Upon differentiation induction of 3T3-L1 preadipocytes by a hormone mixture containing 1-isobutyl-3-methylxanthine, dexamethasone, and insulin, the preadipocytes undergo ~2 rounds of mitotic clonal expansion, which just precedes the adipogenic gene expression program and has been thought to be an essential early step for differentiation initiation. By inducing 3T3-L1 preadipocytes with each individual hormone, it was determined that the mitotic clonal expansion was induced only by insulin and not by 1-isobutyl-3-methylxanthine or dexamethasone. Cell number counting and fluorescence-activated cell-sorting analysis indicated that a significant fraction of 3T3-L1 preadipocytes differentiated into adipocytes without mitotic clonal expansion when induced with the combination of 1-isobutyl-3-methylxanthine and dexamethasone. Furthermore, when normally induced 3T3-L1 preadipocytes were treated with PD98059 (an inhibitor of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1) to block the activation of extracellular signal-regulated kinase (Erk) 1 and Erk2, the mitotic clonal expansion was blocked, but adipocyte differentiation was not affected. These observations were confirmed by bromodeoxyuridine labeling. The differentiated adipocytes induced with 1-isobutyl-3-methylxanthine and dexamethasone or standard hormone mixture plus PD98059 were not labeled by bromodeoxyuridine. Thus, it is evident that 3T3-L1 preadipocytes could differentiate into adipocytes without DNA synthesis and mitotic clonal expansion. Our results also suggested that activation of Erk1 and Erk2 is essential to but not sufficient for induction of mitotic clonal expansion.

Obesity has become a major health hazard in many countries and has been indicated as a risk factor for many physiological disorders, such as diabetes, hypertension, and heart problems.

As the major cellular component in adipose tissue, adipocytes play a key role in obesity. The excessive growth of adipose tissue in obesity has been suggested as expansion of adipocytes both in cell size and in cell number (1, 2). To better understand adipocyte physiology, in vitro cell models, such as 3T3-L1 and 3T3-F442A, have been used. These model cell lines provide a useful tool to study the adipocyte differentiation process (1, 3). 3T3-L1 cells can be induced to differentiate into mature adipocytes in cell culture (4–6). It is one of the most used preadipocyte models to study the adipogenesis process. The adipocyte differentiation program involves several stages. Immediately after hormonal stimulation (IGF-I1 or insulin at a nonphysiologically high concentration, a glucocorticoid, dexamethasone (DEX), and a cAMP phosphodiesterase inhibitor that increases intracellular cAMP, 3-isobutyl-1-methylxanthine (MIX)), postconfluent G0 3T3-L1 preadipocytes reenter a period of the cell cycle called mitotic clonal expansion. The gene expression program leading to terminal adipocyte differentiation is initiated during and after this mitotic clonal expansion period. It has been proposed that this mitotic clonal expansion might facilitate the DNA remodeling for the adipogenesis gene expression program (3).

Whereas it is clear that the mitotic clonal expansion phase precedes the adipogenic gene expression program, whether this cell proliferation process is an obligatory step along the 3T3-L1 adipocyte differentiation process is still not clear. Several studies have indicated that mitotic clonal expansion and 3T3-L1 adipocyte differentiation are both blocked by the DNA synthesis inhibitor aphidicolin (7), the antiproliferation reagent rapanycin (8), calpain inhibitor N-acetyl-leu-leu-norleucinal (9), or tumor necrosis factor α (10). These results supported the view that mitotic growth is a necessary step in the adipocyte differentiation process. However, in our previous studies, we have found that 3T3-L1 adipocyte differentiation can be blocked with the protein tyrosine phosphatase inhibitor vanadate, whereas mitotic clonal expansion is not affected (11, 12). Vanadate inhibits the differentiation induction signal from IGF-I receptor tyrosine kinase by blocking the turnover of tyrosine-phosphorylated c-Crk (12). Further investigation indicates that the effect of vanadate on blocking 3T3-L1 preadipocyte differentiation occurs at a very early stage of differentiation induction, before the initiation of mitotic clonal expansion (12).

* This work was supported by Research Grants 39825107 (to K. L.) and 39825115 (to J. W.) from the Chinese National Nature Sciences Foundation, Research Grant G1998053901 (to J. W.) from the Major State Basic Research Program of China, Grant 98XD14015 (to J. W.) from the Science and Technology Commission of Shanghai Municipal Government, Research Grant 9910 (to K. L. and J. W.) from the Shanghai University of Science and Technology, and State Basic Research Program of China, Grant 98XD14015 (to J. W.) from the Shanghai University of Science and Technology. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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† The abbreviations used are: IGF-I, insulin-like growth factor I; MIX, 3-isobutyl-1-methylxanthine; DEX, dexamethasone; MDI, 3-isobutyl-1-methylxanthine, dexamethasone, and insulin; FBS, fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; BrdUrd, bromodeoxyuridine; DAPI, 4′-6′-diamidino-2-phenylindole; FACs, fluorescence-activated cell sorting; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; Erk, extracellular signal-regulated kinase; MAP, mitogen-activated protein; MDI, 3-isobutyl-1-methylxanthine and dexamethasone.
These results suggest that the activated IGF-I receptor tyrosine kinase induces 3T3-L1 adipocyte differentiation and mitotic clonal expansion by two separate signal transduction pathways. In addition, primary preadipocytes from human adipose tissue enter the differentiation process without mitotic clonal expansion (13). These observations provide an impetus for us to investigate whether mitotic clonal expansion is an essential step during 3T3-L1 the preadipocyte differentiation induction process or a parallel event induced by the differentiation induction hormones.

In the present study, we reported that two rounds of the mitotic clonal expansion cell cycle occurred during 3T3-L1 preadipocyte differentiation induction and that the initiation of clonal expansion required activation of the Erk1 and Erk2 MAP kinases. Of three differentiation induction reagents (MIX, DEX, and a high concentration of insulin in place of IGF-I), only insulin was capable of inducing mitotic clonal expansion. 3T3-L1 preadipocytes induced with MIX and DEX (omitting insulin) or induced with the standard hormone mixture plus MEK inhibitor PD98059 were still able to differentiate into adipocytes, but without DNA synthesis and mitotic clonal expansion. Thus, our results suggest that DNA synthesis and mitotic clonal expansion is not an essential step required for the 3T3-L1 preadipocyte differentiation process; rather, it is a separable event induced by the activated IGF-I receptor tyrosine kinase through a signal pathway of MAP kinases Erk1 and Erk2.

EXPERIMENTAL PROCEDURES

Materials—Anti-Erk and anti-p-Erk (against the critical Tyr residue phosphorylated peptide) antibodies were purchased from Santa Cruz Biotechnology, Inc. Horseradish peroxidase-conjugated secondary antibody (goat anti-mouse IgG, 1:10,000) and horseradish peroxidase-conjugated secondary antibody for 45 min. Target proteins were visualized by enhanced chemiluminescence.

BrdUrd Labeling for DNA Synthesis—Cells were cultured on coverslips until 2-day postconfluence and induced to differentiate with different induction conditions as described in the figure legends. During the 8 phase of the cell cycle, 30 μg/ml BrdUrd was pulsed for 2 h (from 18th to 19th h after (differentiation) to label the S phase. The coverslips were fixed in 70% ethanol for 30 min and then stored in 70% ethanol at 4 °C for immunofluorescence analysis. For cells carried to final differentiation, the BrdUrd labeling medium was replaced with preconditioned medium, which was obtained from a parallel cell culture dish treated with the same induction condition. After 48 h, the medium was replaced with DMEM containing 10% fetal bovine serum with or without a supplement of 1 μg/ml insulin as described in the figure legends. By day 8, cells were fixed with 70% ethanol and stored in the same ethanol solution at 4 °C for immunofluorescence analysis.

BrdUrd labeling was also conducted on day 3 3T3-L1 cells to reveal the effect of the insulin supplementation in the medium. At the 16th h after the medium change, 30 μg/ml BrdUrd was pulsed for 2 h, and the labeling medium was replaced with preconditioned medium obtained from a cell culture dish fed in parallel. On day 4, the cells were fed with normal medium every other day until day 8.

Immunofluorescence—Ethanol-fixed coverslips were incubated in 100% methanol for 10 min at room temperature. The coverslips were then treated with 1.5 M HCl for 30 min, blocked with 0.5% Tween 20 in PBS solution for 5 min, and incubated with anti-BrdUrd primary antibody for 1 h at room temperature. After washing coverslips with Tween-PBS twice for 5 min to remove the secondary antibody, the coverslips were mounted for immunofluorescence microscope analysis (AXIOSKOP 20; Zeiss).

Analysis of Erk activation. For insulin-induced Erk activation, after the medium change (24 h) and pretreatment with 25 μM PD98059 until day 4. For stimulation with individual hormone (MIX plus DEX, or insulin alone) or a two-hormone combination (MIX plus DEX, MIX plus insulin, or DEX plus insulin), 2-day postconfluent preadipocytes were induced by hormone(s) at the above-mentioned concentration for 48 h. Cells were trypsinized from culture dishes, and the cell numbers were counted using a hemocytometer plate. For cells carried to final differentiation, the induction culture medium was replaced with 48 h after with DMEM containing 10% fetal bovine serum supplemented with or without 1 μg/ml insulin as described in the figure legends, and then the normal cell feeding protocol was followed until day 8.

For PD98059 treatment, 20 μM PD98059 was added to the cells with the differentiation induction hormone mixture on day 0. The cells were then cultured following the standard differentiation induction protocol with a supplement of 20 μM PD98059 until day 4.

FACS Analysis, Cell Counting, and Oil-Red-O Staining—3T3-L1 cells (6-cm plate) were trypsinized from the culture dishes and collected by centrifugation. The aliquot was subjected to cell counting using a hemocytometer plate. The cells were then fixed in 70% ethanol, pelleted, and treated with 1 mg/ml RNase A for 30 min at 37 °C. After staining with 20 μg/ml propidium iodide, the DNA content in cells was determined by FACS analysis. For Oil-Red-O staining, 3T3-L1 adipocyte monolayers (usually on day 8) were washed three times with phosphate-buffered saline (PBS) and then fixed for 2 min with 3.7% formaldehyde in PBS. Oil-Red-O (0.5%) in isopropanol was diluted with 1.5 volumes of water, filtered, and added to the fixed cell monolayers for 1 h at room temperature. Cell monolayers were then washed with water, and the stained triglyceride droplets in the cells were visualized and photographed.

RESULTS

Two rounds of mitotic clonal expansion were observed during 3T3-L1 preadipocyte differentiation induction and induced only by insulin. It was observed that after 3T3-L1 preadipocytes...
differentiated into adipocytes, the cell numbers were usually increased by 3–4-fold (Fig. 1A). This is consistent with our previous results and results reported by other researchers (9–12). To ascertain how many cell cycles occurred during the clonal expansion period, we took advantage of the fact that postconfluent 3T3-L1 preadipocytes enter mitotic clonal expansion synchronously after the differentiation induction. By cell number counting and FACS analysis of the cellular DNA content, two rounds of cell cycle were observed, and the cell number increase was matched by the DNA content analysis, especially for the first cell cycle, which is well synchronized (Fig. 1, B and C). Complete DNA synthesis and transition from diploid
to tetraploid were observed (Fig. 1C). Thus, the clonal expansion was full cell cycle mitosis. Because differentiation induction is initiated with three hormones working through three signal pathways (MIX on cAMP, DEX on glucocorticoid receptor, and insulin on IGF-I receptor tyrosine kinase), it is important to know whether the mitotic clonal expansion is the result of the combined effects of three hormones or the result of one of the hormones. By inducing 3T3-L1 cells with individual hormone or a different combination of two hormones on day 0 and counting the cell number after the completion of the first cell cycle, it was clear that once insulin was present, the cell number increased. Even with insulin alone, mitosis still occurred (Fig. 1D). This insulin-activated mitosis was confirmed by analysis of DNA synthesis with BrdUrd labeling. The incorporation of BrdUrd in cells exposed to insulin or to a hormone mixture containing insulin was significantly higher than that in cells exposed to other hormones (Fig. 1E). Because insulin is working through the IGF-I receptor in 3T3-L1 preadipocytes (16, 17), mitotic clonal expansion is induced by the activation of IGF-I receptor.

In the Absence of Insulin, MIX and DEX Could Induce Significant 3T3-L1 Adipocyte Differentiation without the Mitotic Clonal Expansion—Because insulin is the sole factor to induce mitotic clonal expansion, we attempted to induce 3T3-L1 preadipocyte differentiation with MIX and DEX. As shown in Fig. 2A, induction of preadipocytes with MIX, DEX, and insulin led to an almost doubled cell number by day 2, whereas induction with MIX and DEX caused no cell number increase. After the change of medium on day 2, MIX and DEX-induced cells fed with insulin-supplemented medium started mitosis. By day 4, the cell number increased and was close to that induced with the standard differentiation protocol (MDI for 48 h plus insulin...
for an additional 48 h). In contrast, MIX and DEX-induced cells fed with plain medium remained in the quiescent state, and no cell number increase was detected. FACS analysis confirmed the results of cell number counting (Fig. 2B). Only in MIX and DEX-induced cells fed with insulin-supplemented medium was DNA synthesis observed. Thus, induction with MIX and DEX (without insulin) in the first 2 days followed by normal insulin-supplemented medium for an additional 2 days delayed the cells entrance into mitotic clonal expansion, whereas induction only with MIX and DEX for 2 days resulted in the cells staying in the quiescent state without entering into clonal expansion. Furthermore, when 3T3-L1 preadipocytes were induced without insulin for the entire differentiation process (MIX and DEX for the first 2 days and then medium without an insulin supplement), a significant fraction of the preadipocytes differentiated into adipocytes, even though no mitotic clonal expansion occurred; and the differentiated adipocytes were evenly distributed in the entire cell monolayer. The expression of adipocyte marker protein aP2 confirmed the results of Oil-Red-O staining. In all three cases (standard induction condition: MDI for 2 days and insulin for 2 days; clonal expansion delayed condition: MD for 2 days and insulin for 2 days; no clonal expansion: MD for 2 days), expression of aP2 protein started on day 3, and its expression was further increased in fully differentiated adipocytes (Fig. 2D). These results indicated that the differentiation of 3T3-L1 preadipocytes into adipocytes was not related to their mitotic clonal expansion.

DNA Synthesis Is Not Required for 3T3-L1 Preadipocyte Differentiation—The results of FACS analysis and cell number counting indicated that a significant fraction of 3T3-L1 preadipocytes differentiated into adipocytes without undergoing mitotic clonal expansion when induced with MIX and DEX. To
confirm these results, BrdUrd was used to label DNA synthesis. Therefore, if adipocyte differentiation requires mitotic clonal expansion, then most of the differentiated adipocytes will be labeled with BrdUrd. When BrdUrd was added to the cells during the S phase (from the 16th to 18th h after the hormonal stimulation), most MDI-induced cells were labeled by BrdUrd as shown in Fig. 3A and B. In contrast, only a small percentage of cells induced with MIX and DEX were labeled with BrdUrd, and it was the same as the percentage of BrdUrd-labeled control cells fed with DMEM and 10% FBS. Although the adipocyte differentiation in MIX and DEX-induced cells was not as complete as that in MDI-induced cells (37% versus 70% of the cells containing visible triglyceride droplets), only around 15% of differentiated adipocytes with MIX and DEX induction were labeled by BrdUrd. This ratio of BrdUrd incorporation in differentiated adipocytes was in the same range of
MEK-1 Inhibitor PD98059 Blocked Mitotic Clonal Expansion

In the standard differentiation induction protocol, after the initial hormone induction the induction medium was replaced with medium supplemented with insulin. Because of the mitogenic effect of insulin on clonal expansion (Fig. 1), MIX and DEX-induced adipocytes did not undergo DNA synthesis and hence mitosis.

In the standard differentiation induction protocol, after the addition of differentiation inducers (Fig. 1A), cells started to enter S phase 14 h after the activation of Erk1 and Erk2 by MEK-1 during the first 14 h of differentiation induction. To analyze the role of mitotic clonal expansion in the normally induced 3T3-L1 adipocytes, MEK-1 inhibitor PD98059 (18–20) was used to block the activation of MAP kinases Erk1 and Erk2. It has been reported that PD98059 blocks the activation of Erk1 and Erk2 MAP kinases by IGF-I in 3T3-L1 adipocytes and that it also blocks the proliferation of 3T3-L1 adipocytes (21). In addition, it has no effect on the adipocyte differentiation (21, 22). By adding 20 μM PD98059 during the differentiation induction process (from day 0 to day 4), PD98059 almost completely blocked mitotic clonal expansion (Fig. 4A). In the presence of PD98059, the cell number was only slightly increased as compared with noninduced cells. The result of FACS analysis (Fig. 4B) was consistent with the cell number counting. Although PD98059 blocked mitotic clonal expansion, it had no effect on adipocyte differentiation. As shown in Fig. 4C, in the presence of PD98059, 3T3-L1 adipocytes differentiated into adipocytes without any difference from adipocytes induced by standard protocol. Results of BrdUrd labeling confirmed the observation that PD98059 blocked mitotic clonal expansion but not adipocyte differentiation (Fig. 4D and E). The total adipocyte differentiation in the presence of PD98059 was very similar (both around 80% of the cells containing visible triglyceride droplets). However, only 20% of the cells were labeled by BrdUrd in the presence of PD98059, whereas 70% of the cells were labeled by BrdUrd in the standard induced control cells. In differentiated adipocytes, close to 80% of the normally induced adipocytes were labeled by BrdUrd, whereas less than 20% of the adipocytes were labeled by BrdUrd in PD98059-treated cells. Thus, in the presence of PD98059, most differentiated adipocytes had no DNA synthesis and mitosis.

Activation of MAP Kinase Erk1 and Erk2 Is Essential but Not Sufficient for Mitotic Clonal Expansion—During differentiation induction, 3T3-L1 cells started to enter S phase 14 h after the addition of differentiation inducers (Fig. 1C). Thus, the activation of Erk1 and Erk2 by MEK-1 during the first 14 h was investigated. As shown in Fig. 5A, in MDI-induced cells, Erk1 and Erk2 were significantly activated (as shown by the increase in the phosphorylated form of Erk1 and Erk2), whereas the addition of PD98059 to MDI-induced cells greatly diminished the phosphorylation of Erk1 and Erk2. In our previous studies (12), the protein tyrosine phosphatase inhibitor vanadate blocked 3T3-L1 adipocyte differentiation without affecting mitotic clonal expansion. Thus, the effect of vanadate on the activation of Erk1 and Erk2 was also investigated. It was clear that vanadate did not affect the MDI-induced activation of Erk1 and Erk2.

Of MIX, DEX, and insulin, only insulin, acting through the IGF-I receptor (16, 17), was capable of inducing mitotic clonal expansion (Fig. 1). The results of study of the activation of Erk1 and Erk2 by insulin showed a pattern similar to their activation by MDI (Fig. 5B). PD98059 treatment also blocked the activation of Erk1 and Erk2 by insulin, whereas vanadate treatment did not interfere with insulin-activated Erk1 and Erk2 phosphorylation and even slightly increased the phosphorylation of Erk1 and Erk2. MIX alone could also increase the phosphorylation of Erk1 and Erk2, but it caused no mitosis (results not shown). Thus, activation of Erk1 and Erk2 is not essential for adipocyte differentiation, but it is essential for mitotic clonal expansion.
sufficient to promote growth-arrested postconfluent 3T3-L1 preadipocytes to enter mitotic clonal expansion during differentiation induction. Treatment with DEX alone did not activate Erk1 and Erk2 (results not shown).

DISCUSSION

Mitotic clonal expansion during differentiation induction is a rather unique process for adipocyte differentiation. However, its role in the adipocyte differentiation process has not been fully understood. In the debate over whether this cell proliferation is a required step along the differentiation process or a parallel event activated by differentiation inducers, our studies provided evidence for the latter. As indicated in our previous studies with vanadate, which selectively blocks adipocyte differentiation but not mitotic clonal expansion, mitotic clonal expansion is not likely to be an obligatory step in the adipocyte differentiation process because differentiation is blocked at the event(s) preceding mitotic clonal expansion, which, on the other hand, is not inhibited (12).

In the present study, we further analyzed the relationship between 3T3-L1 adipocyte differentiation induction and mitotic clonal expansion during differentiation induction. By cell number counting and FACS analysis, it was found that two rounds of cell division occurred in the clonal expansion period: (a) a well-synchronized first round of the cell cycle; and (b) a not very synchronized second round of the cell cycle (Fig. 1, A–C). By delaying or omitting the addition of insulin during differentiation induction, mitotic clonal expansion could be delayed or completely prevented (Fig. 2, A and B). However, there was still normal adipocyte differentiation under these conditions (Figs. 2 and 3). Thus, mitotic clonal expansion and adipocyte differentiation are two separable events. Importantly, the identification of insulin as the hormone responsible for inducing mitotic clonal expansion (Fig. 1, D and E) indicated that the IGF-I receptor was the important factor responsible for activation of mitotic clonal expansion because insulin acts through the IGF-I receptor in 3T3-L1 cell differentiation induction (12, 16, 17). During the induction of adipocyte differentiation, IGF-I receptor tyrosine kinase signaling was very important. Without the addition of insulin in differentiation induction, 3T3-L1 adipocyte differentiation was only about half of the differentiation induction signal (12). Thus, mitotic clonal expansion occurred in the clonal expansion period: (a) a well-synchronized first round of the cell cycle; and (b) a not very synchronized second round of the cell cycle (Fig. 1, A–C). By delaying or omitting the addition of insulin during differentiation induction, mitotic clonal expansion could be delayed or completely prevented (Fig. 2, A and B). However, there was still normal adipocyte differentiation under these conditions (Figs. 2 and 3). Thus, mitotic clonal expansion and adipocyte differentiation are two separable events. Importantly, the identification of insulin as the hormone responsible for inducing mitotic clonal expansion (Fig. 1, D and E) indicated that the IGF-I receptor was the important factor responsible for activation of mitotic clonal expansion because insulin acts through the IGF-I receptor in 3T3-L1 cell differentiation induction (12, 16, 17). During the induction of adipocyte differentiation, IGF-I receptor tyrosine kinase signaling was very important. Without the addition of insulin in differentiation induction, 3T3-L1 adipocyte differentiation was only about half of the differentiation induction signal (12).

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