Calcium Binding Domains and Calcium-Induced Conformational Transition of SPARC/BM-40/Osteonectin, an Extracellular Glycoprotein Expressed in Mineralized and Nonmineralized Tissues[†]

Jürgen Engel,*,[‡] William Taylor,[§] Mats Paulsson,[‡] Helene Sage,[‖] and Brigid Hogan[⊥]

Department of Biophysical Chemistry, Biozentrum, CH-4056 Basel, Switzerland, Laboratory of Molecular Biology, Department of Crystallography, Birkbeck College, London WC1 E7HX, U.K., Department of Biological Structure, University of Washington, Seattle, Washington 98195, and Laboratory of Molecular Embryology, National Institute for Medical Research, London NW7 1AA, U.K.

ABSTRACT: SPARC, BM-40, and osteonectin are identical or very closely related extracellular proteins of apparent M_r 43 000 (M_r 33 000 predicted from sequence). They were originally isolated from parietal endoderm cells, basement membrane producing tumors, and bone, respectively, but are rather widely distributed in various tissues. In view of the calcium binding activity reported for osteonectin, we analyzed the SPARC sequence and found two putative calcium binding domains. One is an N-terminal acidic region with clusters of glutamic acid residues. This region, although neither γ-carboxylated nor homologous, resembles the γ-carboxyglutamic acid (Gla) domain of vitamin K dependent proteins of the blood clotting system in charge density, size of negatively charged clusters, and linkage to the rest of the molecule by a cysteine-rich domain. The other region is an EF-hand calcium binding domain located near the C-terminus. A disulfide bond between the E and F helix is predicted from modeling the EF-hand structure with the known coordinates of intestinal calcium binding protein. The disulfide bridge apparently serves to stabilize the isolated calcium loop in the extracellular protein. As observed for cytoplasmic EF-hand-containing proteins and for Gla domain containing proteins, a major conformational transition is induced in BM-40 upon binding of several Ca^{2+} ions. This is accompanied by a 35% increase in α-helicity. A pronounced sigmoidicity of the dependence of the circular dichroism signal at 220 nm on calcium concentration indicates that the process is cooperative. In view of its properties, abundance, and wide distribution, it is proposed that SPARC/BM-40/osteonectin has a rather general regulatory function in calcium-dependent processes of the extracellular matrix.

Several model systems are available for studying the assembly and turnover of the extracellular matrix. One is the parietal endoderm of the mouse embryo, which synthesizes a basement membrane (Reichert's membrane) rich in type IV collagen, laminin, entactin/nidogen, and heparan sulfate proteoglycan [for review, see Hogan et al. (1984)]. These very large and complex glycoproteins are also made by the transplantable Engelbreth–Holm–Swarm (EHS) mouse tumor, which provides a convenient and abundant source of purified matrix components for structural and biological studies [reviewed by Timpl et al. (1987)]. Recent work in both model systems has led to the identification of a very abundant, small glycoprotein, named SPARC (Mason et al., 1986a,b) or BM-40 (Dziadek et al., 1986), which migrates with an apparent M_r of 39 000–43 000 in sodium dodecyl sulfate–polyacrylamide gel electrophoresis and contains a single oligosaccharide side chain (Hughes et al., 1987). SPARC is coded for by a single gene, and the complete amino acid sequence of the mouse protein has been deduced from the nucleotide sequence of parietal endoderm cDNAs (Mason et al., 1986a,b). This gives a predicted M_r of 33 062 for the unglycosylated protein after removal of the signal sequence. The amino acid sequence of BM-40 is identical with that of SPARC in all peptides so far analyzed, covering about 50% of the total protein (Mann et al., 1987). Although SPARC was first identified as a major product of parietal endoderm cells, further studies showed that messenger RNA and/or protein are made by a variety of other cell types, including bovine endothelial cells and fibroblasts derived from fetal dermis and ligament (Sage et al., 1984; Sage, 1985, Mason et al., 1986b; Dziadek et al., 1986). The protein made by endothelial cells and isolated by Sage et al. (1984) was initially named 43K albumin binding protein; its identity or close relation with SPARC was demonstrated by Mason et al. (1986a). Moreover, there is now very good evidence that SPARC is identical with the phosphorylated calcium binding protein osteonectin, originally isolated from fetal bovine bone and dentin (Termine et al., 1981a,b). The N-terminal region of both bovine and porcine osteonectin is highly homologous to the predicted N-terminal sequence of SPARC (Romberg et al., 1985; Young et al., 1986). When this comparison is extended to the complete amino acid sequences predicted from the cDNAs of mouse SPARC and bovine osteonectin, 92% sequence identity is observed (Young et al., 1986; M. E. Bolander and J. D. Termine, National Institutes of Health, Bethesda, MD, personal communication). Additional evidence that SPARC and osteonectin are identical comes from in situ hybridization data showing very high levels of SPARC RNA in embryonic and newborn mouse bone (osteoblasts) and teeth (odontoblasts) (Holland et al., 1987). A high-affinity calcium binding site ($K_d \sim 3 \times 10^{-7}$ M) was demonstrated for osteonectin, and hydroxylapatite crystal

[‡] Biozentrum.
[§] Birkbeck College.
[‖] University of Washington.
[⊥] National Institute for Medical Research.

growth was found to be delayed by 50% at about 0.2 μM concentrations of the protein (Romberg et al., 1985). Here we report phosphorylation of SPARC/BM-40/osteonectin in cell cultures and demonstrate a large cooperative and reversible change in α-helicity upon binding of several Ca^{2+} ions. Structural features based on the SPARC sequence are discussed, including the identification of an EF-hand calcium binding domain and putative additional calcium binding sites.

EXPERIMENTAL PROCEDURES

Materials

Isolation of BM-40. Intact BM-40 was extracted from mouse EHS tumor tissue with neutral buffer containing 10 mM EDTA[1] and protease inhibitors [for the preparation of the extract see Paulsson et al. (1987)]. A retarded fraction from Sepharose CL-6B molecular sieve chromatography of this extract contains considerable amounts of BM-40, which was further purified by ion-exchange chromatography on DEAE-cellulose and molecular sieve chromatography on Sephacryl S-200. The initial sample of intact BM-40 isolated from EDTA extracts was a kind gift of Dr. Rupert Timpl, Max-Planck-Institut für Biochemie, Martinsried, FRG. Intact BM-40 migrated as a M_r 40K band with less than 10% of 30K and 10K protease fragments when tested by SDS-PAGE[1] under reducing conditions. Nicked BM-40 was also isolated by the procedure of Dziadek et al. (1986). This material dissociates almost completely into M_r 30K and 10K fragments under the same conditions (Dziadek et al., 1986) and differs from intact BM-40 also with respect to a possible loss of native conformation due to exposure to 6 M guanidine hydrochloride during extraction.

Methods

Metabolic Labeling and PYS Cell Culture. Confluent cultures of the mouse parietal endoderm cell line PYS-2 (Lehman et al., 1974) in tissue culture dishes (5 cm in diameter) were labeled for about 40 h in 2 mL of Dulbecco's minimum essential α medium without phosphate, containing 5% dialyzed fetal bovine serum and 1 mCi [^{32}P]orthophosphoric acid (New England Nuclear). The medium was harvested and made 50 mM in NaF and 100 μM in sodium vanadate to inhibit phosphatase activity. SPARC was recovered by immunoprecipitation using rabbit antipeptide serum and protein A–Sepharose (Pharmacia) as described by Mason et al. (1986a). Samples were analyzed by SDS-PAGE under reducing conditions, and phosphoamino acid analysis of the excised M_r 43K SPARC band was carried out as described (Heath et al., 1986). For glycopeptide analysis the immunocomplex bound to protein A was incubated at 37 °C for 3 days in 0.4% Pronase (Calbiochem) in 50 mM Tris-HCl, pH 7.5, 10 mM CaCl$_2$, and 0.02% NaN$_3$. After incubation at 90 °C for 5 min to inactivate the Pronase and centrifugation to remove the Sepharose beads, the supernatant was applied to a concanavalin A–Sepharose column (Pharmacia) in 10 mM Tris, pH 7.5, 100 mM NaCl, 1 mM MgCl$_2$, 1 mM CaCl$_2$, 1 mM MnCl$_2$, and 0.02% NaN$_3$ [buffer A, Hughes et al. (1987)]. After extensive washing, glycopeptide bound to the concanavalin A was eluted with 10 mM methyl α-glucoside and 500 mM methyl α-mannoside in buffer A (Hughes et al., 1987). For analysis of tryptic cleavage fragments, PYS-2 cells were incubated overnight with 100 μCi/mL [^{35}S]methionine

as described (Mason et al., 1986a). SPARC was recovered from the culture medium with anti-peptide serum and protein A–Sepharose. Immunocomplexes were incubated at 37 °C with 0.1–0.5 μg of trypsin (Sigma, type III) in a 0.17 M NaCl, 3 mM KCl, 9 mM Na$_2$HPO$_4$, and 2 mM KH$_2$PO$_4$, pH 7.4, buffer (phosphate-buffered saline), and the reaction was stopped by adding twice concentrated SDS-PAGE sample buffer at 100 °C. SDS-PAGE was carried out as described (Cooper et al., 1981). Internal ^{14}C-labeled markers were myosin (M_r 212 000), phosphorylase b (M_r 100 000 and 92 500), bovine serum albumin (M_r 69 000), ovalbumin (M_r 46 000), carbonic anhydrase (M_r 30 000), and lysozyme (M_r 14 000) (Amersham International).

Circular Dichroism. A Cary 61 spectropolarimeter (Varian, Zug, Switzerland) was employed. Spectra were recorded in a thermostat-controlled quartz cell (Helma, Müllheim, FRG) of 1-mm path length at 20 °C. The molar ellipticity [θ] expressed in deg·cm^2·dmol^{-1} was calculated on the basis of a mean residue molar mass of 110. Concentrations needed for the calculation of [θ] were determined by quantitative amino acid analysis of aliquots of the sample solutions. The dependence of ellipticity on calcium concentration was measured with a rectangular cell of 2-mm path length which was selected for showing zero ellipticity at 220 nm when filled with buffer. A cell holder was used which assured exact reproducibility of the cell positioning after mixing. The cell temperature was maintained by the thermostated compartment between 20 and 22 °C. The protein was extensively dialyzed against a 10 mM Tris-HCl and 150 mM NaCl, pH 7.4, buffer. The buffer was stirred over night with Chelex 100 resin (Bio-Rad, Richmond, CA) to remove metal ions before use, and it was acertained by atomic absorption analysis that the remaining calcium concentration was less than 10 μm. The calcium analysis was kindly performed by Dr. Hans Günther Seiler, Institute for Inorganic Chemistry, University of Basel. The cell was filled with 0.3 mL of the protein solution (0.33 mg/mL). Aliquots (3–10 μL) of 0.5 mM EDTA in the same buffer were added, and the ellipticity was monitored for 3 min after each addition. After a plateau value was reached, a back-titration was performed with 3-μL aliquots of 0.5 mM CaCl$_2$ in the same buffer. Ellipticities and concentrations of the reagents in the mixture were corrected by dilution factors which did not exceed 1.15.

RESULTS

Analysis of the Sequence. Some salient features of the sequence of SPARC/BM-40/osteonectin are summarized in Figure 1. Clearly, four sequence regions of rather different characteristics can be distinguished, suggesting the presence of at least four conformationally and functionally distinct domains. Domain I, comprising the first 52 residues of the protein, features primarily two segments of 14–15 residues, each of which contains 7–8 glutamic acids in short clusters that impart a negative charge at physiological pH. According to secondary structure prediction these segments are α-helical. It has to be noted, however, that the α-helix is not expected to be stable at this high charge density (see Discussion).

Region II consists of about 85 residues exhibiting the typical features of a disulfide-stabilized domain (Doolittle, 1985). According to the secondary structure prediction in this region, short segments of β-structure alternate with β-bends. Most of the 11 cysteines are probably disulfide linked within the domain, but their uneven number suggests that one of them is connected with a Cys residue (probably Cys-247, see below) of the C-terminal region. A portion of purified BM-40 is nicked, and the small and the large fragment obtained are

---

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; endo-F, endo-β-*N*-acetylglucosaminidase F; Gla, γ-carboxyglutamic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EGF, epidermal growth factor.
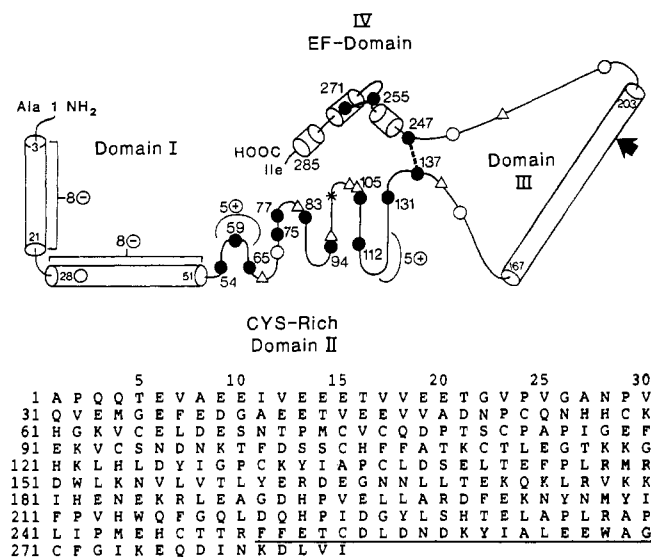
FIGURE 1: Schematic representation of interesting features in the sequence of SPARC. Numbering is according to Mason et al. (1986a) without the signal peptide. The mode of linkage between Cys residues (●) is not yet established. It is suggested from the three-dimensional modeling of the EF-hand calcium binding domain IV (Figure 3) that Cys-255 and Cys-271 are connected (●---●). There is an uneven number of Cys residues in the Cys-rich domain II. The presence of a disulfide bond between one of them and Cys-247 follows from the observation that cleavage at a site in region III leads to fragmentation only after reductive cleavage of one or more disulfide bonds (Figure 7; Sage et al., 1984; Mason et al., 1986a,b; Dziadek et al., 1986). The arrow indicates the site of endogenous cleavage in position Leu-197–Leu-198 (Mann et al., 1987). Assuming that SPARC contains no free thiol groups, it follows that all other Cys residues are disulfide bonded within the Cys-rich domain II. The latter contains two clusters of five positively charged residues HHCKHGK (57–63) and KKGHKLH (118–124), the unique glycosylation site (*) (Asn-98), and five serine residues. (Δ) indicates Ser -70, -82, -95, -103 and -104. In domain I toward the amino terminus there are two regions (6–20 and 32–47), each of which contains eight negatively charged residues. Segments of the sequence for which an α-helical secondary structure is predicted by the method of Chou and Fasman (1974) are indicated by cylinders. The same method predicts short segments of β-structure frequently interrupted by β-turns in the Cys-rich domain and some β-structure as well as β-turns in the rest of the molecule. Positions of all methionines (Met-33, -74, -149. -208, -238) are indicated (O) to show the distribution of [^{35}S]methionine in the material used for the tryptic cleavage experiment.

1. Near both ends of the cysteine-rich domain there are clusters of closely spaced, positively charged residues, while in the central portion is the site of the single glycosylation of SPARC, Asn-98 (Mason et al., 1986a; Hughes et al., 1987). The Cys-rich domain also contains six of the seven serine residues in the protein. These serines are all potential phosphorylation sites, including the sequence DSS (102–104) that is close to Asn-98 (see below).

A cysteine- and proline-free region (domain III) is predominantly α-helical (60–70%) according to the structure prediction. When all of the clearly predicted α-helical segments are summed, the total α-helicity is 31%. This value decreases to 21% if the two Glu-rich segments in the N-terminal region are assumed to be in a randomly coiled conformation at physiological pH. The last region (IV) consists of an EF-hand calcium binding domain connected to region III by a non-α-helical stretch of 32 residues containing 5 prolines.

EF-Hand Ca^{2+} Binding Domain. In Figure 2, residue 248–275 of the SPARC sequence are compared with the consensus motif of an EF-hand calcium binding domain. This motif is based on the sequences of many cytoplasmic calcium binding proteins, of which calmodulin, parvalbumin, and intestinal calcium binding protein are well-known examples. The sequence of domain II, one of four EF-hand motifs in bovine intestinal calcium binding protein, is included in Figure 2 because of the high-resolution X-ray structure that is available for this protein (Szebinyi et al., 1981; Szebinyi & Moffat, 1986). The only clear deviation in the SPARC sequence from the accepted EF-hand motif is the exchange of a normally conserved glycine by lysine (Figure 2). Lysine residues in this position are, however, observed in the recently discovered putative EF-hand domains of another extracellular protein, fibrinogen (Dang et al., 1985). An interesting feature of the EF-hand sequence in SPARC is the occurrence of the two Cys residues in positions of the E and F helices which are occupied by hydrophobic residues in the EF motifs of cytoplasmic proteins. In the intestinal calcium binding protein the hydrophobic side chains of these residues (Leu-53 and Leu-69) are in close contact, and Cys residues in these positions can easily be connected by a disulfide bond (Figure 3). Connection of Cys-255 and Cys-271 by a disulfide bond in the EF calcium binding domain of SPARC leaves Cys-247 as a likely candidate for disulfide bridging to one of the cysteine residues in the Cys-rich domain. This assignment is supported by recent fragmentation studies (Mann et al., 1987). The model in Figure 3 was also used to verify that glycine-59 in intestinal

probably connected by this disulfide bond. The location of the endogenous cleavage site was located to Leu-197–Leu-198 (Mann et al., 1987) and is indicated by an arrow in Figure

```
              E-Helix    Loop       F-Helix

EF-hand motif          hXXhhXXhDXDXDGXIDX-XEhXXhhXXh
                                    *   *
SPARC                  TTRFFETCDLDNDKYIAL-EEWAGCFGIK  248-275
                            ↓         ↓
intestinal Ca++binding protein,bovine  LDELFEELDKNGDGEVSF-EEFQVLVKKI  46-73

fibrinogen  chain precursor,  human  NGMQFSTWDNDNDKFEGNCAEQDGSGWWM  334-363

                             ,rat  NGMHFSTWDNDNDKFEGNCAEQDGSGWWM  334-363

                       ,sea lamprey  NGMLFSTPERDNDKYEGSCAEQDGSGWWM  330-358
```

FIGURE 2: Comparison of a sequence region in SPARC with the consensus sequence for the EF-hand calcium binding domain (Kretsinger, 1979) and related motifs in intestinal calcium binding protein (Fullmer & Wassermann, 1981) and fibrinogen (Dang et al., 1985). In the EF-hand motif Asp (D) can be replaced by Asn (N), and Glu (E) can be replaced by Asp. "h" denotes a hydrophobic residue, and X indicates any residue. Other variations are possible (Kretsinger, 1979, 1980). A deviation of the SPARC sequence from the central portion (boldface letters) of the EF-hand motif is the occurrence of lysine (K) instead of the normally conserved glycine (G) (asterisks). Lysines in this position are however observed in the putative EF hands of fibrinogen γ chains (Dang et al., 1985). Residues that are identical in fibrinogen and SPARC are underlined. The other deviation, alanine (A) instead of D (asterisks), is not inconsistent with an EF hand, since there is only a weak preference for an oxygen-containing side chain at this position (Szebenyi & Moffat, 1986). Residues Leu-53 and Leu-69 (arrows) are in close proximity in the three-dimensional structure of intestinal calcium binding protein (Szebinyi et al., 1981; Szebenyi & Moffat, 1986) and were substituted by cysteines in Figure 3 to test for the possibility of a disulfide bridge formation in this position.
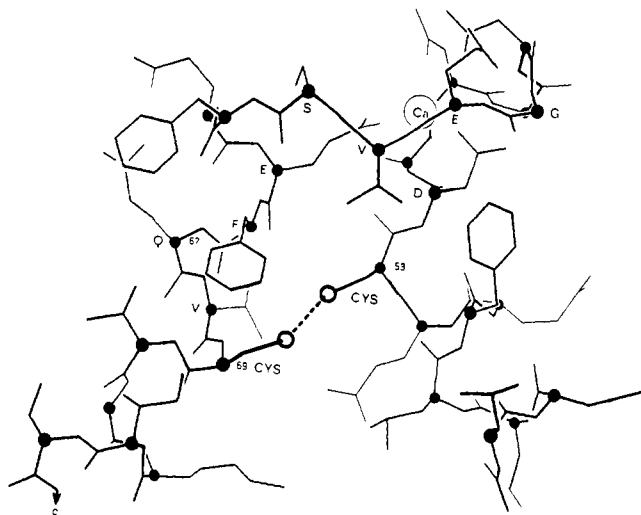
FIGURE 3: Three-dimensional model of an EF-hand calcium binding domain that is stabilized by a disulfide bond. The structure of one of the calcium binding domains in intestinal calcium binding protein (49–70, see Figure 2) was displayed according to the coordinates of Szebinyi et al. (1981) taken from the Brookhaven library (Dayhoff & Baker, 1972). Residues Leu-53 and Leu-69 were replaced by Cys residues which occur in equivalent positions in the EF motif of SPARC (Figure 2). By adjustment of the side chains of the two Cys residues without changes in the positions of their C$_\alpha$ atoms, formation of a disulfide bond (S–S) was easily possible. The bond was slightly stretched (S–S distance of 3 Å and both C–S–S angles greater than 90°), but a slight relative movement of the α-helices would enable an ideal bond geometry to be attained. The diagram was produced by the molecular modeling program FRODO (Jones, 1978). The α-carbon positions are emphasized as filled dots, adjacent to which are indicated the names and numbers of certain residues. The two sulfur atoms are represented as open circles, and the proposed bond between them is dashed. The main chain runs from bottom right to left, and its termini are indicated "n" and "c", respectively.

calcium binding protein can be replaced by lysine without steric interference of the newly introduced side chain with the rest of the structure.

Secondary Structure and Conformational Changes Monitored by Circular Dichroism. BM-40 was extensively dialyzed against Chelex-treated 10 mM Tris-HCl, pH 7.4, and 150 mM NaCl buffer, which according to atomic absorption measurements contained less than 10 μM calcium. Circular dichroism spectra of intact BM-40 recorded at this low concentration of free calcium (Figure 4) were identical with spectra measured in the same buffer containing 1 mM calcium. The pronounced shoulder at 220 nm in the spectrum of BM-40 indicates 25–30% α-helical content. Upon removal of calcium by addition of EDTA to a total concentration of 1 mM the mean residue ellipticity at 220 nm decreases by 35%, indicating a corresponding decrease of the α-helix content to a value of 16–19%. This change is completely reversible upon readdition of calcium, and the cycle can be repeated 3 times without any significant change of the spectra of saturated and free protein. Nicked BM-40 purified from 6 M guanidine hydrochloride extracts exhibits a similar spectrum and calcium-induced change, but the amplitude as well as the magnitude of the change is smaller.

The change of ellipticity was plotted as a function of EDTA or calcium concentration for intact SPARC/BM-40/osteonectin (Figure 5). A cooperative process is indicated by the sigmoidal curve. Data points collected upon addition of EDTA match the points obtained in the back-titration with calcium. As the helix-promoting effect of calcium may in part be due to a compensation of negative charges, we tested whether a similar effect could be induced by lowering the pH. Intact
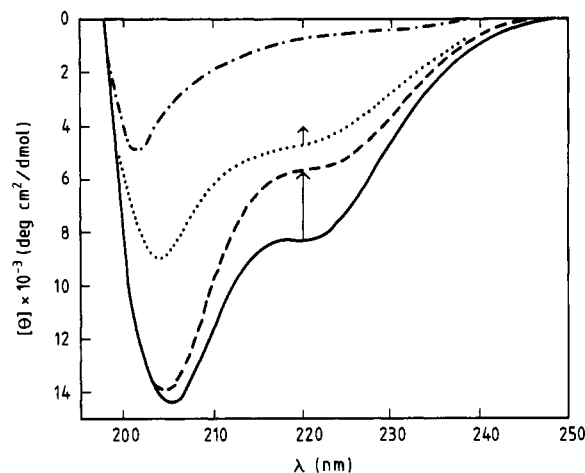

FIGURE 4: Circular dichroism spectra of BM-40 in the presence and absence of calcium. The spectrum of intact BM-40 (—) was measured in Chelex-treated 10 mM Tris-HCl and 150 mM NaCl, pH 7.4. Concentration of free calcium was less than 10 μM. A decrease of negative ellipticity by 35% at 220 nm was observed (arrow) when EDTA was added to a final total concentration of 1 mM (- - -). This change was completely reversed by addition of calcium to a total concentration of 2 mM (2-fold excess over EDTA). The spectrum of nicked BM-40 purified from 6 M guanidine hydrochloride extracts (···) had a similar shape but smaller amplitude, and the changes upon removal and addition of EDTA were less pronounced (change of about 10% at 220 nm, arrow). The protein concentration was 0.33 mg/mL (11 μM), and the temperature was 20 °C. The spectrum of adult bovine osteonectin extracted from bone (−·−) was taken from Romberg et al. (1985). According to these authors, this spectrum is not influenced by addition of EDTA or calcium.
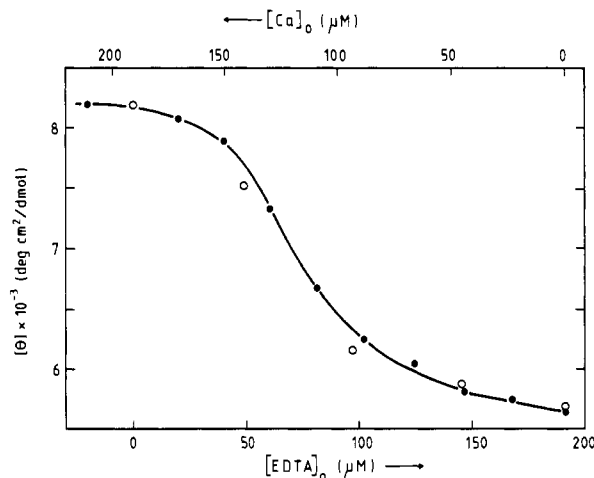

FIGURE 5: Calcium-dependent conformational transition in intact BM-40. The change of ellipticity at 220 nm (see arrow in Figure 4) was monitored as a function of EDTA added to the protein solution (○). At the mean molar residue ellipticity of −5600 deg·cm^2·dmol^{-1} characteristic for the calcium-dependent protein (at 190 μM EDTA), the effect was reversed by successive addition of calcium (upper ordinate, ●). For protein concentration and buffer conditions see legend, Figure 4.

BM-40 was dissolved in a 100 mM NaCl solution containing 1 mM EDTA, and the pH was adjusted by addition of dilute acetic acid. No change of $[\theta]_{220} = -5600$ deg·cm^2·dmol^{-1} was observed between pH 7 and pH 6. When the pH was lowered, however, a sharp sigmoidal increase of negative ellipticity was observed. At pH 5.1 the value was $[\theta] = -6500$. This increase corresponds to one-third of the total change observed upon calcium addition. Between pH 5.1 and pH 4.85 the protein precipitated from the solution but redissolved when the pH was readjusted to pH 5.1.

Phosphorylation. Bovine osteonectin was reported to be phosphorylated (Uchiyama et al., 1986). By incubating PYS
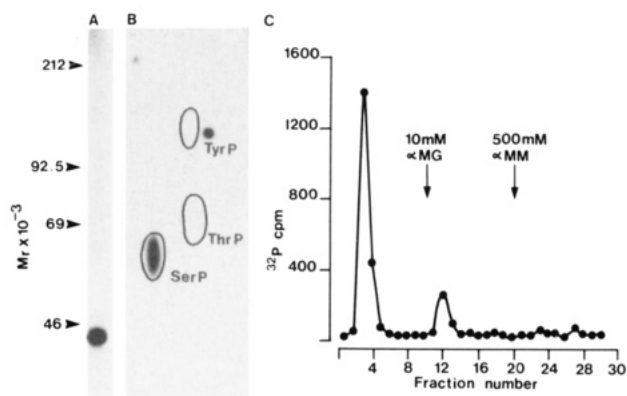
FIGURE 6: Phosphorylation of SPARC secreted by PYS cells. Panel A: PYS cells were incubated with [³²P]orthophosphate for 42 h, and SPARC was immunoprecipitated from the culture medium with anti-peptide serum before analysis by 10% SDS–PAGE under reducing conditions. Panel B: The M_r 43K band shown in panel A was excised and phosphoamino acid analysis carried out as described (Heath et al., 1986). The positions of the internal markers phosphoserine (SerP), phosphothreonine (ThrP), and phosphotyrosine (TyrP) on the thin-layer chromatogram are shown. Autoradiography detected phosphoserine and a trace of phosphothreonine (apparent on original autoradiogram). The spot to the right of the TyrP marker is an artifact. Panel C: Phosphorylation of SPARC glycopeptide. SPARC was immunoprecipitated from the culture medium of PYS cells that were incubated for 42 h with [³²P]orthophosphate. Digestion proceeded for 3 days at 37 °C with 0.4% Pronase in a 50 mM Tris, pH 7.5, 10 mM CaCl₂, and 0.02% NaN₃ buffer. The sample was then chromatographed on a column of concanavalin A–Sepharose as described (Hughes et al., 1987). In this experiment 83% of the radioactivity was eluted from the column in 10 mM Tris, pH 7.5, and 0.1 M NaN₃ (buffer A), while 17% was eluted with 10 mM methyl α-glucoside (αMG) in buffer A. A 500 mM methyl α-mannoside (αMM) concentration released no further peak of radioactivity. The average values over five experiments were 86% and 14%, respectively. In other experiments it was shown that the phosphorylated glycopeptide moieties eluted with 10 mM methyl α-glucoside have an average 12–15 amino acid residue (Hughes et al., 1987).

cells with [³²P]orthophosphate, we also show that SPARC carries this posttranslational modification. Most of the label is recovered in the form of phosphoserine, with only a trace of phosphothreonine and no phosphotyrosine (Figure 6B). The SPARC sequence contains seven serine residues, six of which are in the Cys-rich domain (Figure 1). At present, it is not known whether all of these residues are phosphorylated. However, there is evidence for phosphorylation of at least one residue close to the glycosylation site at Asn-98, on a Pronase-resistant peptide which is bound to concanavalin A–Sepharose and eluted with 10 mM methyl α-glucoside (Figure 6C). From chromatography before and after treatment with endo-F this peptide is estimated to contain between 12 and 15 amino acid residues (Hughes et al., 1987) and would include the sequence DSS (102–104). This sequence and the sequence DSE (139–141) are also found in the protein statherin in which the serines are phosphorylated (Schlesinger & Hay, 1977).

Tryptic Cleavage. Studies with bovine endothelial M_r 43K protein had shown that trypsin cleaved the molecule to a major M_r 30K fragment and smaller peptides after reduction, although the complex migrated as the intact protein on SDS-PAGE under nonreducing conditions (Sage et al., 1984). In contrast, initial tryptic cleavage experiments with SPARC had generated 30K and 10K fragments that dissociated on SDS-PAGE, even under nonreducing conditions [Mason et al. (1986a) and data not shown]. This apparent discrepancy is resolved in the experiment shown in Figure 7 in which milder trypsin cleavage is used. Under these conditions, 30K and 10K fragments held together by one or more disulfide bond(s) were
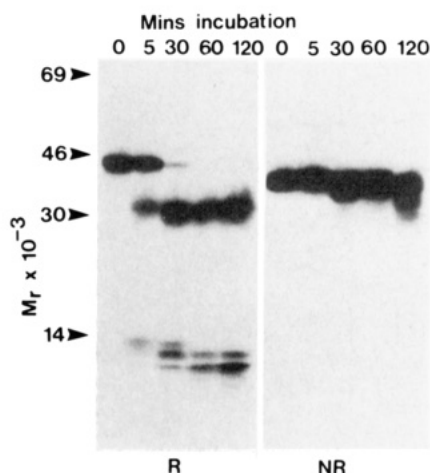

FIGURE 7: Trypsin cleavage of SPARC. [³⁵S]Methionine-labeled SPARC was digested with 0.1 μg of trypsin at 37 °C for 0, 5, 30, 60, and 120 min before analysis on a 10–15% gradient SDS–polyacrylamide gel under reducing (left-hand panel) and nonreducing (right-hand panel) conditions.

obtained. Two lines of evidence suggest that the larger fragment encompasses the Cys-rich domain. First, it contains the glycosylation site of SPARC (Mason et al., 1986a). Second, when this fragment is formed as a result of more extensive trypsin cleavage, its mobility increases under nonreducing conditions (data not shown).

DISCUSSION

SPARC/BM-40/osteonectin contains two potential calcium binding domains, an EF-hand domain at the C-terminus (domain IV) and a glutamic acid rich domain near the N-terminus (domain I). Upon binding of calcium, the protein undergoes a large reversible conformational change corresponding to an increase in α-helicity of about 35%. Comparable transitions have been observed for small cytoplasmic calcium binding proteins which consist of several EF-hand domains. The best studied example is calmodulin [Martin and Bayley (1986) and references cited therein]. Since the EF-hand domain in SPARC/BM-40/osteonectin consitutes only a small fraction of the molecule, the large increase in α-helicity is difficult to explain by a local change in the EF-hand domain alone but is suggestive of a mjaor conformational rearrangement involving, for example, the glutamic acid rich domain I. The two glutamic acid rich sequences in the N-terminal region, in which on average 50% of the residues carry a negative charge, are expected to assume a randomly coiled conformation at physiological pH. This behavior is similar to that of poly(L-glutamic acid), which is randomly coiled at pH values greater than 5.5 and ionic strength 0.2, due to charge repulsion. The onset of the pH-induced coil → α-helix transition observed for BM-40 before it precipitates at pH 4.8–5.0 agrees with that observed for poly(L-glutamic acid) (Wada, 1960; Applequist & Breslow, 1963). Precipitation in this pH range has also been observed for poly(L-glutamic acid) (Schuster, 1965). A coil to α-helix transition of the glutamic acid rich regions in domain I upon binding of calcium would quantitatively account for the observed change in circular dichroism. The observed change in mean molar ellipticity of $[\theta]_{220} = 2700$ deg·cm²·dmol⁻¹ corresponds to a conversion of 25–30 residues from a coiled to an α-helical conformation (Chen et al., 1972), consistent with the total number of 29 residues in the glutamic acid rich clusters in domain I of SPARC.

The pronounced sigmoidicity of the calcium-induced transition curve indicates that more than one Ca²⁺ ion binds and

that the binding and/or the conformational transition are cooperative. Multiple classes of binding sites may exist, and the change in the circular dichroism signal is not necessarily proportional to the degree of saturation. For the equilibrium constants of calcium binding a lower limit of $K > 10^5$ M^{-1} is estimated from the fact that the circular dichroism spectra reminiscent of the liganded state is observed at calcium concentrations as low as 10 μM. Because of the complexity of the titration curve as monitored by circular dichroism only a rough estimate can be obtained for the number of Ca^{2+} ions bound to the protein. Variation of the total EDTA or calcium concentration by 80 μM produces 80% of the total change of the signal at a total protein concentration of 11 μM, which indicates a release or binding of about 7 Ca^{2+} ions. This compares well with an upper limit of 9 which follows from the assumption that at least 2 glutamic acid side chains are needed for the complexing of 1 calcium (Williams, 1977) and that an additional Ca^{2+} ion binds to the EF hand.

No homology was found between domain I and other proteins. However, N-terminal clusters of two glutamic acids flanked by hydrophobic residues are a characteristic feature of the so-called Gla[1] domains in factors VII, IX and X, prothrombin, protein C, and protein S (Hagen et al., 1986; Kurachi & Davie, 1982; Enfield et al., 1978; Magnusson et al., 1975; Fernlund et al., 1978; Dahlbäck et al., 1986a). In these vitamin K dependent proteins of the blood clotting system, a fraction of the Glu residues are γ-carboxylated. However, this modification could not be detected in SPAC/BM-40 from the EHS tumor by using alkaline hydrolysis (Fernlund et al., 1975) followed by separation on an amino acid analyzer (Dahlbäck et al., 1986b). Moreover, the sequence of SPARC lacks the approximately 20 amino acid residues following the signal sequence that are conserved in γ-carboxylated proteins and may act as a recognition signal for the modifying enzyme (Pan & Price, 1985). However, domain I in SPARC/BM-40/osteonectin exhibits a similar number and density of negative charges as the Gla domains. It is therefore of interest to note that, in the crystal structure of fragment 1 of prothrombin, the Gla domain was not resolved in the absence of calcium, a finding consistent with a flexible coiled conformation (Tulinsky et al., 1985). Binding of about 6 Ca^{2+} ions to the Gla domain of prothrombin is cooperative and is accompanied by a conformational transition (Hendriksen & Jackson, 1975; Prendergast & Mann, 1977; Nelsestuen et al., 1981; Deerfield et al., 1986). Calcium binding induces a large increase in α-helicity of prothrombin fragment 1 (Nelsestuen et al., 1981) which is comparable in magnitude to that observed for SPARC/BM-40/osteonectin. Gla domains are also found in bone Gla proteins (or osteocalcins) (Price et al., 1977; Price & Williamson, 1985), and a large coil → α-helix transition is observed for these proteins upon calcium binding (Hauschka & Carr, 1982). No convincing homology exists between these domains and domain I of SPARC, but the SPARC sequence VCELDES (64-70) is similar to the sequence VCELNVS (18-24) in swordfish bone Gla protein in which a fractional γ-carboxylation of Glu (E) has been observed (Price et al., 1977).

In the blood clotting proteins the Gla domain is linked to a C-terminal functional domain by a disulfide-rich domain. This general feature is shared by SPARC/BM-40/osteonectin, but the disulfide-rich domain II is not homologous with either the kringle domains of prothrombin or the EGF-like domains of the clotting factors and protein C but represents a new Cys-rich motif. There is homology of 30-42% identity in 24 residue long overlaps between part of the disulfide-rich domain

II of the SPARC sequence (positions 83-107) and the third domain of ovomucoids (Laskowski et al., 1987). Most interestingly, the homologous region overlaps with the active site region of these PST 1 type protease inhibitors. In addition, two clusters of positively charged residues in the Cys-rich domain of SPARC may be important for an interaction with the negatively charged domain I. Other possible functions are interaction with heparin, which was recently proposed for clusters of similar size in thrombospondin (Lawler & Hynes, 1986), or an involvement in lipid or phosphate binding.

The C-terminal domain IV contains a prominent EF-hand calcium binding domain. This second calcium binding domain meets the standard criteria for EF-hand motifs in many cytoplasmic calcium binding proteins except that a normally conserved glycine is replaced by a lysine. An EF-hand calcium binding domain has been detected in another extracellular protein, fibrinogen (Dang et al., 1985). In this case, lysine is again observed in the same position as in the EF-hand motif of SPARC. A feature that lends additional support to the assignment of domain IV as an EF-hand structure is the putative disulfide bond between Cys-255 and Cys-271 suggested by the three-dimensional model. Substitutions of large hydrophobic residues by Cys are rather frequent. It is interesting to note that in the EF hand of the cytoplasmic protein sorcin (Van der Blick et al., 1986) Cys also occurs in the position that corresponds to Leu-69 in intestinal calcium binding protein. Disulfide stabilization has also been proposed for the putative calcium binding domains in thrombospondin (Lawler & Hynes, 1986). In this case, the polypeptide chain may take on a structure other than the EF hand, since the sequence adjacent to the aspartic acid rich segment are not compatible with α-helices.

A single isolated EF-hand domain as observed for SPARC is unusual. In most cases several EF-hand motifs are repeating in the sequence (Kretsinger, 1979, 1980) and pairwise packing is observed in three-dimensional structures (Szebinyi & Moffat, 1986). The only case in which an isolated EF-hand-like calcium binding domain was described is that of α-lactalbumin (Stuart et al., 1986). Often, however, some of the domains have lost the features of a classical EF hand. We have looked for a second and perhaps degenerate EF hand in the sequence of SPARC but did not find a convincing candidate. Moreover, there is no homology between the nucleotide sequence of the penultimate exon of the mouse SPARC gene, which codes for amino acids 228-278, including the entire EF hand, and the remaining cDNA sequence (J. H. McVey and B. L. M. Hogan, unpublished results).

The single high-affinity calcium binding site in osteonectin that was found by fluorescence titration (Romberg et al., 1985) may be tentatively attributed to the EF-hand domain. Tryptophan-268 located in this domain is a likely source of the fluorescence signal. The osteonectin solubilized by 0.5 M EDTA from bone which was used by Romberg et al. (1985), however, exhibited very little or no α-helicity. Moreover, these authors observed no change in circular dichroism even after addition of 10 mM calcium to the metal-depleted protein. This result is in striking contrast to the observations for SPARC/BM-40 in the present study. A possible explanation is that part of the native conformation of the osteonectin was lost during extraction. It has been observed by us that extraction of BM-40 by 6 M guanidine hydrochloride leads to a decrease of α-helicity and a diminished response to calcium.

At present, the precise function of SPARC/osteonectin/BM-40 is not known. Originally, it was suggested that bovine osteonectin plays a role in initiating matrix mineralization in

bone and teeth by binding to type I collagen and hydroxylapatite (Termine et al., 1981a,b). Studies by a number of laboratories using different techniques have provided evidence for osteonectin/BM-40/SPARC protein and/or messenger RNA expression by a wide variety of nonmineralized tissues (Wasi et al., 1984; Sage, 1985; Mason et al., 1986b; Tung et al., 1985; Young et al., 1986; Dziadek et al., 1986; Holland et al., 1987). Among other things, these results establish a correlation between high levels of SPARC expression and synthesis of basement membrane components (laminin, collagen IV, etc.). These observations clearly demand a reassessment of the original role proposed for osteonectin. One possibility, based on the ability of low concentrations of osteonectin to delay hydroxylapatite-seeded crystal growth (Romberg et al., 1985), is that the protein plays a role in preventing rather than promoting matrix mineralization. This function may be important, for example, in tissues where there may be a risk of ectopic calcification (Russell et al., 1986) and during the early stages of fetal bone morphogenesis when a balance presumably needs to be struck between de novo assembly of the type I collagen fibrils in the matrix and deposition of hydroxylapatite crystals. As osteocytes and bone mature, SPARC/BM-40/osteonectin expression declines (Holland et al., 1987), a process that might allow crystal growth to proceed in an orderly way.

Another possibility is that the large conformational transition induced in SPARC/BM-40 by the binding of several Ca^{2+} ions is involved in some regulatory function involving or mediated by either domains within the same molecule or interaction with other proteins. Intramolecular regulation is exemplified by the blood clotting proteins, which show calcium-mediated binding to lipids and a modulation of the protease activity by conformational changes in the Gla domain (Jackson & Nemerson, 1980). There are numerous studies that emphasize the requirement of an intact and native Gla domain for these functions [Hendriksen and Jackson (1975) and Morita and Jackson (1986) and references cited therein]. Calcium binding to the EF-containing domain of fibrinogen is believed to regulate fibrin polymerization, binding of fibrinogen to platelet membranes, and cross-linking with tissue factor (Dang et al., 1985). Less clearly understood is the functional significance of the large calcium-induced changes in the dimensions of thrombospondin (Dixit et al., 1986).

Troponin C, calmodulin, and other cytoplasmic EF-hand-containing proteins mediate the effect of calcium to other proteins with which they interact, and some proteins of this class also act as buffer proteins for calcium (Kretsinger, 1979, 1980). By analogy, SPARC/BM-40/osteonectin may exert similar functions in the extracellular space in which some processes, for example, the assembly of laminin (Yurchenco et al., 1985; Paulsson et al., 1987) and collagen fibril formation (Drouven & Evans, 1986), are calcium-dependent. Studies are under way to estimate the ability of SPARC/BM-40 to bind to components of the extracellular matrix, to phospholipids, and to cell surfaces and to measure the binding constants and number of metal ions bound to the protein and their dependence on phosphroylation. Furthermore, in view of the homology with ovomucoids, the possibility of a protease inhibitor function is being explored.

REFERENCES

Applequist, J., & Breslow, J. L. (1963) J. Am. Chem. Soc. 85, 2869.

Chen, Y. H., Yang, Y. T., & Martinez, H. M. (1972) Biochemistry 11, 4120–4131.

Chou, P. Y., & Fasman, G. P. (1974) Biochemistry 13, 222–226.

Cooper, A. R., Kurkinen, M., Taylor, A., & Hogan, B. L. M. (1981) Eur. J. Biochem. 119, 189–197.

Dahlbäck, B., Lundwall, A., & Stenflo, J. (1986a) Proc. Natl. Acad. Sci. U.S.A. 83, 4199–4203.

Dahlbäck, B., Lundwall, A., & Stenflo, J. (1986b) J. Biol. Chem. 261, 5111–5115.

Dang, C. V., Ebert, R. F., & Bell, W. R. (1985) J. Biol. Chem. 260, 9713–9719.

Dayhoff, M. O., & Baker, W. C. (1972) in Atlas of Protein Sequence and Structure, National Biomedical Research Foundation, Washington, DC.

Deerfield, D. W., Berkowitz, P., Olson, D. L., Wells, S., Hoke, R. A., Koehler, K. A., Pedersen, L. G., & Hiskey, R. G. (1986) J. Biol. Chem. 261, 4833–4839.

Dixit, V. M., Galvin, N. J., O'Rourke, K. M., & Frazier, W. A. (1986) J. Biol. Chem. 261, 1962–1968.

Doolittle, R. F. (1985) Trends Biochem. Sci. (Pers. Ed.) 10, 233–237.

Drouven, B. J., & Evans, C. H. (1986) J. Biol. Chem. 261, 11792–11797.

Dziadek, M., Paulsson, M., Aumailley, M., & Timpl, R. (1986) Eur. J. Biochem. 161, 455–464.

Enfield, D. L., Ericsson, L. H., Fujikawa, K., Walsh, K. A., Neurath, H., & Titani, K. (1980) Biochemistry 19, 659–667.

Fernlund, P., Stenflo, J., Roepstorff, P., & Thomsen, J. (1975) J. Biol. Chem. 250, 6125–6133.

Fernlund, P., Stenflo, J., & Tufvesson, A. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 5889–5892.

Fullmer, C. S., & Wassermann, R. H. (1981) J. Biol. Chem. 256, 5669–5674.

Hagen, F. S., Gray, C. L., O'Hara, P., Grant, F. J., Saari, G. C., Woodbury, R. G., Hart, C. E., Insley, M., Kisiel, W., Kurachi, K., & Davie, E. W. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 2412–2416.

Hauschka, P. V., & Carr, S. A. (1982) Biochemistry 21, 2538–2547.

Heath, J. K., Mahadevan, L., & Foulkes, J. G. (1986) EMBO J. 5, 1809–1814.

Hendriksen, R. A., & Jackson, C. M. (1975) Arch. Biochem. Biophys. 170, 149–159.

Hogan, B. L. M., Barlow, D. P., & Kurkinen, M. (1984) Ciba Found. Symp. 108, 60–74.

Holland, P. W. H., Harper, S. J., McVey, J. H., & Hogan, B. L. M. (1987) J. Cell Biol. (in press).

Hughes, R. C., Taylor, A., Sage, H., & Hogan, B. L. M. (1987) Eur. J. Biochem. 163, 57–65.

Jackson, C. M., & Nemerson, Y. (1980) Annu. Rev. Biochem. 49, 765–811.

Jones, T. A. (1978) J. Appl. Crystallogr. 11, 268–272.

Kretsinger, R. H. (1979) Adv. Cyclic Nucleotide Res. 11, 1–26.

Kretsinger, R. H. (1980) CRC Crit. Rev. Biochem. 8, 119–174.

Kurachi, K., & Davie, E. W. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 6461–6464.

Laskowski, M., Jr., Ikunoshin, K., Ardelt, W., Cook, J., Denton, A., Empie, M. W., Kohr, W. J., Park, S. J., Parks, K., Schatzley, B. L., Schoenberger, O. L., Tashiro, M., Vichot, G., Whatley, H. E., Wieczorek, A., & Wieczorek, M. (1987) *Biochemistry 26*, 202–221.

Lawler, J., & Hynes, R. O. (1986) *J. Cell Biol. 103*, 1635–1648.

Lehman, J. M., Speers, W. C., Swarzendruber, D. E., & Purie, G. B. (1974) *J. Cell. Physiol. 84*, 13–28.

Magnusson, S., Sottrup-Jensen, L., Petusen, T. E., & Claeys, H. (1975) in *Boerhaave Symposium on Prothrombin and Related Coagulation Factors* (Hemker, H. C., & Veltkamp, J. J., Eds.) pp 25–46, Leiden University Press, Leiden.

Mann, K., Deutzmann, R., Paulsson, M., & Timpl, R. (1987) *FEBS Lett.* (in press).

Martin, S. R., & Bayley, P. M. (1986) *Biochem. J. 238*, 485–490.

Mason, I. J., Taylor, A., Williams, J. G., Sage, H., & Hogan, B. L. M. (1986a) *EMBO J. 5*, 1465–1472.

Mason, I. J., Murphy, D., Kunke, M., Francke, U., Elliott, R. W., & Hogan, B. L. M. (1986b) *EMBO J. 5* 1831–1837.

Morita, T., & Jackson, C. M. (1986) *J. Biol. Chem. 261*, 4015–4023.

Nelsestuen, G. L., Resnick, R. M., Wei, G. J., Pletcher, C. H., & Bloomfield, V. A. (1981) *Biochemistry 20*, 351–358.

Pan, L. C., & Price, P. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6109–6113.

Paulsson, M., Aumailley, M., Deutzmann, R., Timpl, R., Beck, K., & Engel, J. (1987) *Eur. J. Biochem. 166*, 11–19.

Prendergast, F. G., & Mann, K. G. (1977) *J. Biol. Chem. 252*, 840–850.

Price, P. A., & Williamson, M. K. (1985) *J. Biol. Chem. 260*, 14971–14975.

Price, P. A., Otsuka, A. S., & Poser, J. W. (1977) in *Calcium Binding Proteins and Calcium Function* (Wassermann, R. H., Corradino, R. A., Carafoli, E., Kretsinger, R. H., MacLennan, D. H., & Sigel, F. L., Eds.) pp 333–337, North-Holland, New York.

Romberg, R. W., Werness, P. G., Lollar, P., Lawrence Riggs, B., & Mann, K. G. (1985) *J. Biol. Chem. 260*, 2728–2736.

Russell, R. G. G., Caswell, A. M., Hearn, P. R., & Sharrard, R. M. (1986) *Br. Med. Bull. 42*, 435–446.

Sage, H. (1985) *Biochemistry 24*, 7430–7440.

Sage, H., Johnson, C., & Bornstein, P. (1984) *J. Biol. Chem. 259*, 3993–4007.

Schlesinger, D. H., & Hay, D. I. (1977) *J. Biol. Chem. 252*, 1689–1695.

Schuster, T. M. (1965) *Biopolymers 3*, 681–684.

Stuart, D. I., Acharya, K. R., Walker, N. P. C., Smith, S. G., Lewis, M., & Phillips, D. C. (1986) *Nature (London) 324*, 84–87.

Szebinyi, D. M. E., & Moffat, K. (1986) *J. Biol. Chem. 261*, 8761–8777.

Szebinyi, D. M. E., Obendorf, S. K., & Moffat, K. (1981) *Nature (London) 244*, 327–332.

Termine, J. D., Kleinman, H. K., Whitson, S. W., Conn, K. M., McGarvey, J. L., & Martin, G. R. (1981a) *Cell (Cambridge, Mass.) 26*, 99–105.

Termine, J. D., Belcourt, A. B., Conn, K. M., & Kleinman, H. K. (1981b) *J. Biol. Chem. 256*, 10403–10408.

Timpl, R., Paulsson, M., Dziadek, M., & Fujiwara, S. (1987) *Methods Enzymol. 145*, 363–391.

Tufvesson, A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5889–5892.

Tulinsky, A., Park, C. H., & Rydel, T. J. (1985) *J. Biol. Chem. 260*, 10771–10778.

Tung, P. S., Domenicucci, C., Wasi, S., & Sodek, J. (1985) *J. Histochem. Cytochem. 33*, 531–540.

Uchiyama, A., Suzuki, M., Lefteriou, B., & Glimcher, M. (1986) *Biochemistry 25*, 7572–7583.

Van der Blick, A. M., Meyers, M. B., Biddler, J. L., Hes, E., & Borst, P. (1986) *EMBO J. 5*, 3201–3208.

Wada, A. (1960) *Mol. Phys. 3*, 409–411.

Wasi, S., Otsuka, K., Yao, K.-L., Tung, P. S., Aubin, J. E., & Sodek, J. (1984) *Can. J. Biochem. Cell Biol. 62*, 470–477.

Williams, R. J. P. (1977) in *Calcium Binding Proteins and Calcium Function* (Wassermann, R. H., Corradino, R. A., Carafoli, E., Kretsinger, R. H., MacLennan, D. H., & Sigel, F. L., Eds.) pp 3–11, North-Holland, New York.

Young, M. F., Bolander, M. E., Day, A. A., Ramis, C. I., Robey, P. G., Yamada, Y., & Termine, J. D. (1986) *Nucleic Acids Res. 14*, 4483–4497.

Yurchenco, P. D., Tsilibary, E. C., Charonis, A. S., & Furthmayr, H. (1985) *J. Biol. Chem. 260*, 7636–7644.