An endogenous lectin and one of its neuronal glycoprotein ligands are involved in contact guidance of neuron migration

(cerebellum/development/astrocyte/recognition/adhesion)

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ABSTRACT In the central nervous system, postmitotic neurons migrate along astrocytic processes to reach their adult position. The molecular mechanisms of this guided migration are not clearly defined, although some steps have been shown to involve proteases and cell adhesion molecules. We report that monovalent antibodies (Fab fragments) raised against an endogenous cerebellar soluble lectin (CSL) completely inhibit neuronal migration in cultures of cerebellar explants at concentrations as low as 50 μg/ml. A similar inhibition pattern was observed with Fab fragments against one of the endogenous glycoprotein ligands of CSL, the 31-kDa glycoprotein (this glycoprotein is a membrane-bound glycoprotein specifically occurring, in the cerebellum, at the surface of immature neurons). We propose that this lectin–glycoprotein interaction supports the adhesion between neurons and the astrocyte guide during the migration of cerebellar immature neurons.

The formation of brain networks owes its reproducibility to multiple steps involving cell interactions during the various stages of ontogenesis. Guided neuron migration constitutes one such step. The concept of contact guidance of migration emerged primarily from the morphological studies of normal or mutant cerebella deficient in cell migration (1–14). In the cerebellum (see Fig. 1 for illustration), granule cells proliferate in an external zone in the proximity of pia mater, the external germinal layer (egl). Subsequently, cells are ready to migrate in the premigratory zone situated at the transition of the molecular layer and the egl. Then, cells cross the molecular layer and the Purkinje cell layer, finally reaching the internal granular layer (igl) (2–4, 11). Such a migration occurs along preexisting radial glial processes (Bergmann fibers).

The cultures of cerebellar explants provide meaningful tools to understand the molecular basis of contact guidance (12–16). For instance, it was observed in this experimental model that protease inhibitors (12, 15) or Fab fragments derived from antibodies specific for some glycoprotein components of the family of cell adhesion molecules (CAMs) [namely, L1/Ng-CAM molecules (13, 14, 16)] inhibit neuron migration. In this paper, it is reported that, at a low concentration, Fab fragments of a polyclonal antibody specific for endogenous cerebellar soluble lectin (CSL) (17–19) are powerful inhibitors of neuron migration. A similar inhibition is obtained with Fab fragments of a monoclonal antibody specific for a 31-kDa glycoprotein transiently present at the surface of the neuronal plasma membrane, which was shown to interact with lectin CSL (20, 21). The data reported here provide evidence that the endogenous lectin CSL and its 31-kDa endogenous glycoprotein ligand, found at the neuronal surface, play a key role in neuron migration.

MATERIALS AND METHODS

Preparation and Characterization of Molecules and Antibodies. Endogenous lectin CSL was isolated from young rat cerebella and polyclonal antibodies were raised against this protein in rabbits (17). The lectin isolated by immunoaffinity chromatography is a large molecule composed of a large number of subunits of 33 and 31.5 kDa (17). It displays specificity for some mannose-rich glycans and for a relatively few endogenous glycoproteins (21) also binding to the plant lectin concanavalin A. Lectin CSL is widely distributed in the nervous tissues and shows developmental regulation in the cerebellar premigratory zone and in white matter (18). A role in cell adhesion has been reported (22–25) for oligodendrocytes, ependymal cells, Schwann cells, and astrocytes. Anti-CSL Fab fragments were prepared (22) by digestion of IgG on immobilized papain (Miles) in cysteine-containing buffer, followed by elimination of undigested IgG and of Fc fragments on immobilized protein A (IBF). The anti-CSL Fab fragments used in these studies were chosen because, at a concentration of 2 μg/ml, they inhibited agglutination of erythrocytes induced by purified lectin CSL at 0.5 μg/ml (21). Their efficiency has also been tested in other systems (22, 25).

Anti-31-kDa rat monoclonal antibody (clone 194-563) was prepared as described (26) by using as an antigen a membrane glycoprotein fraction solubilized by treatment of thymocytes with phosphatidylinositol-specific phospholipase C (26). In the cerebellum of young rats, the antibody revealed a glycoprotein doublet of 31 and 28 kDa (20). This antigen was transiently expressed at the surface of neuronal cells in the developing nervous tissue and was potentially involved in various ontogenetic processes, including neuronal cell migration (20). In particular, it was present in the area of the surface of migrating neurons in contact with astrocytic processes (20). Fab fragments were produced by a procedure similar to that used for preparing anti-CSL Fab fragments with the difference that soluble mercaptapapain was used (instead of immobilized papain). The activity of the Fab fragments was tested by immunofluorescence labeling on cerebellar primary cultures (data not shown).

Cultures of Cerebellar Explants. Cultures were prepared according to Moonen et al. (12) in a serum-free, hormone-supplemented medium (27) (Seromed, Munich) containing transferrin (100 μg/ml; Sigma), insulin (10 μg/ml; Serva),}

Abbreviations: CAM, cell adhesion molecule; CSL, cerebellar soluble lectin; egl, external germinal layer; GFAP, glial fibrillary acidic protein; igl, internal granular layer.

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fatty acid-free bovine serum albumin (1 mg/ml; Serva), selenium (30 nM; Merck), thyroxine (0.1 nM; Serva), glucose (0.25%; Merck). Explants were sterilly dissected from 10-day-old rat cerebellum. Meninges were carefully removed and cortex pieces (1 × 1 × 2 mm) were quickly excised. Explants were labeled for 90 min in defined culture medium (27) with [3H]thymidine at 1 μCi/ml (6.7 Ci/mmol; 1 Ci = 37 GBq; Amersham) (13, 14) and then washed with unlabeled medium. The culture medium was supplemented with Fab fragments prepared from immune or nonimmune rabbit serum (22). The explants were maintained for 3 days in 3 ml of culture medium at 37°C in an atmosphere of 5% CO2/95% air in 25-ml Erlenmeyer flasks on a rotary shaker (12).

**Quantification of Neuron Migration in Cerebellar Explants.**
Cell migration was determined quantitatively by using short time [3H]thymidine labeling of mitotic cells (see above). Explants were fixed by using a mixture of 4% paraformaldehyde and 0.2% glutaraldehyde in 25 mM sodium phosphate buffer containing 0.15 M NaCl (PBS). Frozen sections (10 μm) were cut in the presence of Fab fragments. Bergmann fibers, the astrocytic support of contact guidance of neuron migration, were still radially oriented when detected by immunostaining for the astrocyte marker GFAP (Fig. 1a). The viability of cells, reported earlier by other investigators in the same culture system (12-16), was assessed by the ultrastructural technique (Fig. 1b); in our experiments, the cerebellar explants preserved their gross initial organization up to 9 days in culture (data not shown).

Cell migration was determined quantitatively by using short time [3H]thymidine labeling of mitotic cells. As shown in Fig. 1c, most of these cells were found initially in the cerebellar egl, indicating that the major contingent of labeled cells consisted of neuroblasts. Very few labeled cells could be found by autoradiography in the molecular layer and in the igl; most of them corresponded to cells in the vicinity of blood vessels and the majority were endothelial cells. In the absence of relevant Fab antibody fragments, after 3 days in culture, cells had migrated from egl to molecular layer and to igl (Figs. 1d and 2). This migration of cells was affected (Fig. 2b) by neither PBS buffer (the buffer containing Fab fragments added in low amount to the cultures), nor (Fig. 2a) by antibodies derived from preimmune rabbit serum (50 or 100 μg/ml). This observation on the inefficiency of normal rabbit serum antibodies or of Fab fragments directed against molecules unrelated to cell adhesion mechanisms to inhibit cell migration has also been confirmed by others (13, 14, 16) using the same experimental model.

When added to the culture medium, Fab fragments derived from anti-CSL antibodies (50 μg/ml) almost completely inhibited cell migration (Figs. 1e and 2a). Raising the Fab fragment concentration to 100 μg/ml did not improve its effects (data not shown). An inhibition was also observed, although less intense, at lower concentrations of Fab fragments (30 μg/ml) (Fig. 2c). We verified ultrastructurally that the inhibition produced by Fab fragments was not due to toxic effects on cerebellar cells (Fig. 1b), as was also observed in earlier reports (13, 14, 16). This demonstrated that the inhibition of migration was not due to cell damage. The nature of the molecular targets of anti-CSL antibodies was identified by isolation of anti-CSL reacting molecules in 10-day-old rat cerebella. As shown in Fig. 3, only protein bands at 31.5 and 33 KDa, corresponding to CSL, were reacting with the antibody. The native material isolated by immunoaffinity chromatography displayed agglutinating activity for erythrocytes (21). Furthermore, the hemagglutination induced by this purified CSL at 0.5 μg/ml could be inhibited by low amounts (2 μg/ml) of the specific anti-CSL Fab fragments used in this study (data not shown).

As shown in Figs. 1f and 2b, Fab fragments derived from monoclonal antibody 194-S63 inhibited almost completely

**RESULTS**

In the culture system described above, the different layers of the cerebellar cortex were preserved after 3 days in culture (Fig. 1) in the presence of Fab fragments. Bergmann fibers, the astrocytic support of contact guidance of neuron migration, were still radially oriented when detected by immunostaining for the astrocyte marker GFAP (Fig. 1a). The viability of cells, reported earlier by other investigators in the same culture system (12-16), was assessed by the ultrastructural technique (Fig. 1b); in our experiments, the cerebellar explants preserved their gross initial organization up to 9 days in culture (data not shown).

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As shown in Figs. 1f and 2b, Fab fragments derived from monoclonal antibody 194-S63 inhibited almost completely
cell migration when included in the culture medium at a concentration of 50 μg/ml. In a total homogenate of 10-day-old rat cerebellum, the antibody revealed a protein doublet at 31 and 28 kDa (Fig. 3d), making it likely that the targeting of these molecules was responsible for the inhibition of migration observed. At a concentration of 50 μg/ml, the inhibition was as efficient as the inhibition obtained with anti-CSL antibodies. When the concentration of the antibody was reduced to 30 μg/ml, the effects were significantly lower than at 50 μg/ml and the inhibition produced by anti-31-kDa Fab fragments was comparable to that obtained with anti-CSL Fab fragments at the same concentration (Fig. 2a and b). Thus, both antibodies seemed to have equivalent capability for inhibiting neuron migration. Moreover, using anti-31-kDa Fab fragments (Fig. 1f), the pattern of distribution of cells was very similar to the one obtained with anti-CSL Fab fragments (Fig. 1e); cells did not penetrate significantly in the molecular layer and they accumulated in the egl. This indi-
cerebellum, and material detection of antigens revealed size markers (kDa), total homogenate of 10-day-old rat cerebellum, and material isolated from 10-day-old rat cerebellum by immunofluorescence and immunoelectron microscopy. The antibody used in this study was Lane d, immunoperoxidase detection of antigens revealed by anti-31-kDa antibody in a total homogenate from 10-day-old rat cerebellum. Protein concentrations were 5 µg in lanes a and c and 15 µg in lanes b and d. Note the difference in size of CSL and 31-kDa subunits (31.5 and 33 kDa for CSL and 28 and 31 kDa for the 31-kDa glycoprotein).

cates clearly that, in both cases, antibodies are blocked in the same area, before they enter the molecular layer. They are not blocked in the upper part of the egl or in the molecular layer or when they enter the igl.

DISCUSSION

The mechanism of contact guidance of cell migration probably involves several molecular mechanisms from the stage where neuroblast precursors of granule cells proliferate in the egl to the final position of granule cells in the igl. From morphological evidence, sustained by immunocytoclochemical studies, the egl is a very compact cerebellar layer (2, 20, 31), with cells in very close apposition. Several mechanisms, including CAMs and endogenous lectins (13, 31-33), have been proposed to explain the cell adhesive capacity in this layer. The compact structure of the cells is suddenly broken when cells are leaving the egl. This transition from a densely compacted to a loosely compacted structure suggests that initial adhesion mechanisms are suppressed. The involvement of proteases in the initial stage of migration (12, 15, 34) is compatible with this change in adhesive capacity.

From the localization of CSL (18) and of its endogenous ligand (clearly not concentrated on the surface of mitotic neurons, but heavily expressed on postmitotic neurons (20)), it is suggested that the effect of Fab fragments we observed is not related to the homotypic adhesion of neuroblasts in the upper part of the egl. In addition, only the 31-kDa glycoprotein, but not CSL, is present in the middle part of the egl (20). Cell adhesion in this area is probably due to CAMs and/or to β-galactoside binding endogenous lectins (31-33). In contrast, it is suggested, from the localization of CSL in the presumptive granule cell lineage, that CSL interactions with 31-kDa glycoprotein take place later (20) and, thus, that the CSL interactions are essentially concerned with the migration step. It is worthy of mention that the 31-kDa glycoprotein has been identified as a phosphatidylinositol glycan anchored molecule (26). This property could be essential for migration, since such glycoproteins, theoretically, may move freely by lateral diffusion on the cell surface, independent of the intensity of the tranverse binding between cells.

It is noteworthy that we have observed a stronger inhibition of migration of postmitotic neurons at much lower antibody concentrations than the ones reported by others using similar approaches (13, 14, 16). For example, in studies involving purified antibodies specific for the antigens of the CAM family, at least 10 times higher concentrations (0.5–4 mg/ml) were used. The observation in this paper, showing that much lower concentrations of antibodies inhibited cell migration, leads us to propose that CSL and its endogenous ligand, the 31-kDa glycoprotein, are involved in a determinant step of the complex phenomenon of neuron migration. It is tempting to speculate that CSL is implicated in a step different from that involving proteases (12, 15, 34) and L1/Ng-CAM molecules (13, 14, 16). But it may not be out of place to suggest that CSL could recognize glycans shared by cell adhesion molecules (22, 24) and particularly those structurally related to L1/Ng-CAM molecules implicated in neuron migration (13, 14, 16).

The inhibition of neuron migration by Fab fragments specific for an endogenous mannose-binding lectin brings about additional information for contact guidance. In Fig. 4, we propose a theoretical model of the mode of interaction of migrating immature neurons with astrocytes by means of CSL. This involves as a distinctive feature the formation of bridges between the astrocyte and neuroblast surface by CSL lectin, which recognizes specific glycan moieties of glycoproteins existing as cell-specific components both on neuroblast and on astrocyte cell surfaces. This is supported by the demonstration that antibodies raised against CSL cause an inhibition of migration. Moreover, this model predicts that antibodies raised against those cell-surface glycoproteins, which subserve as ligands of CSL on the surface of neurons and on the surface of astrocytes, would exert similar inhibitory effects. This is actually the case for the 31-kDa glycoprotein at the neuronal surface, which has been shown to be a ligand of CSL (20, 21). It is noteworthy that in a pioneer paper (38) concerned with the molecular basis of neuron migration in the “weaver” mutant mice, deficient in granule cell migration, it has been observed that a protein doublet at 28 and 31 kDa was absent. This doublet has physical properties (molecular mass, solubility property) identical to those of the 31-kDa glycoprotein.

The ultimate evidence regarding the validity of our hypothesis rests on the discovery of at least one astrocyte constituent exhibiting the required specificity of interaction with CSL. A recent report clearly demonstrates that such
molecules are actually present at the surface of cultured astrocytes and are involved in cell adhesion (25). However, these astrocyte glycoproteins are not yet accessible to immunological techniques.

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