Purification and reconstitution of a collagen-binding heat-shock glycoprotein from L6 myoblasts

John P. VAILLANCOURT and George A. CATES*

Department of Biochemistry, Queen's University, Kingston, Ontario, Canada K7L 3N6

A major collagen-binding heat-shock glycoprotein from L6 myoblasts, designated gp46, was purified by gelatin-agarose chromatography and ion-exchange chromatography. Purified gp46 was functionally active, as shown by its ability to rebind to gelatin-agarose, and was homogeneous as determined by SDS/polyacrylamide-gel electrophoresis. This is the first reported purification of myoblast gp46 in an active state. Triton X-100-soluble gp46 was found to bind preferentially to immobilized pepsin-treated type IV collagen compared with native type I collagen. gp46, reconstituted into phospholipid vesicles, retained collagen-binding activity. This activity could be destroyed by chemical modification with chloramine-T, but was decreased by only 20–30 % following treatment with iodoacetamide or *N*-ethylmaleimide. Since gp46 is a heat-shock protein, we examined the possibility that it may confer protection on type I collagen from thermal denaturation at temperatures above its normal melting temperature of 41 °C. In the presence of gp46 liposomes the apparent melting temperature of type I collagen was marginally increased to 42 °C. This change was considered to be too small to be of physiological significance. We have therefore concluded that the role of gp46 in collagen metabolism is unlikely to be related to any thermal-stabilizing function.

INTRODUCTION

The endoplasmic reticulum of rat skeletal myoblasts as well as a number of other cell types contains a major collagen-binding glycoprotein of *M*_r 46000 which has been designated gp46 (Cates *et al.*, 1984, 1987; Nandan *et al.*, 1988, 1990). This glycoprotein is a heat-shock protein that represents approx. 1 % of the total cell protein in cultured myoblasts active in collagen synthesis (Nandan *et al.*, 1990). Undifferentiated myoblasts synthesize gp46 coincident with type I collagen production, but, following differentiation into multinucleated myotubes, both synthesis of gp46 and collagen declines (Cates *et al.*, 1987). In addition, mutant myoblast lines that are defective in the ability to differentiate show a decreased constitutive expression of gp46, suggesting a correlation between gp46 expression and myogenesis, although no cause-and-effect relationship has been established (Cates *et al.*, 1984, 1987).

It is likely that gp46 is very similar to, if not identical with, the collagen-binding protein colligin, originally described by Kurkinen *et al.* (1984). Antibodies against rat myoblast gp46 cross-react with colligin, but they do not react with the collagen-binding glycoprotein of *M*_r 47000 (hsp47) from chick-embryo fibroblasts (Nagata & Yamada, 1986). The published *N*-terminal sequences of rat myoblast gp46 and chick fibroblast hsp47 have no sequence similarity (Nagata *et al.*, 1988; Nandan *et al.*, 1990); however, binding properties and cellular location would suggest a similar function. The presence of gp46 or related proteins in the endoplasmic reticulum of collagen-secreting cells suggests that gp46 may play a role in intracellular assembly, transport, or secretion of collagen. The fact that it is a heat-shock protein would also suggest that it may be involved in the recognition of abnormal or denatured forms of collagen complexes. Pelham (1986, 1989) has suggested that certain heat-shock proteins serve to recognize malformed proteins that result from cellular stress or heat shock. In addition, Hughes *et al.* (1987) have suggested that colligin may help to stabilize the collagen triple helix.

We have previously purified gp46 from rat myoblasts in a denatured form for a preliminary characterization (Nandan *et al.*, 1990). In order to be able to examine the function of gp46, we have now purified gp46 in an active state which retains its collagen-binding property. The present paper describes the purification of active gp46 and also examines the influence of purified gp46 on the thermal stability of type I collagen *in vitro*.

EXPERIMENTAL

Materials

Type I collagen was isolated from rat tail tendons as described by Chandrakasan *et al.* (1976). Pepsin treatment of bovine type I collagen was done as described by Gelman *et al.* (1979). Pepsin-treated bovine type IV collagen was obtained from Seikagaku America Inc., St. Petersburg, FL, U.S.A. Gelatin-agarose was from Sigma Chemical Co. Bio-Gel P-6DG and horseradish-peroxidase-conjugated antibodies were obtained from Bio-Rad Laboratories. CM-Sepharose was from Pharmacia Canada Inc. Tissue-culture media were from Gibco/BRL Canada, and horse serum was from Bocknek Ltd., Rexdale, Ontario, Canada. Polystyrene microtitre plates were Linbro type EIA II Plus from Flow Laboratories Inc. ¹²⁵I was from Amersham Canada Ltd. Chemicals were of analytical grade.

Cell culture

A highly myogenic subclone of the rat myoblast line, L6, first isolated by Yaffe (1968) and subcloned in the laboratory of Dr. B. D. Sanwal, University of Western Ontario, was used. Cells were cultured in α -modified minimum essential medium supplemented with 50 mg of gentamicin/l, 16 mM-glucose and 100 ml of horse serum/l. Cells were plated at an initial density of 1.5×10^6 cells per 150 mm plate, and were grown to confluence for 72 h at 37 °C. The cells were then heat-shocked at 42 °C for 24 h in order to increase the cell content of gp46.

Abbreviations used: PBS, phosphate-buffered saline; DTT, dithiothreitol; PMSF, phenylmethanesulphonyl fluoride; PepColl I, pepsin-treated type I collagen; TBS, Tris-buffered saline.

* To whom correspondence should be addressed.

Gelatin-agarose affinity chromatography

Cells from 30 150 mm culture plates of confluent heat-shocked L6 cells were used for a typical isolation of gp46. All steps of the isolation were done at 4 °C unless otherwise indicated. Monolayers were washed three times with phosphate-buffered saline (PBS; 0.14 M-NaCl/2.7 mM-KCl/8.1 mM-Na₂HPO₄/7.3 mM-KH₂PO₄, pH 7.4) and scraped from the plates in PBS supplemented with 0.5 mM-phenylmethanesulphonyl fluoride (PMSF) and 2 mg of leupeptin/l. Cells were collected by centrifugation at 2000 *g* for 10 min. The cell pellet was homogenized in 60 ml of homogenizing buffer [5 mM-Hepes/0.15 M-NaCl/Triton X-100 (10 ml/l)/1 mM-dithiothreitol (DTT)/0.5 mM-PMSF/leupeptin (2 mg/l), pH 7.5] by using 20 strokes of a Dounce homogenizer (tight pestle). The cell extract was centrifuged at 17500 *g* for 15 min, and the supernatant was kept for further isolation.

The Triton-soluble extract was added to 2 ml (packed volume) of gelatin-agarose and mixed batchwise for 1 h. The slurry was transferred to a column and washed with 40 ml of homogenizing buffer, followed by 20 ml of the same buffer supplemented with 1 M-NaCl. Finally the column was washed with 1 mM-Hepes/0.15 M-NaCl/Triton X-100 (0.5 ml/l)/1 mM-DTT/0.5 mM-PMSF/leupeptin (2 mg/l), pH 7.5, and then drained of excess buffer. Bound gp46 was eluted with pH 5.5 buffer by incubating the gelatin-agarose batchwise with 2.5 ml of 10 mM-sodium acetate/0.15 M-NaCl/Triton X-100 (0.5 ml/l)/1 mM-DTT/leupeptin (2 mg/l), pH 5.5, for 10 min. The eluate was collected and neutralized by the immediate addition of 50 μ l of 2 M-Hepes, pH 7.5.

CM-Sepharose chromatography

The gelatin-agarose eluate was applied to a Bio-Gel P-6DG gel-filtration column (1 cm $\times$ 17 cm) equilibrated with 10 mM-sodium phosphate/Triton X-100 (0.5 ml/l)/1 mM-DTT, pH 7.5 (phosphate buffer) in order to exchange buffers. The protein fraction eluted from this column was then mixed batchwise for 1 h with 1 ml of CM-Sepharose pre-equilibrated with phosphate buffer. The CM-Sepharose was washed with 10 ml of phosphate buffer, and the gp46 was then eluted with 2.0 ml of phosphate buffer containing 0.5 M-NaCl. The gp46 eluate was then dialysed for a minimum of 4 h against the appropriate buffer before further analysis.

Solid-phase binding assay

Various collagen types were diluted to 10 μ g/ml in Tris-buffered saline [TBS; 20 mM-Tris/0.15 M-NaCl/NaN₃ (0.2 g/l), pH 7.5], and 50 μ l samples were placed into appropriate wells of a 96-well microtitre plate. After overnight incubation at 4 °C, the wells were emptied and blocked with sodium casein (40 g/l) in TBS for 1 h at 37 °C. The wells were washed three times with 5 mM-Hepes/0.15 M-NaCl/Triton X-100 (0.5 ml/l), pH 7.5 (Hepes buffer) and the plates stored in this buffer until ready for use.

In order to demonstrate that equal amounts of type I and type IV collagen were bound to the wells of the microtitre plate, various amounts of ¹²⁵I-labelled collagens were used in one experiment. A 200 μ g portion of either native type I collagen or pepsin-treated type IV collagen was iodinated by using 500 μ Ci of ¹²⁵I and 20 μ g of Iodogen (Pierce Chemical Co.) as described by Fraker & Speck (1978). Specific radioactivities were determined on the basis of trichloroacetic acid-precipitable counts and protein concentration as determined from the absorbance at 280 nm. Incubation with microtitre wells was done as for unlabelled collagen and the radioactivity bound was determined by counting the entire well in a γ -scintillation counter.

gp46 was diluted into Hepes buffer containing 1 g of haemoglobin/l as protein carrier, and 100 μ l of various dilutions were added to the wells of the microtitre plate. Incubations were for 1 h at 25 °C, followed by three washes with Hepes buffer. In order to avoid dissociation of the bound gp46 during subsequent antibody incubations, the gp46-collagen complex was treated with 150 μ l of glutaraldehyde (2.5 g/l) for 15 min. The plate was washed quickly three times with 1 M-ethanolamine, pH 8.0, followed by a single wash with 50 mM-Tris/0.15 M-NaCl/Tween-20 (0.5 ml/l)/sodium casein (10 g/l), pH 7.5 (Tween/casein buffer). Monoclonal anti-gp46 (Cates *et al.*, 1987) diluted to 10 μ g/ml in Tween/casein buffer was added to the wells (150 μ l/well) and incubated for 1 h at 25 °C. The wells were washed three times with Tween/casein buffer and then incubated with 100 μ l of horseradish-peroxidase-conjugated goat-anti-mouse IgG (3000-fold dilution of Bio-Rad stock solution) for 1 h at 25 °C. After the washing with Tween/casein buffer, 200 μ l of freshly prepared colour-development substrate [50 mM-citric acid/100 mM-Na₂HPO₄/o-phenylenediamine (2 mg/ml)/H₂O₂ (0.25 ml/l), pH 5.0] was added to each well. The reaction was terminated after 4 min with 50 μ l of 2.5 M-H₂SO₄ and the absorbance was measured at 490 nm.

Reconstitution of gp46 into liposomes

The reconstitution of gp46 into liposomes was adapted from the detergent dialysis procedure described by Mimms *et al.* (1981). This procedure generates large unilamellar proteoliposomes of about 200–300 nm average diameter. Triton X-100 was exchanged for octyl glucoside during gp46 isolation by substituting 3.4 mM-octyl glucoside for Triton X-100 in the CM-Sepharose phosphate buffer and also supplementing the elution buffer with 50 mM-octyl glucoside instead of Triton X-100. A mixture of phosphatidylcholine/phosphatidylserine (7:3, w/w) in chloroform/methanol (19:1, v/v) was dried under a stream of nitrogen gas in a silicone-coated glass tube. The amount of phospholipid used was adjusted such that the final concentration of lipid, after resolubilization, was 4 mg/ml. Purified gp46 (50 μ g in 2 ml of octyl glucoside buffer) was added to the phospholipids, and the mixture was solubilized by brief agitation of the tube in an ice-cooled bath sonicator. Solubilization was judged to be complete upon loss of turbidity in the protein/detergent/lipid mixture. The solution was then dialysed overnight against TBS containing 1 mM-2-mercaptoethanol to remove the octyl glucoside. The vesicles were collected by ultracentrifugation at 100000 *g* for 30 min, resuspended in 2 ml of TBS and re-centrifuged. The final gp46 liposomes were suspended in 1–2 ml of 50 mM-sodium phosphate, pH 7.5, or 50 mM-Tris/0.15 M-NaCl/1 mM-DTT, pH 7.5.

Chemical modification of gp46 liposomes

Portions (200 μ l) of gp46 liposomes in 50 mM-sodium phosphate, pH 7.5, were treated with 38 mM-2-iodoacetamide, 1.9 mM-N-ethylmaleimide or 1.6 mM-chloramine-T for 30 min at 25 °C. The N-ethylmaleimide treatment was terminated by addition of 20 μ l of 1 M-DTT. The chloramine-T treatment was stopped by addition of 20 μ l of sodium metabisulphite (5 g/l). The liposomes were diluted to 1 ml with TBS and centrifuged at 100000 *g* for 40 min. The pellet was resuspended in 1 ml of TBS and re-centrifuged. Final resuspension was in 200 μ l of 5 mM-Hepes/0.15 M-NaCl/1 mM-DTT, pH 7.5. Solid-phase binding assays using gp46 liposomes were performed in a similar fashion to the assay described for Triton-solubilized gp46. The inclusion of detergent in buffers was omitted until the gp46 had been fixed with glutaraldehyde.

Collagen thermal-stability analysis

Pepsin-treated rat tail tendon type I collagen (PepColl I) was solubilized in 0.1 M-acetic acid, and dialysed against the assay buffer (50 mM-Tris/0.15 M-NaCl/1 mM-DTT, pH 7.5). Insoluble collagen present after dialysis was removed by centrifugation. The assay for collagen thermal stability was based on that of Bonadio & Byers (1985). The gp46 liposomes, suspended in the Tris assay buffer, were combined with PepColl I in a silicone-coated glass tube. The final gp46 and PepColl I concentrations were 0.10 $\mu\text{g}/\text{ml}$ and 0.04 $\mu\text{g}/\text{ml}$ respectively (approx. 16 times molar ratio of gp46 to collagen). The mixtures were preincubated for 30 min at 36 °C, and then the temperature was increased in 1 °C increments at a rate of 6 °C/h to a maximum temperature of 45 °C. At each temperature point, 25 μl portions were removed and rapidly cooled to room temperature. Then 5 μl of trypsin (treated with tosyl-lysylchloromethane; 20 $\mu\text{g}/\text{ml}$) was added. Digestions were carried out for 2 min at room temperature and stopped by addition of 2 μl of 100 mM-PMSF followed by 10 μl of 4 $\times$ SDS electrophoresis sample buffer. The samples were then immediately heated at 100 °C for 4 min. Collagen trypsin-sensitivity was analysed by running the samples on SDS/8.5% polyacrylamide gels.

RESULTS

Purification of functional gp46

Our previous approach (Nandan *et al.*, 1990) to the isolation of gp46 resulted in the denaturation of gp46, which made it unusable for any functional analysis. Subsequent attempts to purify active gp46 by immunoaffinity chromatography, coupled with mild elution conditions, were unsuccessful in retaining gp46–collagen binding activity, presumably because gp46 was bound too tightly to the antibody to allow elution under conditions that retained the collagen-binding function of gp46. Gelatin–agarose chromatography was therefore used as the first step in gp46 purification, since elution could be achieved under weakly acidic conditions. Fig. 1 shows the eluates of identical gelatin–agarose columns incubated with Triton-soluble extracts of L6 myoblasts. The eluates represent elution with acidic buffers using 0.5-pH-unit steps. gp46 was eluted beginning at pH 6.0, although maximal elution was achieved at pH 5.0 or less. A small amount of gp46 was still retained after elution at pH 4.0 and thus could be eluted with 50 mM-diethylamine, pH 12 (results not shown). pH 5.5 was chosen for further experiments, since it resulted in the elution of gp46 that was minimally contaminated with other gelatin-binding proteins such as fibronectin and also could be shown to retain binding activity (see below). More recently, we have found that the higher-molecular-mass contaminants co-eluted with gp46 at pH 5.5 may be decreased by washing the gelatin–agarose extensively with 1 M-NaCl before low-pH elution.

Fig. 2 shows the results of a typical purification of gp46 based upon gelatin–agarose and ion-exchange chromatography. gp46 was the major protein eluted at pH 5.5 (Fig. 2, lane 2), whereas more harsh conditions were required to elute other gelatin-binding proteins, as shown in Fig. 2, lane 4. In order to achieve removal of minor contaminants, gp46 was further purified by CM-Sepharose chromatography. Under the conditions used, gp46 bound readily to CM-Sepharose, whereas contaminants of M_r 72000 and 100000 were present in the column flow-through (Fig. 2, lane 7). gp46 was eluted from CM-Sepharose with NaCl and appeared homogeneous by gel electrophoresis (Fig. 2, lane 9), although some trailing of the gp46 band was seen, owing to overloading of the gel and the presence of high salt in the sample.

Purified gp46 was incubated with gelatin–agarose in order to demonstrate that it had retained its gelatin-binding property

after purification. Fig. 3, lane 1, shows that only a small amount of gp46 was detected in the flow-through of the second gelatin–agarose column. Typically this non-functional gp46 represented 10–15% of the total purified gp46 preparation. Some 85–90% of gp46 retained binding activity, as shown by its retention by the gelatin–agarose column and its elution by high pH (Fig. 3, lanes 2–5). Purified gp46 could be stored at 4 °C for approx. 48 h without significant loss of binding activity, although longer storage or freezing and thawing resulted in loss of activity.

Binding of gp46 to immobilized collagen

In order to be able to assess rapidly the functionality of gp46, we developed a solid-phase binding assay that was sensitive enough to detect gp46 binding to small amounts of immobilized collagen. The binding of gp46 to native type I collagen or to

Fig. 1. pH-dependent elution of gp46 from gelatin–agarose

L6 myoblasts were extracted with Triton buffer as described in the Experimental section and the extracts mixed with 2 ml of gelatin–agarose. The matrix was washed with Triton buffer containing 1 M-NaCl and then divided into eight equal portions. Each portion was washed briefly with distilled water and then incubated batchwise for 10 min with 5 mM-Hepes/5 mM-sodium acetate/0.15 M-NaCl/Triton X-100 (0.5 ml/l), adjusted to the appropriate pH with HCl or NaOH. Equal amounts of the different-pH eluates were analysed on an SDS 8.5%-polyacrylamide gel. Material remaining on the pH 7.5-treated matrix was subsequently eluted with 50 mM-diethylamine, pH 12.0 (last lane). Numbers at the top of the Figure indicate the pH conditions of elution.

Fig. 2. Purification of gp46 from L6 myoblasts

The purification of gp46 from L6 myoblasts was done as described in the Experimental section, and portions of fractions isolated during the course of the purification were analysed on an 8.5%-polyacrylamide gel. Lane 1, flow-through from gelatin–agarose; lane 2, pH 5.5 eluate after washing of the gelatin–agarose with high salt; lanes 3–5, fractions eluted with diethylamine (pH 12) after pH 5.5 elution; lane 6, material applied to CM-Sepharose column; lane 7, flow-through from CM-Sepharose; lane 8, CM-Sepharose wash; lane 9, gp46 eluted from CM-Sepharose with 0.5 M-NaCl.

Fig. 3. Gelatin-rebinding analysis of purified gp46

Purified gp46 was tested following dialysis for ability to rebind to gelatin-agarose as described in the Experimental section. Lane 1, flow-through from gelatin-agarose (non-functional gp46); lanes 2–5, 0.5 ml pulses of 50 mM-diethylamine, pH 12, after washing gelatin-agarose with buffer containing 1 M-NaCl.

Fig. 4. Solid-phase binding of gp46 to different collagen types

Various dilutions of purified gp46 were incubated in microtitre wells precoated with various collagen substrata. Control wells were coated with BSA or sodium casein. Binding was quantified as described in the Experimental section. The absorbance due to non-specific binding was less than 0.05 and was subtracted from the total absorbance for each binding curve. ●, Pepsin-treated type IV collagen; ■, native type I collagen.

pepsin-treated type IV collagen was detected by means of a monoclonal antibody specific for gp46 (Cates *et al.*, 1987). More direct detection of gp46 binding by using gp46 labelled with ^{125}I was unsuccessful, since gp46 invariably lost its collagen-binding property following iodination. Indirect detection of gp46 binding by means of anti-gp46 antibody allowed for a qualitative and rapid assessment of the relative interaction of gp46 with various collagens.

Fig. 4 shows the interaction of purified gp46 with native or denatured type I collagen or with pepsin-treated type IV collagen. The relative affinity of gp46 for type IV collagen was higher than for native type I collagen, with saturation being detected at 25 μg of gp46/ml. In contrast, saturation binding to type I collagen was not achieved, indicating a lower relative affinity of gp46 for type I collagen. The difference in gp46 binding to type I and IV collagens could not be attributed to different amounts of type I or IV collagen binding to the plastic wells, since identical amounts

Fig. 5. Binding of ^{125}I -labelled collagens to microtitre wells

Pepsin-treated type IV collagen (●) or native type I collagen (■) was iodinated as described in the Experimental section and incubated in wells of a microtitre plate under the same conditions as for non-labelled collagens. Bound collagen was determined by counting individual wells in a γ -scintillation counter.

Fig. 6. PAGE analysis of gp46-liposomes

A 1.5 ml suspension of gp46 and proteoliposomes obtained following detergent dialysis was centrifuged as described in the Experimental section in order to separate free gp46 from liposome-incorporated gp46. The liposome pellet was resuspended in 1.5 ml of TBS and re-centrifuged. The final pellet was suspended in 0.35 ml of TBS. Equal volumes of the first supernatant (lane 1) and the final washed gp46-liposome suspension (lane 2) were analysed on an SDS/8.5% polyacrylamide gel.

of iodinated collagens were found to bind to the plastic wells, as shown in Fig. 5.

Binding of gp46 proteoliposomes to immobilized collagen

In order to address the functional role of gp46, we sought to reconstitute purified gp46 into liposomes. This situation would provide a membrane surface for gp46–collagen interaction such as that which would presumably occur under physiological conditions. Dialysis of octyl glucoside-soluble gp46 in the presence of phospholipids resulted in the formation of liposomes containing gp46. Fig. 6 shows the analysis of the liposome pellet and supernatant fractions obtained following dialysis. Washing of the liposomes with either TBS or 0.2 M- NaHCO_3 , pH 10, failed to release gp46 from the liposomes, indicating either incorporation of gp46 into the lipid bilayer or tight association with the phospholipids. Typically, about 15% of the total gp46 was recovered in the liposome fraction, as determined by densitometric analysis.

Fig. 7. Thermal stability of type I collagen in the presence of purified gp46

The thermal stability of pepsin-treated rat tail type I collagen was determined by examining its sensitivity to trypsin digestion after incubation at the temperatures indicated at the top of the Figure. The last lane of each panel represents a control in which the collagen was heated to 45 °C, but no trypsin (NT) was added. These gels were silver-stained in order to increase the sensitivity of detection. (a) Collagen only; (b) collagen plus control liposomes containing no gp46; (c) collagen plus liposomes containing gp46.

These gp46-containing liposomes were tested for binding activity with immobilized type IV pepsin-treated collagen as substrate. In addition, gp46 liposomes were pretreated with various chemical modifiers in order to identify inhibitors of gp46 binding. Such chemically modified inhibited gp46 liposomes would then serve as controls for functional assays.

gp46 liposomes retained binding affinity for type IV collagen. Treatment with the thiol-alkylating agents 2-iodoacetamide or *N*-ethylmaleimide resulted in a 21% or 30% decrease respectively in the maximum binding of gp46 to type IV collagen. Oxidation of gp46 with chloramine-T caused a 91% loss of gp46-binding capacity for type IV collagen. This loss of activity would explain our inability to obtain functionally active iodinated gp46 by using a chloramine-T-based procedure.

Thermal stability of gp46–collagen complexes

One of the possible roles of gp46 in the endoplasmic reticulum of collagen-secreting cells is to provide stability of newly synthesized procollagen, particularly under stress conditions

such as heat shock. To test this hypothesis, we examined the thermal stability of type I collagen in the presence and absence of gp46 liposomes. The temperature at which the α -chains dissociate was determined by the marked increase in trypsin-sensitivity of the collagen. In the absence of liposomes, type I collagen had a melting temperature of 41 °C (Fig. 7a). When liposomes containing active gp46 were present in the incubation mixture there was a small, but reproducible, shift in the melting temperature to 42 °C, as indicated by the presence of trypsin-resistant collagen at 41 °C (Fig. 7c). This shift was not seen either in the presence of liposomes alone (Fig. 7b) or with liposomes containing chloramine-T-inactivated gp46 (results not shown).

DISCUSSION

The collagen-binding glycoprotein, gp46, is a major component of the endoplasmic reticulum of L6 myoblasts, representing about 1% of the total cellular protein of these cells (Nandan *et al.*, 1990). Despite its relative abundance, its functional role is unclear. Its ability to bind to several collagen types and its location in the endoplasmic reticulum would suggest a role in the processing of intracellular collagen. Nakai *et al.* (1990) showed that an M_r -47000 collagen-binding protein in mouse BALB/3T3 cells, which is probably related to gp46, binds type I procollagen *in vivo*. Therefore the collagen-binding property of this class of proteins is clearly of physiological significance, but the purpose of the association with procollagen in the endoplasmic reticulum is unknown. Possible functions for gp46 could include assisting in the folding of procollagen chains, segregation of collagen from other secreted collagen-binding proteins such as fibronectin, or movement of procollagen from the endoplasmic reticulum during secretion, or alternatively gp46 may stabilize newly synthesized procollagen in the endoplasmic reticulum, particularly under heat-shock conditions (Hughes *et al.*, 1987).

The procedure that we have used to isolate gp46 relies on the elution of gp46 from denatured collagen (gelatin)–agarose using relatively mild acidic elution conditions. Optimal retention of gp46–collagen binding was obtained when elution was at pH 5.5, although a significant amount of gp46 was still retained on the affinity matrix at this pH. Elution at lower pH resulted in a loss of gp46-binding activity. Since we were interested in obtaining functionally active gp46, we were forced to compromise yield in favour of activity. The gp46 remaining on the column after pH 5.5 elution may represent aggregates of gp46 that require slightly harsher conditions to achieve elution. Analysis of the phosphorylation state of gp46 eluted at different pH values has shown no difference in the degree of phosphorylation (G. A. Cates, unpublished work); thus it is unlikely that differential binding can be attributed to the phosphorylation of gp46.

pH-dependent elution from gelatin–agarose of a major collagen-binding protein from chick fibroblasts of M_r 47000 has been previously reported (Saga *et al.*, 1987). This protein is probably the avian form of gp46, although it differs in some of its properties. Maximal elution of the chick protein from gelatin–agarose was determined to occur at pH 6.3, which is significantly higher than the pH we have determined for elution of gp46. This probably reflects a lower binding affinity of this chick protein for gelatin than of gp46 for gelatin. This may be an intrinsic difference in the binding property of the protein, or it may reflect different experimental conditions. Unfortunately the partially purified chick protein was not tested for functional binding properties, so it is not known whether it retained activity after elution from gelatin–agarose under the conditions used.

Solid-phase binding analysis of purified gp46 showed a preferential binding to type IV collagen compared with native type I

collagen. It is likely that gp46 binds to the helical region of both collagen types, since gp46 binds to both pepsin-treated and -untreated type I collagen (results not shown). This conclusion is supported by the observation that an M_r -48 000 collagen-binding protein from bovine aortic endothelial cells, which is probably related to gp46, binds only to the collagenous region of type IV collagen and not to the globular NC1 domain (Yannariello-Brown & Madri, 1990). Future studies will be needed to determine more precisely the gp46-binding domain in collagen. Such studies should be directed towards determining the structural difference in the domains recognized by gp46 in type I and type IV collagens.

Purified gp46 was found to be very sensitive to extremes of pH, since either low- or high-pH elution of gp46 from either gelatin-agarose or anti-gp46-agarose resulted in irreversible loss of binding activity. gp46 was only slightly inhibited by thiol-alkylating reagents and was not affected by the presence of DTT, indicating that disulphide bonds are not important for gp46 activity. The loss of gp46 activity seen with chloramine-T treatment may be associated with oxidation of methionine residues (Lundblad & Noyes, 1984), although this is not conclusive. Earlier analysis of the M_r -47 000 gelatin-binding protein colligin from mouse parietal endoderm cells showed that 5 mM-*N*-ethylmaleimide slightly decreased binding of colligin to gelatin-agarose (Kurkinen *et al.*, 1984), which is in agreement with our observation for gp46.

The purification of gp46 in an active state has allowed us to begin exploring the functional role of gp46 with regard to collagen metabolism. Since gp46 synthesis is increased upon heat-shock treatment, it was logical to examine the relationship between increased gp46 expression and collagen stability at temperatures normally used for heat-shock treatment. The type I collagen helix undergoes complete thermal denaturation in solution at temperatures only 4–5 °C higher than physiological temperature (Piez, 1984). The mid-point of the melting transition curve (the melting temperature, T_m) is dependent upon the degree of hydroxylation of proline residues in the collagen chain (Kivirikko & Myllyla, 1984). Overhydroxylation of collagen such as that which is observed in types of osteogenesis imperfecta leads to a decreased thermal stability of type I collagen (Bonadio & Byers, 1985). Thus the primary factor affecting the thermal stability is hydroxylation, but the interaction with other proteins *in vivo* may also confer added stability to the collagen helix, thereby increasing the actual melting temperature in tissues (Piez, 1984). The need for a stable triple helix for normal intracellular trafficking of procollagen is inferred from studies showing increased accumulation of unstable procollagen in the endoplasmic reticulum following inhibition of proline hydroxylation (Schwarz, 1985; Pacifici & Iozzo, 1988). In addition, micro-unfolding of newly synthesized procollagen in the temperature range of 37–40 °C at sites of wound repair or during high fever (Kadler *et al.*, 1988) may be affected by the presence of collagen-binding proteins such as gp46 in the endoplasmic reticulum.

Our reconstitution experiments with gp46 and type I collagen failed to show a significant effect of gp46 on the thermal stability of the collagen helix. It is possible that our reconstituted system failed to reproduce the normal physiological conditions that would occur *in vivo*. Considering the relative abundance of gp46 in the endoplasmic reticulum, particularly after heat-shock

induction, the ratio *in vivo* of gp46 to collagen may be higher than that obtainable in our reconstitution system. Despite this limitation, we would have expected a larger change in the melting temperature of type I collagen, given a molar ratio of gp46 to collagen of 16:1 in our experiments. It must therefore be concluded that gp46 probably does not prevent significant dissociation of the collagen chains upon heat treatment.

We must now consider other possible functions for this relatively abundant collagen-binding protein. Hughes *et al.* (1987) have suggested that colligin, which is probably identical with gp46, may prevent nascent collagen chains from interacting with each other before completion of the C-terminal propeptide sequences. Alternatively, gp46 may be involved in the transport of procollagen from the endoplasmic reticulum of cells. The availability of active reconstituted gp46 will allow us to address these possibilities.

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REFERENCES

- Bonadio, J. & Byers, P. H. (1985) *Nature* (London) **316**, 363–366
- Cates, G. A., Brickenden, A. M. & Sanwal, B. D. (1984) *J. Biol. Chem.* **259**, 2646–2650
- Cates, G. A., Nandan, D., Brickenden, A. M. & Sanwal, B. D. (1987) *Biochem. Cell Biol.* **65**, 767–775
- Chandrakasan, G., Torchia, D. A. & Piez, K. A. (1976) *J. Biol. Chem.* **251**, 6062–6067
- Fraker, P. J. & Speck, J. C. (1978) *Biochem. Biophys. Res. Commun.* **80**, 849–857
- Gelman, R. A., Poppke, D. C. & Piez, K. A. (1979) *J. Biol. Chem.* **254**, 11741–11745
- Hughes, R. C., Taylor, A., Sage, H. & Hogan, B. L. M. (1987) *Eur. J. Biochem.* **163**, 57–65
- Kadler, K. E., Hojima, Y. & Prockop, D. J. (1988) *J. Biol. Chem.* **263**, 10517–10523
- Kivirikko, K. I. & Myllyla, R. (1984) in *Extracellular Matrix Biochemistry* (Piez, K. A. & Reddi, A. H., eds.), pp. 83–118, Elsevier, New York
- Kurkinen, M., Taylor, A., Garrels, J. I. & Hogan, B. L. M. (1984) *J. Biol. Chem.* **259**, 5915–5922
- Lundblad, R. L. & Noyes, C. M. (1984) *Chemical Reagents for Protein Modification*, CRC Press, Boca Raton, FL
- Mimms, L. T., Zampighi, G., Nozaki, Y., Tanford, C. & Reynolds, J. A. (1981) *Biochemistry* **20**, 833–840
- Nagata, K. & Yamada, K. M. (1986) *J. Biol. Chem.* **261**, 7531–7536
- Nagata, K., Saga, S. & Yamada, K. M. (1988) *Biochem. Biophys. Res. Commun.* **153**, 428–434
- Nakai, A., Hirayoshi, K. & Nagata, K. (1990) *J. Biol. Chem.* **265**, 992–999
- Nandan, D., Cates, G. A., Ball, E. H. & Sanwal, B. D. (1988) *Exp. Cell Res.* **179**, 289–297
- Nandan, D., Cates, G. A., Ball, E. H. & Sanwal, B. D. (1990) *Arch. Biochem. Biophys.* **278**, 291–296
- Pacifici, M. & Iozzo, R. V. (1988) *J. Biol. Chem.* **263**, 2483–2492
- Pelham, H. R. B. (1986) *Cell* **46**, 959–961
- Pelham, H. R. B. (1989) *Annu. Rev. Cell Biol.* **5**, 1–23
- Piez, K. A. (1984) in *Extracellular Matrix Biochemistry* (Piez, K. A. & Reddi, A. H., eds.), pp. 1–39, Elsevier, New York
- Saga, S., Nagata, K., Chen, W. T. & Yamada, K. M. (1987) *J. Cell Biol.* **105**, 517–527
- Schwarz, R. I. (1985) *J. Biol. Chem.* **260**, 3045–3049
- Yaffe, D. (1968) *Proc. Natl. Acad. Sci. U.S.A.* **61**, 477–483
- Yannariello-Brown, J. & Madri, J. A. (1990) *Biochem. J.* **265**, 383–392