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Generation of Truncated Forms of the NG2 Proteoglycan by Cell Surface Proteolysis

Akiko Nishiyama,* Xiao-Hong Lin, and William B. Stallcup†

La Jolla Cancer Research Foundation, La Jolla, CA 92037

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NG2 is a chondroitin sulfate proteoglycan that is expressed on dividing progenitor cells of several lineages including glia, muscle, and cartilage. It is an integral membrane proteoglycan with a core glycoprotein of 300 kDa. In the present study we have characterized three molecular forms of the NG2 core protein expressed by different cell lines. Many cell lines that express the full length 300-kDa NG2 core protein also release a 290-kDa form into the medium. This species lacks the cytoplasmic domain but contains almost the entire ectodomain. Two core protein species, the intact 300-kDa form and a truncated 275-kDa form, are expressed at the surface of an NG2-transfected cell line U251NG52. The 275-kDa species lacks the cytoplasmic domain and at least 64 amino acids of the ectodomain. Mild trypsinization of B49 cells also generates the 275-kDa species, suggesting that this component is produced by proteolysis of the 300-kDa form. Conversion of the 300-kDa species to the 275-kDa form in U251NG52 cells is stimulated by reagents such as phorbol esters, which activate protein kinase C. Phorbol esters are also known to induce expression of metalloproteinases such as collagenase and stromelysin, which could be responsible for cleavage of the 300-kDa core protein. Although B49 cells do not spontaneously produce the truncated 275-kDa species, use of monoclonal antibodies against NG2 to block the interaction between NG2 and type VI collagen results in the appearance of the 275-kDa component in these cells. Thus the interaction between NG2 and type VI collagen, which contains a Kunitz-type proteinase inhibitor sequence in the $\alpha 3$ chain, may protect the proteoglycan against proteolysis. This is consistent with the observed deficiency of U251NG52 cells in anchoring type VI collagen at the surface.

INTRODUCTION

NG2 is a large, integral membrane, chondroitin sulfate proteoglycan that was first identified as a cell surface molecule expressed by immature neural cells (Wilson *et al.*, 1981). Subsequently NG2 was found to be expressed by a wide variety of immature cells during development, including cells of the O-2A glial lineage (Levine and Stallcup, 1987; Stallcup and Beasley, 1987) and developing cartilage of the limb (Nishiyama *et al.*, 1991b). A common feature of NG2 expression in these various lineages is that it appears to be preferentially expressed on dividing progenitor cells and then disappears as these cells undergo terminal differentiation into mature cell types. NG2 is also expressed in some proliferative pathological conditions such as glial neoplasms (Schrappe *et al.*, 1991) and reactive gliosis (Levine, 1994).

A large, membrane-spanning proteoglycan such as NG2 has the potential to be involved in multiple cellular functions by interacting with molecules both outside and inside the cell. It was previously shown that the core protein of NG2 can bind type VI collagen (Stallcup *et al.*, 1990), and that expression of NG2 on cells causes anchoring of type VI collagen at the cell surface (Nishiyama and Stallcup, 1993). These findings indicate a role for NG2 in organizing the pericellular matrix by serving as a cell surface receptor for

* Current Address: Department of Neurosciences/NC30, Research Institute, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195.
† Corresponding author.

type VI collagen. Another possible function for NG2 is suggested by recent experiments in which we have shown that NG2 and the α receptor for platelet-derived growth factor can interact on the surface of oligodendrocyte precursors and vascular smooth muscle cells. Disruption of this interaction with antibody against NG2 results in loss of signaling capability through the platelet-derived growth factor α receptor (Grako and Stallcup, 1995; Nishiyama *et al.*, 1996a,b).

In the present study, as part of our effort to correlate the function of NG2 with its structural properties, we have used antibodies against specific domains of the NG2 core protein to characterize three distinct molecular species of the proteoglycan. In addition to the intact 300-kDa species, we have identified a 290-kDa released form and a 275-kDa cell-associated form of the molecule, both of which lack the cytoplasmic domain. Many integral membrane proteins are known to exist both as membrane-bound and soluble truncated forms (see Ehlers and Riordan, 1991 for review), and attempts have been made to assign different functions to these distinct forms of the same protein. Most of these studies have been carried out on growth factor receptor molecules. For example, in Schwann cells a truncated form of low affinity nerve growth factor receptor generated by metalloproteinase cleavage is implicated in regulation of nerve regeneration (DiStefano *et al.*, 1993). Similarly, a soluble form of interleukin-4 (IL-4) receptor has been shown to neutralize the proliferative activity of IL-4 on lymphocytes that express intact IL-4 receptor (Mosley *et al.*, 1989). In contrast, a soluble form of the α receptor for ciliary neurotrophic factor has been shown to promote the biological activity of ciliary neurotrophic factor (Davis *et al.*, 1993). Functions of soluble forms of other integral membrane molecules such as cell adhesion molecules still remain largely unknown. While most truncated forms are readily released in soluble form from cells, there are some examples of released forms of proteins that become incorporated into the extracellular matrix as in the case of *int*-1 protein (Bradley and Brown, 1990; Papkoff and Schryver, 1990). Truncated, soluble forms of integral membrane proteins can arise from separate genes, by alternative splicing of transcripts from the same gene, or by post-translational processing of the intact protein. We present evidence that the truncated 290-kDa and 275-kDa forms of NG2 are derived from the intact 300-kDa core protein by proteolytic processing. In addition, we show that generation of the 275-kDa species is increased under circumstances that favor activation of protein kinase C, conditions that are also known to induce expression of metalloproteinases that might be responsible for proteolysis of the 300-kDa core protein. Finally, we suggest that protection against proteolysis of the 300-kDa core may be provided by interaction of NG2 with type VI collagen, due to the presence of a Kunitz-type proteinase inhibitor domain in the collagen α3 chain.

MATERIALS AND METHODS

Cells

The B49 cell line was derived from an ethylnitrosurea-induced rat brain tumor (Schubert *et al.*, 1974). U251NG52 and U251NG35 cell lines were derived by transfecting U251 MG malignant human glioma cells (Ponten and Westermark, 1978) with the coding region of the rat NG2 cDNA (nucleotides 1 to 7174) as previously described (Nishiyama and Stallcup, 1993). NIH3T3 cells transformed with activated H-*ras* gene (^{61}Leu mutation; Sassone-Corsi *et al.*, 1989) were obtained from Dr. Channing Der (University of North Carolina, Chapel Hill, NC). Cells were maintained in DMEM supplemented with 10% fetal calf serum (Tissue Culture Biologicals, Tulare, CA).

Antibodies

The specificity of anti-NG2 antibodies used in this study are shown schematically in Figure 1. The derivation of monoclonal antibodies against rat NG2 and rabbit antibody 553 against the entire molecule has been previously described (Stallcup *et al.*, 1990). 1466 is a rabbit antibody raised against a β-galactosidase fusion protein containing the cytoplasmic domain of rat NG2 from amino acid 2253–2325 (Nishiyama *et al.*, 1991a). 1657 was raised by immunizing the rabbit with a peptide derived from a region in the ectodomain of the rat NG2 core protein, which lies immediately amino-terminal to the transmembrane domain (amino acids 2161–2179). The peptide was coupled to keyhole limpet hemocyanin before immunization.

Metabolic Labeling and Immunoprecipitation

For immunoprecipitation experiments, cells were labeled for 18 h with 100 μCi/ml of [^{35}S]methionine (TRAN^{35}S-LABEL, ICN Biomedicals, Irvine, CA) in methionine-free DMEM containing 1% dialyzed serum. In some experiments, phorbol 12-myristate 13-acetate (PMA), phorbol 12,13-dibutyrate (PDBu), staurosporin, H-7, or okadaic acid (all obtained from Sigma, St. Louis, MO) were added to the labeling mixture. For the detection of cell surface NG2, cells were labeled with ^{125}I by the lactoperoxidase method (Hubbard and Cohn, 1972). Labeled cells were extracted for 15 min in a buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP40, and 1

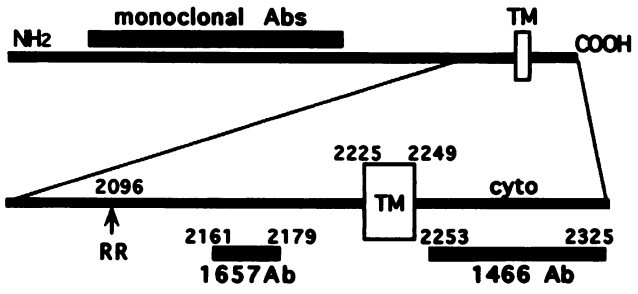

Figure 1. A schematic diagram of the rat NG2 core protein showing the specificity of various antibodies. Ab, antibody; COOH, carboxy terminus; cyto, cytoplasmic domain; NH_2, amino terminus; RR, a double arginine sequence; and TM, transmembrane domain. Numbers above bars indicate amino acid positions in the rat NG2 core polypeptide sequence. The six monoclonal antibodies used in this study recognize distinct epitopes in various locations throughout the ectodomain. Not drawn to scale.

mM phenylmethylsulfonyl fluoride, and insoluble material was removed by centrifugation for 5 min. The lysates were incubated with a mixture of six monoclonal antibodies against the ectodomain of rat NG2 core protein or with rabbit antibodies to rat NG2 for 1 h at room temperature, followed by precipitation with protein G Sepharose (in the case of monoclonal antibodies; Sigma) or protein A Sepharose (in the case of rabbit antibodies; Sigma). The precipitates were washed three times in phosphate-buffered saline (PBS) and then treated with 1 U/ml of chondroitinase ABC (ICN Biomedicals, Costa Mesa, CA) at 37°C for 1 h to remove chondroitin sulfate chains from NG2. The precipitates were then boiled in electrophoresis sample buffer containing 5% 2-mercaptoethanol and 3% SDS and electrophoresed through 6.5% polyacrylamide gels or 3–20% polyacrylamide gradient gels. For electrophoresis under nonreducing conditions, mercaptoethanol was omitted from the sample buffer. The gels were fixed and processed for autoradiography or for fluorography using EN^{3}HANCE (NEN, Boston, MA). For precipitating NG2 from the media, tissue culture supernatant from [^{35}S]methionine-labeled cells was incubated with monoclonal antibodies to NG2 in the presence of 1 mM phenylmethylsulfonyl fluoride and 0.1% NP40 followed by incubation with protein G Sepharose and processed as described above. To quantitate the intensity of radiolabeled bands corresponding to different NG2 core protein species, densitometric analysis was performed on fluorographs using an LKB densitometer.

For dephosphorylation experiments, chondroitinase-treated precipitates were incubated with 7 U/ml of *Escherichia coli* alkaline phosphatase (type III, Sigma) for 1 h at 37°C. To remove complex oligosaccharides from the core protein of NG2, chondroitinase-treated immunoprecipitates were first boiled in a buffer containing 0.4% SDS, 2% 2-mercaptoethanol, and 0.1 M sodium phosphate, pH 6.0. Then NP40 was added to 1%, and the precipitates were incubated with 4 U/ml of *N*-glycanase (Genzyme, Cambridge, MA) for 1 h at 37°C. For removal of immature, high mannose type of oligosaccharides, chondroitinase-treated precipitates were treated with 0.2 U/ml of endoglycosidase H (Miles, West Haven, CT) in 0.1 M sodium phosphate, pH 6.0, for 1 h at 37°C.

Trypsin Treatment

B49 and U251NG52 cells were washed with PBS and treated with 1 μg/ml of trypsin (Calbiochem, La Jolla, CA) for 10 min at room temperature. Trypsin was then inactivated by adding 100 μg/ml of soybean trypsin inhibitor. The samples were processed as above for immunoprecipitation and immunoblotting.

Immunoblotting

Cells were extracted in a small volume of the lysis buffer described above for 20 min at room temperature with the addition of 1.0 U/ml of chondroitinase ABC. For immunoblotting of secreted NG2, B49 cells were incubated in DMEM containing 1% fetal calf serum for 48 h, and NG2 was precipitated from the conditioned media using a mixture of six monoclonal anti-ectodomain antibodies. The precipitates were washed and treated with 1 U/ml of chondroitinase ABC for 1 h at 37°C. The samples were electrophoresed through 6.5% polyacrylamide gels and transferred to Immobilon-P membrane (Millipore, Bedford, MA) in a buffer containing 10 mM 3-(cyclohexylamino)-1-propane sulfonic acid (CAPS) (pH 11.0) and 10% methanol. Membranes were blocked overnight at room temperature in a buffer containing 20 mM Tris-HCl, pH7.4, 0.5 M NaCl, 5% calf serum, and 5% bovine serum albumin (TSS buffer). Then the membranes were incubated with rabbit antibodies against rat NG2 for 2 h at room temperature followed by washing with TSS and incubation with horseradish peroxidase-labeled goat anti-rabbit Igs (1:3000 dilution; Bio-Rad, Richmond, CA) for 1 h at room temperature. Following washes in TSS buffer, reacted proteins were visualized by chemiluminescence (ECL; Amersham, Arlington Heights, IL) or with diaminobenzidine. All antibodies were diluted in TSS buffer.

Pulse-Chase Experiments

For pulse-chase experiments, U251NG52 cells were preincubated for 15 min in methionine-free DMEM. Then the cells were incubated for 30 min at 37°C in methionine-free DMEM containing 200 μCi/ml of [^{35}S]methionine (TRAN^{35}S LABEL) and 1% dialyzed serum in the presence or absence of 50 nM PMA. Following the pulse period, the cells were washed once and incubated for various lengths of time (30 min to 12 h) in DMEM containing 10% fetal calf serum in the presence or absence of 50 nM PMA (chase). After the chase period, the cells were washed with PBS, extracted with 1% NP40, and NG2 was immunoprecipitated with monoclonal anti-NG2 antibodies as described above.

Northern Blot Analyses

Total RNA was extracted from cells by the guanidinium isothiocyanate method (Chirgwin *et al.*, 1979). Poly (A)$^+$ RNA was isolated by oligo(dT) cellulose affinity chromatography of total RNA or directly from cells by using the Micro Fast Track mRNA Isolation Kit from In Vitrogen (San Diego, CA). Two to five micrograms of poly (A)$^+$ RNA were fractionated through 1.2% agarose gels containing 2.2 M formaldehyde and transferred to nitrocellulose membranes. For detecting NG2 mRNA, a 7.2-kb fragment of rat NG2 cDNA encoding the entire coding region was labeled with [α-^{32}P]dCTP by the random priming method and was used for hybridization in a buffer containing 50% formamide.

RESULTS

Three Molecular Forms of NG2

By transfecting rat NG2 cDNA into U251 MG human glioblastoma cells, we have obtained two cell lines, U251NG35 and U251NG52, that stably express rat NG2 at the cell surface. As previously described (Nishiyama *et al.*, 1993), U251NG35 cells express NG2 in moderate levels and are able to anchor type VI collagen at the cell surface in an NG2-dependent fashion. U251NG52 cells express higher levels of NG2, yet fail to anchor type VI collagen at the cell surface.

For biochemical characterization of NG2 in these cells, cultures were metabolically labeled with [^{35}S]methionine. NG2 was immunoprecipitated from 1% NP40 extracts using a mixture of six monoclonal antibodies against rat NG2, all of which recognize different epitopes on the extracellular domain of the core protein. Figure 2 shows immunoprecipitates from extracts of four different cell lines: the B49 rat glioma (Figure 2, B49) that endogenously expresses NG2; the U251CMV line (Figure 2, CMV) obtained by transfecting U251 MG cells with pRc/CMV vector alone; and the two NG2-transfected cell lines, U251NG35 (Figure 2, NG35) and U251NG52 (Figure 2, NG52). No signal was detected from the control-transfected line U251CMV. In the other three cell lines, NG2 was detected as a polydisperse component having a molecular mass of greater than 300 kDa in the absence of chondroitinase ABC treatment (Figure 2, −). Small amounts of core protein without chondroitin sulfate chains were also found in each of these three cell lines. Treatment of the precipitates from B49 and U251NG35 cells with chondroitinase ABC (Figure 2, ch) converted

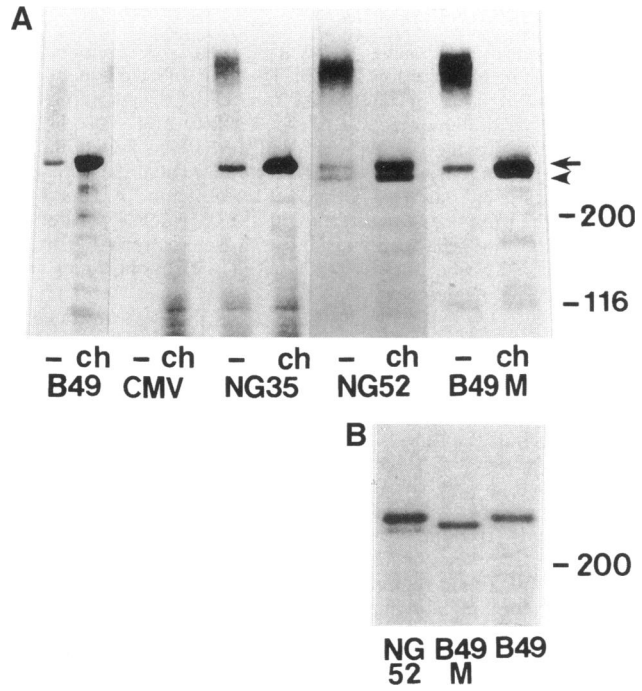

Figure 2. Immunoprecipitation of NG2 from [^{35}S]methionine-labeled cell extracts and media. (A) NG2 was immunoprecipitated using a mixture of six monoclonal anti-NG2 antibodies and fractionated on a 3–20% polyacrylamide gradient gel under nonreducing conditions. ch, precipitates were treated with chondroitinase ABC; −, precipitates were not treated with chondroitinase ABC; B49, B49 rat cells; CMV, U251CMV (vector-transfected) cells; NG35, U251NG35 cells; NG52, U251NG52 cells; and B49 M, medium from B49 cells. Molecular weight standards are indicated on the right (in kilodaltons). arrow, 300-kDa core protein; arrowhead, 275-kDa core protein species detected in U251NG52 cells. A single 300-kDa core protein is detected in B49 and U251NG35 cells, whereas a doublet of 300 and 275 kDa is detected in U251NG52 cells. A 290-kDa core protein is found in the medium from B49 cells. (B) NG2 immunoprecipitates from detergent extracts of U251NG52 cells (NG52), detergent extracts of B49 cells (B49), and tissue culture medium from B49 cells (B49 M) were chondroitinase treated and fractionated on a 5–10% polyacrylamide gradient gel. The difference in sizes of the 300-, 290-, and 275-kDa polypeptides are more readily apparent in this side by side comparison. Position of the 200-kDa molecular weight standard is shown on the right.

the proteoglycan into a discrete core protein of 300 kDa (arrow). Chondroitinase treatment of precipitates from U251NG52 cells resulted in generation of two core protein species of 300 kDa (arrow) and 275 kDa (arrowhead). We have found a similar core protein doublet in cell extracts of NIH3T3 cells transformed with H-*ras* oncogene. Figure 2 shows that NG2 can also be precipitated from tissue culture medium of metabolically labeled B49 cells. This phenomenon is not restricted to B49 cells because we have found released NG2 in the tissue culture medium of several other NG2-positive cell lines that we have examined (our unpublished observations). As with detergent-extracted NG2, released NG2 occurs as a proteoglycan and is sensitive to chondroitinase ABC treatment, which converts it into a core of 290 kDa (Figure 2, B49 M) that migrates between the 300- and 275-kDa bands detected in detergent extracts of U251NG52 cells. Thus we have identified three molecular forms of the NG2 core protein: a 300-kDa form present in extracts from all NG2-expressing cells, a 290-kDa form that is released from many of these same cells, and an additional 275-kDa cell-associated form found in U251NG52 cells and H-*ras*–transfected NIH3T3 cells. Figure 2B shows more clearly the difference in sizes between these three NG2 core protein species.

A Single Transcript for NG2

It is possible that different forms of NG2 could arise at the transcriptional level by alternative splicing. As shown in Figure 3, we attempted to identify alternatively spliced transcripts of NG2 by Northern blotting of RNA from U251NG52 cells (Figure 3, NG52), which produce both the 275-kDa and 300-kDa forms of NG2, and from U251NG35 cells (Figure 3, NG35), which produce only the 300-kDa species. A single transcript of 8.0 kb is detected in both of these cell lines but not in the control-transfected cell line U251CMV (Figure 3, CMV). As previously reported, B49 cells contain a single transcript of 8.9 kb (Nishiyama, *et al.*, 1991a). The smaller size of the NG2 mRNA in the two transfected cell lines is due to the absence of 897 bp from the 3′-untranslated region, which was deleted during construction of the expression plasmid DNA. The blot illustrates the difficulty in separating and distinguishing between transcripts of this size that are similar and yet distinct (8.0 vs. 8.9 kb). Because of this difficulty it

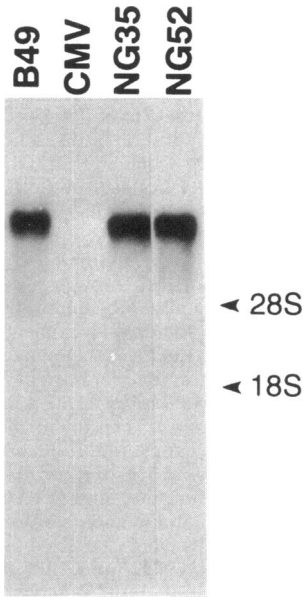

Figure 3. Detection of rat NG2 mRNA in cell lines. Two micrograms (B49 cells and U251NG52 cells) or 5 μg (U251CMV and U251NG35 cells) of poly (A)$^+$ RNA were fractionated through a 1.2% agarose-formaldehyde gel, transferred to a nitrocellulose membrane, and hybridized with a rat NG2 cDNA probe. Positions of 28S and 18S ribosomal RNA are indicated on the right. A single band is detected in B49, U251NG35, and U251NG52 cells but not in U251CMV cells.

is not possible to determine with certainty from Northern blots whether our individual cell lines produce multiple transcripts for NG2. Nevertheless, we consider the existence of multiple NG2 transcripts to be somewhat unlikely. During the molecular cloning of NG2, we isolated cDNA clones from four independent libraries. Sequencing of these clones yielded no evidence for alternatively spliced transcripts for NG2 (Nishiyama *et al.*, 1991a).

Proteolytic Cleavage of the 300-kDa Core Protein Yields the 275- and 290-kDa Species

As suggested above, we suspected on the basis of our cDNA cloning results that the three forms of NG2 (275, 290, and 300 kDa) might arise from a single transcript by post-transcriptional mechanisms. Two initial results of ^{125}I labeling tended to confirm our suspicion. First, both the 275- and 300-kDa species can be detected in extracts of U251NG52 cells surface labeled with ^{125}I. Second, the 290-kDa species can be detected in the supernatant from ^{125}I-labeled B49 cells that are returned to tissue culture for 6 h (Stallcup *et al.*, 1983). These results suggest that all three species arise from cell surface NG2 and are neither secreted directly from the cytoplasm into the medium nor derived from a cytoplasmic pool by detergent lysis of cells. We also found that when immunoprecipitates from U251NG52 cells were treated with 4 U/ml of *N*-glycanase, the 300- and 275-kDa forms were shifted to bands of 280 and 260 kDa, indicating that the two forms do not reflect differences in glycosylation (our unpublished observations). In addition, treatment of the immunoprecipitates with 7 U/ml of bacterial alkaline phosphatase (type III from *E. coli*) did not change the mobility of the two bands, suggesting that they do not result from differential phosphorylation. These findings indicate that the 300-kDa and 275-kDa forms probably differ at the level of the core polypeptide. There are several basic residues in the ectodomain of NG2 that might serve as target sites for cleavage by serine proteases such as trypsin. Therefore, the effects of trypsin on the expression of different forms of NG2 were tested by treating B49 and U251NG52 cells with 1 μg/ml of trypsin for 10 min at room temperature and then immunoblotting cell extracts using the 553 rabbit antibody (Figure 4A, 553). Treatment with trypsin quantitatively converted the 300-kDa form into a 275-kDa form in both cell types. The 275-kDa fragment generated by trypsin (Figure 4, 49T and 52T) had an electrophoretic mobility that was identical to that of the 275-kDa form detected in extracts from untreated U251NG52 cells (Figure 4, 52). These findings suggest that the 275-kDa species may be generated by proteolytic cleavage of the intact 300-kDa form of NG2.

The reactivity of the 275-kDa form with antibodies against the carboxy terminal region of NG2 was next examined. 553, which is a rabbit antibody raised against purified intact NG2, recognized both the 300- and 275-kDa species (Figure 4A, 553). Two other rabbit antibodies, 1466 and 1657, were used to stain identical blots. As shown in Figure 1, the 1466 antibody was raised against a β-galactosidase fusion protein containing the cytoplasmic domain. The 1657 antibody was raised against a peptide derived from a region of the ectodomain near the transmembrane domain. Both the 1466 and 1657 antibodies recognized the 300-kDa species from extracts of B49 and U251NG52 cells (Figure 4A, 49 and 52). However, the 275-kDa species, present in extracts from non-trypsinized U251NG52 cells (Figure 4A, 52) and in extracts from trypsin-treated B49 (Figure 4A, 49T) and U251NG52 cells (Figure 4A, 52T), was recognized only by 553 and not by 1466 or 1657. This suggests that the 275-kDa component lacks the cytoplasmic domain and a small juxtamembrane segment of the extracellular domain.

We next examined the reactivity of the 290-kDa released species with the same three rabbit antibodies. For these experiments, tissue culture supernatant from

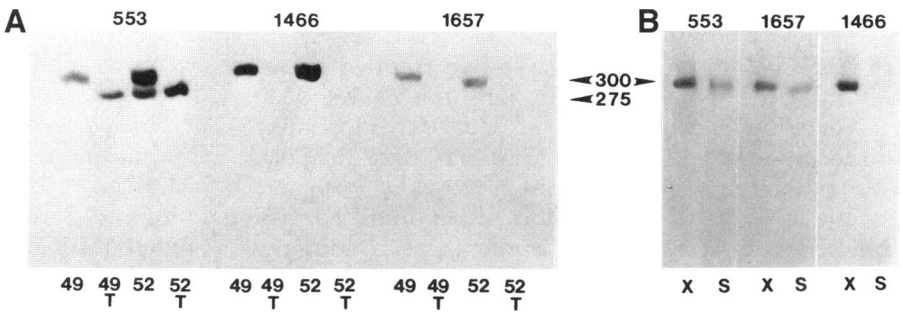

Figure 4. Immunoblot detection of NG2 from cell extracts and media using region-specific antibodies. (A) 1% NP40 extracts from control or trypsin-treated (T) B49 cells (49) or U251NG52 (52) cells were electrophoresed through a 6.5% polyacrylamide gel, transferred to Immobilon-P membrane, and stained with rabbit anti-NG2 antibodies 553 (1:5000 dilution), 1466 (1:500 dilution), or 1657 (1:500 dilution). All extracts were treated with chondroitinase ABC. 553 antibody recognizes both the 300- and 275-kDa forms, whereas 1466 and 1657 antibodies recognize only the 300-kDa form. (B) NG2 was immunoprecipitated from tissue culture media of B49 cells with monoclonal anti-NG2 mixture, treated with chondroitinase ABC, and processed as for panel A. X, cell extracts prepared as described in panel A; S, immunoprecipitates from conditioned media. The 300-kDa core protein from cell extracts of B49 cells is recognized by all three antibodies. The 290-kDa species released from B49 cells is recognized by 553 and 1657 but not by 1466 antibody.

B49 cells was immunoprecipitated with monoclonal anti-NG2 antibody mixture, and the precipitates were then processed for immunoblotting. As shown in Figure 4B, 553 recognized both the 300-kDa cell-associated form (X) and the 290-kDa released form (S). The released form (Figure 4B, S) was also recognized by 1657 but not by 1466 antibody, even though both antibodies recognized the 300-kDa species from cell extracts (Figure 4B, X). These results indicate that the 290-kDa released form of NG2 is also a proteolytically cleaved form of NG2, but that the 290-kDa form is generated by cleavage of the NG2 core protein at a more carboxy terminal site than the site used to generate the 275-kDa form.

The 275-kDa Core Remains Associated with the Transmembrane/Cytoplasmic Stump

The foregoing evidence indicates that the 275-kDa form of NG2, which lacks the cytoplasmic and transmembrane domains and is shorter than the 290-kDa released form, is nevertheless retained at the cell surface. To investigate the nature of the association of the 275-kDa species with the cell surface, NG2 was immunoprecipitated from [^{35}S]methionine-labeled extracts of U251NG52 cells with 553 or 1466 antibody following various treatments of the extracts in an attempt to dissociate complexes containing the 275-kDa form. When untreated control extracts of U251NG52 cells were precipitated with 553 antibody raised against the entire NG2 molecule (Figure 5, N), or with 1466 antibody raised against the cytoplasmic domain (Figure 5, C), both the 300-kDa (Figure 5, arrow) and the 275-kDa (Figure 5, arrowhead) forms could be precipitated with either antibody (Figure 5, cont). The surprising finding that the 275-kDa form, which lacks the cytoplasmic domain, can still be immunoprecipitated with 1466 anti-cytoplasmic antibody indicates that the cleaved 275-kDa component remains associated with the small transmembrane/cytoplasmic fragment under the extraction conditions. Treatment of extracts with 1 M NaCl (Figure 5, 1 M NaCl), 0.5% SDS (Figure 5, SDS), or 1 U/ml of chondroitinase ABC (Figure 5, Ch ABC) before immunoprecipitation resulted in detection of the same two bands with both antibodies. However, when extracts were heated to 65°C for 5 min in the presence of 0.5% SDS (Figure 5, SDS Heated), 1466 antibody precipitated only the 300-kDa form and not the 275-kDa form. Both forms could still be precipitated with 553 antibody under these conditions. These results indicate that after proteolytic cleavage of the 300-kDa form, the newly generated 275-kDa fragment remains associated with the transmembrane/cytoplasmic fragment by a relatively strong, noncovalent interaction that is sensitive to heating in SDS.

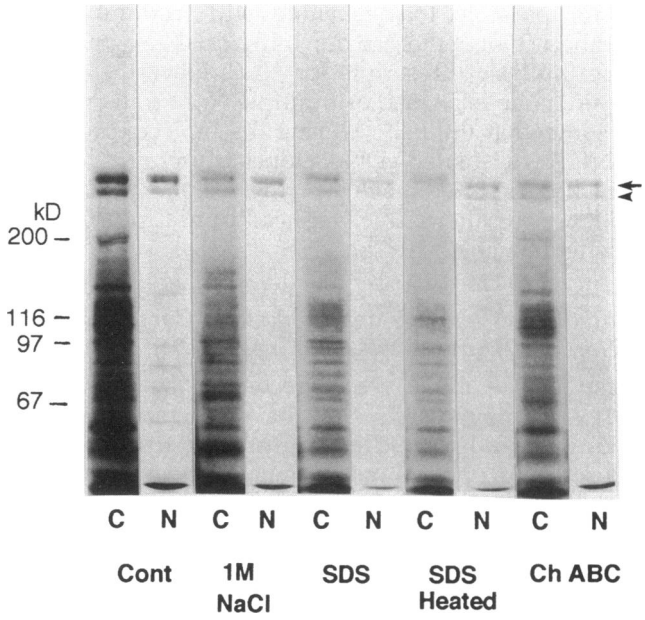

Figure 5. Immunoprecipitation of the 275-kDa core with 1466 anti-cytoplasmic domain antibody. U251NG52 cells were labeled overnight with [^{35}S]methionine. NG2 was immunoprecipitated from 1%NP40 extracts with 553 antibody (N) raised against the entire molecule or with 1466 anti-cytoplasmic domain antibody (C). Before immunoprecipitation, extracts were treated as follows: 1 M NaCl for 30 min at room temperature (1 M NaCl); 0.5% SDS for 30 min at room temperature (SDS); heated for 15 min at 65°C (SDS Heated); and 1.0 U/ml of chondroitinase for 30 min at room temperature (ch ABC). All precipitates were treated with chondroitinase ABC. 1466 antibody failed to precipitate the 275-kDa species only when the extracts were heated in SDS. Arrow, 300-kDa band; arrowhead, 275-kDa band.

Activation of Protein Kinase C Stimulates Conversion of the 300-kDa Form into the 275-kDa Form

During some preliminary attempts to study the state of phosphorylation of NG2, we noticed that treatment of U251NG52 cells with PMA, which is an activator of protein kinase C (Nishizuka, 1984), resulted in a shift in the relative proportion of the two cell-associated forms of NG2. The increase in quantity of the 275-kDa form was accompanied by a decrease in the amount of the 300-kDa form (Figure 6, cont and PMA). The effect of PMA was dose-dependent over a wide range of concentrations, showing maximal effects at 50 and 500 nM. Even at the high concentration of 5 μM, which is known to down-regulate protein kinase C after prolonged incubation (Rodriguez-Pena and Rozengurt, 1984; Nishizuka, 1988), there was a significant shift from the 300- to 275-kDa form. To determine whether the ability of PMA to convert the 300-kDa component into the 275-kDa species resulted from activation of protein kinase C, we incubated U251NG52 cells with

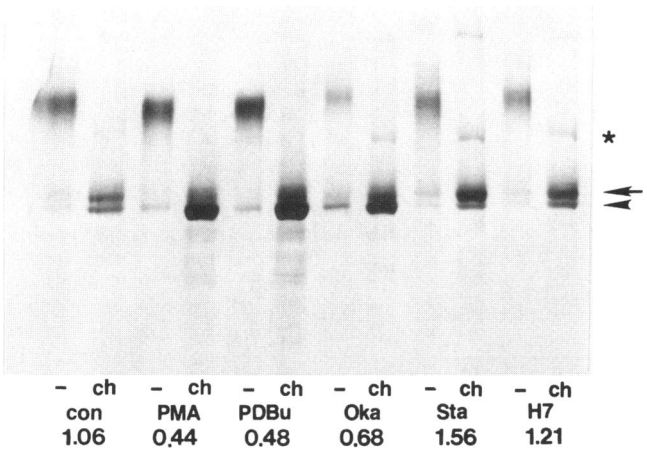

Figure 6. Effects of reagents that modify protein kinase C activity on the relative proportion of the 300- and 275-kDa NG2 core protein species expressed by U251NG52 cells. U251NG52 cells were treated with 50 nM PMA (PMA), 50 nM PDBu (PDBu), 100 nM okadaic acid (oka), 50 μM H-7 (H-7), or 50 nM staurosporin (sta) for 18 h at 37°C while they were labeled with [^{35}S]methionine. NG2 was immunoprecipitated from 1% NP40 extracts using monoclonal anti-NG2 ectodomain antibody mixture, and separated on 3–20% polyacrylamide gels under nonreducing conditions. Cont, cells were not treated with any drugs; −, immunoprecipitates were not treated with chondroitinase ABC; and ch, immunoprecipitates were treated with chondroitinase ABC. PMA, PDBU, and okadaic acid increased the relative intensity of the lower band while H-7 and staurosporin decreased it. Numerical values represent ratios of the intensities of the 300- and 275-kDa components as determined by densitometry. *, 420-kDa band; arrow, 300-kDa band; and arrowhead, 275-kDa band.

other reagents that are known to alter the activity of protein kinase C.

Treatment of U251NG52 cells with 50 nM PDBu, another phorbol ester that binds to and activates protein kinase C (Lee and Bell, 1986), or with 100 nM okadaic acid, which inhibits protein phosphatases 1 and 2A (Bialojan and Takai, 1988), resulted in the same effect as that seen with PMA treatment (Figure 6, PDBu and oka). On the contrary, treatment of cells with 50 μM H-7 or 50 nM staurosporin, both of which are known as high affinity inhibitors of protein kinase C (Hidaka *et al.*, 1984; Tamaoki *et al.*, 1986), resulted in a relative increase in the 300-kDa form over the 275-kDa form (Figure 6, sta and H-7). Numerical values under each set of conditions in Figure 6 represent the ratios between the densitometrically determined quantities of the 300- and 275-kDa components seen in each part of the experiment. Thus treatment of cells with PMA, PDBu, and okadaic acid decreases the ratio from its original value of 1.06, while treatment with staurosporin and H7 increases the ratio.

Under the nonreducing conditions of electrophoresis used in Figure 6, an additional 420-kDa component is consistently observed in chondroitinase-treated NG2 immunoprecipitates. This component is detected most abundantly under conditions that favor the existence of the 300-kDa core protein species as opposed to the 275-kDa species. In Western blotting experiments the 420-kDa component is recognized by the 553 antibody against NG2, indicating that this band may represent a fourth form of the NG2 core protein. Although the exact composition of the 420-kDa component is far from clear, reduction with 2-mercaptoethanol results in its disappearance. This suggests that this species could consist of the NG2 core protein disulfide bonded to an additional polypeptide.

Kinetics of Generation of the 275-kDa Core Protein

To examine the time course of PMA-induced generation of the 275-kDa form of NG2, a pulse-chase experiment was carried out in the presence or absence of PMA (Figure 7A). U251NG52 cells were incubated with [^{35}S]methionine for 30 min (pulse) followed by incubation in chase medium for the indicated lengths of time. NG2 was then immunoprecipitated from cell extracts taken at the various time points. NG2 first appeared as a 275-kDa species, which was quickly converted into the 300-kDa component within 2 h. Up to 4 h after labeling, the 300-kDa form was the predominant NG2 component detected. These findings are consistent with previously reported results of pulse-chase experiments using B49 cells (Stallcup *et al.*, 1983). Starting at 4 h after labeling, a 275-kDa component was again apparent, becoming more prominent by 12 h. This 275-kDa species appeared as a broader band than the initial 275-kDa species. Addition of 50 nM PMA in the pulse and chase media (Figure 7, lane P) did not affect the relative amounts of the initial 275-kDa and 300-kDa forms seen during the first 4 h. In contrast, after 4 h, a larger quantity of the 275-kDa component could be seen in cells treated with PMA. This effect of PMA was most prominently seen 12 h after labeling. To compare the initial 275-kDa form to the 275-kDa form generated at later time points, immunoprecipitates were prepared 1, 4, and 12 h after labeling. These precipitates were subjected to endoglycosidase H (endo H) digestion, which removes only immature, high-mannose types of oligosaccharides. Endo H treatment resulted in a shift of the early-appearing 275-kDa form into a faster-migrating form of 260 kDa (Figure 7B, 1 hr), consistent with previously reported findings (Stallcup *et al.*, 1983). In contrast Endo H treatment did not affect the mobility of the 275-kDa form that appeared at later time points (Figure 7B, 12 hrs) or the mobility of the intact 300-kDa form (Figure 7B, 4 and 12 hrs). These results suggest that the early 275-kDa component is an intact, immature core protein species carrying oligosaccharides that have not yet been processed in the Golgi apparatus. In contrast, the late-appearing 275-kDa component carries mature, processed oligosaccharides and is

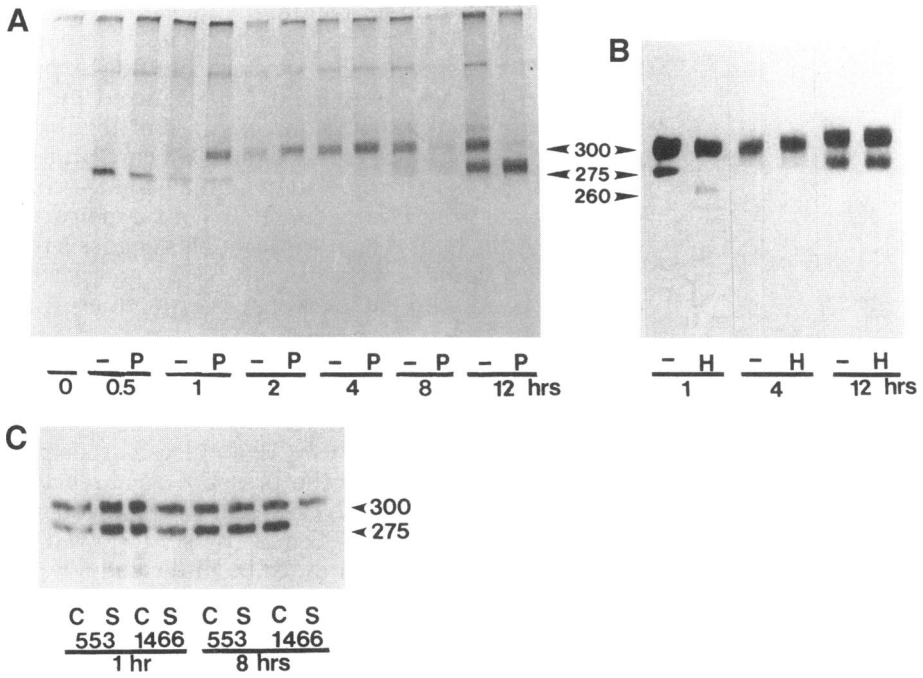

Figure 7. Pulse-chase experiments to determine the time course of generation of the 300- and 275-kDa species in U251NG52 cells. (A) U251NG52 cells were labeled with [^{35}S]methionine for 30 min and chased for the indicated lengths of time in the presence (P) or absence (−) of PMA. At the end of the chase period NG2 was immunoprecipitated from 1% NP40 extracts using monoclonal anti-NG2 antibody mixture. The precipitates were treated with chondroitinase ABC, and resolved on a 6.5% polyacrylamide gel. A 275-kDa form first appears immediately after labeling but disappears by 2 h and is replaced by a 300-kDa species. After 4 h, a broader 275-kDa form appears and increases in intensity through 12 h. (B) Endo H treatment of pulse-chased products. Immunoprecipitates prepared from extracts after 1, 4, and 12 h in the chase medium were treated with chondroitinase ABC followed by treatment with endoglycosidase H. The samples were then separated on a 6.5% polyacrylamide gel under reducing conditions. Treatment of immunoprecipitates with endoglycosidase H (H) increases the mobility of the initial 275-kDa form detected at 1 h after labeling but does not affect the later appearing 275-kDa form that is detected at 12 h. Arrowheads mark positions of the 300-, 275-, and 260-kDa components. (C) 1% NP40 extracts of pulse-chased U251NG52 cells were made after 1 h and 8 h of chase. One-half of each extract was heated for 5 min at 65°C with 0.5% SDS. Denatured (S) and nondenatured (C) extracts were then immunoprecipitated with antibodies against the intact NG2 core protein (553) and with antibodies against the NG2 cytoplasmic domain (1466). Arrowheads mark positions of the 300-and 275-kDa core species.

likely to represent the PMA-sensitive, proteolytically cleaved form of the core protein. Additional support for this conclusion is provided by the pulse-chase experiment shown in Figure 7C. At early time points (Figure 7C, 1 hr) the 1466 antibody against the NG2 cytoplasmic domain is able to immunoprecipitate both the 300- and 275-kDa species, even when they are denatured by heating in SDS (Figure 7C, 1466 S/1 hr). In contrast, at later time points (Figure 7C, 8 hr) the 1466 antibody is unable to immunoprecipitate the denatured 275-kDa species (Figure 7C, 1466 S/8 hr). As expected, the 553 antibody against the whole NG2 core protein is able to immunoprecipitate both components under all sets of conditions. This data shows once again that the 275-kDa component initially synthesized by the U251NG52 cells represents the intact core protein, while the late-appearing 275-kDa component represents the truncated core protein lacking the cytoplasmic and transmembrane domains.

Correlation of Type VI Collagen Retention and the Appearance of the 275-kDa Core Species

We noted previously that U251NG52 cells, which express a mixture of the 275- and 300-kDa NG2 core proteins, retain only a small amount of type VI collagen at the cell surface (Nishiyama and Stallcup, 1993). This is in contrast to the U251NG35 clone, which expresses only the 300-kDa core protein and retains much higher levels of type VI collagen. These two distinct expression patterns are shown in Figure 8, panels C–F. H-*ras*–transformed NIH 3T3 cells, which also express both forms of the core protein, similarly fail to retain much type VI collagen at the cell surface. These correlations provide circumstantial evidence for a cause and effect relationship between the two phenomena of poor type VI collagen anchorage and truncated NG2 core protein expression. To investigate this question further we utilized our observation that NG2-dependent type VI collagen retention can be blocked in NG2-transfected B28 cells by incubation with monoclonal antibodies against NG2 (Nishiyama and Stallcup, 1993). As seen in Figure 8, G–J, this effect is also seen with B49 cells. After trypsinization to remove NG2 and type VI collagen, B49 cells were allowed to recover overnight in the presence or absence of a mixture of three different monoclonal antibodies that recognize epitopes in the NG2 ectodomain. Although NG2 and type VI collagen are re-expressed

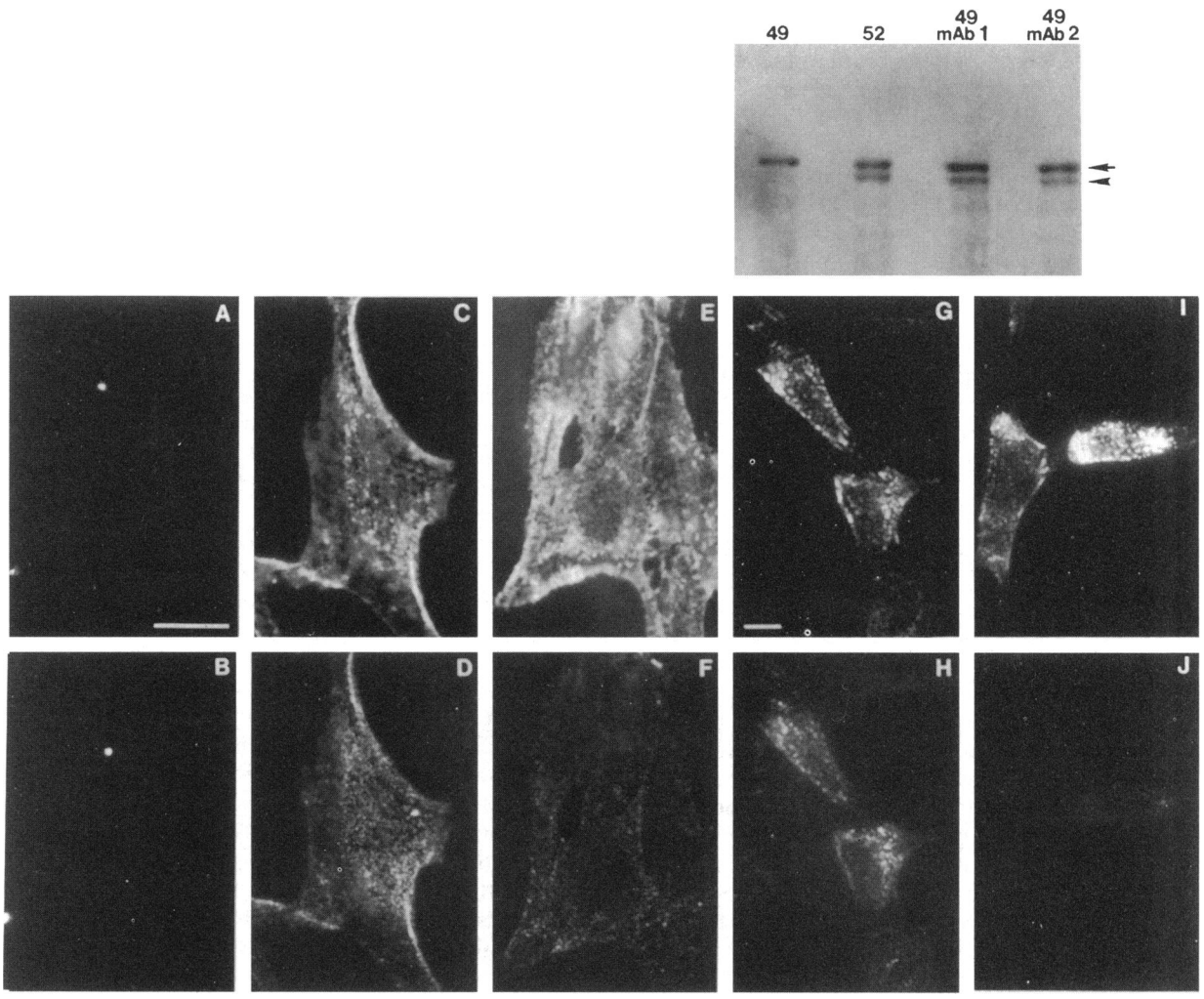

Figure 8. The truncated 275-kDa NG2 core protein is expressed by cells that fail to retain type VI collagen at the cell surface. Cultures were doubled labeled with monoclonal antibodies against NG2 (A, C, E, G, and I) and rabbit antibodies against type VI collagen (B, D, F, H, and J). (A and B) U251 MG cells transfected with vector alone; (C and D) U251NG35 cells; (E and F) U251NG52 cells; (G and H) B49 cells trypsinized and allowed to recover overnight without antibody treatment; and (I and J) B49 cells trypsinized and allowed to recover overnight in the presence of monoclonal antibodies N3, D4, and D31 against NG2. Bars in panels A and G represent 10 μm. Control U251 MG cells are negative for NG2 and do not anchor type VI collagen on their surface. Both U251NG35 and U251NG52 cells express NG2, but clone 52 does not anchor much type VI collagen on the cell surface. B49 cells express both NG2 and type VI collagen in a co-localized fashion. Note that cells with low levels of NG2 retain low levels of type VI collagen (panels G and H). The presence of anti-NG2 antibodies during recovery from trypsinization prevents association of type VI collagen with NG2 (panels I and J). Inset at top right shows Western blot of NG2 core proteins with 553 rabbit antibody. NP40 extracts were treated with chondroitinase ABC and run on 7% SDS-PAGE gels. 49, B49 cells after recovery from trypsinization (comparable to cells in panels G and H); 52, U251NG52 cells (comparable to panels E and F); 49 mAb1, B49 cells after trypsinization and recovery in the presence of mAbs N3, D4, and D31 (comparable to panels I and J); 49 mAb2, B49 cells after trypsinization and recovery in the presence of mAbs N3, D4, and N11. Control B49 cells express only the intact 300-kDa NG2 core protein (arrow). B49 cells treated with a mixture of monoclonal anti-NG2 antibodies express both the 300-kDa and 275-kDa species, similar to the pattern seen with U251NG52 cells.

and co-localized on the control B49 cells (Figure 8, G and H), very little type VI collagen is retained by the antibody-treated cells (Figure 8, I and J). When extracts of the control and antibody-treated cells were compared by Western blotting with rabbit anti-NG2 antibody (553), we found that control cells (Figure 8, 49) contain only the intact 300-kDa form of the NG2 core protein while the antibody-treated cells (Figure 8, 49 mAb1 and 49 mAb2) contain both forms of the core protein, comparable to the pattern seen with U251NG52 cells (Figure 8, 52). This is consistent with the hypothesis that the truncated 275-kDa species is produced in cells that are unable to retain type VI collagen.

DISCUSSION

In the present study we have characterized three different forms of the rat NG2 core protein. The most commonly observed form of the core protein is the mature, intact, 300-kDa species that is found on the surface of all cell types that express NG2. In addition, a spontaneously released form of NG2, with a core protein size of 290 kDa, is detected in the tissue culture supernatant of many of these same cells (Stallcup *et al.*, 1983). Based on immunoblotting experiments, this component is a truncated form that lacks the cytoplasmic domain but contains almost the entire ectodomain. In addition to these two forms, a third species of 275 kDa is found in detergent extracts of U251NG52 cells, which were obtained by transfecting U251 MG human glioma cells with rat NG2 cDNA. This form is not normally expressed by B49 cells nor by the U251NG35 cell line, another NG2-transfected line derived from U251 MG glioma cells, but is found in H-*ras*–transformed NIH 3T3 cells. Immunoblotting analysis with region-specific antibodies indicates that the 275-kDa component lacks the cytoplasmic domain and at least 64 residues of the juxtamembrane region of the ectodomain. Thus the 275-kDa form appears to be truncated at a more amino-terminal site than the 290-kDa released molecule. Several observations suggest to us that the truncated forms of the NG2 core protein are not produced from multiple genes or by alternatively spliced transcripts, but are generated from a single transcript by post-translational proteolysis. These observations are as follows: 1) Southern blot analysis has detected only one copy of the NG2 gene (Nishiyama *et al.*, 1991a). 2) cDNA clones selected from four independent libraries failed to yield evidence for alternatively spliced NG2 transcripts. 3) In pulse-chase experiments with U251NG52 cells the intact core protein species appears at the very beginning of labeling, while the truncated 275-kDa species is not observed until 4–8 h into the experiment. 4) Although a 275-kDa form of NG2 is not normally found in B49 cells, this species can be generated in B49 cells by using anti-NG2 antibodies to block type VI collagen anchorage at the cell surface. 5) Trypsin treatment of intact NG2 yields a 275-kDa species indistinguishable from the truncated component found in U251NG52 cells. 6) Cells surface labeled with ^{125}I are found to release ^{125}I-labeled 290-kDa NG2 into the tissue culture medium in a time-dependent fashion, suggesting that this molecule initially occurs as the intact cell surface form of NG2 before being shed.

The question arises as to how the 275-kDa form, which lacks the membrane anchor, can remain associated with the cell surface. We have observed that under ordinary extraction conditions using 1% NP40 and 150 mM NaCl, the 275-kDa form is immunoprecipitated with the 1466 anti-cytoplasmic antibody, even though this antibody does not detect the 275-kDa species on immunoblots. This suggests that the 275-kDa ectodomain fragment must remain associated with the transmembrane/cytoplasmic fragment after cleavage. Consistent with this notion, heating the extracts to 65°C in the presence of 0.5% SDS before immunoprecipitation abolished the ability of the 1466 antibody to precipitate the 275-kDa form. The ability of 1466 to precipitate the 300-kDa species was retained under these conditions. In Figure 9 we propose a model in which the conformation of NG2 allows a noncovalent association of the 275-kDa ectodomain fragment with the membrane-bound stump even after cleavage near the transmembrane domain. Although

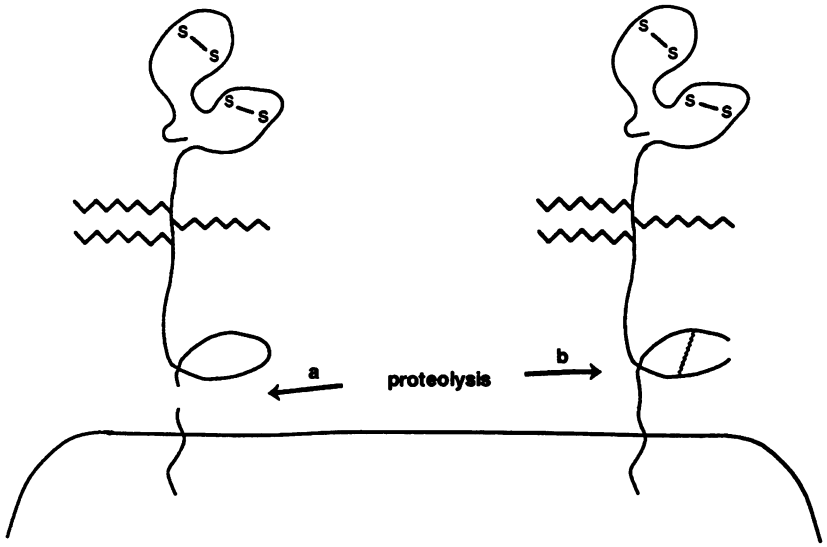

Figure 9. A schematic diagram showing possible conformation of the NG2 proteoglycan. S—S, disulfide linkage in the amino-terminal domain (number of linkages is hypothetical); ∿∿∿, glycosaminoglycan chains (number of chains is hypothetical). ┈┈┈, noncovalent interaction that holds together the 275-kDa ectodomain fragment and the cytoplasmic stump. a, potential cleavage site that is responsible for generating the 290-kDa form secreted by B49 cells; b, potential cleavage site that is responsible for generating the 275-kDa form found on the surface of U251NG52 cells.

little is known about the conformation of the polypeptide in this region of NG2, we suppose that the secondary or tertiary folding of the molecule (symbolized by the loop in Figure 9) allows a stable interaction between part of the ectodomain and the juxtamembrane region. One of the four cadherin-like repeats found in the NG2 core protein lies near this juxtamembrane region, raising the possibility that the conformation of this portion of the molecule might be stabilized by this motif (Nishiyama *et al.*, 1991a). A similar phenomenon of retention of a cleaved fragment at the cell surface has been reported for the cell adhesion molecules L1 or NILE (Sadoul *et al.*, 1988; Prince *et al.*, 1989), and Bravo/NrCAM (Kayyem *et al.*, 1992). In these cases the polypeptide conformations are likely to be stabilized by fibronectin type III repeats located in the juxtamembrane regions. Thus this phenomenon of juxtamembrane cleavage may be a common feature that is shared by many cell surface molecules. This type of cleavage is observed in vivo for several of the cell adhesion molecules, but we have no information at present concerning in vivo occurrence of cleaved forms of NG2. The observation that the 290-kDa form is readily secreted into the medium of B49 cells suggests that in this case cleavage takes place at a site that is not important for the intramolecular interaction between the NG2 ectodomain and the cytoplasmic stump (shown schematically as "a" in Figure 9).

We have observed that the relative proportion of the truncated 275-kDa form of NG2 to the intact 300-kDa form on the surface of U251NG52 is correlated with the activity of protein kinase C. Treatment of U251NG52 cells with phorbol esters that bind to and stimulate protein kinase C or with okadaic acid that increases the net protein kinase activity by inhibiting protein phosphatases 1 and 2A results in a shift from the 300-kDa to the 275-kDa form. Conversely, inhibition of protein kinase activity by staurosporin or H-7 results in a relative increase in the 300-kDa form. We have considered three possible mechanisms by which the 275-kDa form could be generated in a protein kinase C-dependent manner. First, activation of protein kinase C might directly activate a cell surface proteinase by changing its state of phosphorylation. Alternatively, protein kinase C might phosphorylate NG2 and thereby induce a conformational change in the ectodomain, which increases its susceptibility to proteolytic cleavage. However, these two events would be likely to occur very rapidly, as most phosphorylation events take place within minutes. For example, cleavage of the membrane precursor for transforming growth factor-α (TGF-α) by PMA is complete in 15 min (Pandiella and Massague, 1991). Similarly, phosphorylation of amyloid precursor protein following protein kinase C activation results in enhanced processing of the protein within 45 min (Buxbaum *et al.*, 1990). Our pulse-chase experiments show that the 275-kDa form of NG2 does not start to appear until 4 to 8 h after labeling, whereas the intact form is already detectable after 1 h. A third possible mechanism that would account for the delay in appearance of the truncated 275-kDa species is that activation of protein kinase C induces de novo transcription of a gene encoding a proteinase that is responsible for the cleavage of NG2. This must remain speculative until we identify the proteinase responsible for this cleavage.

Although the exact cleavage sites and the enzymes responsible for proteolytic processing of NG2 have not yet been determined, some speculations can be made. Metalloproteinases are zinc-dependent enzymes that degrade various extracellular matrix molecules and are considered to be important in remodeling the extracellular matrix during normal development and in pathological states (Matrisan, 1990; Mauviel, 1993). Phorbol esters stimulate gene expression of two major classes of metalloproteinases, stromelysin and collagenase (Frisch *et al.*, 1987). The promoter regions of these two genes contain a tumor promoter-responsive element (Angel *et al.*, 1987), which binds the transcription factor AP-1. Therefore conditions that increase levels of AP-1, such as stimulation of cells with growth factors, may also lead to enhanced transcription of a metalloproteinase gene that cleaves NG2.

Stromelysin has been shown to cleave the large extracellular proteoglycan aggrecan at an Asn-Phe site (Flannery *et al.*, 1992). Two Asn-Phe sequences are found in NG2, at positions 2020/2021 and 1649/1650. Cleavage at the 2020/2021 site could give rise to a fragment of about 275 kDa in size. Another example of protein kinase C-dependent cleavage of a cell surface molecule has been described for TGF-α by Pandiella and Massague (1991). These authors have shown that phorbol esters activate an elastase-like enzyme that cleaves membrane-anchored pro-TGF-α into soluble TGF-α at an Ala-Val site that is surrounded by small, nonpolar amino acids. A similar sequence of Ala-Val-Ala-Leu-Leu is found at amino acid positions 2157–2161 in the ectodomain of rat NG2. Cleavage at this site would also give rise to an NG2 fragment of about 275 kDa. With regard to our artificial production of a 275-kDa core protein by trypsinization, there are numerous locations in the NG2 ectdomain that could serve as cleavage sites for trypsin, which is known to act specifically at lysine and arginine residues (Bernfield *at al.*, 1992). In particular there is a dibasic Arg-Arg site at amino acid positions 2096/2097 in the NG2 core protein (see Figure 1). Cleavage by trypsin at this site would generate a fragment of about 275 kDa. Because the exact site of cleavage that yields the 290-kDa released form of the NG2 core protein is also unknown, we have no definitive evidence as to whether the same proteinase is responsible for both types of NG2 trunction events. However, the fact that the 290-kDa species is generated in B49 cells under

conditions in which none of the 275-kDa species is seen may be taken as an indication that two different proteinases are responsible for generating these two truncated molecules.

The 275-kDa core protein observed in extracts of H-*ras*–transformed 3T3 cells may also be generated by a mechanism similar to that seen in U251NG52 cells. The H-*ras* oncogene is a potent inducer of metalloproteinases such as collagenase and stromelysin (Ballin *et al.*, 1988; Matrisan, 1990). The fact that these 3T3 cells endogenously express high levels of both the 275- and 300-kDa core species at the cell surface makes it seem less likely that generation of the 275-kDa form is an artifact created by transfection of NG2 cDNA into the U251NG52 cells. However, we cannot rule out the possibility that integration of the NG2 cDNA into the genome of the U251NG52 cells has caused insertional inactivation of the gene for an inhibitor of the proteinase responsible for generating the 275-kDa species or that U251NG52 cells represent a clonal variant that lacks such a proteinase inhibitor. In this regard it is of interest that by treating B49 cells with monoclonal anti-NG2 antibodies during the time when they are recovering from trypsinization, a procedure that blocks association of type VI collagen with newly synthesized NG2 on the cell surface, we can cause the B49 cells to produce the truncated 275-kDa core protein along with the intact 300-kDa core. This indicates that B49 cells have the proteinase necessary for cleavage of NG2, but that it usually is not active, possibly due to interaction with a proteinase inhibitor. Because the α3 chain of type VI collagen contains a sequence characteristic of Kunitz type proteinase inhibitors (Bonaldo and Columbatti, 1989; Chu *et al.*, 1990), it is tempting to speculate that interaction of type VI collagen with NG2 (Stallcup *et al.*, 1990; Nishiyama and Stallcup, 1993) could protect the proteoglycan against proteolytic processing. Blocking the interaction of type VI collagen with NG2 by the use of antibodies would leave the proteoglycan unprotected against the action of the proteinase. This is analogous to the situation that exists in U251NG52 cells which, for unknown reasons, fail to anchor type VI collagen in association with NG2 at the cell surface. Thus the NG2 on these cells would also be susceptible to proteolysis. The validity of this NG2/type VI collagen hypothesis is uncertain in light of the failure, to date, to demonstrate any inhibitory activity associated with the Kunitz-type domain of type VI collagen (Mayer *et al.*, 1994). Nevertheless, there may be other proteinase inhibitors that can substitute for type VI collagen in this hypothesis. One such candidate is the tissue-factor-pathway inhibitor 2 (TFPI-2), a Kunitz-domain–containing protein that has anti-proteolytic activity (Sprecher *et al.*, 1994). Spiro and co-workers have accumulated indirect evidence that TFPI-2 is associated with NG2 on melanoma cell lines (R. Spiro, unpublished results). Development of antibodies against TFPI-2 will allow us to examine this possibility directly.

Proteinases and their inhibitors are involved in a number of biological functions. These include cell differentiation, growth, morphogenesis, migration, and tumor invasion. Many truncated forms of membrane-bound molecules that are important in cell adhesion and cell growth have been shown to be generated by proteolysis. In some cases the proteolytic event is stimulated by protein kinase C (Downing *et al.*, 1989; Lantz *et al.*, 1990), as we have found for the generation of the 275-kDa form of NG2. However, the role of the truncated forms remains unclear in the majority of cases. In our case it will be important to characterize in more detail the regulation of molecular processes that lead to the proteolysis of NG2 and to define any subsequent changes that occur in cell phenotype.

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