A tyrosine-based sorting signal in the $\beta 2$ integrin cytoplasmic domain mediates its recycling to the plasma membrane and is required for ligand-supported migration

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Integrins play pivotal roles in supporting shear- and mechanical-stress-resistant cell adhesion and migration. These functions require the integrity of the short β subunit cytoplasmic domains, which contain multiple, highly conserved tyrosine-based endocytic signals, typically found in receptors undergoing regulated, clathrin-dependent endocytosis. We hypothesized that these sequences may control surface integrin dynamics in statically adherent and/or locomoting cells via regulated internalization and polarized recycling of the receptors. By using site-directed mutagenesis and ectopic expression of the $\alpha L/\beta 2$ integrin in Chinese hamster ovary cells, we found that Y735 in the membrane-proximal YRRF sequence is selectively required for recycling of spontaneously internalized receptors to the cell surface and to growth factor-induced membrane ruffles. Disruption of this motif by non-conservative substitutions has no effect on the receptor's adhesive function, but diverts internalized integrins from a recycling compartment into a degradative pathway. Conversely, the non-conservative F754A substitution in the membrane-proximal NPLF sequence abrogates ligand-dependent adhesion and spreading without affecting receptor recycling. Both of these mutants display a severe impairment in ligand-supported migration, suggesting the existence in integrin cytoplasmic domains of independent signals regulating apparently unrelated functions that are required to sustain cell migration over specific ligands.

Keywords: adhesion receptors/cell migration/endocytosis/integrins/protein sorting

Introduction

Integrins are a large family of heterodimeric type I transmembrane receptors mediating cell–cell or cell–extracellular matrix adhesion (Hynes, 1992). Most functional properties of integrins depend on the integrity of their short cytoplasmic domains, which are highly conserved among the various members of the family and across species, suggesting strong selective pressure for conservation of functionally relevant features (LaFlamme *et al.*,

1997). In spite of their high degree of homology, integrin cytoplasmic domains are devoid of catalytic function and lack defined structural elements found in functionally relevant protein modules. A possible exception is represented by the existence in the cytoplasmic domain of most β subunits of two tyrosine-based motifs, NPXY (where X represents a non-conserved residue), which are tight turn-forming sequences found in receptors (e.g. the LDL receptor) undergoing regulated endocytosis in clathrin-coated pits and vesicles (Chen *et al.*, 1990). Interestingly, integrins selectively expressed by leukocytes, such as $\beta 2$ and $\beta 7$, have an additional tyrosine-based ‘endocytic signal’ in the membrane-proximal region, which conforms to the sequence of the tyrosine-based motif YXX Φ (where X is any amino acid and Φ is an amino acid with a bulky hydrophobic residue) found in the transferrin receptor and other transmembrane receptors (Mellman, 1996; Schmid, 1997). Such a signal has been implicated both in receptor-mediated endocytosis and in protein targeting to post-Golgi compartments (Boll *et al.*, 1996; Wolins *et al.*, 1997).

Why would integrins need multiple internalization signals, typically found in receptors whose ligands are soluble molecules, requiring regulated endocytosis for function? There is indeed evidence for a role of several integrins in the internalization of various microorganisms (Wright and Griffin, 1985; Isberg and Leong, 1990; Wickham *et al.*, 1993). The uptake of the enteroinvasive bacterium *Yersinia pseudotuberculosis* by $\beta 1$ integrins has been studied in detail and shown by mutagenesis to be dependent on the integrity of the membrane-proximal NPIY sequence (Isberg and Leong, 1990). Likewise, the N-terminal NPLY sequence was found to be required for phagocytosis of fibrinogen-coated particles by ectopically expressed $\alpha IIb/\beta 3$ integrin in fibroblastoid cells (Ylanne *et al.*, 1995). However, given the cell- and receptor-specific nature of these interactions, it is unlikely that the primary role of integrin β subunit internalization motifs is to allow efficient phagocytosis of soluble particles.

An alternative role for these conserved sequences could be that of controlling surface integrin dynamics in statically adherent and/or locomoting cells via regulated internalization and polarized recycling of the receptors. Several studies have demonstrated that in a locomoting cell, new integrin-based focal contacts form continuously at the cell front and persist until they reach the cell rear, where integrins are released from their extracellular ligands and the intracellular connections with the actin-based cytoskeleton (Felsenfeld *et al.*, 1996; Lauffenburger and Horwitz, 1996). Recycling of endocytosed receptors (and membrane) to the leading edge is an intriguing possibility, but evidence in its favour remains elusive (Bretscher, 1996a,b; Bretscher and Aguado-Velasco, 1998a). Hopkins *et al.* (1994) have shown that in migrating fibroblasts, endocytosed transferrin receptors loaded by fluorescent

tracers preferentially recycle over the surface of the leading lamellae. In a more recent study, Lawson and Maxfield (1995) have shown that the polarized distribution of $\alpha V/\beta 3$ integrins in migrating neutrophils is maintained by $[Ca^{2+}]_i$ -dependent release of adhesion followed by endocytosis of this integrin and recycling to the leading edge. Finally, a recent report has demonstrated the selective enrichment of recycling receptors in growth factor-induced membrane ruffles (Bretscher and Aguado-Velasco, 1998b).

To address this issue further, we generated site-directed mutants of the $\beta 2$ integrin cytoplasmic domain's internalization motifs and expressed them constitutively in Chinese hamster ovary (CHO) cells. Our results demonstrate that Y735, located in the membrane-proximal YRRF motif, is selectively required for recycling of spontaneously internalized receptors to the cell surface and to growth factor-induced ruffles. Disruption of this motif by non-conservative substitutions does not impair the ability of the mutated receptor to recruit cytoskeletal proteins and effect cell adhesion and spreading on immobilized ligand, but diverts internalized integrins from a recycling compartment into a degradative pathway. This is paralleled by a marked decrease in ligand-supported cell migration, thus lending support to the hypothesis that endocytosis and polarized recycling provide an important contribution to the replenishment of integrin receptors at the leading edge of locomoting cells.

Results

Generation of human $\beta 2$ integrin cytoplasmic domain mutants and expression in CHO cells

We introduced a series of point mutations in the $\beta 2$ integrin putative endocytic signals to analyse their role in constitutive endocytosis and recycling of surface-expressed receptors, relative to the receptor's ability to mediate ligand-induced cell spreading and migration (Figure 1A). Based on previous reports analysing structure–function relationships in membrane receptors carrying tyrosine-based endocytic signals, we introduced both non-conservative (Y to A or F to A) and conservative (Y to F) substitutions in these motifs (Trowbridge *et al.*, 1993; Ohno *et al.*, 1995). The cDNAs coding for either the wild-type or mutant human $\beta 2$ were co-transfected with the wild-type human αL subunit in CHO cells, which do not express endogenous $\beta 2$ integrins (not shown), and stable transfectants were established by drug-based selection and sorting of surface $\alpha L/\beta 2$ -expressing clones. Stable clones were selected that expressed comparable levels of surface-expressed receptor, as judged by immunofluorescence analysis, to an anti- αL monoclonal antibody (mAb) (Figure 1B).

Mutations in the $\beta 2$ putative internalization motifs do not impair constitutive $\alpha L/\beta 2$ endocytosis in CHO cells

To study the effects of the mutations of the $\beta 2$ subunit on its constitutive endocytosis, we determined biochemically the efficiency and kinetics of internalization of surface-expressed receptors in the stable transfectants. Cells were labelled with NHS-SS-biotin at 0°C, incubated for various times at 37°C to allow internalization, and then exposed to a non-membrane-permeable reducing agent, glutathione

(GSH). Using this approach, the endocytosed pool of proteins was protected from reduction and remained biotinylated. $\alpha L/\beta 2$ was then immunoprecipitated from lysed cells and resolved by SDS–PAGE, followed by transfer onto nitrocellulose and detection of the biotinylated fraction with horseradish peroxidase (HRP)–streptavidin (Figure 2). Parallel immunoprecipitations were carried out on each sample with antibodies to a reporter intracellular protein (β tubulin) to normalize the immunoprecipitates for their relative protein content. Figure 3 shows that the spontaneous internalization of wild-type receptors follows a time-dependent increase, with ~30% of the total pool of biotin-labelled $\alpha L/\beta 2$ recovered from the intracellular fraction after a 40 min incubation at 37°C. None of the mutants analysed showed a major impairment of constitutive endocytosis (Figure 3). However, the internalization kinetics of the various mutants differed widely and reproducibly. Notably, the $\Delta 733$ deletion mutant and all mutants carrying the F754A substitution underwent a slower and less efficient internalization compared with the wild-type receptor, with the possible exception of the YAA mutant, which showed, albeit inconsistently, internalization levels at 30 min that were comparable to those of the wild-type receptor. Conversely, mutants carrying amino acid substitutions at positions 735 and 766 did not differ significantly from the wild-type receptor, except for a slightly longer half-time of internalization.

The Y735A mutation diverts the internalized $\alpha L/\beta 2$ from a recycling compartment into a degradative pathway

Using an extension of the biochemical assay described above, we quantified the efficiency and kinetics of recycling to the plasma membrane of the internalized wild-type and mutant $\alpha L/\beta 2$ receptors. Cells were labelled with NHS-SS-biotin at 0°C, incubated at 37°C for 40 min to allow optimal internalization, and treated with GSH to remove the label from the residual surface-expressed receptors. The internalized fraction was then chased at 37°C for various time periods. At each time point, cells were either re-exposed to GSH to remove biotin from receptors that had recycled back to the cell surface, or kept in a non-reducing buffer, to evaluate the total amount of receptors at the various time points (Figure 2).

As shown in Figures 2 and 4, wild-type $\alpha L/\beta 2$ is rapidly and efficiently recycled to the plasma membrane while the total amount of labelled receptor does not vary significantly during the chase period, indicating that it is relatively stable. A similar behaviour was observed in the mutants at positions 754 and 766 (F754A, F766A and YAA) (Figure 4). The deletion mutant ($\Delta 733$) was degraded minimally, in spite of its reduced ability to recycle to the plasma membrane during the observation period (Figure 4). Remarkably, the two mutants carrying non-conservative substitutions at tyrosine 735 (Y735A and AAA) showed a sizeable recycling only at early time points, which was followed by rapid and massive degradation of the mutant receptors at later time points (Figures 2 and 4). In contrast, the conservative Y735F substitution did not alter significantly the extent and kinetics of recycling to the cell surface of the internalized receptor.

Fig. 1. (A) Human $\beta 2$ integrin cytoplasmic domain mutants utilized in this study. Shown is the wild-type amino acid sequence, with the underlined cyto-1, cyto-2 and cyto-3 subregions (Reszka *et al.*, 1992). The introduced amino acid substitutions are shown in bold; the * denotes a nonsense mutation introduced to create a deletion mutant at codon 733. (B) Surface expression of ectopically expressed wild-type and mutant $\beta 2$ integrin in CHO cells. Stable transfectants co-expressing wild-type αL and wild-type or mutant $\beta 2$ were treated with anti- αL mAb, followed by staining with a FITC-conjugated goat anti-mouse Ig antibody and analysis on a FACScanTM flow cytometer. Cells expressing the wild-type receptor were also stained with secondary antibody alone (thick line) as a control for background fluorescence.

To investigate further the rate of internalization and recycling of ectopically expressed $\alpha L/\beta 2$, surface expression of the wild-type or mutant receptors was evaluated by cytofluorimetric analysis after treatment with cycloheximide (Table I). In the absence of newly synthesized proteins, the amount of $\alpha L/\beta 2$ expressed at the cell surface reflects the balance between internalization and either recycling or degradation of the receptor. As shown in Table I, after 12 h of cycloheximide treatment the amount of surface-expressed wild-type receptor was virtually unchanged. In contrast, the expression levels of the Y735A, but not the Y735F mutant showed a marked reduction. These data further confirm that a non-conservative substitution at tyrosine 735 is sufficient to divert the internalized

receptor from a recycling compartment into a degradative pathway. When compared with the Y735A mutant, the triple mutant AAA showed a less dramatic reduction in cell surface expression upon cycloheximide treatment, but the decrease was still considerably larger than in all the other mutants and the wild-type receptor-expressing clones (Table I). This discrepancy might be due to the less efficient internalization of this mutant, which results in a prolonged localization of labelled receptors on the cell surface (Figure 3).

To assess whether receptor degradation in the Y735A mutant is compensated by a higher rate of biosynthesis, we performed a pulse-chase experiment using [³⁵S]methionine in the various clones, followed by immunopre-

Fig. 2. Internalization and recycling of ectopically expressed wild-type and mutant α L/ β 2 receptors in CHO transfectants. To quantify receptor internalization, cells were surface-labelled with NHS-SS-biotin at 4°C and either lysed immediately to evaluate the total amount of labelled receptor (T) or incubated at 37°C for the indicated time points prior to GSH treatment and lysis, as described in Materials and methods. To analyse receptor recycling, NHS-SS-biotin-labelled cells were incubated at 37°C for 45 min to allow optimal receptor internalization and treated with GSH to remove the label from the residual surface-expressed receptors. Cells were then lysed (0 time point) or reincubated at 37°C for the time points indicated to chase internalized receptors, followed by treatment with (+) or without (-) the reducing agent to remove the label from surface-expressed receptors prior to cell lysis. Immunoprecipitation was carried out in all samples using a combination of anti- α L and anti- β 2 mAb. Eluted samples were resolved by SDS-PAGE, transferred onto nitrocellulose membranes and revealed with HRP-streptavidin.

cipitation of the LFA-1 heterodimer (Figure 5). The densitometric analysis of three separate experiments demonstrates that [35 S]methionine incorporation by the Y735A mutant during the pulse is 2.1 ± 0.2 -fold higher than for the wild-type receptor. Both the wild-type and the mutant receptor undergo a steady maturation along the exocytic pathway, as judged by the appearance of slower migrating glycosylated forms of the two subunits (Pardi *et al.*, 1995). At a later time point (24 h), we observed a marked reduction in the levels of the mature Y735A mutant receptors, compared with the wild type (0.5 versus 25.2% of the α L signal detected at time 0, respectively). These findings indicate that the steady-state levels of surface-expressed receptor in this mutant are comparable to those of the wild type as a result of a high rate of synthesis and transport to the plasma membrane of the mutant receptors.

α L/ β 2 receptors carrying the Y735A mutation do not concentrate in epidermal growth factor (EGF)-induced membrane ruffles

The direct visualization of recycling receptors under steady-state conditions is hampered by the fact that the endocytic cycle is an asynchronous process occurring over the entire cell surface. However, a recent study has shown that recycling receptors become enriched in growth factor-induced membrane ruffles, which are visible within minutes of growth factor stimulation in selected cell types, and appear to arise by exocytosis of the internal membrane from the endocytic cycle (Bretscher and Aguado-Velasco, 1998b). We performed a similar experiment in HeLa cells transiently expressing the wild-type or mutant β 2 subunit together with a chimeric α L-green fluorescent protein (GFP) fusion protein, constructed by ligating the GFP moiety C-terminal to the human α L subunit. We had

Fig. 3. Densitometric analysis of spontaneously internalized α L/ β 2 receptors in wild-type and mutant receptor-expressing CHO clones. Internalization of labelled receptors was carried out for the indicated time points as described in the Figure 2 legend. After lysis, samples were subjected to immunoprecipitation using a combination of anti- α L and anti- β 2 mAb plus an anti- β tubulin mAb for normalization, which was carried out as described in Materials and methods. Data are expressed as percentage internalization over the total amount of labelled receptor. Values represent the mean $\pm$ SD of four separate experiments.

preliminarily determined that the chimeric α L-GFP subunit assembled correctly with β 2 and affected neither the ability of this integrin to support ligand-induced adhesion and spreading, nor the extent and kinetics of receptor internalization and recycling (not shown). Prior to EGF stimulation, serum-starved HeLa cells display a poorly organized actin cytoskeleton, consisting of very thin stress fibres without focal accumulation of cortical actin (not shown). Figure 6 shows that upon EGF stimulation, wild-type α L-GFP/ β 2 receptors and those carrying the conservative Y735F mutation become highly enriched in membrane ruffles (characterized by an increased content of F-actin) as early as 10 min after exposure to the growth factor. In contrast, both the Y735A mutant and the Δ 733 mutant display a more diffuse fluorescence pattern, with only a moderate increase in fluorescence intensity in ruffles compared with the rest of the cell surface. Such increase in epi-fluorescence is compatible with an additive signal due to the complex folding of the plasma membrane in ruffles. Note, the Y735A mutant consistently displayed reduced surface expression levels during the transient expression obtained in HeLa cells. A similar experiment was performed using time-lapse videomicroscopy, to follow the receptors' dynamics in living cells. As shown in Figure 7, wild-type receptors were widely distributed over

Fig. 4. Densitometric analysis of recycled versus degraded α L/ β 2 receptors in wild-type and mutant receptor-expressing CHO clones. Recycling and degradation of spontaneously internalized receptors were evaluated for the indicated time points as described in Materials and Methods. Data are expressed as a percentage of the pool of receptors internalized at 45 min, which is recycled to the plasma membrane (closed circles) or degraded (open circles) during the chase period. Values represent the mean $\pm$ SD of four separate experiments.

Table I. Turnover of surface-expressed α L/ β 2 integrin in cycloheximide-treated CHO transfectants^a

Clone	Untreated (n = 3)	Cycloheximide ^b (n = 3)	% variation
Wild type	648 $\pm$ 54	686 $\pm$ 96	+5.5
Δ 733	727 $\pm$ 32	706 $\pm$ 18	-2.9
Y735A	525 $\pm$ 54	132 $\pm$ 39	-74.9
Y735F	462 $\pm$ 28	484 $\pm$ 46	+4.7
F754A	839 $\pm$ 89	864 $\pm$ 89	+2.9
F766A	757 $\pm$ 66	801 $\pm$ 84	+5.8
YAA	420 $\pm$ 36	448 $\pm$ 18	+6.6
AAA	553 $\pm$ 51	426 $\pm$ 44	-23.0

^aSurface expression of α L/ β 2 was evaluated by indirect immunofluorescence on a flow cytometer using an anti- α L mAb. Values are expressed as arbitrary mean fluorescence units $\pm$ 1 SD, n = sample size.

^bCells were left untreated or incubated with 500 μ M cycloheximide for 12 h prior to analysis by immunofluorescence.

the cell body in unstimulated cells and became enriched in ruffles as early as 5 min following EGF stimulation. The enrichment in ruffles was paralleled by an apparent decrease of the fluorescence signal over the rest of the cell body. In contrast, neither the Δ 733 nor the Y735A mutant showed such relocalization in membrane ruffles upon EGF treatment.

Fig. 5. Biosynthesis and degradation of the wild-type and Y735A mutant α L/ β 2 integrin in stable transfectants. Cells were pulsed for 45 min at 37°C with [³⁵S]methionine-cysteine and either lysed (time 0) or chased for the indicated times before lysis. α L/ β 2 was then immunoprecipitated and samples were subjected to SDS-PAGE (upper panel, ³⁵S-met.). To normalize, one-fifth of each sample, after SDS-PAGE, was transferred onto nitrocellulose and blotted with anti- α L, followed by HRP-conjugated goat anti-mouse (lower panel, W.B.). β 2' indicates the immature form of the β 2 subunit.

Selected mutations in the cyto-2 domain of the β 2 subunit affect cell spreading on immobilized ligand

Having identified a critical determinant for the recycling of internalized α L/ β 2 to the cell surface, we asked whether this function could be dissociated from previously established functional properties of integrin receptors, such as the ability to promote cell adhesion and spreading onto the purified ligand ICAM-1. With this aim, the various CHO clones expressing wild-type or mutated α L/ β 2 were plated onto ICAM-1-coated dishes in serum-free medium and incubated at 37°C for 30 min prior to removal of unbound cells. Under these conditions, untransfected CHO cells did not adhere at all to the substratum (not shown), whereas cells expressing wild-type α L/ β 2 adhered firmly and gave rise to a number of lamellipodia (Figure 8) and long membrane projections, which by time-lapse videomicroscopy appeared to be uropodia located at the trailing edge of moving cells (not shown). As expected, based on previous reports by our (Pardi *et al.*, 1995) and other groups (Hibbs *et al.*, 1991), cells carrying the Δ 733 mutation adhered weakly to the immobilized ligand (see below) and did not spread, due to the inability of the deleted β subunit to connect the membrane receptor to the actin-based cytoskeleton. Interestingly, the single amino acid substitution F754A also severely affected adhesion and spreading onto immobilized ICAM-1: all cells bearing this mutation (F754A, YAA, AAA) displayed weak adhesion to the ligand, and showed neither spreading nor membrane projections (Figure 8). Conversely, mutations at the level of tyrosine 735 and phenylalanine 766, irrespective of their conservative (Y735F) or non-conservative (Y735A, F766A) nature, yielded a phenotype comparable to that of the wild-type receptor in this assay (Figure 8). Moreover, using ICAM-1-coated beads to induce receptor clustering, we observed a direct correlation between the ability of wild-type versus mutant α L/ β 2 receptors to recruit α -actinin to the clusters and the efficiency of spreading on immobilized ligand (not shown).

The mutant Y735A has a decreased ability to adhere to and migrate over the immobilized ligand

The functional properties of integrin receptors explored in the assays described above have been reported previously to be relevant for integrin-mediated cell migration. To investigate to what extent ligand-supported adhesion

Fig. 6. Effects of mutations in the $\beta 2$ integrin cytoplasmic domain on $\alpha L/\beta 2$ distribution in EGF-induced ruffles. HeLa cells were transiently transfected with the chimeric αL -GFP cDNA, together with wild-type $\beta 2$ or the $\Delta 733$, Y735A and Y735F mutants. Transfectants were plated on fibronectin-coated glass coverslips, serum-starved for 24 h and incubated with EGF (100 ng/ml) for 10 min. Cells were then fixed, permeabilized and decorated with TRITC-conjugated phalloidin (PHD). Arrowheads indicate ruffles. Scale bar, 15 μ m.

and migration were affected by the mutations introduced in the internalization motifs of $\beta 2$ integrin, we quantified cell adhesion and migration over ICAM-1. Consistent with the findings described above, adhesion to ICAM-1, but not to the $\beta 1$ integrin ligand fibronectin, was defective in the $\Delta 733$ deletion mutant and whenever F754 was non-conservatively substituted (Figure 9A). To assess the specific migration efficiency of the various clones, we utilized a haptotactic migration assay that measures the ability of a cell to move along a concentration gradient of solid phase-bound substrate (Huttenlocher *et al.*, 1996). Cells were plated in the upper chamber of a modified Boyden chamber, in which the lower side of the filter was coated with or without purified ICAM-1 or fibronectin prior to blocking with 1% bovine serum albumin (BSA). The efficiency of migration of all clones over fibronectin-coated filters ranged from 12 to 18% of the total input cell population during the 16 h incubation period, and was reproducible for each clone tested (not shown). Cells

expressing wild-type $\alpha L/\beta 2$, as well as the mutant clones Y735F and F766A, migrated efficiently over immobilized ICAM-1 when compared with the migration of the same clones on fibronectin (Figure 9B). In contrast, the ligand-supported migration of the mutants F754A, YAA and AAA was negligible and comparable to background levels (Figure 9B). Most interestingly, the mutant Y735A, whose only detectable defect was in the recycling to the plasma membrane of the internalized receptor, displayed a marked reduction in the ability to migrate over the immobilized ligand. This defect was not due to a decrease of the steady-state levels of the surface-expressed receptor during the assay, as in the absence of protein synthesis inhibitors they remained stable and were comparable to those observed in the wild-type receptor-expressing clones (not shown). Similar results were obtained by performing shorter-term migration assays (8 h), although the migration values were lower at this time point (control migration ranged between 6 and 8%).

Fig. 7. Wild-type and mutant GFP-tagged α L/ β 2 receptor dynamics in EGF-treated cells. HeLa cells were transiently transfected with the chimeric α L-GFP cDNA, together with the wild-type or Δ 733 and Y735A mutant β 2 subunits. Transfectants were plated on fibronectin-coated glass coverslips, serum-starved for 24 h and analysed by time-lapse videomicroscopy. The images shown were acquired before (untreated) and 10 min after the addition of 100 ng/ml EGF. Arrowheads indicate EGF-induced ruffles. Scale bar, 20 μ m.

Discussion

In this work we utilized site-directed mutagenesis of the β 2 integrin cytoplasmic domain to address the unsolved issue concerning the functional role of the multiple, conserved endocytic signals found in this domain. The most relevant finding reported here is the identification of a sorting signal in the membrane-proximal YRRF motif, which appears to be selectively required for the recycling of spontaneously internalized receptors to the cell surface and to growth factor-induced membrane ruffles. Notably, we show that the disruption of this motif by a non-conservative substitution of Y735 diverts internalized integrins from a recycling compartment into a degradative pathway, while not impairing the ability of the mutated receptor to recruit cytoskeletal proteins and to affect cell adhesion and spreading onto immobilized ligand.

Structural and functional studies performed on a number of receptors undergoing regulated endocytosis have firmly established that short, tyrosine-based linear sequences (YXX Φ - and NPXY-type), located in their cytoplasmic domains, are necessary and sufficient to mediate internalization in clathrin-coated pits and vesicles (Trowbridge *et al.*, 1993). Such motifs appear to mediate the direct

association of the transmembrane receptor with selected components of the AP-2 adaptor complexes, which in turn are connected to the clathrin-based triskelion (Schmid, 1997). The existence in all integrin β subunits of multiple, highly conserved motifs conforming to the above described consensus sequence is difficult to reconcile with the notion that integrin ligands consist mostly of insoluble extracellular matrix components or cell-bound integral membrane proteins. In addition, most integrin functions do not require regulated internalization of the receptor, although evidence exists that in selected cases surface integrins do internalize soluble particles composed of native or opsonized microorganisms (Wright and Griffin, 1985; Isberg and Leong, 1990; Wickham *et al.*, 1993).

We hypothesized that the endocytic signals found in integrin receptors serve a more general function, that of providing a polarized endocytic cycle, aimed at concentrating surface receptors in areas of tight adhesion in statically adherent or locomoting cells. To test this hypothesis, we introduced a series of mutations, alone or in combination, in critical residues belonging to the three endocytic signals of the β 2 subunit. The amino acid substitutions were predicted to be informative on the basis of previous

Fig. 8. Effects of mutations in the $\beta 2$ integrin cytoplasmic domain on cell attachment and spreading onto immobilized ICAM-1. Equal numbers of CHO stable transfectants expressing wild-type or mutated $\alpha L/\beta 2$ receptors were plated onto ICAM-1-coated dishes for 30 min at 37°C, followed by removal of non-adherent cells. Phase contrast micrographs were taken using 240 $\times$ magnification. Scale bar, 20 μ m.

structure–function studies carried out on the analogous sequences found in receptors undergoing regulated endocytosis (Trowbridge *et al.*, 1993).

Our data indicate that the spontaneous internalization of ectopically expressed $\alpha L/\beta 2$ integrin in CHO cells is a very rapid process, considering that >30% of surface-expressed receptors are internalized in 30–40 min, with a half-time of internalization of 10–15 min. However, the rate of internalization of endogenously expressed integrins may differ widely depending on the cell type and individual members of the family, as shown previously by Bretscher (1992). Interestingly, in his report Bretscher showed broad variations in the efficiency of internalization of two leukocyte integrins sharing the common $\beta 2$ subunit ($\alpha L/\beta 2$ and $\alpha M/\beta 2$), which suggests that α subunits may play a fundamental role in the endocytic cycle of these receptors. $\alpha L/\beta 2$ in particular was shown to have a relatively slow turnover in lymphoid cell lines compared with the other members of the leukocyte integrin family. Conceivably, integrin dynamics at the cell surface may be subjected to variations depending on the activation state of the cell and the phenotype, i.e. non-adherent, statically adherent or locomoting, of the cell being studied. Thus, although our model may not be adequate to compare the rate of internalization and recycling of a given ectopically expressed integrin heterodimer with its endogenous coun-

Fig. 9. Ligand-supported cell adhesion and migration of wild-type or mutant $\alpha L/\beta 2$ -expressing CHO transfectants. (A) Adhesion assay. ^{51}Cr -labelled cells were plated onto fibronectin or ICAM-1-coated 96-well ELISA plates. Wells were then thoroughly washed, adherent cells lysed and residual radioactivity analysed with a γ -counter. Results are expressed as the percentage of adherent out of total input cells. (B) Migration assay. Cells were plated over porous (5 μ m diameter) polycarbonate filters coated on the lower side with BSA alone (background), fibronectin (control) or ICAM-1. After 16 h incubation in 1% FCS-containing medium, cells remaining on the upper side of the filter were removed, and cells bound to the lower side of the filter were fixed and stained with crystal violet. Migration was quantified with a colorimetric assay as indicated in Materials and methods. Results are expressed as a percentage migration of each transfectant on ICAM-1 (empty columns) or BSA (background, hatched columns) over control migration on fibronectin-coated filters (ranging from 13 to 18% in the various clones). Values represent the mean $\pm$ SD of five separate experiments.

terpart, it does provide a versatile tool to study the endocytic cycle of these receptors as a function of the adhesive phenotype of the cell.

As reported previously for $\beta 2$ and $\beta 3$ integrins (Wright and Griffin, 1985; Ylanne *et al.*, 1995), none of the introduced mutations had a major effect on the constitutive endocytosis of the surface expressed receptor, although we did observe reduced internalization rates in the $\Delta 733$ deletion mutant and whenever F754, belonging to the membrane-proximal NPXF motif, was mutated non-conservatively. This might suggest that spontaneous endocytosis does require defined structural features in the β subunit cytoplasmic domain. Alternatively, endogenous ligands for the $\alpha L/\beta 2$ integrin present in our ectopic expression system may play a role in promoting receptor clustering and internalization. In favour of this interpreta-

tion, we observed that unlike mock-transfected cells, wild-type α L/ β 2-expressing clones had a strong tendency to form homotypic aggregates when plated on non-adhesive surfaces (not shown). Our findings might therefore be reconciled with previous reports showing that individual endocytic signals in selected integrin β subunits do play a role in ligand-driven internalization of the receptor (Wright and Griffin, 1985; Isberg and Leong, 1990; Ylanne *et al.*, 1995). However, several reports, and our present findings, suggest that the NPXY(F) motif composing the cyto-2 subregion of all integrin β subunits is also essential for the recruitment of the receptor to adhesion sites and its association with intracellular proteins regulating integrin-dependent adhesion, spreading and cytoskeletal rearrangement (Reszka *et al.*, 1992; Cone *et al.*, 1994; Filardo *et al.*, 1995). Given the demonstrated role of membrane–cytoskeletal interactions in phagocytosis (Allen and Aderem, 1996), the defective phenotype produced by mutations in the membrane-proximal NPXY(F) sequence does not allow dissociation of its putative role in receptor internalization from a more general involvement in the receptor's primary function, i.e. its ability to support cell adhesion requiring plasma membrane–cytoskeletal interactions. Indeed, this function could be essential for efficient phagocytosis of ligand-coated particles.

The Y735F mutant does not display any functional defect, when compared with the wild-type receptor, in ligand-induced spreading and cytoskeleton reorganization, thus ruling out that phosphorylation of Y735 plays a significant role in controlling the functions analysed. A recent report addressed the issue of the functional versus structural role of the tyrosine residues found in the integrin β 1A cytoplasmic domain, by expressing mutant integrin subunits in a β 1A-null cell line (Sakai *et al.*, 1998). The results of this study suggest that the phenol group found in these residues' side chains may be specifically required to support migration but not static adhesion over specific ligands, as a Y to F substitution in these positions selectively affects the ability of the cells to migrate in response to growth factors. This raises the possibility that the NPXY sequences are multifunctional motifs that may be involved in mutually exclusive associations with defined sets of proteins, depending on inducible phosphorylation of the critical tyrosine residues. Along this line of thought, it is interesting to note that in the β 2 integrin subunit both of these tyrosines are replaced by phenylalanines, suggesting conservation of the general structure of the motif but loss of its phosphorylation-dependent functions. The normal migration efficiency of the Y735F mutant further confirms that Y735 serves a structural role in these functions, independent of its phosphorylation state. Interestingly, most integrin β subunits are characterized by the presence of a conserved phenylalanine residue in the core sequence of the cyto-1 domain corresponding to Y735 residue of β 2. This suggests that the significance of our study concerning the β 2 subunit may be extended to other integrin β subunits.

In contrast to the F754A mutation, which alone induces a profound impairment in ligand-supported adhesive and migratory functions, the Y735A mutation in the β 2 integrin cytoplasmic region creates a selected defect in the recycling of spontaneously internalized receptors to the cell

surface. Recent studies have provided morphological and biochemical evidence that the sorting of receptors destined to be recycled to the plasma membrane occurs at the level of the early endosome, where they accumulate in tubular extensions that eventually pinch off to yield recycling vesicles (Mellman, 1996). There is now considerable evidence supporting the view that the sorting of endocytosed receptors to recycling vesicles depends on the existence of tyrosine-based sequence motifs similar to, and partially overlapping with coated pit localization signals (Mellman, 1996). By analogy with endocytic signals that direct the association of the receptor with clathrin-bound AP2 complexes, one could postulate that sorting signals involved in recycling also mediate a specific interaction with recycling vesicle-associated adaptor complexes. The absence of such signals may be responsible for the default targeting of endocytosed receptors to a late endosomal compartment and eventually to the lysosomes for degradation, although our observation that the Δ 733 deletion mutant is poorly recycled but minimally degraded suggests that additional, undefined signals may indeed be required for lysosomal targeting of endocytosed receptors. In order to visualize recycling receptors, we utilized a recently proposed approach based on the observation that growth factor-induced ruffles arise by exocytosis of internal membrane from the endocytic cycle (Bretscher and Aguado-Velasco, 1998b). Consistent with our biochemical findings, we observed that removal of a large portion of the β 2 cytoplasmic domain or the single Y735A mutation greatly reduces the ability of the surface-expressed receptors to become enriched in EGF-induced ruffles. Interestingly, the work by Bretscher and Aguado-Velasco (1998b) demonstrates an active involvement of Rac1 in the targeting of recycling receptors to membrane ruffles, further pointing to the relevance of this process in cell motility (Ridley, 1996).

The questions of whether recycling vesicles fuse in a polarized fashion to the plasma membrane of a locomoting cell, and whether polarized recycling of receptors and membrane is required to support locomotion still remain to be addressed. Other groups have shown in the past that short term cell migration proceeds normally when protein synthesis is inhibited (Bretscher, 1996b). This suggests that re-use of internalized integrins, rather than exocytosis of neo-synthesized receptors, is required to support migration, although at present we cannot rule out that Y735 is required for the polarized sorting of newly synthesized receptors to subdomains of the plasma membrane actively involved in cell migration. Alternatively, an undefined signalling defect may underlie the observed impairment in ligand-induced migration observed in the Y735A mutant. The direct visualization of recycling receptors in locomoting cells and the analysis of the migration efficiency of cells displaying selected defects in the endocytic cycle will be needed to establish this point conclusively.

Materials and methods

Antibodies and reagents

MAbs TS1.18 (anti-CD18) and TS1.22 (anti-CD11a) were kindly provided by T.A.Springer (Harvard Medical School, Boston, MA); mAb TUB2.1 (anti- β -tubulin) was purchased from Sigma Chemical Co. (St Louis, MO); mAb anti-LFA-1 α (anti-CD11a) was purchased from

Transduction Laboratories (Lexington, KY). FITC-conjugated and HRP-conjugated goat anti-mouse IgG were purchased from Zymed Laboratories (South San Francisco, CA). Recombinant α z-ICAM-1 was a generous gift from R.Solari (Glaxo-Wellcome, Stevenage, UK). HRP-conjugated streptavidin and protein G-Sepharose were purchased from Amersham Pharmacia Biotech (Roosendaal, The Netherlands). GSH, fibronectin, TRITC-conjugated phalloidin, BSA and cycloheximide were purchased from Sigma Chemical Co. Lipofectamine was from Life Technologies Inc. (Gaithersburg, MD). Ez-Link NHS-SS-Biotin was purchased from Pierce (Rockford, IL). EGF was a kind gift from L.Beguinot (Scientific Institute San Raffaele, Italy).

DNA constructs

The cDNAs containing the entire coding region of the α L and β 2 subunits subcloned into the expression vector pCDM8 were kindly provided by T.A.Springer. The β 2 cytoplasmic deletion Δ 733 was obtained as previously described (Pardi *et al.*, 1995). The β 2 Y735A, Y735F, F754A and F766A point mutations were obtained by using the PCR-based method Quikchange (Stratagene, La Jolla, CA) according to the manufacturer's instructions: synthetic oligonucleotides carrying the desired codon substitution were used for plasmid amplification. Mutants F754,766A (YAA) and Y735A, F754,766A (AAA) were obtained by successive rounds of single codon substitutions. After mutagenesis the correct sequence of the mutant cDNA region spanning the unique restriction sites *Eco*47III and *Not*I was confirmed by dideoxy sequencing (T7 sequencing kit, Pharmacia Biotech, Uppsala, Sweden), and cloned into the same non-PCR-amplified vector. The cDNA coding for α L was cloned into the expression vector pCEP4 (Invitrogen, Carlsbad, CA) by the unique *Not*I and *Hind*III restriction sites. The α L-GFP chimera was composed of the α L integrin chain fused to the GFP at the C-terminus, and was obtained as follows: a restriction site for *Eco*47III was introduced by PCR at nucleotide 3490 of the full-length coding sequence (3512 bp), thereby introducing a six-amino acid deletion at the C-terminus. The modified α L was then extracted with the restriction enzymes *Hind*III and *Eco*47III, blunted with the Klenow fragment and cloned into the vector pEGFP-C3 (Clontech) by the unique restriction site *Eco*47III.

Cell culture and generation of stable transfectants

CHO cells were maintained in Nutrient Mixture F-12 (HAM) medium (Life Technologies Inc.) with 10% fetal calf serum (FCS; Hyclone Laboratories, Logan, UT). HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies Inc.) with 10% FCS. For stable transfectants, CHO cells were grown to 50% confluency in 60 mm dishes. Each β 2 mutant cDNA was cotransfected with the α L chain cDNA (α L/pCEP4) with lipofectamine (5 μ g total DNA). After 72 h, selection with 150 μ g/ml hygromycin (Boehringer Mannheim, Indianapolis, IL) was started. Surviving cells were subcloned, expanded and tested for α L/ β 2 surface expression by flow cytometry as described (Pardi *et al.*, 1995). The selected clones were maintained in the presence of 25 μ g/ml hygromycin.

Internalization and recycling

Internalization and recycling were quantitatively evaluated by a method modified from Bretscher (1989). For the analysis of internalization, cells were plated at 80% confluency on 60 mm diameter dishes, washed twice in phosphate-buffered saline (PBS) and incubated for 1 h on ice in the presence of 0.5 mg/ml NHS-SS-biotin in PBS. Labelled cells were then washed twice with cold PBS and incubated for the times indicated at 37°C in serum-free F-12 medium to allow internalization. The samples were then put back on ice, washed and treated with two successive reductions of 20 min on ice with a reducing solution containing 42 mM GSH, 75 mM NaCl, 1 mM EDTA, 1% BSA and 75 mM NaOH. Cells were then washed carefully in cold PBS, collected by scraping and lysed in 500 μ l of a solution containing 10 mM Tris pH 7.2, 150 mM NaCl, 1 mM CaCl_2 , 1 mM MgCl_2 and 1% Nonidet P-40 for 10 min on ice. α L/ β 2 was immunoprecipitated from lysates to analyse the amount of integrin internalized at each time point. β -tubulin served as a normalization control. For immunoprecipitation, protein G-Sepharose was pre-incubated with 1 μ g/sample of both TS1.22 and TS1.18 and 1 μ l/sample of TUB2.1 liquid ascites in PBS. Beads were then washed twice in PBS, incubated with lysates at 4°C overnight and eluted in non-reducing sample buffer (Laemmli, 1970). Samples were analysed by SDS-PAGE and transferred onto nitrocellulose as described previously (Towbin *et al.*, 1979). The membrane was blocked in 5% BSA and the upper half (containing the α L and β 2 bands) was incubated with HRP-conjugated streptavidin (to identify the internalized fraction of α L/ β 2), while the lower half was immunodecorated with anti- β -tubulin mAb

(1:500 dilutions of TUB2.1 ascites liquid) followed by HRP-conjugated goat anti-mouse Ab. Peroxidase reaction was developed using enhanced chemiluminescence (ECL; Amersham). Band intensity was quantified by densitometry (Molecular Dynamics, Sunnyvale, CA). A sample that was labelled but not reduced was included as a positive control (only one-third of this sample was loaded on the gel) and a sample that was labelled and reduced, prior to incubation at 37°C, was included as a negative control. For the analysis of recycling, optimal internalization was allowed for 45 min at 37°C and then samples were reduced as described above. The internalized fraction was chased by re-incubation at 37°C for 10, 20 or 30 min in duplicate samples. After the incubation, one of the two samples was reduced to quantify the amount of protein that recycled back to the plasma membrane, and the other one was not reduced, to quantify integrin degradation. The samples were then collected and analysed as described above. Recycling and degradation were calculated by subtracting the densitometric values of the residual biotinylated receptor, following incubation at 37°C and treatment with either GSH (recycling) or a non-reducing buffer (degradation), from the total pool of internalized receptors at 45 min. Densitometric analysis of the bands corresponding to β -tubulin was used to normalize the samples for the amount of protein loaded for each sample.

Pulse-chase assay

Pulse-chase experiments were performed as described previously (Pardi *et al.*, 1995). Briefly, 2×10^6 cells/sample of the stable CHO transfectants were incubated in solution for 30 min in methionine-cysteine-free DMEM containing 5% dialysed FCS, followed by incubation in the same medium containing 500 μ Ci/ml [35 S]methionine-cysteine (Redivue Promix, Amersham-Pharmacia Biotech). After 45 min at 37°C, F-12 medium containing 100-fold excess cold methionine and cysteine was added, and the cells were incubated for the indicated chase times. Cell lysis and immunoprecipitation were carried out as described above for the internalization assay. Samples were then subjected to SDS-PAGE, fixed for 20 min in a solution containing 10% acetic acid and 20% isopropanol, incubated for 20 min in Amplify (Amersham), dried and exposed. To normalize, one-fifth of each sample, after SDS-PAGE, was transferred onto nitrocellulose and immunodecorated with 0.5 μ g/ml of anti LFA-1 α (anti-CD11a) mAb, followed by HRP-conjugated goat anti-mouse Ab.

Fluorescence studies

To analyse receptor localization in growth factor-induced membrane ruffles, HeLa cells were grown to 50% confluency in 60 mm dishes. Each β 2 mutant cDNA was cotransfected with the α L-GFP cDNA with lipofectamine. Cells were then plated onto fibronectin-coated glass coverslips. After 48 h, cells were starved by incubation in DMEM without FCS for 24 h prior to ruffling induction. For steady-state studies, cells were treated for 10 min with 100 ng/ml of EGF to induce ruffling, fixed, permeabilized and stained with TRITC-conjugated phalloidin. Cells were then inspected with a Zeiss Axiophot microscope equipped for epifluorescence. For time-lapse videomicroscopy analysis, each coverslip was mounted in a slip dish chamber (Medical Systems Corp., Greenvale, NY), placed over a metal ring stage kept at 37°C by a water recirculation system, and inspected with a Zeiss Axiovert 135 TV microscope equipped with an Orca CCD camera (Hamamatsu, Italy). Photographs were taken before and at 1 min intervals after the addition of 100 ng/ml of EGF, and analysed by the Image Pro Plus software (Media Cybernetics, Silver Spring).

Cell adhesion assays

To analyse ligand-induced cell spreading, 35-mm Petri dishes were precoated for 2 h at room temperature (RT) with 20 μ g/ml human IgG (Sandoglobulina, Sandoz S.A., Basel, Switzerland) in PBS; the dishes were then rinsed twice and incubated further with 2 μ g/ml of recombinant α z-ICAM-1 for 2 h at RT: since the ' α z' tag binds to immunoglobulins, the precoating with human IgG allows a correct orientation of bound ICAM-1. Cells (10^5 /sample) were resuspended in F-12 medium containing 1% nutridoma NS (Boehringer Mannheim), plated on coated dishes and incubated for 30 min at 37°C. Cells were then fixed in 3.7% paraformaldehyde (PFA) for 10 min at RT prior to examination under a phase-contrast microscope. The number of adherent cells was quantified as previously described (Inverardi *et al.*, 1997). Briefly, 96-well ELISA plates were coated with either α z-ICAM-1, as described above, or with fibronectin (20 μ g/ml, 2 h at 37°C), blocked with BSA 1%, and 5×10^4 ^{51}Cr -labelled cells/well were plated in triplicate and incubated for 30 min at 37°C. After washing, adherent cells were lysed and activity of released isotope was counted in a γ -counter (Packard, Canberra, Australia).

Cell migration assay

Cell migration assays were performed in modified Boyden transwell chambers containing 5 µm pore size polycarbonate filters (Costar, Cambridge, MA). The lower surface of the filter was either uncoated (background migration) or coated in PBS (2 h at RT) with either fibronectin (20 µg/ml) or Sandoglobulina (20 µg/ml), followed by recombinant z-ICAM-1 (20 µg/ml). All filters were then blocked with 1% BSA in PBS for 1 h at RT. For each assay, 4×10^4 cells were resuspended in 100 µl of F-12 medium containing 1% FCS, plated on the upper chamber and incubated at 37°C for 8 or 16 h. Cells remaining in the upper surface of the filter at the end of the incubation period were removed with a cotton swab, and the cells that migrated to the lower surface were fixed in 3.7% PFA and stained with 0.5% crystal violet for 10 min at RT. The stained cells were then solubilized in 1% SDS, the solution was transferred to a 96-well plate, and the concentration of crystal violet was quantified by absorbance at 540 nm. Parallel samples containing the total input cell population were fixed, stained and analysed by colorimetry to allow quantitation of the fraction of migrated cells.

Acknowledgements

We thank S. Putignano for her invaluable technical help and I. De Curtis for critical reading of the manuscript. This work was supported in part by grants from Telethon (E492), AIRC and MURST (to R.P.), and by NIH grant R01HL43331 (to J.R.B.).

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Received February 15, 1999; revised June 8, 1999;
accepted July 20, 1999