Abstract. A potential regulatory linkage between the biosynthesis of colligin, a collagen-binding protein of the ER, and procollagen I was examined under a variety of experimental conditions. Cell lines which did not produce a significant amount of procollagen I mRNA also lacked the capacity to produce colligin mRNA. Anchorage-dependent cell lines like L6 myoblasts and normal rat kidney fibroblasts produced both colligin and procollagen I mRNA, but the level of both was concurrently reduced considerably in their ras-transformed counterparts. Similarly, during the differentiation of L6 myoblasts, levels of both colligin and procollagen declined together. Treatment of myoblasts by dexamethasone or EGF led to a decrease in the steady-state levels of procollagen I mRNA, and this was, again, accompanied by a decrease in colligin mRNA synthesis. On the other hand, when the rate of procollagen I synthesis was stimulated by treatment of myoblasts with TGFβ, it led to the concurrent augmentation of both the mRNA and protein levels of colligin. A linkage between the regulation of synthesis of procollagen I and colligin thus seems to exist. The only exception to this generalization is provided by the heat induction behavior of the two proteins. Treatment of myoblasts for a very short period leads to an increase in the synthesis of both the mRNA and protein levels of colligin. This, however, is not accompanied by a change in the mRNA levels of procollagen I. These studies establish that colligin and procollagen are generally tightly co-regulated except after heat shock, suggesting an important functional linkage.

We have earlier characterized a 46-kD glycoprotein (termed gp46) from L6 rat skeletal myoblasts (Cates et al., 1984; Nandan et al., 1990a) and have shown that it binds collagen I, collagen IV, and gelatin (Cates et al., 1987b). We have cloned the mRNA for gp46 from rat myoblasts (Clarke et al., 1991) and human fibroblasts (Clarke and Sanwal, 1992) and have shown that it is closely related to hsp47 described from chick embryo fibroblasts (Hirayoshi et al., 1991) and J6 described from mouse embryonal carcinoma cells (Wang and Gudas, 1990). Since the protein was first reported by Kurkinen et al. (1984) from murine parietal endoderm cells under the name “colligin,” it is appropriate to designate gp46 from rat (Cates et al., 1984), hsp47 from chick (Hirayoshi et al., 1991) and J6 from mouse (Wang and Gudas, 1990) as tissue and organ-specific forms of colligin. Colligin is not only a collagen-binding protein, but is also a serpin, i.e., a member of the serine protease inhibitor family (Carrell and Boswell, 1986; Clarke et al., 1991; Clarke and Sanwal, 1992; Hirayoshi et al., 1991; Wang and Gudas, 1990). As is well known, most of the serpins except leukocyte elastase inhibitor (Potempa et al., 1988) are secreted proteins and most act as serine protease inhibitors, although some, like the cortisol- and thyroxine-binding proteins are noninhibitory (Carrell and Boswell, 1986). Whether colligin belongs to the inhibitory or noninhibitory class of serpins is yet to be determined, the one fact that sets it apart from other serpins is its localization to the ER (Nagata and Yamada, 1986; Nandan et al., 1988). This location coupled to its collagen binding property raises some important questions regarding its function. The collagen binding ability of colligin has led to the suggestion that it may function as a molecular chaperone (Ellis and Van der Veis, 1991; Nakai et al., 1992) for the synthesis of collagen I and IV in various tissues (Cates et al., 1984, 1987b; Kurkinen et al., 1984; Saga et al., 1987), although it might equally well serve some other unknown function in the biosynthesis of collagen. In some preliminary experiments we had shown (Clarke et al., 1991; Nandan et al., 1990b) that the levels of colligin and collagen I and IV are regulated both positively and negatively in parallel under some conditions. Thus, ethyl-3,4-dihydroxybenzoate (an analogue of ascorbate required for posttranslational modification of collagen), a specific inhibitor of collagen I synthesis, also inhibited the synthesis of colligin in skeletal myoblasts (Nandan et al., 1990b). Similarly, when F9 embryonal carcinoma cells were induced to differentiate by retinoic acid and cAMP, they began to produce collagen IV. Under these conditions colligin was also induced in parallel with collagen (Clarke et al.,...
Density of 5 x 10^5 cells/ml in a volume of 50 ml of medium RPMI 1640 (Dak Co., Rochester, NY). Identification of procollagen chains was achieved by using molecular weight standards (Miller and Rhodes, 1982; Sage and Peterkofsky, 1981) by ammonium sulfate precipitation. To extract cellular proteins, cells were homogenized in Laemmli sample buffer (Laemmli, 1970), and the proteins were heated in Laemmli sample buffer for 10 min each and incubated for 3 h. At the end of the incubation period, protease inhibitors (PMSF, 0.5 mM, EGTA, 4 mM and leupeptin, 2 μg/ml) were added, and labeled procollagen was isolated as described previously (Bateman and Peterkofsky, 1981) by ammonium sulfate precipitation. To extract cellular procollagen, labeled cells were washed extensively with cold PBS and extracted with 0.5 M acetic acid containing the mixture of protease inhibitors. Cells were treated three times through a 27-gauge needle and the contents were left on ice for 30 min. The insoluble material was removed by centrifugation. The supernatant was neutralized and lyophilized. This material was heated in Laemmli sample buffer (Laemmli, 1970), and the proteins were separated by 6% SDS-PAGE. The gels were stained, destained, treated with ENHANCE (NEN Research Products, New England Nuclear, Boston, MA) for 45 min, dried, and exposed to Kodak X-Omat film (Eastman Kodak Co., Rochester, NY). Identification of procollagen chains was achieved by using molecular weight standards (Miller and Rhodes, 1982; Sage and Bornstein, 1982).

**DNA Probes**

The EcoRI fragment of pIP, a subclone of pE11 (Clarke et al., 1991), was used as a rat collagen probe. The insert contains 75% of the coding region towards the COOH terminus (amino acid residues 104-400) and contains 140 bp of 3'-untranslated region. Rat collagen proc1(1) (pcsR1) and proc2(1) (pcsR2) cDNA clones were obtained from Dr. D. Rowe (University of Connecticut Medical Center, Farmington, Connecticut). The pCS1 plasmid harbored a 1.3-kb insert coding for amino acids 400 through 943 in the triple helical domain of the proc1(1) chain, ligated into the PstI/BamHI sites of pUC18 (Smith and Niles, 1980). The insert comprised of a 0.9-kb insert coding for the carboxy-terminal extension peptide of proc1(1) ligated into the Psnl site of pUC18. A chicken β-actin cDNA probe (Cates et al., 1987) contained the complete coding and 3'-untranslated regions, was obtained from Dr. D. W. Cleveland (Johns Hopkins University, Baltimore, MD). All probes were isolated away from their respective plasmids before being labeled with (α-32P)-dCTP using a mixture of random hexanucleotides to prime in vitro DNA synthesis (Feinberg and Vogelstein, 1983).

**Northern Blotting and RNA Slot Blots**

For Northerns, poly(A)-tailed RNA or total RNA was denatured with glyoxal and separated on 1% agarose gels containing sodium phosphate (Smith and Niles, 1980). Electrophoresed RNA was then transferred to Bio-trans nylon membranes (ICN Biochemicals, NY). For slot blots, various amounts of total RNA of each sample were slot blotted using the Schleicher and Schuell Minifold II (Schleicher and Schuell, Inc., Keene, NH). The filters were prehybridized at 42°C for 3 h and then hybridized at 42°C for 12-24 h in a solution containing 50% formamide, 5× SSC, 5× Denhardt's solution (Denhardt, 1966), denatured salmon sperm DNA, 0.1% SDS and denatured DNA probes. Blots were washed twice in 2× SSC containing 0.1% SDS at room temperature for 5 min each. Blots probed with rat collagen and β-actin were subsequently washed twice with 0.1× SSC and 0.1% SDS at 42°C for 20 min each. Blots probed with the collagen probes were washed twice with 0.1× SSC containing 0.1% SDS at 60°C for 10 min each. Filters were exposed to Kodak XAR-5 film at −70°C for various lengths of time.

**Western Blotting**

Monolayers were washed once with cold PBS and the cells were scraped into 1% SDS heated to 90°C. Samples were stored frozen at −20°C before use. Protein was quantitated using a modification (Paterson, 1977) of the Lowry procedure (Lowry et al., 1951). Aliquots of cell extracts were run on 5% SDS-polyacrylamide gels as described by Laemmli (Laemmli, 1970). Electrophoresed proteins were then electroblotted onto nitrocellulose membranes for 75 min at 110 V using a Bio-Rad Trans-blot apparatus (Bio-Rad/Analytical Instr. Group, Cambridge, MA) and stained with Amido black. After washing with TBS, the blots were blocked for 1 h at 37°C with 4% BSA, and then incubated overnight at room temperature in buffer containing affinity purified anti-collagen antibody (Cates et al., 1987b) or anti-protein disulfide isomerase antibody (Dimonemis Laboratories, Ontario, Canada). Blots were then washed 4 times with 0.1% Triton X-100 in TBS buffer for 10 min each and incubated for 3 h in 4% BSA in TBS containing 0.1 μCi/ml [125I]-labeled goat anti-mouse IgG antibody. Blots were washed again, dried and autoradiographed at −70°C.

**Densitometry**

Protein and RNA signals in autoradiograms were scanned using an LKB Ultrascan XL laser densitometer. A value of 1 was assigned to the intensity of bands in control lanes and all values were expressed relative to this value. Film responses were in the linear range when quantitation from autoradiograms was performed.

**Results**

**Collagen and Proc collagen I Transcripts in Cell Lines**

Starting with the assumption that cell lines which are not stringent in their requirement for attachment to a surface for growth may lack or have diminished ability to produce collagen I, we tested several such lines and found that they do not have significant amounts of procollagen α1(I) and α2(I) mRNA compared with cell lines which require attachment to substrate for growth (Fig. 1 A). The former types of cell lines also had insignificant amounts of collagen mRNA, while cell lines which produced larger amounts of procollagen I...
mRNA correspondingly had higher levels of colligin mRNA. A striking correlation between the levels of the mRNA of the two proteins was seen in normal L6 myoblasts and NRK fibroblasts and their ras-transformed counterparts (NRKras and L6ras2A; Fig. 1 B). The normal cells require attachment to substratum for growth (and in the case of L6 for differentiation), while the transformed cells can grow on soft agar and are anchorage independent. The mRNA levels of procollagen and colligin are both reduced considerably in the transformed lines. This reduction is not due to general decrease of RNA synthesis in the transformed cells. Levels of ß-actin mRNA, for instance, are unaltered in L6ras2A compared with L6.

**Procollagen and Colligin Transcripts during Myoblast Differentiation**

It is known that skeletal myoblasts produce collagen I, but during their differentiation into myotubes, its level decreases (Nusgens et al., 1986). In view of the correlation observed between colligin and procollagen I levels in various cell lines, we wanted to find if these proteins are co-regulated during differentiation. Under our culture conditions, L6 myoblasts align themselves in arrays on approximately the third day of culture, and begin to fuse on the fifth day. The mRNA levels of procollagen ß1(I) and ß2(I) chains increase significantly on the third day and then decrease to almost half just before fusion. Interestingly, and in conformity with the results described above, the mRNA levels of colligin also follow the same trend as the procollagen chains (Fig. 2). We had earlier demonstrated (Cates et al., 1987b) that the levels of the protein decrease during differentiation. The decrease in the levels of colligin does not reflect a general decline in the biosynthesis of proteins during myoblast differentiation. We have shown earlier (Lorimer et al., 1987) that the DNA/RNA ratios do not significantly change during myogenesis.

**Effect of Dexamethasone on Procollagen I and Colligin Levels in Myoblasts**

It has been shown by several workers in various systems (Raghow et al., 1986; Weiner et al., 1987) that glucocorticoids inhibit the expression of type I collagen through both transcriptional and posttranscriptional regulatory mechanisms. It was of interest to determine if the synthesis of collagen I is also suppressed in myoblasts, and more importantly, if this suppression is accompanied by the inhibition of synthesis of colligin mRNA. Fig. 3 shows that colligin mRNA levels decrease after dexamethasone treatment as do the procollagen I mRNA levels. When myoblasts are exposed to dexamethasone over a period of several days it leads to the inhibition of differentiation and the synthesis of mRNA of differentiation-related proteins such as troponin C and myosin light chain in myoblasts (our own unpublished observations), but levels of the mRNA of a "housekeeping" protein such as ß-actin, both in the short as well as long term, are not affected by the glucocorticoid. The effect on collagen I and colligin mRNA thus seems to be more specific.
Effect of Growth Factors on the Biosynthesis of Procollagen I and Colligin in Myoblasts

There are two well-known growth factors, TGFβ and EGF, which have opposite effects on the biosynthesis of collagen I in various cells. TGFβ not only inhibits myogenesis (Heino and Massagué, 1990; Massagué, 1990; Massagué et al., 1986) but also upregulates the synthesis of collagen I and several other extracellular matrix (ECM) constituents (Massagué, 1990). In addition, it activates production of PAI-1, which, like colligin, is a serpin (Andreasen et al., 1990). EGF, on the other hand, inhibits the transcription of collagen I genes in skin fibroblasts (Kurata and Hata, 1991). The opposite effects of the two growth factors on collagen I synthesis allowed us to ask if the levels of colligin are co-regulated with those of collagen I. In conformity with earlier results (Massagué et al., 1986) TGFβ increased the steady-state levels of procollagen I mRNA in myoblasts (Fig. 4). EGF on the other hand, much like in fibroblasts (Kurata and Hata, 1991), decreased these levels in myoblasts (Fig. 4). The interesting observation, however, is that the mRNA levels of colligin change in the same way as procollagen I, i.e., they increase in the presence of TGFβ and decrease in the presence of EGF (Fig. 4). For a 12-h exposure period for the growth factors, maximum effect was brought about by 0.25 ng/ml TGFβ (Fig. 4) and 50 ng/ml EGF. The change in the steady-state levels of mRNA for both procollagen I and colligin in the presence of growth factors were accompanied by corresponding changes in the protein levels. This is shown in Fig. 5 for TGFβ. We ascertained that the concentration of another protein immediately connected with procollagen synthesis in vivo (Nakai et al., 1992), viz., PDI, a subunit of prolyl hydroxylase, and β-macroglobulin, a housekeeping protein, did not change in the presence of the growth factors (N. Jain, E. Clarke, and B. D. Sanwal, unpublished observations).

Absence of Co-regulation of Procollagen I and Colligin during Heat Shock

It has been demonstrated earlier (Cates et al., 1987b; Nagata et al., 1986; Saga et al., 1987) that colligin is a heat shock protein. Brief exposure of myoblasts (4 h) to 42 and 44°C increases its level. Heat treatment also leads to an increase in the levels of collagen I mRNA (Fig. 6). Other common proteins in myoblasts, such as β-actin, are unaffected by heat treatment. Procollagen I mRNA levels are also not significantly affected by heat (Fig. 6).

Discussion

The aim of this investigation was to find clues to the function of the collagen-binding protein colligin (Clarke et al., 1991; Clarke and Sanwal, 1992; Hirayoshi et al., 1991; Kurkinen

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1. Abbreviation used in this paper: ECM, extracellular matrix.
from each sample was slot blotted onto a nylon filter and probed sequentially with the indicated DNA probes.

e et al., 1984; Wang and Gudas, 1990). Two observations that suggested that colligin may have a role to play in the biosynthesis of procollagen I were: first, that it bound tightly to collagen I and collagen IV (Cates et al., 1984, 1991; Kurkinen et al., 1984; Nagata and Yamada, 1986); and second, it was localized to the lumen of the ER, as judged by the presence of an ER-retention signal, RDEL, at its carboxyl terminus (Clarke et al., 1991), and by immunofluorescence (Nand et al., 1988) and electron microscopic studies (Nagata and Yamada, 1986; Saga et al., 1987) of its localization. Since procollagen is synthesized in the ER, colligin could be involved in any one of the following aspects of procollagen biosynthesis. It could be acting as a chaperone (Nakai et al., 1992) in the intracellular processing of procollagen. Because colligin is a serpin, it could equally well be involved in the protection of the nascent procollagen chains from attack by yet unknown serine protease(s) in the ER. Indeed, there is now firm evidence for the presence of some protein degradative enzymes in the ER compartment itself (Klausner and Sita, 1990; Wikstrom and Lodish, 1992). Results presented here are compatible with either one of these possibilities. Further experimentation will be needed to establish the role of colligin in the synthesis of procollagen I.

We have shown that under a variety of conditions where the level of mRNA for collagen α1(I) and α2(I) decreases, there is also a concurrent decrease in the level of colligin mRNA. Such, for instance, is the case during the differentiation of L6 myoblasts. It has been shown by several workers that during differentiation of myoblasts secretion of collagen I decreases and is replaced by collagen III (Nusgens et al., 1986). This observation is in agreement with our data regarding the diminution of the steady-state levels of mRNA for procollagen I (and concurrently colligin) during differentiation. We had earlier shown that DNA/RNA ratios remain unchanged during myogenesis (Lorimer et al., 1987); the decrease, thus, is not a consequence of a general decrease in RNA synthesis. Similarly, transformed cells are known to produce smaller amounts of procollagen I mRNA (Avvedimento et al., 1981; Sandmeyer et al., 1981), and we have found the same to be true of ras-transformed myoblasts and NRK cells. Here, again, there is a parallel decrease in the content of colligin mRNA, compared with untransformed cells. Further, cells which can grow in suspension and have no apparent need for the presence of ECM components in the medium, produce little or insignificant amounts of procollagen I mRNA. Correspondingly, in these cells colligin mRNA is barely detectable. It has been reported that dexamethasone decreases the levels of procollagen I (Hamalainen et al., 1985; Weiner et al., 1987) by both transcriptional and posttranscriptional mechanisms. We have shown that colligin steady-state mRNA levels are also decreased significantly compared to untreated cells. If present throughout the course of myogenesis in culture, dexamethasone also inhibits differentiation and decreases the levels of mRNA for several proteins, such as myosin light chain and troponin, but short-term exposure of undifferentiated cells to the hormone does not lead to generalized decrease of mRNA synthesis. Growth factor EGF, like the steroid hormones, inhibits the synthesis of both procollagen I mRNA and protein, and this again is accompanied by decrease of mRNA and protein levels of colligin. It is clear that negative regulation of procollagen I is accompanied by negative regulation of colligin. We had also shown earlier (Nandan et al., 1990b) a similar co-relation in the case of ethyl dihydroxybenzoate, a potent inhibitor of procollagen mRNA and protein synthesis.

The relationship between synthesis of procollagen I and colligin also holds for positive regulation. Growth factor TGFβ is known to upregulate the synthesis of procollagen I (and several other ECM constituents) in L6 myoblasts (Ign notz et al., 1987). Our results confirm that finding. However, more interesting from our point of view is that this upregulation is accompanied by increase in the mRNA and protein levels of colligin. Unlike the diversity and numbers of negative regulators, very few positive regulators of procollagen I synthesis are known, and further work will have to be undertaken with tissues where a remodeling of the ECM takes place and procollagen I synthesis becomes dominant (Hay, 1981) to see if appearance of procollagen I is accompanied by colligin synthesis. However, we (Clarke et al., 1991) and others (Hirayoshi et al., 1991) have already shown that during F9 embryonal carcinoma cell differentiation, procollagen IV synthesis increases in parallel with colligin. Undifferentiated cells lack both of these proteins and can grow in suspension. Colligin binds to collagen I and collagen IV equally well (Kurkinen et al., 1984), despite the differences in their structure, and it is very likely that colligin has the same biosynthetic relationship with collagen IV as it has with collagen I.

It may be pointed out that growth factors, such as TGFβ, are known to upregulate the synthesis of several ECM or ECM-related constituents, like fibronectin, tenascin, thrombospondin, and certain proteoglycans (Massagué, 1990), but none of these substances bind to procollagen I, if at all, as specifically and as tightly as does colligin. In fact, it has been shown that in addition to colligin, only PDI, a few stress proteins, and fibronectin are associated with procollagen I chains in vivo, in avian fibroblasts (Nakai et al., 1992) as well as in L6 myoblasts (Clarke, E., N. Jain, A. Brickendennen, E. Ball, and B. D. Sanwal, manuscript in preparation). Obviously proteins other than colligin are involved in procollagen biosynthesis and/or assembly, but their levels are not co-regulated with those of procollagen I chains. TGFβ, for instance, which upregulates procollagen I and colligin levels in parallel, has no significant effect on PDI levels. This is in keeping with results obtained with other cell types (Tsao and Grisham, 1991). Similarly, as we have shown earlier, glucocorticoids downregulate procollagen and colligin levels in parallel, but are known, depending upon the cell type, to have either no significant effect, or upregulate the synthesis of fibronectin (Cutroneo et al., 1986). Thus, all molecules found associated with procollagen in vivo are not regulated in the same way except colligin and procollagen. It is unlikely that this coregulation is entirely fortuitous and without physiological relevance.
The only exception we have so far been able to find where procollagen I levels are not co-regulated with colligin is the heat-induced increase of colligin. Heat induction of colligin was first demonstrated (Nagata et al., 1986) in chick embryo fibroblasts. This is also a characteristic of colligin from myoblasts. Treatment of L6 cells for a few hours at 42 or 44°C leads to an increase in the steady-state levels of colligin mRNA and protein, but levels of procollagen I remain invariant. This observation at a first glance seems to suggest that co-regulation of colligin and procollagen I under different conditions may only be fortuitous. However, if one assumes that co-regulation is due to the sharing of some common regulatory sequences in the genes governing the synthesis of colligin and procollagen I, and heat induction is due to the presence of heat shock elements only in the genetic sequences of colligin, a linkage between the synthesis of colligin and procollagen I would not be evident under some conditions. The heat induction of colligin suggests that it may function in protecting unwound or nascent chains of procollagen in vivo.

Apart from the functional relationship of colligin to procollagen, the regulation of colligin as a member of the serpin family per se by various growth factors, and under different growth conditions, is of great interest because so far only one other member of the serpin family, viz., plasminogen activator inhibitor (PAI-1), has been found to be susceptible to regulation by growth factors (Andreasen et al., 1990). However, while PAI-1 is upregulated by both TGFβ and EGF, procollagen I levels are increased by TGFβ and decreased by EGF. Just as the members of the serpin family differ from each other in regard to their inhibitory characteristics, this observation at a first glance seems to suggest that co-regulation is due to the sharing of some common regulatory sequences in the genes governing the synthesis of colligin and procollagen I, and heat induction is due to the presence of heat shock elements only in the genetic sequences of colligin, a linkage between the synthesis of colligin and procollagen I would not be evident under some conditions. The heat induction of colligin suggests that it may function in protecting unwound or nascent chains of procollagen in vivo.

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