REGULATORY DOMAINS OF C/EBPδ INVOLVED IN p38 MAPK

DEPENDENT TRANSCRIPTIONAL ACTIVATION

BY

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LIST OF ABBREVIATIONS

293T: human transformed kidney cells
AD: activation domain
AP-1: activator protein-1
APP: acute phase protein
APR: acute phase response
ATP: adenosine triphosphate
BR: basic region
BSA: bovine serum albumin
bp: base pairs
bZIP: basic region and leucine zipper domain
Caco-2: human colon carcinoma cells
cAMP: cyclic adenosine monophosphate
C/EBP: CCAAT/enhancer-binding protein
CHOP: C/EBP homologous protein
CO₂: carbon dioxide
cps: counts per second
CREB: cyclic AMP responsive element binding protein
CRP: C-reactive protein
C-terminal: carboxy–terminal
dCTP: deoxyctydine-5’-triphosphate
DMEM: Dulbecco’s modified Eagle medium
DNA: deoxyribonucleic acid
DTT: dithiothreitol
EDTA: ethylenediaminetetraacetic acid
EGTA: ethyleneglycol-bis-(β-aminoethyl ether) tetraacetic acid
EMSA: electrophoretic mobility shift assay
ERK: extracellular signal-regulated kinase
FBS: fetal bovine serum
GSK-3: glycogen synthase kinase-3
GST: glutathion S-transferase
HEPES: 4-(2-hydroxyethyl)-1-piperazineethane sulfate
HSF: heat shock factor
IBD: inflammatory bowel disease
IEC-6: rat intestinal epithelial cells
IFN-γ: interferon-γ
IκB: NF-κB inhibitor
IKK: IκB kinase
IL: interleukin
IL-R: interleukin receptor
IPTG: isopropyl-β-thiogalactopyranoside
IRAK: interleukin receptor-associated kinase
JNK: c-jun N-terminal kinase
LAP: liver-enriched transcriptional activator protein
LB: L-broth
LIP: liver-enriched transcriptional inhibitor protein
LZ: leucine zipper

MALDI-TOF: matrix assisted laser desorption-ionisation/time of flight

MAPK: mitogen activated protein kinase

MEK: MAP/ERK kinase

M KK: MAP kinase kinase

MOPS: sulphonic acid 3-(N-morpholino propane) sodium

MEF2: myocyte enhancer factor 2

mg: milligram

ml: millilitre

mM: millimolar

MSV: murine sarcoma virus

NF-κB: nuclear factor-κB

NP-40: nonidet P-40

nm: nanometre

nt: nucleotide

N-terminal: amino-terminal

PARP-1: Poly(ADP-ribose) polymerase 1

PBS: phosphate buffered saline

PCR: polymerase chain reaction

PEPCK: phosphoenolpyruvate carboxykinase

PKC: protein kinase C

PMSF: phenylmethanesulfonyl fluoride

RNA: ribonucleic acid

RPM: revolutions per minute
RSK: ribosomal S kinase
SAA: serum amyloid A
SAPK: stress-activated protein kinase
SDS: sodium dodecyl sulphate
STAT: signal transducer and activator of transcription
TAB: TAK1 binding protein
TAK: TGFβ-associated kinase 1
TBE: tris borate buffer
TBS: tris buffered saline
TEI-III: transactivation elements I - III
TEMED: tetramethylethylenediamine
TGF: transforming growth factor
TIR: Toll/IL-1 receptor
TLR: Toll-like receptor
TNF: tumour necrosis factor
Tollip: Toll-interacting protein
TRAF: TNF receptor-associated factor
TRIS: tris (hydroxymethyl) aminomethane
µg: microgram
µl: microlitre
UV: ultraviolet
WT: wild type
ZPK: zipper protein kinase
LIST OF PUBLICATIONS

1 - Svotelis A., Désilets A., Doyon G., Rivard N., and Asselin C. Regulatory domains of C/EBPδ involved in p38 MAP kinase dependent transcriptional activation. (in preparation)

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REGULATORY DOMAINS OF C/EBPδ INVOLVED IN p38 MAPK DEPENDENT TRANSCRIPTIONAL ACTIVATION

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ABSTRACT

We have previously shown that C/EBPδ, a transcription factor implicated in various cellular processes, is involved in the intestinal inflammatory response. Indeed, C/EBPδ levels increase in the intestine during the acute phase response. Furthermore, C/EBPδ regulates several inflammatory response genes, such as haptoglobin and α-acid glycoprotein, in the rat intestinal epithelial cell line IEC-6. However, the different C/EBPδ domains involved in transcriptional activation and the kinases implicated in its regulation have not been properly defined. The IL-1 dependent
induction of the haptoglobin-encoded acute phase protein in IEC-6 cells was decreased at the transcriptional level in response to the p38 MAP kinase inhibitor SB203580, as determined by Northern blot. The p38 MAPK inhibitor SB203580 also decreased C/EBPδ transactivation potential in transient transfections. The N-terminal region of C/EBPδ (amino acids 1 to 156), in contrast to the C-terminal region, mostly interacted with p38 MAP kinase through amino acids 70-108. Phosphorylation of C/EBPδ in the N-terminal region of amino acids 151 to 164 was repressed by a specific inhibitor of p38 MAPK. Site-specific mutation of a putative MAP kinase phosphorylation site at serine 160 decreased transcriptional activity, in contrast to the mutation of a second consensus site at threonine 156. These results suggest that phosphorylation of C/EBPδ by p38 MAPK is important for IL-1-induced transcription of haptoglobin, and that the putative MAPK phosphorylation site at serine 160 is important in this transcriptional activation. Through directed mutagenesis, we have obtained several deletion mutants in the N-terminal region of C/EBPδ. Transient transfection assays revealed redundant N-terminal domains essential for optimal C/EBPδ transcriptional activity between amino acids 36 and 108. C/EBPδ may possibly interact in the conserved N-terminal activation domain with the co-activator p300, as is the case for other C/EBP isoforms, and this functional collaboration may increase C/EBPδ transcriptional activity. Further studies will be performed to determine the importance of different domains of C/EBPδ in IL-1β-induced activation of haptoglobin in vivo in intestinal epithelial cells, and the importance of modification of C/EBPδ by phosphorylation. This study has also demonstrated the interaction of novel partners of C/EBPδ, as demonstrated
by GST pull-down assays and identification by mass spectrometry. C/EBPδ interacts with a novel kinase besides the p38 MAPK, a kinase of approximately 120 kD, possibly a kinase associated with stress-dependent signal transduction ZPK (zipper protein kinase) that interacts with other leucine zipper transcription factors. The C-terminal region of C/EBPδ interacts with Ku70 and PARP-1, two factors known to play important roles in nuclear processes, such as DNA repair and transcription.
C/EBPδ est un facteur de transcription induit dans plusieurs tissus, dont l'intestin, lors de la phase aiguë de l'inflammation, et régularise l'expression de gènes de réponse inflammatoire. Les fonctions des différentes régions de cette protéine n'ont pas encore été très bien définies. Nous voulons identifier les domaines fonctionnels de C/EBPδ, et sa régulation par phosphorylation. L'induction de l'expression du gène haptoglobine par l'IL-1 dans les cellules IEC-6 est diminuée après traitement avec l'inhibiteur de MAPK p38, SB203580, tel que déterminé par buvardage Northern. Des transfections transitoires dans les cellules 293T ont démontré que l'activité transcriptionnelle de C/EBPδ est réprimée par cet inhibiteur spécifique. La région N-terminale de C/EBPδ se lie à la MAP kinase p38, entre les acides aminés 70 et 108, ainsi que les isoformes C/EBPα et C/EBPβ. La région N-terminale (acides aminés 151 à 164) de C/EBPδ est phosphorylée *in vitro*, et cette phosphorylation est réprimée par l'inhibiteur spécifique de la MAPK p38. La mutation de la sérine 160, un site putatif de phosphorylation par des MAP kinases, entraîne une diminution de l'activité transcriptionnelle de C/EBPδ. Par contre, la mutation d'un deuxième site consensus (thréonine 156) n'a aucun effet. Ces résultats suggèrent que la phosphorylation de C/EBPδ par la MAPK p38 est essentielle à la transcription du gène haptoglobine dépendante de l'IL-1, et que le site putatif de phosphorylation (sérine 160) est important dans cette activation transcriptionnelle. Par mutagenèse dirigée, nous avons obtenu cinq mutants de délétion de C/EBPδ. En transfectant ces mutants dans
les cellules 293T, nous avons défini des domaines essentiels à l'activité transcriptionnelle de C/EBPδ entre les acides aminés 36 et 108. C/EBPδ pourrait interagir par ces domaines N-terminaux d’activation avec le co-activateur p300, et cette collaboration pourrait induire l’activité transcriptionnelle de C/EBPδ.

L’importance des différents domaines individuels de C/EBPδ dans l'induction des gènes par l'IL-1 et l'importance de la modification de C/EBPδ par la phosphorylation dans l'interaction avec p300 seront éventuellement déterminées in vivo dans des cellules intestinales épithéliales. Cette étude a aussi démontré une interaction de C/EBPδ avec de nouveaux co-facteurs par pull-down GST et identification par spectrométrie de masse. C/EBPδ semble être phosphorylé par une kinase d’approximativement 120 kD, peut-être une kinase de type ZPK (zipper protein kinase) pouvant lier d'autres facteurs de transcription de type leucine zipper. La région C-terminale de C/EBPδ interagit avec Ku70 et PARP-1, deux facteurs connus pour leur rôle dans différents processus nucléaires, notamment la réparation de l'ADN et la transcription.
INTRODUCTION

1 – Intestinal inflammatory response

Intestinal epithelial cells, an integral component of the mucosal immune system, form a critical mucosal barrier between the host’s internal milieu and the external environment (Fiocchi, 1998). Chronic inflammatory bowel diseases (IBD), such as Crohn’s disease and ulcerative colitis, are disorders of the intestine caused by an abnormal and uncontrolled mucosal immune response (Fiocchi, 1998). The inflammatory response in the intestine is characterized by an acute phase response (APR), which uses the concordant responses of cellular and humoral elements, namely cytokines, reactive oxygen radicals, bacterial products, platelet activating factor and eicosanoids (Baumann and Gauldie, 1994). In IBD, there is a characteristic excessive production of cytokines such as Interleukin-1 (IL-1), Tumour Necrosis Factor-α (TNF-α), Transforming Growth Factor-β (TGF-β) and Interleukin-6 (IL-6) (Fiocchi, 1998). The inflammatory response in IBD leads to local tissue destruction, since the inflammation response is not resolved (Fiocchi, 1998). This explains the increased interest in the study of the mechanisms involved in the resolution of the inflammatory response.

Some key targets in APR are the acute phase proteins (APP), of which there are two classes. Both classes have cytokines (regulatory proteins released by the immune system) involved in their induction and regulation (Poli, 1998). The Class I APPs require the combination of the cytokines IL-1 and IL-6 for induction, while the
Class II APPs are dependent on IL-6 and other related cytokines (Poli, 1998). Some examples of APPs are: serum amyloid A (SAA), involved in leukocyte activation, chemotaxis and phagocytosis; haptoglobin, involved in the scavenging of hemoglobin; C-reactive protein (CRP), which enhances or subdues the inflammatory mediator release by interacting with monocytes or macrophages (Suffredini et al., 1999). There are four major classes of regulators of APPs: the IL-6/gp130 family of cytokines, the IL-1 type proinflammatory cytokines including IL-1 and TNF-α, growth factors, for example TGFβ, a major regulator in the APR performing a dual role, proinflammatory in the early stage, with a switch to immunosuppressor activity later in the APR (Koj, 1996; Yu et al., 1999), and corticosteroids (Baumann and Gauldie, 1994; Suffredini et al., 1999). In IBD, the proinflammatory cytokines’ action is increased, as is the effect of the growth factors, assumed to be responsible for mediation of intestinal epithelial repair and defence (Fiocchi, 1998; Morales et al., 1996). IBD is thus a disease in which the normal regulatory pathways involved in the resolution of the inflammatory response is impaired.

Transcription of the APPs is regulated through the induction of four major classes of transcription factors: nuclear factor κB (NF-κB), the signal transducer and activator of transcription (STAT) family, glucocorticoid receptor, and the CCAAT/enhancer binding protein (C/EBP) family (Baumann and Gauldie, 1994; Mackiewicz, 1997). This entire acute phase response, including the different factors involved, is summarized in figure 1. The C/EBP family of transcription factors are the family of most interest to the research in our laboratory. These transcription
The acute phase response (APR) cascade

Simplified cascade of the entire acute phase response (APR), including the different factors involved (APP are acute phase proteins), modified from Koj, 1996.
Triggering Factors (infection/injury)

↓

Local reaction (stress-responsive protein kinase cascade)

↓

Transcription factor induction of transcription of cytokine genes

↓

Production and release of inflammatory cytokines (IL-1, IL-6): the mediators

↓

Induction of stress-responsive protein kinase cascade via cytokines

↓

Secondary systemic reaction: induction of APP via transcription factors such as STAT, glucocorticoid receptor, NF-κB, C/EBP
factors are involved in the basal and induced expression of APPs through TGFβ and IL-6 signalling (Koj, 1996), and, as demonstrated in our laboratory, IL-1 in intestinal epithelial cells (Désilets et al., 2000).

2 – IL-1 signalling during inflammation

One of the major proinflammatory cytokines involved in inflammation is IL-1. Two of the key targets of IL-1 signalling are the MAPK family and the transcription factor NF-κB, also activated by LPS and IL-18 which act through receptors of the IL-1R/TLR superfamily (Kojima et al., 1998; Muzio et al., 1998). During inflammation, IL-1 will bind its receptor (IL-1R) and initiate the activation of several signalling cascades, leading to the transcription of a large number of APPs via the activation of transcription factors.

The IL-1R is a member of the IL-1R/TLR (interleukin-1 receptor/Toll-like receptor) superfamily which is known to be important in inflammation and defence. All members contain a common cytosolic domain, termed the Toll/IL-1 receptor (TIR) domain (O’Neill and Greene, 1998). IL-1R-deficient mice have impaired APR and are incapable of combating inflammation (Labow et al., 1997). Serum levels of IL-1, which are low in normal intestinal cells, are increased in cells isolated from mucosa of patients with intestinal inflammatory diseases such as ulcerative colitis and Crohn’s disease (Mahida et al., 1989). These results underline the importance of IL-1 and its signalling during the APR.
The binding of IL-1 to IL-1R leads to the interaction of the receptor with the protein MyD88A, a member of a subgroup of the IL-1R/TLR superfamily containing non-receptor proteins which possess a TIR domain and are cytosolic (Wesche et al., 1997). MyD88A likely complexes with IL-1R via a TIR–TIR interaction and the protein is thus activated (Takeuchi et al., 2000). The activated MyD88A acts as an adapter protein that will recruit the kinase IRAK-4 (IL-1 receptor associated kinase). Prior to stimulation with IL-1, a complex between Tollip (Toll-interacting protein) and unphosphorylated IRAK-1 is formed in the cytoplasm. This complex is then recruited to the IL-1R complex following stimulation with IL-1 (Burns et al., 2000). IRAK-1 interacts with MyD88A, releasing the IRAK-1 association with Tollip. IRAK-1 is then auto-phosphorylated and phosphorylated by IRAK-4 (Burns et al., 2003). This phosphorylation event reduces IRAK-1 affinity for MyD88A and increases its affinity for the adapter TRAF6 (tumour necrosis factor receptor-associated kinase) (Cao et al., 1996), recruiting it to the IL-1R complex (Jiang et al., 2002) (see figure 2).

The complex of IRAK-1 and TRAF6 leaves the IL-1R complex to associate with the kinase TAK1 (TGFβ-associated kinase 1), and two TAK1 binding proteins (TAB1 and TAB2) at the cellular membrane, inducing the phosphorylation of TAK1 (Jiang et al., 2002). The TRAF6/TAK1/TAB1/TAB2 complex translocates to the cytosol, leaving IRAK-1 at the membrane, where TAK1 is activated by yet unknown protein complexes (Jiang et al., 2002). The activation of TAK1 in the cytosol leads to the activation of the transcription factor NF-κB, as well as the activation of a number
Overview of IL-1β-mediated signalling cascades

IL-1β binding to IL-1R causes the recruitment of the adaptor protein MyD88A and the complex TRAF6/IRAK-1. IRAK-4 is recruited to the complex by MyD88A and stimulates the phosphorylation of IRAK-1. This phosphorylation event activates IRAK-1 and triggers its release, along with TRAF6, from the receptor complex. TRAF6/IRAK-1 then associates with the TAK1/TAB1/TAB2 complex at the cellular membrane and phosphorylates TAK1. The newly formed TAK1/TAB1/TAB2/ TRAF6 complex breaks from IRAK-1 and the membrane to phosphorylate and activate MAPK pathways and NF-κB signalling (modified from Janssens and Beyaert, 2003; Ichijo 1999).
Transactivation of genes implicated in APR
of phosphorylation cascades, namely the p42/p44, JNK and p38 MAPK cascades (see figure 2).

A model for inflammation in hepatocytes was proposed by Poli (1998), involving the activation of the transcription factor NF-κB by IL-1β. TAK1 is known to phosphorylate the (Inhibitor of NF-κB) kinase (IKK) complex (Wang et al., 2001). This phosphorylation event activates IKK, inciting the release of the liaison of NF-κB with the inhibitor IκB by phosphorylation of this inhibitor, targeting it for degradation by the proteasome. NF-κB is then liberated to translocate to the nucleus and bind NF-κB sites on the promoter region of APP genes (see figures 2 and 3).

The activation of the JNK, p42/p44 and p38 MAPK cascades also occurs through the kinase TAK1 (Fig. 2). These MAPK cascades are known for their activation of transcription factors via phosphorylation. TAK1 will activate via phosphorylation MKK7, the upstream kinase activator of JNK (O’Neill and Dinarello, 2000). Phosphorylated JNK, known as a stress-activated protein kinase, will translocate to the nucleus and initiate the transcription of APPs via the transcription factor c-jun (see figure 2) (Pearson et al., 2001). TAK1 can also activate MEK, which is upstream of p42/p44 MAPK (O’Neill and Dinarello, 2000), leading to the phosphorylation and activation of several transcription factors (see figure 2), such as Elk-1 (known to activate transcription of c-jun) (Janknecht et al., 1993) and C/EBPβ (Poli, 1998) (see figure 3). The p38 MAPK is also activated via the activation of upstream kinases MKK3 or MKK6 by TAK1 (O’Neill and
Figure 3

Signalling cascades involved in the hepatocyte inflammatory response

The major signalling cascades involved in APP gene transactivation in hepatocytes during a cytokine-induced inflammatory response, as proposed by Poli (1998).
p38 MAPK is known to activate many transcription factors, such as activating transcription factor (ATF), myocyte enhance factor 2 (MEF2) family, and C/EBPβ (Raingeaud et al., 1995; Tan et al., 1996; Han et al., 1997; Zhao et al., 1999).

The study of the IL-1 signalling pathway indicates its importance in the inflammatory response, as well as the activation of transcription factors implicated in the APR, either directly or via their activation by phosphorylation. One major family of transcription factors that has been studied in IL-1-induced inflammatory response besides NF-κB is the C/EBP family. Studies have demonstrated the implication of C/EBPs in the induction of APPs, as well as the activation of certain isoforms of this family by IL-1 stimulation (Hungness et al., 2002, Gheorghui et al., 2001, Désilets et al., 2000).

3 – The C/EBP family of transcription factors

3.1 – Structure

The C/EBP family of transcription factors was first discovered in 1988 in the laboratory of Steve McKnight (Landschulz et al., 1988a). This family was so named CCAAT/enhancer-binding protein due to its ability to bind to both the CCAAT box of promoters and the core homology sequence TGTGG(A/T)(A/T)(A/T)G in viral enhancers (Landschulz et al., 1988a). The first cloned gene was named C/EBPα (Johnson et al., 1987), with the subsequent isoforms named C/EBPβ (Cao et
al., 1991), C/EBPδ (Cao et al., 1991), C/EBPγ (Roman et al., 1990), C/EBPε (Williams et al., 1991), and C/EBPζ or CHOP (Ron and Habener, 1992).

The different isoforms of the C/EBP family contain a C-terminal domain with a DNA-specific binding region, the basic region, and the leucine zipper: together these two domains are named the bZIP domain (see figure 4) (Wedel and Loms Ziegler-Heitbrock, 1995; Fiocchi, 1998). The basic region determines DNA specificity (Wedel and Loms Ziegler-Heitbrock, 1995). The leucine zipper, a heptad repeat of leucine residues within a 35 amino acid sequence, allows the different isoforms to form homodimers and heterodimers with one another (Landschulz et al., 1988b; Wedel and Loms Ziegler-Heitbrock, 1995). The two α-helices of the separate monomer C/EBP isoforms will dimerize with each other by “zipping” along the hydrophobic residues in the leucine zipper region. This dimerization is necessary for the binding of C/EBPs to DNA, allowing the two basic regions to clasp the DNA (see figure 4) (O’Neill et al., 1990; Ellenberger et al., 1992; Wedel and Loms Ziegler-Heitbrock, 1995).

The N-terminal region of the C/EBPs is known as the transactivation domain of the protein and does contain sequence homologies between the isoforms. The transactivation region contains both structural negative and positive regulatory regions, as well as protein-protein interaction domains. For example, the C/EBPα isoform possesses three known activation domains, TEI, TEII, TEIII (Pei and Shih, 1991, Nerlov and Ziff, 1995) and the C/EBPβ isoform also possesses three activation
The general structure of the C/EBP family

The model of C/EBP structure, formation of C/EBP dimers and the attachment of dimers to DNA.
domains (Kowenz-Leutz et al., 1994) (see figure 5). C/EBPα has one negative regulatory domain (Pei and Shih, 1991, Nerlov and Ziff, 1994), and C/EBPβ has two negative regulatory domains (Kowenz-Leutz et al., 1994, Williams et al., 1995). However, the C/EBPδ isoform has not yet been characterized in the N-terminal region for positive or negative regulatory domains.

3.2 – Expression patterns and functions

The C/EBPs are differentially regulated in various tissues at both the mRNA and protein levels, and thus have separate roles in each tissue. Discrepancies between protein and mRNA expression levels suggest the involvement of modification of the C/EBPs proteins by post-transcriptional regulatory mechanisms. Although the three most studied isoforms, C/EBPα, C/EBPβ and C/EBPδ, are unique in their structure and function, many similarities can be found. There are several sequence homologies that are observed between the different genes (see figure 5), and often they will interact with the same proteins.

C/EBPα is found in two different forms in tissues, either p42 or p30. p30, a truncated form of C/EBPα formed by the use of alternative translation initiation sites, possesses a lower transactivation potential (Landschulz et al., 1988a). C/EBPα is highly expressed in adipose tissue, liver, intestine, lung, adrenal gland, peripheral blood mononuclear cells and placenta (Cao et al., 1991; Williams et al., 1991; Antonson and Xanthopoulos, 1995; Lekstrom-Himes and Xanthopoulos, 1998).
Sequence homologies between C/EBP isoforms α, β, and δ

Amino acid sequences of the three major C/EBP isoforms, C/EBPα, C/EBPβ, and C/EBPδ, with the sequence homologies indicated by dotted line boxes. The solid boxes indicate the activation domains in both C/EBPα and C/EBPβ isoforms; TEI-III for C/EBPα, AD1-3 for C/EBPβ. The basic region of all of the isoforms is indicated as well by a dotted line box.
C/EBPα plays a very important role in the terminal differentiation of adipocytes (Darlington et al., 1998) and hepatocytes (Lekstrom-Himes and Xanthopoulos, 1998). Knockouts of C/EBPα in mice demonstrated that C/EBPα is required for the differentiation of white, not brown, adipose tissue (Linhart et al., 2001). More specifically, C/EBPα induces transcription of several differentiation-specific genes after its induction in pre-adipocytic cells by cyclic AMP (Tae et al., 1995). Knockout mice die of hypoglycemia due to an absence of liver glycogen and gluconeogenic enzymes (Wang et al., 1995). Further studies demonstrated that C/EBPα also works with cyclic AMP in the transactivation of the PEPCK (phosphoenolpyruvate carboxykinase) promoter in hepatocyte differentiation (Roesler et al., 1996). The phosphorylation of C/EBPα is necessary for the maximal transcriptional activity on the PEPCK promoter (Routes et al., 2000), promoting its important role in energy metabolism. During the inflammatory response, it is believed that C/EBPα expression is inhibited (Ramji and Foka, 2002), but studies in knockout mice demonstrated that C/EBPα is absolutely required for STAT3 regulation in the APR in neonatal mice, and cannot be compensated for by other C/EBP family members (Burgess-Beusse and Darlington, 1998).

Many similarities occur between the sequence and expression patterns of the isoforms C/EBPβ and C/EBPδ although these two isoforms are unique. There exists three different forms of C/EBPβ in the cell, LAP* (38 kD), LAP (35 kD) and LIP (20 kD), formed through the use of alternative translation initiation sites. LIP is the dominant negative form in the cell. The C/EBPβ isoform is generally expressed in
the liver, intestine, lung, adipose tissue, spleen, kidney and myelomonocytic cells (Ramji and Foka, 2002). Knockout mice of C/EBPβ have impaired macrophages, mammary glands, carbohydrate metabolism, brown adipose tissue and hepatocyte proliferation following liver injury, giving this isoform roles in inflammation, homeostasis, adipocyte differentiation and tissue repair (Tanaka et al., 1995; Sterneck et al., Seagroves et al., 1998; Tanaka et al., 1997; Croniger et al., 2001; Greenbaum et al., 1998).

The C/EBPδ isoform is mostly expressed in adipose tissue, lung and intestine. Little is known on the functions of the C/EBPδ isoform, but often the functions are related to those of C/EBPβ. C/EBPδ is implicated as well in the differentiation process. Although no obvious phenotype is observed in knockout mice, double C/EBPβ and C/EBPδ knockout mice show impaired adipocyte differentiation (Tanaka et al., 1997). It is thought that the expression of C/EBPβ and C/EBPδ expression early in the differentiation program induces the expression of C/EBPα (Lane et al., 1999).

During the inflammatory response, C/EBPβ and C/EBPδ are induced by several different inflammatory stimuli in various cell types, and binding sites for these isoforms have been identified on the promoters of important APPs and APP regulators (Ramji and Foka, 2002). In hepatocytes, the C/EBP isoforms form both constitutive and inducible complexes on the IL-1- and IL-6-responsive elements of APP genes (Koj, 1996). The sequence of these promoters implicated in C/EBP DNA-
binding is an optimal palindromic sequence, or consensus site, GATTGC-GCAATC (Takiguchi, 1998). The isoforms C/EBPβ and C/EBPδ are upregulated by inflammatory stimuli such as turpentine oil, lipopolysaccharide (LPS), IFN-γ and proinflammatory cytokines (IL-1 and TNF-α) via the activation of MAPK pathways (Poli, 1998, Hungness et al., 2002). The induction of C/EBPδ by these stimuli is more dramatic than that of C/EBPβ (Takiguchi, 1998). Further support for the role of C/EBPβ and C/EBPδ in the inflammatory response has been provided by studies performed in knockout mice. Mice deficient in C/EBPβ have impaired expression of the APPs SAA, α-acid glycoprotein, and TNF-α (Ramji and Foka, 2002). Yet not all of the APP targets of this isoform are impaired, suggesting a possible compensation mechanism via C/EBPδ (Ramji and Foka, 2002).

The activity of these isoforms has been studied extensively in the liver, in hepatocytes and in adipose tissue, yet not much is known for their roles in the intestine. The C/EBP isoforms were found to be expressed during murine intestinal post-natal development and in rat intestines during the APR (Blais et al., 1995), prompting studies in our laboratory concerning the rat intestinal epithelial cell line IEC-6 (Boudreau et al., 1996). Transient transcription of C/EBPβ and C/EBPδ was induced by glucocorticoids, including an increase in C/EBP DNA-binding capacity (Boudreau et al., 1996). Further research in intestinal cells then involved the role of TGFβ in the APR, namely its effect on the regulation of the C/EBP family. Experiments using IEC-6 cells indicated that C/EBP isoforms β and δ were involved both in the TGFβ attenuation and the glucocorticoid-dependent induction of two
APPs, specifically α-acid glycoprotein and haptoglobin (Boudreau et al., 1998; Yu et al., 1999). Recently, C/EBPβ and C/EBPδ have proved to be the most important activators of haptoglobin gene expression in IEC-6 cells in vitro (Désilets et al., 2000) and in vivo (Gheorghiu et al., 2001).

3.3 – Regulation of the C/EBP family of transcription factors

The activity of the C/EBPs is also regulated by post-transcriptional protein modification. The modulation of the transactivation potential of these transcription factors occurs often through different protein-protein interactions or phosphorylation of the protein. As for protein-protein interactions, studies have shown that the C/EBP isoforms differ in transactivation potential (Takiguchi, 1998). For example, the truncated forms of C/EBPα and C/EBPβ are lower transcriptional activators, and thus homo- or hetero-dimerization with these proteins will diminish the total transactivation potential (Descombes, 1991, Ossipow, 1993).

Interaction of the C/EBPs with transcription factors or co-activators can also affect the transcriptional activation potential of the C/EBPs. Transcription factors can either interact through the leucine zipper or the N-terminal region of the protein. For example, C/EBPβ has been shown to interact with the p50 subunit of NF-κB, ATF and the AP-1 complex, these interactions affecting transcriptional activity (LeClair et al., 1992; Vallejo et al., 1993; Hsu et al., 1994). C/EBPα and C/EBPβ have also been shown to bind the nuclear co-activator p300 via several poorly defined N-
terminal regions (see figure 5; Erickson et al., 2001; Schwartz et al., 2003), affecting activity of both C/EBP isoforms. C/EBPα interacts via its N-terminal region as well with the TATA-binding-protein-TFIIB complex (TEI) and the SWI/SNF chromatin remodelling complex (TEII), this interaction activating C/EBPα during adipocyte differentiation (see figure 5; Pederson et al., 2001).

The C/EBPs can also be modulated through ubiquitination of the N-terminal domain. Ubiquitination of a protein will target it for degradation by the proteasome and/or affect transcriptional regulation. Recent studies have revealed that proteasome inhibitors administered to human intestinal epithelial cells will increase C/EBPβ and C/EBPδ protein levels, suggesting that these isoforms are regulated by the proteasome (Hungness et al., 2002; data not shown). Another study demonstrated definitively the degradation of these isoforms by the proteasome in kidney cells, and this degradation could be avoided by dimerization (Hattori et al., 2003).

The most important regulation of C/EBP recognized in research has been the phosphorylation of these proteins. Phosphorylation of the N-terminal region will often affect the transactivation potential of the protein, either activating or inhibiting the C/EBPs transcriptional activity, depending on the area that is phosphorylated. The C-terminal region (the basic region and leucine zipper) can also be phosphorylated, thus often affecting DNA-binding and dimerization.
Several studies have identified phosphorylation sites on the C/EBPα isoform. In adipocytes, the presence of insulin stimulates the dephosphorylation of C/EBPα by inactivating GSK3 (Glycogen Synthase Kinase 3), which phosphorylates the isoform on threonine residues, leading to the degradation of C/EBPα (Ross et al., 1999). C/EBPα can also be phosphorylated at several serine residues by protein kinase C (PKC) in the C-terminal region, decreasing the DNA binding activity (Mahoney et al., 1992). Another study has shown that the phosphorylation of C/EBPα by PKC via the Ras signalling pathway on one of these serines will increase C/EBPα transcriptional activation of the granulocyte colony-stimulating factor receptor promoter, thus inducing granulocyte differentiation (Behre et al., 2002).

C/EBPβ has proven to be a highly phosphorylated protein both in the N-terminal activation region and in the C-terminal region, affecting major cellular activities regulated by C/EBPβ. The phosphorylation of the rat serine 105 of C/EBPβ in hepatocytes by ribosomal S kinase (RSK) allows for TGFα-dependent proliferation (Buck et al., 1999), and helps protect against apoptosis following injury (Buck et al., 2001). PKC is also known to phosphorylate this serine residue, increasing C/EBPβ transactivation potential in hepatocytes (Trautwein et al., 1993). Phosphorylation of a threonine residue in an inhibitory region of the N-terminal domain of the C/EBPβ protein by the p42/p44 MAPK in response to interferon-γ activates the protein (Hu et al., 2001). This threonine residue has also been considered as a target of the p38 MAPK (Engelman et al., 1998).
As for the C/EBPδ isoform, few studies have demonstrated the phosphorylation state of this protein. The transcriptional activity of C/EBPδ is increased in the presence of phosphatase inhibitors in hepatocytes (Ray and Ray, 1994). In inflammatory conditions, dephosphorylation of C/EBPδ will affect its DNA-binding activity to the promoter of the APP genes α₁-acid glycoprotein and serum amyloid A (Ray and Ray, 1994). However, we have detected many sequence homologies between the transactivation domains of C/EBPβ and C/EBPδ (see figure 5). We propose to determine the exact transcriptional activation and/or repression domains important for transcriptional regulation of gene expression by C/EBPδ.

4 – Research project

4.1 – Hypothesis

We propose that C/EBPδ is composed of different N-terminal transactivation domains involved in transcriptional activation or repression during the IL-1 induced inflammatory response, and that these domains are regulated by protein kinases.

4.2 – The p38 MAPK is involved in the regulation of C/EBPδ transcriptional activity

As previously mentioned, the C/EBP isoforms are involved in many important cellular processes. Previous data in our laboratory has indicated that in response to inflammatory stimuli in intestinal epithelial cells the C/EBPδ isoform is the most induced of the C/EBPs (Désilets et al. 2000), strongly suggesting an important role in
the regulation of the intestinal inflammatory response. However, the regulation of C/EBPβ transcriptional activity is not known. The first objective was to determine the kinases involved in C/EBPβ regulation, and determine the region of phosphorylation of the protein. The MAPK family plays an important role in the APR. More specifically, IL-1 can induce the activity of p38 MAPK (O’Neill et al., 2000). The results of GST-pull down assays indicated an interaction between C/EBPβ and p38 MAPK in a domain between amino acids 70 to 108, containing two putative MAPK docking sites. The phosphorylation of the C/EBPβ protein, demonstrated by in vitro kinase assays, occurred in a region between amino acids 151 to 164, in which the serine residue at position 160 is the most important in optimal C/EBPβ transactivation potential. Activation of C/EBPβ by p38 MAPK, as assessed by the use of a specific p38 MAPK inhibitor in luciferase assays, was shown to be necessary for transcriptional activation of the haptoglobin gene.

4.3 – Redundant N-terminal domains are essential for C/EBPβ transcriptional activity

The isoforms C/EBPα and C/EBPβ both contain conserved regulatory domains in their N-terminal regions. Our objective was to prove the existence of such domains in C/EBPβ. Sequential deletions of the transactivation domain of C/EBPβ by mutagenesis followed by transient transfection assays have identified redundant activation domains between amino acids 36 and 108. Thus, transactivation domains characterized in C/EBPβ (amino acids 52-77) (Williams et al., 1995), as well as the region TEII (amino acids 75-82) of C/EBPα (Nerlov and Ziff, 1994) have been
conserved in C/EBPδ (see figure 5). These regions of C/EBPα and C/EBPβ interact with the co-activator p300, increasing transcriptional activity (Erickson et al., 2001; Guo et al., 2001). Therefore, C/EBPδ may possibly interact in the conserved N-terminal transactivation domain with the co-activator p300, and this functional collaboration will increase C/EBPδ transcriptional activity on certain promoters. The p38 MAPK may modulate the interaction of C/EBPδ with p300.

4.4 – C/EBPδ interacts with different novel partners

The existence of co-activator proteins (or partners) of C/EBPδ remained to be determined. We have identified by mass spectrometry novel proteins interacting with C/EBPδ in GST-pull down assays. A novel kinase of approximately 120 kD interacted with and phosphorylated C/EBPδ in vitro. This study also revealed a novel interaction of the C-terminal region of C/EBPδ with Ku70 and PARP-1, two factors known to play important roles in nuclear processes, such as DNA repair and transcription. Their effects on C/EBPδ, separate or in conjunction with one another, remains to be determined.
MATERIALS AND METHODS

1 - Cell Culture

The rat intestinal epithelial cell line IEC-6 was provided by A. Quaroni (Cornell University, Ithaca, NY, USA). The human transformed kidney cell line HEK 293T was provided by A. Nepveu (Department of Oncology, McGill University, Montreal, QC, Canada) and the human colon carcinoma cell line Caco-2 was provided by J.-F. Beaulieu (Department of Anatomy and Cell Biology, Université de Sherbrooke, QC, Canada). Cells were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 5% (IEC-6) or 10% (Caco-2 and 293T) fetal bovine serum (FBS) (BioMédia, Drummondville, QC, Canada), 2 mM of L-glutamine (Invitrogen Life Technologies, Burlington, ON, Canada) and 100 U/ml of penicillin and streptomycin (Invitrogen Life Technologies, Burlington, ON, Canada) at 37°C in an atmosphere containing 95% air and 5% CO₂. IEC-6 cells at 80% confluence were stimulated with IL-1β (10 ng/ml) (R & D Systems, Minneapolis, MN, USA) for 8 or 24 hours. Cells were pre-treated for 1 hour with the p38 MAPK inhibitor SB203580 (20 µM) (Calbiochem, San Diego, CA, USA) or the MEK1/2 inhibitor PD98059 (20 µM) (Calbiochem, San Diego, CA, USA).

2 - Northern Analysis

2.1 – Total RNA extracts

Total cellular RNAs were prepared using IEC-6 cells (100 mm petri dishes) pre-treated for 1 hour with MAPK inhibitors, followed by a treatment of 4 or 24 hours
with IL-1β. Total RNAs were prepared by the guanidinium isothiocyanate-phenol extraction method described by Chomczynski and Sacchi (1987) using TRIZOL reagent (Invitrogen Life Technologies, Burlington, ON, Canada). Cells were first rinsed twice with 3 ml of PBS, collected into sterile tubes using 1 ml of phosphate buffered saline (PBS) and centrifuged for 2 minutes at 1500 RPM. Cells were then resuspended in 800 µl of TRIZOL reagent and left to incubate for 5 minutes. Following incubation at room temperature, 160 µl of chloroform-isoamyl alcohol 24:1 was added and the solution was vortexed for 15 seconds. Following 3 minutes of incubation at room temperature, the tubes were centrifuged for 15 minutes at 13000 RPM at 4°C. The supernatant was then transferred into another sterile tube and 0.5 ml of isopropanol was added. Following a 10 minute incubation at room temperature, the tubes were centrifuged for 10 minutes at 13000 RPM at 4°C. The resulting pellet was washed with 1 ml 70% ethanol, then finally with 100% ethanol. The resulting RNA was resuspended in sterile water and kept at -80°C until utilization for analysis by Northern blot.

2.2 – Northern blot analysis

Total RNA samples were analysed by Northern blot following the protocol described by Fourney et al. (1988). 20 µg of the RNA samples were prepared for electrophoresis by heating at 65°C for 15 minutes, then placed on ice. The samples were separated on a 1% agarose gel containing MOPS buffer (10 mM sodium 3-(N-morpholino propane) sulfonic acid, 50 mM EDTA, 48 mM sodium acetate) and 5% formaldehyde at 70 volts for 5 hours. RNAs were then transferred by capillary action
to a nylon membrane (Nytran, Schleicher and Schuell, Keene, NH, USA) by placing the gel on a sponge saturated with 10X SSC (1.5 M sodium chloride, 150 mM trisodium citrate) overnight as described by Sambrook et al. (1989). The following day the membrane was dried in an 80°C oven for two hours and the RNAs were fixed to the membrane by a two minute UV treatment with a Fotodyne DNA-fixing apparatus (BioCan Scientific, Mississauga, ON, Canada).

2.3 – Preparation of radio-labelled probes

The haptoglobin DNA fragment was isolated by RT-PCR using messenger RNA from rat liver (Pelletier et al., 1998). The C/EBPβ and C/EBPδ DNA fragments originate from expression vectors generously provided by Steve McKnight (Cao et al., 1991), and the murine α-tubulin fragment originates from René St-Arnaud (Shriner’s Hospital, Montréal, QC, Canada). The plasmids were digested for 2 hours at 37°C with different restriction enzymes (Amersham BioSciences, Baie d’Urfé, QC, Canada), and the fragments were isolated using a 0.8% agarose gel and purified. The band in the agarose gel corresponding to the expected fragment was excised and placed in a sterile Eppendorf tube containing mineral wool and holes in the conical bottom of the tube. This tube was placed in another Eppendorf tube and centrifuged for three minutes at 13 000 RPM. The fragment was collected in the bottom tube and purified by phenol-chloroform isoamyl alcohol extraction (25: 24: 1). 1/10 volume of 3 M sodium acetate was added, the DNA was precipitated with cold 100% ethanol and resuspended in sterile water after centrifugation at 13 000 RPM for 15 minutes. The fragments were then labelled by primer extension with the Multiprime DNA
labelling system (Amersham BioSciences, Baie d’Urfé, QC, Canada) in the presence of $\alpha^{-32}$P-dCTP. The genes coding for the C/EBP$\beta$ and C/EBP$\delta$ isoforms were shown to be distinct and specific only for these isoforms (Pelletier et al., 1998).

Table 1: Restriction enzymes used for digestion of DNA fragments

<table>
<thead>
<tr>
<th>FRAGMENT</th>
<th>LENGTH</th>
<th>RESTRICTION SITES</th>
<th>REFERENCES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haptoglobin</td>
<td>545 bp</td>
<td>HindIII, BamHI</td>
<td>Marinkovic and Baumann (1990)</td>
</tr>
<tr>
<td>C/EBP$\beta$</td>
<td>1.5 kb</td>
<td>EcoRI, XhoI</td>
<td>Cao et al. (1991)</td>
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<tr>
<td>C/EBP$\delta$</td>
<td>1.0 kb</td>
<td>EcoRI, BamHI</td>
<td>Cao et al. (1991)</td>
</tr>
<tr>
<td>$\alpha$-tubulin</td>
<td>1.6 kb</td>
<td>PstI</td>
<td>Lemischka et al. (1981)</td>
</tr>
</tbody>
</table>

2.4 – Pre-hybridization and hybridization

The nylon membrane was incubated for 4 hours at 65$^0$C with the pre-hybridization solution (0.12 M Tris-HCl pH 7.4, 0.6 M NaCl, 8 mM EDTA, 0.1 % sodium pyrophosphate, 0.1 % SDS, 0.06% heparin) and then incubated overnight at 65$^0$C with the radioactive-labelled hybridization solution (pre-hybridization solution with 10% dextran sulfate and 0.625 mg/ml heparin). The following day the membrane was washed using a 0.1X SSC solution containing 10% SDS for 30 minutes at 65$^0$C. The membrane was exposed at ~80$^0$C in an autoradiography cassette with a screen and Kodak BioMax MR film (Amersham BioSciences, Baie d’Urfé, QC, Canada).
3 – Analysis of C/EBPδ DNA constructions

3.1 – MSV-C/EBPδ DNA constructions

The C/EBPδ mutants were obtained by PCR (polymerase chain reaction) in collaboration with Antoine Désilets, either by a replacement deletion strategy or by overlap extension using the SK+ C/EBPδ expression vector construct (see figure 6A, C. Asselin, Université de Sherbrooke, Sherbrooke, QC, Canada), in which C/EBPδ is inserted between the 5’ restriction enzyme site EcoRI and the 3’ restriction enzyme site BamHI. The mutants Δ36-164 (nt 108-492), Δ70-164 (nt 210-492), Δ108-164 (nt 324-492), Δ151-164 (nt 453-492) and Δ167-174 (nt 501-522) were obtained by the replacement strategy. The first oligonucleotides used corresponded to the multiple cloning site in the pBLUESCRIPT SK+ vector (Stratagene, La Jolla, CA, USA), either using the restriction enzyme site EcoRI: SK ECO 5’ - ATCAGTAAGCTTGTATCGAATTCC - 3’ or BamHI: SK BAM 5’ - GGCCGCTCTAGAACTAGTGATCC - 3’. The second oligonucleotide used for the PCR was created using C/EBPδ sequences corresponding to the area immediately upstream of the sequence to be deleted as well as the addition of a restriction enzyme site for XhoI, a site found naturally in the C/EBPδ sequence at nucleotide 492, on the 5’ end of the oligonucleotide. The addition of the XhoI site permitted the simple insertion of the mutated fragments into the MSV (murine sarcoma virus)-C/EBPδ vector, provided by Dr. Steve McKnight. The oligonucleotides used were: for MSV-C/EBPδ Δ36-164, SK ECO and DELTA 421 (5’ – GCGCTCGAGCTCGGCCGCGCTGTCCAC - 3’); for MSV-C/EBPδ Δ70-164, SK ECO and DELTA 519 (5’ – GCGCTCGAGCCATGGAGTCAATGTAGGC – 3’);
A) Plasmid map of SK+-C/EBPδ. The mouse C/EBPδ gene was inserted into the pBluescript SK+ vector between the restriction enzyme sites EcoRI and BamHI in the multiple cloning site of SK+ (C. Asselin, Université de Sherbrooke, Sherbrooke, QC, Canada).

B) Plasmid map of MSV-C/EBPδ. The mouse C/EBPδ gene was inserted into the pBluescript MSV vector between the restriction enzyme sites EcoRI and BamHI in the multiple cloning site of MSV (Cao et al., 1991).

C) Plasmid map of MSV-C/EBPδ mutants. The mutated forms of the C/EBPδ gene, Δ36-164 (nt 108-492), Δ70-164 (nt 210-492), Δ108-164 (nt 324-492), Δ151-164 (nt 453-492), Δ167-174 (nt 501-522), Δ50-60 (nt 150-180), Δ60-70 (nt 180-210), Δ75-85 (nt 225-255) and the site-specific mutants T156A and S160A, were inserted into the pBluescript MSV-C/EBPδ vector between the restriction enzyme sites EcoRI and BamHI in the multiple cloning site of MSV.
for MSV-C/EBPΔ Δ109-164, SK ECO and DELTA 637 (5’ - GCGCTCGAGGTCGCTAGGGCCGCCCCTG - 3’); for MSV-C/EBPΔ Δ151-164, Nco199 (5’ - GCCTACATTGACTCCATGGCCGCC - 3’) and Xho (5’ - GCGCTCGAGGCAAGCTCACACTGACTG - 3’); for MSV-C/EBPΔ Δ166-174, SK BAM and DELTA 838 (5’ - GCGCTCGAGGCACAGTCCAGGAAGGG - 3’). The PCR reaction was performed in the following conditions: 100 ng linearized SK+ C/EBPΔ DNA, 10 mM of each deoxyribonucleotide (dATP, dCTP, dGTP, dTTP), 30 pmol of each oligonucleotide, 5 μl of DMSO (dimethylsulfoxyl oxide), 10 μl of 10X Taq DNA polymerase buffer, and 2 units of Taq DNA polymerase (Amersham BioSciences, Baie d’Urfé, QC, Canada). The solution was completed to 100 μl with sterile water. The PCR apparatus used was the PCR Express Thermal Cycler (Hybaid, Franklin, MA, USA).

Table 2: Replacement PCR program

<table>
<thead>
<tr>
<th>NUMBER OF CYCLES</th>
<th>DENATURATION</th>
<th>HYBRIDIZATION</th>
<th>POLYMERIZATION</th>
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</thead>
<tbody>
<tr>
<td>First cycle</td>
<td>1 minute 94°C</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>30 cycles</td>
<td>30 seconds 94°C</td>
<td>30 seconds 55°C</td>
<td>30 seconds 72°C</td>
</tr>
<tr>
<td>Last cycle</td>
<td>-</td>
<td>-</td>
<td>5 minutes 72°C</td>
</tr>
</tbody>
</table>

The C/EBPΔ Δ151-164 construct fragment obtained by PCR was digested with NotI and XhoI and inserted into the C/EBPΔ-SK+ vector digested with the same enzymes. The coding region of the mutated DNA was then transferred into the MSV expression vector using the enzymes EcoRI and BamHI (see figure 6B). For the
other mutants, the fragments obtained by PCR were digested with the restriction enzymes BamHI and XhoI or XhoI and EcoRI and finally inserted into the MSV-C/EBPδ vector digested with the same enzymes (see figure 6C). The ligations were performed overnight at 12°C using the T4 DNA ligase (Amersham BioSciences, Baie d'Urfé, QC, Canada). The DNA was transformed into TOP 10F *Escherichia coli* competent bacteria (Clontech, Mississauga, ON, Canada). The presence of the mutations was verified by restriction enzyme and sequence analysis (sequencing performed by the Sheldon Biotechnology Centre, McGill University, Montreal, QC, Canada).

The deletion mutants Δ50-60 (nt 150-180), Δ60-70 (nt 180-210), Δ75-85 (nt 225-255) and the site-specific mutants T156A and S160A were created using overlap extension PCR as explained by Sambrook and Russell (2001). Two PCR reactions were performed using the same conditions, the first producing two separate overlapping fragments, and the second using the overlapping fragments to create the full mutant. The first oligonucleotides used for the first PCR were the same SK ECO or SK BAM oligonucleotides as for the previous mutation experiments. The second oligonucleotides were created by integrating the mutations into the original C/EBPδ sequence using both the sense and anti-sense strands. The second oligonucleotides used were: for MSV-C/EBPδ Δ50-60, 50-60 sense 5' – CTGGGCTCCACGACTTTTCAGCGCTACATT – 3' and 50-60 AS 5' – AATGTAGGCGCTGAAAAGTCGTTGGAGCCCAAG – 3'; for MSV-C/EBPδ Δ60-70,
60-70 sense 5’ – GACGAGAGCGCCATCGTGCCCACCCTAGAG – 3’ and 60-70 AS 5’ – CTCTAGGGTGGGCACGATGCGCTCTCGTC – 3’; for MSV-C/EBPδ 
Δ75-85, 75-85 sense 5’ – GCCGTGCCACCCTATTCAACAGCAACCAC – 3’ and 75-85 AS 5’ – GTGGTTGCTGTGAATAGGTTGGGACCGG – 3’; for MSV-C/EBPδ T156A, T156A sense 5’ – GCGGTCAGCCCGCTCCACCCACT – 3’ and T156A AS 5’ – AGTGGGTGGAGCGGGCTGAGCCGC – 3’; for MSV-C/EBPδ S160A, S160A sense 5’ – CCCACTCCACCCTGCGCCGGAGCCTCCT – 3’ and S160A AS 5’ – AGGAGGCTCAGGCGCAGTGTTGGTGGAGTGGG – 3’. The sense oligonucleotides were paired with the SK BAM oligonucleotide, and the anti-sense oligonucleotides were paired with the SK ECO oligonucleotide for the first PCR in the following conditions: 100 ng linearized SK+ C/EBPδ DNA, 10 mM of each deoxyribonucleotide (dATP, dCTP, dGTP, dTTP), 30 pmoles of each oligonucleotide, 5 μl of DMSO, 10 μl of 10X Pfu DNA polymerase buffer, and 2 units of Pfu DNA polymerase (Stratagene, La Jolla, CA, USA). The solution was completed to 100 μl with sterile water. The PCR apparatus used was the PCR Express Thermal Cycler (Hybaid, Franklin, MA, USA).

Table 3: Overlap PCR program

<table>
<thead>
<tr>
<th>NUMBER OF CYCLES</th>
<th>DENATURATION</th>
<th>HYBRIDIZATION</th>
<th>POLYMERIZATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 cycles</td>
<td>1 minute 94°C</td>
<td>1 minute 50°C</td>
<td>1 minute 72°C</td>
</tr>
<tr>
<td>Last cycle</td>
<td>1 minute 94°C</td>
<td>-</td>
<td>10 minutes 72°C</td>
</tr>
</tbody>
</table>
The resulting fragments were isolated from a 1.4% agarose gel using the phenol-chloroform extraction method (as described earlier in section 2.3) and used for the second PCR. The second PCR was performed using the resulting fragments and the oligonucleotides SK ECO and SK BAM in the following conditions: 50 ng of each 5’ and 3’ DNA fragments obtained from the first PCR, 10 mM of each deoxyribonucleotide (dATP, dCTP, dGTP, dTTP), 30 pmoles of each oligonucleotide, 5 μl of DMSO, 10 μl of 10X Pfu DNA polymerase buffer, and 2 units of Pfu DNA polymerase (Stratagene, La Jolla, CA, USA). The solution was completed to 100 μl with sterile water. The PCR apparatus used was the PCR Express Thermal Cycler (Hybaid, Franklin, MA, USA) with the same overlap PCR program in table 3 as the first PCR.

The resulting fragments were digested with the restriction enzymes EcoRI and XhoI and purified using the AMICON microcon PCR centrifugal filter devices (Millipore, Bedford, MA, USA) to filter out the oligonucleotides and restriction enzymes. The fragments were inserted into MSV-C/EBPΔ previously digested with the same enzymes and the ligation was performed using the Rapid DNA Ligation Kit (Roche Applied Sciences, Laval, QC, Canada) for 20 minutes at room temperature with 2 units of T4 DNA ligase (see figure 5C). The DNA was transformed into TOP 10F Escherichia coli competent bacteria (Clontech, Mississauga, ON, Canada). The presence of the mutations was verified by restriction enzyme and sequence analysis (sequencing performed by University Core DNA & Protein Services, University of Calgary, Calgary, AB, Canada).
3.2 – GAL4 C/EBPδ DNA constructs

The N-terminal sequences of C/EBPδ (amino acids 1-164: 551 bp, and amino acids 1 to 151: 453 bp) were cloned in frame with the DNA-binding domain of GAL4 in the mammalian expression vector pM2 (see figure 7, Sadowski et al., 1992). For the N-terminal sequence construction, an EGFP-C/EBPδ vector (C. Asselin, Université de Sherbrooke, Sherbrooke, QC, Canada) was digested with the restriction enzymes BglII and XhoI. The GFP peptide added to the N-terminal region of C/EBPδ did not affect normal C/EBPδ transcriptional activity, as determined by luciferase assays with the haptoluc reporter gene (data not shown). The fragment was inserted into the pM2 vector digested with the enzymes BamHI and SalI (see figure 7). The DNA was transformed into TOP 10F Escherichia coli competent bacteria (Clontech, Mississauga, ON, Canada). The DNAs were verified by restriction enzyme and sequence analysis (Sheldon Biotechnology Centre, McGill University, Montreal, QC, Canada).

3.3 – Transient transfections

Transient transfections of the C/EBPδ DNA constructions were performed in 293T cells (60 mm petri dishes) for 24 hours before the nuclear proteins were extracted as described by Stein et al. (1989), or in 293T and Caco-2 (24-well plates) 24 hours before luciferase assays were performed. Cells in confluent 100 mm petri dishes were trypsinised and divided (for 293T cells: one 100 mm dish for 15 60 mm dishes or one 100 mm dish for 96 wells of a 24-well plate; for Caco-2 cells: one 100 mm dish for 60 wells of a 24-well plate). The following day the cells were transfected by
The DNA sequence corresponding to the N-terminal region from the amino acids 1 to 164 of C/EBPδ was cloned in frame with the DNA-binding domain of GAL4 in the mammalian expression vector pM2, from the EGFP-C/EBPδ vector (C. Asselin, Université de Sherbrooke, Sherbrooke, QC, Canada) digested with the restriction enzymes BglII and XhoI, between the restriction enzyme sites BamHI and Sall in the multiple cloning site of pM2.
lipofection. Lipofectamine 2000 (1 μl Lipofectamine 2000 (Invitrogen Life Technologies, Burlington, ON, Canada)/ 50 μl Opti-MEM) was incubated in Opti-MEM medium (Invitrogen Life Technologies, Burlington, ON, Canada) at room temperature for 5 minutes (solution A). The DNA solutions were prepared using 5 μg of DNA in 500 μl of Opti-MEM for 500 μl of solution A (for 60 mm dishes) or 0.1 μg of DNA construct in 50 μl Opti-MEM for 50 μl of solution A (for the 24-well plates). For the transient transfections in the 24-well plates, the reporter gene was added to the DNA solutions in the same concentration as the DNA constructs, either pFRluc (Clontech, Palo Alto, CA, USA) for the GAL-4 constructs or the 395 bp portion of the rat haptoglobin promoter (-396 to -2) inserted in the pGL3 basic luciferase reporter plasmid (Promega, Madison, WI, USA) for the MSV constructs (Marinkovich and Baumann 1990, Pelletier et al., 1998). The DNA solutions were mixed with solution A and incubated at room temperature for 15 minutes. The Lipofectamine 2000-DNA complexes were added directly to the medium. In the case of the 24-well plates, the medium was changed after 4.5 hours, and in some cases replaced with medium containing the p38 MAPK inhibitor SB203580 (20 μM) (Calbiochem, San Diego, CA, USA).

3.4 – Luciferase assays

24 hours after transfection, cells were lysed with 300 μl of lysis buffer (25 mM gly-gly, 15 mM MgSO₄, 4 mM EGTA, 1% Triton X-100, and 1 mM DTT) for 15 minutes with mixing at room temperature. The lysed cells were added to 100 μl of ATP buffer (25 mM gly-gly, 15 mM MgSO₄, 4 mM EGTA, 1% Triton X-100, 15
mM KH$_2$PO$_4$, 6 mM ATP, 3 mM DTT) and 100 µl of 1 mM luciferin solution (Molecular Probes, Eugene, OR, USA). Luciferase activity was measured for 20 seconds in a Lumat LB 9507 luminometer (EG&G Berthold, Gaithersburg, MD, USA). The pRL-SV40 renilla luciferase vector (Promega, Madison, WI, USA) was used as a control for transfection efficiency. Results shown are representative of experiments repeated at least four times in quadruplicate.

3.5 – Statistical analysis

Results were analysed by the student’s t-test and were considered significantly different at $p \leq 0.05$.

3.6 – Extraction of nuclear proteins

24 hours after transfection, the 60 mm plates of cells were washed twice with 1X PBS and collected into sterile microtubes with 1 ml 1X PBS. The cells were pelleted through centrifugation for two minutes at 3 000 RPM. The cellular pellet was resuspended in 100 µl of lysis buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonate pH 7.9, 1 mM ethylenediamine disodium tetraacetate (EDTA), 60 mM KCl, 0.5% NP-40, 1 mM DTT, 1 mM phenylmethysulfonyl fluoride (PMSF)), and incubated on ice for 5 minutes. The solution was centrifuged for 10 seconds at 13 000 RPM to pellet the nuclei, which were then resuspended in 100 µl of nuclear resuspension buffer (0.25 M Tris-HCl pH 7.8, 0.06 M KCl, 1 mM DTT, 1.5 mM PMSF). The resuspended nuclei were submitted to three cycles of 2 minutes each of a flash freeze in liquid nitrogen, and thawing at 37°C, followed by
vortexing. The samples were centrifuged at 4°C for 10 minutes at 13 000 RPM to collect the nuclear protein supernatant, which was flash frozen in liquid nitrogen and conserved at -80°C.

3.7 – Preparation of $^{32}$P radio-labelled oligonucleotides

A double-stranded C/EBP DNA-binding site from the rat haptoglobin promoter hapto A (-166 to -145, 5'- CCAAGTATGAAGCAAGAGCTCA -3') with a GG overhang (Pajovic et al., 1994; Pelletier et al., 1998) was radio-labelled with $^{32}$P-dCTP with the Klenow fragment of DNA polymerase I of *E. coli* (Amersham BioSciences, Baie d’Urfé, QC, Canada).

3.8 – Electrophoretic mobility shift assay

The nuclear extract samples were prepared for electrophoresis by incubation for 30 minutes at room temperature in a final volume of 20 µl containing: 5 µg of nuclear extract, 2 µl of 10X mobility shift buffer (10 mM Tris-HCl pH 7.5, 500 mM NaCl, 10 mM DTT, 10 mM EDTA, 50 % glycerol), 0.25 µg of dIdC (Amersham BioSciences, Baie d’Urfé, QC, Canada), and 1 µl of the radioactive probe at an intensity of 50 CPS (counts per second). For supershift assays, a 30 minute pre-incubation with 1 µl of C/EBP isoform δ rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was performed on the sample solution prior to the addition of the radioactive probe. Following a pre-electrophoresis of the gel for 30 minutes, samples were electrophoresed in a 4% polyacrylamide gel containing 0.5% Tris-borate (TBE) buffer (45 mM Tris-borate pH 8.0, 40 mM boric acid, 1 mM
EDTA) and 2% glycerol for approximately two hours at 150 volts. The gel was then dried on Whatmann paper using a Savant 2000 gel dryer and exposed overnight at −80°C in an autoradiography cassette with a screen and Kodak BioMax MR film (Amersham BioSciences, Baie d’Urfé, QC, Canada).

Table 4: Preparation of the polyacrylamide gel

<table>
<thead>
<tr>
<th>PRODUCT</th>
<th>QUANTITY</th>
<th>FINAL CONCENTRATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide- Bis-acrylamide mix (30:1)</td>
<td>8 ml</td>
<td>4%</td>
</tr>
<tr>
<td>Glycerol 50%</td>
<td>2 ml</td>
<td>2%</td>
</tr>
<tr>
<td>10X TBE buffer</td>
<td>2.25 ml</td>
<td>0.5X</td>
</tr>
<tr>
<td>Distilled water</td>
<td>40.75 ml</td>
<td>-</td>
</tr>
<tr>
<td>Ammonium persulfate</td>
<td>0.01 g</td>
<td>2.2 mg/ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.05 ml</td>
<td>-</td>
</tr>
</tbody>
</table>

4 – Analysis of protein-protein interactions

4.1 – Creation of GST fusion protein constructs

The three C/EBP isoforms C/EBPα, C/EBPβ, and C/EBPδ were cloned in frame, downstream of the glutathion S-transferase (GST) sequence in the IPTG(Isopropyl-β-thiogalactopyranoside)-inducible vector pGEX 4T-2 (Amersham BioSciences, Baie d’Urfé, QC, Canada). For C/EBPα, one construction was created, p42-pGEX (amino acids 1 to 328: 984 bp). For C/EBPβ, one construction was created, LAP-pGEX (the complete protein, 888 bp, amino acids 1 to 296.
Constructions using the sequences coding for the complete C/EBPδ protein (δ-pGEX: amino acids 1-268, 804 bp), the C-terminal region (δ(C)-pGEX: amino acids 165-268, 309 bp), the N-terminal region (δ(N)-pGEX: amino acids 1-164, 492 bp), amino acids 1-151 (δ1-151-pGEX: 453 bp), amino acids 1-108 (δ1-108-pGEX: 324 bp), and amino acids 1-70 (δ1-70-pGEX: 210 bp) were made to study C/EBPδ. For the complete proteins, the EGFP-p42, EGFP-LAP, and EGFP-C/EBPδ plasmids (C. Asselin, Anatomy et Cell Biology, Université de Sherbrooke, QC, Canada) were digested with the restriction enzymes BglII and EcoRI (see figure 8A). The p42 (984 bp), LAP (888 bp), and C/EBPδ (804 bp) fragments were isolated on an agarose gel and ligated in-frame into the pGEX 4T-2 vector digested with BamHI and EcoRI (see figure 8B). For C/EBPδ(N)-pGEX, the EGFP-C/EBPδ plasmid was digested with the enzymes BglII and XhoI, and the fragment of 492 bp corresponding to C/EBPδ(N) was isolated on agarose gel and ligated into the pGEX 4T-2 vector digested with the enzymes BamHI and XhoI (see figure 8C). For C/EBPδ(C)-pGEX, the EGFP-C/EBPδ plasmid was digested with the enzymes XhoI and Sall, and the fragment of 309 bp corresponding to C/EBPδ(C) was isolated on agarose gel and ligated into the pGEX 4T-2 vector digested with the enzyme XhoI (see figure 8D). All ligations for these constructions were performed overnight at 12°C with T4 DNA ligase in the presence of ATP.

For C/EBPδ1-151-pGEX, the C/EBPδ(N)-pGEX plasmid was digested with the enzyme NotI, and was re-circularized by overnight ligation with T4 DNA ligase.
A) **Plasmid map of the pGEX-C/EBP isoform constructs.** The mouse C/EBPα, C/EBPβ, and C/EBPδ genes, from the EGFP-p42, EGFP-LAP, and EGFP-C/EBPδ plasmids (C. Asselin, Anatomy et Cell Biology, Université de Sherbrooke, QC, Canada) digested with the restriction enzymes BglII and EcoRI, were inserted in frame into the pGEX 4T-2 vector (Clontech, Mississauga, ON, Canada) between the restriction enzyme sites BamHI and EcoRI in the multiple cloning site of pGEX.

B) **Plasmid map of the pGEX-C/EBPδ(N) construct.** The DNA sequence corresponding to the N-terminal region from EGFP-C/EBPδ (C. Asselin, Anatomy et Cell Biology, Université de Sherbrooke, QC, Canada) was excised by digestion with the restriction enzymes BglII and XhoI and was inserted in frame into the pGEX 4T-2 vector (Clontech, Mississauga, ON, Canada) between the restriction enzyme sites BamHI and XhoI in the multiple cloning site of pGEX.

C) **Plasmid map of the pGEX-C/EBPδ(C) construct.** The DNA sequence corresponding to the C-terminal region from EGFP-C/EBPδ (C. Asselin, Anatomy et Cell Biology, Université de Sherbrooke, QC, Canada) was excised by digestion with the restriction enzymes XhoI and SalI and was inserted in frame into the pGEX 4T-2 vector (Clontech, Mississauga, ON, Canada) in the restriction enzyme sites XhoI in the multiple cloning site of pGEX.
D) Plasmid map of the pGEX-C/EBPδ1-151 construct. The pGEX 4T-2 construct containing the N-terminal region of amino acids 1 to 151 from the C/EBPδ(N)-pGEX 4T-2 plasmid was digested with the enzyme NotI, and was re-circularized by overnight ligation with T4 DNA ligase (Amersham BioSciences, Baie d'Urfé, QC, Canada) at 12°C.

E) Plasmid map of the pGEX-C/EBPδ1-70 and the pGEX-C/EBPδ1-108 constructs. Fragments from the N-terminal region of the SK+-C/EBPδ plasmid (C. Asselin, Anatomy et Cell Biology, Université de Sherbrooke, QC, Canada) of the amino acids regions 1 to 70 and 1 to 108 were PCR-amplified and inserted in frame into the the pGEX 4T-2 vector (Clontech, Mississauga, ON, Canada) digested with the enzymes BglII and XhoI.
(Amersham BioSciences, Baie d’Urfé, QC, Canada) at 12° C (see figure 8E). For C/EBPδ1-70-pGEX and C/EBPδ1-108-pGEX, fragments from the SK+-C/EBPδ plasmid (C. Asselin, Anatomy et Cell Biology, Université de Sherbrooke, QC, Canada) were PCR-amplified using the oligonucleotide DELTA-UP 5’-GAAGATCTATGTCCGCGGGGGCGCACGGAC - 3’) and either DELTA 519 (5’ - GCGCTCGAGCCATGGAGTCAATGTAGGC - 3’) for C/EBPδ1-70-pGEX, or DELTA 637 (5’ - GCGCTCGAGGGCTCGTAGGGCCGACCCCTG - 3’) for C/EBPδ1-108-pGEX using the overlap PCR conditions in section 3.1 and overlap PCR program as described in table 2. The resulting PCR fragments were digested with the enzymes BglII and XhoI, and were ligated using the Rapid DNA Ligation Kit into the pGEX 4T-2 vector digested with the enzymes BamHI and XhoI (see figure 8F). All of the restriction enzymes were obtained from Amersham BioSciences (Baie d’Urfé, QC, Canada) and were used as per manufacturer instructions. The constructions were transformed in TOP 10F E. coli competent cells for DNA analysis and in BL-21 codon plus E. coli competent cells for protein analysis. The bacteria were conserved at -80°C with 50% glycerol. The presence of the mutations was verified by restriction enzyme and sequence analysis (sequencing performed by University Core DNA & Protein Services, University of Calgary, Calgary, AB, Canada).
4.2 – Verification of the inducibility of the GST-fusion protein-expressing bacteria

The BL-21 codon plus bacteria were spread onto an ampicillin-agar plate and the following day, one colony was selected to incubate overnight in 2 ml of L-Broth (LB) with ampicillin (10 mg/ml NaCl, 10 mg/ml Tryptone Peptone, 5 mg/ml yeast extract, 100 μg/ml ampicillin) and the original colony was conserved at 4°C. 150 μl of the 2 ml overnight culture was taken and incubated in 3 ml LB with ampicillin for three hours at 37°C. The culture solution was then divided equally, one tube for the control and the other induced with 0.1 mM IPTG (Promega, Madison, WI, USA). The cultures were incubated at 37°C for three hours and transferred into sterile microtubes to be pelleted by centrifugation at 13 000 RPM for two minutes. The pellets were resuspended in 40 μl of Laemmli buffer (62.5 mM Tris-HCl, pH 6.9, 2% SDS, 1% β-mercaptoethanol, 10% glycerol and 0.04% bromophenol blue) and boiled at 100°C for three minutes. Proteins were then separated on a polyacrylamide gel containing 10% SDS (SDS-PAGE gel) at 150 volts for approximately one hour.

The gel was then coloured to visualize the proteins with Coomassie blue (0.125% Coomassie Blue, 5% acetic acid, 40% methanol, 55% distilled water) for 15 minutes, then rinsed with decolouring solution (30% methanol, 10% acetic acid, 60 % distilled water) for two to three hours to obtain the proper colouring of the protein bands. The gel was then dried on Whatmann paper in the Savant 2000 gel dryer at 80°C for one hour 30 minutes. The induction of the fusion protein produced by the
bacteria was verified as compared to the control bacteria, and the inducible bacteria were conserved in 50% glycerol at -80°C.

Table 5: Preparation of SDS-PAGE gels (one mini-gel)

<table>
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<tr>
<th>PRODUCT</th>
<th>STACKING</th>
<th>SEPARATOR (10%)</th>
<th>SEPARATOR (12%)</th>
</tr>
</thead>
<tbody>
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<td>3.3 ml</td>
<td>4.0 ml</td>
</tr>
<tr>
<td>Tris-HCl pH 8.8 (1.5M)</td>
<td>-</td>
<td>2.5 ml</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>Tris-HCl pH 6.8 (1.0M)</td>
<td>0.38 ml</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SDS 10%</td>
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<td>0.1 ml</td>
</tr>
<tr>
<td>Sterile water</td>
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<td>4.0 ml</td>
<td>3.3 ml</td>
</tr>
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<td>Ammonium persulfate 10%</td>
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<td>TEMED</td>
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</tr>
</tbody>
</table>

4.3 – GST fusion protein purification

Purification of the C/EBPδ GST fusion proteins was performed according to the GST Gene Fusion System protocol from Amersham BioSciences (Baie d’Urfé, QC, Canada). The inducible bacteria were incubated in 200 ml of LB with ampicillin overnight at 37°C with agitation. The following day, four cultures of 12 ml were extracted and incubated in four 250 ml cultures of LB with ampicillin at 37°C with agitation until the desired optical density of 0.6 at the wavelength of 600 nm was obtained. The bacterial cultures were then induced with 0.1 mM IPTG at 37°C with agitation for three hours. The bacteria were then collected by centrifugation at 5 000
RPM at 4°C, and washed twice with cold 1X PBS. At this point, the bacterial pellet was conserved at −80°C. The following day, 1.2 ml of suspended glutathion-sepharose beads (Amersham Pharmacia Biotech, Baie d’Urfé, QC, Canada) were pelleted by centrifugation at 1 500 RPM for two minutes and washed twice with lysis buffer (1X PBS containing 1% Triton X-100, 10 mM EDTA, 1 mM PMSF, 1/1000 each of leupeptin, pepstatin and aprotinin (all from Sigma-Aldrich, Oakville, ON, Canada)). The beads were then equilibrated for at least one hour at 4°C with 10 ml of lysis buffer. The beads were collected by centrifugation at 1 500 RPM for two minutes and the supernatant was discarded. The bacterial pellets in the meantime were thawed and resuspended in 8 ml of lysis buffer without Triton. The bacterial solution was sonicated for 30 seconds, and left on ice for 10 minutes after adding 1% Triton X-100 and 0.1 volume of lysozyme (Sigma-Aldrich Canada, Oakville, ON, Canada) to help solubilize the proteins. Following a centrifugation at 10 000 RPM for 15 minutes at 4°C, the protein-containing supernatant was added to the prepared glutathion sepharose beads and incubated at 4°C with agitation for 30 minutes. The beads were then washed five times with lysis buffer, and centrifuged for two minutes at 1 500 RPM at 4°C between washes. After the fifth wash, 10 ml of lysis buffer was added to the beads, and the solution was poured into a PolyPrep chromatography column (Bio-Rad Laboratories, Mississauga, ON, Canada). The proteins, in 1 ml aliquots, were eluted with 10 ml of elution buffer (100 mM HEPES pH 7.6, 50 mM reduced glutathion (Sigma-Aldrich Canada, Oakville, ON, Canada)). The aliquots with the highest optical density measured at a 280 nm wavelength (therefore the highest protein concentration) were pooled together. The protein solutions were
concentrated on YM-10 Centricon columns (Millipore, Bedford, MA, USA) by centrifugation at 5 000 RPM for 50 minutes at 4°C, followed by two washes with 1 ml of 100 mM HEPES pH 7.6. The resulting proteins were conserved at −80°C until utilization.

4.4 – Total cell extracts

To prepare total cell extracts, HEK 293T cells (60 mm petri dishes) were transfected with 5 μg of a p38 MAPK expression vector (provided by N. Rivard, Department of Anatomy and Cell Biology, Université de Sherbrooke, QC, Canada) by lipofection (Lipofectamine 2000, Invitrogen Life Technologies, Burlington, ON, Canada). Non-transfected Caco-2 cells (60 mm petri dishes at confluence) were also used in certain experiments. The following day, cells were washed twice with cold PBS and 1 ml of lysis buffer supplemented with protease and phosphatase inhibitors (1% Triton X-100, 50 mM Tris-HCl pH 7.4, 5 mM EDTA, 15 mM NaCl, 5% glycerol, 1 mM PMSF, 40 mM β-glycerophosphate, 50 mM sodium fluoride, 200 μM sodium orthovanadate, 1/1000 each of leupeptin, pepstatin, and aprotinin (all from Sigma-Aldrich, Oakville, ON, Canada)) was placed on the cells. After 15 minute incubation at 4°C, cells were collected in sterile microtubes and pelleted by centrifugation for 5 minutes at 13 000 RPM at 4°C. The supernatant containing the cellular proteins was conserved at −80°C. Protein concentration of cell lysates was determined with the Bio-Rad Protein Assay kit based on the method of Bradford (Bio-Rad Laboratories, Mississauga, ON, Canada).
4.5 – GST pull-down assays

The glutathion-sepharose beads (40 µl) were prepared by washing twice with ice-cold lysis buffer (1X PBS containing 1% Triton X-100, 10 mM EDTA, 1 mM PMSF, 1/1000 each of leupeptin, pepstatin, and aprotinin (all from Sigma-Aldrich, Oakville, ON, Canada)) and centrifuged at 1500 RPM for two minutes between washes. The beads were equilibrated with 15 µg of the prepared GST fusion proteins for one hour at 4°C. The beads were then washed three times with ice-cold lysis buffer and centrifuged at 1500 RPM for two minutes between washes. Total cell protein extracts (1 mg) prepared from 293T cells were then added to the GST proteins coupled to the beads and incubated with agitation for two hours at 4°C. The protein-bead complexes were then washed three times with ice-cold lysis buffer and centrifuged at 1500 RPM for two minutes between washes. Protein complexes were then eluted with Laemmlí buffer (62.5 mM Tris-HCl, pH 6.9, 2% SDS, 1% β-mercaptoethanol, 10% glycerol and 0.04% bromophenol blue). The protein samples were then conserved at −80°C.

4.6 – Western blot analysis

Western blot analysis was performed as described by Laemmli (1970). Protein samples (15 µg) were resolved on SDS-PAGE (10% or 12% acrylamide, see table 5 for specifications) in running buffer (0.025 M Tris-Cl, 0.192 mM glycine, 1% SDS) at 150 volts for approximately 1 hour 30 minutes. The protein ladders used were the Broad Range Pre-stained SDS-PAGE standard (Bio-Rad Laboratories, Missassuaga, ON, Canada) and the Benchmark Protein Ladder (Invitrogen Life
Technologies, Burlington, ON, Canada). The gel was then electroblotted on a PVDF membrane (Roche Diagnostics, Laval, QC, Canada) in transfer buffer (0.025 M Tris-Cl, 0.192 mM glycine, 10% methanol) for two hours at 50 volts. The membrane was then blocked for at least two hours in a blocking solution of 1X TBS (20 mM Tris-Cl pH 7.6, 137 mM NaCl) containing 10% powdered skim milk and 0.05% Tween-20. Incubation with a rabbit polyclonal antibody recognizing total and phosphorylated forms of p38 MAPK (J. Landry, Université Laval, Quebec, QC, Canada) was performed overnight at 4°C at a concentration of 1/1000 in 1X TBS (20 mM Tris-Cl pH 7.6, 137 mM NaCl) containing 10% powdered skim milk and 0.05% Tween-20. The following day, the primary antibody was detected with the secondary rabbit HRP (Horse Radish Peroxidase) antibody (Sigma-Aldrich Canada, Oakville, ON, Canada) at a concentration of 1/1000 in 1X TBS (20 mM Tris-Cl pH 7.6, 137 mM NaCl) containing 10% powdered skim milk and 0.05% Tween-20. Immunocomplexes were visualized with the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

4.7 – Phosphorylation assay

GST fusion proteins (15 µg) coupled to glutathione-sepharose beads (Amersham Biosciences, Baie d'Urfé, QC, Canada) were incubated with 1 mg total cell extracts for 2 hours at 4°C. The beads were then washed three times with ice-cold kinase buffer (20 mM p-nitrophenyl phosphate, 10 mM MgCl₂, 1 mM DTT, and 30 mM HEPES (pH 7.4)) prior to the kinase assay. The kinase reaction was initiated by incubating the beads for 30 minutes at 30 °C in kinase buffer with or without
SB203580 (20 μM) and containing [γ-32P]ATP at 2 μCi/assay. The beads were then washed three times with ice-cold kinase buffer (20 mM p-nitrophenyl phosphate, 10 mM MgCl₂, 1 mM DTT, 30 mM HEPES (pH 7.4)) to remove excess radioactivity and the reaction was stopped by adding Laemmli buffer (62.5 mM Tris-HCl, pH 6.9, 2% SDS, 1% β-mercaptoethanol, 10% glycerol and 0.04% bromophenol blue). Radiolabelled substrates were separated by SDS-PAGE (10% or 12% acrylamide, see table 5 for specifications) in running buffer (0.025 M Tris-Cl, 0.192 mM glycine, 1% SDS) for approximately 1 hour 30 minutes at 150 volts. The gel was then coloured by Coomassie blue (as described in section 4.2) and exposed in autoradiography cassette with a screen and Kodak BioMax MR film (Amersham BioSciences, Baie d'Urfé, QC, Canada) for varying times. The empty GST vector was used as a control.

4.8 – In-gel phosphorylation assay

Purified C/EBPδ(N) and C/EBPδ(C) GST fusion proteins were integrated into an SDS-PAGE gel to determine interactions with kinases present in cellular lysates based on the In-Gel kinase assay method as described by Wooten (2002). The gels were prepared as explained in table 6, and the cellular lysates of 293T (non-transfected) and Caco-2 cells were prepared as in section 4.3. The proteins of the cellular lysates (20 μg) were separated at 150 volts for approximately 1 hour 30 minutes in 1X running buffer (125 mM Tris-Cl pH 8.3, 960 mM glycine, 0.5% SDS). The gel was then washed four times for 30 minutes each in wash buffer (50 mM Tris-Cl, 20% isopropanol). The proteins in the gel were then denatured with 6 M guanidine HCl, 50 mM Tris-Cl, and 5 mM β-mercaptoethanol (pH 8.0) for two hours.
and renatured using 50 mM Tris-Cl, 0.04% Tween 20, and 5 mM \( \beta \)-mercaptoethanol (pH 8.0) overnight with agitation at 4\(^{\circ}\)C. Following the renaturation of the proteins, the gels were equilibrated for 30 minutes at room temperature in an equilibration solution containing 40 mM Hapes, 10 mM MgCl\(_2\), 2 mM DTT, and 0.1 mM EGTA (pH 8.0). The kinase activity was then provoked using the equilibration solution with added 10 mM ATP and 75 \( \mu \)Ci of [\( \gamma \)^{32}P]ATP for 2 hours at room temperature. The reaction was stopped with nine washes of stop buffer (5% TCA, 1% sodium pyrophosphate). The gel was then coloured by Coomassie blue (as described in section 4.2) and exposed in autoradiography cassette with a screen and Kodak BioMax MR film (Amersham BioSciences, Baie d'Urfé, QC, Canada) for varying times.

Table 6: Preparation of polyacrylamide gel containing GST proteins

<table>
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<th>PRODUCT</th>
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<th>SEPARATOR (10%)</th>
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</thead>
<tbody>
<tr>
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<td>0.5 ml</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>Tris-HCl pH 8.8 (1.5M)</td>
<td>-</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>Tris-HCl pH 6.8 (1.0M)</td>
<td>1.25 ml</td>
<td>-</td>
</tr>
<tr>
<td>SDS 10%</td>
<td>0.05 ml</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>C/EBP(\delta)(N) and C/EBP(\delta)(C) GST fusion protein</td>
<td>-</td>
<td>100 ( \mu )g</td>
</tr>
<tr>
<td>Sterile water</td>
<td>3.17 ml</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>Ammonium persulfate 10%</td>
<td>0.150 ml</td>
<td>0.150 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.015 ml</td>
<td>0.015 ml</td>
</tr>
</tbody>
</table>
4.9 – Analysis of unknown interacting proteins by mass spectrometry

The GST pull-down assay was performed as in section 4.4 and the proteins were separated by SDS-PAGE (10% or 12% acrylamide, see table 5 for specifications) in running buffer (0.025 M Tris-Cl, 0.192 mM glycine, 1% SDS) for approximately 1 hour 30 minutes at 150 volts. The bovine serum albumin (BSA) protein was separated with the proteins to be used as a control during mass spectrometry. The gel was then coloured by Sypro Orange (0.04% Sypro Orange Protein Stain (Bio-Rad Laboratories, Mississauga, ON, Canada), 7.5% acetic acid) for 30 minutes at room temperature and decoloured in 7.5% acetic acid for no more than 30 seconds. The gel was then visualized in a UV light box for photography and the desired protein band was excised using utensils carefully cleaned with isopropanol and placed in a sterile microtube previously rinsed with isopropanol. As well as the desired protein, the band corresponding to BSA and a background piece of gel were also excised. Protein sequencing by MALDI-TOF (Matrix-Assisted Laser Desorption/Ionisation - Time Of Flight) mass spectrometry was then performed by Klaus Klarskov (Department of Pharmacology, Université de Sherbrooke, QC, Canada).
RESULTS

1 – The p38 MAPK is involved in the regulation of C/EBPδ transcriptional activity

1.1 – IL-1-mediated induction of haptoglobin expression is decreased by the specific p38 MAPK inhibitor SB203580

We have previously shown that the C/EBPs affect the expression of haptoglobin mRNAs in intestinal epithelial cells after stimulation with IL-1β (Désilets et al., 2000). To determine the role of MAPK pathways on C/EBP-dependent induction of haptoglobin, total RNA extracts were prepared from IEC-6 cells stimulated for 4 or 24 hours with IL-1 with or without the inhibitors SB203580 or PD98059, respectively specific for p38 MAPK and for MEK1/2 (subsequently blocking the p42/p44 MAPK pathway). As determined by Northern blot, the expression of haptoglobin mRNAs was increased after IL-1 treatment (Fig. 9, lanes 1-2, 7-8). In contrast to PD98059, addition of SB203580 decreased IL-1-dependent induction of haptoglobin after 4 and 24 hours (Fig. 9, lanes 2-6, 8-12). As observed previously (Désilets et al., 2000), IL-1β treatment led to an increase in C/EBPβ, and to a greater extent, in C/EBPδ mRNA levels (Fig. 9). Addition of SB203580 did reduce C/EBPβ and C/EBPδ mRNA levels after 4 and 24 hours, but not to the same extent as haptoglobin. While part of the reduction of haptoglobin expression may be due to decreases in mRNA and protein levels of both C/EBPβ and C/EBPδ, we hypothesized that post-translational modification of C/EBPδ could play a major role
in the regulation of C/EBPδ transcriptional activity. The α-tubulin probe was used as a RNA-loading control (Fig. 9).

1.2 – C/EBPδ transactivation potential is decreased by the p38 MAPK inhibitor SB203580

In order to determine whether the p38 MAPK affected C/EBPδ transcriptional activity, we performed transient transfection assays in 293T cells using the promoter pFRluc and the GAL4 constructs. The pFRluc promoter consists of five repeats of the GAL4 DNA-binding element followed by a basic TATA box coupled to the luciferase gene. The N-terminal region of C/EBPδ (amino acids 1 to 164) stimulated transcription of the pFR promoter 10-fold (p < 0.01), and this stimulation was diminished by 33% in the presence of SB203580 (Fig. 10A, p < 0.01). Likewise, while C/EBPδ was able to stimulate transcription of the luciferase gene via the haptoglobin promoter 6-fold (p < 0.01), addition of SB203580 significantly inhibited the transactivation potential by 50% (Fig. 10B, p < 0.05). These results suggest that p38 MAPK may regulate C/EBP δ transactivation potential.

1.3 – The p38 MAPK interacts with C/EBPδ between amino acids 70 and 108

To determine the interaction between C/EBPs and MAPKs, GST constructions were used in pull-down assays with total 293T cell extracts, followed by detection of the kinases by Western blot. C/EBPα (p42), C/EBPβ (LAP) and C/EBPδ did not interact with the MAPK JNK (data not shown). In contrast, the p38 MAPK interacted with the three C/EBP isoforms (Fig. 11A). More specifically for
C/EBPδ, the interaction occurred in the N-terminal region of C/EBPδ, between amino acids 1 and 164 (Fig. 11B). No interaction was observed between the C-terminal region of C/EBPδ and p38 MAPK (Fig. 11B). Further analysis of the smaller N-terminal constructs δ1-70 and δ1-108 showed an interaction of p38 MAPK between amino acids 70 and 108 (Fig. 11C). These results indicate that the p38 MAPK interacts with three C/EBP isoforms, either directly or via an adapter protein. Furthermore, a region of the C/EBPδ isoform between amino acids 70 and 108 is essential for p38 MAPK interaction. This region of C/EBPδ contains two possible interaction sites for p38 with the homologous amino acid motif “LEL”, found in various MAPK docking sites for other transcription factors, such as c-fos (Kallunki et al., 1994), and ETS (Seidel and Graves, 2001) (Fig. 11D).

1.4 – C/EBPδ is phosphorylated by p38 MAPK between amino acids 151 and 164

To determine the phosphorylation state of C/EBP proteins, in vitro kinase assays with GST-fusion proteins were performed with or without SB203580 using 293T or Caco-2 cell extracts. The phosphorylated proteins were separated by SDS-PAGE and autoradiographed. The C/EBPα (p42), C/EBPβ (LAP) and C/EBPδ isoforms were highly phosphorylated with extracts from both cell types (Fig 12A, 12B; phosphorylated forms of the proteins are indicated by the stars). Phosphorylation of C/EBPδ and of the N-terminal 1-164 domain decreased in response to the p38 MAPK inhibitor SB203580 (Fig 12C, lanes 2-3, 6-7). Phosphorylation of the C/EBPδ C-terminal domain was not affected by the addition of SB203580. Interestingly, deletion of amino acids 151 to 164 from the N-terminal
domain construct reduced both the level of phosphorylation and the inhibition by SB203580 (Fig 12C, lanes 8-9). These results suggest that a region between amino acids 151 and 164 may be phosphorylated by p38 MAPK.

1.5 – Mutation of the serine 160 reduces C/EBP\(\delta\) transcriptional activity

The region of C/EBP\(\delta\) between amino acids 151 and 164 contains two consensus MAPK phosphorylation sites, one at threonine (PTPPT) and the other at serine at position 160 (TSPEP) (online NETPHOS results were found at www.cbs.dtu.dk/databases/PhosphoBase/predict.html). To determine whether these amino acids play a role in C/EBP\(\delta\) transactivation potential, both amino acids were mutated to alanines in order to imitate the non-phosphorylated form of the protein on this amino acid. Transcriptional activity of both mutants was assessed by transient transfections in 293T cells. Mutation of threonine 156 to alanine did not affect C/EBP\(\delta\) transactivation potential on the haptoglobin promoter (Fig. 13A, p < 0.05). However, mutation of serine 160 decreased the transactivation potential of C/EBP\(\delta\) significantly by 43% (Fig. 13B, p < 0.05). These results suggest that serine 160 may be an important phosphorylation site in the regulation of C/EBP\(\delta\) transcriptional activity.
2 – Redundant N-terminal domains are essential for C/EBPδ transcriptional activity

To determine other N-terminal transactivation domains essential to C/EBPδ transcriptional activity, 8 mutants were created through deletion by PCR (Fig. 6C) and inserted into the MSV vector. The functionality of the mutant proteins was verified through DNA-binding assay to the haptoA DNA-binding site of the haptoglobin promoter with nuclear extracts of transfected 293T cells. All of the mutated C/EBPδ DNAs were capable of producing functional C/EBPδ proteins in these cells, as assessed by supershift assays with a C/EBPδ antibody (Fig. 14A). Transcriptional activity with the haptoglobin promoter-luciferase constructs was studied in comparison with normal C/EBPδ in the 293T cell line (Fig. 14B). Deletion of amino acids 36 to 164 completely abolished transcriptional activity, whereas addition of amino acids 36 to 70 restored partially transcriptional activity (indicated by mutant Δ70-164, Fig. 14B). Interestingly, the transcriptional activity was restored with the addition of amino acids 70 to 108 (indicated by mutant Δ108-164, Fig. 14B). Deletion of amino acids 151 to 164, and 167 to 174 showed a tendency to decrease C/EBPδ activity in several experiments. We then assayed small deletions in the region between amino acids 36 and 108. These transfection assays demonstrated the presence of redundant elements (see Δ50-60, Δ60-70, Δ75-85, Fig. 14B) important for full C/EBPδ transcriptional activity. These data indicate that C/EBPδ contains
several important elements within the N-terminal activation domain between amino acids 36 and 108.

3 – C/EBPδ interacts with different novel partners

3.1 – Two unknown kinases phosphorylate C/EBPδ

An in-gel kinase assay was performed to determine protein kinases present in 293T and Caco-2 cell extracts that could phosphorylate C/EBPδ. Total cellular extracts from these cell lines were separated on an SDS-PAGE gel, in which the C/EBPδ(N) and C/EBPδ(C) GST fusion proteins had been integrated. The gel was submitted to an in-gel kinase assay allowing phosphorylation of either C/EBPδ(N) or C/EBPδ(C) by renatured kinases. The bands obtained by autoradiography therefore indicate a kinase of that molecular weight that phosphorylates our protein. In all of the cellular extracts, a kinase of approximately 120 kD and a kinase of approximately 38 kD phosphorylated our C/EBPδ protein (Fig. 15).

3.2 – The Ku autoantigen and PARP-1 may interact with the C-terminal region of C/EBPδ

It became apparent, by analysing the data of our phosphorylation assays, that a phosphorylated protein of about 70 kD interacted with C/EBPα (p42), C/EBPβ (LAP) and the C-terminal region of C/EBPδ (see arrows in Fig. 12A and 12B). To identify this protein, pull-down assays were performed using the C/EBPδ(C) GST fusion protein. Proteins were separated by SDS-PAGE, and the gel was coloured with Sypro
Orange, a colorant that does not fix the protein into the gel. Two protein bands were extracted from the gel and analysed by MALDI-TOF mass spectrometry. The first resulting protein was Ku70, also known as ATP-dependent DNA helicase, a protein involved in DNA double-strand break repair (Walker et al., 2001) (Fig. 16). This result prompted further investigation into other possible interacting proteins that could be visualized via pull-down assay. Subsequent pull-down assays demonstrated the interaction of PARP-1 (Poly(ADP-ribose) polymerase 1) with the C-terminal region of C/EBPδ (Fig. 16). This protein is also involved in DNA repair, and has been shown to interact with Ku70 (Galande and Kohwi-Shigematsu, 1999). The exact interplay between these three proteins remains to be determined.
IL-1-mediated induction of haptoglobin expression is decreased by the specific inhibitor of p38 MAP kinase, SB203580

Total RNA extracts were prepared from IEC-6 cells stimulated for 4 or 24 hours with or without (control) IL-1 (10 ng/ml), SB203580 (20 μM) or PD98059 (20 μM), or in combinations of IL-1 with SB203580 or PD98059. Equal quantities of RNA were separated by electrophoresis and Northern blot by hybridization with the following $^{32}$P-labelled probes: haptoglobin, C/EBPβ, C/EBPδ and α-tubulin.
C/EBPδ transactivation potential is decreased by the p38 MAPK inhibitor SB203580

A) Transient transfections with pFR luciferase and GAL4 constructs in 293T cells. Cells were transiently co-transfected by lipofection using the pFR-luc reporter gene and the DNA pM2 (Ctrl) or pM2-C/EBPδ(N) (amino acids 1 to 164). Cells were treated with or without SB203580 5 hours after transfection and luciferase assays were performed 24 hours later. Luciferase activity is expressed in fold induction as compared to the control. The means and standard deviations calculated are representative of experiments repeated four times in quadruplicate.

B) Transient transfection with haptoglobin promoter luciferase and MSV constructs in 293T cells. Cells were transiently co-transfected by lipofection using the Hapto-luc reporter gene and the DNA MSV (Ctrl) or MSV-C/EBPδ (δ wt). Cells were treated with or without SB203580 5 hours after transfection and luciferase assays were performed 24 hours later. Luciferase activity is expressed in fold induction as compared to the control. The means and standard deviations calculated are representative of experiments repeated four times in quadruplicate.
A) pFR luciferase

B) haptoglobin promoter luciferase
The p38 MAPK interacts with C/EBPδ between amino acids 70 and 108

A) The p38 MAPK interacts with the isoforms C/EBPα (p42), C/EBPβ (LAP), and C/EBPδ. C/EBPα (p42), C/EBPβ (LAP) and C/EBPδ GST constructions (15 μg each) were used in pull-down assays with total 293T cell extracts (1 mg per assay). Protein complexes were separated on a 10% SDS-PAGE gel and transferred onto a PVDF membrane for detection of the p38 MAPK with an antibody specific for the p38 MAPK.

B) The p38 MAPK interacts with the isoform C/EBPδ in the N-terminal region. C/EBPδ, C/EBPδ C-terminal (amino acids 165 to 268) and N-terminal (amino acids 1 to 164 and 1 to 151) GST constructions (15 μg each) were used in pull-down assays with total 293T cell extracts (1 mg per assay). Protein complexes were separated on a 12% SDS-PAGE gel and transferred onto a PVDF membrane for detection of the p38 MAPK with an antibody specific for the p38 MAPK.
C) The p38 MAPK interacts with C/EBPδ between amino acids 70 and 108. C/EBPδ C-terminal (amino acids 165 to 268) and N-terminal (amino acids 1 to 164, 1 to 70 and 1 to 108) GST constructions (15 µg each) were used in pull-down assays with total 293T cell extracts (1 mg per assay). Protein complexes were separated on a 12% SDS-PAGE gel and transferred onto a PVDF membrane for detection of the p38 MAPK with an antibody specific for the p38 MAPK.

D) Docking sites of MAPK. The consensus MAPK docking site, known as the D domain, is indicated, as well as the D domain in c-jun for JNK MAPK, as compared to two possible D domains in the interaction region of p38 MAPK on C/EBPδ between amino acids 70 and 108.
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B) Table:

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D) MAPK D domain: LXLXXXF

c-jun: 40 - LNLDPV - 46

C/EBPδ: 73 - T LELCDELFL - 82
97 - G LELQLGTPRP - 108
**Figure 12**

**C/EBPδ is phosphorylated by p38 MAPK between amino acids 151 and 164**

C/EBPα (p42), C/EBPβ (LAP) and C/EBPδ GST constructions (15 µg each) were used in pull-down assays with total 293T cell extracts (A) or Caco-2 cell extracts (B) (1 mg per assay). C/EBPδ, C/EBPδ C-terminal and N-terminal (amino acids 1 to 164 and 1 to 151) GST constructions (15 µg each) were used in pull-down assays with total 293T cell extracts (1 mg per assay) (C). Kinase assays were performed for 30 minutes at 30°C in the presence of [γ-32P]ATP at 2 µCi/assay. Protein complexes were separated on a 10% SDS-PAGE gel. The gel was then coloured by Coomassie blue and exposed in autoradiography cassette. Phosphorylated forms of the proteins are indicated by stars. The arrow indicates the unknown protein sent for mass spectrometry.
Mutation of serine 160 reduces C/EBPδ transcriptional activity

Cells were transiently co-transfected by lipofection in 293T cells using the Hapto-luc reporter gene and the DNA MSV (Ctrl), MSV-C/EBPδ (δ wt) and MSV-C/EBPδ T156A (A) or MSV-C/EBPδ S160A (B). Luciferase activity is expressed in fold induction as compared to the control. The means and standard deviations calculated are representative of experiments repeated four times in quadruplicate.
Redundant N-terminal domains are essential for C/EBPδ transcriptional activity

A) DNA-binding capacity of C/EBPδ mutants. Nuclear extracts of 293T cells transiently transfected for 24 hours with MSV (Ctrl), MSV-C/EBPδ (δ wt), MSV-C/EBPδ Δ151-164, MSV-C/EBPδ Δ36-164, MSV-C/EBPδ Δ70-164, MSV-C/EBPδ Δ108-164, MSV-C/EBPδ Δ166-174, MSV-C/EBPδ Δ50-60, MSV-C/EBPδ Δ60-70 or MSV-C/EBPδ Δ75-85 were incubated with an antibody specific for C/EBPδ for 30 minutes before the 32P-labelled oligonucleotide corresponding to the HaptoA C/EBP DNA-binding site was added.

B) Transcriptional activity of the C/EBPδ mutants. Cells were transiently cotransfected by lipofection in 293T cells using the Hapto-luc reporter gene and the following DNA: MSV (Ctrl), MSV-C/EBPδ (δ wt), MSV-C/EBPδ Δ151-164, MSV-C/EBPδ Δ36-164, MSV-C/EBPδ Δ70-164, MSV-C/EBPδ Δ108-164, MSV-C/EBPδ Δ166-174, MSV-C/EBPδ Δ50-60, MSV-C/EBPδ Δ60-70 or MSV-C/EBPδ Δ75-85. Luciferase activity is expressed in fold induction as compared to the control. The means and standard deviations calculated are representative of experiments repeated four times in quadruplicate.
A) 293T cellular nuclear extracts
- : no antibody
+ : anti-c/ebpδ

B) [Graph showing LacZ reporter activity for different mutants]
Figure 15

Two unknown kinases phosphorylate C/EBPδ

Cellular lysates (20 μg) from 293T, Caco-2 (2 days sub-confluent and 15 days confluent) cells were separated on a 10% SDS-PAGE gel in which purified C/EBPδ(N) and C/EBPδ(C) GST fusion proteins (100 μg each) had been integrated. Kinases present in the cellular lysates were renatured and permitted to phosphorylate C/EBPδ in the presence of 10 mM ATP and 75 μCi of [γ-32P]ATP. The gel was then coloured by Coomassie blue and exposed in an autoradiography cassette.
Figure 16

Two novel proteins interact with C/EBPδ

C/EBPδ and C/EBPδ C-terminal GST constructions (15 μg each) were used in pull-down assays with total 293T cell extracts (1 mg per assay). Protein complexes were separated on a 10% SDS-PAGE gel. The gel was then coloured by Sypro Orange and exposed in a UV light box for visualization and photography. The bands indicated were excised from the gel, along with BSA, and sent to be analysed by mass spectrometry.
DISCUSSION

1 – The p38 MAPK is implicated in the regulation of IL-1 induced haptoglobin expression via the phosphorylation of C/EBPδ

Our results show that specific inhibition of p38 MAPK by the inhibitor SB203580 decreases the IL-1-dependent induction of haptoglobin mRNAs in intestinal epithelial cells. Previous results in our laboratory have demonstrated that the transactivation of the haptoglobin promoter in IL-1-induced cells depended most importantly on C/EBPs (Désilets et al., 2000). The p38 MAPK belongs to the stress-activated protein kinase family of MAPK, and is often implicated in the inflammatory response (New and Han, 1999). More specifically, it has been shown that induction by IL-1 will induce p38 MAPK expression and activity (Freshney et al., 1994). While SB203580 may affect the expression of C/EBPβ and C/EBPδ at the mRNA and protein levels, a decrease in the rapid induction of haptoglobin mRNAs after 4 hours suggests a more direct role for the p38 MAPK. For example, SB203580 decreased the transactivation potential of C/EBPδ on the haptoglobin promoter, as assessed by transient transfection assays. The transactivation potential of the N-terminal 1 to 164 domain was also decreased by SB203580. Thus, these results suggest an important regulatory role for p38 MAPK on C/EBPδ transactivation potential.
Indeed, we have shown that C/EBPδ is a transcription factor that interacts with the p38 MAPK. By GST-fusion protein interactions, we have demonstrated an interaction between p38 MAPK and C/EBP isoforms α, β, and δ. While our results confirm the interaction between C/EBPβ and p38 MAPK (Engelman et al., 1998), interactions of C/EBPα and C/EBPδ with p38 MAPK have never been demonstrated before. Interestingly, a kinase of an approximate molecular weight of 38 kD has been shown to phosphorylate in an in-gel kinase assay.

While the C/EBPα and C/EBPβ docking sites for MAPKs have not been defined, we have determined by pull-down assays that region of C/EBPδ interacting with p38 MAPK was between amino acids 70 and 108. Members of the MAPK family recognize docking sites that vary depending on the substrate and that are specific for transcription factors (Pearson et al., 2001). The docking site common for transcription factors was first discovered in the transcription factor c-Jun between amino acids 30 and 79 (Kallunki et al., 1994). This domain, now known as the D domain, was originally called the δ domain (Kallunki et al., 1996). The D domain was then recognized in other transcription factors such as Elk-1 (Yang et al., 1998) and the members of the MEF2 family (Yang et al., 1999), and is characteristically a cluster of basic amino acid residues followed closely by the motif (L/I)X(L/I) (Yang et al., 1998). The D domain was then discovered to be important in substrate recognition for other MAPK, such as ERK1/2 and p38 (Tanoue et al., 2000). On closer inspection of our area of interaction, two consensus D domains were identified, with the motif LEL, from amino acids 74-76 and 98-100. The putative docking
motifs are not conserved in the isoforms C/EBPα or C/EBPβ. We thus propose that one or both elements may well be involved in direct recruitment of p38 MAPK. These results also suggest that C/EBPδ may be a substrate for p38 MAPK.

Indeed, our results demonstrate that C/EBPδ, as well as the isoforms C/EBPα and C/EBPβ, are phosphorylated by kinases present in both 293T and Caco-2 cells, and by recombinant p38 MAPK (data not shown). The phosphorylation of C/EBPδ is decreased by the addition of SB203580, an inhibitor specific for p38 MAPK. Furthermore, deletion of amino acids 151 to 164 decreases the level of phosphorylation of the N-terminal domain. The remaining phosphorylation of the 1 to 151 region becomes unresponsive to SB203580, as opposed to the 81-164 domain. Interestingly, mutation of the conserved threonine 156 phosphorylation site had no effect on C/EBPδ transactivation potential, as assessed by transient transfection. In contrast, mutation of serine 160, which is conserved in C/EBPβ (see figure 5; Hu et al., 2001; Engelman et al., 1998) and CHOP (Wang et Ron, 1996), decreased C/EBPδ transcriptional activity. Thus our results establish that C/EBPδ is phosphorylated between amino acids 151 and 164 by the p38 MAPK. Of the putative MAPK phosphorylation sites at threonine 156 and serine 160, the mutation of the serine at position 160 most importantly diminished the transactivation potential of C/EBPδ. We can suggest therefore that the effect of p38 MAPK on C/EBPδ transcriptional activity depends on the phosphorylation of serine 160.
The MAPK family is known to phosphorylate serine or threonine residues followed immediately by proline residues, that is (S/T)P (Knighton et al., 1991). Several studies have demonstrated the regulation of the C/EBP family of transcription factors by phosphorylation by the MAPK family (Behre et al., 2002; Hu et al., 2001; Engelman et al., 1998). For the C/EBP family, phosphorylation by MAPK occurs equally on serine and threonine residues. C/EBPα is phosphorylated on rat serine 248 (mouse serine 233) via a Ras signalling pathway (Behre et al., 2002), and C/EBPβ is phosphorylated on the corresponding threonine in its sequence (mouse serine 188) by ERK1/2 MAPK (Hu et al., 2001). This conserved serine residue in another C/EBP family member CHOP is also known to be phosphorylated by p38 MAPK (Wang and Ron, 1996). These regions in C/EBPα and C/EBPβ correspond to amino acid residues between position 151 and 164 in C/EBPβ, the region that was phosphorylated in our kinase assays. Phosphorylation of the N-terminal domains of the C/EBP family of transcription factors has often shown an effect on the transactivation potential of the protein. The transactivation potential of C/EBPβ is induced by phosphorylation via Ras on the C/EBP-responsive promoter-reporter in keratinocytes, while the mutation of an ERK1/2 phosphorylation site at a threonine residue at position 188 abolished this Ras effect (Zhu et al., 2002). As well, phosphorylation of this threonine increases growth hormone-mediated transcription of the c-fos promoter by C/EBPβ (Piwien-Pilipuk et al., 2002).

Many studies indicate the presence of N-terminal activation and repression domains for the isoforms C/EBPα and C/EBPβ, yet few studies have concentrated on
the characterization of C/EBPδ. Deletion mutants of the C/EBPα (Nerlov and Ziff, 1994) and β isoforms (Williams et al., 1995) identified for both isoforms three activation domains. In order to determine the presence of such regulatory regions in C/EBPδ, we created small to large deletion mutations in the N-terminal region of the protein. All of the mutations created functional DNA-binding C/EBPδ proteins, as shown by EMSA. A domain from amino acids 36 to 108 is essential for C/EBPδ transactivation potential, as assessed by transient transfections assays with the C/EBP-dependent promoter haptoglobin. This domain is composed of multiple regions, almost equally affecting C/EBPδ transcriptional activity (Δ50-60, Δ60-70 and Δ75-85).

The activation domains characterized in C/EBPβ (Williams et al., 1995) and C/EBPα (Nerlov and Ziff, 1994) are well conserved in C/EBPδ. For example, the amino acids 52-104 in C/EBPβ (Williams et al., 1995) and the region TEII (amino acids 70-97) of C/EBPα are activation domains for both proteins. There exists a high sequence homology between the three isoforms in this area within the different species forms of C/EBPδ in these regions (Davies et al., 2000). The activation domains of C/EBPα and C/EBPβ also interact with the co-activator p300 (Erickson et al., 2001; Guo et al., 2001). p300 is known to link transcription factors via protein-protein interaction to the basal transcriptional machinery (Mink et al., 1997) and possesses histone acetyltransferase activity, implicating the coactivator in the modification of chromatin (Ogryakoko et al., 1996). The interaction of p300 with C/EBP isoforms α.
Proposed model for IL-1-induced C/EBPδ activation of transcription via phosphorylation by p38 MAPK and the implication of p300
Transactivation of genes implicated in APR
and β is needed to increase the transcriptional activity of both proteins (Erickson et al., 2001; Guo et al., 2001).

We propose that recruitment of p38 MAPK by C/EBPδ may increase the ability of C/EBPδ to interact with p300 (Fig. 17). It has been shown that phosphorylation of C/EBPα and C/EBPβ may alter the conformation of the protein (Williams et al., 1995) enabling an increase in transcriptional activity. Recruitment of p38 MAPK by C/EBPδ may also indirectly lead to phosphorylation of other C/EBP isoforms that form dimers with C/EBPδ. This phosphorylation of dimerization partners has been demonstrated for c-jun: JNK MAPK is known to phosphorylate c-jun readily, and the heterodimerization with JunD, a member of the jun family that lacks the JNK docking site, initiates phosphorylation of JunD (Kallunki et al., 1996). Recruitment of p38 MAPK by C/EBPδ may also directly lead to the phosphorylation of p300. Indeed, such a recruitment of p300 is observed by C/EBPβ, triggering a massive phosphorylation of p300, increasing the co-activator’s activity (Schwartz et al., 2003). Recent studies have also demonstrated the presence of a shift of p300 by C/EBPδ when co-transfected into macrophage cells (Schwartz et al., 2003). However, we have not been able to induce a modification of p300 mobility by co-transfection of C/EBPδ with p300 in 293T cells (Geneviève Doyon, personal communications). Nevertheless, these studies considered in conjunction with our results suggest that C/EBPδ may possibly interact in the conserved N-terminal activation domain with the co-activator p300, and that this functional collaboration, modulated by p38 MAPK, will increase C/EBPδ transcriptional
activity on certain promoters, as is the case for other C/EBP isoforms. Whether the regulation of C/EBPδ depends largely on the interaction between p300 and p38 MAPK remains to be determined.

2 – Novel co-factors may be involved in regulating C/EBPδ transcriptional activity

Our results indicate that C/EBPδ is phosphorylated by a kinase of approximately 120 kD, as assessed by in-gel kinase assay. Further studies will have to be performed to determine the identity of the kinase of molecular weight 120 kD. However, analysis of the kinases of this molecular weight with the kinome (Manning et al., 2002) has suggested ZPK (zipper protein kinase) as a putative kinase of interest. This kinase is associated with stress-dependent signal transduction pathways and contains a leucine-rich region that interacts with different proteins (Reddy and Pleasure, 1994). Interestingly, ZPK forms heterodimers with leucine-zipper containing transcription factors such as CREB and Myc, phosphorylating these proteins (Reddy et al., 1999).

The other two novel co-factors of interacting with the C-terminal region of C/EBPδ, namely Ku70 and PARP-1, were determined on separate occasions. These two factors are known to interact with one another (Galande and Kohwi-Shigematsu, 1999). Both factors are known to play important roles in nuclear processes, such as DNA repair and transcription, and both contain leucine zipper-like motifs (Kioke
2002, Altheus et al., 1990). Ku 70 becomes a functional, stable protein only once it has formed a heterodimer with Ku80 (Jin and Weaver, 1997). The C-terminal region of the C/EBP proteins contains a leucine zipper and often forms heterodimers with different proteins then that of the C/EBP family, which could indicate that the interaction occurs via the leucine zipper.

Previous studies have demonstrated a link between Ku 70 and C/EBPβ. Both factors repress the transcription of heat shock protein 70 (HSP70) in non-stressed conditions, subsequently repressing the activation of heat shock factor 1 (HSF1) and the heat shock response, maintaining homeostasis (Tang et al., 2000; Yang et al., 1996). The Ku heterodimer is also known to recruit DNA-PK (DNA-dependent protein kinase), which phosphorylates IκB, liberating NF-κB (Liu et al., 1998). These results suggest a role for Ku70 in the inflammatory response since NF-κB has been shown to induce the expression of various genes during inflammation (Li and Verma, 2002). The C/EBP family of transcription factors may well be another family of pro-inflammatory regulators affected by Ku.

PARP-1 is also involved in many cellular functions. Most interestingly for this study, it has been shown that PARP-1 induces several inflammatory cytokines, namely IL-1β (Oliver et al., 1999). PARP-1 thus has been identified as a co-activator of NF-κB (Hassa and Hottiger, 1999), and is required for specific NF-κB gene activation through direct interaction with NF-κB (Hassa et al., 2001). The presence of a PARP-1-NFκB complex on DNA activates transcription with the aid of another
positive cofactor that is proposed to bind the same promoter as NF-κB and interact with PARP-1 (Malik and Roeder, 2000).

The specific functionality of these *in vitro* interactions between C/EBPδ, Ku70, and PARP-1 remains to be determined *in vivo*. Since Ku70 and PARP-1 interact with one another, it is possible that only one of the two factors interacts directly with C/EBPδ, bringing the other factor to form a complex. The link between PARP-1-mediated transcriptional activation, NF-κB, and C/EBPδ also requires more investigation.
CONCLUSIONS AND PERSPECTIVES

1 – The p38 MAPK is implicated in the regulation of IL-1 induced haptoglobin expression via the phosphorylation of C/EBPδ

C/EBP isoforms are involved in the regulation of many important cellular processes. Studies have indicated that in response to inflammatory stimuli, in intestinal epithelial cells, the C/EBPδ isoform is the most induced of the C/EBPs (Désilets et al. 2000), strongly suggesting an important role in the regulation of the intestinal inflammatory response. However, the regulation of C/EBPδ transcriptional activity is not known. The principal aim of this research was to understand the role of signalling cascades in C/EBPδ IL-1-induced transactivation of genes.

In this study, we demonstrated that C/EBPδ is a transcription factor that is phosphorylated by the p38 MAPK. This phosphorylation will activate the protein’s ability to transactivate haptoglobin mRNAs expression in response to IL-1. Indeed, we have demonstrated that specific inhibition of p38 MAPK by the inhibitor SB203580 decreases the IL-1-dependent induction of haptoglobin mRNAs in intestinal epithelial cells. We have determined the C/EBPδ docking site for p38 MAPK, between amino acids 70 to 108, containing two putative MAPK docking sites. The importance of amino acids 151 to 164 for phosphorylation was determined by kinase assays. The serine residue at position 160 is the most important in optimal
C/EBPδ transactivation potential. This residue is conserved and phosphorylated by MAPK in the C/EBP family members C/EBPβ and CHOP (Fig 5; Hu et al., 2001; Engelman et al., 1998; Wang et Ron, 1996).

We have identified redundant N-terminal activation domains between amino acids 36 and 108. This region is composed of multiple elements, almost equally affecting C/EBPδ transcriptional activity (Δ50-60, Δ60-70 and Δ75-85). Several studies indicate the presence of N-terminal activation domains for the isoforms C/EBPα (Nerlov and Ziff, 1994) and C/EBPβ (Williams et al., 1995) conserved in C/EBPδ. These regions of C/EBPα and C/EBPβ also interact with the co-activator p300 (Erickson et al., 2001, Guo et al., 2001), increasing the transcriptional activity of both proteins. C/EBPδ may possibly interact in the conserved N-terminal activation domain with the co-activator p300, and this functional collaboration may increase C/EBPδ transcriptional activity.

Further studies will be performed to determine the importance of different domains of C/EBPδ in IL-1β-induced activation of haptoglobin. Retroviral infections of the mutated forms of C/EBPδ in intestinal epithelial cells will be performed and the effect of these mutations on haptoglobin expression, and other C/EBP targets as well, will be analysed. GST-fusion proteins containing mutations of the putative MAPK docking sites of C/EBPδ will be used to determine the specific docking site for p38 MAPK on C/EBPδ. Further studies using the mutant S160A in kinase assays in the presence of SB203580 would further confirm the effect of phosphorylation on
this residue by p38 MAPK. These studies will permit us to determine the role of different C/EBPδ domains in IL-1β-mediated inflammatory response in vivo in intestinal epithelial cells, and the importance of modification of C/EBPδ by phosphorylation.

The involvement of p300 in C/EBPδ transactivation potential remains to be determined. Future co-transfection studies will determine the effect of p300 on C/EBPδ activity. The interaction between p300 and C/EBPδ will also be established via GST-pull down assay. Also, recruitment of p38 MAPK by C/EBPδ may increase the ability of C/EBPδ to interact with p300, and/or trigger the phosphorylation of p300. The exact interplay between p38 MAPK and p300 in C/EBPδ-mediated transcription of APP genes remains to be determined via immunoprecipitation assays and co-transfection experiments in the presence of the p38 MAPK inhibitor SB203580.

Further studies using the C/EBPδ mutants created includes experiments concerning protein stability. Recent studies have revealed that proteasome inhibitors increase C/EBPβ and C/EBPδ protein levels, suggesting that these isoforms are ubiquitinated (Hungness et al., 2002; our data). The degradation of C/EBPβ by the proteasome has been further confirmed in kidney cells (Hattori et al., 2003). The verification of DNA-binding capacity and transcriptional activity of these mutants in the presence of a proteasome inhibitor would indicate if any of these N-terminal regions are important in protein stability and ubiquitination.
2 – Novel co-factors may be involved in regulating C/EBPδ transcriptional activity

This study has demonstrated the interaction of novel partners of C/EBPδ. Our results indicate that C/EBPδ interacts with a novel kinase besides the p38 MAPK, a kinase of approximately 120 kD. Analysis of kinases of this molecular weight by the kinome (Manning et al., 2002) has suggested ZPK (zipper protein kinase), a kinase associated with stress-dependent signal transduction that interacts with other leucine zipper transcription factors such as CREB and Myc (Reddy et al., 1999).

Our results also revealed a novel interaction of the C-terminal region of C/EBPδ with Ku70 and PARP-1, two factors known to play important roles in nuclear processes, such as DNA repair and transcription. These two factors are also known to interact with one another (Galande and Kohwi-Shigematsu, 1999), and both factors contain leucine zipper-like motifs (Kioke, 2002; Altheus et al., 1990). There is evidence that PARP-1 will act as a coactivator of certain transcription factors, namely NF-κB (Hassa and Hottiger, 1999), and Ku70 has also been implicated in the liberation of NF-κB from its inhibitor (Liu et al., 1998).

All of these novel factors are therefore implicated in the transactivation of genes in the inflammatory response, and their effects on C/EBPδ, separate or in conjunction with one another, remains to be determined via co-transfection and immunoprecipitation studies. Further investigation of the unknown kinase will
implicate its identification by mass spectrometry and GST-pull down assays to determine its interaction with C/EBPδ.
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