Transforming Growth Factor β (TGFβ)-Induced Nuclear Localization of Apolipoprotein J/Clusterin in Epithelial Cells†

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ABSTRACT: Apolipoprotein J (apoJ)/clusterin was first identified as an 80 kDa secretory glycoprotein present in most body fluids. It has been implicated in a variety of physiological processes including cellular differentiation and apoptosis. We demonstrate here that in addition to the well characterized secreted form of the protein, there exists an intracellular, nuclear form of apoJ. This intracellular form of the protein is induced to accumulate in the nucleus of two epithelial cell lines (HepG2 and CCL64) in response to treatment with transforming growth factor β (TGFβ). We demonstrate *in vitro* that apoJ protein can be translated from two in-frame ATG sites. Initiation from the first ATG encodes for the secretory form of apoJ and initiation from the second ATG, located 33 amino acids downstream of the first and lacking the hydrophobic signal sequence, encodes for a truncated apoJ protein. This shorter form of apoJ is not recognized by microsomes and therefore not glycosylated, and we postulate that it is retained intracellularly and targeted to the nucleus due to the presence of an SV40-like nuclear localization sequence (NLS). This mechanism of nuclear targeting of apoJ occurs in cells since the protein isolated from nuclei of TGFβ-treated cells and the *in vitro*-translated truncated form are identical by V8 protease analysis. These results suggest that the diverse physiological responses attributed to apoJ may be elicited through a common molecular mechanism involving a previously uncharacterized intracellular form of the protein.

Apolipoprotein J (apoJ)[1] is a heterodimeric glycoprotein found in serum, seminal fluid, and other biological fluids which is constitutively synthesized and secreted by various cell types (Jenne & Tschopp, 1992). It is identical or homologous to several proteins including clusterin (Blaschuk et al., 1983), sulfated glycoprotein-2 (SGP-2) (Collard & Griswold, 1987) and testosterone-repressed prostate message-2 (TRPM-2) (Buttyan at al., 1989). Its cellular distribution is broad; apoJ mRNA is relatively abundant in the testes, brain, liver, and ovary, and also detectable in the kidney, thymus, spleen, and heart (Wong et al., 1993, 1994). The mRNA sequence encodes a 427 amino acid protein of 46 kDa which is expressed in the endoplasmic reticulum as a 56−60 kDa precursor after glycosylation (de Silva et al., 1990a; Burkey et al., 1991). The 56−60 kDa protein undergoes further N-linked glycosylation to a 75−80 kDa precursor. This precursor undergoes intracellular cleavage to yield a disulfide heterodimer of α and β subunits which is ultimately secreted (Burkey et al., 1991).

The physiological role of apoJ is not known. ApoJ is thought to be cytoprotectant at fluid−tissue boundaries, particularly where the fluid contains bioactive amphipathic molecules (Aronow et al., 1993), and to metabolize tissue debris at sites of active remodeling during development and in response to injury (Buttyan et al., 1989; French et al., 1993). It has also been proposed to have roles in complement regulation (Jenne & Tschopp, 1989), sperm maturation (Sylvester et al., 1984), lipid transport (Burkey et al., 1992), neurodegenerative diseases (May et al., 1990), *src*-induced transformation (Michel et al., 1989), and apoptosis (Buttyan et al., 1989). ApoJ protein is abundant in atheromatous lesions (Witte et al., 1993) and has been linked to atherosclerosis (Jenne & Tschopp, 1992). It is implicated in the differentiation and proliferation of epithelial and smooth muscle cells (Hartmann et al., 1991; Thomas-Salgar & Millis, 1991) and is shown to be expressed in a specific manner during heart morphogenesis (Witte et al., 1994). The association of clusterin/apoJ expression with apoptosis, or active cell death (ACD), was first made when TRPM-2 was identified and cloned as the major mRNA species induced during regression of the rat ventral prostate after castration (Montpetit et al., 1986). Since that time apoJ has been associated with other tissues that undergo apoptosis: regressing renal tubular epithelium after injury, developmentally necrosing interdigital tissue of fetal limb bud, and rat thymocytes induced to apoptose by glucocorticoids (Jenne & Tshopp, 1992). It is unclear whether apoJ is directly involved in the apoptotic process or its induction is secondary to apoptosis and the gene is induced as a protective or defense mechanism for the cell.

It is difficult to explain how a secreted glycoprotein could be implicated in such diverse biological responses, and in fact, recent evidence suggests that an intracellular, nonsecreted, form of the protein may exist. First, an intracellular

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[1] Abbreviations: apoJ, apolipoprotein J; TGFβ, transforming growth factor β; PAGE, polyacrylamide gel electrophoresis; NLS, nuclear localization sequence.

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form of the protein can be found in smooth muscle cells where it is thought to have a functional role in their differentiation to the nodular form (Thomas-Salgar & Millis, 1994). Also, immunofluorescence of clusterin/apoJ in MCF-7 cells treated with vitamin D_3, a treatment which induces apoptosis, suggests that the protein is localized in two subcellular compartments, the plasma and nuclear membranes (Wilson et al., 1995). This is supported by the fact that apoJ contains a nuclear localization sequence (NLS), from amino acid 52 to 58 (LEEAKKK), which is identical to that found in the androgen receptor (Wilson et al., 1995). These data suggest that there may exist alternate intracellular targeting routes for apoJ resulting in both a secreted and an intracellular form of the protein.

The expression pattern of apoJ mRNA during cell differentiation, apoptosis, and embryogenesis has been studied in variety of model systems (Buttyan et al., 1989; French et al., 1993; Witte et al., 1994). Regulation of apoJ expression by various growth factors has also been reported. One such such factor is transforming growth factor β (TGFβ) which acts on a wide variety of cell types to regulate cell proliferation, differentiation, and apoptosis (Massague, 1990). For example, it has been shown that TGFβ down-regulates apoJ mRNA expression in porcine smooth muscle cells (Thomas-Salgar & Millis, 1994). However, TGFβ has also been shown to either induce or repress apoJ message in rat astrocytes, depending on total cell-type composition of the cultures (Morgan et al., 1995). When astrocytes are grown in the presence of oligodendrocytes and microglia, astrocytes increase apoJ mRNA levels in response to TGFβ. On the other hand, in monotypic cultures of astrocytes, TGFβ represses apoJ message.

The mink lung epithelial cell line, CCL64, is highly sensitive to TGFβ and is routinely used to study TGFβ-induced cellular responses (Howe et al., 1991; Reddy et al., 1994). It was of interest, therefore, to determine the effects of TGFβ on both apoJ mRNA and its protein in this cell line. In this manuscript we present evidence for the existence of a novel, truncated intracellular form of the apoJ protein. We further show that TGFβ increases the transcription of the apoJ gene in lung epithelial cells and induces the nuclear accumulation of the intracellular form of apoJ.

EXPERIMENTAL PROCEDURES

Northern Blot Analysis. Rapidly growing mink lung (CCL64) cells were treated with 5 ng of TGFβ1/mL for 0−24 h. Total RNA was isolated by guanidium isothiocyanate method (Chomczynski & Sacchi, 1987)). Equivalent quantities of RNA (20 μg) from each time point were separated in 1.2% agarose−formaldehyde gels. The blots were hybridized with random-labeled (Pharmacia) human apoJ cDNA probe and washed extensively with 1× SSC containing 0.1% SDS at room temperature followed by 0.1× SSC containing 0.1% SDS at 55 °C for 15 min. The results were analyzed by autoradiography. The same blot was stripped in a boiling solution of 0.01× SSC containing 0.1% SDS for 30 min and probed with radiolabeled 1B15 (rat cyclophilin; Danielson et al., 1988) probe to ensure equal loading of the RNA.

Antibodies. Antibodies to human apoJ were either a rabbit polyclonal to the β subunit (Burkey et al., 1991) or a monoclonal antibody (mAb11) (de Silva et al., 1990b). The polyclonal anti-apoJβ is highly specific for the human protein and does not recognize mink protein. Sheep anti-rat apoJ or clusterin antibodies were purchased from Quidel Corp. (San Diego, CA). These antibodies recognize both human and mink apoJ proteins.

Metabolic Labeling and Immunoprecipitations. HepG2 cells as well as CCL64 cells were routinely cultured in DMEM-F12 medium containing 10% calf serum. Cells growing in 150 mm tissue culture plates were starved in methionine-deficient medium for 1 h and then pulse labeled for 30 min with 50 μCi of [^{35}S]methionine/mL in methionine-deficient medium. Cells were lysed in lysis buffer [50 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl, 0.5% deoxycholate, 0.1% SDS, and 1% NP-40]. The clarified cell lysates were subjected to immunoprecipitation using anti-apoJ antiserum.

Nuclei Isolation. For nuclear lysate, radiolabeled cells were lysed in TITE buffer (20 mM Tris-HCl, pH 8.0, containing 100 mM NaCl and 0.2% Triton X-100) by Dounce homogenization. The resulting homogenate was layered over a sucrose cushion (10% sucrose in TITE buffer) and centrifuged at 800*g* for 10 min (Wolgemuth & Hsu, 1981). The nuclei were resuspended in lysis buffer and subjected to immunoprecipitations using anti-apoJ antibodies. For the results presented in Figure 6, rapidly growing HepG2 cells were treated with 5 ng of TGFβ1/mL for 0−24 h. Cells were starved in methionine-free media and pulse labeled with [^{35}S]methionine during the last 2 h of the incubation period. Nuclei were isolated, and extracts were prepared as described above. Equal amounts of TCA-precipitable radioactive material from each time point were immunoprecipitated with anti-apoJ antibodies and analyzed on 10% SDS−PAGE followed by fluorography.

In vitro Translations. Either the entire cDNA for apoJ (J1, residues 1−427) or PCR-amplified J2 (corresponding to residues 34−427) was cloned into Bluescript vector (Stratagene). J2 was PCR amplified by using the forward primer 5′-GGATTCATGTCCAATCAGGGAAGTAAGTAC-3′ and the reverse primer 5′-GGATTCTCACTCCTCCCG-GTGCTTTTTGCG-3′. The underlined sequences represent *Bam*H1 adapters for subsequent subcloning. The conditions for the PCR amplification were 95 °C for 15 min followed by 30 cycles of 95 °C for 15 s; 55 °C for 30 s; and 72 °C for 90 s and 65 °C for 10 min. PCR-amplified products were then cloned into the *Bam*H1 site. *In vitro* transcription-coupled translations were performed using the TNT-coupled reticulocyte lysate system (Promega) in the presence of [^{35}S]-methionine. [^{35}S]methionine-labeled products were either directly loaded for SDS−PAGE or subjected to immunoprecipitation by anti-apoJ antibodies.

Overexpression of J1 and J2 in CCL64 Cells. cDNAs for J1 and J2 were also cloned into the eukaryotic expression vector pcDNA3. CCL64 cells were transfected with either J1 or J2 in pcDNA3 by using the ProFection mammalian transfection system (Promega). Stable transfectants were selected in the presence of 500 μg of G418 geneticin/mL. Overexpression of the proteins corresponding to J1 and J2 was confirmed by metabolic labeling and immunoprecipitation, as described above.

V8 Protease Analysis. Both *In vitro*-translated J2 and nuclear J2 from metabolically labeled HepG2 cells were immunoprecipitated by specific antibodies against apoJ. The bands corresponding to J2 were excised from the polyacrylamide gel and subjected to in-gel V8 protease digestion as

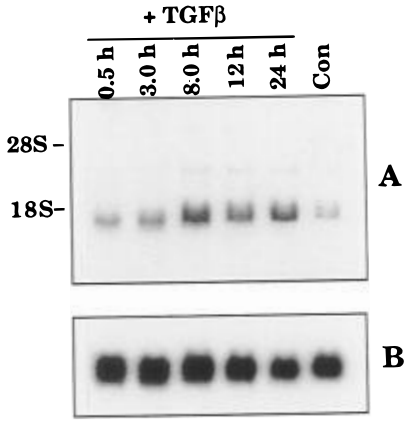

FIGURE 1: Effect of TGFβ on apoJ message. Rapidly growing CCL64 cells were treated with 5 ng of TGFβ1/mL for indicated times. 20 μg of total RNA from each time point was separated on 1% formaldehyde−agarose gels and transferred to Nitran membrane. The blot was probed with random labeled apoJ probe (panel A). Same blot was stripped and reprobed with radiolabeled 1B15 to ensure equal loading (panel B).

previously described (Cleveland et al., 1977). Radiolabeled peptides were separated on 15% SDS−PAGE, and results were analyzed by fluorography.

RESULTS

We first examined the effects of TGFβ on apoJ mRNA expression in mink lung epithelial cells. Rapidly growing CCL64 cells were treated with 5 ng of TGFβ/mL for the indicated times, and total RNA was isolated. Northern blot analysis shows that TGFβ rapidly induces apoJ message (Figure 1). This increase is detectable as early as 3 h after TGFβ treatment and remains elevated for at least 24 h. There is about a 3−4-fold increase in apoJ mRNA level at 24 h post-TGFβ treatment (Figure 1).

We subsequently studied the effect of TGFβ on apoJ protein (Figure 2). Newly synthesized protein was detected by metabolic labeling and immunoprecipitation. HepG2 cells (panel A) and CCL64 cells (panel B) were incubated in the absence or presence of TGFβ for 24 h and labeled with [^{35}S]-methionine (see Experimental Procedures) prior to lysis and immunoprecipitation with anti-human β chain apoJ antisera (Burkey et al., 1991). A 60 kDa protein is observed in HepG2 cell lysates (Figure 2A, lane 2) that is not detected with the preimmune control sera (Figure 2A, lane 1). This 60 kDa protein represents the previously characterized precursor of the secretory form of apoJ. This form of apoJ undergoes glycosylation and an intramolecular cleavage, prior to its secretion (Burkey et al., 1991). In TGFβ-treated HepG2 and CCL64 cells, the 60 kDa form of apoJ was detected, and a prominent band migrating with an apparent MW of 43 kDa was also seen (Figure 2A, lane 3, and 2B, lane 2). We postulated that this lower band present in TGFβ-treated cells represents a second form of apoJ, possibly a non-glycosylated, nonsecretable form of the protein. We performed subcellular fractionation studies from ^{35}S-labeled cellular lysates and determined that in TGFβ-treated cells this form was present in the nucleus (Figure 2A, lanes 4 and 5, and 2B, lanes 3 and 4). Anti-apoJ antibodies immunoprecipitated only the lower molecular weight protein from nuclear lysates of both the cell lines (Figure 2A, lanes 4 and 5, and 2B, lanes 3 and 4). The 43 kDa protein is almost undetectable in the nuclear lysates of cells not treated

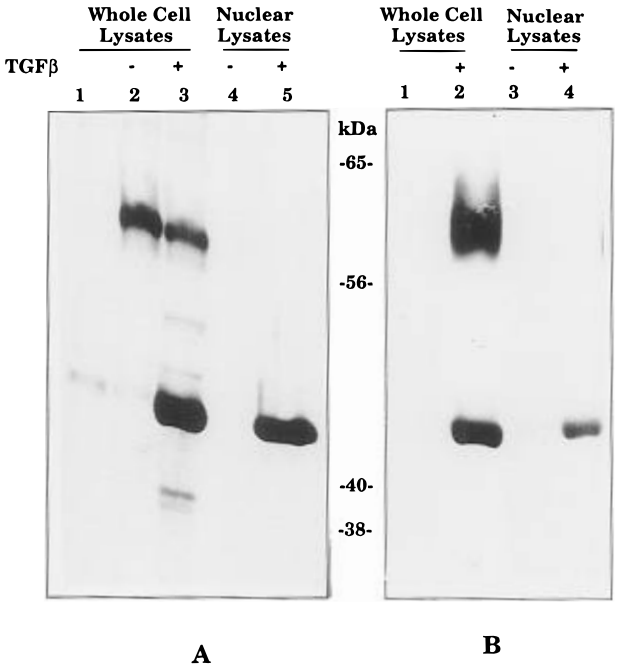

FIGURE 2: Effect of TGFβ on apoJ protein. Panel A: Immunoprecipitation of apoJ from [^{35}S]methionine-labeled HepG2 cells. Lane 1, pre-immune serum; lanes 2−5, rabbit anti-human apoJ antibodies. Immunoprecipitations were performed from either the whole-cell lysates (lanes 1−3) or from nuclear lysates (lanes 4−5). Lanes 3 and 5 represent TGFβ-treated samples. Panel B: Immunoprecipitation of apoJ from radiolabeled CCL64 cells. Lane 1, pre-immune serum; lanes 2−4, sheep anti-rat apoJ antibodies (Quidel Corp., San Diego, CA). Immunoprecipitations were performed from either the whole-cell lysates (lanes 1−2) or from nuclear lysates (lanes 3−4). Lanes 2 and 4 represent TGFβ-treated samples.

with TGFβ. Two different anti-apoJ antibodies immunoprecipitated this 43 kDa band from HepG2 cells: a polyclonal sera raised against the human β chain of the protein (Burkey et al., 1991) and a monoclonal antibody (de Silva et al., 1990b) raised against human apoJ (data not shown). The mink apoJ proteins, either the 60 or the 43 kDa form, are not recognized by antibodies to human protein, and instead a commercially available antibody raised against rat clusterin was employed (see Experimental Procedures). Therefore, recognition of the 43 kDa form of apoJ is not due to a co-immunoprecipitating contaminant of the antibody or unique to a single antibody.

The presence of a 43 kDa protein in the nucleus led us to carefully examine the primary structure of apoJ (Figure 3A). Downstream of the initiation ATG and a secretory signal sequence, there is an additional in-frame ATG at amino acid position 34 in human, porcine, and canine apoJ proteins. Both of these ATG codons have low homology to the Kozak translational sequence (Kozak, 1991), and thus it is possible that the protein may initiate translation at two sites. The second ATG is followed by a consensus nuclear localization signal (NLS) sequence LEEAKKK (residues 52−58; Figure 3, panel A) identical to that found in the androgen receptor (Wilson et al., 1995) and is conserved in all apoJ sequences (Jenne & Tshopp, 1992). We postulated that translation from the first ATG results in the synthesis of a secretory apoJ, whereas initiation from the second ATG results in a truncated, nonglycosylated form of the protein. The latter protein would lack the hydrophobic signal sequence and therefore would not be secreted. To determine whether

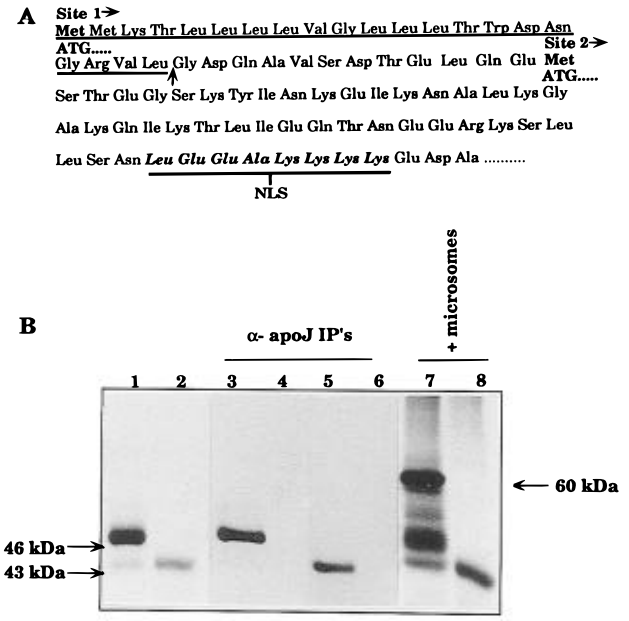

FIGURE 3: Multiple translational initiation sites are present in apoJ mRNA: Panel A: The primary structure of apoJ depicting two putative initiation sites. The first methionine residue and the hydrophobic signal sequence are underlined. The second methionine residue at the second initiation site is shown in bold. The nuclear localization signal (NLS) is shown in italics. Panel B: Immunoprecipitation analysis of the *in vitro*-translated products using anti-apoJ antibodies. Lane 1, *in vitro*-translated J1; lane 2, *in vitro*-translated J2; lane 3, immunoprecipitated J1; lane 4, J1-immunoprecipitation was carried out in the presence of 5 μg of cold purified apoJ; lane 5, immunoprecipitated J2; lane 6, J2-immunoprecipitation was carried in the presence of 5 μg of cold purified apoJ; lane 7, *in vitro*-translated J1 in the presence of microsomes; lane 8, *in vitro*-translated J2 in the presence of microsomes.

multiple forms of the protein could be initiated from the apoJ mRNA, *in vitro* translation studies were performed. Two constructs were made by PCR amplification of the human cDNA, which we termed J1 and J2, representing residues 1−427 and 34−427, respectively. Both cDNAs were subcloned into the pBSK vector (Stratagene), allowing for *in vitro* transcription-coupled translations from the T3 promoter. The results presented in Figure 3, panel B demonstrate that two protein products of 46 and 43 kDa are transcribed and translated from the J1 construct in a reticulocyte lysate (lane 1). The predominant protein is the 46 kDa form; however, the 43 kDa form of apoJ is translated and represents less than 5% of the total, showing that the first ATG is the preferred translational start site. Only the 43 kDa form is translated from the J2 construct (lane 2) which lacks the N-terminal 33 amino acid residues. Both *in vitro*-translated J1 and J2 products can be immunoprecipitated by specific anti-apoJ antibodies (lanes 3 and 5), and cold, excess, purified apoJ protein can compete with labeled *in vitro*-translated apoJ binding to antibodies (lanes 4 and 6). These results show that more than one apoJ protein can be translated from its mRNA. When microsomal membranes are added to the reticulocyte lysates, only the J1 cDNA construct encodes a protein which undergoes glycosylation (lane 7). The 46 kDa protein is glycosylated to a 60 kDa species of apoJ. The 43 kDa form of the protein, encoded by the J2 construct, does not undergo glycosylation (lane 8). The 46 kDa apoJ species that contains an N-terminal hydrophobic sequence is targeted to microsomal

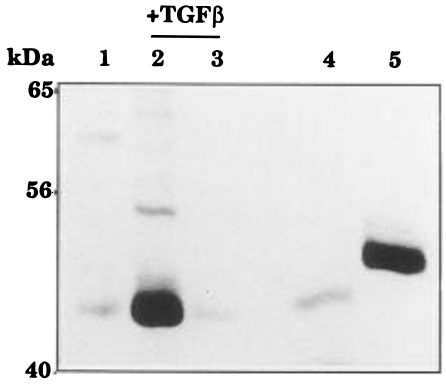

FIGURE 4: Comparison of nuclear apoJ with *in vitro*-translated J1 and J2. Nuclear apoJ was immunoprecipitated from radiolabeled HepG2 cell nuclear lysates using rabbit anti-human apoJ antibodies. Lane 1, control cells; lane 2, TGFβ-treated cells; lane 3, immunoprecipitation was carried in the presence of 5 μg of cold purified apoJ; lane 4, immunoprecipitated J2; and lane 5, immunoprecipitated J1.

membranes and is glycosylated; in contrast the unglycosylated 43 kDa (lane 8) species is not targeted to the endoplasmic reticulum and thus may represent an intracellular form of apoJ.

Two approaches were taken to show that the 43 kDa form of apoJ isolated from nuclei (Figure 2) was the same as the 43 kDa form translated *in vitro*. In a first approach, the 43 kDa protein was immunoprecipitated from control as well as TGFβ-treated cells and compared to the immunoprecipitated *in vitro*-translated J1 and J2 products. As expected, anti-apoJ antibodies immunoprecipitated only the 43 kDa protein from nuclear lysates of TGFβ-treated cells (Figure 4, lanes 1 and 2). Excess cold, purified apoJ from plasma effectively competed with this 43 kDa protein for binding to antibodies (lane 3). The 43 kDa protein from nuclear lysates co-migrates with the *in vitro*-translated J2 protein, indicating similar MW (lane 4). The 46 kDa J1 product, which initiates from the initial ATG in the mRNA and contains the signal sequence, migrates with a higher MW than the nuclear form of the protein (lane 5). These results suggest that the 43 kDa protein present in the nuclear extracts of TGFβ-treated cells is the product of initiation at the second initiation start site at position 34 in the apoJ mRNA. In a second approach, nuclei from TGFβ-treated HepG2 labeled with [^{35}S]methionine were immunoprecipitated with anti-apoJ and subjected to digestion with V8 protease (Figure 5). Comparison of V8 digestion maps of *in vivo*-immunoprecipitated apoJ (panel B) with those of authentic 43 kDa J2 transcribed and translated *in vitro* (panel A) show that the two proteins are identical since they yield indistinguishable peptide fragments (Figure 5). These results conclusively establish that the 43 kDa protein represents a novel intracellular form of apoJ.

We next established the kinetics of the TGFβ-induced accumulation of the 43 kDa form of apoJ in the nucleus. Nuclear lysates from HepG2 cells treated with TGFβ for varying lengths of time were subjected to immunoprecipitation with anti-apoJ antisera (Figure 6, panel A). The results demonstrate that nuclear apoJ protein was not detected until 16 h after TGFβ treatment. Quantitative analysis of the immunoprecipitated bands by Phosphoroimage analysis shows that there is about a 12-fold increase in the nuclear accumulation of apoJ after 24 h of TGFβ treatment (Figure

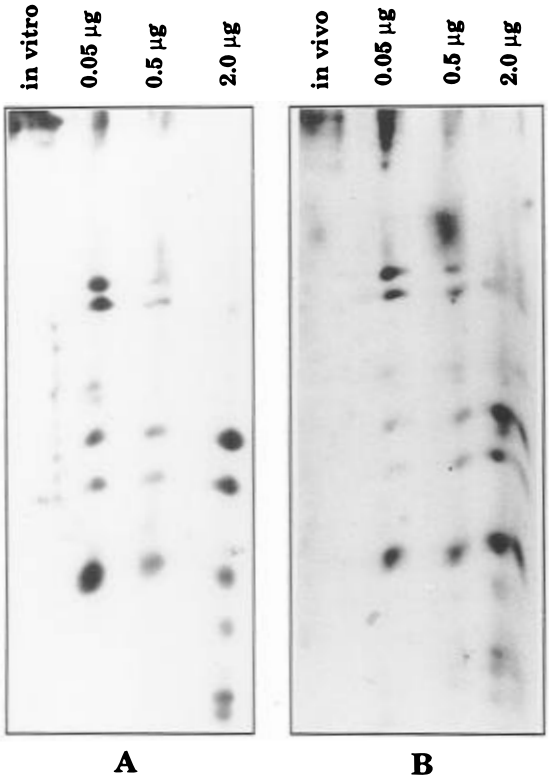

FIGURE 5: V8 protease analysis. Immunoprecipitated *in vitro*-translated J2 (panel A) or nuclear apoJ (panel B) was digested with increasing amounts of V8-protease for 30 min at room temperature and analyzed by SDS−PAGE.

6, panel B). The observed rate of nuclear accumulation of apoJ is in good agreement with other TGFβ-mediated events. Unlike other growth factors such as EGF, PDGF, and NGF, whose responses are very rapid, TGFβ is a relatively slower acting growth factor. TGFβ inhibits cell proliferation in late G1 phase of the cell cycle, and its effects on various cell cycle associated cyclin proteins are not seen until 16 h post-treatment (Howe et al., 1991; Reddy et al., 1994).

To confirm these results by an alternative strategy, we overexpressed human apoJ in CCL64 mink lung epithelial cells. We subcloned both the J1 (entire cDNA) and J2 (truncated apoJ protein lacking the first 33 amino acids) constructs into the eukaryotic expression vector pcDNA3, and stable clones were selected in G418. Overexpression of the protein was analyzed by immunoprecipitation with specific antibodies against human apoJ. These antibodies do not recognize the mink apoJ protein and therefore allow for the analysis of only the overexpressed human forms of apoJ. Figure 7 demonstrates that a 60 kDa protein corresponding to the secretory form of apoJ as well as a 43 kDa protein is seen in cells transfected with the J1 cDNA construct (lane 2). In cells transfected with the J2 construct only the 43 kDa protein is found (lane 3). We then performed immunoprecipitations from nuclear lysates isolated from these overexpressing cells. First, the 60 kDa form of apoJ is not detectable in nuclear lysates from either the control or the overexpressing cells. The 43 kDa apoJ protein, however, is readily detectable in both J1 and J2 overexpressing CCL64 cells (lanes 5 and 6) but not in the control CCL64 cells (lane 4). Although the J2 overexpressing cells accumulate the 43 kDa form of apoJ to a greater extent than the J1 cells, clearly some 43 kDa apoJ is being expressed

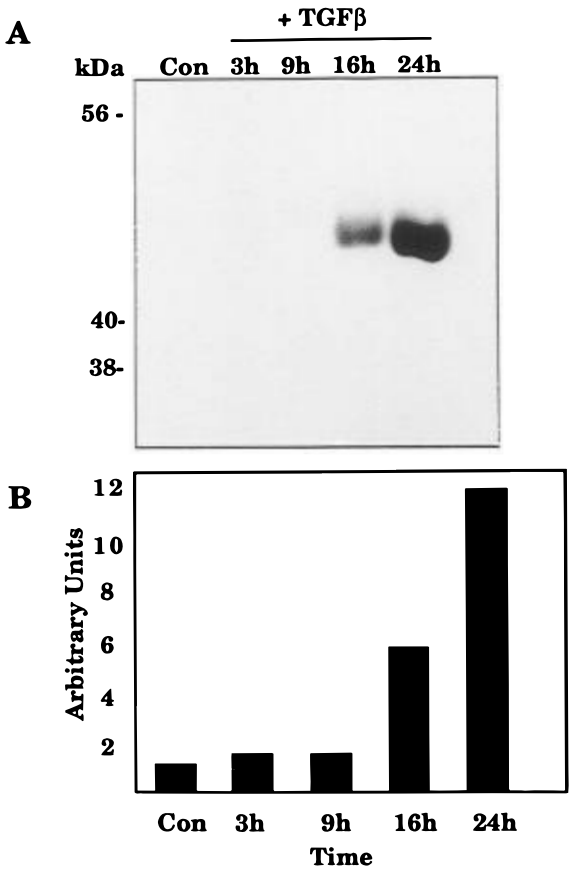

FIGURE 6: Kinetics of TGFβ-induced nuclear accumulation of apoJ. Panel A: HepG2 cells were treated with 5 ng of TGFβ1/mL for the times indicated. The cells were labeled with [^{35}S]methionine during last 30 min. Immunoprecipitations were performed from nuclear lysates, and the results were analyzed by SDS−PAGE followed by fluorography. Panel B: Immunoprecipitated nuclear apoJ was quantitated by 2 phosphoimager and plotted as a function of time.

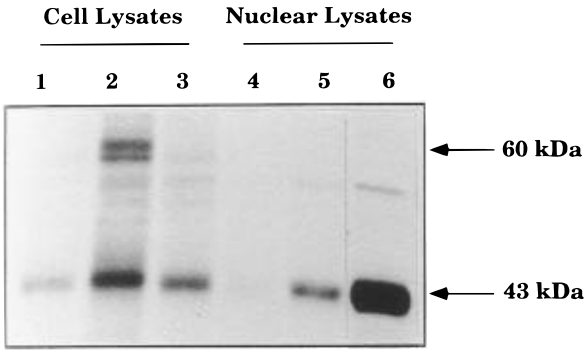

FIGURE 7: Overexpression of apoJ in CCL64 cells. CCL64 cells were transfected with pcDNA3 expression vector containing the cDNA corresponding to J1 (residues 1−427) or J2 (residues 34−427). Expression of J1 and J2 proteins was analyzed by immunoprecipitation with rabbit anti-human apoJ antibodies. Lanes 1 and 4, parental CCL64 cells; lanes 2 and 5, J1 transfected cells. lanes 3 and 8, J2 transfected cells. Lanes 1−3 represent whole-cell lysates, and lanes 4−6 represent nuclear lysates.

from the full-length J1 cDNA and targeted to the nucleus. These results suggest that in the absence of the first initiating ATG, as encoded by the J2 cDNA, the predominant form of apoJ is the 43 kDa protein, which is targeted to the nucleus. If both initiation codons are present, as encoded by the J1 cDNA, both the secreted and nuclear forms of apoJ are expressed.

DISCUSSION

In this manuscript we demonstrate that TGFβ causes the accumulation in the nucleus of an intracellular form of apoJ. This form of the protein migrates with an apparent MW of 43 kDa. It can be immunoprecipitated from mink lung epithelial cells and HepG2 cells treated with TGFβ. The nuclear form of apoJ is recognized by several specific apoJ antibodies and is competed for in immunoprecipitation assays by purified apoJ protein. This 43 kDa form of apoJ represents a truncated form of the secreted and glycosylated protein. Since the truncation removes the first 33 amino acids from apoJ, including the hydrophobic signal sequence, the protein is not recognized by microsomes and therefore is not glycosylated *in vitro*. We propose that the 43 kDa form of the protein isolated from nuclei is also not glycosylated since both the *in vivo*-isolated 43 kDa protein and the *in vitro*-synthesized 43 kDa protein have identical electrophoretic mobilities and, more importantly, identical V8 protease peptide maps. Furthermore, when overexpressed in mink lung epithelial cells, this 43 kDa form of the protein is targeted and accumulates in the nucleus.

Further support for the existence of a nuclear form of the apoJ protein is provided by an examination of its primary sequence (Figure 3A). Two in-frame ATG codons separated by 33 amino acids, including the hydrophobic signal sequence, exist in the apoJ mRNA. Downstream of the second putative initiation site, from amino acid 52−58, is a nuclear localization sequence which is present in the androgen receptor (Wilson et al., 1995). Assignment of the first ATG codon as the translational initiation site is based on the coincidence of predicted and experimental molecular weights of the non-glycosylated protein translated *in vitro* and in tunicamycin-treated cells (Burkey et al., 1991) and on the fact that most secretory proteins have a signal sequence. However, this initiation codon displays poor homology to the Kozak translational consensus sequence (Kozak, 1991), and thus, it is possible that the protein may initiate translation at multiple sites. If translation initiation originates at the second ATG site, then apoJ would lack the N-terminal hydrophobic signal peptide typical of secretory proteins and would instead be retained intracellularly.

A growing number of proteins have been identified with more than one targeting signal in their primary structure (Garcia-Bustos et al., 1990). In such cases, the sequence proximal to the amino terminus determines where the protein is localized in the cell. Secretory and nuclear apoJ differ by two in-frame initiation codons separated by about 99 nucleotides (33 amino acids). Northern blot analysis shows only one species of apoJ mRNA in both control and TGFβ-treated cells (Figure 1). However, it should be noted that it is difficult to detect a difference of 100 nucleotides on regular agarose−formaldehyde gels, and other experiments will have to be performed to address this issue. We do not know whether these two forms of apoJ are generated from the same mRNA or whether TGFβ induces the transcription of a shorter mRNA. Evidence for both of these mechanisms exists in the literature. Expression of avian apoJ, as shown through promoter sequence analysis, is driven from two different promoters (Michel et al., 1995). In this situation it is conceivable that the secretory and nuclear forms of apoJ are encoded by two different mRNA populations. In the case of hepatitis pre-core protein, both secretory and nuclear forms of the protein are translated from the same mRNA and the products have identical primary structures except that the secretory form has an additional 30 amino acids at its N-terminus (Ou et al., 1989). Also, the c-*myc* mRNA gives rise to two species of c-*myc* proteins, also with different N-termini, and differential translational initiation is regulated in a differentiation-dependent manner in myeloid cells (Hann et al., 1988).

Although the secretory form of apoJ is well characterized, its physiological role(s) has not been clearly elucidated. Expression of the protein has been shown to correlate with the induction of apoptosis in several tissues, and interestingly, a nuclear form has been inferred in MCF-7 cells treated with vitamin D3 (Wilson et al., 1995). Also, in a mouse mammary carcinoma model, progression to the androgen independent state is proposed to correlate with a subcellular relocalization of apoJ from the cell membrane to the nucleus (Wilson et al., 1995). In neither of these studies has nuclear apoJ been clearly documented. In the context of the present data, the physiological role of TGFβ-induced nuclear apoJ accumulation is unknown; however, in both CCL64 and in the hepotoma cell line Hep 3B, TGFβ induces apoptosis (Oberhammer et al., 1993; Lin & Chou, 1992), consistent with a role for the 43 kDa apoJ protein in the apoptotic process. It is our current understanding that the 43kDa form of apoJ is somehow involved in TGFβ-induced apoptosis. In this context, we are examining TGFβ-induced apoptosis in normal and apoJ-overexpressing epithelial cells.

The molecular mechanism of action of apoJ has remained elusive despite its purported function in numerous physiological and pathophysiological processes. Also unclear is whether apoJ is truly a multifunctional protein or whether a common mechanism elicits these various processes. Data presented here suggest that an intracellular form of the protein accumulates in the nucleus in response to TGFβ. The existence of this form of the protein suggests that the constitutively expressed secreted form and the TGFβ-regulated form may have different functions. We do not yet know the biological significance for such a TGFβ-induced nuclear accumulation, but an association between TGFβ and apoJ has previously been reported. Indeed, striking similarities in the expression pattern of the two genes has been demonstrated during mouse embryogenesis (French et al., 1993), epithelial cell differentiation (Hartmann et al., 1991), and cardiac valve morphogenesis (Witte et al., 1994). It has even been suggested that apoJ may somehow be involved in mediating TGFβ-induced cellular differentiation (French et al., 1995). Both apoJ and TGFβ are stored in platelets and released during platelet activation at site of tissue injury (Witte et al., 1993), and both are elevated at sites of injury in fibrotic lesions (Buttyan et al., 1989). Further investigation is required to clarify the physiological function of apoJ, but the demonstration of an intracellular form of the protein will perhaps provide new areas of insight and investigation.

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