-II mutant with IGF-I C-domain
BSA  bovine serum albumin
cat#  catalogue number
C1  IGF-II analogue with N-terminal flank of IGF-I C-domain
C2  IGF-II analogue with C-terminal flank of IGF-I C-domain
C3  IGF-II analogue with shortened IGF-I C-domain
CIP  calf intestinal phosphatase
conc.  concentrated
DMEM  Dulbecco’s modified eagles medium
DMSO  dimethyl sulfoxide
DNA  deoxyribonucleic acid
DNAse  deoxyribonuclease
dNTP  deoxyribonucleotide triphosphate
DTPA  diethylenetriaminepentaacetate
DTT  dithiothreitol
EC$_{50}$  half maximal effective concentration
*E. coli*  *Escherichia coli*
ECL  enhanced chemiluminescence
EDTA  ethylenediaminetetraacetic acid
EtBr  ethidium bromide
Eu  europium
FCS  fetal calf serum
FFS  Fast Flow S (buffer)
Fm  FRIES medium
FmH  FRIES medium supplemented with histidine
FmHS  FRIES medium supplemented with histidine and sorbose
FPLC  fast protein liquid chromatography
gDNA  genomic DNA
HCl  hydrochloric acid
HEPES  N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid
hIGF-I  human IGF-I
his  L-histidine
hr  hour(s)
HPLC  high performance liquid chromatography
HRP  horseradish peroxidase
HSMR  Hotspot-mediated recombination
TE  tris EDTA buffer
TBS  tris buffered saline
TBST  tris buffered saline with tween-20
TFA  trifluoracetic acid
Tm  melting temperature
Tris  tris(hydroxymethyl) aminomethane
Triton X-100  iso-octylphenoxypolyethoxyethanol
TSS  transformation and storage solution
Tween 20  polyoxyethylene (20)-sorbitan monolaurate
U  unit of enzyme activity (1 μmol min⁻¹)
UV  ultra violet
V  Volts
VmH  Vogel’s N medium supplemented with histidine
VmHH  Vogel’s N medium supplemented with histidine and hygromycin
vol  volume