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TRANSGENIC MODELS FOR STUDYING EXPRESSION AND FUNCTION OF AXONAL ADHESIVE GLYCOPROTEINS

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INTRODUCTION

Axonal growth and pathfinding are key events in neural development. Both involve homo- or heterophilic interactions among neuronal surface glycoproteins belonging to distinct gene families as the Immunoglobulin Superfamily (IgSf), the Semaphorins, the Integrins, the Cadherins (6, 7, 25, 26, 31, 41). Although provided with similar roles in axonal growth control, these molecules may undergo differential expression during development, which may be closely related to their specific significance in nervous tissue maturation. This is the case for F3/Contactin (F3) (19, 35) and for the Transient Axonal Glycoprotein (TAG-1) (15, 18). F3 and TAG-1 are two glycosylphosphatidylinositol-anchored IgSf components, expressed at the neuronal surface, built of a similar array of Immunoglobulin-type C2 and Fibronectin-type III domains. These molecules display 50% similarity at the aminoacid level and undergo differential expression during neural development. Indeed, while F3 is a component of differentiating neurons, TAG-1 peaks on premigratory elements and, partially, on proliferating precursors (3).

F3/Contactin exerts a complex role in nervous tissue maturation. While its primary function is in axonal growth control (2, 3, 20, 33), its localisation at the synaptic level (16) fits with its ability to modulate specific aspects of synaptic plasticity (30). In addition, myelinating cells also express the molecule (27) whose ability to promote oligodendrocyte differentiation has been recently demonstrated (24). A role for F3 in myelination is also supported by its complex molecular interactions at the paranodal level, which include its cis-association with the Contactin-Associated PRotein CASPR/Paranodin within the axonal surface and its trans-interaction with the IgSF component Neurofascin on the apposing oligodendroglia (4, 9, 21, 34, 36). In the same context, the interaction has been demonstrated between F3 and the Nogo glycoprotein at the paranode (32). Due to the known significance of Nogo as a stop signal for axonal growth (28, 40, 45), F3 could be part of the molecular complex which restricts the growth/regeneration potential of central neurons during development.

Finally, a role has been proposed for F3 in the control of proliferation events of neuronal precursors as its premature expression on these elements affects their ability to enter the cell cycle (3).

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The complex role F3/Contactin plays in neural morphogenesis may correlate with and depend upon its articulated expression in different cell types and developmental steps (43). We report here on specific aspects related to the functional significance of F3 gene regulated expression. First, the activation profile of the F3 promoter in developing cerebellar cortex has been followed through the expression of an F3 promoter/EGFP (Enhanced Green Fluorescent Protein) reporter construct in transgenic mice. Then, the morphological changes have been examined which arise in the cerebellar cortex from F3 gene developmental misexpression under control of a heterologous promoter. For this we have chosen a selected regulatory region from the gene encoding the human relative of the Transient Axonal Glycoprotein TAG-1 (TAX-1). The reason for this choice is that F3 and TAG-1 genes undergo differential developmental activation, TAG-1 being expressed on proliferating and premigratory granule cells precursors while F3 is found on these elements only after they become postmitotic. Furthermore, the two genes undergo differential cell type-specific expression since F3 is detected on all cerebellar neurons, while TAG-1 is silent on Purkinje cells (38, 43). In the generated transgenic mice (TAG/F3 mice), phenotype changes occur in the cerebellar cortex, which strongly supports the view that regulation of the genes encoding axonal adhesive glycoproteins may be provided with key relevance in the control of neural development.

METHODS

Animals and breeding procedures.

All animal experimentation conformed to the EU directive 86/609 EEC.

Transgenic mice were generated, expressing an engineered construct in which a 12.5 Kbp sequence from the F3 gene 5' region, including all the 5' untranslated exons and the surrounding genomic sequences, was fused at the level of the first ATG to an EGFP (Clontech) reporter (Fig. 1C). Previous studies revealed that the corresponding genomic region, which spans more than 100 Kbp, includes three alternative neurospecific promoters undergoing differential activation during postnatal development (12). Due to the large size of this region, only the relevant genomic sequences were assembled together upstream the EGFP reporter.

The generation of the TAG/F3 transgenic mice, expressing F3/Contactin under control of a selected regulatory region from the human TAX-1 gene has been described (3). For this, a fusion construct was generated, including a 17 kb sequence of the TAX-1 gene 5' region (bearing the first two exons and the intervening intron), fused to the full length F3/Contactin cDNA (Fig. 2B).

Morphological analysis.

F3 immunostaining was performed on paraffin embedded sections from mice perfused with 2% paraformaldehyde/0.1% glutaraldeyde as described (43). For Calbindin immunostaining, sections were incubated with a mouse monoclonal antibody (Sigma, St Louis, USA), followed by biotin-conjugated rabbit anti-mouse IgG (DAKO), streptavidin-peroxidase and the AEC kit (Vector laboratories). TAG-1 expression was detected on cryostat sections from mice perfused with 4% paraformaldehyde by using a rabbit antiserum (15), followed by the Alexa 488 goat anti-rabbit IgG (Molecular Probes, the Netherlands). Nuclear staining was done by using the DAPI Nucleic Acid Stain (Molecular Probes). Alternatively, some sections were counterstained with hematoxylin (DAKO).

For double TAG-1/Calbindin immunohistochemistry, 20 μm cryostat sections incubated with both the TAG-1 rabbit antiserum and the Calbindin mouse monoclonal antibody were treated with both Alexa 488 goat anti-rabbit and Alexa 568 goat anti-mouse IgG (Molecular Probes).

For EGFP detection, cryostat sections from mice perfused with 4% paraformaldehyde were immunostained with a rabbit anti-EGFP serum (Santa Cruz), revealed by the Alexa 488 goat anti-rabbit IgG (Molecular Probes).

To estimate cell proliferation in vivo, mice were subcutaneously injected with 5-Bromo-2'-desoxy-Uridine (BrdU, Roche Molecular Biochemicals, Mannheim, Germany). BrdU was then detected with an anti-BrdU mouse monoclonal antibody, revealed by alkaline phosphatase-conjugated sheep anti-mouse IgG (Roche) as described (3).

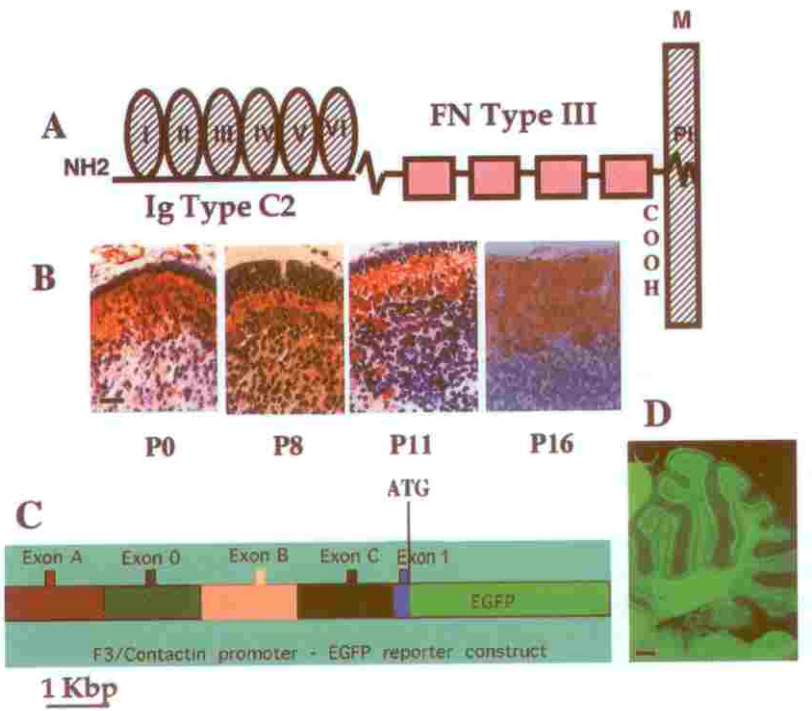

Fig. 1. - *Organization and expression in developing cerebellar cortex of the F3 glycoprotein and of the F3 gene promoter/EGFP reporter construct.*

A. Domain organisation of the mouse F3/Contactin axonal glycoprotein. The location of the Immunoglobulin type C2 domains I to VI (Ig Type C2) and of the Fibronectin type III repeats (FN Type III), separated by a hinge region, is shown. The molecule is associated with the cell membrane via a glycosylphosphatidylinositol anchor (PI).

B. Changes in F3/Contactin expression during development of the cerebellar cortex. The immunostaining of sagittal sections from postnatal day 0 (P0), 8 (P8), 11 (P11) and 16 (P16) mice cerebella are shown. Labelled sections are counterstained with hematoxylin (blue).

C. F3/Contactin promoter/EGFP reporter construct generated to explore the activation profile of the F3/Contactin gene in developing cerebellar cortex. The selected genomic region corresponds to sequences surrounding the 5' untranslated exons from the F3/Contactin gene (exons A, 0, B, C) and the ATG-containing exon 1.

D. Expression profile of the F3 promoter/EGFP reporter construct in the cerebellar cortex of transgenic mice. The data refer to postnatal day 6 cerebellum.

Scale bars: B = 20 μm; D = 100 μm.

RESULTS

Expression profiles of the F3 glycoprotein and of an F3 promoter/EGFP reporter transgene in developing cerebellar cortex.

In Figure 1 the overall organisation (A) and the expression profile in developing cerebellar cortex (B) of the mouse F3 glycoprotein are reported. Although the molecule is expressed all over postnatal life, it is differentially modulated within different classes of cerebellar neurons. At postnatal day 0 (P0), F3 is found on postmitotic granule cells which exit the germinative zone of the external granular layer and migrate towards the inner granular layer. By the end of the first postnatal week (P8), F3 is progressively downregulated on granule cells bodies, a consistent expression remaining on their axonal extensions in the molecular layer. At around the same time (P8-P11), F3 is upregulated on Purkinje neurons (PC), both on their cell bodies and neurite extensions. This is mostly evident on PC dendrites, although the molecule is similarly expressed on axonal extensions (not shown). Finally, starting at the end of the second postnatal week (P16), the F3 gene is progressively downregulated on all cerebellar neurons.

Together, the data show that the F3 gene undergoes a complex developmental regulation which concerns both its overall activation and its differential expression within distinct neuronal populations. Such a complex profile may depend upon the independent activation of distinct promoters, demonstrated by a previous *in vitro* study (12). To verify whether the identified promoter elements were indeed responsible for the definition of the F3 developmental profile *in vivo* we used the corresponding genomic sequences to drive an Enhanced Green Fluorescent Protein (EGFP) reporter in transgenic mice. Since the size of the F3 gene 5' flanking region exceeds 100 Kbp (12), a construct was generated in which only the relevant genomic regions were assembled together upstream the chosen reporter (Fig. 1C). In this construct, the overall size of the assembled genomic region accounted to 12.5 Kbp, much lower as compared to the corresponding region of the endogenous gene (which overcomes 100 Kbp). However, the activation profile of the chosen promoter in developing cerebellar cortex essentially recapitulated the endogenous gene. As shown in Figure 1D at postnatal day 6, reporter gene expression was demonstrated on both granule neurons inside the different folia and on elements outlining them, bearing the position of Purkinje cells. In addition, strong expression was observed in the white matter, consistent with transgene activation on myelinating elements. These data indicate that, at least in the cerebellar cortex, the assembled genomic region carried the cis-acting elements necessary for the definition of the F3 expression profile in both neuronal and non-neuronal cells.

Cerebellar morphogenesis is perturbed in transgenic mice undergoing inappropriate developmental expression of F3/Contactin.

The articulated activation profile of the F3 gene, together with the complex organisation of its regulatory region, suggested a relevant role for the F3 regulated expression in its developmental function. To verify this hypothesis, we asked whether inter-

fering with F3 expression profile could have any consequence on neural morphogenesis. For this, we devised a transgenic model leading to F3 developmental misexpression under control of a regulatory region selected from the TAX-1 gene, which encodes the human homologue of the Transient Axonal Glycoprotein TAG-1. The reason for this choice is shown in Figure 2A. In developing cerebellar cortex, TAG-1 expression is predominant on premigratory elements (in green) while the molecule is strongly downregulated as these precursors enter the inner granular layer. Therefore, TAG-1 expression peaks earlier than F3, as supported by previous studies showing that TAG-1, but not F3, is expressed on proliferating elements within the external granular layer (3). Finally, unlike F3, TAG-1 is absent from Purkinje cells (38), as supported by the double TAG-1/Calbindin immunostaining shown in Figure 2A.

On these bases, the TAG-1 gene was found to differ from F3 in terms of both developmental activation and cell type-specific expression. Therefore, in order to perturb F3 profile in vivo, we decided to express F3 under control of an available genomic sequence, spannig about 16 Kb, arising from the 5' region of the TAX-1 gene (3). This sequence, whose activation profile recapitulated the endogenous TAG-1 gene, was fused to the F3 cDNA at the level of the first ATG (Fig. 2B) and expressed in transgenic mice.

Five different transgenic lines, called TAG/F3 mice, were generated, whose cerebellar phenotypes were independently characterised in comparison to matched control littermates. As shown in figure 2C by TAG-1 immunostaining, at postnatal day 8th a consistent reduction in the cerebellar size was observed in TAG/F3 mice as a result of F3/Contactin developmental misexpression. However, this phenotype alteration underwent developmental regulation, these effects undergoing recovery already at postnatal day 16th (3). Together, these data indicated that F3/Contactin misexpression consistently affected cerebellar development, although the effects were transient in nature.

The observed developmental effects could concern both proliferation and differentiation of neuronal precursors. In Figures 2D and E, the proliferation extent of the main cerebellar neurons, the granule cells, as well as the differentiation profile of Purkinje neurons are compared in TAG/F3 mice versus wild type littermates. At postnatal day (3), granule cells precursor proliferation was consistently affected as deduced by the about 30% reduction of BrdU incorporation in the external granular layer (Fig. 2D). Although these effects underwent recovery at around the 8th postnatal day (3), they were likely to be the most relevant consequence of F3/Contactin developmental misexpression and could account for the decrease in cerebellar size observed at postnatal day 8 (Fig. 2C).

Besides neuronal precursors proliferation, also neuronal differentiation was affected by the chosen misexpression approach. This is typically shown in the case of Purkinje neurons upon Calbindin immunostaining in postnatal day 8 cerebellar cortex (Fig. 2E). Indeed, while no significant differences were observed in the number and position of these elements, their differentiation was consistently affected, as deduced by the reduced extension and branching of their dendritic tree, indicative of delayed terminal differentiation.

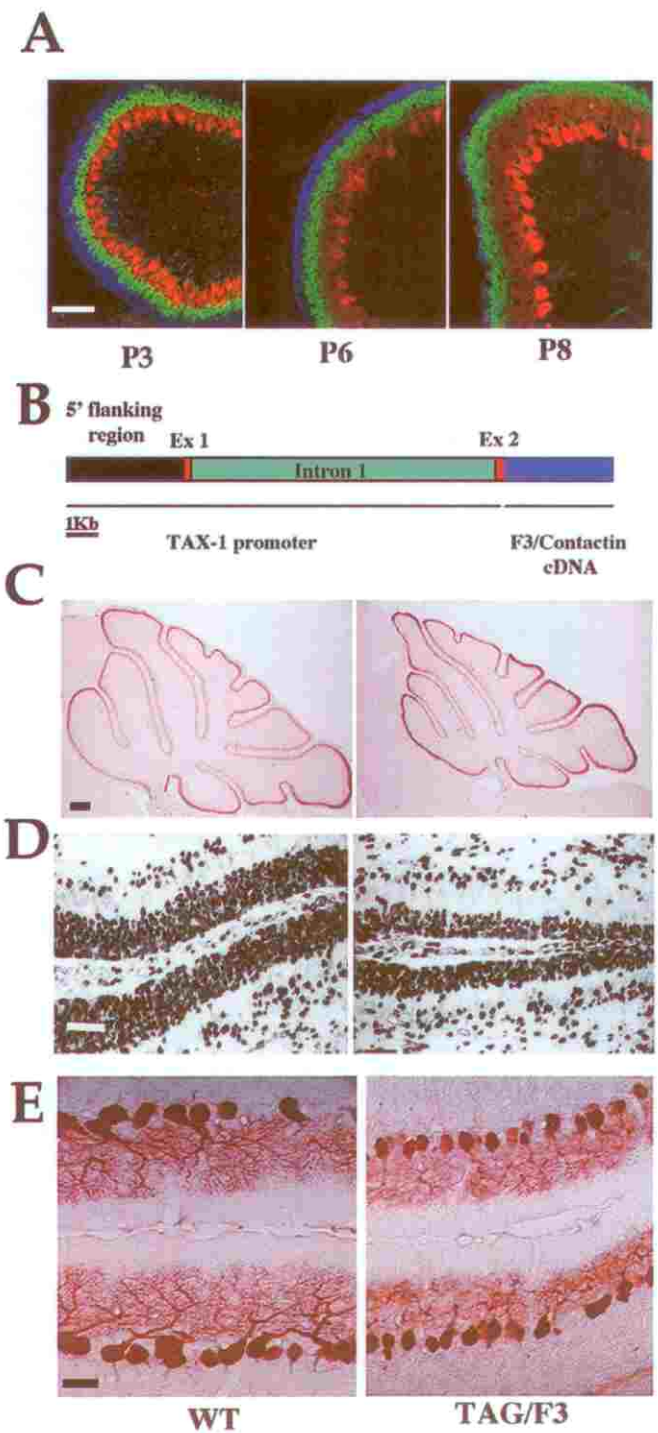

Fig. 2. - *Consequences of F3 misexpression in cerebellar cortex development.*

A. Expression profile of the TAG-1 protein in developing (P3-P6-P8) cerebellar cortex (green), compared to Calbindin as a marker of Purkinje neurons (in red). DAPI staining (in blue) is used to label nuclei (mostly evident in the Extrernal Granular Layer). Scale Bar: 40 μm.
B. Fusion construct of the TAG-1 gene promoter region with the full length F3 cDNA (in blue). The location of the two first TAX-1 exons (in red), of the intervening intron (in green) and of the 5' flanking region of the TAX-1 gene (in black) is indicated.
C. Changes in the cerebellar size observed upon TAG-1 immunostaining in postnatal day 8 TAG/F3 transgenic mice versus control littermates (WT).
D. BrdU incorporation in the external granular layer of developing cerebellar cortex (postnatal day 3) of TAG/F3 mice versus control littermates (WT). Note the consistent reduction in granule cell precursor proliferation in transgenic mice.
E. Differentiation profiles of Purkinje neurons demonstrated upon Calbindin immunostaining in postnatal day 8 cerebellar cortex of TAG/F3 mice and control littermates (WT). Note the consistent reduction in the extension of Purkinje neurons dendritic tree in in TAG/F3 mice.
Scale bars: A, D, E: 40 μm. C: 200 μm.

Together, the data support the view that regulated expression of axonal surface glycoproteins like F3/Contactin may be of key relevance in the control of different aspects of neural development, including both precursor proliferation and differentiation.

DISCUSSION

The functional role of axonal glycoproteins has been largely explored by using both *in vivo* and *in vitro* models (2, 5, 6, 7, 13, 44). This allowed to introduce the definition of neurite growth-promoting molecules on the basis of their ability to exert positive effects on neurite elongation (25, 26, 31, 42). However, further studies suggested more complex functions for these glycoproteins, including control of neuronal precursor proliferation, axonal pathfinding, myelination and modulation of synaptic function (3, 4, 5, 14, 30).

The complex functional role of these molecules may be closely related to the expression profiles of the underlying genes. Different axonal glycoproteins may be co-expressed on the same neuronal elements, thus raising the question of the functional significance of this redundancy. Different explanations may be proposed. First, each adhesive glycoprotein may differentially activate specific signalling mechanisms, each of them resulting in definite developmental effects (10, 39, 41). However, redundancy between such molecules may be also instrumental in order to provide the cell surface with a specific array of adhesion receptors, responsible for the specificity of neuronal connections. The developmental expression of these molecules may be then a crucial component of their ontogenetic function, whose specificity would be strictly related to the activation profile of the underlying genes.

In this study, the topic of the developmental significance of axonal glycoproteins regulated expression has been explored by taking F3/Contactin and TAG-1 as the molecular models. These two glycoproteins differ for their profile of developmental expression, TAG-1 being predominant in earlier steps of brain maturation. In addition, these molecules differ for their cell type-specificity. In the cerebellar cortex, for instance, while both molecules are found on granule cells and on their neurite extensions, only the former is expressed on Purkinje elements (3, 38, 43).

Here we have addressed the functional correlates of these complex expression profiles. We have isolated a large genomic region corresponding to the 5' end of the F3/Contactin gene and found that this region, which displays a large size and a high level of complexity, is necessary and sufficient to recapitulate the F3 gene expression *in vivo*. A potential explanation for the unusual size of this region is that it could be necessary to accommodate and to allow for functional independence of the different elements the F3 promoter is built of. However, a finer dissection of this region will be necessary to elucidate the significance of its cis-acting elements in the regulation of the F3/Contactin expression.

The second hypothesis we wanted to check is that the complexity of the F3/Contactin gene regulatory region is indeed necessary for its ontogenetic function.

According to this hypothesis we found that replacing the F3 promoter for by a TAX-1 gene regulatory region led to premature activation of the F3/Contactin expression and at the same time affected cerebellar development. This concerned both earlier events, as neuronal precursor proliferation, and late steps, including differentiation of both granule (3) and Purkinje elements.

The data clearly support the view that the same adhesive glycoprotein may modulate distinct aspects of neural morphogenesis, depending upon the developmental step in which the underlying gene is activated. In the early development, F3 gene regulated expression may contribute to the control of neuronal precursor proliferation. Indeed, the evidence that F3/Contactin negatively affects this event may justify why the underlying gene is upregulated on postmitotic elements while being silent on proliferating precursors. F3/Contactin misexpression also affects neuronal differentiation, as clearly shown in this report for Purkinje neurons, while a similar effect has been reported for granule cells (3). However, these two neuronal populations may be affected through different mechanisms. Typically, the effects on granule cells should be cell-autonomous in nature and depend upon transgene activation in such elements. Accordingly, the same effects may be observed in dissociated primary cultures (3). On the other hand, the effects on differentiation of Purkinje neurons, in which the transgene is silent (3), should rather arise from perturbed interaction of such elements with granule cells. This is in agreement with several reports showing that granule/Purkinje neurons interactions are important for differentiation of both cell types (1, 8, 22, 23, 29, 37).

Besides than for the structural organization of the neuronal circuits, F3/Contactin developmental misexpression may be relevant also for their functional activation, as demonstrated by the alteration of motor coordination and motor learning observed in TAG/F3 mice in periods in which the morphological changes have fully reversed (11). The suggested role for this molecule in the control of the synaptic function (30) could be involved.

In a general way, the results of this and of previous (3, 11) studies clearly sustain the view that regulated expression of axonal adhesive glycoproteins represents a relevant component of their developmental function. This assumption is further supported by comparing the phenotype changes we observe with those occurring in knockout mice for both F3/Contactin and TAG-1, in which only minor developmental effects were observed (2, 17).

As for the underlying mechanisms, it may be suggested that co-ordinated expression of neuronal surface glycoproteins may be of critical relevance not only in order to build functionally relevant complexes at the neuronal surface, but also for mediating their co-ordinated activation in specific developmental steps.

SUMMARY

In this study, by using two transgenic models, we address the general topic of the significance of axonal glycoproteins regulated expression in nervous tissue matura-

tion. The immunoglobulin superfamily components F3/Contactin (F3) and TAG-1 are used as the molecular models in this respect. First, a minigene including the relevant regulatory sequences of the F3 gene, deduced by a previous in vitro study, has been fused to an EGFP (Enhanced Green Fluorescent Protein) reporter and expressed in transgenic mice, which provided information about the profile of F3 gene developmental activation. In a complementary model, transgenic mice have been generated which express the F3 cDNA under control of a selected regulatory region from the TAG-1 gene. While leading to ectopic expression of F3, this perturbed neuronal precursor proliferation and differentiation. The arising effects were even stronger than those coming from the overall suppression of the F3 or, respectively, TAG-1 genes, thus supporting the hypothesis that the mechanisms underlying axonal glycoprotein regulated expression are themselves endowed with a key significance in neural development.

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