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Disruption of the vimentin intermediate filament system during adipose conversion of 3T3-L1 cells inhibits lipid droplet accumulation

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SUMMARY

During the differentiation of 3T3-L1 pre-adipocytes, vimentin intermediate filaments are reorganized to form cage-like structures around the nascent lipid droplets. Initial studies with 3T3-L1 cells indicated that aggregation of vimentin filaments by nocodazole treatment during or shortly after induction of adipose conversion dramatically reduced the lipid droplet content of 3T3-L1 cells 96-120 hours after induction. Specific but transient disruption of vimentin following anti-IFA antibody injection also resulted in a decrease in lipid droplet formation in differentiating cells. To specifically and stably affect filament organization, 3T3-L1 cells lines were established by transfection with a glucocorticoid-regulatable, dominant negative mutant vimentin cDNA expression plasmid. Treatment of these cells (83ΔC) with dexamethasone resulted in expression of vimentin with a carboxyl-terminal deletion, which led to the disruption of the endogenous filament network. Induction of adipose conversion in 83ΔC cells lead to the formation of lipid droplets comparable to those seen in untransfected 3T3-L1 cells. Addition of dexamethasone during the adipose conversion of 83ΔC cells did not affect the induction of the marker enzyme glycerol-3-phosphate dehydrogenase or the incorporation of [^{14}C]palmitate into triglycerides during a 10 minute pulse label. There was, however, a failure to form prominent lipid droplets and to accumulate [^{14}C]palmitate-labeled triglycerides. Pulse-chase experiments indicated that the failure of these cells to accumulate triglyceride was associated with an increased rate of turnover. These studies indicate that vimentin filaments provide a function that influences lipid stability during adipose conversion of 3T3-L1 cells.

Key words: Vimentin, Intermediate filament, Adipose development, Lipid droplet

INTRODUCTION

Cytoplasmic intermediate filaments (IFs) are composed of individual subunit proteins that are expressed in a complex, cell type-specific pattern (reviewed in Skalli and Goldman, 1991). The biochemical properties of IFs have been extensively described, but the functional significance of this component of the cytoskeleton has, until recently, remained obscure. Experiments with transgenic mice and in the *Xenopus* embryo have begun to illustrate a rather complex picture of the functional significance of IFs in intact animals. The clearest results reported to date have been obtained in studies involving the keratin IFs of epidermal cells. Expression of dominant negative mutant epidermal keratins (reviewed in Fuchs and Coulombe, 1992) or knockout mutations of epidermal keratins (Chan et al., 1994; Rugg et al., 1994; Lloyd et al., 1995) results in cellular fragility, indicating that a normal keratin IF organization is required to protect cells against physical injury (Coulombe et al., 1991). A similar mechanical role has been proposed for desmin IFs characteristic of muscle cells (Cary and Klymkowsky, 1995; Li et al., 1996). Curiously, knockout mutations in other IF proteins have produced more complex effects. Targeted gene disruption of a simple epithelial keratin is associated with embryonic lethality during mid-gestation (Baribault et al., 1993). Intriguingly, the extent of lethality was significantly influenced by the genetic background of the mice, and survivors could be obtained that exhibited a prominent colorectal hyperplasia (Baribault et al., 1994). It is not yet clear if this phenotype observed in mice that lack keratin IFs in simple epithelia is a consequence of decreased mechanical integrity, or perhaps a deficit of some other functional property of keratin IFs. Perhaps the most perplexing results have been the phenotypes of null mutations in vimentin (Colucci-Guyon et al., 1994), which is characteristically expressed in mesenchymal cells, and GFAP (Gomi et al., 1995; Pekny et al., 1995), which is normally expressed by glial cells, where there have been no obvious abnormalities observed in these mice thus far.

In some cell types, the organization as well as the expression of the IF system is developmentally regulated, suggesting that the filament network may play a role in the acquisition of a differentiated phenotype. Vimentin-type IFs, characteristic of many vertebrate cells of mesenchymal origin (Franke et al., 1987), have been shown to undergo a dramatic and specific reorganization during adipocyte differentiation. In culture, 3T3L1 cells have been extensively studied as a

model for the differentiation of adipogenic cells (Ailhaud, 1982). Confluent cultures of 3T3-L1 cells can be induced to express a functional adipose phenotype characterized by accumulation of lipid droplets (Green and Meuth, 1974; Green and Kehinde, 1976). Franke et al. (1987) have shown that during this process, the extended wavy, fibrillar organization of vimentin IFs in undifferentiated cells is altered. The vimentin filaments are translocated to surround the nascent lipid droplets, forming a regularly spaced cage-like structure. Similar cages of IFs surrounding lipid droplets have been described in adipose cells (Luckenbill and Cohen, 1966; Wood, 1967; Novikoff et al., 1980; Franke et al., 1987), cholesterol-loaded macrophages (McGookey and Anderson, 1983), and the foam cells of atherosclerotic lesions (Amanuma et al., 1986).

The unique organization of vimentin IFs around lipid droplets in particular cell types has raised the possibility that vimentin-type IFs may have a specific function associated with lipid droplet formation in adipogenic cells (Evans, 1994). However, Colucci-Guyon et al. (1994) reported no obvious abnormality in the adipose tissue in vimentin null mice. The capacity of vimentin null adipocytes to form lipid droplets could indicate that vimentin is not involved in this process, or that there is some adaptive mechanism that compensates for the absence of IFs, or that the effect of the null mutation is subtle and difficult to appreciate in the experiments that have been done thus far. Another potentially informative approach to examining the role of vimentin in adipose cells would be to interfere with the capacity of cells to normally reorganize an existing vimentin IF network during the differentiation of cultured preadipose cells. Such dominant effects might influence potential adaptive or redundant mechanisms and provide an experimental approach that would be amenable to metabolic studies.

The present experiments were conducted with 3T3-L1 cells to determine the effect of disruption of the vimentin IF network on the process of lipid droplet formation during adipocyte conversion. Perturbation of the organization of the vimentin IFs in these cells appears to significantly reduce the formation of lipid droplets, indicating that vimentin filaments provide an intracellular function that can affect the efficient formation of cytoplasmic lipid droplets during adipocyte differentiation.

MATERIALS AND METHODS

Cells and cell culture

3T3-L1 cells were obtained from the American Type Culture Collection (ATCC CCL 92.1) and maintained in a 1:1 mixture of Ham's F-12:Dulbecco's MEM supplemented with 10 μg/ml gentamicin and 10% fetal bovine serum (FBS). Adipocyte conversion was induced by treating cultures either with 0.25 μM dexamethasone, 0.5 mM isomethylbutylxanthine (IMBX) and 10^{-7} M insulin for 48 hours, or with 2 μg/ml ADD 4743 (gift of Takeda Chemical Industries Ltd) and 10^{-7} M insulin for the duration of the experiment. After induction, the cells were maintained in normal growth medium containing 10% FBS and 10^{-7} M added insulin. G418-resistant cell lines transfected with a steroid hormone-inducible expression plasmid (see below) were routinely cultured in the same basal medium containing 10% controlled process serum replacement (CPSR-1, Sigma) and 400 μg/ml G418. The CPSR-1 was then replaced with 10% FBS in all experiments requiring adipose conversion.

Evaluation of adipose conversion

Lipid accumulation in cells was identified by oil red O (ORO) staining of cells grown on sterile cover slips or glass chamber slides (Lillie and Asburn, 1936). To assess relative levels of lipid droplet accumulation, cells were stained for neutral lipid using ORO, counterstained with Gill's hematoxylin, and scored for the percentage of cells undergoing adipose conversion. Cells were considered to be undergoing adipose conversion if they assumed a round morphology and began to accumulate lipid droplets approximately 3 μm in diameter or greater. Lipid droplets of ≥3 μm in diameter were used as arbitrary markers of adipose conversion, as undifferentiated pre-adipocytes do not usually contain lipid droplets of this size. At least eight 1 mm^2 fields were microscopically examined using an ocular grid. In most experiments 400-700 cells per experimental group were examined. Color photographic exposures were made using either Kodak 320T or EPR 64 film and commercial E6 film processing. The marker enzyme glycerol-3-phosphate dehydrogenase (GPDH) was assayed spectrophotometrically by the method of Kuri-Harcuch and Green (1977).

Anti-filament antibody microinjection

Anti-IFA (Pruss et al., 1981) was purified from ascites fluid by ammonium sulfate precipitation and DE-52 chromatography (Estes et al., 1987). Semi-purified commercial bovine immunoglobulin (Schwarz Mann) was further purified by the same protocol used to isolate anti-IFA for use as a non-specific control. Cells were grown in single-chamber glass chamber slides (Labtek). Approximately 2-3 mm diameter areas were scribed under the lower surface of the glass slide. Purified anti-IFA or bovine IgG was microinjected into all the cells within the scribed areas using an Eppendorf micromanipulator 51780, Eppendorf microinjector 54242 and a Zeiss Axiovert 10 inverted microscope. Microinjection experiments were done using 3.5-10 mg/ml of purified anti-IFA or non-specific IgG in a microinjection buffer containing 114 mM KCl, 20 mM NaCl, 3 mM $MgCl_2$, 3 mM NaH_2PO_4, adjusted to pH 7.0 with KOH. Microinjection was performed by a modification of the method described by Klymkowsky (1981). Sutter 1 mm (OD) borosilicate glass microinjection capillary tubes were pulled on a Sutter pipette puller (Sutter Industries) to produce needles with injection tips <1 μm in diameter. Antibody was delivered by the microinjector at 110 HPA (7.9 psi) for 0.2-0.4 seconds and fluid injection was visually verified. Microinjection was sequentially carried out on each slide, one field at a time, to limit the time that cells were kept out of the incubator (typically 30-60 minutes/injection field). On each slide an additional scribed area was left uninjected. At the indicated times following microinjection, the cells were fixed for either anti-vimentin immunofluorescence or ORO staining.

Mutant vimentin expression plasmid, DNA transfection and isolation of cell lines

The construction of pMMTV-Vim-83ΔC, which contains a truncated mutant vimentin cDNA under the regulation of the promoter and hormone response element of mouse mammary tumor virus, has been described previously (Sarria et al., 1994). The translation product of the mutant sequence lacks the 83 carboxyl-terminal amino acids of wild-type vimentin, including the entire carboxyl-terminal tail and 26 amino acids of the alpha-helical rod domain. Previous studies have shown that when this mutant protein is expressed in human adrenal tumor cells it is assembled into the endogenous vimentin filament network and produces an aberrant filament organization (Sarria et al., 1994). Stable cell lines were obtained by cotransfecting $0.6\text{-}0.8\times10^6$ 3T3-L1 cells in 10 cm dishes with 40 μg pSP64-MMTV-Vim83ΔC and 1.0 μg pSV2Neo by calcium phosphate precipitation (Graham and van der Eb, 1973) and 15% glycerol shock (Parker and Strak, 1979) or lipofection (Lipofectin, Gibco). Stable transfectants were selected in medium containing 400 μg/ml G-418. A total of 91 individual colonies were recovered and examined for altered vimentin filament

organization, in the presence and absence of 10^{-7} M dexamethasone, by indirect immunofluorescence microscopy. Not all 3T3-L1 clones retain the capacity to undergo adipose conversion and therefore all stable clones were evaluated both for hormone-regulated filament disruption and their capacity for adipose differentiation. In the absence of added hormone, the appearance of the vimentin IF network in the transfectant cell lines was indistinguishable from untransfected 3T3-L1 cells. Vimentin organization was unaffected by dexamethasone in untransfected 3T3-L1 cells and most of the 83ΔC transfectant cell lines. However, 12 of the 83ΔC cell lines exhibited some degree of aberrant filament morphology that appeared gradually after exposure to dexamethasone. These transfectant cell lines were examined for their capacity to undergo adipose conversion using the elevation in glycerol-3-phosphate dehydrogenase (GPDH) as a specific and quantifiable marker for adipose conversion. Three stable 83ΔC transfectant cell lines that exhibited a comparable induction of GPDH activity (activity/mg protein) to untransfected 3T3-L1 cells, but differed in the degree of dexamethasone-inducible vimentin filament perturbation, were isolated. The transfectant cell line that showed the greatest dexamethasone-inducible vimentin IF perturbation (83ΔC-9A2) and a cell line that exhibited no observable dexamethasone effect on vimentin organization (83ΔC-5A2, data not shown) were selected for further study.

Fluorescence microscopy

Cells were plated on sterile glass cover slips. In experiments involving immunofluorescence of IFs, the cells were rinsed briefly in phosphate-buffered saline (PBS) and then fixed in acetone:methanol 70:30 (v/v) at −20°C for 10 minutes. The coverslips were rinsed in PBS, and processed for anti-vimentin indirect immunofluorescence as described previously (Sarria et al., 1990).

SDS gel electrophoresis and immunoblotting

Cells were labeled with 25-50 μCi/ml [^{35}S]methionine for 2 hours in methionine-free medium containing 1% FBS. Triton-insoluble proteins were prepared and anti-vimentin immunoblotting was carried out as previously described (Sarria et al., 1994).

Incorporation of [^{14}C]palmitate into cellular lipids

Cells were plated on 60 mm dishes and adipose conversion was induced with ADD 4743 and insulin in the presence or absence of dexamethasone, as described above. [^{14}C]palmitate-BSA was prepared as described by Goldstein et al. (1983) to a specific activity of 1,659 dpm/nmole. The cultures were labeled for the indicated times with 0.04-2.5 μCi/ml [^{14}C]palmitate-BSA in normal growth medium. The medium was removed, the lipids extracted with hexane:isopropanol (3:2) and the neutral lipids were analyzed by thin layer chromatography as previously described (Sarria et al., 1992). TLC plates were scanned for incorporated ^{14}C radioactivity and quantitated using a Bioscan 200 imaging scanner.

RESULTS

Disruption of vimentin IFs with nocodazole inhibits lipid droplet formation during the adipose conversion of 3T3-L1 cells

To initially determine if disruption of the vimentin IF network would affect the capacity of 3T3-L1 cells to accumulate lipid droplets during adipose conversion, cells were treated with 0.2 μg/ml nocodazole for 24 hours at various times after induction of adipose conversion, and then fixed for ORO staining 120 hours after adipose conversion was induced. Drug-induced depolymerization of the microtubule system leads to collapse of extended vimentin IFs into a dense perinuclear coil (Goldman and Knipe, 1972; Geuens et al., 1983), and nocodazole is a reversible agent that reportedly does not affect DNA or protein synthesis (Zieve et al., 1980). As shown in Fig. 1, when the cells were subjected to a 24 hour nocodazole treatment during or immediately after induction of adipose conversion, the percentage of cells that accumulated significant ORO-stained lipid droplets was dramatically reduced compared with untreated controls. Nocodazole treatment at later times after the induction of adipose conversion had progressively smaller effects on lipid droplet accumulation. In addition to accumulation of lipid droplets, 3T3-L1 cells exhibit prominent morphological changes during adipose conversion, characterized by the accumulation of highly spread cells with a more round appearance that were frequently multinucleated. As shown in Fig. 2, while nocodazole treatment inhibited the accumulation of ORO stained lipid droplets, the cells treated with nocodazole for 24 hours during induction exhibited the characteristic changes in morphology associated with adipose conversion.

Anti-vimentin immunofluorescence of the cells treated with nocodazole at 0-24 hours after induction of adipose conversion indicated that although virtually all the cells exhibited drug-induced filament collapse (Fig. 3), this effect was readily reversible, and within 24 hours after nocodazole removal these cells exhibited extended filament networks (not shown). However, the majority of these nocodazole-treated cells failed to exhibit the subsequent filament reorganization observed in the untreated control cells (Fig. 3C), and still contained extended networks of vimentin filaments 120 hours after induction of adipose conversion, although in many cases filament organization appeared to be subtly different from undifferentiated cells (Fig. 3D). Careful examination of these cells after adipose conversion indicated that while all of the small percentage of the nocodazole-treated cells that did accumulate lipid droplets (see Fig. 1) exhibited vimentin filament

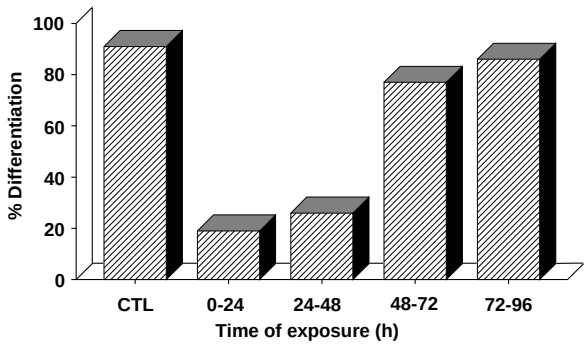

Fig. 1. Effect of nocodazole on lipid droplet formation during adipose conversion of 3T3-L1 cells. Cells were grown to semi-confluence (0 time) and treated with 0.25 μM dexamethasone, 0.5 mM IBMX and 10^{-7} M insulin for 48 hours to induce adipose conversion. Cultures were treated with 0.2 μg/ml nocodazole for 24 hours at the indicated times after induction of adipose conversion and were compared with untreated controls (CTL). All cells were fixed 120 hours after induction and stained with ORO. In each culture at least 250 cells in random fields were examined for accumulation of ORO-stained lipid droplets. Values represent the average percentage of cells with lipid droplets ≥3 μM in diameter, from triplicate cultures.

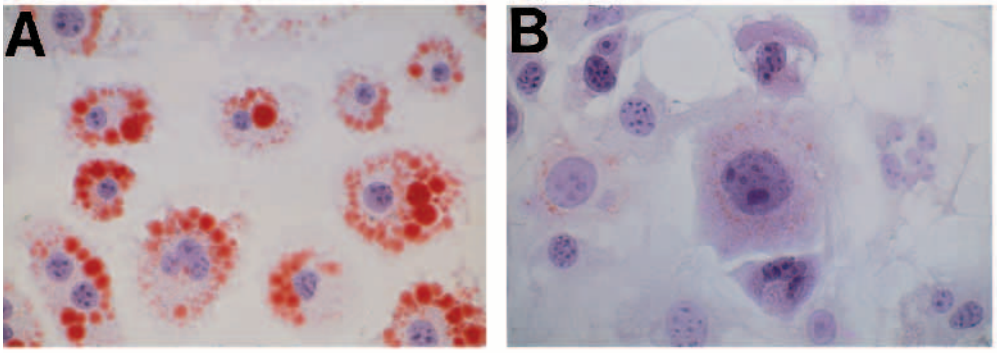

Fig. 2. Nocodazole inhibits lipid droplet formation during adipose conversion of 3T3L1 cells. Cells were treated with 0.25 μM dexamethasone, 0.5 mM IBMX and 10^{-7} M insulin for 48 hours to induce adipose conversion. The figure shows the ORO staining of cultures 120 hours after induction. (A) Untreated; (B) treated with 0.2 μg/ml nocodazole at 0-24 hours after induction of adipose conversion.

reorganization, in no case was lipid droplet accumulation observed in cells with extended vimentin networks. These experiments indicated that nocodazole treatment early after the induction of adipose conversion interfered with the subsequent reorganization of vimentin during adipose conversion, and was consistent with the idea that this reorganization is associated with the accumulation of lipid droplets. Because the effect of nocodazole is not specific for vimentin, it cannot be concluded from these results that the impaired capacity of 3T3-L1 cells to accumulate lipid droplets was due to an effect on vimentin. However, it is interesting to note that Franke et al. (1987) reported that once the vimentin filaments were reorganized to surround the lipid droplets during adipose conversion, they were no longer sensitive to perturbation with anti-microtubule drugs.

Anti-IFA antibody-induced vimentin IF disruption Inhibits lipid droplet formation during adipose conversion

To specifically disrupt the vimentin IF network, 3T3-L1 cells were microinjected with the anti-IFA antibody. This antibody, which recognizes an epitope that is common to most IF proteins (Pruss et al., 1981; Geisler et al., 1983), has been used in previous microinjection studies and causes IF collapse without observably affecting cell division or the distribution of other cytoplasmic organelles in cultured cells (Klymkowsky, 1981; Meyer et al., 1992). Initial studies were conducted to determine the effect of anti-IFA injection on vimentin filament organization, and the duration of this effect. While most anti-IFA-injected 3T3-L1 cells exhibited some degree of IF collapse 24 hours after injection, by 48 hours the number of anti-IFA-injected cells that exhibited aberrant filament organization was reduced by approximately half (Table 1), compared with uninjected cells or cells injected with a similarly purified nonspecific IgG. This result is in agreement with previous studies, which indicated that the antibody-induced perturbation of 3T3 cell vimentin IF organization was transient (Klymkowsky, 1981). Therefore, to maximize the number of cells exhibiting adipose conversion at the earliest possible time after induction during this period of antibody-induced filament disruption, the thiazolidinediones derivative ADD 4743 was utilized as an agent to induce adipose conversion in these studies in place of dexamethasone and IMBX. This compound has been reported to be a more potent inducer of 3T3-L1 differentiation than glu-

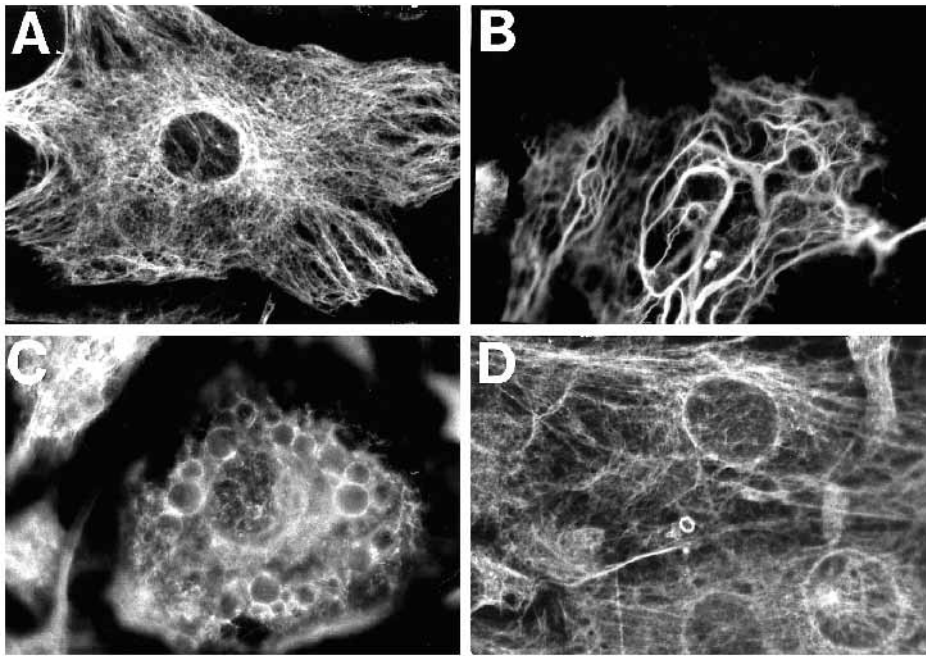

Fig. 3. Effect of nocodazole on the organization of vimentin during adipose conversion of 3T3-L1 cells. Adipose conversion was induced as indicated in Fig. 2. The figure shows the anti-vimentin imunofluorescence of cells at 24 hours (A and B) and 120 hours (C and D) after induction of adipose conversion. (A and C) Untreated controls. (B and D) Treated with 0.2 μg/ml nocodazole at 0-24 hours after induction of adipose conversion.

Table 1. Effect of anti-intermediate filament antibody microinjection on vimentin filament organization in 3T3-L1 cells

Hours after microinjection	% Cells with altered vimentin organization (*n*)*		
	Non-injected	B IgG-injected	IFA-injected
24	17 (176)	14 (225)	82 (149)
48	11 (192)	16 (190)	39 (165)

Cells in adjacent scribed fields were microinjected with either purified non-specific bovine IgG (BIgG) or anti-intermediate filament IFA (IFA) antibodies. At the indicated times, the cells were fixed and processed for anti-vimentin immunofluorescence. The % of the total cells within each field that exhibited observable vimentin IF aggregation was determined.

*Percentage of cells with abnormal vimentin filaments; the value in parenthesis (*n*) gives the total number of cells in the injected field.

cocorticoids (Hiragun et al., 1988; Sparks et al., 1991). Cells within scribed areas on glass chamber slides were microinjected with either anti-IFA or a nonspecific bovine IgG 24 hours after induction with 2 μg/ml ADD 4743 and 10^{-7} M insulin. The effect of antibody microinjection on vimentin filament reorganization was examined at 72 hours post-induction (48 hours after injection). Anti-vimentin immunofluorescence of cells injected with the non-specific IgG did not reveal any obvious difference in the distribution of vimentin compared with non-injected cells (not shown). As shown in Fig. 4, characteristic accumulations of vimentin could be seen around the small lipid droplets that had begun to appear at this relatively early stage of adipose conversion in cells injected with the non-specific IgG. However, in most but not all of the anti-IFA injected cells, the distribution of vimentin was characterized by accumulations of vimentin aggregates ranging from small spot-like structures throughout the cytoplasm to large localized accumulations that were not seen in the control cells (Fig. 4). The effect of this perturbation of vimentin organization on the formation of lipid droplets was then examined. At 72 and 96 hours post-induction (48 and 72 hours after antibody injection, respectively), the cells were fixed and neutral lipid accumulation accessed by ORO staining. Although the percentage of cells that exhibit adipose conversion is less at 72 and 96 hours after induction than at later times, because of the transient nature of the antibody effects on vimentin these time points were chosen as the earliest times that adipose conversion could be clearly distinguished. All of the cells in the microinjection fields were evaluated for adipose conversion. Because the percentage of uninjected control cells that demonstrated adipose conversion and lipid droplet accumulation was found to vary considerably between individual experiments, the results of three separate microinjection experiments are given in Table 2. As shown in Fig. 5, injection of the non-specific bovine IgG had little observable effect on the capacity of ADD 4743-induced cells to form lipid droplets, compared with uninjected cells in an adjacent field. In contrast, antiIFA injection consistently reduced the percentage of cells that exhibited significant lipid droplet accumulation at 48 (not shown) and 72 hours after microinjection, compared with either uninjected control cells or cells injected with non-specific IgG in adjacent fields on the same slide. Although the absolute level of adipose conversion varied between experiments, when the data was expressed as a % of the uninjected control, an approximate 50% reduction in the capacity of anti-IFA injected cells to form lipid droplets was routinely observed (Table 2). While the effect of anti-IFA injection on lipid droplet accumulation was not absolute, it was similar in magnitude to the observed effect of anti-IFA injection on vimentin filament organization (Table 1). It was also observed that although lipid droplet accumulation was affected in the anti-IFA-injected cells, these cells continued to exhibit the other morphological changes associated with adipose conversion, characterized by the accumulation of spread cells with a rounded appearance that were frequently multinucleated (Fig. 5). A similar inhibition of lipid droplet formation in these cells was obtained by electromechanical injection (Kim et al., 1991)

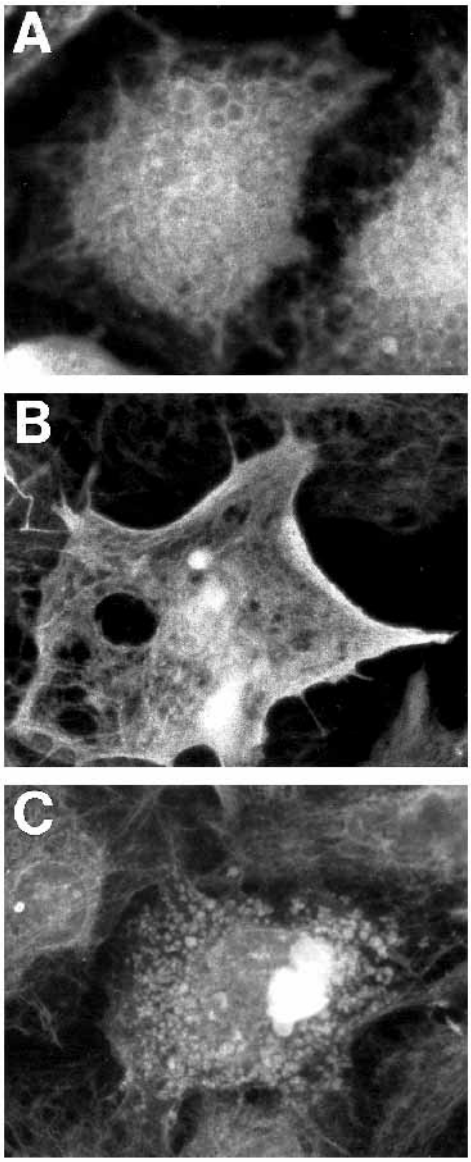

Fig. 4. Effect of anti-IFA antibody injection on the reorganization of vimentin during adipose conversion of 3T3L1 cells. Adipose conversion was induced with 2 μg/ml ADD 4743 and 10^{-7} M insulin for 24 hours and the cells were then injected. The figure shows anti-vimentin immunofluorescence of cells from the same chamber slide injected with a non-specific IgG (A) and cells injected with anti-IFA (B and C), 48 hours after microinjection.

Table 2. Effect of anti-intermediate filament antibody microinjection on lipid droplet accumulation in 3T3-L1 cells

Experiment	Hours after microinjection	Lipid droplet formation				
		Uninjected % (*n*)*	B IgG-injected		IFA-injected	
			% (*n*)*	% of control†	% (*n*)*	% of control†
#1	48	26 (444)	22 (577)	85	7 (1,006)	27
	72	43 (954)	42 (1,112)	98	21 (1,044)	49
#2	48	52 (676)	47 (626)	90	30 (612)	58
	72	50 (1,009)	57 (1,029)	114	28 (947)	56
#3	48	32 (718)	39 (1,345)	121	18 (959)	56
	72	56 (1,288)	63 (1,900)	112	36 (1478)	64

Cells were induced to differentiate with 2 μg/ml ADD 4743 and 10^{-7} M insulin for 24 hours prior to microinjection. Cells in adjacent defined fields were microinjected with either purified bovine IgG (BIgG) or anti-intermediate filament IFA (IFA) antibodies. At the indicated times after injection, the cells were stained with ORO and the number of cells with lipid droplet accumulations in each injected field was determined (see Materials and Methods).

*Percentage of cells lipid droplets ≥3 μm in diameter; the value in parenthesis (*n*) gives the total number of cells in the injection field.

†Data expressed as a percentage of the uninjected control value.

of either anti-IFA or a commercial monoclonal anti-vimentin (clone 3B4, Boehringer Mannheim, data not shown).

Expression of a dominant negative mutant vimentin inhibits lipid droplet formation during adipose conversion

In order to create disruption of the vimentin IF system organization that would be stable during the entire period of adipose conversion on a large population of cells, 3T3-L1 cells were transfected with a hormone-regulatable expression vector containing a truncated mouse vimentin cDNA, lacking the coding sequence for the 83 carboxyl-terminal amino acids of mouse vimentin (83ΔC) (Sarria et al., 1994). A transfectant cell line (83ΔC-9A2), which exhibited a dexamethasone-inducible perturbation in vimentin filament organization, was isolated and characterized for hormone-induced synthesis of an appropriately truncated vimentin polypeptide. Triton-insoluble cytoskeletons were prepared from [^{35}S]methionine-labeled control and dexamethasone-treated 3T3-L1 and 83ΔC cell lines and analyzed by immunoblotting with an anti-vimentin antibody. As shown in Fig. 6, dexamethasone treatment of 3T3-L1 cells had no detectable effect on the pattern of anti-vimentin-reactive Triton-insoluble proteins. However, treatment of 83ΔC cells with dexamethasone resulted in the appearance of an anti-vimentin immunoreactive protein of approximately 48 kDa, which was consistent with the predicted size of the truncated mutant protein. In some experiments, a small amount of the 48 kDa protein was barely detectable in this transfected cell line in the absence of dexamethasone, indicating that there is a low level of basal expression of the mutant protein.

To determine if the expression of the truncated vimentin would affect the reorganization of the vimentin network in the 83ΔC transfectant cells during adipose conversion, anti-vimentin immunofluorescence was carried out on undifferentiated cells and cells that were induced to differentiate with

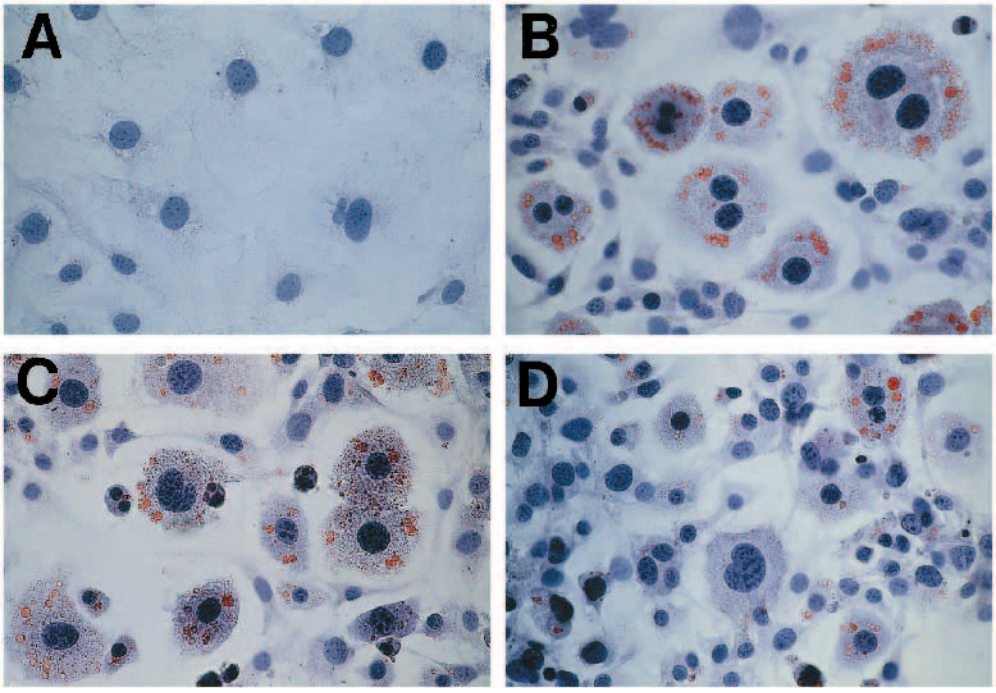

Fig. 5. Anti-IFA antibody injection inhibits lipid droplet formation during adipose conversion of 3T3-L1 cells. Adipose conversion was induced with 2 μg/ml ADD 4743 and 10^{-7} M insulin for 24 hours and the cells were then injected. 72 hours after microinjection, the cells were fixed and stained with ORO and Gill's hematoxylin. ORO staining of (A) untreated (not induced or injected) cells, (B) uninjected cells, (C) non-specific IgG-injected cells and (D) anti-IFA injected cells. Cells in B-D were from the same chamber slide.

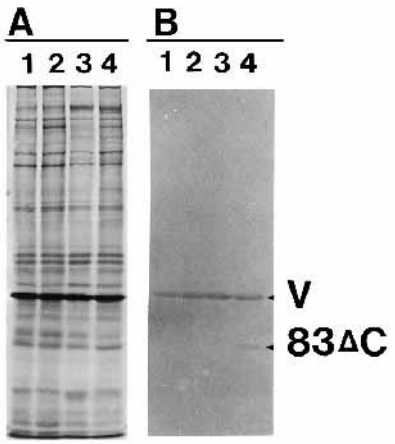

Fig. 6. Dexamethasone-inducible expression of a carboxyl-terminally truncated mutant vimentin in stably transfected 3T3-L1 cells. Triton-insoluble proteins were prepared from [^{35}S]methionine-labeled cells, separated by 7.5% SDS-PAGE, and subjected to immunoblot analysis with an anti-vimentin antiserum. (A) The ^{35}S-autoradiograph; and (B) the anti-vimentin diaminobenzidine staining product for preparations from untreated untransfected 3T3-L1 cells (lane 1), untransfected 3T3-L1 cells treated with 10^{-7} M dexamethasone for 48 hours (lane 2), untreated 83ΔC-9A2 transfectant cells (lane 3), and 83ΔC-9A2 transfectant cells treated with 10^{-7} M dexamethasone for 48 hours (lane 4). The positions of wild-type vimentin (V), and a 48 kDa anti-vimentin immunoreactive that was only detected in the hormone-treated transfectant cells (83ΔC), are indicated.

ADD 4743 in the presence or absence of 10^{-7} M dexamethasone. As shown in Fig. 7, treatment of untransfected 3T3-L1 cells with dexamethasone had no discernible effect on vimentin organization in undifferentiated cells or during adipose conversion (Fig. 7A-D). Dexamethasone also had no effect on the redistribution of vimentin during adipose conversion of 83ΔC-5A2 cells, a transfectant cell line that had no detectable mutant vimentin expression (data not shown). In 83ΔC-9A2 cells in the absence of dexamethasone the distribution of vimentin was not obviously different from 3T3-L1 cells, and during adipose conversion vimentin was reorganized around lipid droplets (Fig. 7E,F). However, when mutant vimentin expression was induced in 83ΔC9A2 cells with dexamethasone, most of the cells exhibited a visible change in vimentin organization, characterized by the formation of aggregates of filaments (Fig. 7G) and, in some cells, the appearance of spot-like accumulations of vimentin (not shown). When adipose conversion was induced in 83ΔC-9A2 cells in the presence of dexamethasone, most of the immunoreactive vimentin appeared in large hazy aggregates and there was little evidence of the redistribution of vimentin associated with discrete structures (Fig. 7H) that was seen in these cells during adipose conversion in the absence of dexamethasone. Although the aberrant distribution of vimentin observed in the dexamethasone-treated 83ΔC-9A2 cells during adipose conversion could not be clearly resolved using immunofluorescence microscopy, expression of the mutant vimentin produced a dominant negative effect on vimentin organization and perturbed the normal redistribution of the filament network.

The effect of the dominant mutant vimentin-induced disruption of vimentin organization on the capacity of 83ΔC transfectant cells to accumulate lipid droplets was then examined. As shown in Fig. 8, treatment of 3T3-L1 control cells with dexamethasone during adipose conversion actually augmented the capacity of these cells to accumulate lipid droplets, compared with cells induced with ADD 4743 and insulin alone. The 83ΔC transfectant cells formed prominent lipid droplets when induced with ADD 4743 and insulin alone, although the size and number of ORO-positive droplets per cell was consistently somewhat less than that observed in the untransfected cells. However, in contrast to the control cells, dexamethasone treatment of the 83ΔC transfectant cells resulted in a dramatic reduction in lipid droplet formation (Fig. 8). This difference in lipid droplet formation was not absolute, and just as the effect of mutant vimentin expression in these cells produced a heterogenous effect on vimentin filaments, some cells in the dexamethasone-treated population did form prominent lipid droplets. Yet the inhibition of lipid droplet accumulation in the hormone-treated cells was clearly visible, even in unstained cells, by phase contrast microscopy. Interestingly, many of the dexamethasone-treated transfectant cells clearly exhibited the morphological changes that are characteristic of adipose conversion, but formed only small lipid droplets, compared with cells induced with ADD 4743 alone. This phenotype is similar to that observed in nocodazole-treated and anti-IFA-injected cells during adipose conversion (compare Figs 2 and 5 with Fig. 8), and indicates that perturbation of the vimentin IF network inhibits the accumulation of lipid droplets in differentiating 3T3-L1 cells. Similar experiments with 83ΔC-5A2, a transfectant cell line that exhibits no observable dexamethasone effect on vimentin organization, indicated that these cells exhibited levels of lipid droplet formation that were comparable to untransfected 3T3-L1 (data not shown).

Dominant negative mutant vimentin expression affects the stability of newly synthesized triglycerides during adipose conversion

The decreased lipid droplet formation observed in 3T3-L1 cells with altered vimentin filament organization reflects a decreased level of neutral lipid accumulation in these cells. Presumably this could result from either a decreased rate of glyceride synthesis or an increased rate of turnover.

It was observed that the induction of glycerol-3-phosphate dehydrogenase (GPDH) in the 83ΔC cells, a relatively late marker of adipose conversion, was generally similar to that observed in 3T3L1 cells, and not significantly affected by dexamethasone (not shown), suggesting that expression of the mutant vimentin did not affect the induction of the lipid synthetic pathway in these cells. Experiments that measured the incorporation of [^{14}C]palmitate into triglycerides in wild-type 3T3-L1 cells after induction of adipose conversion with ADD 4743, consistently demonstrated that dexamethasone augmented the apparent incorporation of [^{14}C]palmitate into triglycerides, regardless of the length of time of radiolabelling. In contrast, during adipose conversion in 83ΔC 9A2 cells, an effect of dexamethasone on the apparent rate of incorporation of [^{14}C]palmitate into triglycerides was affected by the length of the time that the cells were radiolabeled. As shown in Table 3, when cells were labeled for 10 minutes, dexamethasone had no significant ($P \leq 0.05$) effect on the rate of incorporation of [^{14}C]palmitate into triglycerides in 83ΔC cells during adipose

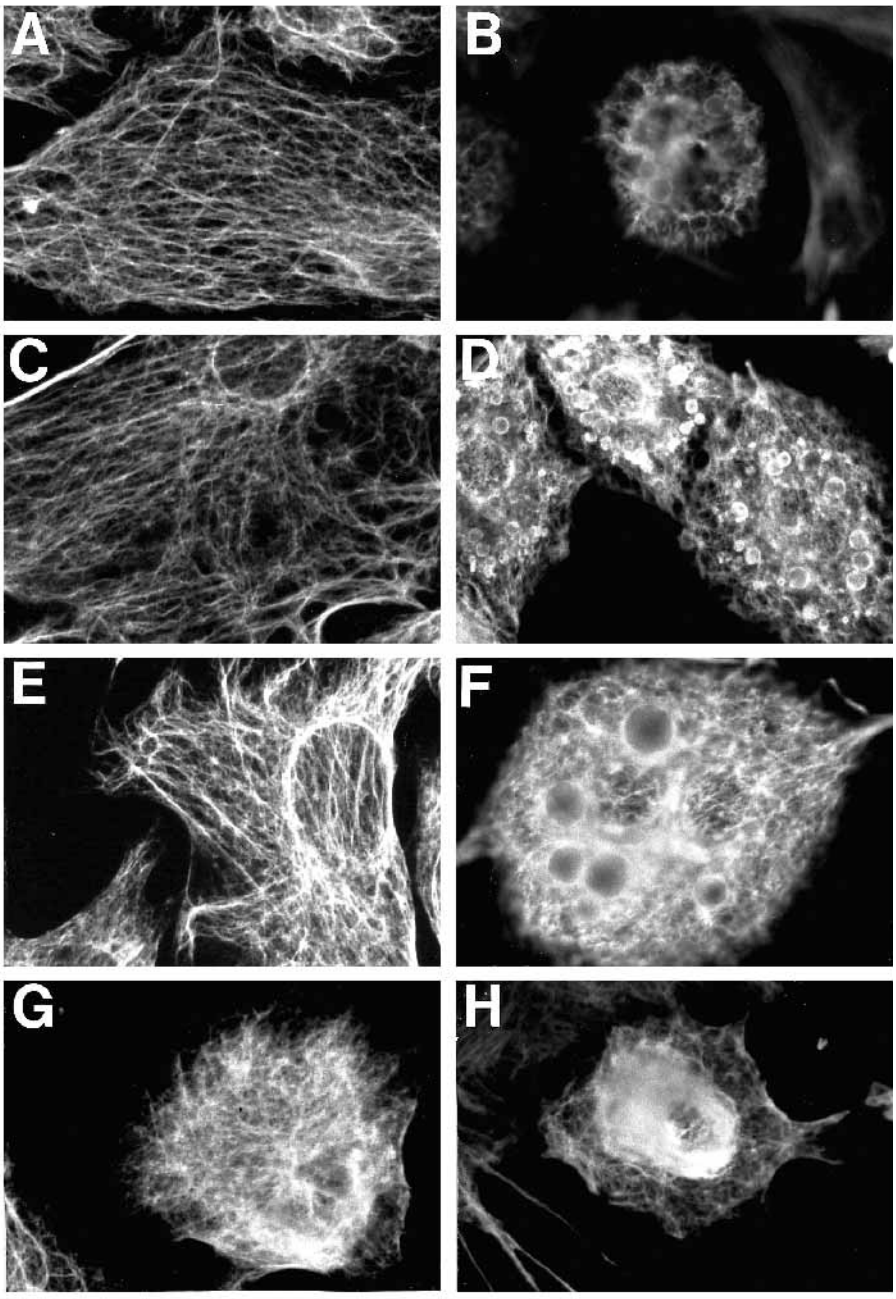

Fig. 7. Hormone-inducible disruption of vimentin filament organization during adipose conversion. The figure shows the anti-vimentin immunofluorescence staining pattern of 3T3-L1 cells (A-D) and 83ΔC 9A2 cells (E-H), that were untreated (A and E), or treated with 10^{-7} M dexamethasone for 96 hours (C and G), and 96 hours after induction of adipose conversion with 2 μg/ml ADD 4743 and 10^{-7} M insulin alone (B and F), or in the presence of 10^{-7} M dexamethasone (D and H).

conversion. However, when cells under identical conditions were radiolabeled for 2 hours, the dexamethasone-treated 83ΔC cells exhibited a significantly ($P \leq 0.05$) lower apparent rate of incorporation of [^{14}C]palmitate into triglycerides than untreated cells. This effect of dexamethasone on the rate of incorporation of [^{14}C]palmitate appeared to be specific for triglyceride metabolism, as incorporation into phospholipids was not significantly affected (Table 3). During adipose conversion of 83ΔC cells, this effect of dexamethasone on the apparent incorporation of [^{14}C]palmitate into triglycerides became larger with longer labeling times, at least up to 24 hours (Fig. 9), reflecting the decreased triglyceride content of these cells. One interpretation of these results was that dexamethasone-induced expression of the dominant negative mutant vimentin had relatively little effect on the rate of triglyceride synthesis, but rather the reduced capacity of these cells to accumulate neutral lipid was associated with an elevated rate of triglyceride turnover.

To determine if perturbation of vimentin organization during adipose conversion might affect the stability of triglycerides, experiments were performed to follow the fate of radiolabeled triglycerides in 83ΔC 9A2 and 3T3-L1 cells that had undergone adipose conversion in the presence or absence of dexamethasone. Cells were induced with ADD 4743 in the presence or absence of dexamethasone and then 120 hours after induction, the cultures were labeled with [^{14}C]palmitate for 10 minutes. The labelling medium was then removed and the level of remaining ^{14}C incorporated into triglycerides was determined before (0 time) and after an 8 and 15 hour chase, either in the presence or absence of dexamethasone. As shown in Fig.

Table 3. Effect of labelling time on the incorporation of [^{14}C]palmitate into lipids during adipose conversion of 3T3-L1 and 83ΔC cells in the presence and absence of dexamethasone

		Incorporation of [^{14}C]palmitate at 120 hours post-induction (μmoles/mg protein/hour)*			
		10 minute labeling		2 hour labeling	
Cell line	Treatment	Triglycerides	Phospholipids	Triglycerides	Phospholipids
3T3-L1	None†	4±1	7±2	30±3	32±2
	+Dex	170±18	73±7	67±10	23±3
83ΔC-9A2	None	42±6	25±2	27±2	25±1
	+Dex	37±3	36±3	15±2	30±3

Adipose conversion was induced with 2 μg/ml ADD 4743 and 10^{-7} M insulin. At 120 hours after induction with (+Dex) or without (None) addition of 10^{-7} M dexamethasone, the cells were labeled with [^{14}C]palmitate for 10 minutes or 2 hours and incorporation into triglycerides and phospholipids determined.

*Values represent the average obtained from triplicate cultures ± s.e.m.

†Examination of ORO-stained cells in this experiment showed that the 3T3-L1 cells without dexamethasone did not exhibit optimal levels of adipose conversion.

10, dexamethasone had no significant ($P \leq 0.05$) effect on the incorporation of [^{14}C]palmitate into triglycerides or phospholipids during a 10 minute pulse in the control 3T3-L1 cells. Although the radioactivity incorporated into triglycerides declined significantly in untreated cultures over the next 15 hours, there was no significant loss of radioactivity from the triglycerides in dexamethasone-treated 3T3-L1 cells during the chase period. In contrast, in 83ΔC 9A2 cells, while dexamethasone did not significantly ($P \leq 0.05$) affect [^{14}C]palmitate incorporation into triglycerides or phospholipids during the pulse, the loss of ^{14}C from triglycerides during the 15 hour chase was significantly ($P \leq 0.05$) greater in the dexamethasone-treated cultures. Again, triglycerides appeared to be specifically affected, as the incorporation of [^{14}C]palmitate into phospholipids was not observably altered by dexamethasone and the ^{14}C incorporated into phospholipids increased during the chase period (Fig. 10). These results indicate that lipid synthesis was not affected by expression of the mutant vimentin, but rather that the impaired capacity of these cells to accumulate lipid droplets was associated with an increased rate of turnover of newly synthesized triglyceride.

DISCUSSION

The alteration in vimentin filament organization that accompanies adipose conversion represents a significant change in the organization of the cytoplasm. The formation of cage-like arrays of vimentin filaments at the surface of nascent lipid droplets, ensheathed by endoplasmic reticulum cisterna, first described by Franke et al. (1987), occurs simultaneously with a large increase in the capacity of the lipid synthetic apparatus of 3T3-L1 cells. While the metabolic processes that are required for lipid accumulation in these cells have been extensively examined (Ailhaud et al., 1992; Smyth et al., 1993), the molecular basis of the movement of lipid components to and from triglyceride-rich lipid droplets is not understood, and the proteins that are associated with the periphery of lipid droplets have only begun to be characterized (see Greenberg et al., 1992; Blanchette-Mackie et al., 1995).

Perturbation of the vimentin filament network in 3T3-L1 cells significantly decreased the lipid-droplet forming capacity of these cells during adipose conversion. The present studies indicate that three different experimental approaches for altering the vimentin filament organization of these cells all produced a similar phenotype, characterized by impaired lipid droplet formation. Any individual method that is used to perturb IF organization has the potential for non-specific effects that could alter adipocyte differentiation. Although it has been reported that expression of a related dominant negative mutant desmin protein can have toxic effects in cells (Yu et al., 1994), we have not observed any indication that the mutant vimentin was toxic to the cells. We have also continuously cultured adrenal tumor cells that constituitively express this truncated vimentin at levels that are similar to the 3T3-L1 transfectants used in these studies (Sarria et al., 1994). In these cells, the mutant protein is expressed heterogeneously and the presence or absence of the mutant vimentin does not seem to confer any selective disadvantage for the growth of the cells. Furthermore, the effects of these agents on 3T3-L1 cells appeared quite specific to lipid droplet formation, and other morphological changes that are characteristic of adipose conversion seemed to be unaffected. Finally, it seems unlikely that disruption of the normal vimentin filament organization using an anti-microtubule drug, anti-filament antibody microinjection, and expression of mutant filament protein, would all produce the same non-specific effects. We therefore conclude that the maintenance of a normal filament organization during adipocyte conversion provides some function or functions that affect efficient lipid droplet formation.

The initial examination of triglyceride metabolism in the 3T3-L1 cells that express a dominant negative mutant vimentin indicated that the impaired capacity to accumulate lipid droplets was not associated with a decrease in triglyceride synthesis. Induction of glycerol-3-phosphate dehydrogenase, a late marker of adipose conversion (Kuri-Harcuch and Green, 1977), and the rate of triglyceride synthesis, as assessed by [^{14}C]palmitate pulse labeling, were not affected by perturbation of vimentin IFs. Instead, the effect of dexamethasone-induced expression of the mutant vimentin was associated with an increased rate of triglyceride turnover. This suggests that the impaired capacity of these cells to accumulate lipid droplets is not associated with an effect of vimentin on the ability of the cells to synthesize triglycerides, but seems to reflect a decrease in the stability of newly synthesized triglyceride in the cells that express elevated levels of the mutant vimentin. How vimentin IFs might influence the stability of triglycerides in 3T3-L1 cells during adipose conversion obviously remains

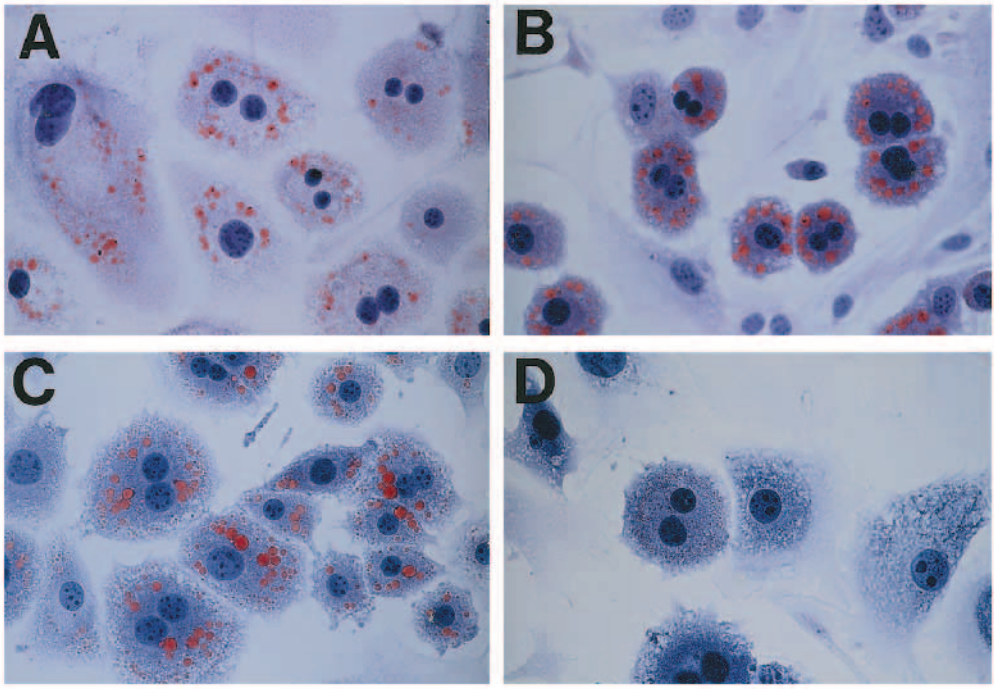

Fig. 8. Induction of a dominant negative mutant vimentin with dexamethasone inhibits lipid droplet formation. ORO staining of wild-type 3T3L1 cells (A and B) and 83ΔC-9A2 cells (C and D) 120 hours after induction of adipose conversion with 2 μg/ml ADD 4743 and 10^{-7} M insulin in the absence (A and C) or presence of 10^{-7} M dexamethasone (B and D).

a matter of conjecture. Lipid droplets appear to form from specialized areas of endoplasmic reticulum involved in triglyceride synthesis (Novikoff et al., 1980). Recently, using confocal microscopy, Blanchette-Mackie et al. (1995) observed that while vimentin was present around small to medium-sized lipid droplets, it was more difficult to detect or even absent from the surface of large lipid droplets in 3T3-L1 cells. Perhaps vimentin IFs provide a function that affects lipid stability during the initial formation of lipid droplets. The rate-limiting step in triglyceride lipolysis is catalyzed by hormone-sensitive lipase. It has been reported that the distribution of hormone-sensitive lipase and other lipid droplet components are altered under conditions of increased triglyceride turnover. In isoproterenol-stimulated 3T3-L1 cells, Egan et al. (1992) have described a translocation of hormone-sensitive lipase to the surface of the lipid droplets and speculated that lipid droplet surface proteins such as perilipin, a lipid droplet component that is expressed during adipose conversion, might serve to regulate this interaction. Perhaps perturbation of the vimentin IF structure during the formation of lipid droplets interferes with some aspect of this process and allows an inappropriate hydrolysis of triglycerides. It will therefore be intriguing to learn if the distribution of hormone-sensitive lipase is affected by perturbation of vimentin IFs during adipose conversion in these cells.

To what extent might the effect of an alteration in vimentin cage structure on 3T3-L1 cell triglyceride stability indicate a more general function for vimentin IFs in other cell types? Recent studies have indicated that vimentin filaments are closely associated with cholesteryl ester-rich lipid droplets in steroidogenic cells (Almahbobi et al., 1992, 1993), although the vimentin filaments in these cells apparently do not undergo the reorganization observed in 3T3-L1 cells (Evans, 1994). Examination of SW-13 adrenal tumor cell lines that either contain vimentin IFs or lack a detectable IF system has shown that the presence or absence of a vimentin IF system affects the capacity of SW-13 cells to transport lysosomally derived cholesterol (Sarria et al., 1992). In these studies, adrenal tumor cells that contained vimentin IFs exhibited an increased capacity to accumulate lysosomally derived cholesteryl esters, compared with highly related cells that lacked an IF network. However, this does not appear to be associated with an increased rate of cholesteryl ester turnover. Gillard et al. (1994) have recently shown that vimentin expression in SW-13 cells

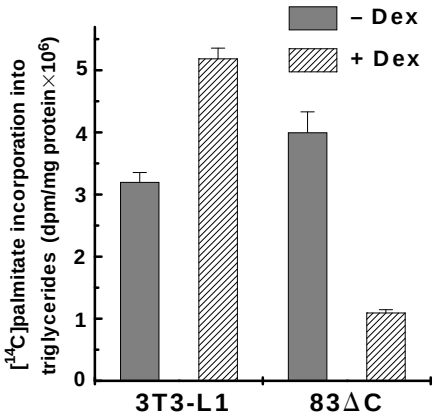

Fig. 9. The effect of dexamethasone on the accumulation of [^{14}C]palmitate-labeled triglycerides during adipose conversion in 3T3-L1 cells and 83ΔC 9A2 cells that express the hormone-inducible dominant negative mutant vimentin. Adipose conversion was induced with 2 μg/ml ADD 4743 and 10^{-7} M insulin in the absence (–Dex) or presence (+Dex) of 10^{-7} M dexamethasone. At 96 hours after induction the cells were radiolabeled with [^{14}C]palmitate for 24 hours and incorporation into triglycerides determined. The values represent the average obtained from triplicate cultures ± s.e.m.

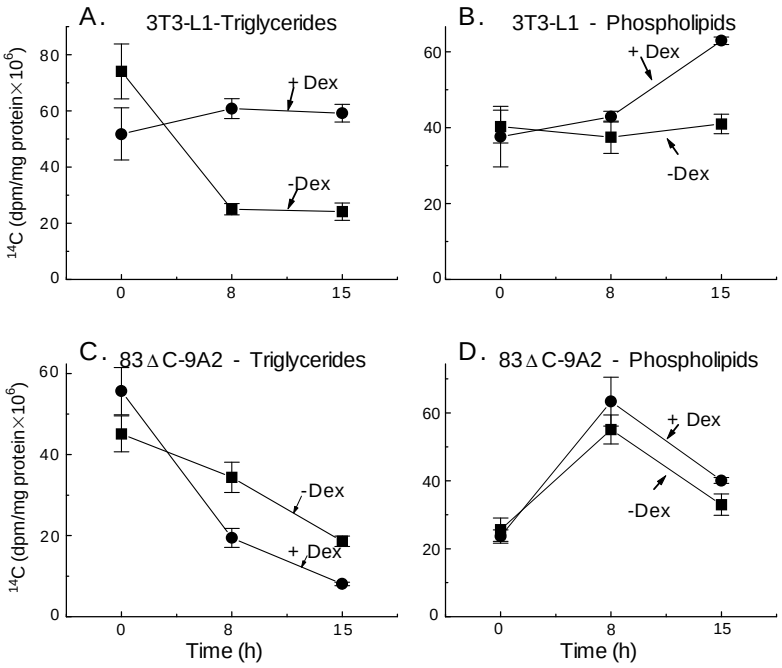

Fig. 10. The effect of dexamethasone on the incorporation of [^{14}C]palmitate into triglycerides and phospholipids during a 10 minute pulse label and a 15 hour chase. Adipose conversion was induced with 2 μg/ml ADD 4743 and 10^{-7} M insulin in the absence (–Dex) or presence (+Dex) of 10^{-7} M dexamethasone. At 120 hours after induction the cells were radiolabeled with [^{14}C]palmitate for 10 minutes (time 0). The labeling medium was removed and the cells incubated for an 8- or 15-hour chase period. The figure shows ^{14}C incorporated in triglycerides (A and C) and phospholipids (B and D) in 3T3-L1 cells (A and B) and 83ΔC-9A2 cells (C and D) at time 0, 8 and 15 hours after removal of the labeling medium. Values represent the average obtained from triplicate cultures ± s.e.m.

also affects the synthesis of glycosphingolipids, characterized by an increased capacity of cells that contain vimentin to recycle sphingosine through the Golgi network (Gillard et al., 1996). Since the lipid composition of the droplets in adipose cells is primarily triglyceride, and therefore differs from the cholesteryl ester-rich lipid droplets characteristic of steroidogenic cells, it is not immediately apparent if these observations are related. However, it is tempting to speculate that vimentin IFs may have a more general function that is seen in both adrenal and adipose cells.

Finally, since perturbation of vimentin filament organization had such a prominent effect on adipose conversion of cultured 3T3-L1 cells, why has no obvious defect in adipose development been observed in vimentin null mice? There has been some speculation that the lack of an apparent phenotype in vimentin null animals could be related to some redundancy of function (Colucci-Guyon et al., 1994), but no redundant structure has yet been identified. Another possibility is that there might be an adaptive response to the absence of a filament system. It should be considered that disruption of vimentin organization during adipose conversion of cells in culture represents an acute situation where a relative difference in lipid stability results in an observable effect on lipid accumulation, while differences in lipid stability in animals or primary cultures might be considered to be a chronic condition that might be expected to have more subtle effects on lipid accumulation. It is also possible that there is an inherent difference between the effects produced by the absence of an IF network compared with dominant effects on an existing vimentin network. The possible effects of dominant mutations in vimentin have not been examined in transgenic mice. We have tried to isolate a preadipose cell line from embryo fibroblasts derived from vimentin null mice, but these efforts have so far been unsuccessful, and therefore it has not been possible to directly address these questions using cultured cells. Clearly, future studies will begin to examine these issues, and the availability of cultured cell models will be an important tool in characterizing the functional significance of vimentin-type IFs.

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