Interaction of the alphaNAC coactivator with its c-Jun target

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ABSTRACT

Nascent polypeptide-associated complex And Coactivator alpha (αNAC) is a coactivator that potentiates AP-1-dependent transcription. αNAC has been shown to interact with the homodimeric transcription factor c-Jun, and more specifically in the N-terminal activation domain of the protein. This interaction leads to a greater stabilization of c-Jun on the AP-1 consensus site and consequently, an increased rate of transcription. αNAC is a substrate of the Integrin-Linked Kinase (ILK), which is activated through integrin-extracellular matrix interactions and stimulates c-Jun-mediated gene transcription.

By means of protein pulldown assays, we have demonstrated that phosphorylation of αNAC by ILK results in an enhanced interaction of phospho-αNAC with the phosphorylated form of c-Jun. Additionally, the c-Jun interaction domain on αNAC was identified. Affinity chromatography involving αNAC deletion mutants revealed that the c-Jun-binding site is located in the middle part of the protein, between amino acids 89 and 129. These results are consistent with earlier data from our laboratory demonstrating an interaction between αNAC and c-Jun as well as increased c-Jun-mediated transcription by the coactivating function of αNAC.

RÉSUMÉ

Nascent polypeptide-associated complex And Coactivator alpha (αNAC) est un coactiveur qui augmente le niveau de transcription des gènes contrôlés par l'élément Activator Protein-1 (AP-1). αNAC démontre une interaction avec le facteur de transcription dimérique c-Jun, dans le domaine N-terminal d'activation de la protéine. Cette interaction accroît la stabilité de c-Jun sur le site AP-1 des gènes, et produit une augmentation du niveau de transcription αNAC. αNAC est phosphorylé par Integrin-Linked Kinase (ILK) suite à des interactions entre intégrines et la matrice extra-cellulaire. En conséquence, la forme activée de ILK incite l'expression de gènes contrôlés par c-Jun.

Par l'entremise d'essais protéine-protéine, nous avons démontré que la phosphorylation de αNAC par ILK rehausse l'interaction entre phospho-αNAC et phospho-c-Jun. De plus, le domaine d'interaction avec c-Jun a été identifié sur la protéine αNAC. La technique de chromatographie par affinité avec l'utilisation de mutants de délétions de αNAC a délimité le domaine d'interaction entre les acides aminés 89 et 129 de αNAC. Ces résultats appuient les données antécédentes de notre laboratoire qui démontrent une interaction entre αNAC et c-Jun de même que l'augmentation de la transcription contrôlée par c-Jun et coactivée par αNAC.

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ABBREVIATIONS AND SYMBOLS

αNAC :	Nascent polypeptide-associated complex And Coactivator alpha
AP-1 :	Activator Protein-1
APC :	Adenomatous Polyposis Coli
bZIP :	basic domain leucine-ZIPper
BTF3b :	Basic Transcription Factor 3b
CBD :	Chitin-Binding Domain
CBP :	CREB-Binding Protein
CMV :	CytoMegaloVirus
CREB/ATF :	cAMP Response Element-Binding protein/Activating Transcription Factor
dsRNAi :	double-stranded RNA interference
DTT :	1, 4-DiThioThreitol
ECM :	ExtraCellular Matrix
ERK :	Extracellular-Regulated Kinase
ES Cells :	Embryonic Stem Cells
GSK-3 :	Glycogen Synthase Kinase-3
GST :	Glutathione-S-Transferase
GTFs :	General Transcription initiation Factors
HAT :	Histone AcetylTransferase
HIF-1 :	Hypoxia-Inducible Factor-1
HSV-TK :	Herpes Simplex Virus-Thymidine Kinase
ICBD :	Intein Chitin-Binding Domain

IL-6 :	InterLeukin-6
ILK :	Integrin-Linked Kinase
IPTG :	IsoPropyl-Thio-β-D-Galactopyranoside
JAB1 :	Jun Activation domain-Binding protein 1
JNK :	Jun N-terminal Kinase
LEF-1/TCF :	Lymphocyte Enhancer-binding Factor-1/T-Cell Factor
MaBP :	Maltose-Binding Protein
MAP3K :	Mitogen Activated Protein Kinase Kinase Kinase
MAPK :	Mitogen Activated Protein Kinase
MEF :	Mouse Embryonic Fibroblast
MyBP :	Myelin Basic Protein
PAK :	Protein Activated Kinase
PDK-1 :	3'-Phosphoinositide-Dependent Kinase-1
Pi(3)K :	Phosphoinositide-3-OH Kinase
PKC :	Protein Kinase C
PVDF :	PolyVinyldene DiFluoride
SAPK :	Stress-Activated Protein Kinase
SDS-PAGE :	Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis
SRC-1 :	Steroid Receptor Coactivator-1
STAT3 :	Signal Transducer and Activator of Transcription 3
TAF :	TBP-Associated Factors
TBP :	TATA-Binding Protein
TFIIX :	Transcription Factor II (A, B, D, E, F, H, J)
VEGF :	Vascular Endothelial Growth Factor

INTRODUCTION

Transcriptional Control

Initiation of transcription by RNA polymerase II is dependent on General Transcription initiation Factors (GTFs) (1,2). In the promoter region of most genes, a short 8 base pair (bp) sequence named the TATA box can be found and is usually located 25 bp upstream of the transcriptional startpoint. This A/T sequence is initially recognized by Transcription Factor II D (TFIID) of the GTFs (1,2), which contains two types of components. One of the factors is TATA-Binding Protein (TBP), which binds to the TATA box and bends the DNA slightly. This allows the GTFs and RNA polymerase II to form a closer association than would be possible on linear DNA (2,3). TBP-Associated Factors (TAFs), the second component of TFIID, interact with TBP and bind DNA (2,3). TFIIA then joins the complex, followed by the binding of TFIIB downstream of the TATA box. TFIIF and RNA polymerase II as well as TFIIE, TFIIH and TFIIJ assemble sequentially and the polymerase synthesizes the first nucleotide bonds in RNA. The initiation process of transcription is followed by elongation of the RNA chain and transcriptional termination.

RNA polymerase II along with TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH and TFIIJ constitute the basal apparatus (1-3). However, transcription also requires DNA-binding transcriptional activators, also referred to as transcription factors. A transcription factor may be tissue-specific or ubiquitously expressed and activates a promoter that contains a common target sequence known as a consensus site. They are

categorized into several classes or families according to their modular domains. A first protein motif enables transcription factors to recognize and bind specific target sequences located in promoters or other regulatory elements that affect a target gene (4). A second motif is the activation domain, whereby the particular function of the transcription factor is exercised by binding to accessory proteins or the basal apparatus. Transcriptional activators can bind several different general transcription factors, including TFIIB and TFIID. A variety of protein structures form the DNA-binding domain of transcription factors such as homeodomains, zinc-finger motifs, whinged-helix motifs or forkhead domains and helix-loop-helix domains (1,2,4). A well-characterized structure for DNA binding is the basic domain leucine-ZIPper motif (bZIP). bZIP proteins have coiled coils of alpha helices with preserved leucine residues at every 7^{th} residue. Dimerization of two bZIP proteins positions the basic domains of the proteins so that they can bind DNA.

Although GTFs and transcriptional activators are sufficient for basal-level transcription, coactivators are required to enhance or potentiate the transcriptional response (5,6). Coactivators are proteins that potentiate, in a selective manner, the stimulatory activity of transcription factors and general transcription factors by binding to both proteins. Several coactivators have been identified and described in the literature. One example is TBP-Associated Factors (TAFs), which provide protein interfaces to link transcriptional activators to the basal transcriptional machinery (3,7). Some subunits also possess specific enzymatic activities that are necessary for enhanced gene transcription (8,9). It has been demonstrated that some transcriptional coactivators exhibit an intrinsic Histone AcetylTransferase (HAT) activity enabling them to mediate long-range transcriptional control (9,10). Histones are conserved DNA-binding proteins

that form the nucleosome, the basic subunit of chromatin. Chromatin, in its normal state, is transcriptionally repressed and blocks the association of TFIID and RNA polymerase II with the DNA. However, acetylation of histone tails by coactivators correlates with transcriptional activity in many genes and may involve decondensation of the chromatin in the regions of promoters (11-13). The removal of acetyl groups from nucleosomes causes repression of gene expression (14,15). It is thought that transcription factors recruit HAT complexes to the promoters of target genes in order to acetylate histones, thereby increasing DNA accessibility to transcription factors.

Transcriptional control through means of transcription factors and coactivators is essential since different proteins are expressed in different cells at different times in development. One way to ensure regulation is by signal transduction pathways.

Mitogen Activated Protein Kinase (MAPK) Signalling Pathway

Signal transduction generates differential responses to stimuli such as activating different pathways, varying the length of the activation and modifying the intensity of the outcome. One of the important features of signal transduction pathways is that they diverge and converge, thus allowing different but overlapping responses to be triggered in different circumstances (2,4). Signalling at the plasma membrane can lead to the activation of numerous cascades (divergence) whereas different extracellular signals can activate the same pathway (convergence). For the most part, signal transduction pathways consist of an exchange of information between proteins through means of phosphorylation and dephosphorylation (16). Protein kinases catalyze the covalent

addition of a phosphate group to specific amino acid residues. Conversely, phosphatases remove phosphate groups from substrates. The degree to which a protein is phosphorylated or dephosphorylated correlates with the activity of particular kinases and phosphatases, respectively (17,18). The amino acids that are targeted by kinases and phosphatases are serines/threonines (ser/thr), tyrosines, lysines and histidines. One well-characterized MAPK pathway is the Ras pathway (figure 1).

The Ras signalling cascade is induced by the binding of a growth factor to a specific transmembrane receptor tyrosine kinase, which allows for dimerization and activation of the cell surface receptor. The activation occurs by autophosphorylation of several tyrosine residues within the cytoplasmic tail of the receptor (2,19). Adapter proteins then associate with the activated receptor tyrosine kinase, which results in the activation of the monomeric guanine nucleotide-binding protein Ras. Activated Ras recruits the ser/thr kinase Raf to the plasma membrane where it is activated. A series of phosphorylation events then occurs with Raf phosphorylating MEK, a MAP kinase kinase, which in turn phosphorylates the MAP kinase Extracellular-Regulated Kinase (ERK) (2,19). This leads to the phosphorylation of transcription factors such as c-Myc that trigger changes in cell growth and differentiation.

Activation of Ras can also regulate another important MAPK pathway, the Jun N-terminal Kinase (JNK) pathway (figure 1). Although Ras activation consequently leads to the activation of JNK, exposure to cellular stresses and cytokines have been more thoroughly investigated as inducers of the pathway. The JNK signalling cascade is also referred to as a Stress-Activated Protein Kinase (SAPK) pathway, whereby the numerous JNK isoforms are classified as SAPKs (20). Several stresses can affect cellular processes such as UV radiation, osmotic shock, protein synthesis inhibitors and

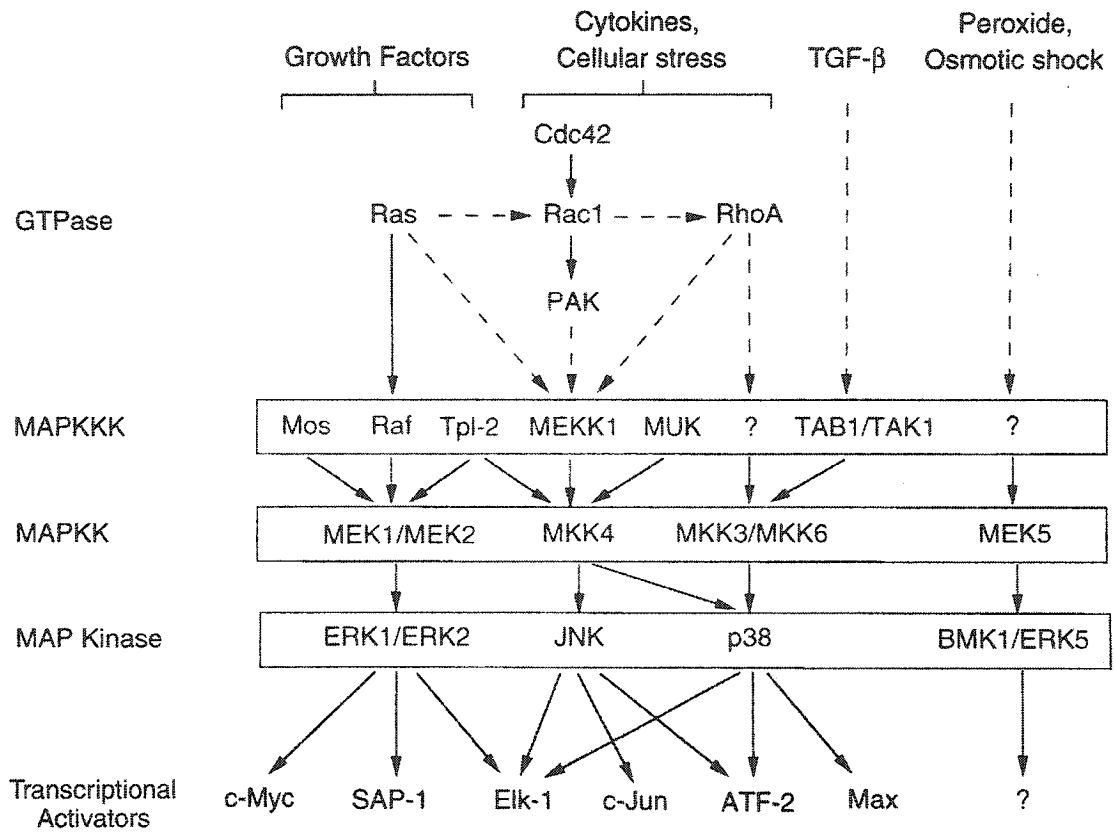

FIGURE 1 : Mammalian Mitogen Activated Protein Kinase (MAPK) signal transduction pathway. Bold arrows represent a direct activation of downstream components; dashed arrows are possible signalling routes. Of interest for the work presented here is the Jun N-terminal Kinase (JNK) MAP Kinase pathway (middle). Modified from Whitmarsh, A. J., and Davis, R.J. (1996) *J. Mol. Med.* **74**, 589-607.

heat shock (19,21-23). The extracellular stimuli are received by the Ras-related Rho family of GTPases, a group of small G proteins, which include Cdc42 and Rac1. These two proteins can bind to and activate p21-Activated ser/thr Kinase (PAK), which in turn phosphorylates the MAP Kinase Kinase Kinase (abbreviated to MAP3K) MEKK1. Once MEKK1 is activated, it phosphorylates the dual specificity protein kinase MKK4 (also known as SEK1/JNKK). JNK is a substrate of MKK4 and is therefore phosphorylated on the tyrosine and threonine residues within the tripeptide motif Thr-Pro-Tyr located in one of the subdomains (24). Activated JNK targets several nuclear transcription factors, including the AP-1 family member c-Jun. c-Jun is phosphorylated by JNK on serines 63 and 73 (25,26) and this results in the expression of early immediate AP-1 responsive genes (27-29).

c-Jun

c-Jun is a transcription factor belonging to the AP-1 (Activator Protein-1) family. Robert Tijan and colleagues (30) identified several proteins regulating the expression of the human metallothionein promoter, which they named AP-1 proteins. By means of DNA affinity chromatography, the proteins were purified along with others, which were later discovered to be members of the fos and jun proto-oncogene families. c-jun is found on human chromosome 1 (31) and is an immediate early gene, a class of genes that are rapidly induced when resting cells are treated with mitogens. The protein is 334 amino acids in length and regulates genes involved in initiating or promoting growth (31). Initial findings demonstrated that the avian sarcoma virus-17 v-jun oncogene, as

well as its c-jun cellular counterpart, had a domain that was similar to the DNA-binding domain of the yeast transcription factor GCN4 (32,33). On account of the similarity, c-Jun was thought to function as a DNA-binding transcriptional regulator. Further experiments revealed that c-Jun was indeed a transcription factor and was part of the AP-1 family (34).

AP-1 transcription factors are formed by the dimerization of members of the Jun and Fos protein families. Both families are basic domain leucine-ZIPper (bZIP) DNA-binding proteins that dimerize to form a coiled-coil structure, bringing together two regions rich in basic amino acids and essential for DNA binding. The leucine-zipper motif was identified by Landschulz et al. (35) upon investigating similarities within the Fos family. c-Jun, JunB and JunD are the AP-1 members forming the Jun family and c-Fos and Fos-Related Antigens 1 and 2 (Fra-1, Fra-2) form another family. Whereas c-Fos must heterodimerize, c-Jun can homodimerize (36) or bind to several other partners such as Fos family members or the cAMP Response Element-Binding Protein/Activating Transcription Factor (CREB/ATF) (37). The dimerized protein complex binds to DNA at the AP-1 consensus sequence [TGA(C/G)TCA] in the promoter region of various genes. The heterodimer c-Fos-c-Jun has recently been crystallized (38). The crystal structure demonstrates continuous α-helices in both subunits, an asymmetric coiled-coil in the C-terminal regions and base-specific contacts with DNA in the N-terminal regions. Increased expression of Fos and Jun proteins as well as post-translational modifications of Fos and Jun by phosphorylation cause AP-1 activation (28,39).

c-Jun activity is regulated through phosphorylation by Jun N-terminal Kinase (JNK). JNK interacts with c-Jun in its δ domain found in the N-terminal region

(20,25,26). Upon signalling, JNK is phosphorylated on residues 183 and 185 (20), and it then phosphorylates the N-terminal activation domain of c-Jun on serines 63 and 73. This results in a prolonged half-life and activated c-Jun translocates to the nucleus where it binds to an AP-1 consensus site in the promoter of various genes (40,41).

Protein deactivation and degradation are also essential for proper cellular functions. In the absence of a stimulus, JNK does not become activated and is therefore not able to phosphorylate c-Jun. Consequently, JNK targets c-Jun for ubiquitination (51,52), which involves the attachment of several ubiquitins to the protein. The c-Jun-ubiquitin complex is then shuttled to the 26S proteasome, a multicatalytic proteolysis system. The proteasome-dependent protein degradation pathway has been shown to modulate the intracellular levels of several regulatory proteins implicated in the control of key cellular functions including cell cycle progression, signal transduction, differentiation, apoptosis and regulation of transcription (52,53). Stated differently, the magnitude and duration of the activity of several proteins, including c-Jun, are regulated by ubiquitin-dependent proteolysis by the 26S proteasome.

c-Jun is also regulated by the activity of Glycogen Synthase Kinase-3 (GSK-3). Studies have demonstrated that phosphorylation mediated by GSK-3 is involved in negative regulation of c-Jun DNA-binding function (54). Specifically, GSK-3 phosphorylates three amino acids on c-Jun in a region proximal to the C-terminal DNA-binding domain, resulting in decreased DNA binding (54,55). Transcriptional activation elicited by c-Jun is also decreased once c-Jun is phosphorylated by GSK-3.

In addition to the SAPK cascade, c-Jun-mediated AP-1 transcription is also modulated by the integrin signalling pathway. Integrins are a large family of cell surface receptors composed of two subunits, the α and β chains, of molecular weight between

95 and 220 kDa (42). Most integrins have the ability to recognize several proteins from the ExtraCellular Matrix (ECM). Additionally, ECM proteins such as fibronectin, vitronectin, laminins and collagens can interact with various integrins (43). Integrins are transmembrane receptors and do not possess enzymatic functions in their cytoplasmic tails. In order to accomplish their function as signal transducers, the tails interact with adapter proteins that connect the integrins to the cytoskeleton, cytoplasmic kinases and transmembrane growth factor receptors. Signalling can occur from the inside-out, where the binding of ECM proteins is controlled from inside the cell. Conversely, signals stemming from the interaction of ECM proteins with the integrins are transduced into the cell (outside-in signalling) (44).

Integrin-Linked Kinase (ILK)

ILK is an ankyrin-repeat containing serine-threonine protein kinase that interacts directly with the β1 and β3 cytoplasmic domains of integrins and is therefore implicated in integrin-mediated signal transduction (45-47). ILK was identified from a yeast two-hybrid genetic screen by using the cytoplasmic domain of the β1 integrin subunit as bait (48). The activity of ILK is regulated by interactions with components of the ECM, such as fibronectin, or by integrin clustering. ILK is rapidly and transiently stimulated in a Phosphoinositide-3-OH-Kinase [Pi(3)K]-dependent manner upon integrin-fibronectin interactions (46,47). Integrin-mediated interactions with fibronectin also lead to the stimulation of c-Jun AP-1 transcription, where ILK is stimulated and GSK-3 is inhibited (49, figure 2). The group of Dedhar (49) analyzed the ability of c-Jun to

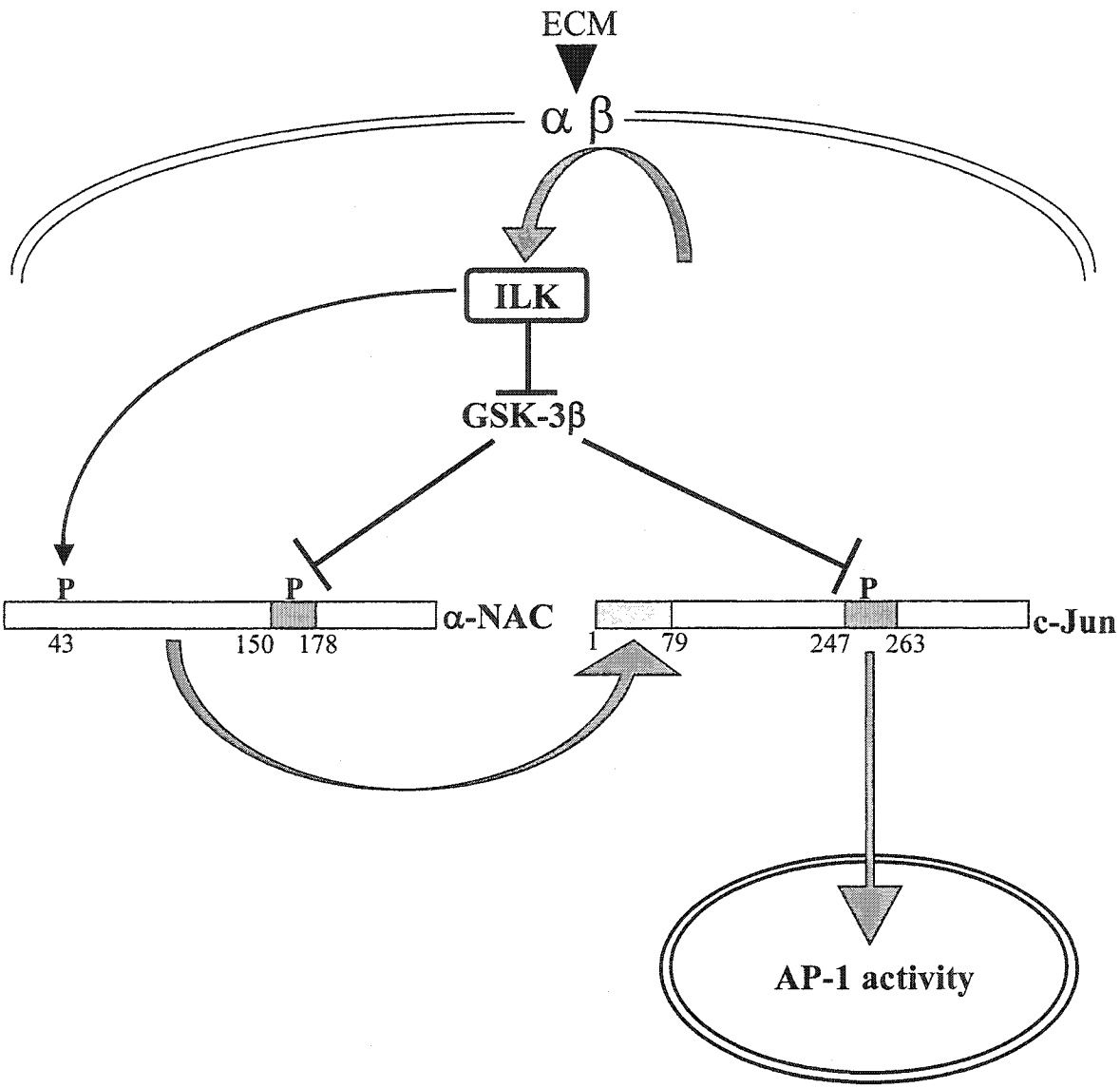

FIGURE 2 : Schematic diagram of the Integrin-ILK signalling pathway. Interaction of the integrins with ExtraCellular Matrix (ECM) components stimulate ILK activity, which phosphorylates αNAC and GSK-3β. Phosphorylation of GSK-3β inhibits its activity, leading to prolonged half-lives for αNAC and c-Jun. The ILK-phosphorylated αNAC protein also has increased affinity for c-Jun, leading to translocation into the nucleus and stimulation of AP-1 transcriptional activity. αβ chains represent the integrins; ILK, Integrin-Linked Kinase; GSK-3β, Glycogen Synthase Kinase-3β; αNAC, Nascent polypeptide-associated complex And Coactivator α; AP-1, Activator Protein-1.

form a complex with an AP-1 consensus oligonucleotide in the presence of ILK and GSK-3, a kinase that negatively regulates DNA-binding of c-Jun. Transient transfections using an AP-1 reporter gene, ILK recombinant DNA, GSK-3 and ILK-Kinase Dead (KD) recombinant DNA were performed in human embryonic kidney HEK-293 cells. Luciferase assays as well as gel mobility shift assays demonstrated increased AP-1 activity due to ILK expression and a decrease of this activity once co-transfected with GSK-3 or ILK-KD (49). A protein complex was induced in the cells transfected with ILK cDNA. A supershift assay revealed that c-Jun was abundantly present in the complex. The results show that ILK activates the binding of c-Jun to its AP-1 consensus site and that this stimulation requires the inhibition of GSK-3 (49).

ILK has also been shown to induce the translocation of β-catenin, a coactivator and member of the Wnt signalling pathway, to the nucleus (46). This results in the formation of interactions between Lymphocyte Enhancer-binding Factor 1/T-Cell Factor (LEF-1/TCF) transcription factor and β-catenin, and consequently, the enhancement of LEF-1/TCF transcriptional activity (46,50).

AP-1 and Integrins in Bone Development

The interactions of bone cells with their surrounding extracellular environment is mediated by integrins. Bone remodeling, a process involving *de novo* bone formation and resorption of bone tissue requires three cell types. The first type, the chondrocytes, form cartilage and are necessary for endochondral or long bone formation. Osteoblasts, characterized as the bone-forming cells that synthesize and secrete extracellular matrix,

are involved in both endochondral and intramembranous (flat bones) ossification (94). Finally, resorption of bone is performed by osteoclasts in order to maintain a constant bone mass. Normal human bone cells displayed preferential adhesion to fibronectin over other extracellular matrix proteins such as collagen types I and IV and vitronectin (95). Amongst others, integrin heterodimers alpha 1 beta 1 and alpha 2 beta 1 were found to be constitutively expressed on the cellular surface of the human bone cells.

Disrupting the adhesion of the integrins to the extracellular matrix leads to impaired bone formation. Upon assessing the role of integrins in mature osteoblasts *in vivo*, a dominant-negative integrin subunit (beta-1-DN) consisting of the beta 1 subunit cytoplasmic and transmembrane domains, driven by the osteoblast-specific osteocalcin promoter, was expressed in mice (96). A culture of immature osteoblasts taken from the transgenic animals differentiated normally initially. However, once the osteocalcin promoter became active, detachment of the osteoblasts occurred, potentially as a result of the beta-1-DN transgene compromising adhesion of the mature osteoblasts (96). Additionally, the transgenic mice demonstrated reduced bone mass, with increased cortical porosity in long bones and thinner flat bones in the skull.

Several members of the AP-1 family of transcription factors have been identified as modulators of bone cell proliferation and differentiation. For example, transgenic mice overexpressing c-fos post-natally acquired transformed osteoblasts and osteosarcomas (97). Measurements of the expression levels of c-jun in the c-fos-induced osteosarcomas highly suggest that c-fos heterodimerized with c-Jun. Interestingly, c-Jun has been shown to be expressed during all stages of osteoblastic differentiation (98) as well as in early embryogenesis (99). Within the same series of experiments, double Fos-Jun transgenic mice developed a higher frequency of osteosarcomas than the Fos-

induced mice (97), which supports that Jun is implicated in proper bone development. Whereas an overexpression of c-Fos develops osteosarcomas, minimal or no expression leads to osteopetrosis since the c-Fos knockout mice lack osteoclasts (102,103).

During embryogenesis, high levels of the c-fos transgene affect chondrocytes and lead to chondrogenic tumors (100). As well, overexpression of Jun family members, namely c-Jun and JunD, affects the maturation of chondrocytes (101).

Perhaps the most recent documented findings of AP-1 regulation in bone development demonstrate the involvement of two members of the Fos family, Fos-Related Antigen 1 (Fra-1; known previously as FOSL1) and ΔFosB. Jochum and colleagues (104) generated transgenic mice having elevated levels of Fra-1 in various organs. The Fra-1-induced mice developed osteosclerosis, a disorder of increased bone mass of the entire skeleton. Fra-1 expression levels increased the numbers of mature osteoblasts *in vivo* and accelerated osteoblast differentiation *in vitro* (104). Similarly, overexpression of ΔFosB, a naturally occurring truncated form of FosB which occurs from alternative splicing, causes osteosclerosis by deregulating osteoblast function in the same manner as Fra-1 (105). It has been stipulated that the increased levels of ΔFosB potentially favor osteoblastogenesis over adipogenesis in order to increase bone formation.

Nascent polypeptide-associated complex And Coactivator alpha (αNAC)

Nascent polypeptide-associated complex And Coactivator alpha (αNAC) is a coactivator of 215 amino acids in length. αNAC was initially purified in 1994, where it was reported to form a complex with Basic Transcription Factor 3b (BTF3b) and bind to nascent-polypeptide domains emerging from ribosomes (56). NAC (αNAC and BTF3b) was stated to prevent short polypeptides from inappropriately interacting with proteins in the cytosol. However, BTF3b is involved in the control of gene transcription (57), suggesting a link between NAC and transcription. Our laboratory has been involved in the characterization of the transcriptional regulatory function of αNAC. αNAC was identified in our lab during a screen for proteins specifically expressed in terminally differentiated osteoblasts, the bone-forming cells (58). Differential display of messenger RNA (mRNA) amplified by the polymerase chain reaction (differential display PCR) was performed in order to compare genes expressed in mineralizing murine osteoblasts with those expressed in de-differentiated, non-mineralizing cells of the same lineage. From this technique, αNAC was identified as a gene expressed in differentiated osteoblasts during development (58). Northern blot assays using murine poly $(A)^+$ RNA from 7-, 11-, 15- and 17-day postconception (p.c.) whole mouse embryos demonstrated αNAC mRNA expression at 15 and 17 days p.c. (59). Additionally, immunohistochemistry detected the αNAC protein in the nucleus of differentiated osteoblasts at 14.5 days p.c., suggesting a role in signalling events that could lead to a transcriptional response. By means of anti-αNAC antibodies, our lab demonstrated differential entry of the protein into the nucleus (58).

Perhaps the most convincing evidence establishing αNAC as a coactivator was the discovery of its interactions with transcription factors as well as the basic transcriptional machinery. Transient transfection assays using the 5Ga14-E1b-CAT reporter plasmid showed that full-length αNAC, also referred to as clone 1.9.2, enhanced the transcriptional response mediated by the chimeric activator GAL4/VP16 by 10-fold (58). The enhancement was also seen at the mRNA level of the reporter gene, identifying the potentiation by αNAC to have occurred at the transcriptional level. Furthermore, αNAC was also shown to enhance transcription mediated by the c-Jun homodimer (59). AP-1 family members were utilized to identify potential interactions with αNAC. Transient transfection assays revealed that the c-Jun homodimer stimulation of the luciferase reporter gene under the control of an AP-1 binding site was potentiated by 9-fold once αNAC was present (59). However, αNAC could not enhance the transcriptional response of the c-Fos-c-Jun heterodimer. Further investigations by means of protein pulldown assays confirmed an interaction between αNAC and c-Jun. Specifically, αNAC was shown to interact with the N-terminus of c-Jun at amino acids 1-89 and had the ability to bind both non-phosphorylated and JNK-phosphorylated c-Jun (59). Affinity chromatography of crude nuclear extracts from serum-stimulated osteoblasts on a glutathione sepharose column loaded with recombinant GST-αNAC fusion protein, as well as immunoblotting with the anti-phospho-serine 73 antibody displayed a strong interaction between αNAC and phospho-c-Jun. Phospho-c-Jun, having been retained on the column by interacting with αNAC, was eluted with 0.5 M salt (59).

Mammalian two-hybrid assays confirmed that αNAC interacts with c-Jun *in vivo* (59). Full-length αNAC was linked to the yeast GAL4-DNA-binding domain and did not significantly influence the expression of the GAL4-dependent reporter gene, stating that αNAC cannot potentiate transcription without a transcriptional activator being present. However, co-transfection of c-jun and GAL4-αNAC stimulated expression of the reporter gene. Further experiments with several c-Jun mutants confirmed the binding of αNAC to amino acids 1-89 of the c-Jun protein that was initially revealed by protein pulldown assays (59). More recently, results in the lab have identified PIN/ATF4 as another target for αNAC (data not published).

By performing affinity chromatography of crude nuclear extracts on glutathione sepharose columns loaded with the GST-αNAC fusion protein, TBP, a general transcription factor, was also identified as an αNAC interaction partner (58). Figure 3 depicts a model of the αNAC potentiation of c-Jun-activated transcription. The upper panel demonstrates the c-Jun basic leucine-zipper homodimer binding to the AP-1 consensus site. Similarly to αNAC, c-Jun can also interact with TBP but the binding is less strong (81). Basal-level transcription occurs in the absence of αNAC. However, once αNAC is recruited to the complex, it binds c-Jun and TBP, thereby stabilizing the complex onto AP-1 and strengthening the contact between the two proteins. Consequently, αNAC potentiates the transcriptional response of c-Jun-activated genes by increasing the rate of transcription (59).

The sequence and structure of αNAC have revealed further evidence identifying αNAC as a component of transcription. By means of computational analysis, sequence similarities between αNAC and transcriptional regulatory proteins have been found as

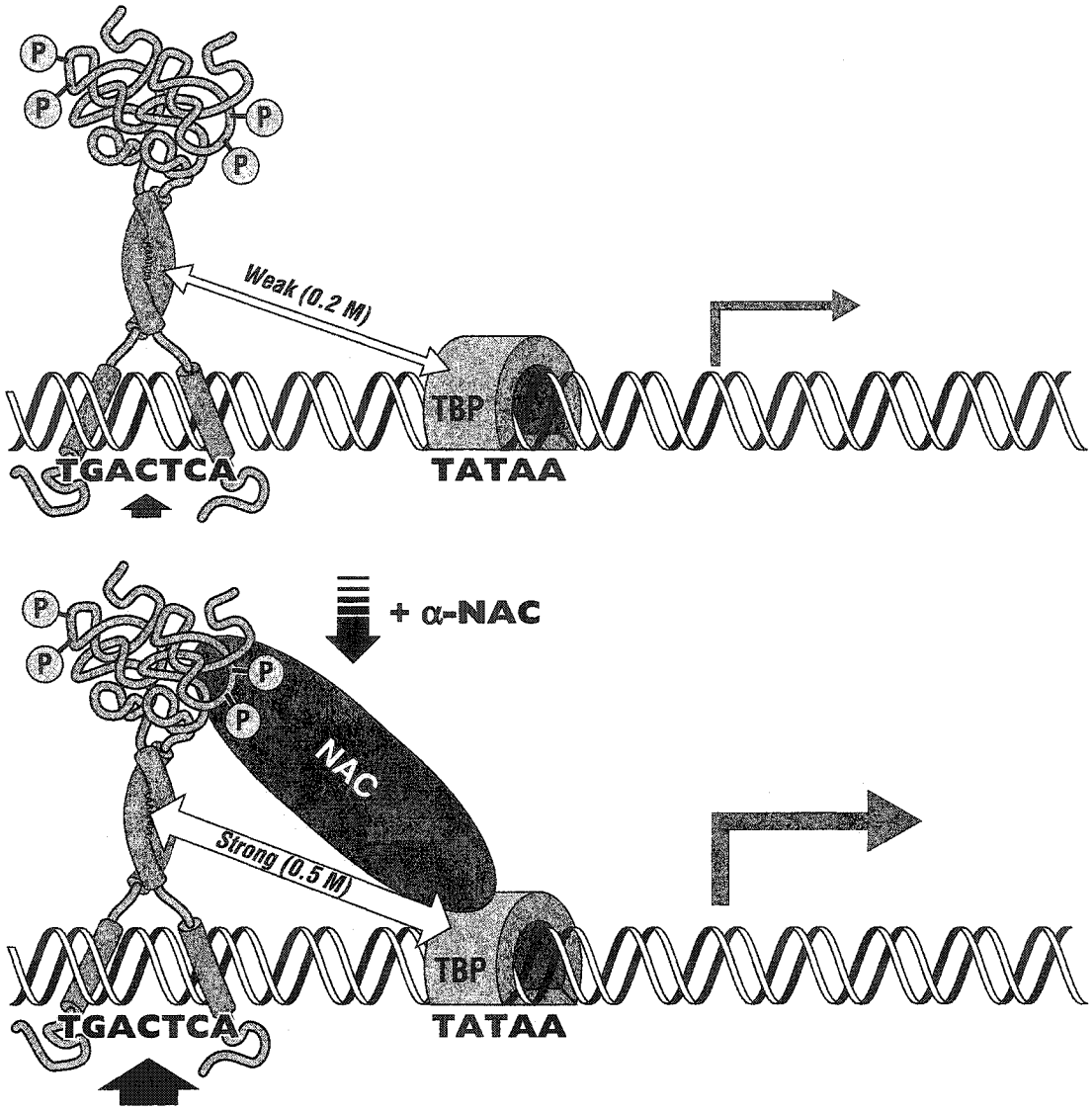

FIGURE 3 : Model of the αNAC potentiation of c-Jun-activated transcription. (Upper panel) The c-Jun homodimer binds the AP-1 site and interacts weakly with TATA-Binding Protein (TBP). (Lower panel) The αNAC coactivator interacts with the N-terminal domain of c-Jun, stabilizing the complex on the AP-1 site. αNAC also binds TBP strongly and strengthens the contacts between c-Jun and the basal transcriptional machinery. Taken from Moreau, A., Yotov, W. V., Glorieux, F. H., and St-Arnaud, R. (1998). *Mol. Cel. Biol.* **18**(3), 1312-1321.

well as several putative serine and threonine phosphorylation sites. Several domains have been delineated on αNAC. An acidic domain is located in the N-terminal end of the protein as well as a glutamine/alanine (Q/A) rich domain. Further down the sequence in the central part of αNAC lies a calcium-binding motif referred to as EF-hand. Although structure analysis has confirmed known functions of αNAC, an important domain remains to be determined. αNAC has been shown to interact with c-Jun in the N-terminal domain of the protein (59). However, the region on αNAC that interacts with c-Jun has not been identified.

Other coactivators have been shown to interact with AP-1 family members and more specifically, c-Jun. Similar to αNAC, Jun Activation domain-Binding protein 1 (JAB1) is a coactivator that has been shown to bind c-Jun in its N-terminal activation end (60). Interactions of both coactivators with c-Jun lead to stability of the homodimer on the AP-1 consensus site. αNAC and JAB1 can interact with both the JNK-phosphorylated and non-phosphorylated forms of c-Jun. The coactivator Thyroid hormone Receptor-Binding Protein/Activating Signal Cointegrator-2 (TRBP/ASC-2) specifically interacts with c-Jun, where the c-Jun-binding domain is located in its N-terminal end (61). Unlike αNAC, TRBP/ASC-2 can also interact with c-Fos and coactivators Steroid Receptor Coactivator-1 (SRC-1) and CREB-binding protein (CBP). CBP is yet another example of an AP-1 coactivator which shares similarities with αNAC. Studies have demonstrated that the interaction of CBP with c-Jun occurs in the N-terminal activation domain (62). CBP can also interact with the basic transcriptional machinery protein TBP (62). Although CBP potentiates c-Jun-activated transcription, it can also enhance the transcriptional activity of the c-Fos/c-Jun heterodimer and

possesses an intrinsic HAT activity (63), which differentiates this coactivator from αNAC.

In order to increase the specificity of transcriptional responses, the activity of coactivators is regulated by means of post-translational modifications and DNA binding. Similarly to the activation of transcription factors, coactivators can also be recruited and activated via phosphorylation. CBP and its homolog p300 are large nuclear proteins which require phosphorylation of its signal-regulated domain by calcium/calmodulin-dependent protein kinase IV in the presence of calcium for stimulating transcription (64,65). CBP interacts with the transcription factor CREB, and can also bind to c-Jun. The interaction involves the N-terminus of c-Jun and the CREB-binding domain of CBP (63). Specificity of gene transcription can also be mediated by DNA-binding activity. One example is the B cell-specific coactivator Bob-1, which binds to members of the POU family of transcription factors, namely Oct-1 and Oct-2 (66). Because of the sequence-specific DNA-binding activity of Bob-1, the ternary complex DNA-Oct-Bob-1 is restricted to particular promoters (67,68), thus establishing another role for coactivators.

The coactivation of αNAC may also be regulated by phosphorylation and DNA binding. It has been demonstrated that αNAC binds DNA (80), although it is not yet known whether this function is required for maximal coactivating activity. However, several protein kinases have been shown to target αNAC. Protein Kinase C (PKC) phosphorylates αNAC on serine 72 whereas the putative GSK-3 phosphoacceptor site is localized to position 159 (data not published). Casein Kinase II (CKII) phosphorylates αNAC on residues 25, 27, 29 and 34 (Quélo, I. and St-Arnaud, R., submitted). αNAC is

phosphorylated by yet another protein kinase. By means of yeast two-hybrid assays, an interaction between αNAC and Integrin-Linked Kinase (ILK) was identified (data not published). Moreover, *in vitro* kinase assays performed with recombinant αNAC, recombinant ILK and γ-[^{32}P]-ATP have shown that αNAC is indeed a substrate of ILK.

The ILK kinase interacts with the β chain of integrins and is therefore involved in the integrin signalling pathway (45-47). Amongst several proteins, c-Jun is a downstream effector of ILK-mediated signal transduction (49). ILK has been shown to stimulate c-Jun AP-1 transcription by negatively regulating GSK-3 (49). αNAC has also proven to be a substrate of both GSK-3β and ILK, and interacts with c-Jun in the N-terminal activation domain of the protein (59). Given that the phosphorylation of αNAC by ILK leads to the modulation of c-Jun transcriptional activity, αNAC is proposed to be a factor in the regulation of bone development.

Although αNAC potentiates c-Jun transactivation and has been shown to be a target of ILK, the effect that ILK-phosphorylation has on αNAC-c-Jun interactions remains to be determined. Moreover, the c-Jun interaction domain on αNAC has not been established and requires further investigation in order to delineate the region where c-Jun contacts αNAC.

HYPOTHESIS AND SPECIFIC AIMS

The phosphorylation of αNAC by ILK leads to a specific interaction with the phosphorylated form of c-Jun and consequently, the potentiation of the AP-1 transcriptional response.

Aim 1: Determine the effect of αNAC phosphorylation by ILK on the αNAC-c-Jun interactions

Aim 2: Localize the region on αNAC which interacts with c-Jun

MATERIALS AND METHODS

In-vitro Kinase Assays

GST-c-Jun (1-89) fused to glutathione sepharose beads (SAPK/JNK assay kit, New England Biolabs-NEB-, Mississauga, ON) was phosphorylated using c-Jun N-terminal Kinase (JNK), which was purchased from Stratagene (La Jolla, CA). At 30°C, 1 μg of GST-c-Jun (1-89) fusion protein beads, 0.8 μg of JNK and 100 μM of ATP were incubated in JNK reaction buffer (250 mM HEPES (pH 7.5), 100 mM $C_4H_6MgO_4$, 500 μM ATP) for 2 hours.

Full-length recombinant proteins αNAC and the negative control MaBP were produced using the Impact purification system (NEB; see protein purification) while His-ILK was produced using the Ni-NTA metal-chelating purification system by Qiagen (Mississauga, ON) according to the manufacturer's protocol. For 30 min, 2 μg of αNAC or MaBP, 0.2 μg of ILK and 5 μCi of γ-[^{32}P]-ATP (Amersham Pharmacia Biotech, Baie d'Urfé, QC) were incubated in ILK reaction buffer (50 mM HEPES (pH 7.0), 10 mM $MnCl_2$, 10 mM $MgCl_2$, 2 mM NaF) at 30°C in a total volume of 20 μl.

Protein Pulldown Assays

Phosphorylated and non-phosphorylated forms of both αNAC and GST-c-Jun were mixed together in varying ratios in 450 µl of binding buffer (250 mM NaCl, 50 mM HEPES (pH 7.5), 0.5 mM EDTA, 0.1% (vol/vol) Nonidet P-40, 0.2 mM PMSF, 1 mM DTT, 100 µg/ml BSA). Overnight incubation was performed at 4°C with gentle rocking. The negative control MaBP was also incubated with GST-c-Jun. After four washes in binding buffer (4°C, 2 x 500 µl, 2 x 250 µl), bound proteins recovered by centrifugation of the beads at 12 000 rpm for 30 sec were eluted by boiling for 5 min in SDS sample buffer and resolved by 12% SDS-PAGE. The gel was then dried and autoradiography with Kodak X-OMAT AR film (Kodak, Rochester, NY) was performed for 48 h at -80°C.

Protein Expression and Purification

The system utilized to purify recombinant αNAC and MaBP proteins was the Impact T7 system from New England Biolabs (figure 4). The gene of interest is inserted in frame with the N-terminus (Cys1) of the intein gene, which in turn is bound to chitin-binding domain (CBD) in the pTYB2 vector. The intein incorporates an asparagine to alanine substitution at codon 454 in its C-terminus, which blocks the splicing reaction between the intein and the chitin-binding domain. An N-S acyl rearrangement at Cys1

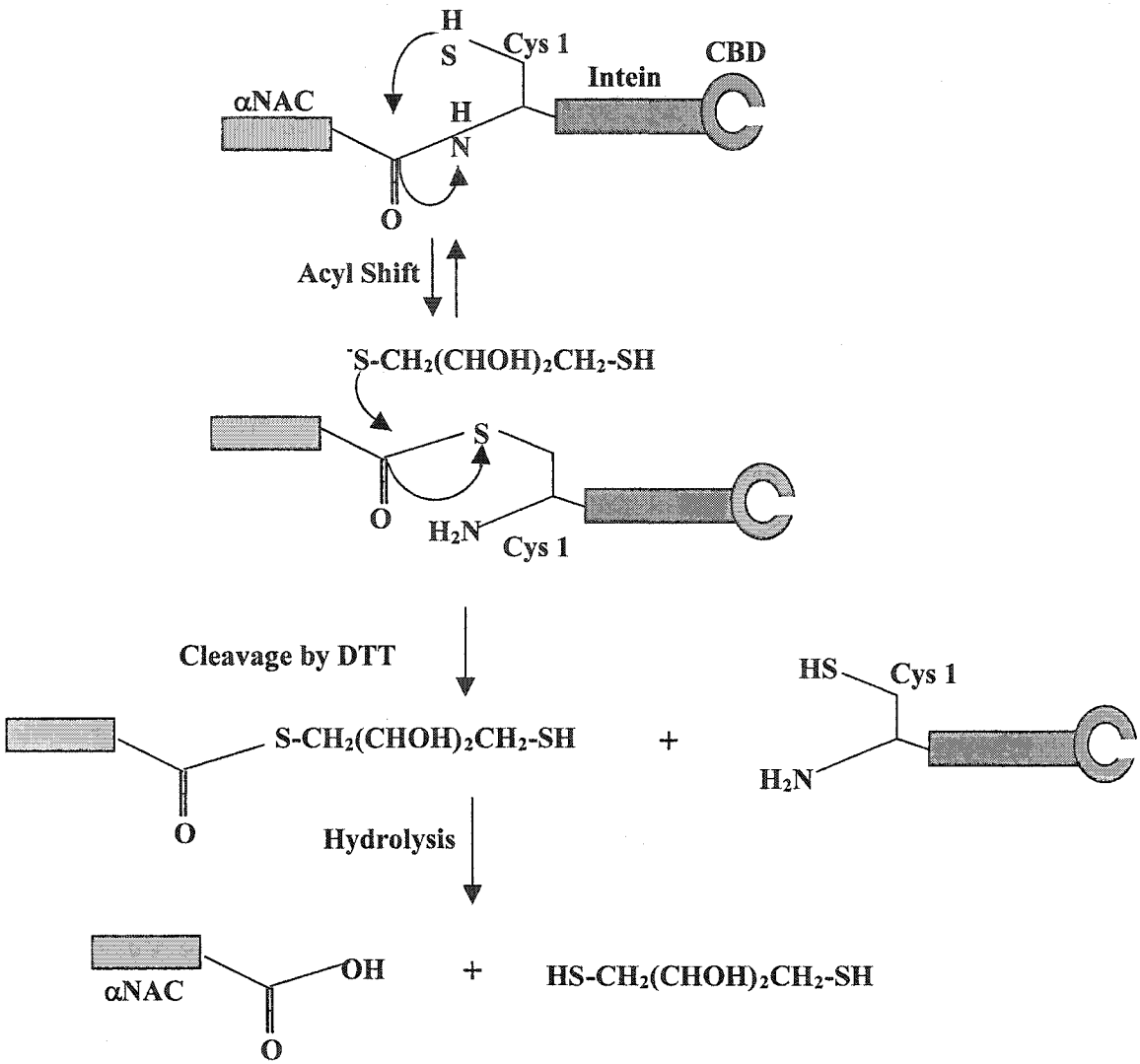

FIGURE 4 : Cleavage reaction induced by 1, 4-DiThioThreitol (DTT) in the Impact T7 protein purification system from New England Biolabs (NEB). Cys1, cysteine at the N-terminus of the intein; CBD, Chitin-Binding Domain. Modified from the instruction manual provided by NEB.

of the intein occurs, creating a thioester bond between the intein and the target protein. Exposure to 1, 4-dithiothreitol (DTT) cleaves this bond and releases the protein of interest once the newly formed non-stable thioester bond between the thiol compound and the protein is hydrolyzed. One cysteine residue remains at the C-terminus of αNAC after cleavage.

The fusion gene was inserted into *E. coli* ER2566 bacterial cells by electroporation, in a 1 mm cuvette (see plasmid preparations for protocol) and plated onto Luria Bertoni medium (LB agar: (pH 7.0) 10 g peptone, 5 g yeast extract, 10 g NaCl, 7.5 g agar) agar plates supplemented with ampicillin (100 μg/ml) and left to grow overnight at 37°C (figure 5). One colony was chosen and grown in 5 ml of LB medium in the presence of ampicillin for 16 h. Two hundred ml of LB + ampicillin were then inoculated with the mixture of bacteria incorporating the fusion gene and the culture was incubated at 37°C to an A_{600} of 0.5-0.6. The fusion protein was induced with 0.3 mM IPTG at 30°C for 3 hours followed by cell harvesting for 10 min at 6500 rpm, at 4°C. The cell pellet was resuspended in 5 ml of column buffer (20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 0.1 mM EDTA, 0.1% Triton x-100) in presence of anti-proteases (1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin A and 1 mM PMSF), lysed by sonication and clarified by centrifugation at 10 000 rpm for 30 min at 4°C. One ml of chitin beads was loaded into a column (Econocolumn, Bio-Rad), and the beads were washed with 10 ml of column buffer. The cell extract was then poured into the chitin column and washed three times with 10 ml of column buffer. The column was quickly flushed with 6 ml of cleavage buffer (20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.1 mM EDTA, 30 mM DTT freshly diluted), plugged and left at 4°C overnight. The protein of

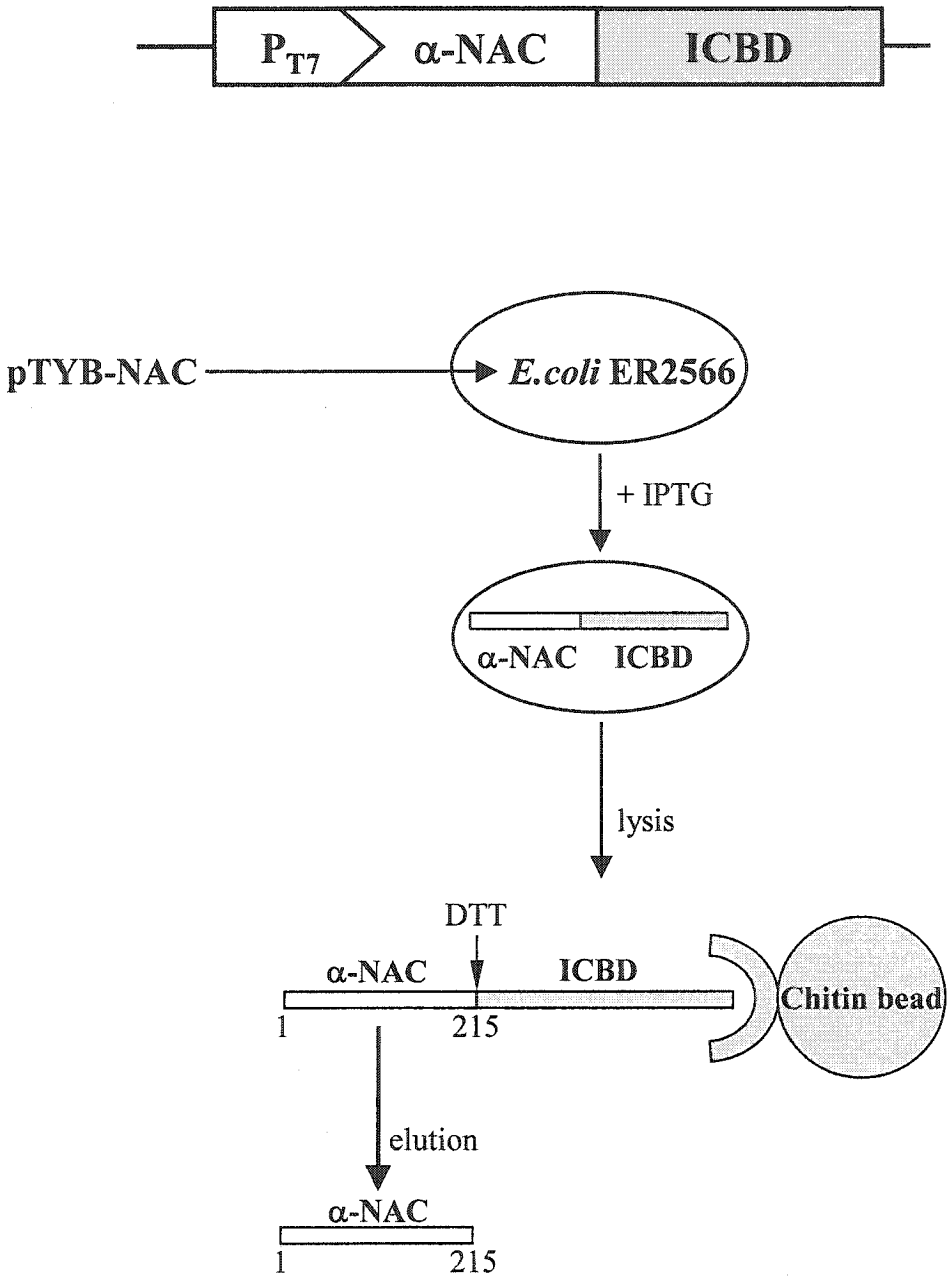

FIGURE 5 : Schematic diagram of the steps describing the Impact T7 protein purification system from NEB The addition of DTT leads to self-cleavage of the fusion protein, allowing to purify the recombinant target protein (αNAC) devoid of any fusion moiety. ICBD, Intein Chitin-Binding Domain; P_{T7}, T7 promoter driving the αNAC-ICBD fusion gene; IPTG, IsoPropyl –Thio-β-D-Galactopyranoside; DTT, 1, 4-DiThioThreitol.

interest, cleaved from its ICBD partner, was eluted using additional cleavage buffer without DTT in 4 x 1 ml fractions (figure 5). Centricon centrifugal concentrators from Millipore (Bedford, MA) with a cut-off of 10 kDa were used to enrich the first fraction of the protein sample at a concentration of 0.5 μg/μl. The approach of this system involves centrifugal force to drive the solvent and low molecular weight solutes from the sample through the hydrophilic membrane, while retaining the protein of interest. The protein elution was added to the sample reservoir and centrifuged at 6500 rpm for 1 h at RT. The sample reservoir was then reversed and the retentate vial added before eluting the concentrated solute by centrifuging at 3000 rpm for 3 min. The proteins were snap frozen in a dry-ice/ethanol bath and kept at -80°C.

L-[^{35}S]-Cysteine Labelling

In the case of αNAC, initial experiments incorporated covalent labelling with L-[^{35}S]-cysteine at its C-terminus before concentrating the protein. This was accomplished by the formation of a thioester bond between DTT and the C-terminal residue of αNAC. The thioester is not stable and therefore hydrolyzes to yield a free C-terminus in the protein. The addition of radiolabelled cysteine forms a thioester bond with αNAC that is followed by a spontaneous S-N shift leading to the formation of a peptide bond. As previously described, αNAC was produced and purified using the Impact T7 purification system. αNAC was released from its ICBD moiety by overnight exposure to DTT. Immediately following elution, 900 μl of αNAC and 1.1 mCi of L-[^{35}S]-cysteine were

incubated at 4°C overnight. This led to the incorporation of a radiolabelled cysteine residue at the αNAC terminus. The protein sample was then enriched using centricon concentrators that also served to remove traces of free L-[^{35}S]-cysteine.

Cell Culture

C_2C_{12}, a pluripotent mesenchymal precursor cell line (82), was maintained in high glucose DMEM (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS: Cansera, lot #SF70926, Rexdale, ON) at 37°C in 5% CO_2. Upon confluency, the cells were washed twice with 4 ml of Dulbecco's 1X PBS (2.68 mM KCl, 1.47 mM KH_2PO_4, 137 mM NaCl, 8.06 mM $Na_2HPO_4.7H_2O$), and were incubated with 1 ml of 0.05% trypsin-EDTA (Gibco BRL) at 37°C for no longer than 5 min. Once the cells were detached from the p100 dish, the trypsin was inactivated by adding 4 ml of serum-containing DMEM. The cells were triturated 20 times in order to break up the clumps and 10 µl of cells were deposited on a haemocytometer for counting. Only cells within the limitations of the haemocytometer and those having maintained their rounded appearance were counted. The concentration was calculated and 5.0 x 10^5 cells were seeded per p100 plate in a total volume of 10 ml of serum containing DMEM.

Cell lines including C3H10$T_{1/2}$ murine pluripotent mesenchymal cells (84), C1 osteogenic mouse teratocarcinoma-derived cells (69) and COS-7 African green monkey kidney cells (83) were also maintained in culture in a manner similar to what was previously described. C1 and COS-7 cells were grown in high and low glucose DMEM,

respectively, supplemented with 10% FBS while C3H10$T_{1/2}$ cells were maintained in Basal Medium Eagle (BME) culture media (both media purchased from Gibco BRL) + FBS at 37°C and 5% CO_2. All three cell lines were harvested using 0.25% trypsin (Gibco BRL).

Murine undifferentiated pluripotent Embryonic Stem (ES) cells were grown in high glucose DMEM complete medium (1% glutamax, 1% penicillin-streptomycin (pen-strep), 1% non-essential amino acids, 1% leukemia inhibitory factor (LIF), β-mercaptoethanol (βME: 0.7 μl/100 ml), 15% FBS) at 37°C in 5% CO_2. ES cells require LIF as well as feeder layers of irradiated fibroblasts to be able to grow and self-renew in an undifferentiated state *in-vitro* without losing pluripotency.

Feeder layers were made from Mouse Embryonic Fibroblasts (MEFs). A pregnant female mouse that had mated with the CYP24 male knockout mouse expressing the neomycin selection gene in all tissues (85) was sacrificed on day 14 of gestation by cervical dislocation and sterilized in ethanol before undergoing a caesarean section. The uterine horn was transferred to a p100 plate where the embryos were removed and placed into a fresh dish. The heads of the embryos were removed as well as the internal organs and the carcasses were placed into a new plate containing 10 ml of PBS. Once rinsed, each carcass was transferred to its individual dish containing 5 ml of 0.25% trypsin, minced into fine bits and incubated at 37°C for 30 min. The tissues were homogenized by vigorous pipetting after which 5 ml of high glucose DMEM with 10% FBS and 1% pen-strep were added. The cells were centrifuged at 1000 rpm for 5 min, the pellets were resuspended in medium and each resuspension was plated into three p100 dishes. Approximately 48 h later, 6 of the confluent plates were trypsinized and

frozen down at -80°C in DMEM with pen-strep/25% FBS/10% DMSO while the remaining 3 dishes were harvested in trypsin and each split into 6 x p100 dishes. The confluent cells were plated onto 72 plates in total and allowed to grow to confluency after which the medium was aspirated and replaced with 6 ml of DMEM/10% FBS supplemented with mitomycin C (10 μg/ml) to mitotically inactivate them. The cells were incubated for 2 to 3 h at 37°C in 5% CO_2 followed by 3 x 5 ml washes with serum-free DMEM. After trypsinizing and inactivating the trypsin with DMEM supplemented with 10% FBS and pen-strep, the cells were centrifuged at 1000 rpm for 5 min and resuspended in DMEM with pen-strep/25% FBS/10% DMSO for freezing. As 1 x p100 dish of confluent cells was placed in 1 cryovial, the procedure allowed for 72 vials of feeder layers to be stored and used for future maintenance of ES cells.

Feeder layers were plated at a concentration of 9 x 10^5 cells the day prior to splitting the ES cells on p60 dishes that were coated with 0.1% (w/v) gelatin for 30 min. Once the ES cells were bathed in 0.5 ml of 0.25% trypsin for 5 min, the harvested cells were added to 10 ml of complete medium, triturated and counted using a haemocytometer. The ES cells were then plated onto the feeder layers at a concentration of 1 x 10^5 to 4 x 10^5 cells and incubated at 37°C in CO_2.

Plasmid Preparations

Escherichia coli (*E. coli*) XL_1 cells were transformed using electroporation for the purpose of purifying plasmids. The bacterial cells were thawed and placed on ice after which 35 μl of bacteria were placed in a pre-chilled micro-centrifuge tube. One μl

or less of a specific plasmid having a concentration of 50 ng/µl or more was mixed with the bacterial cells and then placed in the electroporation cuvette, ensuring that all air bubbles were removed. Once in the *E.coli* pulser (Bio-Rad, Mississauga, ON), exponential pulses of 1.8 kV were delivered to the cells in order to allow the DNA to pass through the plasma membrane and enter the cells. The transformed cells were then incubated in 1 ml of LB medium for 1 h at 37°C and 200 µl of the transfected bacteria were plated on LB agar plates supplemented with ampicillin (100 µg/ml) and incubated at 37°C for 16 h. One colony on the LB agar plate was chosen and placed in 3 ml of LB medium with ampicillin at 37°C and shaking for 8 h. Two hundred µl of the culture were then incubated in 40 ml of LB with ampicillin at 37°C for 16 h with shaking, after which midi-preparations of the plasmid were performed using the Qiagen plasmid purification kit (Qiagen Inc, Mississauga, ON). The bacterial cells were harvested by centrifugation at 6000 rpm for 15 min at 4°C and the pellet was resuspended in 4 ml of resuspension buffer P1 (50 mM Tris-Cl (pH 8.0), 10 mM EDTA, 100µg/ml Rnase A). Four ml of lysis buffer P2 (200 mM NaOH, 1% SDS (w/v)) were added and the solution was mixed gently and incubated at RT for 5 min. Neutralization of the lysis buffer was achieved by adding 4 ml of chilled neutralization buffer P3 (3.0 M $KC_2H_3O_2$ (pH 5.5)), followed by mixing and incubating the bacteria on ice for 15 min. The lysate was then centrifuged at 13 000 rpm for 30 min at 4°C and the supernatant was centrifuged once again for 15 min. Qiagen-tip 100 columns were equilibrated by flushing 4 ml of equilibration buffer QBT (750 mM NaCl, 50 mM MOPS (pH 7.0), 15% $CH_3CHOHCH_3$ (v/v), 0.15% Triton X-100 (v/v)) and the cleared supernatant was loaded onto the column. The Qiagen-tip was washed with 2 x 10 ml of wash buffer QC (1.0 M NaCl,

50 mM MOPS (pH 7.0), 15% $CH_3CHOHCH_3$ (v/v)) and the DNA was eluted with 5 ml of elution buffer QF (1.25 M NaCl, 50 mM MOPS (pH 8.5), 15% $CH_3CHOHCH_3$ (v/v)). In order to precipitate the DNA, 3.5 ml of RT isopropanol were added followed by centrifugation at 11 000 rpm for 30 min at 4°C. The DNA pellet was washed with 2 ml of RT 70% ethanol and centrifuged at 11 000 rpm for 10 min at 4°C. Once dry, the DNA pellet was resuspended thoroughly in 50 or 100 µl of TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA), depending on the amount of DNA recovered. The DNA concentration was determined by UV spectrophotometry and diluted with additional TE buffer to an appropriate concentration.

Transient Transfection Assays

The day following seeding of C_2C_{12} cells at 5.0 x 10^5 cells in p100 dishes, the expended medium was replaced with 8 ml of fresh high glucose DMEM supplemented with 10% FBS. A mixture of 750 µl of serum free medium, 4 µg of pCi-c-jun (full-length c-jun cDNA under the control of the CMV promoter) and 12 µl of Fugene 6 (Roche Molecular Biochemicals, Laval, QC) were added to the cells bathing in the fresh complete medium and left overnight at 37°C and 5% CO_2. The cellular extracts were harvested 24 h post-transfection by washing the cells twice with 2 ml of PBS, scraping the cells in 1 ml of the saline and then centrifuging at 1500 rpm for 5 min at 4°C. The supernatant was discarded and the cell pellet was resuspended in 250 µl x the number of

p100 plates of 0.2 M NaCl column buffer. Sonication was performed in order to burst the cells and recover the intracellular proteins.

Transient transfections were performed using other cell lines, including C3H10$T_{1/2}$ cells, COS-7 cells and ES cells. C3H10$T_{1/2}$ cells were plated in 6-well dishes the previous day at a density of 1.5 x 10^5 cells in BME culture medium supplemented with 10% FBS. In order to use the dual-luciferase reporter assay system by Promega (Madison, WI), the AP1-TK-luc reporter vector, in which one AP-1-binding site [5'-TGACTCA-3'] was subcloned upstream of the minimal herpes simplex virus-thymidine kinase (HSV-TK) promoter which drives the firefly luciferase gene, was utilized. To control for transfection efficiency variations, pRL-TK was chosen as the internal control and contains a cDNA (R*luc*) encoding the *Renilla* luciferase. Full-length c-jun and αNAC cDNAs driven by the cytomegalovirus (CMV) promoter as well as the phagemid vector pBS used to boost the amount of DNA to 3 μg were also utilized during the transient transfections. More specifically, 50 ng of pRL-TK, 100 ng of pAP1-TK-luc, 700 ng of pCi-cjun and 500 to 1000 ng of pBK-NAC/1.9.2 were mixed with 15 μl/well of genePORTER transfection reagent (Gene Therapy Systems, San Diego, CA) and incubated in 1 ml of serum-free BME at RT for 30 min. The culture medium was then aspirated from the cells and 1 ml of serum-free BME was added as well as the 1 ml of DNA/genePORTER complex and left at 37°C in 5% CO_2 for 5 h. In each well, 2 ml of BME containing 20% FBS were added to acquire normal culture conditions followed by incubation at 37°C in 5% CO_2 for 16 h. Results of the experiment were assayed using the Monolight 2010 Luminometer (Analytical Luminescence Laboratory, San Diego, CA).

ES cells were plated at a concentration of 4.0 x 10^5 cells in a total of 2 ml on feeder layers in 6-well dishes the day prior to transfection. The PathDetect *in vivo* signal transduction pathway trans-reporting system from Stratagene was utilized to support αNAC's coactivating role in the MAPK cascade. The system incorporates the activation domain of the transactivator protein c-Jun fused with the DNA-binding domain of the yeast GAL4 in the mammalian expression vector pFA-CMV (pFA2-cjun). The pFRluc reporter plasmid contains a synthetic promoter with five tandem repeats of the yeast GAL4-binding sites that control expression of the firefly luciferase gene and allow pFA2-cjun to bind and activate transcription of the luciferase gene. pSi-FlagαNAC, having a simian virus 40 (SV40) enhancer and early promoter driving the αNAC gene bound to the Flag epitope tag, was cotransfected with the reporter and transactivator plasmids in order to demonstrate enhanced transcriptional levels of the luciferase gene. Also involved were the negative controls for the pFA and pSi plasmids, pFC2-dbd and pSi-FLAG+2, so as to ensure the effects observed were not due to the GAL4-DNA-binding domain or the Flag epitope, respectively. A total of 4.05 μg of DNA broken down into 10 ng of pRL-TK, 1 μg of pFRluc, 0.5 ng of pFC2-dbd, 0.5 ng of pFA2-cjun, 1 μg of pSi-FLAG+2, 0.5 to 2 μg of pSi-FlagαNAC and pBS were mixed with Fugene-6 (2 μl Fugene-6 : 1 μg DNA) in DMEM without FBS, pen-strep and β-mercaptoethanol and incubated for 15 min. The expended medium in the 6-well dishes was replaced with 2 ml of medium without pen-strep and βME and the various DNA/Fugene-6 complexes were added to the cells for 24 h before undergoing a dual-luciferase reporter assay.

Affinity Chromatography

Cellular Extract Preparation

At 24 h post-transfection, the C_2C_{12} cells were washed twice with 1X PBS, harvested and pooled before undergoing centrifugation as previously described. The cell pellet was resuspended in column buffer having a concentration of 0.20 M NaCl, sonicated and centrifuged at 10 000 rpm for 30 min at 4°C in order to remove cellular debris. The cellular extracts were then pre-cleared into a 1 ml chitin column with the intention of eliminating proteins or other solutes that bind to the chitin beads. This clarified extract enriched with transfected full-length c-Jun was then used to interact with αNAC and its deletion mutants.

Column Preparation

Full-length recombinant αNAC and its C-terminal and N-terminal deletion mutants were produced using the Impact purification system as previously described. Once resuspended in cold 0.20 M NaCl column buffer, sonicated and centrifuged, 700 µl of the bacterial extract were loaded onto the 1 ml chitin column. The chitin-binding domain of the fusion protein bound to the chitin beads and was retained in the column. The column was then washed 3 times with 10ml of 0.20 M NaCl column buffer at 4°C.

Interaction

The interaction was achieved by adding 1 ml of c-Jun cellular extracts to the chitin-bound αNAC columns and placed at 4°C, gently rocking overnight. Bound

proteins were released from the column by adding 75 µl of 0.7 M NaCl cleavage buffer without DTT for one hour, followed by elution from the column.

SDS-PAGE and Western Immunoblotting

The eluted proteins were resolved on a 10% Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) and transferred to a hybond P PVDF membrane (Amersham Pharmacia Biotech, Piscataway, NJ) where the bands were detected by immunoblotting. The gel consisted of a 10% separating gel (10% acrylamide/0.267% bisacrylamide, 1X Tris-Cl/SDS (pH 8.8), 0.1% Ammonium PerSulfate (APS), 0.1% TEMED) and a 4% stacking gel (7.5% acrylamide/0.2% bisacrylamide, 1X Tris-Cl/SDS (pH 6.8), 0.1% APS, 0.1% TEMED). Twenty-two µl of sample proteins were mixed with 8 µl of 3X SDS sample buffer (187.5 mM Tris (pH 6.8), 6% SDS, 30% glycerol, 0.03% BromoPhenol Blue (BPB)) and loaded in the wells of the stacking gel. The gel was initially run at 100 V and then at 150 V once the bands entered the separating gel for a duration of approximately 1 h. The proteins were then transferred to a PVDF membrane by blotting at 100 V for 45 min in transfer buffer (39 mM glycine, 48 mM Tris, 0.037% SDS, 20% methanol). The PVDF membrane was soaked in 100% methanol for 10 sec, washed in H_2O for 5 min and equilibrated in transfer buffer for 10 min prior to blotting. The non-specific-binding sites on the membrane were blocked in a 5% skimmed milk 1X TBS (50 mM Tris-HCl, 150 mM NaCl (pH 7.4)) solution containing 0.2% Tween-20 (TBS-T) for 16 h. After 1 x 15 min and 2 x 10 min washes in TBS-T, the membrane was bathed in 4 ml of TBS-T

supplemented with an anti-phospho-c-Jun (ser63) antibody (SAPK/JNK assay kit, NEB) diluted to 1: 1000 for 1 h at RT with shaking. Once thoroughly washed with TBS-T for 1 x 15 min and 2 x 5 min, the blot was incubated with a goat anti-rabbit HRP-conjugated secondary antibody (Amersham Pharmacia Biotech) at a dilution of 1: 50 000 for 1 h at RT with shaking followed by three washes of 15 min and 1 of 5 min in TBS-T. The bands were detected by soaking the membrane in the ECL Plus western blotting reagents (Amersham Pharmacia Biotech) for 5 min at RT and exposed on hyperfilm ECL autoradiography film (Amersham Pharmacia Biotech) for duration of 5 sec to 5 min.

Modified Experimental Procedures

In 300 µl of chitin bead columns, 200 µl of αNAC or its deletion mutants were incubated overnight in cleavage buffer at 4°C. The eluted proteins were resolved by 10% SDS-PAGE and the bands were stained using Gel Code blue stain reagent according to the manufacturer's protocol (Pierce, Rockford, IL) (figure 13). The band representing full-length αNAC was assigned a value of 1 and all other bands were compared to it. According to their intensities, more or less volume of protein was used to perform the last affinity chromatography experiment. The volumes used were as follows: 0.5 ml chitin beads, 0.5 ml cellular extracts containing transfected c-Jun and 350 µl αNAC, 127 µl m4, 334 µl m2, 1.79 ml ΔEF-hand, 850 µl Δ12-69, 575 µl Δ46-61, 1.33 ml Δ4-45 or 350 µl ICBD. Once the proteins bound to αNAC or the deletions mutants were released using 0.7 M NaCl cleavage buffer without DTT for 1 h, a 10%

SDS-PAGE was performed as previously described. The proteins were then transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech) and western blotting was carried out as stated earlier. Detection of bands was accomplished using the Supersignal west femto maximum sensitivity substrate (Pierce). The blot was immersed in the working solution of the product for 5 min and exposed onto Hyperfilm ECL autoradiography film for 5 sec to 2 min.

Dual-Luciferase Reporter Assays

Promega's dual-luciferase reporter assay system incorporates simultaneous expression and measurement of two individual reporter enzymes, the experimental reporter displaying the effect of specific experimental conditions and the co-transfected control reporter providing an internal control. By normalizing the activity of the experimental reporter to the activity of the internal control, experimental variability caused by differences in cell viability and transfection efficiency can be minimized. Through bioluminescent reactions, the firefly luciferase within the experimental vector can be measured, and once quantified, the reaction is quenched in order to activate the glow-type reaction of the *Renilla* luciferase in the control plasmid. The internal control used in the various experiments performed was pRL-TK and the experimental reporter was pFRluc as previously described. The growth medium was removed from the C3H10$T_{1/2}$ cells or ES cells in the 6-well dishes, and 2 ml of PBS were added and removed before dispensing 500 µl of 1X passive lysis buffer (PLB). The culture plates were placed on a rocking platform and incubated at RT for 20 min. The lysate was then

recovered by thoroughly washing the adherent cells with the 500 µl of PLB/lysate solution and transferred to micro-centrifuge tubes. Prior to beginning the assay, the single-sample luminometer was warmed-up and 100 µl of luciferase assay reagent II (LAR II) was allotted in polypropylene tubes. Twenty µl of the cell lysate were added to the LAR II and mixed by pipetting 3 times before being placed in the luminometer. The program involved a 2 sec pre-measurement delay, followed by a 10 sec measurement period for each enzyme in the samples. Once the reading of the firefly luciferase activity from pFRluc was achieved and recorded, 100 µl of 'stop and glo' reagent were added manually, mixed thoroughly with a pipette, and replaced in the luminometer for measurement of the *Renilla* luciferase activity. The reaction tube was discarded and all other cell lysate samples were systematically analyzed in the same manner.

RESULTS

ILK phosphorylation increases the specificity of the interaction between αNAC and c-Jun

Protein pulldown assays utilizing c-Jun (1-89) protein fusion beads followed by SDS-PAGE demonstrate that c-Jun interacts with αNAC, once both proteins are phosphorylated by their respective kinases (figure 6). In this experiment, αNAC and its negative control MaBP were phosphorylated with γ-[^{32}P]-ATP by ILK in wells 1, 3, 4 and 6. The possible interactions between αNAC and c-Jun or phospho-c-Jun in wells 2 and 5 could not be detected, as the non-phosphorylated αNAC and c-Jun molecules were not labelled. The smear visible in lane 6 most likely results from non-specific interactions, as it was not observed in lane 3 as well as in other experiments (data not shown). It should be noted that the molecular size of MaBP is 40 kDa, and no band is visible at that position in the gel. The only visible band on the gel was in the lane demonstrating interaction between phospho-c-Jun and phospho-αNAC. Thus, once αNAC has been phosphorylated by ILK, it preferentially binds to the phosphorylated form of c-Jun. This result can also be seen in figure 7 (compare lanes 3 and 6). Attempting to eliminate the smearing pattern shown with MaBP, another control, Myelin Basic Protein (MyBP), was utilized. As well, labelling of αNAC with L-[^{35}S]-cysteine was performed in order to demonstrate the possible interactions between non-phosphorylated αNAC and phosphorylated or non-phosphorylated c-Jun. Two different

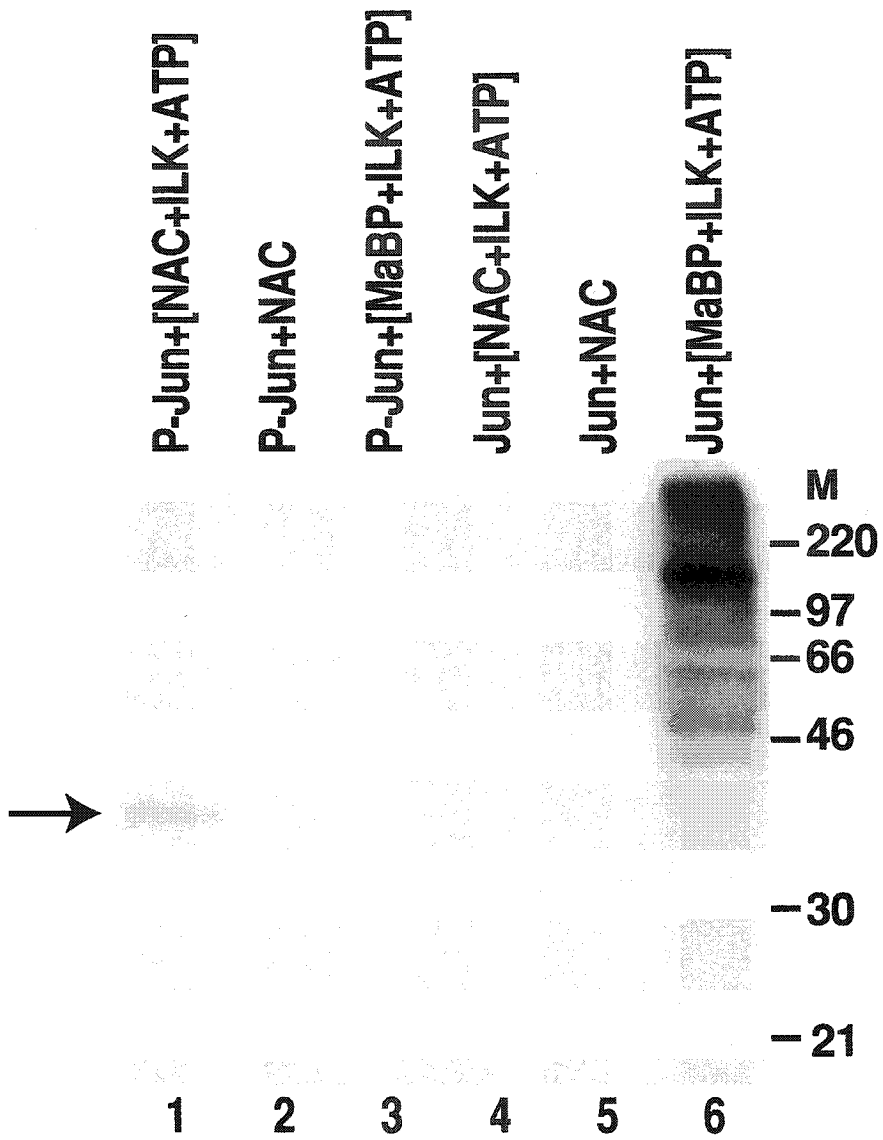

FIGURE 6 : Phosporylation of αNAC by ILK enhances the interaction with JNK-phosphorylated GST-c-Jun. 12% SDS-PAGE of GST-c-Jun (1-89) and αNAC protein pulldown assays. In lanes 1-3, GST-c-Jun was phosphorylated with ATP by its kinase Jun N-terminal Kinase (JNK). αNAC and the negative control Maltose-Binding Protein (MaBP) were phosphorylated by Integrin-Linked Kinase (ILK) with γ-[^{32}P]-ATP. Autoradiography was performed 48 h post-exposure at -80°C. The only visible αNAC signal was shown in lane 1 at 37 kDa, whereby αNAC phosphorylated by ILK interacted with phospho-c-Jun.

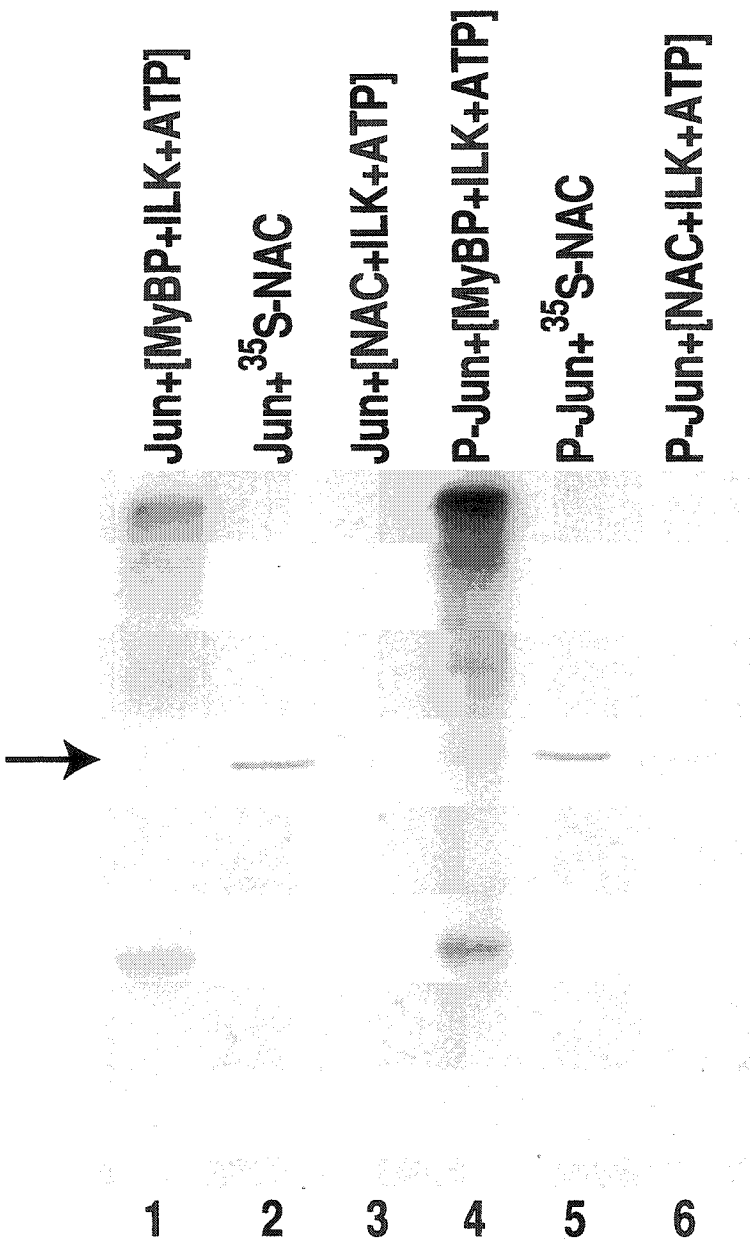

FIGURE 7 : Interactions of cysteine-labelled αNAC and ILK-phosphorylated αNAC with GST-c-Jun. 12% SDS-PAGE of GST-c-Jun (1-89) and αNAC protein pulldown assays. Procedures followed were similar to those explained in legend to figure 6. However, the negative control was changed to Myelin Basic Protein (MyBP) (lanes 1, 4) and αNAC was labelled with L-[^{35}S]-cysteine in lanes 2 and 5. After 48 h at -80°C, the film was autoradiographed. Once again, phosphorylation of αNAC by ILK signalled specificity with the phosphorylated form of c-Jun.

types of radioactivity, L-[^{35}S]-cysteine and γ-[^{32}P]-ATP, were used in this experiment and therefore the signals from both types could not be compared with one another. However, it was possible to compare interactions between reactions that utilized the same radioactive tracer. Once again, αNAC phosphorylated by ILK preferentially interacted with phospho-c-Jun, as seen in lane 6 but not in lane 3. In addition, αNAC bound with comparable affinity (lanes 2 and 5) to both c-Jun and phospho-c-Jun. MyBP proved to be a positive control, unlike MaBP. These interactions between αNAC and c-Jun support earlier results achieved in the lab whereby αNAC was shown to bind to both c-Jun and c-Jun phosphorylated by JNK (59) and reveals a novel interaction between αNAC phosphorylated by ILK and phospho-c-Jun. In summary, the results shown in figures 6 and 7 support the hypothesis that phosphorylation of αNAC by ILK leads to an enhanced interaction with the phosphorylated form of c-Jun.

c-Jun-Binding Domain in αNAC

Protein Pulldown Assays

As previously mentioned, αNAC interacts with c-Jun at its N-terminus, and more specifically within the first 89 amino acids (59). Conversely, c-Jun binds to αNAC. We next investigated the region on αNAC which makes contact with c-Jun. This was first attempted by utilizing L-[^{35}S]-cysteine-labelled C-terminal and N-terminal αNAC deletion mutants in protein pulldown assays. Deletions spanning the entire αNAC protein were produced by Dr. Isabelle Quélo from our laboratory, creating an array of

αNAC mutants (figure 8), which were used in the protein pulldown assays in order to locate the c-Jun-binding domain. We initiated the search by utilizing mutants having large deletions in the C-terminus of the αNAC protein. Figure 9 shows the interactions between c-Jun or phospho-c-Jun and the various αNAC mutants m1, m2, m4 and ΔEF-hand. All four αNAC deletion mutants made contact with c-Jun and phospho-c-Jun as shown in lanes 2, 3, 4 5 and 7, 8, 9, 10. These results suggested that the c-Jun-binding domain was not located between amino acids 89 and 215 and therefore would reside within the first 88 amino acids of αNAC. Consequently, N-terminal αNAC deletion mutants Δ4-45, Δ46-69 and Δ69-80 were then tested. As shown in figure 10, deleting amino acids 4 to 45, 46 to 69 and 69 to 80 had no effect on the interaction between the two proteins as all αNAC mutants bound to c-Jun. The observed results delineated the regions of interest from amino acids 1-3 and amino acids 81-88. Another αNAC mutant of 78 amino acids and having a serine to glycine substitution at position 70 was utilized in order to verify whether the c-Jun interaction domain on αNAC was located between amino acids 81-88. We also decided to test whether L-[^{35}S]-cysteine-αNAC could bind to the glutathione sepharose beads without the c-Jun moiety since it interacted with the c-Jun fusion protein in all deletion pulldown assays attempted to date. Figure 11 displays αNAC deletion mutant m4-S70G interacting with c-Jun and phospho-c-Jun. More importantly, the results showed an interaction between αNAC and the glutathione sepharose beads. These results were surprising since possible interactions between αNAC and GST-glutathione sepharose beads had been tested previously and shown to be negative (59).

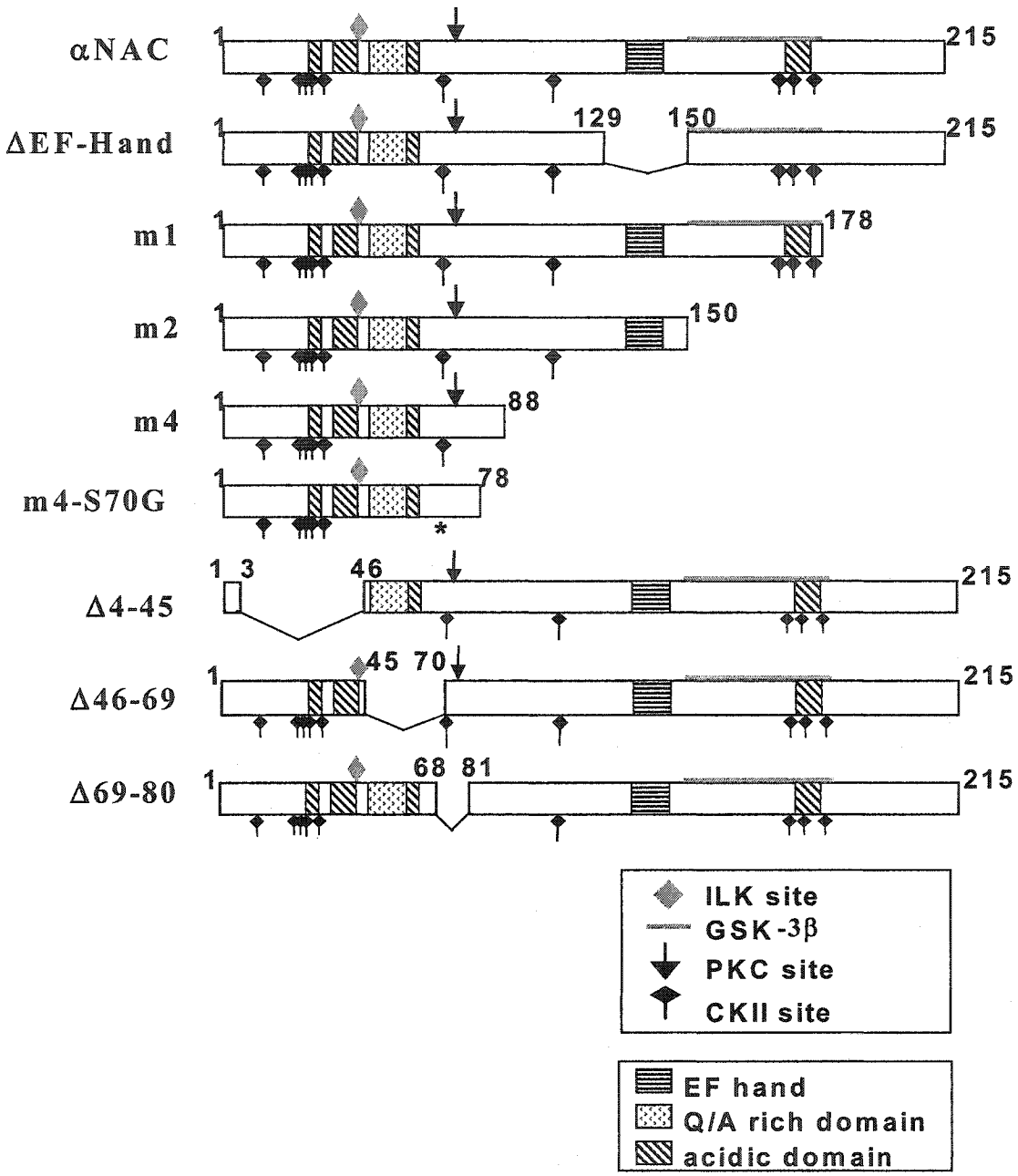

FIGURE 8 : Schematic diagram of the αNAC protein and its N-terminal and C-terminal deletion mutants. Integrin-Linked Kinase (ILK), Protein Kinase C (PKC) and Casein Kinase II (CKII) phosphorylation sites on αNAC are shown. The calcium-binding (EF-hand motif), glutamine and alanine rich (Q/A) and acidic domains are also delineated. m1, m2 and m3 mutants are C-terminal truncations. m4-S70G is 78 amino acids in length and has a serine to glycine substitution at position 70.

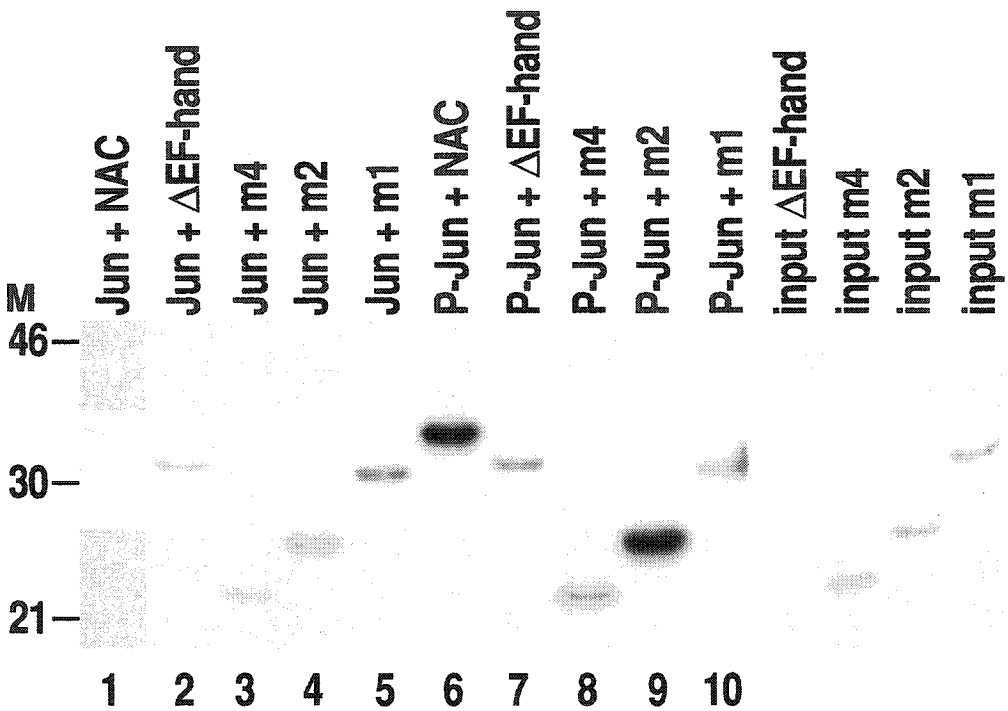

FIGURE 9 : Interactions of C-terminal cysteine-labelled αNAC deletion mutants with GST-c-Jun. 12% SDS-PAGE of GST-c-Jun (1-89) and L-[^{35}S]-cysteine-αNAC protein pulldown assays. Phosphorylated and non-phosphorylated forms of both αNAC and GST-c-Jun were mixed together in binding buffer, incubated overnight and αNAC deletion mutants were cleaved from the c-Jun columns thereafter. GST-c-Jun was phosphorylated with ATP by JNK in lanes 6-10. Inputs (1/800) serve as a control. Autoradiography was performed 72 h post-exposure at -80°C. Note that αNAC as well as all deletion mutants tested interacted with GST-c-Jun (lanes 1-5) and GST-phospho-c-Jun (lanes 6-10).

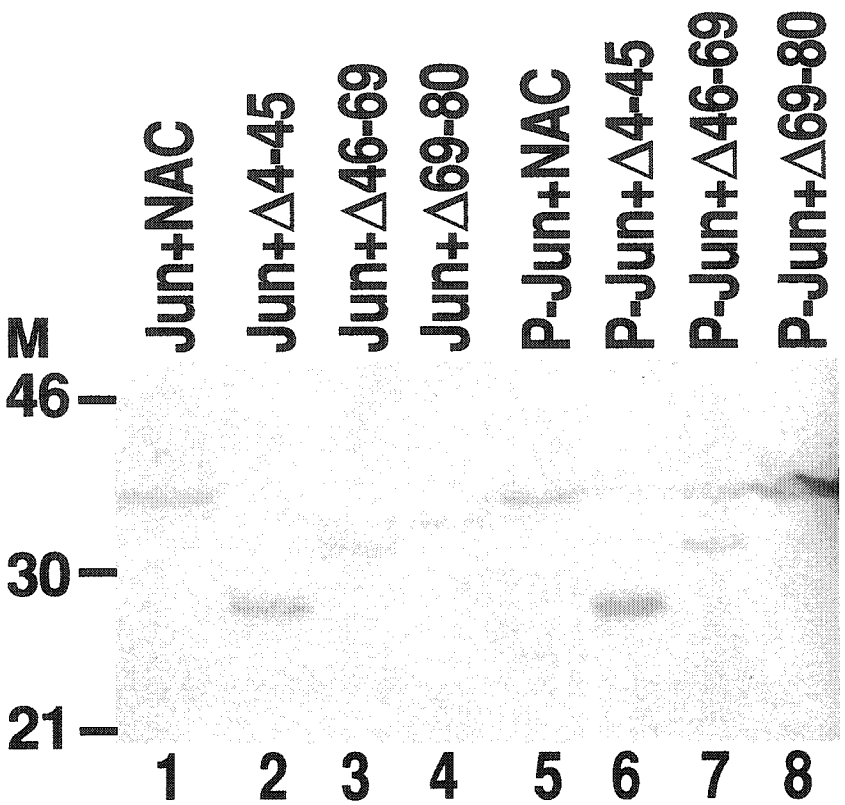

FIGURE 10 : Interactions of N-terminal cysteine-labelled αNAC deletion mutants with GST-c-Jun. 12% SDS-PAGE of GST-c-Jun (1-89) and L-[^{35}S]-cysteine-αNAC protein pulldown assays. Similar procedures to those explained in legend to figure 9 were performed. All N-terminal αNAC deletion mutants labelled with L-[^{35}S]-cysteine interacted with GST-c-Jun, as demonstrated in lanes 2-4 and 6-8, which places the c-Jun interaction domain of αNAC between amino acids 80-88 or amino acids 1-3.

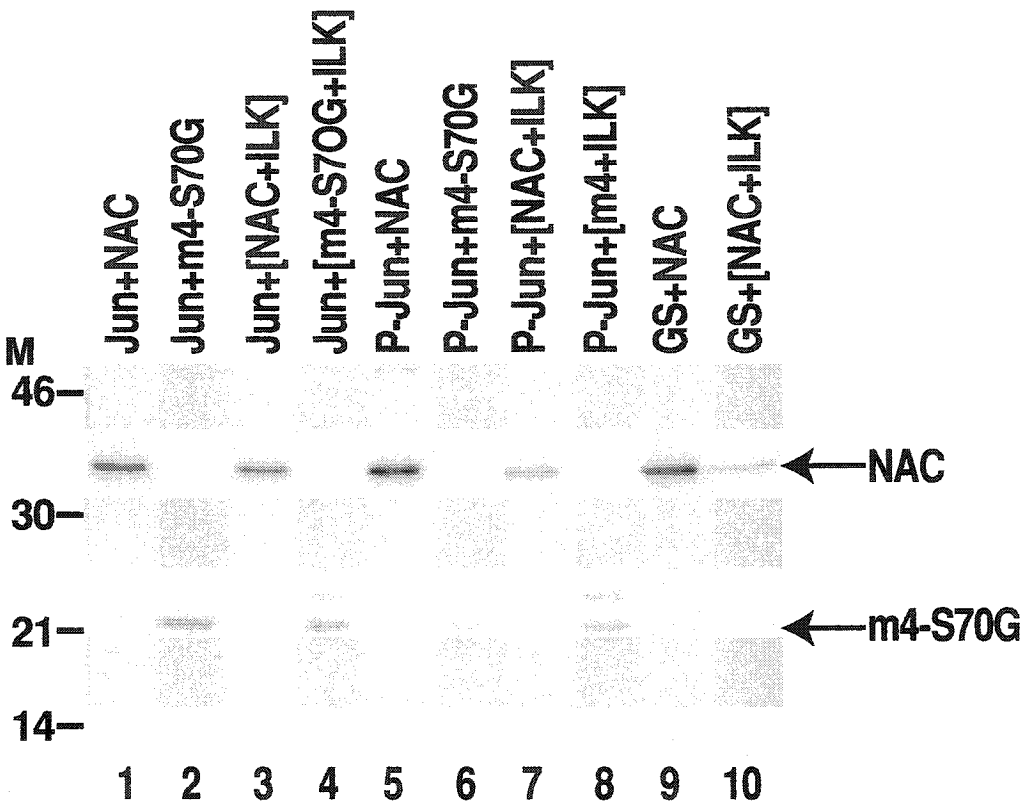

FIGURE 11 : Binding of cysteine-labelled αNAC with the glutathione sepharose beads. 12% SDS-PAGE of GST-c-Jun (1-89) and L-[^{35}S]-cysteine-αNAC protein pulldown assays, following procedures mentioned in legend to figure 9. The film was exposed at -80°C for 10 days. L-[^{35}S]-cysteine-αNAC interacted with the glutathione sepharose beads in the absence of GST-c-Jun (lanes 9, 10).

We conclude that the labelling of αNAC with L-[^{35}S]-cysteine modified the binding properties of αNAC such that it interacted non-specifically with the resin, precluding the use of GST pulldown assays to delineate the c-Jun interaction domain on the αNAC molecule.

Affinity Chromatography

Affinity chromatography was next used to eliminate L-[^{35}S]-cysteine labelling during protein-protein interaction assays. The αNAC-ICBD fusion protein and its deletion mutants were produced using the Impact protein purification system described in Methods and run on chitin columns. Cellular C_2C_{12} extracts containing transfected c-Jun were loaded on the αNAC chitin columns. The proteins retained on the column were eluted and analyzed by SDS-PAGE and western immunoblotting with an anti-phospho-c-Jun antibody. As seen in figure 12, c-Jun interacted with full-length αNAC (lane 5). As well, c-Jun bound to αNAC deletion mutants m2, m4 and Δ12-69. However, c-Jun was not retained in the control column containing the Intein Chitin-Binding Domain (ICBD) without the αNAC moiety (lane 1). To ensure that similar amounts of αNAC fusion protein were being retained in the chitin columns, an equal volume of αNAC and its deletion mutants were passed through chitin columns, cleaved from its ICBD moiety overnight, and eluted. The eluted proteins were separated on SDS-PAGE and the intensities of the different bands were compared. Amongst the differences displayed in figure 13, a greater concentration of the mutant m4 was retained in the column as compared to full-length αNAC. Despite the uneven loading, mutant m4 bound significantly less c-Jun than the other αNAC molecules (figure 12, lane 3),

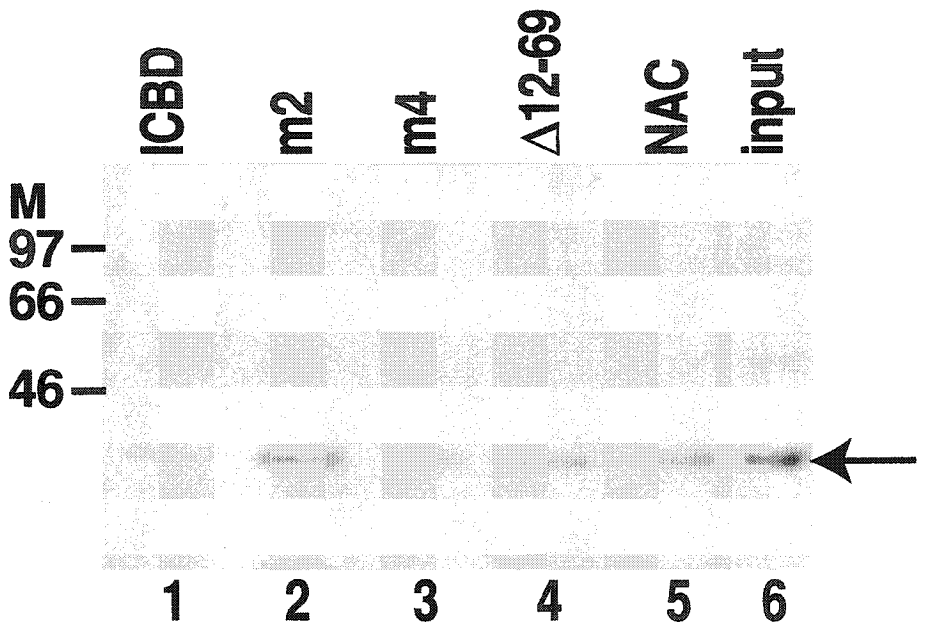

FIGURE 12 : 10% SDS-PAGE of affinity chromatography between αNAC chitin-bound columns and transfected full-length c-Jun. Intein Chitin Binding Domain (ICBD) was used as a negative control (lane 1). c-Jun was retained and therefore interacted with the αNAC deletion mutant columns to a similar or lesser extent (lanes 2-4) than with full-length αNAC.

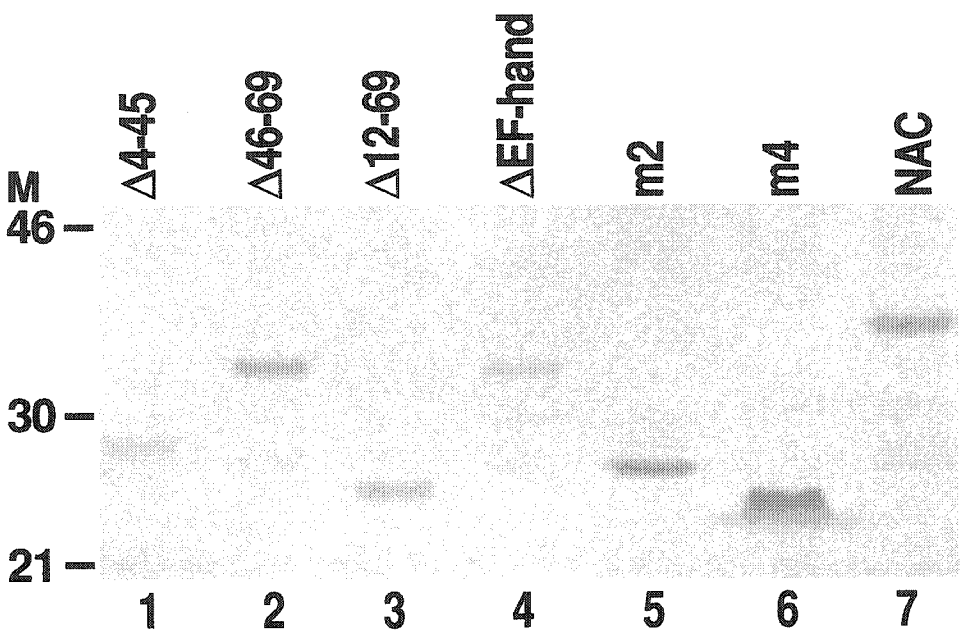

FIGURE 13 : Variable concentrations of αNAC and its deletion mutants retained in the chitin columns. 10% SDS-PAGE of αNAC cleaved from the chitin-bound columns. The proteins were revealed by Gel Code Blue Stain reagent. The concentration of αNAC and its deletion mutants retained in the chitin-bound columns were not identical and therefore required adjustment.

suggesting that mutant m4 had significantly reduced affinity for c-Jun. This was tested by performing a second affinity chromatography with adjusted volumes of protein, ensuring that equal concentrations of protein were loaded on each column. Figure 14 demonstrates interactions of c-Jun with αNAC and its deletion mutants. c-Jun interacted with full-length αNAC (lane 8) but not with ICBD alone (lane 1). Δ4-45, Δ46-69, Δ12-69 and ΔEF-hand (Δ130-149) interacted with c-Jun while m2 interacted to a lesser extent with the transcription factor. However, c-Jun was not retained in the column containing m4 (lane 7), suggesting that this shortened αNAC protein can no longer bind to c-Jun. ΔEF-hand, which is composed of amino acids 1-129 and 150-215 and m2 having amino acids 1-150 showed an interaction with c-Jun (lanes 5 and 6). As well, removing sections in the first 69 amino acids of the αNAC protein did not hinder binding (lanes 2, 3 and 4). However, once amino acids 89-129 were removed (mutant m4, figure 8), the αNAC protein did not interact with c-Jun, as seen in lane 7. From these results, we conclude that the c-Jun-binding domain on αNAC resides in its central part, between amino acids 89 to 129.

Potentiation of the AP-1 Transcriptional Activation by αNAC Coactivation

In order to demonstrate the coactivating role of αNAC on the AP-1 transcriptional response, several transient transfections were performed in a number of different cell lines, including C1 osteogenic mouse teratocarcinoma-derived cells, C3H10$T_{1/2}$ murine pluripotent mesenchymal cells, ES pluripotent mouse cells and

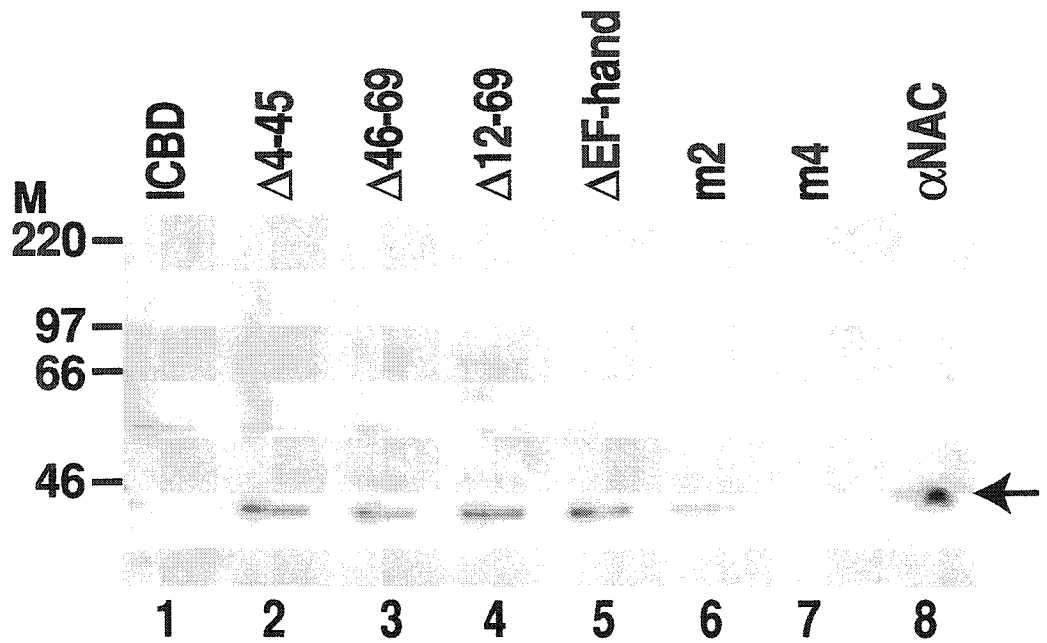

FIGURE 14 : Identification of the c-Jun interaction domain on αNAC. 10% SDS-PAGE of affinity chromatography between chitin-bound αNAC columns and transfected full-length c-Jun. The volumes of the αNAC deletion mutants were adjusted according to the results of figure 13 to ensure identical concentrations with the full-length αNAC protein. c-Jun was shown to interact with most αNAC deletion mutants (lanes 2-6). However, c-Jun did not interact with m4, a mutant composed of amino acids 1-88 (lane 7). Intein Chitin-Binding Domain (ICBD) was utilized as a negative control (lane 1).

COS-7 African green monkey kidney cells. The effect of αNAC was analyzed by performing dual-luciferase assays described in Methods. As αNAC is selectively expressed in developing bone during embryogenesis, we initially utilized the C1 osteogenic mouse teratocarcinoma-derived cell line (69) to show the coactivating function of αNAC. Full-length αNAC, together with c-Jun, the *Renilla* control luciferase and the firefly reporter luciferase were expressed into the C1 cells and a dual-luciferase assay was performed. pCi-cjun and pBK-NAC/1.9.2 are expression vectors for c-jun and αNAC, respectively, based on the CMV promoter. pAP1-TK-luc is the expression vector consisting of AP-1 response elements for the reporter gene. pRLTK is the expression vector for the *Renilla* luciferase, which controls efficiency of the transfection assays. As shown in figure 15, c-Jun potentiated the AP-1 transcriptional response to a 3.88 fold induction. Upon adding αNAC, expression of the luciferase protein was reduced slightly to a 2.76 induction and seemed to show a dose-dependent decrease. These results contrast with the previously published potentiation of c-Jun activity by αNAC (59). Several attempts using various conditions were not successful using the C1 cells. The experiment was thus attempted with a different cell line.

C3H10$T_{1/2}$ cells are a pluripotent mesenchymal line (84), which can differentiate into bone tissue but have not undergone the many internal mutational changes that have occurred in the previously utilized carcinoma line. It is also the cell line in which the coactivation of αNAC was established earlier in the lab. Figure 16 shows that transcription mediated by c-Jun occurred (lane 2) with a fold induction of 3.21, however, the addition of αNAC reduced the potentiation to below the arbitrarily ascribed AP1-TK-luc reporter value of 1 (lanes 3, 4 and 5). This result was not what we had expected

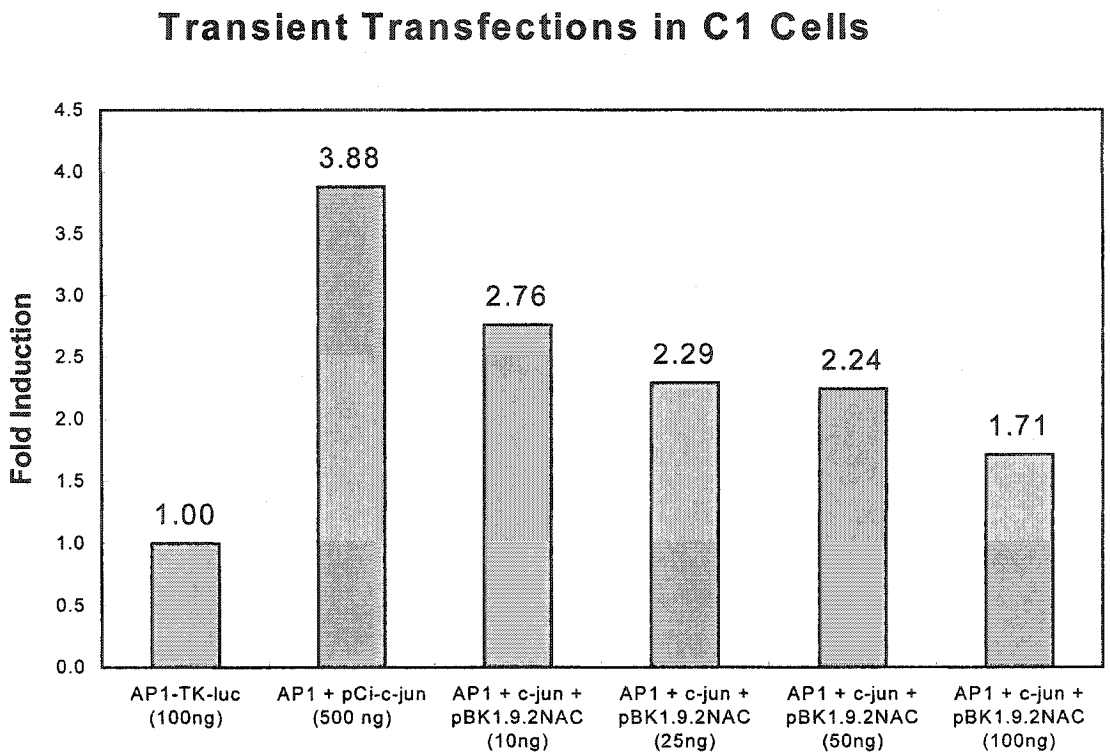

FIGURE 15 : Transcriptional response of the luciferase gene driven by an AP-1 consensus element inserted upstream of the thymidine kinase (TK) minimal promoter. c-Jun binds to the AP-1 response elements and induces transcription. The αNAC gene (NAC/1.9.2) and c-jun are driven by the cytomegalovirus (CMV) promoter. Contrary to previous work in our laboratory, αNAC decreases c-Jun induction slightly (lanes 3-6).

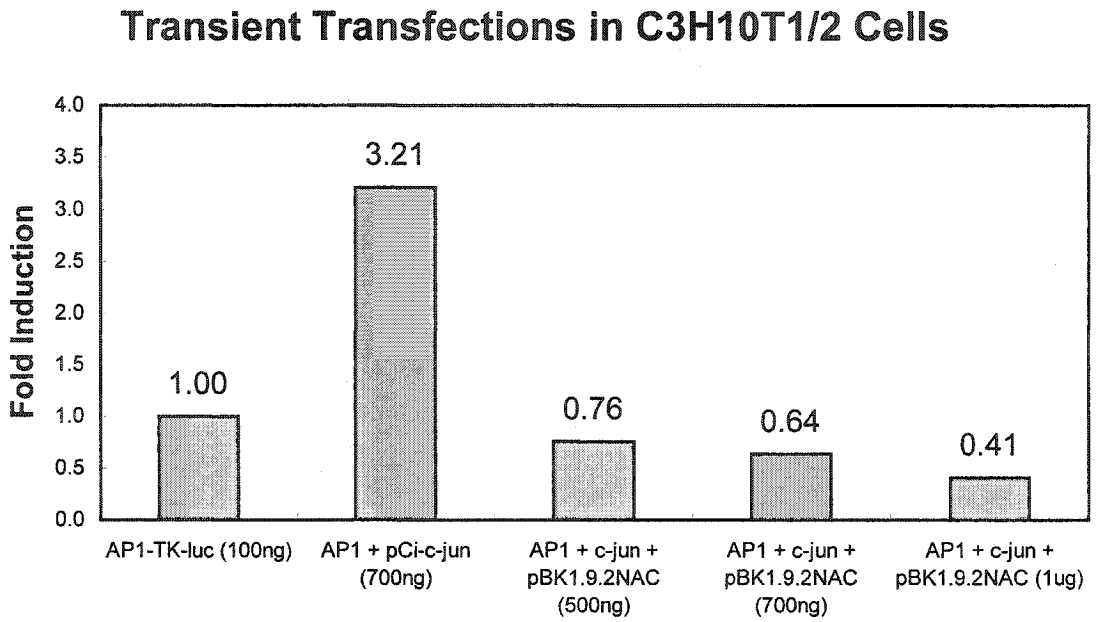

FIGURE 16 : Transcriptional response of the luciferase gene driven by an AP-1 consensus element inserted upstream of the thymidine kinase (TK) minimal promoter. c-Jun binds to the AP-1 response elements and induces induction. The αNAC gene (NAC/1.9.2) and c-jun are driven by the cytomegalovirus (CMV) promoter. Once more, αNAC decreases c-Jun induction of the control reporter plasmid (lanes 3-5).

to see as the same concentrations of plasmids were used and the cell line utilized during the experiment was indeed C3H10$T_{1/2}$. At this point, we thought that endogenous levels of αNAC and c-Jun as well as other endogenous proteins might be impinging on the concentrations added to the system and therefore sought a different strategy.

A commercially available trans-reporting system by the name of PathDetect was purchased from Stratagene. This system is designed for assessment of activation of specific transcriptional activators and can also identify whether a gene of interest is involved in a given signal transduction pathway. As previously described in Materials and Methods, the reporter plasmid pFRluc carrying the luciferase gene was transiently transfected within the cells, as well as the activator c-Jun and the coactivator αNAC. With the PathDetect system, only the c-Jun protein transcribed and translated from the pFA2-c-jun transfected vector can bind and activate transcription of the luciferase gene as it contains a GAL4 yeast DNA-binding domain which interacts with the GAL4-binding sites within the pFRluc promoter controlling transcription of the luciferase gene. As GAL4 yeast DNA is not found in mammalian cells, endogenous c-Jun cannot interact with the reporter plasmid and therefore cannot have an impact on the results of the experiment. Endogenous αNAC, however, can contribute to the outcome as the gene utilized does not have a GAL4-binding domain. In order to eliminate possible fluctuations in αNAC concentrations, undifferentiated Embryonic Stem (ES) cells were selected because they do not express the αNAC protein (Yotov and St-Arnaud, unpublished observations). Although the αNAC gene is present in these cells, the protein is not expressed until the cells start to differentiate, which made them an ideal candidate for our purposes. Figure 17 shows the results of the experiment using the

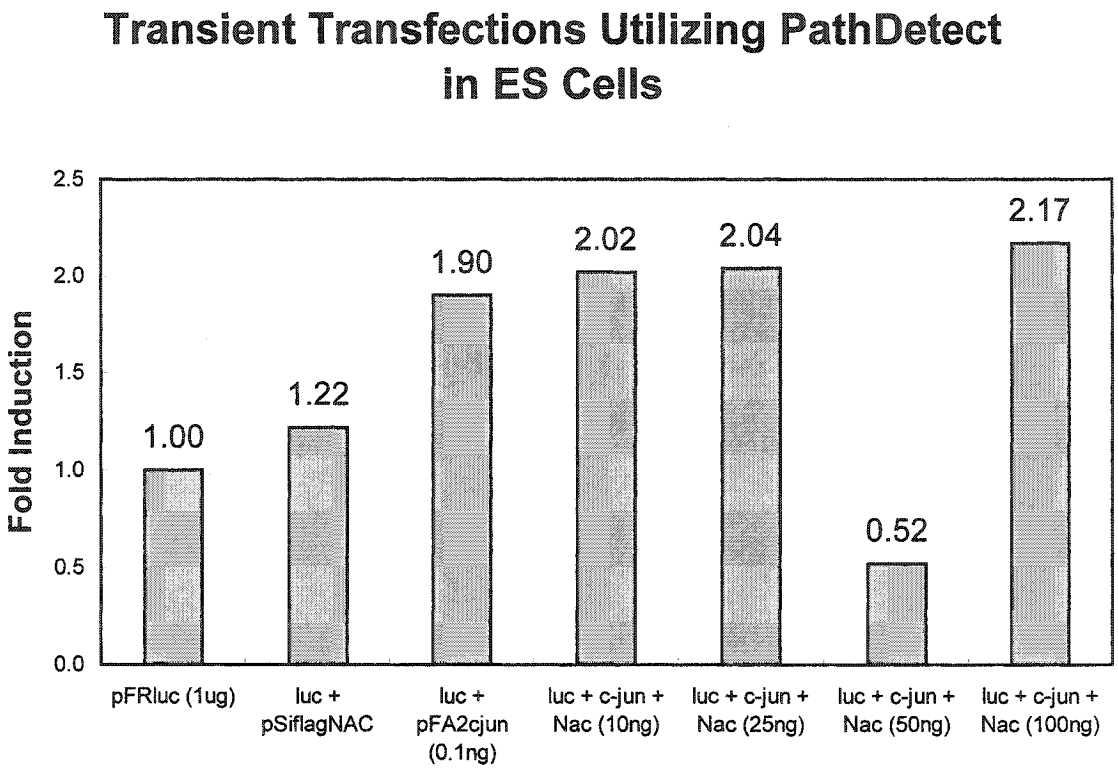

FIGURE 17 : Transcriptional response of the luciferase gene under the control of tandem yeast GAL4-binding sites. c-jun is driven by the CMV promoter whereas αNAC is driven by the simian virus 40 (SV40) enhancer and promoter. c-Jun carries a GAL4-binding domain and interacts with the reporter. αNAC shows no significant induction (lanes 4-7) over the luciferase expression by c-Jun (lane 3). The low expression level of αNAC coactivating function in lane 6 is most likely a result of technical difficulties.

PathDetect system in ES cells. Although the expression of the luciferase gene was induced with c-Jun (lane 3), αNAC at varying concentrations did not significantly potentiate this level of expression (lanes 4, 5, 6 and 7). The conditions of the experiment were modified several times, however, similar results were observed.

Experiments involving ILK were next tested using the PathDetect system. An expression vector for the ILK S343D mutant was transiently transfected in COS-7 cells along with pFRluc, pFA2-cjun and pSiFlagNAC. The serine to aspartic acid substitution at codon 343 constitutively activates ILK (78). We initially chose to try the experiments in COS-7 African green monkey kidney cells as opposed to ES cells as the former are easily transfected and do not require delicate growth conditions. As shown in figure 18, addition of αNAC and/or ILK without c-jun did not activate transcription (lanes 2, 3 and 4). Upon adding c-jun, transcription of the luciferase reporter gene was induced 2.33 fold (lane 5). Co-expressing ILK S343D further induced luciferase gene expression (lane 7). Although αNAC alone had no effect on the c-Jun induction (lane 6), it did increase the induction to 4.03 in combination with ILK (lane 8). This result complements our novel finding earlier mentioned in this section that phosphorylation of αNAC by ILK leads to a specific interaction with the phosphorylated form of c-Jun, and consequently, enhancement of the AP-1 transcriptional response.

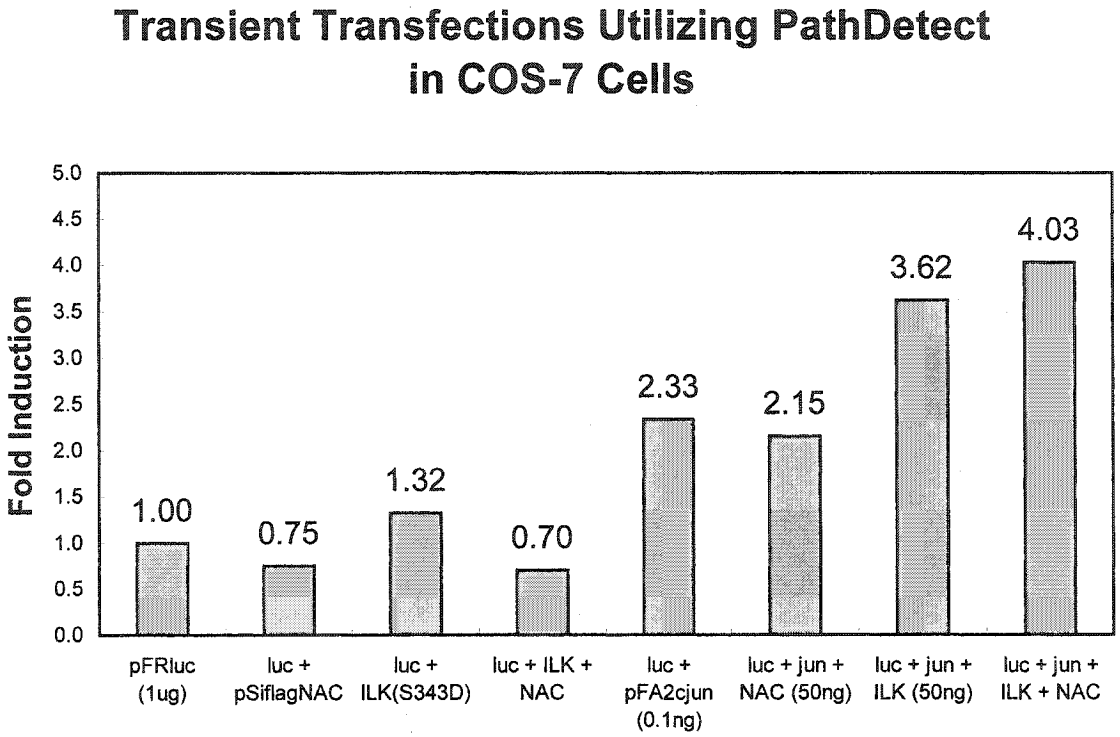

FIGURE 18 : Transcriptional response of the luciferase gene under the control of tandem yeast GAL4 binding sites. c-jun is driven by the CMV promoter whereas αNAC is driven by the simian virus 40 (SV40) enhancer and promoter. c-Jun carries a GAL4-binding domain and interacts with the reporter. Constitutively active ILK (S343D) is under the control of the EF promoter. ILK increases the c-Jun induction 3.62 fold (lane 7). αNAC and ILK together increase luciferase expression 4.03 fold (lane 8).

DISCUSSION

The results presented here suggest that the ILK-phosphorylated form of αNAC preferentially interacts with JNK-phosphorylated c-Jun. Our data further show that residues 89 to 129 of αNAC constitute the interaction domain of the molecule with c-Jun. Since ILK has been shown to potentiate the c-Jun-mediated AP-1 response (49), we hypothesize that the effect of ILK on AP-1-dependent gene transcription involves the phosphorylated form of αNAC. We have begun to establish a suitable transient transfection system to test this hypothesis, which is explained further in the section.

Integrin-mediated interactions of cells with components of the extracellular matrix are known to regulate cell survival, cell proliferation, cell differentiation and cell migration (70,71). These regulations are achieved by controlling the activity of transcription factors such as AP-1. It has been shown that ILK stimulates the binding of c-Jun to the AP-1-responsive element (49), thereby implicating this kinase in MAPK signal transduction. It has also been established that αNAC is a substrate of ILK (Quélo, I. and St-Arnaud, R., manuscript in preparation). Previous *in-vitro* and *in-vivo* experiments have shown that αNAC interacts with the N-terminus of c-Jun and stabilizes the AP-1 complex (59). Since αNAC is a substrate of ILK and can interact with c-Jun, we hypothesized that the phosphorylation of αNAC by ILK could lead to an enhanced interaction with the phosphorylated form of c-Jun. *In-vitro* kinase assays were performed with ILK and Jun N-terminal Kinase (JNK) in order to phosphorylate αNAC and c-Jun, respectively. Phosphorylated and non-phosphorylated αNAC and c-Jun proteins were then utilized in protein pulldown assays to test the hypothesis. Our results

have shown that the phosphorylation of αNAC by ILK increases the specific interaction with phospho-c-Jun. Post-translational modifications regulating protein associations have been reported in the literature and can be compared to this novel interaction of phospho-αNAC and phospho-c-Jun via ILK signalling.

CREB is a leucine-zipper transcription factor involved in the cAMP transduction pathway (86-88). A hormonal response regulates the activity of Protein Kinase A (PKA) by means of intracellular cAMP, which then leads to the phosphorylation of CREB at serine 133, located in the N-terminal transactivation domain of the protein. Once CREB is phosphorylated by PKA, it interacts with the coactivator CREB-Binding Protein (CBP), which bridges the CRE/CREB complex to components of the basal transcriptional machinery. PKA-dependent phosphorylation of CREB therefore stimulates transcription of various genes having a cAMP Response Element (CRE) in their promoter (86-88). CREB activity has also been shown to be regulated by mitogenic pathways. The major MAPK cascades reported include the ERK or Ras-Raf-MAPK pathway (72,93), the JNK/SAPK pathway (91,92) and the stress-related p38 pathway (89,90).

Phosphorylation can also greatly affect the stability and half-life of various proteins. One example of this control is JNK/SAPK. JNK requires phosphorylation on amino acids 183 and 185 by the upstream kinase MKK4 (also referred to as JNKK/SEK1) for its activation. On account of the added phosphate group, JNK is stimulated and can phosphorylate AP-1 transcription factor c-Jun. Likewise, the phosphorylation of c-Jun regulates its activity and also stabilizes the protein in order to escape ubiquitination and consequently, early proteasomal degradation (25,27).

Another example of protein regulation by post-translational modifications is that of β-catenin. β-catenin is a component of the cadherin cell adhesion system and the Wnt signalling pathway (50). Through interactions with cadherins at the plasma membrane, β-catenin binds to a complex consisting of Axin, Adenomatous Polyposis Coli (APC) and GSK-3β in the cytoplasm (50). GSK-3β phosphorylates β-catenin, which triggers its ubiquitination. However, if the activity of the APC-Axin-GSK-3β complex is inhibited by Wnt signalling and/or integrin signalling, β-catenin is no longer phosphorylated by GSK-3β and its stability and half-life are therefore increased. Consequently, β-catenin accumulates in the nucleus and binds to the transcriptional factors Lymphoid Enhancer Factor/T-Cell Factor (LEF-1/TCF), thereby activating transcription (50).

Our results show that phosphorylation of αNAC by ILK signals specificity with the phosphorylated form of c-Jun. These results support the notion that αNAC is involved in the integrin signalling cascade. As demonstrated in figure 19, integrins interact with components of the ECM, such as fibronectin, in order to activate gene expression (42-44). ILK interacts with the β chain of the integrin and is activated by the fibronectin-integrin interaction, in a Phosphoinositide-3-OH Kinase [Pi(3)K]-dependent manner (46,47). Activated ILK, along with constitutively active 3'-Phosphoinositide-Dependent Kinase-1 (PDK-1), can phosphorylate Protein Kinase B (PKB/AKT), which in turn leads to the phosphorylation and inactivation of GSK-3β. GSK-3β can also be inactivated through other means, one of them involving direct interaction with ILK. Once ILK is activated by cell attachment to the matrix, it can phosphorylate and negatively regulate the activity of GSK-3β (49). GSK-3β can presumably no longer

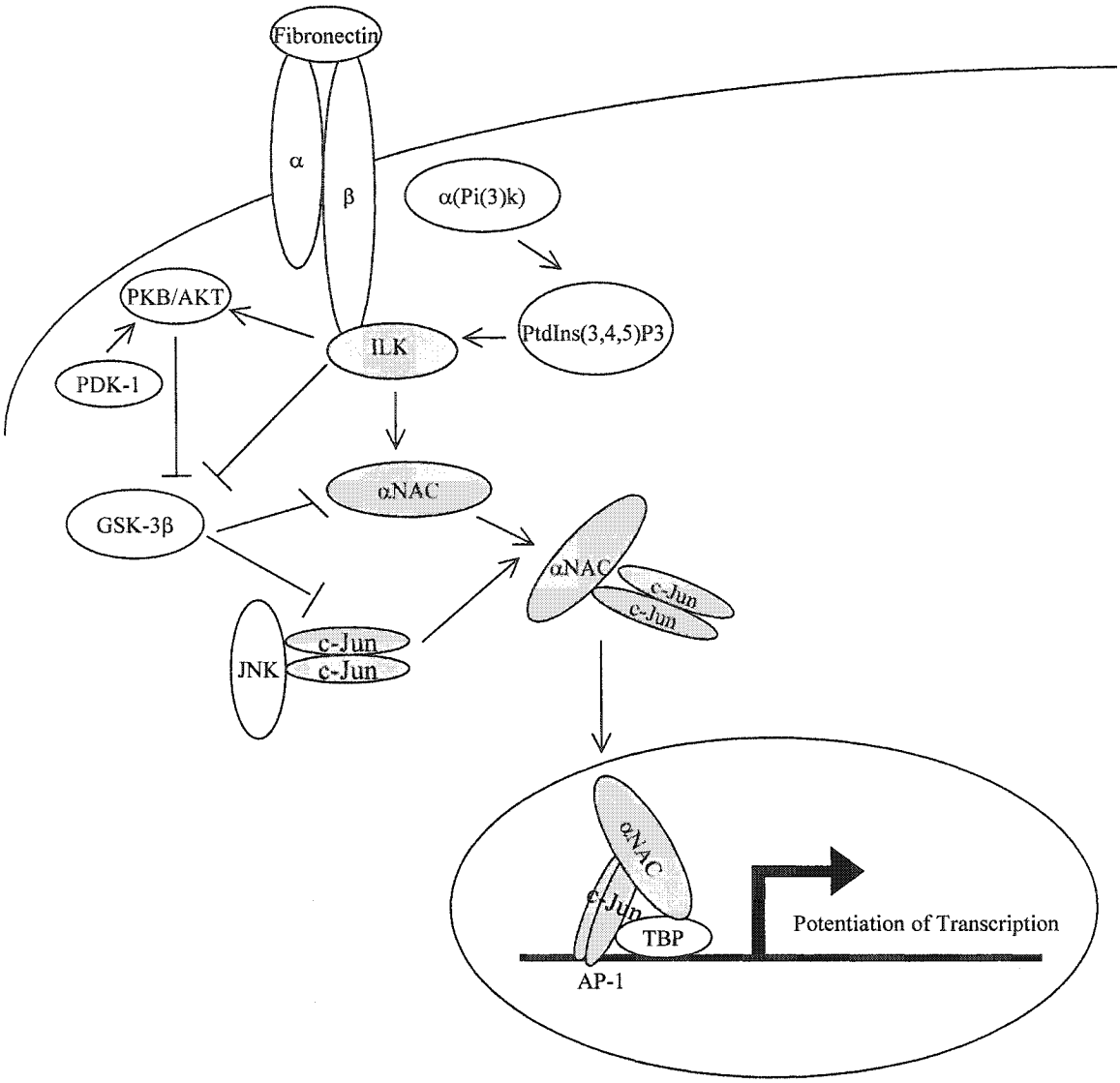

FIGURE 19 : Schematic diagram of the Integrin-Linked Kinase (ILK) signalling pathway. Fibronectin, a component of the ExtraCellular Matrix (ECM), interacts with an integrin (α β chains) and activates ILK in a Phosphoinositide-3-OH Kinase [Pi(3)K)]-dependent manner. Consequently, a series of interactions involving activation and deactivation of downstream proteins occur and result in a AP-1 transcriptional response. Through previous results and novel findings described in this thesis, αNAC likely integrates into the ILK cascade, and potentiates c-Jun-mediated transcription.

phosphorylate c-Jun (49) and αNAC (data not published), which enables both proteins to evade degradation by the 26S proteasome. We are currently establishing ES cells that specifically target the αNAC gene in osteoblasts to gain further knowledge about the role of αNAC in bone development.

Activated ILK can also phosphorylate αNAC on serine 43 (figure 2, Quélo, I. and St-Arnaud, R., manuscript in preparation). The phosphorylation by ILK may act as a hinge-type mechanism by which the αNAC protein undergoes a conformational change and therefore allows for certain domains, such as the c-Jun-binding domain, to be increasingly exposed. We reported here that the c-Jun interaction domain on αNAC is between amino acids 89 and 129. We further stated that phosphorylation of αNAC by ILK results in an enhanced interaction with the phosphorylated form of c-Jun. On account of the post-translational modification caused by ILK, the 3-dimensional structure of αNAC may be changed in such a way that the c-Jun-binding domain becomes more easily accessible to c-Jun. Phosphorylation of c-Jun by JNK in the N-terminal activation domain could also allow for increased interaction with αNAC in the same manner. Hence, this could explain why αNAC and c-Jun interact at increased levels once phosphorylated by ILK and JNK, respectively. Phospho-αNAC and phospho-c-Jun are then presumed to enter the nucleus as a complex, where c-Jun binds to the AP-1-responsive element and activates gene expression (19). αNAC interacts with both c-Jun and TATA-binding protein (TBP), leading to stabilization of the complex and potentiation of the c-Jun-mediated AP-1 transcriptional response (figures 2, 19).

Post-translational modifications such as phosphorylation generally increase the specificity between a coactivator and either the phosphorylated or non-phosphorylated form of a transcriptional activator. For example, only the phosphorylated form of c-Jun can interact with the coactivator CBP (63). However, αNAC was shown to bind both c-Jun and JNK-phosphorylated c-Jun (59 and figure 7, lanes 2, 5). To date, one AP-1 coactivator that binds both forms of c-Jun has been identified and reported in the literature. The cofactor Jun Activation domain-Binding protein 1 (JAB1) was initially characterized as a novel coactivator that increases AP-1 gene activation by interacting with c-Jun and JunD (60). In these experiments, JAB1 showed an interaction with c-Jun and c-Jun phophorylated by JNK. Recent data demonstrate that JAB1 may be more intimately involved in the regulation of protein stability (73). The reported interactions of JAB1 with both forms of c-Jun therefore support our results that αNAC binds to both c-Jun and phospho-c-Jun, although αNAC may be the first true coactivator to demonstrate this behavior.

In addition to the phosphorylation of αNAC by ILK leading to a specific interaction with phospho-c-Jun, our experiments have identified the c-Jun-binding domain on αNAC. The C-terminal αNAC mutant m2 was shown to bind to c-Jun regardless of its 151-215 amino acid deletion. However, deleting a further 62 amino acids from the C-terminal end of m2 disrupted the interaction with c-Jun since the αNAC mutant m4 did not have an affinity for c-Jun (figure 14, lane 7). The αNAC mutant ΔEF-hand, having amino acids 129 to 150 removed, proved to bind to c-Jun, which delineated the interaction site to a region of 40 amino acids. The c-Jun-binding domain is therefore localized to amino acids 89-129 on the αNAC protein.

Computer modeling showed the charge distribution of the various amino acids within the 89-129 domain to be mostly uncharged, although a small positive-charged cluster is present between residues 93 and 100. Computational analysis also suggests that the secondary structure of the c-Jun interaction domain is a β-sheet. Similarly, the N-terminal activation domain of c-Jun seems to also be structured as a β-sheet. The two β-sheets may therefore be closely positioned to one another, which would allow for hydrogen bonding and maximized protein-protein affinity.

To establish the c-Jun-binding domain, we labelled αNAC with L-[^{35}S]-cysteine. This resulted in non-specific binding as all cysteine-labelled αNAC mutants interacted with c-Jun. In effect, appropriate controls showed that the cysteine-labelled αNAC interacted non-specifically with the glutathione sepharose resin used in GST-c-Jun pulldowns (figure 11). One possible mechanism to explain these results is conformational change. Labelling the protein added one extra amino acid on the C-terminal end of αNAC. The added cysteine may have changed the 3-dimensional structure of αNAC, which caused an unnatural binding to the glutathione sepharose beads. Perhaps the conformational change in αNAC involved exposure of several side chains that may have interacted with those of the resin. A difference in protein charge could also be another mechanism explaining why L-[^{35}S]-cysteine labelling of αNAC led to all mutants binding to the resin. Although cysteine does not carry a charge and can therefore not change the overall charge of the protein, it is composed of a sulfur molecule, which may affect the physico-chemical properties of the protein.

Affinity chromatography was the technique that delineated the c-Jun-binding domain on αNAC. This strategy eliminated the use of L-[^{35}S]-cysteine labelling and

therefore non-specific interactions between αNAC and the resin. Identification of two αNAC-interacting partners, VP16 and c-Jun, has been accomplished by means of affinity chromatography earlier in our laboratory (59,60) and therefore remains a good technique to establish novel protein-protein interactions. Another strategy that could have uncovered the c-Jun interaction domain on αNAC is yeast two-hybrid. The αNAC deletion mutants fused to the yeast GAL4-DNA-binding domain could be used as bait proteins while c-Jun would be fused to the transcriptional activation domain. Interaction between αNAC and c-Jun would lead to the expression of a reporter gene controlled by GAL4-binding sites and would therefore confirm the presence of the c-Jun interaction domain on αNAC. Conversely, an absence of expression of the reporter gene would establish a loss of the binding domain. The region on αNAC necessary for c-Jun interactions would therefore be identified.

It is of importance to contrast the localization of the c-Jun- and αNAC-binding domains of αNAC and c-Jun, respectively, to other interaction domains of coactivators and transcription factors. Prior to our investigations, αNAC was known to bind c-Jun in its N-terminal activation domain and potentiate the transcriptional response. Several coactivators, such as JAB1, SRC-1 and TRBP/ASC-2, have also been shown to interact with activators and consequently, increase transcription.

Similar to αNAC, studies have demonstrated that JAB1 can bind to c-Jun in its N-terminal activation end (60). This interaction stabilizes c-Jun and increases the specificity of AP-1 target gene activation. JAB1 can also interact with the transcriptional activator Hypoxia-Inducible Factor-1 (HIF-1), which is involved in inducing transcription of genes responsive to low cellular oxygen tension (74). Under

hypoxia, JAB1 enhances the activity of HIF-1, which leads to the increased expression of particular proteins, such as Vascular Endothelial Growth Factor (VEGF) (74). Although the HIF-1-binding domain on JAB1 has not been determined, the potentiation of the transcriptional response is due to the interaction of JAB1 with HIF-1, which increases the stability of the latter. αNAC shares similarities with this coactivator. αNAC interacts with c-Jun in its N-terminus and potentiates the activity of the homodimeric activator. Analogous to JAB1, αNAC accomplishes the potentiation by stabilizing the AP-1 complex formed by c-Jun (59). Additionally, results from our laboratory have demonstrated that αNAC interacts with other transcriptional regulators, namely PIN/ATF4 (unplublished results).

Steroid Receptor Coactivator-1 (SRC-1) is another coactivator that potentiates AP1-mediated transcription. SRC-1 can interact with the AP-1 members c-Jun and c-Fos. Once bound to the heterodimer, SRC-1 enhances AP-1-mediated transactivations in a dose-dependent manner (75). The c-Jun- and c-Fos-binding domain on SRC-1 has been established and is localized to the C-terminal domain of the protein. This is also the region that encompasses the previously described histone acetyltransferase- and receptor-binding domains (76). SRC-1 has also been studied in the context of Interleukin-6 (IL-6) cytokine stimulation. STAT3 transcription factors are cytoplasmic proteins that induce gene activation in response to cytokine receptor stimulation. Following tyrosine phosphorylation, STAT3 proteins dimerize, translocate to the nucleus and activate specific target genes (77). Similar to interactions between αNAC and c-Jun, SRC-1 binds STAT3 in its activation domain and potentiates the transcriptional response. The STAT3-binding domain is located in the N-terminus of the

protein, and therefore differs from the location of the c-Jun- and c-Fos-binding site on SRC-1. We have demonstrated that the c-Jun interaction domain on αNAC is localized to its middle section, that of amino acids 89-129. Coactivators can therefore associate with transcription factors along various positions on the protein and the location of the interaction domain may depend largely on the transcriptional activator involved.

Thyroid hormone Receptor-Binding Protein/Activating Signal Cointegrator-2 (TRBP/ASC-2) is another AP-1 coactivator. TRBP/ASC-2 stimulates the AP-1 transcriptional response once MEKK activates the JNK signalling pathway (78). Further studies demonstrated an interaction between TRBP/ASC-2 and c-Jun as well as c-Fos (61). Although TRBP/ASC-2 interacts with c-Fos unlike αNAC, they both bind c-Jun and the interactions of TRBP/ASC-2 and αNAC with c-Jun were both confirmed through glutathione-S-transferase (GST) pulldown assays.

Transient transfections were performed in C1, C3H10T½ and ES cells to investigate the effect of ILK on αNAC-coactivated c-Jun-mediated gene transcription. Since substantial data results were not obtained using C1 and C3H10T½ cells, we switched to undifferentiated ES cells that do not express the αNAC protein. We believed at the time that endogenous levels of αNAC and c-Jun affected the transcriptional outcome of the reporter gene. We also used the PathDetect signal transduction trans-reporting system from Stratagene to eliminate contributions from endogenous c-Jun on the outcome. However difficulties in establishing αNAC potentiation of AP-1-dependent gene transcription persisted. We then utilized the constitutively active form of ILK, ILK S343D (78), in COS-7 cells and our initial findings showed co-expression of ILK and αNAC potentiated the transactivation of c-

Jun on the reporter gene (figure 18, lane 8). Statistical analyses were not performed on the preliminary data, however, these experiments have assisted our laboratory in further experimental designs whereby mathematical significance was assessed. Although ILK was used in several transfection experiments in the various cell lines (data not shown), αNAC potentiation was only demonstrated once the active form of ILK was used. We believe that difficulties arose because the ILK signalling cascade was not activated. Once activated ILK was introduced into the cells, the cascade involving a series of phosphorylation/dephosphorylation events was most likely initiated, leading to enhancement of c-Jun gene transcription by αNAC. This strategy has now permitted us to characterize the αNAC nuclear entry mechanism (Quélo, I. and St-Arnaud, R., manuscript in preparation), the control of the half-life of αNAC (Quélo, I. and St-Arnaud, R., submitted), and the nuclear export mechanism for αNAC (Quélo, I. and St-Arnaud, R., submitted). Controlling these mechanisms by adding the proper expression vectors in the cellular systems have proven to be essential for the coactivating function of αNAC in AP-1 transcription.

Although our reasoning behind utilizing ES cells for transient transfection assays was well-suited for the purpose of eliminating the effects caused by endogenous αNAC, manipulation of the cell line proved to be restrictive. Feeder cells, although necessary for proper growth as well as to keep the ES cells from differentiating, were an added layer of cells that potientially affected the outcome of our transfection assays. Recently, Yang et al. (79) have developed a technique which eliminates possible contributions made by feeder cells during transient transfection assays in ES cells. Upon confluency, ES cells grown on feeder cells were trypsinized, pipetted extensively, suspended in

additional medium, plated on a gelatinized dish and incubated at 37°C for 45 min. With the majority of the feeder cells having adhered to the plate, the ES cells in the suspension were harvested and transient transfections were performed similarly to our explanations in Methods. To ensure activation of the integrin signalling pathway, the ES cells can be plated on fibronectin. Furthermore, our laboratory has recently engineered cells having no functional copy of the αNAC gene by incorporating mutations in both alleles of the gene. This true gain-of-function system would be a supplementary method to show the effect of ILK on the coactivating activity of αNAC in c-Jun-mediated transcription.

Although progress has been made regarding the impact that ILK has on the coactivating function of αNAC in AP-1 transcription (figure 18), further work is needed. Due to time constraints, we were not able to accommodate all modifications necessary for adapting the PathDetect trans-reporting kit to our system. Consequently, the experiments were continued by Dr. Isabelle Quélo, a post-doctoral researcher of the lab. The experiments involved the cotransfection of activated ILK and αNAC, as well as controlling the entry and exit of αNAC from the nucleus. Results show a reproducible increase in c-Jun-mediated AP-1 transcriptional response once both ILK and αNAC are involved (figure 4 in Appendix). The αNAC mutant m4, comprised only of the first 88 amino acids of the full-length protein, was also tested and proved to decrease the c-Jun-ILK-αNAC induction (lane 3). These findings compliment our results of the c-Jun-binding domain localized to amino acids 89-129 of the αNAC protein.

Experiments are presently being performed in the lab in order to further our knowledge of the regulations and function of αNAC. Antibodies recognizing the

phosphorylated and non-phosphorylated forms of αNAC are being produced in collaboration with the McGill Animal Care Centre. Although an antibody recognizing αNAC has already been produced in our lab and serves as a valuable tool in several experimental assays, it proved to be ineffective for our purposes. This antibody was created against part of the GST-αNAC fusion protein, which consequently recognizes both moieties. Since our work involved protein-protein interactions between GST-c-Jun glutathione sepharose beads and αNAC, the antibody identified both αNAC and GST-c-Jun (data not shown). Therefore, this αNAC antibody does recognize its intended target, that of αNAC, but should not be used if a GST moiety is involved. Producing an antibody that specifically identifies the phosphorylation of αNAC on serine 43 by ILK could confirm our protein pulldown results. We had found that the phosphorylation of αNAC by ILK led to a specific interaction with the phosphorylated form of c-Jun (figure 6). This was shown by a signal being present during phospho-c-Jun/phospho-αNAC interactions (lane 6) and absent during c-Jun/phospho-αNAC interactions (lane 3). To perform these experiments, two types of radioactivity were needed, that of γ-[^{32}P]-ATP for αNAC phosphorylation by ILK and L-[^{35}S]-cysteine for all other lanes not involving ILK. Antibodies specifically recognizing the αNAC protein would eliminate the use of radioactivity all together and would provide comparisons between the various unphosphorylated/phosphorylated c-Jun and αNAC interactions (figure 7, lanes 2,3,5,6). Furthermore, the use of a second antibody identifying only αNAC proteins that have been phosphorylated by ILK could support our finding of an enhanced interaction between phospho-c-Jun and phospho-αNAC via ILK signalling.

Another means of assessing the contribution of αNAC in ILK signalling is by mimicking the natural activation of the ILK kinase. Cell surface integrins bind to protein components of the ECM, such as fibronectin, and mediate chemical and mechanical signals (70,71). Integrin adhesion to fibronectin stimulates the activities of both AP-1 and ILK (49). Future experiments in our lab will consist of plating cells over a layer of fibronectin in order to activate ILK through ECM-integrin interactions. Immunocytochemistry could then be performed to determine the effect of ILK activation on the subcellular localization of αNAC. Since we know that αNAC coactivates the c-Jun-mediated AP-1 transcriptional response (59) and have shown that phosphorylation of αNAC by ILK confers specificity with phospho-c-Jun, we think that integrin-mediated activation of ILK could lead to nuclear translocation of αNAC. This hypothesis is also founded on previous results demonstrating nuclear translocation of αNAC once GSK-3β, a downstream effector of ILK, can no longer phosphorylate αNAC (data not published). By utilizing the newly produced αNAC antibodies, it would be possible to determine whether the nuclear translocation of cytoplasmic and perinuclear αNAC is a consequence of ILK phosphorylation.

CONCLUSION

By means of protein pulldown assays, we have demonstrated that phosphorylation of αNAC by the ILK kinase leads to an enhanced interaction with the phosphorylated form of c-Jun.

Additionally, we have localized the c-Jun interaction domain on αNAC to the middle part of the protein, that of amino acids 89 to 129. A series of N-terminal and C-terminal αNAC mutants labelled with L-[^{35}S]-cysteine were initially utilized in protein pulldown assays in order to delineate the c-Jun-binding domain. The interaction domain on αNAC was finally revealed by means of affinity chromatography.

Preliminary experiments have shown that αNAC further potentiates c-Jun-mediated transcriptional activation via ILK signalling. Our efforts have contributed to the development of a potential transient transfection system to test the hypothesis that the effect of ILK on AP-1-dependent gene transcription involves the phosphorylated form of αNAC.

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APPENDIX

Quélo, I., Hurtubise, M., and St-Arnaud, R. (2002). αNAC requires an interaction with c-Jun to exert its transcriptional coactivation. *Gene expression* **10**, 255-262.

In the following manuscript, I did the experiments shown in figure 2 and contributed to the data shown in figures 1 and 4. Dr. Isabelle Quélo engineered all mutants and performed subsequent experiments described in the article. Dr. René St-Arnaud supervised the work and contributed to the writing of the article.

Dear Ms. Tauber:

This is to request the authorization of GENE EXPRESSION to reproduce the article: "alphaNAC requires an interaction with c-Jun to exert its transcriptional coactivation" by Quélo, I., M. Hurtubise, and R. St-Arnaud, GENE EXPRESSION manuscript No. L02-2224, in press.

The article would be photocopied and attached as an appendix in the M.Sc.thesis of my student, Ms. Melanie Hurtubise, who is an author on the manuscript.

I thank you for your attention to this question. A rapid reply would be appreciated.

Yours truly.

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αNAC requires an interaction with c-Jun to exert its transcriptional coactivation.

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Running title: αNAC interacts with c-Jun.

Abbreviations: αNAC, Nascent Polypeptide Associated Complex and Coactivator alpha.

ABSTRACT

αNAC is a transcriptional coactivator known to interact with the N-terminal activation domain of the c-Jun transcription factor. In this paper, we describe the identification of the c-Jun interaction domain within the αNAC protein. Deletion analysis of αNAC indicated that the c-Jun binding site was located in the middle part of the protein, between residues 89 and 129. The deletion of the C-terminal end of αNAC, including the c-Jun interacting domain, induced a nuclear translocation of the mutated coactivator. Despite its presence in the nucleus, this deletion mutant did not retain the capacity to coactivate an AP-1 response. These results demonstrate that the interaction between αNAC and c-Jun was necessary for the potentiation of the AP-1 transcriptional activity. These data are consistent with a mechanism by which αNAC acts as a coactivator for c-Jun-dependent transcription by interacting with the c-Jun N-terminal activation domain.

Key words : αNAC, c-Jun, coactivation, transcriptional activation.

INTRODUCTION

The Fos and Jun proteins, members of the AP-1 family of transcription factors, regulate a wide variety of cellular processes including cell proliferation, differentiation, apoptosis and oncogenesis (6; 9; 20). Fos and Jun proteins function as dimeric transcription factors that bind AP-1 regulatory elements in the promoter and/or enhancer regions of numerous genes (8). Jun family members (c-Jun, JunB, JunD) can homodimerize, as well as form heterodimers amongst themselves or with partners of the Fos or ATF families (14; 17). Fos proteins, on the other hand, are obligate heterodimers. The dimeric complexes bind DNA on AP-1 sites with high affinity and CRE elements with low affinity.

Both Fos and Jun proteins interact with coactivators to potentiate transcription. The proteins CBP (CREB-binding protein) (3), JAB-1 (Jun-Activation domain-Binding protein 1) (7), SRC-1 (Steroid Receptor Coactivator-1) (18), TRBP/ASC-2 (Activating Signal Cointegrator-2) (19) and αNAC (Nascent polypeptide associated complex And Coactivator alpha) (21) were characterized as coactivators of AP-1-mediated transcription. Several coactivators characterized to date share functional properties. But some of them have unique functions such as histone acetyltransferase for CBP, p300 (4) and SRC-1, kinase for $hTAF_{II}250$ (10), or specific DNA binding for $dTAF_{II}150$ (24). αNAC also appears to exhibit specific DNA binding activity (26), although the physiological relevance of this property remains to be explored.

The Nascent polypeptide-associated complex And Coactivator alpha (αNAC) gene was first identified as a modulator of translation (27) and purified as an heterodimer with ßNAC/BTF3b, previously identified as a transcriptional factor in yeast (16) and higher eukaryotes (28). α and ß NAC subunits were, moreover, shown to enter the nucleus in yeast (12),

and αNAC enters the nucleus in eukaryotic cells (25). We characterized the αNAC subunit as a transcriptional coactivator of the chimeric Gal4-VP16 activator and of c-Jun homodimers *in vivo* (25; 21). αNAC provides a protein bridge between these transcription factors and the basal transcriptional machinery by contacting the general transcription factor TBP (TATA-binding protein) (25). The current model of the αNAC coactivator function is to promote an interaction between the transcription factors bound to DNA and the basal transcriptional machinery, therefore stabilizing the transcription factors on DNA and resulting in an enhanced transcription rate. To exert its coactivation function, αNAC enters the nucleus (12), and the subcellular localization of the protein appears regulated (25). We have previously identified the 1-89 c-Jun N-terminal domain as the region interacting with αNAC (21). We were then interested in charaterizing the domain of αNAC that interacts with c-Jun.

In this report, we identified the c-Jun-interacting domain of αNAC within the mid-part of the molecule, more precisely between residues 89 and 129. Deletion of the C-terminal part of the molecule, including the c-Jun interacting domain, induces the nuclear translocation of αNAC. This nuclear patterning presumably occurs by passive diffusion due to the small size of the mutant molecule. Despite its nuclear patterning, this deletion mutant molecule does not retain the coactivating ability of αNAC, showing that the interaction between αNAC and c-Jun is required for the potentiation of the c-Jun transcriptional response.

MATERIALS AND METHODS

Plasmids and constructs (subcloning details and vector maps available on request)

Full-length αNAC (WT) and mutants cDNAs (see Figure 1) were subcloned in-frame at their C-termini with the Intein-Chitin Binding Domain (ICBD) of the $pTYB_2$ expression vector (NEB, Mississauga, ON) to give $pTYB_2$-NAC plasmids. The Flag epitope was inserted into the pSI mammalian expression vector (Promega, Madison, WI) to give the pSI-Flag plasmid. The cDNAs encoding wild-type or m4 mutated αNAC were inserted in-frame into pSI-Flag to yield the pSI-NAC-Flag expression vectors. The c-Jun cDNA was cloned into the pCI mammalian expression vector (Promega) to yield pCI-c-Jun. The expression vector for the constitutively active form of ILK (pcDNA3.1/V5-His-ILK S343D) was kindly provided by Dr. S. Dedhar (23).

Cell culture and transfection

COS-7 african green monkey kidney cells were maintained in Low Glucose DMEM supplemented with 10% fetal bovine serum at 37°C in 5% CO_2. All transient transfections were performed using 5 µl per µg of DNA of the GenePorter transfection reagent, according to the manufacturer's procedure (Gene Therapy System, San Diego, CA).

C_2C_{12} cells, a pluripotent mesenchymal cell line, were grown in High Glucose DMEM supplemented with 10% fetal bovine serum at 37°C in 5% CO_2. Transient transfections were performed with Fugene 6 (3 µl/µg DNA) (Roche, Laval, QC).

Protein production

The $pTYB_2$ and $pTYB_2$-NAC constructs were transformed into *E.coli* ER2566 cells. The Intein-Chitin Binding Domain (ICBD) from $pTYB_2$, αNAC WT and mutant proteins were produced and purified following the manufacturer's procedure (NEB). Briefly, after IPTG induction, the bacterial cell extract was passed through a chitin column. Following extensive

washes, the αNAC moiety was cleaved from the ICBD moiety in the presence of DTT and eluted from the column. The eluted proteins were concentrated using Centricon 10 columns (Millipore, Bedford, MA) and protein concentration was measured by Bradford assay (Bio-Rad, Hercules, CA). The purified proteins were run onto a 12% SDS-polyacrylamide gel and stained with the Gel code blue staining reagent (Pierce, Rockford, IL) to monitor yield and quality.

Affinity chromatography

Twenty-four hours before transfection, the C_2C_{12} cells were seeded at $5x10^5$ cells per 100 mm plates in complete medium. Transient transfections were performed for 24 hours in complete medium with 4 µg of pCI-c-Jun expression vector in the presence of 12 µl of Fugene 6 (Roche). C_2C_{12} transfected cells were lysed by sonication in 0.2 M NaCl column buffer (20 mM Tris-Cl pH 8, 200 mM NaCl, 0.1 mM EDTA, 0.1% Triton X-100) in the presence of 1 µg/ml anti-proteases (leupeptin, pepstatin, aprotinin) and 1 mM PMSF. Total C_2C_{12} cell extracts were precleared onto a chitin column (NEB) to reduce unspecific binding. Bacterial extracts, obtained from *E.coli* cells transformed with $pTYB_2$ and $pTYB_2$-NAC plasmids, and containing an equivalent quantity of proteins (data not shown), were immobilized on the chitin columns. After washes, 1 ml of precleared total extracts from pCI-c-Jun-transfected C_2C_{12} cells was loaded on the αNAC-Chitin affinity columns and incubated overnight at 4°C. After extensive washes with column buffer, the bound proteins were eluted with elution buffer (20 mM Tris-Cl pH 8, 700 mM NaCl, 0.1 mM EDTA). The eluted proteins were loaded onto a 10% SDS-polyacrylamide gel and transferred to nitrocellulose membranes (Amersham-Pharmacia). The specific signal was detected with the anti-phospho-c-Jun (S63) antibody (NEB) and revealed with the Supersignal West femto maximum sensitivity substrate kit (Pierce).

Immunocytochemistry

The COS-7 cells were plated at $1.2x10^5$ cells/35 mm plate, on gelatin-coated cover slips, and transiently transfected with 0.4 µg of pSI-NAC-Flag, or mutant, and 1.6 µg of the pBluescript plasmid (Stratagene, La Jolla, CA). Twenty-four hours post-transfection, the cells were fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and the endogenous peroxidase activity was quenched with 1% H_2O_2. Following blocking with 1% Blocking Reagent (Roche) supplemented with 0.2% Tween-20, the cells were incubated with the anti-Flag M2 antibody (Sigma, Oakville, ON), then with biotinylated secondary anti-mouse IgG antibody (Vector Lab. Inc., Burlingame, CA). After washes, the cells were immersed in the Avidin Biotin peroxidase reagent (Vector Lab. Inc.). The peroxidase staining was revealed with DAB reagent and visualized on a Leica DM-R microscope at 200X.

Luciferase assays

The COS-7 cells were transiently cotransfected with 500 ng of wild-type αNAC or m4 mutant, 500 ng of a constitutively active form of the Integrin-Linked Kinase (pcDNA3.1/V5-His-ILK S343D) (an activator of the AP-1 pathway) (23) and plasmids from the PathDetect c-Jun *trans*-reporting system (Stratagene). The PathDetect plasmids were as followed: 500 ng of pFR-Luc reporter plasmid containing 5xGal4 binding sites fused to the luciferase gene and 50 ng of pFA2-cJun expression vector (c-Jun-GAL4 DNA binding domain fusion, Gal4-c-Jun) or pFC2-dbd as a negative control (Gal4-DNA binding domain). Corresponding empty vectors served as controls. Transient transfections were carried out in COS-7 cells for 48 hours in 0.5% FBS containing DMEM. Subsequently, the cells were lysed 20 min in the reporter gene assay lysis buffer (Roche). Twenty µl of cell lysate were used for single luciferase reporter assays following the manufacturer's procedure (Promega) and analyzed with a Monolight 2010 luminometer

(Analytical Luminescence Laboratory, San Diego, CA). The presence of the transfected αNAC-Flag proteins was controlled by immunoprecipitation with anti-Flag M2 beads (Sigma) followed by immunoblotting with the anti-NAC antibody (26). The Gal4-c-Jun proteins were detected immunoblotting with the anti-Gal4 antibody, recognizing the DNA binding domain of Gal4 (Santa Cruz Biotechnology Inc., Santa Cruz, CA).

RESULTS

αNAC is a small protein of 215 aminoacids, with an apparent molecular weight of 37 kDa. We have engineered N-terminal and C-terminal deletion mutants cDNAs by PCR (primer sequences available upon request) and introduced them in bacterial and eukaryotic expression vectors. Figure 1A shows a schematic representation of αNAC (WT) and the deletion mutant proteins that were used in this study. One μg of each recombinant protein (wild-type αNAC and mutants) was loaded onto a 12% denaturing polyacrylamide gel and the purified proteins were revealed with the Gel code blue staining reagent. This data showed that all the proteins were produced. They were of good quality and purity, and had the predicted sizes (Fig. 1B). Mutant m4 always exhibited a diffuse pattern when revealed by staining of the SDS-PAGE gels (lane 4).

[INSERT FIGURE 1 HERE]

To localize the domain of αNAC interacting with c-Jun, we used recombinant αNAC proteins for affinity chromatography. αNAC WT and mutants, as well as ICBD moiety as a negative control, were immobilized onto chitin beads to prepare affinity columns. C_2C_{12} cells were transiently transfected with c-Jun expression vector before retrieving the cellular extracts. The c-Jun-containing cell extracts were precleared on chitin columns, then loaded on the αNAC affinity columns. After overnight incubation, the proteins interacting with αNAC were eluted with high salt buffer and controlled for the presence of phosphorylated c-Jun by immunoblotting with the anti-phospho-c-Jun antibody.

The immunoblotting revealed that αNAC interacted with the activated, phosphorylated form of c-Jun (Fig. 2, lane 8). Deleting residues 4 to 45 (Δ4-45), 46 to 69 (Δ46-69), or 12 to 69 (Δ12-69) did not affect the interaction of αNAC with the phosphorylated form of c-Jun (lanes 2, 3 and 4, respectively). We observed differences in the interaction between c-Jun and αNAC

when the C-terminal region of the protein was deleted. While deleting residues 130 to 149 (Δ130-149), or 150 to 215 (m2), did not affect interaction with c-Jun (lanes 5 and 6), a further deletion to aminoacid 88 (deletion 89 to 215, mutant m4), almost completely abolished interaction with phospho-c-Jun (lane 7). Since deleting up to residue 130 was neutral (Δ130-149 and m2, lanes 5 and 6) but further deleting to residue 88 abolished interaction (m4, lane 7), we concluded that residus 89 to 129 of the αNAC protein are essential for its interaction with the c-Jun activator.

[INSERT FIGURE 2 HERE]

We further checked whether the loss of interaction between αNAC and c-Jun could interfere with the subcellular localization of αNAC. For that purpose, we performed immunocytochemistry in COS-7 cells transfected with pSI-NAC-Flag WT and the m4 construct (Fig. 3). Specific signals were detected with the anti-Flag M2 antibody. As shown in panel a, background signal from cells transfected with the inert vector was barely dectectable. The subcellular localization of WT αNAC was cytosolic and mostly perinuclear (panel b), whereas the m4 mutant had an exclusively nuclear staining pattern (panel c). The m4 mutant, despite its loss of c-Jun interaction, can nevertheless enter the nucleus of cells.

[INSERT FIGURE 3 HERE]

αNAC was previously characterized as a coactivator of c-Jun homodimers (21). We showed above that the m4 mutant was not able to directly interact with c-Jun, but could still enter the nucleus. To study the coactivation potency of the m4 deletion mutant, we performed transient transfections in COS-7 cells. The c-Jun expression vector contained the N-terminal part of c-Jun fused to the Gal4-DNA binding Domain, while the luciferase reporter gene was under the control of Gal4 binding sites. The 1-89 N-terminal domain of c-Jun was previously shown to be

sufficient for transactivation (15) and for interaction with αNAC (21). The c-Jun pathway was activated by overexpression of ILK (Integrin Linked Kinase) (23). Luciferase activity was measured after 2 days of culture in 0.5% serum to avoid the activation of the endogenous AP-1 cascade or other pathways. The luciferase signal detected was dependent upon the transfected expression vectors. In this experimental system, αNAC moderately but reproducibly potentiated the Gal4-c-Jun response (Fig. 4A). The m4 mutant was not able to potentiate the c-Jun response, demonstrating the importance of a direct interaction between the transcription factor and its coactivator. Immunoblotting with anti-Gal4 and anti-NAC antibodies demonstrated that the lack of response with the m4 mutant was not due to an absence of one of the two partners Gal4-c-Jun and m4, respectively (Fig. 4B).

[INSERT FIGURE 4 HERE]

DISCUSSION

It was established that αNAC is a transcriptional coactivator (21; 25) that interacts with the c-Jun transcription factor, a member of the AP-1 family (21). But the structural motifs mediating the αNAC interaction remained to be identified. In this study, we have identified the aminoacid residues that are essential for allowing αNAC to bind c-Jun, and addressed the role of the c-Jun-αNAC interaction in the function of αNAC as a transcriptional coactivator.

Using internal, N-terminal, and C-terminal deletion mutants, the c-Jun interaction domain was localized to a short region of 40 aminoacids corresponding to the middle part of the αNAC protein. This region was necessary for the interaction with the activated form of c-Jun. c-Jun is activated by phosphorylation in its N-terminal domain by the Jun N-terminal kinases (JNKs) on Serines 63 and 73. This phosphorylation enhances c-Jun transactivation properties by recruiting coactivator proteins such as CBP (22; 1).

c-Jun coactivators, such as CBP and JAB-1, also contact the c-Jun N-terminal activation domain (3; 7; 1). CBP is a coactivator of c-Jun that stimulates the activity of c-Jun *in vivo*, and a reduced binding between CBP and c-Jun abolishes the increase of transcription *in vivo* (2). It is interesting to note the similarities between the mechanism of action of these coactivators and that of αNAC, despite the absence of sequence similarity. Both JAB-1 and αNAC proteins interact with the N-terminal part of c-Jun and can bind the phosphorylated as well as the unphosphorylated forms of c-Jun (7; 21; and our data). The CBP-c-Jun interaction, however, is observed only with the phosphorylated form of the transcription factor (2). The αNAC coactivator interacts with the c-Jun N-terminal activation domain and therefore coactivates its response. As for CBP, the loss of binding between c-Jun and αNAC was detrimental for the

further augmentation of AP-1 response. These data are consistent with a mechanism by which αNAC coactivates c-Jun activity by interacting with the c-Jun activation domain.

We also observed that deletion of the c-Jun interaction domain resulted in nuclear translocation of the αNAC mutant protein. This nuclear patterning could result from the loss of interaction between αNAC and c-Jun, although this interpretation appears unlikely. We hypothesize that the nuclear patterning of the m4 mutant was due to the large deletion within the molecule. The resulting mutant protein was short (104 aminoacids including Flag tag epitope) and small enough to passively diffuse from the cytosol to the nucleus. Indeed, the cutoff for efficient passive diffusion into the nucleus is 20-30kDa (13).

Despite the apparent passive diffusion of the m4 deletion mutant, we do not believe that the wild-type αNAC protein could localize to the nucleus by the same mechanism. Structure-function analysis of the αNAC molecule did not reveal the presence of a functional nuclear localization signal (data not shown). We favor the model that the interaction of αNAC with c-Jun is the signal for a controlled nuclear entry of the coactivator, as c-Jun nuclear import itself is regulated (11; 5).

In conclusion, we identified the c-Jun interaction domain within the αNAC coactivator. We demonstrated that nuclear localization of αNAC and coactivation can be functionally separated from one another, however interaction between c-Jun and its coactivator are fundamental for the potentiation of the c-Jun transcriptional response in cells.

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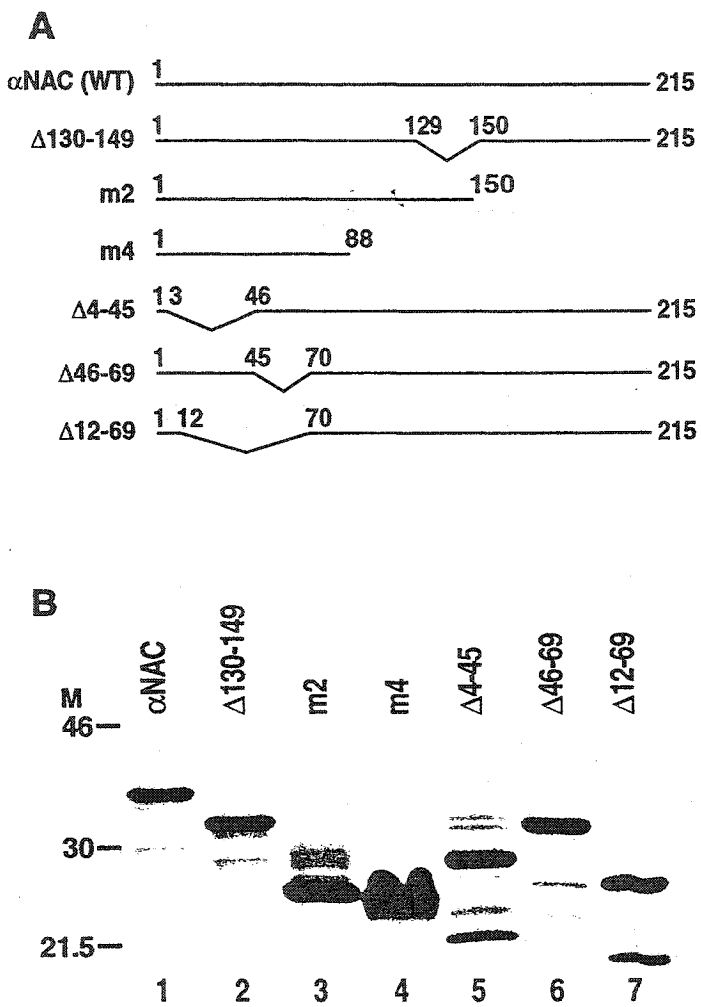
LEGENDS

Figure 1 : *αNAC mutants.* **A)** Schematic representation of αNAC wild-type (WT) and deletion mutant proteins. **B)** The WT, C-terminal and N-terminal deletion mutants of αNAC were cloned into $pTYB_2$ expression vector and the proteins produced and purified following the manufacturer's procedure (NEB). An aliquot of each purified protein was run onto a 12% SDS-PAGE and revealed by Gel code blue staining. 1: αNAC (WT), 2: Δ130-149, 3: m2 (Δ150-215), 4: m4 (Δ89-215), 5: Δ4-45, 6: Δ46-69, 7: Δ12-69.

Figure 2: *Identification of the c-Jun-interacting domain.* αNAC fusion proteins (WT or mutants), or ICBD as a negative control, were immobilized onto chitin beads columns. Total extracts of C_2C_{12} cells transfected with a c-Jun expression vector were loaded on the affinity columns. The proteins interacting with αNAC were eluted with high salt concentration buffer. Immunoblotting of the eluates with the anti-phospho-c-Jun antibody revealed a specific interaction between αNAC proteins and the active, phosphorylated form of c-Jun. A specific c-Jun signal was detected with αNAC WT (lane 8), C-terminal and N-terminal deletion mutant proteins (lanes 2 to 6). No significant binding was observed with the control Intein Chitin Binding Domain (ICBD, lane 1). A highly significant decrease was observed with the m4 mutant (lane 7).

Figure 3: *Nuclear localization of the m4 mutant.* Immunocytochemistry was performed on COS-7 cells transiently transfected with Flag-taggeg αNAC WT or m4 mutant expression vectors, or inert vector. The detection was performed using the anti-Flag M2 antibody and revealed by DAB staining. The cells transfected with the inert vector presented very low background (panel a). The WT αNAC protein had a cytosolic and perinuclear pattern (panel b), whereas the m4 mutant localized to the nucleus of cells (panel c).

Figure 4: *Decreased coactivation activity of the m4 mutant.* **A)** COS-7 cells were transiently transfected with expression vectors for αNAC WT or m4 mutant, Gal4-c-Jun and ILK, and a luciferase reporter gene. After 48 hours in 0.5% serum, single luciferase assays were performed. WT αNAC protein coactivates c-Jun transcriptional activity (black bar) compared to activated c-Jun transcriptional level (white bar). Deletion of the c-Jun interacting domain (m4 mutant) blocks the coactivation capacity of αNAC (hatched bar). The results shown (mean ± SEM) are representative of 3 independent experiments performed in triplicate. The results were statistically analyzed using ANOVA and the Tukey post-test (** $p<0.01$, *** $p<0.001$). **B)** Total cell extracts obtained from tranfected COS-7 cells were probed with the anti-Gal4 antibody to detect the expression of Gal4-c-Jun (upper panel). After immunoprecipitation with anti-Flag M2 beads, the expression of αNAC WT or m4 mutant were revealed with the anti-NAC antibody (middle and lower panels, respectively).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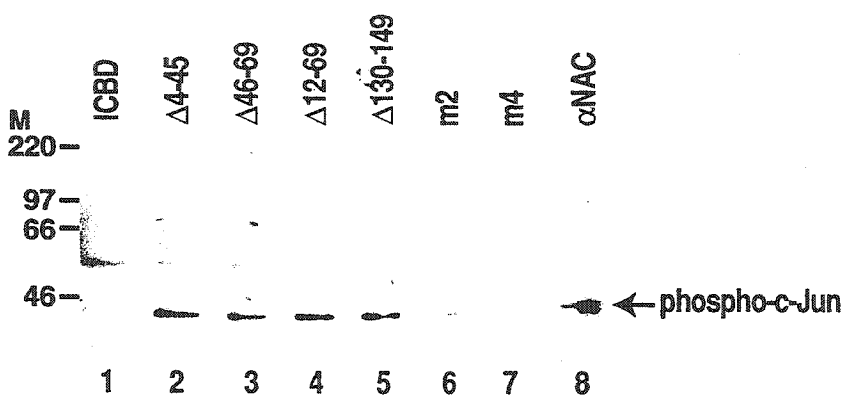

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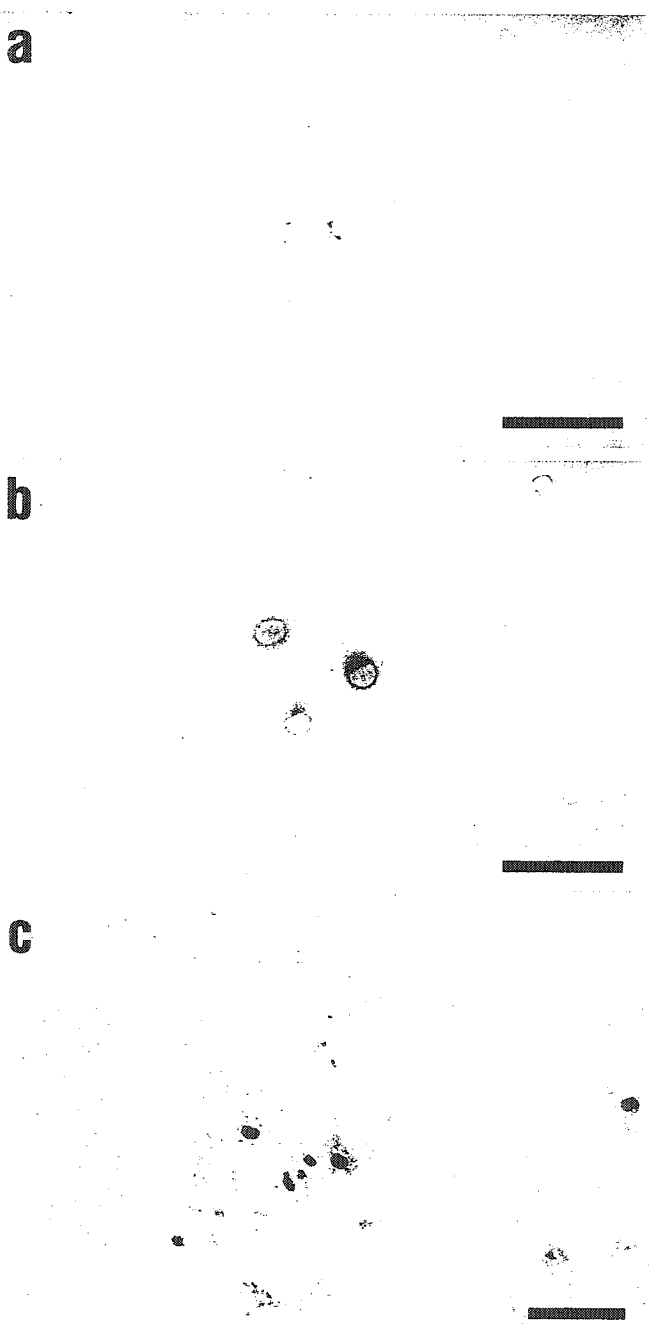

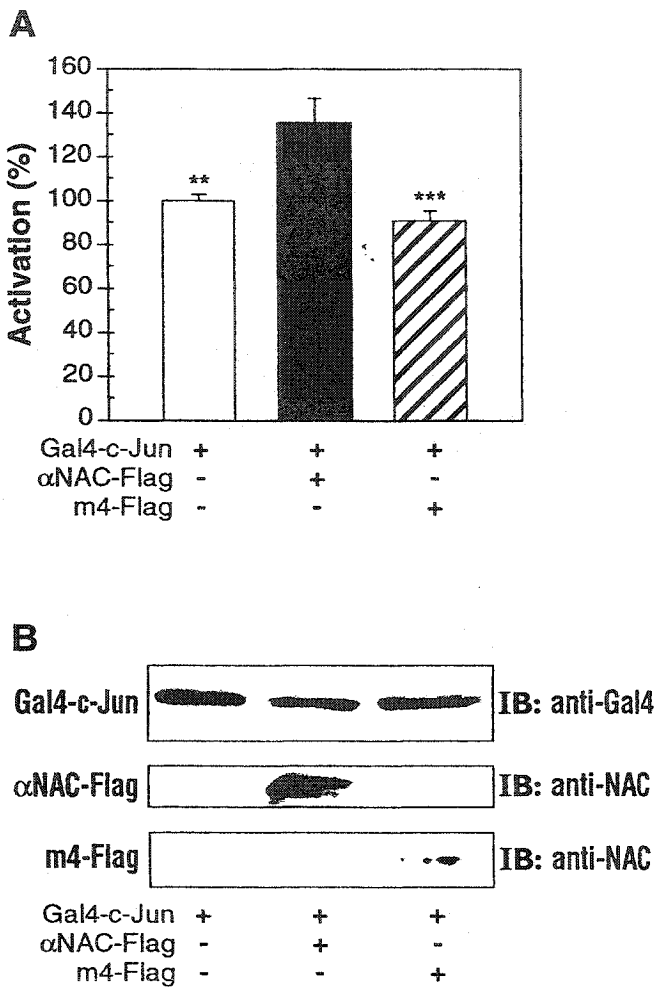
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