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Mapping Regions of the β1 Integrin Cytoplasmic Domain Involved in Migration and Survival in Primary Oligodendrocyte Precursors Using Cell-Permeable Homeopeptides

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The mapping of regions within integrin cytoplasmic domains responsible for the different effects on cell behaviour is an important part of an analysis of integrin-mediated signalling. In order to facilitate this analysis in primary cells, we have used cell-permeable homeopeptides to deliver sequences mimicking parts of the integrin β1 cytoplasmic domain into the cell. In a study using oligodendrocyte precursors, the cells that give rise to myelin-forming oligodendrocytes during CNS development, we show that these peptides can be used to manipulate β1 integrin signalling and that the regions of the cytoplasmic domain involved in migration and survival are distinct. Peptides mimicking the N-terminal portion of the cytoplasmic domain previously implicated in binding to Focal Adhesion Kinase (FAK) induce apoptosis, while peptides mimicking more C-terminal sequences do not cause cell death. In contrast they show that the NPIY sequence, the N-terminal one of two NPXY motifs previously implicated in signalling, is involved in migration. Peptides containing this sequence promote migration while alteration of NPIY to NPIA makes the peptide inhibitory to migration. Our results show that these peptides represent a novel approach to integrin signalling that allow rapid definition of critical cytoplasmic sequences in primary cells.

Key Words: **integrins; cell-permeable peptide; oligodendrocyte precursor; myelin; migration; NPXY motif; apoptosis; FAK; PDGF; FGF-2.**

Integrins are a family of cell-surface receptors for extracellular matrix and cell-surface ligands. Each functional integrin molecule is formed by a dimer of one α and one β chain, with the combination of α and β chains determining ligand specificity (reviewed in (1)). Both the α and β chains have short cytoplasmic domains which have been implicated in two forms of signaling: outside-in signaling, where ligand occupancy can activate a number of different downstream signaling molecules including focal adhesion kinase (FAK), mitogen-activated protein kinase (MAPK) and P-I-3 kinase (2-6); and inside-out signaling where the affinity of the integrin for extracellular ligands is regulated by interactions with cytoplasmic molecules including H-ras, R-ras, CD98 and β3-endonexin (7-10). Experiments using antibodies or peptides to perturb ligand binding to integrins have suggested important roles for integrins in cell proliferation, migration, survival and differentiation during development (11-15), and also in events associated with tumour formation and repair such as angiogenesis (16,17). In the case of the β1 subunit, some of these roles such as extracellular matrix assembly and cell proliferation have also been supported by genetic ablation experiments using cells derived from knock-out mice (18,19). These results point to a central role for β1 integrins in the regulation of cell behaviour and emphasise the need to understand how these integrins interact with downstream adaptor and signaling molecules to control intracellular regulatory pathways.

An important initial step in an analysis of β1 signaling pathways is to establish the precise regions of the cytoplasmic domain that initiate activation of the different signaling molecules. Two approaches have been

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taken to these mapping studies. First, short peptides mimicking the different regions of the cytoplasmic domain have been used *in vitro* to establish whether they bind directly to molecules known to contribute to adhesion complexes (20,21); these methods have demonstrated binding to the molecules FAK, paxillin and α-actinin (22-24). Second, cells have been transfected with mutant β1 integrins with point mutations or deletions within the cytoplasmic domain and the subunits analysed for their ability to participate in normal adhesion complex formation and signaling (25-32). These experiments have been performed either by over-expression of β1 subunits from different species localised using species-specific antibodies or by transfecting cells derived from the β1 integrin knock-out mouse. However, all these approaches have significant disadvantages; analysis of the effect of peptides on cell behaviour has required direct injection of peptide into individual cells, while the transfection experiments require that cell lines are established, making it difficult to use primary cells. However, it is obviously important to analyse integrin function in primary cells as they exhibit the full behavioural phenotype of cells *in vivo* to a greater degree than established cell lines.

Our interests are focused on the function of integrins in central nervous system development, using the oligodendrocyte precursor (OPC) that gives rise to myelin-forming oligodendrocytes as a model system. These cells migrate, proliferate and then differentiate or undergo programmed cell death *in vitro* just as they do *in vivo* (33-38). We have shown previously that these cells express α6β1, αvβ1, αvβ3, αvβ5 and αvβ8 integrins, with developmental switching of the αv integrins during differentiation (39,40). Antibody blocking experiments have shown that β1 integrins have roles in oligodendrocyte precursor migration and survival ((12) and unpublished observations), and the aim of this present study was to initiate an analysis of the signaling pathways involved by mapping the regions of the β1 cytoplasmic domain involved in these two responses. In order to overcome the problems above, we have used a novel approach to β1 integrin signaling in which short peptide sequences mimicking different regions within the cytoplasmic domain are internalised by synthesising them in conjunction with a 16 amino acid sequence derived from the homeodomain of the antennapedia protein (41). Such homeopeptides are able to translocate across cell membranes and have been used previously to examine FGF receptor-mediated signaling (42), while peptides using a hydrophobic, signal peptide sequence to render them cell-permeable have been used to block β3 integrin function (43). Here we show that this approach allows rapid functional mapping of the β1 integrin cytoplasmic domain in primary cells.

EXPERIMENTAL PROCEDURES

Peptide synthesis. The antennapedia internalisation sequence, forming the first (NH2) portion of all peptides, was RQIKIWFQNRRMKWKK. Peptides Hom1, Hom2 and Hom3 were synthesised on a 431A Applied Biosystem peptide synthesiser using p-Hydroxymethylphenoxymethyl polystyrene resin and standard Fmoc chemistry as previously described (44,45). Other peptides were synthesised by Genosys and by MWG Biotech Ltd, followed by HPLC purification to >95% purity. The amino acid sequences of the integrin-derived sequences that consituted the COOH portion of the peptides are shown in Table 1.

Cell culture. Minimal essential medium (MEM), Dulbecco's MEM (DMEM, with glucose, sodium pyruvate and pyridoxine) and Hanks Buffered Saline Solution (calcium and magnesium-free, HBSS) were from Sigma. Sato solution was DMEM supplemented with bovine insulin (Sigma, 5μg/ml), human transferrin (Sigma, 50μg/ml), bovine serum albumin (BSA) fraction V (Sigma, 100μg/ml), progesterone (Sigma, 6.2ng/ml), putrescine (Sigma, 16μg/ml), sodium selenite (Sigma, 5ng/ml), T3 (Sigma, 400ng/ml), T4 (Sigma, 400ng/ml), L-glutamine (Sigma, 4mM), penicillin and streptomycin (Sigma). SFG and DFG solutions were made by adding 10% fetal calf serum (FCS, Sigma) and L-glutamine (Sigma, 4mM) to Sato and DMEM respectively. Differentiation medium consisted of Sato solution plus 0.5% heat-inactivated FCS (TCS Biologicals) and 20ng/ml of TGFβ1 (human recombinant, R&D Systems). Trypsin-EDTA solution contained 9mls of HBSS with 1ml of 10x Trypsin-EDTA solution (Sigma, 25mg/ml trypsin, 10mg/ml EDTA). Ovomucoid trypsin inhibitor (OTI) solution was made from L15 medium (Sigma) with 0.6mg/ml bovine serum albumin (Sigma, fraction V) and 1.125mg/ml of trypsin inhibitor (Boeringher-Mannheim). Poly-D-lysine (PDL) pre-coating of flasks and dishes was with PDL solution (Sigma >300kD molecular weight, 5μg/ml in sterile distilled water (dH_2O)) for at least 1 hour at room temperature, and washed once with dH_2O.

All cell culture was carried out at 37°C with 7.5%CO_2. Purified oligodendrocyte precursors were obtained from neonatal rat forebrains by the method of McCarthy and de Vellis (46) with modifications. Briefly, forebrains from post-natal day 0-2 Sprague-Dawley rat pups were dissected free of meninges, and minced with fine scissors. They were then incubated for one hour in a solution of 30U/ml papain (Worthington), 0.24mg/ml cysteine (Sigma) and 40μg/ml DNAase I type IV (Sigma) in 1ml MEM (as described in (38)). Supernatant was then removed, 1ml of OTI solution was added and the cells dissociated by trituration through a 1ml pipette tip, followed by a 21-gauge needle. Cells were then centrifuged and resuspended in DFG solution and plated out in Falcon flasks pre-coated with PDL. Medium was exchanged every 3 days. After 10 to 20 days of culture, the flasks were placed on a rotatory shaker (New Brunswick Orbital) at 250rpm for 2 hours to remove any loosely adherent microglia. A subsequent prolonged shake for 20-24 hours served to dislodge large numbers of oligodendrocyte precursors from the underlying monolayer (consisting predominantly of astrocytes and fibroblasts) into the supernatant. The supernatant was retrieved and contaminating microglia were removed from it by their differential adherence, during a 30 minute incubation, to non-tissue culture plastic petri dishes (Media). This cell suspension was then used for survival and migration assays.

Survival assays. The cell suspension obtained as above was centrifuged, triturated briefly in 1ml of Sato solution, and resuspended in SFG solution, in the presence of FGF-2 (Peprotech, recombinant human, 10ng/ml). This suspension (10mls per shaken flask) was plated into a Nunc tissue culture dish pre-coated with PDL, and cultured for 4-5 days at 37°C; FGF-2 was replaced daily at 10ng/ml. Cells form these enriched OPC cultures were used on day 4 or day 5 for survival assays. To obtain these cells, the cultures were trypsinised in 6ml Trypsin-EDTA solution for 6 minutes at 37°C, before being stopped with 2mls of OTI solution. The resulting cell

suspension was then centrifuged and resuspended in Sato solution. For all assays cells were then plated at 5000/well in 96 well Microtest III tissue culture plates (Falcon), with cell numbers calculated using a haemocytometer. Prior to plating, wells were precoated with poly-D-lysine (Sigma) at 10μg/ml overnight, before being washed once with dH_2O. Merosin (human placental, GibcoBRL-Life Technologies) was diluted to 25μg/ml in PBS prior to coating for 4 hours at 37°C. Wells were then blocked for at least 30 minutes with 0.3% heat-inactivated BSA (fraction V, Sigma) before being washed once with PBS and plated with cells. BSA was heat-inactivated by incubation in a water bath at 80°C for 30 minutes. Cells were added in 100μl aliquots to wells already containing 100μl of Sato solution with growth factors PDGF and FGF-2 to produce final concentrations each of 25ng/ml. The plates were spun for 2 minutes at 500rpm in a Sorvall RT6000B centrifuge to obtain an even spread of cells before being incubated at 37°C in 7.5% CO_2 either overnight or for 4 days depending upon the assay.

The number of live cells was assessed by MTT assay as described by Barres et al (38). Briefly, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma) was dissolved in PBS at 5mg/ml and filter sterilised. This was added to the cultures at 1:20 dilution for 20 minutes at 37°C; a dark blue formazan reaction product is produced by the mitochondria of live cells, allowing surviving cells to be counted. One or two low power fields per well were counted by phase at 48 hours, peptides were then added, and the same field(s) counted again by MTT 6 hours later. Surviving cells 6 hours after addition of peptide are given as a percentage of the number of cells in the same field immediately prior to addition. In each experiment values were averaged from duplicate wells for each condition, and each experiment was repeated three times. Results are given as mean ± standard error; statistical significance was assessed by paired Students' t-test.

TUNEL assay. For TUNEL assays enriched OPCs were plated out as above, but in SFG solution and in 16-well chamber slides rather than 96-well plates. At the end of 4 days, an equal volume of 4% paraformaldehyde was added and the cells fixed for 30 minutes at room temperature. The chambers were then removed and the assay performed on the fixed cells. In the TUNEL assay nick-end labelling of the fragmented DNA within apoptotic nuclei relies on the Terminal deoxynucleotide transferase (Tdt) enzyme; the assay was carried out using the Apoptag Kit from Oncor/Appligene with methods according to the manufacturer's instructions. Staurosporin as control in these assays was used at 2μM as described by Jacobson et al (47).

Migration assays. Cell migration was measured using the agarose drop migration assay (12). Briefly, the oligodendrocyte precursors were obtained as described above from whole brain cultures, the suspension was centrifuged and resuspended in 20μl of SATO media containing 10% FCS and 0.3% low melting point agarose (Sigma) maintained at 37°C to prevent setting of the agarose. A 24 well culture dish (Nunc) was pre-coated with poly-L-ornithine (Sigma) overnight at room temperature, before washing once with dH_2O. The cell suspension was then applied in 1.5μl drops to the centre of each well, and this was placed at 4°C for 10 minutes to allow the agarose to partially solidify. The cell drops were then immediately surrounded by 50μl of a 50:50 solution of SATO media to DFG in order to prevent the cell drops drying. The cooled drops were then covered with 0.45ml of serum-free SATO media containing both PDGF and FGF-2 to a final concentration of 10ng/ml. Cell migration was measured at 24 hour intervals over a period of five days using a phase microscope at low power with a calibrated eyepiece graticule. Cells tended to migrate out to form a uniform corona around the drop. At any one time point, the distance between the edge of the drop and the leading edge of the processes of migrating cells within the corona was recorded on four sides of the drop, and a mean calculated using these figures. Any apparent anomalies such as individual cells which may have migrated ahead of the corona or dislodged cells were not included in the measurement of migration. Homeopeptides were added on day 1 after plating, by removing 120μl of the surrounding media from each well and adding solutions of varying concentrations of each homeopeptides. Within single experiments, each condition was tested in triplicate. The mean migration was calculated for each experiment, and the results were expressed as the mean ± standard error of three separate experiments. Statistical significance was assessed by using the Student's paired t-test.

Pre-incubation with Hom3. In order to exclude the possibility of Hom3 acting extracellularly, OPCs were also pre-incubated with Hom3, before resuspension in agarose. After the initial centrifugation the cells were resuspended in SATO media (defined above) containing Hom3 at concentrations of 0.7, 1.4 or 2.8μM or an equivalent volume of phosphate buffer solution (control), and incubated for 30 minutes. The cells were washed once in SATO media prior to a final centrifugation and suspension in an agarose drop and plating as before. Migration of the cells was assessed as above, with the experiment repeated three times.

RESULTS

In order to analyse the role of the β1 integrin cytoplasmic domain in oligodendrocyte behaviour we examined the effect of cell-permeable peptides corresponding to different portions of the cytoplasmic domain on cell survival and on cell migration. In our initial experiments three peptides linked to a 16 residue sequence from the drosophila antennapedia protein known to be capable of transporting peptides across cell membranes (41) were synthesised with sequences of 20 residues in length corresponding respectively to the membrane-proximal sequence, the central sequence and the C-terminal sequence of the β1 cytoplasmic domain. These peptides (termed Hom1, Hom2 and Hom3) are shown in Table 1.

To test the effect of the peptides on cell survival, they were added to cultures of OPCs previously grown in the presence of FGF-2 for 4-5 days; these cells are synchronised by FGF-2 at a stage of precursor differentiation at which they remain responsive to the effects of PDGF-AA (48) and do not express markers of differentiation such as O1 and MBP (49). OPCs were plated in the presence of FGF-2, PDGF and different concentrations of the three homeopeptides onto substrates of merosin (laminin-2); this substrate provides a ligand for the $\alpha 6\beta 1$ integrin which is expressed on the cells (39), and which is implicated in survival signaling by antibody blocking studies (Frost, Buttery, Milner and ffrench-Constant, unpublished observations). Survival of the cells was ascertained 6 hours after plating by the MTT assay as described in materials and methods. The membrane-proximal peptide Hom1 caused cell death at concentrations at which both Hom2 and Hom3 have no effect on survival (Fig. 1). To assess whether cells were undergoing apoptosis in the presence of Hom1, cells were exposed to the peptide or to staurosporine (2μM), known to induce apoptosis in oligodendrocyte precursors (47). After 4 days, cells were then fixed and stained by Apoptag assay. As shown in Fig. 2, both cells exposed to staurosporin and Hom1 undergo apoptosis.

TABLE 1

Sequence of β1 Integrin Cytoplasmic Domain Peptides

KLLMIIHDRREFAKFEKEKMNAKWDTGENPIYKSAVTTVVNPKYEGK	β1 integrin cytoplasmic domain
MIIHDRREFAKFEKEKMNAK	Hom1
KMNAKWDTGENPIYKSAVTT	Hom2
ENPIYKSAVTTVVNPKYEGK	Hom3/BPEP3
ENPIAKSAVTTVVNPKYEGK	BPEP4 (A/Y)
ENPIYKSAVTTVVNPKAEGK	BPEP5 (Y/A)
ENPIAKSAVTTVVNPKAEGK	BPEP6 (A/A)
ENPIYKSAVAAVVNPKYEGK	BPEP7 (AA)

Note. The sequence of the β1 integrin cytoplasmic domain peptides is given at the top, with the -COOH terminal at the right. All peptides were attached at their -NH2 terminal to the antennapedia sequence. Peptides Hom1, Hom2 and Hom3 were synthesized to >60% purity without HPLC purification; other peptides were HPLC-purified to >95% purity.

In order to test the effect of the peptides on cell migration, they were added to OPCs in an agarose drop migration assay; this assay has been used previously in this laboratory to show that the $\alpha v\beta 1$ integrin is required for oligodendrocyte precursor cells migration (12). Experiments were carried out in the presence of growth factors PDGF and FGF-2 to prevent differentiation and stimulate migration. As shown in Fig. 3A, at 1.4μM Hom2 and Hom3 enhanced migration over control whereas Hom1 had an inhibitory effect. This inhibitory effect was observed at a lower concentration than that sufficient to cause apoptosis (Fig. 1). In order to show that the effects of the peptides on migration were specific and a consequence of peptide internalization rather than an effect on the substrate, we preincubated the cells in Hom3 for 30 minutes and then washed the peptide out of the tissue culture medium before plating the cells in the migration assay. The pro-migratory effect of Hom3 was still observed, with the cells showing enhanced migratory speeds for at least 3 days after exposure to the peptide (not shown).

Our finding that both Hom3 and, to a lesser extent, Hom2 promoted migration focuses attention on the amino acids within the overlap as being involved in integrin-mediated migration. Previous studies have shown that, within this region, the NPIY sequence (which represents the N-terminal one of two NPXY motifs that are highly conserved in several integrin β cytoplasmic domains) is important for migration in other systems (31,50), while the two threonines (-TT-) have been implicated in cell adhesion (32). For our next set of experiments we therefore generated a purified peptide (>95% purity following HPLC) corresponding to the original Hom3 which contained both NPXY motifs (called BPEP3—see Table 1), and also purified peptides with mutations of the tyrosines in either or both NPXY motifs (BPEP4-6—see Table 1). Using such peptides we were able to observe significant differences in the effects on migration. Altering the N-terminal NPIY to NPIA resulted in a peptide that was now inhibitory to migration (Fig. 3B); this result was duplicated by the substitution of the tyrosines of both of the NPXY motifs (i.e. NPIY to NPIA, and NPKY to NPKA). However, an isolated change of the C-terminal NPKY to NPKA, or changing the -TT- to -AA- in the centre of the peptide (BPEP7—see Table 1), generated peptides that, in contrast to Hom3, had no effect on migration (Fig. 3B).

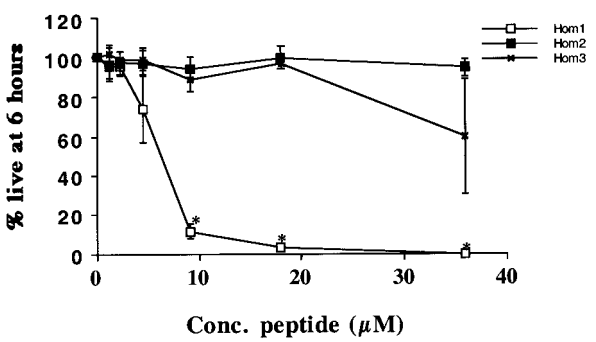

FIG. 1. Effect of Hom1, Hom2 and Hom3 on survival. Peptide was added to cell on day 2 after plating in Sato medium with growth factors PDGF and FGF-2 each at 25ng/ml. Survival was assessed 6 hour after plating by MTT assay. Results are mean ± standard error. Statistical significance from conditions without peptide are calculated by paired Student's t-test (* = $p<0.05$).

DISCUSSION

Three significant conclusions can be drawn from this work. First, cell permeable peptides can be used to investigate integrin signaling in primary cells by mapping the regions of the cytoplasmic domain involved in different aspects of cell behaviour. Second, the region of the cytoplasmic domain adjacent to the membrane is involved in cell survival signaling in oligodendrocyte precursor cells. Third, the N-terminal NPXY motif plays an important role in the control of cell migration in these cells.

A peptide corresponding to the membrane-proximal segment of the β1 integrin cytoplasmic domain caused the cells to undergo apoptosis. Two lines of evidence suggest that this is a sequence-specific effect of this peptide. First, two other peptides mimicking integrin

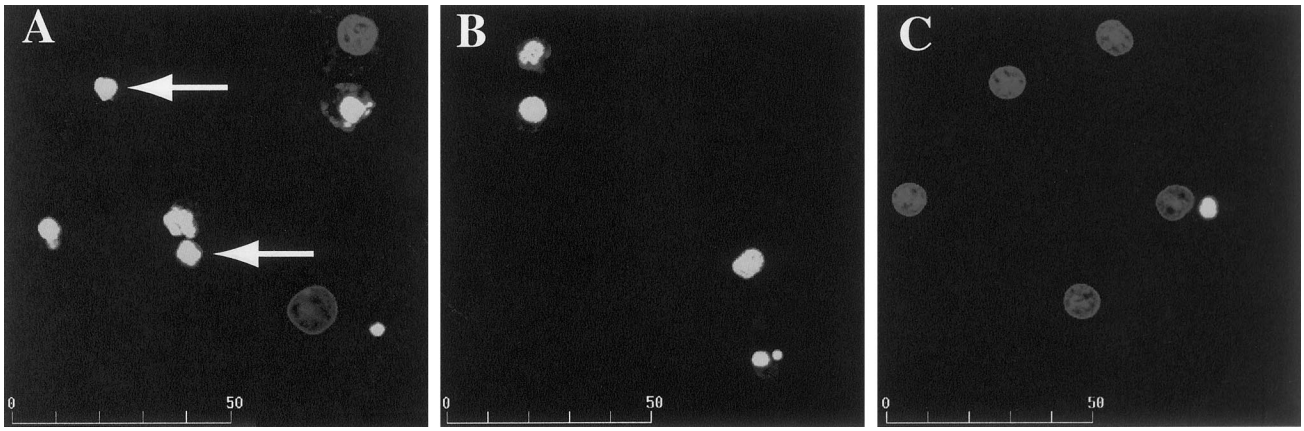

FIG. 2. Oligodendrocyte precursor cells exposed to Hom1 undergo death by apoptosis. Examples of Apoptag staining in cells treated with Hom1 (A, 30μg/ml), staurosporin (B, 2μM), or PBS (C). Homeopeptides were added at day 1 to cells in SFG medium and fixed on day 4; they were then stained with Apoptag assay kit. Imaging was by laser confocal microscopy (scale is in μm). Note that many intensely stained nuclei (arrows in A) showing apoptosis in this assay are seen in Hom1 and staurosporin (A and B) but only occasional apoptotic cells are seen in PBS (C).

cytoplasmic domain sequences (Hom 2 and 3), synthesised in an identical manner, had no effect on survival in the conditions used for our assay. Second, experiments with 12 other peptides synthesised using methods identical to Hom 1-3, and containing the antennapedia sequence coupled to sequences mimicking parts of other intracellular signalling molecules, have not shown any effects on cell survival at concentrations equal or greater than 10μM (P Doherty, unpublished observations). Hom 1, in contrast, caused more than 80% of the cells to die at this concentration. Our results with Hom 1 corroborate previous findings using injected peptides that a C-terminal sequence is important for anchorage-dependent cell survival in fibroblasts, possibly through an interaction with the protein kinase pp125-FAK (51). Just as in our study where peptides entered the cell as a result of the attached homeodomain sequence, these authors found that injected peptides mimicking more carboxy-terminal regions of the cytoplasmic domain had no effect on survival. Hypothetically, the exogenous peptide could compete with endogenous β1 integrin sequences for binding to FAK, paxillin or other cytoplasmic proteins important for formation of integrin-based signaling complexes. The identification, using *in vitro* assays, of two acidic amino acids (aspartic and glutamic acid) in the sequence KLLMIIHDRREFA that are required for FAK binding (24) suggests that further studies using Hom1 peptides with point mutations in these and other residues will enable the integrin-associated signaling molecules to be identified.

The two more C-terminal peptides, Hom2 and Hom3, enhance migration of the precursors under conditions in which differentiation of the cells is inhibited by the presence of PDGF and FGF-2 in the medium. The most C-terminal peptide Hom3 shows the most dramatic enhancement of migration, and it is this peptide that includes the two NPXY motifs, previously termed cyto-2 and cyto-3 (27), that have been shown in transfection studies to be important for integrin-mediated adhesion and focal adhesion localisation as well as cell migration (26,27,50,52-54). Cell permeable peptides have previously been used in cell lines and in macrophages to show that the C-terminal region of the β1 and β3 cytoplasmic domains are important for cell adhesion and spreading (43,55). In both of these previous studies the region defined has included both of these C-terminal tyrosine residues, but the critical sequences were not further defined. Our study significantly extends this approach in a different integrin by mapping two different integrin-mediated effects to different regions of the cytoplasmic domain and also by defining a functional role for the N-terminal NPXY motif, whose sequence is NPIY. This latter conclusion stems from the two findings. First, Hom 2, which contains the N-terminal but not the C-terminal NPXY motif, also promotes migration. Second, our structural analysis in which either or both of the two tyrosines are changed to alanine show that mutations in the C-terminal motif produce peptides that have no effect on migration (as do peptides with a double mutation of two central threonine residues) while those in the N-terminal motif now inhibit migration.

Our poor understanding of the mechanisms by which the two NPXY motifs interact with signaling molecules involved in both inside-out and outside-in signaling makes it difficult generate a precise model to explain all our results. Models based on the peptides stimulating migration directly by triggering downstream pathways or indirectly by altering cell adhesion are equally feasible. In the former case, Hom3 itself could initiate downstream signaling by binding pro-migratory sig-

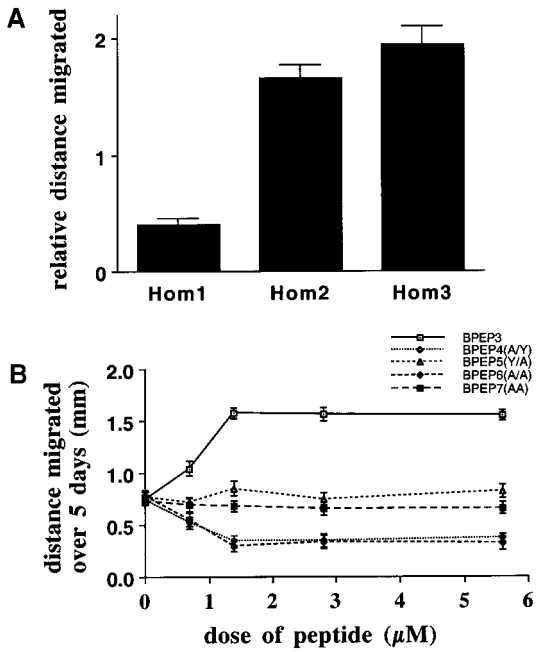

FIG. 3. (A) Inhibition and stimulation of OPC migration by different β1 integrin cytoplasmic domain peptides. Migration was assayed by agarose drop assay as described over 5 days. Peptides were added 1 day after initial plating, each at a concentration of 1.4μM. Distance migrated over 5 days under each condition is given relative to distance migrated without peptide. Results are plotted as mean $\pm$ s.e. of three separate experiments. (B) Inhibition and stimulation of OPC migration by the C-terminal β1 integrin cytoplasmic domain peptide Hom3/BPEP3 and by mutated peptides. All peptides were purified to >95% purity. Migration of OPCs was assayed by agarose drop assay as described, over 5 days. Peptides were added 1 day after initial plating, and distance migrated by the cells over 5 days is plotted against dose of peptide used on day 1. Results are plotted as mean $\pm$ s.e. of three separate experiments for each dose of peptide.

nalling molecules and then inducing conformational changes such as phosphorylation, which in turn lead on to the activation of downstream molecules. The effect of the NPIA mutation in inhibiting migration could then be explained by the peptide binding the pro-migratory molecules while not allowing subsequent phosphorylation (so acting as a dominant negative for migration signalling), while the lack of effect of the peptides with mutations in the threonines or in the C-terminal NPXY could reflect their inability to bind the pro-migratory molecules. In the latter case, in which changes in migration are secondary to changes in adhesion, at least two further models are possible. First, studies on inside-out signaling have shown that the N-terminal NPXY sequence plays an important role in this respect as mutations of this sequence (NPIY to IPIY/NPIA/YTRF) can abolish activation of the β1 integrin as measured by binding of activation-specific antibodies to chimeric integrin subunits with β1 cytoplasmic domains (56). The resulting changes in cell adhesion could promote migration by optimizing cell-substrate adhesion for motility (57). Second, previous work from β1-/- cells has led to the suggestion that controlled phosphorylation/de-phosphorylation events of the tyrosines in the two NPXY motifs is important for regulating the association of individual integrin receptors with adhesion complexes, with the phosphorylated receptors unable to join such complexes (31). The pro-migratory effect of Hom3/BPEP3 could then be explained on the basis of peptide-mediated inhibition of receptor aggregation (possibly through peptide binding to focal adhesion components such as talin (53)), with unaggregated receptors then diffusing freely in the membrane and contributing to migration. Potentially this is also in agreement with previous observations that cell-permeable peptide covering the C-terminal region of β1 and β3 cytoplasmic domain inhibit integrin-mediated adhesion (43).

Further experiments using mutated peptides combined with immunoprecipitation studies to examine complex formation will distinguish these possibilities and add significantly to our understanding of integrin signalling. These experiments and those examining integrin signalling in other cell types will be greatly facilitated by the ease with which the cell-permeable peptides can be used to introduce the different sequences into primary cells. In addition, the ability of the peptides to manipulate distinct integrin-mediated functions as we describe here suggests that they may be extremely useful therapeutic tools. Current therapeutic strategies based on inhibiting integrin function by blocking ligand binding will inhibit all downstream functions, including those that may be beneficial. As a result, unwanted side effects may emerge. The ability to dissect apart the different integrin effects by targetting the cytoplasmic domain directly using cell-permeable peptides will allow the development of more effective drug strategies.

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REFERENCES

1. Hynes, R. O. (1992) *Cell* **69**(1) 11–25.
2. Kornberg, L. J., Earp, H. S., Turner, C. E., Prockop, C., and Juliano, R. L. (1991) *Proc. Natl. Acad. Sci. USA* **88**(19), 8392–6.
3. Wary, K. K., Mainiero, F., Isakoff, S. J., Marcantonio, E. E., and Giancotti, F. G. (1996) *Cell* **87,** 733–743.
4. Mainiero, F., Murgia, C., Wary, K. K., Curatola, A. M., Pepe, A., Blumemberg, M., Westwick, J. K., Der, C. J., and Giancotti, F. G. (1997) *EMBO J.* **16**(9), 2365–2375.
5. Wei, J., Shaw, L. M., and Mercurio, A. M. (1998) *J. Biol. Chem.* **273**(10), 5903–5907.
6. Farrelly, N., Lee, Y.-J., Dive, C., and Streuli, C. H. (1998) In press.
7. Zhang, Z., Vuori, K., Wang, H.-G., Reed, J. C., and Ruoslahti, E. (1996) *Cell* **85,** 61–69.
8. Hughes, P. E., Renshaw, M. W., Pfaff, M., Forsyth, J., Keivens,

V. M., Schwartz, M. A., and Ginsberg, M. H. (1997) *Cell* **88,** 521–530.

9. Fenczik, C. A., Sethi, T., Ramos, J. W., Hughes, P. A., and Ginsberg, M. H. (1997) *Nature* **390,** 81–85.
10. Kashiwagi, H., Schwartz, M. A., Eigenthaler, M., Davis, K. A., Ginsberg, M. H., and Shattil, S. J. (1997) *J. Cell. Biol.* **137**(6), 1433–1443.
11. Chang, A. C., Salomon, D. R., Wadsworth, S., Hong, M. J. P., Mojcik, C. F., Otto, S., Shevach, E. M., and Coligan, J. E. (1995) *J. Immunol.* **154**(2), 500–510.
12. Milner, R., Edwards, G., Streuli, C., and ffrench-Constant, C. (1996) *J. Neurosci.* **16,** 7240–7252.
13. Dowling, J., Yu, Q.-C., and Fuchs, W. (1996) *J. Cell. Biol.* **134,** 559–572.
14. Frade, J. M., Marti, E., Bovolenta, P., Rodriguez-Pena, M. A., Perez-Garcia, D., Rohrer, H., Edgar, D., and Rodriquez-Tebar, A. (1996) *Development* **122,** 2497–2506.
15. Sastry, S. K., Lakonishok, M., Thomas, D. A., Muschler, J., and Horwitz, A. F. (1996) *J. Cell. Biol.* **133**(1), 169–184.
16. Felding-Habermann, B., Mueller, B. M., Romerdahl, C. A., and Cheresh, D. A. (1992) *J. Clin. Invest.* **89**(6), 2018–22.
17. Brooks, P. C., Clark, R. A., and Cheresh, D. A. (1994) *Science* **264**(5158), 569–71.
18. Fassler, R., and Meyer, M. (1995) *Genes & Dev.* **9,** 1896–1908.
19. Brakebusch, C., Hirsch, E., Potocnik, A., and Fassler, R. (1997) *J. Cell Sci.* **110,** 2895–2904.
20. Burridge, K., Fath, K., Kelly, T., Nuckolls, G., and Turner, C. (1988) *Ann. Rev. Cell Biol.* **4,** 487–525.
21. Miyamoto, S., Teramoto, H., Coso, O. A., Gutkind, J. S., Burbelo, P. D., Akiyama, S. K., and Yamada, K. M. (1995) *J. Cell. Biol.* **131**(3), 791–805.
22. Otey, C. A., Pavalko, F. M., and Burridge, K. (1990) *J. Cell. Biol.* **111**(2), 721–9.
23. Otey, C. A., Vasquez, G. B., Burridge, K., and Erickson, B. W. (1993) *J. Biol. Chem.* **268**(28), 21193–7.
24. Schaller, M. D., Otey, C. A., Hildebrand, J. D., and Parsons, J. T. (1995) *J. Cell. Biol.* **130**(5).
25. Hayashi, Y., Haimovich, B., Reszka, A., Boettiger, D., and Horwitz, A. (1990) *J. Cell. Biol.* **110**(1), 175–84.
26. Marcantonio, E. E., Guan, J. L., Trevithick, J. E., and Hynes, R. O. (1990) *Cell Regul.* **1**(8), 597–604.
27. Reszka, A. A., Hayashi, Y., and Horwitz, A. F. (1992) *J. Cell. Biol.* **117**(6), 1321–30.
28. Solowska, J., Guan, J. L., Marcantonio, E. E., Trevithick, J. E., Buck, C. A., and Hynes, R. O. (1989) *J. Cell. Biol.* **109**(2), 853–61.
29. Lewis, J. M., and Schwartz, M. A. (1995) *Mol. Biol. Cell* **6,** 151–160.
30. Belkin, A. M., Retta, S. F., Pletjushkina, O. Y., Balzac, F., Silengo, L., Fassler, R., Koteliansky, V. E., Burridge, K., and Tarone, G. (1997) *J. Cell. Biol.* **139,** 1583–1595.
31. Sakai, T., Zhang, Q., Fassler, R., and Mosher, D. F. (1998) *J. Cell. Biol.* **141**(2), 527–538.
32. Wennerberg, K., Fassler, R., Warmegard, B., and Johansson, S. (1998) *J. Cell Sci.* **111,** 1117–1126.
33. Abney, E. R., Bartlett, P. F., and Raff, M. C. (1981) *Dev. Biol.* **83,** 301–310.
34. Raff, M. C., Miller, R. H., and Noble, M. (1983) *Nature* **303,** 390–396.
35. Temple, S., and Raff, M. C. (1985) *Nature* **313,** 223–225.
36. Gard, A. L., and Pfeiffer, S. E. (1989) *Development* **106**(1), 119–32.
37. Hardy, R., and Reynolds, R. (1991) *Development* **111,** 1061–1080.
38. Barres, B. A., Hart, I. K., Coles, H. S., Burne, J. F., Voyvodic, J. T., Richardson, W. D., and Raff, M. C. (1992) *Cell* **70**(1), 31–46.
39. Milner, R., and ffrench-Constant, C. (1994) *Development* **120**.
40. Milner, R., Frost, E., Nishimura, S., Delcommenne, M., Streuli, C., Pytela, R., and ffrench-Constant, C. (1997) *Glia* **21,** 350–360.
41. Derossi, D., Joliot, A. H., Chassaing, G., and Prochiantz, A. (1994) *J. Biol. Chem.* **269**(14), 10444–10450.
42. Hall, H., Williams, E. J., Moore, S. E., Walsh, F. S., Prochiantz, A., and Doherty, P. (1996) *Curr. Biol.* **6**(5), 580–587.
43. Liu, K.-Y., Timmons, S., Lin, Y.-Z., and Hawiger, J. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 11819–11824.
44. Hall, D. E., Neugebauer, K. M., and Reichardt, L. F. (1987) *J. Cell. Biol.* **104,** 623–634.
45. Williams, E. J., Dunican, D. J., Green, P. J., Howell, F. V., Derossi, D., Walsh, F. S., and Doherty, P. (1997) *J. Biol. Chem.* **272,** 22349–354.
46. McCarthy, K. D., and de Vellis, J. (1980) *J. Cell. Biol.* **85,** 8790–8802.
47. Jacobson, M. D., Burne, J. F., King, M. P., Miyashita, T., Reed, J. C., and Raff, M. C. (1993) *Nature* **361,** 365–369.
48. McKinnon, R. D., Smith, C., Behar, T., Smith, T., and Dubois-Dalcq, M. (1993) *Glia* **7**(3), 245–54.
49. Bansal, R., Kumar, M., Murray, K., Morrison, R. S., and Pfeiffer, S. E. (1996) *Mol. Cell. Neurosci.* **7,** 263–275.
50. Filardo, E. J., Brooks, P. C., Deming, S. L., Damsky, C., and Cheresh, D. A. (1995) *J. Cell. Biol.* **130,** 441–450.
51. Hungerford, J. E., Compton, M. T., Matter, M. L., Hoffstrom, B. G., and Otey, C. A. (1996) *J. Cell. Biol.* **135**(5), 1383–1390.
52. Balzac, F., Belkin, A. M., Koteliansky, V. E., Balabanov, Y. V., Altruda, F., Silengo, L., and Tarone, G. (1993) *J. Cell. Biol.* **121**(1), 171–8.
53. Vignoud, L., Albiges-Rizo, C., Frachet, P., and Block, M. R. (1997) *J. Cell Sci.* **110,** 1421–1430.
54. Romzek, N. C., Harris, E. S., Dell, C. L., Skronek, J., Hasse, E., Reynolds, P. J., Hunt, S. W., and Shimizu, Y. (1998) *Mol. Biol. Cell* **9,** 2715–2727.
55. Boissy, P., Machuca, I., Pfaff, M., Ficheux, D., and Jurdic, P. (1998) *J. Cell Sci.* **111,** 2563–74.
56. O'Toole, T. E., Ylanne, J., and Culley, B. M. (1995) *J. Biol. Chem.* **270**(15), 8553–8558.
57. Palecek, S. P., Loftus, J. C., Ginsburg, M. H., Lauffenburger, A., and Horwitz, A. F. (1997) *Nature* **385,** 537–540.