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β_8 Integrins Mediate Interactions of Chick Sensory Neurons with Laminin-1, Collagen IV, and Fibronectin

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Integrins are major receptors used by cells to interact with extracellular matrices. In this paper, we identify the first ligands for the β_8 family of integrins, presenting evidence that integrin heterodimers containing the β_8 subunit mediate interactions of chick sensory neurons with laminin-1, collagen IV, and fibronectin. A polyclonal antibody, anti-β_8-Ex, was prepared to a bacterial fusion protein expressing an extracellular portion of the chicken β_8 subunit. In nonreducing conditions, this antibody immunoprecipitated from surface-labeled embryonic dorsal root ganglia neurons a M_r 100 k protein, the expected M_r of the β_8 subunit, and putative α subunit(s) of M_r 120 k. Affinity-purified anti-β_8-Ex strongly inhibited sensory neurite outgrowth on laminin-1, collagen IV, and fibronectin-coated substrata. Binding sites were identified in a heat-resistant domain in laminin-1 and in the carboxyl terminal, 40-kDa fibronectin fragment. On substrates coated with the carboxyl terminal fragment of fibronectin, antibodies to β_1 and β_8 were only partially effective alone, but were completely effective in combination, at inhibiting neurite outgrowth. Results thus indicate that the integrin β_8 subunit in association with one or more α subunits forms an important set of extracellular matrix receptors on sensory neurons.

INTRODUCTION

Integrins are a widely expressed family of heterodimers that are important receptors for extracellular matrix (ECM)[1] molecules (Hynes, 1992). Each $\alpha\beta$ heterodimer forms a transmembrane complex, its extracellular domain able to interact with ligands and its cytoplasmic domain able to interact with elements of the cytoskeleton. The 15 α and 8 β subunits characterized by cDNA sequencing have been shown to noncovalently associate into at least 20 different heterodimers. Additional variants generated by alternative splicing of subunit mRNAs are also known. Although some individual integrin heterodimers have been shown to bind only one ligand, others are less specific, and some recognize cell adhesion molecules, such as the ICAMs or VCAM-1.

The development of the nervous system depends on cell migration and process outgrowth by neurons, much of which occurs in the presence of the ECM (Reichardt and Tomaselli, 1991; Venstrom and Reichardt, 1993). Several ECM glycoproteins, including laminin-1, collagen IV, and fibronectin, seem likely to be important for neuronal development because they have been shown to promote neuronal attachment, migration, and neurite outgrowth in vitro and have been localized in developing embryos at positions appropriate for exerting similar actions in vivo.

Laminins comprise a family of related trimers composed of α, β, and γ glycoprotein chains (Timpl and Brown, 1994). The best characterized laminin, the laminin-1 isoform, has been isolated from the Engelbreth-Holm-Swarm sarcoma and is composed of an α_1, β_1, and γ_1 chain (A, B1, and B2 chains in original nomenclature). Receptors for laminin-1 include integrin family members $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$, $\alpha_9\beta_1$, $\alpha_v\beta_3$, and perhaps $\alpha_6\beta_4$ (Ignatius and Reichardt, 1988; Gehlsen *et al.*, 1989; Kramer *et al.*, 1990, 1991; de Curtis *et al.*, 1991; Forsberg *et al.*, 1994). Cell binding domains

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[1] Abbreviations used: DRG, dorsal root ganglion; ECM, extracellular matrix; KLH, keyhole limpet hemacyanin; MAb, monoclonal antibody; NGF, nerve growth factor; RGD, arginine-glycine-aspartic acid; PCR, polymerase chain reaction.

in laminin-1's cruciform structure have been localized to the bottom of the cross (in fragment E8) and to a short arm (in fragment E1X) (Timpl and Brown, 1994). In recent studies $\alpha_1\beta_1$ and $\alpha_3\beta_1$ have been shown to be functionally important as laminin-1 receptors on embryonic dorsal root ganglion (DRG) neurons (Tomaselli *et al.*, 1993), whereas $\alpha_6\beta_1$ functions as a laminin-1 receptor on retinal neurons (Cohen and Johnson, 1991; de Curtis and Reichardt, 1993).

Collagen IV is a major structural component of basement membranes and has been shown to promote neurite outgrowth by both sympathetic and DRG neurons in vitro. The $\alpha_1\beta_1$ integrin has been shown to function as a collagen receptor on each of these neurons (Lein *et al.*, 1991). Additional integrins, most notably $\alpha_2\beta_1$, can also mediate interactions of other cells with collagen (Takada and Hemler, 1989) .

Fibronectin contains several distinct functional domains. Near the middle of fibronectin is a series of fibronectin type III domains, one of which contains an arginine-glycine-aspartic acid (RGD) sequence that is recognized by several integrins, including $\alpha_5\beta_1$, $\alpha_3\beta_1$, and several α_v integrins (Pytela *et al.*, 1985; Cheresh and Spiro, 1987; Charo *et al.*, 1990; Dedhar and Gray, 1990; Vogel *et al.*, 1990; Elices *et al.*, 1991; Busk *et al.*, 1992; Gu *et al.*, 1994; Weinacker *et al.*, 1994). The carboxyl terminal domain of fibronectin contains a major heparin binding region and an alternatively spliced variable region. Sites in this region have been shown to be recognized by the integrins $\alpha_4\beta_1$ and $\alpha_4\beta_7$ (Takada and Hemler, 1989; Wayner *et al.*, 1989; Chan *et al.*, 1992). Both the RGD and carboxyl terminal domains of fibronectin support neurite outgrowth by peripheral neurons (Humphries *et al.*, 1988).

The ligand specificities of β_8-containing heterodimers, the most recently identified family of integrins (Moyle *et al.*, 1991), have not been characterized. Motivated by the reported expression of β_8 in rabbit brain (Moyle *et al.*, 1991), we initiated a study of its function in the nervous system. In this paper we present evidence showing that heterodimers containing the β_8 subunit function as collagen IV, laminin-1, and fibronectin receptors on embryonic DRG neurons. The binding sites were further mapped to a heat-resistant domain of laminin-1 and to the CS-1–containing, carboxyl terminal 40-kDa fragment of fibronectin.

MATERIALS AND METHODS

cDNA Cloning

Total RNA extracted from adult chicken brain (Chirgwin *et al.*, 1979) was used as a template for preparation of cDNA, using SuperScript reverse transcriptase (Life Technologies, Gaithersburg, MD), and was amplified using the GeneAmp polymerase chain reaction (PCR) reagent kit (Cetus, Emeryville, CA) and degenerate oligonucleotides (1 μM) based on the published human and rabbit sequences (ATGCACAA C/T AATATAGA A/G AAA and TC T/C T T A/G TACCA A/G TG A/G AA T/C TG) (Moyle *et al.*, 1991). A temperature of 94°C was used to denature, followed by 55°C to anneal and 72°C to extend. The sequence amplified during PCR, starting from nucleotide #1156 and ending with #1738 in the human sequence, is noted in bold in Figure 1. A 584-bp fragment of the chicken β_8 integrin sequence, shown in Figure 1, was subcloned into the TA cloning vector, PCR II, according to the manufacturer's instructions (Invitrogen, San Diego, CA). The fragment was then sequenced using a kit (United States Biochemical, Cleveland, OH); α-^{35}S for sequencing was purchased from Amersham Arlington Heights, IL. Using the *Eco*RI site in the β_8 integrin sequence and the one in the TA cloning vector, the chicken sequence was subcloned into PGEX-3X (Pharmacia, Piscataway, NJ). The resulting plasmid contained the *taq* promoter followed by the complete coding sequence of glutathione-*S*-transferase, a polylinker containing the *Eco*RI site, the 584-base β_8 partial sequence, another *Eco*RI site, and a stop codon. This vector fused the ~27-kDa β_8-Ex protein with the ~27-kDa glutathione-*S*-transferase protein to yield the ~45-kDa fusion protein when expressed in the DH5α strain of *Escherichia coli* bacteria. This protein was isolated from bacteria after induction with 0.1 mM isopropyl-β-thiogalactopyranoside. The induced bacteria were sonicated, and the pellet was resuspended in 1.5% *N*-lauryl-sarcosine and 2% Triton X-100 (Greico *et al.*, 1992). The detergent soluble β_8-Ex fusion protein was collected on glutathione agarose beads (Pharmacia). Antigen was sent to Caltag (South San Francisco, CA) for antibody production in rabbits. The antisera produced will be referred to as anti-β_8-Ex.

Integrin Antibody Purification

In general, Ig from rabbit sera or from mouse ascites, including the anti-β_1 monoclonal antibody (MAb) W1B10 (Hayashi *et al.*, 1990), was purified using MAC protein G disks (Amicon, Beverly, MA) according to the manufacturer's instructions; however, Ig from mouse ascites containing MAb antibody anti-α_v, Chav-1 (Neugebauer *et al.*, 1991), was purified by affinity chromatography on protein A-Sepharose Cl-4B according to the manufacturer's instructions (Pharmacia). JW-2, an anti-laminin-1 rabbit polyclonal antibody, was used at 20 μg/ml (Lander *et al.*, 1985). Anti-collagen IV rabbit polyclonal sera used at 1 to 100 dilution was purchased from Chemicon, Temecula, CA. All Ig preparations were dialyzed extensively against phosphate-buffered saline (PBS). The polyclonal Ig preparations were further dialyzed against F-12 medium and were sterilized by filtration.

To affinity purify anti-β_8 Ex Ig, one liter cultures of bacteria expressing the β_8-Ex fusion protein or a "control" fusion protein (anti-α_8-Ex, also expressed in the same vector) were induced with 0.1 mM isopropyl-β-thiogalactopyranoside for 4 h. The bacterial pellets were collected and resuspended in 9 ml PBS with protease inhibitors, sonicated, and centrifuged for 10 min at 10,000 × *g*. The resultant pellets were solubilized in 1.5% *N*-lauroyl-sarcosine (Sigma Chemical, St. Louis, MO) and 2% Triton-X-100 (Sigma Chemical) in PBS and protease inhibitors. This solution was extracted on ice for 1 h and centrifuged for 10 min at 10,000 × *g*. The supernatant was withdrawn and a 10-cm^2 piece of nitrocellulose membrane (Schleicher and Schuell, Keene, NH) was immersed in the supernatant for 1 h. The β_8 Ig preparation was first incubated with the control (anti-α_8-Ex) *E. coli* extract immobilized on nitrocellulose membrane and then with similarly prepared β_8 fusion protein–containing membrane. Before use, each membrane was washed extensively with PBS, pre-eluted with 0.1 M glycine, pH 2.7, equilibrated with PBS and incubated with Ig for 1 h. After washing with PBS, affinity-purified anti-β_8-Ex was eluted from the β_8 fusion protein–containing membrane and control Ig was eluted from the α_8-Ex–containing membranes with 0.1 M glycine, pH 2.7, and was immediately neutralized with 1 M Tris-Cl, pH 8.5. This was dialyzed against PBS followed by F-12 medium and was sterilized by filtration.

To generate Fab' fragments, purified Ig was dissolved in PBS and dialyzed against 100 mM sodium acetate, pH 5.5. Papain-

agarose (Sigma Chemical) was incubated with the dialysate for 1.5 h at 37°C. The reaction was stopped with 75 mM iodoacetimide (Sigma Chemical) for 30 min at room temperature. The Fab' fragment solution was then dialyzed against PBS and subsequently against F-12 medium.

Substrate Preparation

Linbro Titertek 96-well plastic dishes (ICN Flow, McLean, VA) were first coated with nitrocellulose as described previously (Lagenauer and Lemmon, 1987). Then 100 μl/well of laminin-1 (10 μg/ml in PBS), mouse collagen IV (13 μg/ml; Collaborative Research, Waltham, MA), human fibronectin (10–25 μg/ml; Collaborative Biomedical Products, Bedford, MA, #4008), human fibronectin fragment (120 kDa [10 μg/ml] or 40 kDa [50 μg/ml]; Calbiochem, La Jolla, CA), collagen I (10 μg/ml, Celtrix, Santa Clara, CA, vitrogen-100 #0712), human vitronectin (5 μg/ml, Life Technologies, #X017), or tenascin-C (30–40 μg/ml) was added in PBS and incubated overnight at 4°C. The tenascin-C was purified using a protocol based on previously described methods (Huber *et al.*, 1986; Vaughan *et al.*, 1987). The CS-1 peptide was synthesized by Dr. Chris Turck (Howard Hughes Medical Institute, UCSF). This peptide, DELPQLVTLPHPNLHGPEILDVPSTC, was coupled to keyhole limpet hemocyanin (KLH) using sulfo-MBS according to the manufacturer's instructions (Pierce Chemical, Rockford, IL). The coupled peptide or KLH alone was plated at 100 μg/ml overnight at 4°C. Laminin-1 was purified from Engelbreth-Holm-Swarm sarcoma tumors using published procedures (Kleinman *et al.*, 1982). Heat-resistant laminin-1 was prepared by heating laminin-1 for 10 min at 80°C and was then plated overnight at 29–30 μg/ml (Goodman *et al.*, 1987).

Cell Culture

For chick sensory neuron cultures, embryonic 7- to 8-day chick dorsal root ganglia were dissected and dissociated into single cells by incubating in 0.05% trypsin and 0.2% versene in 1 g/l glucose, 0.2 g/l EDTA, 0.58 g/l $NaHCO_3$ (UCSF cell culture, San Francisco, CA) for 10 min at 37°C followed by trituration. Dissociated cells were collected by centrifugation and resuspended in F-12 containing 10% fetal bovine serum (Life Technologies). To enrich for neuronal cells, cell suspensions were plated onto 60-mm-diameter tissue culture dishes (Falcon, Fisher, Santa Clara, CA) for 1–3 h. Neurons were pipetted from the culture dishes, centrifuged, and resuspended in DRG growth medium [F-12 containing 2% bovine serum albumin (Serva, Heidelberg, Germany) and 100 ng/ml nerve growth factor (NGF) (Mobley *et al.*, 1976)] at an appropriate density (1–2 × 10^3 cells per well). Fifty microliters of this cell suspension was added to 50 μl of warmed and equilibrated 2× concentrated antibody in F-12. Neurons were gently centrifuged onto the dish and incubated for 4–7 h at 37°C in a 5% CO_2 atmosphere.

Immunoprecipitations

Dissociated DRG neurons were cultured for 24 h on two 100-mm^2 Falcon tissue culture dishes previously coated with 5 μg/ml laminin-1 overnight. After removal of growth medium and washing with PBS, cultures were surface labeled with sulfo-NHS-biotin (Pierce). Lysates were prepared by adding 1.2 ml lysis buffer (1% Triton-X-100 and 0.1% sodium dodecyl sulfate [SDS] in PBS containing 1 mM Ca^{2+} and 1 mMg^{2+} and protease inhibitors) to the cultures. Lysed material was then scraped from the dish with a cell scraper, extracted for 15 min on ice, and centrifuged at 20,000 × *g* for 20 min at 4°C. After pre-clearing twice with 100 μl of protein A-Sepharose, supernatants were incubated for 3 h with anti-β_8-Ex Ig coupled to protein A-Sepharose (50 μl beads per culture) or 5 μg W1B10 or Chav-1 Ig not coupled to protein A-Sepharose. Antibodies were coupled to protein A-Sepharose with dimethylpimelimidate (Pierce) using the standard procedure (Harlow and Lane, 1988). Beads were washed six times with 1.0 ml of lysis buffer. Immunoprecipitated proteins were separated on 6% SDS-polyacrylamide gel electrophoresis (PAGE). Protein was transferred by Western blot to nitrocellulose (Schleicher and Schuell). After blocking the nitrocellulose for 1 h in 5% nonfat dry milk, 3% bovine serum albumin (Sigma Chemical), and 0.1% Tween (Sigma Chemical) in PBS, blots were incubated with strepavidin-horse radish peroxidase (Zymed, South San Francisco, CA) for another hour in PBS with 0.1% Tween. The biotinylated proteins were visualized with electrochemical luminescence (Amersham, Arlington Heights, IL).

Immunohistochemistry

Embryos were placed in 4% paraformaldehyde for 1 h, gently washed with PBS, and placed in 30% sucrose for 3–5 days. Embryos were then frozen in Tissue Tek OCT (Baxter, McGaw Park, IL) and immediately sectioned to 10 μm with a Leica cryostat. Sections were rehydrated with PBS, incubated with 10% normal goat, 10% bovine serum albumin, 1% glycine, and 0.4% Triton-X-100 for 1 h, and were then incubated with purified Ig (10 μg/ml) overnight at 4°C. Sections were extensively washed and immunoperoxidase stained according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA). Slides were examined with a Nikon Microphot (Melville, NY) and photographed with Kodak T-Max-100 (Rochester, NY).

Cell cultures in 96-well dishes were fixed with 4% paraformaldehyde for 10 min, carefully washed, and incubated for 2 h with the following antibodies: anti-β_8-Ex affinity-purified or pre-immune Ig (10 μg/ml) diluted in PBS and 5% normal goat serum. Cells were washed extensively and stained according to the instructions included in the Vectastain kits. Cells were examined with a Nikon inverted Diaphot-TMD and photographed with Kodak T-MAX-100.

Quantitation of Neurite Outgrowth

For each condition, the percentage of DRG cells with neurites was determined in duplicate and the results were averaged (Figures 5 and 7). This mean was normalized in each experiment to the mean percentage of neurite outgrowth in "no antibody" control wells. In control conditions, about 50% of neurons extended neurites on laminin-1, 30% on collagen IV, 17% on heated laminin-1, 25% on fibronectin 120, 20% on fibronectin 40, and 8% on CS-1 (a domain in fibronectin 40). A neurite was counted when it was greater than two cell body diameters in length. However, on the CS-1 peptide, neurites were counted if they had a length greater than or equal to one cell diameter because of the poor neurite outgrowth on this peptide substrate. The number of experiments was greater than or equal to three, except for anti-α_v and affinity-purified control and pre-immune controls on CS-1 and fibronectin and Fab' fragments on fibronectin 40 where n equaled 2 (Figure 5b). In addition, the control for heated laminin (Figure 5a) was no antibody, not anti-β_8-Ex pre-immune. The error bars represent the SD from the mean (Figures 5–7).

To measure neurite length (Figure 6), cultures were viewed with an inverted Olympus IMT2 microscope with phase optics. The Student t-test was performed on the mean lengths of neurites from four to six wells per substrate (Glantz, 1992). A total of 80–120 neurites per substrate were measured. Microscope images were collected with a cooled CCD camera (series 200, Photometrics, Tuscon, Arizona) equipped with a 1024 × 1024 pixel CCD imaging device (Texas Instruments, Dallas, Texas, TC215) and stored on the hard disk in a VAX 3200 computer. Processes longer than 20 μm were measured using the Prism program (Chen *et al.*, 1989).

RESULTS

Chicken β_8 Integrin cDNA Cloning and Polyclonal Antibody Production

PCR was used to clone a portion of the extracellular domain of the chicken β_8 integrin subunit (see MATERIALS AND METHODS). Degenerate oligonucleotide primers based on the published rabbit and human sequences (Moyle *et al.*, 1991) were used to amplify the corresponding sequence from chicken RNA. The sequence of this fragment of the chicken β_8 is shown in Figure 1. The deduced amino acid sequence of this PCR clone of chicken β_8 in this region is ~87% identical to the human and rabbit sequences. This clone was expressed in bacteria as a glutathione-*S*-transferase fusion β_8 protein and injected into two rabbits. Although sera from both rabbits appeared to have similar properties including recognition of native β_8 heterodimers, all experiments were performed using Ig from one serum, named anti-β_8-Ex.

Characterization of Chicken β_8 by Immunoprecipitation

To identify β_8 and its associated α subunit(s), DRG cells were cultured overnight in the presence of NGF and surface labeled with biotin. Detergent extracts were immunoprecipitated with anti-β_8-EX, anti-α_v, or anti-β_1 integrin antibodies (Figure 2). In β_8 immunoprecipitates, two bands of M_r 100 k and 120 k were observed under nonreducing conditions (Figure 2, lane 1). The M_r 100 k (Figure 2, see arrow lane 1) band appears to migrate at M_r 110 k in reducing conditions (Figure 2, see arrow lane 4). This shift is characteristic of β integrin subunits. The low M_r smear in lane 4 could be breakdown products of β_8 released by reduction, as they were not seen in nonreducing gels (Figure 2, lane 1). Clearly the predominant integrin α(s) subunit(s) associated with β_8 appears to be a doublet migrating at M_r 120 k that could represent more than one protein (Figure 2, lane 1). When reduced, this putative subunit(s) also appeared to migrate with the M_r 110 k band (Figure 2, lane 4), suggesting that it is a complex of disulfide-linked large and small chains, similar to many other integrin α subunits. The identity of this subunit(s) is not known, but it is not the same M_r as α_v (Figure 2, compare lanes 1 and 2), the only α subunit previously detected in association with β_8 (Moyle *et al.*, 1991). Consistent with this, anti-α_v does not precipitate detectable amounts of protein at the same M_r as β_8 (Figure 2, lanes 1 and 2). Although there is some homology in the fragment of β_8 used as Ag and the corresponding region of β_1, anti-β_8-Ex clearly precipitates proteins distinct from

START OF ß8 EXTRACELLULAR PORTION OF THE FUSION PROTEIN

atg	**cac**	**aac**	**aat**	**ata**	**gag**	**aaa**	ctg	aat	tcc	gtt	gga	ttt	gct	tta	tca	aag	aag	atg	gag	aat	att	tct	ctt	gat	ttt	cga	ctt	ggg	ttt	gga	tcc	tat	gtg	gat	aaa
M	**H**	**N**	**N**	**I**	**E**	**K**	L	N	S	V	G	F	A	L	S	K	K	M	E	N	I	S	L	D	F	R	L	G	F	G	S	T	V	D	K
114												N	D			R			A	F	F		R												
act	gtg	tca	ccc	tat	atc	tgc	att	cac	cca	ggt	aga	atc	cat	aac	caa	tgc	agc	gat	tat	aat	tta	gat	tgt	atg	ccg	cct	cat	ggt	tat	att	cat	gtg	cta	tcc	ctg
T	V	S	P	Y	I	C	I	H	P	G	R	I	H	N	Q	C	S	D	Y	N	L	D	C	M	P	P	H	G	Y	I	H	V	L	S	G
150						S			E																										
act	gac	aat	ata	gca	gaa	ttt	aga	aat	gca	gtg	aat	aaa	caa	aaa	att	tct	ggg	aat	att	gat	aca	cct	gaa	ggg	ggt	ttt	gat	gca	atg	ctc	caa	gca	gct	gtt	tgc
T	D	N	I	A	E	F	R	N	A	V	N	K	Q	K	I	S	G	N	I	D	T	P	E	G	G	F	D	A	M	L	Q	A	A	V	C
186	E			T			E	K			H	R																							
cag	agc	cac	att	gga	tgg	cgg	aaa	gaa	gcc	aag	cgg	cta	ctt	ctt	gtg	atg	aca	gac	caa	act	tcc	cat	cta	gcc	cta	gat	agt	aaa	tta	gca	ggg	atc	gtg	att	ccc
Q	S	H	I	G	W	R	K	E	A	K	R	L	L	L	V	M	T	D	Q	T	S	H	L	A	L	D	S	K	L	A	G	I	V	I	P
E																																		V	
cat	gag	cca	tct	ctt	ggg	cag	ctc	cgc	gaa	aaa	ttg	att	gat	aat	aac	atc	aat	gag	cat	cca	tct	ctt	ggg	cag	ctc	cgc	gaa	aaa	ttg	att	gat	aat	aac	atc	aat
H	D	F	N	C	H	L	K	D	N	V	Y	V	K	A	T	S	N	E	H	P	S	L	G	Q	L	R	E	K	L	I	D	N	N	I	N
N								N						S		T										S									
gtc	att	ttt	gca	gtt	caa	gga	agc	**cag**	**ttc**	**cat**	**tgg**	**tac**	**aag**	**ga**																					
V	I	F	A	V	Q	G	S	**Q**	**F**	**H**	**W**	**Y**	**K**																						
294							K																												

Figure 1. The PCR-cloned chicken β_8 sequence: nucleotide sequence on top, deduced amino acid sequence in the middle, and the differing amino acids in the human sequence on the bottom for comparison. The amino acid number of the corresponding human sequence is given at the left margin. The *Eco*RI site used to construct the β_8-Ex fusion protein is marked with an arrow. The oligonucleotide primer sequences amplified during the PCR reaction are in bold.

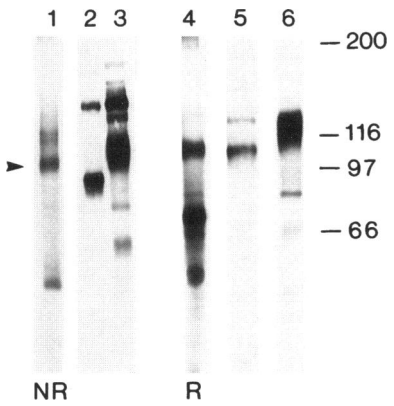


Figure 2. Immunoprecipitation of surface biotinylated dorsal root ganglion cells. Lanes 1–3 were resolved by SDS-PAGE in nonreducing conditions, and lanes 4–6 were resolved in reducing conditions. Lanes 1 and 4 show proteins precipitated by anti-β_8-Ex, lanes 2 and 5 show proteins precipitated by anti-α_v (MAb Chav-1), and lanes 3 and 6 show proteins precipitated by anti-β_1 (MAb W1B10). The β_8 subunit was ~100 k in nonreduced (lane 1) and 110 k in (lane 3) reduced conditions. The prominent M_r 120-k putative α band(s) appeared to collapse into the 110-k β_8 band when reduced. Surprisingly, there did not appear to be a band with the M_r of α_v associated with β_8 in the DRGs. Correspondingly, the α_v antibody did not seem to immunoprecipitate a β integrin subunit the size of β_8 in nonreduced conditions (lane 2). In addition, β_8 (lane 4) migrated with a lower M_r than β_1 when reduced (lane 6). Lower M_r bands precipitated with β_8-Ex in reducing conditions (lane 4) are of unknown identity, but may be proteolytic products of β_8 or associated subunits.

those precipitated by an anti-β_1 MAb (Figure 2, compare lanes 1, 3, 4, and 6). The M_r of the putative β_8 bands, 100 k nonreduced and 110 k reduced, migrate with lower mobility than β_1 bands, 110 k nonreduced and 120 k reduced. Therefore, anti-β_8-Ex does not appear to cross-react with the β_1 subunit.

Expression of β_8 Integrin

To identify populations of DRG neurons expressing individual integrin subunits, neurons were cultured overnight in the presence of NGF and stained with several integrin subunit-specific antibodies. The expression of β8 by DRG neurons cultured on fibronectin is shown in Figure 3A. Most neurons (ca 90%) expressed integrin subunits β_8, β_1, and α_v. In the presence of pre-immune serum or in the absence of primary antibody, neurons remained unstained. Similar results were obtained when these neurons were cultured on laminin-1. To investigate the expression pattern in vivo of β_8, transverse sections through embryonic day 8 chicken embryos were stained with anti-β_8-Ex affinity-purified Ig (Figure 3, C-F). Neurons in the DRG (Figure 3, C-D) and spinal cord (Figure 3, E-F) were heavily stained. The strongest staining in the spinal cord appeared to be concentrated in the ventral spinal cord. In addition, embryonic muscle and brain cells appeared to express β_8 (our unpublished observations).

Identification of β_8 as a Receptor for Laminin-1, Collagen IV, and Fibronectin

The β_8-Ex fusion protein used to make the anti-β_8-Ex antibody includes the domain homologous to the ligand binding region, which has been mapped in other integrin β subunits (Shih *et al.*, 1993). In an effort to identify potential ligands, anti β_8-Ex was tested for inhibitory effects by adding it to cultures of embryonic chick DRG's plated on extracellular matrix molecules (Figure 4). Results were quantified by counting the percentage of cells with neurites (Figure 5) in the absence or presence of anti-β_8-Ex affinity-purified Ig (25 μg/ml). Affinity-purified anti-β_8-Ex had strong, but partial inhibitory effects on laminin-1 (to ~56% shown in Figure 5 and Figure 4, A and B) and collagen IV (to ~58% shown in Figure 5 and Figure 4, C and D). Although neurite lengths were significantly affected in the presence of anti-β_8-Ex (see below) at the time when the cultures were fixed (4–7 h), the most striking effect was that close to one-half of the neurons that normally would have extended neutrites remained rounded (Figure 4). In addition, the neurites growing out in the presence of anti-β_8-Ex looked thinner (possibly less attached to the substrate).

Neurons were also cultured on laminin and collagen IV in the presence of function-inhibiting antibodies to the integrin $\beta_1$or α_v subunits. As expected, the anti-β_1 MAb, W1B10, completely inhibited neurite outgrowth by sensory neurons on laminin-1 or collagen IV. (Figure 5A). In the presence of this antibody, all neurons remained rounded and appeared detached from the substrate. Anti-β_1 integrin-specific MAbs including W1B10, have been shown previously to inhibit neuronal interactions with laminin-1 and collagen IV (Bozyczko and Horwitz, 1986; Hall *et al.*, 1987; Lein *et al.*, 1991; Tomaselli *et al.*, 1993). In contrast, the anti-α_v MAb had no detectable effect on either laminin-1 or collagen IV-coated substrates, but did inhibit outgrowth on other substrates (see below). The anti-α_v-specific MAb Chav-1 has been shown to inhibit interactions of α_v subunit–containing integrins with vitronectin (Neugebauer *et al.*, 1991).

The domain of laminin-1 recognized by β_8 integrin heterodimer was partially characterized by examining interactions with heat-inactivated laminin-1. Although cell binding sites in the foot of the laminin-1 cruciform structure (fragment E8) are destroyed by heat, the cell adhesion sites in the top part of the laminin-1 cross, the E1X domain, have been shown to be resistant to heating at 80°C for 10 min (Goodman *et al.*, 1987). DRG neurons remain able to extend neurites on heat-inactivated laminin-1. Neurite outgrowth is strongly in-

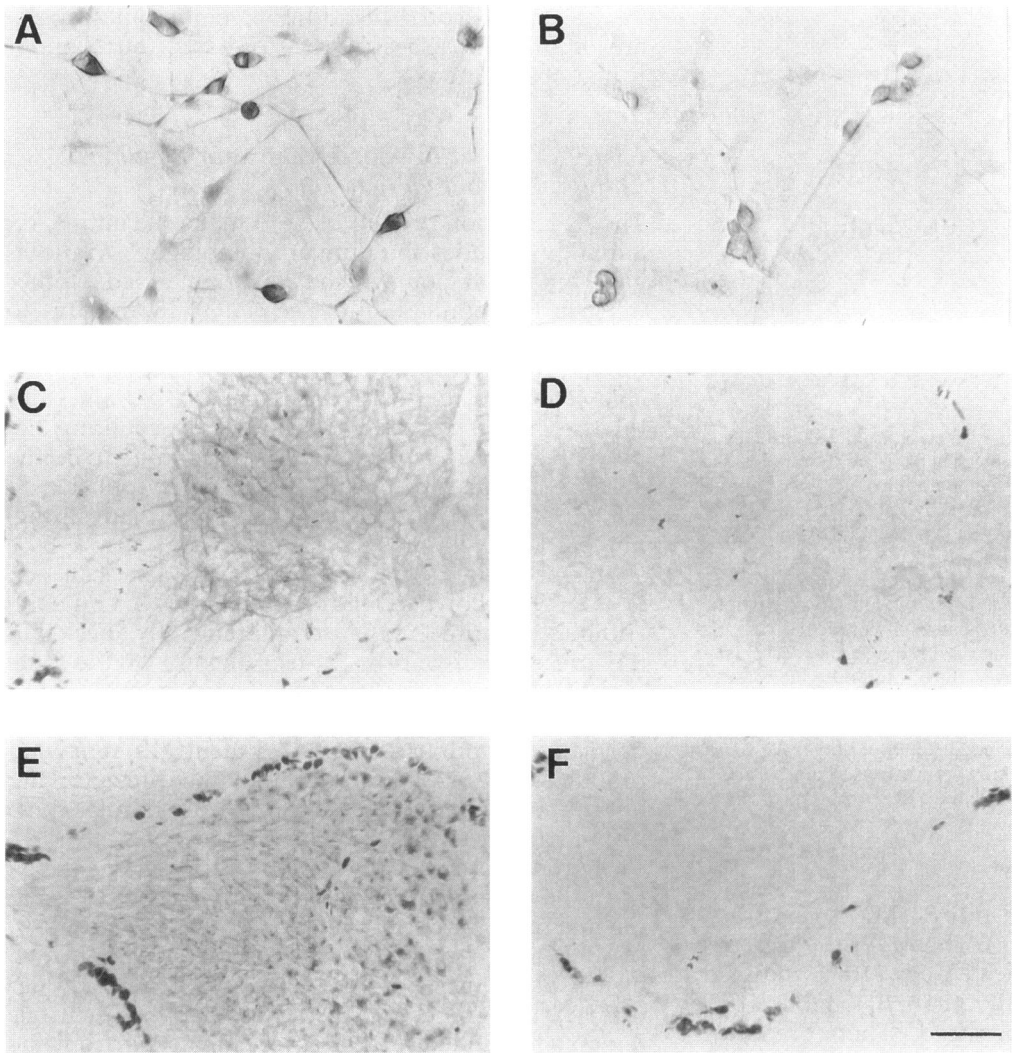


Figure 3. β_8 integrin is expressed on embryonic day 8 chicken DRG neurons. The neurons were cultured on fibronectin in an overnight culture and stained with 5 μg/ml β_8-Ex affinity-purified (A) or pre-immune Ig (B). The immunoperoxidase reaction product is dark. Over 90% of the neurons with neurites counted in the cultures stained positively for the β_8-Ex antibody. Transverse sections through embryonic day 8 chick spinal chords were prepared and stained with β_8-Ex (C and E) and preimmune for β_8-Ex (D and F) with 10 μg/ml Ig. B_8-Ex antibody appears to stain the spinal cord and is concentrated in the ventral spinal cord (C and D) and is abundant in the DRG (E and F). Dark cells in controls (D and F) are red blood cells. Bar for all panels is 50 μm and is depicted in panel F.

hibited by affinity-purified anti-β_8-Ex antibody on heat-inactivated laminin-1 (to ~40% shown in Figure 5A), demonstrating that a heat-resistant cell binding site in laminin-1, most likely in E1X, is recognized by a β_8 integrin heterodimer. Cell attachment on E1X is known to be mediated by $\alpha_1\beta_1$ and $\alpha_v\beta_3$ (Kramer *et al.*, 1990; Goodman *et al.*, 1991). Similarly, DRG neurite outgrowth on intact laminin-1 is thought to be mediated by $\alpha_1\beta_1$ (Tomaselli *et al.*, 1993). When intact laminin-1 is heat inactivated, a cryptic binding site for α_v integrins is exposed. This is likely to be the same site exposed during preparation of the E1X fragment (Sung *et al.*, 1993). Neurite outgrowth was inhibited completely by anti-β_1 (perhaps because of poor control neurite outgrowth) and strongly by anti-α_v Ig (to ~36% shown in Figure 5A). Assays on laminin-1 fragments will be needed to map more definitive binding sites for β_8 integrins.

Binding of β_8 integrins to domains of fibronectin is displayed in Figure 5B. On intact fibronectin, neurite outgrowth was partially inhibited by anti-β_8-EX (to ~64%), anti-β_1 MAb (to ~36%), or anti- α_v MAb (to ~65%). The domains recognized by each subset of integrins (β_1, α_v, and β_8 heterodimers) was more precisely mapped by plating neurons on two cell binding fragments: fibronectin 120, containing the RGD sequence; and fibronectin 40, the carboxyl terminal, containing the variable domains, CS-1 sequence, and heparin-binding site(s). β_8-Ex antibody inhibited neurite outgrowth on the fibronectin 40 fragment (to ~57% shown in Figure 5B and Figure 4, E and F), but not on the fibronectin 120 fragment (Figure 4, G and H and Figure 5B). On fibronectin 40, anti-β_1 and anti-β_8 antibodies were partially inhibitory alone (to ~55% and ~57%, respectively), and much more effective in combination (to ~11%). Anti-α_v MAb was not inhibitory

alone or additive in combination with a β_1 MAb. This result maps the site recognized by β_8 integrins to the fibronectin 40 fragment where binding sites for $\alpha_4\beta_1$ and $\alpha_4\beta_7$ have previously been reported (Wayner *et al.*, 1989; Chan *et al.*, 1992; Haugen *et al.*, 1992b). On fibronectin 120, which contains the RGD site, anti-α_v and anti-β_1 MAbs were partially effective alone (to ~59% and ~25%, respectively) and completely effective in combination (to ~1%). Anti-β_8-Ex was not inhibitory alone and did not potentiate the effects of anti-β_1 MAbs. Thus, in agreement with previous publications, β_1 and α_v integrin heterodimers recognize a site in fibronectin-120 (Pytela *et al.*, 1985; Bozyczko and Horwitz, 1986; Hall *et al.*, 1987; Charo *et al.*, 1990; Vogel *et al.*, 1990; Busk *et al.*, 1992; Dedhar *et al.*, 1994; Weinacker *et al.*, 1994). This supports the immunoprecipitation data shown in Figure 2 that α_v must associate with a β subunit distinct from β_1 on these cells.

In an effort to map the binding site of β_8 integrins in fibronectin 40, neurite outgrowth was examined on the CS-1 peptide, an alternatively spliced amino acid sequence in this fragment of fibronectin (Humphries *et al.*, 1988) (Figure 5B). On this substrate, the percent of neurons with neurites was greatly reduced in the presence of anti-β_8-Ex Ig. KLH, the protein coupled to the CS-1 peptide, did not promote any detectable neurite outgrowth either in the presence or absence of anti-β_8 antibodies. In addition, anti-α_v Ig or affinity-purified control Ig (see MATERIALS AND METHODS) did not block neurite outgrowth on the CS-1 peptide. The failure of anti-α_v antibodies to inhibit outgrowth provides further evidence that $\alpha_v\beta_8$ is not the receptor mediating this interaction. As previously published, neurite outgrowth on CS-1 was completely inhibited by addition of anti-β_1 antibodies (Haugen *et al.*, 1992b). Experiments on lymphoid cells have suggested that $\alpha_4\beta_1$ and $\alpha_4\beta_7$ interact with the CS-1 peptide (Wayner *et al.*, 1989; Chan *et al.*, 1992). Recent results indicate that a subset of embryonic mouse DRG cells express the α_4 integrin receptor (Sheppard *et al.*, 1994). Function-blocking antibodies to chicken α_4 would be needed to further investigate the heterodimers mediating this interaction.

Neurite Length Is Reduced in the Presence of β_8-Ex

In addition to reducing the percentage of neurons with neurites, anti-β_8-Ex also reduced the length of neurites extended by neurons on substrates coated with the same ECM components. The mean length of neurites extended by DRG neurons grown in the presence of the anti-β_8-Ex affinity-purified Ig was reduced by 24 ± 9% on laminin-1, 24 ± 5% on fibronectin 40, and 26 ± 7% on collagen IV, when compared with cultures incubated with control media (Figure 6). The Student t-test was performed on the mean neurite lengths of four to six cultures per substrate (Glantz, 1992). These results were statistically significant ($p < 0.002$), except in the case of fibronectin 120, the control substrate. Cumulative frequency distribution plots showed an apparently continuous reduction in neurite lengths despite DRG heterogeneity. Consistent with previous results demonstrating that the β_8 integrin subunit was expressed by almost all DRG neurons (Figure 3), this suggests that neurite outgrowth by almost all sensory neurons was inhibited by the anti-β_8-Ex Ig in these cultures.

Anti-β_8-Ex Antibody Effects Are Substrate Specific

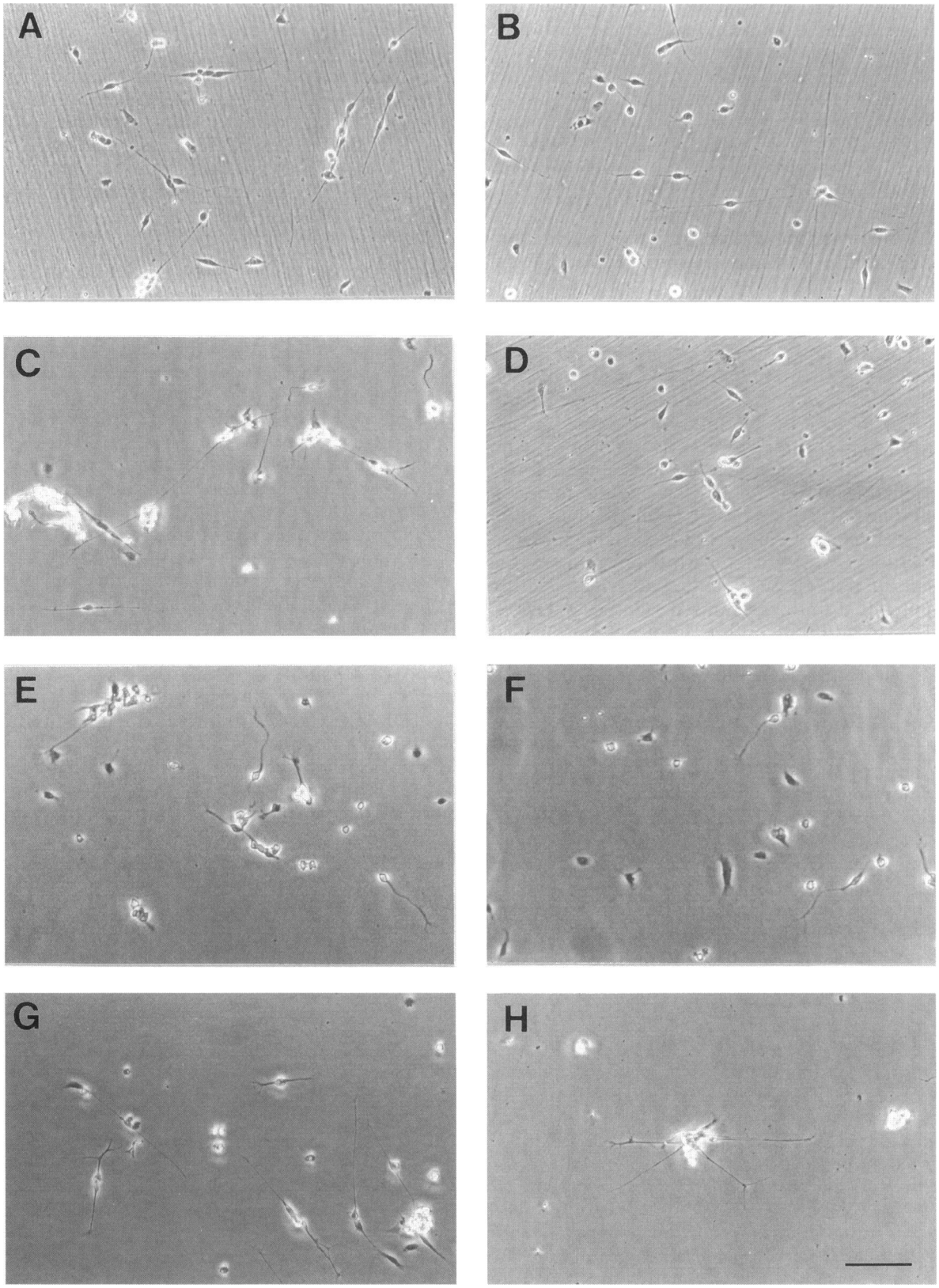
Collagen I, vitronectin, and tenascin were tested and found to support normal neurite outgrowth in the presence of anti-β_8 antibodies (Figure 7). This result demonstrates that β_8 has a unique pattern of substrate preference differing from β_1 integrin. Anti-β_1 antibodies inhibited DRG neurite outgrowth strongly and in some cases completely on these substrates (K. Venstrom, unpublished observations; Figure 5; Varnum-Finney *et al.*, 1995). In addition, inhibitory effects of anti-β_8-Ex were not seen with neurite outgrowth on the fibronectin 120-k fragment (Figure 5b).

As additional controls, anti-β_8-Ex Fab' fragments (300 μg/ml) were similarly inhibitory on laminin-1, collagen IV, and the fibronectin 40 fragment (Figure 5). This indicates that inhibition was direct and not caused by receptor cross-linking. In addition, adsorption with a control bacterial extract was shown not to reduce the inhibitory effects of anti-β_8-Ex Ig. As a third control, Ig prepared from the anti-β_8-Ex pre-immune serum did not inhibit outgrowth of DRG neurons (Figure 5A). In addition, another function-blocking integrin polyclonal antibody made in the same way, anti-α_8-Ex, was used as a control in the same and similar experiments (Muller *et al.*, 1995; Varnum-Finney *et al.*, 1995; our unpublished observations). The anti-α_8-Ex, used at the same concentration, did not inhibit neurite outgrowth on fibronectin 40, laminin-1, or collagen IV. These controls demonstrate that inhibition does not result from nonspecific toxic effects or from the presence of nonspecific or other integrin-specific antibodies.

As a substrate control, JW-2, an anti-laminin-1 polyclonal antibody (Lander *et al.*, 1985), was used to block neurite outgrowth on laminin-1 almost completely. This serum did not significantly inhibit outgrowth on collagen IV. Similarly, an anti-collagen IV polyclonal sera blocked almost all neurite outgrowth on collagen IV but did not significantly affect neurite outgrowth on laminin-1.

DISCUSSION

In this study, we identify for the first time ligands for integrins containing the β_8 subunit. Using an affinity-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purified subunit-specific antibody, prepared with the use of a fusion protein expressing the putative ligand binding domain of β_8, we show that neuronal interactions with laminin-1, collagen IV, and fibronectin are mediated, in part, by this family of integrins. Neurite outgrowth on each substrate was strongly inhibited by anti-β_8 Ex. Fab' fragments of anti-β_8 Ex were equally potent, demonstrating that inhibitory effects were not indirect consequences of receptor cross-linking. The binding site on laminin-1 was mapped to a heat-resistant domain, most likely in the E1X fragment. The binding domain on fibronectin was mapped to the carboxyl terminal 40-k fragment that contains the CS-1 amino acid sequence. On fibronectin, we show that β_1 and α_v integrins cooperate to mediate neurite outgrowth on the 120-k fragment that contains the RGD attachment site. Several β_1 and α_v integrins have previously been shown to recognize this attachment site (Pytela *et al.*, 1985; Cheresh and Spiro, 1987; Charo *et al.*, 1990; Dedhar and Gray, 1990; Vogel *et al.*, 1990; Elices *et al.*, 1991; Busk *et al.*, 1992; Gu *et al.*, 1994; Weinacker *et al.*, 1994). On the carboxyl terminal 40-k fragment, which has previously been shown to contain binding sites for $\alpha_4\beta_1$ and $\alpha_4\beta_7$ (Wayner *et al.*, 1989; Chan *et al.*, 1992; Ruegg *et al.*, 1992), we show that β_8 and β_1 integrins cooperate to mediate neurite outgrowth.

The role of β_8 integrins in promoting neurite outgrowth was clearly apparent in the DRG neuron response to fibronectin 40. Although anti-β_1 and anti-β_8 antibodies inhibited neurite outgrowth by DRG neurons only partially when added individually, in combination these antibodies inhibited neurite outgrowth almost completely (Figure 5). Anti-α_v and anti-β_1 had similar cooperative inhibitory effects on neurite outgrowth on fibronectin-120. Because DRG neurons express both α_v and β_8 in addition to β_1 integrins, it does not seem surprising that they are able to initiate neurite outgrowth in the presence of anti-β_1 antibodies. More surprising is the observation that anti-β_1 completely inhibits sensory neurite outgrowth on laminin-1, heat-inactivated laminin, collagen IV, and CS-1, despite the presence of β_8 integrins able to interact with these proteins. It seems possible that adhesion must reach a "threshold" before it is detected in neurite outgrowth assays.

Identification of these ligands for β_8 heterodimers depends critically upon the specificity and inhibitory function of the antibody, anti-β_8-Ex, used in this paper. This antibody was made to a fusion protein containing part, but not all of the extracellular domain. The region present in the fusion protein includes a sequence believed to participate in ligand binding, because it corresponds to the regions in the integrin β_1 and β_3 sequences that have been shown directly to mediate ligand binding by cross-linking and mutagenesis, respectively (D'Souza *et al.*, 1988; Smith and Cheresh, 1988; Takada *et al.*, 1992). Thus, before initiation of the experiments presented in this paper, there were strong expectations that an antiserum to this region of the β_8 subunit would inhibit ligand binding. Because the ligand binding regions of different integrin β subunits are homologous (see Moyle *et al.*, 1991), it was also necessary to verify the specificity of the β_8-Ex antibodies. In this paper, we show that these antibodies immunoprecipitate a unique set of proteins, clearly distinct from those immunoprecipitated by anti-β_1 or anti-α_v antibodies. The M_r in nonreducing SDS-PAGE of the chick β_1, β_3, and β_5 subunits have been determined (see, e.g. Delannet *et al.*, 1994). Each is clearly different than that of β_8 bands immunoprecipitated by anti-β_8-Ex. Although the molecular weight of β_4 has not been determined in chick, in other vertebrates its M_r is ca 200 k, far larger than the size of any protein recognized by anti-β_8 (see, e.g. Reichardt and Tomaselli, 1991). The β_2 subunit has never been detected outside the immune system and each of the three α subunits that associate with β_2 has an M_r larger than that of the largest protein immunoprecipitated by anti-β_8-Ex. Although the molecular weights of chicken β_6 and β_7 are also not known, they can associate with α_v and, therefore, if present, may have been detected in anti-α_v immunoprecipitates. None of the proteins precipitated by anti-α_v had the same M_r as those precipitated by anti-β_8-Ex in our experiments. Although β_6 and β_7 are reported to be fibronectin receptors, they have not been reported to bind to laminin or collagen IV (Busk *et al.*, 1992; Chan *et al.*, 1992; Ruegg *et al.*, 1992); therefore, the biochemical evidence strongly suggests that anti-β_8-Ex is specific for the β_8 subunit.

In agreement with the biochemical characterization of proteins precipitated by anti-β_8-Ex, this antibody has function-blocking effects that distinguish it from anti-β_1 or other anti-β or anti-α subunit antibodies, such as anti-α_v. For example, anti-β_1 antibodies

Figure 4. Anti-β_8-Ex reduced neurite outgrowth of sensory neurons on laminin-1 (A and B), collagen IV (C and D), fibronectin 40 (E and F), but not on fibronectin 120 (G and H). The left panels (A, C, E, and G) illustrate neurons cultured for 4–7 h in control conditions and the right panels (B, D, F, and H) illustrate neurons cultured with β_8-Ex affinity-purified antibody (10 μg/ml). In the presence of β_8-Ex antibodies, cultures on laminin-1, collagen IV, or fibronectin 40 had a lower percentage of neurons with neurites and reduced neurite length and thickness compared with controls (also see Figures 5 and 6). Neurons on fibronectin 120 (G and H), were not inhibited in the presence of anti-β_8-Ex antibody. Control antibodies that did not inhibit neurite outgrowth on laminin-1, collagen IV, and fibronectin 40 included cell surface binding integrin antibodies (anti-α_8-Ex polyclonal and anti-α_v MAb), as well as nonspecific controls (anti-β_8-Ex pre-immune and affinity purified control Ig). Bar for all panels is depicted in panel H and equals 100 μm.

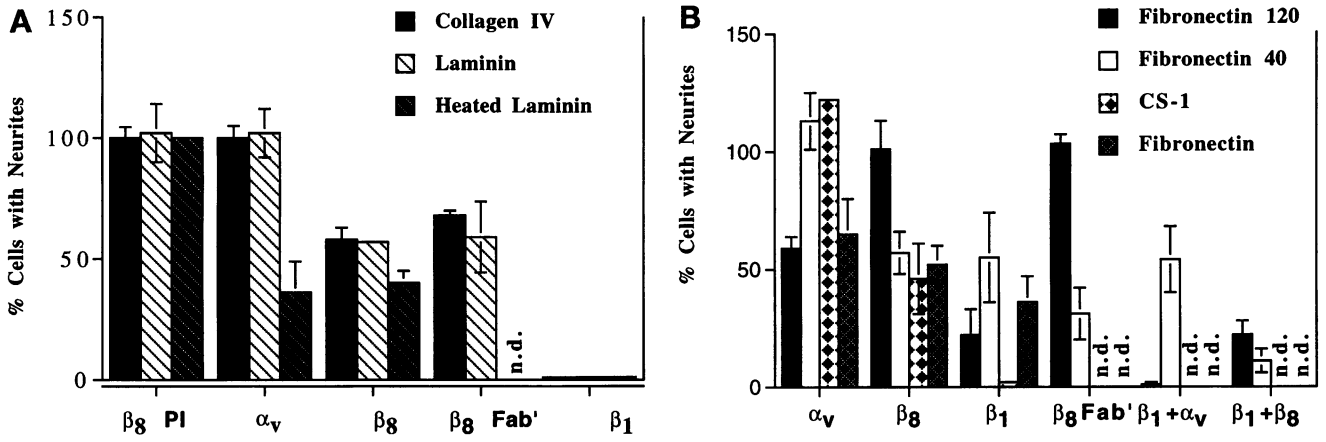

Figure 5. Effect of anti-β_8-Ex and other integrin antibodies on the normalized percent of DRG cells that developed neurites in culture. Cultures were incubated in the presence of the following integrin antibodies: β_8 pre-immune Ig (PI) (25 μg/ml for affinity purified or 300 μg/ml for Fab' Ig), β_8-Ex affinity-purified Ig (25 μg/ml), β_8-Ex Fab' Ig (300 μg/ml), β_1 (W1B10, MAb, 50 μg/ml), α_v (Chav-1, MAb, 50 μg/ml). (A) Affinity-purified β_8-Ex reduced the number of neurons with neurites on collagen IV, laminin-1, and heated laminin-1. After heat inactivation of the laminin-1, neurites were strongly inhibited with anti-α_v antibodies. Neurite outgrowth on all three substrates was completely inhibited by anti-β_1 antibodies and Fab' fragments were not tested on heat-inactivated laminin. (B) On fibronectin 120, neurite outgrowth was reduced by the α_v and β_1 antibodies; however, when combined, neurite outgrowth was virtually blocked (to ~1%). Neurite outgrowth on fibronectin 120 was not inhibited in the presence of anti-β_8-Ex or additionally inhibited with coincubation of anti-β_1 antibodies. Neurite outgrowth on fibronectin 40 was partially inhibited by β_8-Ex affinity-purified, anti-β_8-Ex Fab' Ig, and anti-β_1; however, coincubation of anti-β_1 and β_8-Ex gave almost complete inhibition of neurite outgrowth. On the CS-1 peptide, the anti-β_8-Ex antibody strongly reduced neurite outgrowth and anti-β_1 antibodies completely inhibited outgrowth. In addition, anti-α_v antibodies did not inhibit neurite outgrowth CS-1 or fibronectin 40. Neurite outgrowth was partially inhibited on whole fibronectin by anti-β_8-Ex, anti-α_v antibodies, and to a greater extent with antibodies to β_1 integrin.

strongly inhibit sensory neurite outgrowth on collagen I, tenascin, fibronectin 120, and vitronectin (Figures 5 and 6; Varnum Finney *et al.*, 1995; Venstrom, unpublished observations). Anti-β_8-Ex does not inhibit neurite outgrowth on any of these substrates (Figures 5 and 6). Anti-β_8-Ex does inhibit sensory neurite outgrowth on laminin-1, collagen IV, and fibronectin (Figures 5 and 6). No integrin containing the β_2-β_7 subunits has been described that functions as a receptor for these three proteins. Thus both the biochemical and functional studies in the present paper provide strong evidence that anti-β_8-Ex recognizes only one integrin β subunit. Controls for possible nonspecific and toxic effects also show that anti-β_8-Ex is specific in both biochemical and functional studies.

Additional experiments are needed to more precisely define the domains on ECM proteins that are recognized by β_8 integrins. We observed that β_8 integrins recognize a site in laminin-1 that is resistant to heat denaturation. In previous work, cell attachment sites in E8, derived from the foot of laminin's cruciform structure, have been shown to be heat labile, whereas sites in E1X, a fragment derived from the center of laminin's cruciform structure, were shown to be heat resistant (Goodman *et al.*, 1987). This suggests that β_8 integrins recognize a site in E1X, but this needs to be confirmed in studies using laminin fragments. The carboxyl terminal region of fibronectin is known to contain at least three sites recognized by $\alpha_4\beta_1$, one of which is the CS-1 sequence (Haugen *et al.*, 1992a,b). Which of these function as major attachment sites for binding by β_8 integrins is not yet certain. We observed comparatively weak neurite outgrowth on the CS-1 peptide compared with fibronectin 40, suggesting that other sites may be more important. Finally, the NC1 domain of collagen IV has been shown to support outgrowth of sympathetic neurons (Lein *et al.*, 1991). It will be interesting to see if this fragment of collagen IV also contains a β_8 integrin binding site.

The major α subunit(s) associating with the β_8 subunit in sensory neurons remain unidentified. In some cells, β_8 has been shown to associate with α_v (Moyle *et al.*, 1991). In sensory neurons, however, our analysis of immunoprecipitates using β_8 or α_v-specific antibodies provides strong evidence that they are not primarily associated with each other. Functional assays support the same conclusion. In contrast to anti-β_8, anti-α_v did not inhibit sensory neurite outgrowth on intact laminin-1, collagen IV, or fibronectin 40, but was inhibitory on fibronectin 120. The only substrate shared between heterodimers containing these two subunits was heat-treated laminin. Thus, both biochemical and functional assays argue that β_8 is not associated with α_v in sensory neurons. In sensory neurons, a β_8-associated M_r 120 k band was observed in nonreducing SDS-PAGE that appeared to have an M_r of 110 k when

fractionated in reducing conditions. Of known integrin α subunits, this M_r is most similar to that of α_7 (Kramer *et al.*, 1991; Song *et al.*, 1992); however, α_7 has only been detected in association with β_1 so far. Consequently, the identity of this β_8-associated M_r 120-k band is not certain, but it may well represent a novel integrin α subunit(s).

The embryonic DRG contains several distinct classes of sensory neurons, all of which can be maintained in culture in the presence of appropriate trophic factors. Because our cultures included NGF, our assays monitored the behavior of the approximately 70% of the neurons that are NGF responsive. These mediate nociceptive sensation and express the peptide transmitters CGRP and substance P (reviewed in Scott, 1992). Because our assays were for short times, however, other subclasses of sensory neurons may have been present also. Studies using subtype-specific markers would be needed to determine this definitively. Our results show conclusively, however, that the NGF-supported population of DRG neurons present in these cultures expresses β_8 integrins (Figures 2 and 3). In previous work, this same population of sensory neurons has also been shown to express at least two receptors for laminin-1 ($\alpha_1\beta_1$ and $\alpha_3\beta_1$), one receptor for collagen IV ($\alpha_1\beta_1$), and three receptors for fibronectin ($\alpha_5\beta_1$ and α_4 and α_v-integrins) (Lein *et al.*, 1991; Tomaselli *et al.*, 1993; Sheppard *et al.*, 1994). With the identification of a β_8 heterodimer as another receptor for each of these ECM proteins, it is clear that sensory neurons express many integrins able to interact with the same protein. The reason for such a high degree of functional redundancy is not clear. The cytoplasmic domain of β_8, however, has very low homology to the cytoplasmic domains of other β subunits (Moyle *et al.*, 1991), so binding of ligands to β_8 integrins may induce cellular responses that are unique. Studies on the β_1, β_3, and β_5 integrins have shown that different cytoplasmic tails induce distinct patterns of integrin distribution on the cell surface, regulate the efficiency with which integrins are recruited into focal adhesions, and determine the effectiveness with which ligand binding promotes cell migration (Wayner *et al.*, 1991; Akiyama *et al.*, 1994; Pasqualini and Hemler, 1994).

In conclusion, the present paper demonstrates that β_8 integrins are expressed in the embryonic nervous system and identifies at least some of their ligands. In future work it will be important to characterize this receptor family, its expression pattern, and its ligands more completely. It will also be important to determine its functions in vivo.

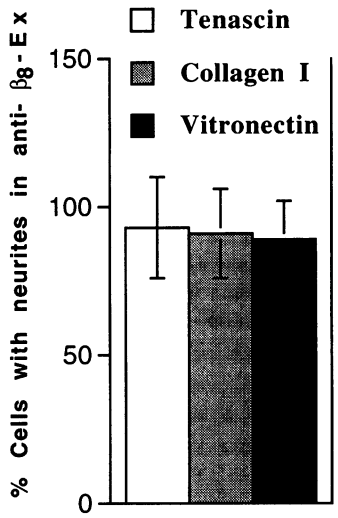

Figure 7. β_8 integrins did not appear to mediate neurite outgrowth on tenascin, collagen I, or vitronectin. Neurons were cultured for 4–7 h in the presence of anti-β_8-Ex Ig (300 mg/ml, or affinity purified 25 mg/ml). The number of experiments was greater than four. Error bars represent the SD from the normalized mean values.

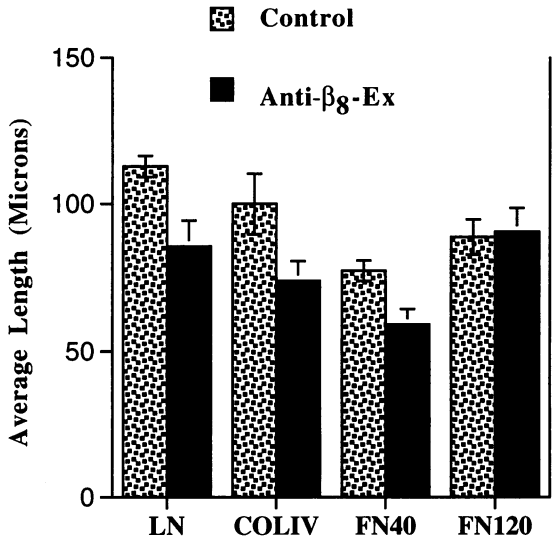

Figure 6. Effect of anti-β_8-Ex on neurite lengths of embryonic day 8 DRG neurons cultured approximately 4–7 h. Compared with no antibody controls, anti-β_8-Ex affinity-purified Ig (25 μg/ml) reduced mean neurite length on laminin-1 by ~24%, on collagen IV by ~26%, and on fibronectin 40 by ~24%. The t-test was calculated on the mean lengths and SD of the means between control and anti-β_8-Ex wells. This result was statistically significant ($p < 0.002$) for laminin-1, collagen IV, and fibronectin 40, but not for the control substrate, fibronectin 120. Consistent with the expression of anti-β_8-Ex on 90% of the cultured DRG neurons (Figure 2), anti-β_8-Ex appeared to reduce the lengths of all classes of neurites at this developmental age. Each mean is based on the measurement of 20 neurites from duplicate wells from two to three representative experiments. The error bars represent the SE of the means. Neurites were measured with lengths greater than two cell bodies.

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