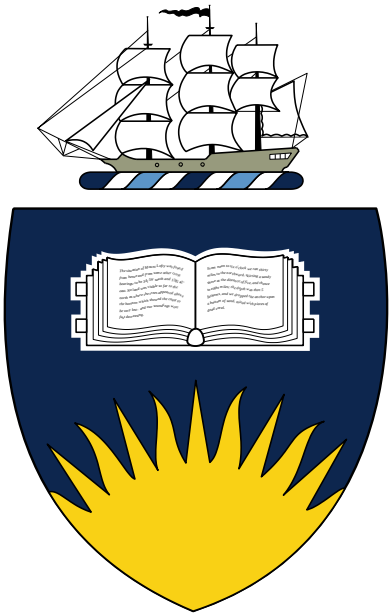
Validating dipeptidyl peptidase (DP) 8 and DP9 potential substrates and investigating the effects of DP8 and DP9 overexpression and silencing on adenylate kinase (AK) 2 in ovarian cancer cells



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Thesis submitted in fulfillment of the requirement for the degree of Doctor of Philosophy

DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously 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.

…….. …….
Dono Indarto Date

I hereby certify that this statement is correct, that this thesis is properly presented and is of sufficient standard, *prima facie*, worthy examination.

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(Co-Supervisor of this study)

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ABSTRACT

The dipeptidyl peptidase (DP) 4 gene family is a serine protease family that cleaves various biopeptides at the post-prolyl bond. DP4 is the most studied member of the family and is involved in regulating metabolism, the immune system and cell signaling. Fibroblast activation protein (FAP) is another member of the DP4 gene family that contributes to tissue proliferation during cell malignancy and wound healing. In contrast to DP4 and FAP, the biological functions of DP8 and DP9 are still under investigation although their mRNA and protein are ubiquitously expressed in human tissues. Previous work in the laboratory using a proteomics approach identified adenylate kinase 2 (AK2) and other proteins as potential *in vivo* DP8 and DP9 substrates and revealed a role for DP8 and DP9 in cell metabolism. AK2 is an enzyme that is localized in the intermembrane space of mitochondria. This enzyme has a pivotal role in catalyzing reversibly ATP and AMP to 2ADP, maintaining cellular energy homeostasis. The major aim of this study was to further validate the substrates identified and to investigate the role of these proteases in cellular metabolism, in particular in regulating the function of AK2.

Immunocytochemistry and western blotting were used for studying DP8 and DP9 co-localization with AK2 and calreticulin in ovarian cancer cell lines (SKOV3) overexpressing DP8 and DP9, which were tagged with enhanced green fluorescent protein (EGFP). MALDI-TOF mass spectrometry (MS) was then used to test a further 12 substrates for cleavage by DP8 and DP9 that were previously identified using a recent proteomics approach. Cleavage activity of DP8 and DP9 towards AK2 in these SKOV3 cells was evaluated using proliferation rate and AK assays. Changes in adenine nucleotide levels (ATP, ADP and AMP) and cellular energy charge were

assessed by using high performance liquid chromatography (HPLC). DP8 and DP9 small interfering RNA (siRNA) were used to silence these proteases in OVCA 429, OVCA 432 and SKOV3 cell lines in order to test the effects of each DP gene on AK2 protein and enzyme activity.

Immunofluorescence confocal microscopy was used to demonstrate that DP8 and DP9 and their substrates AK2 and calreticulin are in close proximity. No difference in substrate localization was observed in cells expressing wt or mt proteases. The work also revealed that N-terminal oligopeptides of seven of 12 potential substrates are cleaved by DP8, DP9 and DP4. However cleavage rates differed between the three enzymes, reflecting differences in their active sites. These differences reflect different amino acids that line entry to the catalytic site and the site itself between the three proteases. In the first six hrs after sub culture in a 96 well plate cells expressing wt and mt DP8 had increased viability compared to vector expressing cells, while the proliferation rate of SKOV3 cells with wt and mt DP9 overexpression was lower. In contrast in a T75 flask it was observed that both wt and mt DP8 took longer to reach confluence and that vector and wt and mt DP9 expressing cells (four days compared to seven). In these SKOV3 cells overexpressing wt and mt DP9, ATP levels and adenylate energy charge decreased significantly, compared to those in SKOV3 cells overexpressing vector control. Meanwhile, ADP and AMP levels in SKOV3 cells overexpressing wt and mt DP9 increased but a significant increase in AMP level was only observed in SKOV3 cells overexpressing wt DP9. ADP/ATP and AMP/ATP ratios also increased in wt and mt DP9 overexpression. This data combined suggests opposite roles for both DP8 and DP9 in cell growth and proliferation that are independent of their enzyme activity.

DP8 and DP9 were silenced in three different ovarian cancer cell lines. The results were more similar in OVCA 423 and SKOV3 cells than in OVCA 429 cells, this difference probably reflects the different phenotypes of these cells to start with. Intriguing data was obtained using silencing that suggests that *in vivo* in SKOV3 cells that AK2 is a substrate of DP8 but not DP9. DP8 silencing led to a decrease in AK specific activity and an increase in AMPK phosphorylation. A similar result was observed when AK2 was silenced in these cells. In contrast DP9 silencing had no significant effect on AK specific activity but did appear to increase the expression of AK2 and had no effect on AMPK phosphorylation

In summary this work has provided additional evidence for the role of DP8 and DP9 in maintaining cellular metabolism. However, further work is required. Important questions that need to be answered is what effect does DP9 and or DP8 overexpression have on activated AMPK and the pathways downstream including glucose uptake and lactate production. In addition more work needs to be performed to investigate the effect of DP8 and DP9 silencing on genes downstream of AMPK. DP8 and DP9 may modulate AMPK an important player in both cellular metabolism and cancer growth. Further understanding of this role may lead to development of DP8/DP9 inhibitors or activators that may be used to treat cancers such as ovarian cancer.

ABBREVIATIONS

ADA	adenosine deaminase
AK	adenylate kinase
AK2	adenylate kinase 2
ADP	adenosine diphosphate
AMP	adenosine monophosphate
AMPK	adenosine monophosphate kinase
ANOVA	analysis of variance
ATCC	American type tissue collection
ATP	adenosine triphosphate
APS	ammonium persulphate
BCA	bicinchoninic acid
BSA	bovine serum albumin
C	celsius
Ca	calcium
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CD3	cluster of differentiation 3
CD4	cluster of differentiation 4
CD26	cluster of differentiation 26
CD47	cluster of differentiation 47
CD91	cluster of differentiation 91
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1propanesulfonate
CO_2	carbon dioxide
C terminus	carboxyl terminus
CXCl12	chemokine ligand 12
DAPI	4'-6-diaminidino-2-phenylindole
DMEM	Dulbecco's modified eagle medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DP	dipeptidyl peptidase
DP8	dipeptidyl peptidase 8
DP9	dipeptidyl peptidase 9

DP6	dipeptidyl peptidase 6
DP4	dipeptidyl peptidase 4
DP10	dipeptidyl peptidase 10
DPD	dihydropyrimidine dehydrogenase
DTT	dithiothreitol
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylene diamine tetraacetic acid
EGFP	enhanced green fluorescent protein
EGF	epithelial growth factor
EGTA	ethylene glycol tetraacetic acid
FACS	fluorescence activated cell sorter
FAP	fibroblast activation protein
FBS	foetal bovine serum
FDA	food and drug administration
g	gram
GIP	gastric inhibitory polypeptide
GLP-1	glucagon like peptide1
GLP-2	glucagon like peptide 2
GLUT	glucose transporter
h	hour
HCl	hydrochloric acid
HEK 293	human embryonic kidney cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high performance liquid chromatography
HRP	horse radish peroxidase
HT-29	human colon adenocarcinoma grade II cell line
IBC	Institutional Biosafety Committee
IL-1	interleukin-1
IL-2	interleukin-2
IgG	immunoglobulin G
IP10	inflammatory protein-10
ITAC	interferon-inducible T cell chemo-attractant
K_2HPO_4	dipotassium hydrogen phosphate

K_3PO_4	potassium phosphate
KCl	potassium chloride
kDa	kilo dalton
KH_2PO_4	monopotassium phosphate
KOH	potassium hydroxide
LDH	lactate dehydrogenase
M	molar
mAb F19	monoclonal antibody against the human FAP antigen
MALDI-TOF	matrix-assisted laser desorption ionization time of flight
Mg	magnesium
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
MHC	major histocompatibility complex
min	minutes
ml	milliliter
mm	millimeter
mM	millimolar
Mn	manganese
mU	milliunit
mt	mutant
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MTT	3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide
Na_2HPO_4	disodium hydrogen phosphate
NaCl	sodium chloride
NADP	nicotinamide adenine dinucleotide phosphate
NADH	nicotinamide adenine dinucleotide
NaOH	sodium hydroxide
NaN_3	sodium azide
nM	nanomolar
NK	natural killer
NPY	neuropeptide Y
N terminus	amino terminus
OD	optical density

OVCA 429	ovarian cancer cell line
OVCA 432	ovarian cancer cell line
SDS-PAGE	SDS polyacrylamide gel electrophoresis
pAMPK	phosphorylated adenosine monophosphate kinase
PBS	phosphate buffered saline
pEGFPN1	cloning vector containing EGFP
PEP	phosphoenol pyruvate
PK	pyruvate kinase
PKA	protein kinase A
PVDF	polyvinylidene difluoride
PYY	peptide YY
RANTES	regulated on activation normal T cell expressed and secreted
RFU	relative fluorescence intensity
RNA	ribonucleic acid
RPMI 1640	Roswell Park Memorial Institute1640
RT	room temperature
RT-PCR	reverse transcript polymerase chain reaction
SCDFα and β	stromal cell-derived factors 1α and 1ß
SCID	severe combined immunodeficient
SDS	sodium dodecyl sulphate
SEM	standard error mean
siRNA	small interfering RNA
SKOV3	ovarian cancer cell line
SOD	superoxide dismutase
SPSS	statistical package for the social science
TAILS	terminal amine isotopic labelling of substrates
TFA	trifluoroacetic acid
THF	tetrahydrofolate
TNFα	tumor necrosis factor α
TSP-1	thrombospondin-1
TEMED	N,N,N,N tetramethylenediamine
µM	micromolar
UCSF	University of California, San Francisco
UK	United Kingdom

USA	United States of America
TIM	translocase of the inner membrane
TOM	translocase of the outer membrane
wt	wildtype