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# Analysis of Interactions of the Adhesion Molecule TAG-1 and Its Domains with Other Immunoglobulin Superfamily Members

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**Cell adhesion molecules of the immunoglobulin superfamily promote cell aggregation and neurite outgrowth via homophilic and heterophilic interactions. The transient axonal glycoprotein TAG-1 induces cell aggregation through homophilic interaction of its fibronectin repeats. We investigated the domains responsible for the neurite outgrowth promoting activity of TAG-1 as well as its interactions with other cell adhesion molecules. Binding experiments with Fc-chimeric proteins revealed that TAG-1 interacts with L1, NrCAM, and F3/contactin. The membrane-associated as opposed to the soluble form of TAG-1 behaves differently in these assays. We demonstrate that both the immunoglobulin as well as the fibronectin domains promote neurite outgrowth when used as substrates. Furthermore we investigated the putative role of L1 and NrCAM as the neuronal TAG-1 receptors mediating neurite extension. DRG neurons from L1-deficient mice were found to extend neurites on TAG-1 substrates and blocking NrCAM function did not diminish the TAG-1-dependent neurite outgrowth. These results indicate that neither L1 nor NrCAM are required for TAG-1-elicited neurite outgrowth.**

## INTRODUCTION

During development of the nervous system a number of critical and finely tuned processes take place. Accurate neuronal connections require the correct navigation of growing axons over long distances. This complex process involves adhesion and axon extension over diverse substrates. The molecular cues encountered by the developing growth cones involve chemoattractive and chemorepulsive molecules, as well as contact repulsion and attraction mediated by surface molecules present on substrate cells (Tessier-Lavigne and Goodman, 1996). Among the latter the immumoglobulin/fibronectin type III (Ig/FNIII) family includes molecules that contain a number of domains (usually four to six) bearing homology to the C2 portion of immunoglobulins (Ig), followed by a number of domains homologous to the type III repeat of the fibronectin molecule (FNIII).

The rodent glycoprotein TAG-1 (Dodd *et al.*, 1988; Furley *et al.*, 1990; Felsenfeld *et al.*, 1994) and its homologs TAX-1 in the human (Hasler *et al.*, 1993; Tsiotra *et al.*, 1993) and axonin-1 in chick (Stoeckli *et al.*, 1991; Rader *et al.*, 1993) is a cell adhesion molecule belonging to the Ig/FNIII family (Tessier-Lavigne and Goodman, 1996), a group that also includes N-CAM, L1/NgCAM, NrCAM, neurofascin, and F3/F11/contactin. Structurally, TAG-1 is composed of 6 Ig domains followed by 4 FNIII repeats and is anchored to the membrane via a glycocyl-phosphatidyl-inositol (GPI) tail (Furley *et al.*, 1990). TAG-1 is expressed transiently during CNS and PNS development both as a GPI-anchored and as a soluble form (Dodd *et al.*, 1988; Karagogeos *et al.*, 1991).

Members of the Ig/FNIII group undergo complex interactions with other members of the same family. They can either act as ligands or receptors, in a context specific manner (Brummendorf and Rathjen, 1996). TAG-1/axonin-1 interacts with L1/NgCAM in *cis*-posi-

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tion (i.e., when the partners are expressed in the same cell) (Rader *et al.,* 1996). This interaction triggers intracellular signalling (Dhar Malhotra *et al.,* 1998). On chick peripheral neurons TAG-1 acts as an NrCAM receptor mediating neuron-glia interactions (Suter *et al.,* 1995) and neurite outgrowth (Lustig *et al.,* 1999). In the developing chick spinal cord, NrCAM has been implicated as one of the receptors for axonin-1 present on commissural growth cones, in mediating pathfinding (Stoeckli and Landmesser, 1995; Stoeckli *et al.,* 1997). Finally, TAG-1 overrides the inhibitory action of F3/contactin on the outgrowth of neurites from mouse cerebellar granule cells (Buttiglione *et al.,* 1998).

In the last few years several studies have focused on linking the structural domains of Ig/FNIII members to specific functions (Brummendorf and Rathjen, 1996). The study of axonin-1, the chick homolog of TAG-1, has revealed that the homophilic activity of the molecule probably maps to several places both in the Ig and the FNIII portions (Kunz *et al.,* 1998). In a different aspect, it was shown that deletion of the first four Ig domains of axonin-1 does not abolish the ability of the molecule to promote neurite outgrowth, whereas the four Ig domains alone do not support this function (Rader *et al.,* 1996). In the case of human TAG-1, we have previously demonstrated that the homophilic activity of the molecule is mediated through its FNIII repeats (Tsiotra *et al.,* 1996). However, the domains that are responsible for its neurite outgrowth activity have been elusive so far. In this study we set out to identify these domains as well as to investigate *in vitro* the interactions of TAG-1 and its domains with other members of the Ig/FNIII superfamily.

## RESULTS

### *Binding Experiments Reveal a Complex Hierarchy of Interactions between Members of the Ig/FNIII Family*

Homophilic and heterophilic interactions in *trans*-position were tested using soluble molecules produced as Fc-chimeras (Figs. 1A, 1B, and 2) and conjugated to fluorescent covaspheres. Fluorospheres of two different colors were each conjugated to a given soluble protein, mixed, incubated, and visualized under the confocal microscope. The association of the protein molecules led to the formation of bead aggregates of single and/or double color (Figs. 2 and 3).

TAG-1-Fc displays homophilic interactions by forming self aggregates of moderate size (14 beads, Fig. 2A and Table 1). In the case of the isolated Ig domains the few aggregates observed are small (Fig. 2B and Table 1). In contrast, for the FNIII domains of TAG-1, very large clusters are observed, larger than those for the complete TAG-1 molecule (Fig. 2C and Table 1). The homophilic interaction between the TAG-1(FNIII)-Fc molecules appeared to be so strong that aggregates formed prior to the assay could not be disrupted by sonication thus limiting the experimental potential for the FNIII domains in this assay. These observations are in agreement with our previous work indicating that no homophilic interaction is mediated through the Ig domains whereas the FNIII domains are sufficient for this function (Tsiotra *et al.,* 1996).

We analyzed the heterophilic interactions of TAG-1 and its isolated domains with other members of the Ig/FNIII family: L1, NrCAM and F3/contactin. Fluorescent covaspheres of one color coupled with Fc-chimeras of TAG-1 or its subdomains were mixed with fluorospheres of a different color coupled to soluble L1-Fc, F3-Fc, or NrCAM-Fc. Representative images are shown in Fig. 3 and the frequencies of heterophilic interactions are summarized in Table 2. L1-Fc and NrCAM-Fc displayed homophilic interactions whereas F3-Fc did not (Figs. 3A, 3C, and 3E; see also Faivre-Sarrailh *et al.,* 1999). TAG-1-Fc beads formed mixed aggregates with NrCAM-Fc beads but not with L1-Fc or F3-Fc beads (Figs. 3A, 3C, and 3E). When TAG-1-Fc and L1-Fc beads were mixed mostly homophilic bead aggregates were observed (Fig. 3A and Table 2). When TAG-1(Ig)-Fc beads were incubated with NrCAM-Fc or L1-Fc beads, mixed aggregates were formed (Figs. 3B and 3D). It is of particular interest that although there was limited interaction between L1 and the total extracellular domain of TAG-1, a significant number of mixed aggregates were formed between beads coupled with L1 and the Ig domains of TAG-1 (compare Fig. 3A with 3B). This indicates that an association between TAG-1 and L1 may depend on conformational constraints, which are relaxed by the deletion of the FNIII domains.

In a second type of experiment, we investigated the interactions between soluble and membrane-expressed adhesion molecules. The soluble Fc-chimeras were preclustered via the anti-Fc antibody to enhance the binding activity as previously described (Faivre-Sarrailh *et al.,* 1999) and were incubated with CHO cells stably expressing the various adhesion molecules in a membrane-associated form (Figs. 4 and 5). While most interactions between soluble and membrane-expressed Ig/FNIII family members reflected those obtained in the bead assay described above, some differences were also

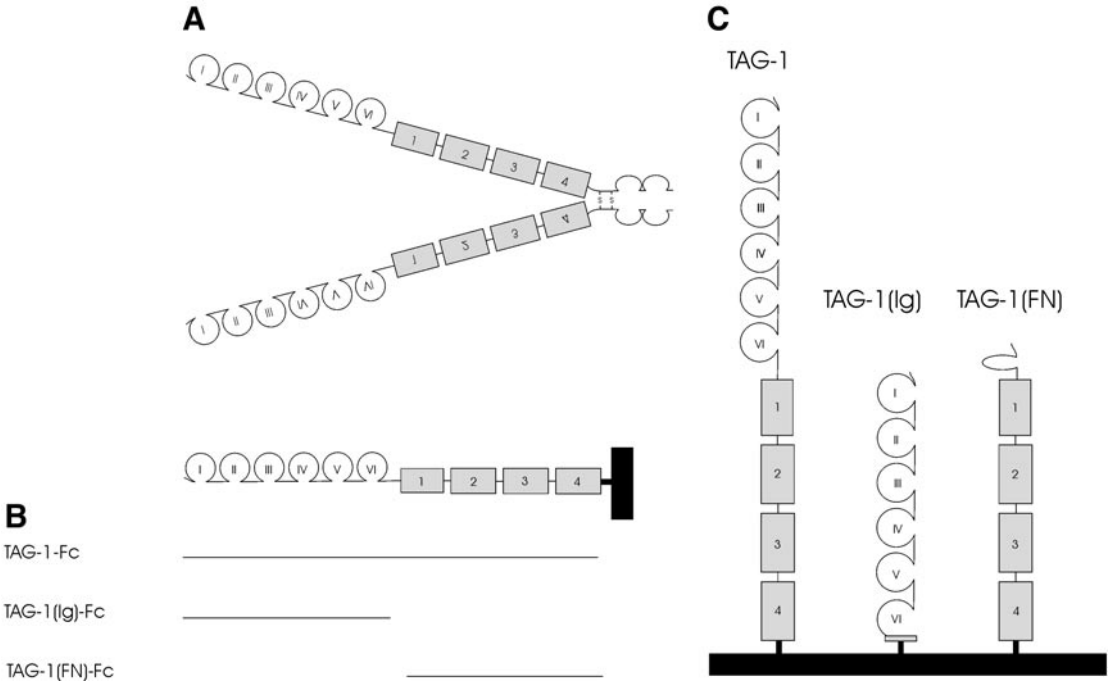



**FIG. 1.** The TAG-1 constructs used for the production of soluble and membrane-bound proteins. (A) A schematic representation of TAG-1-Fc is shown as an example of the structure of the Fc chimeric proteins used in this study. The molecules dimerize via the disulfide bonds of the Fc portion (indicated by S). The Ig domains of TAG-1 are indicated by half circles numbered I-VI. The FNIII domains are represented by rectangles numbered 1–4. (B) The Fc-chimeric constructs used in this study. A schematic of the native TAG-1 molecule is shown at the top. Listed below are the names of the various deletion constructs, with a horizontal bar indicating the domains included in each one. (C) Structure of the protein molecules produced by the CHO stably transfected lines. The total extracellular domain of TAG-1 or its Ig or FNIII domains are expressed as GPI-anchored proteins.

observed. For example CHO cells expressing transmembrane NrCAM showed an interaction with Fc-chimeras containing either the total extracellular domain of TAG-1 or its Ig domains only (Fig. 5C and Table 3). On the other hand, we could detect an interaction of soluble F3-Fc or F3(Ig)-Fc with CHO cells expressing GPI-anchored TAG-1 or its domains (Fig. 5I and Table 3). In the reverse experiment, where the CHO substrate expressing F3 was incubated with preclustered soluble TAG-1-Fc or TAG-1(Ig)-Fc, no binding was observed (Fig. 5E, Table 3). Thus soluble F3 binds to TAG-1 only if TAG-1 is presented in membrane-associated form. This indicates a different behavior of the soluble versus the membrane form of TAG-1. Additional evidence that soluble protein might behave differently from the membrane form came from the observation that, in the same type of assay, soluble NrCAM-Fc bound CHO cells expressing F3 or TAG-1 but it did not bind to membrane NrCAM presented in substrate-bound form (Table 3). In contrast, soluble NrCAM forms homophilic mixed-bead aggregates (Faivre-Sarrailh *et al.*, 1999).

### *Both the Ig and FNIII Domains of TAG-1 Promote Neurite Extension from DRG Neurons*

In order to understand which domains of TAG-1 promote neurite extension, stably transfected CHO lines expressing the total extracellular domain of TAG-1 or its Ig and FNIII portions (Fig. 4) were used as monolayers in coculture with sensory neurons. The experiment shown in Fig. 6A was performed to investigate whether there would be a difference in the ability of either rat or human TAG-1 to promote neurite outgrowth of sensory neurons. Both molecules stimulated neurite outgrowth to a similar extent when compared with the control CHO monolayer.

We monitored the degree of DRG neurite outgrowth on transfected cell lines as well as parental CHO cells. The parental cell line provided basal levels of adhesion and neurite outgrowth. Thus, the percentage of cells displaying neurite extension over the total number of adherent cells was evaluated on the parental as well as on cell lines expressing either the total extracellular

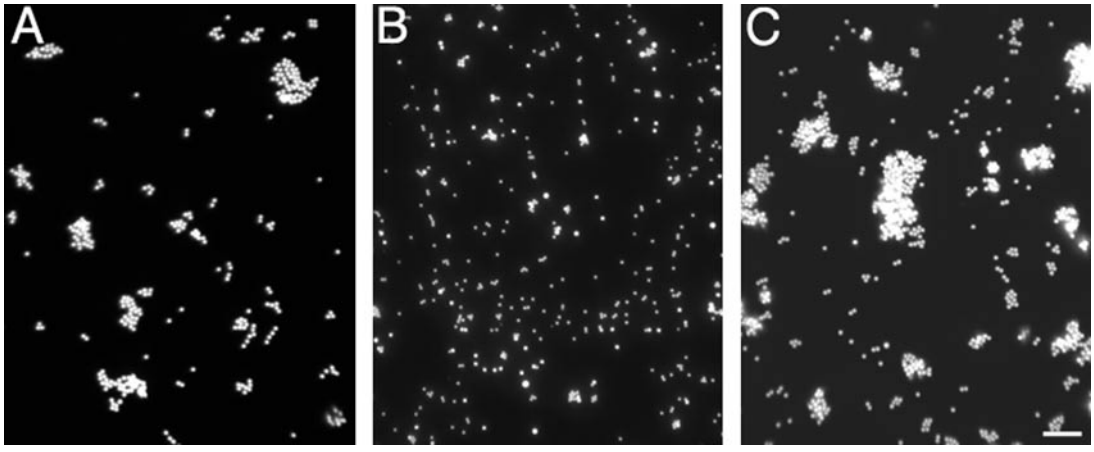



**FIG. 2.** Confocal microscopy analysis of the homophilic bead aggregates. Soluble protein in the form of Fc-chimera was conjugated to fluorescent covaspheres via an anti-human Fc antibody. Protein-bearing fluorospheres were incubated in solution for 1 h at 37°C and observed under the confocal microscope for the formation of bead clusters. See also Table 1 for quantification of the aggregates formed. (A) TAG-1-Fc beads forming aggregates. (B) TAG-1(Ig)-Fc beads displaying lack of homophilic interactions. The few aggregates formed are small. (C) TAG-1(FN)-Fc beads form very large clusters. Bar, 10 $\mu$m.

domain of TAG-1 or its Ig portion only. On the CHO parental cell line only about 15% of all cells that adhered extended neurites (Fig. 6B). In contrast, approximately 60% of all cells that adhered on cell lines expressing either the total extracellular domain of TAG-1 or its Ig domains, extended neurites (Fig. 6B). Thus, the neurite outgrowth curves on CHO substrates for all the graphs presented in this work are generated by about only 15% of adherent cells, while the respective curves on experimental substrates by 60% of adherent cells or more. Therefore, the total extracellular domain of TAG-1 and its Ig domains provide a potent substrate for the initiation of neurite outgrowth (Fig. 6B).

We next tested the ability of both the Ig and the FNIII domains to promote neurite outgrowth. We observed that both types of domains stimulate neurite outgrowth of chick sensory neurons to the same extent as the total extracellular domain of TAG-1 (Figs. 6C and 6D). Similar results were obtained when the sensory neurons were obtained from mouse embryos (Figs. 6E and 6F), indicating that there is no species barrier for the ability of TAG-1 to promote neurite outgrowth.

### *L1 and NrCAM Are Not Required as Receptors for TAG-1-Mediated Neurite Outgrowth*

All DRG neurons used in this study are known to express TAG-1 (Wolfer *et al.*, 1994), or its homolog axonin-1, in the case of avian sensory neurons (Halfter *et al.*, 1994; K.T. and D.K., unpublished observations). However, previous work has shown that TAG-1-mediated neurite outgrowth does not depend on homophilic interactions (Felsenfeld *et al.*, 1994). L1 and NrCAM are known binding partners of TAG-1 and are both expressed by DRG neurons (Karagogeos *et al.*, 1991; Suter *et al.*, 1995; Stoeckli *et al.*, 1996; Grumet, 1997; Sonderegger, 1997; Lustig *et al.*, 1999). Previous work by Felsenfeld and colleagues (1994) employing the use of polyclonal antibodies against L1, implicated an L1-like molecule in TAG-1-mediated neurite outgrowth. We, therefore, analyzed the direct involvement of L1 in TAG-1-mediated neurite outgrowth by performing experiments with sensory neurons from L1-deficient mice on CHO cells expressing the total extracellular domain of TAG-1. As shown in Fig. 7A there was no significant difference in the degree of neurite outgrowth of sensory neurons that were from wild type, heterozygous, or mutant embryos grown on TAG-1. Similar results were obtained when the L1-deficient DRG neurons were grown on CHO cells expressing just the Ig domains of TAG-1 (data not shown), ruling out any homophilic TAG-1 involvement in this assay, in agreement with the results of Felsenfeld and colleagues (1994) mentioned above.

The role of NrCAM as a receptor for TAG-1-mediated neurite outgrowth was also investigated given previous reports that NrCAM might be acting as a receptor on peripheral ganglia for axonin-1, the chick homolog of TAG-1 (Suter *et al.*, 1995; Lustig *et al.*, 1999). Neurite outgrowth experiments were performed with chick sen-

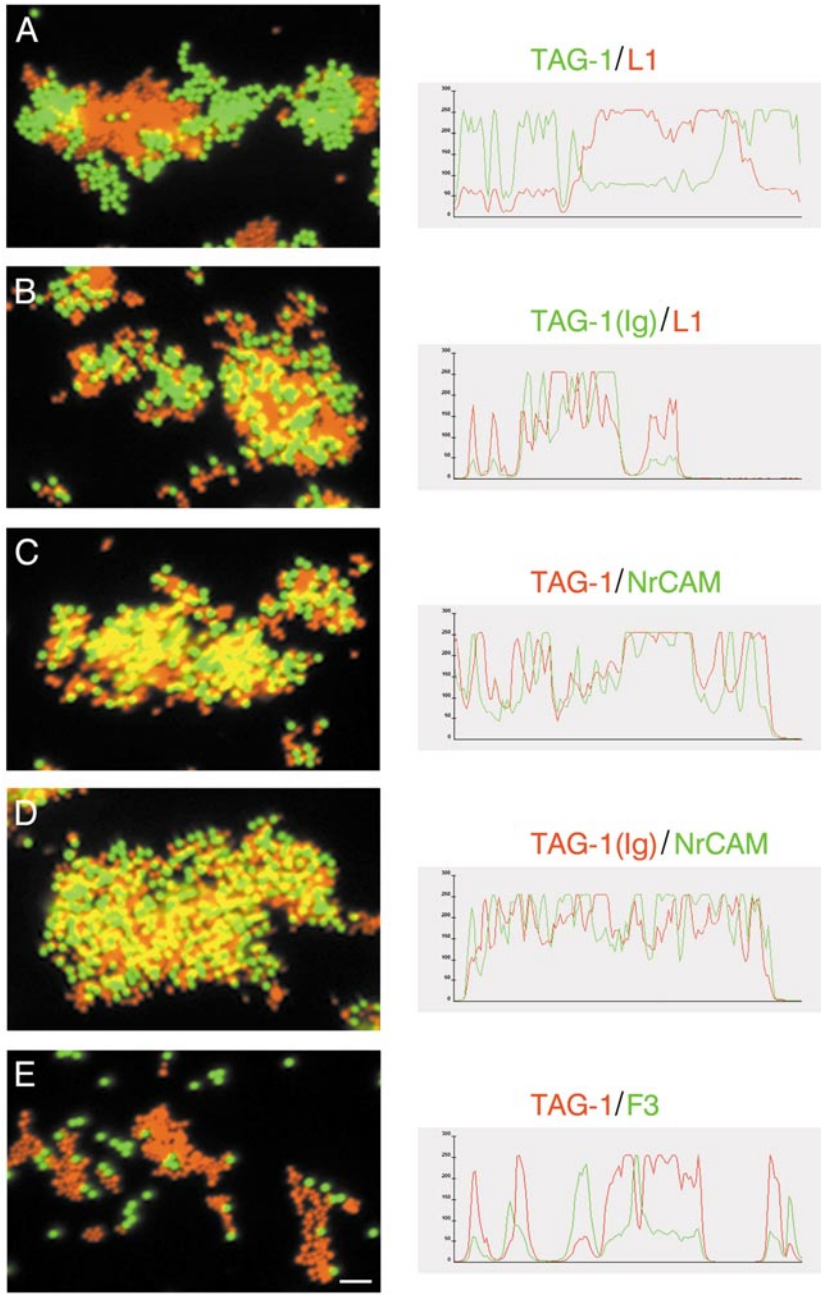



**FIG. 3.** Confocal microscopy analysis of the heterophilic mixed-bead aggregates. Fluorospheres of two different colors (red and green) conjugated with the different protein partners were mixed in a 1:1 ratio, incubated at 37°C for 1 h and observed under the confocal microscope for the formation of mixed-bead clusters (indicated by yellow color). The line histograms correspond to the distribution of the fluorescence intensities (grey-levels in y-axis) along a 150 pixel-long horizontal line crossing the aggregates (pixels in x-axis). A bead diameter is approximately 3 pixels. The Visiolab 2000 software (Biocom, France) was used. For a quantification of the relative frequency of interactions see Table 2. (A) TAG-1-Fc beads (green) mixed with L1-Fc beads (red). Homophilic aggregates are formed and very rare heterophilic interactions are observed. (B) TAG-1(Ig)-Fc beads (green) mixed with L1-Fc beads (red). Mixed aggregates are formed. (C) TAG-1-Fc beads (red) mixed with NrCAM-Fc beads (green). Mixed aggregates are observed. (D) TAG-1(Ig)-Fc beads (red) are forming mixed aggregates with NrCAM-Fc beads (green). (E) No heterophilic interaction is observed between TAG-1-Fc beads (red) and F3-Fc beads (green). Bar, 5 $\mu$m.

**TABLE 1**
Quantitative Analysis of the Homophilic Bead Aggregation

| Homophilic binding | Percentage of beads in aggregates | Size of aggregates (mean bead number/aggregate) |
|---|---|---|
| TAG-1Fc | 75.6 ± 1.9 | 14.3 ± 2.4 |
| TAG-1(Ig)-Fc | 16.8 ± 2.1 | 6.6 ± 0.1 |
| TAG-1(FN)-Fc | 86.5 ± 4.5 | 21.3 ± 2.9 |

*Note.* The percentage of beads found in homophilic aggregates was calculated using Visiolab 2000 software (Biocom, France). An aggregate was considered to be composed of a minimum of 5 beads; means ± SEM from three experiments.

**TABLE 2**
Relative Frequency of Heterophilic Interactions

| | |
|---|---|
| TAG-1/L1 | 3.25 ± 0.25 |
| TAG-1(Ig)/L1 | 9.7 ± 1.1 |
| TAG-1/NrCAM | 14.0 ± 1.3 |
| TAG-1(Ig)/NrCAM | 17.2 ± 1.7 |
| TAG-1/F3 | 1.0 ± 0 |

*Note.* From the histograms presented in Fig. 3, we quantified the number of bead contacts along the 150-pixel line. To analyze the relative frequency of interactions between red and green beads we counted the number of intersections between the red and green curves occuring above the intensity of the 150 grey-level (see Fig. 3); means ± SEM from three experiments.

sory neurons on TAG-1 expressing CHO cells in the presence of blocking antibody for NrCAM. No significant reduction in the extent of neurite outgrowth was observed in the presence of anti-NrCAM antibody at concentrations used in similar experiments (Faivre-Sarrailh *et al.*, 1999) (Fig. 7B).

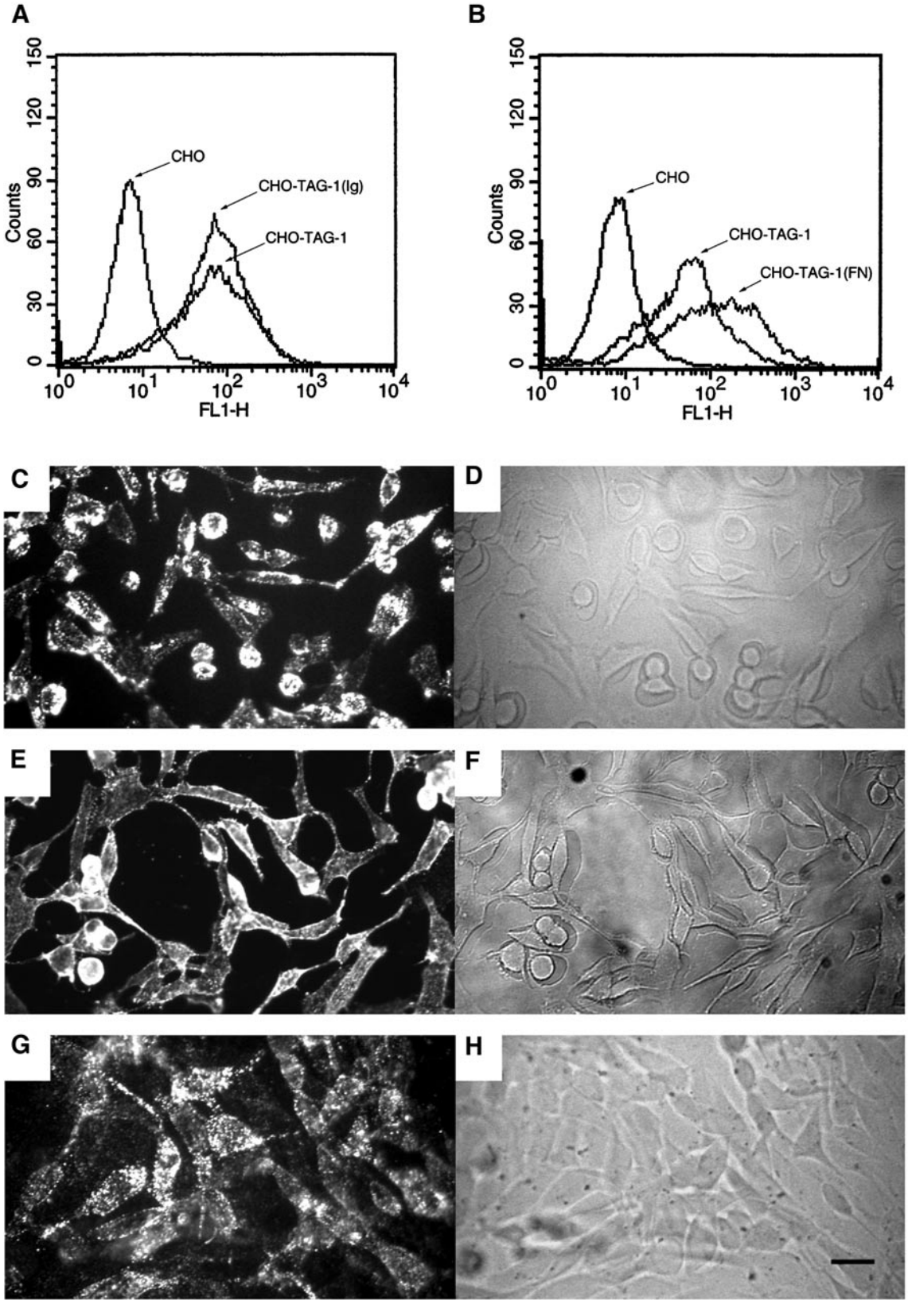
## DISCUSSION

The cell adhesion molecule TAG-1 has been implicated in several developmental processes such as adhesion, neurite outgrowth and axonal pathfinding. The mechanisms by which TAG-1, and its chick homolog axonin-1, mediate these processes appear complex and specific for the particular cellular environments. In this study we investigated the components involved in mediating neurite outgrowth on a TAG-1 substrate *in vitro* and showed novel types of interactions between TAG-1 and related cell adhesion molecules. We demonstrate that TAG-1 harbors a very complex set of interactions with other members of the Ig/FNIII family whose functional relevance may depend on the molecule's tertiary structure and the cellular context.

### *Both Types of Domains of the TAG-1 Molecule Are Equally Able to Promote Neurite Outgrowth But Play a Different Role in Homophilic Recognition*

The two-domain structure of TAG-1 originally suggested to us that each domain may contribute differently to the dual roles of TAG-1 in adhesion and neurite outgrowth promotion. We have previously shown that the FNIII portion of the molecule is by itself sufficient to promote homophilic binding and cell adhesion while the Ig domains are not (Tsiotra *et al.*, 1996). However, it was not known whether the Ig or the FNIII domains of TAG-1 were required for neurite outgrowth. Work on axonin-1 has shown that deletion of the first four Ig domains does not abolish the ability of the molecule to promote neurite outgrowth, whereas the four Ig domains alone do not support this function (Rader *et al.*, 1996). Here we provide evidence that both the Ig and the FNIII portions are able to promote neurite extension as efficiently as the total extracellular domain of TAG-1. If we presume that similar mechanisms are employed by TAG-1 and axonin-1 and in reference to the work of Rader and colleagues (1996) it is possible that the deletion of the last two Ig domains abolished the ability of axonin-1 to promote neurite outgrowth. On the other

**FIG. 4.** The CHO stable lines expressing TAG-1 or its domains. Stably transfected CHO lines expressing the intact TAG-1 or its subdomains as membrane proteins were assayed for recombinant protein levels by FACS analysis (A, B) and immunofluorescence staining (C–H). (A) FACS analysis of the parental CHO cells and the CHO-TAG-1 and CHO-TAG-1(Ig) cell lines with monoclonal antibody 1C12. Curves for all relevant controls, including antibody isotype, secondary antibody, and unstained cell controls fall at the same level as the parental CHO curve shown here. CHO-TAG-1 and CHO-TAG-1(Ig) express comparable levels of recombinant protein. (B) FACS analysis of the parental CHO cells and the CHO-TAG-1 and CHO-TAG-1(FN) cell lines with polyclonal antibodies against TAG-1. Curves for all relevant controls, including antibody isotype, secondary antibody, and unstained cell controls fall at the same level as the parental CHO curve shown here. CHO-TAG-1(FN) has a broader expression profile and slightly higher levels than CHO-TAG-1. (C) The CHO line expressing intact TAG-1 stained with monoclonal antibody 4D7. (D) Phase contrast of C. (E) The CHO line expressing TAG-1(Ig) stained with monoclonal antibody 4D7. (F) Phase contrast of E. (G) The CHO line expressing TAG-1(FN) stained with polyclonal antibodies against TAG-1. (H) Phase contrast of G. Bar, 20 $\mu$m (C–H).


A
Counts
FL1-H
CHO
CHO-TAG-1(Ig)
CHO-TAG-1
B
Counts
FL1-H
CHO
CHO-TAG-1
CHO-TAG-1(FN)
C
D
E
F
G
H

hand it is also possible that there are differences between the two molecules in terms of the domains modulating each function. With regards to the homophilic activity of axonin-1 it was mentioned in an earlier publication that it resides in domains Ig1 and FN4 of the molecule (cited as submitted in Kunz *et al.,* 1998), in contrast to TAG-1 whose FNIII domains are sufficient for homophilic binding (Tsiotra *et al.,* 1996)

Experiments with both mouse and chick sensory neurons demonstrated that there is no species barrier on the action of TAG-1 or its isolated domains. The degree of neurite extension observed, together with the significant percentage of cells exhibiting such outgrowth suggests that TAG-1 acts both in initiating the formation of neurites and in promoting their extension.

Although the two structural modules of TAG-1 retained the ability of the total extracellular domain of the molecule to promote neurite outgrowth, they displayed different homophilic activities (see also (Tsiotra *et al.,* 1996)). In all assays we performed with soluble molecules, the Fc-fusion proteins were conjugated to microspheres via anti-Fc antibodies. This presumably exposed the protein in a configuration more similar to the membrane-bound form. This approach differs from the one adopted in previous work where the protein was directly adsorbed onto microspheres (Kuhn *et al.,* 1991; Rader *et al.,* 1996), with a fraction only of the conjugated molecules expected to be appropriately oriented (Rader *et al.,* 1996). In the experiments performed with soluble Fc-chimeras we observed homophilic interaction of the FNIII domains of TAG-1 and very limited *trans*-interaction between the Ig domains (Figs. 2B and 2C).

### *L1 and NrCAM Interact with the Ig Domains of TAG-1 But Do Not Mediate Neurite Outgrowth on TAG-1 Substrates*

In bead binding assays we observed interaction of the Ig domains of TAG-1 with both NrCAM and L1. However, the total extracellular domain of TAG-1 displayed limited interaction with L1. The interaction of TAG-1/axonin-1 with L1/NgCAM described previously has been documented to occur in *cis*-position (Buchstaller *et al.,* 1996; Rader *et al.,* 1996; Dhar Malhotra *et al.,* 1998). In the experiments presented in our study all interactions were tested in *trans*-position, in solution, between Fc-chimeras coupled to beads via anti-Fc antibodies. The observation that only the Ig domains of TAG-1 bind to L1 in *trans*-position might be explained by one of two possible scenarios. In the first, soluble TAG-1 is in a conformation such that its binding in *trans*-position to L1 is not possible. The deletion of the FNIII domains induces a conformational change such that the remaining portion is now available to interact with L1. This type of conformational change has been proposed to occur in TAG-1/axonin-1, when Ig domains 5 and 6 were deleted (Rader *et al.,* 1996). This is, in turn, consistent with a model in which binding of TAG-1 with L1 is facilitated by homophilic TAG-1 binding via its FNIII domains (Dhar Malhotra *et al.,* 1998). Alternatively, it is possible that a hierarchy of interactions exists with the homophilic adhesion of TAG-1 being the strongest, thus competing the TAG-1/L1 interaction. With the deletion of the FNIII domains and, thus, the disruption of the homophilic interaction, the interaction between TAG-1 and L1 is revealed. In the present study we show, indeed, that TAG-1/L1 *trans*-interactions become evident upon domain deletion. Previous work has proposed the presence of masked binding sites on TAG-1/axonin-1 (Rader *et al.,* 1996) and the existence of overlapping domains through which axonin-1 and F3/F11/contactin interact with L1 (De Angelis *et al.,* 1999). We, thus, propose a complex array of interacting proteins that is modulated by the relative affinities, the folding arrangement, and hidden sites revealed.

L1 and NrCAM are known binding partners of TAG-1 and they have been implicated in guidance choices (Dodd *et al.,* 1988; Stoeckli and Landmesser, 1995; Stoeckli *et al.,* 1997; Fitzli *et al.,* 2000). In our assay we used sensory neurons from L1 deficient mice and, thus, ruled out the requirement for L1 in TAG-1-mediated neurite outgrowth. When NrCAM was tested for involvement in TAG-1-mediated neurite outgrowth, we did not observe any decrease in the extent of neurite outgrowth by inhibiting the action of NrCAM by the use of a blocking antibody. These results are noteworthy in view of recent observations on axonin-1/TAG-1 regarding the preference of commissural axons for a mixed NrCAM/NgCAM substrate over a NgCAM substrate (Fitzli *et al.,* 2000). This preference resulted in different guidance but not altered neurite outgrowth of commissural axons. So far, all evidence obtained for axonin-1 suggests that interaction of these three molecules *in vivo* modulates pathfinding choices, which are distinct from neuritic growth (Lustig *et al.,* 1999; Fitzli *et al.,* 2000; Perrin *et al.,* 2001). In the present study, we have shown a *trans*-interaction of TAG-1 with NrCAM while the *trans*-interaction of TAG-1 with L1 apparently depends on conformational constraints. Taken together, we suggest that the TAG-1/NrCAM interaction does not mediate the neurite outgrowth of DRG neurons on a TAG-1 substrate, but it might serve a different function such as pathfinding. Previous work on axonin-1, which has reported the involvement of axonin-1/Nr-

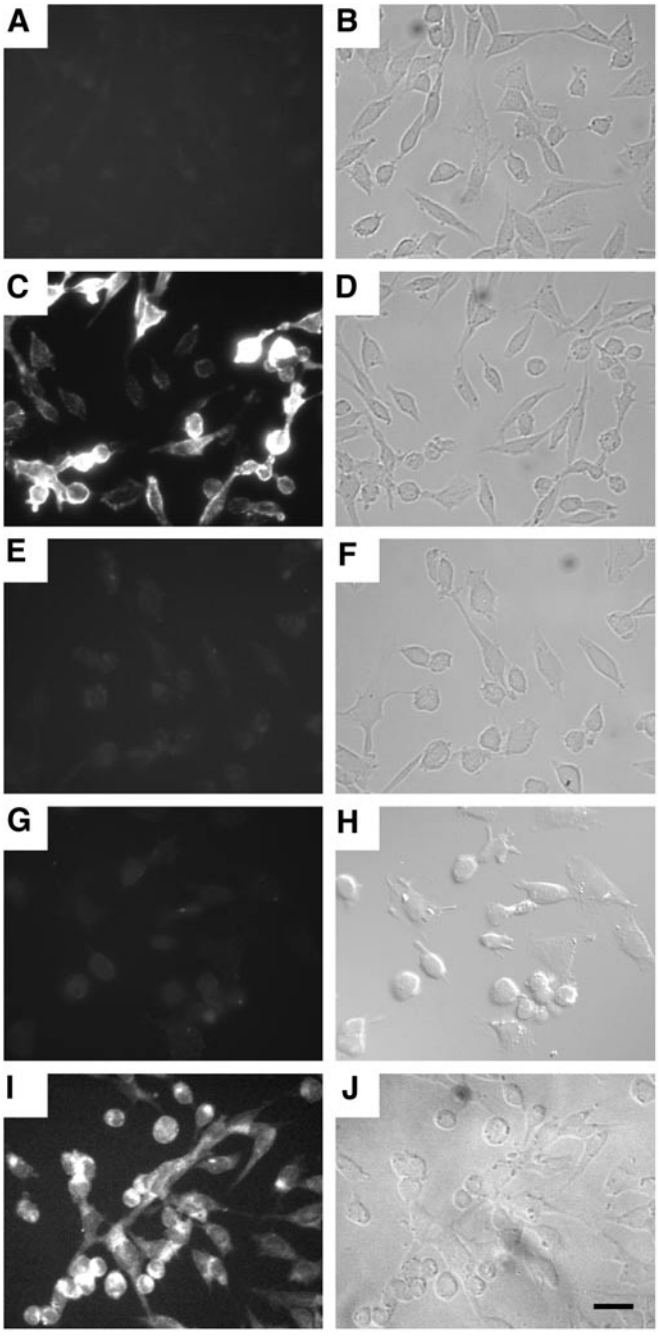



**FIG. 5.** Trans-interactions between soluble and substrate proteins. Soluble proteins in the form of Fc chimeras were clustered via the anti-human Fc antibody and incubated with cell monolayers expressing membrane proteins. Interaction between the soluble and membrane partners was visualized by indirect immunofluorescence with a FITC-conjugated antibody directed against the anti-human Fc. (A) Incubation of preclustered TAG-1(Ig)-Fc with parental CHO cells. No immunofluorescence staining is observed. (B) Phase contrast of A. (C) Binding of preclustered TAG-1(Ig)-Fc to NrCAM-expressing CHO cells. (D) Phase contrast of C. (E) Incubation of preclustered TAG-1(Ig)-Fc with F3-expressing CHO cells. No immunofluorescence staining is observed. (F) Phase contrast of E. (G) Incubation of preclustered F3-Fc with parental CHO cells. No immunofluorescence staining is observed. (H) Phase contrast of G. (I) Binding of preclustered F3-Fc to TAG-1(Ig)-expressing CHO cells. (J) Phase contrast of G. Bar, 20 $\mu$m (A–J).

CAM interaction in neurite outgrowth, differs in the way the two molecules are presented (Lustig *et al.*, 1999). In that particular case the substrate upon which peripheral ganglia grow is NrCAM and its presumed cell receptor mediating outgrowth is axonin-1. In our assay, the substrate monolayer expresses surface TAG-1 and the blocking antibody masks the neuronal NrCAM molecules. It is possible that there is an assymetry in the interaction of TAG-1 / axonin-1 with NrCAM in terms of which protein is the substrate and which is the neuronal receptor. A different kind of asymmetry is demonstrated in this work between TAG-1 and F3 presented as substrate-bound or soluble molecules as discussed below.

Our observations indicate that L1 and NrCAM may not be the sole receptors for TAG-1 and that direct interaction of TAG-1 with either of the two proteins does not imply that L1 and NrCAM are involved in TAG-1-mediated neurite outgrowth. These results reinforce the notion that neurite outgrowth on a TAG-1 substrate is mediated via a complex set of interacting proteins.

### *Different Behavior of the Soluble versus the Membrane-Associated Form of TAG-1 Implies Different Functions in Vivo*

Of particular interest are the results obtained with the binding assay of preclustered soluble molecules to cells. When coupled to fluorescent microspheres, the Fc chimeras of TAG-1 and F3 do not interact with each other. In contrast, we have previously observed that the two molecules expressed at the membrane of transfected CHO cells can associate as analyzed by coclustering and coimmnoprecipitation experiments (Buttiglione *et al.*, 1998). Our present data indicate that the membrane anchored TAG-1 is capable of direct interaction with F3. Therefore, we can hypothesize that TAG-1 is presumably subject to specific steric constraints at the membrane that allow interaction with F3. In addition we observe that substrate-exposed TAG-1 interacts with soluble F3-Fc or F3(Ig)-Fc, but substrate-exposed F3 does not interact with soluble TAG-1-Fc. In an *in vivo* context this might imply separate mechanisms for dif-

**TABLE 3**
Interactions Observed between Preclustered Soluble Proteins and Membrane-Bound Substrate Molecules

| Substrate | TAG-1-Fc | TAG-1(Ig)-Fc | F3-Fc | F3(Ig)-Fc | NrCAM-Fc |
|---|---|---|---|---|---|
| CHO | − | − | − | − | − |
| CHO-TAG-1 | * | − | + | + | + |
| CHO-TAG-1(Ig) | − | +/− | + | + | N.D. |
| CHO-TAG-1(FN) | * | +/− | + | + | N.D. |
| CHO-L1 | − | − | − | N.D. | − |
| CHO-F3 | − | − | − | N.D. | + |
| CHO-NrCAM | + | + | + | N.D. | − |

*Note.* The first column (substrate) denotes the membrane-bound proteins expressed by the various CHO cell lines. The first row lists the preclustered soluble proteins used in Fc chimeric form. *Denotes only clusters observed, indicating homophilic complexes formed by the soluble protein prior to binding to the substrate. N.D., not determined.

ferent recognition events. TAG-1 and other members of the Ig/FNIII family exist *in vivo* in both a soluble and a membrane-bound form, linked via a GPI anchor, with the two forms differentially regulated during development (Dodd *et al.*, 1988; Furley *et al.*, 1990; Karagogeos *et al.*, 1991; Faivre-Sarrailh and Rougon, 1997). It is possible that the two forms serve different functions *in vivo*, interacting with separate receptors; the membrane-bound form with axonal and substrate molecules, *in cis* and *trans*-position, and the soluble form, *in trans*-position with the ability to act at a distance. This notion is consistent with the results presented here as it becomes apparent that TAG-1 shows differences in its interaction with other molecules when presented as a membrane-associated versus a soluble form. These differences could be explained by a change in the tertiary structure of TAG-1 which renders the molecule recognizable by different receptors. Our present data raise the possibility of a direct binding between TAG-1 and F3 that may account for their functional interaction, which we reported in a previous study (Buttiglione *et al.*, 1998).

## EXPERIMENTAL METHODS

***Antibodies.*** Antibodies used for immunofluorescence of neurite outgrowth experiments were 3A10 for neurofilament-associated antigen (Furley *et al.*, 1990) and 5A5 for PSA-NCAM (Dodd *et al.*, 1988). Immunofluorescence and FACS analysis of TAG-1 stably expressing CHO lines were carried out with monoclonal antibodies 1C12 and 4D7 and polyclonal antibodies against TAG-1 (Yamamoto *et al.*, 1986; Dodd *et al.*, 1988). Polyclonal antibody for chick NrCAM (Suter *et al.*, 1995) was used in neurite outgrowth assays with chick sensory neurons. Secondary antibodies (FITC, Cy3) were purchased from Roche (Mannheim, Germany), Sigma-Aldrich (Hellas), and Jackson Immunoresearch (West Grove, PA).

***Constructs.*** All the results presented here refer to the human homolog of TAG-1 (TAX-1) unless otherwise noted. As the experiments showing the homophilic interactions between the FN domains were performed with the human homolog of TAG-1 (Tsiotra *et al.*, 1993, 1996) we continued, for reasons of uniformity, to study structure–function relationships reported here with the same molecule. Figure 1A shows the structure of dimerized soluble TAG-1 proteins in Fc-chimeric form. Figure 1B shows the deletions of TAG-1 that were used for the construction of Fc chimeras. Figure 1C shows the molecules used for the production of CHO lines stably expressing TAG-1 and its domains at the cell surface.

***Production of soluble molecules.*** TAG-1 cDNA and two deleted constructs of it were cloned into the pIG-1 vector in fusion with the human Fc region (hinge, CH2, CH3) (Simmons, 1993). The two deletion constructs included the Ig portion or the FNIII domains (Fig. 1B). The inserts were obtained by PCR using as template pRmHA-TAG, pRmHa-FN and pRmHA-Ig (Tsiotra *et al.*, 1996) and were cloned into the EcoRV/BamHI sites of pIG-1. Soluble F3-Fc and NrCAM-Fc proteins were produced from pIG-F3 (Buttiglione *et al.*, 1998) and pIG-NrCAM (Faivre-Sarrailh *et al.*, 1999) plasmids, respectively. The Fc-chimeric constructs were used for transient transfection of Cos7 cells using lipofectamine (Life Technologies, Gaithersburg, MD). The soluble chimeric protein accumulated in the culture medium (without FCS, at approximately 3 $\mu$g/ml) and could be detected by Western blot analysis. Soluble L1-Fc was

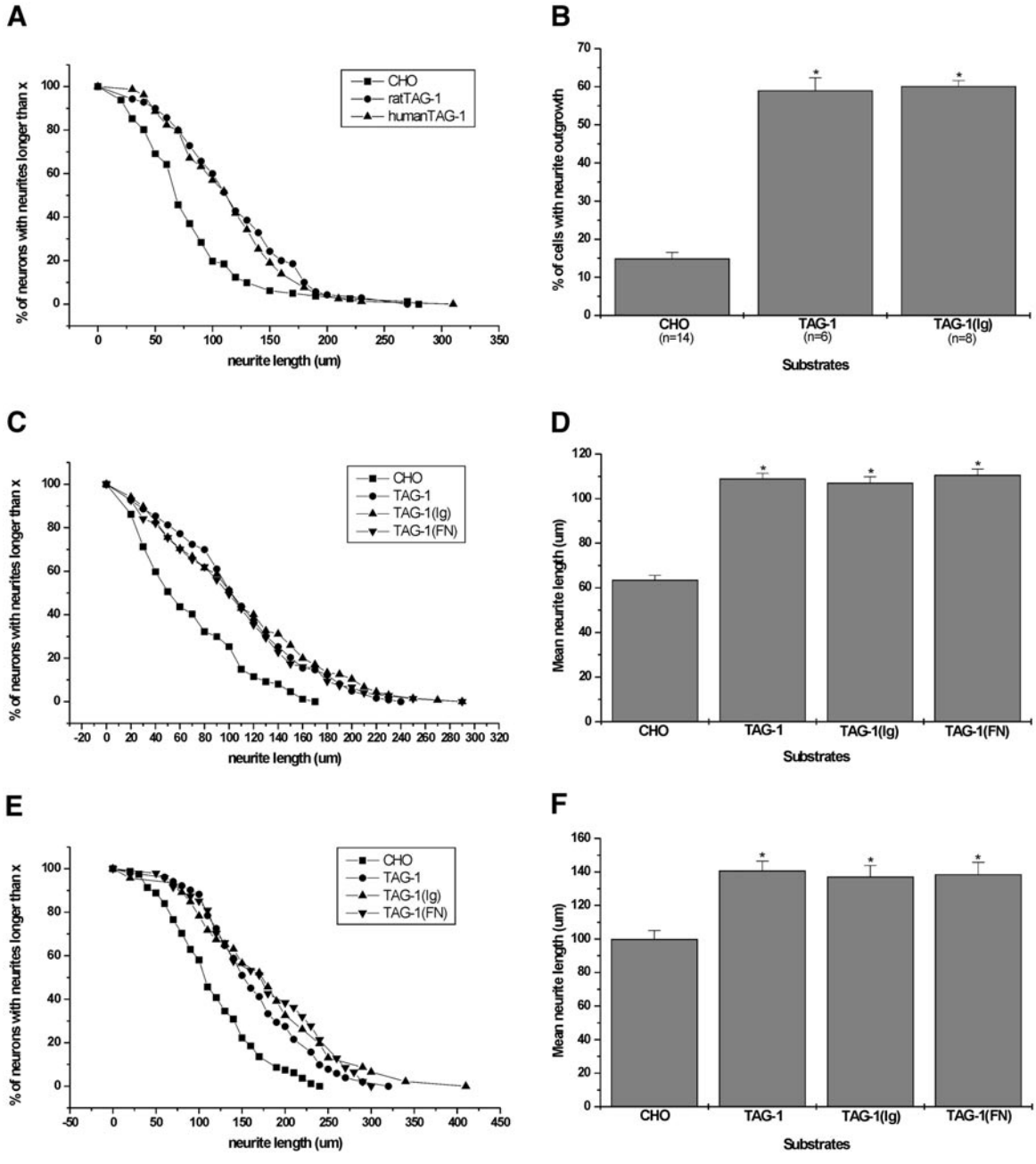



**FIG. 6.** TAG-1 and its isolated Ig and FNIII domains are able to promote neurite outgrowth of avian and mouse sensory neurons. (A) Neurite outgrowth of chick sensory neurons on rat and human TAG-1. Cumulative neurite length histogram showing the distribution of neurite lengths for each substrate after 7 h *in vitro*. Measurements were made on more than 70 neurons in each experimental condition. Distribution was plotted as percentage of neurons with longest neurite (y axis) longer than a given length (x axis). (B) TAG-1 is a potent substrate for the initiation of neurite outgrowth. Proportion of mouse sensory neurons with neurites grown on control, TAG-1 and TAG-1 (Ig) substrates after 3 h *in vitro*. The graph quantifies the percentage of cells that extended neurites over the total number of cells that adhered on the different substrates. Mean values ± SEM. The number of separate experiments examined for each experimental condition (*n*) is indicated. The total number of neurons examined for each substrate is at least 2500. *Significant difference ($P < 0.01$) with CHO values using ANOVA. (C) Neurite outgrowth of chick sensory neurons grown on TAG-1 and its isolated domains. Cumulative neurite length histogram from one representative experiment out of three. In the particular experiment measurements were made on more than 90 neurons for each substrate after 7 h *in vitro*. Similar results were obtained for longer culture times (14 h *in vitro*). (D) Mean neurite length of chick sensory neurons on the different substrates. Mean values ± SEM from three independent experiments. Measurements were made on more than 280 neurons for each experimental condition. *Significant

obtained from a CHO line stably expressing this molecule (Chen *et al.*, 1999).

***Production of CHO lines stably expressing cell surface-associated adhesion molecules.*** The CHO line stably expressing rat TAG-1 has been described previously (Buttiglione *et al.*, 1998). The constructs used for the other cell lines were prepared as follows: for human TAG-1 and its Ig domains the complete constructs were cloned using a two-step approach. First the EcoRI/Xbal insert from pRmHa-TAG-1 and pRmHa-Ig (Tsiotra *et al.*, 1996) was cloned into pCDNA3. Finally, a 400-bp *Eco*RI fragment was cloned into the *Eco*RI site of the intermediate construct and its orientation verified. The FNIII insert was obtained by PCR using pRmHa-FN (Tsiotra *et al.*, 1996) as template and cloned into pcDNA3/EcoRI/Xbal. The constructs (Fig. 1C) were used for the transfection of CHO cells using lipofectamine (Life Technologies). The cells were grown in the presence of G418 (Life Technologies), plated at limiting dilutions and individual colonies were selected. Several clones were picked for each construct and tested for expression levels using immunofluorescence. The stably transfected cell lines chosen for further experiments expressed high levels of surface protein on 90–100% of all cells, as judged by immunofluorescence using monoclonal antibodies 4D7 and 1C12 (for CHO-TAG-1 and CHO-TAG-1(Ig)) and polyclonal antibodies against TAG-1 for (CHO-TAG-1(FN)) (Figs. 3C, 3E, and 3G). The monoclonal antibodies recognize epitopes in the Ig portion of the molecule and the polyclonal antibodies recognize better the FNIII domains. The levels of the expressed proteins were measured using fluorescence activated cell sorting (FACS) analysis after immunofluorescence labeling. FACS analysis of the transfected lines was carried out with antibody 1C12 for CHO-TAG-1 and CHO-TAG-1(Ig) and with polyclonal antibodies to TAG-1 for CHO-TAG-1 and CHO-TAG-1(FN). Briefly, approximately $10^6$ parental or transfected CHO cells were harvested in PBS, pelleted, and resuspended in PBS/1%BSA/0.02% $NaN_3$ and incubated with antibody 1C12 (1:10,000) or polyclonal antibodies to TAG-1 (1:50) for 30 min on ice. The cells were washed, incubated with FITC-conjugated anti-mouse

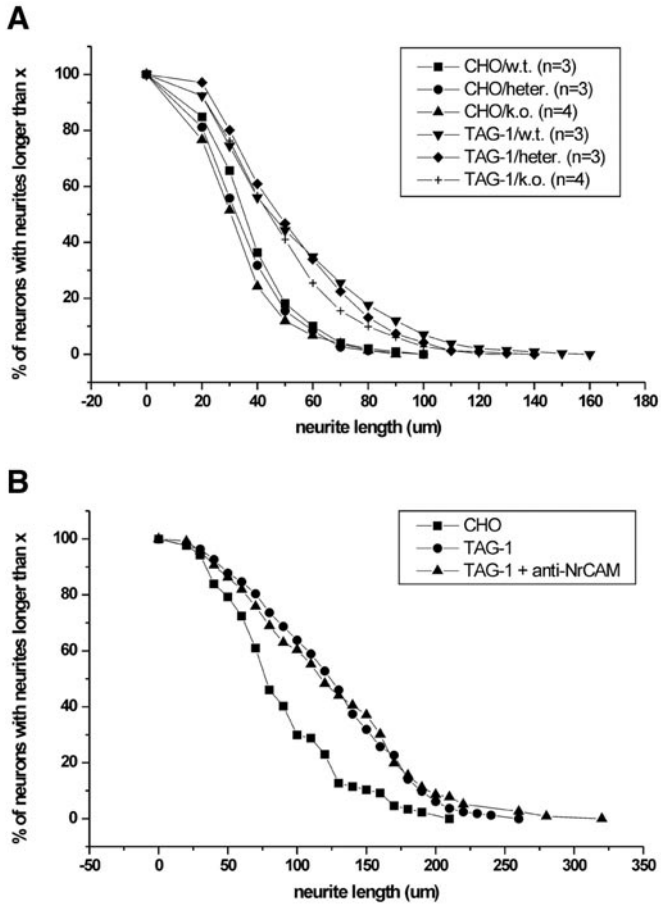



**FIG. 7.** L1 and NrCAM are not required for TAG-1-mediated neurite outgrowth. (A) Neurite outgrowth on TAG-1 does not require neuronal L1. Cumulative neurite length histogram from sensory neurons of wild-type and mutant mouse embryos grown on TAG-1 substrate. In the particular experiment, measurements were made on more than 300 neurons for each genotype after 3 h *in vitro*. Numbers of separate animals examined per genotype are indicated on the graph (*n*). w.t., wild type; heter., heterozygous; k.o., L1-deficient mice. (B) Neurite outgrowth on TAG-1 is unaffected by the presence of anti-NrCAM blocking antibody. Chick sensory neurons were grown for 7 h on TAG-1 substrate with anti-NrCAM blocking antibody added to the culture medium. Cumulative neurite length histogram in a representative experiment of two. In the particular assay, measurements were made on more than 80 neurons for each experimental condition.

difference ($P < 0.01$) with CHO values using ANOVA. (E) Neurite outgrowth of mouse sensory neurons grown on TAG-1 and its isolated domains. Cumulative neurite length histogram after 7 h *in vitro* from one representative experiment of two. In the particular experiment measurements were made on more than 50 neurons for each substrate. Similar results were obtained for shorter culture times (3 h *in vitro*). (F) Mean neurite length of mouse sensory neurons on the different substrates. Mean values ± SEM from two independent experiments. Measurements were made on more than 90 neurons for each experimental condition after 7 h *in vitro*. *Significant difference ($P < 0.01$) with CHO values using ANOVA.

IgG (1:200) (Jackson Immunoresearch, U.S.A.) or anti-rabbit IgG (1:200) (Jackson Immunoresearch), for 30 min on ice, and analyzed with a FACSscan flow cytometer (Becton and Dickinson, U.S.A.). Appropriate controls included unstained cells, incubation with FITC-conjugated antibody only, or unrelated first antibody of the same isotype. As indicated in Figs. 4A and 4B the transfected lines express significant amounts of recombinant protein. CHO-TAG-1(FN) has a broader FACS profile and slightly higher levels than CHO-TAG-1 (Fig. 4B).

The CHO line expressing NrCAM was obtained as follows: the coding sequence for rat Nr-CAM (Davis *et al.*, 1996) was inserted into pIRES at the *Eco*RI cloning site and stably transfected CHO cells were obtained after G418 selection. Expression was checked by Western blot analysis and immunofluorescence using a polyclonal rabbit anti-NrCAM12 antibody raised against the 6xHis-tagged fusion protein with the Ig2–6 domains of rat NrCAM12. The CHO lines expressing L1 (Chen *et al.*, 1999) and F3 (Durbec *et al.*, 1994) have been described previously.

***Binding assays with soluble proteins.*** The soluble Fc-chimeras (15 $\mu$g/ml) were coupled to fluorescent microspheres (fluorospheres, 1-$\mu$m diameter, Molecular Probes, Eugene, OR) as described previously (Buttiglione *et al.*, 1998). Briefly, microspheres were first coated with 50 $\mu$g/ml of anti-human Fc Ig and then incubated until saturation with the various Fc chimeras. Therefore the amount of coated Fc chimera was identical for all the adhesion molecules tested. The amount of bound chimera was estimated to be 5 $\mu g/10^9$ microspheres. For the binding assay, soluble proteins coupled to beads of two different colors were mixed and incubated for 1 h at 37°C as described previously (Faivre-Sarrailh *et al.*, 1999). Aggregates were visualized on a Leica TCS-NT laser scanning confocal microscope.

For the detection of interactions between soluble and substrate-attached molecules the chimeras (15 $\mu$g/ml) were crosslinked using an anti-human Fc antibody (50 $\mu$g/ml) (Jackson Immunoresearch) for 1 h at 37°C and used for binding studies on cultures of transfected CHO cells. Culture supernatant containing soluble chimeras preclustered with the anti-human Fc antibody (rabbit IgG) was incubated with CHO cells stably expressing TAG-1, NrCAM, L1, F3, or subdomains of them, for 1 h at 37°C. The interaction between the soluble and the substrate proteins was visualized using FITC-conjugated anti-rabbit IgG antibody (Jackson Immunoresearch) (Faivre-Sarrailh *et al.*, 1999).

***Neurite outgrowth assays.*** Transfected and control CHO cells were plated and grown to confluency in 35-mm petri dishes or on glass coverslips in 24-well culture plates prior to plating of sensory neurons. Dorsal root ganglia neurons (DRG) from embryonic day (E) 12- to 14-day mouse embryos, or from E5 chick embryos were dissected, trypsinized and plated on the CHO monolayers and cultured for 3–7 h (mouse) or 7–14 h (chick). The neurons were stained for neurofilament-associated antigen and PSA-NCAM and visualized using a FITC or Cy3 conjugated secondary antibody (as described by (Furley *et al.*, 1990; Karagogeos *et al.*, 1991; Felsenfeld *et al.*, 1994). Experiments with L1-deficient embryos were carried out with E12–14 embryos from the breeding of an L1 heterozygous female with a C57BL/10 wild-type male. Each embryo was dissected and processed separately and genotyped for the status of the L1 allele by PCR. Experiments with polyclonal anti-NrCAM blocking antibody were carried out with DRG neurons from E5 chick embryos since the antibody recognizes only chick NrCAM (Suter *et al.*, 1995). Purified anti-NrCAM IgG was included at 0.125–0.250 mg/ml in the culture medium for the entire coculture period (7 h) (Faivre-Sarrailh *et al.*, 1999).

***Quantification of neurite lengths.*** Systematic scan of the culture plates included capturing of the cell images on a Hamamatsu C5985 CDC camera. Cells chosen for quantification were isolated ones that did not make contact with other cells. The neurofilament or PSA-NCAM immunolabeled neurites scored were equal to or longer than two cell body diameters. In the case of multiple neurites per cell body only the longest one was measured. Cells without neurites were not included in the plots. Analysis of images and measurement of neurites was performed on a Macintosh Power PC using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image). Plotting and analysis of the data were performed with the Cricket Graph III and Microcal Origin 5.0 software.

***Scoring the percentage of cells with neurite outgrowth.*** The experiments scored for the degree of neurite extension versus adhesion were from assays with sensory neurons grown on the parental CHO cell line or on CHO cells expressing the total extracellular domain of TAG-1 or its Ig domains. The images, taken as described above, were scored for the portion of cells extending neurites over the total number of cells that adhered to the monolayer. The criterion for neurite outgrowth was again length equal to or greater than two cell bodies.

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