An Antibody to the Tetraspan Membrane Protein CD9 Promotes Neurite Formation in a Partially α3β1 Integrin-Dependent Manner

Shilpi A. Banerjee, Michael Hadjiargyrou, and Paul H. Patterson

Division of Biology, California Institute of Technology, Pasadena, California 91125

The tetraspan cell surface glycoprotein, CD9, has been implicated in cellular signaling during growth and differentiation in the hematopoietic and nervous systems. Because CD9 expression is induced early in development in sensory and sympathetic neuroblasts, we investigated the role of CD9 in neurite outgrowth. We plated dissociated cells from neonatal sympathetic ganglia or immobilized anti-CD9 antibodies or antibodies against other cell surface molecules. We show here that B2C11, an anti-CD9 antibody that has been shown previously to activate Schwann cells in vitro, promotes robust neurite outgrowth from sympathetic neurons that is greater than that on other antibody surfaces and is comparable to neurite outgrowth on a collagen substratum. In addition, B2C11 causes dramatic morphological changes in neurons and glia from dissociated ganglia, including a flattening of these cells.

Because CD9 interacts with integrins in many cell types including Schwann cells, and specifically with the α3β1 integrin in some cells, we tested whether the effect of B2C11 on neurite outgrowth is mediated by this integrin. An anti-α3β1 antibody, Ralph 3–1, attenuates the extent of neurite outgrowth on B2C11 and collagen, but not on laminin. Because the α3β1 integrin has been shown to mediate neurite outgrowth on different substrates, these results provide a functional significance for the CD9–α3β1 interaction; downstream signaling may be activated by this cis interaction on the cell surface in response to external cues that promote neurite outgrowth.

Key words: CD9; tetraspan proteins; antibody perturbation; neurite outgrowth; sympathetic neurons; α3β1 integrin
MATERIALS AND METHODS

Antibodies. mAbs utilized in these experiments were described previously (DeFreitas et al., 1995; Hadjiargyrou et al., 1996). Briefly, mAbs to rat surface proteins, CD9 (ROCA1, ROCA2 and B2C11), p75NTR (192-IgG), Thy-1 (OX-7), a heparan sulfate proteoglycan (pg22), and the α3β1 integrin (Ralph-1) are all mouse IgGs. They were purified from hybridoma supernatants using the mAb Trap-G kit (Pharmacia) and stored at −20°C in a solution containing 1 M glycine-HCl, pH 2.7, and 60 mM Tris-HCl, pH 9 (final, pH 7.6). The Ralph-1 mAb was a generous gift of Dr. Louis Reichardt (University of California, San Francisco, CA), and purified IgGs and F(ab) fragments of an NCAM mAb were a generous gift of Dr. Urs Rutishauser (Case Western Reserve University, Cleveland, OH).

Sympathetic neuronal cultures. Superior cervical ganglia were dissected from neonatal rats and enzymatically dissociated as described previously (Banerjee and Patterson, 1995). Dissociated cells were plated on various surfaces and grown in complete medium: L15-CO2 containing fresh vitamin mix (Hawrot and Patterson, 1979), 5 µg/ml bovine insulin (Sigma, St. Louis, MO), 100 µg/ml transferrin (Sigma), and 100 ng/ml NGF (Boehringer–Mannheim, Indianapolis, IN).

Neurite outgrowth assays. For quantitation of neurite outgrowth, 8-well glass slides (Roboz Surgical Instrument Co. Inc., Rockville, MD) were

Figure 1. Immunostaining of SCG cultures with various mAbs. All mAbs used here stain sympathetic neurons, and most also stain glial cells. Dissociated SCGs were plated on collagen, cultured for 16 hr, and immunostained with pg22 (B), OX-7 (D), 192-IgG (F), ROCA2 (H), or B2C11 (J). The corresponding phase contrast micrographs are shown in A, C, E, G, and I, respectively. The arrows and arrowheads point to glial cells and demonstrate that whereas 192-IgG, ROCA2, and B2C11 recognize Schwann cells, pg22 and OX-7 do not seem to do so. Bar, 100 µm.
sterilized by first being immersed in 95% ethanol, followed by flaming. Each well of the 8-well slide was coated with 5 μl of a solution of 5 cm² of type BA85 nitrocellulose (Schleicher & Schuell, Keene, NH) dissolved in 6 ml methanol (Lagenaur and Lemmon, 1987), and allowed to dry in a tissue culture hood. Each purified mAb (5 μg/ml), which was diluted in 100 mM carbonate buffer, pH 9.6, and rat tail collagen (Hawrot and Patterson, 1979), were added to the wells (50 μl total volume). The mAbs were allowed to bind to the nitrocellulose for 2–4 hours at room temperature (RT), followed by 2 washes with 1/3 PBS. To prevent nonspecific cell binding, the wells were then blocked for 1 hr at 37°C with a 5% BSA solution (in PBS), and washed twice with PBS. Dissociated SCG cells were either directly added to each well at a

Figure 3. B2C11 and OX-7 promote neurite outgrowth. Dissociated sympathetic neurons that had been cultured for 16 hr and stained for peripherin display robust neurite outgrowth on collagen, OX-7, and B2C11. On pg22, ROCA2, and 192-IgG, however, there is little or no neurite outgrowth. Bar, 25 μm.
Peripherin immunohistochemistry was performed by incubating the fixed cultures with anti-peripherin polyclonal Ab (Chemicon International, Temecula, CA) diluted 1:1000 in PBS containing 2% goat serum and 0.1% NP-40. After washes in PBS, cells were incubated with an FITC-conjugated anti-rabbit secondary Ab (Vector Laboratories), washed, and mounted in glycerol containing n-propyl galrate, as described above.

RESULTS

B2C11 and control Abs recognize cultured sympathetic neurons and glia

All of the purified mAbs used in these experiments bind well to living, dissociated cells from neonatal rat sympathetic ganglia. In addition to the CD9-activating mAb (B2C11; Hadjiargyrou and Patterson, 1995; Kaprielian et al., 1995), other mAbs used recognize CD9 (ROCA1 and ROCA2), the low affinity NGF receptor (192-IgG), Thy-1 (OX-7), and a heparan sulfate proteoglycan (pg22), and were chosen because of their reported ability to recognize sympathetic neurons (Mason and Williams, 1980; Chandler et al., 1984; Matthew et al., 1985; Mahanthappa and Patterson, 1992). All, also the antigens are cell surface molecules, and the corresponding mAbs are of the IgG isotype. As shown in Figure 1, pg22, OX-7, 192-IgG, and ROCA2 recognize and strongly immunostain the surface of neurons, as defined by morphology shown in the respective phase contrast micrographs. In addition, 192-IgG and ROCA2 immunostain the surface of Schwann cells (Fig. 1, E–H, arrows). The anti-CD9 mAb ROCA1 is an exception; it does not label sympathetic neurons (data not shown), consistent with the report by Kaprielian et al. (1995) that although ROCA1 immunoprecipitates CD9 and recognizes CD9 on Western blots, it does not stain the surface of living cells. B2C11 recognizes both Schwann cells and neurons in dissociated SCG cultures (Fig. 1). The mAb concentration used for histological staining, 5 μg/ml, is the same as that used to coat surfaces in the experiments described below.

Neurons adhere to immobilized B2C11

Using the experimental paradigm used to show that immobilized B2C11 activates Schwann cells (Hadjiargyrou and Patterson, 1995), sympathetic ganglia were dissociated and plated either on rat tail collagen type I or on various mAbs that had been immobilized on a nitrocellulose surface. Cells that had been cultured for 16 hr on the various surfaces were washed gently and fixed. Neurons were identified by immunostaining for peripherin, a neuron-specific intermediate filament protein (Portier et al., 1984). The number of peripherin-immunoreactive cells that remain on each surface after washing was determined. As shown in Figure 2, neurons adhere well on a variety of surfaces; numbers of neurons on B2C11, OX-7, and 192-IgG are comparable to those on the adhesive ECM protein, rat tail collagen type I. Adhesion is somewhat less on immobilized pg22 and ROCA2. In two other experiments, cells adhered well on collagen, B2C11, 192-IgG, and OX-7, and less well on pg22 and ROCA2 (approximately three- to fivefold more cells adhered to B2C11 than to pg22 and ROCA2), although the total number of cells on each surface varied somewhat from experiment to experiment. In experiments where the cultures were not washed before fixation, the numbers of cells on all surfaces were comparable (data not shown).

Neurons extend processes on B2C11

After 16 hr in culture, sympathetic neurons display robust neurite outgrowth on certain surfaces. Shown in Figure 3 are representative examples of neurite outgrowth on each surface tested. As
would be expected, extensive neurite outgrowth is observed on rat tail collagen, a surface well characterized as favorable to neurite outgrowth. In contrast, neurons on pg22, 192-IgG, and ROCA2 display limited neurite outgrowth. Consistent with a previously demonstrated role for Thy-1 in neurite outgrowth (Leifer et al., 1984, 1991; Mahanthappa and Patterson, 1992), many neurons put out neurites on immobilized OX-7. Extensive neurite outgrowth that is more robust than that on OX-7, and comparable to that observed on collagen, is observed on B2C11.

To quantitate the extent of neurite outgrowth, the percentage of neurons bearing neurites, as well as the length of neurites, was determined on each surface. The number of neurons with processes more than 100 μm in length were categorized as neurite-bearing cells. The percentages of neurite-bearing cells on each surface were consistent in four separate experiments. As shown in Figure 4, the percentage of neurons that extend neurites on immobilized B2C11 is similar to that on rat tail collagen type I. A smaller percentage of neurons extend neurites on OX-7. Neurons did not extend neurites on immobilized pg22 or ROCA2 in this experiment, and only a small percentage of cells had neurites on 192-IgG. In three other experiments, the mean percentages of neurite-bearing neurons were 40–62% on collagen, 41–54% on B2C11, 0–15% on pg22, 0–10% on ROCA2, 23–32% on OX-7, and 3–20% on 192-IgG. In experiments where the cultures were

Figure 5. Neurite length on B2C11 is equivalent to that on collagen. Neurite length was measured using an eyepiece reticule and was divided into classes, and the number of neurons with neurite lengths in each class was plotted for each condition. Neurite length on B2C11 is almost as long as that on collagen and much longer than that on OX-7. In this experiment, no neurites were observed from neurons on pg22 or ROCA2.
not washed before they were fixed and immunostained, numbers of neurons on pg22 and ROCA2 were comparable to the other surfaces. In these cases as well, the percentage of neurons with neurites were 7–17%, suggesting that fewer cells adhering to pg22 after washing does not result in an underestimation of the percent outgrowth on these surfaces.

The length of the neurites on each of the surfaces was also quantitated. The representative data shown in Figure 5 were obtained from the same experiment shown in Figures 2 and 4, and the data were pooled from quadruplicate cultures in a single experiment. These experiments were repeated three times, with the same overall trends shown in Figure 5. The neurite lengths were pooled in bins, and the number of neurons with neurite lengths in each bin in one experiment are shown in Figure 5, where each bin is represented as a bar. In three experiments, 38–62% of neurons on B2C11 have neurites in the smallest length bin, and 2–15% of neurons have neurites in the longest length bin. On rat tail collagen, the corresponding values are 23–40% (smallest length bin) and 3–23% (longest length bin). On OX-7, the corresponding values are 50–55% and 0–13%. These profiles indicate that neurons on B2C11 have neurite lengths shorter than those on collagen type I and longer than those on OX-7. In all experiments, the majority of neurite lengths of neurons on pg22, 192-IgG, and ROCA2 were confined to the bin with shortest length neurites (Fig. 5; data not shown).

A mAb against the α3β1 integrin attenuates neurite outgrowth on B2C11

To determine whether the effects of B2C11 depend on a previously demonstrated association of CD9 with the α3β1 integrin, we tested the effect of a function-blocking mAb to α3β1, Ralph 3–1 (DeFreitas et al., 1995), on the extent of sympathetic neurite outgrowth on various surfaces. To do so, we preincubated dissociated SCG cells with either Ralph 3–1 or a variety of control mAbs, then plated them on either rat tail collagen, B2C11, OX-7, or laminin, which results in neurite outgrowth that is comparable to that on B2C11 and collagen, at the concentrations used in this study. As shown in Figure 6, Ralph 3–1 decreases the extent of neurite outgrowth by 38% on B2C11, 45% on OX-7, and 36% on collagen type I. Ralph 3–1 does not significantly modify the extent of neurite outgrowth on laminin, consistent with a previous report (DeFreitas et al., 1995). F(ab) fragments of an anti-NCAM mAb were used as one control and do not show any effect on outgrowth on any surface. In addition, the intact
anti-NCAM mAb and an anti-pg22 mAb also do not show any effect on neurite outgrowth (data not shown).

**Morphology of neurons and Schwann cells is altered on B2C11**

In addition to the robust neurite outgrowth observed on B2C11, this mAb induces dramatic morphological changes in the neurons. Unlike the round, phase-bright morphology seen on all other surfaces (Fig. 3), a large percentage of neurons flatten out on B2C11, and a peripherin-positive cytoskeleton in the flattened soma is easily seen (Fig. 7). In addition, neurites and growth cones also assume a flattened morphology on B2C11 (Fig. 7, arrow). A small percentage of neurons and neurites do, however, retain their usual three-dimensional morphology. Because peripherin is an intermediate filament, these results suggest a dramatic rearrangement of the cytoskeleton induced by neurons binding B2C11.

To determine whether this altered morphological phenotype on B2C11 is restricted to neurons, we also tested B2C11 on ganglionic glial cells. S-100-positive glia have the expected bipolar morphology on collagen, as well as on 192-IgG, ROCA2, pg22, and OX-7 (Fig. 8). In contrast, on B2C11 (Fig. 8F), Schwann cells lose the bipolar shape and flatten out. A thinning of the cytoplasm, probably because of excessive spreading of the cell membrane, is observed, causing gaps in the cytoplasm in some cases; these areas are apparently devoid of cell membrane (Fig. 8F, arrow). These results are consistent with previously observed spreading of the S-16 Schwann cells on B2C11, observed starting at 2 hr after plating and extending up to 72 hr (Hadjiargyrou and Patterson, 1995).

**DISCUSSION**

CD9, a member of the tetraspan family of cell surface molecules, has been implicated as a signaling molecule in various cellular processes such as adhesion, growth, motility, and differentiation in the hematopoietic and nervous systems (Wright and Tomlinson, 1994; Anton et al., 1995; Banerjee and Patterson, 1995; Hadjiargyrou and Patterson, 1995). The ability of activating anti-CD9 mAbs to cause cellular changes was utilized to elucidate many of these functions. Here we show that B2C11, an anti-CD9 mAb that has previously been demonstrated to stimulate adhesion, migration, and proliferation of Schwann cells (Anton et al., 1995; Hadjiargyrou and Patterson, 1995), induces neurite outgrowth in sympathetic neurons and morphological changes in neurons and glial cells from dissociated sympathetic ganglia.

Neurite outgrowth on B2C11 is comparable to that on rat tail collagen type I and enhanced compared to other mAb surfaces used in our assays, in both the percentage of neurons with neurites as well as in the length of neurites (Figs. 3–5). Moreover, striking morphological changes in neurons and glial cells are observed only when these cells are plated on B2C11, indicating a reorganization of cytoskeletal components induced by B2C11 activation (Figs. 7, 8).

The neurite outgrowth and morphological changes are not a general consequence of the presence of IgG molecules immobilized on the culture surface because ROCA1 (data not shown), which does not recognize the neuronal surface under these conditions, or pg22, ROCA2, and 192-IgG, which do bind the neurons, cause any significant changes. The observation that ROCA2 does not activate the cells, even though it recognizes CD9 with an affinity similar to B2C11 (Hadjiargyrou and Patterson, 1995), suggests that the effects of B2C11 are a result of an epitope-specific perturbation of CD9. In addition to collagen type I and B2C11, another mAb used as a control in our experiments, OX-7, which recognizes Thy-1, also induces neurite outgrowth. This result is consistent with previous studies showing that OX-7 enhances sympathetic neurite outgrowth when added in solution (Mahanthappa and Patterson, 1992; Doherty et al., 1993). Furthermore, OX-7 and a different anti-Thy-1 mAb, 2G12, enhance neurite outgrowth from retinal ganglion cells when immobilized on glass (Leifer et al., 1984, 1991). The spreading phenotype of both Schwann cells and neurons observed on B2C11 is, however, unique to this surface and is not seen on OX-7, rat tail collagen, or on any other surface tested. Spreading of S-16 Schwann cells has also been observed on B2C11 but not on 192-IgG (Hadjiargyrou and Patterson, 1995).

CD9 perturbation with anti-CD9 mAbs has been used extensively in both the hematopoietic and nervous systems to explore possible functions of CD9. For example, anti-CD9 mAbs cause platelet aggregation (Griffith et al., 1991 and references therein), homotypic adhesion of pre-B cell lines (Maselli-Smith et al., 1990), adhesion of pre-B cells to bone marrow fibroblasts (Maselli-Smith and Shaw, 1994), neutrophil adhesion to endothelium (Forsyth, 1991), increased adhesion of primary Schwann cells, increased adhesion and proliferation of a Schwann cell line (Hadjiargyrou and Patterson, 1995), and enhanced Schwann cell migration (Anton et al., 1995). The ability of anti-CD9 mAbs to activate cells may be a result of the ability of the mAb to mimic a putative ligand for CD9. In this model, the mAb replicates the action of a CD9 ligand. A similar mode of activation has been...
proposed for neurite outgrowth triggered by the OX-7 mAb, where it may mimic the natural ligand for endogenous Thy-1 (Doherty et al., 1993). An alternative model in which the anti-CD9 mAb works by causing the aggregation and/or internalization of CD9 seems unlikely in view of the fact that in our experiments the ROCA2 mAb, which binds CD9 very well, does not activate the cells.

The dramatic effects of B2C11 on neurite outgrowth and cell morphology support a previously suggested developmental role for CD9 (Tole and Patterson, 1993). During embryogenesis, CD9 is expressed on several neuronal populations (Tole and Patterson, 1993; Kaprielian et al., 1995). In sympathetic neurons, CD9 is expressed at E12, very soon after neural crest cells have formed ganglia. Although sympathetic neuroblasts continue to divide at this stage, neuronal differentiation is concurrent with mitosis (Rohrer and Thoenen, 1987; DiCicco-Bloom et al., 1990). Thus, CD9 expression is correlated with very early neuronal differentiation (Tole and Patterson, 1993). Sensory ganglia express CD9 at E15, which also corresponds to very early neurite outgrowth, which is a postmitotic event in these cells. (Rohrer and Thoenen, 1987; Tole and Patterson, 1993). This correlation is particularly striking in the ventral horn of the spinal cord, where CD9 expression parallels the transient expression of other surface proteins involved in neurite outgrowth such as TAG-1 (Dodd et al., 1988) and DM-GRASP/SC1 (Burns et al., 1991; Tanaka et al., 1991; Tole and Patterson, 1993). CD9 expression in myelinating Schwann cells during development and after injury in vivo also suggests that immature Schwann cells do not express CD9, whereas differentiating Schwann cells do so (Banerjee and Patterson, 1995; Kaprielian et al., 1995).

Our results provide further experimental evidence that CD9 may play an early role in neurite outgrowth. It is therefore interesting that in many cell types, CD9 interacts with various integrins, receptors that are critical for the neuronal response to outgrowth-promoting molecules and that are associated with downstream signaling events leading to cytoskeletal rearrangements and process extension (Reichardt and Tomasselli, 1991). Induction of platelet aggregation by an anti-CD9 mAb causes a specific association of CD9 with the GPIIb-IIIa integrin, and this association is necessary for cell activation (Higashihara et al., 1985; Slupsky et al., 1989). CD9 associates with the α3, α6, and β1 integrins in Schwann (Hadjiargyrou et al., 1996) and neuroblas-

Figure 8. B2C11 induces altered glial morphology. Dissociated SCG cells that had been cultured for 16 hr were immunostained for S100, a glial cell marker. Glia grown on collagen (A), 192-IgG (B), ROCA2 (C), pg22 (D), and OX-7 (E) have the normal bipolar morphology. On B2C11, in contrast, glial cells flatten out, with a thinning of the cytoplasm (F). Bar, 25 μm.
Thus, integrin α3β1 is involved with CD9-mediated neurite outgrowth. It also mediates sympathetic neurite outgrowth on thrombospondin (DeFreitas et al., 1995) and type I rat tail collagen (this report), but not on collagen IV (DeFreitas et al., 1995) or laminin (DeFreitas et al., 1995, this report).

Integrins αβ1 (Muller et al., 1995) and GPIIb-IIIa (Pelletier et al., 1995) have also been implicated in cell spreading. In our experiments, the cell spreading phenotype was not significantly modified by the presence of the Ralp 3–1 mAb. Thus, the interaction of B2C11 with CD9 leading to cytoskeletal rearrangement and flattening could act by mimicking a putative CD9 ligand that directly causes downstream signaling events, or it may involve a different integrin. In addition, the B2C11-CD9 interaction may induce a cis interaction with the α3β1 integrin, triggering an integrin-associated signaling cascade leading to other cellular changes. The possibility that CD9 signaling may involve interactions with another set of cell surface receptors, the integrins, suggests that CD9 may be part of a larger cell surface complex that mediates interactions with the extracellular environment. Such multicomponent complexes have been reported in various cell lines (Berditchevski et al., 1996), including S-16 Schwann (Hadjiargyrou et al., 1996) and N2A neuroblastoma cells (Schmidt et al., 1996). In addition, the ability of CD9 to interact with different integrins in distinct systems raises the possibility that this may be a mechanism that underlies the specificity of CD9 functions.

REFERENCES


