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Role of calpain in adipocyte differentiation

(3T3-L1 adipocytes/calpastatin/cAMP/*N*-acetyl-Leu-Leu-norleucinal/CCAAT)

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ABSTRACT **Evidence is presented that the calcium-activated protease, calpain, is required for differentiation of 3T3-L1 preadipocytes into adipocytes induced by methylisobutylxanthine (a cAMP phosphodiesterase inhibitor), dexamethasone, and insulin. Calpain is expressed by preadipocytes and its level falls during differentiation. Exposure of preadipocytes to the calpain inhibitor *N*-acetyl-Leu-Leu-norleucinal or overexpression of calpastatin, a specific endogenous inhibitor of calpain, blocks expression of adipocyte-specific genes, notably the CCAAT/enhancer-binding protein (C/EBP)α gene, and acquisition of the adipocyte phenotype. The inhibitor disrupts the differentiation-inducing effect of methylisobutylxanthine (by means of the cAMP-signaling pathway), but is without effect on differentiation induced by dexamethasone or insulin. *N*-acetyl-Leu-Leu-norleucinal, or overexpression of calpastatin, inhibits reporter gene expression mediated by the C/EBPα gene promoter by preventing C/EBPβ, a transcriptional activator of the C/EBPα gene, from binding to the promoter. These findings implicate calpain in the transcriptional activation of the C/EBPα gene, a process required for terminal adipocyte differentiation.**

Terminal differentiation involves the coordinate expression of a cell type-specific set of genes that gives rise to a new and unique phenotype. The mouse 3T3-L1 preadipocyte system provides a well-characterized cell culture model for the study of adipocyte-specific terminal differentiation (1–7). During adipocyte differentiation, CCAAT/enhancer-binding protein (C/EBP)α functions as a plieotropic transcriptional activator of numerous adipocyte genes (2, 3). The promoters of these genes possess C/EBP regulatory elements that mediate transactivation by C/EBPα. Moreover, the proximal promoter of the C/EBPα gene itself contains a C/EBP regulatory element that mediates transactivation by other members of the C/EBP family, notably C/EBPβ, which is expressed before C/EBPα in the differentiation program (8–11). C/EBPα has an essential function in the differentiation process as indicated by the fact that forced expression of C/EBPα is sufficient to trigger differentiation of 3T3-L1 preadipocytes in the absence of the exogenous inducers usually required (11, 12). Moreover, blocking expression of C/EBP with antisense C/EBPα RNA prevents adipocyte differentiation (13).

Growth-arrested (confluent) 3T3-L1 preadipocytes can be induced to differentiate by treatment with a combination of inducers including methylisobutylxanthine (MIX), dexamethasone (DEX), insulin, and fetal bovine serum (FBS) (14), hereafter referred to as the MDI protocol. On exposure to these inducers, preadipocytes reenter the cell cycle and undergo several rounds of cell division (15), which mimics differentiation-associated mitotic clonal expansion (2). Clonal expansion is followed by the coordinate expression of a subset of genes that confer the morphological and biochemical adipocyte phenotype (3, 16, 17). Whereas each component of the MDI protocol is capable of inducing a limited degree of differentiation, the combination of all three inducers produces a maximal rate and extent of differentiation (1–3). These inducers activate multiple signal transduction pathways, which may be redundant or which may involve crosstalk between pathways, because any one of the three components alone can promote differentiation, albeit weakly. At least three second-messenger pathways have been implicated in the induction of differentiation of 3T3-L1 preadipocytes, i.e., the cAMP-dependent protein kinase—the glucocorticoid—and the insulin-like growth factor-I/insulin-signaling pathways (2, 3, 18–20).

The goal of this research is to identify the intermediaries in the second messenger-signaling pathways that link the pathway(s) to the genes that activate (or derepress) the differentiation program. In this paper, we provide evidence that calpain functions between the cAMP-signaling pathway and the expression of the C/EBPα gene, a critical step in the adipocyte differentiation program. Calpain is a ubiquitous heterodimeric protease, known to be involved in signal transduction (21–23) and the differentiation of myoblasts, osteoblasts, and chondrocytes (24–26), which, like adipocytes, are derived from a common mesenchymal progenitor (3).

EXPERIMENTAL PROCEDURES

Cell Culture. 3T3-L1 preadipocytes were cultured in DMEM containing 10% calf serum until 2 days after reaching confluence (day 0). Differentiation was induced on day 0 as previously described (14) by addition of 0.5 mM MIX, 1 μM DEX, 1 μg/ml insulin, and 10% FBS in DMEM. After 48 h (day 2), the medium was replaced with DMEM containing 1 μg/ml insulin and 10% FBS. After day 4, the cells were fed every other day with 10% FBS in DMEM. Where indicated, 26 μM *N*-acetyl-Leu-Leu-norleucinal (ALLN) was added at the time of induction to inhibit calpain activity.

Transfection. 3T3-L1 preadipocytes were transiently cotransfected on day 0 by using calcium phosphate-precipitated DNA with a C/EBPα promoter-luciferase construct (27), with or without ALLN, or with or without a pCDNA-I-hemagglutinin (HA)-tagged human calpastatin expression vector (generously provided by M. Maki, Nagoya University, Nagoya, Japan.). Cells were then maintained in medium containing DMEM and 10% calf serum for 24 h. Differentiation was induced as described above. Cell lysates were prepared 24 h after induction and assayed for luciferase activity, which was normalized to that of MDI-treated cells.

The tetracycline (TET)-regulated (TET-OFF) Expression System, (GIBCO) was used to induce expression of calpastatin

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Abbreviations: MIX, methylisobutylxanthine; DEX, dexamethasone; MDI protocol, MIX/DEX/insulin protocol; ALLN, *N*-acetyl-Leu-Leu-norleucinal; C/EBP, CCAAT/enhancer-binding protein; FBS, fetal bovine serum; TET, tetracycline; HA, hemagglutinin; EMSA, electrophoretic mobility-shift assay; TET-OFF, tetracycline regulated.

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in 3T3-L1 cells. Fifty percent confluent preadipocytes were cotransfected with a pTet-Splice-human-calpastatin expression vector, a pTAK expression vector, and a SV40-NEO expression vector. Clonal cell lines were selected for G418 resistance and were maintained in medium containing tetracycline. Cells harboring the transgenes were induced to differentiate, as described above, in the presence or absence of tetracycline until day 7.

Analysis of RNA. Total RNA was isolated by the acid-phenol guanidine isothiocyanate method (28). Total cellular RNA (10 μg) was separated by electrophoresis in 1.2% agarose gels containing formaldehyde, transferred overnight to Hybond-N (Amersham), and covalently crosslinked to the membrane with ultraviolet light. Membranes were prehybridized as described (29). cDNA fragments of Scd-2 (a 235-bp *Pst*I fragment), 422/aP2 (a 700-bp *Pst*I fragment), C/EBPα (full-length C/EBPα cDNA), calpain (a 1,215-bp *Eco*RI fragment), actin (full-length actin cDNA), and 18S rRNA (a 236-bp *Pst*I fragment) were used to probe for the corresponding mRNAs. Probes were labeled to high specific activity by random priming (30).

Immunoblotting. Cell monolayers were washed with PBS, lysed in 1% SDS/60 mM Tris·Cl, pH 6.8, buffer and incubated at 100°C for 10 min. Lysates were subjected to SDS/PAGE (10% acrylamide) and transferred to Immobilon-P membranes (Millipore). Membranes were incubated with a rabbit calpain antiserum (generously provided by E. Hogan and N. Banik, Medical University of South Carolina, Charleston, SC) or HA mouse antiserum to detect epitope (HA)-tagged human calpastatin followed by a horseradish peroxidase-conjugated secondary antibody (Sigma). Immunoreactive protein was visualized by enhanced chemiluminescence (Amersham).

Electrophoretic Mobility Shift Assay (EMSA). Nuclear extracts were prepared as described (31). EMSA was performed as described by MacDougald *et al.* (29) by using a double-stranded oligonucleotide probe (20 bp) corresponding to the C/EBP site in the C/EBPα promoter.

Staining of Cytoplasmic Triglyceride with Oil Red O. Cell monolayers were washed twice with PBS and fixed with 3.7% formaldehyde for 2 min. A 0.2% Oil Red O–isopropanol solution was added to the cell monolayers for 1 h, which were then washed several times with distilled water (32).

RESULTS AND DISCUSSION

Recent evidence has implicated calpain in the differentiation of myoblasts, osteoblasts, and chondrocytes (24–26). Because these cell lineages are derived from the same mesenchymal pluripotent progenitor as adipocytes, the possibility was considered that calpain is involved in adipocyte differentiation.

Expression of Calpain During Adipocyte Differentiation. To determine whether calpain is differentially expressed during adipocyte differentiation, 3T3-L1 preadipocytes were subjected to the MDI differentiation protocol. Expression of calpain mRNA and protein was monitored by Northern and Western blotting procedures, respectively, during differentiation. Northern blot analysis revealed that calpain mRNA is expressed by preadipocytes and that the levels decline during differentiation (Fig. 1). The expression of calpain protein during the differentiation program closely followed that of the calpain message (Fig. 1).

Effect of Calpain Inhibitors on Differentiation. To determine whether calpain plays a role in adipocyte differentiation, its catalytic activity was inhibited by exposing 3T3-L1 preadipocytes to a calpain inhibitor, ALLN, during the course of differentiation. Preadipocytes were subjected to the MDI protocol for 48 h (day 0 to day 2) in the presence or absence of ALLN. On day 7 of the differentiation program, cells were fixed and stained with Oil Red O. As illustrated in Fig. 2*A*, control preadipocytes, treated with MDI, accumulated massive amounts of cytoplasmic triglyceride as visualized by Oil Red O staining. Cells treated with MDI and ALLN, however, failed to accumulate significant amounts of cytoplasmic triglyceride exhibiting little Oil Red O staining (Fig. 2*A*). Staining of ALLN-treated 3T3-LI preadipocytes was similar to that of confluent preadipocytes maintained in calf serum for 7 days without differentiation inducers (Fig. 2*A*).

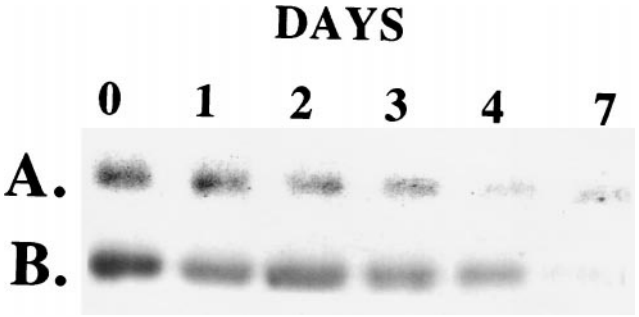

FIG. 1. Expression of calpain during differentiation of 3T3-L1 preadipocytes. Total cellular RNA and protein were isolated from two-day postconfluent (day 0) preadipocytes and various time points after induction of differentiation. Ten μg of RNA was subjected to Northern blot analysis by using a calpain cDNA as probe. Cellular proteins were extracted from cell monolayers and subjected to SDS/PAGE and Western blot analysis by using antisera against calpain.

Because ALLN is not a strictly specific inhibitor of calpain, overexpression of calpastatin, a specific endogenous inhibitor of calpain, was also tested. 3T3-L1 preadipocytes, stably transfected with a TET-OFF calpastatin expression vector, were induced to differentiate in the presence or absence of tetracycline. Results with a representative cell line harboring the expression vector are shown in Fig. 2 *B* and *C*. Cells maintained in medium containing tetracycline (+TET) did not express calpastatin (Fig. 2*C*) and differentiated to the same extent as untransfected cells, as indicated by Oil Red O staining (MDI in Fig. 2*A* and +TET Fig. 2*B*). In contrast, when the same transfected cell line was maintained in medium without tetracycline (-TET), expression of calpastatin was induced (Fig. 2*C*), and differentiation was dramatically curtailed (by >90%; Fig. 2*B*). Thus, specific inhibition of calpain activity by calpastatin prevented differentiation of 3T3-L1 preadipocytes.

3T3-L1 preadipocytes acquire the adipocyte phenotype by expressing a subset of adipocyte genes including the C/EBPα (3), the 422/aP2 (a fatty acid-binding protein) (33), and SCD-2 (stearoyl-CoA desaturase) genes (34). Northern blot analysis was performed to determine whether preadipocytes subjected to the differentiation protocol with ALLN express these adipocyte-specific mRNAs. As shown in Fig. 3*A*, preadipocytes exposed to ALLN for 48 h during induction by the MDI protocol failed to express C/EBPα, aP2/422, or SCD-2 mRNAs, but did express actin mRNA and 18S rRNA, which are known to be constitutively expressed during differentiation. In contrast, cells subjected to the differentiation protocol in the absence of the inhibitor expressed the adipocyte-specific mRNAs; cells maintained in medium without differentiation inducers did not (Fig. 3*A*). The level of actin mRNA in cells treated with ALLN was similar to that of cells maintained in medium without differentiation inducers. [The partial down-regulation of actin mRNA in MDI-treated preadipocytes is known to occur as a consequence of adipocyte differentiation (15)]. Thus, it can be concluded that ALLN inhibits adipocyte-specific gene expression.

To locate the time window during which the differentiation program can be inhibited by ALLN, two-day postconfluent 3T3-L1 preadipocytes were exposed to ALLN for different time intervals during the course of differentiation. It was determined (results not shown) that the action of ALLN is

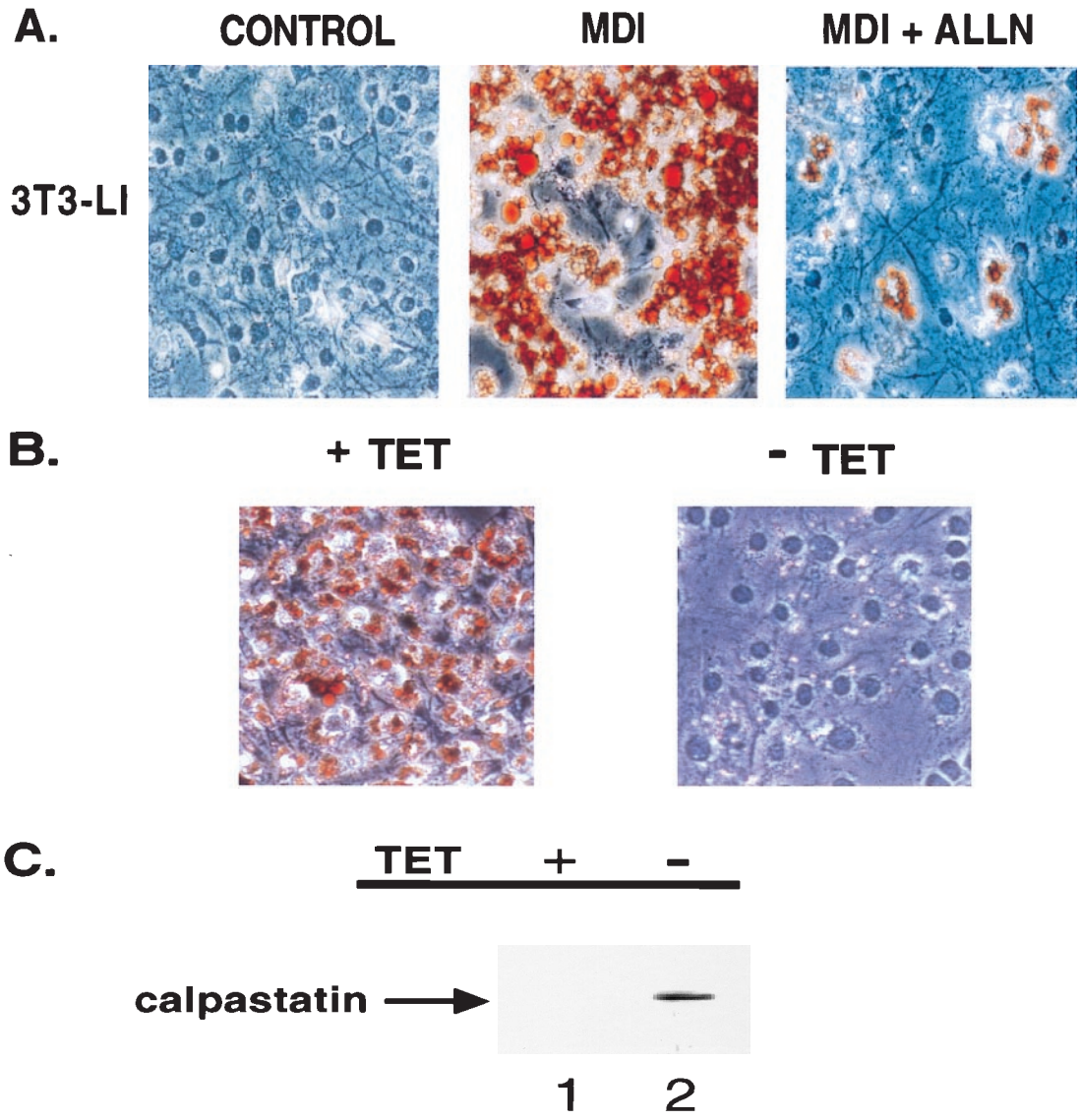

FIG. 2. Effect of inhibition of calpain on differentiation of 3T3-LI preadipocytes. (*A*) Two-day postconfluent (day 0) 3T3-L1 preadipocytes were induced to differentiate by using either the standard MDI protocol (MDI) or MDI and 26 μM ALLN (MDI + ALLN) for 48 h and then stained with Oil Red O on day 7. Confluent preadipocytes maintained in 10% calf serum for 7 days (control) were also stained with Oil Red O on day 7. (*B*) A representative 3T3-L1 cell line (one of four) harboring the human calpastatin gene under the control of a tetracycline-regulated promoter (TET-OFF System) was induced to differentiate as described in *Experimental Procedures*, in the presence (+TET) or absence (-TET) of tetracycline for 7 days. Oil Red O staining was performed to determine the extent of differentiation (*B*) and Western blot analysis was performed with HA mouse antisera to detect the expression of a transfected HA-tagged human calpastatin transgene (*C*) on day 7.

required for only a limited period (between 6 and 24 h after addition of the differentiation inducers) to inhibit differentiation.

The inhibition of differentiation by ALLN is reversible. Preadipocytes treated with ALLN and MDI for 48 h and then allowed to remain in culture for an additional 5 days were again subjected to the differentiation protocol on day 7 (in the absence of the inhibitor). Cells whose differentiation was arrested (by ALLN treatment) retain the capacity to reenter the differentiation program when reinduced, as indicated by their capacity to accumulate cytoplasmic triglyceride (Fig. 3*B*) and express adipocyte-specific mRNAs (results not shown) to the same extent as preadipocytes treated with MDI alone. Cells treated with MDI and ALLN for 48 h and then maintained in 10% FBS for an additional 7 days (MDI + ALLN) did not differentiate. Thus, ALLN arrests differentiation rather than merely delaying its onset, and this arrest is reversible.

Differentiation Inducer-Initiated Signaling Pathway Disrupted by Inhibition of Calpain. To identify the differentiation inducer-initiated signaling pathway disrupted by calpain inhibitors, differentiation was induced with each component of the MDI protocol in the presence or absence of ALLN. Previous studies had shown that exposure of preadipocytes to any one of the inducers alone results in a limited extent of differentiation compared with that obtained with the combination of all three inducers (Table 1). The extent of differentiation of preadipocytes induced by DEX or insulin alone is not affected by ALLN (Table 1); however, differentiation induced by MIX alone is inhibited. These findings indicated that ALLN interferes with a step(s) in the MIX-activated second-messenger pathway that leads to adipocyte differentiation. Because MIX is a cAMP phosphodiesterase inhibitor, the effects of other agents, e.g., forskolin (an adenylate cyclase agonist) or dibutryl-cAMP, which activate the cAMP-signaling pathway, were also examined. ALLN blocked differentiation

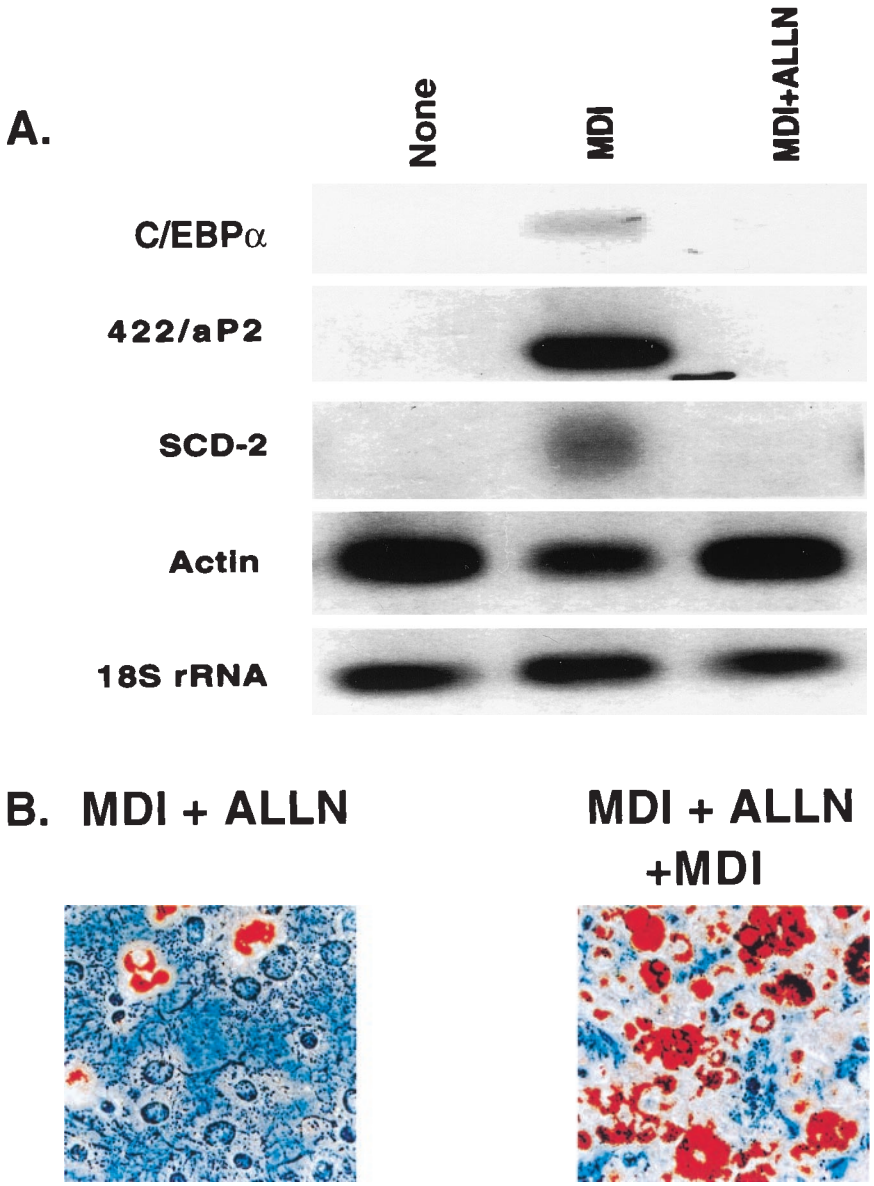

FIG. 3. ALLN blocks adipocyte gene expression and arrests differentiation. (*A*) 3T3-LI preadipocytes were either induced to differentiate by the standard protocol (MDI) or were maintained in 10% calf serum (none) for 7 days. Cells induced to differentiate were either treated or not with 26 μM ALLN for 48 h. Total cellular RNA was isolated from day 7 adipocytes and subjected to Northern blot analysis with cDNA probes for C/EBPα, 422(aP2), SCD-2, actin, and 18S ribosomal RNA. Results are representative of three independent experiments. (*B*) Confluent 3T3-LI preadipocytes, treated with MDI and 26 μM ALLN for 48 h, were maintained to day 7 as described in *Experimental Procedures* and then were either maintained for 7 days in 10% FBS (MDI + ALLN) or induced to differentiate again (with MDI) by the standard protocol (MDI + ALLN + MDI). Cells were fixed and stained with Oil Red O 7 days later (i.e., on day 14).

triggered by forskolin- and dibutyryl-cAMP when these factors replaced MIX in the MDI protocol (results not shown) or when tested alone (Table 1). Therefore, it appears that the target(s) of calpain lies downstream of cAMP in the MIX-induced signaling pathway of the differentiation program.

Inhibition of Calpain Disrupts Activation of the C/EBPα Promoter. Previous studies have shown that blocking expression of C/EBPα with C/EBPα anti-sense RNA prevents adipocyte differentiation (13) and that ectopic expression of C/EBPα in 3T3-L1 preadipocytes is sufficient to trigger adipocyte differentiation (11, 12). Because inhibition of calpain action with ALLN prevents expression of C/EBPα during differentiation (Fig. 3*A*), the possibility was considered that the inhibitor interferes with the transcriptional activation of the C/EBPα gene. To assess this possibility, 3T3-L1 preadipocytes were transfected with a C/EBPα promoter-luciferase transgene, after which the cells were treated with MDI or MDI and ALLN. As shown in Fig. 4, reporter gene expression was markedly decreased (by >75%) by treatment with ALLN. To verify that calpain *per se* is involved in the activation of the C/EBPα gene promoter, the effect of overexpressing calpastatin on C/EBPα promoter-mediated reporter expression was assessed. Expression of calpastatin by MDI-treated preadipocytes also caused substantial (>50%) expression of luciferase (Fig. 4). Thus, inhibition of calpain, either by ALLN treatment or overexpression of calpastatin, curtails expression of a C/EBPα promoter-luciferase transgene. These findings suggest that calpain plays a role in the transcriptional activation of the C/EBPα gene promoter during 3T3-L1 adipocyte differentiation.

The C/EBPα gene promoter possesses a C/EBP binding site (8) that mediates transactivation by members of the C/EBP family of transcription factors including C/EBPβ (35). C/EBPβ is expressed shortly after (within 4 to 6 h) induction

Table 1. ALLN inhibits cAMP-induced adipocyte differentiation

Treatment	Relative extent of differentiation	
	−ALLN	+ALLN
None	−	−
MDI	++++	−
MIX	+++	−
DEX	++	++
INS	+	+
dibutyryl-cAMP	+++	−
Forskolin	+++	−

Two-day postconfluent 3T3-LI preadipocytes were treated or not (None) for 2 days with MDI, MIX, DEX, INS, 100 μM dibutyryl-cAMP (cAMP) or 100 μM forskolin in the absence or presence of 26 μM ALLN. The extent of differentiation, estimated by Oil Red O staining of cytoplasmic triglyceride (+ symbols indicate the relative extent of cytoplasmic triglyceride accumulation) was assessed on day 7, (−) symbol indicates <5% differentiation by day 7. Results are representative of three individual experiments.

of differentiation and is thought to transcriptionally activate the C/EBPα gene, which is expressed shortly thereafter (9, 10). Importantly, C/EBPβ is expressed in the same time window (between 6 and 24 h after induction of differentiation) during which ALLN can inhibit differentiation (see above). Also important is the fact that the cAMP-signaling pathway, which activates expression of C/EBPβ, is the signaling pathway inhibited by ALLN (see above and Table 1). Whereas ALLN has no effect on the expression of C/EBPβ *per se* (results not shown), the inhibitor drastically decreases the ability of both isoforms of C/EBPβ[†] to bind to an oligonucleotide corresponding to the C/EBP regulatory element in the C/EBPα gene promoter (Fig. 5). That the nuclear protein–oligonucleotide complexes detected by EMSA contain C/EBPβ is shown by the nearly complete supershift of these complexes by antibody directed against the C terminus of C/EBPβ. Thus, a calpain is required for C/EBPβ to acquire the capacity to bind to the C/EBPα gene promoter and thereby to activate transcription of the gene.

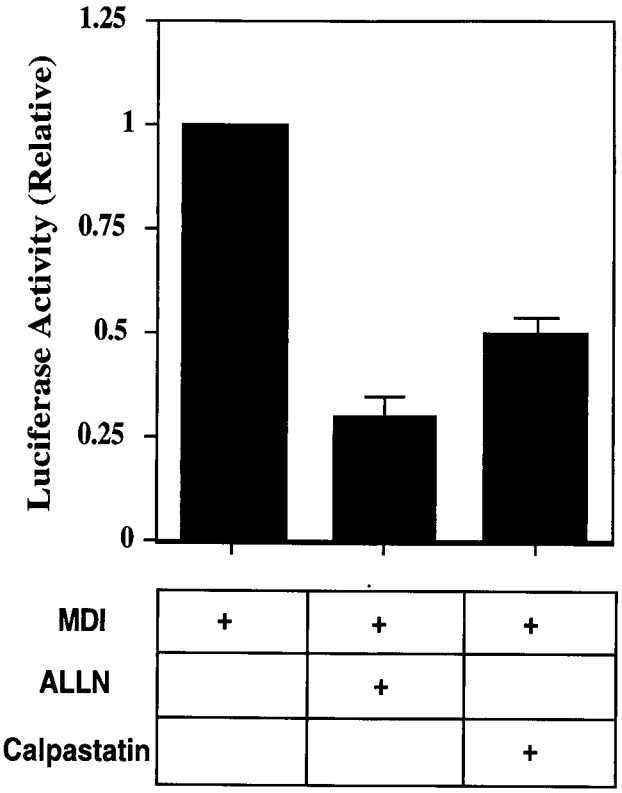

FIG. 4. Inhibition of calpain prevents reporter gene expression mediated by the C/EBPα gene promoter. Two-day postconfluent preadipocytes were transiently cotransfected with a C/EBPα-luciferase expression vector and with or without a CMV-human calpastatin expression vector. Twenty-four hours later, transfected cells were induced to differentiate with medium containing MDI or MDI and 26 μM ALLN (as indicated) for an additional 24 h. Cell lysates were analyzed for total cellular protein and luciferase activity was normalized to values from MDI-treated cells.

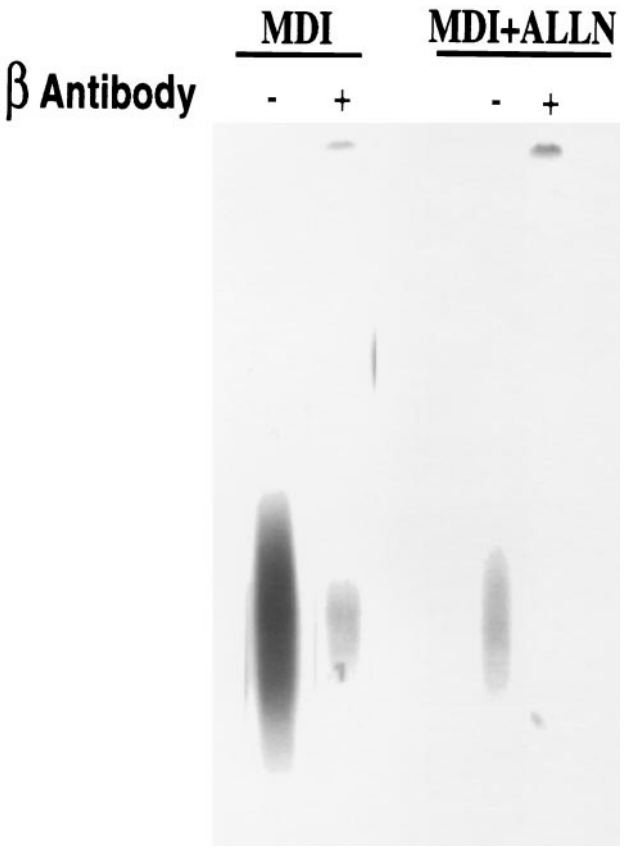

FIG. 5. Calpain is required for acquisition of C/EBPβ binding activity. Nuclear extracts were prepared from preadipocytes treated with MDI or MDI and ALLN for 24 h. EMSA was performed by using an oligonucleotide corresponding to the C/EBP binding site in the C/EBPα promoter and 10 μg of nuclear extract protein. Protein–DNA complexes were resolved on 6% polyacrylamide gels. Supershift analysis was performed by using antiserum against a peptide corresponding to the C terminus of C/EBPβ.

Compelling evidence provided by McKnight's group (9, 10) showed that two of the adipocyte differentiation inducers, i.e., MIX, which increases cellular cAMP, and DEX, a glucocorticoid, initiate a signaling cascade involving members of the C/EBP family of transcription factors. Cyclic AMP rapidly activates expression of C/EBPβ, and glucocorticoid rapidly activates expression of C/EBPδ (10). Both of these C/EBPs can transcriptionally activate the C/EBPα gene promoter (35). In the next segment of the cascade, C/EBPα functions as a plieotropic transcriptional activator of the adipocyte genes that give rise to the adipocyte phenotype (3). In the present paper, we provide evidence that calpain acts at an early step(s) in this cascade and that calpain inhibitors disrupt this step(s) by

[†]C/EBPβ has two isoforms, LAP and LIP, arising from alternative translational start-sites. LAP and LIP bind to DNA either as homo- or heterodimers. Thus, C/EBPβ gives rise to a complex EMSA pattern of protein-oligonucleotide homo- and heterodimers (LAP-LAP, LAP-LIP, and LIP-LIP) by EMSA, as shown in Fig. 5.

preventing the binding of C/EBPβ to the C/EBP regulatory element in the C/EBPα gene promoter. The latter, in turn, would prevent the expression of C/EBPα. The mechanism by which the calpain inhibitors prevent binding of C/EBPβ to the promoter of the C/EBPα gene is as yet unknown. Because calpain is a protease, it may be required for the turnover of a protein(s) required for the covalent modification of C/EBPβ or for the release of C/EBPβ from a "sequestered" state, which would render the transcription factor unavailable for binding. In this connection it was recently reported (36) that Rb can bind/sequester C/EBPβ and thereby cause loss of function. Further work will be required to determine the mechanism by which calpain acts.

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