# Interaction of the NG2 Chondroitin Sulfate Proteoglycan with Type VI Collagen

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*Abstract.* The NG2 chondroitin sulfate proteoglycan is a membrane-associated molecule of ~500 kD with a core glycoprotein of 300 kD. Both the complete proteoglycan and a smaller quantity of the 300-kD core are immunoprecipitable with polyclonal and monoclonal antibodies against purified NG2. From some cell lines, the antibodies coprecipitate NG2 and type VI collagen, the latter appearing on SDS-PAGE as components of 140 and 250 kD under reducing conditions. The immunoprecipitation of type VI collagen does not seem to be due to recognition of the collagen by the antibodies, but rather to binding of the collagen to NG2. Studies on the NG2-type VI collagen complex suggest that binding between the two molecules is mediated by protein–protein interactions rather than by ionic interactions involving the glycosaminoglycans.

Immunofluorescence double labeling in frozen sections of embryonic rat shows that NG2 and type VI collagen are colocalized in structures such as the intervertebral discs and arteries of the spinal column. In vitro the two molecules are highly colocalized on the surface of several cell lines. Treatment of these cells resulting in a change in the distribution of NG2 on the cell surface also causes a parallel change in type VI collagen distribution. Our results suggest that cell surface NG2 may mediate cellular interactions with the extracellular matrix by binding to type VI collagen.

PROTEOGLYCANS constitute a rapidly expanding family of glycoproteins exhibiting a high degree of variability in both their polypeptide and polysaccharide components (reviewed by Hassell et al., 1986; Gallagher, 1989; Ruoslahti, 1988*a*, 1989). In keeping with the diversity of their structures, these complex glycoproteins have been found to be capable of interacting with a variety of different types of molecules. Interactions with proteoglycans have been reported for most, if not all, extracellular matrix molecules, including collagens (Ruoslahti and Engvall, 1980; Scott, 1988; Smith et al., 1985), laminin (Martin and Timpl, 1987; Frenette et al., 1989; Lander et al., 1985), fibronectin (Oldberg and Ruoslahti, 1982; Saunders and Bernfield, 1987; Ruoslahti 1988*b*), vitronectin (Suzuki et al., 1984, 1985), and thrombospondin (Dixit et al., 1984; Sun et al., 1989). Cell surface molecules such as the neural cell adhesion molecule can also have binding sites for proteoglycans (Cole and Burg, 1989). In addition, proteoglycans are efficient at binding some types of growth factors (Gospodarowicz et al., 1987; Berman et al., 1987, Segarini and Seyedin, 1988; Cheifetz et al., 1988). Most of these interactions appear to be ionic and are probably mediated by the highly charged glycosaminoglycan chains of the proteoglycans. In some cases, however, direct protein–protein interactions are responsible for the binding phenomena. This appears to be true in the case of decorin binding to fibronectin (Schmidt et al., 1987) and to collagen (Vogel et al., 1984, 1986). Similarly, cytotactin can bind to the core protein of the cytotactin-binding proteoglycan (Hoffman et al., 1988), the cytoplasmic domain of syndecan can bind to actin (Rapraeger et al., 1986), and the core protein of the cartilage proteoglycan aggrecan has a site for interaction with the link protein (Neame et al., 1987; Kiss et al., 1987). Finally, the aggrecan core protein is able to bind to hyaluronic acid, a property it appears to share with versican (Zimmerman and Ruoslahti, 1989).

We have described a proteoglycan called NG2 which appears to have a small number of chondroitin sulfate chains (<5) coupled to a core glycoprotein of 300 kD. Because of its tight association with the cell and the need to use nonionic detergent for its solubilization, we have proposed that NG2 is a membrane-bound molecule (Stallcup et al., 1984). NG2 is found in the rodent central nervous system on glial precursor cells of the 0-2A lineage (Raff et al., 1984). On one branch of the 0-2A pathway, type 2 astrocytes continue to express NG2, while on the other branch oligodendrocytes become NG2 negative. Immunohistochemical staining allows identification of NG2-positive cells in primary cultures of many different parts of the rat central nervous system (Stallcup, 1981; Stallcup and Beasley, 1987; Levine and Stallcup, 1987), and NG2-positive cells are seen in frozen sections of both immature and adult brain (Stallcup et al., 1984; Levine and Card, 1987). As shown in this manuscript, we are now discovering NG2 expression in other tissues of the developing rat, especially some types of blood vessels and cartilaginous structures of the head, limbs, and spine.

Cell surface, membrane-bound proteoglycans have been proposed to serve as a means of linking the interior and exterior environments of the cells. On the inside they may interact with cytoplasmic or cytoskeletal elements, while on the outside they may provide a means of linkage with the extracellular matrix (Hook et al., 1984; David and Van Den-Berghe, 1985; Rapraeger et al., 1986). In this report we present evidence that NG2 binds to type VI collagen via a protein–protein interaction and thus can serve as a linkage point for NG2-positive cells with the matrix.

## Materials and Methods

### Cell Lines

Three rat cell lines, B49, B111, and B28 (Schubert et al., 1974), and two human cell lines, IMR-90 (Nichols et al., 1977) and M21 (Bumol and Reisfeld, 1982), were grown in DME supplemented with 10% FCS (Tissue Culture Biologicals, Tulare, CA). Cells were usually plated on standard tissue culture dishes (Falcon Labware, Becton, Dickinson & Co., Oxnard, CA), but in some cases were grown on tissue culture dishes coated by overnight incubation with a 25-μg/ml solution of poly-L-lysine (Sigma Chemical Co., St. Louis, MO). All cell lines tested negatively for mycoplasma contamination using an assay for adenosine phosphorylase (Gibco Laboratories, Grand Island, NY; Bethesda Research Laboratories, Gaithersburg, MD).

### Purification of NG2

NG2 was extracted from B49 cells (4 ml of packed cells) by homogenization in 20 ml of 150 mM NaCl, 10 mM Tris-HCl, 5 mM EDTA, pH 8.0, containing 1% NP-40, 1 mM PMSF, and 50 μg/ml soybean trypsin inhibitor. After removal of insoluble material by centrifugation, a sample of B49 extract labeled with $^{125}$I by the lactoperoxidase technique was added to the supernatant to serve as a tracer. This greatly facilitated the identification of protein-containing fractions during the final two steps in the purification when optical density at 280 nm was extremely low. The NP-40 extract was adjusted to 0.6 M NaCl and applied to a DEAE-Sephadex A-50 column (50 ml bed volume). After thorough washing with the 0.6 M NaCl solution, the column was eluted with the standard buffer (10 mM Tris HCl, 5 mM EDTA, 1 mM PMSF, 0.02% NP-40, pH 8.0) containing 1 M NaCl. Eluted material was passed over a Sepharose CL4B column to remove any Sepharose-adherent components, and the flow-through was agitated gently overnight at 4°C with Sepharose CL4B derivatized with either the D31.10 or D120.43 mAb against NG2 (Stallcup et al., 1984; Levine and Card, 1987). This mixture was poured into a column and washed first with the standard Tris-EDTA buffer containing 1 M NaCl, then with Tris-EDTA containing 1 M urea. NG2 was eluted with Tris-EDTA containing 7 M guanidine-HCl, concentrated in an Amicon filtration device, and subjected to gel filtration on Sepharose CL4B in the guanidine-containing buffer. Two pools of protein-containing fractions were made: a leading peak and a trailing shoulder of this peak. These pools were dialyzed extensively against 10 mM $NH_4OAc$, pH 7.4, and concentrated by lyophilization. Material in the leading peak from the CL4B column migrated as a broad band of ~500 kD on SDS-PAGE, while material from the trailing shoulder ran as a faster moving smear of ~400 kD. Treatment of these two pools with chondroitinase ABC before electrophoresis resulted in production of well-defined components of 300 and 200 kD, respectively. We believe the 300-kD component represents the intact core glycoprotein of NG2, since it matches the 300-kD species seen previously in rapid biosynthetic pulse–chase experiments (Stallcup et al., 1984). The 200-kD component appears to be a proteolytic fragment of the NG2 core protein, since it is still recognized by rabbit anti-NG2 antibody. The corresponding 400-kD smear seen before treatment with chondroitinase ABC apparently represents this core fragment with the glycosaminoglycan chains attached.

### Antibodies

Rabbit and mouse polyclonal antisera were prepared against purified NG2. mAbs against NG2 were the result of fusions between the P3×63 Ag 8.653 cell line (Kearney et al., 1979) and spleen cells taken from Balb/c mice immunized with purified NG2. Fusions and HAT selection of hybridoma colonies were performed as described previously (Levine et al., 1984). Supernatants from hybridomas resulting from the selection process were screened for binding to purified NG2 immobilized in Falcon flexible microtest wells. An $^{125}$I-labeled goat antibody against mouse immunoglobulins was used to detect mouse antibody bound to the NG2-coated wells. While the detailed properties of most of these mAbs will be presented elsewhere, a few relevant results with three of the clones (N92, N109, and N143) are included in this manuscript.

The F84 mAb against a 95-kD neural glycoprotein has been previously described (Stallcup et al., 1985). Rabbit antisera against type IV collagen and laminin were gifts from Dr. Eva Engvall (La Jolla Cancer Research Foundation, La Jolla, CA). Rabbit antibody against human type VI collagen was prepared by Dr. Engvall by immunization with material eluted from mAb affinity columns (Hessle and Engvall, 1984). This antiserum was precipitated with 40% ammonium sulfate and further purified by absorption with human serum proteins coupled to CNBr-activated Sepharose CL4B. Affinity-purified rabbit antifibronectin was provided by Dr. Erkki Ruoslahti (La Jolla Cancer Research Foundation, La Jolla, CA). Rabbit antitenascin was a gift from Dr. Mario Bourdon (California Institute of Biological Research, La Jolla, CA). Normal mouse serum and goat antibody against mouse immunoglobulin were purchased from Calbiochem-Behring Corp. Fluorescein and rhodamine-labeled antibodies were obtained from Tago Inc. (Burlingame, CA).

### Immune Precipitation and Gel Electrophoresis

Cells were labeled with $^{125}$I using the lactoperoxidase technique (Hubbard and Cohn, 1972). Extracts of the labeled cells were prepared using 1% NP-40 in 10 mM Tris HCl, 5 mM EDTA, pH 7.4, containing 1 mM PMSF (Calbiochem-Behring Corp., La Jolla, CA). Insoluble material was removed after 10 min by centrifugation. Aliquots of the extracts were incubated for 2 h at room temperature with the desired primary antibodies. In the case of rabbit antibodies, immunoprecipitation was accomplished by subsequent incubation with protein A–Sepharose (Sigma Chemical Co.) for 2 h. For mouse mAbs we added normal mouse serum as carrier followed by goat antibody against mouse immunoglobulin to form an immune precipitate. In both cases the precipitates were washed three times with PBS containing 0.2% NP-40 and 0.02% SDS, then dissolved in electrophoresis sample buffer containing 20% glycerol and 3% SDS. In cases where reduction of disulfide bonds was desired, the sample buffer included 5% 2-mercaptoethanol.

SDS-PAGE was performed using slab gels with a gradient of 3–20% acrylamide (Laemmli, 1970). A stacking gel of 2.5% was used. Gels were dried under vacuum and used for autoradiography with Kodak XAR-5 film.

### Immunofluorescence

Immunofluorescence with cell lines was performed using living cells on tissue culture dishes. All antibodies were diluted into Hepes-buffered DME containing 2% FCS. The Hepes-DME-FCS solution was also used for the washes. Cells were incubated with primary antibodies for 30 min, then washed three times. They were incubated with secondary antibodies for another 30 min, washed three more times, then fixed with 95% ethanol for 5 min. After drying, they were coverslipped in 90% glycerol, 0.1% M Tris, pH 8.0.

For immunofluorescence with frozen sections we used embryonic day 16 rat tissue fixed by immersion for 6 h in cold 2% paraformaldehyde, pH 7.5. After additional overnight incubation in cold 2% paraformaldehyde containing 25% sucrose, the tissue was embedded in OCT and frozen in liquid nitrogen. 15-μm sections were cut on a Reichardt cryostat and mounted on gelatin-coated slides. Sections were incubated overnight at 4°C with primary antibody diluted in potassium PBS (KPBS) containing 2% normal goat serum and 0.1% Triton X-100. After three 5-min washes in the KPBS-NGS-Triton solution, the sections were incubated for another 2 h with secondary antibody. Finally, the sections were washed three times in KPBS-NGS-Triton, rinsed once in $H_2O$, and coverslipped in glycerol-Tris as above.

Immunolabeled preparations were examined with a Nikon optiphot microscope equipped for epifluorescence and phase contrast optics. Photographs were taken using Kodak Tri-X 400 film.

## Results

### Coimmunoprecipitation of NG2 and Type VI Collagen

We previously showed that when detergent extracts of $^{125}$I-labeled cell lines were treated with anti-NG2 antibodies, two

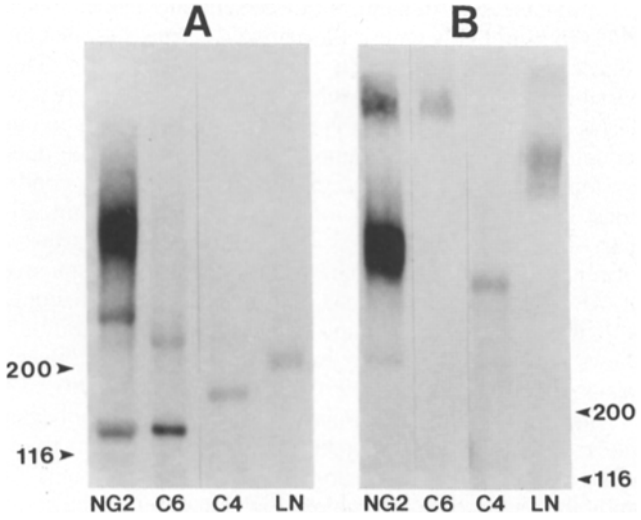


*Figure 1.* Immunoprecipitation of $^{125}$I-labeled NG2, type VI collagen (*C6*), type IV collagen (*C4*), and laminin (*LN*) from B49 cells. B49 cells were $^{125}$I labeled using the lactoperoxidase technique and extracted with 1% NP-40. Rabbit antibodies against NG2, type VI collagen, type IV collagen, and laminin were used to prepare immunoprecipitates from this extract. Immunoprecipitates were analyzed by SDS gel electrophoresis followed by autoradiography to identify labeled components. (*A*) Reducing conditions; (*B*) nonreducing conditions. Molecular mass standards (in kD) are shown at left and right. Under both reducing and nonreducing conditions the extra bands in NG2 immunoprecipitates appear identical to type VI collagen.

major components could be immunoprecipitated and resolved under reducing conditions on SDS-PAGE. The first was a glycoprotein of 300 kD, and the second was a chondroitin sulfate proteoglycan which migrated as a broad smear with an approximate size of 500 kD. Treatment of the immune precipitates with chondroitinase ABC quantitatively converted the 500-kD proteoglycan into the 300-kD core protein (Stallcup et al., 1984). Subsequently, we have found that treatment with chondroitinase AC produces an identical effect, suggesting that NG2 may not contain dermatan sulfate.

With a few NG2-positive cell lines such as B49, an additional major component of 140 kD is consistently seen in rabbit anti-NG2 immunoprecipitates. Occasionally, a less distinct band of 250 kD is also observed (Fig. 1 *A*). Under nonreducing conditions (Fig. 1 *B*), the 140- and 250-kD components are not present and are replaced by a very high molecular mass band that does not migrate very far into the gel. Since the mobility of the NG2 proteoglycan and core protein are not affected by the nonreducing conditions, it seems unlikely that the unknown component(s) is structurally related to NG2 (for example, as a proteolytic fragment). Instead, it is probably a distinct molecule stabilized by disulfide bonds. We were able to rule out a number of possibilities by performing immune precipitations with antibodies against extracellular matrix molecules known to be highly aggregated under nonreducing conditions. The examples of type IV collagen and laminin are shown in Fig. 1. Fibronectin, tenascin, and thrombospondin are other molecules that did not match the components seen in the NG2 immunoprecipitate. Antibodies against type VI collagen, however, immunoprecipitated components that were identical to the unknown bands on both reducing and nonreducing gels. The heavily labeled 140-kD band corresponds to type VI collagen light chains, while the 250-kD band matches the heavy chain (Hessle and Engvall, 1984; Engvall et al., 1986).

mAbs against human type VI collagen (Hessle and Engvall, 1984; Engvall et al., 1986) failed to cross react with rat type VI collagen in our immunoprecipitation experiments. However, when used with $^{125}$I-labeled extracts from the human melanoma cell line M21, antitype VI collagen monoclonals such as 3C4 precipitated a component which, under reducing conditions, migrated identically to the 140-kD species seen in anti-NG2 precipitates prepared from B49 cells (not shown). Under nonreducing conditions this 140-kD

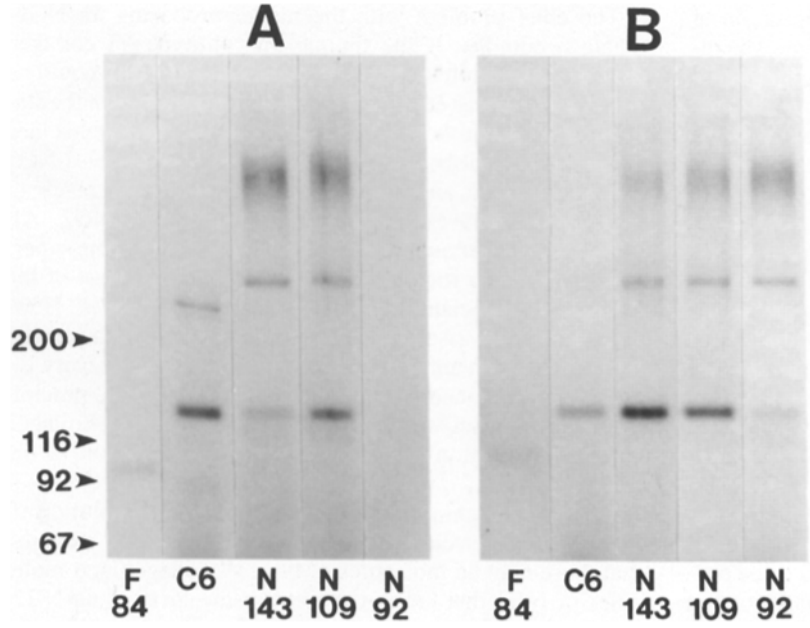


*Figure 2.* Immunoprecipitations of $^{125}$I-labeled NG2, type VI collagen (*C6*), and F84 glycoprotein from B49 cells. N143, N109, and N92 are mAbs against NG2. (*A*) Precipitations from 1% NP-40; (*B*) precipitations from 1% NP-40 and 0.5% SDS. All electrophoresis was done under reducing conditions. Molecular mass standards (in kD) are shown at left. Type VI collagen (seen here chiefly as 140-kD light chains) is immunoprecipitated only when NG2 is immunoprecipitated.

band from M21 cells was not present, being replaced by a high molecular mass component that comigrated with the large aggregates shown in Fig. 1 *B* for B49 cells. These mAb data support our identification of the anti-NG2 immunoprecipitable material as type VI collagen. We cannot explain with certainty why the 250-kD heavy chain is often absent from the autoradiograms in the case of both B49 and M21 cells. One possible explanation is that, relative to the light chain, the heavy chain is poorly labeled by the lactoperoxidase iodination procedure, so that it does not show up well on the autoradiograms. The fact that it is present, albeit in an unlabeled state, is indicated by the existence of the very large aggregates on SDS gel electrophoresis performed under nonreducing conditions. The $^{125}$I label in the case of the unreduced aggregate is supplied by the light chains. We have therefore continued to use the presence of the 140-kD light chains on our autoradiograms as an indicator of the presence of type VI collagen, even in cases where labeled heavy chain is not seen.

Several possibilities exist to explain the ability of rabbit anti-NG2 to precipitate type VI collagen along with NG2. (*a*) The rabbit antiserum might be contaminated with anti-type VI collagen activity; (*b*) type VI collagen could be nonspecifically bound to or trapped in the immune precipitates; (*c*) NG2 and type VI collagen might share an antigenic determinant; (*d*) the NG2 core protein might be a type VI collagen heavy chain; or (*e*) NG2 and type VI collagen could form a specific complex that is immunoprecipitated by anti-NG2.

To evaluate possibility *a* we performed immune precipitations with several mAbs against NG2. These should be monospecific and uncontaminated with activity against type VI collagen. Fig. 2 shows that mAbs N143 and N109 precipitate both NG2 and type VI collagen, just as seen with rabbit anti-NG2. mAb N92 fails to recognize any components in the NP-40 extract. However, if the extract is partially denatured with 0.5% SDS, mAb N92 also precipitates both NG2 and type VI collagen. An unrelated mAb that immunoprecipitates the 95-kD F84 glycoprotein does not immunoprecipitate type VI collagen. These results with mAbs rule out the possibility that contaminating antibodies against type VI collagen are responsible for the coimmunoprecipitation of NG2 and type VI collagen. They also show that type VI collagen is not nonspecifically bound to immunoprecipitates (possibility *b*), since type VI collagen is not seen in the case of F84 or in the case of mAb N92 in the absence of SDS. Type VI collagen is only immunoprecipitated in cases where NG2 is immunoprecipitated.

One further inference can be made from the mAb data. While all three of the monoclonals recognize epitopes on the 300-kD NG2 core protein, only N109 recognizes an epitope on the 200-kD proteolytic fragment obtained as a side product of NG2 purification (see Materials and Methods). Furthermore, as shown in Fig. 2, N143 recognizes an epitope present on the native polypeptide, while N92 recognizes an epitope that is exposed only after partial denaturation of the polypeptide in SDS. Therefore, we believe that N109, N143, and N92 recognize three distinct determinants on the NG2 core protein. Preliminary immunoblotting experiments using CNBr fragments of NG2 also support this conclusion. Thus, if alternative *c* concerning shared determinants is to be correct, NG2 and type VI collagen would have to share not just one antigenic determinant, but three separate determinants. This seems unlikely unless alternative *d* is true, i.e., that the NG2 core protein is actually a collagen heavy chain. This possibility also appears improbable, since the NG2 core protein is distinguishable from the collagen heavy chain on our reducing gels, and more importantly the core protein does not form part of a large aggregate under nonreducing conditions, as one would expect for a collagen heavy chain. Finally, the available amino acid sequence for the carboxy-terminal half of NG2, deduced from the nucleotide sequence of cDNA clones (Nishiyama, A., K. Dahlin, J. Johnstone, J. Prince, and W. Stallcup, manuscript in preparation), shows little or no similarity to that of the type VI collagen heavy chain (Chu et al., 1990; Bonaldo et al., 1990).

This leaves us with alternative *e*, that type VI collagen binds to NG2 and is coprecipitated as part of an NG2-collagen complex. We performed several experiments to study the nature of this complex. Fig. 3 shows that the complex is not disrupted by incubation with 1 M NaCl or with 0.5% SDS. However, boiling in the presence of 0.5% SDS before immune precipitation results in loss of type VI collagen from the complex. Apparently, this treatment is sufficient to disrupt the NG2–collagen interaction. These results suggest that the binding between the two molecules is too strong to be mediated by ionic interactions between collagen and the chondroitin sulfate chains of NG2. This hypothesis is strengthened by the finding that the absence of chondroitin sulfate from the NG2 core protein does not affect its ability to bind type VI collagen during immune precipitation (Fig. 3 *B*). For this experiment we used B49 cells grown in the presence of p-nitrophenylxyloside to prevent attachment of the glycosaminoglycan chains to the core protein. As a result of this treatment we see the disappearance of the heterodisperse proteoglycan band along with a large increase in the quantity of 300-kD core protein. The quantity of type VI collagen in the precipitates is unchanged, however, as reflected by the continued presence of the 140-kD light chain component. (The 250-kD heavy chain is not visible on this autoradiogram.) Identical results were obtained when we treated B49 cells with chondroitinase ABC before $^{125}$I labeling, extraction with NP-40, and immunoprecipitation.

The chief problem with the model proposing an NG2-collagen complex is the finding that antitype VI collagen does not coprecipitate NG2. We suspected that this could be due to a disruptive effect of the collagen antiserum on the stability of the putative NG2-collagen complex. To test this idea we performed clearing experiments with both the anti-NG2 and anticollagen antibodies. Fig. 4 *A* shows the result of clearing the B49 extract with antibody against NG2. Although a small amount of type VI collagen is left in the supernatant after two rounds of anti-NG2 clearing, most of the collagen is associated with NG2 in the precipitates. However, in the converse experiment, after clearing of a parallel B49 extract with antibody against type VI collagen, very little collagen is found to be associated with the NG2 precipitate. This is what we would expect if antitype VI collagen causes dissociation or interferes with reassociation of the complex.

The fact that some type VI collagen remains in solution after NG2 has been cleared from the extract in Fig. 4 *A* suggest that there may be molecules of type VI collagen and molecules of NG2 that are not present in the form of an NG2-

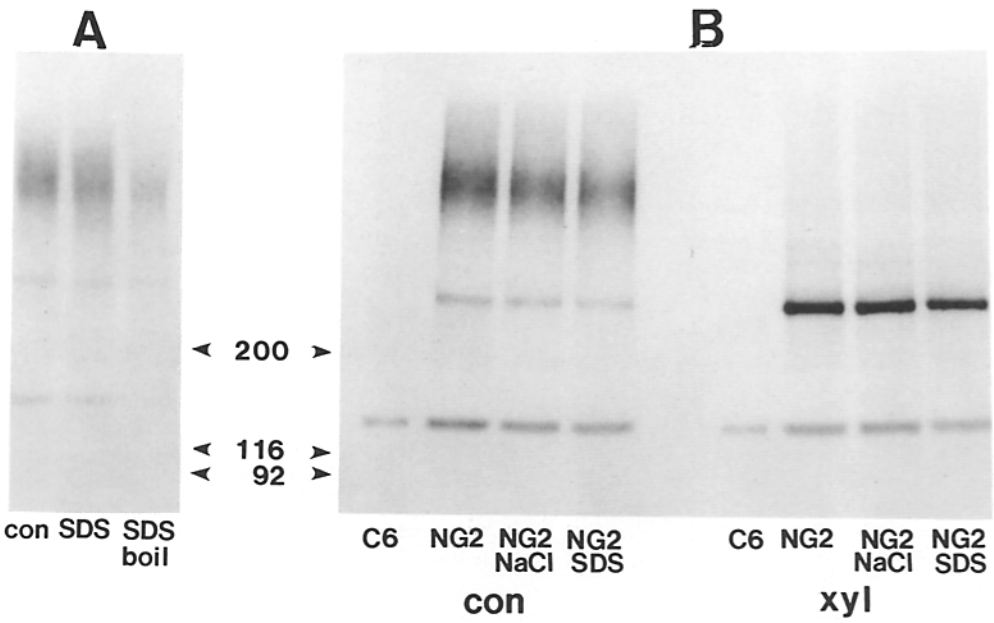


*Figure 3.* Immunoprecipitation of $^{125}$I-labeled NG2 and type VI collagen (*C6*) from B49 cells. (*A*) Precipitations were done from control extracts containing 1% NP-40 (*con*), extracts containing 1% NP-40 plus 0.5% SDS (*SDS*), and extracts boiled in 1% NP-40 and 0.5% SDS (*SDS boil*). The 140-kD component is no longer seen in the immunoprecipitate after boiling of the extract with SDS. (*B*) Precipitations were done using control B49 cells (*con*) and B49 cells grown for 4 d in 2 mM p-nitrophenylxyloside (*Xyl*). Some NG2 precipitations were done from 1% NP-40 (*NG2*), some from 1% NP-40 and 1 M NaCl (*NG2 NaCl*), and some from 1% NP-40 and 0.5% SDS (*NG2 SDS*). The absence of chondroitin sulfate chains from the 300-kD core protein from xyloside-treated cells does not alter the coprecipitation of the 140-kD type VI collagen band.

collagen complex. The existence of this non-NG2-precipitable pool of type VI collagen in Fig. 4 *A* also reinforces our earlier conclusion that anti-NG2 does not directly recognize type VI collagen, but precipitates it indirectly as part of a complex.

### *Colocalization of NG2 and Type VI Collagen*

If there is an interaction between NG2 and type VI collagen, we would expect this to be reflected in the distribution of the two molecules on cells that express both components. We

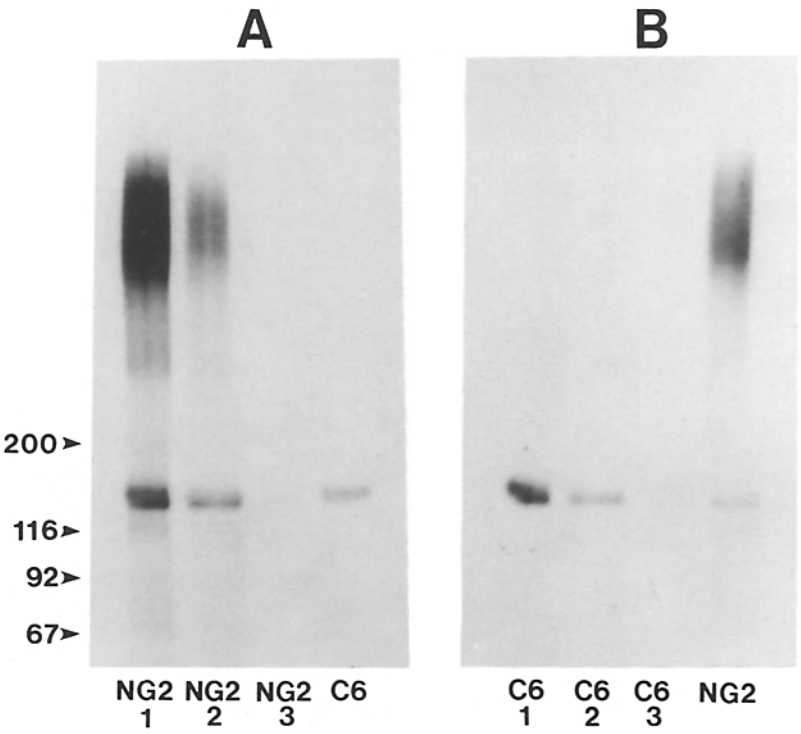


*Figure 4.* Clearing experiments using anti-NG2 and antitype VI collagen (*C6*) antisera. (*A*) Half of the $^{125}$I-labeled B49 cell extract was cleared twice (*NG2 1* and *NG2 2*) with rabbit anti-NG2. The supernatant was divided in half and subjected to further precipitation with rabbit anti-NG2 (*NG2 3*) or rabbit antitype VI collagen (*C6*). (*B*) The other half of the B49 extract was cleared twice with rabbit antitype VI collagen (*C6 1* and *C6 2*). The supernatant was divided in half and subjected to further precipitation with antitype VI collagen (*C6 3*) or anti-NG2 (*NG2*).

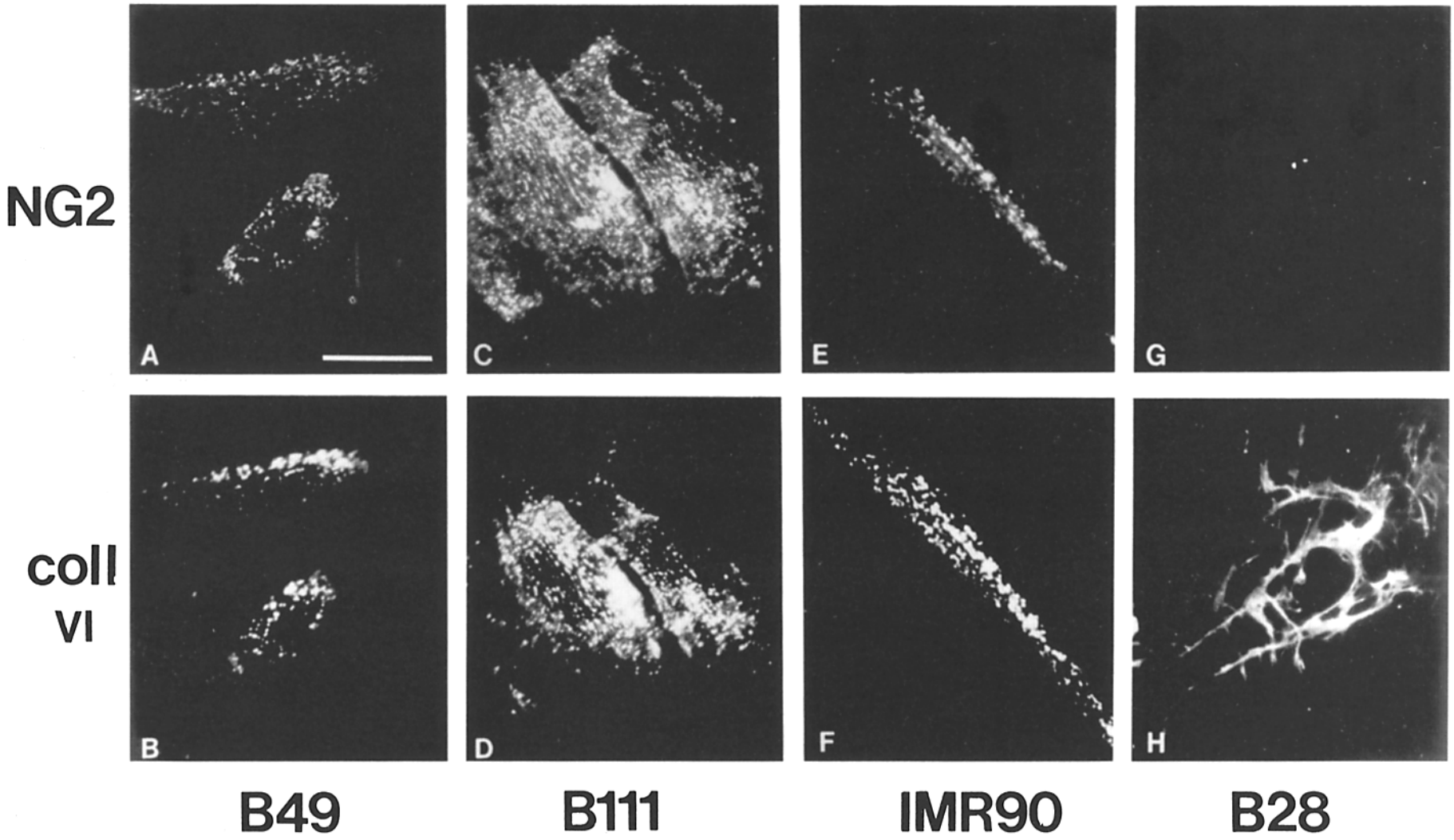


*Figure 5.* B49 (*A* and *B*), B111 (*C* and *D*), IMR 90 (*E* and *F*), and B28 (*G* and *H*) cell lines double labeled with mouse anti-NG2 (and FITC-GaMIg) and rabbit antitype VI collagen (and TRITC-GaRIg). The labeling patterns for NG2 and type VI collagen are quite similar on B49, B111, and IMR-90 cells. Bar, 10 $\mu$m.

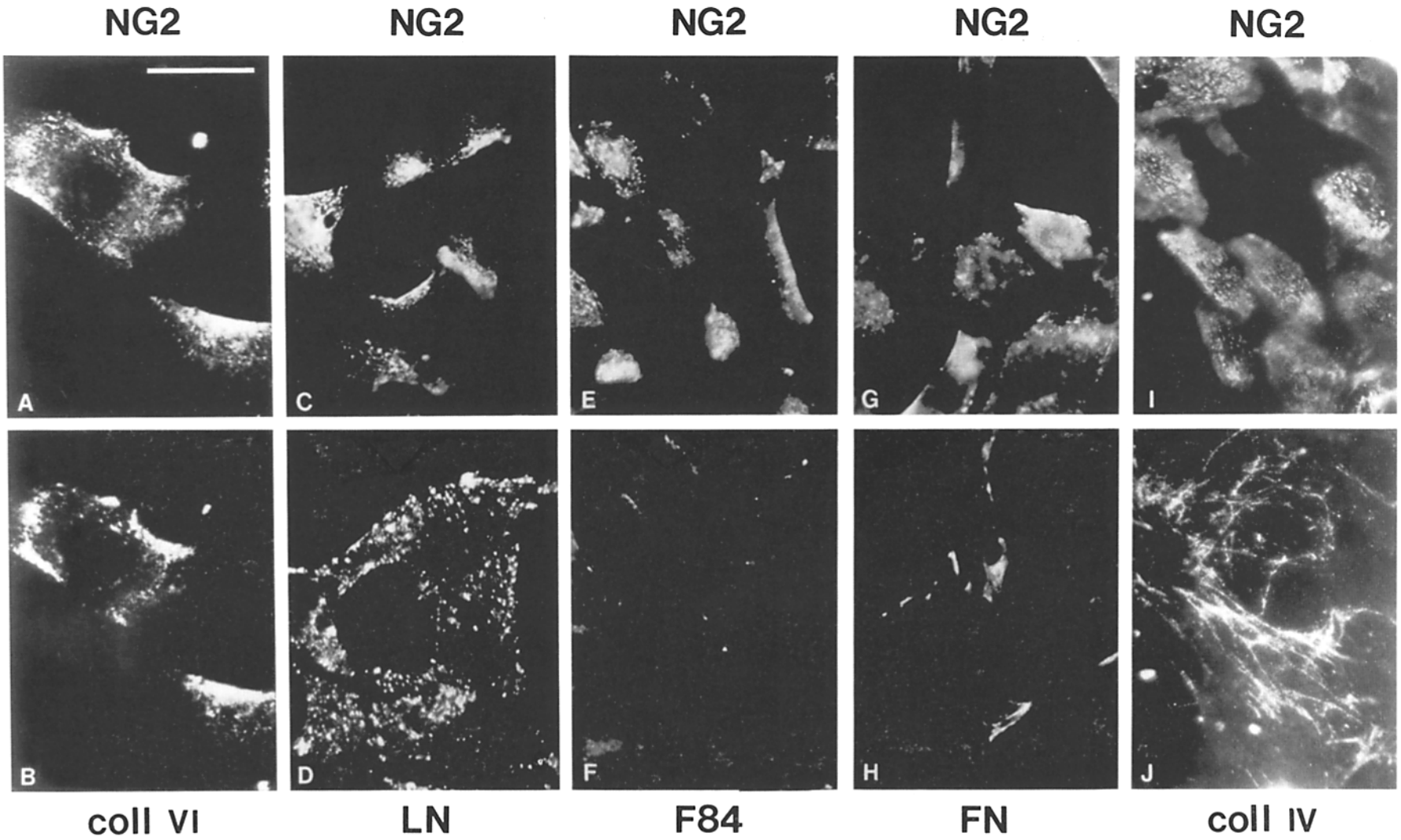


*Figure 6.* B49 cell double labeled with the following combinations of antibodies: (*A* and *B*) mouse anti-NG2 and rabbit antitype VI collagen; (*C* and *D*) mouse anti-NG2 and rabbit antilaminin; (*E* and *F*) rabbit anti-NG2 and mouse Mab F84; (*G* and *H*) mouse anti-NG2 and rabbit antifibronectin; and (*I* and *J*) mouse anti-NG2 and rabbit antitype IV collagen. A combination of FITC-GaMIg and TRITC-GaRIg was used to localize the primary antibodies. The codistribution of NG2 and type VI collagen is not seen with the other combinations of molecules. Bar, 10 $\mu$m.

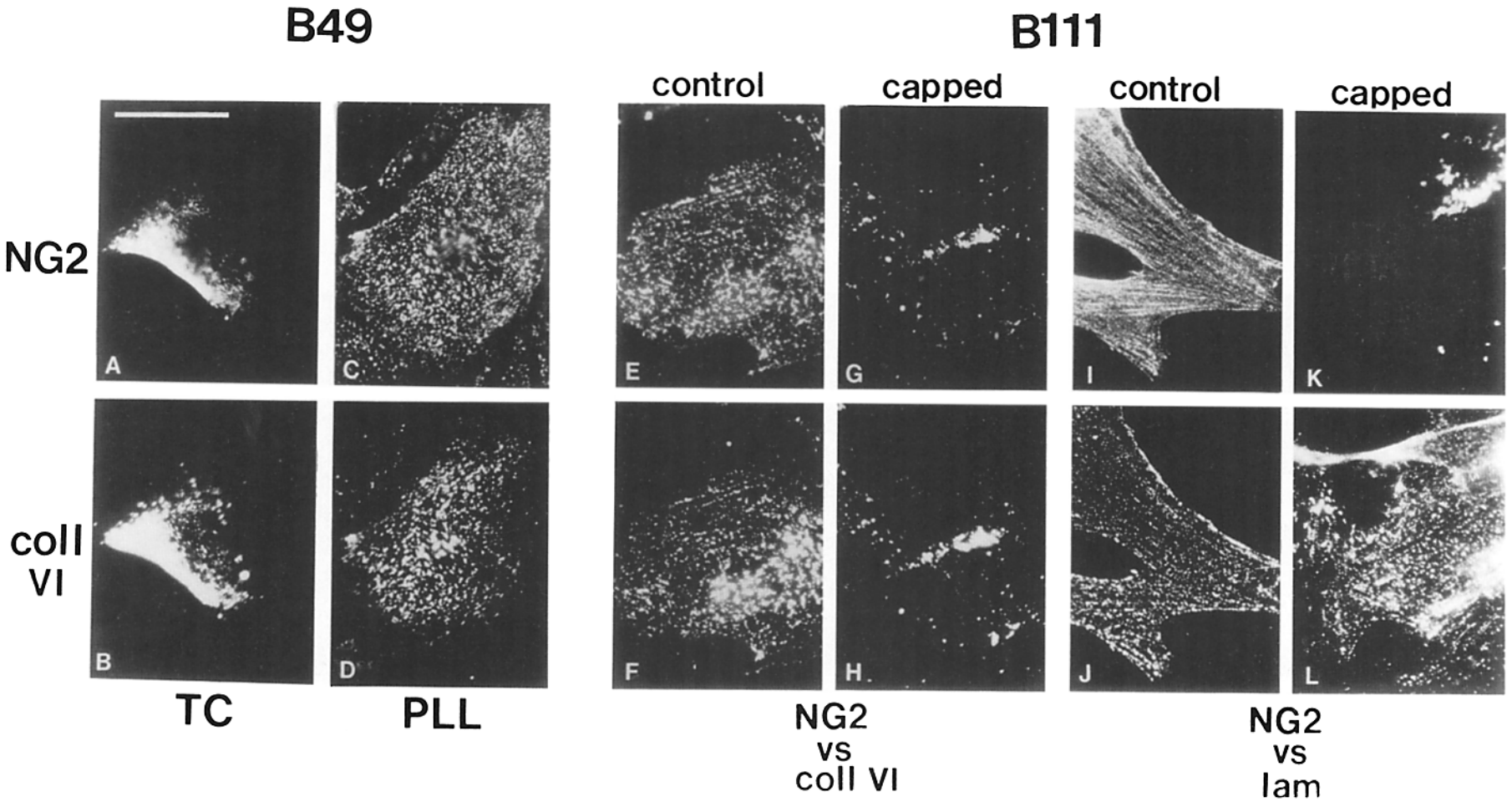


*Figure 7.* Redistribution of NG2 and type VI collagen. B49, cells. (*A* and *B*) Tissue culture dishes. Mouse anti-NG2 (*A*) and rabbit antitype VI collagen (*B*). (*C* and *D*) Poly-L-Lysine–coated dishes. Mouse anti-NG2 (*C*) and rabbit antitype VI collagen (*D*). B111 cells. (*E* and *F*) Control cells. Mouse anti-NG2 (*E*) and rabbit antitype VI collagen (*F*). (*G* and *H*) Cells treated overnight with mouse anti-NG2. Mouse anti-NG2 (*G*) and rabbit antitype VI collagen (*H*). (*I* and *J*) Control cells. Mouse anti-NG2 (*I*) and rabbit antilaminin (*J*). (*K* and *L*) Cells treated overnight with mouse anti-NG2. Mouse anti-NG2 (*K*) and rabbit antilaminin (*L*). A combination of FITC-GaMIg and TRITC-GaRIg was used to localize the primary antibodies. Changes in the distribution of NG2 are matched by changes in the distribution of type VI collagen. Bar, 10 $\mu$m.

used immunofluorescence double staining to examine the distribution of NG2 and type VI collagen on several cell lines (Fig. 5). We found the staining patterns for NG2 and type VI collagen to be very similar on three cell lines, B49, B111, and IMR-90, that express both molecules. The staining patterns were punctate in appearance and colocalized to a high degree. Culturing B49 cells in the presence of ascorbic acid, a cofactor for prolyl and lysyl hydroxylases (Engvall et al., 1986), increased the intensity of type VI collagen staining, but did not change the punctate nature of the staining or the colocalization with NG2. In contrast, the NG2-negative cell line B28 stained quite differently with antitype VI collagen; the collagen is present in a very fibrillar pattern. Using B49 cells we compared the distribution of NG2 to that of several other cell surface and matrix molecules (Fig. 6). Type IV collagen, fibronectin, and F84 are quite different in distribution from NG2. Laminin has a somewhat similar punctate appearance, but is not colocalized with NG2 to the degree seen with type VI collagen.

If NG2 and type VI collagen form complexes on the cell surface, we would expect changes in the distribution of NG2 to affect the distribution of collagen. One means of altering the distribution of NG2 is to grow the B49 cells on substrates of different adhesivity. On B49 cells grown in regular tissue culture dishes NG2 is often found to be concentrated along the edges of cells. On B49 cells attached to poly-L-lysine–coated dishes, however, NG2 is much more evenly distributed over the cell surface (Fig. 7, *A–D*). In both of these cases, type VI collagen codistributes with NG2. A more drastic change in NG2 distribution can be obtained by overnight incubation of the cells with anti-NG2 antibody. As shown in Fig. 7, *E–L* with B111 cells, this treatment causes patching or capping of NG2 into large clusters. Type VI collagen is also colocalized in these clusters after anti-NG2 treatment. In contrast, laminin remains spread over the cell surface and is not cocapped with NG2. It should be noted that antitype VI collagen will cause capping of type VI collagen without producing cocapping of NG2. This appears analogous to the failure of this antiserum to coprecipitate NG2 in our earlier results.

Finally, if the NG2-collagen interaction is relevant for normal cells, we should find examples of colocalization of the two molecules in vivo. We were unable to find type VI collagen associated with the NG2-positive glial progenitor cells in the central nervous system. In fact, no type VI collagen was seen in the brain. However, some of the blood vessels associated with the membranes surrounding the brain were stained by both anti-NG2 and antitype VI collagen. This was also seen in the periphery where a number of arteries showed striking colabeling with the two antibodies. The example of arteries in the spinal column of the embryonic day 16 rat is shown in Fig. 8, *A–C* and *G–I*. The intervertebral disks of the spine provide another good example of co-localization of NG2 and type VI collagen (Fig. 8, *A–C* and *D–F*). These pictures also show examples of structures such as the developing vertebrae which are NG2-positive, type VI collagen-negative, and connective tissue which is NG2-negative, type VI collagen positive. Thus, as with the cell lines, there are cases in which the two molecules are not coexpressed. However, there are clearly good examples of in vivo colocalization to validate our studies with the cell lines.

## *Discussion*

We have presented two types of evidence for an interaction of the NG2 proteoglycan with type VI collagen: coimmunoprecipitation and immunofluorescence colocalization. The immunoprecipitation experiments show that anti-NG2 antibodies coprecipitate type VI collagen along with NG2 proteoglycan and core protein. The fact that this coprecipitation is observed with both rabbit anti-NG2 and several different mAbs against NG2 argues that the phenomenon is not caused either by contamination of the rabbi antiserum with anticollagen activity or by recognition of an epitope that is shared by NG2 and type VI collagen. Furthermore, since type VI collagen is not seen in immunoprecipitations with antibodies against molecules other than NG2, the interaction of NG2 and type VI collagen must be a specific one that is not caused by general stickiness of the two molecules. Since we have looked only at the immunoprecipitation of molecules that are labeled with $^{125}I$ by the lactoperoxidase method, our experiments do not rule out the possibility that other unlabeled molecules may also bind to the NG2 proteoglycan. This is, of course, a subject of some interest which needs to be pursued.

Attempts to understand the nature of the NG2-collagen complex suggest that the glycosaminoglycan chains of NG2 are not required for binding. For example, a complex of NG2 core protein and type VI collagen is still seen in NP-40 extracts of cells treated with chondroitinase ABC or cells grown in the presence of p-nitrophenylxyloside. This is consistent with experiments which show that the complex in 1% NP-40 is not affected by addition of 1M NaCl or 0.5% SDS. However, boiling in 1% NP-40 plus 0.5% SDS dissociates the complex so that only NG2 is precipitated by anti-NG2 antibodies. Thus, the binding is probably mediated by protein–protein interactions rather than by weaker interactions involving chondroitin sulfate chains.

The interaction between NG2 and type VI collagen is reflected in their codistribution on several cell lines that we examined using immunofluorescence double staining. The fact that there is not a perfect one to one correspondence between the NG2 and type VI collagen staining patterns may indicate that not all type VI collagen is complexed with NG2 (or vice versa). Our immunoprecipitation clearing experiments (Fig. 4) also suggests that this is the case. It is interesting to note that the fibrillar distribution of type VI collagen on the NG2-negative cell line B28 is quite different from its punctate distribution on NG2-positive cell lines. NG2 might be responsible for organizing collagen molecules on these cell lines. The close colocalization of NG2 and type VI collagen is in sharp contrast to the lack of correspondence between NG2 and several other matrix molecules we examined. Type IV collagen, fibronectin, and laminin have noticeably different patterns of expression from that seen with NG2. Furthermore, perturbation of NG2 distribution on the cell surface (by plating cells on different substrates or by antibody-induced capping) results in parallel changes in type VI collagen distribution, suggesting once again a linkage between the two molecules.

One of our primary foci with NG2 to date has been the expression of the proteoglycan in the central nervous system on cells of the 0-2A lineage. Using immunofluorescence double labeling we have been unable to detect type VI colla-

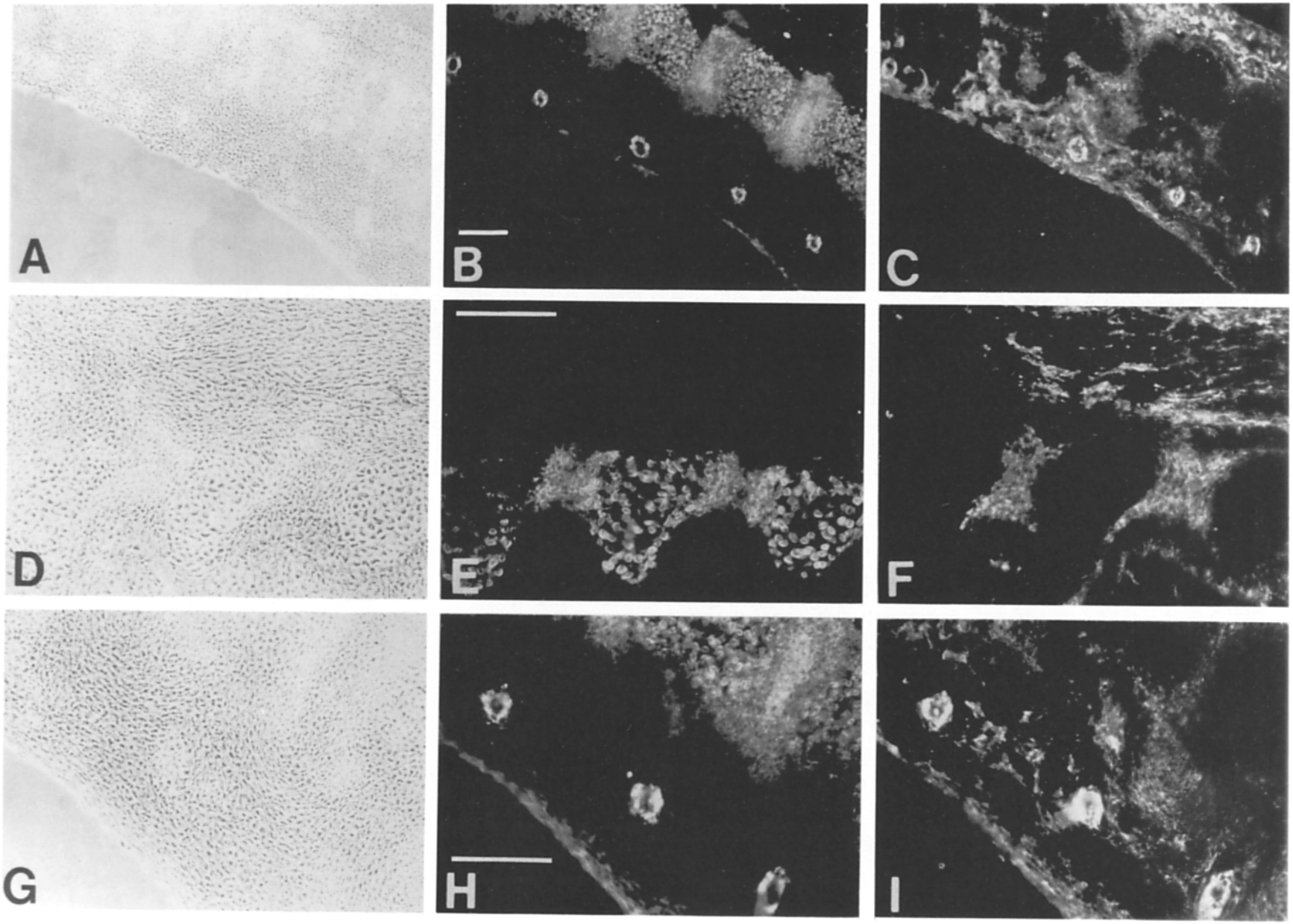


*Figure 8.* Localization of NG2 and type VI collagen in frozen sections from embryonic day 16 rat spine. (*A*, *D*, and *G*) Phase contrast; (*B*, *E*, and *H*) NG2; (*C*, *F*, and *I*) type VI collagen. NG2 and type VI collagen are colocalized in arteries and intervertebral discs of the spinal column. Bars, 150 $\mu$m.

gen expression on these NG2-positive glial cells. During these studies, however, we found NG2 and type VI collagen immunoreactivity in several other tissues of the developing rat. A detailed description of the distribution of NG2 will appear elsewhere, but for the present discussion it is important to note that there are areas in which NG2 and type VI collagen are colocalized. This occurs most prominently in some of the blood vessels in the thoracic cavity, in the spinal column, and in the intestines. Coexpression is also found in the intervertebral discs of the spine as well as in the tissue surrounding cartilaginous structures in the head. These examples show that our findings with clonal cell lines are relevant to interactions that can occur in vivo. It will be important to examine the in vivo preparations in more detail to determine what cell types are responsible for coexpression of NG2 and collagen.

The NG2-type VI collagen interaction may be important for organization of the extracellular matrix, for binding of cells to the matrix, and for determination of cell morphology in relation to the matrix. Type VI collagen is thought to be an important component of thin 3–5-nm microfibrils that interact with larger interstitial fibers containing types I and III collagen (Sakai et al., 1986; Keene et al., 1988). It has been suggested that the type VI collagen present in ligaments is associated with chondroitin sulfate proteoglycans (Bray et al., 1990). Binding to NG2 would provide the collagenous network with a point of attachment on the cell surface. Additional points of attachment are provided by molecules such as fibronectin and vitronectin which bind both to collagens and to cell surface receptors (or integrins) anchored in the cell membrane (Ruoslahti, 1988*b*). Since integrins can interact with the cytoskeleton, the collagen-fibronectin-integrin linkage provides one means of signal transmission from the extracellular matrix across the cell membrane to the cytoskeleton. Based on its detergent solubility (Stallcup et al., 1984) and our tentative identification of a membrane-spanning domain near the carboxy terminus (Nishiyama, A., K. Dahlin, S. Johnstone, J. Prince, and W. Stallcup, manuscript in preparation), we believe NG2 is a membrane-intercalated molecule. If so, then, the collagen-NG2 linkage may provide additional machinery for transmembrane signaling.

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