Molecular characterization of the CP2-related transcription factor, CRTR-1

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Doctor of Philosophy

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DECLARATION

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ABBREVIATIONS

β-ME	β-mercaptoethanol
Ac	acetate
AP	alkaline phosphatase
APP	amyloid precursor protein
APS	ammonium persulphate
BOM	brother-of-MGR
BSA	bovine serum albumin
cDNA	complementary DNA
chIP	chromatin immunoprecipitation
CMV	cytomegalovirus
CRM1	chromosome region maintenance 1
CRTR-1	CP2-related transcriptional repressor 1
d.p.c.	days post coitum
DAPI	4',6'-diamidino-2-phenylindole dihydrochloride
DBD	DNA-binding domain
dct	distal convoluted tubule
dCTP	deoxycytosine triphosphate
ddPCR	differential display polymerase chain reaction
DIG	digoxygenin
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DT	diphtheria
DTT	dithiothreitol

E. coli	*Escherichia coli*
ECF	enhanced chemifluorescence
EDTA	ethylenediamine tetra acetic acid
EF	elongation factor
EGFP	enhanced green fluorescent protein
EMSA	electromobility shift assay
EPL	early primitive ectoderm-like
ES	embryonic stem
EtBr	ethidium bromide
FAM	carboxyfluorescein
FCS	foetal calf serum
FLB	Ficoll loading buffer
fw	forward
g	gravity
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GLB	gel loading buffer
GR	glucocorticoid receptor
GRH	grainyhead
GSK-3	glycogen synthase kinase-3
HDAC	histone deacetylase
HEK	human embryonic kidney
HEPES	N-2-hydroxyethyl piperazine-N-ethane sulphonic acid
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
HSV	herpes simplex virus
ICM	inner cell mass
Ig	immunoglobulin
IL	interleukin

IP	immunoprecipitation
IRES	internal ribosome entry sequence
kb	kilobase
kD	kilodalton
LB	luria broth
LIF	leukaemia inhibitory factor
LMB	leptomycin B
LTR	long terminal repeat
LUC	luciferase
MAP	mitogen-activated protein
MEL	murine erythroleukemia
MGR	mammalian grainyhead
MOPS	3-[N-Morpholino] propanesulfonic acid
MQ H_2O	Milli-Q water
MR	mineralcorticoid receptor
mRNA	messenger RNA
mut	mutant
MW	molecular weight
NaAc	sodium acetate
NEB	New England Biolabs
NEM	N-ethylmaleimide
NES	nuclear export sequence
NLS	nuclear localization signal
NP-40	Nonidet P-40
OD_n	optical density at a wavelength of *n* nm
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PB	phosphate buffer

PBS	phosphate buffered saline
PBT	phosphate buffered saline + 0.1% Tween-20
PCR	polymerase chain reaction
PDSM	phosphorylation-dependent sumoylation motif
PFA	paraformaldehyde
PIAS	protein inhibitor of activated STAT
PML	promyelocytic leukemia
PMSF	phenylmethylsulphonyl fluoride
PREX	positive regulatory element for XRE
puro	puromycin
PVA	polyvinyl alcohol
PVDF	polyvinylidene fluoride
rcf	relative centrifugal force
Rev	reverse
RNA	ribonucleic acid
RNAi	RNA interference
RNase	ribonuclease
rpm	revolutions per minute
RT	reverse transcription
SAP	shrimp alkaline phosphatase
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
siRNA	small interference RNA
SOM	sister-of-MGR
SRF	serum response factor
SSE	stage selector element
STAT	signal transducer and activator of transcription
SUMO	small ubiquitin-related modifier

SV40	Simian Virus 40
TAE	tris acetate EDTA
TAP	tandem affinity purification
TBE	tris borate EDTA
TBS	tris buffered saline
TBST	tris buffered saline + 0.1% Tween-20
TE	tris EDTA
TEMED	N, N, N', N'-tetra methylethylenediamine
TK	thymidine kinase
TS	thymidylate synthase
Tween-20	polyoxyethylenesorbitan monolaurate
Ubc9	ubiquitin-conjugating enzyme 9
UTP	uridine triphosphate
WT	wild type
XRE	xenobiotic responsive element
YY1	ying-yang 1

SUMMARY

CRTR-1 is a member of the CP2 family of transcription factors. Unlike other CP2 family members, *CRTR-1* expression is regulated developmentally. Major sites of expression in the embryo include the pluripotent inner cell mass (ICM) of the pre-implantation blastocyst and the developing kidney. It is also expressed in embryonic stem (ES) cells, which are derived from the ICM of blastocysts, and is downregulated as these cells differentiate into early primitive ectoderm-like (EPL) cells. This expression pattern suggests that CRTR-1 plays a role in early pluripotent populations. This thesis aims to characterize the transcription factor CRTR-1 at the molecular level and analyses the role of sumoylation on CRTR-1 function to develop a better understanding of the molecular role of CRTR-1 in ES cells.

Luciferase reporter assays show that CRTR-1 is able to regulate the activities of other CP2 family members: CP2, NF2d9 and altNF2d9. It enhances CP2- and NF2d9-mediated activation but suppresses altNF2d9-mediated activation. To map the functional domains in the CRTR-1 protein, transactivation studies using CRTR-1 deletion mutants fused to the GAL4 DNA binding domain and a GAL4-responsive reporter system were performed. These studies map repressor activity to amino acids 48-200, but fail to identify a transactivation domain within the CRTR-1 protein.

In order to understand the mechanisms by which CRTR-1 regulates the transcriptional activities of CP2 family members, a number of approaches are taken, including co-immunoprecipitation to show that CRTR-1 interacts with other CP2-like proteins, EMSA which demonstrate that CRTR-1 forms DNA binding complexes with CP2 family members, and subcellular protein localisation studies which reveal the ability of CRTR-1 and other family members to shuttle between the nucleus and cytoplasm via a CRM1-dependent

pathway. In addition, the subcellular localisation of CRTR-1 appears to be cell type specific, with an exclusively nuclear localisation pattern in ES cells, a predominantly cytoplasmic localisation pattern in HEK293T cells, and a cytoplasmic and nuclear speckle localisation pattern in COS-1 cells. Co-expression of CRTR-1 with CP2 or NF2d9 results in the re-localisation of CRTR-1 to the cytoplasm in ES cells.

The sumoylation enzymes Ubc9 and PIAS1 have previously been identified as CP2-interacting proteins (Kang et al., 2005a). Given the identification of two potential sumoylation sites within CRTR-1, $FK^{30}QE$ and $LK^{464}AE$, and the ability for sumoylation to regulate transcription factor function, the possibility that CRTR-1 is regulated by sumoylation is investigated in this thesis. Immunoprecipitation experiments show that CRTR-1 is modified by SUMO-1 and that lysine 30 is the critical residue for this modification. Mutation of lysine 30 to alanine, which abolishes CRTR-1 sumoylation, results in enhancement of transactivation by CRTR-1, suggesting that sumoylation of CRTR-1 blocks maximal activation. Unexpectedly, however, overexpression of Ubc9, PIAS1, or SUMO-1 results in enhancement of CRTR-1 transcriptional activity, indicating that a more complex mechanism of regulation of CRTR-1 activity is likely.

This thesis presents several novel properties of CRTR-1 and other CP2 family members, including the ability of CRTR-1, previously characterized as a repressor, to activate transcription. It is also the first demonstration that CP2 proteins are regulated by sumoylation and that they shuttle between the nucleus and cytoplasm via a CRM1-dependent mechanism.