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

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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 Neugenesis 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)imino-tris(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

hyg	hygromycin
IB/s	inclusion body/bodies
IC_{50}	half maximal inhibitory concentration
IGF	insulin-like growth factor
IGF-I	insulin-like growth factor I
IGF-II	insulin-like growth factor II
IGF-IR	insulin-like growth factor I receptor
IGF-IIR	insulin-like growth factor II receptor
IPTG	isopropyl-beta-D-thiogalactopyranoside
IR	insulin receptor
IR-A	insulin receptor A isoform
IR-B	insulin receptor B isoform
kb	kilobase pair
LB	Luria broth
LB/amp	Luria broth with ampicillin (100µg/ml)
LG	(*Neurospora*) linkage group
mAB	monoclonal antibody
min	minute(s)
miniprep	miniscale plasmid preparation
midiprep	midiscale plasmid preparation
mqH_2O	milli-Q water
NEB	New England Biolabs
nIGF-I	*Neurospora* RIP-optimised human IGF-I coding sequence
NMR	nuclear magnetic resonance spectroscopy
NSB	non-specific binding
NSmH	*Neurospora* secretion medium supplemented with histidine
OD_{XXXnm}	optical density at wavelength in nm
OPM	orbits per minute
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PIC	Protease inhibitor cocktail (Sigma)
PMSF	phenylmethylsulfonyl fluoride
RIP	repeat-induced point mutation
RNA	ribonucleic acid
RNase	ribonuclease
HPLC	(reverse phase) high performance liquid chromatography
RT	room temperature
s	second(s)
SC	synthetic crossing medium
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SGF	sorbose, glucose & fructose
SOC	super optimal catabolite repression medium
SSC	salt sodium citrate buffer
TAE	tris acetate EDTA buffer
TCA	trichloroacetic acid

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 (1µmol 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