Human Translocation Liposarcoma-CCAAT/Enhancer Binding Protein (C/EBP) Homologous Protein (TLS-CHOP) Oncoprotein Prevents Adipocyte Differentiation by Directly Interfering with C/EBPβ Function*

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Human translocation liposarcoma (TLS)-CCAAT/enhancer binding protein (C/EBP) homologous protein (CHOP) is a fusion oncoprotein found specifically in a malignant tumor of adipose tissue and results from a t(12;16) translocation that fuses the amino-terminal part of TLS to the entire coding region of CHOP. Being that CHOP is a member of the C/EBP transcription factor family, proteins that comprise part of the adipocyte differentiation machinery, we examined whether TLS-CHOP blocked adipocyte differentiation by directly interfering with C/EBP function. Using a single-step retroviral infection protocol, either wild-type or mutant TLS-CHOP were co-expressed along with C/EBPβ in naive NIH3T3 cells, and their ability to inhibit C/EBPβ-driven adipogenesis was determined. TLS-CHOP was extremely effective at blocking adipocyte differentiation when expressed at a level comparable to that observed in human myxoid liposarcoma. This effect of TLS-CHOP required a functional leucine zipper domain and correlated with its ability to heterodimerize with C/EBPβ and inhibit C/EBP DNA binding and transactivation activity in situ. In contrast, the TLS-CHOP basic region was dispensable, making it unlikely that the inhibitory effect of TLS-CHOP is attributable to unscheduled gene expression resulting from TLS-CHOP’s putative transactivation activity.

Another adipogenic transcription factor, PPARγ2, was able to rescue TLS-CHOP-inhibited cells, indicating that TLS-CHOP interferes primarily with C/EBPβ-driven adipogenesis and not with other requisite events of the adipocyte differentiation program. Together, the results demonstrate that TLS-CHOP blocks adipocyte differentiation by directly preventing C/EBPβ from binding to and transactivating its target genes. Moreover, they provide strong support for the thesis that a blockade to normal differentiation is an important aspect of the cancer process.

Adipogenesis is a process in which an undifferentiated mesenchymal cell capable of proliferation matures into a post mitotic, fat-laden adipocyte. This differentiation process results in dramatic changes in gene expression and a spectacular alteration in cell morphology. The early phase of adipocyte differentiation is accompanied by the induction of transcription factors that promote cell cycle withdrawal and activation of cell-specific genes, two key aspects of terminal cell differentiation.

CCAAT/enhancer binding protein α (C/EBPα),1 a basic-leucine zipper protein, is among the transcription factors that are induced prior to the onset of morphological differentiation. C/EBPα was the first regulatory protein demonstrated to play a central role in promotion of the adipogenic program. We (1, 2) and others (3–6) demonstrated that C/EBPs was both necessary and sufficient to promote adipogenesis in fibroblastic cells such as NIH3T3 and 3T3-L1. These observations, coupled with the fact that mice deficient in C/EBPα completely lack mature white or brown adipose tissue (7), demonstrate unequivocally the pivotal role of this transcription factor in adipogenesis.

Following the same approach, several other transcription factors, including another member of the C/EBP family, C/EBPβ (8, 9), and PPARγ2, a member of the nuclear hormone receptor superfamily (10, 11), proved capable of converting NIH3T3 cells into morphologically and biochemically differentiated adipocytes. It is now clear that all of the aforementioned transcription factors play an important and sequential role in the adipocyte differentiation process. In the 3T3-L1 preadipocyte model, induction of C/EBPβ and C/EBPα are the earliest events to occur upon treatment with differentiation inducers (8). These factors, in conjunction with adipogenic hormones, induce the expression of PPARγ2 (9). The next event, which coincides with cell cycle withdrawal and commitment to the adipocyte differentiation program, is the induction of C/EBPα. The presence of C/EBP and PPAR binding sites in the C/EBPα promoter suggest that its expression may be regulated by C/EBPβ and PPARγ2 (12).

In addition to transcription factors that induce and maintain the mature adipocyte phenotype, there are also factors that negatively regulate adipogenesis. Interestingly, one of these negative regulators, CHOP (C/EBP homologous protein), a stress and DNA damage-induced transcription factor, is a member of the C/EBP family (13–15). Unlike other C/EBPs that can bind sequence-specific DNA as homodimers, CHOP homodimers cannot bind sequence-specific DNA due to a non-consensural sequence in its basic region. However, CHOP is able to bind weakly to a restricted subset of high affinity C/EBP binding sites (GCAAT) when complexed as a heterodimer with

1 The abbreviations used are: C/EBP, CCAAT/enhancer binding protein; CHOP, C/EBP homologous protein; TLS, translocation liposarcoma; PCR, polymerase chain reaction; CAT, chloramphenicol acetytransferase.
other C/EBP proteins, such as C/EBPβ (16). Although CHOP participates in the stress signal in response to metabolic injuries, its downstream effectors are yet to be described.

Interestingly, an oncogenic variant of CHOP, called TLS-CHOP (TLS, translocation liposarcoma), is found specifically in myxoid liposarcoma, a malignant tumor of adipose tissue. TLS-CHOP results from a t(12;16) chromosomal translocation that fuses the amino-terminal part of TLS to the entire coding sequence of CHOP (17, 18). Recently, a second chromosomal translocation (EWS-CHOP, Ewing sarcoma) specific for myxoid liposarcoma was also found to involve CHOP (19). That two fusion oncoproteins involving CHOP are consistently (in over 90% of cases) and specifically associated with myxoid liposarcoma raises the possibility they are perturbing C/EBP function and causing a blockade in adipocyte differentiation. Although a previous study demonstrated that induction of endogenous CHOP by glucose deprivation inhibited 3T3-L1 adipogenesis (20), the underlying mechanism of this inhibition is unclear.

CHOP-expressing cells failed to induce normal levels of C/EBPα and C/EBPβ, two factors which are required to drive the 3T3-L1 adipogenic program. Thus, it is not possible to discern from that study whether the failure of CHOP-expressing cells to differentiate was attributable to inhibition of C/EBP function by CHOP or a lack of required C/EBPα and C/EBPβ expression. Moreover, the effect of TLS-CHOP, the oncogenic form of CHOP, on adipocyte differentiation remains unexplored.

In the present study we investigate whether TLS-CHOP can inhibit C/EBPβ-driven adipogenesis. Three lines of evidence make C/EBPβ the likely molecular target of TLS-CHOP in myxoid liposarcoma; (i) C/EBPβ protein is expressed in myxoid liposarcoma cells whereas C/EBPα is not (17)2; (ii) C/EBPβ is induced early in the adipogenic pathway and is likely to trigger many of the downstream events (8, 9); and (iii) C/EBPβ is the preferred heterodimerizing partner of TLS-CHOP (13). We demonstrate here that TLS-CHOP completely blocks C/EBPβ-driven adipogenesis by directly interfering with the normal function of the C/EBPβ protein. The results support the thesis that a blockade to normal differentiation is important in the development of the cancer phenotype.

MATERIALS AND METHODS

Retroviral and Plasmid Constructs—The parental vector, pLNI(−φ−), was derived from pLXSN (21). The encephalomyocarditis virus IRES sequence from pWZLneo (2) was introduced into pLXSN as an EcoRI-BamHI fragment following PCR amplification. The mouse C/EBPβ coding sequence was amplified by PCR from pMEX-CRP2 (22) and cloned into the EcoRI restriction site of pLNI(−φ−) to generate pLN(C/EBPβ−CHOP). pLN(C/EBPβ−CHOP) and pLN(C/EBPβ-TLS-CHOP) were constructed by insertion of PCR-amplified human CHOP- and TLS-chromosomal sequences into the BamHI site of pLNI(C/EBPβ−φ−), respectively. Human TLS-CHOP-coding sequences were obtained by PCR amplification of a 402/91 myxoid liposarcoma (17) (obtained from P. Aman) AZAP (Stratagene) cDNA library. The two TLS-CHOP mutant versions were engineered by PCR-mediated mutagenesis. Mutated regions were verified by DNA sequencing. TLS-CHOP/BR was found to carry a Gln to Gly mutation at amino acid 386. To generate pWZLhygroPPAR−2, the mouse PPAR-γ2-coding sequence was PCR-amplified from pBluescript-PPAR−2 (10) (obtained from B. Spiegelman) and blunt end-ligated into pWZLhygro between the BamHI and EcoRI sites. pWZLhygro is identical to pWZLneo (2) except that it contains the hygromycin resistance gene in place of the neomycin resistance gene, p(α2p2),TATA-chloramphenicol acetyltransferase (CAT) was created by cloning the double-stranded oligonucleotide 5′-AGCTTTTCACACCTTTGGGCTTTCATCTTTTTCCGTTTGGG-3′ into HindIII/BamHI-digested TATA-CAT (23).

Cell Culture and Gene Transfection—Retroviruses were produced by transient transfection of the Bosc 23 ectotropic packaging cell line (24). Cells (5 × 10⁶, 60-mm diameter dish) were transfected by the calcium phosphate precipitation method using 10 μg of plasmid DNA. Viral supernatants were harvested 48 h later and filtered through a 0.45-μm filter syringe. NIH3T3 cells (ATCC) were cultivated in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (growth medium). NIH3T3 cells (3 × 10⁶, 60-mm diameter dish) were infected with either of the two different retroviruses. NIH3T3 cells infected with WZLhygro and WZLhygroPPAR−2 were treated in exactly the same manner except cells were selected in growth medium containing 400 μg/ml hygromycin B for 7 days. To test for differentiation ability, each pool was seeded at 3 × 10⁵ cells/dish (60-mm diameter) and treated 3 days later (confluence) with 500 μg/ml hygromycin B for 7 days. To test for differentiation ability, each pool was seeded at 3 × 10⁵ cells/dish (60-mm diameter) and treated 3 days later (confluence) with differentiation medium consisting of Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 1 μg dexamethasone, 0.5 mM methylisobutylxanthine, and 10 μg/ml insulin. After 48 h of treatment, the medium was changed and cells were maintained thereafter in the same medium but containing only insulin. Seven days later, cells were either fixed with 3.7% formaldehyde and stained with Oil Red O or processed for whole cell protein analysis. NIH3T3 cells expressing PPAR−2 were induced to differentiate by the same protocol was used to prepare nuclear extracts from 3T3-L1 and 402/91 cells. Nuclear extracts were prepared from actively dividing cell cultures. Briefly, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and collected by centrifugation, and the cell pellet was resuspended in 500 μl of Nonidet P-40 lysis buffer (10 mM Tris, pH 7.4, 6.6 mM NaCl, 3 mM MgCl₂, 0.5% Nonidet P-40, 50 μM phenylmethylsulfonyl fluoride). After a 15 min incubation on ice, the suspension was homogenized, and cell debris was removed by centrifugation. The crude nuclei were washed in 500 μl of the same buffer, collected by microcentrifugation, lysed in Laemmli sample buffer, and boiled for 10 min. The same protocol was used to prepare nuclear extracts from 3T3-L1 and 402/91 cells. Nuclear extracts were resolved by SDS-polyacrylamide (12%) gel electrophoresis, and specific proteins were detected by Western blotting using the following antibodies: mouse anti-C/EBPα (Santa Cruz), anti-C/EBPβ antibody (R20, Santa Cruz) prior to the addition of the anti-CHOP antibody (R20, Santa Cruz).

The underlying mechanism of this inhibition is unclear.
EDTA. The following \textsuperscript{32}P-labeled, double-stranded oligonucleotides were used as probes in DNA-binding reactions: aP2, 5'-agcttgTTTCT-CAACTTTGa-3'; SAAB4, 5'-agcttgAAATGCAATCGCCa-3'.

**RESULTS**

**TLS-CHOP Inhibits C/EBPβ-driven NIH3T3 Adipogenesis**—To determine whether TLS-CHOP could inhibit C/EBPβ function in adipogenesis, we developed a system where both the inducer of differentiation (C/EBPβ) and the potential inhibitor (TLS-CHOP) could be co-expressed simultaneously in naive NIH3T3 cells. This was accomplished through the use of triple gene retroviral vectors in which the C/EBPβ and TLS-CHOP proteins are produced from the same bicistronic mRNA driven by the proviral long terminal repeat, and expression of the selectable marker gene (neo) is driven by an internal (SV40) promoter (Fig. 1). This approach has several conceptual and technical advantages: (i) unlike the 3T3-L1 system, the differentiation of NIH3T3 cells is completely dependent on the introduction of an exogenous transcription factor(s) making it possible to express the factor constitutively, (ii) use of the triple gene vectors eliminates the need to perform sequential infections and selections thereby avoiding the generation of clonal tangles, and (iii) co-translation of the C/EBPβ and TLS-CHOP proteins from the same bicistronic mRNA greatly increases the likelihood that they will be expressed stoichiometrically within the same cell. To obtain insight into which structural domains of TLS-CHOP are required for its inhibitory effect, two mutants were generated. TLS-CHOP/BR contains three site-directed mutations in its basic region (Arg407, Lys410, and Arg412) and was constructed to investigate the requirement of the heterodimerization domain. A retroviral vector encoding the normal CHOP protein was also generated and its two mutants are expressed at a roughly equal level.

Western blot analysis of G418-resistant NIH3T3 pools was performed to determine whether TLS-CHOP could inhibit C/EBPβ-driven adipogenesis. As shown in Fig. 2A, the five cell pools infected with the vectors encoding C/EBPβ either alone or in conjunction with CHOP, TLS-CHOP, and the TLS-CHOP mutants, express the same amount of the 36-kDa C/EBPβ (LAP) protein (lanes 2–6). This amount is significantly higher than the endogenous level of C/EBPβ found in NIH3T3 cells infected with the control vector (lane 1). Importantly, the level of C/EBPβ protein expressed in the NIH3T3 pools (lanes 2–6) is identical to that observed in 3T3-L1 adipoblasts on day 2 of differentiation (lane 8), whereas its level of expression in cells infected with the control vector (lane 1) is similar to that of undifferentiated 3T3-L1 cells (lane 7). Similarly, the level of CHOP expression in cells stably infected with the C/EBPβ-CHOP retrovirus (Fig. 2B, lane 3) is comparable to that observed in 3T3-L1 cells grown in low glucose for 2 days (lane 8). Likewise, TLS-CHOP and its two mutants are expressed at a roughly equal level (lanes 4–6), which is slightly greater than that observed in the 402/91 human myxoid liposarcoma cell line (lane 9). These results demonstrate that in this model system, the expression of C/EBPβ, CHOP, and TLS-CHOP closely mimics the normal or pathological conditions occurring in adipose tissue.

NIH3T3 cells expressing exogenous C/EBPβ can be induced to differentiate in a manner very similar to 3T3-L1 cells (8, 9). To determine the adipogenic potential of the various cell pools,
confluent cell monolayers were treated with adipogenic hormones for two days and the extent of differentiation was scored at day ten. As expected, the differentiation of NIH3T3 cells was completely dependent on ectopic expression of C/EBPβ (Fig. 3A, top, compare φ-φ to C/EBPβ-φ). Under these conditions, C/EBPβ was able to convert essentially 100% of the cell monolayer into mature, fat-laden adipocytes. Co-expression of CHOP (C/EBPβ-CHOP) strongly inhibited C/EBPβ-driven adipogenesis as only a low percentage of the cells acquired small fat droplets. Similarly, TLS-CHOP abolished C/EBPβ-driven adipogenesis and proved to be slightly, but consistently, better than CHOP. The extent of differentiation of TLS-CHOP-expressing cells (C/EBPβ-TLS-CHOP) was essentially identical to that of cells infected with the parental vector (φ-φ). Importantly, this inhibitory effect of TLS-CHOP was not attributable to its transforming activity as only a minute percentage (∼< 1%) of cells co-expressing C/EBPβ and TLS-CHOP showed signs of morphological transformation (fusiform morphology or foci formation). On the contrary, the vast majority of these cells displayed a morphology which was indistinguishable from that of control cells. Surprisingly, the TLS-CHOP basic region mutant (C/EBPβ-TLS-CHOP/BR) blocked adipogenic conversion by C/EBPβ as effectively as CHOP. By contrast, TLS-CHOP bearing point mutations in its heterodimerizing domain (C/EBPβ-TLS-CHOP/LZ) had no effect on C/EBPβ-driven differentiation.

These morphological observations were corroborated by examining the expression of aP2, a late differentiation marker. As shown in Fig. 3B (bottom), expression of aP2 was strongly induced upon differentiation of the C/EBPβ-φ and C/EBPβ-TLS-CHOP/LZ cell pools. In contrast, there was little or no induction of aP2 in those pools that failed to differentiate morphologically. Importantly, the failure of the CHOP-, TLS-CHOP-, and TLS-CHOP/BR-expressing cell pools to differentiate can not be attributed to reduced expression of the transcription factor driving the adipogenic program as C/EBPβ was expressed equally among these cell pools (see Fig. 2A).

Because the leucine zipper domain of TLS-CHOP was required for its ability to inhibit C/EBPβ-driven adipogenesis, we examined whether this effect of TLS-CHOP correlated with its ability to heterodimerize with C/EBPβ in situ. Both TLS-CHOP (Fig. 4, lane 4) and its basic region mutant (lane 5) could be co-immunoprecipitated from nuclear extracts with C/EBPβ-specific antibodies, indicating that these proteins were stably complexed with C/EBPβ. As expected, mutation of the leucine zipper domain of TLS-CHOP (lane 6) abolished its ability to heterodimerize with C/EBPβ. Thus, the ability of TLS-CHOP to inhibit C/EBPβ-driven adipogenesis requires its leucine zipper domain and correlates with its ability to form a stable heterodimer with C/EBPβ in situ.

Ectopic Expression of PPARγ2 Can Rescue TLS-CHOP-inhibited Cells—The data presented thus far are consistent with a mechanism in which TLS-CHOP inhibits C/EBPβ-driven adipogenesis by directly interfering with C/EBPβ function. However, they do not rule out the possibility that TLS-CHOP may block other requisite events which are independent of C/EBPβ function. To address this possibility, we examined whether ectopic expression of PPARγ2, another adipogenic transcription factor (10, 11), could rescue TLS-CHOP-inhibited cells from their differentiation block. NIH3T3 pools expressing φ-φ and C/EBPβ-TLS-CHOP were infected with a control retrovi-
rus (WZLhygro) or one encoding PPARγ2 (WZLhygroPPARγ2). Following a brief selection in hygromycin B, cells were pooled and examined for expression of PPARγ2 and their ability to differentiate into adipocytes. Immunofluorescence studies demonstrated that 80% of cells infected with WZLhygroPPARγ2 expressed PPARγ2 in the nucleus (not shown). By contrast, cells infected with the control virus and uninfected C/EBPb-TLS-CHOP cells (see Fig. 8, below right) did not express PPARγ2. Results of the differentiation assays demonstrated clearly that PPARγ2 was able to rescue TLS-CHOP-inhibited cells from their differentiation block (Fig. 5). Indeed, following treatment with the PPARγ2 activator pioglitazone (11), C/EBPβ-TLS-CHOP cells expressing PPARγ2 differentiated as well as PPARγ2-expressing control (φ-φ) cells. The results demonstrate that TLS-CHOP-inhibited cells are capable of adipogenesis, suggesting that TLS-CHOP does not interfere with other requisite C/EBPβ-independent events.

**FIG. 6. C/EBPβ DNA binding activity in NIH3T3 pools.** Electrophoretic mobility shift assays using the aP2 probe. φ-φ (lanes 1–6), C/EBPβ-φ (lanes 7–12), C/EBPβ-CHOP (lanes 13–18), C/EBPβ-TLS-CHOP (lanes 19–24), C/EBPβ-TLS-CHOP/BR (lanes 25–30), and C/EBPβ-TLS-CHOP/LZ (lanes 31–36). No nuclear extract (lanes 1, 7, 13, 19, 25, and 31); nuclear extract with no additions (lanes 2, 8, 14, 20, 26, and 32); addition of nonspecific (Sp1 binding site) competitor DNA (lanes 3, 9, 15, 21, 27, and 33); addition of specific (aP2 binding site) competitor DNA (lanes 4, 10, 16, 22, 28, and 34); addition of anti-C/EBPβ antibodies (lanes 5, 11, 17, 23, and 35); and addition of anti-CHOP antibodies (lanes 6, 12, 18, 24, 30, and 36). The positions of the C/EBPβ-C/EBPβ homodimer, supershifted complexes, and free aP2 probe are indicated.

**TLS-CHOP Blocks C/EBPβ function by Preventing Its Binding to Target DNA Sequences**—To gain a better understanding of how TLS-CHOP inhibits C/EBPβ function, we examined the possibility that TLS-CHOP blocks C/EBPβ from binding to its DNA target sites. In a first test, the amount of C/EBPβ DNA binding activity in each NIH3T3 pool was determined by the electrophoretic mobility shift assay using the aP2 probe (28). Ectopic expression of C/EBPβ resulted in an increase in the amount of complex formed between C/EBPβ and the aP2 probe (Fig. 6, compare lane 2 to lane 8). The presence of C/EBPβ in this complex is demonstrated by the fact that an anti-C/EBPβ antibody could completely “supershift” this complex (lane 11). Although the five cell pools overexpressing C/EBPβ contain the same amount of C/EBPβ protein in the nucleus (Fig. 2A), the C/EBPβ DNA binding activity in cells co-expressing CHOP (lane 14), TLS-CHOP (lane 20), or TLS-CHOP/BR (lane 26) was significantly reduced relative to...
C/EBPβ-φ cells (lane 8). In contrast, C/EBPβ DNA binding activity was unaffected in cells co-expressing TLS-CHOP/LZ (lane 32).

The C/EBPβ-CHOP heterodimer was recently shown to bind a subset of high affinity C/EBP sites (AAATGC*AAATCC, SAAB4 site) (16). Thus, the same nuclear extracts were examined for C/EBPβ DNA binding activity using the SAAB4 probe. The results were essentially the same as for the aP2 probe, except that a relatively minor complex was observed below the C/EBPβ-CHOP homodimer complex in cells co-expressing C/EBPβ and CHOP (not shown). This complex was the C/EBPβ-CHOP heterodimer, as it is eliminated by both anti-C/EBPβ and anti-CHOP antibodies. No additional complexes were observed with extracts co-expressing C/EBPβ and TLS-CHOP (or TLS-CHOP/LZ), suggesting that unlike the C/EBPβ-CHOP heterodimer, the C/EBPβ-TLS-CHOP heterodimer can not bind to the SAAB4 sequence.

This issue was examined further by measuring C/EBPβ transactivation activity in the different NIH3T3 pools. For this purpose, each cell pool was transfected with a CAT reporter gene driven by a C/EBP-responsive promoter containing a tri-merized aP2 binding site. A modest (average of 4-fold, n = 5), but reproducible, increase in CAT reporter gene activity was observed in cells overexpressing C/EBPβ (C/EBPβ-φ) relative to control (φ-φ) cells (Fig. 7, compare lanes 1 and 2). Importantly, TLS-CHOP completely abolished C/EBPβ transactivation activity, as cells co-expressing C/EBPβ and TLS-CHOP showed the same level of CAT activity as cells expressing the parental vector (compare lane 4 to lane 1). As expected, TLS-CHOP mutated in its leucine zipper had no effect on C/EBPβ transactivation activity (compare lane 6 to lane 2). CHOP (lane 3) and TLS-CHOP/BR (lane 5) were less effective than wild-type TLS-CHOP (lane 4) at inhibiting C/EBPβ transactivation activity, demonstrating that these results qualitatively paralleled those of the differentiation assays (Fig. 3).

**TLS-CHOP Blocks Induction of the Adipogenic Transcription Factor PPARγ2**—Two indirect lines of evidence suggest that the PPARγ2 gene is a downstream target of C/EBPβ. Conditional expression of C/EBPβ results in the induction of PPARγ2 mRNA (9), and the mouse PPARγ2 promoter contains potential C/EBP binding sites (29). Because PPARγ2 is an important component of the adipocyte differentiation machinery, we examined whether TLS-CHOP blocked the induction of PPARγ2 in C/EBPβ-expressing cells following treatment with differentiation inducers. Cells expressing C/EBPβ either alone (C/EBPβ-φ) or with TLS-CHOP (C/EBPβ-TLS-CHOP) were grown to confluence, treated with differentiation inducers, and examined for expression of PPARγ2 by immunofluorescence 2 days later. As expected, cells expressing C/EBPβ alone showed clear evidence of PPARγ2 expression in the nucleus (Fig. 8, left). In contrast, cells co-expressing C/EBPβ and TLS-CHOP (right) exhibited only background immunofluorescence that was evenly distributed over the entire cell. This uniform background immunofluorescence was similar to that observed with C/EBPβ-φ cells prior to treatment with differentiation inducers (not shown). Only an occasional C/EBPβ-TLS-CHOP cell showed weak nuclear expression of PPARγ2 following treatment with differentiation inducers (Fig. 8, right). Together, the results demonstrate that TLS-CHOP effectively blocks C/EBPβ function, induction of PPARγ2, and development of the mature adipocyte phenotype.

**DISCUSSION**

Using a differentiation model that closely mimics physiological conditions and where the expression of C/EBPβ is constitutive and sufficient to drive the adipogenic program, we demonstrate here that TLS-CHOP functions as a potent inhibitor of C/EBPβ-driven adipogenesis. Unlike the 3T3-L1 system where a battery of transcription factors, including at least three C/EBP family members, cooperate to trigger the onset of differentiation, the simple model system described here allowed us to demonstrate that TLS-CHOP blocks adipocyte differentiation by directly interfering with C/EBPβ function.

Based on the result that three key mutations in the TLS-CHOP basic region have little effect on its anti-adipogenic activity, and unlike for CHOP (16), there is no evidence that the C/EBPβ-TLS-CHOP heterodimer can bind sequence-specific DNA, we conclude it is unlikely that the inhibitory effect of TLS-CHOP is attributable to unscheduled gene expression that prevents adipocyte differentiation. The fact that ectopic expression of PPARγ2 can rescue TLS-CHOP-inhibited cells strongly supports this conclusion. Nor can the inhibitory effect of TLS-CHOP be attributed to its weak oncogenic activity (30), as the vast majority of C/EBPβ-TLS-CHOP-expressing cells showed no signs of morphological transformation. On the contrary, our results indicate that the ability of TLS-CHOP to inhibit adipocyte differentiation is mediated largely through its ability to heterodimerize with and inhibit the function of C/EBPβ. As shown here, this interaction inhibits C/EBPβ DNA binding, resulting in a decrease in its transactivation ability. Our results with the TLS-CHOP basic region mutant are somewhat at odds with the results of Ron and colleagues (20) who reported that the basic region of CHOP was required for its ability to inhibit 3T3-L1 adipogenesis. We believe the most likely explanation for this discrepancy is that the CHOP basic region mutant used in that study was poorly expressed relative to wild-type CHOP (see Fig. 3B in Batchvarova et al. (20)). In contrast, the TLS-CHOP basic region mutant used here is expressed equally well relative to wild-type TLS-CHOP and heterodimerizes equally well with C/EBPβ. Unfortunately, because there is no evidence that TLS-CHOP homodimers or heterodimers can bind sequence-specific DNA, we could not demonstrate that the introduced mutations had the expected effect on TLS-CHOP’s presumed DNA binding activity. However, given that the three basic amino acids altered are critical for C/EBPβ DNA binding activity it is likely that if TLS-CHOP is capable of binding sequence-specific DNA, this mutant of TLS-CHOP would lack this activity.

The finding that TLS-CHOP, the product of a chromosomal translocation found only in a malignancy of adipose tissue, involves a member of the C/EBP family makes much sense in light of the pivotal role of the C/EBP transcription factors in...
adipocyte differentiation. Results from four laboratories have shown that C/EBPα is both sufficient (1–3) and necessary (5–7) for adipogenesis in vitro and in vivo. C/EBPβ and C/EBPδ, two proteins closely related to C/EBPα, and PPARγ2, a transcription factor belonging to the nuclear hormone receptor superfamily, are also important factors in the adipogenic pathway (8–11). The apparent redundancy of these transcription factors was initially somewhat puzzling; however, their respective roles in adipocyte differentiation are becoming clearer. In the 3T3-L1 model system, induction of C/EBPβ is one of the first events to occur following treatment with adipogenic hormones (8). Expression of C/EBPβ, in collaboration with C/EBPα, generates a second wave of transcriptional activation that leads to the induction of PPARγ2 (9). Although it is not known whether the induction of PPARγ2 expression is a direct effect of C/EBPβ, the presence of C/EBP binding sites in the PPARγ2 promoter makes it likely that PPARγ2 is a downstream target of C/EBPβ. The regulation of C/EBPα expression is more complex and subject to both positive and negative control. The presence of PPAR and C/EBP binding sites in the C/EBPα promoter (12), coupled with the fact that C/EBPβ (LAP) can transactivate this promoter in transfection assays (our unpublished results), suggests that induction of C/EBPα results from a cooperation between C/EBPβ and PPARγ2. Whereas the induction of C/EBPβ and PPARγ2 occurs early (by 24 h) and at least 1 day prior to any signs of morphological differentiation, the induction of C/EBPα (by 48 h) coincides precisely with cell cycle withdrawal and commitment to the adipocyte differentiation program (1, 31). These observations, coupled with the facts that C/EBPα is known to inhibit cell growth (1, 2, 4, 32) and is required for adipogenesis both in vitro and in vivo (5–7), make it likely that induction of C/EBPα is the critical event which commits adipoblasts to the differentiation program. However, the fact that C/EBPβ, but not C/EBPα, is expressed in myxoid liposarcoma suggests that TLS-CHOP is blocking differentiation of this lineage at a point prior to induction of C/EBPα, making C/EBPβ the likely target. Given that PPARγ2 and C/EBPα are likely downstream targets of C/EBPβ, inhibition of C/EBPβ function by TLS-CHOP would prevent their induction and commitment to the adipocyte differentiation pathway.

Although the studies described here are directly relevant to the genesis of myxoid liposarcoma, we believe they may have even broader significance. TLS-CHOP belongs to a growing family of fusion oncoproteins resulting from chromosomal translocations. That most of these fusion proteins are specific for a given type of cancer suggests they are impinging on a mechanism which is specific for that particular cell lineage (e.g. differentiation machinery, cell-specific signal transduction pathway) and not some general proliferation mechanism (cell cycle machinery). Although incomplete differentiation is a hallmark characteristic of the cancer cell, there is little direct proof that the inability to differentiate normally is in fact important in the cancer process. Indeed, because of the well known reciprocal relationship between proliferation and differentiation, it was often argued that the inability of a cancer cell to differentiate completely was simply an indirect consequence of uncontrolled proliferation. Cancer cells can not irreversibly exit the cell cycle, which is required for terminal differentiation. This argument has been very difficult to challenge as it is buttressed by the fact that many of the genes known to be mutated in human cancer play a role in the regulation of cell proliferation. The discovery of oncoproteins such as TLS-CHOP and others (e.g. PML-RARα), whose molecular targets are integral components of the cell differentiation machinery, however, significantly weakens this argument, at least for those cancers that involve them. On the contrary, the fact that these oncoproteins directly target and inhibit the cell differentiation machinery argues strongly that a blockade in differentiation is important, but clearly not sufficient, for development of the malignant phenotype. Indeed, it is likely that the other component of the TLS-CHOP fusion oncoprotein (i.e. TLS) provides an important function required for malignant transformation in vivo, such as a continuous growth signal to differentiation-arrested cells.

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FIG. 8. TLS-CHOP-inhibited cells fail to induce PPARγ2. Confluent cell monolayers expressing either C/EBPδ alone (C/EBPδ−, left) or co-expressing C/EBPβ and TLS-CHOP (C/EBPβ-TLS-CHOP, right) were treated with differentiation inducers for 2 days and examined for expression of PPARγ2 by immunofluorescence. Cells on the right were intentionally overexposed to show the uniform background immunofluorescence and lack of nuclear staining. Cells were photographed at a magnification of 500×. Differentiation assays were repeated three times with similar results.
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