Molecular mechanisms of neural crest cell attachment and migration on types I and IV collagen

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SUMMARY

We have examined the mechanisms involved in the interaction of avian neural crest cells with collagen types I and IV (Col I and IV) during their adhesion and migration in vitro. For this purpose native Col IV was purified from chicken tissues, characterized biochemically and ultrastructurally. Purified chicken Col I and Col IV, and various proteolytic fragments of the collagens, were used in quantitative cell attachment and migration assays in conjunction with domain-specific collagen antibodies and antibodies to avian integrin subunits. Neural crest cells do not distinguish between different macromolecular arrangements of Col I during their initial attachment, but do so during their migration, showing a clear preference for polymeric Col I. Interaction with Col I is mediated by the $\alpha_1\beta_1$ integrin, through binding to a segment of the $\alpha_1(I)$ chain composed of fragment CNBr3. Neural crest cell attachment and migration on Col IV involves recognition of conformation-dependent sites within the triple-helical region and the noncollagenous, carboxyl-terminal NC1 domain. This recognition requires integrity of inter- and intrachain disulfide linkages and correct folding of the molecule. Moreover, there also is evidence that interaction sites within the NC1 domain may be cryptic, being exposed during migration of the cells in the intact collagen as a result of the prolonged cell-substratum contact. In contrast to Col I, neural crest cell interaction with Col IV is mediated by β_1-class integrins other than $\alpha_1\beta_1$.

Key words: neural crest cells, migration, collagen, adhesion

INTRODUCTION

Although collagens are primary components of the extracellular matrix deposited along neural crest migratory pathways, very little is known about their function during neural crest development. Due to their intimate association with migrating neural crest cells in situ, as revealed by ultrastructural observations (Löfberg et al., 1980; Perris et al., 1990), collagens have been proposed to participate in the regulation of neural crest cell migration in vivo. For instance, the uniform parallel orientation of collagen fibrils deposited on the dorsolateral aspect of neural tube prior to the onset of neural crest cell movement has been correlated with the polarization and initial guidance of the cells in their migratory direction (Löfberg et al., 1980; Newgreen, 1989; Perris et al., 1990). At least ten collagen (Col) types, including types I-VI, VIII, IX, XI and XII are known to be expressed during neural crest development (Perris and Bronner-Fraser, 1989; Erickson and Perris, 1993; Perris et al., 1993a,b). All these collagen types occur transiently within or in association with basement membranes and throughout the somitic sclerotome, which corresponds to

the primary neural crest migratory pathway (Newgreen and Erickson, 1986; Kosher and Solursh, 1989; Duband and Thiery, 1987; Perris et al., 1990, 1991, 1993a,b).

Col IV, which is typically associated with basement membranes, displays an unusual distribution in interstitial fibrils of the sclerotome where it is transiently expressed at times of neural crest cell migration (Duband and Thiery, 1987; Perris et al., 1990, 1991). At present, it is unclear what constituent chains of Col IV are expressed in the early avian embryo, but it is likely that most Col IV molecules have a classical $\alpha_1(IV)_2 \alpha_2(IV)$ composition, since it has not been possible to immunolocalize the $\alpha_3(IV)$ or $\alpha_4(IV)$ chains during neural crest development (Perris, unpublished observations).

Neural crest cells are well-known to interact with collagens in vitro (Tucker and Erickson, 1984; Newgreen and Erickson, 1986; Bilozur and Hay, 1988; Perris and Johansson, 1990; Perris et al., 1991). More recently, we have shown that Col I, IV and VI promote significant neural crest cell motility, independently of other matrix molecules and without invoking cell surface fibronectin (Perris and Johansson, 1990; Perris et al., 1991, 1993a,b). While the

molecular interaction of neural crest cells with Col VI has been extensively examined (Perris et al., 1993a,b), the molecular basis for the interaction of neural crest cells with Col I and Col IV remains largely unknown.

In this study we have examined the molecular mechanisms involved in the interaction of avian neural crest cells with Col I and IV in vitro in an attempt to localize the responsible sites within the molecules and identify the cell surface receptors recognizing these sites. For this purpose, we isolated native forms of chicken Col IV and used intact and fragmented Col I and Col IV in combination with domain-specific antisera and anti-integrin antibodies in quantitative cell adhesion and motility assays.

MATERIALS AND METHODS

Purification of chicken Col IV

Cleaned chicken gizzard (100 g) was cut into cubes and homogenized in 500 ml 0.15 M NaCl, 50 mM Tris-HCl, pH 7.4, containing 2 mM PSMF and 2 mM N-ethylmaleimide at 4°C and centrifuged at 10,000 g for 20 minutes. After repeating this washing step, the pellet was resuspended in 250 ml 0.15 M NaCl, 50 mM Tris-HCl, pH 7.4, containing the above protease inhibitors and 10 mM EDTA, and stirred for 1 hour at 4°C. Then, following centrifugation, extraction in the presence of EDTA was repeated and the residual insoluble material was suspended in 250 ml 0.5 M acetic acid and further extracted for 3 days at 4°C. The pellet resulting from the subsequent centrifugation was dissolved in 100 ml 0.5 M acetic acid and lyophilized. A typical yield was 15 g of dry lyophilized material.

7S-linked Col IV tetramers were prepared from this dry material by resuspension in 0.5 M acetic acid (5 g material in 400 ml 0.5 M acetic acid) and digestion with pepsin at an enzyme:substrate ratio of 1:200 for 3-4 hours at 4°C. Insoluble material was removed by centrifugation and the pH of the supernatant was adjusted to 7.5 by addition of NaOH. Most of the proteins in the supernatant were precipitated by addition of 1.7 M NaCl for 2 hours in the cold and collected by centrifugation. Under these conditions Col IV is selectively precipated (Miller and Rhodes, 1982) as expected for a major component of the original proteolytic digest. The precipitate was redissolved in 50 mM Tris-HCl, pH 8.6, with 2 M urea and dialyzed extensively against the same buffer. Dialyzed material was then chromatographed on a DEAE-cellulose column (DE52, Whatman), equilibrated with the same urea-Tris buffer, and the flowthrough fractions were pooled, dialyzed against 0.5 M acetic acid and lyophilized.

Dimers of chicken Col IV were isolated by trypsin digestion according to a modification of the procedure described for mammalian Col IV (Risteli et al., 1980). Extracted and lyophilized gizzard (5 g) was homogenized in 125 ml 0.2 M ammonium hydrogen carbonate, pH 7.9, and incubated for 4 hours at room temperature with 50 mg (223 units/mg protein) TPCK-treated trypsin (Worthington). The reaction was stopped by addition of 5 mg of L-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone hydrochloride (TLCK, Serva) and insoluble material was removed by centrifugation. Col IV was precipitated from the supernatant by adding 2 M NaCl for 2 hours at 4°C and the precipitate was collected by centrifugation, resuspended in urea-Tris buffer and passed over DEAE-cellulose as described for 7S-linked Col IV tetramers. Pooled flowthrough fractions were dialyzed against 0.2 M ammonium hydrogen carbonate, pH 7.9, and lyophilized.

Other intact and fragmented collagens

Purified collagens and their fragments were obatined as follows:

Chick skin Col I from Dr Charles Little, Department of Anatomy and Cell Biology, University of Virginia, Charlottesville, VA; intact human Col IV dimers and 7S-linked Col IV tetramers from Dr Klaus Kühn, Max-Planck Institute for Biochemistry, Martinsried, Germany; cyanogen bromide-derived fragments from the 1(I) chain, CNBr3, CNBr7 and CNBr8, from Dr Helene Sage; cyanogen bromide-derived fragments from the 2(I) chain, CNBr3-5 and CNBr4 from Dr John Harding, Nuffield Laboratory of Ophtalmology, University of Oxford, England; bovine and human hexameric NC1 fragment (Col IV) from Drs Jörgen Wieslander, Department of Nephrology, University Hospital, Lund, Sweden, and Klaus Kühn; bovine 7S fragment (Col IV; Dixit et al., 1981) from Dr Saryu Dixit, The Veterans Administration Lakeside Medical Center, Northwestern University, Chicago, IL; human placental and skin Col III from Telios Pharmaceuticals, Inc. and Collaborative Biochemicals Inc.

Reversible and irreversible unfolding of collagens

Col I was dialyzed against PBS in the cold at concentrations below 1 mg/ml and unfolded by heating at 60°C for 10-15 minutes. Col III and intact Col IV dimers were dialyzed against 0.2 M Tris-HCl buffer, pH 7.4, containing 0.4 M NaCl and 5 mM EDTA with and without 6 M urea. Complete reduction of disulfide bonds under denaturing (in the presence of urea) and nondenaturing conditions was achieved for mouse and chicken Col IV dimers by incubation of 0.05-1 mg of the collagen preparations with 25-50 mM DTT overnight at 12°C. Following reduction, samples were alkylated with a 4-fold molar excess of N-ethylmaleimide for 1 hour at room temperature and dialyzed extensively against 0.1 M sodium bicarbonate buffer, pH 8.0, containing 0.2 M NaCl and 5 mM EDTA.

Preparation of Col IV triple-helical fragment

Since purified chick dimers cannot be obtained in quantities large enough to carry out reproducible fragmentation of the molecule, Col IV dimers from mouse EHS tumor were used for production of triple-helical fragments. For this purpose mouse Col IV dimers were reduced and alkylated as described above and digested with pepsin at an enzyme-substrate ratio of 1:50 overnight at 8°C, followed by dialysis against 50 mM Tris-HCl, pH 8.0, with 200 mM NaCl and 5 mM EDTA in the cold. The digested collagen then was chromatographed on a Sephacryl S-400 column (Pharmacia) equilibrated with the same buffer and fractions containing exclusively the triple-helical region of the collagen were identified by ELISA using antibodies to NC1, 7S and triple-helical domains, and by SDS-PAGE (not shown). These fractions were pooled and concentrated by centrifugation using the Amicon Centriprep concentration filter units (M_r cut-off 12,000).

Antibodies

Affinity-purified antibodies to the 7S and NC1 domains of Col IV were obtained from Dr Jörgen Wieslander. Production and specificity of these antisera have been described in previous publications (Risteli et al., 1980; Wieslander et al., 1985). CSAT and JG22-producing hybridoma cell lines were purchased from the Developmental Studies Hybridoma Bank (Iowa City, IW) and the IgGs were purified by chromatography on Protein A-Sepharose (Pharmacia). These antibodies have been shown to be similarly effective in blocking neural crest cell adhesion and migration on fibronectin, laminin and Col VI (Lallier and Bronner-Fraser, 1991, 1992; Perris et al., 1993b), and were therefore used interchangeably throughout the study. Production and specificity of the antiserum against the 1 integrin subunit has been described previously (Syfrig et al., 1991).

ELISA

Microwell plates (96, Nunclon) were coated with mouse and chick

dimers and 7S-linked tetramers of Col IV, and bovine 7S and NC1 fragments in Voller's buffer as earlier described (Perris et al., 1989, 1991). Uncoated plastic was blocked with 1% ovalbumin/2% BSA in Voller's buffer for 2 hours at room temperature; the wells were extensively rinsed and further incubated with decreasing concentrations of the anti-NC1 and anti-7S antisera diluted in 0.1 M phosphate buffer containing 0.15 M NaCl and 0.1% BSA. Binding of the antisera was detected using horseradish peroxidase-conjugated antibodies diluted 1:1000 in the same buffer as for primary antibodies, and a previously described colorimetric reaction for detection of the peroxidase activity (Perris et al., 1989, 1991).

SDS-polyacrylamide gel electrophoresis

Electrophoresis was performed according to Laemmli (1970) on gradient gels of 3% to 15% polyacrylamide in the presence of SDS. When desired, the samples were reduced with 2% -mercaptoethanol in sample buffer. Proteins were detected by staining with Coomassie Brilliant Blue R.

Glycerol spraying/rotary shadowing electron microscopy

Electron microscopy was performed on collagen samples diluted to 0.5 µg/ml with 0.1 M acetic acid. Glycerol spraying and rotary shadowing was done following standard procedures (Engel and Furthmayr, 1987).

Cell adhesion assay

The quantitative cell adhesion assay used in this study is based on centrifugation and has been described previously (Lallier and Bronner-Fraser, 1991, 1992, Perris et al., 1993a,b). Briefly, neural tube-neural crest explants were isolated from 2- to 2.5-day-old quail embryos (*Coturnix coturnix japonica*) as previously described (Perris et al., 1989, 1991), plated onto fibronectin-coated cell and tissue culture dishes (35 mm, Nunc) and cultured for 12-16 hours in Minimal Essential Medium with Earl's salts (MEM) containing 0.1% BSA (grade V, Sigma). Neural tube explants were mechanically removed with the aid of tungsten needles and the spread neural crest cells were metabolically labelled by incubation overnight with 50 µCi [³H]leucine (ICN Biochemicals). Labelled cells were detached from the fibronectin substratum by addition of 5 mM EDTA and samples were placed in strips of 96-microwell flexible plates (PVC, Falcon Flexiplate III), which had been previously coated with the collagen molecule to be tested as previously described (Perris et al., 1989, 1991). Relative amounts of protein bound to plastic have been determined in these previous studies using biotinylated molecules. Wells were extensively washed with MEM containing 0.5% ovalbumin and incubated with the same medium for 2-4 hours at room temperature. Plate strips then were centrifuged at 50 *g* for 5 minutes and subsequently incubated for 10 minutes at 37°C. A similar set of plate strips filled with ovalbumin-containing MEM was sealed on top of the strips containing the substratum-bound cells and the assemblies were inverted and recentrifuged at 150 *g* for 5 minutes to allow the nonbound and weakly bound cells to be dislodged to the wells opposite to those containing the substratum molecules. The tips of bottom (substratum) and top wells of the assemblies then were cut off, placed in scintillation vials and counted separately. The ratio between bottom rpm counts (bound cells) and total counts was calculated and adopted to denote the percentage adherent cells out of the total.

Migration assay

Neural crest/neural tube explants isolated as described above were plated onto the various collagenous substrata, prepared as described for cell adhesion assays, in MEM containing 0.1% BSA, and grown for up to 16 hours. Assessment of neural crest cell migration after this culture period was as previously described (Perris et al., 1989, 1991, 1993a,b).

RESULTS

Neural crest cell attachment and migration on Col I

Previous studies have shown that Col I is the only fibril-forming collagen supporting significant neural crest cell movement and that this occurs in a manner that is dependent upon the supramolecular organization of the collagen (Perris et al., 1991). In order to further determine the importance of the macromolecular assembly of Col I for both initial neural crest cell attachment and migration, we reconstituted polymeric Col I substrata in which the collagen concentration was varied to yield different fibril sizes and organizations, or in which the macromolecular architecture of the fibril network was altered by air drying (Newgreen, 1982).

Neural crest cells attached equally well to monomeric and polymeric Col I (Fig. 1), but did not adhere to denatured Col I (Fig. 1). Neural crest cells also adhered in a comparable manner to hydrated polymeric Col I and dehydrated polymeric Col I (Fig. 1). Attachment to both hydrated and dehydrated Col I was not influenced by the concentration of Col I in the substratum (Fig. 1), indicating that it was not directly determined by the fibril size and/or organization within the network. In contrast, neural crest cell motility on both hydrated and dehydrated Col I was markedly affected by the concentration of the collagen, showing a sharp ascending phase and a less-steep descending phase with increasing concentrations of Col I in the substratum (Fig. 1). Moreover, when compared at their respective optimal concentrations, dehydrated polymeric Col I was significantly more effective in supporting neural crest cell movement than the corresponding hydrated form (Fig. 1). This difference indicates a bias for a specific supramolecular arrangements of Col I, rather than a preference of the cells for partially unfolded collagen, since migration on native monomeric Col I was less extensive than on hydrated or dehydrated polymeric Col I (Fig. 1), and cells were unable to migrate on denatured Col I (Fig. 1).

To localize the cell-binding sites within the Col I molecule, we tested a number of fragments produced from its constituent   chains by cyanogen bromide cleavage. Neural crest cell attached and migrated with high efficiency on fragment CNBr3 from the   1(I) chain and to some extent on fragment CNBr 7 from the same   chain (Fig. 2). Conversely, cells did not respond to fragments   1(I) CNBr8 or CNBr 2-5 and CNBr4 from the   2(I) chain (Fig. 2). When compared in molar equivalents, neural crest cell attachment and migration on CNBr 3 was similar to that seen on monomeric Col I. Moreover, coating onto plastic of fragments CNBr3 and CNBr7 in the presence of 6 M urea abolished all their ability to support neural crest cell attachment and migration (not shown), indicating that refolding of the triple helix was neccessary for optimal cell interaction.
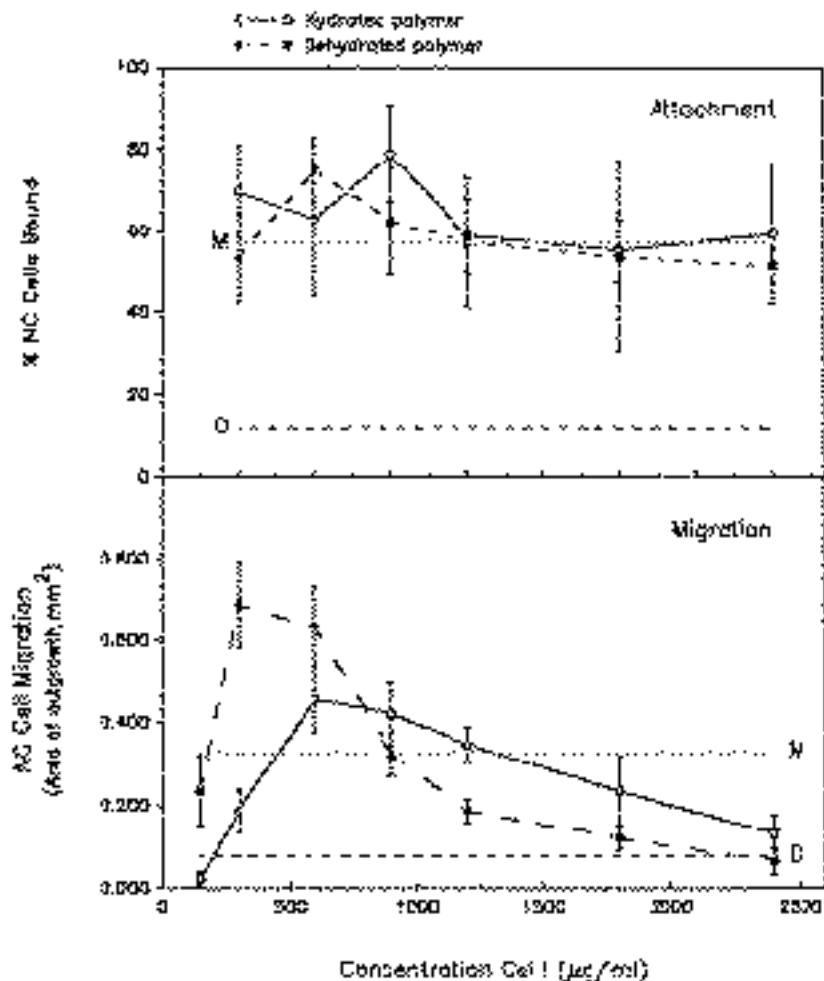
Fig. 1. Dose-dependency of neural crest cell attachment and migration on hydrated and dehydrated chick skin Col I. Reference values for cell attachment and migration on monomeric and denatured Col I are given by the dotted and broken lines, respectively. Comparable results were obtained with rat tail Col I also comprising the teleopeptides, whereas Col I from bovine skin and human placenta lacking such peptides were significantly less effective (data not shown).

Isolation and characterization of chicken Col IV

As our cell biological assays were performed with avian cells we decided to prepare a set of overlapping fragments of chicken Col IV to ascertain that the activities seen with well-characterized preparations of Col IV of human and murine origin were representative also for the avian neural crest. Previously available procedures for isolation of avian Col IV were designed to obtain parts of the major triple helix (Mayne and Zettergren, 1980), and would not yield Col IV fragments containing all potential binding sites. Pepsin digestion of pre-extracted chick gizzard gave tetrameric fragments containing the 7S domain (Fig. 3B,b), similar to tetrameric fragments isolated from human placenta by an analogous procedure (Fig. 3B,a; and Timpl et al., 1981). Trypsin digestion of gizzard yielded a dimeric fragment connected by the NC1 domains (Fig. 3B,c) and comprising variable portions of the 7S domain. The latter result was somewhat surprising as a similar procedure yields 7S-containing tetramers when applied to mammlian tissues (Risteli et al., 1980). Both kinds of fragments contained long stretches of the major triple helix, and it can be taken for granted that any binding site occurring in a molecule of Col IV would be recovered in at least one of the two overlapping fragments. When compared, results obtained with mammalian Col IV were qualitatively similar to those obtained with avian Col IV preparations, indi-

cating that cell-binding sites in this collagen are well conserved in evolution.

Neural crest cell attachment and migration on Col IV

The sites in Col IV acting on neural crest cells were mapped by use of a variety of proteolytic fragments and well-characterized chemical treatments of Col IV. The fragments and procedures used are summarized in Fig. 4. Neural crest cells attached and migrated significantly more extensively on intact dimers of Col IV than on 7S-linked tetramers (Fig. 5; Table 1). Neural crest cell adhesion to intact dimers was unaffected by complete native reduction and alkylation of disulfide bonds, causing disruption of the dimers into monomeric units, but was dramatically diminished by reduction and alkylation under denaturing conditions (Fig. 5). Conversely, the extent of neural crest cell migration on dimers was affected by native reduction and alkylation of the dimers and virtually all their motility-promoting activity was abolished by simultaneous reduction/alkylation and denaturation (Fig. 5; Table 1). Neural crest cells avidly attached and migrated on the isolated hexameric NC1 domain of Col IV, but were unable to adhere and migrate on the isolated 7S domain (Table 1). When computed in molar equivalents after alignment of dose-dependent adhesion and migration curves (Fig. 5), the NC1 domain
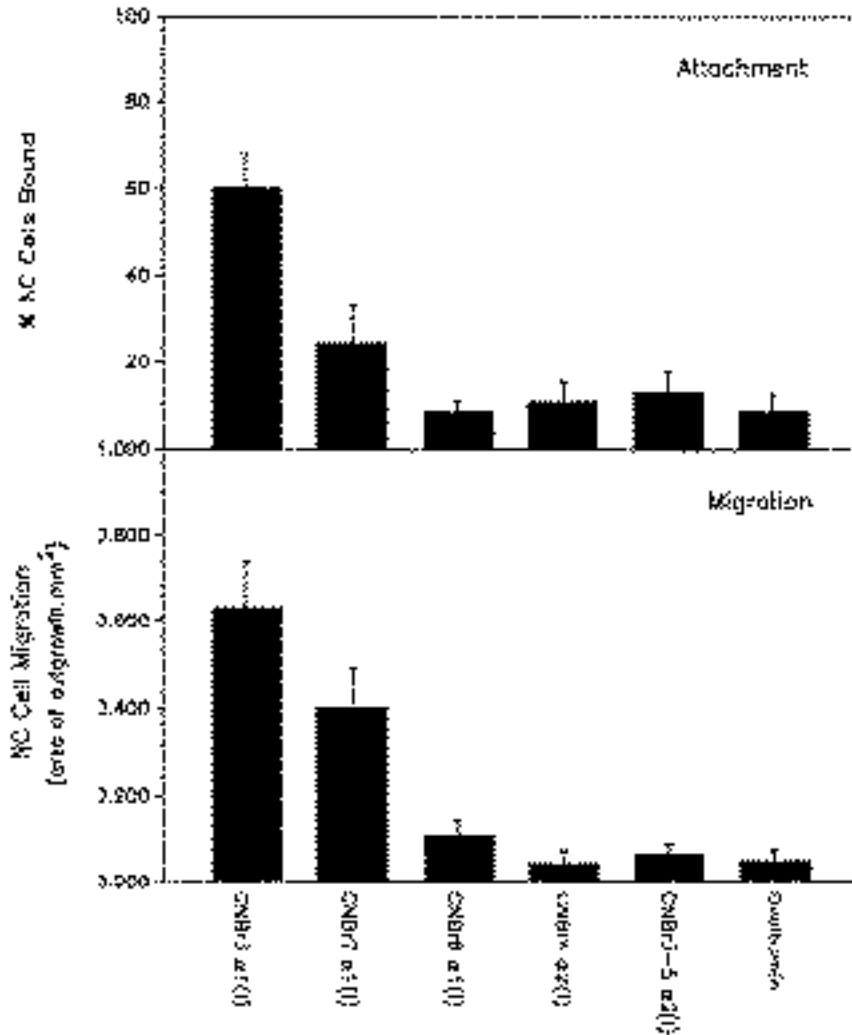
Fig. 2. Neural crest cell attachment and migration on CNBr fragments from α1(I) and α2(I) chains of rat and bovine Col I.

accounted for 80% of the total adhesive activity and 100% of the total migration-promoting activity (based on M_r 170,000 for the hexameric NC1 fragment and M_r 800,000 for the intact Col IV dimer). The NC1 was effective in supporting neural crest cell attachment and migration only in its hexameric molecular arrangement, since reduction and alkylation under native or dissociative conditions reduced its adhesive properties and virtually eliminated its motility-promoting properties (Table 1). The pepsin-derived triple-helical fragment of Col IV promoted some neural crest cell attachment, but was largely unable to promote significant neural crest cell movement (Table 1), and thereby paralleled the behavior of the 7S-linked tetramers.

Substrata of intact Col IV dimers were pre-incubated with antisera specific for the 7S and NC1 domains (Fig. 6; Wieslander et al., 1985) and tested for their ability to support neural crest cell adhesion and movement. Pre-incubation of Col IV dimers with an antiserum to the NC1 and 7S domains had no effect on neural crest cell adhesion and migration, whereas anti-NC1 antibodies affected to some extent cell migration (Table 1). Conversely, pre-incubation of the isolated hexameric NC1 fragment with the anti-NC1 antiserum strongly reduced neural crest cell binding and migration on the fragment (Table 1). As shown by ELISA

(Fig. 6), this discrepancy cannot be due to a lower affinity of the antiserum for the isolated NC1 domain versus the NC1 domain contained within the intact Col IV molecule.

Effect of anti-integrin antibodies on neural crest cell adhesion and migration on Col I and IV

To identify integrin receptors on neural crest cells that could be involved in their interaction with Col I and Col IV we used the monoclonal antibodies CSAT and JG22 against the avian β1 integrin subunit and an affinity-purified antiserum against the avian β1 integrin subunit (Syfrig et al., 1991). Neural crest cell adhesion to polymeric hydrated Col I, monomeric Col III and Col I fragments CNBr3 and CNBr7 was dose-dependently inhibited by CSAT and JG22 antibodies (Fig. 7A), demonstrating that neural crest cell interaction with interstitial collagens is entirely mediated by the β1 class of integrin receptors. The anti-β1 antiserum similarly blocked in a dose-dependent manner cell attachment to polymeric Col I and CNBr3 fragment from the α1 (I) chain, but did not affect neural crest cell adhesion to CNBr7 fragment and monomeric Col III (Fig. 7B). Neural crest cell adhesion to Col IV dimers, 7S-linked tetramers and the isolated NC1 domain was strongly inhibited by
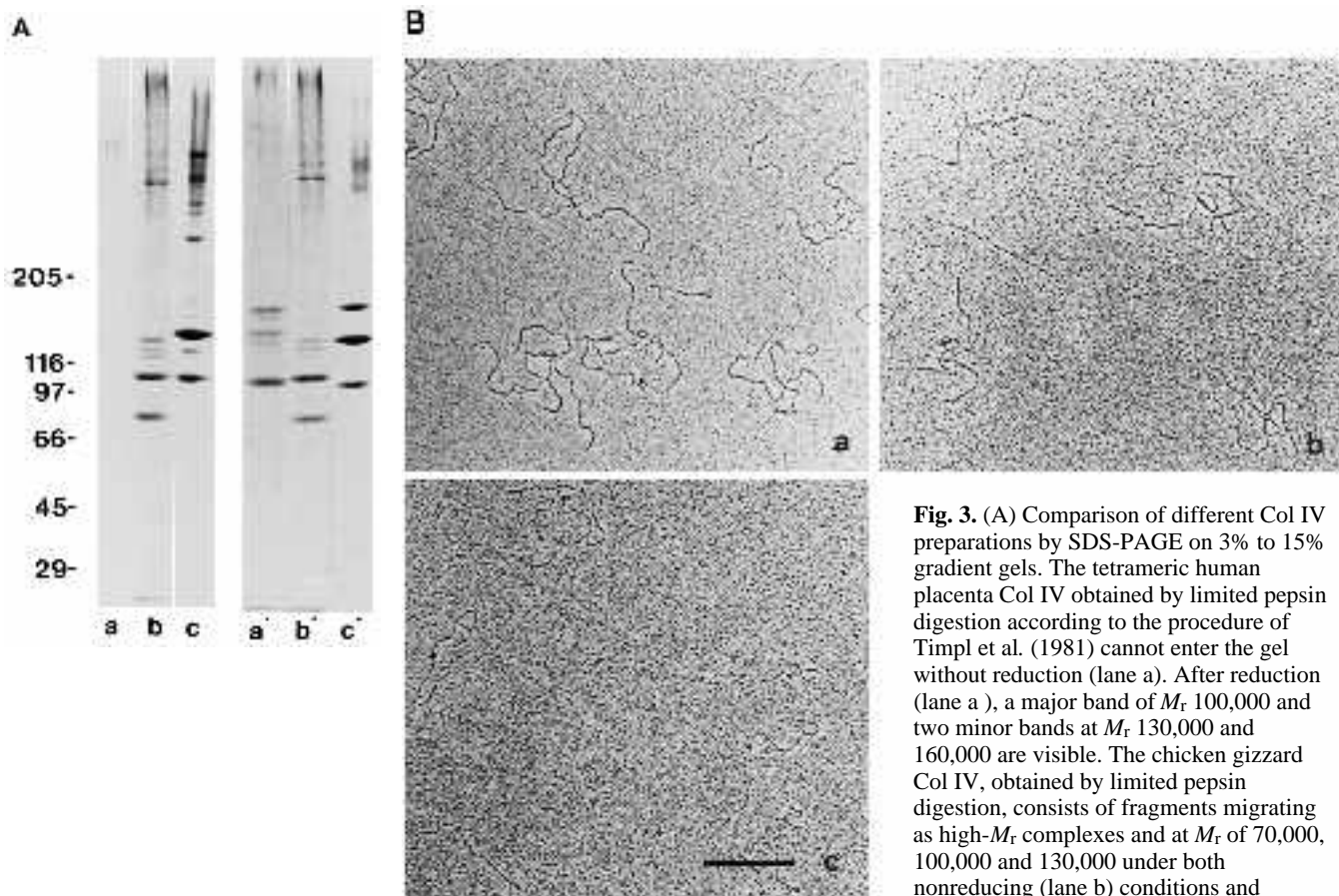
Fig. 3. (A) Comparison of different Col IV preparations by SDS-PAGE on 3% to 15% gradient gels. The tetrameric human placenta Col IV obtained by limited pepsin digestion according to the procedure of Timpl et al. (1981) cannot enter the gel without reduction (lane a). After reduction (lane a), a major band of M_r 100,000 and two minor bands at M_r 130,000 and 160,000 are visible. The chicken gizzard Col IV, obtained by limited pepsin digestion, consists of fragments migrating as high-M_r complexes and at M_r of 70,000, 100,000 and 130,000 under both nonreducing (lane b) conditions and reducing conditions (lane b). Col IV obtained by tryptic digestion of gizzard, contains fragments migrating at M_r 100,000 and 130,000 under nonreducing conditions (lane c) and fragments migrating at M_r 100,000, 130,000 and 160,000 under reducing conditions (lane c). In addition larger complexes are seen under both conditions. The M_r ($\times 10^{-3}$) of globular standard proteins is given to the left. (B) Rotary-shadowing electron microscopy of different Col IV preparations. Human placenta Col IV obtained after limited pepsin digestion (a) consists mainly of intact tetramers. Also chick gizzard Col IV prepared by limited pepsin digestion (b) contains a large proportion of tetramers. Tryptic digestion of gizzard (c) yields a Col IV preparation consisting mainly of NC1-containing dimers. Bar, 300 nm.

CSAT/JG22 (Fig. 7C), whereas it was not affected by the anti- ₁ antiserum (Fig. 7C).

Addition of the anti- ₁ antiserum and CSAT/JG22 antibodies to cells that had migrated on CNBr3 fragment and Col IV dimers for 12 hours caused detachment and rounding up of the majority of the cells within 2-3 hours (Fig. 8). Neural crest cell migration on various macromolecular forms of Col I, active fragments of Col I, and intact and fragmented forms of Col IV, was markedly reduced by the JG22 antibody (Fig. 9). Moreover, similar to its effect on neural crest cell adhesion, the anti- ₁ antiserum significantly blocked migration on polymeric Col I and CNBr3 fragment, but did not affect movement on CNBr7 fragment and Col IV (Fig. 9).

DISCUSSION

This study examines the molecular mechanisms of neural crest cell interaction with Col I and IV. Collagens may be involved in neural crest cell migration because of their abundant expression along the migratory pathways and their intimate contact with migrating neural crest cells. We have previously shown that of the 12 collagen types expressed during neural crest development, only Col I, Col IV and Col VI support significant neural crest cell movement in vitro (Perris et al., 1991; Erickson and Perris, 1993; Perris et al., 1993a,b). Neural crest cells preferentially interact with fibrillar polymeric Col I, displaying only a weaker ability to recognize monomeric collagen (Perris et al., 1991; and present data). This observation indicates that optimal neural crest cell interaction with Col I may require a 'native' macromolecular arrangement of the molecule and prompted us to further investigate this requirement. The extent of neural crest cell movement observed on hydrated and dehydrated polymeric Col I, in which the macromoleular configuration was modulated by varying the collagen concentration, revealed that cell motility may be dependent upon the relative size of the Col I fibrils, as well as by the macromolecular architecture of the fibril network. Interestingly, initial neural crest cell adhesion to Col I does not exhibit the same dependence, suggesting that the differences in adhesive and migratory responses to different macromolecular arrangements of the collagen may be due
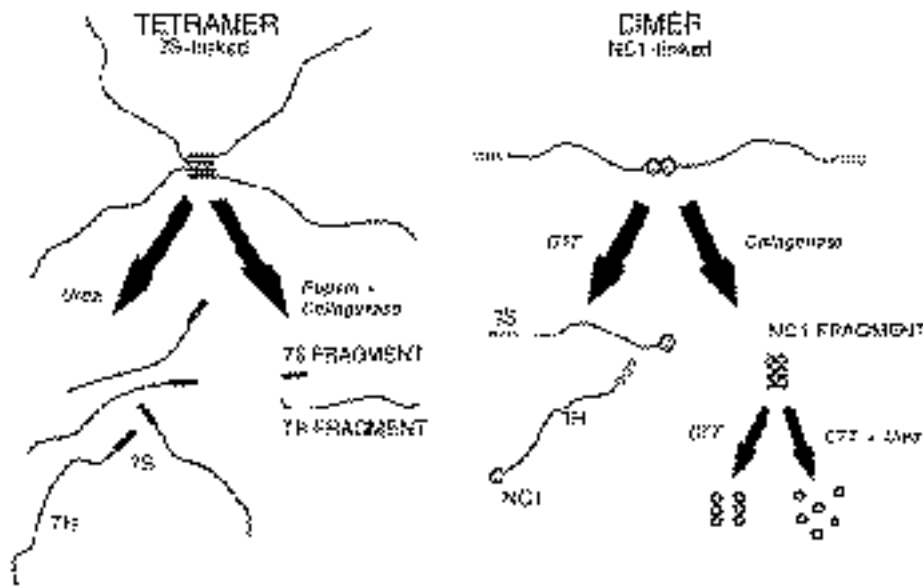
Fig. 4. Schematic diagaram illustrating the molecular properties of Col IV. Col IV can be extracted from tissues in two forms, both of which retain their triple-helical region intact. Pepsin digestion of tissues liberates a tetrameric form of the molecule in which each monomer is held together at its amino-terminal 7S end region by disulfide crosslinks and possibly by additional electrostatic forces between the apposing triple helices (Risteli et al., 1980; Timpl et al.; 1981; Madri et al., 1983; Weber et al., 1984; Siebold et al., 1987, 1988; Yurchenco and Furthmayr, 1984; Yurchenco and Ruben, 1987). In contrast, differential salt extraction followed by collagenase digestion releases dimeric Col IV molecules, which are linked at their carboxy-terminal ends, NC1 domain, linked by disulfide bridges (Timpl et al., 1981; Weber et al., 1984; Dolz et al., 1988) and containing variable portions of the 7S domain. If the extraction is done in denaturing conditions, there is a tendency to retain larger portions of the 7S domain. The carboxy-terminal NC1 domain can be isolated from Col IV by harsher collagenase digestion and is retained as a protein structure consisting of six cross-linked polypetides (Timpl et al., 1981; Weber et al., 1984; Dölz et al., 1988). Reduction and alkylation produces dimeric/tetrameric NC1 fragments, and reduction and alkylation in urea yields dissociation of the hexameric unit into monomeric and dimeric polypetide fragments. The 7S domain can be isolated by collagenase treatment of pesin-digested basement membranes following a combination of molecular sieve and ion-exchange chromatograpy (Risteli et al., 1980; Timpl et al., 1981; Dixit et al., 1981; Madri et al., 1983; Siebold et al., 1987, 1988). Fragments comprising the triple-helical (TH) region are intermediary proteolytic products resulting from collagenase and/or pepsin digestion of dimers and 7S-linked tetramers.

to modifications of the substratum by the migrating neural crest cells. Although the mechanical stability of the Col I network may vary with different relative sizes and arrange-

Table 1. Neural crest cell adhesion and migration on various Col IV substrata

Substratum	Addition	Adhesion (%)	Migration (mm^2)	% Activity vs control*	
Col IV dimers	–	64.1±5.3	0.42±0.07	100	100
Col IV tetramers	–	26.3±4.1	0.20±0.03	41†	48†
Col IV dimers	Anti-7S	55.1±6.1	0.40±0.06	14	95
Col IV dimers	Anti-NC1	58.2±3.3	0.29±0.04	91	69†
NC1	–	68.4±4.8	0.68±0.12	100	100
NC1	Anti-NC1	19.4±7.2	0.11±0.02	28†	16†
NC1 Red/Alk	–	52.5±4.8	0.32±0.04	77	47†
NC1 Red/Alk/Den	–	16.7±6.5	0.07±0.06	24†	10†
7S	–	11.2±3.9	0.06±0.03	17†	14†
THF‡ (Col IV)	–	28.1±3.4	0.18±0.02	44†	43†

Col IV dimers and tetramers are from chicken; NC1 and 7S fragments derive from bovine Col IV; and the THF fragment is from EHS mouse tumor Col IV (see Materials and Methods). Col IV dimeric substrata were pre-incubated with antisera to NC1 and 7S domains, using antibody dilutions giving saturation of the binding according to ELISA (Fig. 6), prior to be tested in cell adhesion and cell migration assays.

*% Activity vs control indicates the percentage adhesive and motility-promoting activity retained when compared with the control substrata (Col IV dimers or isolated NC1 in the case of Red/Alk and Red/Alk/Den NC1 fragment).

†Denotes that the values are significantly different than the controls as indicated in % Activity vs control and according to the two-tailed Student's t-test, $P<0.001$. $n=18$-24 for adhesion and 11-17 for migration.

‡THF, triple-helical fragments of Col IV.

ments of the single constituent fibrils, it is unlikely that such modifications are solely mechanical, since the tractional force exerted on the substratum by migrating neural crest cells is minimal (Tucker et al., 1985).

Although neural crest cells preferentially interact with fibrillar Col I, a strong response was seen to fragment CNBr3 of the α1(I) chain of the collagen. This fragment is known to contain interaction sites recognized by a number of cell types via the $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins (Rubin et al., 1981; Staatz et al., 1990; Santoro et al., 1991; Gullberg et al., 1992). Consistent with these findings we show that neural crest cells recognize Col I through their $\alpha_1\beta_1$ integrin (Lallier et al., 1992), which specifically binds to the CNBr3 fragment. However, it is unlikely that the interaction site recognized by the neural crest $\alpha_1\beta_1$ integrin can be reduced to a single, linear amino acid sequence as that described for the $\alpha_2\beta_1$ integrin (Santoro et al., 1992). This since neural crest cells are unable to interact with both denatured Col I and the CNBr3 fragment in cases where correct refolding of the fragment was prevented during adsorbance to plastic.

To examine the molecular interaction of avian neural crest cells with Col IV, the primary effort of this study was to isolate native 7S-linked tetramers lacking the NC1 domain, and native dimers held together by their NC1 domains, from chicken tissues. Previously, chick Col IV has been isolated as pepsin-resistant fragments, comprising incomplete portions of the triple-helical region and devoid of 7S and NC1 domains (Mayne and Zettergren, 1980; Mayne et al., 1982, 1983, 1984). Neural crest cells attached

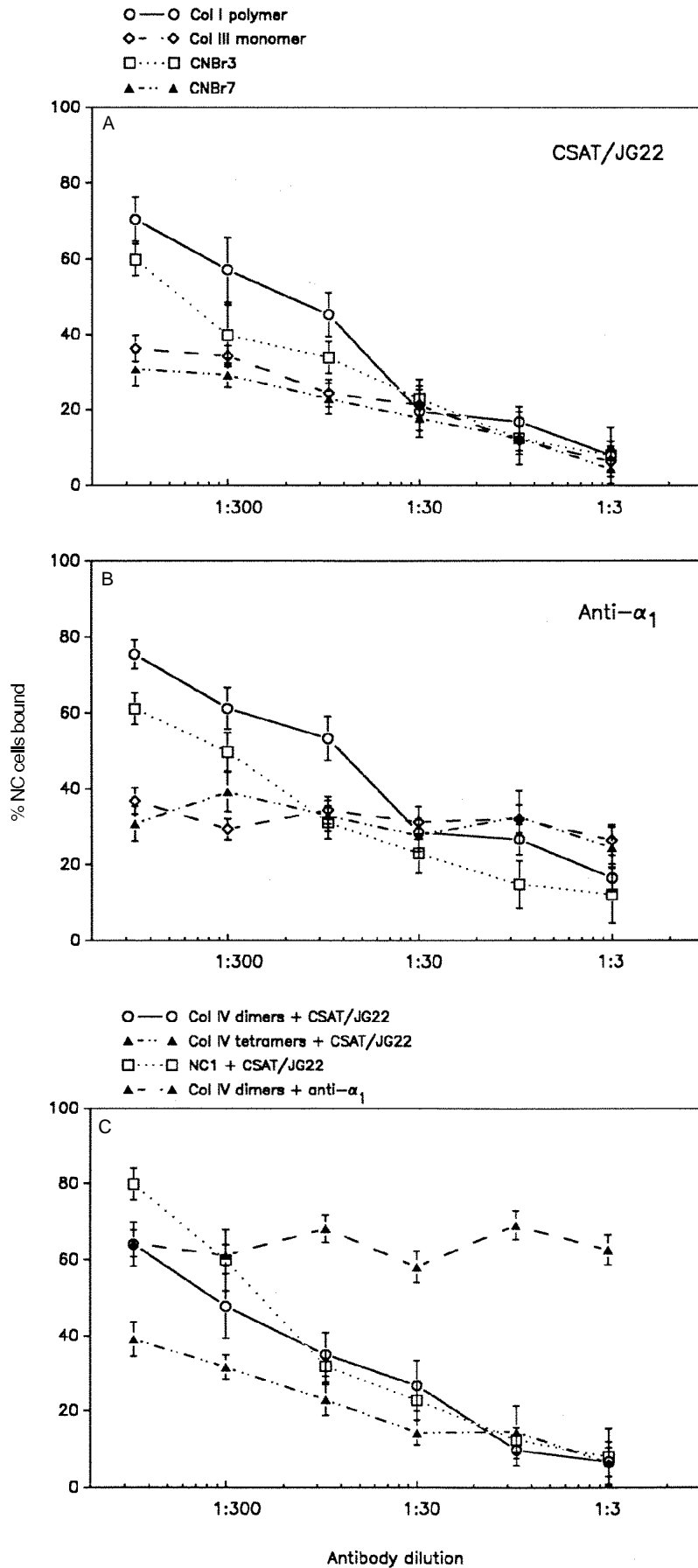**Fig. 5.** Dose-dependency of neural crest cell attachment and migration on intact avian Col IV dimers and 7S-linked tetramers; avian Col IV dimers reduced and alkylated under native (Red/Alk) and denaturing (Den-Red/Alk) conditions; and bovine hexameric NC1 fragment. Similar results were obtained with intact dimers and 7S-linked tetramers from human placenta, whereas dimers isolated from the EHS mouse tumor by guanidine extraction were somewhat less efficient (data not shown).
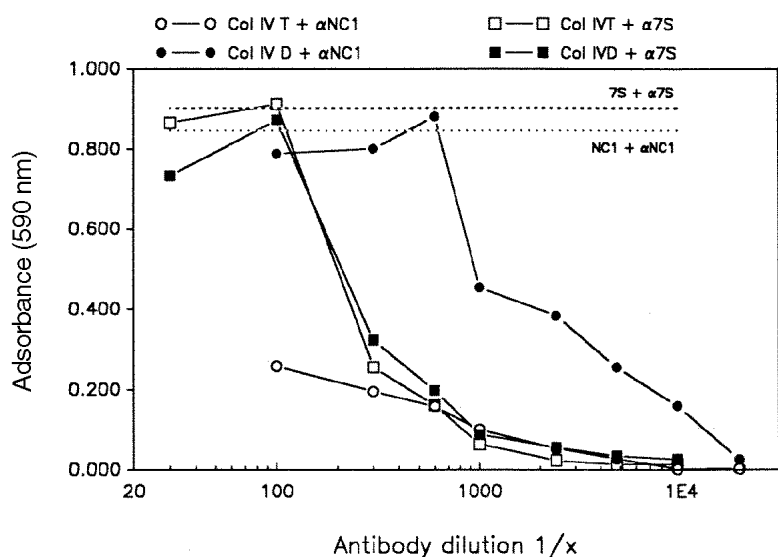

Fig. 6. ELISA using avian and murine dimers, avian 7S-linked tetramers, and bovine NC1 and 7S fragments as antigens and affinity-purified antisera to these fragments. Values for the binding of the antisera to the corresponding immunogens are shown for reference. No cross-reactivity was seen for the anti-NC1 and 7S antisera with the heterologous antigens.
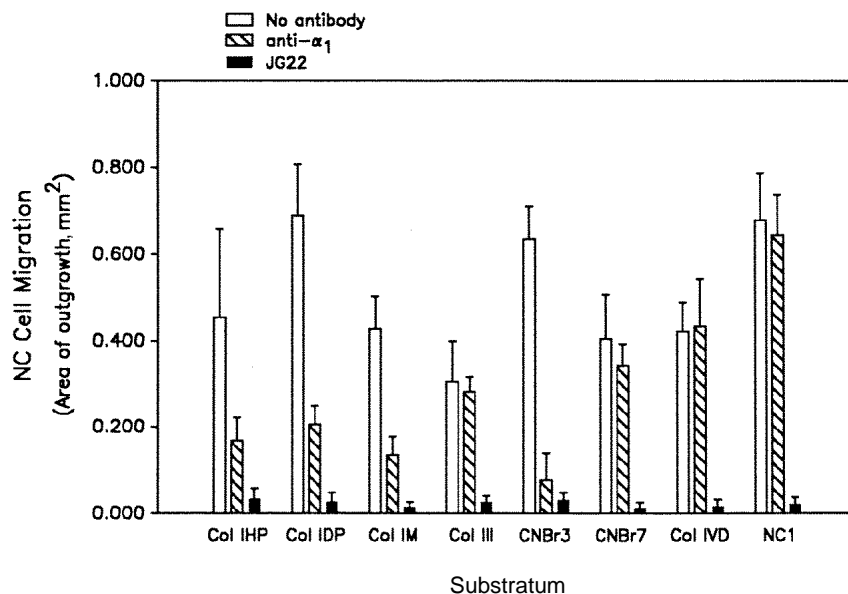
Fig. 7. (A) Dose-dependent inhibition of neural crest cell attachment to polymeric chick skin Col I, monomeric human placental Col III and CNBr fragments from rat tail Col I by anti- ₁-integrin monoclonal antibodies CSAT and JG22. (B) Dose-dependent inhibition of neural crest cell attachment to chick polymeric Col I, monomeric human placental Col III and CNBr fragments from the 1 (I) chain of rat tail Col I by the anti- ₁-integrin antiserum. (C) Dose-dependent inhibition of neural crest cell attachment to chick gizzard Col IV dimers, 7S-linked tetramers and the bovine hexameric NC1 fragment by anti- ₁-integrin monoclonals CSAT and JG22. No effect was seen with the anti- ₁-integrin antiserum on neural crest cell attachment to both dimers (shown above) and tetramers (not shown).
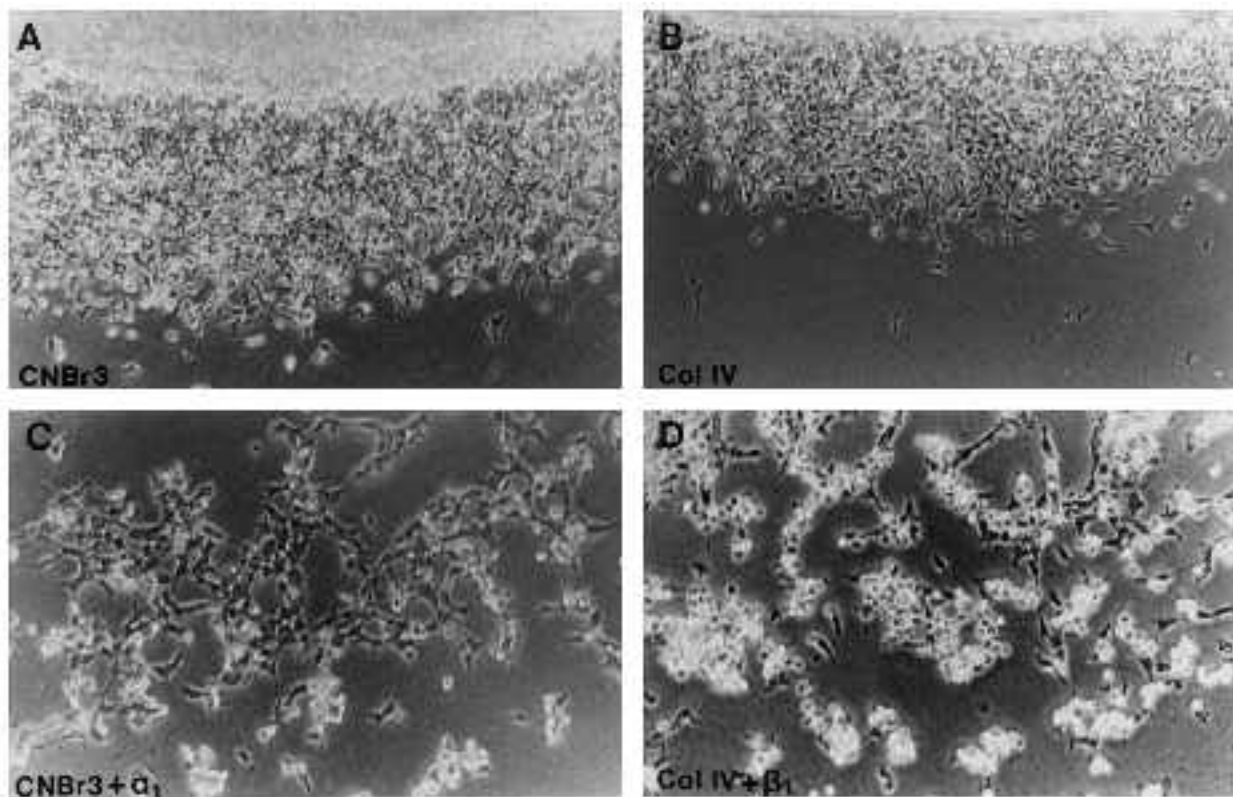
Fig. 8. Phase-contrast photographs showing representative cases of detachment of neural crest cells that had migrated for 12 hours on the CNBr3 fragment from the α1 (I) chain of Col I (A) and dimers of Col IV (B), 2 hours after addition of the anti-α_1-integrin subunit (C) and the anti-β_1 monoclonal JG22 (D).

Fig. 9. Inhibition of neural crest cell migration on hydrated polymeric (HP), dehydrated polymeric (DP), and monomeric chick skin Col I; monomeric human placental Col III; CNBr fragments (3 and 7) from the α1 (I) chain of rat tail Col I; intact chick gizzard Col IV dimers; and bovine hexameric NC1 fragment after addition of the anti-α_1-integrin antiserum (1:10) and the anti-β_1 monoclonal JG22 antibody (1:10 using supernatant), 1 hour after emigration of neural crest cells from the neural tube.

and migrated substantially more efficiently on intact Col IV dimers than on 7S-linked tetramers, suggesting that vital cell interaction sites had been lost in the tetrameric preparation. Moreover, experiments with Col IV reduced and alkylated under native and denaturing conditions showed that native monomers of Col IV were significantly less active than the dimeric counterparts and denatured monomers were entirely inactive. This finding indicates that the interaction of neural crest cells with Col IV requires integrity of the triple helix as well as integrity of the carboxyl- and amino-terminal domains.

The isolated NC1 domain, missing in 7S-linked tetramers, effectively supported neural crest cell adhesion and migration, and prevailed as the primary region of the

molecule for cell interaction. This notion was further sustained by the fact that, as for intact dimers, dissociation of the hexameric NC1 complex into dimeric-monomeric units strongly reduces neural crest cell adhesion and migration. A central role in mediating cell attachment and locomotion has been shown for the NC1 domain also in other cell types (Herbst et al., 1988; Tsilibary et al., 1990; Lein et al., 1991). Moreover, in some cells the interaction with the NC1 domain of Col IV has been shown to occur through the $\alpha_1\beta_1$ integrin (Lein et al., 1991), whereas the $\alpha_1\beta_1$ of other cell types recognizes a triple-helix-associated site (Hall et al., 1990; Clyman et al., 1990; Vandenberg et al., 1991; Syfrig et al., 1991), which may or may not be distinct from those found in synthetic peptides (Chelberg et al., 1990; Tsilibary et al., 1990). As previously suggested (Lallier et al., 1992), avian neural crest cells markedly differ from these cell types in that the $\alpha_1\beta_1$ is not involved in the interaction of the cells with Col IV. The molecular mechanism through which the same integrin expressed in two avian cell types may differ in ligand specificity remains unknown. However, a comparison between the present results and those of Syfrig et al. (1991) obtained with chick gizzard cells and the isolated $\alpha_1\beta_1$ integrin complex clearly demonstrates the differential specificity. While the anti-β_1 antiserum perturbs the interaction of chick gizzard myocytes with Col IV, but not with Col I, the opposite result is obtained when the same antiserum is tested on quail neural crest cells. Plausible explanations include the expression of accessory modulating proteins in one cell type and not in the other, and the occurrence of an unidentified alternative splicing of the avian β_1 integrin chain, yielding functionally different isoforms.

We found that an antiserum to the NC1 domain completely blocks neural crest cell adhesion and migration on the isolated NC1, but only weakly perturbs cell migration and does not affect cell attachment on intact dimers. One possible explanation for this observation is that the neural crest interaction site(s) embodied by the NC1 domain is conformation-dependent, being 'activated' in the isolated domain and being largely inactive in the intact Col IV dimers. An alternative explanation is that the proteolytic digestion used to generate the hexameric fragment NC1 unfolds a latent interaction site, which in the intact collagen could be prone to unfolding during prolonged contact of the cells with the substratum molecule. Finally, the weaker activity of 7S-linked tetramers and triple-helical fragments indicates that additional neural crest interaction sites are contained by the triple-helical region of the collagen. Our results demonstrate that these sites act to a different extent in cooperation with sites contained within the NC1 noncollagenous domain during neural crest cell adhesion and migration on Col IV. The identity of these sites and the nature of the β_1 integrins mediating the interaction of neural crest cells with Col IV remain to be determined.

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