A Defective Cell Surface Collagen-binding Protein in Dermatosparactic Sheep Fibroblasts

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Abstract. Fibroblasts from dermatosparactic sheep fail to contract collagen gels and show a reduced attachment to collagenous substrates. By comparing collagen-binding membrane proteins of normal (+/+) homozygote (−/−), and heterozygote (+/−) fibroblasts, we present evidence that the interaction of normal fibroblasts with native type I collagen involves a protein of apparent Mr = 34,000 which is absent from dermatosparactic fibroblasts and seems to be related to anchorin CII. This conclusion was reached from the following experiments: (a) On a blot of membrane proteins from normal fibroblasts radioactively labeled type I collagen bound predominantly to a protein band of 34 kD; dermatosparactic membranes revealed only a small amount of binding to a component with a molecular mass of 47 kD. (b) After separation of normal fibroblast membrane proteins on type I collagen-Sepharose, a collagen-binding component of 34 kD was found which was absent from the corresponding fraction of dermatosparactic membranes. (c) Antibodies to anchorin CII stained the surface of normal (+/+) fibroblasts and labeled a 34-kD component after immunoblotting of normal fibroblast membrane proteins. (d) After metabolic labeling of fibroblasts with [35S]methionine and immunoprecipitation with anti-anchorin CII, 40- and 34-kD components were precipitated from extracts of normal fibroblasts, while the latter component was absent from affected cells.

Similar differences were found after immunoblotting of membranes from whole normal or affected skin. These data indicate that dermatosparaxis of sheep involves a molecular defect of a collagen-binding protein. Therefore this disease represents a model to study the complex interaction of cells with the extracellular matrix on a molecular level.

Interactions of cells with the surrounding extracellular matrix are a prerequisite for the normal functioning of connective tissue. They play a major role in the control of cellular activities in development, tissue repair and wound healing. Attempts to elucidate mechanisms and to identify molecules involved in cell-matrix interaction have been hampered by the fact that cells probably interact with the matrix components by multiple mechanisms (for review see Yamada et al., 1985, Ruoslahti et al., 1985). Adhesion of most mesenchyme-derived cells to native and denatured collagen is mediated by fibronectin (for review see Yamada and Akiyama, 1983; Kleinman et al., 1981) and a receptor for fibronectin could recently be identified (Pytel et al., 1985; Hasagawa et al., 1985; Tamkun et al., 1986).

However, direct binding of cells to native collagen via cell surface receptors has been reported (Goldberg, 1979; Grinnell and Minter, 1977; Linsenmayer et al., 1978) and a 68-kD collagen receptor was identified on platelet membranes (Chiang and Kang, 1982). Dedhar et al. (1987) have isolated a collagen-binding protein complex from fibroblast membranes on the basis of its affinity for the cell-binding peptide RGDT, incorporated into a triple-helical (Gly-Pro-X), collagen-like fragment. Alternative mechanisms of collagen binding include also a membrane-intercalated heparan sulfate proteoglycan with affinity for native type I, III, and V collagen (Koda et al., 1985), and anchorin CII which is a type II collagen-binding protein of 34 kD originally isolated from membranes of chicken chondrocytes (Mollenhauer and v.d. Mark, 1983).

The study of inherited connective tissue disorders has had an important impact on the understanding of collagen structure and biosynthesis as well as for identifying the biological role of various posttranslational modifications (Prockop and Kivirikko, 1984). Similarly, the search for defective cell adhesion molecules offers a potential model to shed light on the complex mechanism of cell-matrix interaction on a molecular level.

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Dermatosparaxis, an autosomal recessively inherited disease of connective tissue in calf and sheep is characterized by extreme fragility of the skin (Hanset, 1971; O'Hara et al., 1970; Fjælstad and Helle, 1974). The affected animals usually die shortly after birth due to infection of the extensive cutaneous wounds. In dermatosparactic cattle lack of a specific procollagen N-proteinase has been found to be responsible for the accumulation of a collagen precursor in the dermis (Piérad and Lapierre, 1973; Lapierre et al., 1971). This results in the formation of abnormal collagen fibrils owing to steric hindrance by the N-peptide (Bailey and Lapierre, 1973). Similar alterations have been observed in sheep skin, where pN collagen accumulates (Becker et al., 1976, 1977; Rohde et al., 1976; Wick et al., 1978). However, here defective activity of the pN-proteinase has never been directly demonstrated.

Recently, it has been shown that fibroblasts from dermatosparactic animals also differ from healthy controls in their interaction with extracellular matrix proteins. Dermatosparactic fibroblasts of calf and sheep (Delvoye et al., 1985, 1986) fail to contract collagen gels, while normal fibroblasts seeded in a collagen lattice retracted until a dense collagenous matrix is formed. In monolayer culture the cells show an epithelioid morphology, and in a previous study we have shown that fibroblasts of dermatosparactic sheep and calves attach less well to collagens I and IV than normal controls whereas the attachment to non-collagenous substrates (fibronectin, laminin) was unaltered (Mauch et al., 1986).

In the present study we have investigated differences in the collagen-binding cell surface proteins of normal and dermatosparactic sheep fibroblasts with biochemical and immunochromical techniques. We present evidence that the defective interaction of dermatosparactic fibroblasts with collagen results from a defect in a collagen-binding protein related to anchorin CII.

**Materials and Methods**

**Cell Culture and Metabolic Labeling**

Cultures of fibroblasts from homozygote dermatosparactic (+/−), heterozygotes (+/−), and normal (+/+ ) sheep were established as described previously (Wiestner et al., 1982). Biopsies were taken from the back skin of the Republic of Germany, and 10% FCS (Gibco).

**Preparation of Plasma Membranes**

8.5% succrose-TEA buffer, and pelleted by centrifugation at 25,000 g for 1 h, resuspended in 8.5% succrose-TEA buffer and separated by centrifugation on a discontinuous sucrose gradient of 8.5, 17, and 40% sucrose at 130,000 g for 3 h. Under these conditions the membranes were enriched at the 17/40% interface (Cates and Holland, 1978).

Membranes were then pelleted again by centrifugation at 25,000 rpm for 1 h to remove the sucrose, and resuspended in 10 mM TEA and stored at −20°C until use.

Plasma membranes were also prepared from authentic skin material. Hairs were removed with a razor, and the skin was cut into small pieces, homogenized in 8.5% succrose-TEA buffer with an ultraturrax homogenizer (Jance and Kunekl, FRG), and centrifuged at 1,500 g. This procedure was repeated three times, and from the homogenate membranes were isolated as described above.

**Binding Assays**

Binding of Collagen I to Plasma Membranes in Suspension. Pepsin-treated collagen I from calf skin was prepared according to Weber et al. (1984) and labeled with 32P (sp act 600 MBq/μg) by the chloramin-T procedure (Greenwood et al., 1963) to a specific radioactivity of 107 cpm/μg protein. 0.1 ml of plasma membranes, resuspended in 10 mM TEA-buffer were incubated for 2 h at 25°C with 107 cpm of [32P]collagen. After incubation the mixture was centrifugated on a sucrose gradient (40:7:8.5:−4:4:3 ml) to separate membrane-bound from unbound [32P]collagen. The membranes were collected at the 17/40% interphase while free collagen remained on top of the gradient. The gradient was eluted drop-wise from the bottom, and the fractions were counted for radioactivity (Mollenhauer and v.d. Mark, 1983).

**Affinity Chromatography**

50 mg acid soluble, native I collagen from calf skin was coupled to 50 ml of CNBr-activated Sepharose 4B as described earlier (v.d. Mark et al., 1976).

Plasma membranes suspended in 10 mM TEA-buffer containing 0.1% NP40 were sonicated and applied to a collagen-Sepharose column which had been equilibrated in the same buffer. After a 50 mM NaCl step, the column was eluted with a linear salt gradient (0.05-0.5 M NaCl) over a total volume of 400 ml. The fractions were collected, pooled, dialyzed against 10 mM TEA-buffer and kept frozen or analyzed by SDS-PAGE.

**Protein Blotting of Plasma Membranes**

Membrane proteins were subjected to electrophoresis on 12% PAGE containing 0.1% SDS, electroblotted to nitrocellulose membrane according to Towbin et al. (1979). Strips carrying transferred proteins were incubated with [1H]proline-labeled collagen (107 cpm in 2.5% BSA-TBS). After extensive washing strips were cut in 2.0-mm pieces, and bound radioactivity was determined.

**Immunochromical Methods**

**Immunofluorescence.** Antiserum against chick anchorin CII was raised in rabbits as described earlier (Mollenhauer et al., 1984). Fibroblasts in monolayer culture were washed with PBS and incubated unfixed with anti-anchoirn antisemur as described previously (Dessau et al., 1981). Cells were washed three times with PBS and stained with FITC-conjugated anti-rabbit antibodies. Cells were photographed with a Zeiss inverted microscope (ICM 405).

**Immunoprecipitation.** 35S-labeled cell extract (0.5 ml) was preincubated with Staphylococcus aureus for 30 min on ice. After centrifugation the supernatant was incubated for 2 h with 10 μl rabbit anti-anchoirn antisemur (25°C). Anchorin was isolated from chicken cartilage and the antisemur prepared as described earlier (Mollenhauer et al., 1984). The antigen-antibody complex was precipitated with Staphylococcus aureus according to Kessler et al. (1976) and the proteins were analyzed by electrophoresis on a 12% polyacrylamide slab gel. The gels were treated for autoradiography according to Bonner and Lasky (1974).

**Immunoblot.** Fibroblast membrane proteins were separated on a 12% SDS polyacrylamide slab gel and electroblotted onto nitrocellulose paper (BioRad Laboratories, Richmond, CA; 0.2 μm). Nitrocellulose sheets were incubated with anti-anchoirn CII and the protein bands visualized by the peroxidase method according to Towbin et al. (1979).

**PAGE.** One-dimensional slab-gel electrophoresis in 0.1% SDS was carried out on 12% polyacrylamide gels according to the method of Laemmli.

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1. Abbreviations used in this paper: BSA-TBS, bovine serum albumin in Tris-buffered saline; TEA, triethanolamine.
Mauch et al. Collagen-binding Proteins in Dermatosparaxis 207

Results

Binding of Plasma Membranes to Collagen

Plasma membranes were prepared from cultured normal (+/+) and dermatosparactic (-/-) fibroblasts and used in a suspension assay to estimate the binding of 125I-labeled collagen I. About 42% of the total labeled collagen was bound by normal and also by heterozygous fibroblast membranes, whereas the binding of collagen to plasma membranes isolated from fibroblasts obtained from dermatosparactic cells was severely reduced to 10% (Fig. 1).

To characterize collagen-binding proteins present in fibroblast membranes, membrane preparations were analyzed by SDS–PAGE, and subsequently blotted onto nitrocellulose paper. After incubation of strips with native 3H-labeled type I collagen, specific binding to a protein band of ~34 kD was found in cell membranes from normal sheep fibroblasts (+/+) (Fig. 2). In contrast, after incubation of cell membranes isolated from dermatosparactic cells with radiactively labeled collagen I the binding to proteins in the 34-kD range was insignificant, however, binding was obtained in the Mr range of 47–50 kD which was not observed in membranes obtained from control fibroblasts (+/+). Altogether, the amount of radioactive collagen bound to dermatosparactic membrane proteins (-/-) was much lower than to membrane proteins isolated from control fibroblasts (+/+).

Purification of Collagen-binding Proteins from Fibroblasts after Metabolic Labeling

Normal (+/+) and dermatosparactic (-/-) fibroblasts were metabolically labeled with [35S]methionine and the plasma membranes chromatographed on a native type I collagen-Sepharose column. Elution of bound proteins with a linear salt gradient revealed a similar pattern for both cell strains (Fig. 3). The material eluting from the column was then
Figure 3. Purification of collagen-binding proteins after metabolic labeling by affinity chromatography on type I collagen-Sepharose. Membranes were solubilized in 0.1% NP40 in 10 mM TEA buffer, pH 7.4, and passed over a type I collagen-coupled Sepharose 4B. After washing the column with 0.05 M NaCl in TEA-NP40 buffer, bound proteins were eluted with a linear 0.05–0.5 M NaCl gradient over a total volume of 400 ml. The peaks were then analyzed in a 12% SDS acrylamide gel. Peak II of normal and dermatosparactic membranes differed in the presence of a 34-kD protein band (inset in Fig. 3).

Figure 4. Immunoblotting of dermatosparactic (a), chick chondrocyte (b), and normal sheep fibroblasts (c) membrane proteins with anti-anchorin CII serum. Plasma membranes were separated by SDS-gel electrophoresis and transferred to nitrocellulose by electroblotting. Strips were incubated with anti-anchorin CII serum, bound antibody was stained with peroxidase-conjugated goat anti-rabbit immunoglobulin. Most protein bands were present in both the dermatosparactic (−/−) and control (+/+) fibroblasts. However, one protein with a molecular mass of 34 kD, present in normal membranes in small amounts, was missing in dermatosparaxis and could also not be detected in other peaks. No difference in the protein pattern of peak I and III could be observed between normal and dermatosparactic fibroblasts (not shown).

Identification of the 34-kD Band as an Anchorin-related Protein

For further identification and characterization of the 34-kD protein cell membranes of chicken chondrocytes, normal, and dermatosparactic sheep fibroblasts were separated by SDS–PAGE and analyzed in a Western blot for reactivity with anti-anchorin. As shown in Fig. 4 a protein band of 34 kD reacted with the antibodies in chondrocytes (Fig. 4 b) as well as in normal chicken (not shown) and sheep fibroblasts (Fig. 4 c). An additional band of 36–38 kD was found in the fibroblast preparation (Fig. 4 c). In dermatosparactic fibroblasts, however, no protein band could be detected by the antibodies (Fig. 4 a).

Using the anti-anchorin CII antiserum reacting with the 34 kD protein of chicken and sheep plasma membranes the staining pattern of normal and dermatosparactic fibroblasts was then investigated by indirect immunofluorescence. Normal sheep fibroblasts showed a punctate surface fluorescence...
Figure 7. Immuno blot of collagen-binding proteins from normal (N) and dermatosparactic (D) sheep skin. Collagen-binding proteins of peak II (see Fig. 3) obtained after chromatography of plasma membrane proteins on type I collagen-coupled Sepharose 4B were separated on a 12% SDS-polyacrylamide slab gel, and stained. Parallel samples were then transferred to nitrocellulose and incubated with anti-anchorin CII. While the silver staining still demonstrated a complex pattern which was similar for membrane proteins isolated from normal and dermatosparactic sheep only a few bands with a Mr of 34,000–38,000 were visualized in the immunoblot in normal (b), but not in affected skin (d).

Discussion

Specific interactions of fibroblasts with components of the extracellular matrix are a requirement for normal development, wound healing, and the maintenance of the macro-molecular organization of connective tissue. Recently, we have shown that skin fibroblasts from sheep with dermatosparaxis, an inherited disease characterized by fragile skin, adhere poorly to collagenous substrates but normally to laminin and fibronectin (Mauch et al., 1986). In the present study we demonstrate that the impaired adhesiveness of
dermatosparactic fibroblasts to collagen is reflected by a marked reduction in the binding of their membranes to radioactively labeled collagen, and that this defect is associated with the absence of a collagen-binding protein of \( M_r = 34,000 \).

This protein is present in normal sheep fibroblasts and is related to anchoring CII, a collagen-binding protein which was originally isolated from chondrocyte membranes (Mollenhauer and von der Mark, 1983; Mollenhauer et al., 1984) but recently found also in other connective tissue cells (von der Mark et al., 1984, 1985, 1986). The conclusion is based on the following observations: (a) In an overlay assay using membranes of normal fibroblasts (+/+ ) a significant collagen-binding activity could be assigned to a 34-kD component. (b) A protein band in this molecular mass range was recognized by an antiserum to chicken anchorin CII in Western blot analysis, and a 34-kD protein was immunoprecipitated from metabolically labeled normal fibroblasts with the same antiserum. The 34-kD component was virtually absent in homozygous dermatosparastic animals (−/−). Instead, blotted membrane proteins from homozygous fibroblasts (−/−) showed some binding of \(^{3}H\)collagen to a band of apparent molecular mass of 47–50 kD; since a collagen-binding component of that size was not found in the overlay binding assay with normal fibroblast membranes, the possibility existed that the genetic defect in dermatosparastic animals resulted in a modification in size of the 34 kD protein to a 47 kD protein. However, anti–anchorin serum did not recognize a component of this molecular mass range in membranes of dermatosparastic fibroblasts (−/−), neither by immunoblotting nor by immunoprecipitation of metabolically labeled membranes, indicating that the 47-kD protein is not related to the 34-kD protein. Whether it represents collagen, a collagen-binding heat-shock protein (Kurkinen et al., 1984; Nagata et al., 1986) remains questionable in view of the recent localization of collagen to the endoplasmic reticulum (Hughes et al., 1987).

In contrast to chick chondrocytes, chick and sheep fibroblast membranes seem to contain additional collagen-binding proteins of approximate molecular masses of 38 and 40 kD which bind to type I collagen–Sepharose and coelute with the 34-kD component. Proteins of the same size are also present in rat tumor cells (Wirl, G., M. Pffaffle, and K. v. d. Mark, manuscript in preparation). They probably represent the Ca2+- and phospholipid-binding protein P39 (calpactin) (Saris et al., 1986; Glenney et al., 1987), or related representatives of this protein family, which are homologous to anchorin CII and show immunological crossreactivity (Pffaffle, M., and K. v. d. Mark, manuscript in preparation).

The question remained open whether the defect in the 34-kD protein in dermatosparastic fibroblasts is restricted to cell culture conditions for a selected population of fibroblasts, or whether it is a systemic effect. However, immunoblotting of membrane proteins extracted from normal skin confirmed the presence of the 34-kD protein in normal and its absence from dermatosparastic animals.

Our findings suggest a correlation between the deficiency in the 34-kD protein in dermatosparastic fibroblasts and their inability to attach to collagen (Mauch et al., 1986) and to contract collagen gels (Delvoye et al., 1985, 1986). However, the question remains whether the 34-kD protein is exclusively responsible for the anchorage of normal fibroblasts in the extracellular collagen matrix and whether it is a requirement for the contraction of collagen gels. Attempts to inhibit collagen gel contraction of normal fibroblasts by anti–anchorin antibodies have failed so far, which may be due to internalization or inactivation of the antibody during the culture period of several days or due to competitive cell interaction with collagen via serum fibronectin or an RGDbinding protein complex described by Dedhar et al. (1987).

The fragility of dermatosparastic skin has been ascribed to the formation of defective collagen fibrils, owing to an accumulation of pN-collagen (Bailey et al., 1973, Lapierre et al., 1971, Becker et al., 1976, 1977, Rohde et al., 1976, Wick et al., 1978), which was thought to result from a lack in the pN-collagen proteinase in the affected animals. However, there is indication that the situation is more complex (Shinkai and Lapierre, 1983). Although Minor et al. (1986) recently demonstrated that there is some pN-proteinase activity in the medium of cultured cells, it is also possible that processing of procollagen on the cell surface requires a close interaction between pN-collagen proteinase and collagen-binding proteins such as the 34-kD protein. Accumulation of pN-collagen in the dermatosparastic skin could therefore result from a defect of the pN-proteinase as well as in collagen-binding proteins which could function as “presenter” of procollagen to the enzyme.

It remains to be seen whether the defect in pN-proteinase and the 34-kD protein are primary defects, or a reflection of more pleiotropic alterations of the fibroblasts present in dermatosparastic skin. Heterogeneities of the molecular defects involving the processing of procollagen have already been reported in the Ehlers-Danlos syndrome type VII, where a defect of the pN-proteinase, as well as structural modifications of the pro \( \alpha_1 \)-chain have been demonstrated (Lichtenstein et al., 1973, Minor et al., 1986, Steinmann et al., 1980). Thus, both lack of pN-collagen proteinase and 34-kD protein may represent only two out of many phenotypic alterations caused by the genetic defect in dermatosparaxis.

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