Role of the CCAAT/Enhancer-binding Protein NFATc2 Transcription Factor Cascade in the Induction of Secretory Phospholipase A2 *S

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Inflammatory cytokines such as interleukin-1 and tumor necrosis factor-α modulate a transcription factor cascade in the liver to induce and sustain an acute and systemic defense against foreign entities. The transcription factors involved include NF-κB, STAT, and CCAAT/enhancer-binding protein (C/EBP). Whether the NFAT group of transcription factors (which was first characterized as playing an important role in cytokine gene expression in the adaptive response in immune cells) participates in the acute-phase response in hepatocytes is not known. Here, we have investigated whether NFAT is part of the transcription factor cascade in hepatocytes during inflammatory stress. We report that interleukin-1 or tumor necrosis factor-α increases expression of and activates NFATc2. C/EBP-mediated NFATc2 induction is temporally required for expression of type IIA secretory phospholipase A2. NFATc2 is also required for expression of phospholipase D2 and the calcium-binding protein S100A3. Thus, a C/EBP-NFATc2 transcription factor cascade provides an additional means to modulate the acute-phase response upon stimulation with inflammatory cytokines.

The inflammatory response is a part of a protective means to restore cellular homeostasis and to maintain normal physiological processes after invasion by foreign entities (reviewed in Refs. 1–3). In reaction to tissue injury and infection, inflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) are released to mediate an acute and systemic defense to restore cellular homeostasis. Both immune and nonimmune cells are actively involved in these processes. For example, differentiation of CD4 T cells into a specific T-helper cell lineage and increased production of plasma proteins in hepatocytes are part of these protective changes.

Upon stimulation with inflammatory cytokines, the expression of plasma proteins, proteases, and procoagulants in hepatocytes is increased in part by pre-existing and newly synthesized transcription factors (4, 5). Proteins involved in this transcription factor cascade include NF-κB, STAT (signal transducer and activator of transcription), and CCAAT/ enhancer-binding protein (C/EBP) family members. Cooperative action mediated by NF-κB and C/EBP is also important for the induction of early phase inflammatory-responsive genes (6–8).

In addition to the transcription factors that induce plasma proteins and proteases involved in the complement and coagulation cascades, early phase responsive genes also include transcription factors C/EBPβ and C/EBPδ (9). The induction of C/EBPβ and C/EBPδ suggests an autoregulation in gene expression among the members of the C/EBP family. The newly synthesized C/EBP proteins may then sustain and/or induce additional anti-inflammatory target genes to uphold transcription activation initiated by the inflammatory cytokines. Whether the expression of other transcription factors, in addition to C/EBP proteins, is induced and participates in the later stage of the inflammatory response to provide necessary metabolic changes to re-establish cellular homeostasis remains elusive. Understanding the basic mechanism of activation of the transcription factor cascade mediated by inflammatory cytokines will shed new light on the treatment of chronic inflammation such as sepsis and atherosclerosis.

The NFAT (nuclear factor of activated T cell) group of proteins was first characterized to play an important role in cytokine gene expression in immune cells (reviewed in Refs. 10–12). Subsequent studies demonstrated that NFAT participates in multiple physiological processes. Recently, NFAT activity has been demonstrated in cardiac hypertrophy, adipocyte differentiation, and learning and memory (13–18). Despite the critical function of NFAT in the adaptive immune response in T and B cells (reviewed in Refs. 19 and 20), the role of NFAT in the acute-phase response in hepatocytes is not known.

Four distinct genes encoding closely related, calcium/calcineurin-regulated NFAT proteins (NFATc1/NFATc/NFAT2, NFATc2/NFATp/NFAT1, NFATc3/NFAT4/NFATx, and NFATc4/NFAT3) have been identified (reviewed in Refs. 21 and 22). Alternative mRNA splicing of these four genes further generates multiple NFAT polypeptides. The function of these alternatively spliced NFAT isoforms remains elusive. However, all NFAT family members contain a highly conserved N-terminal NFAT homology domain for calcium/calcineurin regulation and a C-terminal Rel homology region for DNA binding. The presence of conserved regulatory domains suggests functional redundancy among different NFAT family members, although specific regulation mediated by individual NFAT family members on selective targets remains elusive.

The purpose of this study was to examine the role of NFAT in hepatocytes during inflammatory stress. Because inflammatory cytokines mediate acute and systemic responses by regulating transcription factor expression/activation, we ask whether NFAT is part of the transcription factor cascade during inflammatory stress. Here, we report the induction and activation of NFATc2, but not other NFAT family members, by the inflammatory cytokine IL-1 or...
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TNF-α. Mechanistically, IL-1 induces C/EBP binding to NFATc2 transcription loci and mediates gene expression. C/EBP-mediated NFATc2 induction is temporally required for the expression of type II A secretory phospholipase A$_2$ (sPLA$_2$-IIA). NFATc2 is also required for the expression of phospholipase D$_1$ (PLD$_1$) and the calcium-binding protein S100A3. The C/EBP-NFATc2 transcription factor cascade provides an additional means to modulate the acute-phase response in the liver.

EXPERIMENTAL PROCEDURES

Reagents—The human NFATc2 (−1 to −2000 bp) and sPLA$_2$-IIA (−1 to −1200 bp) gene promoters were amplified from human genomic DNA and subcloned into the pG3L3-luciferase reporter plasmid (Promega) using the KpnI and Xhol sites and the Mhel and Xhol sites, respectively. Deletions and mutations in the NFATc2 and sPLA$_2$-IIA promoters were generated by PCR. The expression vectors for C/EBPα, C/EBPβ, C/EBPγ, and NFATc2 have been described (13, 23). The human NFATc2-specific short hairpin RNA (shRNA) was obtained from Open Biosystems (GenBank accession numbers NM_012340). Anti-sPLA$_2$-IIA polyclonal antibody and a human sPLA$_2$-IIA enzyme-linked immunosorbent assay kit were purchased from Cayman Chemical Co. Anti-NFATc2 antibody was generously provided by Dr. Nancy Rice (antibody 1777) (24). Control rabbit and mouse IgG and anti-NFAT, anti-C/EBP, and anti-YY1 antibodies were purchased from Santa Cruz Biotechnology, Inc., and/or Affinity BioReagents. Anti-β-actin Monoclonal antibody was purchased from Sigma. Anti-acetylated histone H3 polyclonal antibody was obtained from Upstate.

Cell Culture—HepG2 hepatoma cells were cultured in minimum Eagle’s medium. HEK293 and Huh7 cells were cultured in Dulbecco’s modified Eagle’s medium. Primary hepatocytes from C57BL/6 mouse liver were prepared by collagenase digestion and cultured in Dulbecco’s modified Eagle’s medium in the presence of 1 μM dexamethasone and 10 μg/ml insulin as described (25). All media were also supplemented with 10% fetal calf serum, 2 mM 1-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Cells were transfected using Lipofectamine (Invitrogen). Cells were stimulated with IL-1 (4 ng/ml) or TNF-α (5 ng/ml) for the indicated times before harvest. To generate NFATc2 knockdown HepG2 cells, NFATc2 shRNA was transfected and selected under puromycin (0.1 mg/ml) for stably transfected clones.

A similar protocol was followed to generate HepG2 cells expressing a dominant-negative (dn) NFAT inhibitor, except that cells were selected for neomycin resistance (1 mg/ml). Huh7 cells stably integrated with NFAT-luciferase reporter plasmids encoding triple repeats of the peroxisome proliferator-activated receptor-γ2 (PPARγ2) proximal or distal NFAT-binding elements were similarly selected for neomycin resistance. Results were obtained with both pooled and single-cell clones. Representative of isolated single-cell clones are presented.

Microarray Analysis—Microarray analysis was designed to profile gene expression upon administration of the inflammatory cytokine IL-1. The role of calcineurin/NFAT in IL-1-regulated genes was also investigated by pretreatment with the calcineurin inhibitor cyclosporin A (CsA). Glass oligonucleotide arrays manufactured in our genomic facility were used. These glass oligonucleotide arrays contain 34,580 70-mer probe sets with controls (Qiagen Human Genome Oligo Set Version 3.0). Total RNA isolated from IL-1-treated (12 h) or untreated HepG2 cells in the presence and absence of CsA was labeled with Cy5- or Cy3-labeled dUTP and hybridized to glass oligonucleotide arrays. Gene annotation and function were determined using the Database for Annotation, Visualization and Integrated Discovery at NIAMD, National Institutes of Health (available at david.niaid.nih.gov/david/) with assistance from our microarray facility. Candidate genes were selected with a difference of >1.5-fold and are reported (see supplemental material).

Chromatin Immunoprecipitations—Nuclear factors that were associated with chromatin in IL-1-stimulated or unstimulated HepG2 cells were cross-linked to DNA using 1% formaldehyde. Cells were harvested, and cross-linked chromatin was sheared by sonication. Sonicated cell lysate was immunoprecipitated using antibodies as indicated. IgG was used as a control. DNA present in the immunoprecipitated chromatin was isolated using protein G-agarose, after reversed cross-link and proteinase K digestion, and PCR was performed to examine the presence of the NFATc2 gene promoter (5’-GAATTGGCGTCAGTCTCT-3’ and 5’-ATAAGCATTGTAATACATC-3’), the sPLA$_2$-IIA promoter (5’-AGGATAGGCTCTGCTTTTTCAGGTGTTAG-3’ and 5’-GAGATTGGGAAACC-TTACGTGTCTGACTG-3’), or the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter (5’-AGCTCCAGGCTCAGACCTTT-3’ and 5’-AAGAAGATGCAGGCTGACTG-3’).

DNA Affinity Binding Assays—Streptavidin-agarose-precleared HepG2 cell extracts were incubated overnight with double-stranded biotinylated oligonucleotides (10 pmol) at 4 °C in the presence of 1 μg of poly(dI-dC). Protein-DNA complexes were precipitated with 20 μl of streptavidin-agarose at 4 °C for an additional 2 h. After three washes with Triton lysis buffer (20 mM Tris (pH 7.4), 137 mM NaCl, 2 mM EDTA, 1% Triton X-100, 25 mM β-glycerophosphate, 1 mM sodium vanadate, 2 mM sodium pyrophosphate, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin), bound proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. C/EBP or NFAT in the DNA precipitates was detected by immunoblot analysis. Competition was performed using a 5-fold excess of the non-biotinylated oligonucleotide encoding the consensus C/EBP-binding (5’-TG-CAGATTTGCCAATCTGCA-3’) or NF-κB-binding (5’-CAGAGGG-GACTTTCCGAGGAC-3’) sequence.

Gel Mobility Shift Assays—Nuclear extracts were prepared from HepG2 cells as described previously (13, 14). Double-stranded oligonucleotides for gel mobility shift assays were labeled with [α-32P]dCTP. The sequence for the C/EBP-binding site of the NFATc2 promoter is 5’-CCCTATTCAGTTGTTGCCAAATGTACCTG-3’. The sequence for the NFAT-binding site of the IL-2 antigen receptor-responsive element is 5’-AGAAGGAGAAACACTGTTCATACAAAGG-3’. The sequences for the NFAT-binding sites of sPLA$_2$-IIA are 5’-GAGGATTGGGAAACCTTACGTGTCAGTA-3’ (proximal), 5’-GAGGGCGTTTTCGAGTTTCCATGAGTA-3’ (middle), and 5’-AGGATAGGCTCTGCTTTTCCAAGTTTAG-3’ (distal).

Binding reactions for gel mobility shift assays were carried out at room temperature in gel shift buffer (1 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM HEPES (pH 7.9), 50 mM NaCl, 15 mM β-mercaptoethanol, 10% glycerol, 0.1 μM bovine serum albumin, and 1 μg/ml poly(dI-dC) for 30 min. Protein-DNA complexes were separated on 5% nondenaturing polyacrylamide gels in 25 mM Tris, 200 mM glycine, and 1 mM EDTA and visualized by autoradiography. For supershift analysis, antibody was preincubated with a nuclear extract for 30 min at before addition of the labeled probe. For competition analysis, an excess amount of the unlabeled oligonucleotide (50 fmol) was incubated together with the labeled probe before addition of the nuclear extract.

Semiquantitative Reverse Transcription (RT)-PCR—Total RNA was isolated from HepG2 cells using TRIzol reagent (Invitrogen). The isolated RNA (2 μg) was reverse-transcribed with mouse mammary tumor virus reverse transcriptase (Promega), and the cDNA prepared was amplified by PCR, separated, and visualized by agarose gel electrophoresis. The intensity of PCR products was quantified using ImageQuant Version 5.0 software (Adobe Systems, Inc.). The primers used for PCR amplification were as
follows (all primers target human cDNA sequence unless indicated otherwise): NFATc2, 5'-AGAAACTCGGCTCCAGAATCC-3' and 5'-H11032 TGGTGCCCTCATGTTT-3'; NFATc3, 5'-GCAGATCTTGAGCCAGATGAT-3' and 5'-H11032 CCGGTAGGATGGCTCAAGAG-3'; sPLA2-IIA, 5'-ATGAAGACCCTCCTACTGTTG-GCA-3' and 5'-H11032 TCAGCAACGAGGGGTGCTCCCTCT-3'; PLD 1, 5'-TGCTCTACAGGCAATCATGC-3' and 5'-H11032 GCACTGTAGCCGA-GTCCTC-3'; and S100A3, 5'-ACACCCGAACTGGTCAACTC-3' and 5'-H11032 GAGCGCACATACTCCACAAA-3'. The expression of GAPDH was used as a control (5'-H11032 ACCTGACCTGCCGTCTAGAA-3' and 5'-H11032 TCCACCACCCTGCTGCTGA-3').

Luciferase Assays—The NFATc2 or sPLA2-IIA promoter plasmid (0.3 μg) was cotransfected with the pRSV-β-galactosidase control plasmid (0.1 μg) or the C/EBP or NFATc2 expression plasmid (0.3 μg) as indicated. Luciferase and β-galactosidase activities were measured 48 h after transfection. The data are presented as relative luciferase activity calculated as the ratio of luciferase activity to β-galactosidase activity (means ± S.E., n = 4).

RESULTS

Inflammatory Cytokine IL-1 or TNF-α Induces NFATc2 Expression—To investigate the role of NFAT during inflammatory stress in hepatocytes, we tested whether inflammatory cytokines such as IL-1 and TNF-α regulate NFAT expression. Stimulation of HepG2 hepatoma cells with IL-1 or TNF-α increased NFATc2 expression (Fig. 1A). Accumulation of NFATc2 protein was evident after 8 h of IL-1 or TNF-α stimulation, although NFATc2 induction was detected as early as 4 h. However, the expression of other NFAT family members (NFATc1, NFATc3, and NFATc4) was similar in treated and untreated cells. It is interesting that NFATc2 exhibited a modest increase in electrophoretic mobility,
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whereas the electrophoretic mobility of other NFAT family members was similar. As reported previously, the expression of C/EBPβ was elevated upon IL-1 or TNF-α stimulation. The expression level of β-actin was used as a control. In addition to HepG2 cells, a similar induction of NFATc2 (but not other NFAT family members) upon IL-1 stimulation was observed in HuH7 hepatoma cells, HEK293 embryonic kidney cells, Caco-2 colorectal carcinoma cells, and human dermal microvascular endothelial cells (data not shown). These data demonstrate that inflammatory cytokines such as IL-1 and TNF-α increase NFATc2 expression.

Next, we tested whether the induction of NFATc2 by IL-1 requires de novo mRNA transcription and protein synthesis. Administration of either the transcription inhibitor actinomycin D or the protein synthesis inhibitor cycloheximide blocked the induction of NFATc2 by IL-1 (Fig. 1B). These data demonstrate that IL-1 modulates NFATc2 at least at the mRNA level.

To determine the amount of NFATc2 induction, we performed semi-quantitative RT-PCR analysis using various PCR amplification cycles (Fig. 1C). IL-1 stimulation increased NFATc2 expression by ~5-fold in HepG2 cells. However, the expression of NFATc3 and GAPDH was similar in the presence and absence of IL-1 stimulation. These data demonstrate that IL-1 selectively increases NFATc2 expression in HepG2 cells.

Next, we confirmed the induction of NFATc2 by IL-1 in primary hepatocytes. Stimulation of primary hepatocytes with IL-1 increased NFATc2 mRNA expression (Fig. 1D). However, the expression of GAPDH was similar in the presence and absence of IL-1 stimulation.

We also confirmed the induction of NFATc2 protein expression in primary hepatocytes by immunoblot analysis (Fig. 1E). Similar to what was observed in HepG2 cells, NFATc2 protein accumulated upon IL-1 treatment in primary hepatocytes. A similar amount of β-actin was used as a control. Together, these data demonstrate that inflammatory cytokines such as IL-1 increase NFATc2 expression in primary hepatocytes as well as in hepatoma cell lines.

NFAT Activity Is Not Required for IL-1-induced NFATc2 Expression—An increase in NFAT electrophoretic mobility has been used as a hallmark for NFAT nuclear accumulation and activation (13, 26, 27). Next, we confirmed that the modest increase in electrophoretic mobility (Fig. 1) promoted NFATc2 nuclear accumulation. We performed subcellular fractionation and determined the amount of NFATc2 in nuclear extracts upon IL-1 stimulation (Fig. 2A). Immunoblot analysis demonstrated increased NFATc2 accumulation in the nuclear extracts upon IL-1 stimulation. However, the amount of NFATc3 and YY1 in the nuclear extracts remained similar upon IL-1 stimulation. These data demonstrate that IL-1 stimulation specifically increases and activates NFATc2.

A previous study demonstrated that the expression of NFATc1 is induced upon NFAT activation (28). Autoregulation in the expression of NFAT proteins may be analogous to the induction of C/EBPβ and C/EBPβ expression upon C/EBP activation during inflammatory stress. Thus, we examined whether the IL-1-mediated induction of NFATc2 expression requires NFAT activation using the immunosuppressant drug CsA or FK506 analog FK520, which inhibit calcineurin phosphatase and block NFAT-mediated transcription. Administration of CsA or FK520 had a minimal effect on NFATc2 induction upon IL-1 stimulation (Fig. 2B), although IL-1-mediated NFAT activation was CsA-sensitive, as there was a modest decrease in NFATc2 electrophoretic mobility. In addition, luciferase reporter assay demonstrated that IL-1-induced NFAT transcription activity was blocked by CsA (Fig. 2C). These data demonstrate that IL-1 increases the expression of and activates NFATc2. Unlike the induction of NFATc1, that of NFATc2 is not NFAT-dependent.

C/EBP Binds to and Regulates the NFATc2 Promoter—Sequence analysis indicated that the NFATc2 promoter encodes sequences resembling the consensus DNA-binding element for C/EBP (Fig. 3A). To investigate whether C/EBP is recruited to the NFATc2 loci upon IL-1 stimulation, we performed chromatin immunoprecipitation assays using anti-C/EBP antibodies (Fig. 3A). In resting untreated HepG2 cells, there was minimal C/EBP association with the NFATc2 loci. Stimulation with IL-1 increased the association of C/EBPα or C/EBPβ with the NFATc2 promoter. However, neither C/EBPα nor C/EBPβ was associated with the GAPDH promoter. These data demonstrate that C/EBP associates with the NFATc2 promoter upon IL-1 stimulation in vivo.

To investigate binding of C/EBP to the NFATc2 promoter in vitro, we performed DNA affinity binding assays (Fig. 3B). Using biotinylated oligonucleotides encoding the putative C/EBP-binding site of the NFATc2 promoter, we found both C/EBPα and C/EBPβ in the DNA precipitates. Specific binding of C/EBP was demonstrated by successful competition by the consensus C/EBP sequence, but not by the NF-kB-binding elements. In addition, C/EBP was not present in the precipitates in the absence of oligonucleotides. These data demonstrate that C/EBP binds to the NFATc2 promoter.
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We further established binding of C/EBP to the NFATc2 promoter by gel mobility shift assays. Similar C/EBP-DNA complexes were detected using either a 32P-radiolabeled NFATc2 C/EBP-binding site or a consensus C/EBP DNA-binding element as a probe (Fig. 3C). Competition assays demonstrated that the C/EBP-DNA complex from the NFATc2 C/EBP-binding site was competed by the unlabeled NFATc2 C/EBP-binding site (i.e. self-competition) or the consensus C/EBP-binding element, but not by the mutant C/EBP-binding site. The C/EBP-DNA complex from the NFATc2 C/EBP-binding site was also supershifted by anti-C/EBP antibodies. A similar C/EBP-DNA complex was also detected using the consensus C/EBP-binding element as a probe. Together, these data demonstrate that C/EBP binds to the NFATc2 promoter.

We also examined the role of C/EBP in NFATc2 promoter activity. Administration of IL-1 increased transcription activity mediated by the NFATc2 promoter (−1 to −2000 bp) (Fig. 3D). Similarly, the expression of C/EBPβ increased NFATc2 promoter activity (Fig. 3E). However, mutation or deletion (−1 to −1000 bp) of the C/EBP-binding site reduced transcription activity mediated by the NFATc2 promoter upon IL-1 stimulation or C/EBPβ coexpression. The expression of C/EBPβ caused minimal activation of the promoterless pGL3-Basic control. Together, these data demonstrate that IL-1 stimulation promotes C/EBP association with the NFATc2 promoter. Recruitment of C/EBP is likely to play a role in NFATc2 induction by IL-1 stimulation.

C/EBP Activity Is Required for IL-1-mediated NFATc2 Induction—Next, we investigated the requirement for C/EBP in the induction of NFATc2. Among the C/EBP family members, at least C/EBPα and C/EBPβ bind to the NFATc2 promoter (Fig. 3). To inhibit C/EBP-mediated gene transcription, we expressed the transcription repressor C/EBPγ (also known as Ig/enhancer-binding protein) in cells. C/EBPγ lacks a transcription activation domain (29), although it contains a similar basic leucine zipper DNA-binding motif and interacts with other C/EBP family members. Thus, C/EBP-mediated transcription activation is abrogated by C/EBPγ. The expression of C/EBPγ blocked IL-1-mediated NFATc2 promoter activity (Fig. 4A). Notably, the expression of C/EBPγ reduced IL-1-mediated NFATc2 expression (Fig. 4B). A similar reduction in NFATc2 promoter activity and expression was also found upon expression of a dominant-negative C/EBP inhibitor (A–C/EBP) (data not shown). These data demonstrate that C/EBP regulates NFATc2 promoter activity and gene transcription. Activation of the C/EBP-
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NFATc2 transcription factor cascade may be critical in mediating inflammatory stress.

IL-1-induced Transcription Regulation of sPLA₂-IIA—What are the targets regulated by the C/EBP-NFATc2 transcription factor cascade? We performed microarray analysis and determined the gene expression profile that was regulated by IL-1. Candidate genes with a difference of >1.5-fold were considered significant and are reported (see supplemental material). The ranges of fold induction and suppression are 1.51–19.47 and 0.70 to 0.05 in the absence of CsA and 1.52–4.38 and 0.70 to 0.04 in the presence of CsA. Microarray analysis indicated that the expression of 245 genes were induced and that of 820 genes was reduced after 12 h of IL-1 stimulation. Upon administration of CsA, 32 induced genes and 312 reduced genes elicited by IL-1 were affected. These data demonstrated that calcineurin/NFAT signaling contributes to IL-1-mediated transcription regulation.

Among the IL-1-induced genes, the expression of sPLA₂-IIA was reduced upon CsA treatment (see supplemental material). We elected to further investigate sPLA₂-IIA transcription regulation because sPLA₂-IIA contributes to arachidonic acid release upon phospholipid hydrolysis (30–34). Released arachidonic acid plays a critical role in the production of leukotrienes and prostaglandins through the lipoxygenase and cyclooxygenase pathways, respectively. Our focus on sPLA₂-IIA induction was further catalyzed by the role of NFAT in inducible cyclooxygenase COX2 expression (35–39), which may constitute an inflammatory cascade in leukotriene and prostaglandin production through transcription regulation of the sPLA₂-IIA and COX2 genes by NFAT. In addition, sPLA₂-IIA exhibits antibacterial properties (9) and characteristics of receptor ligands (42). Thus, understanding the regulation of sPLA₂-IIA expression during inflammatory stress is important.

To examine the role of calcineurin/NFAT in IL-1-induced sPLA₂-IIA expression, we performed immunoblot analysis using cell extracts prepared from IL-1-stimulated HepG2 cells pretreated or not with the calcineurin inhibitor CsA or FK520. IL-1 stimulation increased sPLA₂-IIA protein expression (Fig. 5A). Pretreatment with CsA or FK520 abolished IL-1-mediated sPLA₂-IIA induction. Similarly, administration of the calcineurin inhibitor reduced sPLA₂-IIA mRNA levels upon IL-1 stimulation (Fig. 5B). IL-1-regulated sPLA₂-IIA induction at the mRNA level was further confirmed using the transcription inhibitor actinomycin D or the protein synthesis inhibitor cycloheximide. The induction of sPLA₂-IIA by IL-1 was sensitive to pretreatment with actinomycin D or cycloheximide (Fig. 5C). Together, these data demonstrate that NFATc2 regulates sPLA₂-IIA expression at least at the mRNA level.

We also ascertained the role of the calcineurin/NFAT signaling pathway in sPLA₂-IIA induction using HepG2 cells stably expressing the dnNFAT inhibitor (Fig. 6A). We have demonstrated previously that dnNFAT blocks calcineurin targeting (40). Therefore, NFAT nuclear accumulation and subsequent transcription are abolished. Stimulation with IL-1 or TNF-α increased sPLA₂-IIA expression in parental control HepG2 cells (Fig. 6, B and C). However, the expression of sPLA₂-IIA...
protein and mRNA was attenuated in dnNFAT-expressing cells. Together, these data demonstrate that the calcineurin/NFAT signaling pathway contributes to sPLA₂-IIA induction.

**Temporal Requirement for the Calcineurin/NFAT Signaling Pathway in IL-1-mediated sPLA₂-IIA Induction**—Next, we examined the kinetic profile of sPLA₂-IIA induction. Administration of IL-1 increased sPLA₂-IIA expression and subsequent secretion into the media (Fig. 5D). Notably, a marked increase in sPLA₂-IIA expression was observed in the later phase (after 12 h) of IL-1 stimulation, although induction was detected as early as 6 h after stimulation. To delineate the temporal requirement for the calcineurin/NFAT pathway in IL-1-mediated sPLA₂-IIA induction, we administered the calcineurin inhibitor CsA at various times during IL-1 stimulation (Fig. 7A). Pretreatment with CsA (i.e. administration of CsA 1 h before stimulation with IL-1 (i.e. -1 h)) reduced sPLA₂-IIA protein (Fig. 7B) and mRNA (Fig. 7C) expression after 24 h of IL-1 stimulation. Secretion of sPLA₂-IIA into the media was also reduced (Fig. 7B). Administration of CsA after 8 or 12 h of IL-1 stimulation reduced sPLA₂-IIA expression to a lesser extent. Administration of CsA in the last 8 h (i.e. 16 h after the initial IL-1 stimulation), before determining sPLA₂-IIA expression and secretion after 24 h of IL-1 stimulation, exhibited a minimal inhibitory effect. These data demonstrate that the calcineurin/NFAT pathway is temporally required for the expression of sPLA₂-IIA upon inflammatory stimulation.

**NFAT Binds to and Regulates the sPLA₂-IIA Promoter**—What is the molecular basis of NFAT in sPLA₂-IIA expression? Sequence analysis indicated that there are three putative NFAT-binding sites in the sPLA₂-IIA promoter. These three putative NFAT-binding sites are located at −200 (proximal), −250 (middle), and −500 (distal) bp from the sPLA₂-IIA transcription start region (Fig. 8A). To examine whether NFAT binds to these sites, we performed gel mobility assays using nuclear extracts prepared from NFATc2-expressing COS cells (Fig. 8B) or IL-1-treated HepG2 cells (Fig. 8C). Distinct NFAT-DNA complexes were detected in all three sPLA₂-IIA NFAT-binding sites. The specificity of the NFAT-DNA complexes was determined by competition analysis using consensus NFAT-binding elements from the IL-2 and PPARγ2 promoters. Supershift analysis further demonstrated the presence of NFAT in the protein-DNA complexes. In addition, we performed chromatin immunoprecipitation assays to demonstrate binding of NFATc2 and acetylated histone H3 (but not NFATc3) to the sPLA₂-IIA promoter (Fig. 8D). Neither NFATc2 nor NFATc3 was present in...
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A sequence comparison of NFAT-binding sites (proximal, middle, and distal) in the sPLA₂-IIA promoter with consensus NFAT-binding sites in the IL-2 and PPARγ promoters. The consensus binding sequences for NFAT partners (AP-1 and C/EBP) are also indicated. B and C, NFAT binds to the sPLA₂-IIA promoter in vitro. Nuclear extracts prepared from COS cells expressing NFATc2 (B) or IL-1-induced HepG2 cells (C) were incubated with 32P-labeled sPLA₂-IIA NFAT-binding oligonucleotides (proximal, middle, and distal). Formation of the NFAT-DNA complex was examined by gel mobility shift assays and visualized by autoradiography. The specificity of the NFAT-DNA complex was demonstrated by antibody supershift analysis (anti-NFATc2 antibody; see asterisks). D, association of NFATc2 with the sPLA₂-IIA promoter. Nuclear proteins associated with chromatin in HepG2 cells were cross-linked with 1% formaldehyde. Chromatin was sheared and immunoprecipitated with anti-NFATc2, anti-NFATc3, or anti-acetylated histone H3 (Ac-H3) antibody. DNA in the NFATc2, NFATc3, or acetylated histone 3 precipitate was purified, and the presence of the sPLA₂-IIA and GAPDH promoters was determined by PCR. Rabbit IgG was used as a control. E, NFAT positively regulates sPLA₂-IIA promoter activity. The sPLA₂-IIA promoter (−1 to −1200 bp) was cotransfected with the NFATc2 expression vector (gray bars) or empty vector (white bars) in HepG2 cells. Cell extracts were harvested after 36 h of transfection, and the measured luciferase activity was normalized to β-galactosidase activity and is presented (n = 4). The effect of deletion of NFAT-binding elements (−1 to −150 bp) on sPLA₂-IIA promoter activity was also examined. A promoterless luciferase reporter plasmid (pGL3-Basic) was used as a control.

The GAPDH promoter. These data demonstrate that NFAT binds to the sPLA₂-IIA promoter.

Binding of NFATc2 may regulate sPLA₂-IIA gene transcription. Using luciferase reporter assays, we tested whether NFAT regulates the sPLA₂-IIA gene promoter. The expression of NFATc2 increased sPLA₂-IIA promoter activity (Fig. 8E). Deletion of NFAT-binding sites to −150 bp abrogated increases in sPLA₂-IIA promoter activity. Together, these data demonstrate that NFAT binds to and regulates the sPLA₂-IIA gene promoter.

Induction of NFATc2 Is Required for IL-1-increased sPLA₂-IIA Expression—Because NFAT binds to and regulates sPLA₂-IIA expression (Fig. 8), we hypothesized that the induction of NFATc2 by IL-1 is required for sPLA₂-IIA expression. One approach to study sPLA₂-IIA expression in the absence of NFATc2 is to utilize NFATc2 null mice, especially because NFATc2 null mice in a C57BL/6 background seem to exhibit hypersensitivity upon lipopolysaccharide stimulation. However, sPLA₂-IIA is not expressed in mouse liver (41, 42). It is expressed only in mouse small intestine. Indeed, sPLA₂-IIA is expressed only in the small intestine in a certain mouse background (43). Thus, the available NFATc2 null mouse (mainly in mouse strains C57BL/6 and 129/SvJ, which are defective in sPLA₂-IIA expression) is not appropriate for analyzing the induction of sPLA₂-IIA upon inflammatory cytokine challenge.

To circumvent the lack of sPLA₂-IIA expression in mouse liver and to address the role of NFATc2 in sPLA₂-IIA expression, we exploited RNA interference technology using shRNA targeting NFATc2 in HepG2 cells (Fig. 9A). Cells stably transfected with shRNA targeting NFATc2 exhibited reduced expression of NFATc2 compared with the parental control HepG2 cells. The expression of NFATc3 and NFATc4 was similar, however. Notably, targeted reduction of NFATc2 abolished IL-1-induced NFATc2 expression. These data demonstrate that shRNA specifically reduces NFATc2 expression in resting and IL-1-stimulated HepG2 cells.

Next, we examined the expression of sPLA₂-IIA in NFATc2 knockdown and parental control HepG2 cells (Fig. 9, B–E). Stimulation with IL-1 (Fig. 9B) or TNF-α (Fig. 9C) induced sPLA₂-IIA protein expression.

3 T. T. C. Yang, R. Y. L. Yu, and C.-W. Chow, unpublished data.
in parental control HepG2 cells. The expression of sPLA2-IIA in NFATc2 knockdown cells was abolished, however. Targeted reduction of NFATc2 expression also decreased sPLA2-IIA mRNA expression (Fig. 9D). Notably, kinetic analysis demonstrated that the induction and subsequent secretion of sPLA2-IIA were abolished in NFATc2 knockdown cells (Fig. 9E).

Together, these data demonstrate that the induction of NFATc2 by IL-1 is required for sPLA2-IIA expression.

Induction of NFATc2 Is Required for IL-1-increased PLD1 and S100A3 Expression—Our microarray analysis revealed that, in addition to sPLA2-IIA, the IL-1-mediated induction of PLD1 and the calcium-binding protein S100A3 was sensitive to CsA inhibition (see supplemental material). In conjunction with sPLA2-IIA, PLD1 hydrolyzes phospholipids (e.g. phosphatidylcholine) to lysophosphatidic acid (LPA), arachidonic acid, and choline (44, 45). LPA is a potent G-protein-coupled receptor agonist that may further modulate inflammatory signals (46, 47). In association with annexins, S100A3 may modulate membrane dynamics and intracellular trafficking (e.g. exocytosis, endocytosis, and membrane architecture and remodeling) (48). Unlike the role of sPLA2-IIA, that of PLD1 and S100A3 during inflammatory stress has yet to be demonstrated.

Sequence analysis indicated that there are nine and five putative NFAT-binding sites (GGAAA) in the PLD1 and S100A3 promoters, respectively. In the PLD1 gene, these NFAT-binding sites are located at /H11002 /H11002 /H11002 /H11002 /H11002 /H11002 /H11002 /H11002 /H11002 and parental control HepG2 cells were stimulated (+) or not (−) with IL-1. The expression levels of PLD1 and S100A3 mRNAs were determined by semiquantitative RT-PCR. The expression level of GAPDH was used as a control.

Next, we investigated the role of NFATc2 in IL-1-induced PLD1 and S100A3 expression. RT-PCR analysis demonstrated that IL-1 increased PLD1 and S100A3 expression (Fig. 10A). However, the induction of PLD1 and S100A3 was abrogated by CsA. Targeted reduction of
NFATc2 by shRNA also eliminated the induction of PLD$_1$ and S100A3 upon IL-1 stimulation (Fig. 10B). Together, these data demonstrate that, similar to sPLA$_2$-IIA expression, IL-1-induced PLD$_1$ and S100A3 expression is NFATc2-dependent.

**DISCUSSION**

**Role of NFAT in the Inflammatory Response in Nonimmune Cells**—In this study, we have demonstrated that inflammatory cytokines induce NFATc2 expression in hepatocytes. C/EBP-mediated NFATc2 induction plays a critical role in the expression of sPLA$_2$-IIA, an important phospholipase in the regulation of arachidonic acid release and the subsequent leukotriene and/or prostaglandin pathway. These results provide a mechanism for NFATc2 expression and expand the repertoire of NFAT function to the acute-phase response in the liver. Previously, we demonstrated that IL-6 increases NFATc2 expression, which then promotes IL-4 expression and subsequent Th2 cell differentiation (49, 50). Together, these results demonstrate that the transcription factor NFAT contributes to the restoration of cellular homeostasis in both nonimmune and immune cells upon the induction of inflammatory stress by the invasion of foreign entities.

Our microarray analysis demonstrated that 32 genes induced by IL-1 stimulation are sensitive to CsA administration. Among the CsA-sensitive IL-1-induced genes, we have confirmed the regulation of sPLA$_2$-IIA and PLD$_1$ by NFATc2. In addition, the calcium-binding protein S100A3 is also regulated by NFATc2. These data indicate that calcineurin/NFAT signaling can contribute to feedback regulation of phospholipid hydrolysis and calcium mobilization upon IL-1 challenge.

Our microarray analysis also demonstrated that transcription repression mediated by IL-1 is sensitive to CsA. The expression of 312 genes suppressed by IL-1 stimulation is sensitive to CsA. These genes include transcription factors, signaling mediators, and secretory proteins. Further characterization of these targets is warranted to provide a comprehensive analysis of the role of calcineurin/NFAT during inflammatory stress in hepatocytes.

In addition to hepatocytes, adipocytes have also been implicated to play a role in the acute-phase response (51, 52). How adipocytes contribute to the acute-phase response remains to be determined. Adipocytes may modulate glucose and lipid metabolism in response to invasion by foreign entities. Induction and subsequent secretion of adipose-specific peptides/hormones (adipokines) may also participate in part in antibacterial/antifungal function and macrophage recruitment. Indeed, analogous to the role of NFAT in cytokine gene transcription in immune cells, NFAT also regulates adipokine gene expression in adipocytes. Hence, adipocytes may provide an additional means to enable necessary metabolic changes during inflammatory stress.

It is intriguing that the transcription factor C/EBP plays a critical role in gene transcription in both hepatocytes and adipocytes (4, 53, 54). C/EBP-mediated NFATc2 induction in hepatocytes may be extrapolated to adipocytes during inflammatory stress. Similar NFATc2 induction may also be required for the role of NFAT in adipocytes (13, 14, 18, 55), especially because a C/EBP transcription factor cascade is important for the initiation of adipocyte differentiation (56–61). In addition, the extent of adiposity and insulin resistance correlates with the degree of inflammation (62–64). Thus, the C/EBP-NFATc2 transcription factor cascade may provide a mechanism for gene transcription regulation in hepatocytes and adipocytes.

In addition to HepG2 hepatoma cells, Huh7 hepatoma cells, HEK293 embryonic kidney cells, Caco-2 colorectal carcinoma cells, and human dermal microvascular endothelial cells also exhibited NFATc2 induction upon IL-1 stimulation (data not shown). The induction of NFATc2 may be a conserved mechanism in nonimmune cells to combat invasion by foreign entities in response to IL-1 stimulation. It is interesting that components of the IL-1 signaling pathway are highly conserved in multiple species (65, 66), whereas NFAT is expressed only in vertebrates (67). Integration of these two apparent evolutionarily independent pathways may provide diverse responses to restore cellular homeostasis in vertebrates.

**NFAT Versus NF-κB in the Inflammatory Response**—Previous studies demonstrated that IL-1 activates NF-κB and mediates immediate responses during inflammatory stress (68–71). The induction of target genes such as iκBα and MCP1 is mediated by NF-κB within 1 h of stimulation with inflammatory cytokines (70, 72, 73). Activation of NF-κB is subsequently down-regulated by the newly synthesized iκB. Here, we have demonstrated that NFAT temporally modulates sPLA$_2$-IIA expression upon stimulation with inflammatory cytokines. These data suggest that NF-κB and NFAT may mediate distinct responses upon inflammatory cytokine stimulation.

It is interesting that both NFAT and NF-κB are Rel domain-containing transcription factors (67, 74). NFAT interacts with NFAT partners (e.g. Fos)un-AP-1 complex, C/EBP, and GATA proteins), whereas NF-κB binds as a homo- or heterodimer to mediate gene transcription. The distinct regulation mediated by NFAT and NF-κB in gene transcription may account for the temporally discrete redundant role in response to inflammatory cytokine stimulation.

With respect to NFAT partners, the striking differences in various NFAT-DNA complexes formed in the sPLA$_2$-IIA promoter (Fig. 8) imply that NFAT cooperates with distinct nuclear factors to mediate gene transcription during inflammatory stress. These nuclear factors may include C/EBP, STAT, and Forkhead members (4, 5, 75, 76). Dimerization of NFAT may also contribute to transcription cooperation (77, 78). The presence of distinct NFAT-DNA complexes is also found in the PPAR2 gene promoter upon adipocyte differentiation (13, 14). Because dissociation of NFAT-DNA complexes is regulated by NFAT partners (14, 55), interaction with unique nuclear factor is likely to modulate the extent of NFAT activation to provide a dose-dependent regulation of inflammation and adipocyte differentiation.

The dose-dependent regulation of NFAT activation may also be mediated by the co-localization of multiple but distinct NFAT-DNA complexes in the sPLA$_2$-IIA and PPAR2 gene promoters upon transcription. Hence, a graded response, in part due to interaction with various NFAT partners and/or the number of recruited NFAT-DNA complexes in the active transcription loci, may arise and contribute to a threshold regulation of NFAT target genes. A goal for future research will be to identify NFAT targets regulated by the threshold mechanism.

**sPLA$_2$-IIA Induction and Disease**—In this study, we have demonstrated that induction of NFATc2 regulates sPLA$_2$-IIA expression. Two possible mechanisms may account for the induction of sPLA$_2$-IIA by NFATc2. First, NFATc2 may selectively activate sPLA$_2$-IIA. In this mechanism, NFAT DNA-responsive elements in the sPLA$_2$-IIA gene may selectively bind NFATc2, but not other NFAT family members. Further characterization of these sPLA$_2$-IIA NFAT elements may shed new light on the molecular basis of NFATc2-specific target genes. Alternatively, newly synthesized NFATc2 may be selectively activated and account for the sPLA$_2$-IIA induction. This may be due in part to differential regulation by upstream NFAT kinases and/or phosphatases to modulate NFAT subcellular localization and DNA binding, transactivation, and/or degradation.

Second, accumulation of NFAT proteins per se may account for the induction of sPLA$_2$-IIA. Hence, the purpose of increased NFATc2 expression is to provide sufficient proteins to be activated by and to
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References

1. Burger, D., and Dayer, J. M. (2002) Ann. N. Y. Acad. Sci. 966, 464–473
2. Ramadori, G., and Christ, B. (1999) Semin. Liver Dis. 19, 141–155
3. Baumann, H., and Gaudel, J. (1994) Immunol. Today 15, 74–80
4. Takiguchi, M. (1998) Int. J. Exp. Pathol. 79, 369–391
5. Poli, V. (1998) J. Biol. Chem. 273, 29279–29282
6. Matusaka, T., Fujikawa, K., Nishio, Y., Mukaida, N., Matushima, K., Kishimoto, T., and Akira, S. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10193–10197
7. Betts, J. C., Chesshire, J. K., Akira, S., Kishimoto, T., and Woo, P. (1993) J. Biol. Chem. 268, 25624–25631
8. Lee, Y. M., Mian, L. H., Chang, C. J., and Lee, S. C. (1996) Mol. Cell. Biol. 16, 4257–4263
9. Lekstrom-Himes, J., and Xanthopoulous, K. G. (1998) J. Biol. Chem. 273, 28554–28588
10. Horsley, V., and Pavlath, G. K. (2002) J. Cell Biol. 156, 771–774
11. Crabtree, G. R., and Olson, E. N. (2002) Cell 109, (suppl.) 567–579
12. Hossain, P. G., Chen, L., Nardone, J., and Rao, A. (2003) Genes Dev. 17, 2205–2232
13. Tang, Y. T. C., Xiong, Q., Enslen, H., Davis, R. J., and Chow, C. W. (2002) Mol. Cell. Biol. 22, 3892–3904
14. Yang, Y. T. C., and Chow, C. W. (2003) J. Biol. Chem. 278, 15874–15885
15. Molkentin, J. D., and Dorn, I. G., II (2001) Ann. Rev. Physiol. 63, 391–426
16. Graef, I. A., Meremelstein, P. G., Stankunas, K., Neilson, J. R., Detschower, K., Tisen, R. W., and Crabtree, G. R. (1999) Nature 401, 703–708
17. Molkentin, J. D., Lu, R., Antos, C. L., Markham, B., Richardson, J., Robbins, J., Grant, S. R., and Olson, E. N. (1998) Cell 93, 215–228
18. Hori, I. C., Kim, J. H., Rooney, J. W., Spiegelman, B. M., and Glimcher, L. H. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 15537–15541
19. Rao, A., and Avni, O. (2000) Br. Med. Bull. 56, 969–984
20. Diehl, S., and Rincon, M. (2002) Mol. Immunol. 39, 531–536
21. Crabtree, G. R. (2001) J. Biol. Chem. 276, 2313–2316
22. Rao, A., Luo, C., and Hogan, P. G. (1997) Annu. Rev. Immunol. 15, 707–747
23. Hoey, T., Sun, Y. L., Williamson, K., and Xu, X. (1995) Immunity 2, 461–472
24. Lyakh, L., Ghosh, P., and Rice, N. R. (1997) Mol. Cell. Biol. 17, 2475–2484
25. Neufeld, D. S. (1997) Methods Mol. Biol. 75, 145–151
26. Chow, C. W., Rincon, M., Cavanagh, J., Dickens, M., and Davis, R. J. (1997) Science 278, 1638–1641
27. Lee, C., Shaw, K. T., Carew, J., Viola, J. P., Luo, C., Perrino, B. A., and Rao, A. (1996) J. Biol. Chem. 271, 10884–10891
28. Zhou, B., Cron, R. Q., Wu, B., Genin, A., Wang, Z., Liu, S., Robson, P., and Baldwin, H. S. (2002) J. Biol. Chem. 277, 10704–10711
29. Parkin, S. E., Baer, M., Copeland, T. D., Schwartz, R. C., and Johnson, P. F. (2002) J. Biol. Chem. 277, 25683–25727
30. Gilroy, D. W., Newsom, J., Sawmynaden, P., Willoughby, D. A., and Croxall, J. D. (2006) FASEB J. 19, 489–498
31. Touqui, L., and Alouei-El-Azhneri, M. (2001) Curr. Mol. Med. 1, 793–754
32. Bingham, C. O., III, and Austen, K. F. (1999) Proc. Assoc. Am. Physicians 111, 516–524
33. Buckland, A. G., and Wilton, D. C. (2000) Biochim. Biophys. Acta 1488, 71–82
34. Lambeau, G., and Lazdunski, M. (1999) Trends Pharmacol. Sci. 20, 162–170
35. de Gregorio, R., Iniguez, M. A., Fresno, M., and Alemany, S. (2000) J. Biol. Chem. 275, 7003–7009
36. Hernandez, G. L., Volpert, O. V., Iniguez, M. A., Martinez-Martinez, S., Grau, F., Fresno, M., and Redondo, J. M. (2001) J. Biol. Chem. 277, 1930–1939
37. Iniguez, M. A., Martinez-Martinez, S., Punzon, C., Redondo, J. M., and Fresno, M. (2000) J. Biol. Chem. 275, 23627–23635
38. Granja, A. G., Nogal, M. L., Hurtado, C., Vila, V., Carrascosa, A. L., Salas, M. L., Fresno, M., and Revilla, Y. (2004) J. Biol. Chem. 279, 53736–53746
39. Duque, J. F., Fresno, M., and Iniguez, M. A. (2005) J. Biol. Chem. 280, 8666–8673
40. Chow, C. W., Rincon, M., and Davis, R. J. (1999) Mol. Cell. Biol. 19, 2300–2307
41. Valentin, E., Ghomashchi, F., Gelb, M. H., Lazdunski, M., and Lambeau, G. (1999) J. Biol. Chem. 274, 31195–31202
42. Valentin, E., and Lambeau, G. (2000) Biochim. Biophys. Acta 1488, 59–70
43. Kennedy, B. P., Payette, P., Mudgett, J., Vadás, P., Pruzanski, W., Kwan, M., Tang, C., Rancourt, D. E., and Croomlish, W. A. (1996) J. Biol. Chem. 270, 22378–22385
44. Aoki, J. (2004) Semin. Cell Dev. Biol. 15, 477–489
45. Ishi, I., Fukushima, N., Xu, Y., and Chun, J. (2004) Annu. Rev. Biochem. 73, 321–354
46. Graier, M. H., and Goetzl, E. J. (2002) Biochim. Biophys. Acta 1582, 168–174
47. Svetlov, S. I., Sautin, Y. Y., and Crawford, J. M. (2002) Biochim. Biophys. Acta 1582.
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251–256
48. Gerke, V., Creutz, C. E., and Moss, S. E. (2005) Nat. Rev. Mol. Cell. Biol. 6, 449–461
49. Rincón, M., Anguita, J., Nakamura, T., Fikrig, E., and Flavell, R. A. (1997) J. Exp. Med. 185, 461–469
50. Diel, S., Chow, C.-W., Weiss, L., Palmetshofer, A., Twardzik, T., Rounds, L., Serfling, E., Davis, R. J., Anguita, J., and Rincón, M. (2002) J. Exp. Med. 196, 39–49
51. Lin, Y., Lee, H., Berg, A. H., Lisanti, M. P., Shapiro, L., and Scherer, P. E. (2000) J. Biol. Chem. 275, 24255–24263
52. Coppack, S. W. (2001)
53. MacDougald, O. A., and Mandrup, S. (2002)
54. Ramji, D. P., and Foka, P. (2002)
55. Yang, T. T. C., Xiong, Q., Graef, I. A., Crabtree, G. R., and Chow, C.-W. (2005)
56. Cao, Z., Umek, R. M., and McKnight, S. L. (1991)
57. Yeh, W. C., Cao, Z., Classon, M., and McKnight, S. L. (1995)
58. Cowherd, R. M., Lyle, R. E., and McGehee, R. E., Jr. (1999)
59. Spiegelman, B. M. (1997)
60. Rangwala, S. M., and Lazar, M. A. (2000)
61. Lin, Y., Rajala, M. W., Berger, J. P., Moller, D. E., Barzilai, N., and Scherer, P. E. (2001)
62. Rothermel, B. A., Vega, R. B., and Williams, R. S. (2003) Trends Cardiovasc. Med. 13, 15–21
63. Lyon, C. J., Law, R. E., and Hsueh, W. A. (2003)
64. Grimble, R. F. (2002)
65. Akira, S., Takeda, K., and Kaisho, T. (2001)
66. Dunne, A., and O’Neill, L. A. (2003) Sci. STRE 2003, RE3
67. Graef, I. A., Gastier, J. M., Francke, U., and Crabtree, G. R. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 5740–5745
68. Baldwin, A. S., Jr. (1996) Annu. Rev. Immunol. 14, 649–683
69. Karin, M., and Ben-Neriah, Y. (2000) Annu. Rev. Immunol. 18, 621–663
70. Hayden, M. S., and Ghosh, S. (2004) Genes Dev. 18, 2195–2224
71. Bowie, A., and O’Neill, L. A. (2000) Biochem. Pharmacol. 59, 13–23
72. Rothwarf, D. M., and Karin, M. (1999) Sci. STRE 1999, RE1
73. Li, Q., and Verma, I. M. (2002) Nat. Rev. Immunol. 2, 725–734
74. Wolfe, S. A., Zhou, P., Dotsch, V., Chen, L., You, A., Ho, S. N., Crabtree, G. R., Wagner, G., and Verdin, G. L. (1997) Nature 385, 172–176
75. Hromas, R., and Costa, R. (1995) Crit. Rev. Oncol. Hematol. 20, 129–140
76. Streett, K. L., Wustefeld, T., Klein, C., Manns, M. P., and Trautwein, C. (2001) Cell. Mol. Biol. (Noisy-Le-Grand) 47, 661–673
77. Griffin, M. J., Stroud, J. C., Bates, D. L., von Koenig, K. D., Hardin, J., Chen, L., Jin, L., Sliz, P., Macfian, F., Rao, A., Hogan, P. G., and Harrison, S. C. (2003) Nat. Struct. Biol. 10, 800–806
78. Lin, L., Sliz, P., Chen, L., Macfian, F., Rao, A., Hogan, P. G., and Harrison, S. C. (2003) Nat. Struct. Biol. 10, 807–811
79. Ryeom, S., Greenwald, R. J., Sharpe, A. H., and McKeon, F. (2003) Nat. Immunol. 4, 874–881
80. Rothermel, B. A., Vega, R. B., and Williams, R. S. (2003) Trends Cardiovasc. Med. 13, 15–21
81. Vega, R. B., Rothermel, B. A., Weinheimer, C. J., Kovacs, A., Naseem, R. H., Bassel-Duby, R., Williams, R. S., and Olson, E. N. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 669–674
82. Rothermel, B., Vega, R. B., Yang, J., Wu, H., Bassel-Duby, R., and Williams, R. S. (2000) J. Biol. Chem. 275, 8719–8725
83. Kingsbury, T. J., and Canningham, K. W. (2000) Genes Dev. 14, 1595–1604
84. Holvoet, P. (2004) Acta Cardiol. 59, 479–484
85. Jaross, W., Ecker, R., and Menschikowski, M. (2002) Eur. J. Clin. Invest. 32, 383–393
86. Bak, C. E., Anderson, C. M., Hanglow, A. C., and Morgan, D. W. (1991) Agents Actions 34, 81–83
87. Lin, M. K., Farewell, V., Vadás, P., Bookman, A. A., Keystone, E. C., and Pruzanski, W. (1996) J. Rheumatol. 23, 1162–1166
88. Coussens, L. M., and Werb, Z. (2002) Nature 420, 860–867
89. Clevers, H. (2004) Cell 118, 671–674
90. Sved, P., Scott, K. F., McLeod, D., King, N., Singh, J., Tsatsralis, T., Nikolov, B., Boulas, J., Nallan, L., Gelb, M. H., Sainjovic, M., Graham, G. G., Russell, P. J., and Dong, Q. (2004) Cancer Res. 64, 6934–6940
91. Laye, J. P., and Gill, J. H. (2003) Drug Discov. Today 8, 710–716
92. Wendum, D., Swiecik, M., Rigau, V., Boelle, P. Y., Sebbagh, N., Parc, R., Masliah, J., Trugnan, G., and Flejou, J. F. (2003) Med. Pathol. 16, 130–136
93. Cushing, J., Tugwell, P., Weinblatt, M., and Yocum, D. (1999) J. Rheumatol. 26, 1176–1186
94. Kountouras, J., Zavos, C., and Chatzopoulos, D. (2004) J. Cell. Mol. Med. 8, 317–328