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Reexpression of Vimentin in Differentiated Neuroblastoma Cells Enhances Elongation of Axonal Neurites

Maya Dubey,[1] Sadaf Hoda,[1] Walter K.-H. Chan,[1] Aurea Pimenta,[2] Daniela D. Ortiz,[1] and Thomas B. Shea[1]*

[1]Center for Cellular Neurobiology and Neurodegeneration Research, Department of Biological Sciences, University of Massachusetts Lowell, Lowell, Massachusetts
[2]Department of Pharmacology, Vanderbilt University, Nashville, Tennessee

Vimentin (Vm) is initially expressed by early neuronal precursors in situ and in culture. Vm is essential for neuritogenesis at least in culture and is gradually replaced by neurofilaments (NFs) because of downregulation of Vm expression. This period is accompanied by a slowing of axonal elongation. We examined whether continued expression of Vm could foster continued axonal elongation. NB2a/d1 cells differentiated with dibutyryl cAMP were transfected with constructs expressing Vm or the middle-molecular-weight NF subunit (NF-M) each conjugated to green fluorescent protein (GFP). Axonal neurites of cells expressing GFP-Vm were 30% longer than those of nonexpressing cells, or cells expressing GFP-M, and exhibited a decrease in neurite caliber. Expression of GFP-M did not enhance axonal neurite length but significantly increased caliber. These findings provide further evidence of a role for Vm in axonal outgrowth. Culturing of nontransfected cells on laminin increased neurite length, but cells expressing GFP-Vm demonstrated an equivalent increase whether cultured on laminin or culture plastic. Axonal neurites of cells expressing GFP-Vm turned to avoid a nonfavorable substrate (nitrocellulose), but culturing of these cells on nitrocellulose did not impair axonal outgrowth. These latter findings indicate that the more robust outgrowth following reexpression of Vm is independent of a favorable or nonfavorable substrate but that axonal neurites of these cells still interact with the substrate to the extent that the substrate can influence directionality.
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Key words: vimentin; neurofilaments; neuronal differentiation; axonal outgrowth; phosphorylation; cytoskeleton

Vimentin (Vm) is initially expressed by nearly all neuronal precursor cells in situ and declines shortly before division ceases (Cochard and Paulin, 1984). Studies with cultured neurons and differentiating neuroblastoma cells have demonstrated that Vm is required for axonal initiation (Shea et al., 1993; Boyne et al., 1996). Compensatory mechanisms for this in vitro requirement for Vm clearly exist in situ (Colucci-Guyon et al., 1994). Nevertheless, these findings in culture indicate a transitional importance of Vm in facilitating axonal outgrowth. Vm initially concentrates within axonal neurites but then undergoes a developmental decline and is replaced by neurofilaments (NFs), which coincides with a slowing of the rate of axonal outgrowth in culture (Shea, 1990; Shea and Beermann, 1994). The shift in predominance of Vm within nascent axonal neurites to NFs characteristic of mature axons has been considered to reflect the changing dynamics of nascent, rapidly elongating neurites to stabilized axons (for review see Nixon and Shea, 1992). Whether the transient expression of Vm is instrumental in the initial faster growth rate of axonal neurites is unclear. We tested here whether continued expression of Vm beyond its normal developmental decline could also maintain the rapid outgrowth rate associated with initial axonal outgrowth.

MATERIALS AND METHODS

NB2a/d1 cells were cultured on uncoated plates in DMEM (high-glucose formulation) containing 10% horse serum. Differentiation and outgrowth of axonal neurites was induced by the addition of 1 mM dibutyryl cyclic AMP (dbcAMP; Shea et al., 1988). Additional cells were cultured and differentiated on plates with half of the plate coated with 1 mg/ml laminin, which enhances the length of axonal neurites of NB2a/d1 cells (Hammarback et al., 1985; Shea et al., 1992a,b); allowed to dry; and then rinsed three times with DMEM. This allowed quantification within the same culture plate of the influence of laminin or culture plastic on neurite

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*Correspondence to: Thomas B. Shea, Center for Cellular Neurobiology and Neurodegeneration Research, Department of Biological Sciences, University of Massachusetts Lowell, One University Avenue, Lowell, MA 01854. E-mail: thomas_shea@uml.edu

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length. Additional cells were cultured on laminin-coated plates on which a nonfavorable surface region had been created; a 1-mm-diameter disk of nitrocellulose (NC) paper had been placed, then dissolved with a drop of methanol, allowed to dry, and rinsed three times with DMEM.

Cells were transfected with lipofectamine 2 days after db treatment with constructs expressing the middle-molecular-weight neurofilament subunit (NF-M) or Vm conjugated to green fluorescent protein (GFP-M and GFP-Vm, respectively) as described previously (Yabe et al., 1999, 2003). Briefly, constructs (2 μg) were resuspended in 100 μl serum-free DMEM, then combined with 8 μl lipofectamine that had been resuspended in 100 μl serum-free DMEM and allowed to equilibrate for 45 min at room temperature. An additional 800 μl of serum-free DMEM was added, and this mixture was added to cultures. After incubation of cultures for 3.5 hr, an additional 1 ml DMEM containing 10% serum was added (without removing the DNA:lipofectamine mixture), and was incubation continued for 24 hr, after which the medium was replaced with 2 ml fresh DMEM containing 10% serum. Cultures were first viewed at this time, because prior studies (Yabe et al., 1999) demonstrated that 18–24 hr were required for transfected cells to accumulate sufficient GFP-M for visualization; accordingly, cells were first viewed following 3 days of dbcAMP treatment. Some cells were fixed and extracted under conditions that retain only cytoskeleton-associated polymers and eliminate noncytoskeleton-associated subunits, and further promote splaying of cytoskeletal elements, to evaluate whether GFP-Vm was incorporated into cytoskeleton-associated polymers (Brown, 1997).

Phase-contrast and epifluorescent images of individual cells were captured via a Dage CCL-72 camera connected to a Scion LG-3 frame grabber housed in a Macintosh 7100AV operated by NIH Image software. Images were then stored as TIFF or PICT files. The length and caliber of axonal neurites were measured on phase-contrast images by using NIH Image software, and data were exported to Microsoft Excel for further calculations. Fusion of Vm and NF-M with GFP provided an easy means of identifying cells expressing significant levels of these proteins; such cells were defined as "expressing GFP-Vm" or "expressing GFP-M" and were scored separately from cells in transfected cultures not expressing detectable GFP. Cells from nontransfected cultures were referred to as "controls." Statistical analyses were carried out with ANOVA of mean and quantified length of axonal neurites in phase-contrast images.

RESULTS

As shown previously (Yabe et al., 2003), GFP-Vm forms filamentous profiles throughout perikarya and axonal neurites that are retained following extraction with Triton, indicating that some GFP-Vm has been incorporated into cytoskeletal-associated filaments (Fig. 1). Expressions of GFP-Vm and GFP-M each dramatically altered axonal neurite dynamics, as evidenced by changes in length and caliber (Fig. 2). Axonal neurite length was increased by approximately 25% in cells expressing GFP-Vm compared with nontransfected cells ($P < .05$). In addition, the caliber of axonal neurites expressing GFP-Vm was decreased by approximately 20% compared with that of neurites of nontransfected cells ($P < .05$). By contrast, axonal neurites of cells expressing GFP-M were identical in length to those of nontransfected cells but, consistently with prior studies (Nakagawa et al., 1995), demonstrated an approximate 38% increase in caliber compared with axons of nontransfected cells and an approximately 70% increase over that of cells expressing GFP-Vm ($P < .05$ for both). The enhanced length and caliber cannot be artifactually derived from transfection alone, because cells from nontransfected cultures and cells from cultures transfected with either construct but not expressing detectable GFP exhibited identical length and caliber (not shown; see Yabe et al., 1999). Moreover, these two constructs yielded complementary effects: Transfection with GFP-VM increased length but not caliber, whereas transfection with GFP-M increased caliber but not length (Fig. 2).

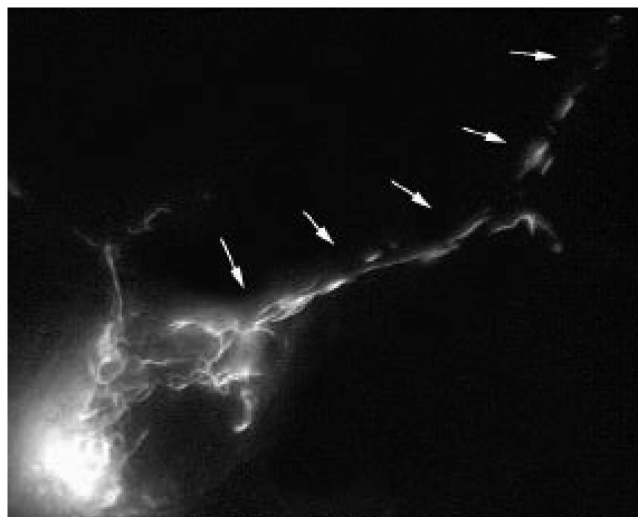

Fig. 1. GFP-Vm forms filamentous profiles within perikarya and axonal neurites following transfection of NB2a/d1 cells. Epifluorescent micrograph of a representative cell differentiated for a total of 7 days and transfected 24 hr previously with a construct expressing Vm conjugated to GFP ("GFP-Vm"), after which the culture was fixed and extracted under conditions that promote splaying of cytoskeletal elements, which more closely reveals fibrillar structures. Note the retention of GFP within a fibrillar pattern within the perikaryon and along the axonal neurite (arrows).

Although enhancement of axonal neurite outgrowth has potential importance for regeneration, it remained possible that reexpression of Vm fostered unregulated growth that might overcome responses to environmental cues. To examine this possibility, we next compared the responses of cells expressing GFP-Vm and nontransfected control cells to favorable and nonfavorable culture surfaces. Consistently with prior studies (Shea et al., 1992a,b), laminin enhanced the length of axonal neurites of nontransfected cells vs. the length on culture plastic by approximately 30% (Fig. 2). The axonal neurites of cells expressing GFP-Vm on culture plastic were already as long as those of nontransfected cells cultured on laminin; cul-

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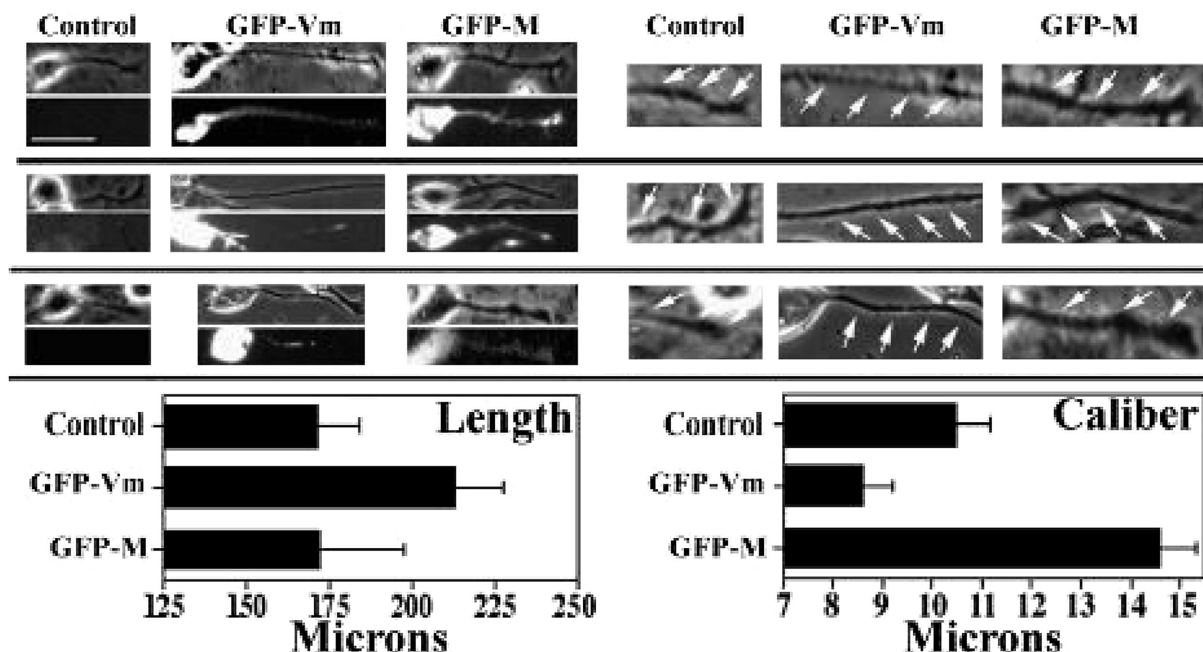

Fig. 2. Reexpression of Vm increases axonal outgrowth, whereas increased expression of NF-M increases axonal caliber. Panels at left present phase-contrast and epifluorescent images of cells differentiated for a total of 7 days and transfected 24 hr previously with constructs expressing GFP-Vm or GFP-M as indicated. Note that nontransfected control cells exhibit no epifluorescence. Panels at right present higher magnification of portions of phase-contrast images of axonal neurites (arrows) of panels at left to denote differences in caliber more clearly. The accompanying graphs present mean values for axonal length and caliber (±SEM) compiled from ≥10 nontransfected ("Control") cells or cells expressing GFP-Vm or expressing GFP-M from three separate experiments (total n = 30 for each condition). Note that cells expressing GFP-Vm demonstrated increased axonal length and decreased caliber compared with control cells or cells expressing GFP-M ($P < .05$ for length and caliber, ANOVA), whereas axons of cells expressing GFP-M were identical to those of control cells in length but demonstrated increased caliber compared with both control cells and cells expressing GFP-Vm ($P < .05$). Scale bar = 100 μm.

turing on laminin did not further enhance their length (Fig. 3). With NC dissolved on top of laminin, 100% of axonal neurites of both Vm-transfected and nontransfected cells turned away from the NC disk (Fig. 4; n = 50 cells each). Neurites of cells cultured on the edge of the NC disk were invariably oriented away from the disk (Fig. 4). However, the axonal neurites of Vm-transfected cells within more central areas of the NC disk (and therefore unable to extend off of the disk) were as long as those cultured on laminin or plastic; neurites of Vm-transfected cells on NC disks averaged 123.8 ± 32.5 μm, whereas those on laminin averaged 139.0 ± 27 μm, and those on culture plastic averaged 140.4 ± 30.1 μm (mean ± SD; Fig. 4). Although apparently a less-favorable surface, NC did not inhibit the elongation of axonal neurites that accompanied reexpression of Vm.

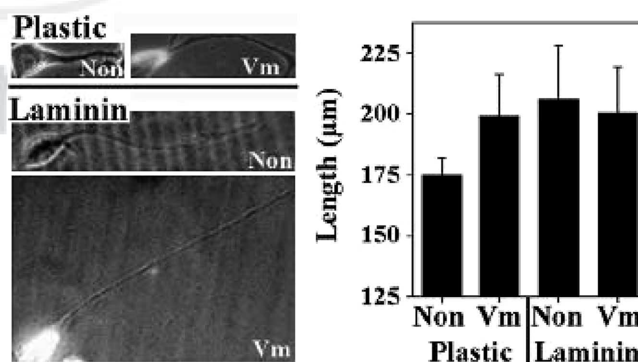

Fig. 3. Influence of laminin on axonal neurite length. Panels present merged phase-contrast and epiflourescent images of nontransfected ("Non") and Vm-transfected ("Vm") cells cultured on uncoated plastic or laminin as indicated. Expression of GFP-Vm is confirmed by perikaryal fluorescence in the merged image. The graph presents the length of axonal neurites (mean ± SEM) for ≥50 cells expressing GFP-Vm and nonexpressing cells from two independent experiments cultured on laminin or culture plastic. Note that culturing on laminin enhanced the average length of nonexpressing cells but did not enhance the average length of cells expressing GFP-Vm.

DISCUSSION

Our prior studies demonstrated that Vm was required for initiation of axonogenesis in culture (Shea et al., 1993; Boyne et al., 1996). Here we have demonstrated that reexpression of Vm after its normal developmental decline maintains a rapid elongation rate for axonal neurites beyond the time when their outgrowth would normally have slowed or stopped. This finding was relatively

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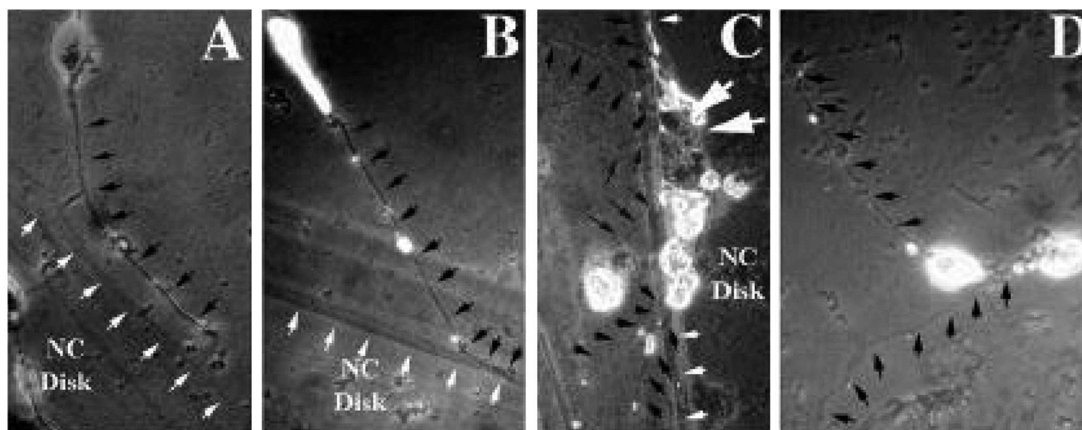

Fig. 4. Vm-transfected cells exhibit surface selectivity. Panels present merged phase-contrast and epiflourescent images of cells cultured on laminin onto which an NC disk had been dissolved as described in Materials and Methods to establish favorable and nonfavorable adjacent culture surfaces. The edge of the NC disk is denoted in **A–C** by small white arrows. Axonal neurites are indicated by black arrows. Axonal neurites of both nontransfected (A) and Vm-transfected (B) cells cultured on laminin turned when encountering the edge of the NC disk. Axonal neurites of transfected and nontransfected cells that were cultured on the edge of the NC disk elongated away from the disk (transfected cells are indicated by perikaryal fluorescence; the single nontransfected cell is indicated by large white arrows). **D** shows two transfected cells within the central region of the NC disk that have elaborated axonal neurites characteristic of Vm-transfected cells on laminin or plastic.

specific for Vm; increased expression of NF-M did not enhance axonal elongation but instead increased axonal neurite caliber, which has previously been attributed to NF-M (Nakagawa et al., 1995; Pant and Veeranna, 1995). These data provide additional evidence of a role for Vm in axonal outgrowth.

Beyond this basic finding, however, restoration or maintenance of axonal elongation by reexpression of a developmentally regulated cytoskeletal protein that facilitates initiation of axonal outgrowth is consistent with the possibility that reexpression of Vm might be useful for axonal regeneration in situ. This possibility is supported by the continued expression of Vm by olfactory receptor neurons throughout life, which has been suggested to contribute to the unique plasticity and regenerative capacity of these neurons (Gorham et al., 1991). In additional to fostering increased elongation of axonal neurites, the behavior of neurites of cells expressing GFP-Vm in response to favorable and nonfavorable substrates is also consistent with this possibility. Axonal outgrowth and pathfinding are dependent on the extracellular matrix during development (see, e.g., Hammarback et al., 1985). Subsequent critical developmental changes render the extracellular matrix nonpermissive for axonal outgrowth, which has hampered progress in axonal regeneration (for reviews see Evans, 2000; West et al., 2001). We observed here that expression of GFP-Vm enhanced the length of axonal neurites to the same degree as culturing on laminin did for nontransfected cells. Notably, culturing on laminin did not further enhance the length of axons of cells expressing GFP-Vm, which may reflect a maximal length that axonal neurites of these cells can achieve under these conditions of culturing and differentiation. Culturing on NC, a less-favorable culture surface, did not inhibit the enhanced outgrowth of axonal neurites of cells expressing GFP-Vm. These findings indicate that Vm-transfected cells do not require favorable surfaces to achieve their enhanced outgrowth and that the more robust growth provided by continued expression of Vm might help axons to elongate over areas that are otherwise nonsupportive for axonal regeneration. Such efforts may help with the difficulties encountered regarding the nonpermissive nature of the extracellular matrix in nerve regeneration (see, e.g., Evans, 2000; West et al., 2001; Jones et al., 2003). Furthermore, avoidance of NC indicates that axonal neurites of cells expressing GFP-Vm still interact with their environment; this latter finding suggests that, although the more robust outgrowth following reexpression of Vm may eliminate or reduce the requirement for a favorable substrate, it apparently remains compatible with axonal pathfinding and directionality. Reexpression of Vm and/or other developmental cytoskeletal proteins may augment other approaches to support axonal regeneration. For example, recent studies demonstrate that fibronectin mats support directionalized growth of axons within experimentally injured spinal cord (King et al., 2003); combinatorial approaches utilizing transfection with Vm accompanied by favorable surfaces, including laminin (Hammarback et al., 1985) and fibronectin, as well as supplementation with nonneuronal cells and factors derived from them (see, e.g., Liu et al., 2001; Pellitteri et al., 2001; Schlosshauer et al., 2003; and references therein), may have synergistic effects on axonal regeneration.

The mechanism by which Vm supports initial axonal outgrowth (Shea et al., 1993; Boyne et al., 1996) or elongation as demonstrated herein remains unclear. Vm an NFs overlap spatially and temporally within the same axons (Tapscott et al., 1981; Bignami et al., 1982; Jacobs et al., 1982;

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Houle and Fedoroff, 1983; Ziller et al., 1983; Cochard and Paulin, 1984; Shea et al., 1988; Shea and Nixon, 1988; Shea, 1990; Boyne et al., 1996). However, recent studies (Yabe et al., 2003) demonstrate that at least some GFP-Vm transiently coassembles with NFs in axonal neurites. It is therefore possible that Vm may faciliate NF deposition within newly elaborated neurites (or regions of neurites) in a side-by-side manner (Sun et al., 1997) and/or by transient formation of Vm/NF heteropolymers (Yabe et al., 2003), which could serve as nucleation sites for NF assembly, as previously suggested with coexpression of Vm and keratins (Georgatos, 1993; for review see Steinert and Liem, 1990). Of interest, and essential for consideration of Vm reexpression as a means to facilitate axonal regeneration, would be to determine 1) the extent to which reexpression/continued expression of Vm facilitates axonal outgrowth in primary neurons, 2) whether expression of Vm is compatible with target recognition and synaptogenesis, and 3) whether after synaptogenesis axons expressing exogenous Vm undergo stabilization as evidenced by accumulation of phospho-NFs. In addition, axonal elongation, but not initiation or maturation, is facilitated by alpha-internexin in these cells (Shea and Beermann, 1999), and peripherin is essential for neurite outgrowth in PC12 cells (Helfand et al., 2003). Determining the interplay of reexpression of vimentin with these additional neuronal intermediate filament species will be of interest.

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