Interaction of procollagen I and other collagens with colligin

Neeraj JAIN, Anne BRICKENDEN, Ian LORIMER, Eric H. BALL and Bishnu D. SANWAL*

Department of Biochemistry, University of Western Ontario, London, Canada N6A 5C1

Colligin is a collagen-binding glycoprotein of molecular mass 46000 Da localized to the endoplasmic reticulum (ER) of diverse kinds of cells that produce collagen I. In order to help define its role in collagen biosynthesis and to study the interaction of colligin with procollagen I in detail, the binding characteristics of colligin purified from L6 myoblasts have been studied. A total of 3 mol were found to bind/mol of procollagen I, with a K_d of about 25 nM. Both pure and separated $\text{pro}\alpha_1(\text{I})$ and procollagen $\alpha_2(\text{I})$ chains were able to compete with procollagen I for binding to colligin. However, colligin binds to $\text{pro}\alpha_2(\text{I})$ with higher affinity than to $\text{pro}\alpha_1(\text{I})$. To find if the binding activity of colligin was altered during purification, an assay to measure colligin binding to procollagen in crude myoblast cell extracts was developed. This procedure gave the same binding parameters as

did the highly purified colligin. Among different collagen types, colligin was found to bind to collagen I and collagen IV, but not to collagen III. In order to examine whether glycosylation or phosphorylation of colligin were required for the binding of colligin to procollagen I and to obtain enough colligin for further studies, recombinant protein was produced in *Escherichia coli*. An immunoaffinity purification scheme was used to get virtually pure protein in milligram yields. Comparison of the recombinant colligin with that isolated from L6 myoblasts showed that both types existed in solution as monomers and dimers. In addition, both types of colligins showed identical properties with regard to their binding to procollagen I and the isolated $\text{pro}\alpha_1(\text{I})$ and $\text{pro}\alpha_2(\text{I})$ chains. Post-translational modifications of colligin were thus not essential for binding to procollagen I.

INTRODUCTION

Kurkinen et al. (1984) were the first to describe a 47 kDa glycoprotein from murine parietal endoderm cells which bound specifically to gelatin (denatured collagen), collagen I and collagen IV which they named 'colligin'. Similar glycoproteins were later described in L6 rat myoblasts (gp46) (Cates et al., 1984), 3T3 fibroblasts, keratinocytes (Taylor et al., 1985; Hughes et al., 1987), and chick embryo fibroblasts (termed hsp47) (Nagata et al., 1986). The close similarity of these glycoproteins became evident with the cloning of the mRNA for colligin from L6 myoblasts (Clarke et al., 1991), human fibroblasts (Clarke and Sanwal, 1992), chick embryo fibroblasts (Hirayoshi et al., 1991) and F9 embryonal carcinoma cells (Wang and Gudas, 1990). Human colligin, for instance, shows 98% identity with colligin from rat skeletal myoblasts, 97% with the protein from mouse cells, and 80% identity with the avian protein (Clarke and Sanwal, 1992).

Colligin is localized to the endoplasmic reticulum (ER) of both myoblasts (Nandan et al., 1988) and avian fibroblasts (Saga et al., 1987). It has an RDEL sequence at its C-terminus, which is known to serve as an ER retention signal (Pelham, 1990). Colligin is glycosylated and phosphorylated *in vivo* (Nagata et al., 1986; Cates et al., 1987b) and is heat-inducible (Saga et al., 1987; Nandan et al., 1990b). Where known, the colligins seem to be glycoproteins with high-mannose type of glycan chains, and are capable of binding to concanavalin A (Cates et al., 1984; Cates et al., 1987b) and fetuin (Saga et al., 1987). It is also a member of the serine-proteinase inhibitor family (serpins) and is unique among serpins in its cellular localization. Almost all the members of the serpin family with the exception of leucocyte elastase inhibitor (Potempa et al., 1988) are secreted proteins and most act as serpins, although some, like the cortisol-

and thyroxine-binding proteins, are non-inhibitory (Carrell and Boswell, 1986). However, we do not know the physiological significance of colligin as serpin in the ER.

The localization of colligin in the ER together with its ability to bind collagen I and IV has led to the suggestion that it may have a role to play in the biosynthesis of collagen as a molecular chaperone (Clarke et al., 1991; Nakai et al., 1992). Since recent findings suggest the presence of a proteolytic system in the ER that is separate from the lysosomal pathway (Klausner and Sitia, 1990), an alternative possibility is that colligin protects procollagen chains from proteolysis before or after triple-helix formation. Being a serpin it would be suited to play such a role. It is generally believed that the assembly of procollagen I in the ER takes place by an initial folding of carboxy-propeptides of two $\text{pro}\alpha_1(\text{I})$ and one $\text{pro}\alpha_2(\text{I})$ chains and an unaided triple-helix, heterotrimer assembly from the C- to N-terminus in a zipper-like fashion (Olsen, 1991). To understand the involvement of colligin during this assembly process, we have developed an *in vitro* assay to study the interaction of colligin with procollagen I. One question arising is whether colligin interacts with the separate procollagen chains as well as the mature protein. Also, colligin is glycosylated and phosphorylated *in vivo*, but whether these modifications serve a regulatory function and change the binding properties of the protein is unknown. One approach to questions of this nature involves production and characterization of unmodified protein in a prokaryotic system. We therefore attempted to overexpress and isolate the unmodified colligin from *Escherichia coli* and compare it with native colligin isolated from rat skeletal myoblasts. In the present paper we describe some properties of colligin produced in myoblasts and *E. coli* and show that colligin can bind to isolated $\text{pro}\alpha_1(\text{I})$ and $\text{pro}\alpha_2(\text{I})$ chains of procollagen I as well as to collagen IV heterotrimers *in vitro*.

Abbreviations used: DSP, dithiobis(succinyl propionate); ER, endoplasmic reticulum; IPTG, isopropyl β -D-thiogalactoside; PDI, protein disulphide-isomerase; PMSF, phenylmethanesulphonyl fluoride; serpin, serine-proteinase inhibitor.

* To whom correspondence should be sent.

MATERIALS AND METHODS

Materials

Restriction enzymes and DNA-modifying enzymes were purchased from Pharmacia or Promega. Isopropyl β -D-thiogalactoside (IPTG), *o*-phenylenediamine and poly-L-lysine were obtained from Sigma. 125 I-labelled rabbit anti-mouse IgG from NEN was used for antibody detection in Western-blotting procedures. Anti-colligin monoclonal antibody was produced as described previously (Cates et al., 1987b). A full-length colligin cDNA, pEF4, was constructed from rat colligin cDNA fragments p1P1 and pE52 as described previously (Clarke et al., 1991). Single-stranded DNA sequencing was performed with Sequenase Version 2.0 (USB), using the manufacturer's protocol. Gene Clean II was purchased from Bio 101 Inc. (La Jolla, CA, U.S.A.). Immobilion transfer membrane was purchased from Millipore. [3 H]Proline and the scintillator EN 3 HANCE were purchased from du Pont (NEN Research Products).

Cell culture

The rat skeletal myoblast cell line L6 was grown in α -minimum essential medium supplemented with 50 μ g/ml gentamycin, 16 mM glucose and 10% horse serum as described previously (Cates et al., 1987b). For the growth of primary human skin fibroblasts (cell line 449, kindly donated by Dr. T. Rupar, University of Western Ontario), 20% fetal-calf serum replaced horse serum. Cells were plated at an initial density of 3.5×10^5 /100 mm plate and were grown to confluence for 72 h at 37 °C.

Labelling and cell extracts

Subconfluent cultures were rinsed with growth medium lacking methionine and then labelled for 3 h at 37 °C with 0.1 mCi/ml of [35 S]methionine. At the end of this period, cultures were rinsed twice with cold 0.15 M PBS, pH 7.5. Cells were lysed by adding 4 ml of lysis buffer [20 mM Tris/0.15 M NaCl/1% Triton X-100/2 μ g/ml leupeptin/0.5 mM phenylmethanesulphonyl fluoride (PMSF), pH 7.5] to the monolayers, and incubating on ice for 20 min. The cell extract was centrifuged and the supernatant used for binding or other studies.

Bacterial strains and plasmids

E. coli RZ1034 (*dut*⁻, *ung*⁻), used for generation of single-stranded uracil-containing templates, was obtained from Dr. Michael Smith, University of British Columbia. Plasmid pGEM3zf+ was obtained from Promega, and the prokaryote expression vector pTrc99A was obtained from Pharmacia. JM101 (*dut*⁺, *ung*⁺) strain was obtained from Dr. G. Chaconas, University of Western Ontario. Helper phage R408 was from Stratagene.

Expression plasmid construction

An expression vector (pTrc46K) was designed to express the mature form of colligin in *E. coli*. The cDNA sequence for the signal peptide of colligin was deleted by site-directed mutagenesis. The cDNA coding for mature colligin was then ligated into the expression vector pTrc99A such that the ATG start codon was

situated at an optimal distance from the ribosome-binding site. Transcription in this vector is driven by the strong inducible bacterial promoter *Trc*, which is a hybrid between the *Trp* and *lac* promoters (Amann and Brosius, 1985). Site-directed mutagenesis was performed using single-stranded uracil-containing templates as described previously (Kunkel et al., 1987; Lorimer et al., 1992). A 30-base oligonucleotide:

5'-CACCTCCCAGCCATGGCCGAGGTGAAGAAA-3'

was used to delete the cDNA sequence coding for the colligin signal peptide (Clarke and Sanwal, 1992) as follows. Single-stranded uracil-containing template was prepared from RZ1034 transformed with pEF4, using the helper phage R408. A 0.5 μ g portion of template (0.3 pmol) was mixed with 3 pmol of phosphorylated oligonucleotide in a final volume of 10 μ l of a buffer containing 40 mM Tris, pH 7.5, 20 mM MgCl₂ and 50 mM NaCl. This mixture was heated to 65 °C for 3 min and allowed to cool slowly to room temperature. A 5.6 μ l volume of water, 0.7 μ l of 30 mM ATP, 1.0 μ l of 4 mM each dNTP, 1.0 μ l of 0.1 M dithiothreitol, 1 μ l (3.0 units) of T4 DNA ligase and 0.7 μ l of Sequenase (1 unit, diluted 1:8 according to the manufacturer's recommendations) were added and the mixture was incubated at 37 °C for 20 min. The reaction was stopped with the addition of 0.5 μ l of 0.5 M EDTA, and 2 μ l were used to transform competent *E. coli* JM101.

Deletion of the signal sequence creates an *Nco*I restriction site around the ATG start codon. Colonies from the transformation were initially screened by restriction digestion with *Nco*I and *Bam*HI. Those containing the new restriction site were further analysed by DNA sequencing. The 1300 bp *Nco*I/*Bam*HI fragment was purified using agarose-gel electrophoresis and Gene Clean II. This fragment was ligated into the expression vector pTrc99A, which had been linearized with the same restriction enzymes and similarly purified. *E. coli* RR1 was transfected with the ligation mixture, and clones containing the insert were identified by restriction digests.

Isolation of procollagen and colligin

To isolate procollagen I, human skin fibroblasts were grown in the presence of [2,3,4,5- 3 H]proline (25 μ Ci/ml) for 48 h. Radioactively labelled procollagen I was isolated from the medium as described by Uitto et al. (1980). The pro α_1 (I) and pro α_2 (I) chains were separated by a chromatographic procedure (Uitto et al., 1980), and purity of the isolated chains established by SDS/PAGE. The concentration of procollagen was measured by hydroxyproline assays (Kivirikko et al., 1967), assuming that procollagen I contains 10.11% (w/w) hydroxyproline by weight for procollagen I (Fiedler-Nagy et al., 1982). 35 S-labelled or unlabelled colligin was isolated from L6 rat myoblasts by the procedure of Nandan et al., 1990a,b,c). Anti-GRP78 antibody was kindly donated by Dr. Linda Hendershot, St. Jude Children's Hospital, Memphis, TN, U.S.A. Anti-(type I collagen) antibody was obtained from Seva laboratories, Chemicon, Temecula, Canada. Anti-[protein disulphide-isomerase (PDI)] antibodies were purchased from Dimension laboratories, Toronto, Canada. Protein was measured by a modification of the Lowry procedure (Peterson, 1977).

Purification of recombinant colligin

Routinely, cultures were set up for overnight growth in Luria-Bertani medium with 100 μ g/ml carbenicillin and 1 mM IPTG at 37 °C. After 18 h of growth, bacterial pellets were isolated by centrifugation at 3400 g for 10 min at 4 °C, washed twice with

PBS and stored frozen at 70 °C, if not used immediately. When needed, the pellets were resuspended on ice in lysis buffer [50 mM Tris/HCl (pH 8.0)/1 mM EDTA/100 mM NaCl/10 mM β -mercaptoethanol/0.5 mM PMSF/2 μ g/ml leupeptin], and the cells were lysed by the addition of SDS stock solution to a final concentration of 1% and heating in a boiling-water bath for 10 min. The lysate was cooled on ice, and then subjected to sonication to shear DNA. Insoluble material was removed by centrifugation at 140 000 *g* for 10 min at 4 °C. The supernatant was harvested and diluted in binding buffer [10 mM Mops (pH 7.6)/10 mM NaCl/1 mM EDTA/5 mM β -mercaptoethanol/0.5 mM PMSF/1 g/ml leupeptin and 0.1% Triton X-100] to a final SDS concentration of 0.1%.

A column (5 cm $\times$ 1.5 cm) of Sepharose 4B coupled to anti-colligin antibody (Cates et al., 1984; Cates et al., 1987b) was pre-cycled with a bacterial lysate from pTrc99A-transformed cells before use in purification. The beads were recovered, placed in a 50 ml tube, and equilibrated with binding buffer. Lysate from the pTrc46K-transformed cells was incubated with the beads on a rotator for 2 h at 4 °C. Sepharose with bound colligin was recovered by centrifugation at 800 *g* for 5 min at 4 °C, washed twice with binding buffer without detergents, and again packed in the column. Colligin was eluted from the column with 50 mM diethylamine, pH 11.9, and adjusted to pH 7.6 with 2 M Tris/HCl. The eluate was dialysed overnight against 2 litres of binding buffer without detergents or β -mercaptoethanol at 4 °C, followed by concentration in an Amicon filtration unit over a YM 10 filter. Routinely, 4 mg of purified recombinant colligin was recovered from a 1 g (wet wt.) *E. coli* pellet.

Sephacryl S-300 gel filtration

A gel-filtration column (1.5 cm $\times$ 48 cm) was prepared with Sephacryl S-300 (Pharmacia) beads. The column was run with S-300 buffer [10 mM Mops (pH 7.6)/50 mM NaCl/1 mM EDTA]. Standard gel-filtration molecular-mass markers (Sigma) were used for calibration.

To determine the status of colligin in L6 myoblasts, cells were grown in 150 mm tissue-culture plates and the cell lysate was prepared in lysis buffer [20 mM Tris/HCl (pH 7.5)/0.15 M NaCl/0.1% Triton X-100], containing a proteinase-inhibitor cocktail and 10 mM *N*-ethylmaleimide. The cell lysate was centrifuged at 435 680 *g* for 15 min, the supernatant loaded on to the Sephacryl S-300 gel-filtration column, and 1 ml fractions were collected. The purified recombinant colligin sample (2.5 mg) was treated with Triton X-100 (final concn. 0.1%) to disrupt the insoluble aggregates and centrifuged at 435 000 *g* for 15 min at 4 °C. The supernatant was harvested, loaded on to the column and eluted with S-300 buffer without β -mercaptoethanol. The presence of colligin in the fractions was detected by Western-blot analysis.

Cross-linking and immunoprecipitation

In vivo cross-linking of colligin to proteins was performed as described by Roth and Pierce (1987). Briefly, L6 myoblasts cultured in 35 mm plastic dishes were labelled with [³⁵S]methionine and treated with 0.15 mM dithiobis(succinyl propionate) (DSP) in PBS on ice for 30 min. The cells were rinsed with PBS containing 2 mM glycine, lysed in the lysis buffer, and the supernatant used for immunoprecipitation. For this purpose, 100 μ l of the lysate was incubated with 25 μ l of CNBr-Sephacryl beads (Pharmacia Fine Chemicals) coupled to anti-colligin antibody for 1 h at 4 °C. The precipitated immune complexes were washed twice with 0.5 ml of 20 mM Tris/HCl (pH 7.5)/

0.6 M NaCl and once with 5 mM Hepes buffer, pH 7.5. The samples were resuspended in sample buffer (Laemmli, 1970) containing β -mercaptoethanol, boiled for 3 min and analysed by SDS/PAGE. The gels were exposed on Kodak X-ray film after treatment with EN³HANCE.

Solid-phase binding assays

Radioactivity assay

Small immobilon discs [Millipore; poly(vinylidene difluoride)-type; pore size 0.45 μ m] were cut to fit the wells of a 96-well polypropylene plate. These were immersed in methanol and then washed and soaked for 20 min in distilled water. The discs in wells of a 96-well plate were incubated with 40 μ l/per disc of gelatin or procollagen I (100 μ g/ml) for 2 h at room temperature. After removal of the unbound protein, discs were blocked with 4% casein in TBS [20 mM Tris/HCl (pH 7.5)/0.15 M NaCl], then incubated for 2 h at 4 °C in an angled rotator with 50 μ l of ³⁵S-methionine-labelled cell lysate with or without competing protein. The discs were then removed to tubes containing 0.5 ml of cold lysis buffer and rinsed two times with the same buffer, then with 0.5 ml of lysis buffer containing 0.6 M NaCl for 5 min. Finally, the discs were rinsed rapidly with 5 mM Hepes buffer, pH 7.5, and bound proteins eluted by boiling in sample buffer. The samples were run on SDS/10% polyacrylamide gels. The gels were stained, destained, treated with EN³HANCE (NEN Products) and autoradiographed. The signals were quantified by densitometric scanning (LKB ultrascan XL laser densitometer). Using highly purified labelled colligin we ascertained that recoveries of the bound protein using the aforementioned procedure were at least 90%. Film responses were in the linear range when quantification from autoradiograms was performed.

E.l.i.s.a. assay

A quantitative assessment of the interaction of colligin with procollagen I, pro α_1 (I) and pro α_2 (I) was performed using an e.l.i.s.a.-type assay (Hedbom and Heinegård, 1989). All steps were carried out at room temperature. Polystyrene microtitre wells (Corning e.l.i.s.a. plates) were precoated with 100 μ l of poly-L-lysine (10 μ g/ml) for 30 min. Procollagen I (10 μ g/ml in 0.05 M Na₂CO₃/NaHCO₃, pH 9.7) was applied in 0.1 ml volumes to the wells, incubated for 3 h and then washed three times with buffer A [0.02 M Tris/HCl (pH 7.4)/0.15 M NaCl/0.05% Tween 20]. The wells were then blocked for 2 h with 0.2 ml of BSA (10 mg/ml) and different concentrations of colligin applied. After 4 h, wells were washed three times with buffer A, and bound colligin was quantified by an e.l.i.s.a. assay using anti-colligin antibody and rabbit anti-mouse IgG-horseradish peroxidase conjugate as described previously (Vaillancourt and Cates, 1991). The absorbance readings at 490 nm, after colour development with *o*-phenylenediamine reagent, were corrected for non-specific binding. Colligin binding to procollagen was analysed by using an iterative fit to the equation:

$$A = A_{\max} \cdot (C/K_d + C)$$

where *A* is the measured absorbance, *A*_{max} is the absorbance at saturation, *K*_d is the apparent dissociation constant, and *C* is the concentration of free ligand in solution. This analysis assumes that the surface-associated procollagen represents a single class of non-interacting binding sites and that the free concentration of colligin and the total (added) concentration of colligin are essentially the same.

RESULTS

Binding of myoblast colligin to procollagen I

Procollagen I was isolated from human skin fibroblast cells in order to study colligin interaction with purified procollagen I chains. Also, $\text{pro}\alpha_1(\text{I})$ and $\text{pro}\alpha_2(\text{I})$ chains were purified and separated for use in binding assays (Figure 1a). Freshly purified colligin binds to procollagen I in an e.l.i.s.a.-based binding assay (Figure 1b) with an apparent K_d of 2.5×10^{-8} M. Isolated $\text{pro}\alpha_1(\text{I})$ and $\text{pro}\alpha_2(\text{I})$ chains are able to compete with procollagen heterotrimer for binding to colligin (Figure 1b). The reduced binding of colligin was not due to the masking of colligin binding sites on procollagen I by the isolated chains. We ascertained that $[\text{H}]\text{pro}\alpha_1(\text{I})$ or $[\text{H}]\text{pro}\alpha_2(\text{I})$ do not themselves bind to procollagen-treated wells. Since $\text{pro}\alpha_2(\text{I})$ was a more effective inhibitor of colligin binding to procollagen I, $\text{pro}\alpha_2(\text{I})$ chains must bind with a higher affinity to colligin than the $\text{pro}\alpha_1(\text{I})$ chains. To estimate the stoichiometry of the colligin–procollagen I interaction, we determined the amount of procollagen coated to microtitre wells by using labelled procollagen. Under our ex-

Figure 1 Binding of colligin to procollagen I in the presence and absence of isolated $\text{pro}\alpha_1(\text{I})$ and $\text{pro}\alpha_2(\text{I})$ chains

(a) Isolation of $\text{pro}\alpha_1(\text{I})$ and $\text{pro}\alpha_2(\text{I})$ chains. The procollagen I (lane 2), $\text{pro}\alpha_1(\text{I})$ (lane 3) and $\text{pro}\alpha_2(\text{I})$ (lane 4) were run on SDS/6%-polyacrylamide gels and the gel was stained with Coomassie Blue. Lane 1, molecular-mass standards. (b) e.l.i.s.a.-type binding assay. The binding of highly purified colligin to procollagen-coated wells was assessed by e.l.i.s.a. assay and data analysed as described in the text. The apparent dissociation constant of colligin calculated from fits to the binding equation is 25 nM in the absence of the isolated chains (+). Equal amounts (1.0 μM) of the isolated $\text{pro}\alpha_1(\text{I})$ (Δ) and $\text{pro}\alpha_2(\text{I})$ ($\circ$) chains are used as competitors in the binding assay. Each data point in the Figure represents the average of four separate readings. The variation between individual readings never exceeded $\pm 10\%$.

Figure 2 Analysis of procollagen binding protein in crude extracts

Radiolabelled proteins bound to procollagen I discs were analysed by SDS/PAGE and autoradiography. Molecular-mass markers are indicated on the left side of the Figure. (a) The 46 kDa procollagen-binding protein is colligin. Lane 1, proteins from labelled L6 cells; lane 2, proteins from colligin-depleted L6 cell extract; lane 3, after low-pH release (pH 6.0); lane 4, after ATP (1 mM) release. (b) Immunoprecipitation of procollagen-bound protein; lane 1, immunoprecipitation of labelled L6 cell extract with anti-colligin antibody; lane 2, procollagen I-binding protein from solid-phase binding assay. (c) Binding protein from heat-shocked cells. Lane 1, L6 cells cultured at 37 °C; lane 2, L6 cells cultured at 42 °C. Equal amounts of protein were run in both the lanes. The arrow marks position of colligin.

perimental conditions an average of 0.13 μg of procollagen was bound to each well. A maximum of 0.046 μg of colligin was bound to the immobilized procollagen. Using these values and molecular masses of 415000 Da for procollagen I and 46000 Da for colligin, it can be calculated that approx. 3 mol of colligin bind/mol of procollagen.

Interaction of native colligin with procollagen

It is known that some serpins are activated by interaction with other protein molecules. Vitronectin, for instance, enhances the inhibition of plasminogen activator by interacting with plasminogen-activator inhibitor (PAI-1), a secreted serpin in some cells (Ehrlich et al., 1990). In order to find out whether the binding activity of colligin is modified by unknown proteins in the cell, we decided to examine the binding ability and specificity of unpurified colligin in crude extracts. Incubation of extracts of $[\text{S}]\text{methionine}$ -labelled L6 myoblasts with nitrocellulose discs coated with procollagen I resulted in specific binding of labelled colligin. At least 80 % of the bound radioactivity was found to be due to colligin. As expected, colligin could be selectively eluted from the procollagen-coated discs by low-pH treatment (Figure 2a, lane 3), but not by ATP (Figure 2a, lane 4), conditions which we (Nandan et al., 1990a,b,c) and others (Nagata et al., 1986) have previously shown to be characteristic of colligin binding to gelatin. Insignificant amounts of radioactivity were bound to procollagen discs when extracts depleted of colligin (by treatment of L6 extracts with anti-colligin antibody) were used in the binding assay (Figure 2a, lane 2). The mobility of bound colligin during SDS/PAGE was also the same as colligin immunoprecipitated from $[\text{S}]\text{methionine}$ -labelled L6 extracts (Figure 2b). When L6 cells were heat-shocked prior to $[\text{S}]\text{methionine}$ labelling, the amount of colligin bound to the discs from cell extracts increased more than 3-fold (Figure 2c). It had previously been demonstrated (Nagata et al., 1986; Cates et al., 1987b; Nandan et al., 1990b) that heat shock increases the level of colligin. These

Figure 3 Interaction of colligin with pro α_1 (I) and pro α_2 (I) chains

Nitrocellulose discs coated with procollagen I were incubated with [35 S]methionine-labelled L6 cell extract in the presence or absence of various concentrations of either pro α_1 (I) (lanes 1–5) or pro α_2 (I) (lanes 6–10): lanes 1 and 6, none; lanes 2 and 7, 0.75 μ M; lanes 3 and 8, 1.50 μ M; lanes 4 and 9, 2.25 μ M; lanes 5 and 10, 3.0 μ M. Bound colligin was detected by running SDS/10%-polyacrylamide gels and exposing the gels to X-ray films (a). Results were analysed by densitometric scanning of autoradiographic signals. Normalized values were used to plot the histogram (b).

Figure 4 Comparison of interaction of colligin with various types of collagen

Procollagen I-coated nitrocellulose discs were incubated with [35 S]methionine-labelled L6 cell extract with various concentrations of collagen I, collagen III, collagen IV and gelatin. Results were analysed by densitometric scanning of autoradiographic signals. Normalized values were used to plot the histogram.

experiments make it clear that the major procollagen-binding protein in L6 cells is colligin.

Having ascertained that crude L6 extracts could be used to examine the parameters of colligin binding to procollagen, we used increasing amounts of L6 extracts to measure the amount of colligin binding to a fixed amount of procollagen I on nitrocellulose discs. In agreement with the e.l.i.s.a. assays, we found approx. 3 mol of colligin binding/mol of procollagen I at saturation. The apparent K_d obtained from the binding plots was about 10^{-8} M, a value not significantly different than that

Figure 5 Purification of recombinant colligin

Colligin was immunopurified by using anti-colligin monoclonal-antibody column from the cell lysate of RR1 strain of *E. coli* containing the pTrc46K vector. (a) Silver staining of proteins separated by SDS/PAGE. Lane 1, molecular-mass markers; lane 2, *E. coli* strain RR1 containing vector pTrc99A incubated with 1 mM IPTG, the whole-cell lysate (10 μ g) loaded on to the SDS/polyacrylamide gel; lane 3, *E. coli* (containing vector pTrc46K) cell extract (10 μ g) without IPTG induction; lane 4, cell lysate (10 μ g) from *E. coli* containing pTrc46K and induced with 1 mM IPTG; lane 5, 2 μ g of purified recombinant colligin. (b) Western-blot analysis using monoclonal anti-colligin antibody. Lane 1, 30 μ g of the same sample as in lane 2 of (a); lane 2, 30 μ g of the same sample as in lane 3 of (a); lane 3, 30 μ g of the same sample as in lane 4 of (a); lane 4, 10 μ g of the same sample as in lane 5 of the (a). The left-hand-side arrows indicate the positions of the molecular-mass markers; the arrow at the right indicates the position of recombinant (R') colligin.

obtained when using purified colligin. In the disc-binding assay, isolated pro α_1 (I) and pro α_2 (I) chains and other collagens are also able to compete with procollagen. As shown in Figure 3, pro α_2 (I) chains are better competitors than pro α_1 (I) chains. Competition experiments also show that collagen I and collagen IV bind to colligin with similar affinity, while collagen III does not bind to colligin significantly (Figure 4).

Recombinant colligin and its binding to procollagen

As mentioned above, colligin isolated from animal tissues is both glycosylated (Cates et al., 1984) and phosphorylated (Nagata and Yamada, 1986; Cates et al., 1987a). It was conceivable that the binding affinity of colligin to procollagen I is altered by these modifications. Accordingly, we made attempts to produce an unglycosylated and unphosphorylated form of colligin in *E. coli*. Analysis of *E. coli* RR1 transformed with pTrc46K (Figure 5a) showed that a protein of the expected molecular mass was expressed at high levels in an IPTG-inducible manner. Western blotting with a specific monoclonal antibody (Cates et al., 1987b) confirmed that this protein was colligin (Figure 5b). In the presence of 1 mM IPTG, colligin was expressed at a level of about 1% of total *E. coli* protein.

The scheme for purifying recombinant colligin took into consideration several features of the overexpressed protein which were determined in preliminary experiments: (1) recombinant colligin was present in a mixture of soluble and insoluble forms when cells were lysed in buffers without detergents or chaotropes; (2) the soluble protein was very susceptible to proteolysis in crude extracts; we therefore chose to use denaturing conditions (1% SDS with boiling) to solubilize the protein and inactivate *E. coli* proteinases. The detergent was then diluted with buffer so that a monoclonal-antibody-affinity column could be used to

Figure 6 Comparison of L6 and recombinant colligin binding to procollagen

Various concentrations of L6 and recombinant colligin were incubated with precoated procollagen I in 96-well polystyrene plates. The binding of colligin to procollagen was measured by a modified e.l.i.s.a. assay using anti-colligin antibody and rabbit anti-mouse IgG-horseradish peroxidase conjugate. Each observation was a mean of quadruplicates of each of the concentrations of colligin used.

purify recombinant colligin. The one-step antibody-affinity column procedure developed here is a modification of a method we have described previously for the purification of colligin from tissue-culture cells (Cates et al., 1987b; Nandan et al., 1990a).

Recombinant colligin isolated by this method appeared as a single band with molecular mass of about 41 kDa in Coomassie Blue- or silver-stained SDS/polyacrylamide gels (Figure 5a). However a very minor band also appeared at about molecular mass of 42 kDa. Both of the bands cross-reacted with anti-colligin antibody in Western blots (Figure 5b), indicating that both were forms of colligin differing in molecular mass. This was further substantiated by amino acid sequencing. The sequence of the first ten N-terminal amino acids in both cases was:

Ala-Glu-Val-Lys-Lys-Pro-Val-Glu-Ala-Ala

This sequence is identical with the sequence of rat and human colligin we published previously (Clarke et al., 1991; Clarke and Sanwal, 1992). The N-terminal methionine expected for proteins produced in *E. coli* was obviously removed (Ben-Bassat and Bauer, 1987). The origin of the second form of recombinant colligin is uncertain. Although we are unable to rule out proteolysis, it is possible that the 42 kDa form results from a read-through past the first stop codon. Termination at the second UAG of the cDNA would add 1.3 kDa of peptide to the C-terminus.

Recombinant colligin isolated by our procedure was approx. 98% pure, as judged by scanning densitometry of silver-stained SDS/polyacrylamide gels (Figure 5). Yields were typically about 4 mg of colligin/g wet weight of pelleted *E. coli* cells. The molecular masses of 41 and 42 kDa for the purified protein correspond to the expected molecular-mass range for the unglycosylated protein (calculated molecular mass 42856 Da). We assayed the ability of purified recombinant colligin to bind procollagen I using the e.l.i.s.a.-type assay as described in the Materials and methods section. The results (Figure 6) show that colligin purified from L6 myoblasts and recombinant colligin have similar affinities for procollagen I, binding with a K_d of about 25 nM. This shows that recombinant colligin is functional and implies that neither the glycosylation of colligin (Cates et al., 1984; Kurkinen et al., 1984), nor its modification by phos-

Figure 7 Polymerization state of colligin

(a) Colligin under reducing and non-reducing conditions. Colligin was purified from L6 cells and run on SDS/8.5%-polyacrylamide gels under reducing and non-reducing conditions, and colligin was analysed by Western-blot analysis using anti-colligin antibody. Lane 1, 5 μ g of colligin with β -mercaptoethanol; lane 2, 5 μ g of colligin without β -mercaptoethanol. (b) Column chromatography of myoblast colligin. L6 myoblast cell extract was loaded on to a calibrated Sepharose S-300 gel-filtration column, and fractions were collected as described in text. Samples (50 μ l) of each fraction was electrophoresed on SDS/polyacrylamide gels and Western-blotted using anti-colligin antibody. The autoradiograms were scanned, and the values were used to plot the elution profile of the various forms of colligin. The numbers on top of each peak represent the fraction number. Arrows indicate the position of each marker protein and various forms of colligin. Abbreviations: ADH, alcohol dehydrogenase; CANH, carbonic anhydrase; Cyt. c, cytochrome c; 'Monomer', 'Dimer' and 'Complex' refer to the various forms of colligin.

phorylation (Cates et al., 1987a; Nagata and Yamada, 1986), are required for its procollagen-binding function.

Polymerization state of colligin in solution

In preliminary experiments we noted that myoblast colligin exhibited two bands when electrophoresed in SDS/polyacrylamide gels without β -mercaptoethanol (Figure 7a). To examine the aggregation state of colligin in solution, we performed gel-filtration chromatography. Recombinant colligin gave two peaks (in the absence of β -mercaptoethanol) of estimated molecular masses 42000 and 90000 Da, suggesting the existence of both monomeric and dimeric forms in solution. For comparison, we also analysed colligin in crude L6 myoblast extracts by gel filtration. Again we observed two peaks, of estimated molecular masses 45000 and 96000 Da. In L6 cell extract, a third high-molecular-mass peak was also observed that was eluted very early (Figure 7b). Immunoprecipitation from this fraction with anti-colligin antibody revealed that the complex contained procollagen I and a glucose-regulated protein (GRP78) (results not

shown). It is well known that GRP78 participates in the correct folding of several multimeric proteins in the ER (Hendershot, 1990; Gething and Sambrook, 1992). In *in vivo* cross-linking experiments (results not shown) we could also demonstrate that procollagen I is associated with GRP78, PDI and an unidentified 68 kDa protein.

DISCUSSION

Our studies of colligin binding specificity (in both crude and purified fractions) show selective interactions with certain types and forms of collagen. We have examined procollagen I as well as its separated chains and find that colligin interacts with $\text{pro}\alpha_1(\text{I})$, $\text{pro}\alpha_2(\text{I})$ as well as with procollagen I. This observation is important, because procollagen rather than collagen is found in the ER. Previous work (Vaillancourt and Cates, 1991) has been limited to the qualitative binding of mature collagens with colligin. However, the binding of colligin to cleaved, mature collagen indicates that at least one of the sites that colligin recognizes in procollagen lies in the central helical region. Interaction of colligin with both the separated chains of procollagen implies binding also to non-triple helical areas, since the $\text{pro}\alpha_2(\text{I})$ chain does not form homotrimers. These characteristics are compatible with a variety of possible roles of colligin at various steps of procollagen I folding process.

Binding studies also showed that colligin binds specifically to procollagen I and collagen type IV, but not to collagen type III. Interestingly, colligin levels in cells parallel those of collagen I and IV under several circumstances (Clarke et al., 1991). Further, during differentiation of myoblasts, synthesis and secretion of collagen I decreases and is replaced by collagen III (Nusgens et al., 1986), but the levels of colligin decrease pointing to a connection with types I and IV collagen, but not III. Recent experiments using *in situ* hybridization also showed that colligin is localized in tissues expressing procollagen I and not procollagen III (Shroff et al., 1993). Thus colligin is not essential for, or involved with, all types of collagen.

Since some serpins are known to be regulated by interaction with specific cellular proteins, we examined the binding parameters of colligin in crude cell extracts in the hope that any interaction with unknown activators will be revealed by a change in the binding parameters compared with those of purified colligin. Since the stoichiometry of binding of colligin to procollagen and K_d values in crude and purified preparations did not alter significantly, it is likely that the binding activity of colligin is not altered by other proteins in the cell. This, however, remains to be proven.

Preparation of a recombinant protein from *E. coli* has many advantages, including greater yields and cheaper starting materials. Expression of colligin indeed gave large quantities, but the protein was subject to proteolysis in the lysate. Immediate denaturation with SDS solved this problem, and an antibody column allowed rapid purification. The apparent ready renaturation of the protein may be a property of many serpins, as shown by the 're-activation' of plasminogen-activator inhibitor-1 (Hekman and Loskutoff, 1985) following denaturation. In any case the recombinant colligin proved to be active with kinetic parameters virtually identical with those of the natural protein. It is well known that *E. coli* lacks the machinery for glycosylation and phosphorylation of heterologous proteins, and colligin isolated from the bacterial cells, unlike normal colligin, is unglycosylated and unphosphorylated. Since the binding activities of the recombinant protein are similar to the natural protein, it is clear that post-translational modifications do not play any essential role in the binding of colligin to procollagen I.

Both cross-linking in living cells (see also Nakai et al., 1992) and the presence of a high-molecular-mass complex in cell extracts indicate the existence of a multicomponent procollagen complex in the ER containing (at least) PDI, GRP78 and colligin. The latter three proteins probably bind to procollagen I independently, without interacting with each other (Nandan et al., 1990a). Both PDI and GRP78 are known to participate in the correct folding of other oligomeric secretory proteins (Gething and Sambrook, 1992; Bardwell and Beckwith, 1993), consistent with a similar role for colligin. Interaction of colligin with the mature collagen distinguishes it from the others, however, and may indicate additional or alternative functions. [In preliminary experiments we have obtained evidence that colligin binds triple-helical procollagen *in vivo* as well as *in vitro* (N. Jain, E. H. Ball and B. D. Sanwal, unpublished work)]. It may remain associated at later stages of procollagen assembly, perhaps acting in the ER-to-Golgi transfer as well.

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