Neural precursor cell chain migration and division are regulated through different β1 integrins

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SUMMARY

Proliferation and tangential migration of neural precursor cells are essential determinants of CNS development. We have established cell culture models of both these processes using neural precursor cells grown as neurospheres. The pattern of migration that we observe in these cells is homotypic and occurs in the absence of a glial or neuronal scaffold, and is therefore equivalent to that previously described as chain migration. To determine the role of integrins in proliferation and migration, we have analysed the expression pattern of integrins on neurosphere cells and then performed blocking peptide and antibody experiments. Neurosphere cells express five major integrins, α5β1, α6β1, αvβ1, αvβ5 and αvβ8 and, in addition, express low levels of α6β1. Chain migration is inhibited by blocking the α6β1 integrin. Proliferation, by contrast, is inhibited by blocking the other β1 integrins, αvβ1 and α5β1. These results show that integrins are important regulators of neural precursor cell behaviour, with distinct β1 integrins regulating proliferation and migration. They also demonstrate a novel role for the α6β1 integrin in the cell-cell interactions underlying homotypic chain migration.

Key words: Central nervous system, Chain migration, Neural precursor, Neurosphere, Neuroepithelium, Proliferation, Immunoprecipitation, Integrin

INTRODUCTION

The cells of the vertebrate central nervous system arise from a neuroepithelium surrounding the ventricles termed the ventricular zone (VZ; The Boulder Committee, 1970). During embryonic development, these VZ cells undergo substantial proliferation (The Boulder Committee, 1970; Frederiksen and McKay, 1988) and migration (Fishell et al., 1993; Walsh and Cepko, 1993; O'Rourke et al., 1995, 1997; Walsh and Reid, 1995). Examples of radial and tangential migrations show that they differ in their substrata for movement. Radial migration occurs along glial fibres that act as a scaffold to guide migrating cells (Rakic, 1990). Cells undergoing tangential migration, by contrast, appear not always to use a different cell type as a scaffold for migration (Ono and Kawamura, 1989; Rakic, 1990; O'Rourke et al., 1992). Lois et al. (1996) examined the migration of neuronal precursors within the rostral migratory stream and showed that these cells migrated in contact with one another without a glial scaffold, a form of migration they termed chain migration, and similar chains of neuronal precursors have been described in the adult mouse subventricular zone (Doetsch and Alvarez-Buylla, 1996).

The molecular and cellular cues that regulate the proliferation and two forms of migration of VZ and SVZ cells are therefore essential determinants of normal CNS
development. Previous work has suggested a role for soluble extracellular molecules such as growth factors (e.g. EGF, bFGF) (Bartlett et al., 1995; Johe et al., 1996; Weiss et al., 1996), wnt-related molecules (Dickinson et al., 1994) and neurotransmitters (glutamate, GABA) (LoTurco et al., 1995; Antonopoulos et al., 1997) in regulating the proliferation of neuroepithelial precursors. Studies on tangential migration show a role for the cell adhesion molecule polysialated N-CAM, expressed at high levels on neuronal precursor cells migrating towards the olfactory bulb (Bonfanti and Theodosis, 1994; Rousselot et al., 1995). Mice lacking the form of NCAM that can be polysialated (N-CAM 180) show abnormalities in the migration of these cells (Tomasiwicz et al., 1993), and this defect is also produced by enzymatic removal of PSA from N-CAM in normal mice (Ono et al., 1998). However, the contribution of other cell surface adhesion molecules to the regulation of neuroepithelial cell behaviour is unknown despite the substantial evidence for an important role of these molecules elsewhere during development (Hynes, 1994).

One important group of such adhesion molecules are the integrins (Hynes, 1992). These are heterodimeric cell surface glycoproteins consisting of distinct α and β chains. They bind to both extracellular matrix molecules and other cell-surface molecules such as some members of the immunoglobulin family (such as V-CAM-1) and the ADAMs family of disintegrin- and metalloprotease-related molecules (Hynes, 1992; Wolfsberg et al., 1995). Integrins can mediate intracellular signals that regulate behaviour, as their ligands alter the proliferation of a wide range of cells and specific anti-integrin antibodies and peptides block these effects (Davis et al., 1990; Chang et al., 1995; Hurley et al., 1995). Integrins have also been shown to regulate the migration of a range of neural precursor cells, including neural crest cells (Bronner-Fraser, 1993), Schwann cells (Milner et al., 1997b), oligodendrocyte precursors (Milner et al., 1996) and neuronal precursors within the optic tectum (Galileo et al., 1992). Taken together, these different experiments suggest that integrins are important candidate molecules for the regulation of neural precursor cell proliferation and migration.

A study analysing the expression of integrins in neural precursors of the VZ and SVZ would be difficult to perform in intact tissue as the majority of available antibodies react against individual α or β integrin subunits rather than specific heterodimers. This is of particular importance in the analysis of αv and β1 expression as each of these subunits will associate with a variety of different partners and each heterodimer can have different functional properties (Hynes, 1992). We have therefore used immunoprecipitation techniques that define the dimeric pairing of each subunit but require a large number of cells for surface labelling. To obtain these numbers, we have used enriched cell cultures of EGF-dependent neural precursors grown in culture as neurospheres (Reynolds and Weiss, 1992). These are spheres of undifferentiated neural cells that proliferate in the presence of epidermal growth factor (EGF) and have the phenotype and developmental potential of cells found in the germinal zones in vivo (Reynolds and Weiss, 1996). They therefore represent a model system to allow the study of the molecular mechanisms regulating neural precursor behaviour. Our approach has been first to establish assays for proliferation, survival and migration of neurosphere cells, second to establish their pattern of integrin expression and third to examine the effects on cell behaviour of blocking or stimulating the different integrins using antibodies and peptides.

**MATERIALS AND METHODS**

**Neurosphere cell culture**

EGF-driven spheres of neural precursors were grown from dissociated postnatal day 0-3 rat forebrain in DMEM/Hams-F12 with B27 supplement (Gibco) using previously described methods (Reynolds and Weiss, 1992; Svendsen et al., 1995). The cells were grown on untreated tissue culture flasks (Falcon) at 37°C in 7.5% CO₂. The spheres were passaged every 3 days.

**Immunostaining of neurosphere cells to examine differentiation**

To label intact spheres after 7-11 days in vitro (DIV), neurospheres were fixed in 4% paraformaldehyde (PF) in phosphate-buffered saline (PBS) for 2 hours at 4°C, incubated overnight with 50% normal goat serum, 1% Triton X-100 (Sigma) in PBS at 4°C and washed twice in PBS before incubating in either polyclonal anti-nestin (1 in 500; 130-Dahlstrand et al., 1995; a kind gift of Dr Urban Lendahl, Stockholm) or monoclonal anti-glial fibrillary acidic protein (GFAP, 1 in 25; Boehringer Mannheim) in 5% FCS, 1% Triton X-100, PBS (FT) at 4°C overnight. The nestin-stained spheres were incubated in goat anti-rabbit fluorescein (FITC) antibody (1 in 100 in FT; Caltag Laboratories) overnight. The GFAP-stained spheres were incubated first in goat anti-mouse IgG/M biotin-conjugated antibody (1 in 100 in FT) and then in a streptavidin-FITC conjugate (1 in 100 in FT). Finally, the spheres were incubated for 1 hour at 4°C in propidium iodide (5 µg/ml, Sigma). The spheres were mounted in cavity slides and viewed by confocal microscopy (BioRad).

To label dissociated sphere cells, the spheres were dissociated and plated on PDL-coated chamber slides for 24 hours at 37°C. Slides to be stained for neurofilament and GFAP were fixed for 15 minutes at room temperature in 4% PF in PBS, incubated at 4°C in 50% normal goat serum, 0.1% Triton X-100 in PBS for 12 hours and then incubated at 4°C overnight in either anti-GFAP monoclonal antibody (1 in 25) or anti-neurofilament (1 in 1000; Affiniti) in FT prior to labelling with FITC-conjugated secondary antibodies (see above). Slides stained for galactocerebroside (Gal-C; Ranscht et al., 1982) were incubated in anti-Gal-C monoclonal supernatant (1 in 2 in 5% FCS, L15) and secondary antibodies prior to fixation for 15 minutes and staining with propidium iodide. 10 random fields of view from each well were counted for total live cell nuclei (judged by nuclear morphology) and for the number of differentiated cells. At least 400 cells per experiment were counted and each experiment was repeated from three separate cultures. The results are expressed as mean ± s.e.m.

**Neurosphere cell proliferation**

After 9-11 days in vitro (DIV) and 2 passages, neurospheres were dissociated into a single cell culture and plated at 6 × 10⁵ cells/ml in 96-well plates precoated with ECM as described below. After 25 hours incubation at 37°C, tritiated thymidine (specific activity 70-90 Ci/mmol, Du Pont NEN) was added to give a final concentration of 5 µCi/ml. After a further 8 hours incubation at 37°C, the cells were lysed with SDS (final concentration 1%) and precipitated with 1/3 volume of 30% trichloroacetic acid (Sigma) overnight at 4°C. The precipitates were washed on glass-fibre filters (Whatman), dried and 0.5 ml of Optiscint Hisafe II scintillant (Wallac) added for counts per minute (cpm) measurement. Results comprise at least 3 separate cultures with more than 2 wells per culture and experimental condition.

For ECM coating, 96-well plates or 16-well chamber slides (Nunc)
were coated first with poly-D-lysine (5 μg/ml) for 1 hour at room temperature. The dishes were then coated with fibronectin, laminin or tenasin-C. Coating with fibronectin (from bovine plasma, Sigma) or laminin (EHS, Sigma) was for 3 hours at 37°C at a concentration of either 50 μg/ml (for the initial experiments on cell proliferation, differentiation and death) or 10 μg/ml (for the blocking experiments with RGD peptides and antibodies) Tenasin-C (a kind gift from Dr A. Faisstner, Heidelberg, extracted from mouse brain) was coated at 42 μg/ml at 37°C overnight. Wells coated with fibronectin and tenasin-C were coated first with fibronectin for 3 hours and then with tenasin-C or PBS alone (controls) overnight at 37°C.

Neurosphere cell migration
After 9-11 DIV, intact rat or mouse neurospheres were plated on uncoated tissue culture 96 well plates at 200 μl/well at approximately 6x10^5 cells/ml. At 1 day and 3 days after plating migration was scored by counting the number of chains emanating from each sphere in 4 random fields (10x objective) per well. In order to determine the motility of individual cells, cultures established for 2-3 days were recorded using time-lapse videomicroscopy. A field of view was recorded every 30 seconds for approximately 25 hours. The total distance moved by each cell in the field of view was measured over the course of the second 12.5 hours of this 25 hours period. All experiments were repeated in triplicate with independent cultures. To examine the immunocytochemistry of migrating cells the cultures were fixed by adding 4% PF overnight and antibodies against neurofilament, nestin, tubulin βIII and GFAP were used as above. For the experiments examining integrin expression, we used immunoperoxidase techniques to visualise labelled cells following incubation in either anti-α6A (a kind gift from Dr V. Quaranta) or αv (Chemicon) antisera (1 in 100).

Cell labelling, immunoprecipitation and immunodepletion
Neurospheres were washed in PBS and then incubated in 0.1 mg/ml NHS-LC-Biotin (Pierce) in PBS for 1 hour at 37°C. Immunoprecipitations and immunodepletion were then performed as described by Milner and ffrench-Constant (1994) and Milner et al. (1997a,b), using the following antibodies. Antiserum against the cytoplasmic domains of α1, α2, α3, α5, αv and β1 were purchased from Chemicon and used at a final concentration of 1/250. A monoclonal antibody against α4 (Chemicon) was used at 1/50. Antibodies against α6A and α6B were a gift from Dr V Quaranta, used at 1/50. Two antibodies against β3 were used; an antiserum against the cytoplasmic domain (a gift from Dr K Venstrom) and a mouse monoclonal against the extracellular domain (F11, a gift from Dr M Horton), both used at 1/250. A mouse monoclonal antibody against β5 (P3G2, a gift from Dr D Cheres) was used at 1/50. An antiserum against β8 was generated against the cytoplasmic domain (S. N. et al., unpublished data) and used at 1/25. The specificity of these antibodies for the appropriate subunits is described in Milner and ffrench-Constant (1994) and Milner et al. (1997a,b).

Antibodies and peptides used in function-blocking studies
The anti-β1 integrin blocking antibody was raised in rabbits against purified mouse α6β1 integrin and provided as purified IgG. The antibody was reconstituted in PBS and used at 62 μg/ml. Control cultures were treated with normal rabbit IgG (Sigma) in PBS at the same concentration. The anti-α6β1 blocking monoclonal antibody GoH3 (Sonnenberg et al., 1988; Hall et al., 1990; Serotoc) was dialysed in PBS before use to remove azide and was used at a final concentration of 20 μg/ml. Control cultures were treated with an equal volume of PBS. The sequence of the RGD and RGE peptides (Life technologies, Gibco BRL) were used: RGD: GRGDSP and RGE: GRGESP. They were reconstituted in PBS and used at a final concentration of 100 μg/ml. The P3 peptide and scrambled P3 (SP3) was a kind gift of Dr K Sekiguchi (Osaka Medical Centre, Japan, Murayama et al., 1996). The sequences were; P3: VSVFSGRRHYSPPFAVS and SP3: RFSVAVSHYPFWRS. The peptides were reconstituted in 0.02 % acetic acid and used at 0.5 mM.

RESULTS
Characterization of neurospheres
To confirm that our neurospheres represent a valid model of neural precursor cell behaviour in vivo, we grew spheres as described in Materials and Methods and then examined their proliferation, differentiation, survival and migration in our cultures. Labeling of neurospheres with anti-BrdU antibodies following growth in the presence of EGF and with BrdU added to the medium showed large numbers of labelled cells, suggesting that the majority of cells proliferate in response to this growth factor (Fig. 1). To quantify this, we measured 3HT uptake into the spheres. This was increased in the presence of EGF in the culture medium (Fig. 1), in keeping with the observations of others that EGF is a mitogen for neurosphere cells (Reynolds and Weiss, 1992). To confirm that 3HT incorporation accurately reflected DNA synthesis in these cultures, all experiments were performed in parallel with control cultures treated with the DNA polymerase inhibitor, aphidicolin (Spadari et al., 1982). Under these conditions, 3HT incorporation fell to background levels (Fig. 1).

As described by other groups, we found that the majority of live cells in the intact spheres expressed nestin, a marker of neuroepithelial precursors, when visualised by confocal microscopy while few of the cells expressed the astrocyte marker, GFAP (Fig. 1; Reynolds et al., 1992; Reynolds and Weiss, 1992, 1996; Svendsen et al., 1995). We confirmed that the majority of the neurosphere cells were undifferentiated by plating dissociated sphere cells on PDL-coated slides for 20 hours in the presence of EGF, before staining with antibodies against markers of astrocytes (GFAP), oligodendrocytes (Gal-C) and neurons (NF). We found that few cells expressed any of these markers (GFAP 6.5±2.2%, GalC 0.2±0.2%, NF 0.3±0.1%, n=3). However, in agreement with the results of others (Reynolds et al., 1992; Reynolds and Weiss, 1992, 1996; Svendsen et al., 1995), spheres differentiated into astrocytes, neurons and oligodendrocytes over the course of 9 days following plating to an adhesive substratum (fibronectin or PDL) in the absence of EGF (not shown).

Migration of neural precursor cells was seen when neurospheres were plated at high density in 96 well plates. We found that the spheres spontaneously adhered to the bottom of the wells in the absence of added extracellular matrix and cells then migrated away from the spheres. The migrating cells had a simple unipolar or bipolar morphology and formed chains of cells extending from the spheres (Fig. 2). Cells lying within these chains could be seen to migrate in time-lapse videomicroscopy experiments by extending short processes, displacing their nucleus within the cytoplasm and then often retracting the trailing cytoplasm. The migration results from cell-cell interaction, as the cells maintained contact with the chains during their migration by extending their processes over the other cells in the chains. Occasionally cells extended processes out of the chain; such cells always remained attached to the original chain unless they made contact with an adjacent chain, following which the cell would move into the new chain. To determine whether the cells were migrating on an axonal or
Characterization of the neurospheres.

Fig. 1. Characterization of the neurospheres. (A) A sphere grown in EGF and in the presence of BrdU labelled with anti-BrdU antibodies visualised using fluorescein optics; note that the majority of cells appear to be labelled. This effect is quantified in the graph, which shows that EGF increases tritiated thymidine incorporation and that this incorporation is inhibited by the DNA synthesis inhibitor aphidicolin, confirming that it represents stimulation of cell division in response to the growth factor. The insets B and C show confocal sections through two rat neurospheres stained for nestin (B) and GFAP (C), shown in green, and counterstained with propidium iodide to show nuclei, shown in red. The majority of the cells are nestin+, while few express GFAP. Note that many cells in the center of the spheres are dead (arrows in B), as shown by the condensed, fragmented nuclei.

Neurosphere cells express β1 and αv integrins

In order to determine the phenotype of the migrating cells, we immunostained these cultures 3-4 days after plating with antibodies against nestin and tubulin βIII, a neuronal marker expressed very early after commitment to the neuronal lineage. We found that all the cells in the chains were nestin positive but very few cells within the chains expressed the marker tubulin βIII (Fig. 3). Therefore, we concluded that the majority of cells in the chain had the phenotype of undifferentiated neural precursors while a minority showed evidence for neuronal differentiation.

In order to determine the role of integrins in the regulation of proliferation and migration of these cells, we first analysed the integrins expressed by these cells using immunoprecipitation as described in Materials and Methods. Immunoprecipitation with an αv antibody brought down bands of ~85 kDa, 95 kDa, 110 kDa and 130 kDa (Fig. 4, Panel A). The highest of these bands was identified as αv based on its molecular weight and because it comigrated with the αv band from mixed glial cultures. The 110 kDa band comigrated with a 110 kDa band found in β1 immunoprecipitates (see below). To confirm that this band was the β1 subunit, lysates were depleted using an anti-β1 antibody and then an anti-αv antibody was used to immunoprecipitate the remaining αv integrins; in these experiments, the αv immunoprecipitations no longer coprecipitated a 110 kDa band (Fig. 4A). The 95 kDa band in the αv immunoprecipitation had the molecular weight of the β5 integrin subunit, and immunoprecipitation with a specific anti-αvβ5 antibody brought down two bands, one of 95 kDa band and one that comigrated with the putative αv band, confirming that the cells express αvβ5 integrin (not shown). The lower band in the αv immunoprecipitation ran with the expected molecular weight of the β3 or β8 subunits.

Immunoprecipitations with an anti-β8 (Fig. 4B), but not with two different anti-β3 antibodies (not shown), brought down a band of 85 kDa and a band that comigrated with the putative αv band. This suggests that the 85 kDa band comprises entirely β8, with no contribution from β3. To confirm this, we depleted lysates with multiple rounds of immunoprecipitation with the anti-β8 antibody. Subsequent immunoprecipitation with an anti-αv antibody no longer co-precipitated an 85 kDa band (Fig. 4B). We conclude, therefore, that the major αv integrins expressed by neurospheres cells are αvβ1, αvβ5 and αvβ8.

Immunoprecipitation with antibodies against β1 brought down three subunits of ~110 kDa, 130 kDa, 150 kDa (Fig. 4C). As described above, the 110 kDa band was identified as the β1 subunit. In order to identify the other subunits that were co-precipitated with β1, we performed immunoprecipitations with a panel of antibodies to a subunit known to associate with the β1 subunit. Immunoprecipitation with an anti-α5 antibody brought down bands of 110 kDa and 150 kDa that comigrated with the β1 band and the 150 kDa band in the β1 immunoprecipitations respectively (Fig. 4D).
Immunoprecipitation with an anti-α6A antibody brought down a band of 110 kDa and a band of 130 kDa comigrating with the β1 and 130 kDa bands in the β1 immunoprecipitation (Fig. 4D,E). As two structural variants of α6 (α6A, and α6B) have been described, differing in their cytoplasmic domains as a result of alternative splicing (Cooper et al., 1991; Heirck et al., 1993; Hogervorst et al., 1993), we also examined expression of α6B. Immunoprecipitation with an anti-α6B antibody brought down low levels of a band of 130 kDa (Fig. 4E), showing that both forms of α6 are expressed but that α6A predominates. In control experiments, we found that mixed glial cultures (G) and oligodendroglial cells (O) expressed α6A but not α6B, confirming the specificity of α6B expression on the neurosphere cells (Fig. 4E). In contrast, immunoprecipitation with antibodies known recognise α2 and α4 subunits on other cells did not precipitate detectable levels of integrin from neurosphere cells (Fig. 4C,F). Immunoprecipitation with antibodies against α1 and α3 integrin precipitated very low levels of these integrins (Fig. 4C). We conclude, therefore, that major β1 integrins expressed by neurosphere cells are α5β1, α6Aβ1 and αvβ1 with lower levels of α6Bβ1.

These immunoprecipitations were performed on spheres biotinylated as intact clusters of cells. To confirm that the patterns of integrins that we found on these cells did not represent labelling of cells at the surface of the spheres only, the αv, α5 and β1 immunoprecipitations were repeated using dissociated spheres in single cell cultures. These immunoprecipitations revealed the same pattern of integrin expression as on intact spheres (not shown). This result also shows that, at least in the short term, alteration of the cell-cell interactions in these cells does not alter the pattern of integrin expression.

Integrins regulate the migration of neural precursors

To determine if the migration of neurosphere cells observed in our cultures was regulated by integrins, we plated the spheres in conditions that promote chain migration as

![Image](image-url)
described above, in the presence of an anti-β1 integrin antibody or RGD peptides. The anti-β1 antibody completely blocked the extension of the chains, showing a role for β1 integrins in migration (Fig. 5). In contrast, RGD peptides had no effect on the extension of such processes (Fig. 5); as RGD peptides would be expected to block α5β1 and all αv integrins (Haas and Plow, 1994), the most likely candidate β1 integrin that these cells express that might regulate migration is therefore α6β1. To examine the role of α6β1 further, we used the anti-α6β1 blocking monoclonal antibody GoH3 (Sonnenberg et al., 1988). These experiments were performed on mouse neurospheres, as the GoH3 rat monoclonal antibody recognises mouse but not rat α6β1.

When added to the cultures, we found that GoH3 blocked the extension of the migrating chains of mouse spheres (Fig. 5), confirming the requirement for the α6β1 integrin in chain migration in our experiments. We also examined the expression of β1 integrins during chain migration from rat neurospheres in vitro. In experiments on fixed cells using polyclonal antisera, we showed that α6β1 was expressed on cells migrating in the chains, with highest levels seen at points of cell-cell contact rather than at the leading edge of the cells where they contact the substratum. αv, in contrast, was spread equally over the cells and expressed at lower levels (Fig 5).

If α6β1 is involved in the molecular mechanisms regulating neural precursor cell migration in our cultures, then ligands for α6β1 should also alter rates of migration. α6β1 is a receptor for members of the laminin family of extracellular matrix molecules and the ADAMs family of cell surface molecules (Hynes, 1992; Almeida et al., 1995). While neural precursor cells did migrate well on laminin (not shown), this ligand also binds to a wide range of non-integrin receptors (Reichardt and Tomasedelli, 1991). As a result, these experiments with a laminin substratum do not provide a specific ligand for α6β1. Therefore, we plated the cells in the presence of a short peptide specifically recognised by α6β1 (Murayama et al., 1996). This peptide when added to the tissue culture medium significantly enhanced the formation of chains between the spheres without increasing the number of cells migrating independently from the chains (Fig. 6). The number of chains increased by 352.4±83% over a scrambled peptide (SP3) control (P<0.05, n=3) in paired experiments. The scrambled peptide itself had no significant effect on migration, with the extent of migration in PBS being the same (128±43%, n=6) as in the presence of SP3, confirming a role for α6β1 in chain migration.

To establish that the effect of the anti-integrin antibodies on chain migration was direct and not secondary to alterations in substratum adhesion that might compromise the ability of neural precursor cells to leave the sphere and form chains, the chains were established by plating the spheres for 2-4 days in the absence of the antibody after which the anti-β1 antibody was added. While some of the chains retracted over the course of these experiments, many remained and we were able to measure the motility of the remaining cells within the unretracted chains by time-lapse videomicroscopy. While

**Fig. 4.** Integrin expression by neurosphere cells examined by immunoprecipitation. (A) A β1 immunodepletion (ID) experiment. The first lane (Con-ID αv) show an αv immunoprecipitation following a series of mock depletions with a control serum. The remaining lanes show four sequential IDs and a subsequent αv immunoprecipitation. Note the absence of α1 following the immunodepletion but the continued presence of β5 and β8 in association with αv. (B) A similar immunodepletion experiment using β8 antisera; note the specific loss of the lower band confirming it represents β8 only. (C,D) Immunoprecipitations on neurospheres using anti-integrin antibodies shown under each lane. (E) Immunoprecipitations with α6A and α6B antibodies on neurospheres (S), mixed glial cultures (G) and oligodendrocyte precursors (O). Note that neurospheres but not glial cells express α6B. (F) Immunoprecipitations with α4 and β1 antibodies on neurospheres (S), microglia (MG) and the JM T-cell line (JM). Note that there is no expression of α4 on the neurospheres in contrast to control cells.
Integrins regulate neural precursor proliferation

In order to determine the role of integrin receptors in regulating cell proliferation, we measured cell proliferation in the presence of ECM integrin ligands with or without integrin-blocking antibodies and peptides. We plated dissociated neurospheres on different extracellular matrix molecules for 24 hours before measuring their proliferation. The same number of viable cells were plated in each well in all experiments, with the cell density identical to that used in the migration experiments so as to allow a valid comparison of integrin function in the two sets of experiments. All wells were precoated with poly-D-lysine so as to minimise the effects on proliferation of any differential adhesion to the different ECM substrata. Non-adherent cells were also included in the analysis to avoid selection of specific populations on the different ECM substrata. We found that the ECM molecules fibronectin (Fig. 7A) and laminin (not shown) produced a 2-fold increase in neurosphere cell proliferation compared to a non-specific adhesive substratum, poly-D-lysine (PDL). In contrast, the ECM molecule tenasin-C alone had no effect on proliferation (Fig. 7A). To determine how a mix of ECM ligands regulated cell proliferation, we compared the effects of fibronectin and tenasin-C alone with a combined substratum of both molecules. While tenasin-C alone did not alter cell proliferation, it did block the proliferative effect of fibronectin (Fig. 7A).

To confirm that the neurosphere cells remained undifferentiated in these experiments and the observed effects did not reflect ECM-induced changes in rates of differentiation or survival, dissociated cultures were grown on fibronectin for 24 hours and then immunostained with antibodies against markers of astrocytes (GFAP), oligodendrocytes (Gal-C) and neurons (NF). Apart from a small but significant decrease in the number of GFAP* cells on fibronectin (6.5±2.2% on PDL; 4.5±1.8% on fibronectin, P<0.05 Student’s paired t-test), no change in the number of labelled cells was seen as compared with our experiments on PDL substrata described above. In addition, we were unable to detect an effect of fibronectin on cell death in these cultures as determined by propidium iodide exclusion after 24 hours.

Having demonstrated a proliferative effect of fibronectin and laminin, we next examined the role of integrins. RGD peptides but not RGE peptides completely blocked the effect of fibronectin on cell proliferation (Fig. 7B). Of the integrins that we identified on the neurospheres, αvβ1, α5β1 and αvβ8 have been reported to bind fibronectin in an RGD-dependent manner (Haas and Plow, 1994; Venstrom and Reichardt, 1995). In order to further define which integrins are responsible for the increase proliferation, we measured proliferation in the presence of anti-β1 antibody. We found that this antibody produced a partial block of the fibronectin-induced proliferation (Fig. 7B) indicating a role for either αvβ1 or α5β1 integrins in the proliferation of neurospheres.

The enhanced proliferation of neurosphere cells when plated on laminin, a ligand for α6β1 integrin, suggested that this integrin might play a role in regulating proliferation as well as migration. However, there are several putative receptors for laminin including non-integrin receptors. To determine if laminin was mediating a proliferative signal through α6β1, we plated mouse cells on laminin in the presence of the specific α6β1 blocking antibody GoH3, which blocked neurosphere migration as described above. GoH3 had no effect on laminin-induced proliferation (not shown). This shows that proliferation signals, unlike those involved in migration, are not mediated by α6β1 integrin and that different integrin-mediated signalling pathways are used for these two aspects of cell behaviour.

DISCUSSION

Our results analysing integrin expression on neural precursor cells grown as neurospheres in culture show that these cells express predominantly α5β1, α6αβ1, αvβ1, αvβ5 and αvβ8 and lower levels of α6β1β1. The β1 integrins expressed on the neurosphere cells contribute to the regulation of cell migration and proliferation. Moreover, different β1 integrins are involved in two aspects of cell behaviour; migration is regulated by α6β1 but this integrin does not play a role in regulating proliferation.

In agreement with previous work (Reynolds and Weiss, 1996; Weiss et al., 1996), we have shown that the neurospheres used in our study are multipotential, being able to differentiate in astrocytes, oligodendrocytes and neurons as do VZ/SVZ cells in vivo. We have not shown in this study that neurospheres contain stem cells rather than committed precursors for all three lineages, but clonal analysis experiments by Reynolds and Weiss (1996) provide strong evidence for the presence of stem cells in these spheres. While the precise nature of the cell type in vivo corresponding to the neurosphere cells is unclear, three lines of evidence point to the precursor cells of the SVZ. First, neurospheres can be grown from the postnatal and adult CNS (Reynolds and Weiss, 1992), a time at which proliferation within the VZ has ceased while proliferation continues in the SVZ. Second, the neurosphere cells have the phenotype of undifferentiated SVZ cells, being nestin* but almost entirely GFAP+, GalC+ or neurofilament+ (Reynolds et al., 1992; Reynolds and Weiss, 1992, 1996; Svendsen et al., 1995). Third, the neurosphere cells used in our study and those of others (Reynolds and Weiss, 1992, 1996; Weiss et al., 1996) proliferate in response to EGF, as do subependymal zone cells (the remnant of the SVZ) in the adult mouse CNS (Craig et al., 1996). While we cannot determine whether the neurosphere cells represent all or only a subpopulation of the SVZ cells, the conclusion that they have the properties of multipotential neural precursors makes them a useful model system in which to study the role of integrins in the regulation of neural precursor behaviour.

The migration that we have observed in our neurosphere cultures appears to be similar to the previously described chain migration from SVZ explants (Lois et al., 1996; Wichterle et al., 1997). First, it occurs in the absence of a neuronal or glial...
scaffold as evidenced by the lack of any neurofilament or GFAP+ processes within the migrating chains. Second, the cells migrate as cords or chains over one another rather than over tissue culture substratum, suggesting that the substratum for migration is present on the cell surface of similar cells. Third, cells show periods of stationary behaviour and movement in both directions (T. S. J., unpublished observations) as do cells from the SVZ explants. There are, however, interesting differences between our experiments and those of Wichterle et al. (1997). First, migrating cells from the SVZ explants were labelled by the TUJ1 monoclonal antibody against tubulin (Wichterle et al., 1997), suggesting that this population was already committed to form one of the special classes of neurons (olfactory bulb neurones that migrate in the rostral migratory stream) that develop from the SVZ cells. Cells from the neurospheres, in contrast, were not labelled by a monoclonal antibody against this tubulin isoform but were labelled by an antiserum against nestin. Nestin is a marker of less differentiated cells, suggesting that chain migration may also be a property of neural precursor cells within the SVZ that have not yet committed to differentiation down a neuronal lineage. Second, sustained chain migration from the SVZ explants was only seen in Matrigel substrata (Wichterle et al., 1997), while we observed chain migration over tissue culture plastic. While this may reflect a genuine difference in the extracellular requirements for migration, it may also reflect the different culture conditions used. We found that chain formation only occurred when spheres were plated at high density (T. S. J. unpublished observations) and it is therefore possible that migration is stimulated by a soluble extracellular growth or other factor produced by the neurosphere cells and also present in the Matrigel.

Previous studies have shown widespread expression of α6 in the neuroepithelium of early Xenopus (Lallier et al., 1996) and chick embryos (Bronner-Fraser et al., 1992). Based on studies using antisense oligonucleotides in Xenopus embryos, roles for α6β1 have been proposed in retinal neuronal differentiation and the maintenance of neuroepithelial integrity and induction (Frade et al., 1996; Lallier et al., 1996). Our study makes the novel observation that the α6β1 integrin plays an important role in the interactions regulating chain migration in these cells. This conclusion is based on three observations. First, antibodies against β1 but not RGD peptides (that block α5β1 and αvβ1, the other β1 integrins on the cells) block migration. Second, antibodies against α6β1 show this integrin to be present at sites of cell contact involved in homotypic migration and also block this migration. Third, the soluble P3 peptide that binds to α6β1 stimulates migration. This final observation was interesting, as small peptides that represent monovalent ligands for integrins (such as the RGD peptide) are more usually found to either stimulate or inhibit cell adhesion when present on the substratum or in solution, respectively. Our results show enhanced chain migration without any effect on cell-substratum adhesion (as judged by the lack of any increase in the number of chains per sphere).
of cells migrating individually over the substratum). This points to a role for α6β1 in the signalling pathways of neural precursor cell migration that is independent of any adhesive function, as has also been shown for this integrin in murine ES cell migration (Domanico et al., 1997).

Two previous studies have examined integrin function in the other major form of migration in the developing CNS, radial migration. Galileo et al. (1992) used a retroviral approach to transfect chicken tectal neuroepithelial cells in vivo with an antisense construct to β1 integrin. They found that the subsequent radial migration of neuronal precursors was reduced in cells expressing the antisense construct, presumably as a consequence of reduced β1 expression; however, this in vivo study does not allow precise characterization of the integrin responsible for migration as one cannot distinguish between different β1 integrins. We have previously used an in vitro approach to examine the role in migration of integrins expressed on another radially migrating cell population, oligodendrocyte precursor cells (Milner and ffrench-Constant, 1994; Milner et al., 1997a). We found that the migration of oligodendrocyte precursors is regulated by αvβ1 while, in contrast to the neural precursor cells examined in the present study, α6β1 appears not to contribute (Milner et al., 1996). Although this and our present in vitro study are limited in that the migratory substratum may differ from that in vivo, they do provide an example of cell populations migrating in different axes within the CNS using different integrins for migration. This may reflect the different cellular and extracellular substrata used for migration; as discussed above, neural precursor cells migrate tangentially on one another within the neuroepithelium while oligodendrocyte precursors migrate away from the neuroepithelium as individual cells and may use extracellular matrix ligands such as vitronectin present within pathways of migration.

The principle ECM ligand for α6, laminin, has been shown to stimulate proliferation of embryonic neuroepithelial cells (Drago et al., 1991). We also found that laminin stimulated proliferation in neurosphere cells; however, this effect is not mediated by α6β1 as the blocking monoclonal antibody GoH3 had no effect on proliferation. Our present studies therefore suggest that α6 is not implicated in the regulation of proliferation of these cells. RGD peptides and anti-β1 antibodies did block proliferation, suggesting a role for αvβ1 and/or α5β1 in proliferation. Both integrins will bind fibronectin, an extracellular matrix ligand which we have shown to stimulate proliferation of neural precursor cells and which is known to be expressed in the ventricular region of the central nervous system (Stewart and Pearlman, 1987; Sheppard et al., 1991, 1995). We have been unable to distinguish which of the two β1 integrins are predominantly responsible for the proliferative effect as appropriately well-characterised blocking antibodies to the two integrin heterodimers are not available. However, these results showing that different β1 integrins regulate proliferation and migration do provide further evidence for distinct functional roles for the individual integrins in each cell type.

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