Bravo/Nr-CAM Is Closely Related to the Cell Adhesion Molecules L1 and Ng-CAM and Has a Similar Heterodimer Structure

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Abstract. Diverse cell-surface molecules of the nervous system play an important role in specifying cell interactions during development. Using a method designed to generate mAbs against neural surface molecules of defined molecular weight, we have previously reported on the surface protein, Bravo, found in the developing avian retinotectal system. Bravo is immunologically detected on developing optic fibers in the retina, but absent from distal regions of the same fibers in the tectum.

We have isolated cDNA clones encompassing the entire coding region of Bravo, including clones containing five alternative sequences of cDNA. These putative alternatively spliced sequences encode stretches of polypeptide ranging in length from 10–93 amino acids and are predicted to be both extra- and intracellular. The deduced primary structure of Bravo reveals that, like the cell adhesion molecules (CAMs) chicken Ng-CAM and mouse L1, Bravo is composed of six Ig-like domains, five fibronectin type III repeats, a transmembrane domain, and a short cytoplasmic region. Recently, the cDNA sequence of a related molecule, Nr-CAM, was reported and its possible identity with Bravo discussed (Grumet, M., V. Mauro, M. P. Burgoon, G. E. Edelman, and B. A. Cunningham. 1991. *J. Cell Biol.* 113:1399–1412). Here we confirm this identity and moreover show that Bravo is found on Müller glial processes and end-feet in the developing retina. In contrast to the single polypeptide chain structure of Nr-CAM reported previously, we show that Bravo has a heterodimer structure composed of an α chain of M_r 140/130 and a β chain of 60–80 kD. As with L1 and Ng-CAM, the two chains of Bravo are generated from an intact polypeptide by cleavage at identical locations and conserved sites within all three molecules (Ser-Arg/Lys-Arg). The similar domain composition and heterodimer structure, as well as the 40% amino acid sequence identity of these molecules, defines them as an evolutionarily related subgroup of CAMs. The relationship of Bravo to molecules known to be involved in cell adhesion and process outgrowth, combined with its pattern of expression and numerous potential isoforms, suggests a complex role for this molecule in cell interactions during neural development.

THE development of a functioning nervous system involves the creation of a complex network of cells and cell processes. In vertebrates, this often requires axons born in one area to project and form synapses in distant areas of the nervous system. The visual system of lower vertebrates is particularly well-suited to studies of patterned neuronal projections (Crossland et al., 1975). In general, fibers from retinal ganglion cells grow towards the optic fissure, exiting the retina as the optic nerve which crosses the chiasm and innervates the optic tectum. A topographic map of the retina is formed on the tectum with neighboring retinal cells projecting to neighboring tectal cells.

The retinotectal system lends itself well to molecular analysis because cells and cell processes in this system interact with each other and with components of the extracellular matrix (ECM)[1] via a wide variety of known molecules. An increasing body of primary structure data has revealed that, in general, these cell-surface and ECM molecules are "mosaic" proteins (Doolittle, 1985) assembled—presumably by exon shuffling—from a number of different structural units or domains (see reviews in Bork, 1991). The cadherins, for example, are integral membrane glycoproteins composed of four repeated external domains that promote homophilic cell adhesion in a Ca^{2+}-dependent manner (Takeichi, 1991; In-

1. *Abbreviations used in this paper*: ECM, extracellular matrix; FNIII, fibronectin type III homology repeat; CAM, cell adhesion molecule; N-CAM, neural-CAM: Ng-CAM, neuron-glia CAM; Nr-CAM, Ng-CAM related CAM; OFL, optic fiber layer; PCR, polymerase chain reaction; PFA, paraformaldehyde; RT, room temperature.

uzaka et al., 1991). The diverse molecules of the extracellular matrix such as collagen, laminin, fibrinogen, fibronectin, thrombospondin, vitronectin, and von Willebrand factor (reviewed in Reichardt and Tomaselli, 1991) also demonstrate a mosaic nature—being composed of regions with extensive internal homology repeats and homologies with other known proteins (Engel, 1991).

Another important group of cell-surface molecules found in the developing nervous system includes several neural cell-surface members of the immunoglobulin superfamily collectively known as Ig-CAMs (Williams and Barclay, 1988; Salzer and Colman, 1989). The neural cell adhesion molecule (N-CAM) is the most abundant and well-studied of the Ig-CAMs, promoting homophilic cell–cell adhesion via a Ca^{2+}-independent mechanism. N-CAM has been proposed to play a major role in a variety of developmental events including axon–axon adhesion, neuroblast migration, stabilization of synaptic junctions, and varied roles in other tissues including muscle, kidney, heart, and feathers (Edelman, 1986; Crossin et al., 1985; Chuong et al., 1985). N-CAM is composed of five immunoglobulin-like domains and two fibronectin type III homology repeats (FNIIIs).

Molecules with multiple Ig-domains and FNIIIs define an ancient family (older than the divergence of arthropods and chordates) of Ig-CAMs (Edelman, 1987; Jessell, 1989). Many of these molecules have been shown to be involved in cell–cell adhesion and axon fasciculation and in the promotion of neurite outgrowth and cell migration. In addition, the restricted pattern of expression of some of these molecules, TAG-1, fasciclin II, and neuroglian, suggest they might serve as cell or axonal guidance molecules (Dodd et al., 1988; Harrelson and Goodman, 1988; Bieber et al., 1989).

In an attempt to identify additional members of the Ig-CAM family, we generated mAbs specifically against cell-surface molecules of the same molecular weight as many of these family members, ~135 kD (Kayyem et al., 1992). Using this approach we identified not only all of the previously identified chicken Ig-CAMs, but also a number of other molecules including the novel neural cell-surface molecule Bravo (de la Rosa et al., 1990; Kayyem, J. F., J. M. Roman, E. J. de la Rosa, U. Schwarz, and W. J. Dreyer. 1990. *J. Neurosci. Abstracts*. 16:1010).

Bravo shows a striking pattern of topological restriction in the embryonic chicken retinotectal system (de la Rosa et al., 1990). Bravo is present on the retinal portion of optic fibers during the period of time when optic fibers are growing towards the tectum and seeking their targets, but it is not detectable on tectal regions of these same optic fibers. Preliminary amino acid sequence analysis suggested Bravo is a member of the immunoglobulin superfamily most closely related to mammalian L1 and chicken neuron-glia (Ng)-CAM (Moos et al., 1988; Rathjen et al., 1987; Burgoon et al., 1991).

Here we show results from protein analysis and molecular cloning of Bravo cDNAs which confirm this relationship to the immunoglobulin superfamily and reveal Bravo's identity with Ng-CAM related CAM (Nr-CAM) (Grumet et al., 1991). In contrast to results reported on Nr-CAM, we show that Bravo is a heterodimer composed of an α chain of M_r 130/140 noncovalently attached to a β chain of 60–80 kD. A similar heterodimer structure has been proposed for both L1 and Ng-CAM (Burgoon et al., 1991; Faissner et al., 1985). These α and β chains are derived from intact polypeptides by proteolytic cleavage at identical locations and conserved sites on each of these molecules. Furthermore, we have identified five putative alternatively spliced sequences.

The nature of the restricted pattern of Bravo expression as well as the sequence and structural relationship to L1 and Ng-CAM suggests Bravo is involved in fiber contacts and fasciculation during development. The possible controlled splicing of alternative cDNA sequences identified here and in Grumet et al. (1991) offers the intriguing possibility that differential processing of Bravo mRNA significantly increases the potential functions of this cell-surface molecule.

Materials and Methods

Immunohistochemistry

Eight anti-Bravo mAbs and one anti-Ng-CAM mAb used in these experiments were generated using a novel method for isolating, fractionating, and generating a vigorous murine immune response against tectal surface components of approximately M_r 135 kD (Kayyem et al., 1992). Embryonic day eight (E8) chick embryos were fixed with paraformaldehyde (PFA) by overnight immersion at room temperature (RT) in 4% PFA in 0.1 M sodium phosphate buffer (pH 7.0) followed by soaking in 30% sucrose for 4 h. Retinotectal tissue was sliced horizontally into 12-μm sections using a freezing microtome, adhered to poly-lysine–coated slides, and allowed to air dry overnight at RT.

Sections were stained using a 1:1,500 dilution of mAb ascites fluid in PBS with 10% FCS (Gibco Laboratories, Grand Island, NY) or using undiluted culture supernatant fluid for 1 h at RT. Sections were washed three times in PBS for 5 min at RT, followed by a 1-h incubation with fluorescein-labeled goat anti-mouse Ig (Cappel Laboratories, Malvern, PA) diluted 1:200 in PBS. Sections were washed again as before and post-fixed by incubation with 4% PFA in 0.1 M sodium phosphate buffer, pH 7.0, for 2 min at RT. Finally, stained sections were mounted in glycerol and photographed with a Photomicroscope (Carl Zeiss, Oberkochen, Germany).

Purification of Antigen by Immunoaffinity Chromatography

Before its immobilization on a column, anti-Bravo mAb 2F12 and anti-Ng-CAM mAb 2B8 were purified from mouse ascites by chromatography on a CM Affi-Gel Blue column (Bio-Rad Laboratories, Cambridge, MA), according to the manufacturer's instructions. Purified mAb (10 mg) was bound to 3 ml of Affi-Gel 10 (Bio-Rad Laboratories) by overnight incubation with gentle agitation in PBS at 4°C as recommended by the manufacturer. A column of each solid support was pre-run with the various buffers to be used for antigen purification (see below).

Bravo and Ng-CAM proteins were purified from post-hatching day 1 (P1) chicken brains which were first washed three times for 5 min in 140 mM NaCl, 5 mM KCl, 5 mM glucose, 7 mM $NaHCO_3$, 1.5 mM $MgSO_4$, and 1.5 mM $CaCl_2$ with iodoacetamide (2 mg/ml), PMSF (0.2 mg/ml) and soybean trypsin inhibitor (50 μg/ml) added as protease inhibitors. Brains were lysed by shaking tissue for 15 min at RT in lysis buffer (3 ml per brain): 10 mM Hepes, pH 7.5, 140 mM NaCl, 4 mM EDTA, 2.5% NP-40 (Sigma Chemical Co., St. Louis, MO), 2.5% zwittergent 3-14 (Calbiochem-Behring Corp., San Diego, CA), supplemented with 0.02% azide and 0.5 μl/ml aprotinin in addition to the same protease inhibitors used for washing (Updyke and Nicolson, 1984). After centrifugation (50,000 *g*, 30 min, 4°C) the supernate from 200 brains was gravity loaded onto the affinity column at a flow rate of ~30 ml/h. To remove nonspecifically and weakly bound material, the 3-ml column was washed as follows: (*a*) 50 ml PBS containing 0.5% NP-40; (*b*) 20 ml of 20 mM Tris-HCL, pH 8.0, containing 140 mM NaCl, 0.5% NP-40 and 0.5% Zwittergent 3-14; (*c*) 10 ml of 50 mM Tris-HCl, pH 8.0, containing 0.5 M NaCl and 0.5% NP40; and (*d*) 10 ml of 50 mM Tris-HCl, pH 9.0, containing 0.5 M NaCl and 0.1% NP-40. The antigen was eluted with 12 ml of 50 mM triethanolamine, pH 11.5,, containing 150 mM NaCl and 0.1% NP-40. Fractions of 1.2 ml were collected in tubes containing 0.3 ml of 1 M Tris-HCl, pH 6.7. The fractions were analyzed using SDS-PAGE and peak fractions were concentrated in a centricon-30 (Ami-

con Corp., Danvers, CO) following the manufacturer's instructions. Finally, the column was regenerated by washing with 20 ml of 20 mM Tris-HCl, pH 8.0, containing 140 mM NaCl, and 0.025% azide.

Protein Analysis

Purified proteins were separated on SDS-PAGE (6 or 7.5% acrylamide) using standard methods (Laemmli, 1970) and silver stained using the method of Ansorge (1985). DTT (75 mM) or iodoacetamide (100 mM) was added to the protein samples before analyses by SDS-PAGE to produce reducing or nonreducing conditions, respectively. Molecular weights were determined by comparison with molecular weight standard from Bio-Rad Laboratories: 200 kD, myosin; 116 kD, β-galactosidase; 93 kD, phosphorylase B; 66 kD, BSA; and 45 kD, ovalbumin.

For Western blots, proteins were transferred to Hybond-C (Amersham Corp.) using essentially the method of Towbin et al., 1979. After blocking sites on the membrane overnight with 5% BSA in PBS, the membranes were labeled with mAb ascites fluid at a 1/1,000 dilution for 1 h, or with a 1/20 dilution of the anti-HNK-1 mAb "Anti-Leu 7" from Beckton-Dickinson & Co., Mountain View, CA. Reactive proteins were subsequently visualized using standard biotin/avidin sandwich methods with biotinylated goat anti-mouse immunoglobulin (Amersham Corp.; 1:200) and HRP-conjugated streptavidin (Amersham Corp.; 1:200) visualized with 4-chloronapthol (0.5 mg/ml) and H_2O_2 (0.015%) in PBS.

Amino-terminal and internal protein sequence information was gathered using a protein sequenator (model 477A; Applied Biosystems, Foster City, CA) with on-line PTH analysis. Antigens (30–200 pmole) were purified by affinity chromatography as described above, and subsequently electroblotted onto polyvinylidene difluoride (PVDF) membrane following SDS-PAGE (Matsudaira, 1987). Alternatively, peptides were generated from the affinity purified proteins by treatment with V8 protease (Sigma Chemical Co., St. Louis, MO) overnight at 37°C at a protein/enzyme ratio of 50:1 (wt/wt), before separation on a C18 (Pharmacia Fine Chemicals, Piscataway, NJ) HPLC column (Faissner et al., 1985). Peak fractions were collected while monitoring absorbance at 215 nm, and then loaded directly onto the protein sequenator.

DNA Cloning and Sequencing

Total RNA was prepared from E7 retina and from E17 cerebella by homogenization of tissue in cesium trifluoroacetic acid (Carter et al., 1983). Poly $(A)^+$ RNA was prepared using oligo (dT) spin columns from Pharmacia Fine Chemicals. cDNA libraries were constructed in λZapII (Stratagene, La Jolla, CA) from the poly (A)+ retinal RNA using the RNAse H method (Gubler and Hoffman, 1983) and random oligonucleotides (Promega Biotec, Madison, WI) as primers. A library was also prepared by Stratagene from the poly $(A)^+$ cerebellar RNA using random oligonucleotides as primers.

All three libraries were screened with a mixture of eight anti-Bravo mAbs: 2B3, 2F12, 3A10, 1C10, 3E11, 3G9, 1C4, and 1F3 (Young and Davis, 1983). The mAbs were pooled and diluted in PBS such that each mAb was represented at $1/1{,}000^{th}$ the concentration of its ascites fluid. Five putatively Bravo plaques were identified using HRP-conjugated anti-mouse Ig. Only one of these (from the Stratagene E17 cerebellar poly $[A]^+$ library) was reactive with Bravo mAbs after plaque purification to homogeneity (Maniatis et al., 1982).

This clone, Bravo-1, was subcloned into the plasmid pBluescript using an in vivo excision technique as per Stratagene's instructions. The cDNA was partially sequenced from pBluescript using dideoxy sequencing and Sequenase 2 (United States Biochemical, Cleveland, OH) (Sanger et al., 1977), and its identity with Bravo confirmed by comparison with amino acid sequence data generated from a Bravo peptide. After EcoRI excision of the Bravo-1 insert and subsequent subcloning into Pmob vector (kindly supplied by Dr. Michael Strathmann, University of California, Berkeley, CA), the remainder of Bravo-1 was sequenced using a transposon based strategy (Strathmann et al., 1991).

To identify remaining Bravo cDNA sequences, Bravo-1 insert was labeled with ^{32}P-dCTP by random oligonucleotide priming (Feinberg and Vogelstein, 1984) and used to screen the cerebellar cDNA library. Oligonucleotide primers (Caltech Microchemical Facility, Pasadena, CA) matching either the 5′ or the 3′ end of the Bravo-1 insert were used in conjunction with vector primers (M13 universal and reverse primers) in PCR reactions to determine which of the positive clones contained the most new 5′ and 3′ sequences, respectively. N1 and C1 were derived in this way (see Fig. 4 *a*).

For sequencing, N1 and C1 were purified to homogeneity and subcloned into pBluescript as before. Synthetic oligonucleotide primers were used in both the sense and anti-sense directions to determine the new sequences (with some overlap with Bravo-1) of these clones. To identify sequences encoding the COOH terminus of Bravo, the 3′ end of C1 was amplified and labeled by PCR. Using primers spaced 300-bp apart, a band was PCR amplified from C1 DNA and gel purified using standard protocols. The purified fragment was then labeled using the "single-stranded PCR" technique by amplification through 35 linear cycles with the sense PCR primer incorporating ^{32}P-dCTP as the only source of cytosine. This single-stranded probe was used to screen the cerebellar cDNA library, and positive clones were mapped by PCR. Two clones, C2 and C3, encoding alternative COOH termini of the protein were identified in this way, and sequenced as described above.

cDNA sequences were compiled using the GCG system software package (Devereux et al., 1984). Translated sequences from Bravo and from the GenBank database were compared pairwise using the FASTA algorithm (Pearson and Lipman, 1988) to determine percent sequence identities.

Results

mAbs raised against E8 tectal surface proteins of ~135 kD stained retinotectal tissue sections in a variety of patterns. At least nineteen patterns of reactivity have been identified from mAbs of this fusion, including a group of mAbs we originally called Alpha (using the international phonetic alphabet). The Alpha staining pattern is identical to that of mAbs against the neural cell-surface molecule Ng-CAM, which is also known as 8D9 and G4 (Grumet and Edelman, 1984; Lemmon and McLoon, 1986; Rathjen et al., 1987). Immunocross-reactivity studies on purified antigen, and NH_2-terminal protein sequencing revealed that the Alpha antigen is identical to Ng-CAM (data not shown).

Another group of mAbs we call Bravo were found to stain tissue in a pattern similar to but distinct from Ng-CAM. In contrast to the large number (>100) of Alpha mAbs that were generated in this fusion, only eight Bravo mAbs were detected, perhaps reflecting the relative abundance or antigenicity of these two molecules. Three anti-Bravo mAbs (2B3, 2F12, and 3E11) were used to characterize the Bravo protein and its distribution.

Distribution of Antigen

Anti-Bravo mAbs stain all fiber layers of the retina as soon as they are morphologically identifiable, whereas, Ng-CAM staining in the retina is primarily confined to the ganglion cell fibers of the optic fiber layer (OFL) (Rathjen et al., 1987). We have previously shown that Bravo (and not Ng-CAM) labeling of optic fibers is restricted along the axon length to the proximal region of the retinal axons and absent or dramatically reduced from distal regions in the tectum (de la Rosa et al., 1990). Outside the retinotectal system, both Bravo and Ng-CAM appear simultaneously on fibers emerging from the early neural tube, and in the developing cerebellum, both Ng-CAM and Bravo are found primarily on fibers in the external granule cell layer (data not shown).

In contrast to results reported previously on Nr-CAM (Grumet et al., 1991), we have found Bravo, but not Ng-CAM, expressed on glial processes in the retina. In the OFL, Müller glial processes and end-feet are lined up in parallel rows with bundles of retinal axons running between them toward the optic fissure. In most immunohistologically stained sections, dense labeling of the optic fiber fascicles would obscure any staining of the glia and their end-feet in the OFL. However, certain sections situated along rows of glial

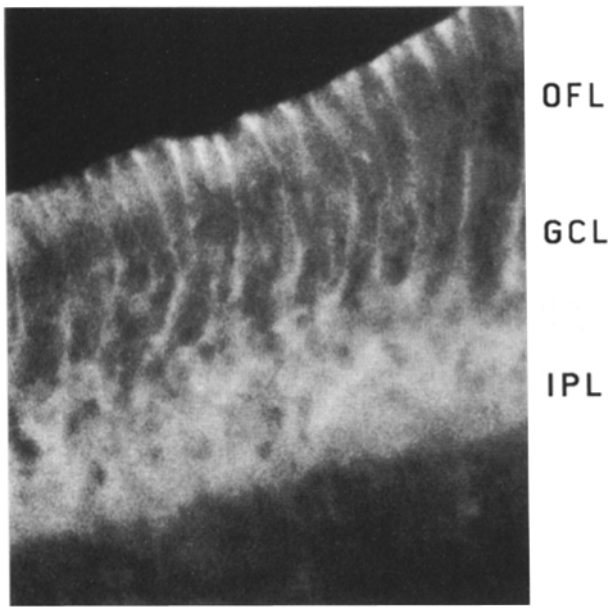

Figure 1. Immunofluorescence staining of chicken retina showing labeling of Müller glial end-feet. A region of a cryostat section of an E7.5 retina was found relatively free of optic fiber fascicles. Labeling of this region with anti-Bravo mAb 2B3, and FITC-conjugated anti-mouse Ig reveals intense staining of glial end-feet in the OFL, as well as staining of processes in the IPL and ganglion cell layer (GCL).

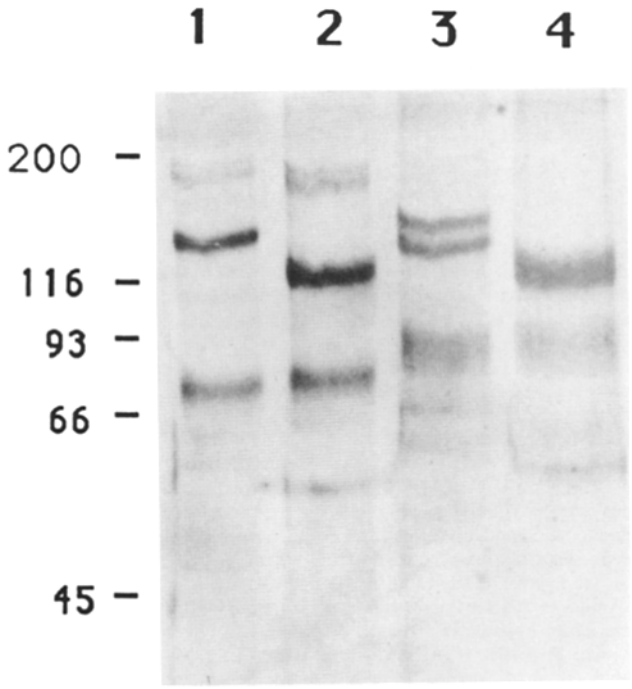

Figure 2. Ng-CAM and Bravo proteins resolved by SDS-PAGE. Immunoaffinity isolates using anti-NgCAM mAbs and anti-Bravo mAbs were separated by 7.5% SDS-PAGE and visualized by silver staining. Ng-CAM protein was treated to reduce disulphide bonds (lane *1*) or treated to protect these bonds (lane *2*); similarly, immunopurified Bravo protein is shown reduced (lane *3*) and unreduced (lane *4*). Note that the Ng-CAM 135-kD band and the Bravo 140/130-kD doublet appear to contain internal disulphide bonds that cause lower mobility under reducing conditions. A similar effect is seen with the less abundant Ng-CAM 190-kD intact polypeptide, but not with the lower molecular weight Ng-CAM or Bravo 60–80-kD bands.

processes show a clear pattern of Bravo staining indicative of the labeling of Müller glial processes and end-feet (Fig. 1). Also, the possible labeling of ventricular cell processes and end-feet in the tectum has been implicated in the Bravo staining of tectal structures in the vicinity of the *Stratum opticum* (de la Rosa et al., 1990).

Bravo Protein Characterization

Bravo mAbs were used to immunopurify chicken antigens from detergent lysates of E8 and E12 tecta, E17 cerebella and post-embryonic day 1 (P1) whole brain using anti-Bravo solid supports. The column eluates were analyzed by SDS-PAGE and in each case showed a prominent doublet at 14/130 kD, as well as a diffusely migrating set of bands centered around 60 and 80 kD (Fig. 2). Thus, no obvious differences exist in the pattern of the SDS gel bands of Bravo isolated from these different tissues.

This pattern of bands on SDS-PAGE is nearly identical to the patterns found with mouse L1 (Sadoul et al., 1988) and with chicken Ng-CAM (Rathjen et al., 1987)—shown here using Alpha mAb 1E12 (Fig. 2, lane *1*). The major components purified by the anti-L1 mAb are a 140-kD band and a broad band of ~80 kD. Ng-CAM mAbs immunopurify a 135-kD band and diffuse bands around 80 and 65 kD. The 140/130-kD doublet purified by Bravo mAbs (Bravo-140/130) and the higher molecular weight bands of Ng-CAM have faster mobilities under nonreducing conditions than under reducing conditions (Fig. 2), implying that these molecules are stabilized by internal disulphide bridges. This apparent molecular weight shift has been described for L1 and NILE, the rat L1 equivalent, and suggests that intrachain disulphide bonds, typical of those characteristic of immunoglobulin domains, are involved in stabilizing the Bravo protein (Prince et al., 1989).

Both Ng-CAM and L1 are found predominantly as heterodimers, an α polypeptide chain of ~140 kD noncovalently attached to a smaller β chain of ~80 kD (Fig. 2) (Sadoul et al., 1988; Burgoon et al., 1991). The pattern of Bravo bands in Fig. 2 suggests a similar dimer structure. Anti-Bravo mAbs immunopurify both the 140/130 kD and the 60–80-kD Bravo polypeptides, but only the higher molecular weight bands react with Bravo mAbs on immunoblots, arguing strongly for the heterodimer structure of Bravo (Fig. 3, *a* and *b*). This suggests that the immunopurification of the 60–80-kD polypeptides is the result of their strong association with the 130/140-kD polypeptides, as is the case for L1 and Ng-CAM. In the case of L1, the 140- and 80-kD components of the major heterodimer structure represent products of proteolytic processing of an intact polypeptide chain with an apparent molecular weight of 200 kD. Similarly, Ng-CAM is synthesized as a 200-kD chain, and subsequently processed into the 135-kD α chain and the 80-kD β chain components. L1 (Sadoul et al., 1988) and Ng-CAM mAbs (Fig. 2) can be used to immunopurify these higher molecular weight intact molecules. In contrast, no higher molecular weight proteins were immunopurified using Bravo mAbs. Nonetheless, a Bravo-reactive band of M_r 190 kD was found by immuno-

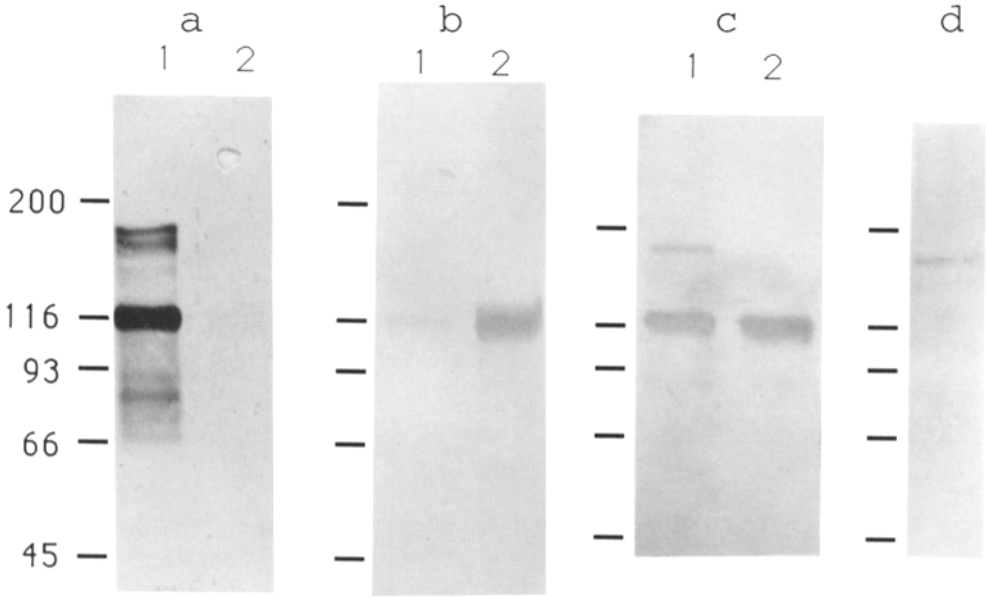

Figure 3. Comparison of immunoblots of Ng-CAM and Bravo. The immunoaffinity isolates (*a*, *b*, and *c*) using anti-NgCAM (lane *1*) and anti-Bravo (lane *2*) were resolved by 6% SDS-PAGE under nonreducing conditions, transferred to nitrocellulose, and analyzed by mAb staining with anti-NgCAM (*a*), anti-Bravo (*b*), and anti-HNK-1 (*c*). Proteins solubilized from the tectal lysate pellet (see Materials and Methods) were resolved by 6% SDS-PAGE, transferred to modified nitrocellulose membrane, and subjected to mAb analysis with anti-Bravo (*d*). Binding of all antibodies was visualized using biotinylated second antibodies bound to HRP-conjugated streptavidin. The positions of molecular weight standards (reduced) are shown to the left of each panel.

blotting components of the tectum insoluble in our lysis buffer (Fig. 3 *d*). Presumably, this Bravo precursor is more thoroughly or rapidly processed into the α and β chains than is the case for L1 and Ng-CAM.

Immunoblotting of purified Bravo protein also reveals that this molecule carries the HNK-1 carbohydrate epitope found on several other neural molecules as well. The HNK-1 epitope is associated with cell-adhesion molecules of the vertebrate nervous system, and may itself play a role in adhesion (Kunemund et al., 1988; Kadmon et al., 1990). L1, Ng-CAM, myelin-associated glycoprotein (MAG), and N-CAM among others, are known to carry this epitope (Cole and Schachner, 1987; Kruse et al., 1984). As shown in Fig. 3 *c*, it is the 130–140-kD components of both Bravo and Ng-CAM – not the 60–80 kD ones – that carry this epitope, analogous to the situation with L1 (Faissner et al., 1985).

Isolation of cDNA Clones

The initial identification and isolation of a Bravo cDNA clone was achieved by screening an unamplified λZapII cDNA library prepared using mRNA from E17 chicken cerebella. Eight anti-Bravo mAbs were pooled and used to select Bravo-1 which was subsequently plaque purified and found to react with four of the eight mAbs.

After excision of the insert and DNA sequencing, Bravo-1 was found to encode an open reading frame with neither a start methionine nor a stop codon. Therefore, we rescreened the cerebellar library with the Bravo-1 insert and isolated several clones. These clones were mapped by PCR using specific Bravo-1 primers in conjunction with vector primers to determine which clones had inserts containing the greatest amount of new sequence data. N1 and C1 were derived in this way and contained sequences more NH_2- and COOH-terminal to Bravo-1, respectively. Another rescreen with C1 produced C2 and C3 which contained alternative 3′ coding regions (Fig. 4 *a*).

The five overlapping Bravo clones (Bravo-1, N1, C1, C2, and C3) were sequenced, and the amino acid sequences of the corresponding polypeptides were deduced. The deduced amino acid sequence was then compared to the protein sequence data generated by analyses of immunopurified Bravo polypeptides. Microsequencing of the first nineteen residues of each component of the purified Bravo 140/130 doublet yielded identical sequences for the Bravo α chains. Peptide sequencing of two proteolytic fragments generated by V8 protease treatment of purified Bravo α chains yielded 35 residues of unambiguous amino acid sequence data, and NH_2-terminal sequencing of the 60–80-kD Bravo β chain yielded 35 residues of amino acid sequence information. The sequences of these components were found in the translated sequence of Bravo cDNAs, with two amino acid residue exceptions, which we conclude represent amino acid sequencing errors. These putative sequencing errors excepted, the remaining protein data matched the derived sequence exactly at 87 out of 87 sites (Fig. 5). We conclude that the cDNAs we have identified encode Bravo.

cDNA Sequence Analysis and Comparison to L1 and Ng-CAM

As reported earlier (de la Rosa et al., 1990), Bravo is a member of the immunoglobulin superfamily with close relationship to L1 and Ng-CAM. Another molecule similar to Ng-CAM was recently characterized and given the name Nr-CAM (Grumet et al., 1991). The cDNA sequence of Nr-CAM contains many regions identical in sequence to Bravo, though several novel regions of alternatively spliced sequences exist as well. The cDNA sequence determined by Grumet et al. (1991) can be found in the EMBL/GenBank/

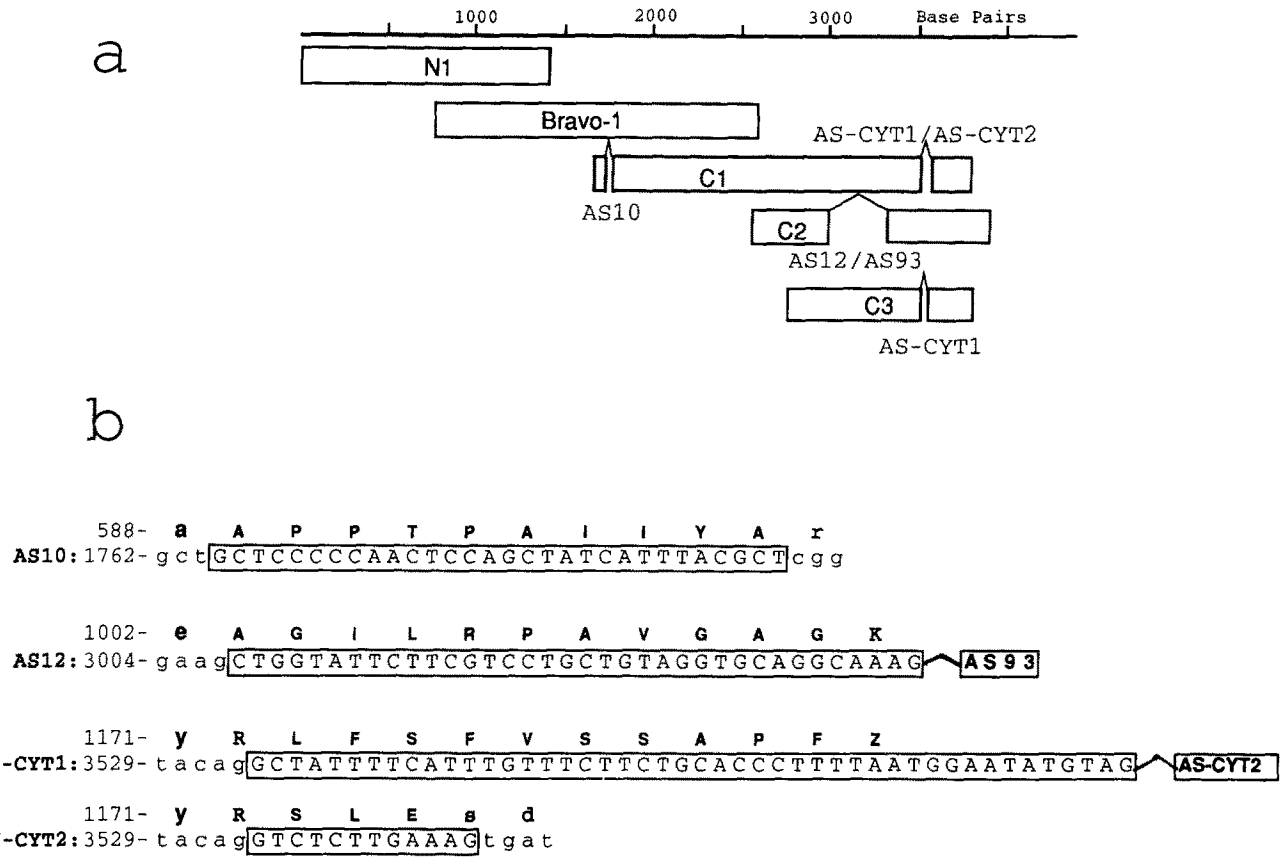

Figure 4. Schematic representation of Bravo cDNA clones and sequences of novel alternatively spliced regions. (*a*) The five cDNA clones used to determine the sequence of Bravo are shown. Sequences absent from one or more clones, suggesting alternative processing of messenger RNA, are indicated by angled lines, and labeled AS10, AS12, AS93, AS-CYT1, and AS-CYT2. Bravo-1 was sequenced in its entirety using a transposon based strategy for spreading primer sequences throughout the clone. The remaining clones were sequenced using synthetic oligonucleotide primers in both sense and anti-sense directions. (*b*) The cDNA (*boxed*) and derived protein sequences of novel putative alternatively spliced regions AS10, AS12, AS-CYT1, and AS-CYT2 are shown in upper case. Flanking Bravo sequences in lower case are shown for positioning. AS12 and AS-CYT1 were found associated with and and abutting alternative sequences AS93 and AS-CYT2, respectively. Note that these sequences, and AS-CYT2 as well, terminate with the bases AG and contain tracks of polypyrimidines which are associated with intron branch points and 3′ intron splice sites, suggesting that these sequences may represent incompletely processed mRNA.

DDBJ under accession number X58482. Novel sequences identified in this laboratory are shown in Fig. 4 *b*. Here, we concentrate on results not shown previously, such as the heterodimer nature of Bravo and the novel regions of putative alternative mRNA splicing.

A comparison of Bravo amino acid sequence to those in the translated Genbank sequence database using the FASTA rapid homology search showed that Bravo is most closely related to L1 (Moos et al., 1988) and to Ng-CAM (Burgoon et al., 1991). The extracellular portion of Bravo encodes six immunoglobulin-like domains and alternatively four or five FNIIIs. The complete protein sequence of Bravo/Nr-CAM is shown aligned with L1 and Ng-CAM in Fig. 5. Bravo cDNAs as well as those encoding Nr-CAM show regions of common sequence interrupted by stretches of sequence unique to one or more clone but absent from others, presumably resulting from alternative processing of mRNA. For example, between the second and third immunoglobulin domains lies a stretch of amino acids identified in one Nr-CAM clone and not found in any Bravo clones. This presumably alternatively spliced sequence encodes 20 amino acids and is given the name Alternative Sequence, encoding 20 amino acids (AS20) (Figs. 4, 5, and 6). In addition, following the sequence encoding the six Ig domains is a 30-bp stretch of novel sequence found in Bravo clone C1 and N1 but not in either Bravo-1 or C2. This sequence, highly hydrophobic in profile (Kyte and Doolittle, 1982) encodes ten amino acids (Ala-Pro-Pro-Thr-Pro-Ala-Ile-Ile-Tyr-Ala) and is denoted AS10. AS10 lies in a region analogous to the hinge region of the neural cell adhesion molecule N-CAM (Becker et al., 1989). This region has been implicated in cis-interactions of N-CAM with both itself and other membrane molecules such as L1. In addition, antibodies against this region disrupt optic fiber fascicles and cause misrouting of fibers in the retina (Pollerberg, G. E., and W. J. Dreyer, manuscript submitted for publication). After this alternatively spliced sequence are five FNIII repeats, similar to the repeats found in fibronectin but far more closely related to those of the neural CAMs. The fifth fibronectin repeat (AS93) (also identified as a putative alternatively spliced sequence by Grumet et al., 1991) is missing from Bravo clone C2, as is AS12, an additional stretch of novel sequence abutting the NH_2 terminus of AS93 (Figs. 4, 5, and 6).

The deduced Bravo sequence and comparison to that of L1

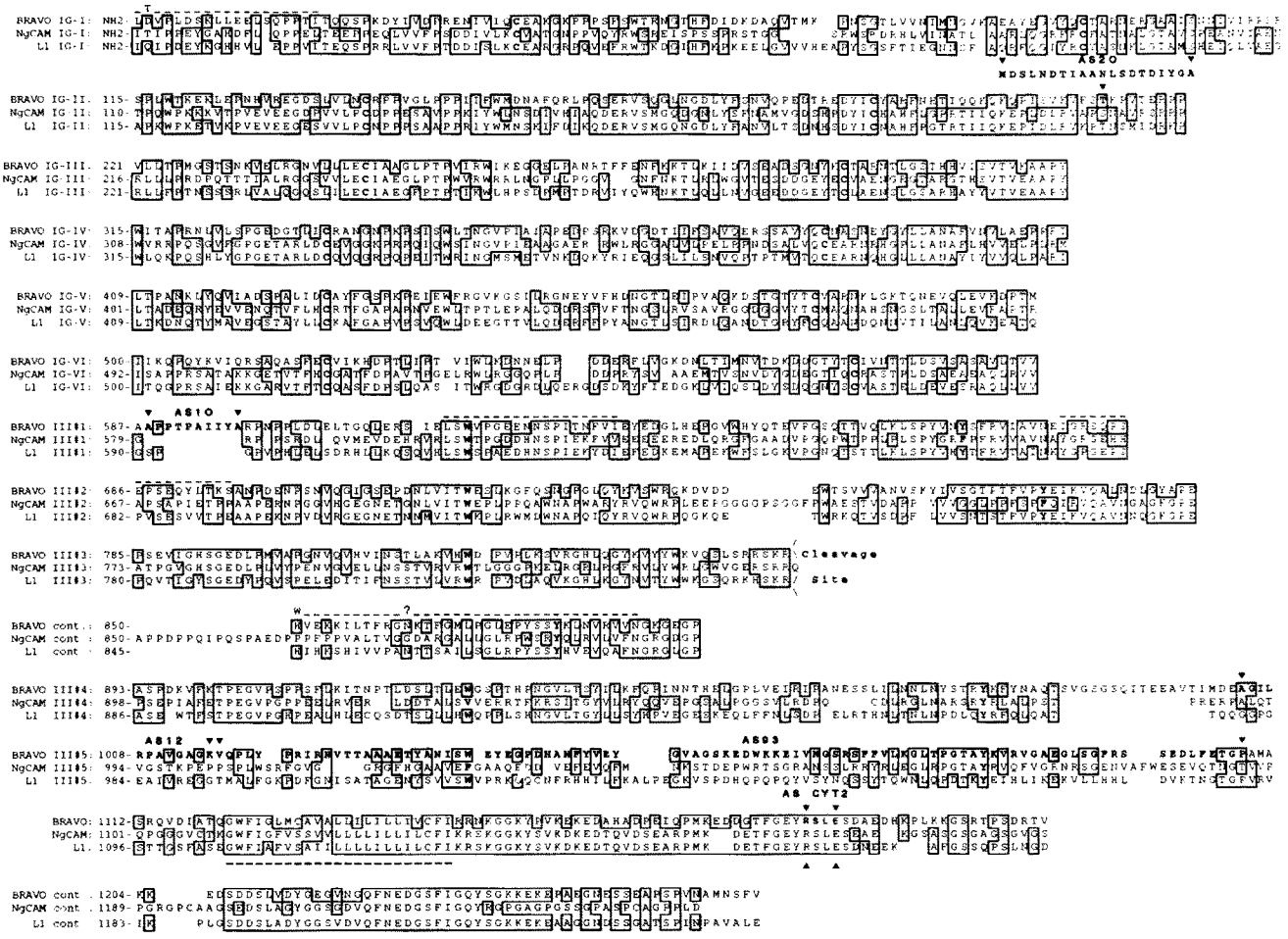

Figure 5. Alignment of the complete amino acid sequences of Bravo, Ng-CAM, and L1. The cDNA derived sequence of Bravo is identical to that of Nr-CAM except for the identification of the putative alternatively spliced sequences, AS20 uniquely in Nr-CAM (Grumet et al., 1991) and AS10, AS12, AS-CYT1, and AS-CYT2 uniquely here. AS93 was identified in both laboratories. The Bravo sequence (shown missing AS-CYT1 which encodes a stop codon and therefore results in a truncated Bravo protein) was aligned with L1 and Ng-CAM by eye and gaps introduced where appropriate to maximize identity. Identical residues are boxed. The six immunoglobulin-like domains in all three molecules are labeled Ig I-Ig VI with characteristic cysteines and tryptophans in bold type. The five FNIII repeats in all three molecules are labeled FNIII#1–FNIII#5 and characteristic tryptophans and tyrosines (or phenylalanines) are also in bold type. The location of the common proteolytic cleavage site is indicated. The NH_2 terminus of the Ng-CAM β chain is removed by one amino acid (Gln, single letter amino acid code Q) from this conserved site. Sequences confirmed by protein and peptide sequencing of Bravo are overlined with two misidentified amino acid residues indicated at positions 2 and 850. A nonstandard amino acid residue labeled "?" was identified at position 861, which is a potential asparagine-linked glycosylation site. The transmembrane domain in all three molecules is underlined with dashed lines. Putative alternatively spliced sequences of Bravo are labeled (e.g., **AS20**) in bold type and bordered by inverted triangles. The sequence found alternatively spliced in rat and human equivalents of L1, and identical to Bravo AS-CYT2, is bordered by triangles.

and Ng-CAM suggest a common mechanism for the generation of the α and β chains from an intact polypeptide. The third fibronectin repeat of Bravo contains the sequence previously identified by amino acid sequencing as the NH_2 terminus of the 60–80-kD β chain of Bravo. As expected from the similarity of Bravo to L1 and Ng-CAM, immediately NH_2-terminal to this site is a conserved series of amino acids culminating in the sequence Ser-Lys-Arg in both L1 and Bravo, and Ser-Arg-Arg in Ng-CAM, suggesting cleavage at this site in all three molecules by a trypsin-like protease. Thus, not only is the heterodimer structure of Bravo similar to that of L1 and Ng-CAM, but so is the mechanism that generates the α and β chains: proteolytic cleavage of an intact precursor molecule at the COOH-terminal end of the consensus sequence Ser-Arg/Lys-Arg. Bravo contains this putative consensus sequence tandemly repeated at this conserved cleavage site (i.e., Ser-Arg-Arg-Ser-Lys-Arg), which may explain the absence of detectable intact Bravo 190-kD precursor in normal tissue lysates.

The deduced Bravo sequence reveals numerous potential sites of N-linked glycosylation (Asn-X-Ser/Thr) spread throughout the extracellular portion of Bravo. These sites number 18 within the Bravo sequence proper, and two within the putative alternatively spliced region AS20. The deduced Bravo sequence also contains a transmembrane domain and a relatively short cytoplasmic domain much of which is highly conserved with Ng-CAM and L1 but which has no known structural motif. Because L1 is phosphorylated in vivo, and thought to co-purify with at least one kinase (Faissner et al., 1985; Sadoul et al., 1989; Schuch et al., 1989), the primary sequence of Bravo was analyzed for potential kinase substrates (Glass et al., 1986; Kishimoto et al., 1985;

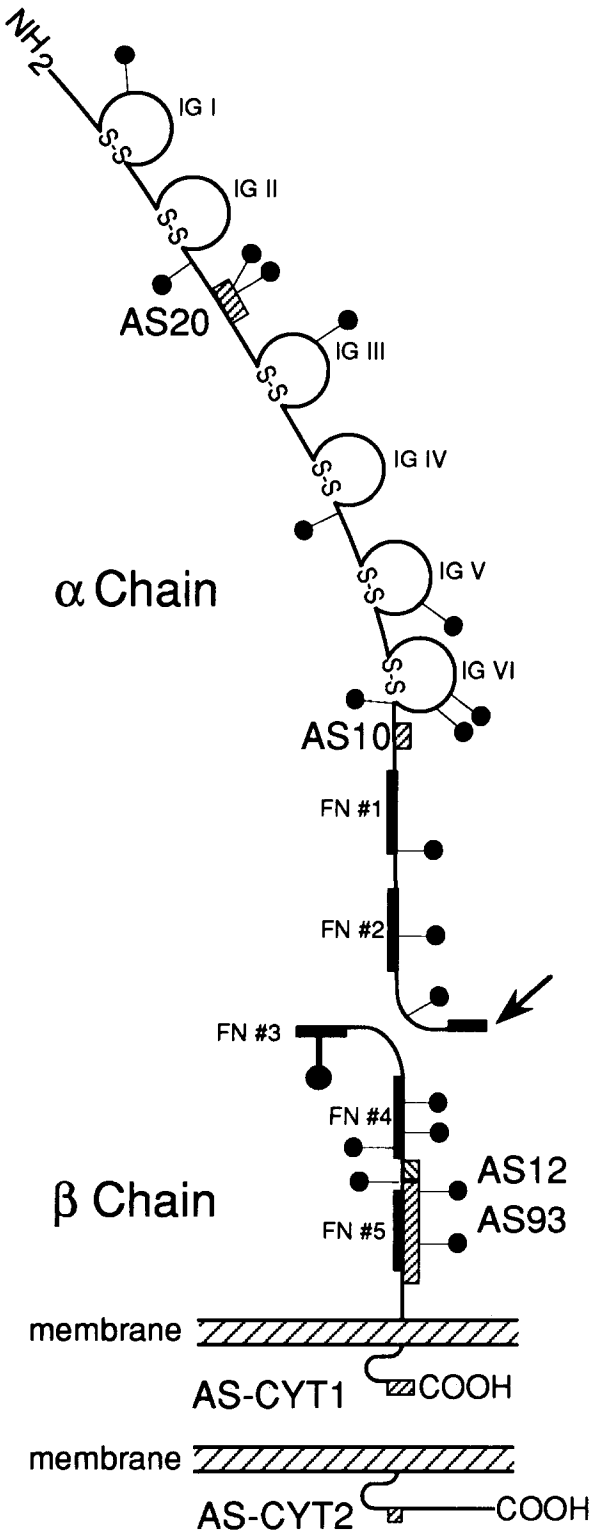

Figure 6. Topological model of Bravo including alternatively spliced sequences. The six Ig domains of Bravo are shown as loops linked by disulphide (*S–S*) bonds. The five FNIIIs are shown as darkened boxes. The six potential alternatively spliced regions are shown by hatched boxes labeled AS20, AS10, AS12, AS93, AS-CYT1, and AS-CYT2. Two alternative cytoplasmic domains are shown, one terminating within AS-CYT1 and resulting in a truncated Bravo isoform. Putative asparagine-linked glycosylation sites are indicated with circle-and-stick symbols. The oversized circle-and-stick symbol within FNIII#3 of the Bravo β chain denotes the location of a potential glycosylation site confirmed by amino acid sequence analysis to contain a nonstandard amino acid residue, presumably a glycosylated asparagine. The location of the cleavage site within FNIII#3 is indicated by an arrow. The domains involved in the noncovalent association of the α and β chains are not known.

Kuenzel et al., 1987). Eight potential phosphorylation sites are present within the cytoplasmic region where L1 has been shown to be phosphorylated. Three of Bravo's eight potential cytoplasmic phosphorylation sites may be conserved: two align with sites on L1 (Bravo serines 1,225 and 1,248) and an additional one matches with sites on both L1 and Ng-CAM (Bravo threonine 1,173).

The putative alternatively spliced cytoplasmic sequences, AS-CYT1 and AS-CYT2, contain no consensus sequence for known kinases. AS-CYT1 produces a shift of frame that results in a stop codon within the cytoplasmic region of Bravo, and AS-CYT2, 12-bp long, inserts the amino acid sequence Arg-Ser-Leu-Glu (RSLE, using the one letter amino acid code) into Bravo's cytoplasmic domain. This tetrapeptide sequence aligns with the identical sequence in the derived protein sequence of Ng-CAM and is found alternatively spliced in both rat and human L1 (Miura et al., 1991; Kobayashi et al., 1991).

Discussion

We have characterized the Bravo glycoprotein (de la Rosa et al., 1990) and found that it is related in structure and primary sequence to the cell-surface adhesion molecules Ng-CAM and L1. While the primary structure of Bravo has been described under the name Nr-CAM, the structure shown below differs from the structure previously predicted (Grumet et al., 1991), specifically with regard to the apparent molecular weight of the Bravo protein, its heterodimer nature and the potential diversity of alternatively spliced forms.

Bravo is composed of two different chains. The α chain is found as a doublet of 140/130 kD, containing the six Ig-like domains and two complete and one partial FNIII. The Bravo β chain contains two complete and one partial FNIII as well as the transmembrane spanning domain and COOH-terminal cytoplasmic sequences. These two chains are found associated by noncovalent interactions, and are generated from an intact precursor molecule by proteolytic cleavage following the sequence Ser-Lys-Arg with FNIII#3 (Fig. 6).

In addition to conserved structural features, the molecular weight of Bravo matches that of L1 and Ng-CAM. The apparent molecular weight as determined by SDS-PAGE of the α and β chains ranges from 190 kD (α chain of 130 kD plus β chain of 60 kD) to 220 kD (α chain of 140 kD plus β chain of 80 kD). These apparent molecular weight determinations are significantly higher than the predicted molecular weights of polypeptides encoded by the cDNA sequences. The predicted molecular weight for Bravo lacking any alternatively spliced sequences is 127 kD. The Bravo protein containing all four of the extracellular alternatively spliced sequences identified has a predicted molecular weight of 142 kD. The difference in these molecular weights from the apparent protein values is presumably due largely to glycosylation. Consistent with this proposal, Bravo carries the HNK-1 carbohydrate epitope, and its sequence contains twenty potential

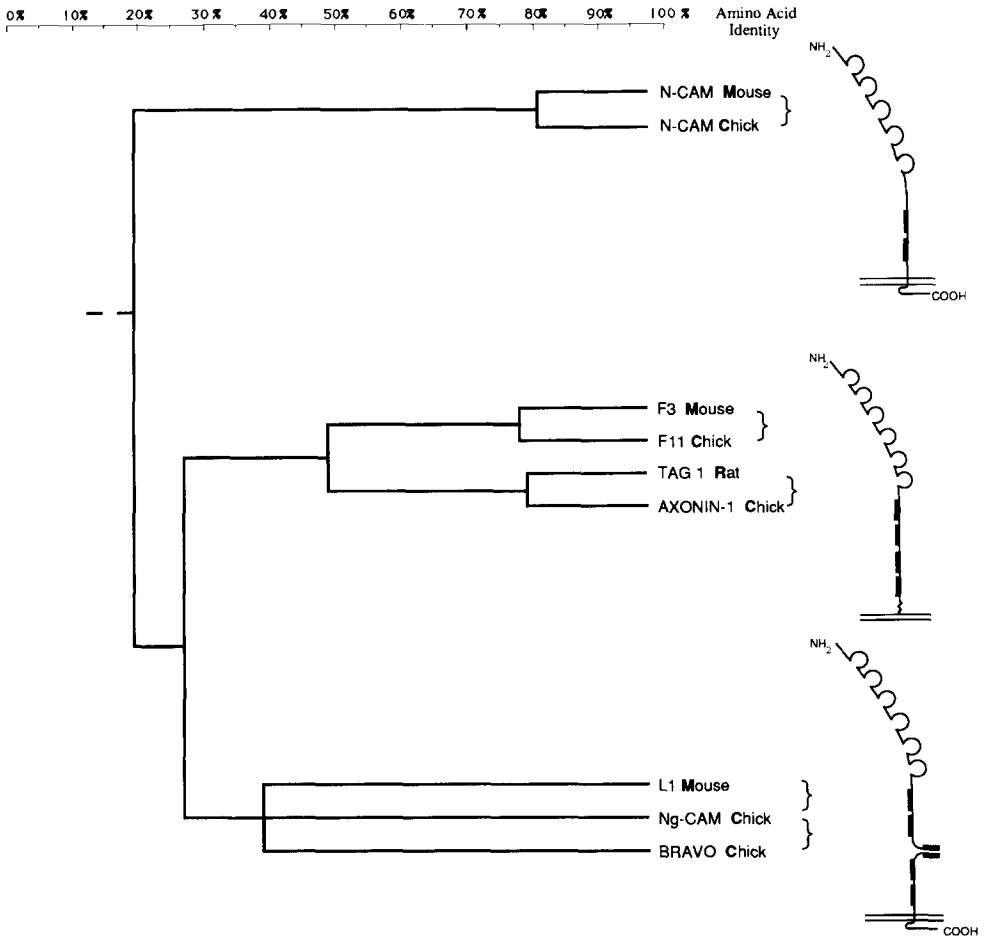

Figure 7. Gene tree representing subgroups of Ig-CAMs identified by amino acid sequence identity and domain structure. Protein sequences for each of the molecules were compared pairwise using the FASTA program of Pearson and Lipman (1988). A tree of relatedness was constructed with lines branching at positions corresponding to percent sequence identity (±1.5%). Models of the three characteristic subgroups are shown to the right. Hypotheses about the relationship among Bravo, Ng-CAM, and L1 (shown as a "tri-furcation" of the tree in this figure) are included in the text.

sites for N-linked glycosylation. The determination of the Nr-CAM molecular weight on SDS PAGE as only 145 kD (Grumet et al., 1991) presumably results from the absence of the 60–80-kD β chain in their protein analyses, and the subsequent failure to account for the substantial increase in apparent molecular weight of the polypeptide caused by glycosylation.

The cleavage of the intact chains of L1, Ng-CAM, and Bravo at identical locations (within FNIII#3) and at conserved sequences (Ser-Arg/Lys-Arg) may suggest these molecules are substrates for a common protease. Investigations of the processing of pro-albumin and parathyroid prohormone and several viral structural proteins (Rice and Strauss, 1981) imply the presence in the Golgi apparatus of proteases that cleave specifically after two basic amino acids, as is found with L1, Ng-CAM, and Bravo. Indeed, the cleavage sites of Bravo and Ng-CAM exactly match proteolytic cleavage sites found in alphavirus and flavivirus glycoproteins, respectively (Strauss et al., 1987). The cleavage at such a dibasic site within a FNIII may be a common mechanism to produce noncovalently associated heterodimers. The insulin receptor, a mosaic cell-surface protein that includes multiple FNIIIs, is found as a heterodimer generated from an intact polypeptide by cleavage following the sequence: Ser-Arg-Lys-Arg-Arg, remarkably similar to Bravo's Ser-Arg-Arg-Ser-Lys-Arg (Ebina et al., 1985). While the function of cleavage at this site and the possibly different roles of intact versus heterodimer structures in these molecules has not yet been elucidated, it appears in these cases that this dibasic site is used for processing of a pre-protein form.

The similar structures of Bravo, Ng-CAM, and L1, including the proteolytic cleavage site, creates a subgroup of neural Ig-superfamily members. Pairwise sequence comparisons support such a grouping, as these three molecules (and their equivalents) are more closely related to each other than to any as yet reported molecules. The results of a pairwise comparison of these and other vertebrate neural molecules are shown graphically represented as a "gene tree" (Fig. 7). Quantitative sequence identity comparisons (shown by the left to right position of "branch" points) supported by the arrangement of conserved domains among these molecules suggest the division of neural Ig-superfamily members into three subgroups. N-CAM represents the first subgroup. It is characterized by five Ig-like domains and two fibronectin type III repeats (Cunningham et al., 1987; Santoni et al., 1987). The next subgroup consists of two pairs of molecules. F3 and Contactin/F11 are thought to be equivalent molecules in mouse and chicken, respectively (Gennarini et al., 1989; Ranscht et al., 1988; Brümmendorf et al., 1989). Likewise, rat TAG-I and chicken axonin-1 are also thought to be equivalent, although the complete characterization of the axonin-1 cDNA has not yet been reported (Furley et al., 1990). The layout of these molecules is six Ig domains, four FNIIIs and a glycosylphosphatidylinositol linkage to the membrane. With each of these pairs, the equivalent molecules are roughly 80% identical to each other.

In contrast, the pairwise comparisons of L1, Ng-CAM, and Bravo do not reveal any equivalency among these molecules. The gene tree in Fig. 7 represents clearly that neither Ng-CAM nor Bravo is equivalent to L1 as the "tri-furcation" of these three molecules is displaced far to the left (lower percent identity) of the bifurcation of the other molecules. Mouse L1 is equally related at the level of amino acid sequence identity (40%) to both chicken Ng-CAM and Bravo. The application of molecular clocks to related molecules in setting up phylogenetic trees relies on the premise that equivalent molecules diverge at a relatively constant rate over a given length of time in a particular group of organisms (Dayhoff et al., 1969). The roughly 20% divergence of N-CAM, TAG-1, and F3 from their chicken equivalent over the same amount of time conforms to this premise. The much larger disparity (60%) of L1 and Ng-CAM and of L1 and Bravo suggests that neither Ng-CAM nor Bravo is the L1 equivalent.

The close relationship of Bravo, Ng-CAM, and L1, especially at the level of higher order protein structure, suggests their roles may be similar. L1 and Ng-CAM have both been implicated in neuron–neuron adhesion, axon fasciculation and neurite outgrowth (Chang et al., 1987; Fischer et al., 1986; Grumet and Edelman, 1988; Grumet et al., 1984; Lagenaur and Lemmon, 1987; Lemmon et al., 1989; Stallcup and Beasley, 1985), and the drosophila molecule neuroglian, of a similar structure to the L1 subgroup of Ig-CAMs discussed here, facilitates cell–cell adhesion when transfected into non-adhesive cell lines (Hortsch and Bieber, 1991). Therefore, it seems likely that Bravo plays a role in neural adhesion and fasciculation.

This close relationship of Bravo and Ng-CAM suggests the possibility that these two molecules interact heterophilically (as do L1 and Ng-CAM with the same 40% sequence identity) (Lemmon et al., 1989). This is consistent with the observation that Ng-CAM mediates neuron–glia adhesion via heretofore unidentified glial components which may include Bravo (Grumet et al., 1984). Given the results described here, it will be possible to test the role of the different Bravo isoforms in neuron–neuron and neuron–glia adhesion by means of several new assays (e.g., Hortsch and Bieber, 1991; Lee and Lander, 1991). The restricted distribution of Bravo along retinal axons suggests that it might be involved in both the strong fasciculation of retinal fibers seen in the retina and the reduced fasciculation of these same fibers seen in the tectum where Bravo is not detected. The absence or reduced levels of Bravo protein on distal axons in the tectum (de la Rosa et al., 1990) might play a role in reducing the cohesiveness of retinal axons for each other, allowing less hindered target seeking by the growth cones. In addition, Bravo protein found on glial end-feet in the retina (and possibly in the tectum) might interact with the retinal axons that track along this glial substrate.

The possibly extensive use of alternative splicing suggests a complex and developmentally regulated role for Bravo. In analyzing only five clones in this laboratory, five putative alternatively spliced sequences were identified, not counting AS20 identified previously (Grumet et al., 1991). Alternative splicing of mRNA precursors can provide an important mechanism for generating developmentally programmed functional diversity of proteins involved in cell-surface recognition (e.g., Smith et al., 1989). The specific sequences that may be included or excluded in a particular isoform have, in several examples, been characterized as specific functional protein domains that contribute to different cell adhesion properties (Huhtala et al., 1990: Komoriya et al., 1991; Siri et al., 1991; Weller et al., 1991). The programmed alternative splicing of FNIIIs, in particular, as well as numerous other functional protein domains is a powerful mechanism for generating functional diversity among cell-surface receptors during development (Kimizuka et al., 1991). Therefore, it is interesting to note that both Bravo and Nr-CAM cDNA clones have been identified with sequences either containing or missing FNIII#5, and that the Bravo protein β chain shows molecular weight heterogeneity (60–80 kD) suggestive of the presence of these two isoforms.

Bravo cDNA clones reveal alternative sequences within the cytoplasmic region as well. All alternative cDNA sequences will require genomic DNA sequencing to ensure that they do not represent cloning artifacts, but in particular the sequence of AS-CYT1 suggests the need for further analysis, as it encodes a frameshift resulting in a stop codon within the highly conserved cytoplasmic domain. In addition, AS-CYT1 and AS-CYT2 both show similarity to 3′ regions of introns, including potential polypyrimidine tracks and termination with the nucleotide sequence AG (Smith et al., 1989). OIne or both of these sequences might, therefore, represent incompletely processed mRNA. On the other hand, in support of the notion that AS-CYT2 is not an artifact of cloning but actually encodes a functional Bravo variant, it is important to note that both human and rat versions of L1 contain alternatively spliced sequences corresponding exactly to the tetrapeptide encoded by AS-CYT2, Arg-Ser-Leu-Glu. Furthermore, L1 from rat shows a tissue-specific regulation of this alternative cytoplasmic sequence: the isoform missing this sequence is predominantly expressed in the brain, while the RSLE isoform is found in the peripheral nervous system (Miura et al., 1991). Investigations of the distribution of Bravo polypeptides (specifically of the isoforms containing or missing AS-CYT2) might reveal a similar spatial restriction and suggest some roles for alternative splicing in this region, possibly involving alterations of interactions with cytoplasmic or cytoskeletal proteins critical for influencing neurite outgrowth and cell migration.

The large number of domains and alternatively spliced sequences (both extra- and intracellular) suggests numerous diverse functions for Bravo proteins. In the developing nervous system, just as in the immune system, it appears that a number of molecules are generated by RNA or DNA splicing to provide functional diversity among molecules containing Ig domains.

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