The Fibronectin Domains of the Neural Adhesion Molecule TAX-1 Are Necessary and Sufficient for Homophilic Binding*

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Cell adhesion molecules belonging to the immunoglobulin superfamily promote cell aggregation and neurite outgrowth. These proteins are multidomain molecules comprising a number of distinct modules, notably Ig domains of the C2 class and fibronectin type III repeats. A subgroup of these neural adhesion molecules are linked to the membrane with a glycosylphosphatidylinositol anchor and show a more restricted pattern of expression in the embryo. Among them, the human homologue of the transient axonal glycoprotein, named TAX-1, shares a great degree of similarity at the protein level with rodent TAG-1. In the present study we set out to determine which domains of TAX-1 are involved in promoting the homophilic, adhesive properties of the molecule. We established stable Schneider-2 cell lines expressing the intact molecule, the fibronectin, or the immunoglobulin domains. The fibronectin domains were necessary and sufficient to mediate homophilic binding and induce cell aggregation, a response also observed with cells expressing the intact TAX-1 molecule. Aggregation was inhibited by the secreted form of the TAG-1 protein. On the other hand, the immunoglobulin domains by themselves were not able to induce cell aggregation. In addition, TAX-1 was localized in areas of cell contact among aggregating cells, justifying its role as an adhesion molecule.

During the establishment of the body plan but also during the development of the nervous system, selective adhesive interactions are responsible for cell segregation. Through the specific expression of adhesive or anti-adhesive components, the development of organized layers and the establishment of boundaries in the central nervous system is also achieved. Moreover, adhesion, both on ECM and on cell surfaces, seems to be a prerequisite of axon outgrowth and fasciculation (1, 2).

Cell adhesion molecules (CAMs) belonging to the immunoglobulin superfamily (IgSF), among others, have been implicated to act both as receptors on the growth cone and as substrates for growing axons (3–6). Some of these molecules participate in axonal guidance and migration, as has been demonstrated by antibody perturbation or genetic studies in vivo (7–10).

CAMs belonging to the IgSF group are multidomain molecules comprising a number of distinct modules. Proteins with Ig domains of the C2 class (IgC2) and fibronectin type III repeats (FNIII), constitute a subfamily and are expressed mainly, but not exclusively, in the nervous system. Members include N-CAM, contactin/F11/F3, Ng-CAM/G4, Nr-CAM, and TAG-1/axonin-1, among many others (5, 11).

The complexity of the molecular structure of IgSF molecules may point to the possibility that distinct functions are mediated by different domains. There is growing evidence that different members of this family interact with themselves (homophilic binding) or with yet unidentified receptors (heterophilic binding) in order to promote adhesion and axon outgrowth (12–21). Most members of the neural adhesion subfamily have been shown to possess both such adhesive and axon outgrowth activities in vitro (5, 11). In various molecules studied, these activities have been mapped to both the IgC2 and FNIII or independently on either one of the two domains (17, 22–25).

A subgroup of neural adhesion molecules of the IgSF group are linked to the membrane with a glycosylphosphatidylinositol (GPI) tail and show a more restricted pattern of expression in the embryo. TAG-1 and F3 in rodents and their chick homologs, axonin-1, and F11/contactin are included in this subgroup (6). Recently, TAG-1 has been shown to mediate homophilic as well as heterophilic binding to an L1-like molecule and β1 integrins (20) and axonin-1 interacts with itself but also recognizes an Ng-CAM molecule (14, 26).

We have cloned and characterized the human homologue of TAG-1, which we call TAX-1 (for TAG-1 and axonin-1) (27). TAX-1 is very similar to rat TAG-1, and its gene is localized on chromosome 1q32 (27). Deletion of an area including 1q32 has been demonstrated to occur in some cases of microcephaly and van der Woode syndrome (28–30). The expression pattern of TAX-1 in the developing human embryo suggests that it is involved in early steps of axonogenesis. In the present study we set out to determine which domains of TAX-1 are involved in promoting the homophilic, adhesive properties of the molecule. Our data demonstrate that the fibronectin type III domains are necessary and sufficient to mediate homophilic binding and induce cell aggregation. This response is inhibited by 3,3′,3′-dioctadecyl-3,3,3′,3′-tetratethylindocarbocyanine perchlorate.

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‡ The abbreviations used are: CAM, cell adhesion molecule; FNIII, fibronectin type III; GPI, glycosylphosphatidylinositol; IgSF, immunoglobulin superfamily; PI-PLC, phosphatidylinositol-specific phospholipase C; S2, Scheider type 2 cell line; TAB-1, transient axonal glycoprotein; bp, base pair(s); kbp, kilobase pair(s); FCS, fetal calf serum; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; DI, 3,3′-dioctadecylxcarbocyanine perchlorate; DIo, 1,1′-dioctadecyl-3,3,3′,3′-tetratethylindocarbocyanine perchlorate.

§ K. Theodorakis and O. Pourquie, unpublished observations.

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the secreted form of the protein. On the other hand, the immunoglobulin domains by themselves are not able to induce this activity.

EXPERIMENTAL PROCEDURES

Generation of the Full-length TAX-1 cDNA and Deletion Constructs—Human cDNA clones encoding TAX-1 were isolated as described before (27). The cloning strategy as well as the resulting proteins are depicted schematically in Fig. 1. The constructs were cloned into the HumancDNA clones encoding TAX-1 were isolated as described before (27). The cloning strategy followed. The diagram on the left shows a 3-kbp Ncol-AhaIII fragment of the TAX-1 cDNA encoding the whole TAX-1 protein ligated into vector pRmHa-3. The diagram in the middle shows a 1.6-kbp Ncol-AhaIII TAX-1 cDNA fragment encoding the signal sequence (Ncol-Xmnl) in frame to the FNIII repeats together with the GPI anchor, ligated into the same vector. The diagram on the right shows the HT-IgC2 construct, which contains the signal sequence and the IgC2 domains in frame with a fragment containing the GPI anchorage signal. The bottom diagram schematically depicts the structural features of the resulting proteins. IgC2 domains are shown as loops, and FNIII domains as dark boxes.

Stable S2 Cell Lines Expressing Different Domains of TAX-1 Protein—Schneider line 2 (S2) cells (a gift of Dr. P. Cherbas) expressing TAX-1 and its truncated forms were generated essentially as described (31). S2 cells were maintained in M3 or Schneider's medium supplemented with 12.5% heat-inactivated fetal calf serum (FCS, Life Technologies, Inc.), 100 units/ml gentamycin, at 25 °C with air as the gas phase. In order to obtain S2 cells expressing high levels of TAX-1, S2 cells were co-transfected with either one of the three constructs (TAX-1, HT-IgC2, or HT-FNIII) together (in a 1:5 ratio) with the plasmid pHGO, which confers methotrexate resistance (a gift of Dr. L. Cherbas). Cells were left for 2 days to express the dihydrofolate reductase gene, and then they were plated in limiting dilutions in the presence of methotrexate. The cells were selected over a period of 6 weeks when individual clones were assayed either by Western blotting or immunofluorescence for the highest level of expression of recombinant proteins. These cell lines were designated S2/TAX-1, S2/HT-FNIII, and S2/HT-IgC2, respectively. In the mixing experiments, transient transfections were performed before cell aggregation, in order to obtain even higher levels of expression.

S2 Cell Aggregation Assay—For the cell aggregation assay, cells were plated at 10^6 cells/ml in 100-mm Petri dishes and were induced for 14–24 h, with 0.7 mM CuSO4. After induction, cells were washed, dissociated by gentle pipetting, and resuspended in PBS with 2 mg/ml bovine serum albumin (Sigma). Then they were plated at a density of 10^6 cells/well in a 24-well plate and allowed to aggregate in a rotation platform, at 120 rpm for 60 min, a time determined to be optimal for the aggregation procedure. At the end of the aggregation period, cells were fixed by the addition of 0.5 ml of 4% paraformaldehyde in PBS and were left to rotate for another 10 min. Cell aggregates were visualized and photographed on an Olympus microscope with phase contrast optics.

In cell adhesion assays that required the mixing of two cell lines expressing different forms of the TAX-1 molecule, cell labeling was done as follows: 1 μl of 2.5 mg/ml DiI (3,3'-dioctadecylxocarbocyanine perchlorate) or DiO (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) fluorescent lipophilic dyes (in ethanol, Molecular Probes), was added to 1 ml of cells for a period of 1 h. Then cells were washed three times with PBS to remove excess dye, and the two populations were mixed at a concentration of 2 x 10^6 each and left to

FIG. 1. Construction of TAX-1 cDNAs containing the intact protein, the FNIII repeats, or the IgC2 domains. The top diagrams show the cloning strategy followed. The diagram on the left shows a 3-kbp Ncol-AhaIII fragment of the TAX-1 cDNA encoding the whole TAX-1 protein ligated into vector pRmHa-3. The diagram in the middle shows a 1.6-kbp Ncol-AhaIII TAX-1 cDNA fragment encoding the signal sequence (Ncol-Xmnl) in frame to the FNIII repeats together with the GPI anchor, ligated into the same vector. The diagram on the right shows the HT-IgC2 construct, which contains the signal sequence and the IgC2 domains in frame with a fragment containing the GPI anchorage signal. The bottom diagram schematically depicts the structural features of the resulting proteins. IgC2 domains are shown as loops, and FNIII domains as dark boxes.
aggregate as above. Samples were visualized on a Zeiss Axiophot microscope by epifluorescence and phase contrast optics. Alternatively, cell labeling was done by staining with two different antibodies recognizing rat TAG-1 protein (32). Incubation with a monoclonal antibody recognizing only the IgC2 domains for 1 h, was followed by a polyclonal antiserum for an additional hour. Finally secondary antibodies conjugated with either rhodamine or fluorescein (Boehringer Mannheim) were used, and samples visualized on a Zeiss Axiophot microscope by epifluorescence and phase contrast optics.

Soluble TAG-1 used in aggregation assays was a gift of Dr. T. Jessell (20).

Quantitation Analysis of Cell Aggregation—At selected time points during the period of aggregation, aliquots of cells were placed on slides and analyzed for cell adhesion while the remaining cells were processed for immunostaining. Serial, nonoverlapping fields were counted at 150-fold magnification. Aggregation was quantified as the loss of single cells over time. To exclude nonspecific adhesion, only cell clusters that contained more than 4 cells were counted. The number of cells in the large aggregates (greater than 80 cells) was difficult to count precisely. This approach does not take into account the aggregate size, which varies depending on the percentage of cells expressing the proteins as well as with expression levels in each cell line.

PI-PLC Treatment—Phosphatidylinositol-specific phospholipase C (PI-PLC) from Bacillus aureus was obtained from Boehringer Mannheim. After induction, S2 cells were incubated with 80 milliunits of PI-PLC/4 × 10^6 cells for 30 min at 37°C in 60 mM Tris-ethanolamine. When the incubation period was over, cells were collected by centrifugation at 900 × g and the supernatant was kept for the detection of the proteins by Western blotting. The remaining cells were diluted in PBS and tested in aggregation assays as above.

Isolation and Analysis of the S2 Recombinant Proteins—6–8 × 10^5 S2 cells were induced overnight with 0.7 mM CuSO4. Cell lysates were prepared after homogenization on ice for 15 min, in 0.05 M Tris-Cl pH 7.2, 1 mM phenylmethylsulfonyl fluoride, 5 mM MgCl2, 1% Nonidet P-40, followed by centrifugation at 10,000 × g for 10 min. Proteins were fractionated on 8% SDS-PAGE, under reducing conditions, followed by overnight transfer on nitrocellulose filters (Schleicher & Schuell). The filters were incubated with a polyclonal antibody against the rat TAG-1 protein (a gift of Dr. T. M. Jessell; Ref. 32), followed by peroxidase-conjugated secondary antibodies (Boehringer Mannheim). The samples were visualized by the Enhanced Chemiluminescence (ECL) detection system (Amersham).

Immunocytochemical Localization—For immunostaining, cells were fixed with 2% paraformaldehyde in PBS for 10 min. Cells were then washed twice with PBS supplemented with 1% FCS and were incubated with primary antibodies (diluted in PBS, 1% FCS) for 60 min at room temperature.
temperature. Monoclonal antibody 1C12 and four different polyclonal antisera recognizing rat TAG-1 were used (32). Samples were washed twice in PBS, 1% FCS before the incubation with the fluorescein isothiocyanate-conjugated secondary antibodies diluted in PBS, 1% FCS for 30–60 min at room temperature. Cells were washed with PBS, 1% FCS and mounted in 4% o- n-propylgallate in glycerol. Samples were visualized on a Zeiss Axiophot microscope by epifluorescence and Nomarski optics.

SamplestobeanalyzedonaCoulterEpicsCytometerwerepreparedasfollows;106 cellswereinducedforproteinexpression24hbeforeuse. After two washes in PBS, 1% FCS, they were incubated at room temperature with primary antibodies for 30 min, washed twice with PBS, and incubated with fluorescein-conjugated secondary antibodies diluted in PBS, 1% FCS for 30 min. After two final washes, they were analyzed on a Coulter Epics Cytometer.

RESULTS

Construction and Characterization of Cell Lines Expressing TAX-1 and Its Truncated Forms—In order to assay the in vitro cell adhesion activity of TAX-1 and to map the functional domains responsible for this activity, we devised an experimental model in which a recombinant TAX-1 protein carrying either all or some of the TAX-1 domains is expressed in a nonadherent cell line. Fig. 1 describes the strategy used to construct the cDNAs to be transfected into the D. melanogaster S2 cell line. We chose this line because its nonadherent nature provides us with a reliable indicator of binding activities and also because any putative TAG-1 receptor is unlikely to be expressed in Drosophila cells (33, 34). The S2 assay is likely to reflect the normal mode of presentation of TAX-1 on the neuronal cell surface, rather than protein immobilized on an inert substrate. This assay has also been utilized to show both homophilic and heterophilic interactions between a number of invertebrate proteins such as Delta, Notch (35), Boss, Sevenless (36), and more recently neuroglian (37), as well as vertebrate ones such as TAG-1 and the receptor tyrosine kinase ARK (20, 38).

The stable cell lines expressed high levels of protein as shown in Figs. 2 and 3. The construct TAX-1 produced a protein with a molecular mass of 132 kDa (Fig. 2, A and B, lane 2), slightly lower than the rat protein (135 kDa). This could be attributed to incomplete glycosylation in S2 cells, as noticed in other cases (38). Constructs HT-FNIII (FNIII molecule) and HT-IgC2 (IgC2 molecule) produced proteins of 60 (Fig. 2A, lane 1, filled arrow) and 70 kDa, respectively (Fig. 2B, lane 1, filled arrow). The FNIII protein always produces an immuno reactive doublet, probably indicating different glycosylation products.

In order to show that the recombinant molecules are all...
linked to the cell membrane via a GPI anchor, thus maintaining a configuration as close to the native protein as possible, we analyzed S2 cell extracts and supernatants after PI-PLC digestion. Recombinant TAX-1 protein is found solely in the supernatant fraction after digestion with PI-PLC, thus verifying that the protein is attached to the membrane via a GPI anchor. The same was true for FNIII and IgC2 recombinant proteins (data not shown; see also Refs. 20 and 39). No immunoreactive TAX-1 band is detected in untransfected S2 cells (data not shown).

The intact molecule as well as the truncated proteins IgC2 and FNIII were detected with monoclonal and polyclonal antibodies to rat TAG-1 (Fig. 3, panel A, a–c). Immunofluorescence assays showed that 70% of cells expressing TAX-1 were immunoreactive, as compared to 48% of cells expressing FNIII and 50% of cells expressing IgC2 only. As has been noticed for TAG-1 in vivo and in vitro (40), both TAX-1 and HT-FNIII are concentrated at junctions between S2 cells (Fig. 3, panel B, a and b, arrows).

**TAX-1 Can Mediate Homophilic Aggregation Which Is Abolished by PI-PLC and Soluble TAG-1—**S2 cells that express TAX-1 form large aggregates of a few hundred cells within 15 min (Fig. 4, left panels). The aggregation process reaches a saturation point at 60 min. All the cells within the aggregates strongly expressed TAX-1 while cells not incorporated in the aggregates did not express detectable amounts of the protein (Fig. 3, panel B; see also the following section and Fig. 8). In order to verify the specificity of the reaction, S2/TAX-1 cells were mixed with parental S2 cells transfected with the vector only, labeled with the lipophilic dyes DiO and DiI, respectively. Selective aggregation of TAX-1-expressing cells was observed, their aggregates completely excluding vector-transfected S2 cells (data not shown).

In addition, when S2/TAX-1 cells (Fig. 4, left panels) were treated with the enzyme PI-PLC before the adhesion assay was performed, no cell aggregation was evident in the treated cells (Fig. 4, right panels). Addition of soluble TAG-1 (20 μg/ml) during the adhesion assay abolished the aggregation response (Fig. 5, a and b).

**The Fibronectin Domains of TAX-1 Are Necessary and Sufficient for Homophilic Binding—**To determine which domains of the TAX-1 molecule are involved in cell binding and aggregation, we used S2 cells expressing the FNIII domains of TAX-1 in aggregation assays (Fig. 6, left panels). The adhesive properties of these cells were comparable to cells expressing full-length TAX-1. Again, PI-PLC completely abolished aggregation of these cells (Fig. 6, right panels). Thus the FNIII domains of TAX-1 are able to promote cell aggregation on their own.

To test whether adhesion is determined solely by the FNIII domains, we used the S2/HT-IgC2 cells in the same type of experiment. The results shown in Fig. 7 (top two panels) demonstrate that no significant aggregation was observed in cells expressing the IgC2 domains of TAX-1, even at a 60-min time point. S2 cells transfected with the vector only showed no aggregation at all (Fig. 7, bottom panels).

Since the above experiments do not exclude the possibility of additional interactions between the FNIII and the IgC2 domains, we performed a mixing experiment between DiI-labeled S2/FNIII and DiO-labeled S2/IgC2 cells. Fig. 8 (top panels) showed that no IgC2-expressing cells (panel c) participate in the aggregate formed by FNIII cells (panel b). In addition, mixing of S2/TAX-1 and S2/FNIII cells labeled with two antibodies, a monoclonal one that recognizes an epitope on the IgC2 domains only, and a polyclonal one, showed that all aggregates observed contained both types of cells (Fig. 8, bottom panels, b and c). These results further support the conclusion that homophilic binding of TAX-1 involves primarily the FNIII domains and that the FNIII region interacts equally well with the intact TAX-1 molecule. Most of the aggregates contain small clusters of S2/FNIII cells within S2/TAX-1 clusters, probably due to the lower percentage of immunopositive cells in the

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**Fig. 8.** The FNIII repeats can not interact with the IgC2 domains but they aggregate with TAX-1-expressing cells. The top panel shows DiI-labeled S2/FNIII cells mixed with an equal number of DiO-labeled S2/IgC2 cells. The S2/FNIII cells form aggregates excluding the S2/IgC2 cells which do not aggregate: a, phase-contrast micrograph; b, same field under rhodamine epifluorescence, showing S2/FNIII cells; and c, same field under fluorescein filter showing S2/IgC2 cells. Bar, 20 μm. The bottom panel shows S2/TAX-1 cells co-aggregating with S2/FNIII cells: a, phase-contrast micrograph of three different aggregates; b, same field under the fluorescein filter. A monoclonal antibody was used that recognizes only the IgC2 domains, followed by a fluorescein-conjugated anti-mouse antibody, thus marking S2/TAX-1 cells alone in the aggregates, and same field under the rhodamine filter (c). A polyclonal antibody that detects both cell lines was used, followed by rhodamine-conjugated anti-rabbit Ig. Arrows point to FNIII-positive S2 cells inside the aggregate. Bar, 60 μm.
**DISCUSSION**

CAMs are thought to play an important role in the development and maintenance of the nervous system by promoting morphological plasticity and maintaining stable contacts between cells. CAMs belonging to the Ig superfamily comprise different structural motifs that may act as distinct functional units. Individual domains from the IgC2 or FNIII repeats can interact and participate in the adhesive activities either in a homophilic or heterophilic fashion.

The _in vitro_ studies presented here show that TAX-1, the human homologue of rat TAG-1 and chick axonin-1, can bind in a homophilic fashion thus promoting cell aggregation. Homophilic binding is detected when the FNIII domains of TAX-1 are expressed on the cell surface of the non-adherent _D. melanogaster_ S2 cell line. In contrast, S2 cells that express the IgC2 domains of TAX-1 are not able to induce cell aggregation. The kinetics and capacity of adhesion (Fig. 9A) of cells expressing intact TAX-1 and FNIII domains only, are comparable, thus leaving little room for a significant contribution of the IgC2 domains. In addition, cell mixing experiments provide further evidence that the FNIII domains alone are actually mediating the homophilic interaction observed. Whether the IgC2 domains are involved in the putative neurite outgrowth activity of TAX-1, as has been shown to be the case for F3, is currently under investigation.

How are homophilic adhesive events relevant to developmental processes? A number of mechanisms have been postulated (10, 39, 42–44). The simplest idea would be that TAG-1/TAX-1 may provide positional information to another cell or axon. Thus, a homotypic interaction may provide a mechanism of recognition and fasciculation. _In vivo_, TAG-1 is expressed in a patchy distribution on the surface of apposing parallel fibers of cerebellar granule cells (40), consistent with the possibility of a homophilic interaction. In line with these observations, it has been proposed that TAG-1 serves a recognition/fasciculation function in these parallel fibers that develop synchronously (40, 45). The observation that TAX-1 is localized in areas of close cell contact in aggregated cells (Fig. 3, panel B) supports the idea that TAX-1-mediated interactions, possibly both in _cis_ and _trans_, result in a planar movement of molecules in the membrane. This may be followed by an increase in the local concentration of TAX-1 molecules in order to establish stable cell-cell interactions.

Other (i.e. heterophilic) types of interactions seem to operate between the membrane-bound form of TAG-1 and other axonal receptors (20), thus also influencing axonal growth. The released form of TAG-1/TAX-1 could interrupt these (homotypic or heterotypic interactions) as has been shown by us (Fig. 5) and others (20) _in vitro_. Thus, it is reasonable to postulate that _in vivo_, axonal growth may be modulated by differential regulation of the GPI-linked and released form of the protein, as has been observed previously _in vitro_ (46).

In our assay we measure only effects of recognition of the membrane forms of TAX-1 on apposing cellular surfaces, as no detectable soluble protein is produced after transfection of the different constructs in S2 cells. Even in permanently expressing lines, the level of expression of TAX-1 is variable (as is the case with F3 transfected lines, see Ref. 25; refer to Fig. 3). Since we observe the same number of _low versus_ high expressors in all three lines and the same intensity of fluorescence among the high expressors, we assume that overall, the number of molecules per cell is not dramatically different in these lines.

Cell aggregation may not involve a direct interaction with intracellular signaling molecules, as was demonstrated in the case of neuroglian, the _D. melanogaster_ homologue of L1, and the murine tyrosine kinase receptor ARK (37, 38). The homophilic binding activities of these molecules are maintained even if the cytoplasmic kinase domain is deleted, an observation consistent with the existence of GPI-anchored proteins able to
mediate homophilic interactions. However, some signaling potential in the case of GPI-linked molecules cannot be excluded.

In general, the mechanisms whereby neuronal IgSF proteins transduce recognition signals intracellularly are not established yet. Some evidence suggests that several tyrosine kinases and phosphatases are involved in the signal transduction machinery of IgSF members (44, 47–52). It would be of interest to determine the transduction mechanism(s) of the TAG-1/TAX-1 proteins.

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