Expression and Regulation of Neuronal Apoptosis Inhibitory Protein During Adipocyte Differentiation

Rick Magun, AnneMarie Gagnon, Zahra Yaraghi, and Alexander Sorisky

We have used the 3T3-L1 and 3T3-F442A preadipocyte cell lines to examine the expression and regulation of neuronal apoptosis inhibitory protein (NAIP) during adipocyte differentiation. When 3T3-L1 preadipocytes differentiated into adipocytes, they developed resistance to apoptosis induced by growth factor deprivation, as assessed by terminal deoxynucleotide transferase (TdT)-mediated dUTP nick end labeling. Protein expression of NAIP was markedly elevated in 3T3-L1 and 3T3-F442A adipocytes compared with that in their fibroblast-like precursors. NAIP was also present in rat white adipocytes. In 3T3-L1 cells, the increase in NAIP occurred by day 4 of the 8-day differentiation protocol, which includes exposure of confluent preadipocytes to insulin, dexamethasone, and isobutylmethylxanthine. Incubation of confluent 3T3-L1 preadipocytes with any of these components alone had no effect on NAIP expression. When 3T3-C2 cells, a control cell line that does not differentiate, were subjected to the differentiation protocol, the low NAIP levels remained unaltered. Addition of rapamycin, a p70 S6 kinase inhibitor that blocks adipocyte differentiation, to the 3T3-L1 differentiation medium prevented the rise in NAIP expression. These data demonstrate for the first time that NAIP is expressed in adipocyte cell lines and primary adipocytes. The differentiation-dependent augmentation of NAIP protein levels in 3T3-L1 adipocytes is closely correlated with the development of resistance to apoptosis induced by growth factor deprivation, suggesting a potential role for NAIP in these cells. *Diabetes* 47:1948–1952, 1998

Deletions in the gene encoding neuronal apoptosis inhibitory protein (NAIP) are implicated in the pathogenesis of spinal muscular atrophy, a disease caused by motor neuron death (1). NAIP is one of a number of mammalian proteins, known as inhibitors of apoptosis (IAPs), that suppress apoptosis (2). These include HIAP-1, HIAP-2, and XIAP (2). One of the common structural motifs in IAP family members is an 80–amino acid domain known as the baculovirus IAP repeat (BIR), and NAIP possesses three of these repeats (2). This region may play a critical role in mediating cell survival (3). Overexpression of NAIP in CHO, Rat-1, or HeLa cells prevents the death of cells challenged by growth factor withdrawal, menadione, or tumor necrosis factor (2). Enhancing local NAIP levels by an adenovirus vector conferred protection against ischemic cell death in the rat hippocampus (4).

Expression of NAIP has been reported in the central nervous system, as well as in the liver and placenta (1). In this report, we show for the first time that high levels of NAIP are found in 3T3-L1 and 3T3-F442A adipocytes, as well as in rat white adipocytes. Furthermore, NAIP expression is dramatically upregulated during adipocyte differentiation. These novel observations are of particular interest, given that 3T3-L1 adipocyte differentiation is also associated with an acquired resistance to apoptosis induced by growth factor deprivation (5).

RESEARCH DESIGN AND METHODS

Cell culture and differentiation. The 3T3-L1 (American Type Tissue Collection), 3T3-F442A, and 3T3-C2 cell lines (kind gift of H. Green, Harvard University) are derived from the Swiss 3T3 mouse (6). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 10% calf serum, all from Gibco-BRL (Burlington, Canada). At 2 days postconfluence, 3T3-L1 preadipocytes were induced to differentiate (day 0) with addition of insulin (100 nmol/l; Boehringer Mannheim, Indianapolis, IN). Dexamethasone (0.25 µmol/l; Steraloids, Wilton, NH) and isobutylmethylxanthine (IBMX) (0.5 mmol/l; Sigma, St. Louis, MO) were added to the medium for the first 48 h only. From days 2 to 6, the medium was supplemented with 100 nmol/l insulin only. The cells were then switched back to 10% calf serum from days 6 to 8. The 3T3-C2 cells (resistant to differentiation) were subjected to the same differentiation protocol. For 3T3-F442A preadipocytes, confluent cultures were switched to 10% fetal bovine serum (day 0) and were induced to differentiate with addition of insulin (1µmol/l) until day 6. Insulin was omitted from days 6 to 8. For experiments using rapamycin (100 nmol/l; Calbiochem, La Jolla, CA) or vehicle control , the medium was replaced every 24 h; otherwise, it was replaced every 48 h.

Western blot analysis. All cells were lysed in Laemmli sample buffer (7). Equal amounts of solubilized cellular protein were resolved by 7.5% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane (Bio-Rad, Mississauga, Canada). Nonspecific antigenic sites were blocked, and membranes were probed with either anti-NAIP (Z.Y., A. MacKenzie, unpublished observations; the rabbit polyclonal antibody was prepared by immunization with a mouse NAIP/glutathione S-transferase (GST) fusion protein) or anti-HIAP-2 (8) polyclonal antibodies. The antibodies were generously provided by Drs. A. MacKenzie and R. Korneluk of Apoptogen (Ottawa, Canada). Rat white adipocyte homogenates were kindly made available to us by Drs. M. Ghorbani and J. Himms-Hagen. Detection was by enhanced chemiluminescence (Amersham, Arlington Heights, IL) using horseradish peroxidase-conjugated anti-rabbit secondary antibody (Amersham). Protein was measured with the Sigma Protein Assay kit according to manufacturer's instructions, using bovine serum albumin as a standard.

Assessment of apoptosis. 3T3-L1 preadipocytes were grown on glass cover slips and subjected to differentiation as described above. 3T3-L1 preadipocytes and their fully differentiated counterparts were deprived of growth factors (serum withdrawal) for either 24 or 48 h, as indicated. The cells were then fixed for 30 min at room temperature with 4% paraformaldehyde in phosphate-buffered saline. Apo-

From the Departments of Medicine and Biochemistry (R.M., A.G., A.S.), Loeb Research Institute, Ottawa Hospital, and the Molecular Genetics Laboratory (Z.Y.), Children's Hospital of Eastern Ontario, University of Ottawa, Ottawa, Ontario, Canada.

Address correspondence and reprint requests to Alexander Sorisky, MD, Loeb Research Institute, 725 Parkdale Ave., Ottawa, Ontario, Canada K1Y 4E9. E-mail: sorisky@civich.ottawa.on.ca.

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BIR, baculovirus IAP repeat; IAP, inhibitor of apoptosis protein; IBMX, isobutylmethylxanthine; NAIP, neuronal apoptosis inhibitory protein; PPAR-γ, peroxisome proliferator-activated receptor-γ; SMA, spinal muscular atrophy; TdT, terminal deoxynucleotide transferase; TUNEL, TdT-mediated dUTP nick end labeling.

ptosis was measured by TdT-mediated dUTP nick end labeling (TUNEL) using the In Situ Cell Death Detection kit (Boehringer Mannheim) according to manufacturer's instructions. Cells were visualized with a Zeiss fluorescence microscope.

Assessment of differentiation. The protein expression of peroxisome proliferator-activated receptor-γ (PPAR-γ) was by Western blot analysis using a monoclonal anti-PPAR-γ antibody, which detects the shorter γ1 and longer γ2 isoforms (Santa Cruz Biotech, Santa Cruz, CA). Cellular triglyceride was quantified as described previously (9,10).

RESULTS

Our laboratory has recently demonstrated that as 3T3-L1 cells differentiate from preadipocytes to adipocytes, they acquire a resistance to apoptosis induced by growth factor deprivation (5). Apoptosis was measured by phase-contrast microscopy, nuclear staining with Hoechst dye, and DNA fragmentation (5). We have confirmed these observations here, using TUNEL. Figure 1 shows photomicrographs of 3T3-L1 cells (preadipocytes: Fig. 1*A–C*; adipocytes: Fig. 1*D–F*) with corresponding fields that were examined under fluorescence microscopy to detect the labeling of 3′-OH DNA ends with fluorescein-tagged dUTP (preadipocytes: Fig. 1*G–I*; adipocytes: Fig. 1*J–L*). Phase-contrast microscopy revealed cell shrinkage and a phase-bright refractile appearance characteristic of membrane blebbing in the serum-starved preadipocytes (Fig. 1*B* and *C*); these morphological changes were not seen in serum-starved (24 or 48 h) adipocytes (Fig. 1*E* and *F*). After 24 or 48 h of serum withdrawal for preadipocytes, positive staining by TUNEL indicative of apoptosis was easily observed in 40% (Fig. 1*H*) and 60% (Fig. 1*I*) of the cells, respectively. In contrast, the in vitro differentiated adipocytes did not exhibit any positive staining for the same durations of growth factor deprivation (Fig. 1*K* and *L*).

As part of our search for apoptosis-related genes in the preadipocyte/adipocyte whose expression might be differentiation-dependent, we investigated whether NAIP might be expressed in established adipocyte cell lines (Fig. 2; immunoblot analysis). For 3T3-L1 and 3T3-F442A preadipocytes (Fig. 2*A* and *B*, *lanes 1* and *2*), a barely detectable ~150-kD band corresponding to NAIP was seen. However, upon differentiation (day 8; ~90% of cells acquired the adipose phenotype) of either of these preadipocyte cell lines, there was a huge increase (>20-fold) in NAIP expression (Fig. 2*A* and *B*, *lanes 3* and *4*). Additional evidence of adipocyte differentiation was provided (Fig. 2*B*) by measuring the induction of PPAR-γ, a marker of adipogenesis (11). These changes in NAIP levels are significantly larger than the twofold increases seen in neuronal systems examining NAIP immunoreactivity and neuroprotection (4).

The 3T3-C2 cell line, also derived from the Swiss 3T3 mouse embryo, is relatively resistant to adipocyte differentiation (6). When 3T3-C2 cells were subjected to the same differentiation protocol as the 3T3-L1 cells, they did not differentiate or express PPAR-γ, as expected, nor was there any increase in the level of NAIP expression (barely detectable) after 8 days (Fig. 2*C*). In contrast to NAIP, levels of HIAP-2, another member of the IAP family, remained unchanged during adipocyte differentiation of 3T3-L1 cells.

Figure 3 illustrates NAIP and HIAP-2 expression during the 8-day differentiation period. In 3T3-L1 cells, NAIP levels rose

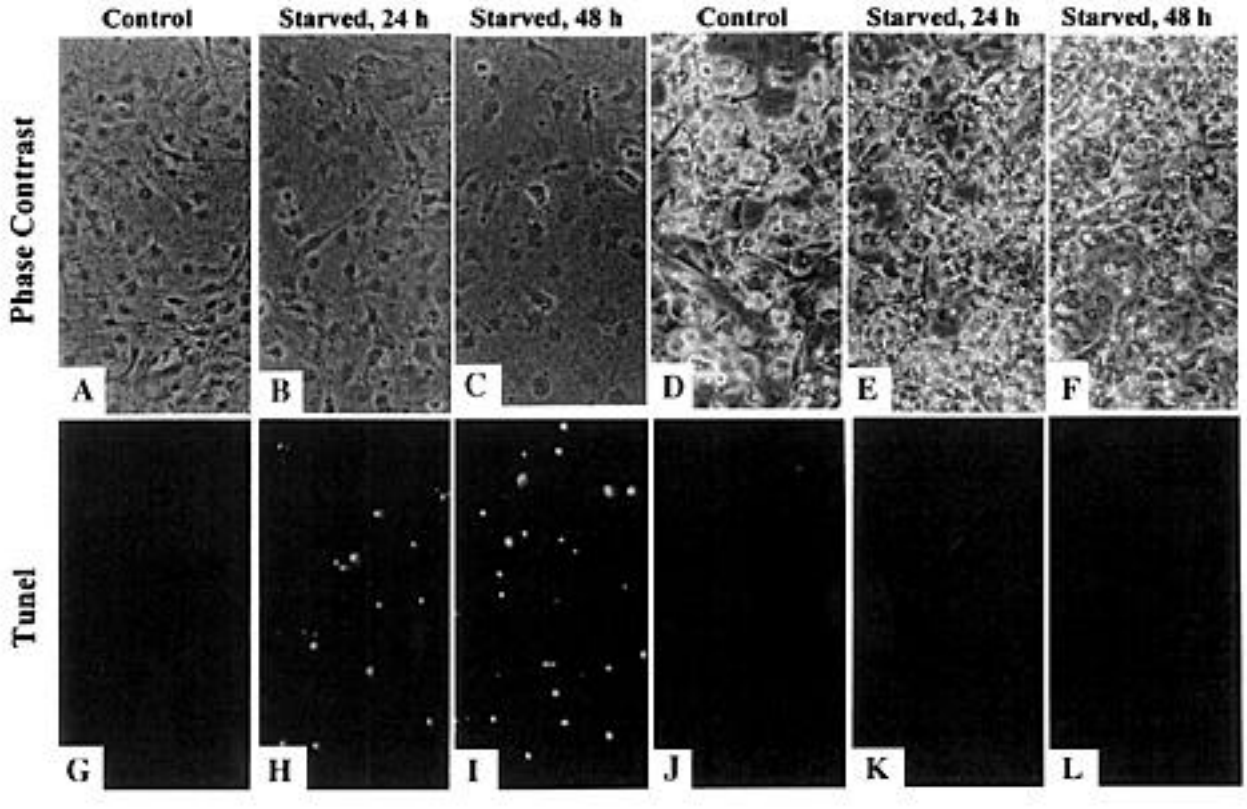

FIG. 1. Apoptosis induced by growth factor deprivation occurs in 3T3-L1 preadipocytes, but not in 3T3-L1 adipocytes. Phase-contrast photomicrographs (original magnification ×100) of 3T3-L1 preadipocytes (*A–C*) and differentiated adipocytes (*D–F*) were maintained in 10% calf serum (*A* and *D*) or serum-free medium for 24 (*B* and *E*) or 48 h (*C* and *F*). TUNEL staining of the same fields is shown (*G–L*).

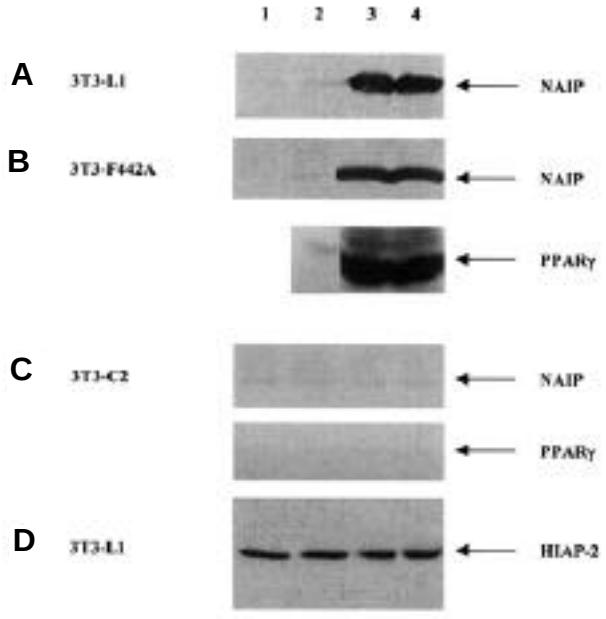

FIG. 2. Expression of NAIP and HIAP-2 during adipocyte differentiation. Confluent preadipocytes (*A* and *D*, 3T3-L1; *B*, 3T3-F442A; *C*, 3T3-C2) were maintained as preadipocytes (*lanes 1* and *2*; duplicates) or subjected to the appropriate differentiation protocol (*lanes 3* and *4*; duplicates). Solubilized protein from equivalent numbers of cells were immunoblotted with anti-NAIP (*A–C*), anti-HIAP-2 (*D*), or anti-PPAR-γ (*B* and *C*) antibody, as described. The immunoblots shown are representative of two to four experiments, each done in duplicate.

very slightly at day 2, increased dramatically by day 4, and persisted at these high levels until day 8. HIAP-2 levels remained constant throughout the 8-day differentiation protocol. PPAR-γ expression was maximal by day 2.

The active inducers of differentiation for the 3T3-L1 cell line include insulin, and for the first 48 h, dexamethasone and IBMX. We tested these agents to determine if they could act individually to upregulate NAIP expression (Fig. 4). Confluent 3T3-L1 preadipocytes were exposed to either insulin, dexamethasone, or IBMX for 4 days. Insulin (Fig. 4, *lane 3*), dexamethasone (Fig. 4, *lane 2*), and IBMX (Fig. 4, *lane 4*) did not induce NAIP expression on their own. As expected, the cells showed no evidence of early morphologic changes of differentiation. In comparison, when preadipocytes were exposed to insulin, dexamethasone, and IBMX together for 2 days, followed by insulin alone for another 2 days (as in the standard differentiation protocol), NAIP expression increased (Fig. 4, *lane 5*). There was also evidence of early differentiation, including cell rounding and the start of lipid droplet formation, in ~80% of the cells and upregulation of PPAR-γ, as seen earlier (Fig. 3).

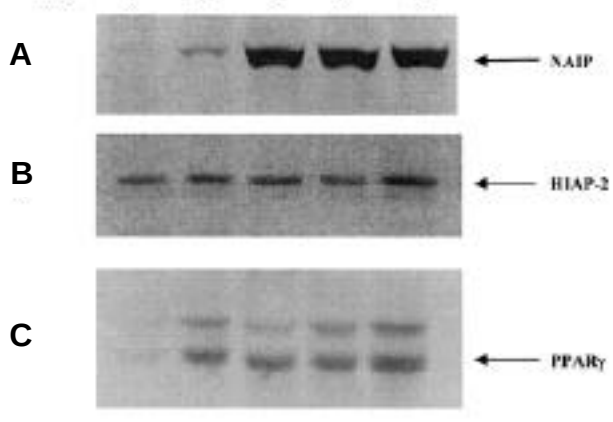

FIG. 3. Time course of NAIP and HIAP-2 expression during 3T3-L1 adipocyte differentiation. Confluent 3T3-L1 preadipocytes were induced to differentiate as described. At the indicated times, total cell lysates were prepared, and solubilized protein from equivalent numbers of cells was immunoblotted with anti-NAIP (*A*), anti-HIAP-2 (*B*), or anti-PPAR-γ (*C*) antibody, as described. The immunoblots shown are representative of 3 experiments.

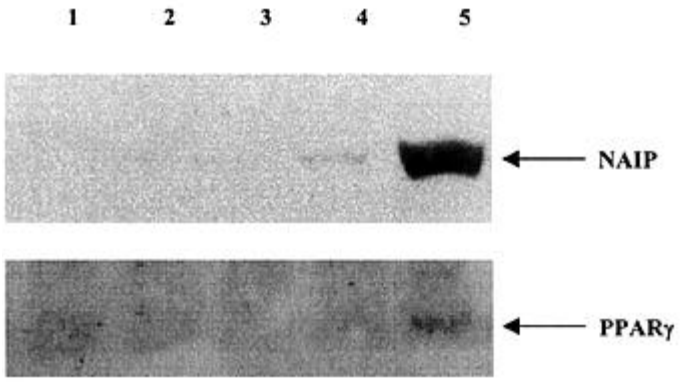

FIG. 4. Expression of NAIP in response to insulin, dexamethasone, or IBMX alone. Confluent 3T3-L1 preadipocytes were incubated for 4 days with either insulin (*lane 2*), dexamethasone (*lane 3*), or IBMX (*lane 4*). Unstimulated 3T3-L1 preadipocytes (*lane 1*) and 3T3-L1 preadipocytes that progressed through 4 days of the standard differentiation protocol (*lane 5*) are shown for comparison. On day 4, solubilized protein from equivalent numbers of cells were immunoblotted with anti-NAIP or anti-PPAR-γ antibody, as described. The immunoblots shown are representative of two experiments.

Rapamycin, when present throughout the differentiation protocol, inhibits insulin-induced 3T3-L1 adipocyte differentiation, predominantly by blocking the induction of a critical adipogenic transcription factor, C/EBPα (12). Blockade of critical postconfluent mitoses by rapamycin (clonal expansion phase) appears to account for the inhibitory effect of rapamycin on differentiation. When we added rapamycin to the 3T3-L1 differentiation medium, the induction of glycerol phosphate dehydrogenase normally seen during adipogenesis (13,14) was blocked by 90%, as expected. To examine the effects of rapamycin on NAIP regulation, we treated 3T3-L1 preadipocytes undergoing differentiation (days 0–4, at which point NAIP levels have reached their maximum) with rapamycin (100 nmol/l) added at the times indicated (Fig. 5). When rapamycin was present from days 0 and 4 (Fig. 5, *lane 2*), there was no increase in NAIP expression at day 4. Morphologically, the cells maintained the preadipocyte phenotype. When the presence of rapamycin was restricted to either days 0–2 (Fig. 5, *lane 3*) or days 2–4 (Fig. 5, *lane 4*), it was still sufficient to blunt the usual large increase in NAIP expression, as well as to inhibit differentiation.

To confirm that NAIP expression is not confined to immortalized cell lines, we performed Western blots on rat white adipocyte homogenates (15). As shown in Fig. 6, NAIP was detected in samples from two animals.

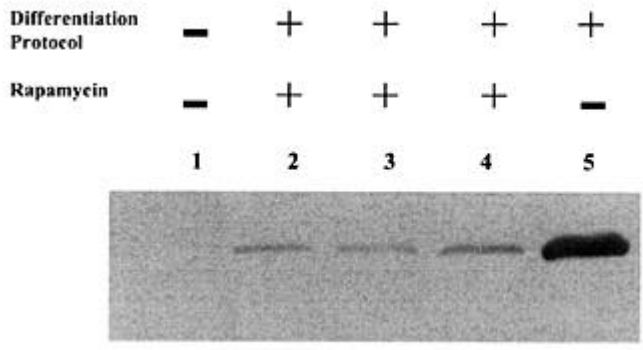

FIG. 5. Effect of rapamycin on NAIP expression during adipocyte differentiation. Confluent 3T3-L1 preadipocytes were induced to differentiate into adipocytes (from day 0 to 4), as described, either in the absence of rapamycin (*lane 5*) or in the presence of rapamycin (days 0–4, *lane 2*; days 0–2, *lane 3*; days 2–4, *lane 5*). Control preadipocytes are shown in *lane 1* for comparison. On day 4, solubilized protein from equivalent numbers of cells were immunoblotted with anti-NAIP antibody, as described. The immunoblots shown are representative of two experiments.

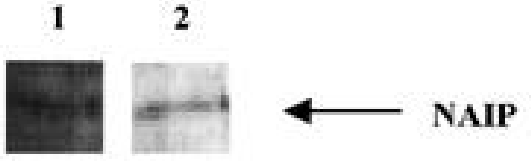

FIG. 6. NAIP expression in rat adipocytes. Equal amounts of protein of rat white adipocyte homogenate from two animals (*lanes 1* and *2*) were immunoblotted with anti-NAIP antibody, as described.

DISCUSSION

Our data show that NAIP, originally identified as a neuronal survival factor, is expressed in the 3T3-L1 and 3T3-F442A adipocyte cell lines, as well as in rat white adipocytes. Whereas NAIP protein is very low in preadipocytes, there is an enormous increase in its expression during adipocyte differentiation. Insulin, dexamethasone, or IBMX alone do not upregulate NAIP in the absence of adipose conversion. Rapamycin, a known inhibitor of 3T3-L1 adipocyte differentiation, also abrogates the increase in NAIP expression. We therefore conclude that NAIP is highly expressed during differentiation and that its induction is suppressed when differentiation is inhibited.

The development of established preadipocyte cell lines has greatly facilitated the study of adipocyte differentiation. Two commonly used cell lines for in vitro studies of adipocyte differentiation are the 3T3-L1 and the 3T3-F442A, cell lines originally prepared from a disaggregated 17- to 19-day-old Swiss 3T3 mouse embryo (13). The terminally differentiated adipocytes produced by this in vitro differentiation protocol share many characteristics with adipocytes in vivo (13,16,17). The 3T3-C2 cell line is derived from the same source, but these cells are resistant to differentiation and thus serve as controls for the preadipocyte lines. The observation that NAIP upregulation occurs exclusively in cell types undergoing differentiation signifies that its expression is differentiation-dependent.

For the 3T3-L1 cell line, dexamethasone and IBMX are included, for the first 48 h only, to accelerate the differentiation program (18). IBMX and dexamethasone may act by inducing the adipogenic transcription factors C/EBPβ and C/EBPδ, respectively (19). In contrast, 3T3-F442A preadipocytes require only insulin to elicit adipocyte differentiation, which may be due to differences in the stage at which preadipocyte development was arrested during isolation of these two cell lines (13). In both cases, despite the differences in the differentiation protocol, NAIP upregulation accompanied adipocyte differentiation, suggesting a specific effect of the differentiation process per se on NAIP expression. Also in support of a differentiation-dependent effect on NAIP expression are the data showing that insulin, dexamethasone, and IBMX, when added individually, are inadequate to alter NAIP levels. Finally, the rapamycin experiments demonstrate that increases in NAIP expression can be blocked by inhibiting adipocyte differentiation. Possibly, the modulation of transcriptional or translational factors during adipocyte differentiation are crucial for increasing NAIP protein expression.

The time course studies reveal that the differentiation-dependent events that affect NAIP expression occur between days 2 and 4, the mid-phase of adipogenesis. Key adipogenic transcriptional regulators, such as PPAR-γ and CEB/Pα (20,21), are themselves induced by this time point and could conceivably be involved in NAIP gene expression. Alternately, it is possible that the increase in NAIP protein observed here depends on accelerated translation. More detailed knowledge about the upstream regulatory elements of the NAIP gene and the translational control governing NAIP protein synthesis will be required to study precisely how NAIP expression is governed during adipogenesis.

NAIP was the first human IAP to be identified and was isolated based on its involvement with the neurodegenerative disorder, spinal muscular atrophy (SMA) (1). SMA patients lose their motor neurons over time, which eventually results in voluntary muscle wasting. Therefore, the deletion of the apoptotic suppressor gene NAIP in individuals with SMA is consistent as a pathogenic mechanism. NAIP displays two important differences from other IAP family members: NAIP contains three BIR domains rather than two, and it does not contain a COOH-terminal RING zinc finger domain, as seen in other IAP proteins. The reasons for these differences have yet to be determined, but nevertheless, NAIP is fully capable of suppressing apoptosis as previously described (2,4).

An unanswered question is the mechanism through which NAIP promotes the suppression of apoptosis. Recent evidence has shown that HIAP-1, HIAP-2, and XIAP directly inhibit caspase-3 and caspase-7 (3,22). NAIP, however, did not inhibit these specific caspases or others, such as caspase-1, caspase-6, or caspase-8 (3). This suggests that NAIP may function along a separate pathway from its family members, with distinct targets that have yet to be identified. Interestingly, our data reveal a specific upregulation of NAIP, but not of HIAP-2. Adipocyte differentiation apparently is not associated with a general induction of all IAPs, but instead may exert specific influences on individual members of this family of genes. The basis for such specificity remains undetermined at present. Because we have observed the presence of HIAP-2 in preadipocytes, which are susceptible to apoptosis, it may be that the distinguishing molecular attributes

of NAIP mentioned above allow it to play a more pivotal role in apoptosis suppression in adipocytes.

It has been shown previously that overexpression of NAIP in CHO cells suppressed apoptosis in response to serum withdrawal (2). Using the TUNEL assay, we have demonstrated here that serum withdrawal induces apoptosis in 3T3-L1 preadipocytes, but not in terminally differentiated adipocytes, in agreement with our previous work (5). Our data indicate that upregulation of NAIP during 3T3-L1 adipose cell differentiation is consistent with an acquired resistance to apoptosis induced by growth factor deprivation. These novel data on NAIP expression in adipocyte cell lines set the stage for future studies to delineate whether adipocyte resistance to apoptosis can be reversed by deleting or inactivating NAIP.

Finally, it will be instructive to investigate the effects of other apoptotic-inducing stimuli in the preadipocyte/adipocyte. Prins et al. (23) have recently reported that tumor necrosis factor-α triggers apoptosis of cultured human preadipocytes, as well as of adipocytes within adipose explants (cultured adipocytes were not studied). Determining the relative susceptibilities of preadipocytes and adipocytes to known and potential apoptotic stimuli should be a focus of research into the regulation of adipose tissue accumulation.

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