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Name of Candidate: MAJ Thomas Ceremuga
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Dissertation and Abstract Approved:

Brian Cox, Ph.D.
Department of Pharmacology
and Neuroscience Program
Committee Chairperson

3/13/03

Date

Joseph McCabe, Ph.D.
Department of Anatomy, Physiology & Genetics
and Neuroscience Program
Committee Member

3/13/03

Date

Juanita Anders, Ph.D.
Department of Anatomy, Physiology & Genetics
and Neuroscience Program
Committee Member

3/13/03

Date

Thomas Cote, Ph.D.
Department of Pharmacology
and Neuroscience Program
Committee Member

3/13/03

Date

Thomas Darling, M.D., Ph.D.
Department of Pharmacology
Committee Member

3/13/03

Date

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14. ABSTRACT

Protein degradation by ubiquitination and the 26S proteasome is used to modulate the steady-state levels of proteins and to regulate cellular processes. Proteins become targets of the proteasome by covalent attachment of polyubiquitin chains, which requires three main enzymes (E1, E2, and E3). It is the E3 ubiquitin ligases that control the selection and specificity of substrate ubiquitination. Cullin-5 (Cul-5), a member of the cullin family of E3 ubiquitin ligases, remains obscure. The goals of this research project were to characterize Cul-5, and investigate its response to cellular stresses of water deprivation and hemorrhagic shock in the rat. Northern blotting of poly(A)+ RNA from various rat tissues demonstrated the cul-5 transcript is approximately 6.3 kb. Reverse transcription-polymerase chain reaction (RT-PCR) indicated cul-5 mRNA is present in twelve tissues examined: brainstem, cerebral cortex, cerebellum, hypothalamus, aorta, gastrointestinal tract, heart, kidney medulla, liver, lung, skeletal muscle, and spleen. Quantitative realtime PCR confirmed RT-PCR results that Cul-5 mRNA is ubiquitously expressed and that levels are similar in all tissues. Cellular specificity examination showed cul-5 mRNA expression in rodent neuronal, glial, and vascular endothelial cells in the central nervous system (CNS) via RT-PCR. We corroborated these data by immunocytochemical techniques demonstrating Cul-5 protein presence in neurons, astrocytes, blood vessels, and choroid plexus in rat. Functional assays measured cul-5 mRNA expression responses to water deprivation and hemorrhagic shock. Quantitative realtime PCR showed significant cul-5 mRNA elevations in the rat cerebral cortex (3 fold, $p < 0.001$), hypothalamus (2 fold, $p < 0.007$), and kidney (1.5 fold, $p < 0.04$) following 48 hours of water deprivation. Water deprivation for 24 hours or rehydration (24 hours access to water following 48 hours of water deprivation) also increased kidney cul-5 mRNA levels (1.5 fold, $p < 0.04$ and 3 fold, $p < 0.001$ respectively). Hemorrhagic shock was used as a second in vivo cellular stress model. Rats were subjected to volume controlled (27 ml/kg) hemorrhage over 10 minutes and kept in shock for 60 minutes. Levels of cul-5 mRNA were significantly increased in the brainstem and cerebellum (1.6 fold, $p < 0.01$ and 1.5 fold, $p < 0.05$ respectively), and decreased in the hypothalamus (0.5 fold, $p < 0.05$) compared to sham-treated rats. We determined that Cul-5 is synthesized in all tissues and organs we examined, and in neurons, glia, and endothelial cells in the CNS. Using two paradigms of cellular stress, we found cul-5 mRNA levels in the CNS are altered by water deprivation and by hemorrhagic shock. However, much remains to be revealed concerning what precise physiological role(s) Cullin-5 plays in various cellular processes.

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MAJOR THOMAS E. CEREMUGA
Program in Neuroscience
Uniformed Services University

ABSTRACT

CULLIN 5 EXPRESSION IN THE RAT: CELLULAR AND TISSUE DISTRIBUTION, AND CHANGES IN RESPONSE TO WATER DEPRIVATION AND HEMORRHAGIC SHOCK

Major Thomas E. Ceremuga

directed by Joseph T. McCabe, Ph.D., Vice-Chair of Department of Anatomy,
Physiology & Genetics; and Professor of Molecular and Cellular Biology, and
Neuroscience

Protein degradation by ubiquitination and the 26S proteasome is used to modulate the steady-state levels of proteins and to regulate cellular processes. Proteins become targets of the proteasome by covalent attachment of polyubiquitin chains, which requires three main enzymes (E1, E2, and E3). It is the E3 ubiquitin ligases that control the selection and specificity of substrate ubiquitination. Cullin-5 (Cul-5), a member of the cullin family of E3 ubiquitin ligases, remains obscure. The goals of this research project were to characterize Cul-5, and investigate its response to cellular stresses of water deprivation and hemorrhagic shock in the rat.

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**CULLIN 5 EXPRESSION IN THE RAT: CELLULAR AND TISSUE
DISTRIBUTION, AND CHANGES IN RESPONSE TO WATER DEPRIVATION
AND HEMORRHAGIC SHOCK**

by

Major Thomas E. Ceremuga

US Army Nurse Corps

Dissertation submitted to the faculty of the Graduate Program in Neuroscience of the
Uniformed Services University of the Health Sciences in partial fulfillment of the
requirements for the degree of Doctor of Philosophy 2003

DEDICATIONS

To **GOD** in whom all things are possible, I dedicate this work. **GOD** gives me *strength, perseverance, purpose* and all my *blessings*.



To the United States Military Personnel, for all of their sacrifice and service.

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
AO	aorta
APC	anaphase promoting complex
AQP	aquaporin
ATP	adenosine triphosphate
BS	brainstem
bp	base pair
BUN	blood urea nitrogen
bv	blood vessel
b.w.	body weight
C	control
°C	degrees celsius
Ca ²⁺	calcium
CB	cerebellum
cc	corpus callosum
CC	cerebral cortex
cDNA	complementary deoxyribonucleic acid
Cl ⁻	chloride
Ct	cycle threshold
ctx	cerebral cortex
CNS	central nervous system

Cul-1	cullin-1
Cul-2	cullin-2
Cul-3	cullin-3
Cul-4A	cullin-4A
Cul-4A	cullin-4B
Cul-5	cullin-5 protein
cul-5	cullin-5 mRNA
Cul-7	cullin-7
CYC	cyclophilin
DAB	diaminobenzidine tetrachloride
DNA	deoxyribonucleic acid
E1	ubiquitin-activating enzyme
E2	ubiquitin-conjugating enzyme
E3	ubiquitin-ligase enzyme
E4orf6-E1B55K	adenoviral protein
E6-AP	E6-associated protein
E-Cdk2	E-cyclin dependent kinase 2
ECL	enhanced chemiluminescent
ENaC	epithelial Na ⁺ channels
g	gram
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFAP	glial fibrillary acidic protein
GI	gastrointestinal

GPC	glycerophosphorylcholine
H ₂ O ₂	hydrogen peroxide
HCO ₃	bicarbonate
HCT	hematocrit
HIF1- α	hypoxic inducible transcription factor
HECT	homologous to E6-AP C-terminus
HPVs	human papilloma viruses
HT	heart
hp	hippocampus
HS	hemorrhagic shock
HY	hypothalamus
I κ B	inhibitor of nuclear factor kappa B
ISR-2	insulin receptor substrate protein
K ⁺	potassium
kb	kilobases
kDa	kilodalton
kg	kilogram
KD	kidney
KM	kidney medulla
LG	lung
LI	liver
ln	natural log
Mg ²⁺	magnesium

μg	microgram
μl	microliter
mg	milligram
ml	milliliter
mm	millimeter
mM	millimolar
mmHG	millimeters of mercury
mRNA	messenger ribonucleic acid
MAPKs	mitogen-activated protein kinase
Muf1	elongin BC protein
Na ⁺	sodium
NaCl	sodium chloride
NeuN	neuronal nuclear protein
NEDD8	neural precursor cell-expressed, developmentally down-regulated
NFκB	nuclear factor kappa B
NIH	National Institute of Health
p53	tumor suppressor
PBS	phosphate buffered saline
PCO ₂	partial pressure of carbon dioxide
PO ₂	partial pressure of oxygen
PCR	polymerase chain reaction
PIT	pituitary gland
PVDF	polyvinylidene difluoride membrane

Qty	quantity
Rbx1	RING-box protein
RING	Really Interesting New Gene finger proteins
RNA	ribonucleic acid
ROC1	regulator of cullins
ROS	reactive oxygen species
RT	reverse transcription
RT-PCR	reverse transcription-polymerase chain reaction
SCF	Skp1 Cullin F-box protein complexes
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SM	skeletal muscle
SO ₂	oxygen saturation
SP	spleen
SOCS	suppressor of cytokine signaling box
TBS	tris buffered saline
TTBS	tween-20 tris buffered saline
TonEBP	tonicity-response enhancer binding protein
TSNRP	TriService Nursing Research Program
Ub	ubiquitin
USUHS	Uniformed Services University of the Health Sciences
VHL	von Hippel-Lindau
VACM-1	vasopressin-activated, calcium-mobilizing protein; same as Cul-5

12WD	12 hour water deprivation
18S	18S ribosomal RNA
24WD	24 hour water deprivation
48WD	48 hour water deprivation
48WD+24W	48 hours of water deprivation followed by 24 hours of rehydration

CHAPTER 1

Introduction

Cullin 5 (Cul-5) is a member of the cullin gene family involved in ubiquitination and protein degradation (Kipreos et al., 1996). Cul-5 was first described as vasopressin-activated, calcium-mobilizing (VACM-1) receptor in the rabbit kidney medulla (Burnatowska-Hledin et al., 1995) and further analysis indicated this protein is homologous with Cul-5 (Byrd et al., 1997). Many questions remain concerning the role(s), function(s), and characterization of Cul-5. Inconsistencies concerning its cellular specificity and localization have been reported as histological studies showed Cul-5 protein primarily restricted to vascular endothelial cells (Burnatowska-Hledin et al., 1999). Other researchers described cul-5 mRNA distribution as extensive and predominantly neuronal (Hurbin et al., 2000). Currently, evidence identifies Cul-5 as a component of large protein complexes, E3 ubiquitin ligases (Kamura et al., 2001) that covalently bind ubiquitin to targeted proteins for degradation by the 26S proteasome.

Protein degradation is essential for competent cell function

Proteins are the foundation of all cells and consist of both structural and enzymatic molecules. Most proteins are replaced every few days and various proteins are degraded at an assortment of rates. Some proteins have half-lives as short as 20 minutes, while others may remain stable for days to weeks. These degradation rates can vary according to the dynamic conditions in the organism (Goldberg et al., 2001). This degradative process, therefore, has to be highly selective and tightly regulated (Goldberg,

2000). As the cell's major mechanism for protein degradation, the complex ubiquitin-proteasome pathway is an important regulator of almost all biological activities responsible for normal cell growth and metabolism (Hochstrasser, 1995; Deshaies, 1999).

Although traditionally seen as no more than a means of eliminating no longer needed cell products, research in the last decade has demonstrated protein degradation is essential to life. Recent work has shown it is critical to the control of basic cellular processes such as cell cycle progression, signal transduction, degradation of regulatory transcription factors, and the removal of damaged and misfolded proteins (Ciechanover, 1994; Laney and Hochstrasser, 1999). Ubiquitination and proteasomal degradation, then, participates in all important cellular processes, including cell cycle and division, modulation of the cellular response to stress, neuronal development, ion channel and cell-surface receptor downregulation, DNA repair, regulation of the immune and inflammatory responses, and organelle genesis (Ciechanover et al., 2000). Moreover, ubiquitination in these biological pathways is not only extremely complex, but we remain almost completely uninformed about how certain molecules of the proteasome complex identify, and determine when, certain cell proteins and messengers must be destroyed (Ciechanover et al., 2000).

Recent investigations are beginning to reveal the basic designs whereby the cell can modify biochemical reactions to meet its needs, and regulate various cellular mechanisms and pathways via degradation of critical enzymes or regulatory proteins. One example is the rapid destruction of cyclins, the regulatory proteins that control the transitions through various steps of the cell cycle (Hershko and Ciechanover, 1998; Goldberg, 2000). Another example is the regulation of the nuclear factor- κ B (NF κ B)

pathway via proteolysis of its inhibitor, I κ B. When the cytosolic inhibitor of NF κ B is degraded, NF κ B translocates to the nucleus inducing the transcription and the production of inflammatory factors (Schwartz and Ciechanover, 1999). Tumor suppressors (p53) and cell surface receptors (growth hormone receptor and T cell receptor) are also known to be targeted and degraded by the proteasome (Ciechanover et al., 2000).

Proteasomal protein degradation also is involved in a host of regulatory mechanisms that underlie body metabolism. In times of starvation or disease, for example, the proteasome becomes more active in its breakdown of proteins, whereby amino acids can then be used in gluconeogenesis, synthesizing glucose for energy (Lee and Goldberg, 1998). Proteolysis is also important for immune system function. When the proteasome degrades foreign proteins into amino acid fragments, these antigenic peptides are presented on the cell surface by the major histocompatibility complex class I molecules, triggering the host immune system to attack infected cells (Rock and Goldberg, 1999; Ulrich, 2002). Abnormal, aberrant, or damaged proteins are also targets for destruction by the proteasome such as in cystic fibrosis. In this disease the mutated chloride channel proteins are targeted for rapid degradation (Ciechanover et al., 2000; Goldberg et al., 2001). This gives rise to chronic pulmonary obstruction and dysfunction of pancreatic processes. Ironically, it has been shown that in many cases if the mutated channel was not so rapidly eliminated, its insertion into the membrane would result in “normal” functions. The *failure* of the proteasome to destroy abnormal misfolded proteins may also contribute to pathologic conditions. The neurological diseases of Parkinson’s, Alzheimer’s, and Huntington’s arise in part from the accumulation of

abnormal proteins that cause neurodegeneration (van Leeuwen et al., 1998; Ciechanover et al., 2000; Goldberg et al., 2001).

Biochemical steps of protein degradation

Broadly, the targeting of proteins for degradation by the proteasome is a two-step process. The cell must first deem what proteins must be eliminated, and then undertake the process of proteolysis. Proteins destined for degradation by the proteasome are first tagged by ubiquitin, a highly conserved 76 amino acid protein. Ubiquitin attaches to larger proteins via long chains (polyubiquitination), which act as signals for entry into the proteasomal protein degradation pathway (Ciechanover et al., 2000).

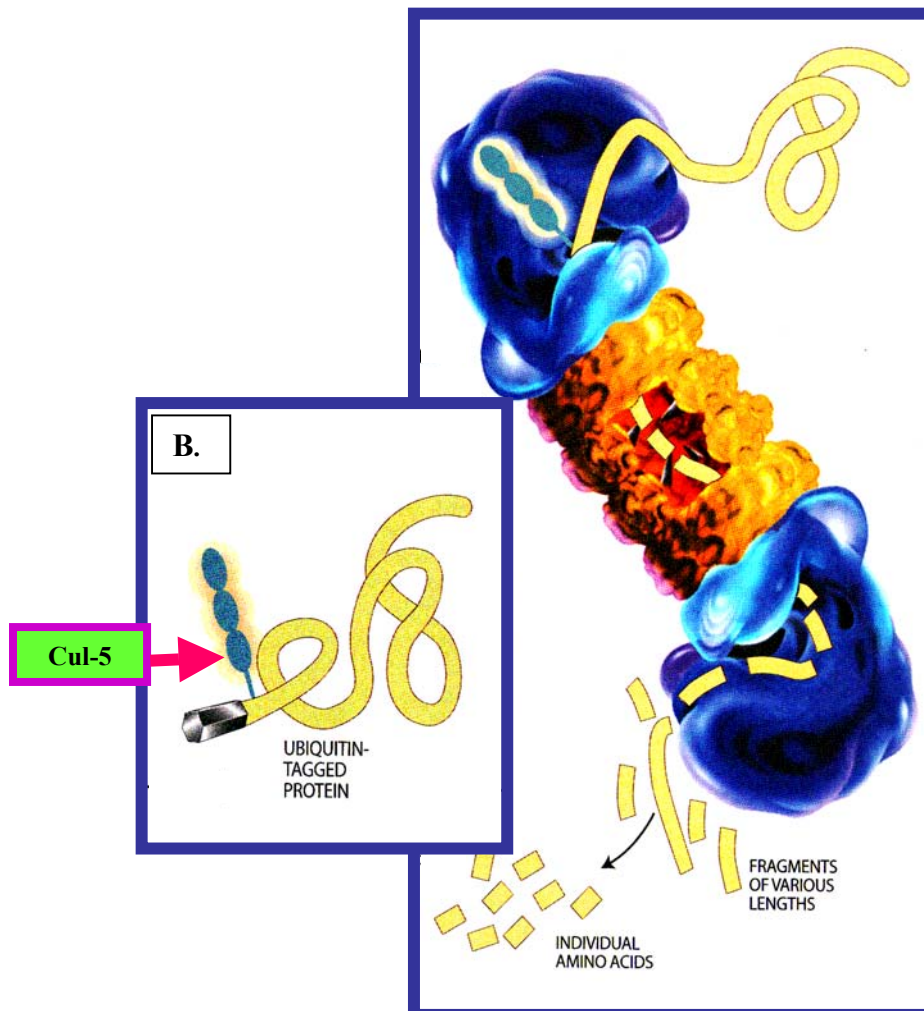
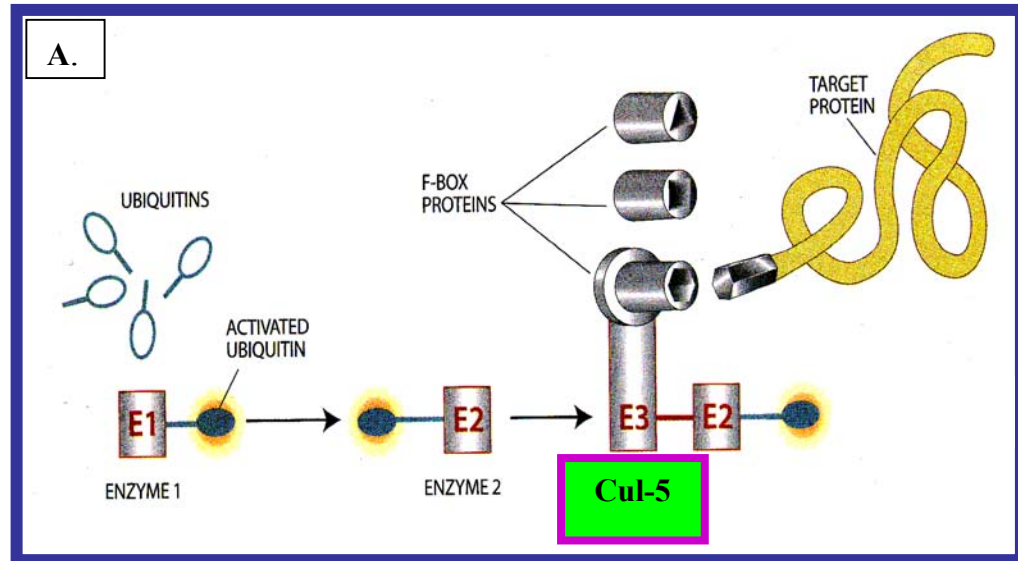
Ubiquitination of proteins requires three main enzymes. In an ATP-dependent manner, the first enzyme (E1), binds to and activates an ubiquitin molecule and then delivers it to the second enzyme (E2), which then binds to the third enzyme, E3 (Figure 1A). Ubiquitination typically results in the formation of a bond between the C-terminus of ubiquitin and the ϵ -amino group of a substrate lysine residue. E1, the ubiquitin-activating enzyme, forms a thiol ester with the carboxyl group of ubiquitin's glycine⁷⁶, thereby activating the C-terminus of ubiquitin for nucleophilic attack; E2, the ubiquitin conjugating enzyme, then transiently carries the activated ubiquitin molecule as a thiol ester; and an E3, the ubiquitin ligating enzyme or ligase, transfers the activated ubiquitin from the E2 to the substrate lysine residue (Hershko et al., 1983; Pickart, 2001).

When an E3 binds to a protein, the ubiquitin carried by the E2 enzyme is transferred to the protein. This cycle repeats until the targeted protein is tagged with a chain of ubiquitins. The ubiquitin chain then attaches to the proteasome, where enzymes

unfold the protein, direct it into the proteasome digestive core where it is chopped up into little pieces (Figure 1B) (Goldberg et al., 2001). Therefore, the E3 proteins are responsible for the selection of proteins for ubiquitination and ultimately proteosomal degradation (Pickart, 2001).

The E3 reaction involves at least two distinct steps: E3 binding to the substrate via the ubiquitination signal, and the covalent ligation of one or more ubiquitins to the substrate. Many E3s catalyze both initial ubiquitin-substrate and subsequent ubiquitin-ubiquitin ligation (Pickart, 2001). The first ubiquitin is bound to a ϵ -NH₂ group of a lysine residue of the protein substrate to create an isopeptide bond. Once the first ubiquitin molecule is bound, a polyubiquitin chain is created by progressive transfer of additional activated ubiquitins to the lysine of a previously conjugated ubiquitin molecule. This polyubiquitin chain is thought to serve as recognition marker for the 26S proteasome (Ciechanover et al., 2000).

Figure 1. Ubiquitin proteasomal protein degradation pathway. (A) The E1 binds and activates ubiquitin, then E2 conjugates and transfers activated ubiquitin to the E3 ubiquitin ligases (e.g. Cul-5). E3 ubiquitin ligases, which consist of receptor-like subunits (F-box proteins), covalently bind ubiquitin to the targeted protein. (B) The ubiquitin chain then attaches to the proteasome, where enzymes unfold the protein and direct it into the proteasome digestive core, where it is hydrolyzed up into amino acid fragments. Figure is adapted with permission (Goldberg et al., 2001).



In summary, E1 enzymes begin the ubiquitination pathway by activating and transferring ubiquitin to E2 enzymes, which then transfer activated ubiquitin to the E3 ubiquitin ligases. Usually there is a single E1 enzyme, many variants of E2 enzymes, and multiple classes of E3 ubiquitin ligase complexes (Hershko and Ciechanover, 1998).

E3 ubiquitin ligases play a key role in identification of proteins targeted for degradation

The E3 ubiquitin ligases recognize the substrates for ubiquitination by ubiquitin signals. Research is only beginning to determine what specific signals in proteins make them targets for degradation. Many rapidly degraded proteins often contain sequences abundant in PEST (Proline, Glutamine, Serine, and Threonine) and when these residues are phosphorylated they become targets for ubiquitination and degradation (Rogers et al., 1986; Rechsteiner and Rogers, 1996). Another important signal for ubiquitination of some cell cycle regulatory proteins is a conserved nine amino acid sequence named the destruction box (Yamano et al., 1996; Pickart, 2001). These destruction-box cell cycle regulators are ligated to ubiquitin by the APC (Anaphase Promoting Complex) and are degraded at late mitosis, after the conversion of the APC to the active form (Hershko and Ciechanover, 1998). There is not much known concerning other markers for proteolysis of other cellular proteins, however, phosphorylation appears to be one means to target proteins for ubiquitination and proteasomal degradation (Hershko and Ciechanover, 1998).

The proteasome

Proteasomes, numbering approximately 30,000 in a typical cell (Goldberg et al., 2001), are large multienzymatic complexes consisting of many proteases that cleave proteins into many smaller peptides. In brief, the 26S proteasome is a very large multicomponent protein that weighs approximately 2.4 megadaltons with more than 30 distinct subunits (Goldberg, 2000). Each 26S proteasome consists of a core, the 20S proteasome that resembles a canal, and two smaller regulatory particles, 19S, that resemble caps and are positioned at the ends. The core compartment consists of four stacked rings each comprised of seven subunits forming a central passage that serves as the degradation channel. The caps (19S) appear to be selective regulatory components and gatekeepers, or guards, which only permit the entrance of targeted proteins into the core channel. These regulatory components recognize, bind, and unfold targeted proteins and consequently guide them into the degradation channel (Ciechanover et al., 2000; Goldberg et al., 2001).

During protein degradation, ubiquitin is released from lysine residues of end-protein degradation products, catalyzed by ubiquitin-C-terminal specific proteases and isopeptidases. This is done in order to disassemble polyubiquitin chains, proofread wrongly ubiquitinated proteins, and potentially shorten unusually long polyubiquitin chains so they may be identified by the ubiquitin binding areas of the 19S complex. This release of ubiquitin aids in maintaining a free pool of cellular ubiquitin (Ciechanover et al., 2000).

The E3 ubiquitin ligases

Although E3 ubiquitin ligases have been shown to be primarily responsible for the selectivity, specificity and regulation of ubiquitin-mediated protein degradation, there remains a huge void in our understanding of these protein complexes and their mechanism of action. Very little is known regarding the E3 ubiquitin ligases and this is partly as a result of diversification of various E3 ligase families. They have been classified into four groups.

The first group, the “N-end rule” E3, is approximately 200 kDa protein and the best characterized E3 ubiquitin ligase. It identifies and binds “N-end rule” target proteins by their basic or bulky-hydrophobic N-terminal amino acid residues (Hershko and Ciechanover, 1998).

The HECT (homologous to E6-AP C-terminus) domain E3 ligases are the second class of E3 ligases. The first member of the HECT family was identified as E6-AP (E6-associated protein) and used by the human papillomavirus E6 in conjugating p53 via the viral protein cofactor, E6. The E6-AP involved formation of a high energy thiolester bond with ubiquitin and transfer of the activated ubiquitin from the cysteine residue to the substrate or the previously conjugated ubiquitin molecule of the polyubiquitin chain. Members of the HECT family of E3 ubiquitin ligases contain a C-terminus domain homologous to E6-AP. All of these HECT family members contain a conserved cysteine residue near the C-terminus that appears to accept an ubiquitin. The N-terminus is variable and probably acts like a substrate recognition domain (Ciechanover et al., 2000).

The third subset of E3 ligases is the multisubunit complexes involved in the degradation of cyclins. The best characterized is the APC (anaphase promoting complex)

which has ubiquitin ligase activity specific for cell cycle regulatory proteins that have a 9 amino acid proteolytic sequence known as a destruction box. Identified APC substrates consist of mitotic cyclins, certain anaphase inhibitors, and spindle-associated proteins. All of these proteins are destroyed at the end of mitosis.

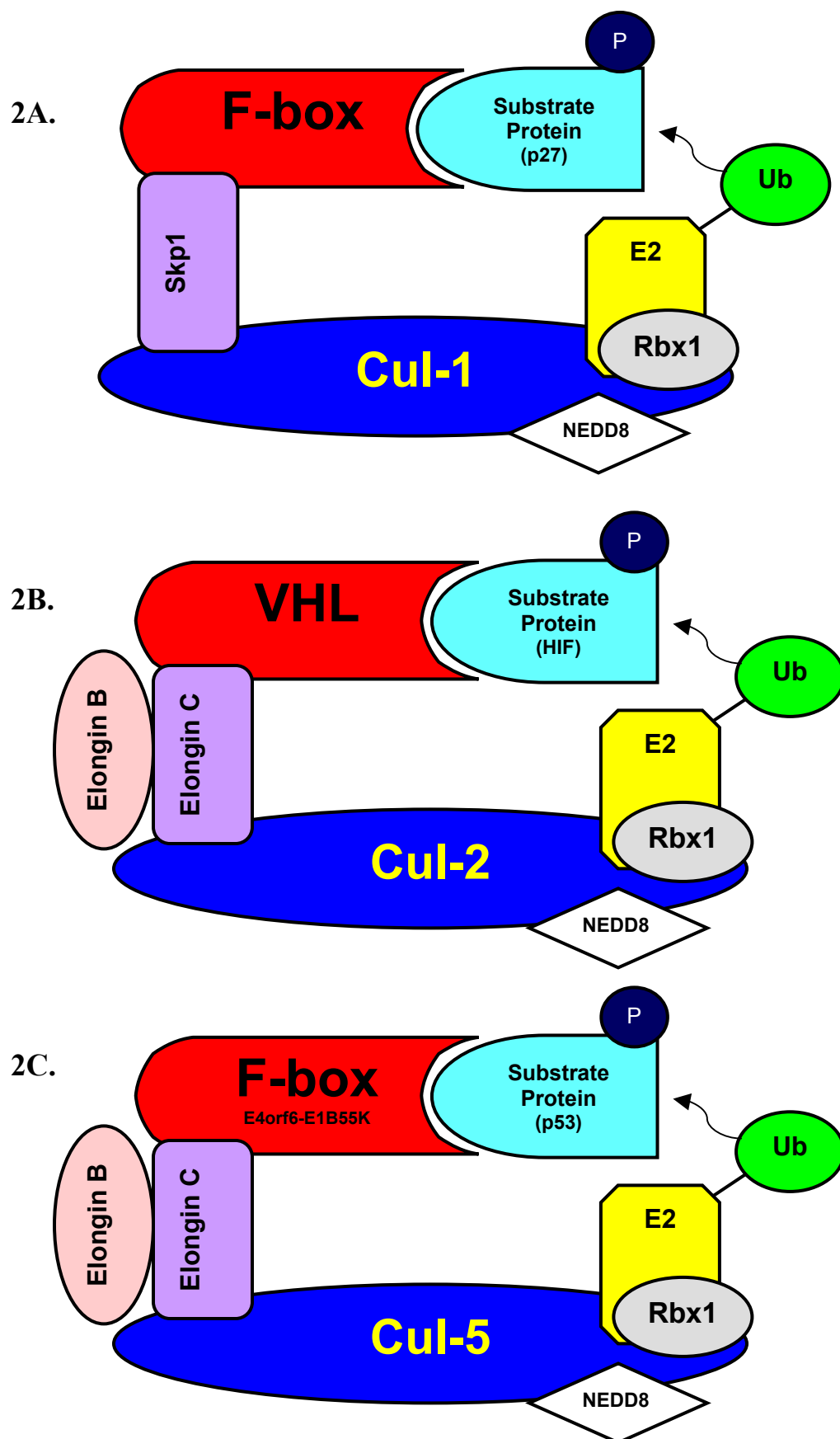
The fourth group of E3 ubiquitin ligases, originally identified in yeast, consists of large multimeric complexes responsible for the proteolysis of other cell cycle regulators and various proteins. The ortholog mammalian complexes have been described as SCF (Skp1, Cullin, F-box protein) complexes. These multiprotein complexes contain subunits that possess different roles, such as ligase activity, substrate recognition, E2 enzyme recognition, and adaptor functions. Cullin (Cul) is the core subunit of SCF complexes and many have been identified in both yeast and mammals that serve to ubiquitinate many phosphorylated substrate proteins, which indicates that a similar SCF mechanism is conserved across species (Deshaies, 1999). A subunit of the APC E3 complex also shares a sequence similar to cullin (Yu et al., 1998; Zachariae et al., 1998; Ohta et al., 1999), which further emphasizes an important role for this molecule in the ubiquitin protein degradation pathway. Cul is the subunit that binds an E2 enzyme and an adaptor protein called Skp1, which recruits the substrate recognition subunit, the F-box (Ulrich, 2002). Recently discovered RING (Really Interesting New Gene) finger proteins, represented as Rbx1 (RING-box protein), also known as ROC1 (regulator of cullins) were shown to interact with all cullin E3 ubiquitin ligases in catalyzing the ubiquitination of phosphorylated substrates (Kamura et al., 1999b; Ohta et al., 1999; Seol et al., 1999; Skowyra et al., 1999; Tan et al., 1999). This domain contains an area with many cysteine residues that enclose two structurally necessary zinc ions. Rbx1 may

act as a linker or adapter protein as it interacts with cullins, the E2 enzyme, and F-box proteins of the SCF complex (Tyers and Jorgensen, 2000).

Thus, presently there are four known subunits of SCF, which are assembled to form a heterotetrameric ubiquitin ligase (Figure 2A) (Deshaies, 1999). F-box proteins have been demonstrated to bind Skp1, and Skp1 in turn binds directly to Cul-1. Since Skp1 binds both to F-box proteins and Cul-1, it is suggested that their link may function to position these two subunits optimally for transferring of ubiquitin from the E2 enzyme to the substrate (Deshaies, 1999). However, Skp1 is only present in the Cul-1 complex, whereas other cullin homologs contain a similar protein, elongin C (Michel and Xiong, 1998).

In summary, the Skp1, cullin, and Rbx1 (RING finger) subunits are common core components of the SCF complex, while the F-box proteins of numerous different proteins function as substrate receptors and thus provide substrate specificity (Katayama et al., 2002). The RING domain, Rbx1, and Cul-1 form the catalytic core complex that recruits the E2, the variable F-box protein subunit binds the substrate and Skp1 serves as an adapter that links the F-box protein to Cul-1 (Skowyra et al., 1997; Zheng et al., 2002). The quaternary structure of the SCF E3 ubiquitin ligase was recently described as elongated with Rbx1 and Skp1-F-box complexes separated at the opposite ends (Zheng et al., 2002) and Cul-1 acting as a scaffold and interacting with all three subunits (Rbx1-Skp1-F box) (Figure 2A) (Zheng et al., 2002).

Figure 2. Hypothesized models for various cullin (Cul) E3 ubiquitin ligases (modified with permission (Querido et al., 2001)). Parallels between the Cul-1 (A), Cul-2 (B), and Cul-5 (C) ubiquitin ligase complexes. Abbreviations: 2A. (P) phosphorylation, (F-box) substrate recognition subunit or receptor, (Skp1) adaptor protein, (Rbx1) ring box protein, (E2) ubiquitin conjugating enzyme, (NEDD8) neural precursor cell-expressed and developmentally down-regulated modification, (Ub) ubiquitin. 2B. Same as in 2A and (VHL) von Hippel-Lindau substrate recognition subunit, (HIF1- α) hypoxic inducible transcription factor, (Elongin B and C) adaptor proteins. 2C. Same as in 2A ,2B, and (E4orf6-E1B55K) adenoviral F-box protein substrate recognition subunit for p53.



The Cullins

Cullins are a conserved family of genes with at least five members in *Caenorhabditis elegans* and seven in humans (Kipreos et al., 1996; Dias et al., 2002). As first described in yeast, Cdc53, the homologue to Cul-1, was known to function as a ubiquitin ligase, therefore the cullin gene family was named after the verb “cull,” which means to examine carefully as to select or reject, like the ubiquitin ligase selects which proteins are degraded (in conversation with Edward T. Kipreos). Currently there are seven identified mammalian cullin genes, sharing homology of approximately 200 amino acids at the C-terminus, the evolutionarily conserved cullin domain (Yu et al., 1998). Cullins interact with Rbx1 at the cullin domain to form the core ubiquitin protein ligase units that connect to the E2 ubiquitin conjugating enzymes and other known E3 ubiquitin ligase subunits to facilitate ubiquitin transfer to substrates (Patton et al., 1998; Kamura et al., 1999b; Ohta et al., 1999; Seol et al., 1999; Kamura et al., 2001).

Most of the cullin homologs remain poorly understood, except that Cul-2 has been shown to have similar function in assembling a ubiquitin ligase complex that includes Rbx1, elongin C, and SOCS-box family protein VHL. Homology to cullins was also found in the APC complex that is responsible for ubiquitination of mitotic regulatory proteins (Yu et al., 1998; Zheng et al., 2002).

The majority of biochemical and structural investigation of the cullins has been conducted on Cul-1, the original cullin discovered (Kipreos et al., 1996). Recently, the Cul-1 ubiquitin ligase pathway has been dissected with the recognition of many components. Cul-1 C-terminus, which is the sequence homologous with other cullins, is

necessary for both nuclear localization and NEDD8 (neural precursor cell-expressed and developmentally down-regulated) modification. Rbx1 indirectly supports NEDD8 conjugation by promoting Cul-1 nuclear accumulation. Mammalian cullins have been shown to require post-translational modification with NEDD8 (Hori et al., 1999). This covalent NEDD8 conjugation of cullins was found to be dependent on Rbx1 (Kamura et al., 1999a) and a requirement for ligase activity of mammalian SCFs, for without NEDD8, cullin ubiquitin ligase activity decreased significantly (Morimoto et al., 2000; Osaka et al., 2000; Podust et al., 2000; Read et al., 2000).

Since the C-terminal sequence, Rbx1 binding, and NEDD8 modification are similar and conserved by cullin family members, the ubiquitin ligase pathways identified with these components may be similarly used by other cullins (Furukawa et al., 2000). The C-terminal sequence and binding with Rbx1 are both required for efficient nuclear accumulation of cullin (Furukawa et al., 2000; Furstenthal et al., 2001), where then NEDD8 modification occurs and activates the ubiquitin ligase activity of the cullin complex. Furukawa et al. showed that transfected human Cul-1 in U2OS cells displayed a predominant nuclear pattern (81.9%), a small group of cells with high proportion in the cytoplasm (6.5%), and equal distribution of cytoplasmic and nuclear localization of Cul-1 in 11.6% of the cells. They report similar subcellular localization for Cul-2, Cul-4A, and Cul-5 where localization was predominantly in the nucleus of most cells and in the cytoplasm in smaller cell populations (Furukawa et al., 2000).

Functional roles of the cullins

Cullins are diversified in their ubiquitin ligase function as evidenced by the various cellular biochemical pathways they participate in regulating. The most well known and characterized cullin, Cul-1, is a component of the E3 ubiquitin ligase complex that degrades proteins (p27 or Kip1) that inhibit cyclin E-Cdk2 responsible for the transition from G1 to the S phase in the cell cycle (Furstenenthal et al., 2001). Cul-1 has also been demonstrated to be involved in the proteolysis of myc and the inhibitor to NFκB (Ciechanover et al., 2000). It has been shown to be crucial for survival, as mice with Cul-1 deletion did not survive past embryonic day 6.5 (Wang et al., 1999).

Cullin-2 (Cul-2), second to Cul-1 in characterization, binds elongin C, which shares homology with the N-terminal region of Skp1 (Lonergan et al., 1998); and elongin C binds to elongin B and the von Hippel-Lindau (VHL) tumor suppressor protein (Figure 2B) (Takagi et al., 1997). Cul-2 was shown to bind to VHL by the connector complex elongin B and C, and in association with Rbx1 resembles an ubiquitin ligase complex similar to the SCF ligases. The VHL protein acts as the substrate recognition and binding subunit, similar to the F-box protein (Iwai et al., 1999). In an analogy to the SCF E3 ubiquitin ligase, in the VHL-elongin C-elongin B complex, VHL serves as the F box receptor and elongin C as Skp1 subunits (Deshaies, 1999).

VHL contains a sequence shared with other proteins, known as the suppressor of cytokine signaling (SOCS) box. The SOCS was originally identified in proteins that negatively regulate cytokine-inducible signaling through the Jak/STAT pathway. SOCS box sequences from VHL, SOCS-1 and SOC-3 are necessary and sufficient to mediate

binding to elongin C (Kamura et al., 1998; Stebbins et al., 1999). Thus Cul-2 and elongin C may form the core ubiquitin ligase complex that also consists of elongin B, Rbx1 (Kamura et al., 1999b) and any one of a number of SOCS box containing proteins. The VHL complex in connection with Cul-2 and Rbx1 was described as an ubiquitin ligase, which targets the hypoxic inducible transcription factor (HIF1- α) for proteolysis (Tyers and Jorgensen, 2000).

Cullin-3 (Cul-3) was identified as a constituent in a pathway that controls cyclin E ubiquitination and in controlling S phase in mammalian cells (Singer et al., 1999). It was found to localize to the nucleus and Golgi, and homozygous deletion of the Cul-3 gene resulted in no viable progeny and elevated levels of cyclin E protein (Singer et al., 1999).

Cullin-4A (Cul-4A) and cullin-4B (Cul-4B) are poorly defined, but a recent report showed Cul-4A targeted ultraviolet damaged DNA-binding proteins believed to play a role in DNA repair, for ubiquitination and protein degradation (Chen et al., 2001). In addition, overexpression of Cul-4A was described in human breast cancer and other tumor types (Chen et al., 1998; Hori et al., 1999).

Similar to the other cullins, characterization of Cul-5 remains incomplete. Cul-5 was first identified as vasopressin-activated, calcium-mobilizing (VACM-1) receptor in the rabbit kidney medulla by Burnatowska-Hledin and associates. They describe this molecule to have affinity for vasopressin, resulting in intracellular calcium movement (Burnatowska-Hledin et al., 1995). Further analysis indicated this protein is homologous with Cul-5 (Byrd et al., 1997). A discrepancy in the literature concerning the cellular specificity and localization of Cul-5 ensued as histological studies demonstrated that the Cul-5 protein is primarily limited to vascular endothelial cells of the kidney medulla and

other organs examined (Burnatowska-Hledin et al., 1999). Other investigators, however, describe cul-5 mRNA distribution as extensive and primarily neuronal (Hurbin et al., 2000). Other findings suggest a possible Cul-5 role in different stages of the cell cycle as Cul-5 was reported to disappear during S phase and localize to the cytosol during cell division (Burnatowska et al., 2001).

Most recent investigation and evidence indicate Cul-5 is involved in cellular protein degradation mechanisms akin to other cullin gene members. Cul-5 has been reported to be a part of a multimeric protein complex that possesses ubiquitin ligase activity (Kamura et al., 2001) and its relationship with vasopressin uncertain. Another study investigating the degradation of p53, a cellular tumor suppressor, found Cul-5 to be a component of a protein complex that is necessary for the adenoviral protein, E4orf6-E1B55K, mediated p53 degradation (Kamura et al., 2001). They showed that Cul-5 localized to the nucleus with E4orf6 and that NEDD8 modification was critical for ubiquitination to occur. Their findings demonstrated that assembly of the viral protein, E4orf6, into a multiprotein complex containing E1B55K, elongins B and C, Cul-5, and Rbx1 is required for E4orf6-E1B55K dependent p53 turnover in cells. They conclude that Cul-5 is a player in the multiprotein machinery required for this mechanism of p53 *in vitro* ubiquitination and degradation. A model was proposed (Figure 2C) showing that the E4orf6-E1B55K complex is very similar to SCF and VHL E3 ubiquitin ligase complexes, in that each contains a substrate recognition module (F-box or VHL) linked to a cullin-Rbx1 subcomplex by either Skp1 or the Skp1-like elongin B/C. The interaction between the E4orf6 and the Cul-5/elongin BC-Rbx1 complex has not yet been determined, however it has been proposed that the E1B55K functions as the principal

substrate recognition component, similar to the F-box subunit of the SCF ubiquitin ligase (Harada et al., 2002). Another finding showed Cul-5-elongin BC-Rbx1 complex associates with yet another novel protein (Muf1) a leucine-rich BC box protein, to form an active ubiquitin ligase (Kamura et al., 2001).

Cullin-7 (Cul-7) is the newest member of the cullin gene family. It has recently been identified as the seventh mammalian cullin containing a cullin domain that is responsible for binding Rbx1, and assembles a SCF-Rbx1 like E3 ubiquitin ligase complex consisting of Skp1, Cul-7, the Fbx29 (F-box protein), and Rbx1 (Dias et al., 2002). Incidentally, since the homologue of Cul-1 in *Caenorhabditis elegans* was recently named Cul-6, this novel protein was given the name Cul-7 (Dias et al., 2002).

Research plan

The investigation of ubiquitination and protein degradation via the proteosomal pathway is a relatively new field and many unknowns remain. Consequently, a huge void exists pertaining to various molecules involved in this protein regulatory process. There continues to be a considerable lack of knowledge regarding cullins and their definitive roles in proteolysis as the cullin gene family was just described in 1996 (Kipreos et al., 1996) with Cul-1 receiving the focus of most examination. Since Cul-5 was originally described as a vasopressin activated molecule and we were interested in examining the environmental and cellular stressors related to the military (e.g. water deprivation and hemorrhagic shock), we embarked on a research project that investigated Cul-5. We planned to characterize Cul-5 whereby clarifying its distribution and localization. We also desired to examine its expression response in cellular stressors, since ubiquitination

and proteasomal degradation participate in many important cellular processes including modulation of the cellular response to stress (Ciechanover et al., 2000).

The goals of this research project were to investigate and characterize a novel protein, Cul-5, and its response to cellular stressors that are extremely pertinent to the health and well-being of military troops. The hypothesis tested was that Cul-5 is located in multiple areas of the brain and that it responds to cellular stressors related to altered water homeostasis. The objectives of this research project were to: (1) identify what organs of the body express Cul-5, (2) determine the neuroanatomical distribution of Cul-5 in the central nervous system (CNS), and (3) examine Cul-5 expression patterns in relation to cellular stressors of water deprivation and hemorrhagic shock.

Experimental approaches

Characterization of cul-5 mRNA expression and distribution in the CNS and in various tissues and organs of the laboratory rat was accomplished employing Northern blotting and polymerase chain reaction (PCR). The size of the cul-5 transcript in the rat is approximately 6.3 kb and quantitative realtime PCR showed that expression levels are similar in all 12 tissues examined. These data provide an extensive description of transcript size and prevalence of cul-5 mRNA in the laboratory rat (Chapter 2) (Ceremuga et al., 2001). Cul-5 was further characterized by pinpointing its cellular specificity and localization, showing that cul-5 mRNA is present in various cell types in the rodent CNS and Cul-5 protein is expressed ubiquitously in the rat brain (Chapter 3) (Ceremuga et al., 2003a).

Quantitative real-time PCR was used in order to investigate and measure cul-5 mRNA expression in response to cellular stressors of altered water homeostasis (water deprivation and hemorrhagic shock). Significant increases in cul-5 mRNA levels in the rat CNS and kidney were demonstrated in response to *in vivo* hyperosmotic stress (Chapter 4) (Ceremuga et al., 2003c). Whereas, significantly altered cul-5 mRNA levels were found in various CNS regions in the rat following hemorrhagic shock (Chapter 5) (Ceremuga et al., 2003b).

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CHAPTER 2

VASOPRESSIN-ACTIVATED CALCIUM-MOBILIZING (VACM-1) RECEPTOR mRNA IS PRESENT IN PERIPHERAL ORGANS AND THE CENTRAL NERVOUS SYSTEM (CNS) OF THE LABORATORY RAT

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Thomas E. Ceremuga^{1,2*}, Xiang-Lan Yao², and Joseph T. McCabe^{1,2}

*To whom correspondence should be addressed:

Email: tceremuga@usuhs.mil

Tel: 301-295-9366

FAX: 301-295-1715

¹Graduate Program in Neuroscience

²Department of Anatomy, Physiology and Genetics

F. Edmund Hébert School of Medicine

Uniformed Services University of the Health Sciences

4301 Jones Bridge Road

Bethesda, MD 20814

U.S.A.

Short title for page headings: *“Vasopressin-activated calcium-mobilizing receptor localization”*

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ABSTRACT

The distribution and physiological role of the neuropeptide, arginine vasopressin (AVP), and its three receptor subtypes, V_{1a} , V_{1b} and V_2 , has been well described. A fourth AVP receptor, VACM-1, was recently discovered and appears to be a member of the cullin gene family. The objective of this research is to characterize VACM-1 receptor mRNA expression in the CNS as well as in various tissues and organs of the laboratory rat.

Northern blotting of poly(A)⁺ RNA from various tissues demonstrated the size of VACM-1 mRNA in the rat is approximately 6.3 kb. RT-PCR indicated the transcript is present in all twelve tissues examined: brainstem, cerebral cortex, cerebellum, hypothalamus, aorta, gastrointestinal tract, heart, kidney medulla, liver, lung, skeletal muscle, and spleen. Quantitative realtime PCR confirmed RT-PCR results that VACM-1 mRNA is in all organs and tissues and expression levels are similar in all tissues examined. The transcript encoding VACM-1, a novel AVP receptor, appears to be ubiquitously expressed in various tissues of the laboratory rat. VACM-1 shares some similarities with both V_1 and V_2 receptors, as it binds AVP analogues that independently recognized either of these receptors. Therefore, many functions ascribed to activation of the previously known AVP receptors could at least in part be mediated by VACM-1.

INTRODUCTION

The neuropeptide, arginine vasopressin (AVP), regulates a variety of metabolic functions that maintain homeostasis. Synthesized in magnocellular neurons in the hypothalamus,

AVP is secreted from the posterior pituitary into the circulation following adequate stimuli, such as plasma hyperosmolality and hypovolemia. Parvocellular neurons of the paraventricular nucleus also synthesize AVP and neurons from this site control neuroendocrine and autonomic responses to stress. AVP receptors are distributed in various tissues of the body, where the three AVP receptor subtypes, V_{1a} , V_{1b} , and V_2 , activate their vast array of physiological activities. The V_1 receptor's response to AVP is quite pervasive and is associated with the regulation of blood flow (1), gluconeogenesis (2), platelet aggregation (3), learning (4), stress (5), antipyretic activity (6), development (7), and mitogenesis (8,9). The role of the V_2 receptor appears more defined and is associated with regulating water reabsorption in the kidney (10), although V_2 receptors may have other functions associated with their localization in other tissues and organs (11,12). Recently, Burnatowska-Hledin and associates identified VACM-1 in the rabbit kidney medulla. This receptor has strong affinity for AVP, results in intracellular calcium movement, and is inhibited by V_1 receptor antagonists and V_2 receptor agonists (13). Additional analysis of VACM-1 indicates this protein is homologous with another protein, cullin-5. The cullin gene family encodes proteins that mediate cell cycle regulation (14-17).

The amino acid sequence of the VACM-1 protein shows no homology to any of the known AVP receptors (13), but it exhibits wide tissue distribution. VACM-1 mRNA transcripts are reported in the rabbit kidney medulla, brain, heart, and ovaries, as well as in the human kidney, heart, placenta, skeletal muscle, and brain (18). Histological studies demonstrated that VACM-1 is localized in the vascular endothelial cells of the kidney medulla (18). Another group, using *in situ* hybridization to investigate the distribution of

VACM-1 mRNA, found the receptor to be widely distributed throughout the brain (19). In addition, they propose that VACM-1 is primarily located on neurons, dissimilar to the original studies (13,18) that localized VACM-1 to vascular endothelial cells. Further accounts of this receptor are needed. We report here our initial characterization of the VACM-1 transcript and its tissue distribution in the laboratory rat.

MATERIALS AND METHODS

Animals

Six male Long-Evans rats (275-300g b.w., Harlan Sprague Dawley Laboratories) received intraperitoneal injections of Ketaset (80 mg/kg b.w.) and Rompun (13 mg/kg). When unresponsive to paw pinch, they were decapitated to obtain tissue samples of the CNS (brainstem, cerebellum, cerebral cortex, hypothalamus) and organ tissue from aorta, gastrointestinal (small intestine), heart, kidney medulla, liver, lung, skeletal muscle, and spleen. These tissues were collected, weighed, frozen, and stored in liquid nitrogen.

VACM-1 DNA Cloning

Reverse transcription polymerase chain reaction (RT-PCR) was used to create cDNA from total RNA from rat hypothalamus and kidney medulla. We designed specific primers according to the rat sequence for VACM-1 (19) as follows: 5'-GAAGCAAAAAGGAGTGGGGTTG-3' (5' primer) and 5'-

CTAAAACTTACGACCACGAACC-3' (3' primer); and rat glyceraldehyde-3-phosphate dehydrogenase GAPDH; (20): 5'-CAACGACCCCTTCATTGACCTC-3' (5' primer) and 5'-CGGTAGTGACGGTGAGTCTTCT-3' (3' primer). These primers produced amplified products of 442 base pairs (bp) and 461 base pairs, respectively. The amplified PCR products for both VACM-1 and GAPDH were then purified and subcloned into a pGEM-T Easy Plasmid vector (Promega) and restriction enzyme digestion verified appropriate sizes. Plasmid DNA sequence also confirmed the PCR product for VACM-1 and GAPDH.

RNA Extraction and Northern Analysis

Tissue RNA was isolated from the following organs and tissues: brainstem, cerebellum, cerebral cortex, hypothalamus, aorta, gastrointestinal (small intestine), heart, kidney medulla, liver, lung, skeletal muscle, and spleen using the Totally RNA™ Kit (Ambion). In brief, frozen tissue was crushed with mortar and pestle in liquid nitrogen and then dispersed in denaturation buffer (guanidine thiocyanate solution) with a sonicator. The RNA was extracted using phenol/chloroform and precipitated with isopropanol. Agarose gel electrophoresis (2 µg total RNA) was employed to visualize the quality of ribosomal RNA bands.

For Northern blot analysis, tissue total RNAs were isolated, which were then used to obtain poly(A)⁺ RNA using a MicroPoly(A)Pure™ mRNA Isolation Kit (Ambion). Northern blot analysis was conducted using standard lab methods (21) and according to the manufacturer's recommendations of NorthernMax™ Kit (Ambion). In brief, for the

detection of VACM-1 and GAPDH mRNAs, respectively, 7-10 µg or 0.75 µg of poly(A)⁺ RNA from the 12 rat tissues described above were separated on a 1% agarose formaldehyde gel, transferred to BrightStar-PlusTM nylon membranes (Ambion), and cross-linked by ultraviolet light using a UV StratalinkerTM 1800 (Stratagene). Northern blots were prehybridized with ULTRAhybTM (Ambion) for one hour at 42°C, followed by hybridization for 24 hours at 42°C with the same solution and 1×10^6 counts⁻¹ x ml⁻¹ of the [α -³²P]dCTP-labeled denatured probes for either VACM-1 or GAPDH. The blots were washed twice with Low Stringency Wash Solution #1 (Ambion) for 5 minutes at room temperature, and washed twice with High Stringency Wash Solution #2 (Ambion) for 15 minutes at 42°C. The hybridized blots were then apposed to autoradiography film (Kodak BiomaxTM MS). The size of hybridized transcripts was then compared to the migration of a 0.24-9.5 kb RNA Ladder (Gibco BRL, Life Technologies, Inc.). The log₁₀ of the known standards were plotted on semi-log paper. These points were connected with a smooth curve, which was then used to estimate the transcript size of the hybridized band.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

For PCR amplification, total RNA from the aforementioned tissues and organs was treated with 10 units of DNase I per 20µg of RNA as recommended by the manufacturer for DNA-freeTM reagents (Ambion) to prevent spurious amplification of genomic DNA. RT-PCR was performed using 2 µl (1.4 µg) of total RNA in a 20µl standard RT reaction

using the GeneAmp® RNA PCR kit (Applied Biosystems) to synthesize cDNA, carried out at 42°C for 15 minutes, 99°C for 5 minutes, and 5°C for 5 minutes. Similar reactions without MuLV Reverse Transcriptase were performed simultaneously to assess genomic DNA contamination. The primers used for probe preparation for Northern blotting were again used to amplify tissue VACM-1 and GAPDH mRNAs. Gel electrophoresis and DNA sequencing was used to verify PCR products for VACM-1 (442 bp) and GAPDH (461 bp).

Quantitative Realtime PCR (QRT-PCR)

Total RNA from CNS, peripheral tissues and organs was used to prepare cDNA using MuLV reverse transcriptase as described earlier. To rule out DNA contamination, similar DNase I treatment was done and reactions were performed simultaneously, but without inclusion of MuLV reverse transcriptase. Using PrimerExpress software (Applied Biosystems), primers were designed for rat VACM-1 (19), GAPDH (20), and cyclophilin (22) as follows: VACM-1: 5'-GCTGATCGAGCACAAGTACATCA-3' (5' primer) and 5'-GTATATGTACCGCATCGACTTAGG-3' (3' primer); GAPDH: 5'-TGCCAAGTATGATGACATCAAGAA-3' (5' primer) and 5'-TGATTTCCTCGTAGGACCCGA-3' (3' primer); and CYC: 5'-TGTGCCAGGGTGGTGACTT-3' (5' primer) and 5'-TTCAGGTAGATGCCTCTCTTTAAACT-3' (3' primer). Templates (three replicates per sample) were loaded into a MicroAmp Optical 96-Well Reaction Plate (Applied

Biosystems). In brief, 5µl of cDNA template was placed in microwells for each tissue sample, followed by the addition of 17.5µl of a master mix [12.5µl SYBER Green (Applied Biosystems), 2.5µl distilled H₂O, 2.5µl (3µM) of each forward and backward primer set]. The GeneAmp 5700 Sequence Detection System machine (Perkin-Elmer) was used for amplification and employed a two-temperature cycling program (denaturation at 95°C for 15 seconds, followed by annealing and extension cycles at 60°C for 1 minute) for a total of 40 cycles. At the completion of the amplification run, GeneAmp software was used to inspect the dissociation curve plots to assess possible instances of contamination or the generation of multiple PCR products. Amplification plots, also generated by the software, were inspected to assign values for the “cycle threshold” (Ct), a value above baseline (random) amplification where SYBER Green fluorescence with each subsequent amplification is within the exponential phase of PCR amplification. In parallel with measures of mRNA quantities in each tissue sample, standard curves were generated to correlate Ct values with dilutions of cDNA samples (see ABI Prism 7700 Sequence Detection System User Bulletin #2, 1997, for details concerning the standardization procedure). In brief, decreasing amounts (3-fold dilutions beginning with 20 µl of cDNA from 1.4 µg of sample RNA, 3 replicates per dilution amount) of cDNA from rat lung tissue was used to generate relative standards for each target mRNA (VACM-1, GAPDH and CYC mRNAs). Values for measures of GAPDH and CYC mRNA were used as endogenous references to control for differences in harvested RNA samples across various tissue samples (3 replicates per sample). RNA measures for VACM-1, GAPDH and CYC were determined for each of the 12 tissues

from three rats and values obtained for VACM-1 were standardized to GAPDH and CYC.

Statistical Analyses

As a means for reducing numerical variability amongst the tissue types and amongst values obtained from independent PCR assays, measures of VACM-1 mRNA (Qty values, see Applied Biosystems software) from each sample (per measures of GAPDH and CYC) were converted to natural logarithmic (ln) values. A single factor analysis of variance was then employed (SPSS Version 10.0.5 for Windows software) and the Newman-Keuls test for *post hoc* comparisons was used to assess the prevalence of VACM-1, relative to GAPDH and to CYC, in the 12 tissue samples. Data in the figures summarize the results of three independent experiments, and values are standardized to make the experiments comparable to each other. This was achieved by computing mean ratios of VACM-1 to GAPDH and CYC and then subtracting the mean values for VACM-1 (relative to GAPDH or CYC) across the 12 samples within each separate experiment. The mean and standard error of the mean for each tissue was then computed, and the antilog of the arithmetic average was computed as the geometric mean for each tissue sample. The standard error of the geometric mean was computed by multiplying the geometric mean values for each tissue by the ln of the standard error of the mean [see Casella and Berger, 1990, p. 330, for additional information (23)].

RESULTS

Northern blot analysis of 30 µg of total RNA from various rat tissues produced no visible band when hybridized with a [α - ^{32} P]dCTP labeled probe for VACM-1; although GAPDH displayed a strong signal on the autoradiograph (data not shown). We then used 7-10 µg poly(A)⁺ RNA from the 12 tissue types: brainstem, cerebellum, cerebral cortex, hypothalamus, aorta, gastrointestinal (small intestine), heart, kidney medulla, liver, lung, skeletal muscle, and spleen to perform Northern blot analysis. All tissues we examined showed a prominent VACM-1 transcript of approximately 6.3-kb. At the same time, 0.75 µg poly(A)⁺ RNA from the 12 tissue types were probed for GAPDH mRNA as an internal control. All tissues showed an appropriate transcript size of 1.4-kb for GAPDH mRNA (Figure 1).

In accord with Northern blotting results, RT-PCR of total RNA from the same 12 rat tissues, plus from whole kidney, indicated VACM-1 transcripts are present in all samples. Consistent with the reported sequence in the rat, our primers amplified a product of 442 bases in length (Figure 2), and sequencing verified the product was homologous with the rat sequence. Amplification of the same samples of total RNA with GAPDH primers resulted in the amplification of a product size of 461 bases, in accordance with the known sequence for GAPDH. Gels from RT-PCR reactions where no RT enzyme or no tissue RNA was added to the reaction, showed no visible band (data not shown).

Quantitative realtime PCR was used to measure the abundance of VACM-1 mRNA in various tissues and organs. When standardized with GAPDH or with cyclophilin, there were significant differences in the amounts of VACM-1 mRNA across

tissue samples (Figure 3). When standardized with GAPDH measures, *post hoc* analysis indicated there was significantly greater amounts of VACM-1 mRNA in the lung (LG in Figure 3, left panel), than in all other tissue samples, while VACM-1 mRNA levels in the other 11 tissues were not different. Normalization with cyclophilin, however, indicated levels of VACM-1 mRNA were greater in the aorta (AO in Figure 3, right panel) and in the heart (HT), than in other tissues, with the exception of skeletal muscle (SM). Levels of VACM-1 mRNA in skeletal muscle was greater than the amounts observed in all other tissues (SM Figure 3, right panel). The use of both GAPDH and cyclophilin as a standard for control of variability in the amount of input template underscores the need for inclusion of multiple standards (cf. Table 1). A separate series of three experiments (Experiment 2 in Table 1), where levels of GAPDH and cyclophilin were measured simultaneously in the same tissue samples, indicated the proportion of GAPDH to cyclophilin in tissues can vary widely (Figure 4). Levels of GAPDH (relative to cyclophilin) in samples of skeletal muscle, for example, were almost 70-fold greater than in most other tissues, with the exception of the heart where levels of GAPDH to cyclophilin were about 4 times greater than in other tissues.

DISCUSSION

This report provides an extensive description of transcript size and prevalence of VACM-1 mRNA in the laboratory rat. Northern blotting indicates VACM-1 mRNA is approximately 6.3kb in nucleotide length in all tissues, suggesting there are no predominant splice variants. This size is analogous to the reported size (6.4 kb) in the rat

(17) and rabbit (13), but is shorter than the human 7.5kb VACM-1 sequence originally reported by Burnatowska and Byrd and their colleagues (13,14). Northern blot results by Hori and coworkers, however, find Cul-5 (VACM-1) mRNA is less than 7.5kb in human tissue samples (24).

PCR of total RNA samples from 12 tissues indicate the transcript is ubiquitous. Our results are in agreement with findings that describe the presence of VACM-1 mRNA in rat cerebral cortex, cerebellum, hippocampus, liver and kidney (19). In the rabbit, VACM-1 mRNA was not found in the liver, gastrointestinal or aorta (13). In human samples of poly(A)⁺ RNA, Northern blotting failed to detect VACM-1 (Cul-5) mRNA in lung tissue samples (18,24). Our RT-PCR and realtime PCR findings, however, verified our Northern blot data that, at least in the rat, VACM-1 mRNA is present in all of these tissues.

The use of GAPDH and cyclophilin mRNAs as endogenous controls for variation in the amount of input total RNA permitted us to determine that levels of VACM-1 mRNA are similar in various tissue samples. When GAPDH mRNA is used to estimate VACM-1 mRNA, levels were approximately the same in all tissues with the exception of the lung, where levels were significantly greater than all other tissue samples. The apparent higher levels of VACM-1, compared to GAPDH, in the lung may be related to the density of vasculature. As originally shown, VACM-1 expression has been localized to endothelial cells (18). In the rat, endothelial cells are the largest cell population in the lung, comprising as much as 43% of all cell types (25). VACM-1 may also be expressed in additional cell types in the lung further raising VACM-1 mRNA levels in this tissue. Another explanation may be due to the expression levels of GAPDH mRNA that were

lowest in lung tissue samples (as shown in Table 1), and the use of GAPDH as a comparator may have artificially inflated our estimate of VACM-1 mRNA levels in lung tissue. It is known that GAPDH mRNA abundance varies across tissues in the rat. GAPDH mRNA levels are higher, for example, in skeletal muscle and heart, compared to liver, brain and kidney (26). No comparisons of GAPDH levels in tissues relative to lung, however, have been reported. When cyclophilin was employed as a means for comparing total RNA levels, lung tissue was estimated to contain levels of VACM-1 mRNA that were not significantly different than other tissues. In this case, levels of VACM-1 mRNA were higher in skeletal muscle, aorta and heart tissues. Again, levels of cyclophilin are known to vary from tissue to tissue, and the latter may explain these results. It was also shown by Danielson (27) that cyclophilin mRNA levels were lowest in skeletal muscle in rat tissues. We observed levels of cyclophilin were lowest in skeletal muscle, aorta and heart, in line with these tissues exhibiting the highest levels of VACM-1 mRNA (Table 1).

Numerous reports using Northern blots, RT-PCR and gene microarrays conclude, as was employed in the present study, that normalization of input amounts of RNA is required to establish a basis for comparison (28). Ideally, one would examine several housekeeping genes to use as a comparator and choose as the “correct” endogenous control, a transcript that does not exhibit variation across experimental treatment samples. For comparisons across a wide variety of tissue types, however, there may be no ideal comparator (29). The recent study by Zhong and colleagues (30), for example, compared levels of GAPDH, β -actin, 28s rRNA, and cyclophilin among various cell lines and reported 28s levels are the most consistent in various samples, including when the cells

were stressed by incubation under hypoxic conditions. Shinohara and colleagues (31) report that GAPDH (*ccg-7*) mRNA levels in *Neurospora* exhibits a circadian rhythm, but that cell cultures did not exhibit variations in GAPDH as a function of the application of cell stressors (heat shock, osmotic stress, etc.). In the present study, we have employed an approach suggested by several authors (22,32), where two comparators were used to assess the relative levels of VACM-1 mRNA in various tissues.

In conclusion, this report has characterized the size and abundance of VACM-1 mRNA in various tissues and organs of the laboratory rat. All of the 12 tissues examined produced positive patterns of expression of VACM-1 that leads us to hypothesize that this molecule is ubiquitous. Additionally, these data support the need for caution in regard to appropriate internal control utilization when normalizing RNA, as there appears to be differences in the amount of VACM-1 mRNA across tissue types that are dependent upon using GAPDH or cyclophilin as endogenous controls. This report emphasizes the need for prudence and judicious use of internal control genes as essential factors when making accurate quantitative transcription measurements.

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Table 1. Average (ln Mean $\pm$ SEM) transcript levels of GAPDH, CYC and VACM-1 in rat tissues and organs*

Experiment 1				Experiment 2	
<u>Tissue Type</u>	<u>GAPDH</u>	<u>CYC</u>	<u>VACM-1</u>	<u>GAPDH</u>	<u>CYC</u>
BS	6.25 $\pm$ 1.19	5.23 $\pm$ 0.44	3.57 $\pm$ 0.33	6.12 $\pm$ 0.19	5.47 $\pm$ 0.32
CB	5.45 $\pm$ 0.90	4.51 $\pm$ 0.60	4.95 $\pm$ 0.45	4.81 $\pm$ 0.25	4.99 $\pm$ 0.31
CC	5.83 $\pm$ 1.37	5.10 $\pm$ 0.95	4.93 $\pm$ 0.59	6.13 $\pm$ 0.65	5.59 $\pm$ 0.27
HY	5.22 $\pm$ 0.53	4.82 $\pm$ 0.32	4.68 $\pm$ 0.16	5.35 $\pm$ 0.20	5.07 $\pm$ 0.33
AO	2.34 $\pm$ 2.02	0.16 $\pm$ 0.72	1.77 $\pm$ 0.60	1.06 $\pm$ 1.60	-0.47 $\pm$ 1.03
GI	5.38 $\pm$ 1.50	4.32 $\pm$ 0.41	3.40 $\pm$ 0.50	5.08 $\pm$ 0.60	4.33 $\pm$ 0.28
HT	5.44 $\pm$ 0.71	1.65 $\pm$ 0.72	3.84 $\pm$ 0.63	4.42 $\pm$ 1.08	1.85 $\pm$ 0.55
KM	4.98 $\pm$ 1.13	4.37 $\pm$ 0.68	3.73 $\pm$ 0.22	5.18 $\pm$ 0.27	3.67 $\pm$ 0.52
LI	4.44 $\pm$ 0.73	4.47 $\pm$ 0.58	4.38 $\pm$ 0.26	4.81 $\pm$ 0.26	4.47 $\pm$ 0.32
LG	1.42 $\pm$ 2.10	2.90 $\pm$ 0.65	3.72 $\pm$ 0.55	2.73 $\pm$ 0.92	3.38 $\pm$ 0.45
SM	2.75 $\pm$ 2.87	-1.38 $\pm$ 1.48	1.67 $\pm$ 1.34	3.92 $\pm$ 2.00	-1.28 $\pm$ 1.40
SP	2.91 $\pm$ 0.19	3.27 $\pm$ 0.40	2.64 $\pm$ 0.59	2.17 $\pm$ 1.01	3.60 $\pm$ 1.02

* Experiment 1 data refers to findings summarized in Figure 3; Experiment 2 data refers to findings summarized in Figure 4. See legend of Figure 3 for abbreviations.

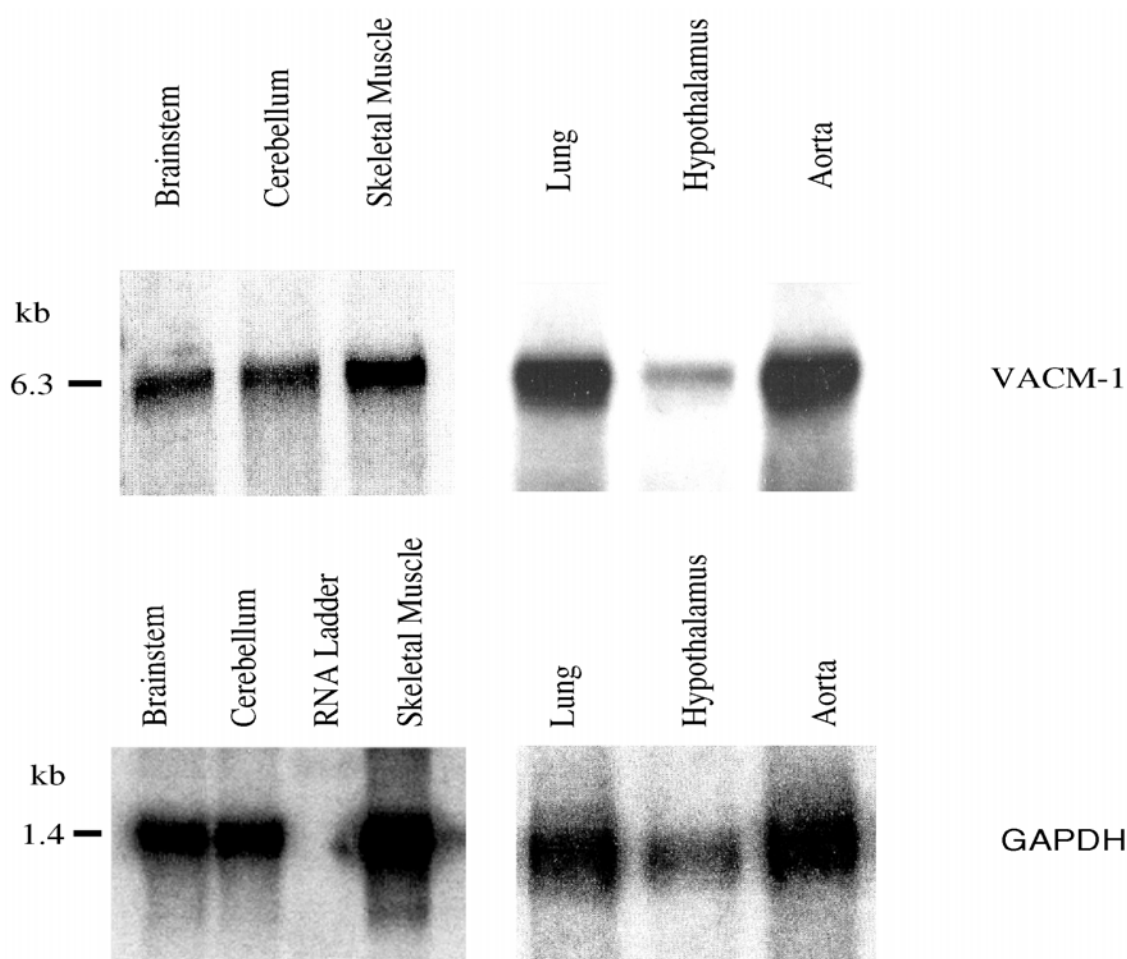


Figure 1. Representative samples of Northern blots showing hybridization for VACM-1 mRNA in the poly(A)⁺ samples from brainstem, cerebellum, skeletal muscle (left top blot) and in the lung, hypothalamus, and aorta (top right blot). Tissue poly-A⁺ RNA was also probed in the corresponding tissues for GAPDH (bottom blots). The third lane in the left bottom blot was a RNA ladder. VACM-1 mRNA was ~6.3kb in length and was detected in all 12 tissue samples tested (all data not shown).

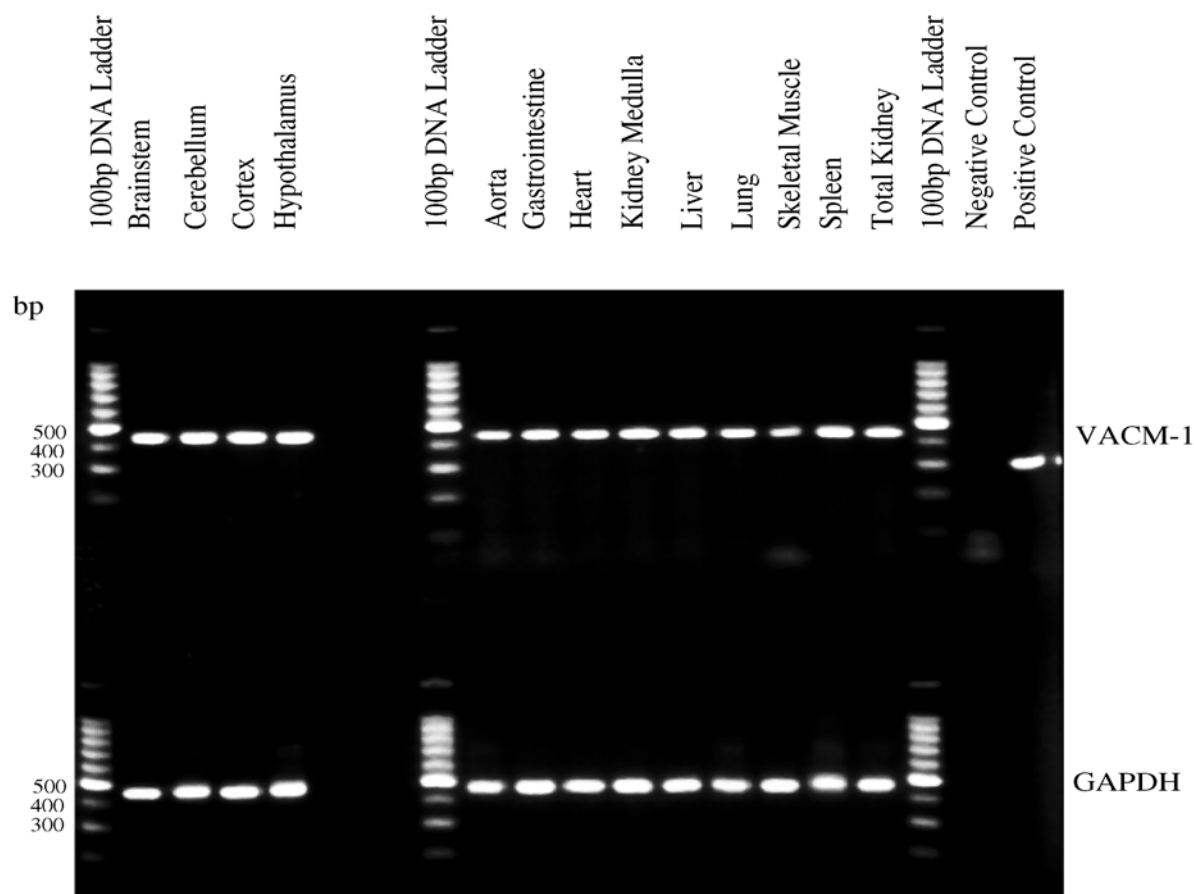


Figure 2. Reverse transcription-polymerase chain reaction amplification of VACM-1 and GAPDH. Total RNA (1.4 μ g) samples from brainstem, cerebellum, cerebral cortex, and hypothalamus, as well as from aorta, gastrointestinal, heart, kidney medulla, liver, lung, skeletal muscle, spleen, and total kidney all exhibited a strong amplification of VACM-1 sequence cDNA (top panel). Primers homologous to GAPDH also indicated a strong amplified product. A negative control (water) failed to produce an amplified product, while primers to amplify a positive control (pAW109) produced a band of 308 bp (Perkin Elmer). DNA sequencing of amplified product confirmed the VACM-1 primers amplified the appropriate target sequence.

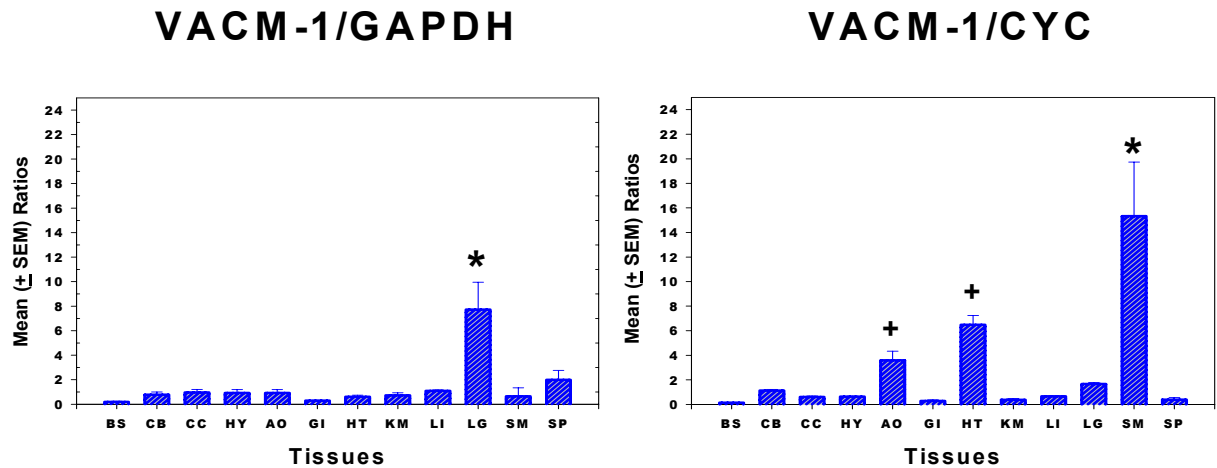


Figure 3. Measurement (mean $\pm$ SEM) of VACM-1 mRNA in various tissues, relative to GAPDH (left graph) or cyclophilin (CYC) (right graph). When GAPDH was used as a comparator, levels of VACM-1 were significantly greater in the lung (LG) than levels in all other tissues (left graph, * p <0.05). When cyclophilin (CYC) mRNA levels were used as a comparator, VACM-1 mRNA levels in skeletal muscle (SM) samples were significantly higher than in all other tissues (* p <0.05), while levels in the aorta (AO) and heart (HT) were greater than levels in all other tissues (+ p <0.05) with the exception of skeletal muscle. Abbreviations: brainstem (BS), cerebral cortex (CC), cerebellum (CB), hypothalamus (HY), aorta (AO), gastrointestinal (GI), heart (HT), kidney medulla (KM), liver (LI), lung (LG), skeletal muscle (SM), and spleen (SP).

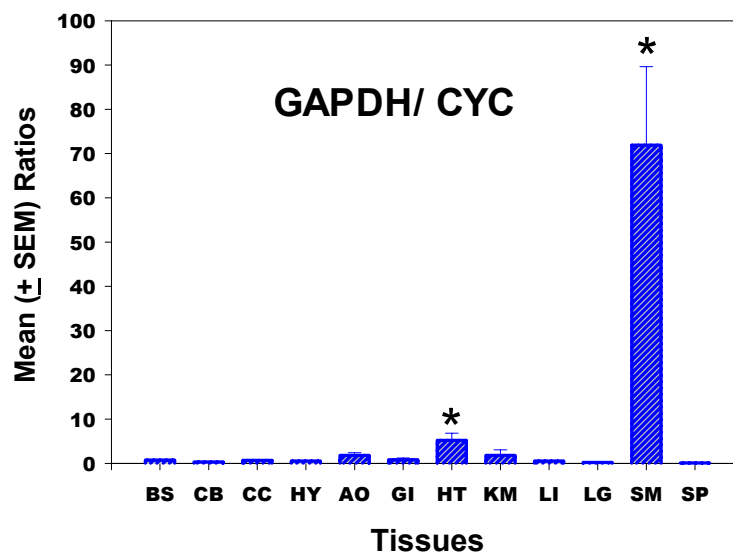


Figure 4. Expression pattern of GAPDH mRNA in various tissues, relative to levels of cyclophilin (CYC) mRNA. Levels of GAPDH mRNA were significantly greater in skeletal muscle (SM) than in all other tissue samples (* $p < 0.05$). Levels of GAPDH were also higher in the heart (HT), aorta (AO) and kidney medulla (KM) than in all other tissues, while levels were lowest in the spleen (SP) and lung (LG). Abbreviations are listed in the legend of Figure 3.

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CHAPTER 3

Cullin-5 is Ubiquitous in the Rat Brain

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Thomas E. Ceremuga^{1,2*}, Xiang-Lan Yao², and Joseph T. McCabe^{1,2}

*To whom correspondence should be addressed:

Email: tceremuga@usuhs.mil

Tel: 301-295-9366

FAX: 301-295-1715

¹Graduate Program in Neuroscience

²Department of Anatomy, Physiology and Genetics

F. Edmund Hébert School of Medicine

Uniformed Services University of the Health Sciences

4301 Jones Bridge Road

Bethesda, MD 20814

U.S.A.

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Title: Cullin-5 is Ubiquitous in the Rat Brain

Abstract

Cullin-5 (Cul-5), an E3 ubiquitin ligase that covalently binds ubiquitin to proteins targeted for degradation via the proteasome, was examined for its localization and distribution in the rat central nervous system (CNS). We showed cul-5 mRNA expression in rat neuronal, glial, and vascular endothelial cells by reverse transcription-polymerase chain reaction (RT-PCR) and corroborated these data by Cul-5 immunostaining in neurons, astrocytes, blood vessels, and choroid plexus of the laboratory rat. Widespread and ubiquitous expression of Cul-5 in the brain suggests that it may have a vital role(s) in cellular activities of the CNS.

Keywords: Cullin, cul-5, gene expression, immunocytochemistry, ubiquitin ligase, proteasome

Protein degradation is an important and complex mechanism for the regulation of various cellular activities. A chain of ubiquitins (**Ub**), a 76 amino acid, highly conserved protein, is attached to proteins destined for degradation by a series of molecular reactions. The assembly of the polyubiquitin chain involves an E1 (Ub-activating) enzyme, an E2 (Ub-conjugating) enzyme, and an E3 (Ub-ligase) enzyme. The E3 ligase is responsible for the selectivity of protein degradation by covalently binding polyubiquitin to proteins targeted for degradation by the proteasome [6,9]. Cullin-5 (**Cul-5**), a member of the cullin gene family [12], has been identified as an E3 ubiquitin ligase [11].

Although our laboratory previously showed that the cul-5 transcript is present in the CNS (brainstem, cerebellum, cerebral cortex, hypothalamus), aorta, small intestine, heart, kidney, liver, lung, skeletal muscle, and spleen [4], inconsistencies concerning cellular specificity of Cul-5 remain. Conflicting findings have been described in previous investigations in the CNS, where Cul-5 localization was reported to be exclusive to vascular endothelial cells in the cortex via immunocytochemistry [2]. While another group, using *in situ* hybridization, reported primarily neuronal distribution of cul-5 mRNA in the rat brain [10]. Our goal was to further characterize Cul-5 by pinpointing its cellular specificity and localization. We found that cul-5 mRNA is present in various cell types of the rodent CNS and Cul-5 protein is expressed ubiquitously in the rat brain.

Four CNS related cell lines were utilized for evaluation of cul-5 mRNA expression. Rat primary astrocyte cell cultures (a gift from Dr. Regina Armstrong's laboratory, USUHS) and rat glial tumor cells, C6, (ATCC # CCL-107; cDNA gift from Dr. Albert Dobi, USUHS) represented rat glial cell specificity. Rat pheochromocytoma cell line, PC12, (ATCC# CRL-1721) represented rat neuronal cells and endothelioma

cells from mouse brain (ATCC #CRL-2299) depicted vascular endothelial cell specificity. The total RNA from each cell line was isolated using TriReagent (Molecular Research Center, Inc.).

Long-Evans rats (300-350g b.w., Harlan Sprague Dawley Laboratories) were maintained for six days with *ad libitum* access to food and water. All animals received intraperitoneal injections of Ketaset (80 mg/kg b.w.) and Rompun (13 mg/kg) and when unresponsive to paw pinch, were decapitated to obtain tissue samples of the CNS: corpus callosum (primarily glial cells), frontoparietal cerebral cortex, and basilar skull blood vessels. All CNS tissue was snap frozen in liquid nitrogen and stored in liquid nitrogen.

Tissue RNA was isolated from rat corpus callosum, cerebral cortex, and basilar blood vessels using the Totally RNATM Kit (Ambion). Agarose gel electrophoresis (2 µg total RNA) was employed to visualize the quality of ribosomal RNA bands. For PCR amplification, total RNA from the three CNS regions and four cell lines were treated with 10 units of DNase I per 20 µg of RNA as recommended by the manufacturer for DNA-freeTM reagents (Ambion) to prevent spurious amplification of genomic DNA. RT-PCR was performed using 2 µl (1.4 µg) of total RNA in a 20 µl standard RT reaction using the GeneAmp® RNA PCR kit (Applied Biosystems) to synthesize cDNA, carried out at 42°C for 15 minutes, 99°C for 5 minutes, and 5°C for 5 minutes. Similar reactions without MuLV Reverse Transcriptase were performed simultaneously to assess genomic DNA contamination. Specific primers were designed for the rat cul-5 sequence [10] as follows: 5'-GAAGCAAAAAGGAGTGGGGTTG-3' (5' primer) and 5'-CTAAAACTTACGACCACGAACC-3' (3' primer); and rat glyceraldehyde-3-phosphate dehydrogenase GAPDH; [7]: 5'-CAACGACCCCTTCATTGACCTC-3' (5' primer) and

5'-CGGTAGTGACGGTGAGTCTTCT-3' (3' primer). Since cul-5 and GAPDH gene sequences were homologous in both rat and mouse genomes, both sets of primers were appropriate for PCR amplification. These primers produced amplified products of 442 base pairs (bp) for cul-5 and 461 bp for GAPDH. RT-PCR of total RNA from the three rat CNS regions and four rodent cell lines showed that cul-5 transcripts are present in all samples (Fig. 1). Amplification of the same samples of total RNA with GAPDH primers resulted in the amplification of a product size of 461 bases, in accordance with the known sequence for GAPDH. Gels from RT-PCR reactions where no RT enzyme or no tissue RNA was added to the reaction, showed no visible band (data not shown).

Animals used in the immunocytochemistry studies were euthanized as described above, and when unresponsive to paw pinch, a sternotomy was performed and rats were transcardially perfused with 100 mM phosphate buffered saline (PBS) and then with 4% paraformaldehyde in PBS. Frozen coronal sections (40 μ m thickness) of the cerebrum, cerebellum, and brainstem were obtained (Vibratome 1000 Series Sectioning System), and incubated for 30 minutes in PBS containing 0.3% H₂O₂. Subsequently, according to Vector Elite ABC kit instructions, sections were washed two times for 5 minutes in 0.1 M PBS, and immersed for 30 minutes in blocking solution (0.1 M PBS containing normal goat serum diluted 1:10). Sections were incubated overnight at 4°C with rabbit anti-Cul-5 polyclonal antisera (gift from Dr. Michael Fay, Department of Pharmacology, Midwestern University, Grove, IL) diluted 1:1000 with PBS, or with rabbit glial fibrillary acidic protein (GFAP) polyclonal antibody (Chemicon International) diluted 1:10,000, or mouse anti-NeuN monoclonal antisera (Chemicon International) diluted 1:1000. Subsequent antibody detection was carried out by using Vectastain *Elite* ABC kit anti-

rabbit or –mouse IgG (Vector Labs, Burlingame, VT) with 3,3'-diaminobenzidine tetrachloride (DAB) as a peroxidase substrate. Specificity of Cul-5 antisera immunodetection was demonstrated by preabsorption with peptide antigen and preimmune serum, which abolished immunoreactivity (per correspondence with Dr. Michael Fay, Department of Pharmacology, Midwestern University, Grove, IL). Negative results were obtained by omission of primary antibodies, and Western blot analysis of Cul-5 on rat brain demonstrated the anticipated size band, approximately 80 kDa (data not shown).

Immunocytochemical results were consistent with our RT-PCR findings. Immunolabeling for Cul-5 was present throughout the brain and appears in all different cell types examined in the CNS (Fig. 2). Immunostaining was evident in cerebral blood vessels (bv) and parietal cortex neurons (Fig. 2A); cytoplasmic and nuclear subcompartments of motor neurons in the motor cortex (Fig. 2B); cerebral cortex (ctx), corpus callosum (cc), and hippocampal (hp) regions (Fig. 2C); and choroid plexus of the lateral ventricle (Fig. 2D).

Double immunofluorescence labeling was employed with rabbit anti-Cul-5 polyclonal antisera (gift from Dr. Michael Fay, Department of Pharmacology, Midwestern University, Grove, IL) in combination with (GFAP) polyclonal antibody (Chemicon International) diluted 1:10,000, or mouse anti-NeuN monoclonal antisera (Chemicon International) diluted 1:1000. Brain sections were incubated in rabbit anti-Cul-5 polyclonal antibody diluted 1:1000 with PBS for 16-20 hours and then incubated in the secondary anti-rabbit IgG conjugated to Cy3 (1:200, Jackson ImmunoResearch Laboratories, Inc.). The second primary antibody for GFAP (diluted 1:10,000) was then

applied, following rinsing in PBS, incubated for 16-20 hours, and then sections were incubated in the appropriate secondary IgG conjugated with FITC (1:200 Jackson ImmunoResearch Laboratories, Inc.) for GFAP. Other sections were used for double immunofluorescence staining by first incubating with rabbit anti-Cul-5 polyclonal antisera in combination with mouse anti-NeuN monoclonal antisera (diluted 1:1000) for 16-20 hours at 4°C. Secondary anti-rabbit IgG conjugated to Cy3 for Cul-5 and secondary anti-mouse IgG conjugated to AMCA (1:100 Jackson ImmunoResearch Laboratories, Inc.) for NeuN were then used (1 hour) for detection of Cul-5 and NeuN. Immunofluorescent sections were captured with a Spot 2 digital camera (Diagnostic Instruments, Sterling Heights, MI) using single channel filter sets for Cy3, FITC, and AMCA. Six different rats were examined and displayed similar results to the representative data shown. Double-labeled immunofluorescent staining (Fig. 3) confirmed the presence of Cul-5 in different CNS cell types; showing colocalization with NeuN, a neuronal nuclei marker (Figs. 3C,H) and GFAP, specific for intermediate filament protein in astrocytes (Figs. 3F,G).

We have shown that cul-5 mRNA is present in neuronal, glial, and vascular endothelial cells and these findings are corroborated by Cul-5 immunostaining in neurons, astrocytes, blood vessels, and choroid plexus. This report provides a comprehensive description of Cul-5 cellular specificity and distribution in the laboratory rat CNS and clarifies reported inconsistencies, i.e., exclusively vascular endothelial cell expression [2] or primarily neuronal localization [10]. Our characterization of Cul-5's distribution, may assist in understanding its functional and biological role. The implications of these results are significant as protein degradation is a vital regulator of

cellular functions, i.e., cell cycle, signal transduction, transcriptional regulation, and tumor suppression [6,13].

In brief, the ubiquitination-proteasome protein degradation pathway begins with the E1 enzymes activating and transferring ubiquitin to E2 enzymes, which then transfer activated ubiquitin to the E3 ubiquitin ligases. Usually there is a single E1 enzyme, many variants of E2 enzymes, and multiple classes of E3 ubiquitin ligase complexes [8]. Although E3 ubiquitin ligases have been shown to be primarily responsible for the selectivity, specificity, and regulation of ubiquitin-mediated protein degradation, there remains a huge void in our understanding of these protein complexes and their mechanism of action. Very little is known regarding E3 ubiquitin ligases and this is partly as a result of diversification of various E3 ligase families.

Similar to other E3 ubiquitin ligases, the precise physiological role of Cul-5 remains to be elucidated by future investigations, however evidence implicates this protein as having a regulatory role in protein degradation in adenovirus-infected cells. Cul-5 was identified as a component of an E3 ligase that mediated p53 *in vitro* ubiquitination and degradation [14]. Other findings suggest a possible Cul-5 role in different stages of the cell cycle [1]. Cul-5 may be intricately involved in degradation of proteins that are specific to certain cells in various conditions and treatments. Our recent work found cul-5 mRNA expression pattern changes in the CNS in response to cellular stresses of hemorrhagic shock [3] and water deprivation [5]. Further work is needed, for example, to determine the specific functional role of Cul-5 and what proteins are specifically targeted by this E3 ubiquitin ligase. It is interesting to speculate that this

molecule may be intricately involved in degradation of proteins that play key roles in CNS function and regulation of cellular homeostasis.

In conclusion, we have demonstrated the presence of cul-5 mRNA in all CNS cell types and brain regions of the laboratory rat via RT-PCR. Immunocytochemistry indicates Cul-5 is present in the major cell types of the CNS, suggesting this E3 ligase is ubiquitous throughout the rat CNS.

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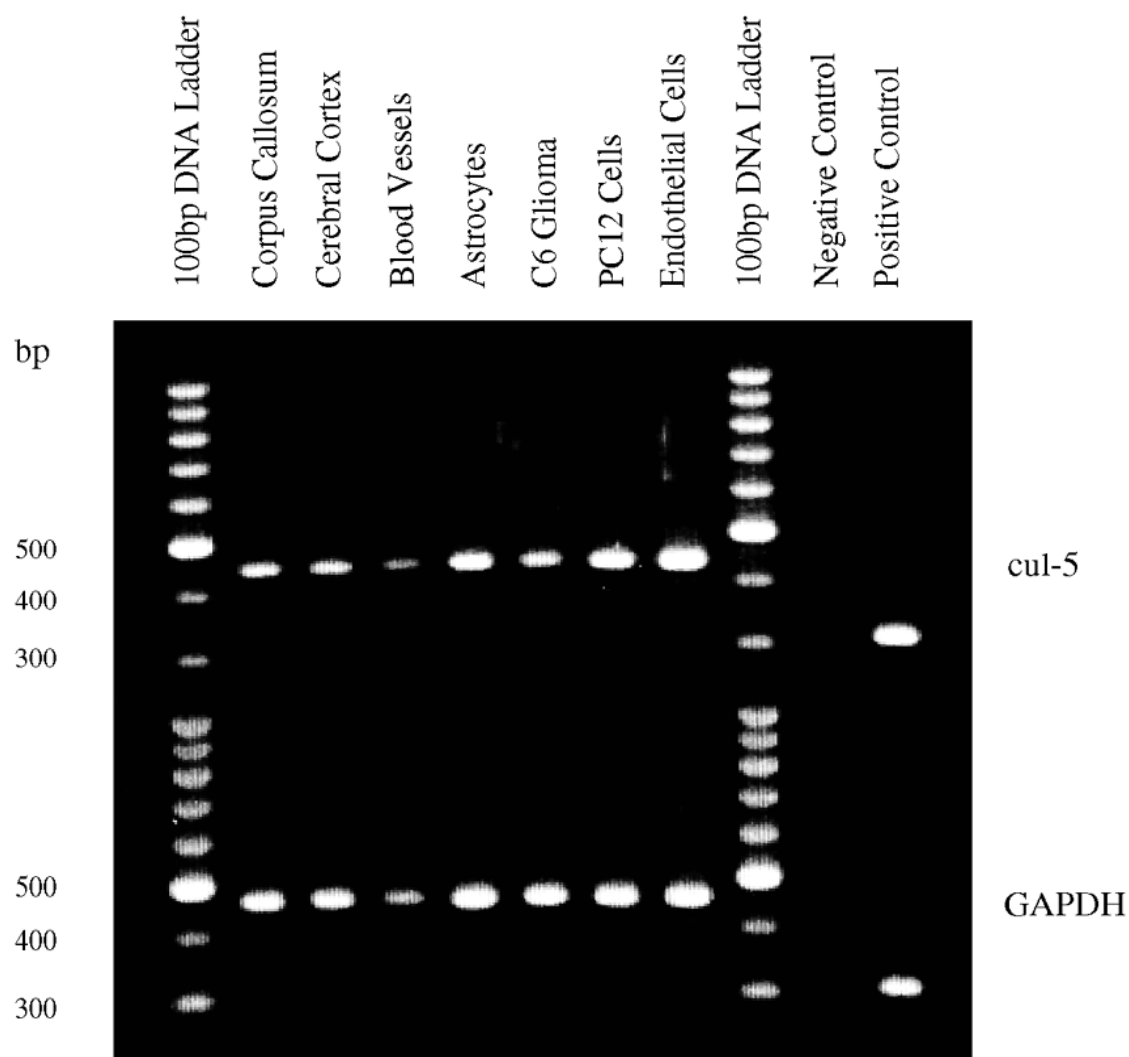


Fig. 1. Reverse transcription-polymerase chain reaction amplification of *cul-5* and GAPDH mRNA. Total RNA (1.4 μ g) samples from rat corpus callosum, cerebral cortex, basilar blood vessels, primary astrocytes, C6 glioma cell line, PC12 pheochromocytoma cell line, and a mouse cerebral vascular endothelioma cell line, all exhibited a strong amplification of *cul-5* cDNA (top panel). Primers homologous to GAPDH also indicated a strong amplified product (bottom panel). A negative control (corpus callosum RT-PCR reaction devoid of Reverse Transcriptase enzyme) failed to produce an amplified product, while primers to amplify a positive control (pAW109) produced a band of 308 bp (Perkin Elmer).

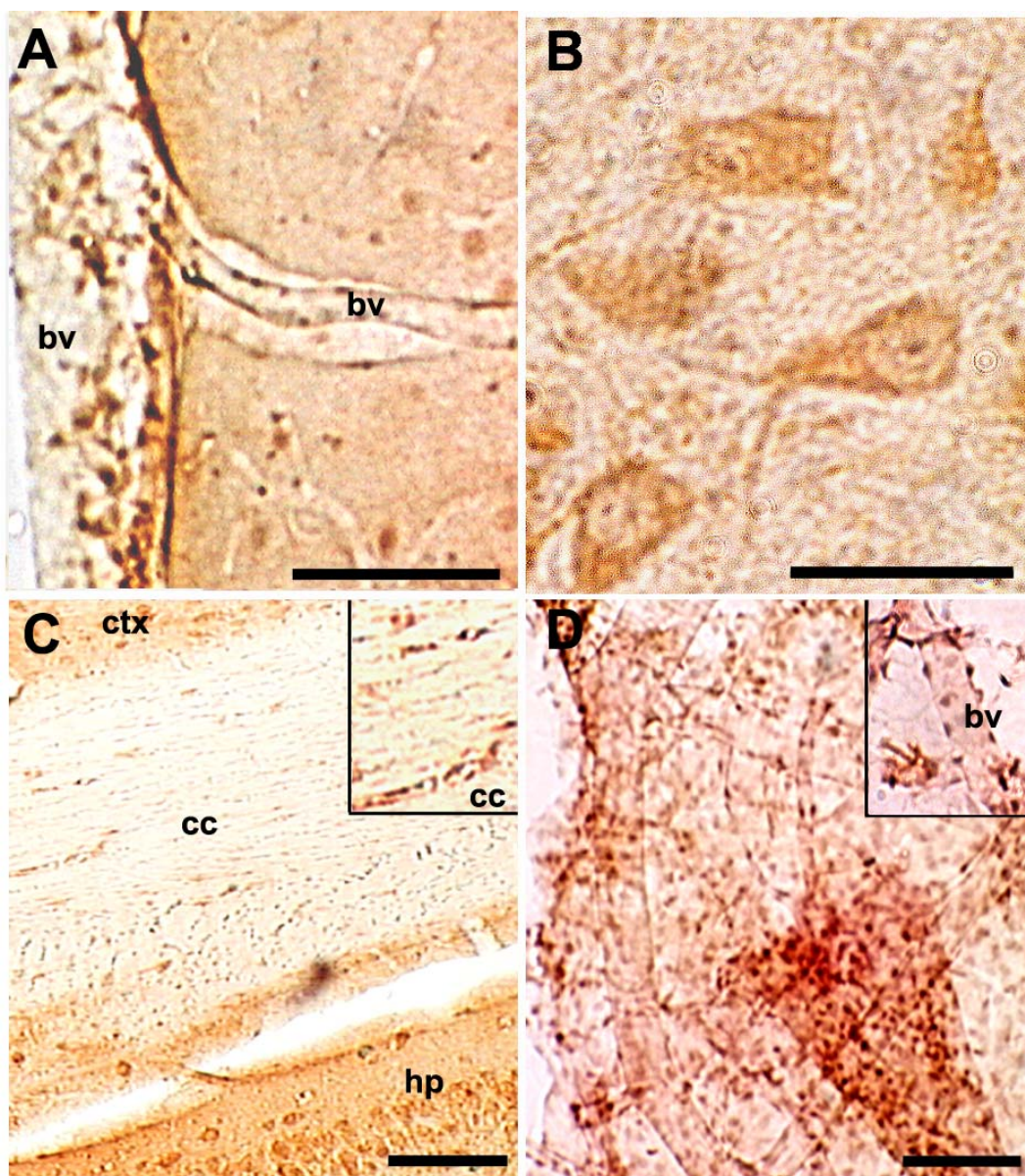


Fig. 2. Photomicrographs exhibiting Cul-5 immunoreactive regions and structures of rat brain. (A) Cerebral cortex neurons and blood vessels (bv); (B) cytoplasmic and nuclear reactivity in large motor neurons in the frontoparietal motor cortex; (C) cerebral cortex (ctx), corpus callosum (cc), hippocampal (hp) areas and with enlarged inset of cc; and (D) choroid plexus of the lateral ventricle with enlarged inset of choroid plexus bv. Scale bar in B = 50 μ m, all other scale bars = 100 μ m.

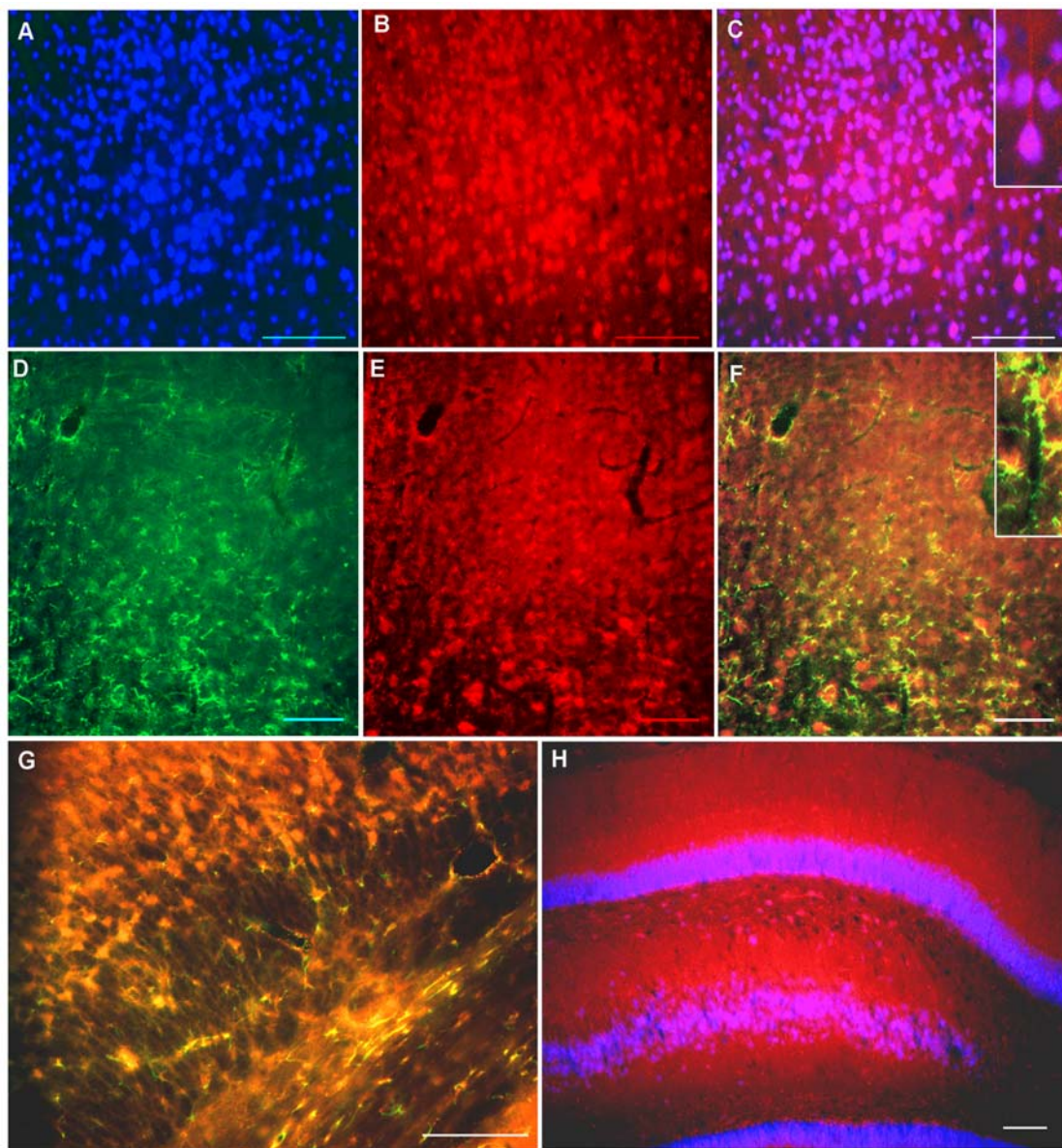


Fig. 3. Immunofluorescent staining of Cul-5 (B,E) in red; neuronal nuclei marker NeuN (A) in blue; and astrocyte marker GFAP in green (D). Double-label overlapped images for Cul-5 and NeuN (C,H) show dual labeling of neurons (purple, enlarged in inset). Double-label overlapped images for Cul-5 and GFAP (F,G) demonstrate dual labeling of astrocytes (yellow, enlarged in inset). Scale bars = 100 μ m.

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CHAPTER 4

Osmotic stress increases cullin-5 (cul-5) mRNA in the rat cerebral cortex, hypothalamus, and kidney

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Thomas E. Ceremuga^{1,2*}, Xiang-Lan Yao², Yang Xia², Dhritiman Mukherjee^{1,2}, and Joseph T. McCabe^{1,2}

*To whom correspondence should be addressed:

Email: tceremuga@usuhs.mil

Tel: 301-295-9366

FAX: 301-295-1715

¹Graduate Program in Neuroscience

²Department of Anatomy, Physiology and Genetics

F. Edmund Hébert School of Medicine

Uniformed Services University of the Health Sciences

4301 Jones Bridge Road

Bethesda, MD 20814

U.S.A.

Key words: cullin, cul-5, water deprivation, osmotic stress, ubiquitination, protein degradation

Abstract

Cullin-5 (cul-5), a member of the cullin gene family, may have a role in proteolysis and cell cycle regulation. Our recent study demonstrated that cul-5 mRNA is ubiquitously expressed in the central nervous system and many peripheral organs. The present study used quantitative realtime polymerase chain reaction to measure changes in cul-5 mRNA expression as a consequence of osmotic stress *in vivo*. Cul-5 mRNA levels were significantly increased in the rat cerebral cortex, hypothalamus, and kidney following 48 hours of water deprivation. Water deprivation for a shorter time period (24 hours) or rehydration (24 hours access to water following 48 hours of water deprivation) also elevated kidney cul-5 mRNA levels. Water deprivation did not significantly alter cul-5 mRNA levels in the brainstem, cerebellum, hippocampus, lung, or liver. Since cul-5 appears to be linked to proteasome-mediated protein degradation, it may have a role in protein regulation under conditions of osmotic stress.

1. Introduction

Maintenance of adequate body fluid is vital to optimal cellular metabolic activity. Hyperosmolality arising from water deprivation can dramatically alter cellular function, but organisms have evolved a number of adaptive strategies that ensure intra- and extra-cellular fluid homeostasis. The hypothalamoneurohypophysial system, for example, maintains whole body fluid balance by releasing vasopressin (antidiuretic hormone) to regulate renal antidiuresis. Central nervous system (CNS) pathways engage thirst and drinking responses to replenish body fluid. Hyperosmotic stress also stimulates intracellular activation, whereby various ion pumps and channels respond to osmotic shrinkage by the uptake of Na^+ , K^+ and Cl^- . Epithelial Na^+ channels (ENaC), for example, increase Na^+ conductance with osmotic stress, reestablishing cellular volume (Graf and Haussinger, 1996; Wehner et al., 2000). Aquaporins (AQP), a family of membranous water channels, are distributed throughout many tissues, and function to maintain intracellular osmotic balance. AQP2 and AQP3 mRNA levels are increased in response to osmotic stress (Umenishi et al., 1996), and hyperosmolality elevates AQP1 protein stability and expression (Leitch et al., 2001). Cellular response also includes changes in cell signaling mechanisms. Mitogen-activated protein kinases (MAPKs) and stress-activated protein kinases are set in motion by osmotic stress (Matsuda et al., 1995; Nahm et al., 2002), that in turn can activate cellular changes and alter gene transcription (Burg et al., 1996; Glasgow et al., 2000). The cellular uptake of organic osmolytes forms an important adaptation to hyperosmotic states, especially in the mammalian kidney and brain (Yancey et al., 1982; Star, 1990). Additionally, recent evidence indicates

ubiquitination may be a significant cellular response to osmotic stress (Staub et al., 1997; Leitch et al., 2001; Rui et al., 2001).

Cullin-5 (cul-5) was first identified in the rabbit kidney medulla by Burnatowska-Hledin and associates (Burnatowska-Hledin et al., 1995) as vasopressin-activated, calcium-mobilizing protein (VACM-1). Additional analysis indicated VACM-1 is homologous with cullin-5 (Byrd et al., 1997). Six human cullins have been identified (Kipreos et al., 1996) and most of these proteins have been described as components of large protein complexes, E3 ligases, that bind proteins targeted for degradation by the 26S proteasome (Ciechanover et al., 2000). Cul-1 is a part of the Skp/Cul-1/F-box complex (Deshaies, 1999), Cul-2 is a fraction of the von Hippel-Lindau tumor suppressor complex (Iwai et al., 1999), Cul-3 binds to and increases ubiquitination of cyclin E (Singer et al., 1999), and Cul-4A has recently been reported to target UV-damaged DNA-binding proteins for ubiquitination and degradation (Chen et al., 2001). Cul-5 has been reported to also be a part of protein complexes that exhibit ubiquitin ligase activity (Kamura et al., 2001). Querido and coworkers identified cul-5 as part of a protein complex that facilitates adenoviral protein-mediated p53 degradation. They report that the viral protein E4orf6-E1B55K binds to p53, a tumor suppressor, thus targeting p53 for degradation, allowing viral replication by inhibition of p53-mediated apoptosis (Querido et al., 2001).

Histological studies demonstrated cul-5 is primarily restricted to vascular endothelial cells of the kidney medulla (Burnatowska-Hledin et al., 1999). Other investigators, however, describe the distribution of cul-5 mRNA as widespread and primarily neuronal (Hurbin et al., 2000). In our previous work (Ceremuga et al., 2001),

we found that the cul-5 transcript is present in the CNS (brainstem, cerebellum, cerebral cortex, hypothalamus), aorta, small intestine, heart, kidney medulla, liver, lung, skeletal muscle, and spleen. Since cul-5 mRNA appears to be expressed in all tissues, the role and function of cul-5 must be identified. We are interested in studying the effects of water deprivation and rehydration on cul-5 mRNA expression in the central nervous system and peripheral tissues *in vivo*. Cul-5 mRNA expression was significantly increased in the cerebral cortex, hypothalamus, and kidney. In addition, cul-5 mRNA levels in kidney were also significantly elevated after rats were water-deprived and rehydrated.

2. Materials and Methods

2.1. Animal treatments

For six days prior to experimental treatment, male Long-Evans rats (300-350 g b.w., Harlan Sprague Dawley Laboratories) were handled daily and had *ad libitum* access to food and water. All methods were performed in accordance with University Laboratory Animal Review Board guidelines. Each animal was then deprived of water (6 rats/group) for 12, 24, or 48 hours, or for 48 hours followed by 24 hours of access to water. Eight additional rats were not deprived of water, and two of these animals were each sacrificed concurrently with one of the four groups of water-deprived animals. Animals were euthanized by intraperitoneal injection of Ketaset (80 mg/kg b.w.) and Rompun (13 mg/kg). When unresponsive to paw pinch, a sternotomy was performed and blood was obtained from intracardiac puncture using a 21-gauge needle. Blood samples were evaluated for Na⁺, Cl⁻, blood urea nitrogen (BUN), glucose, osmolality, and hematocrit plasma levels. The anesthetized rats were then decapitated to obtain tissue

samples of the CNS: brainstem, cerebellum, cerebral cortex, hippocampus, and hypothalamus. Cerebral cortex, cerebellum, and hippocampal samples were prepared by microdissection with a clean razor blade and forceps. Samples of the hypothalamus included the entire hypothalamus and a small portion of the caudal preoptic area. In brief, coronal sections through the diencephalon were made with a clean razor blade. The tissue block extended from the caudal-most region of the anterior commissure (Bregma -0.40 mm, see ref. (Paxinos, 1986)) to the midportion of the infundibulum (Bregma -3.30 mm), extending lateral +2.0 mm from midline, and dorsally (+2.00 mm) to just above the third ventricle (Paxinos, 1986). Brainstem samples extended from the region just caudal to the inferior colliculi (Bregma -9.00 mm) to the level of the obex (Bregma -13.30 mm). Kidney, liver, and lung organ tissues were also harvested. All tissues were snap frozen in liquid nitrogen and stored in liquid nitrogen. Serum electrolytes and osmolality levels were assayed (Clinical Pathology Laboratory, USUHS) using a Vitros 250 Chemistry System (Johnson & Johnson Clinical Diagnostics); hematocrit was determined by use of a Cell-Dyn 3500R machine (Abbott).

2.2 RNA extraction and reverse transcription (RT)

Tissue RNA was isolated using the Totally RNA Kit (Ambion). In brief, frozen tissue was crushed with mortar and pestle in liquid nitrogen and then dispersed in denaturation buffer (guanidine thiocyanate solution) with a sonicator (Kontes micro-ultrasonic cell disrupter). The RNA was extracted using phenol/chloroform and precipitated with isopropanol. Total RNA (2 µg) was electrophoresed on an agarose gel to ensure quality of RNA by visualization of ribosomal RNA bands. To prevent amplification of genomic DNA, total RNA was treated with 10 units of DNase I per 20

µg of RNA using DNA-*free* reagents (Ambion). Reverse transcription was performed on total RNA (1.4 µg/2 µl) in a 20 µl standard RT reaction using the GeneAmp RNA PCR kit (Applied Biosystems) to synthesize cDNA (25°C for 10 minutes, 48°C for 30 minutes, and 95°C for 5 minutes). To rule out DNA contamination, samples were performed simultaneously, but without inclusion of MuLV reverse transcriptase.

2.3. *Quantitative realtime polymerase chain reaction (QRT-PCR)*

Using PrimerExpress software (Applied Biosystems), primers were designed for rat cul-5 (Hurbin et al., 2000) and 18S ribosomal RNA (18S) (Torczynski et al., 1983): as follows: cul-5: 5'-GCTGATCGAGCACAAGTACATCA-3' (5' primer) and 5'-GTATATGTACCGCATCGACTTAGG-3' (3' primer); 18S: 5'-CGGCTACCACATCCAAGGAA-3' (5' primer) and 5'-GCTGGAATTACCGCGGCT-3' (3' primer). As described in our previous work (Ceremuga et al., 2001), templates (three replicates per sample) were loaded into a MicroAmp Optical 96-Well Reaction Plate (Applied Biosystems). Each experimental 96-well plate included samples for the generation of standard curves for the cul-5 mRNA and 18S rRNA. The plates also included triplicate samples of one specific tissue type from all six rats of the control group and from one of the four treatment group samples (water-deprived for either 12, 24, 48, or 48 with 24 hours rehydration), and negative control samples (water). To generate relative standards for the target mRNA (cul-5), decreasing amounts (3-fold dilutions beginning with 20 µl of cDNA from 1.4 µg of sample RNA, 3 replicates per dilution amount) of cDNA from rat lung tissue were used. cDNA samples of 1:100 dilutions were also prepared to measure 18S rRNA levels as an

endogenous reference to control for differences in harvested RNA samples across various tissue samples. The cycle threshold (Ct) values were used to determine the amount of cul-5 mRNA and 18S rRNA for all 8 tissues from each rat in every treatment group, and values for cul-5 mRNA were standardized to 18S rRNA.

2.4. Statistical analyses

The mean Ct value of triplicate samples of the RNA of interest (cul-5 mRNA and 18S rRNA) from each rat was computed to then determine the ratio of cul-5 mRNA per 18S rRNA in each sample. For statistical analyses, the ratios were converted to natural logarithm (ln) values to attain homogeneity of error variance (see ref. (Winer, 1971; Ceremuga et al., 2001) for discussion). After adjusting for differences among plates, a two-factor analysis of variance (ANOVA) was used to assess differences among treatments in each tissue and Fisher's test for *post-hoc* comparisons was used to compare each treatment to the control group (SPSS version 10.0.5 for Windows software). For each tissue and treatment, the mean ratio of cul-5 mRNA to 18S rRNA was obtained by first adjusting the mean ln of the ratio for differences among plates, and then taking the antilog of each adjusted mean to yield the adjusted geometric mean. The standard error of the adjusted geometric mean was computed by multiplying the geometric mean by the ln of the standard error of the adjusted mean (Casella and Berger, 1990). Plasma data were analyzed by one-way ANOVA, while a two-way, repeated measures ANOVA on one factor was used to assess body weight changes as a function of osmotic stress (SigmaStat software). The Student-Newman-Keuls test was then used to assess mean body weight changes before and after experimental treatment.

3. Results

3.1. Physiologic findings

Water deprivation (and rehydration) had a substantial impact upon physiological measures (Table 1). While analysis of variance indicated there was no significant difference amongst the groups for pre-treatment body weights, animals lost a significant amount of weight after they were deprived of water for 24 or 48 hours. Rehydration (48 hour water deprivation + 24 hours rehydration) allowed the animals to regain weight, and this group of animals exhibited a significant weight gain relative to their pre-treatment levels. The plasma sodium (Na^+), hematocrit (HCT), osmolality, and blood urea nitrogen (BUN) levels were all significantly increased after 24 and/or 48 hours of water-deprivation compared to the hydrated groups (Table 1). All glucose levels were above the normal range (50-135 mg/dl) for rats (Mitruka and Rawnsley, 1977), but displayed no differences between groups.

3.2. Measurement of *cul-5* mRNA levels following water deprivation

In five tissues (brainstem, cerebellum, hippocampus, lung, and liver), mean *cul-5* mRNA levels did not significantly change (Table 2) after water deprivation (ANOVA, $p > 0.05$). In the cerebral cortex, hypothalamus, and kidney, however, water deprivation resulted in an increase in *cul-5* mRNA (Fig. 1). In the cerebral cortex and the hypothalamus, *post hoc* tests indicated that the levels of *cul-5* mRNA were significantly greater in tissue samples from rats that had been deprived of water for 48 hours (levels compared to tissue specific control tissue, $p < 0.001$ and 0.007 , respectively). In the kidney, water deprivation for either 24 or 48 hours resulted in increased *cul-5* mRNA levels (both $p < 0.04$). Levels in the kidney remained significantly elevated in animals

that had been rehydrated ($p < 0.001$). That is, compared to the control animals that were not water deprived, cul-5 mRNA levels were still higher in tissue samples of rats that were given water for 24 hours following 48 hours of water deprivation.

4. Discussion

Our study demonstrated that *in vivo* hyperosmotic stress significantly increases cul-5 mRNA levels in the CNS and kidney. Cul-5 transcripts were elevated following 48 hours of water deprivation above the levels observed in rats that had free access to water. A more robust change was observed in the kidney. Water deprivation for 24 hours, or for 48 hours followed by 24 hours with access to water, also increased renal cul-5 mRNA levels. The increase in cul-5 mRNA levels in the CNS and kidney in response to water deprivation suggests a role for this protein in the regulation of cellular response to osmotic stress.

While cul-5 bears wide distribution (Burnatowska-Hledin et al., 1999; Ceremuga et al., 2001) only a few reports thus far have investigated this molecule's function. Cul-5 has been reported to be a part of a protein complex that contains ubiquitin ligase activity (Kamura et al., 2001). In adenovirus-infected cells, Cul-5 was identified as a component of an E3 ligase that mediated p53 *in vitro* ubiquitination and degradation (Querido et al., 2001). Considering numerous studies that indicate p53 mediates neuron cell death (Morrison and Kinoshita, 2000), Cul-5 may “protect” cells from apoptosis arising from osmotic stress.

It is important to note that investigations of the ubiquitination and proteasomal degradation mechanisms are relatively new and there is much to be discovered. However, ubiquitination and proteasomal degradation has been reported as a protein

regulatory mechanism in fluid homeostasis and hyperosmolar states *in vitro*. Staub and colleagues (Staub et al., 1997) reported ubiquitination and protein degradation regulates the function and stability of the plasma membrane epithelial Na⁺ channel, which plays a vital role in fluid and sodium balance. Osmotic stress was also shown to stimulate ubiquitination and protein degradation of the insulin receptor substrate protein (ISR-2) via the 26S proteasome (Rui et al., 2001). Ubiquitination and protein degradation, as a post-transcriptional regulatory mechanism, has also been reported to control protein expression of the renal water channel, AQP1 (Leitch et al., 2001).

Cellular uptake of organic osmolytes is a beneficial adaptation mechanism in hyperosmotic states, particularly in the mammalian kidney and brain (Yancey et al., 1982; Star, 1990; Gullans and Verbalis, 1993). Renal cells respond to hyperosmolality by accumulating organic osmolytes glycerophosphorylcholine (GPC), betaine, *myo*-inositol, sorbitol and free amino acids (Beck et al., 1998). Accumulation of amino acids has also been shown to function as volume-regulating osmolytes in the canine kidney cells (Horio et al., 1997). The accumulation of organic osmolytes protects the cellular environment from the deleterious and perturbing effects of high concentrations of intracellular electrolytes. We speculate that Cul-5 may have a role in pathways that lead to the accumulation of organic osmolytes. The accumulation of organic osmolytes occurs via various mechanisms, such as increased uptake and synthesis, decreased degradation, and reduced release. Many of these mechanisms that lead to organic osmolyte accumulation require multiple enzymes and transporter proteins that regulate these molecules (Beck et al., 1998). Thus, a possible interpretation for the elevation of cul-5 mRNA levels in the kidney is that Cul-5 may participate in the regulation of proteins in

these pathways that result in the accumulation of organic osmolytes. Ubiquitination of proteins that interact with the tonicity-response enhancer binding protein (TonEBP), for example, enables TonEBP to induce transcription of the sodium chloride-betaine cotransporter and the sodium *myo*-inositol cotransporter (Woo et al., 2000). It is also interesting to speculate that Cul-5 may be involved in the breakdown of proteins into amino acids via the 26S proteasome. These free amino acids from proteolysis would then be available to potentially function as an adaptive response to osmotic stress.

There was an overall trend of increased cul-5 mRNA expression levels at 48 hours of water deprivation in all CNS regions examined, however only the cerebral cortex, hypothalamus, and kidney showed significant elevation. Organic osmolyte accumulation plays an important role in cellular osmoregulation in the rat brain and during chronic hypernatremia (Lohr et al., 1988). We speculate that the significant increase in cul-5 mRNA expression in the cerebral cortex and hypothalamus is related to the levels of organic osmolytes present in these specific CNS sites of the rat. Organic osmolytes were found to vary in their distribution in rat brain (Lien, 1995). Thus, an increase in cul-5 mRNA expression in these areas during water deprivation may reflect a cellular adaptation response of organic osmolyte accumulation in order to preserve crucial areas of the CNS that are essential for survival. To confirm this possibility, anatomically specific measures of each osmolytes and the enzymes involved in their transport and synthesis is needed.

A possible explanation for the continued elevation of cul-5 mRNA in the kidney 24 hours after rehydration may be attributed to the increased metabolic activity of the kidney in response to changes in fluid/osmolality status from water deprivation and then

rehydration. Since the kidney is the primary organ responsible for maintaining fluid and electrolyte balance, it maintains a high metabolic rate that may require increased ubiquitination and proteolysis of various proteins that respond to dehydration and rehydration. Although Cul-5 has only been implicated in the ubiquitination and degradation of one specific substrate protein, p53, we presume that it is responsible for the regulation of many different proteins involved in the mediation of cellular activities. The investigation of Cul-5 is in the early stages and many aspects remain unknown. Further studies will be necessary to define Cul-5's specific cellular localization and compartmentalization; exact physiologic function; its specific protein-protein interactions; and the precise protein substrates targeted by Cul-5.

In summary, protein degradation is one biological mechanism that can affect protein expression and stability in order to maintain homeostasis. Cul-5 may indeed serve a functional role as a post-translational mediator of proteins via E3 ligase activity for maintenance of cellular osmotic regulation. This is the first *in vivo* report showing significant increases in gene expression of cul-5 in response to osmotic stress.

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Table 1. Body weight (grams) and plasma values (mean $\pm$ SEM) of rats following water deprivation and rehydration[¶]

Group	Pre Weight	Post Weight	Weight Difference	Na ⁺	HCT	Osmolality	BUN	Glucose
Control (n=8)	331.00 $\pm$ 4.52	344.64 $\pm$ 4.97*	13.64 $\pm$ 2.92	141.14 $\pm$ 0.83	38.76 $\pm$ 1.17	279.71 $\pm$ 1.61	17.71 $\pm$ 0.61	194.71 $\pm$ 15.61
24WD (n=6)	322.57 $\pm$ 9.15	297.71 $\pm$ 8.11*	-24.86 $\pm$ 1.36	144.29 $\pm$ 0.42†	40.73 $\pm$ 2.31	284.14 $\pm$ 1.22	20.43 $\pm$ 0.48†	157.29 $\pm$ 14.60
48WD (n=6)	312.86 $\pm$ 2.65	276.36 $\pm$ 2.51*	-36.50 $\pm$ 1.49	145.29 $\pm$ 0.61†	47.02 $\pm$ 1.18†	289.14 $\pm$ 1.70†	21.43 $\pm$ 0.61†	201.14 $\pm$ 14.71
48WD+24W (n=6)	318.57 $\pm$ 5.91	325.29 $\pm$ 7.33*	8.14 $\pm$ 1.72	141.86 $\pm$ 0.70	38.10 $\pm$ 0.63	280.14 $\pm$ 0.91	16.57 $\pm$ 1.00	186.43 $\pm$ 12.24

[¶]Abbreviations: 12 hour water deprivation (12WD), 24 hour water deprivation (24WD), 48 hour water deprivation (48WD), 48 hours of water deprivation followed by 24 hours of rehydration (48WD+24W), blood urea nitrogen (BUN), hematocrit (HCT), standard error of the mean (SEM)

* $p < 0.05$: Comparison of pre weight and post weight (grams) per treatment group

† $p < 0.05$: Comparison of treatment group to control group

Table 2. *cul-5* mRNA (geometric mean $\pm$ SEM) of rat tissue following water deprivation and rehydration

Tissue Type	Control	24WD	48WD	48WD+24W	Overall p Value
Brainstem	2.53 $\pm$ 0.35	3.62 $\pm$ 1.12	3.17 $\pm$ 0.98	3.68 $\pm$ 1.14	0.437
Cerebellum	0.67 $\pm$ 0.08	0.84 $\pm$ 0.21	1.12 $\pm$ 0.29	0.96 $\pm$ 0.25	0.164
Cerebral Cortex	1.23 $\pm$ 0.15	1.02 $\pm$ 0.28	3.79 $\pm$ 1.02*	2.04 $\pm$ 0.55	0.003
Hippocampus	0.54 $\pm$ 0.07	0.91 $\pm$ 0.26	0.67 $\pm$ 0.19	0.52 $\pm$ 0.15	0.344
Hypothalamus	0.93 $\pm$ 0.07	0.88 $\pm$ 0.15	1.57 $\pm$ 0.26*	0.83 $\pm$ 0.14	0.050
Kidney	0.84 $\pm$ 0.07	1.39 $\pm$ 0.30*	1.39 $\pm$ 0.30*	1.93 $\pm$ 0.42*	0.001
Liver	0.30 $\pm$ 0.05	0.59 $\pm$ 0.22	0.64 $\pm$ 0.24	0.30 $\pm$ 0.11	0.150
Lung	0.64 $\pm$ 0.07	0.62 $\pm$ 0.15	1.16 $\pm$ 0.28	0.55 $\pm$ 0.13	0.166

¶Abbreviations: 24 hour water deprivation (24WD), 48 hour water deprivation (48WD), 48 hours of water deprivation followed by 24 hours of rehydration (48WD+24W), SEM = standard error of the mean

*Statistically significant when overall $p < 0.05$ with a two-factor analysis of variance (ANOVA) and Fisher's test for post-hoc comparisons was used to compare each treatment to the control group

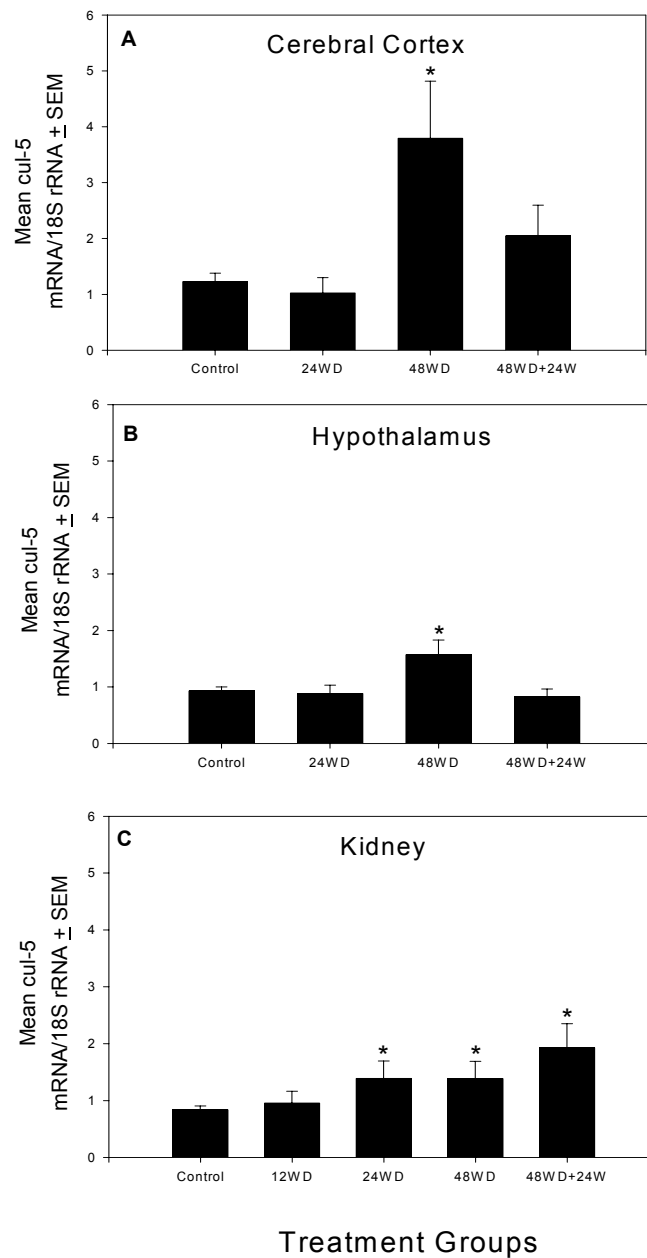


Fig. 1. Measurement (geometric mean $\pm$ SEM) of cul-5 mRNA in cerebral cortex, hypothalamus, and kidney relative to 18S rRNA. Cul-5 levels were significantly increased in cerebral cortex (A) and hypothalamus (B) after 48 hours of water deprivation ($*p < 0.05$). Significantly greater levels of cul-5 mRNA were also found in the kidney (C) when rats were water deprived for either 24 or 48 hours; and levels in the kidney were still significantly elevated in animals that had been rehydrated for 24 hours after 48 hours of water deprivation ($*p < 0.05$). Abbreviations: see Table 1.

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CHAPTER 5

ALTERATIONS OF CULLIN-5 mRNA LEVELS IN THE RAT CENTRAL NERVOUS SYSTEM FOLLOWING HEMORRHAGIC SHOCK

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Thomas E. Ceremuga^{1,2*}, Xiang-Lan Yao², Hasan B. Alam³, and Joseph T. McCabe^{1,2}

*To whom correspondence should be addressed:

Email: tceremuga@usuhs.mil

Tel: 301-295-9366

FAX: 301-295-1715

¹Graduate Program in Neuroscience

²Department of Anatomy, Physiology and Genetics

³Department of Surgery

F. Edmund Hébert School of Medicine

Uniformed Services University of the Health Sciences

4301 Jones Bridge Road

Bethesda, MD 20814

U.S.A.

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ABSTRACT

Hemorrhagic shock is a clinical syndrome that manifests as hypoperfusion, hypoxia, and ischemia initiating various cellular stress responses involved in the synthesis and release of an assortment of pro-inflammatory molecules, cytokines, chemokines, and reactive oxidant species (ROS). The ROS have been shown to oxidize and damage proteins making them targets for ubiquitination and proteasomal degradation. Cullin-5 (Cul-5), an E3 ligase that binds ubiquitin to proteins targeted for degradation via the proteasome, was investigated for its gene expression during hemorrhagic shock. Male Long-Evans rats were subjected to volume controlled (27 ml/kg) hemorrhage over 10 minutes and kept in shock for 60 minutes. Quantitative realtime polymerase chain reaction showed *cul-5* mRNA levels were significantly increased in the brainstem and cerebellum, and decreased in the hypothalamus of rats as a result of hemorrhagic shock (n=6) compared to sham-treated rats (n=6). *cul-5* mRNA levels in the cerebral cortex, small intestine, kidney, liver, lung, or pituitary gland did not significantly change after hemorrhagic shock. This is the first report of *cul-5* mRNA regulation by hemorrhagic shock. Evidence indicates this protein may have a regulatory role in ubiquitin-proteasomal protein degradation in response to hemorrhagic shock.

INTRODUCTION

Hemorrhagic shock is a clinical syndrome that can progress to organ dysfunction and death¹. Unable to meet metabolic demands, hemorrhagic shock initiates various cellular stress responses, including the synthesis and release of an assortment of pro-inflammatory molecules, cytokines, and chemokines¹. Cellular dysfunction also decouples the mitochondrial electron transport system to give rise to reactive oxidant species (**ROS**)²⁻⁴ and free radical damage⁵, which then activate various lethal pathways that damage DNA, cellular lipids, and proteins (as reviewed in the literature^{1,6,7}). Although some aspects of this process are not fully understood, the cell manages damaged and oxidized proteins through the ubiquitin-proteasome pathway^{8,9}. As the cell's major mechanism for degradation, it is an important regulator of almost all biological activities responsible for normal cell growth and metabolism.

The initial step in the selection of proteins for destruction is ubiquitination. In brief, a chain of ubiquitins (**Ub**), a 76 amino acid, highly conserved protein, is attached to proteins targeted for degradation by a series of molecular reactions. These reactions are controlled by an E1 (Ub-activating) enzyme, an E2 (Ub-conjugating) enzyme, and an E3 (Ub-ligase) enzyme that assembles a polyubiquitin chain. It is the E3 ligase that is responsible for the selectivity of protein degradation by covalently binding polyubiquitin to proteins targeted for proteasome degradation¹⁰⁻¹³.

Cullin-5 (**cul-5**), one of six human cullins¹⁴, has been identified as an E3 ligase¹⁵. It was initially identified as vasopressin-activated, calcium-mobilizing protein (**VACM-1**)¹⁶ but further investigation showed VACM-1 and Cul-5 are homologous¹⁷. Our earlier work¹⁸ showed that the *cul-5* transcript is present in the central nervous system

(brainstem, cerebellum, cerebral cortex, hypothalamus), aorta, small intestine, heart, kidney, liver, lung, skeletal muscle, and spleen of the laboratory rat. *In vivo* study of Cul-5 is necessary to ascertain its possible role in cellular homeostasis, particularly in states that give rise to oxidized proteins.

Protein degradation has gained considerable interest lately. Weih and colleagues¹⁹ found that oxidized proteins were degraded by the proteasome in rat primary cortical neurons deprived of oxygen and glucose. Since ischemic injury ensuing from hemorrhagic shock results in oxidation and eventual degradation of proteins and Cul-5 seems to participate in protein degradation, we were interested in investigating the effects of hemorrhagic shock on *cul-5* mRNA expression in the central nervous system (CNS) and peripheral tissues. We found significantly altered *cul-5* mRNA levels in various CNS regions in the laboratory rat following hemorrhagic shock.

MATERIALS AND METHODS

Animal treatment

For six days prior to experimental treatment, a total of 12 male Long-Evans rats (275-300 g b.w., Harlan Sprague Dawley Laboratories) were handled daily and had *ad libitum* access to food and water. All methods were performed in accordance with the USUHS Laboratory Animal Review Board guidelines. Animals were exsanguinated utilizing the hemorrhage model employed in previous experiments^{20,21}. Six Long-Evans rats (275-300g) were anesthetized in an induction chamber with 5% isoflurane until unconscious (approximately 15 seconds). The animals were then placed on a heating pad and rectal thermometers inserted. Anesthesia was maintained throughout the experiment

with isoflurane at 1.0% via a rodent nose cone with a scavenging system to maintain a constant level of anesthesia throughout the experiment. A surgical cutdown was performed to the right groin for isolation and cannulation of the right femoral artery. Polyethylene catheters (PE-50, Clay Adams, Piscataway, NJ) were inserted using aseptic technique and connected to a transducer for cardiovascular monitoring, blood drawings, and exsanguination. The right femoral arterial line was connected to a pressure transducer and computerized physiograph system (Ponemah Physiology Platform, Gould Instrument Systems, Valley View, OH) for continual blood pressure and heart rate recordings. Arterial blood samples were obtained and analyzed by a Stat Profile 2 Blood Gas and Electrolyte Analyzer (Nova Biomedical, Waltham, MA) at Baseline, 10 minutes after exsanguination (27ml/kg: approximately 40% of total blood volume based on body weight as previously performed^{21,22}), and after 60 minutes of hemorrhagic shock. After the 10 minute hemorrhage, the animals remained in hypovolemic shock for 60 minutes (shock period). The six rats in the Sham group received identical surgical treatment except exsanguination.

Rats were then euthanized, and brainstem, cerebellum, cerebral cortex, small gastrointestinal, heart, hypothalamus, kidneys, liver, lungs, and pituitary gland were harvested. Cerebral cortex and cerebellum samples were prepared by microdissection with a clean razor blade and forceps. Samples of the hypothalamus included the entire hypothalamus and a small portion of the caudal preoptic area. In brief, coronal sections through the diencephalon were made with a clean razor blade. The tissue block extended from the caudal-most region of the anterior commissure (Bregma -0.40 mm, see reference²³) to the midportion of the infundibulum (Bregma -3.30 mm), extending lateral

+2.0 mm from midline, and dorsally (+2.00 mm) to just above the third ventricle²³.

Brainstem samples extended from the region just caudal to the inferior colliculi (Bregma -9.00 mm) to the level of the obex (Bregma -13.30 mm). All tissues were snap frozen in liquid nitrogen and stored in liquid nitrogen.

RNA extraction and reverse transcription

Tissue RNA was isolated using the Totally RNA Kit (Ambion). In brief, frozen tissue was crushed with mortar and pestle in liquid nitrogen and then dispersed in denaturation buffer (guanidine thiocyanate solution) with a sonicator (Kontes micro-ultrasonic cell disrupter). The RNA was extracted using phenol/chloroform and precipitated with isopropanol. Total RNA (2 µg) was electrophoresed on an agarose gel to ensure quality of RNA by visualization of ribosomal RNA bands. To prevent amplification of genomic DNA, total RNA was treated with 10 units of DNase I per 20 µg of RNA using DNA-*free* reagents (Ambion). Reverse transcription was performed on total RNA (1.4 µg/2 µl) in a 20 µl standard reverse transcription reaction using the GeneAmp RNA PCR kit (Applied Biosystems) to synthesize cDNA (25°C for 10 minutes, 48°C for 30 minutes, and 95°C for 5 minutes). To rule out DNA contamination, samples were performed simultaneously, but without inclusion of MuLV reverse transcriptase.

Quantitative realtime polymerase chain reaction (QRT-PCR)

Using PrimerExpress software (Applied Biosystems), primers were designed for rat *cul-5*²⁴ and 18S ribosomal RNA (18S rRNA)²⁵: as follows: *cul-5*: 5'-GCTGATCGAGCACAAGTACATCA-3' (5' primer) and 5'-GTATATGTACCGCATCGACTTAGG-3' (3' primer); 18S: 5'-CGGCTACCACATCCAAGGAA-3' (5' primer) and 5'-GCTGGAATTACCGCGGCT-3' (3' primer). As described in our previous work (Ceremuga et al., 2001), templates (three replicates per sample) were loaded into a MicroAmp Optical 96-Well Reaction Plate (Applied Biosystems). Each experimental 96-well plate included samples for the generation of standard curves for the *cul-5* mRNA and 18S rRNA. The plates also included triplicate samples of one specific tissue type from all six rats of the Sham group and from the Hemorrhagic Shock group, and negative control samples (water). To generate relative standards for the target mRNA (*cul-5*), decreasing amounts (3-fold dilutions beginning with 20 µl of cDNA from 1.4 µg of sample RNA, 3 replicates per dilution amount) of cDNA from rat lung tissue were used. cDNA samples of 1:100 dilutions were also prepared to measure 18S rRNA levels as an endogenous reference to control for differences in harvested RNA samples across various tissue samples. The cycle threshold (Ct) values were used to determine the amount of *cul-5* mRNA and 18S rRNA for all 10 tissues from each rat in every treatment group.

Statistical analyses

The mean Ct value of triplicate samples of the RNA of interest (*cul-5* mRNA and 18S rRNA) from each rat tissue was computed to determine the ratio of *cul-5* mRNA per

18S rRNA in each sample. A two-factor analysis of variance (ANOVA, factors: Hemorrhagic Treatment and Tissue) was performed to assess changes in *cul-5* mRNA/18S rRNA levels in various brain regions and tissues as a function of hemorrhagic shock (SigmaStat for Windows, Version 2.03, SPSS, Chicago, IL). *Post-hoc* tests on simple main effects²⁶ were used to ascertain which tissues exhibited significant changes from hemorrhagic shock. Hemodynamic parameters (blood pressures and heart rates) and blood values: pH, partial pressure of oxygen (PO₂), partial pressure of carbon dioxide (PCO₂), oxygen saturation (SO₂), bicarbonate (HCO₃⁻), hematocrit (HCT), sodium (Na⁺), potassium (K⁺), calcium (Ca²⁺), magnesium (Mg²⁺), glucose, lactate, base excess, and osmolality were evaluated using a two-factor ANOVA (factors: Hemorrhagic Treatment and Time) with repeated measures for the Time factor, and simple main effects were examined for changes at each time point between Sham and Hemorrhagic Shock animals by the Tukey Test (SigmaStat software).

RESULTS

Hemodynamic and physiologic findings

Hemorrhagic shock had a substantial impact on cardiovascular parameters and blood values. Mean arterial blood pressure and heart rate were significantly lowered as a result of exsanguination, where mean arterial blood pressure gradually increased, but remained significantly lower during the entire 60 minute shock period. Heart rate was

gradually regained, 50-60 minutes after shock initiation, but still remained approximately 15% below the levels in Sham animals (Figure 1).

Hemorrhagic shock had a substantial impact upon physiological measures (Table 1). Analysis of variance indicated there was no significant difference between the groups for arterial PO₂, PCO₂, SO₂, osmolality, calcium, or potassium levels at each time point. Plasma sodium and hematocrit levels however were significantly decreased, while magnesium levels were significantly increased at the 0 and 60 minute time points post-exsanguination in the Hemorrhagic Shock group compared to the Sham group (Table 1). Glucose levels were significantly elevated after the initial exsanguination and at the 60 minute time point. The pH, base excess, and bicarbonate decreased; and lactate levels increased in the Hemorrhagic Shock group compared to the Sham group indicative of an ischemic/acidotic milieu (Table 1).

Measurement of *cul-5* mRNA levels following hemorrhagic shock

Since the ANOVA indicated there was a significant interaction between hemorrhagic shock treatment and tissue type ($p=0.038$), further analysis was performed with *post hoc* tests. Although there appears to be a trend of increased levels of *cul-5* mRNA after hemorrhagic shock, seven tissues (cerebral cortex, small gastrointestinal, heart, kidney, lung, liver, and pituitary gland) did not show significant changes in *cul-5* mRNA expression (ANOVA, $p>0.05$). However, brainstem and cerebellum demonstrated significant *cul-5* mRNA elevations in response to hemorrhagic shock compared to sham tissue ($p<0.01$ and 0.05 respectively; Figure 2); while *cul-5* mRNA

levels in the hypothalamus decreased when rats were subjected to hemorrhagic shock ($p<0.05$) compared to Sham rats.

DISCUSSION

The ischemic and hypoxic consequences of hemorrhagic shock result in activation of many systemic and cellular pathways in an attempt to maintain optimal cellular metabolic activity and thwart organ failure¹. Mitochondrial dysfunction from failure of oxidative-phosphorylation and insufficient ATP production^{27,28} has been described as a major factor in cellular dysfunction occurring in hemorrhagic shock^{29,30}. Ischemia progression leads to depletion of high energy stores and decoupling of the electron transport system with the production of ROS²⁻⁴. ROS have been shown to cause damage in brain^{2,31}, heart³², and lung³³ tissues. The ROS damaged proteins become targets for ubiquitination and proteasomal degradation⁸. Proteasomal degradation of oxidized proteins has been reported in human hematopoietic cells³⁴, liver epithelial cells³⁵, and fibroblasts³⁶. More recently Weih and associates¹⁹ showed degradation of oxidized proteins in oxygen and glucose deprived rat primary cortical neurons via the proteasome.

Our present work investigated the effect of hemorrhagic shock on Cul-5, an E3 ligase¹⁵, which covalently binds Ub to a protein targeted for proteasomal degradation^{11,13}. In order to evaluate initial responses of *cul-5* gene expression in hemorrhagic shock, a simple hemorrhagic shock paradigm (27 ml/kg over 10 minutes) without a resuscitative treatment or reperfusion was employed. This straightforward model avoided the multitudinous factors and variables associated with several resuscitative regimens and reperfusion. The 60 minute time-point was used to investigate early transcriptional

responses of *cul-5* and establish a foundation for further studies regarding alterations of *cul-5* gene expression following hemorrhagic shock.

Our study demonstrates significantly altered *cul-5* mRNA expression in regions of the CNS after hemorrhagic shock. *cul-5* transcripts in the cerebellum and brainstem were increased after 60 minutes of hemorrhagic shock with a greater increase found in the brainstem. Different changes were observed in the hypothalamus, where *cul-5* mRNA levels decreased. The modulation of *cul-5* mRNA levels in the CNS after hemorrhagic shock suggests it is an important component of gene transcription programs that ameliorate the impact of cytotoxic proteins generated by hypoxia, ischemia, ROS, and free radical damage. The precise physiological role of Cul-5, however remains to be elucidated. Further work is needed, for example, to determine what proteins are specifically targeted by this E3 ligase.

CONCLUSION

Protein degradation is one mechanism that can influence protein expression and stability in order to maintain cellular homeostasis. There is a huge void in information concerning the role of the ubiquitin-proteasome protein degradation pathway in hemorrhagic shock. The proteasome may serve an active role in cellular defenses against oxidative stress after cerebral ischemia¹⁹ and Cul-5 may function as a post-translational mediator of proteins in cellular adaptation/survival responses to hemorrhagic shock. Our findings demonstrate *in vivo* regulation of *cul-5* gene expression, a molecule involved in the ubiquitination-proteosomal pathway, in response to hemorrhagic shock.

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Table 1. Arterial Blood Values (mean $\pm$ SEM) of Rats with Hemorrhagic Shock or Sham Treatment

Group Time Point	pH	HCO ₃ ⁻ ¶	Base Excess	Lactate	Na ⁺	HCT ¶	Glucose	Mg ⁺⁺
Sham Baseline	7.46 $\pm$ 0.01	33.42 $\pm$ 0.72	9.20 $\pm$ 0.64	1.65 $\pm$ 0.18	143.58 $\pm$ 0.86	33.33 $\pm$ 0.95	222.20 $\pm$ 10.43	0.30 $\pm$ 0.01
Shock Baseline	7.43 $\pm$ 0.01	35.20 $\pm$ 0.59	9.25 $\pm$ 1.44	1.73 $\pm$ 0.18	144.13 $\pm$ 0.42	33.43 $\pm$ 1.31	214.00 $\pm$ 6.74	0.30 $\pm$ 0.01
Sham Time 0 minutes	7.43 $\pm$ 0.01	32.88 $\pm$ 0.87	8.42 $\pm$ 0.86	2.00 $\pm$ 0.56	144.27 $\pm$ 0.46	31.83 $\pm$ 0.75	203.00 $\pm$ 10.70	0.28 $\pm$ 0.02
Shock Time 0 minutes	7.45 $\pm$ 0.01	30.29 $\pm$ 0.90	6.03 $\pm$ 0.80	3.30 $\pm$ 0.50	142.04 $\pm$ 0.73*	28.29 $\pm$ 0.81†	259.50 $\pm$ 11.56*	0.33 $\pm$ 0.01*
Sham Time 60 minutes	7.42 $\pm$ 0.01	32.32 $\pm$ 1.36	7.70 $\pm$ 1.35	3.35 $\pm$ 0.74	141.82 $\pm$ 0.24	31.83 $\pm$ 0.83	174.80 $\pm$ 016.69	0.33 $\pm$ 0.01
Shock Time 60 minutes	7.37 $\pm$ 0.03*	27.16 $\pm$ 2.26†	1.96 $\pm$ 2.28†	5.73 $\pm$ 0.97*	139.70 $\pm$ 0.88*	26.50 $\pm$ 0.43†	321.00 $\pm$ 039.46†	0.40 $\pm$ 0.03*

¶ HCO₃⁻ = Bicarbonate, HCT = Hematocrit, SEM = Standard error of the mean

*p< 0.05: Comparison of Hemorrhagic Shock group at the specific time point to the corresponding Sham group time point

†p< 0.01: Comparison of Hemorrhagic Shock group at the specific time point to the corresponding Sham group time point

Time 0 minutes = End of 10 minute exsanguination time point

Time 60 minutes = End of 60 minutes of hemorrhagic shock period and animal sacrifice time point

No significant changes were observed in arterial blood measurements of PCO₂, PO₂, SO₂, calcium, potassium, or osmolality levels (data not shown)

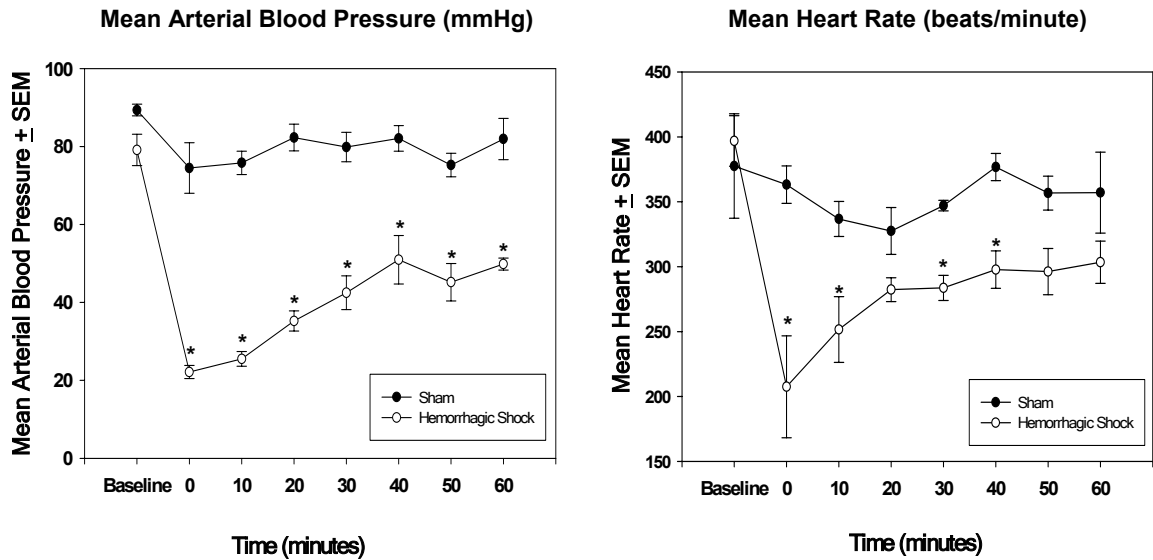


Figure 1: Measurements of mean arterial blood pressure (mmHg) and heart rate (beats per minute) in Sham and Hemorrhagic Shock rats. Values are mean $\pm$ standard error of the mean (SEM). There was an overall significance between the two groups (ANOVA, $p < 0.001$). The asterisk symbols indicate significant difference from the Sham values obtained at the same time point using the Tukey Test (ANOVA, $*p < 0.05$).

Impact of Hemorrhagic Shock upon *cul-5* mRNA Levels

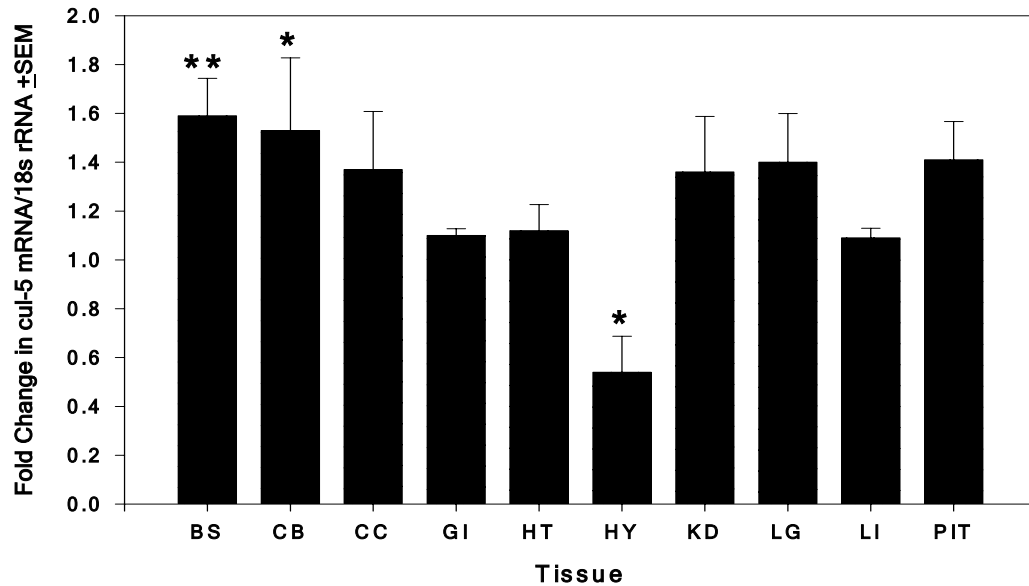


Figure 2: Measurement (mean $\pm$ SEM) of *cul-5* mRNA in brainstem (BS), cerebellum (CB), cerebral cortex (CC), small intestine (GI), heart (HT), hypothalamus (HY), kidney (KD), lung (LG), liver (LI), and pituitary gland (PIT) relative to 18S rRNA. *cul-5* levels were significantly increased in brainstem (** $p < 0.01$) and cerebellum (* $p < 0.05$), and decreased in the hypothalamus (* $p < 0.05$) after hemorrhagic shock (27 mL/kg). All Hemorrhagic Shock tissue types were compared to their corresponding Sham tissue types adjusted to equal 1.0.

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Addendum to Chapter 5 - Hemorrhagic Shock Data

In addition to the data presented in the preceding manuscript, we evaluated the impact of hemorrhagic shock on protein levels of Cul-5 in the brainstem of the laboratory rat using Western blot analysis. Seven Male Long-Evans rats were subjected to volume controlled (27 ml/kg) hemorrhage (HS1-HS7) over 10 minutes and kept in shock for 60 minutes (see detailed description of animal treatment methods, page 90-92). The seven rats in the Sham or control group (C1-C7) received identical surgical treatment except exsanguination.

Western blot method consisted of the following:

Tissue to isolate protein was lysed in lysis buffer containing 1x TBS, 0.1% triton-x-100 and protease inhibitor tablet. The protein concentration was estimated by using Protein Assay Kit (Biorad) according to the manufacturer's instructions. Appropriate amount of protein lysate was mixed with Laemmli's sample buffer and boiled for 5 minutes at 100°C in a water bath. The samples were loaded on to a SDS-polyacrylamide gel and electrophoresis was performed at 125V in a Novex mini gel unit for 90 minutes. After electrophoresis the gel was subjected to transfer on to a PVDF Immobilon membrane in a Novex electroblotter, overnight at 40°C. The transfer buffer consisted of 25mM Tris-HCL, 192mM glycine and 20% methanol. The PVDF membrane was blocked in blocking buffer consisting of 5% dry nonfat milk in TTBS (20mM Tris-HCL, pH 7.4, 150mM NaCl and 0.1% Tween-20) which was followed by incubation in rabbit anti-Cul-5 polyclonal antisera (gift from Dr. Michael Fay, Department of Pharmacology, Midwestern University, Grove, IL) diluted 1:1000 in TTBS overnight at 4°C. The membranes were washed 4 times (15 minutes/wash) in TTBS. This was followed by incubation in secondary antibody diluted in TTBS and 5% nonfat milk

for 1 hour at room temperature. After 4 wash cycles in TTBS, the membranes were incubated in ECL substrate solution (according to the manufacturer's instructions) and exposed to X-ray film for the desired amount of time to visualize the immunoreactive bands.

The band size (approximately 80 kD) is the anticipated size and is in congruence with previous reports investigating Cul-5 protein expression (Burnatowska et al., 2001). Western blot analysis indicates that there is no substantial quantitative difference in Cul-5 protein expression levels in brainstem tissue between the hemorrhagic shock treatment group and the Sham group (Figure 3).

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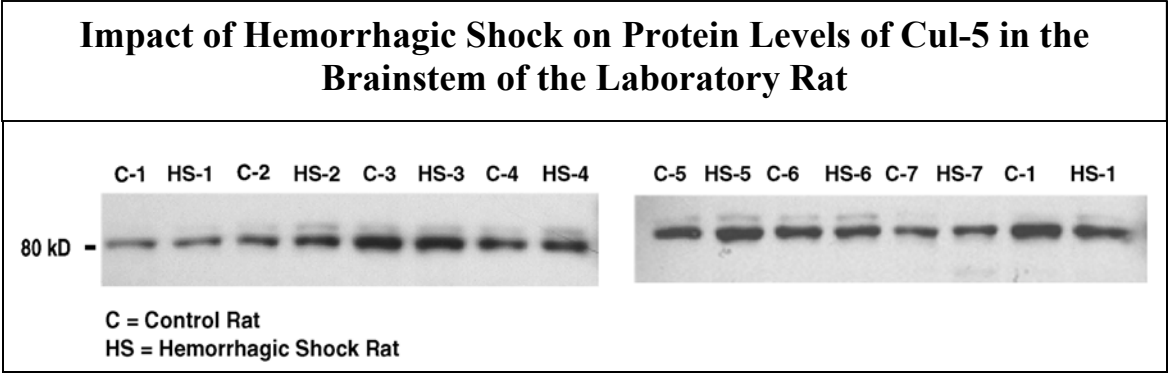


Figure 3. Western results of Brainstem in Hemorrhagic Shock

CHAPTER 6

Discussion

Characterization of cullin-5 in the laboratory rat

The objective of this thesis research project was to characterize cullin-5 (Cul-5), and evaluate its response to cellular stressors. Cul-5 remains an enigma. Currently, there are only *nine* reports in the literature that specifically investigated this molecule or attempted to clarify its role(s) and function(s).

Cullin-5 (Cul-5), first identified as vasopressin-activated, calcium-mobilizing protein (VACM-1) in the rabbit kidney medulla (Burnatowska-Hledin et al., 1995), was shown to be homologous with Cul-5 (Byrd et al., 1997). One histological study reported Cul-5 is primarily restricted to vascular endothelial cells of the kidney medulla (Burnatowska-Hledin et al., 1999), whereas another report described widespread, primarily neuronal, distribution of cul-5 mRNA in the CNS (Hurbin et al., 2000).

Our research determined the anatomical distribution of Cul-5. Northern blotting of poly(A)⁺ RNA from various tissues demonstrated the size of cul-5 mRNA in the rat is approximately 6.3 kb. RT-PCR indicated the transcript is present in all twelve tissues examined: brainstem, cerebral cortex, cerebellum, hypothalamus, aorta, gastrointestinal tract, heart, kidney medulla, liver, lung, skeletal muscle, and spleen. Quantitative realtime PCR validated RT-PCR findings that cul-5 mRNA is in all organs and tissues, and demonstrated expression levels that are comparable in all tissues (Ceremuga et al., 2001).

Although we showed that the cul-5 transcript is present in the CNS and all peripheral organs investigated, we wanted to further describe Cul-5, by examining cellular specificity and protein expression in the CNS. By RT-PCR, we showed there is cul-5 mRNA expression in rodent neuronal, glial, and vascular endothelial cells. Immunostaining of Cul-5 corroborated these data; the protein is present in neurons, astrocytes, blood vessels, and the choroid plexus (Ceremuga et al., 2003a). This work provided a comprehensive description of Cul-5 cellular specificity and distribution in the laboratory rat CNS, and clarified reported inconsistencies, i.e., exclusively vascular endothelial cell expression (Burnatowska-Hledin et al., 1999) or primarily neuronal localization (Hurbin et al., 2000).

Alterations of cullin-5 transcript levels by cellular stress: Water deprivation

The widespread and ubiquitous expression of Cul-5 in the brain suggests that it may have a vital role(s) in cellular activities of the CNS. The next series of experiments examined the function of Cul-5, a presumed E3 ubiquitin ligase, to cellular stress; we ascertained cul-5 mRNA expression in the context of water deprivation and hemorrhagic shock.

Adequate body fluid is critical to optimal cellular metabolic activity, and hyperosmolality arising from water deprivation can dramatically alter cellular function (Yancey et al., 1982; Star, 1990). Therefore, we measured the effect of *in vivo* osmotic stress on cul-5 mRNA expression using quantitative realtime PCR. There was an overall trend of increased cul-5 mRNA expression levels at 48 hours of water deprivation in all CNS regions examined, however only the cerebral cortex, hypothalamus, and kidney

showed significant elevation. Water deprivation for a shorter time period (24 hours) or rehydration (24 hours access to water following 48 hours of water deprivation) also elevated kidney cul-5 mRNA levels. Water deprivation did not significantly alter cul-5 mRNA levels in the brainstem, cerebellum, hippocampus, lung, or liver (Ceremuga et al., 2003c).

Ubiquitination and proteosomal degradation has been reported as a protein regulatory mechanism in fluid homeostasis and hyperosmolar states *in vitro*. Osmotic stress was shown to stimulate ubiquitination and protein degradation regulation of the insulin receptor substrate protein (ISR-2) via the 26S proteasome (Rui et al., 2001); the stability of the plasma membrane epithelial Na⁺ channel protein (which plays a vital role in fluid and sodium balance) (Staub et al., 1997), and protein expression of the renal water channel, aquaporin-1 (Leitch et al., 2001).

Hyperosmotic states promote the uptake of organic osmolytes as a beneficial adaptation mechanism in the mammalian kidney and brain (Yancey et al., 1982; Lohr et al., 1988; Star, 1990; Gullans and Verbalis, 1993). The accumulation of organic osmolytes protects the cellular environment from deleterious and perturbing effects of increased concentrations of intracellular electrolytes. Many mechanisms, such as increased uptake and synthesis, decreased degradation, and reduced release, that lead to organic osmolyte accumulation require multiple enzymes and transporter proteins that regulate these molecules (Beck et al., 1998). Thus one role of Cul-5 may be participation in pathways that lead to the accumulation of organic osmolytes.

A suggested interpretation of the increase in cul-5 mRNA expression in the cerebral cortex and hypothalamus is related to the levels of organic osmolytes present in

these specific CNS sites of the rat. Organic osmolytes were found to vary in their distribution in rat brain (Lien, 1995). Thus, an increase in cul-5 mRNA expression in these areas during water deprivation may reflect a cellular adaptation response of organic osmolyte accumulation in order to preserve crucial areas of the CNS that are essential for survival. However, to confirm this possibility, anatomically specific measures of each osmolyte and the enzymes involved in their transport and synthesis is needed.

Renal cells respond to hyperosmolality by accumulating organic osmolytes glycerophosphorylcholine (GPC), betaine, *myo*-inositol, sorbitol and free amino acids (Beck et al., 1998). Accumulation of amino acids has also been shown to function as volume-regulating osmolytes in the canine kidney cells (Hori et al., 1999). A possible interpretation for the elevation of cul-5 mRNA levels in the kidney is that Cul-5 may participate in the regulation of proteins in these pathways that result in the accumulation of organic osmolytes. Ubiquitination of proteins that interact with the tonicity-response enhancer binding protein (TonEBP), for example, enables TonEBP to induce transcription of the sodium chloride-betaine cotransporter and the sodium *myo*-inositol cotransporter (Woo et al., 2000). It is also interesting to speculate that Cul-5 may be involved in the breakdown of proteins into amino acids via the 26S proteasome. These free amino acids from proteolysis would then be available to potentially function as an adaptive response to osmotic stress.

A possible explanation for the continued elevation of cul-5 mRNA in the kidney 24 hours after rehydration may be attributed to the increased metabolic activity of the kidney in response to changes in fluid/osmolality status from water deprivation and then rehydration. Since the kidney is the primary organ responsible for maintaining fluid and

electrolyte balance, it maintains a high metabolic rate that may require increased ubiquitination and proteolysis of various proteins that respond to dehydration and rehydration.

The increase in cul-5 mRNA levels in the CNS and kidney in response to water deprivation suggests a role for this protein in the regulation of cellular response to osmotic stress. Cul-5 may indeed serve a functional role as a post-translational mediator of proteins via E3 ubiquitin ligase activity for maintenance of cellular osmotic regulation.

Alterations of cullin-5 transcript levels by cellular stress: Hemorrhagic shock

Hypovolemia resulting from hemorrhagic shock can be devastating to troops and the military mission and is the most frequent cause of traumatic death in combat casualties, representing approximately 50% of the total killed in action (Maughon, 1970; Bellamy, 1998). We found cul-5 mRNA expression was significantly altered in regions of the CNS in the laboratory rat in response to hemorrhagic shock. Quantitative realtime PCR showed cul-5 transcripts in the cerebellum and brainstem were increased after 60 minutes of hemorrhagic shock with a greater increase found in the brainstem. Different changes were observed in the hypothalamus, where there was a decrease in cul-5 mRNA levels. There was no significant change in cul-5 mRNA levels in the cerebral cortex, small intestine, kidney, liver, lung, or pituitary gland after hemorrhagic shock (Ceremuga et al., 2003b).

The ischemic and hypoxic consequences of hemorrhagic shock result in activation of many systemic and cellular pathways in an attempt to maintain optimal cellular metabolic activity and thwart organ failure (Peitzman et al., 1995). Mitochondrial

dysfunction from failure of oxidative-phosphorylation and insufficient ATP production (Hirasawa et al., 1978; Chaudry, 1983) has been described as a major factor in cellular dysfunction occurring in hemorrhagic shock (White et al., 1973; Moreno-Sanchez and Torres-Marquez, 1991). Ischemia progression leads to depletion of high energy stores and decoupling of the electron transport system with the production of reactive oxidant species (ROS) (Proctor et al., 1982; Zhang and Piantadosi, 1992; Rosenthal et al., 1995). ROS have been shown to cause damage in brain (Patt et al., 1988; Rosenthal et al., 1995), heart (Brown et al., 1988), and lung (Grosso et al., 1989) tissues and the ROS damaged proteins become targets for ubiquitination and proteasomal degradation (Ciechanover, 1994). Proteasomal degradation of oxidized proteins has been reported in human hematopoietic cells (Grune et al., 1996), liver epithelial cells (Grune et al., 1995), and fibroblasts (Sitte et al., 1998). More recently Weih and associates (Weih et al., 2001) showed degradation of oxidized proteins in oxygen and glucose deprived rat primary cortical neurons via the proteasome. This is the first *in vivo* report of cul-5 mRNA regulation by hemorrhagic shock in the CNS, suggesting it is an important component in regulating processes that ameliorate the impact of cytotoxic proteins generated by hypoxia, ischemia, ROS, and free radical damage.

Prospects for modulation of proteasome activity in human disease states

The ubiquitin-proteasomal degradation of regulatory proteins has important implications in cellular pathways: the transition and progression in the cell cycle (Koepp et al., 1999), increases in the inflammatory response, antigen presentation, and policing abnormal or aberrant proteins (Ciechanover, 1994; Ciechanover et al., 2000). Many of

these crucial cellular processes, such as p53 degradation and proteasomal muscle breakdown (Lee and Goldberg, 1998) appear to be regulated by proteasomal proteolysis. This has prompted investigation relating to pharmacological targeting of the proteasomal pathway's elements. For example, although proteasome inhibitors in high doses are lethal to all cells, cancer cells seem to be more vulnerable. This has led to experimental trials with proteasome inhibitors in several cancers by Millennium Pharmaceuticals (Adams et al., 1999). Similarly, there is testing of proteasomal inhibitors as a possible treatment in stroke and myocardial infarction in humans by Millennium Pharmaceuticals (Goldberg et al., 2001). Although proteasome inhibitors have profound anti-inflammatory and anticancer effects, more selective inhibition of the ubiquitin-proteasomal pathway, in theory, may be achieved by blocking specific ubiquitin enzymes.

Continual and frequent discoveries about the importance of E3 ubiquitin ligases in disease are rapidly emerging. Several E3 enzymes have also been identified as tumor suppressors, linking ubiquitination with the onset of cancer. An example is the von Hippel Lindau (VHL) tumor suppressor, an E3 ubiquitin ligase that is often mutated in kidney tumors. VHL restricts cell growth by limiting blood vessel development in tissues. However, a mutation in VHL enables newly developed tumors to develop a rich vasculature and rapid tumor growth. In addition, an inherited form of Parkinson's disease occurs from a mutated E3 ubiquitin ligase enzyme that results in the accumulation of certain proteins in brain cells (Shimura et al., 2000). Moreover, viruses have gained the means to hijack the process of ubiquitination and protein degradation for their own survival. Human papilloma viruses (HPVs) have developed the ability to

synthesize a protein that binds both to p53, a tumor suppressor protein, and an E3 ubiquitin ligase enzyme. This leads to the ubiquitination and proteasomal destruction of cellular tumor control (p53) and an increased susceptibility to cancer (Goldberg et al., 2001).

Although currently there are no E2 or E3 inhibitors, these enzymes are likely to be targeted for pharmaceutical development in the future. Uncovering the molecular nature and regulation of E3 ubiquitin ligases is critically important in comprehending protein degradation regulation and continues to undergo extensive exploration. Since it is believed that each E3 ubiquitin ligase is responsible for the degradation of a certain number of proteins, specific inhibitors of E3 ubiquitin ligases may be highly selective with fewer untoward effects, versus blockade of the entire proteasome. Recent discovery of various categories of E3 ubiquitin ligase enzymes has initiated new approaches for drug discoveries (Goldberg et al., 2001).

Future Directions

The structural organization of Cul-5 may be identified by similar methods (x-ray crystallography) used by Zheng et al. in characterizing the quaternary structure of Cul-1 (Yu et al., 1998; Zheng et al., 2002). Cul-5's specific protein-protein interactions and precise protein substrates need to be delineated in an attempt to potentially dissect out various other molecules that may be involved in this E3 ubiquitin ligase's activity. This may be accomplished by utilizing various protein assays, such as protein purification, mass spectrometry based sequencing, and immunoprecipitation techniques. These methods have been used by other researchers investigating cullin E3 ubiquitin ligase

complexes and yielded significant findings, such as discovering the essential subunit Rbx1 (Seol, Feldman et al. 1999). Likewise, Querido et al. identified the only known targeted protein substrate (p53) of the Cul-5 E3 ubiquitin ligase complex. They purified an adenovirus multiprotein E4orf6- E1B55K associated complex known to degrade p53 and found that it consisted of an E3 ubiquitin ligase containing Cul-5, elongins B and C, and Rbx1 (Querido et al., 2001).

In summary, the implications of these results are significant as protein degradation is a vital regulator of cellular functions, i.e., cell cycle, signal transduction, transcriptional regulation, and tumor suppression (Ciechanover et al., 2000; Pickart, 2001). Our recent work found cul-5 mRNA expression pattern changes in the CNS in response to cellular stresses of hemorrhagic shock (Ceremuga et al., 2003b) and water deprivation (Ceremuga et al., 2003c). Further work is needed, for example, to determine the precise physiological role of Cul-5 and what proteins are specifically targeted by this E3 ubiquitin ligase. While the precise physiological role of Cul-5 remains to be elucidated by future investigations (Querido et al., 2001), this molecule may be intricately involved in degradation of proteins that play key roles in the regulation of cellular homeostasis.

These investigations may help us gain understanding of many cellular processes that rely on protein stability, and understanding the controls of this stability will answer many of biology's mysteries.

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