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MAPK signaling triggers transcriptional induction of cFOS during amino acid limitation of HepG2 cells☆

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ABSTRACT

Amino acid (AA) deprivation in mammalian cells activates a collection of signaling cascades known as the AA response (AAR), which is characterized by transcriptional induction of stress-related genes, including FBJ murine osteosarcoma viral oncogene homolog (cFOS). The present study established that the signaling mechanism underlying the AA-dependent transcriptional regulation of the cFOS gene in HepG2 human hepatocellular carcinoma cells is independent of the classic GCN2-eIF2-ATF4 pathway. Instead, a RAS-RAF-MEK-ERK cascade mediates AAR signaling to the cFOS gene. Increased cFOS transcription is observed from 4-24 h after AAR-activation, exhibiting little or no overlap with the rapid and transient increase triggered by the well-known serum response. Furthermore, serum is not required for the AA-responsiveness of the cFOS gene and no phosphorylation of promoter-bound serum response factor (SRF) is observed. The ERK-phosphorylated transcription factor E-twenty six-like (p-ELK1) is increased in its association with the cFOS promoter after activation of the AAR. This research identified cFOS as a target of the AAR and further highlights the importance of AA-responsive MAPK signaling in HepG2 cells.

1. Introduction

Amino acid (AA) deprivation in mammalian cells activates several signaling cascades collectively known as the AA response (AAR), which is characterized by translational and transcriptional induction of a wide-range of stress-related genes aimed at restoring cellular homeostasis [1–3]. The protein kinase general control non-derepressible 2 (GCN2) is currently the only well characterized sensor for AA deficiency and the GCN2-eIF2-ATF4 pathway is the predominant AAR signaling mechanism in mammalian cells. GCN2 senses AA limitation by binding uncharged tRNAs, which activates its kinase activity resulting in phosphorylation of eIF2. The p-eIF2 leads to a slower ribosomal assembly and a suppression of general translation while increasing the translation of specific mRNA species containing short upstream opening reading frames [4,5]. Activating transcription factor 4 (ATF4) is a primary target of this translational regulatory mechanism and an effector of many genes involved in the AAR [6,7]. ATF4 induces transcription by binding to C/EBP-ATF response elements (CARE) within hundreds of targeted genes, including asparagine synthetase (ASNS) and C/EBP homology protein (CHOP) [8,9]. However, emerging evidence suggests that there are many AA-responsive genes that are regulated by GCN2-independent mechanisms. This was demonstrated through a global expression array analysis of GCN2 knockout mouse embryonic fibroblasts (MEF) [10] and independently observed on a single gene basis for FOXA2 and FOXA3 [11], cJUN [12], and EGR1 [10,13]. The present report extends this list to include the FBJ murine osteosarcoma viral oncogene homolog (cFOS) gene.

An expression array investigation performed in HepG2 human hepatocellular carcinoma cells showed that AAR activation increased expression of cFOS mRNA [14] and that observation was confirmed in a subsequent study [12]. cFOS is a transcription factor and proto-oncogene involved in cellular proliferation and differentiation that contains a basic leucine zipper (bZIP) region, which facilitates DNA binding and dimerization with other bZIP proteins [15–17]. cFOS associates with the known pro-apoptotic factor CHOP [18], a well-characterized target gene for the AAR [reviewed in 1]. cFOS is a member of a larger class of genes termed “immediate early response genes” based on their rapid and transient induction following extracellular stimulation by growth factors and stress [16,19,20]. Although the functions of cFOS are diverse, context dependent, and not completely understood [15], it is clear that elevated cFOS expression can contribute to

Abbreviations: AA, amino acid; AAR, AA response; ActD, actinomycin D; ASNS, asparagine synthetase; ATF4, activating transcription factor 4; cFOS, FBJ murine osteosarcoma viral oncogene homolog; DOX, doxycycline; eIF2, eukaryotic initiation factor 2; ERK, extracellular-signal regulated kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GCN2, general control non-derepressible 2; HisOH, histidinol; JNK, JUN N-terminal kinase; MAPK, mitogen-activated protein kinase; MEF, mouse embryonic fibroblasts; MEK, MAPK/ERK kinase; qPCR, quantitative real time PCR; TET, tetracycline

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cellular transformation and tumor growth [16,17]. However, to illustrate the complexity of its functions, cFOS has also been linked to tumor suppression in some circumstances [21].

Transcriptional regulation of the cFOS gene is induced by a wide variety of stimuli that trigger mitogen-activated protein kinase (MAPK) signal transduction pathways. For example, activation of the mitogen-activated extracellular kinase (MEK)/extracellular-regulated kinase (ERK) pathway is crucial for cFOS induction in response to growth factors, mitogens, and cell stress, most of which trigger increased transcription via a cluster of sequences in the cFOS promoter often referred to as the serum response element (SRE) [16,19,20]. Two of the sequences within the SRE region are the CArG element (CC-A/T_n-GG), known to bind serum response factor (SRF), and the E-twenty six (ETS) motif (GGA-A/T) that is bound by ternary complex factor (TCF) members, such as (ETS)-like factor 1 (ELK1) [22,23]. The induction of the cFOS gene triggered by the ERK pathway involves phosphorylation of constitutively bound SRF and/or ELK1, which is associated with chromatin remodeling and increased transcription [19,20,24,25]. One of the hallmarks of the immediate early response genes is a rapid onset of transcriptional activation that is of short duration. Typical of this group, after exposure of the cells to stimulus, a high degree of cFOS transcription occurs within 15 min and the return to near basal rate occurs within 90 min [16,19].

Although GCN2-eIF2-ATF4 is the best characterized AAR signaling pathway and the predominant mechanism for AA-responsive transcriptional control in mammalian cells, a recent ChIP-sequencing analysis for ATF4 binding sites did not identify functional ATF4-responsive genomic elements associated with the cFOS gene [26]. The present study investigated GCN2-independent AAR target genes in HepG2 human hepatocellular carcinoma cells cultured in medium deficient for the essential AA histidine to activate the AAR. The results document that cFOS was among a number of genes that are induced in a GCN2- and ATF4-independent process following AA limitation. For cFOS in particular, AA-responsive transcription was dependent on the RAS-RAF-MEK-ERK arm of MAPK signaling. Association of the ERK-phosphorylated transcription factor p-ELK1 with the cFOS promoter was increased after activation of the AAR, whereas the abundance of total or phosphorylated SRF was not increased. The latter result is consistent with additional data distinguishing the induction by AA limitation from that of serum replenishment. The results indicate that the ELK1 transcription factor and ETS genomic sequences must be added to the list of AA-responsive genomic signaling mechanisms that contribute to the overall AAR program in mammalian cells. Furthermore, this work extends our understanding of the role that MAPK pathways play during amino acid stress.

2. Materials and methods

2.1. Reagents

Actinomycin D (ActD) (#A1410), thapsigargin (TG) (#T9033), and tetracycline (TET) (#T3258) were from Sigma-Aldrich Co. (St. Louis, MO). All PCR primers used were obtained from Sigma-Aldrich and are listed in Table 1. The siRNA siGENOME SMARTpool constructs for non-targeting siRNA Pool #2 (siCtrl) (#D-001206-14-05), siH-RAS (#M-004142-00-0005), siK-RAS (#M-005069-00-0005), siN-RAS (#M-003919-00-0005), si-ERK1 (#L-003592-00-0005), and siERK2 (#L-003555-00-0005), siELK1 (#L-003885-00-0005) were purchased from Dharmacon/Thermo Scientific. Transient siRNA transfections with 25 nM for each siRAS member or 50 nM each for siERK1 + siERK2 (a total of 100 nM) were performed in 12-well plates according to the manufacturer's protocol using DharmaFECT4 Transfection Reagent (T-2004-02) 48-72 h prior to activating the AAR. The following inhibitors were diluted in dimethylsulfoxide. MEK inhibitor (PD98059, #P215) and c-RAF inhibitor (GW5074, #G6416) were obtained from Sigma-Aldrich. The p38 inhibitor (SB203580, #559389) was from EMD Millipore and the JNK inhibitor (SP600125, #S1076) was from Selleck Chemicals (Houston, TX).

Table 1
qPCR Primers and shRNA Sequences (Human).
Primers were obtained from Sigma-Aldrich and shRNA constructs from Open Biosystems/Thermo Scientific.

qPCR Primers:	
GAPDH mRNA	Sense, TTGGTATCGTGGAAGGACTC
	Anti-sense, ACAGTCTTCTGGGTGGCAGT
cFOS mRNA	Sense, GGAGGAGGGAGCTGACTGATA
	Anti-sense, GGCAATCTCGGTCTGCAA
cFOS hnRNA	Sense, ATGGAGGTGATGGCAGACACTTTTAC
	Anti-sense, TCTTATTCCTTTCCCTTCGGATTCTC
ASNS mRNA	Sense, GCAGCTGAAAGAAGCCCAAGT
	Anti-sense, TGTCTTCCATGCCAATTGCA
ATF4 mRNA	Sense, GGGACAGATTGGATGTTGGAGA
	Anti-sense, ACCCAACAGGGCATCCAAGT
GCN2 mRNA	Sense, GAAATGGTAAACATCGGGCAAACTC
	Anti-sense, TTCACAAGAGCCAGGAGAATCTTCAC
ERK1 mRNA	Sense, CGCTTCCGCCATGAGAATGTC
	Anti-sense, CAGGTCAGTCTCCATCAGGTCCTG
ERK2 mRNA	Sense, CGTGTTGCAGATCCAGACCATGAT
	Anti-sense, TGGACTTGGTGTAGCCCTTGGAA
ATF3 mRNA	Sense, GAGCGGAGCCTGGAGCAAAA
	Anti-sense, GGGGACGATGGCAGAAGCACT
CHOP mRNA	Sense, CATCACCACACCTGAAAGCA
	Anti-sense, TCAGCTGCCATCTCTGCA
EGR1 mRNA	Sense, AGAAGGACAAGAAAGCAGACAAAAGTGT
	Anti-sense, GGGGACGGGTAGGAAGAGAG
cJUN mRNA	Sense, TTCTATGACGATGCCCTCAACGC
	Anti-sense, GCTCTGTTTCAGGATCTTGGGGTTAC
CAT1 mRNA	Sense, TCATCTGGAGGCAGCCCGAG
	Anti-sense, CATCATGAGATAGACGTTCACGAAGATG
IL-8 mRNA	Sense, TCTCTTGGCAGCCTTCCTGATTTC
	Anti-sense, GGGGTGGAAAGGTTTGGAGTATGT
KRAS mRNA	Sense, CTAGAACAGTAGACACAAAACAGG
	Anti-sense, CGAACTAATGTATAGAAGGCATC
HRAS mRNA	Sense, TACGGCATCCCCTACATCGAGAC
	Anti-sense, CACCAACGTGTAGAAGGCATCCTC
NRAS mRNA	Sense, GAGTTACGGGATTCCATTCATTGAAAC
	Anti-sense, TGGCGTATTTCTCTTACCAGTGTGTAAAA
ELK1 mRNA	Sense, CTGACCCCATCCCTGCTTCCTA
	Anti-sense, GAAGTGAATGCTAGGAGGCAGCG
ChIP Primers:	
P1	Forward: TTTCACCTCTGCCTGTGACAGGG,
	Reverse: GGGGATTCGTGGAACTGGGC
P2	Forward: CCATCCCCGAAACCCCTCAT,
	Reverse: GCGTGTCCTAATCTCGTGAGCATTT
P3	Forward: GTGGTTGAGCCCGTGACGTTTA,
	Reverse: TCTTGGCTTCTCAGATGCTCGC
P4	Forward: GTAAGGCAGTTTCATTGATAAAAAGCGAG,
	Reverse: CACTTGCTTGAAAGGGGGTTTGTTATA
P5	Forward: CCAACCTGCTGAAGGAGAAGGAAA,
	Reverse: GATCAAGGGAAGCCACAGACATCTC
P6	Forward: GCATTGTGGTTTCTGGTTTCTCTAATACC,
	Reverse: CCCACTTCCGCCCACTATAAACTG
Anti-sense shRNA:	
shCtrl (non-silencing)	CUUACUCUCGCCCAAGCGAGAG
shATF4 (TRIPZ)	UAAACUUUCUGGGAGAUGG
shGCN2 (GIPZ)	UCAUUGCAAUCUUCAUCCU

2.2. Cell culture

HepG2 and a HepG2 subclone (C3A) human hepatocellular carcinoma cells, HC-04 immortalized human hepatocytes, U87 human glioblastoma cells, and HEK293T human embryonic kidney cells were cultured in high glucose (4.5 g/L) Dulbecco's modified Eagle's medium (DMEM, pH 7.4) supplemented with 10% fetal bovine serum, 1X non-essential AAs, 2 mM glutamine, 100 μg/ml streptomycin sulfate, 100 units/ml penicillin G, and 0.25 μg/ml amphotericin B in a 37 °C incubator with 100% humidity and 5% CO2. For experimental treatment, cells were plated at a density

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