

Two Type XII-like Collagens Localize to the Surface of Banded Collagen Fibrils

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Abstract. Two recently identified collagen molecules, termed twelve-like A and twelve-like B (TL-A and TL-B) have properties similar to type XII collagen. These molecules have been localized in human and calf tissues by immunoelectron microscopy. The observations strongly suggest that both molecules are located along the surface of banded collagen fibers. The epitopes recognized by the antibodies are contained in large, nontriple-helical domains at one end of the collagen helix. The epitopes are visualized at a distance from the surface of the banded fibers roughly equal to the length of the nonhelical domains, suggesting that the nonhelical domains extend from the fibril, while the triple-helical domains are likely to bind directly to the fibril surface. Occasionally, both TL-A and TL-B

demonstrate periodic distribution along the fibril surface. The period corresponds to the primary interband distance of the banded fibrils. Not all fibrils in a fiber bundle are labeled, nor is the labeling continuous along the length of labeled fibrils. Simultaneous labeling of TL-A and type VI collagen only rarely shows colocalization, suggesting that TL-A and TL-B do not mediate interactions between the type VI collagen beaded filaments and banded collagen fibrils. Also, interfibrillar distances are approximately equivalent in the presence and absence of these type XII-like molecules. While the results do not directly indicate a specific function for these molecules, the localization at the fibril surface suggests that they mediate interactions between the fibrils and other matrix macromolecules or with cells.

THE structure of the banded collagen fiber has been found to be far more complicated than previously recognized. When the multiplicity of collagen types was first recognized, it was generally believed that each of the collagens formed separate structures. This concept was supported by *in vitro* fibrillogenesis studies that indicated that fibers could be independently reconstituted from type I, II, or III collagen molecules that demonstrated the major features of the *in vivo* fiber. It is now believed that most, if not all, fibers contain two or more collagen types. The fibers of chick cornea have been shown to contain types I and V collagens, where the type V is present in the fibril interior but is not found on the fibril surface (Birk et al., 1988). In cartilage, type XI collagen is located in the interior of the banded fibers (Mendler et al., 1989). In human skin, tendon, and ligament, and in other tissues as well, type III collagen is present in all fibers, regardless of the fiber diameter (Keene et al., 1987). Recently, it has been suggested that type III collagen is present predominantly on the surface of the fiber in skin (Fleischmajer et al., 1990). The isolated triple-helical domains of these banded fiber-forming collagens are capable of self assembly into the quarter-staggered array characteristic of collagen fibers. This ability is conferred by the uniform length and uninterrupted Gly-X-Y triplet repeat common to the triple-helix of all these molecules (Kuhn, 1987).

Previous work has demonstrated that type IX collagen is present on the cartilage fiber surface (Vaughan et al., 1988). However, the structure of type IX collagen differs significantly from the typical fiber-forming collagens. The triple helical domain of type IX is only ~60% the length of the analogous type I collagen domain. Furthermore, this short domain is discontinuous: the triple helix is divided into three regions (COL1, COL2, and COL3) by two nonhelix-forming sequences (NC2 and NC3). A chain of chondroitin sulfate of variable length is attached to $\alpha 2(\text{IX})$ chain at the NC3 domain (Vaughan et al., 1985). The $\alpha 1(\text{IX})$ chain is considerably longer than either the $\alpha 2(\text{IX})$ or $\alpha 3(\text{IX})$ and forms a globular domain (NC4) at the NH_2 -terminus of the molecule that appears as a globule by rotary shadowing imaging (Muller-Glausner et al., 1986; Vaughan et al., 1988). Type IX collagen appears to adhere to the banded cartilage fibril by interactions of the COL1 and COL2 domains with the surface of the fibril. This interaction is stabilized by a crosslink between the COL2 domain and type II in the fibril (Eyre et al., 1987; van der Rest and Mayne, 1988; Shimokomaki et al., 1990).

Recently, type XII collagen has been identified (Gordon et al., 1987; Dublet and van der Rest, 1987). This molecule is composed of three identical chains that form a molecule with five subdomains (Dublet et al., 1989; Gordon et al., 1989). The triple-helical domain is only one-quarter that of type I

collagen and is divided into two distinct regions (COL1 and COL2) by a nonhelical interruption (NC2). Another small nonhelical domain, NC-1, is present at the COOH-terminus of COL1. The large NH₂-terminal NC3 region of each chain forms an independent domain that by rotary shadowing appears as three thickened arms, each ~60 nm in length. The COL1 region of chick type XII is 50% identical in amino acid and nucleotide sequence to chick α 1(IX) COL1 (Gordon et al., 1989). The COL2 domains of α 1(IX) and α 1(XII) are dissimilar, however, a portion of the α 1(XII) NC3 domain shares 46% nucleotide identity with the NC4 domain of α 1(IX). The type IX NC3 domain that contains the chondroitin sulfate attachment site and the site of crosslinking to the banded cartilage fibril does not exist in type XII collagen. The striking homologies between regions of type IX and type XII have led to the speculation that, like type IX, type XII binds to the banded fibril surface through the COL1 domain, but does not become crosslinked to the fibril (Gordon et al., 1990). An mAb directed against a synthetic peptide predicted from sequences in the NC-1 domain indicates that chicken type XII is present in tendon, ligament, periosteum, and perichondria, but not in cartilage and bone (Sugrue et al., 1989). A type XII collagen homologue has also been observed in bovine skin (Dublet and van der Rest, 1990).

We have reported (Lunstrum et al., 1991) the identification of two collagen molecules, termed twelve-like A and twelve-like B (TL-A and TL-B), in bovine skin that share many characteristics with type XII molecule but have a broader tissue distribution than that reported for chicken type XII (Sugrue et al., 1989). In this study we examine the ultrastructural localization of these two molecules in several tissues. In all cases, both molecules appear to be located on the surface of the banded fibrils.

Materials and Methods

Antibody Production and Characterization

mAbs to bovine TL-A and TL-B collagens have been previously described (Lunstrum et al., 1991). The CIJ antibody reacts with both bovine and human TL-A collagen, while the 1011G antibody does not crossreact with human TL-B collagen (data not shown). Hybridomas were grown in media containing 5% fetal bovine serum which had been depleted of endogenous IgG by affinity chromatography on protein G-Sepharose (Pharmacia LKB Inc., Piscataway, NJ). mAbs were then purified from clarified hybridoma media on protein G-Sepharose. Polyclonal antiserum to human TL-A, pTL-A, was raised in rabbits to an electrophoretic gel band derived from WISH cell culture medium. The uppermost electrophoretic band described by Lunstrum et al. (1986) in Fig. 1 A, lane 2 of that publication was excised for antibody production as described there. Polyclonal antibodies were purified from rabbit serum by chromatography on protein G-Sepharose. Antibodies were tested for cross-reactivity by ELISA (see Fig. 1) as previously described (Sakai et al., 1986). The mAb 5C6, recognizing type VI collagen, has been previously described (Engvall et al., 1986). Direct gold conjugation of antibodies was performed using 15-nm colloidal gold according to the manufacturer's instructions (Janssen Life Sciences Products, Piscataway, NJ).

Immunoelectron Microscopy

Human tissue sources included neonate foreskin collected shortly after circumcision, 30 wk fetal skin and femur collected within 8 h after death, tendon collected from an 11-y-old individual during surgery, and tendon col-

lected from an 80-y-old within 24 h after death. Fetal calf tissues, obtained within 30 h after death, included cornea and skin.

En bloc immunolocalization of antigens was performed as previously described (Sakai et al., 1986) with some modification. Briefly, tissues were washed for ~2 h in PBS, pH 7.4 at 4°C, incubated in primary antibody diluted 1:5 in PBS overnight at 4°C, rinsed in several changes of PBS over ~6 h, incubated in 5- or 15-nm gold-conjugated secondary antibody (Janssen Life Sciences Products) diluted 1:3 in BSA (20 mM Tris-HCl, 0.9% NaCl, 1 mg/ml BSA, 20 mM Na₂S₂O₃), pH 8.0, overnight at 4°C, and then washed as above. Control antibodies included those recognizing type III (Keene et al., 1987), collagen type VI (Keene et al., 1988), and collagen type VII (Sakai et al., 1986). Tissues were then fixed in 1.5% glutaraldehyde, 1.5% paraformaldehyde followed by 1% OsO₄, dehydrated in a graded series of ethanol dilutions, exposed to propylene oxide, and embedded in Spurr's epoxy. Tissues immunolabeled with antibody directly conjugated to gold particles were treated similarly, except that incubation in secondary gold conjugate was omitted. Some tissues were prefixed for 30 min in ice-cold acetone (calf skin, 11 and 80 year human tendon) or in 0.1% glutaraldehyde, 0.1% paraformaldehyde (calf skin) and rinsed in buffer before incubation in primary antibody. Those tissues initially fixed in acetone were also dehydrated in acetone before embedding. One sample of immunolabeled calf skin was not exposed to any solvent dehydrant during preparation, and was embedded in nanoplast water soluble resin (Bio-Rad Laboratories, Cambridge, MA). Cornea obtained from fetal calf was fixed in ice-cold acetone and immunolabeled and fixed as described above, except that the aldehyde was modified to contain 0.15% Ruthenium red and the wash buffer and OsO₄ contained 0.04% Ruthenium red. A sample of 30 week human fetal rib bone was obtained at autopsy and stirred in 0.2 M EDTA in 50 mM tris-HCl for several months at 4°C with several buffer changes. After decalcification, bone samples were rinsed in PBS and immunolabeled en bloc without prefixation as described above.

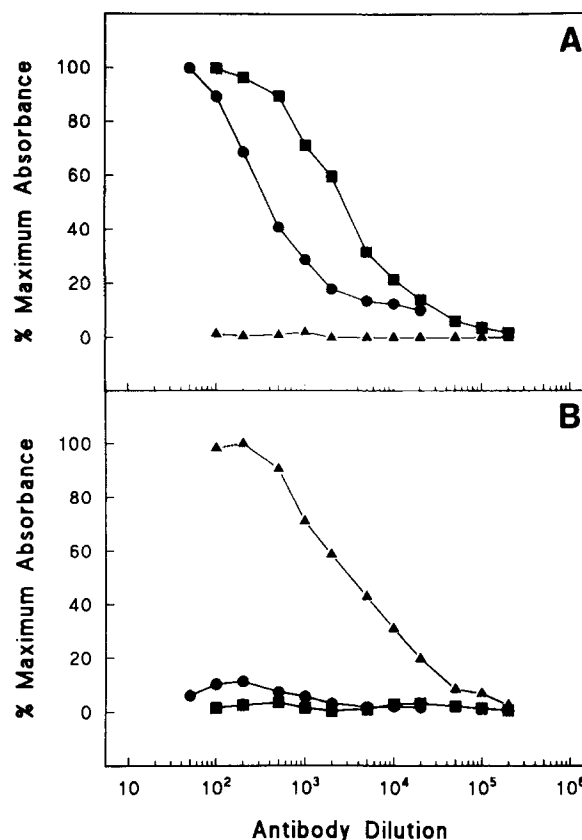


Figure 1. Comparison of antigen specificity for various antibodies. ELISA plates were coated with either TL-A (A) or TL-B (B), 0.6 ng/well, purified as described previously (Lunstrum et al., 1991). Monoclonal and polyclonal antibodies, 1 mg/ml, were tested at the indicated dilutions. pTL-A (circles); CIJ (squares); 1011G (triangles).

1. Abbreviations used in this paper: TL-A and TL-B, twelve-like A and B, respectively.

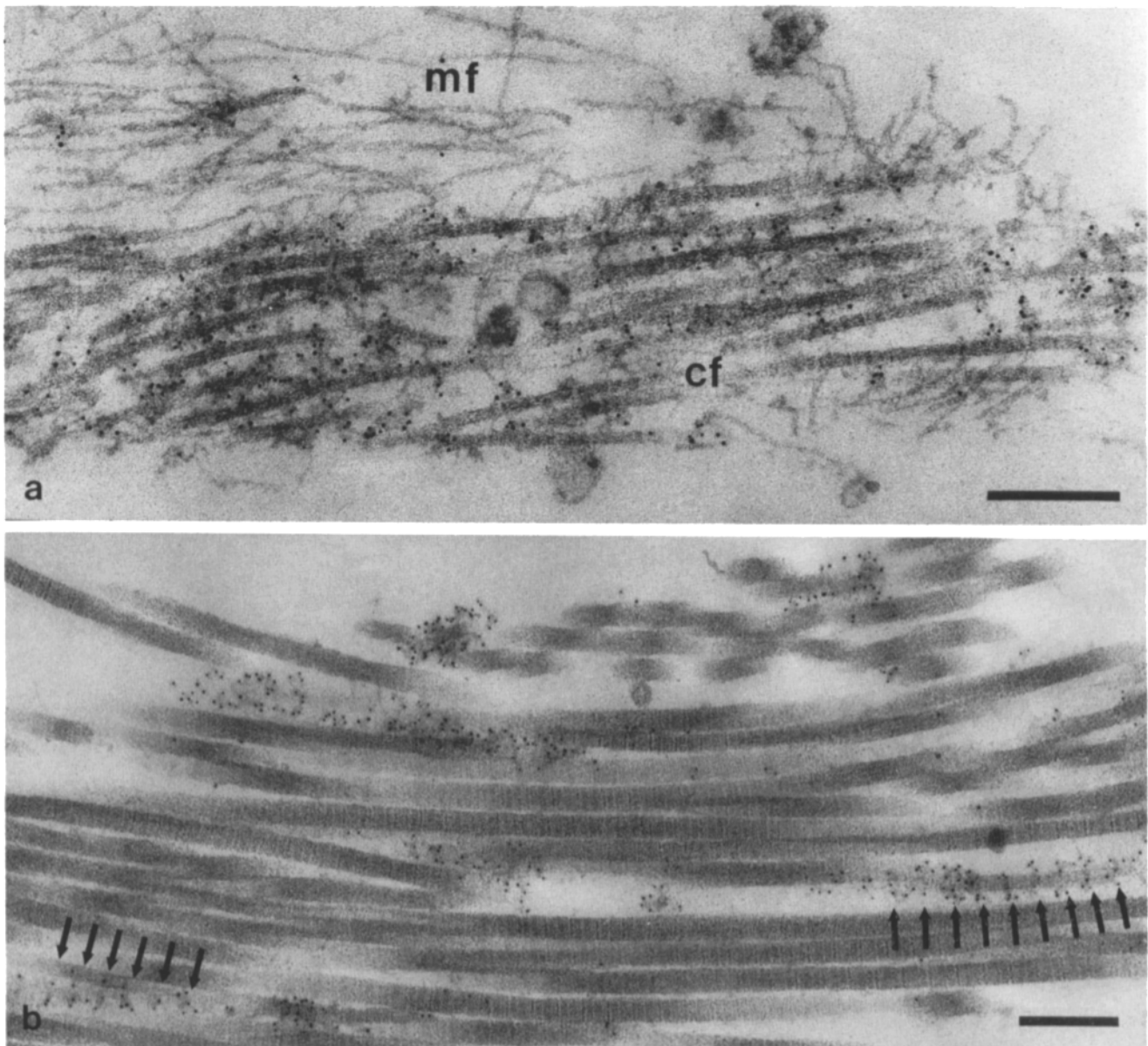


Figure 2. Localization of TL-A in calf skin and fetal human skin. (a) TL-A was localized to the region of banded collagen fibers (*cf*) in the papillary dermis using mAb CIJ after acetone prefixation. Microfibrils (*mf*) are not labeled. (b) mAb CIJ against TL-A was localized to the surface of banded collagen fibers in human fetal skin using a 5-nm colloidal gold-conjugated second antibody. The gold appears to demonstrate a period of ~ 56 nm (*arrows*). Bars, 200 nm.

For routine TEM examination, 60–90-nm thick sections were cut on a Reichert ultramicrotome using diamond knives and examined using a Philips 410 LS operated at 60 KV after contrasting in uranyl acetate and Reynolds lead citrate (Reynolds, 1963). For stereo pair microscopy, sections were cut at $0.5 \mu\text{m}$ and observed either with a Philips 410 LS operated at 100 KV or with the JEM 1000 located at the High Voltage Facility at the University of Colorado, operated at 1 MEV. Tilt angles for stereo pairs differed by 10° .

Results

We have described the partial characterization of two type XII-like collagens, TL-A and TL-B, isolated from fetal bovine skin (Lunstrum et al., 1991). In previous work, in which we reported that type VII procollagen is synthesized by a human epithelium-derived cell line (Lunstrum et al.,

1986), two additional high molecular weight ($M_r > 350,000$), collagenase-sensitive peptides were observed. Polyclonal antibodies (pTL-A) produced against the larger of these two peptides recognize bovine TL-A collagen. The pTL-A antisera and mAbs, CIJ and 1011G, were tested for cross-reactivity using bovine TL-A and TL-B as substrates (Fig. 1). As shown, these antibodies are specific in their reaction with the large globular domain of TL-A or TL-B collagen.

TL-A and TL-B collagens are easily extractable from fetal calf skin with 0.2 M NaCl (Lunstrum et al., 1991), indicating that neither molecule is covalently fixed in the matrix of the fetal calf. Similar results have been obtained for both molecules isolated from human tissues. Therefore, in order to localize these molecules within tissues by immunoelectron microscopy, we felt it was important to perform these

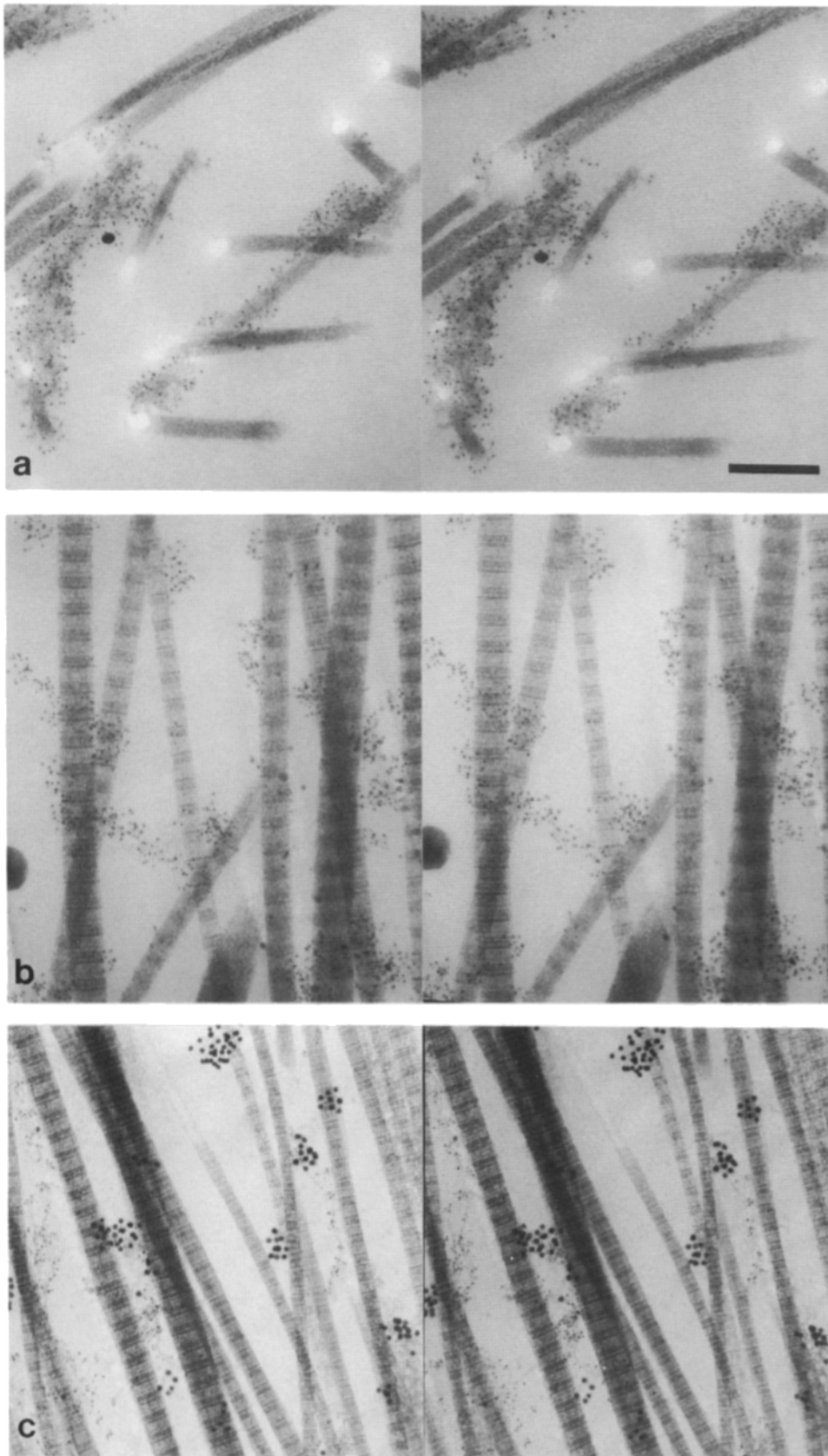


Figure 3. Stereo-pair visualization of TL-A in human skin and tendon. (a) In unfixed human skin, TL-A appears to surround a portion of the banded fibers. The CIJ antibody was used, followed by a 5-nm gold-conjugated secondary antibody. (b) TL-A, localized in human tendon from an 11-y-old individual after acetone fixation occasionally shows bridging of two fibers by TL-A collagen. The pTL-A antibody was used, followed by a 5-nm gold-conjugated secondary antibody. (c) Simultaneous localization of polyclonal pTL-A (15 nm) and type VI collagen (5 nm) in acetone-prefixed human tendon from an 80-y-old individual. Although some colocalization is observed, most often the two collagens localize independently. Bar, 200 nm.

studies under a variety of fixation conditions. The stability of the epitope to fixation was determined by immunofluorescent assay of prefixed tissues (data not shown). Of the conditions tested, the epitope was stable after fixation with

acetone and with 0.1% glutaraldehyde and 0.1% paraformaldehyde. In all cases, by EM colloidal gold deposition directed by anti-TL-A collagen antibodies is seen closely associated with collagen fibrils as shown in Fig. 2 a, after mild

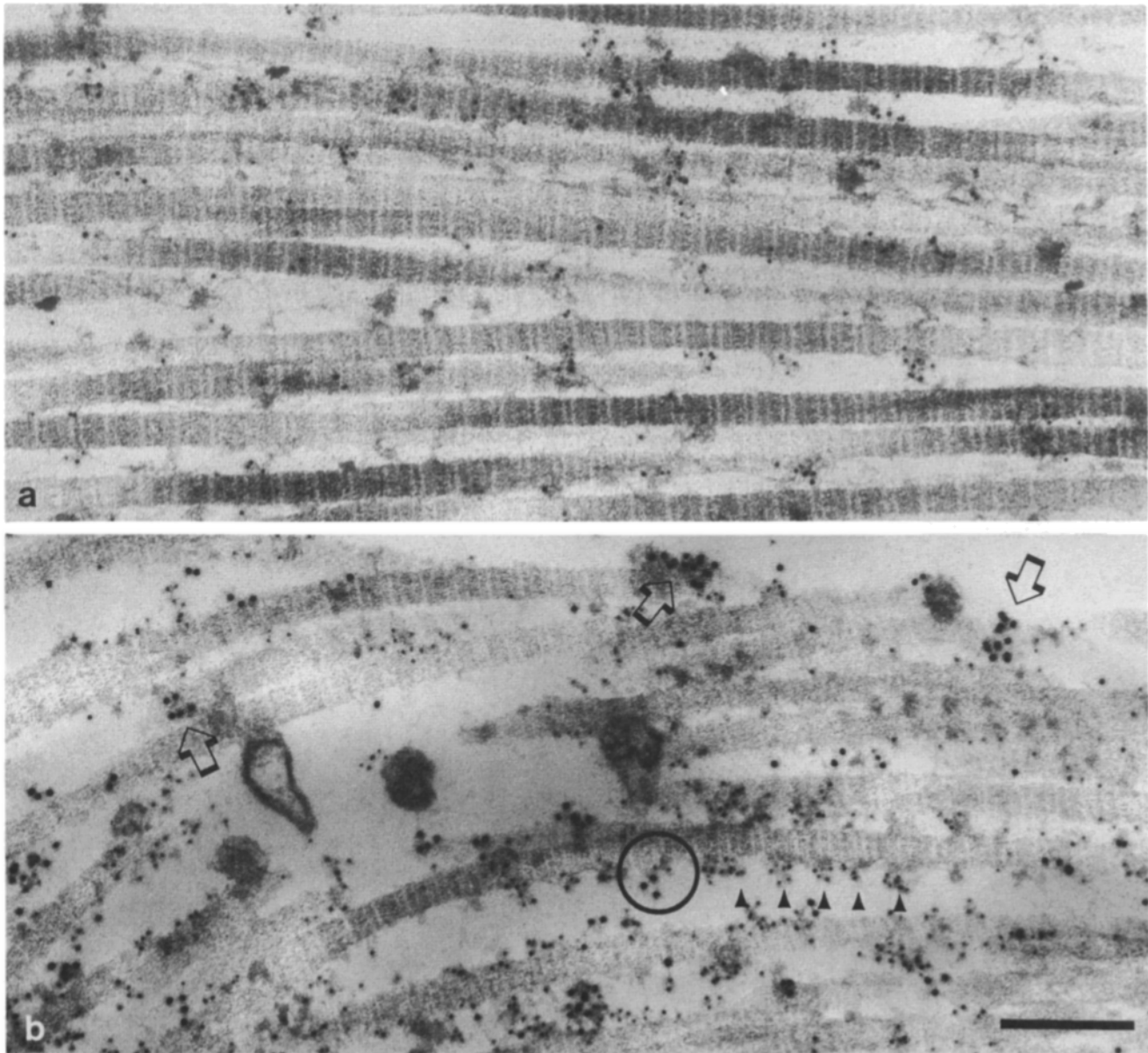


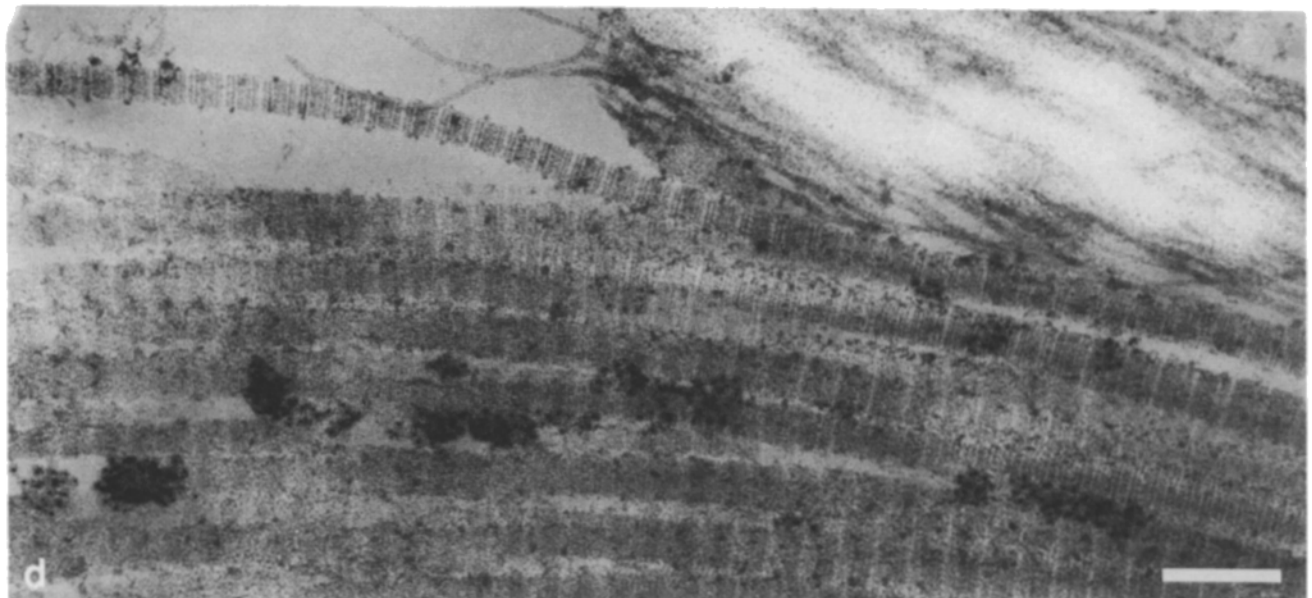
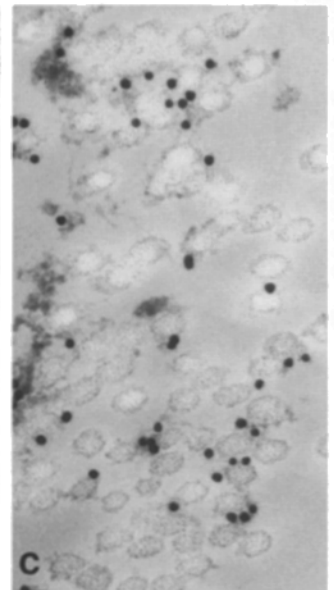
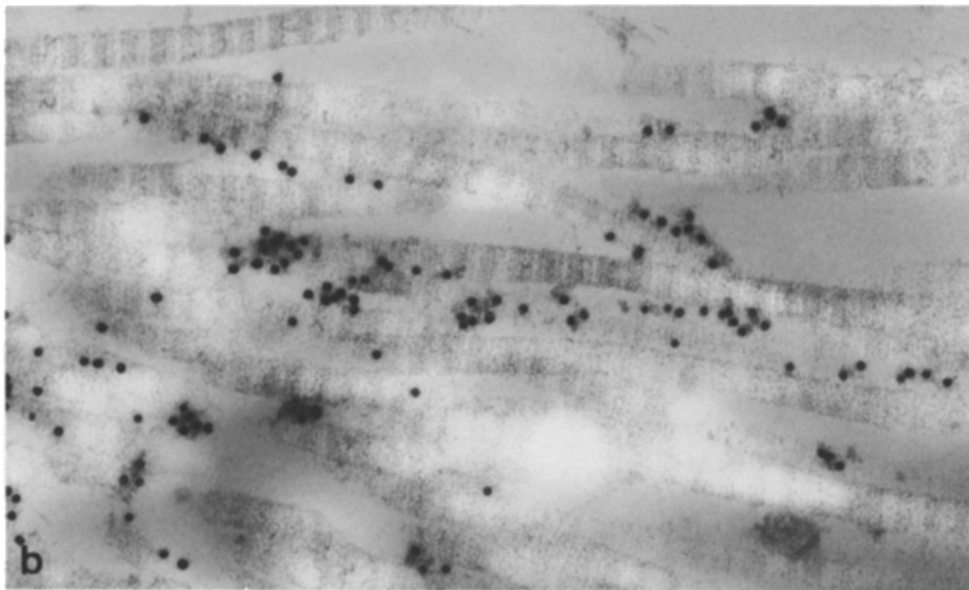
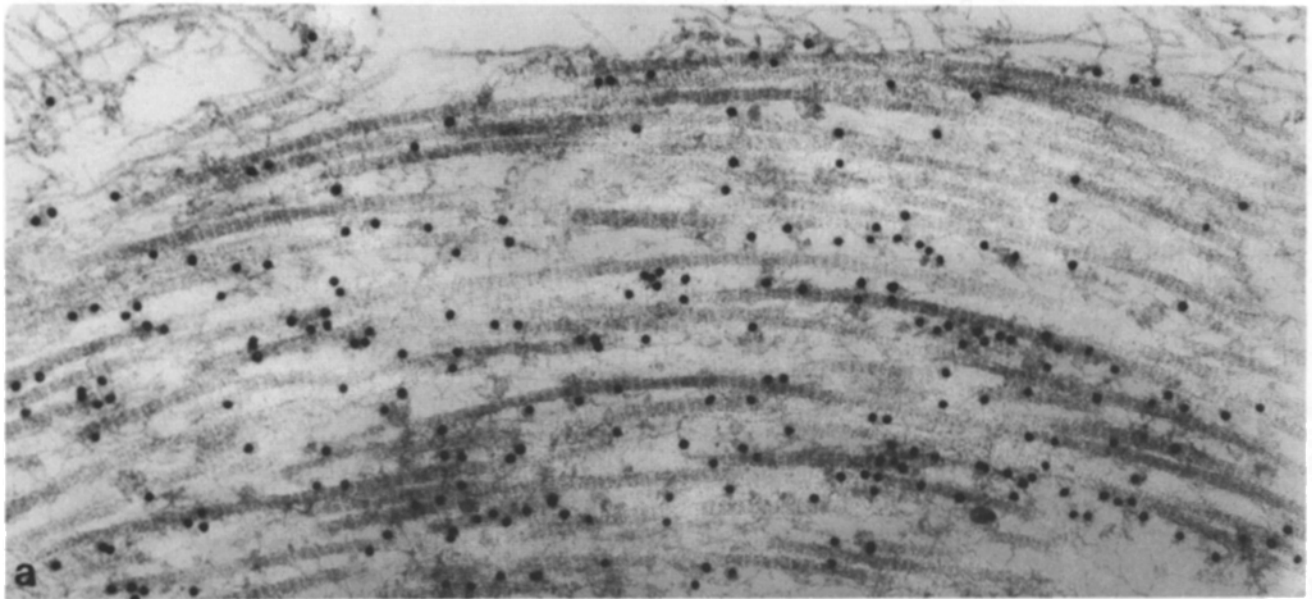
Figure 4. Localization of TL-B in fetal calf skin. (a) mAb 1011G was used to localize TL-B at the junction of the papillary and reticular dermis in acetone-prefixed calf skin. As seen with TL-A, the 5-nm secondary antibody localizes to the surface of the banded collagen fibers. (b) TL-A and TL-B were simultaneously localized in calf skin after 0.1% glutaraldehyde, 0.1% paraformaldehyde fixation using polyclonal PTL-A and mAb 1011G. The patterns shown by TL-A (15 nm) and TL-B (5 nm) are very similar, but the two collagens distribute independently along the fiber surface (*large arrows* indicate clusters of TL-A). Infrequently, mixed clusters of TL-A and TL-B are seen (*circled*), where the two antigens are not likely to have been bound to adjacent fibers. In some micrographs, a 56-nm periodic localization of TL-B is also detected (*small arrowheads*), as was observed for TL-A. Bar, 200 nm.

acetone fixation. This association is independent of the method of sample preparation, although labeling is less extensive in nonfixed tissues (not shown). Antibody-directed gold labeling within the papillary dermis (Fig. 2 a) can be seen along collagen fibrils, but not along the nearby microfibrils. The gold deposits are not continuous along single fibrils. In the reticular dermis, TL-A labeling is far less frequent, and the gold often appears in larger clusters (not shown), but again, these clusters appear randomly distributed along the fibrils, and only a portion of the fibrils contained within the field are labeled.

In fetal human skin (Fig. 2 b) the distribution of TL-A resembles that seen in calf. Infrequently, gold particles ap-

pear to show a measured periodicity approximating 56 nm (*arrows*), very near the value of the D-period of the collagen fibril after preparation of EM using the cited procedures. When directly compared to the collagen interband distance in the micrographs, the period of the gold labeling is nearly identical to the collagen period. In many micrographs the gold appears to surround a given fibril in a helical pattern continuous along the fibrils for a considerable length. This is particularly apparent when the labeling is visualized by stereo pair analysis (Fig. 3 a).

Like TL-A, in fetal calf skin TL-B also localizes along and between collagen fibrils (Fig. 4 a). In agreement with the immunofluorescent localization results (Lunstrum et al., 1991),



sparse labeling was seen with TL-B in the papillary dermis (not shown), but labeling of the reticular dermis was extensive. Some periodicity of the gold deposits is occasionally seen along the fibrils (Fig. 4 *b*, *solid arrowheads*) as was observed with TL-A, again with a repeat distance near that of the collagen fibrillar D-period. These observations suggest that both TL-B and TL-A associate with the banded fibrils by a similar mechanism. At the interface of the papillary dermis and reticular dermis, TL-A and TL-B localize to the same fibril bundles. As seen in Fig. 4 *b*, TL-A and TL-B do not often appear in the same clusters, although the distribution of both species along the fibrils is similar. Rarely, TL-A and TL-B localize to the same area (*circled* in Fig. 4 *b*).

The distribution of TL-A in human tendon parallels its location in skin. Antibody-directed gold is closely associated with the fibril surface, even in regions where considerable separation between fibrils is evident (not shown). In some micrographs, gold clusters appear to span two separated fibrils (Fig. 3 *b*). Neither TL-B nor TL-A appear to form a fibril system entirely independent from the banded fibrils.

The distribution of TL-A and TL-B in skin and in tendon suggest that they may provide an interaction interface between banded fibrils and type VI collagen filaments. However, double-labeling studies (Fig. 3 *c*; TL-A = 15 nm; type VI = 5 nm) do not support this hypothesis. While in some cases TL-A is present near where a type VI filament approaches a banded fibril, more often it is absent, or may also be present along the banded fibril in the absence of type VI filaments.

Unlike chicken type XII collagen, TL-A and TL-B are present in cartilage and bone as well as periosteum and perichondrium (Lunstrum et al., 1991). The location of TL-A in periosteum (not shown) is identical to that observed in tendon and in skin. In bone, the label shows the same distribution along the fibrils (not shown), but is much less abundant than seen in other tissues. The relative paucity of TL-A in bone is likely to be a function of the need to decalcify this tissue before ultrastructural examination. We have previously reported that the type VI and fibrillin networks in bone appear to be disrupted by the extensive decalcification in EDTA (Keene et al., 1991). Because of the solubility of TL-A, it is likely that only a part of the TL-A initially present in bone remains after decalcification. In cornea TL-A labeling is similar to that seen in other tissues (not shown). The addition of Ruthenium red to the preparatory procedure does not reveal a significant change in the appearance of the immuno-complex as compared to samples not treated with Ruthenium red, suggesting that the immunolabeled sites are not particularly rich in glycosaminoglycans.

In the micrographs presented thus far, antibody-directed gold is sometimes visualized at variable distances from the banded collagen fibers. To better assess the distance of the

epitopes from the fibril surface, 15 nm gold particles were directly conjugated to monoclonal anti-TL-A antibodies and utilized for electron microscopic localization (Fig. 5, *a-c*). These results show the same general distribution as seen before using a secondary antibody conjugate, but the gold particles are now very close to the fibril surface. This observation is consistent with the proposed association of the triple-helical domain of TL-A being associated with the fibril surface and the arms, which are ~50 nm in length (Lunstrum et al., 1991), projecting into the interfibrillar spaces.

After en bloc immunolabeling, if tissues are embedded in water-soluble media without exposure to solvent dehydration, it is our impression that although the general staining pattern remains unchanged from that described above, the gold particles seem more tightly packed within clusters associated with fibrils (Fig. 5 *d*). The periodicity of collagen fibrils is also greater after embedding by this technique, indicating less tissue shrinkage.

Discussion

Readily solubilized antigens are problematic for localization studies such as those reported here. In an attempt to circumvent the potential problem of antigen rearrangement or precipitation during processing, we have examined the localization patterns under a variety of mild fixation conditions. In each case, the localization pattern observed is invariant, although labeling is less intense under conditions lacking a fixative. We cannot exclude the possibility that bivalent antibody causes some unknown degree of clustering of the long nonhelical arms of TL-A and TL-B. Immunoblotting studies indicate that these arms contain the epitopes localized in these studies.

The simplest interpretation of the localization results is that both TL-A and TL-B associate with the surface of the banded collagen fibers. In tissue regions where the fibrils appear well separated, the label remains within 50 nm of the fibrils using directly conjugated primary antibody, and does not appear as an independent network between fibrils as has been shown for type VI collagen. This result might be predicted if clusters of TL-A or TL-B were present between fibrils or surrounding fibril bundles and were precipitated upon the fibrils by dehydration. However, two observations argue against this possibility. First, if TL-A and TL-B were randomly precipitated upon fibrous tissue elements, then one would predict that localization upon elastic microfibrils or type VI microfilaments would be observed, at least when they are adjacent to labeled banded fibril bundles, and this is not the case. Secondly, a periodic labeling of both TL-A and TL-B is occasionally seen along the fibrils that closely approximates the collagen D-period. This strongly suggests that there is a specific association of TL-A and TL-B with

Figure 5. En bloc localization of TL-A using monoclonal anti-TL-A directly conjugated with 15-nm colloidal gold, or with secondary gold conjugates in tissues embedded in water soluble embedding media. The direct conjugate was used for TL-A localization in calf skin within the papillary (*a*) and reticular dermis (*b* and *c*). The gold particles lie closer to the banded fiber surface than when a secondary antibody was employed. All samples were acetone prefixed before localization. (*d*) Unfixed calf skin was immunolabeled en bloc with polyclonal pTL-A antibody followed by 5-nm secondary gold conjugate, and then embedded in Nanoplast water soluble resin without exposure to solvent dehydration. The pattern of antibody-directed gold label is generally the same as that following solvent dehydration, although clusters of gold label are more tightly packed. Bar, 200 nm.

the fibril surface at least in some instances. The suggested direct association of TL-A and TL-B with the fibril surface is also consistent with the striking structural similarity of TL-B and TL-A to type XII collagen (Lunstrum et al., 1991). Type XII has been shown to contain a portion of the triple-helical domain homologous to the region of type IX collagen which lies along the surface of cartilage banded collagen fibrils. Therefore, the results reported here are consistent with the published hypothesis that collagenous molecules with the structural characteristics of type IX and type XII, which are shared by TL-A and TL-B, associate with the surface of banded collagen fibrils through the triple-helical domain, and the nontriple-helical arms occupy the interfibrillar space (Olsen, 1989). The results also suggest that the COL1 domains of both type XII collagen and of TL-A and TL-B are sufficient to allow attachment of these collagen to banded fibers, since type XII lacks a region equivalent to the COL2 region of type IX.

In some fields (for example, Fig. 3 a), TL-A shows a helical distribution around the collagen fibrils, suggesting an association of the nonhelical arms of the molecules. These interactions could be direct associations of the nonhelical arms of these molecules, or could be mediated by an unidentified ligand. In some cases, the molecules appear to bridge adjacent fibrils (for example, Fig. 3 b). These results suggest that the interactions of the nonhelical arms may in some cases supersede the interactions of the triple-helical portion of the molecules with the fibril surface, forming a network somewhat independent of the direct interactions with the fibrils. While both types of interactions are observed, arrays entwining the fibrils or bridging them are relatively rare observations.

The functions of TL-A and TL-B are not obvious from these localization studies. It would be reasonable to predict that a fibril-associated molecule might either cross-link and separate collagen fibrils, thus, determining or maintaining interfibrillar distances. This function appears unlikely in the case of TL-A or TL-B, since one would predict they should be present uniformly along all or most fibrils. Such molecules might also be predicted to secure the collagen fibril to the elastic network or the type VI collagen network. While interactions of TL-A and TL-B with type VI collagen are not excluded by these studies, there is neither obvious coincidence of TL-A with type VI where it directly contacts the banded fibrils, nor concentration of TL-A or TL-B between the banded fibril bundles and the elastic fibers. Although not obvious in the micrographs included in this report, it is our definite impression that both TL-A and TL-B are more concentrated on the fibril bundles near cells. By en bloc technology, intense labeling of intracellular compartments containing electron-dense contents is often observed where cells have ruptured during processing. Together with the apparent lack of covalent attachment of TL-A and TL-B to the fibrils, this observation suggests that these molecules may in some way be involved with the interaction of cells with the fibrils.

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