The Expression and Regulation of STATs during 3T3-L1 Adipocyte Differentiation*

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Jacqueline M. Stephens, Ron F. Morrison, and Paul F. Pilch‡

From the Department of Biochemistry, Boston University Medical Center, Boston, Massachusetts 02118

STATs (Signal Transducers and Activators of Transcription) comprise a family of transcription factors that reside in the cytoplasm of resting cells. In response to a variety of stimuli, STATs become tyrosine-phosphorylated and translocate to the nucleus where they mediate transcriptional regulation. We have used the 3T3-L1 murine cell line to examine the expression of STAT proteins as a function of their differentiation into adipocytes. The expression of STATs 1, 3, and 5, but not of STAT 6, is markedly elevated in adipocytes as compared with their fibroblast precursors. Exposure of 3T3-L1 preadipocytes to tumor necrosis factor α (TNFα) blocks their differentiation into adipocytes. Therefore, we examined STAT expression as a function of differentiation in the presence of this cytokine. The expression of STATs 1 and 5 is markedly attenuated in the presence of TNFα, whereas STAT 3 expression is unaffected by this treatment. Only STAT 1 is down-regulated by TNFα in fully differentiated cells. Thus, although the expression of STATs 1, 3, and 5 is markedly enhanced upon differentiation, only STAT 5 expression is tightly correlated with the adipocyte phenotype. These data suggest that STAT 5, and possibly STAT 1, could be potential inducers of tissue-specific genes, which contribute to the development and maintenance of the adipocyte phenotype.

The 3T3-L1 cell line differentiates under the controlled conditions of cell culture from fibroblasts, or preadipocytes, into cells with the morphological and biochemical properties of adipocytes (Green and Kehinde, 1974; Green and Kehinde, 1976) in a process that closely resembles the development of adipose tissue in vivo. Upon differentiation, these cells acquire sensitivity to hormones and exhibit a coordinate increase in the activities of numerous enzymes in the lipolytic, lipogenic, and glycolytic pathways (Smas and Sul, 1995). To date, members of two transcription factor families, C/EBP (C/AAAT Enhancer Binding Proteins) and PPAR (Peroxisome Proliferator Activated Receptors) have been shown to be induced during adipocyte differentiation and are thought to play a significant role in the regulation of fat-specific gene expression.

The STAT (Signal Transducers and Activators of Transcription) family of transcription factors is comprised of six family members (STATs 1–6) that, in response to stimulation of various receptors, mainly those for cytokines, are phosphorylated on tyrosine residues, which causes their translocation to the nucleus. Each STAT family member shows a distinct pattern of activation by cytokines, has a unique tissue distribution, and upon nuclear translocation can regulate the transcription of particular genes (Schindler and Darnell, 1993; Ihle, 1995). The likely order of events for STAT activation can be described as follows: 1) ligand binding of cell surface receptor; 2) receptor association with a JAK (Janus kinase) kinase family member; 3) JAK tyrosine phosphorylation of STAT proteins; 4) dimerization of the STATs; 5) translocation to the nucleus; and 6) DNA binding. STATs have been shown to bind at least three different consensus sequences, and this binding regulates the transcription of specific genes (Schindler and Darnell, 1993; Ihle, 1995).

One of the first identified inhibitors of adipocyte differentiation was tumor necrosis factor-α (TNFα), a cytokine that elicits a wide range of biological effects including the regulation of growth and differentiation. In addition, TNFα has been shown to down-regulate the insulin responsiveness of fully differentiated adipocytes (Stephens and Pekala, 1991; Hotamisligil et al. 1993). Because regulation of the STATs is mainly cytokine-mediated, TNFα could be a mediator of STAT expression during and/or after adipocyte differentiation. Most of the studies on the STAT family of transcription factors have focused on their tyrosine phosphorylation and DNA binding. In this report, we demonstrate that another level of regulation of these proteins exists as they are induced during the differentiation of adipose cells in culture. Moreover, we demonstrate that inhibition of differentiation by TNFα completely suppresses the expression of two STAT family members. We interpret these data to indicate that STAT family members may play a role in the regulation of genes that contribute to the phenotype of the mature adipocyte.

EXPERIMENTAL PROCEDURES

Cell Culture—Murine 3T3-L1 preadipocytes were cultured, maintained, and differentiated as described previously (Cornelius et al., 1990). Briefly, cells were plated and grown to 2 days postconfluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf bovine serum. Differentiation was then induced by changing the medium to DMEM supplemented with 10% fetal bovine serum (FBS), 0.5 mM 3-isobutyl-1-methylxanthine, 1 mM dexamethasone, and 1.7 mM insulin. After 48 h, the differentiation medium was replaced with maintenance medium containing DMEM supplemented with 10% FBS. The maintenance medium was changed every 48 h until the cells were utilized for experimentation. Human recombinant TNFα (Quality Control Biochemical) was resuspended in phosphate-buffered saline containing 0.1% fatty acid-free and growth factor-depleted bovine serum albumin (Sigma).

Proliferating Adipocytes—Adipocytes were isolated from the epididymal fat pads of male Sprague-Dawley rats (150–175 g) by collagenase digestion as described (Rodbell, 1964). Cell fractionation was performed as described previously (Simpson et al., 1983), and the cytosol fraction was utilized for experimentation. Human recombinant TNFα (Quality Control Biochemical) was resuspended in phosphate-buffered saline containing 0.1% fatty acid-free and growth factor-depleted bovine serum albumin (Sigma).

The abbreviations used are: TNFα, tumor necrosis factor-α; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; C/EBP, C/AAAT enhancer binding protein; PPAR, peroxisome proliferator-activated receptor; PAGE, polyacrylamide gel electrophoresis.
FIG. 1. The expression of STATs in adipocytes. Panel A, whole cell extracts were prepared from 3T3-L1 adipocytes and the cells listed below. In addition, cytosolic extract was prepared from the adipocytes of rat epididymal fat pads. Positive controls for STAT immunoblotting were provided by Transduction Laboratories and were as follows: A431 cells for STAT 1, human fibroblasts for STAT 3, RSV-3T3 mouse fibroblast cell line for STAT 5, and Jurkat cells derived from acute T-cell leukemia for STAT 6. Cell extracts from 3T3-L1 adipocytes (lanes 1, 4, 7, and 10) and cytosol from rat epididymal fat (lanes 2, 5, 8, and 11) were divided and blotted simultaneously, while a different positive control (lanes 3, 6, 9, and 12) was used to examine the expression of STAT family members. The whole gel for each STAT family member is shown in panel A, while the remainder of the figures only includes the part of the blot that had a signal as these antibodies do not have any cross-reactivity as shown in this panel. Panel B, whole cell extracts were isolated from growing 3T3-L1 preadipocytes (lanes 1, 4, 7, and 10) and cytosol from rat epididymal fat (lanes 2, 5, 8, and 11) were divided and blotted simultaneously, while a different positive control (lanes 3, 6, 9, and 12) was used to examine the expression of STAT family members. The whole gel for each STAT family member is shown in panel A, while the remainder of the figures only includes the part of the blot that had a signal as these antibodies do not have any cross-reactivity as shown in this panel.

FIG. 2. Early induction of STATs during 3T3-L1 differentiation. Whole cell extracts were prepared from 3T3-L1 cells at various times following the induction of differentiation. Cells were induced to differentiate at 2 days postconfluence with the addition of a differentiation mixture containing 10% fetal bovine serum (FBS), 0.5 mM 3-isobutyl-1-methylxanthine, 1 mM dexamethasone, and 1.7 μM insulin. After 48 h this medium was replaced with DMEM supplemented with 10% FBS, and cells were maintained in this condition throughout the remainder of the analysis. Samples were processed and results were visualized as described in Fig. 1.

Fig. 3. The 96- and 94-kDa doublet has been consistently identified in various other cell types and postulated to be alternatively spliced gene products of STAT 5 (Schindler and Darnell, 1995). The higher molecular mass band at 110 kDa has been speculated to be a phosphorylated form of STAT 5 (Barahmand-pour et al., 1995). Alternatively, this protein product could be another form of STAT 5 or even an unidentified STAT family member. Monoclonal antibodies for STAT 3 and STAT 6 reacted with single protein products with molecular masses of 92 and 100 kDa, respectively.

RESULTS

The expression in adipocytes of the various STAT proteins, as detected by Western blot, is depicted in Fig. 1. The profile in panel A illustrates that STAT 1, STAT 3, STAT 5, and STAT 6 have similar levels of expression in the whole cell extracts from 3T3-L1 cultured murine adipocytes (lanes 1, 4, 7, and 10) and from the cytosol of rat epididymal fat cells (lanes 2, 5, 8, and 11). The third lane of each panel shows STAT expression in cellular extracts (provided by Transduction Laboratories) from cells known to express these proteins at substantial levels (lanes 3, 6, 9, and 12). It can be seen that STAT expression in adipocyte extracts was equivalent to or greater than cells known to express these proteins. The expression of STAT 4 protein was undetectable in either cultured adipocytes or adipose tissue when compared with positive controls (data not shown), and species-specific antibodies for STAT 2 are not commercially available at this time. Panel A also illustrates that the 91- and 84-kDa proteins of STAT 1, reported as alternatively spliced products of the same gene (Schindler et al., 1995), were detectable in both cultured adipocytes and adipose tissue. The monoclonal antibody for STAT 5 reacted with three protein products from cultured adipocytes (best illustrated in...
TNFα-activated receptors (PPARs), have been shown to be induced by enhancer binding proteins (C/EBPs) and peroxisome proliferated at the level of their expression as well as by their cytosolic localization. This indicates that this family of transcription factors can be regulated by TNFα.

The expression of STATs 1, 3, and 5 is induced during the differentiation of 3T3-L1 cells from fibroblast cell lines can promote adipogenesis (Tontonoz et al., 1994), while expression of C/EBPα antisense RNA blocks the differentiation of 3T3-L1 adipocytes and the expression of some fat-specific genes (Lin and Lane, 1992). PPARγ is a recently cloned member of the peroxisome proliferator-activated receptor family and has been identified as a component of the adipogenic transcription factor complex (ARF6), which regulates transcription of the fat-specific gene aP2/422 (Tontonoz et al., 1994a). PPARγ is expressed primarily in adipocytes and is induced very early in the process of adipocyte differentiation. Like C/EBPα, when ectopically expressed in a number of fibroblast cell lines, PPARγ can induce adipogenesis (Tontonoz et al., 1994b). We show here that three members of the STAT family of transcription factors, STATs 1, 3, and 5, are induced during differentiation in a manner similar to C/EBPα and PPARγ. Therefore, these STATs could potentially play a critical role in both the development of the adipose phenotype and the regulation of expression of fat-specific genes.

Since STAT 1 and STAT 5 are highly induced during differentiation and their accumulation is repressed when differentiation is inhibited, it is likely that these STAT family members could be transcriptional regulators involved in the development and/or maintenance of the adipose phenotype. However, the repression of STAT 1 and STAT 5, which occurs when inhibiting differentiation with TNFα, could be due to a direct effect of TNFα on STAT expression. In fact, this may be the case for STAT 1 whose expression is severely down-regulated when fully differentiated 3T3-L1 adipocytes are exposed to prolonged TNFα treatment (Fig. 4, see also, next paragraph). TNFα has no effect on STAT 5 accumulation in fully differentiated adipocytes (Fig. 4), and STAT 5 expression strongly correlates with the degree of adipocyte differentiation when this process is manipulated by subtraction of differentiation mixture elements (data not shown). STAT 3 expression is increased upon conversion of preadipocytes to adipocytes (Fig. 2), but its induction is not inhibited with TNFα, which also inhibits differentiation (Fig. 3), thus suggesting that its increased expression is unrelated to this process. However, STAT 3 is still likely to have a function in the terminally differentiated adipocyte as it is clearly present in the fully differentiated adipocytes and in rat fat cells (Fig. 1). STAT 1 expression also correlates with the degree of adipocyte differentiation, albeit to a lesser extent than STAT 5 (data not shown).

The transient down-regulation of STAT 1 and STAT 5 during differentiation occurs between 24 and 72 h after the induction of differentiation. This time frame overlaps with the presence of the differentiation-inducing mixture (0–48 h), and it is possible that the combination of hormones present in the mixture may be responsible for this temporary down-regulation of the STATs.
STAT1 and STAT5 protein levels, either directly or indirectly, by inducing the expression of some additional effector (inhibitor) of STAT expression. We are currently examining the effects of specific components of the induction mixture on STAT expression during differentiation.

Exposure of fully differentiated adipocytes to TNF\(\alpha\) results in a highly specific and significant decrease in STAT 1 expression (Fig. 4). As previously shown, this exposure to TNF\(\alpha\) did not result in dedifferentiation or a loss of lipid content (Stephens and Pekala, 1991). Recent studies have demonstrated that TNF\(\alpha\) treatment of fully differentiated cultured adipocytes results in insulin resistance, which is accompanied by the down-regulation of the insulin-sensitive glucose transporter (GLUT4) and the insulin receptor (Stephens and Pekala, 1991; Hotamisligil et al., 1993). Furthermore, the observed decrease in STAT 1 parallels the TNF\(\alpha\)-induced repression of GLUT4.2 Given that the induction of STAT 1 expression is concomitant with the acquisition of insulin sensitivity of 3T3-L1 adipocytes and is down-regulated by TNF\(\alpha\) in a condition of insulin resistance, we hypothesize that STAT 1 expression and function may be contributing to the regulation of genes involved in insulin sensitivity in 3T3-L1 adipocytes. We are in the process of experimentally addressing this and other hypotheses concerning the physiological role of STATs 1, 3, and 5 in adipocyte differentiation and gene expression.

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