

Role of cdk2 in the sequential phosphorylation/activation of C/EBP β during adipocyte differentiation

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Upon induction of differentiation, growth-arrested (G₁ phase) 3T3-L1 preadipocytes express CCAAT/enhancer binding protein- β (C/EBP β), initiating a transcriptional cascade. C/EBP β immediately undergoes a priming phosphorylation (on Thr₁₈₈) by MAPK/ERK. However, the acquisition of DNA binding and transactivation capacity of C/EBP β is delayed until further phosphorylation (on Ser₁₈₄ or Thr₁₇₉) by GSK3 β occurs. Phosphorylation by glycogen synthase kinase-3 β (GSK3 β) induces S phase entry and thereby mitotic clonal expansion (MCE), a requirement for terminal differentiation. Because MAPK activity is down-regulated before S phase is completed, we sought to identify the kinase that maintains C/EBP β in the primed phosphorylated state throughout S phase and MCE. We show here that cdk2/cyclinA, whose expression is activated at the onset of S phase, functions in this capacity. *Ex vivo* and *in vitro* experiments show that cdk2/cyclinA catalyzes this delayed priming phosphorylation. Mass spectrometric analysis revealed that cdk2/cyclinA phosphorylates C/EBP β on Thr₁₈₈ and is required for phosphorylation (on Ser₁₈₄ or Thr₁₇₉) of C/EBP β by GSK3 β and maintenance of DNA binding activity. Suppression of cdk2 activity by RNA interference or pharmacologic inhibitor disrupts subsequent events in the differentiation program. Thus, MAPK and cdk2/cyclinA act sequentially to maintain Thr₁₈₈ of C/EBP β in the primed phosphorylated state during MCE and thereby progression of terminal differentiation.

3T3-L1 adipocyte | adipose | cell cycle | mitotic clonal expansion | obesity

When induced to differentiate, growth-arrested 3T3-L1 preadipocytes in G₁ phase synchronously reenter the cell cycle, undergo two rounds of mitosis [(mitotic clonal expansion (MCE)], then exit the cell cycle and commence terminal differentiation into adipocytes (1, 2). Fourteen hours after induction, preadipocytes traverse the G₁/S checkpoint as evidenced by the expression/activation of cdk2 and cyclinA, the turnover of p27/kip1, hyperphosphorylation of Rb protein, translocation of glycogen synthase kinase-3 β (GSK3 β) into the nucleus and cyclinD1 from the nucleus, and incorporation of [³H]thymidine into DNA (1). Blocking these steps disrupts MCE and progression of the differentiation program. The synchrony by which 3T3-L1 preadipocytes proceed through MCE has been invaluable in delineating the sequence of events that occur early in the adipocyte differentiation program (2, 3).

Upon induction of differentiation CCAAT/enhancer binding protein- β (C/EBP β) is expressed immediately (≤ 2 h), triggering a transcriptional cascade by transcriptionally inducing the expression of C/EBP α and peroxisome proliferator-activated receptor γ (PPAR γ) (4–7). C/EBP α and PPAR γ are pleiotropic transcriptional activators of genes that give rise to the adipocyte phenotype (4, 8–10). Although C/EBP β is expressed ≤ 2 h after induction, it lacks DNA-binding and transactivation activity (11). These activities are acquired only after a long (≥ 14 h) lag. Acquisition of DNA binding activity occurs concomitant with the entry of S phase at the G₁/S checkpoint and the onset of MCE (11). This lag appears to be required, because C/EBP α is

antimitotic (12–14) and its premature expression would otherwise prevent MCE, during which chromatin remodeling is thought to provide access of C/EBP α and PPAR γ to adipocyte gene promoters. It should be noted that sequestration of C/EBP β with a dominant-negative A-Zip blocks its entry into the nucleus, preventing MCE and thus terminal differentiation (15). Likewise, MCE and terminal differentiation are disrupted in C/EBP β ^{-/-} mouse embryo fibroblasts (16).

Previous studies have shown that C/EBP β is sequentially phosphorylated during differentiation of 3T3-L1 adipocytes (3), first by MAPK (on Thr₁₈₈) in G₁ phase and later by GSK3 β (on Ser₁₈₄ or Thr₁₇₉) at the onset of S phase concurrent with the translocation of GSK3 β into the nucleus. Phosphorylation of Thr₁₈₈ appears to prime C/EBP β for subsequent phosphorylation on Ser₁₈₄ or Thr₁₇₉, which lie immediately upstream of the priming site. Recent studies indicate that this dual phosphorylation induces a conformational change in C/EBP β that allows dimerization through its C-terminal leucine zipper domain (17). Dimerization brings the adjacent apposing basic regions into position to hold the C/EBP regulatory element of the gene in a “scissors-like” grip as suggested by McKnight and colleagues (18). Together, these actions are believed to facilitate acquisition of DNA-binding activity and transcription.

However, because expression of MAPK activity is transient and abruptly decreases 14–16 h after induction, the following question must be considered: How is the “priming” phosphorylation at Thr₁₈₈ maintained during the subsequent period of S phase and MCE when dual phosphorylation by GSK3 β occurs? This issue is addressed in the present paper. Here we show that in both cellular and *in vitro* contexts, C/EBP β undergoes phosphorylation on Thr₁₈₈ by cdk2/cyclinA, which primes the target region for further phosphorylation by GSK3 β . Together, these phosphorylations give rise to DNA binding activity. Our results indicate that MAPK and cdk2/cyclinA act sequentially to maintain Thr₁₈₈ of C/EBP β in the primed phosphorylated state for phosphorylation by GSK3 β throughout MCE.

Results

C/EBP β Is Phosphorylated on Thr₁₈₈ by cdk2/cyclinA During Differentiation of 3T3-L1 Preadipocytes. Consistent with findings in ref. 3, C/EBP β is rapidly (≤ 4 h) expressed and phosphorylated on

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Abbreviations: cdk2, cyclin-dependent kinase; C/EBP, CCAAT/enhancer-binding protein; GSK3 β , glycogen synthase kinase-3 β ; LC, liquid chromatography; MCE, mitotic clonal expansion; MS/MS, tandem MS; PPAR, peroxisome proliferator-activated receptor; P-ERK, phospho-ERK; rC/EBP β , recombinant rC/EBP β .

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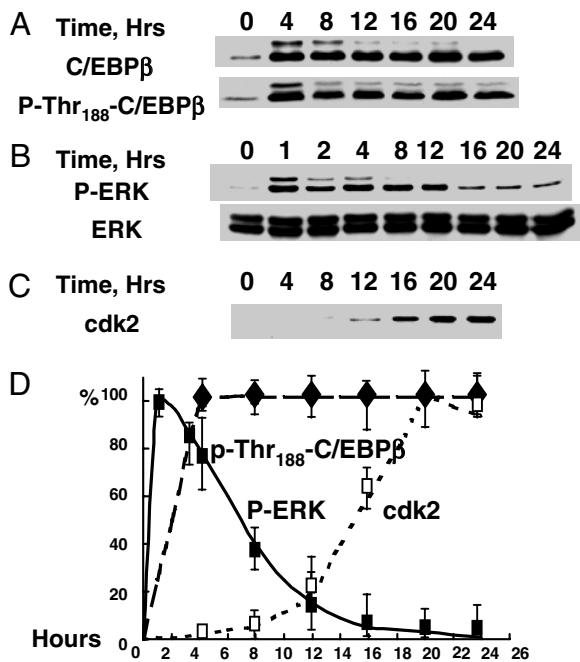


Fig. 1. Changes in the levels of C/EBP β , ERK, and cdk2 and phosphorylation states of C/EBP β /Thr₁₈₈ and ERK during differentiation of 3T3-L1 preadipocytes. Two-day postconfluent 3T3-L1 preadipocytes were induced to differentiate, and cell extracts were prepared, subjected to SDS/PAGE, and immunoblotted with antibodies to C/EBP β , P-Thr₁₈₈-C/EBP β (A), ERK, P-ERK (B), and cdk2 (C); relative levels are plotted (D).

Thr₁₈₈ after induction of differentiation (Fig. 1A). This priming phosphorylation at Thr₁₈₈ is catalyzed by MAPK and required for subsequent phosphorylation by GSK3 β and the acquisition of DNA binding activity by C/EBP β (3). MAPK/ERK itself is rapidly (≤ 1 h) phosphorylated/activated after induction of differentiation (Fig. 1B) (3). Although phosphorylation of C/EBP β on Thr₁₈₈ persists at a high level for >24 h (Fig. 1A and ref. 3), the phosphorylation/activity of MAPK [phospho-ERK (P-

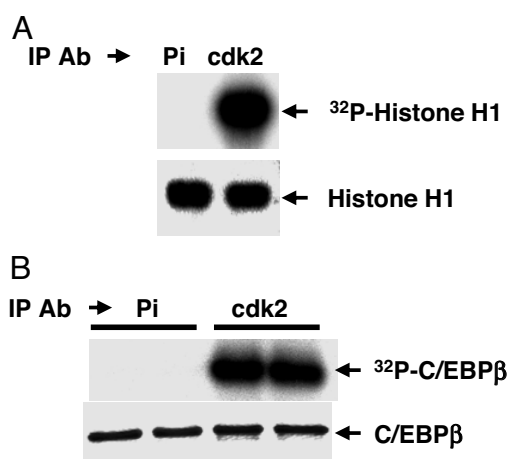


Fig. 2. C/EBP β is a substrate for cdk2/cyclinA. Two-day postconfluent 3T3-L1 preadipocytes were induced to differentiate, and 20 h later nuclear extracts were prepared and cdk2/cyclinA was immunoprecipitated and incubated with purified histone H1 (A) or purified recombinant C/EBP β (B) in the presence of [γ -³²P]ATP. After SDS/PAGE, [³²P]histone H1 (A Upper) or [³²P]C/EBP β (B Upper) was detected by autoradiography. Total histone H1 (A Lower) and C/EBP β (B Lower) were detected by Coomassie blue staining. Pi refers to preimmune serum.

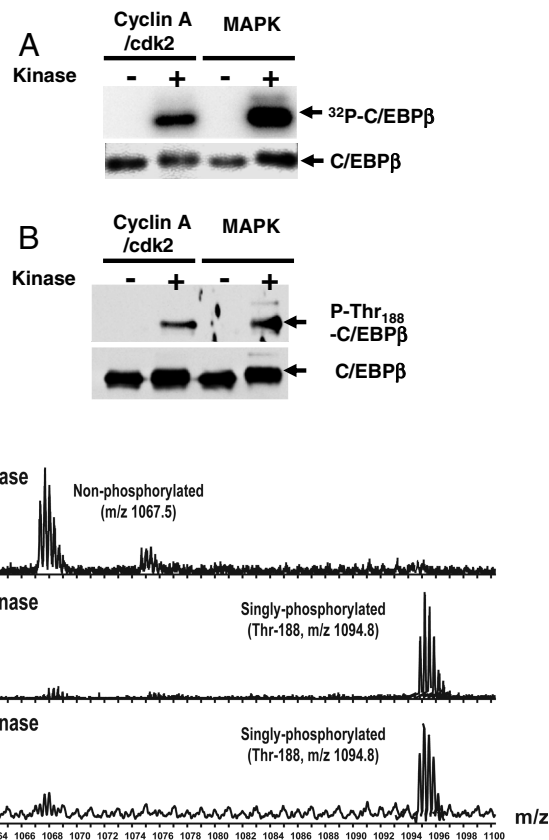


Fig. 3. Thr₁₈₈ of C/EBP β is phosphorylated both by MAPK and cdk2/cyclinA. (A) rC/EBP β was incubated without or with MAPK or cdk2/cyclinA and [γ -³²P]ATP. After SDS/PAGE, [³²P]C/EBP β was detected by autoradiography (Upper), and total C/EBP β was detected by Coomassie blue staining (Lower); similar experiments were performed as above but with cold ATP. (B) Immunoblotting was with anti-P-Thr₁₈₈-C/EBP β (Upper) or anti-C/EBP β (Lower) antibodies. (C) The C/EBP β band was excised from the gel, digested by trypsin, and analyzed by nanoLC-MS/MS. A singly phosphorylated peptide species from C/EBP β was observed (m/z 1,094.8) with MAPK or cdk2/cyclinA (Middle and Bottom, respectively). No phosphorylation was observed in the same peptide (m/z 1,067.5) without kinase treatment (Top).

ERK]) falls off abruptly after 10–12 h. This finding raises the question of how this priming phosphorylation, which is required for subsequent phosphorylation by GSK3 β (3), is maintained throughout S phase and MCE. Several findings suggested cdk2 as the candidate priming kinase for S phase progression. First, cdk2 is known to phosphorylate rat C/EBP β on Thr₁₈₉ [equivalent to Thr₁₈₈ in mouse C/EBP β (19)]. Furthermore, the expression of both cdk2 and cyclinA is coordinately activated, concomitant with the turnover of p27/kip (a cdk2/cyclinA inhibitor) during this time window of the differentiation program (1).

To determine whether cdk2/cyclinA has the potential to act as the priming kinase, cdk2/cyclinA was immunoprecipitated from 3T3-L1 cells 20 h after induction and the decline of P-ERK/MAPK (Fig. 1B). It is evident that, at 20 h, cdk2/cyclinA is active as indicated by its ability to phosphorylate both histone H1 (Fig. 2A) and purified rC/EBP β (Fig. 2B). Furthermore, treatment of the cells with roscovitine, a potent cdk2/cyclinA inhibitor, rapidly reduces the phosphorylation of C/EBP β (see below).

In vitro experiments with recombinant C/EBP β and [γ -³²P]ATP verified that cdk2/cyclinA phosphorylates C/EBP β (Fig. 3A) that phosphorylation occurs on Thr₁₈₈ (Fig. 3B) as it does with MAPK (3). To ascertain the number of sites phosphorylated by cdk2/cyclinA (or MAPK), the reaction products

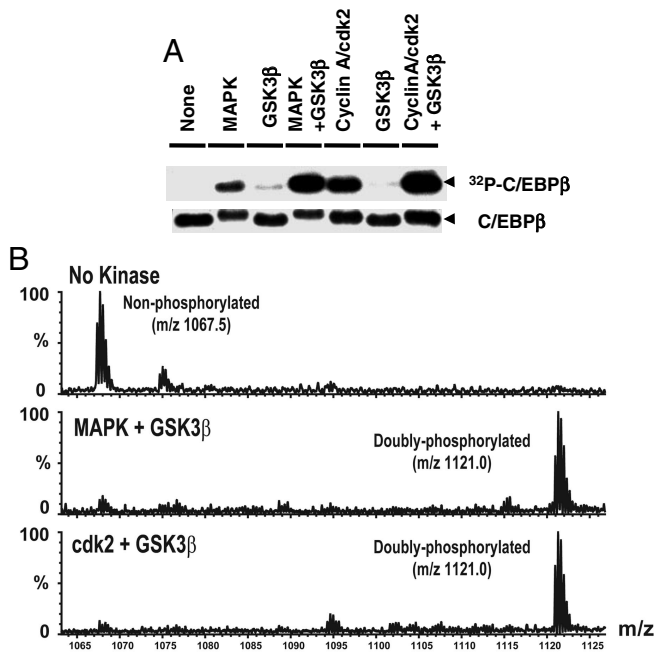


Fig. 4. Phosphorylation by either MAPK or cdk2/cyclinA primes C/EBPβ for phosphorylation by GSK3β. (A) rC/EBPβ was incubated without or with MAPK or cdk2/cyclinA or/and in combination with GSK3β in the presence of [γ - 32 P]ATP. After SDS/PAGE [32 P]C/EBPβ was detected by autoradiography (Upper), and protein was detected by Coomassie blue staining (Lower). Similar experiments were performed but with cold ATP. (B) The C/EBPβ band was excised from the gel, digested with trypsin, and analyzed by nanoLC-MS/MS. A doubly phosphorylated peptide species was observed (m/z 1,121.0) with C/EBPβ incubated with MAPK plus GSK3β (Middle) or cdk2/cyclinA plus GSK3β (Bottom). No phosphorylation of the peptide (m/z 1,067.5) occurred without kinase (Top).

were separated by SDS/PAGE, and the protein band corresponding to C/EBPβ was excised, digested with trypsin, and subjected to mass spectrometry. Nanospray MS showed that phosphorylation of rC/EBPβ by either cdk2/cyclinA or MAPK gave rise to a singly phosphorylated triply charged species of the correct mass (m/z , 1,094.8) (Fig. 3C Middle and Bottom). Identification of the phosphorylation site was also verified by mass spectrometry. The site phosphorylated by cdk2/cyclinA [supporting information (SI) Fig. 8] or MAPK (data not shown, see ref. 3) was identified as Thr₁₈₈.

Phosphorylation of Thr₁₈₈ by cdk2/cyclinA Primes C/EBPβ for Phosphorylation by GSK3β. Phosphorylation of C/EBPβ by MAPK at Thr₁₈₈ is required for hyperphosphorylation of C/EBPβ at Thr₁₇₉ or Ser₁₈₄ by GSK3β (3). Likewise, phosphorylation at Thr₁₈₈ by cdk2/cyclinA primes C/EBPβ for phosphorylation by GSK3β. Thus, incubation of cdk2/cyclinA (or MAPK), GSK3β, and [γ - 32 P]ATP leads to phosphorylation of purified rC/EBPβ, whereas in the absence of cdk2/cyclinA, GSK3β alone does not support phosphorylation (Fig. 4A). Incubation with cdk2/cyclinA (or MAPK) in combination with GSK3β produced a level of phosphorylation approximately twice that by cdk2/cyclinA or MAPK alone (Fig. 4A). This finding indicates that phosphorylation by cdk2/cyclinA (or MAPK) primes C/EBPβ for further phosphorylation by GSK3β. It should also be noted that phosphorylation by cdk2/cyclinA (or MAPK) slows the mobility of C/EBPβ slightly (Figs. 4A and 5A) and that dual phosphorylation by cdk2/cyclinA (or MAPK) and GSK3β slows it even more (Fig. 5A).

The extent of phosphorylation of rC/EBPβ by the combined kinases was verified by mass spectrometry. After separation by

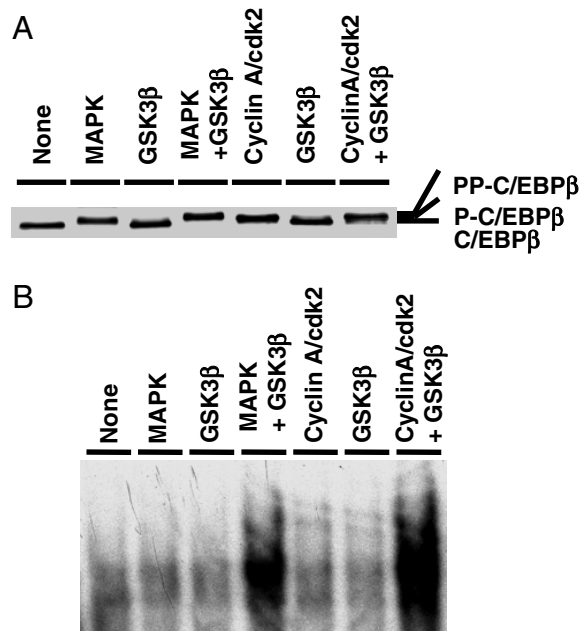


Fig. 5. Phosphorylation of C/EBPβ by cdk2/cyclinA and GSK3β leads to the gain of DNA binding activity. *In vitro* phosphorylation of C/EBPβ was performed (A) and DNA binding activity was assessed (B).

SDS/PAGE, the protein band corresponding to C/EBPβ was excised, digested with trypsin, and subjected to nano liquid chromatography (LC)-MS/MS analysis. This analysis identified a doubly phosphorylated peptide (m/z , 1,121.0) when C/EBPβ was incubated with both cdk2/cyclinA and GSK3β or both MAPK and GSK3β (Fig. 4B). To identify the specific amino acids in rC/EBPβ phosphorylated *in vitro*, experiments similar to those above were performed. MS/MS analysis of C/EBPβ incubated with cdk2/cyclinA and GSK3β revealed two doubly phosphorylated peptide species, one phosphorylated at Thr₁₈₈ and Ser₁₈₄ (SI Fig. 9) and another phosphorylated at Thr₁₈₈ and Ser₁₇₉ (data not shown). No phosphorylation product was detected with GSK3β alone. Together, these findings show that C/EBPβ can also be phosphorylated at Thr₁₈₈ by cdk2/cyclinA and that this phosphorylation serves as the priming site for phosphorylation at Ser₁₈₄/Thr₁₇₉ by GSK3β *in vitro*. It should be noted that we showed in ref. 3 that 20–24 h after induction of differentiation of 3T3-L1 preadipocytes, the time window in which MAPK activity is low and cdk2/cyclinA activity is high (Fig. 2 A and B), both doubly phosphorylated C/EBPβ peptide species (Thr₁₈₈ and Ser₁₈₄ or Thr₁₇₉) are present.

Dual Phosphorylation of C/EBPβ by cdk2/cyclinA and GSK3β Leads to Acquisition of DNA-Binding Activity. To determine the effect of phosphorylation of rC/EBPβ by cdk2/cyclinA with or without GSK3β on DNA-binding activity, an experiment identical to that described in Fig. 4 was performed. DNA binding activity was assessed by EMSA with a labeled oligonucleotide corresponding to the C/EBP-binding site in the C/EBPα gene promoter (7). DNA-binding activity was acquired only when C/EBPβ was phosphorylated by both cdk2/cyclinA and GSK3β (Fig. 5B). Unphosphorylated C/EBPβ or C/EBPβ phosphorylated by cdk2/cyclinA or MAPK alone had no effect on DNA-binding activity. Together, these results show that dual phosphorylation of C/EBPβ on Thr₁₈₈ by cdk2/cyclinA and on Ser₁₈₄ or Thr₁₇₉ by GSK3β is required for the acquisition of DNA-binding activity and that phosphorylation by cdk2/cyclinA alone is insufficient to increase DNA binding activity.

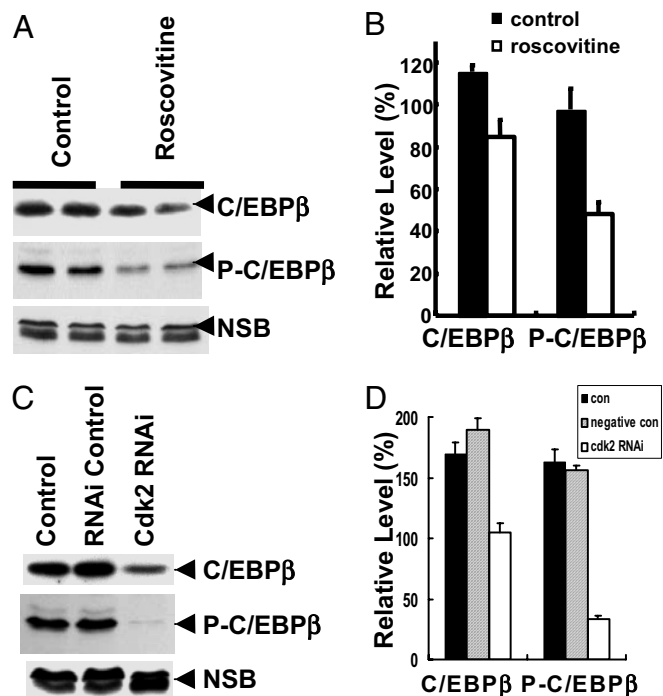


Fig. 6. Suppression of cdk2 activity with roscovitine or by RNA interference decreases phosphorylation of C/EBPβ on Thr₁₈₈. (A and B) Day-0 3T3-L1 preadipocytes were treated with or without a cdk2 inhibitor (roscovitine, 25 μM) 1 h before induction of differentiation. (A) Cell extracts, prepared 20 h after induction, were subjected to SDS/PAGE and immunoblotted with anti-C/EBPβ (Top) or anti-P-Thr₁₈₈-C/EBPβ (Middle) antibodies. (B) Relative levels were quantified. (C and D) 3T3-L1 preadipocytes were treated with 40 nM of cdk2 RNAi or an irrelevant RNAi control, and the effects on C/EBPβ expression (C Top) or phosphorylation of Thr₁₈₈ (C Middle) were quantified (D). (A Bottom and C Bottom) Shown is a nonspecific loading control (NSB).

Suppression of cdk2 Activity or Expression Decreases Phosphorylation of C/EBPβ at Thr₁₈₈ and Blocks MCE and Differentiation. Brief exposure (4 h) of 3T3-L1 preadipocytes to the potent cdk2 inhibitor, roscovitine, as the cells enter S phase (1) markedly lowers phosphorylation of Thr₁₈₈ in C/EBPβ (Fig. 6A and B). Although the level of C/EBPβ was also partially lowered by inhibitor treatment, the level of phosphorylation was more drastically affected, raising the possibility that the turnover of C/EBPβ may be preceded and accelerated by phosphorylation. It should be noted that, in previous experiments (1), we showed that treatment with roscovitine in this time window blocked MCE, the expression of differentiation markers, and acquisition of adipocyte characteristics.

Reducing the expression of cdk2 by RNA interference had similar effects. Several siRNA oligonucleotides (23 mers), corresponding to diverse regions in the cdk2 mRNA sequence, were tested initially for their ability to silence cdk2 expression. The most active of these oligonucleotides virtually abolished expression of cdk2 (Fig. 7A). Proliferating 3T3-L1 preadipocytes were exposed to this oligonucleotide and later treated with differentiation inducers (at confluence) under conditions similar to those of the standard differentiation protocol. As shown in Fig. 6C and D, lowering cdk2 by RNA interference almost completely blocked the phosphorylation of Thr₁₈₈ in C/EBPβ. Consistent with the results with roscovitine (see above), blocking phosphorylation of Thr₁₈₈ only partially reduced the level of C/EBPβ (Fig. 6A and 7A). Moreover, suppressing expression of cdk2 or C/EBPβ by RNA interference blocked MCE (Fig. 7B), suppressed the expression of differentiation markers (Fig. 7D), and prevented the accumulation of cytoplasmic triglyceride (Fig.

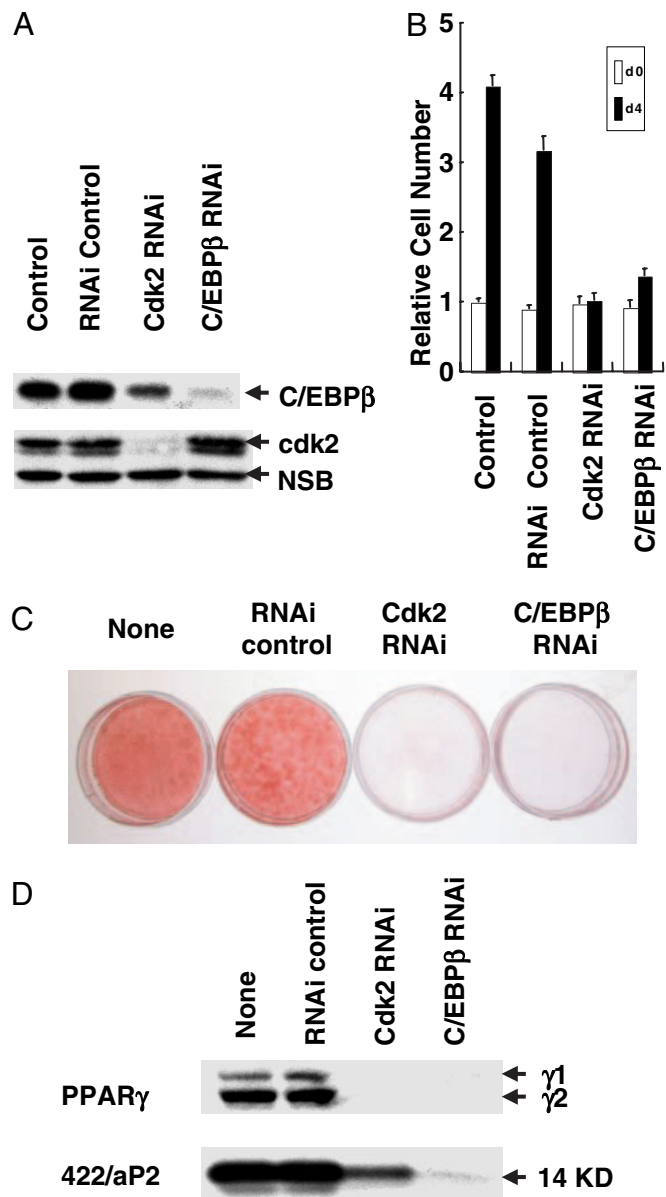


Fig. 7. RNA interference with cdk2 or C/EBPβ siRNA prevents MCE and differentiation. 3T3-L1 preadipocytes were treated with cdk2 or C/EBPβ siRNA (40 nM) at 30–50% confluency by using Lipofectamine RNAi Max, and 24 h later cells were trypsinized and replated at confluent cell density. After 24 h, cells were induced to differentiate. (A) Twenty hours after induction, cell extracts were prepared, and the effects on the expression of cdk2 (Middle) and C/EBPβ (Top) were analyzed. (Bottom) Shown is a nonspecific loading control (NSB). (B) Cell number on day 4. (C) Oil red O staining on day 8. (D) Expression of PPARγ and 422/aP2 on day 6.

7C), an indicator of adipogenesis. Taken together, these findings suggest that cdk2, which catalyzes the priming phosphorylation of Thr₁₈₈ in C/EBPβ (Figs. 3–5), is required for MCE and differentiation. It should be noted that because cdk2 has other roles, it is possible that its inactivation also acts at another site in the cell cycle.

Discussion

During differentiation of 3T3-L1 preadipocytes C/EBPβ undergoes a priming phosphorylation on Thr₁₈₈ followed by an additional phosphorylation on Ser₁₈₄ or Thr₁₇₉ by GSK3β to produce dually phosphorylated species that bind to and transactivate

genes that produce the adipocyte phenotype (4, 8–10). In this paper, we show that the priming phosphorylation occurs at two points in the differentiation program: an initial phosphorylation by MAPK followed much later by phosphorylation by cdk2/cyclinA when MAPK activity has disappeared. Both priming phosphorylations occur independently on Thr₁₈₈ as demonstrated in *in vitro* experiments with rC/EBP β and the purified kinases (Figs. 2–4). Phosphorylation at this site is required but is insufficient for the acquisition of DNA binding function; DNA binding requires a further phosphorylation by GSK3 β (Fig. 5), as discussed above. We have shown that dual phosphorylation of Thr₁₈₈ and of Ser₁₈₄ induces a conformational change that facilitates dimerization of C/EBP β monomers, creating a DNA binding pocket that can bind the C/EBP regulatory element in DNA and support transactivation (17).

The phosphoryl group (phospho-Thr₁₈₈) on C/EBP β , introduced by MAPK, turns over rapidly during the period (2–12 h after induction of differentiation) in which MAPK/P-ERK activity decreases. Rapid turnover of phospho-Thr₁₈₈ is indicated by its rapid rate of decline when cdk2 is down-regulated by RNA interference (Fig. 7A and B). Because the level of P-Thr₁₈₈-C/EBP β remains relatively constant during this time window (Fig. 1D), it is evident that normally cdk2 takes over this function (phosphorylation of C/EBP β on Thr₁₈₈), thereby compensating for the loss of MAPK activity. The effectiveness of the RNAi knockdown of cdk2 is indicated by the fact that the terminal steps in the differentiation program including MCE (Fig. 7B), i.e., the expression of key adipocyte differentiation markers (Fig. 7D) and acquisition of the adipose phenotype, are blocked (Fig. 7C) when cdk2 expression is suppressed (Fig. 7A). This finding also shows that priming by MAPK cannot replace priming by cdk2/cyclinA. The requirement of MAPK for the successful initiation of terminal differentiation has also been demonstrated in earlier studies by the use of potent MAPK inhibitors (1). Together, these findings illustrate the importance of both priming phosphorylations of C/EBP β in orchestrating the differentiation program. It is now clear that maintaining Thr₁₈₈ in the primed/phosphorylated state throughout MCE, is necessary to ensure phosphorylation by GSK3 β . Because cdk2 has other roles, it is possible that its inactivation could act at another point in the cell cycle and thereby affect differentiation. It is worthy of note that C/EBP β can also be phosphorylated by cdk2 at other sites, i.e., S₆₄ and T₁₈₉, in another context, i.e., in Ras-transformed NIH cells (19).

It should be noted that this is unlike the situation in mouse liver where phosphorylation at a different site (S₂₃₉) in C/EBP β causes exit from the nucleus to the cytoplasm (20). This change of intracellular localization was attributed to phosphorylation at a site adjacent to/within the nuclear localization sequence (nls). Hence, exit from the nucleus correlated with phosphorylation. Because cdk2 phosphorylates C/EBP β on T₁₈₈, which is immediately succeeded by further phosphorylation (as above) on Ser₁₈₄ or Thr₁₇₉ sites not located at the nls, a change in localization would not be expected. We have shown that dimerization of C/EBP β with A-C/EBP, which obscures the nls, prevents nuclear entry C/EBP β and thereby a blockade of subsequent events in the differentiation program that involve C/EBP β , e.g., mitotic clonal expansion, expression of adipocyte genes, and the accumulation of cytoplasmic fat (15).

It is formally possible that one of these kinases targets an additional, as-yet unidentified phosphorylation site in C/EBP β . Although only a single tryptic peptide product, phosphorylated (on Thr₁₈₈) of MAPK or cdk2/cyclinA, was detected in our analysis (Fig. 4), it is possible that another phosphopeptide species has eluded detection by mass spectrometry. Although we have searched for other phosphorylated tryptic peptide species by mass spectrometry, to date only one phosphopeptide, derived

from kinase-treated rC/EBP β , has been identified, i.e., that phosphorylated on Thr₁₈₈.

It should also be noted that C/EBP β has two known functions in the adipocyte differentiation program, i.e., the induction of MCE (15, 16) and transactivation of C/EBP α and PPAR γ (4–7). Thus, it is conceivable that these distinct functions require phosphorylation at two different sites. Were this the case, different phosphorylated species C/EBP β might function at these points in the differentiation program. Whether this occurs must await further investigation.

Materials and Methods

Cell Culture and Induction of Differentiation. 3T3-L1 preadipocytes were propagated and maintained in DMEM containing 10% (vol/vol) calf serum as described in ref. 21. To induce differentiation, 2-day postconfluent/G₁ phase preadipocytes (designated day 0) were fed DMEM containing 10% (vol/vol) FBS (FBS), 1 μ g/ml insulin, 1 μ M dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine (MDI) until day 2. Cells were then fed DMEM supplemented with 10% FBS and 1 μ g/ml insulin for 2 days, after which they were fed every other day with DMEM containing 10% FBS. Adipocyte gene expression and acquisition of the adipocyte phenotype begins on day 3 and is maximal by day 8.

Western Blotting. To follow changes in the level of C/EBP β , P-Thr₁₈₈-C/EBP β , MAPK(ERK), P-ERK and cdk2 proteins after induction of differentiation, 2-day postconfluent (day 0) 3T3-L1 preadipocytes were treated with MDI in 10% FBS as above. At various times thereafter, cell monolayers (6-cm dishes) were washed once with cold PBS (pH 7.4) and then scraped into lysis buffer containing 1% SDS and 60 mM Tris-HCl (pH 6.8). Lysates were heated at 100°C for 10 min, were clarified by centrifugation, and equal amounts of protein were subjected to SDS/PAGE and immunoblotting with antibodies against C/EBP β , P-Thr₁₈₈-C/EBP β , ERK, P-ERK, and cdk2. Antibody against C/EBP β was prepared in this laboratory; antibody against P-Thr₁₈₈-C/EBP β was from Cell Signaling Technology (Beverly, MA). Antibodies against MAPK and P-MAPK (Thr₂₀₂/Tyr₂₀₄) were from Upstate Biotechnology (Lake Placid, NY). Antibody against cdk2 was from Santa Cruz Biotechnology (Santa Cruz, CA).

Preparation of Recombinant C/EBP β (LAP) Protein. The cDNA encoding full-length C/EBP β (LAP) protein was cloned downstream of the GST gene in pGEX-6P (Amersham Pharmacia Biotech, Piscataway, NJ) and transformed into *Escherichia coli* strain BL21(DE3)pLysS (Novagen, San Diego, CA). A single colony was propagated overnight in 3 ml of LB media containing ampicillin and chloramphenicol and was then diluted (1:100) into 500 ml of fresh LB media the next day and cultured until an A₆₀₀ of 0.6–0.7 was reached. Expression of the fusion proteins was induced by addition of 0.5 mM isopropyl β -D-thiogalactoside (IPTG) for 3 h, and the cells were harvested and resuspended in 25 ml of PBS containing 1% Triton X-100. After lysis by one cycle of freeze–thawing, the cell suspension was treated with DNase I and RNase A/T1 and then incubated on ice for 10 min in the presence of 0.5 M NaCl and 5 mM DTT to dissociate the DNA–protein complex. After centrifugation at 12,000 \times g for 10 min, 250- to 500- μ l bead volume of GSH-Sepharose (GE Healthcare, Piscataway, NJ) was added to the supernatate and mixed overnight at 4°C. Beads were washed three times with PBS, once with PreScission cleavage buffer (50 mM Tris-Cl, pH 7.5/150 mM NaCl/1 mM EDTA/1 mM DTT), and then treated with 80 units of PreScission Protease (GE Healthcare) in 960 μ l of buffer. The rC/EBP β cleavage product was further purified by CM-Sepharose chromatography (GE Healthcare), and after extensive washing with a low salt buffer, the highly purified recombinant protein eluted 250–300 mM of NaCl. Protein was

determined by Bradford assay, and purity of the fusion protein was verified by SDS/PAGE.

Immunoprecipitation and *in Vitro* Kinase Reaction. Preadipocytes were induced to differentiate. Nuclear extracts (1) were prepared at 20 h after induction, cdk2/cyclinA was immunoprecipitated from nuclear extracts (50 μ g of protein) with mouse monoclonal anti-cdk2 antibody, and the immune complex was subjected to the *in vitro* kinase assay with Histone H1 or rC/EBP β as substrates as described in ref. 22.

***In Vitro* Phosphorylation and Mass Spectrometric Analysis of Full-Length C/EBP β (LAP).** Two micrograms of rC/EBP β (LAP) was incubated with (i) activated MAPK (Calbiochem, San Diego, CA); (ii) cdk2-Cyclin A (Upstate); (iii) GSK3 β (Upstate); or (iv) the combination of MAPK (or cdk2/cyclinA) with GSK3 β in buffer containing 50 mM Hepes (pH 7.0), 10 mM MgCl₂, 1 mM DTT, and 20 μ Ci [γ -³²P]ATP (1Ci = 37 GBq) at 30°C for 30min. Phosphorylation was detected by autoradiography after SDS/PAGE. For identification of phosphorylation sites on C/EBP β , similar experiments were performed with 0.5 mM ATP. The protein band containing C/EBP β (38 kDa) was excised from the gel, digested with trypsin, and analyzed by nanoLC-MS/MS as described in ref. 23.

EMSA. EMSA was performed essentially as described in ref. 2 with minor modification. Reaction mixtures containing 50,000 cpm of ³²P-labeled C/EBP site probe, 0.1 μ g of poly[d(I-C)], 4 μ g of BSA, and 1–10 ng of rC/EBP β protein in 30 μ l of buffer (10 mM Hepes/1 mM EDTA/7% glycerol/1 mM MgCl₂/100 mM NaCl) were incubated on ice for 20min, and proteins were separated electrophoretically on 4% polyacrylamide gels with

0.25 \times TBE buffer. The labeled probe included a double-stranded oligonucleotide corresponding to the sequence of the C/EBP regulatory element in the C/EBP α gene promoter (2), 5'-G₁₉₁CGTTGCGCCACGATCTCTC_{172-3'}.

RNAi of cdk2 and C/EBP β with siRNA. Synthetic siRNA oligonucleotides specific for regions in the cdk2 and C/EBP β mRNAs were designed and synthesized by Invitrogen (Carlsbad, CA) Stealth RNAi. The silencing effects of several siRNA oligonucleotides were screened and tested initially for their ability to silence cdk2 or C/EBP β expression. The most active of these oligonucleotides for cdk2 (5' to 3': GCUCGACACUGAGACUGAAGGUGUA) and for C/EBP β (5' to 3': CCCUGCGGAACUUGU-CAAGCAGCU) almost completely blocked the expression of cdk2 or C/EBP β . 3T3-L1 preadipocytes in 60-mm dishes at 30–50% confluency were transfected with siRNA oligonucleotides by using Lipofectamine RNAiMAX (Invitrogen). Twenty-four hours later, the cells were trypsinized and plated into 35-mm dishes at cell density of 5 \times 10⁵ cells per dish to generate confluent monolayers (23). Twenty-four hours later, the cells were subjected to the standard differentiation protocol, and at various times thereafter, cell extracts were prepared for analysis. Two control transfections were performed, one with Lipofectamine RNAiMAX and the other was with Stealth RNAi Negative Control Duplexes (Invitrogen).

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