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Circulating integrins: $\alpha_5\beta_1$, $\alpha_6\beta_4$ and Mac-1, but not $\alpha_3\beta_1$, $\alpha_4\beta_1$ or LFA-1

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The $\alpha_5\beta_1$, $\alpha_6\beta_4$ and Mac-1 integrins all participate in the endocytotic cycle. By contrast, $\alpha_3\beta_1$, $\alpha_4\beta_1$ and LFA-1 do so much more slowly, or not at all, in the cell lines examined. This indicates that the α-chains appear to determine whether an integrin cycles or not, and that $\alpha_5\beta_1$, $\alpha_6\beta_4$ and Mac-1 can be brought to the leading edge of a moving cell by endocytosis and recycling.

Key words: cell locomotion/cell recognition/cell surface receptors/endocytosis/integrins

Introduction

The integrins are a family of cell surface molecules which have profound functions in a wide variety of contexts (Hynes, 1987). They serve as the principal cell anchors to the extracellular matrix (Ruoslahti, 1988), as sites in the plasma membrane for stress fibre or muscle attachments (Burridge *et al.* 1988; Leptin *et al.*, 1989) and, especially in the immune system, are involved in binding one cell to another (Springer, 1990). For all these functions, it seems likely that they are required solely at the cell surface, and there is no doubt that that is where most of them are, most of the time. Integrins also attach cells to the substratum during locomotion, both *in vitro*, and *in vivo* (e.g. see Bronner-Fraser, 1985); in this situation it might be advantageous to the cell if the integrins circulated into the cytoplasm and back to the cell surface—that is, if they participated in the endocytotic cycle. This is because those integrins holding the cell to the matrix will move rearwards on the cell as the cell advances over, or through, a matrix. Further attachments at the cell's leading edge will require replenishment of matrix receptors to move the cell forward: this could be achieved by endocytosis of integrins from the rear regions of the cell, transport through the cytoplasm in vesicular form, followed by exocytosis at the leading edge for re-use (Bretscher, 1984).

The ease of studying the circulating properties of surface proteins has been increased by the introduction of cleavable tags for labelling them (Bretscher and Lutter, 1988; see also Le Bivic *et al.*, 1990; Reid and Watts, 1990). Use of such labels enabled me to show that a fibronectin receptor on CHO cells circulates (Bretscher, 1989). Here I have extended these observations to a variety of other integrins.

Results

The cycling properties of several different integrins have been determined using a reagent designed for the purpose (Bretscher and Lutter, 1988; Bretscher, 1989). The essence of the method is to label the cells' surfaces at 0°C (where endocytosis and exocytosis are halted in mammalian cells) with an impermeant iodinated reagent *N'*-hydroxyphenyl propionyl *N*[sulphosuccinimdyl di(thiopropionyl) glycyl] cystine (DPSch). This label can be removed again at 0°C, but only from the cells' surfaces, by subsequent reduction with an impermeant thiol, cysteine. Incubation of the cells in suspension at 37°C between labelling and reduction allows labelled proteins to endocytose and thus become protected from subsequent removal of the label by cysteine at 0°C. Here, the integrins under study have been followed by isolating them from cell extracts with monoclonal antibodies.

$\alpha_5\beta_1$ fibronectin receptor (VLA 5)

I have previously shown that a fibronectin receptor circulates into, and out of, CHO cells (Bretscher, 1989). This was done with a β1-specific monoclonal, PB1 (Brown and Juliano, 1985). As there are now several known β1-containing receptors for fibronectin—$\alpha_3\beta_1$, $\alpha_4\beta_1$ and $\alpha_5\beta_1$—the molecular identity of this CHO cell receptor has been examined. Using anti-peptide antisera to the C-terminal regions of α_3 and α_5, kindly provided by Dr R.Hynes, I find that the major β_1-containing integrin on these cells is $\alpha_5\beta_1$: this molecule circulates as previously described (Figure 1), with an internalization rate of ~1.3%/min, and an intracellular pool of ~14% (Bretscher, 1989).

This integrin is also weakly present on the surface of A431 carcinoma cells (Wayner *et al.*, 1988) and has been examined using a commercial anti-human α5 monoclonal antibody (P1D6). As shown in Figure 2, (lane 2, compared with the control, lacking anti-α5 antibody, lane 1) two major bands are present having molecular weights of ~100 kDa (β) and 120 kDa (α). Incubation of labelled cells at 37°C (lanes 3–6: note that these lanes have been autoradiographed for longer than lanes 1 and 2) leads to the internalization of $\alpha_5\beta_1$: after 20 min some 14% (lane 6) has become resistant to reduction, with an initial internalization rate of ~1.1%/min. Inclusion of 2 mM primaquin, which retards exocytosis (see Reid and Watts, 1990), leads to an internal pool after 20 min of ~23% (lane 7). In separate experiments (not shown), the majority of labelled and internalized $\alpha_5\beta_1$ (as in lane 6) returns to the cell surface upon subsequent incubation at 37°C.

The cycling properties of $\alpha_5\beta_1$ in A431 cells and CHO cells are very similar.

$\alpha_3\beta_1$ (VLA 3)

This integrin is quite abundant on A431 cells (Wayner *et al.*, 1988), and has been examined using a commercial anti-human α3 monoclonal antibody (P1B5). As shown in Figure 3 (lane 2; control, without monoclonal, lane 1), two main bands (α_3, ~120 kDa; β_1, ~100 kDa) are isolated from labelled cells. Reduction of cells at 0°C (lane 3) is imperfect and some label remains which provides an unhelpful background. Incubation of labelled cells at 37°C

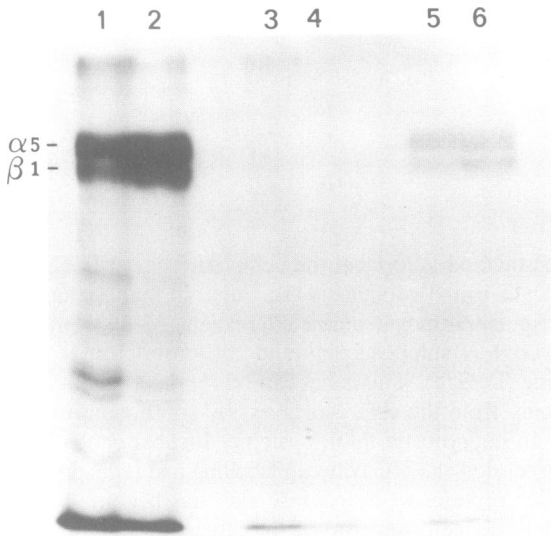

Fig. 1. Internalization of $\alpha_5\beta_1$ in CHO cells. Lanes 1 and 2: total labelled receptor from an aliquot of cells held at 0°C. Lanes 3 and 4: as lanes 1 and 2, but reduced at 0°C with cysteine. Lanes 5 and 6: as lanes 3 and 4 but cells incubated for 10 min at 37°C between labelling and reduction. In lanes 1, 3 and 5, $\alpha_5\beta_1$ isolated with anti-α_5 peptide antiserum (Hynes *et al.*, 1989), or (lanes 2, 4 and 6) with anti-β_1 monoclonal antibody, PB1 (Brown and Juliano, 1985). Pairs of lanes are indistinguishable, showing that the receptor internalized is $\alpha_5\beta_1$.

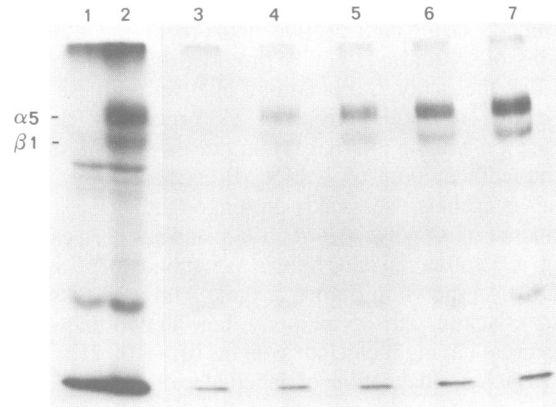

Fig. 2. Internalization of $\alpha_5\beta_1$ in A431 cells. Lane 2: total labelled receptor isolated from cells held at 0°C, using PID6 anti-α_5 monoclonal antibody. Lane 1: as lane 2 but omitting PID6. Lanes 3–7: as lane 2, but cells incubated at 37°C for 0 min (lane 3), 5 min (lane 4), 10 min, (lane 5) or 20 min (lanes 6 and 7) and then reduced at 0°C. In lane 7, 2 mM primaquin was present during the 37°C incubation. Autoradiography: lanes 1–2, 6 days; lanes 4–7, 30 days. Radioactivities in the region of α, β bands (after subtraction of background in lane 3): lane 2, 1800 c.p.m.; lanes 4–7: 100, 160, 260 and 420 c.p.m. respectively.

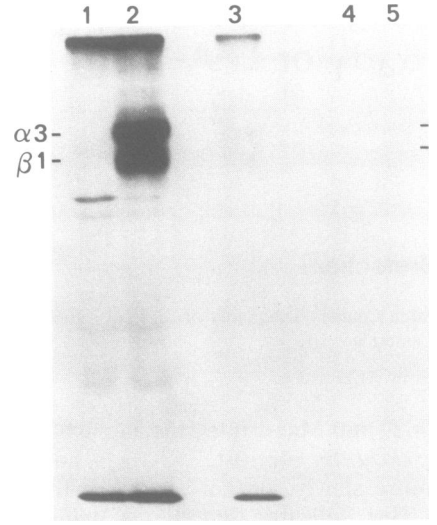

Fig. 3. Internalization of $\alpha_3\beta_1$ in A431 cells. Lane 2: total labelled receptor isolated from cells held at 0°C, using P1B5 anti-α_3 monoclonal antibody. Lane 1: as lane 2 but omitting P1B5. Lanes 3–5: as lane 2, but cells incubated at 37°C for 0 min (lane 3), 10 min (lane 4) or 20 min (lane 5) and then reduced at 0°C. Radioactivities in the region of α, β bands (after subtraction of an equal area of adjacent gel in the same lane) in lanes 2–5: 11 200, 290, 310 and 385 c.p.m. respectively.

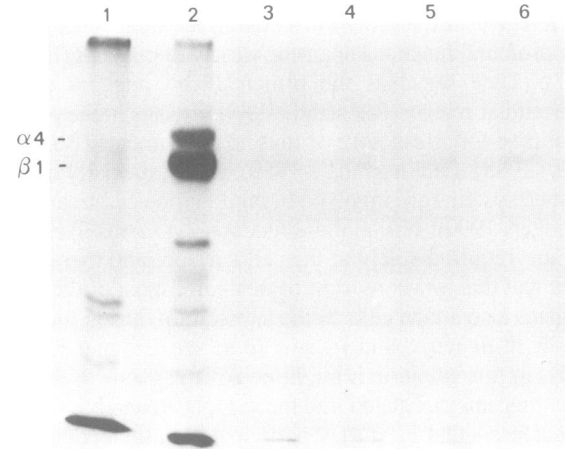

Fig. 4. Internalization of $\alpha_4\beta_1$ in Jurkat cells. Lane 2: total labelled receptor isolated from cells held at 0°C, using HP2/4 anti-α_4 monoclonal antibody. Lane 1: as lane 2, but omitting HP 2/4. Lanes 3–6: as lane 2, but cells incubated at 37°C for 0 min (lane 3), 2 min (lane 4), 5 min (lane 5) or 20 min (lane 6) and then reduced. Autoradiography: lanes 1–2, 4 days; lanes 3–6 (7 days). Radioactivities in the region of α, β bands: lane 2, (less than that in lane 1): 7900 c.p.m.: lanes 2–5: 240, 250, 230 and 250 c.p.m. respectively.

for 10 or 20 min does not reveal much endocytosis (lanes 4 and 5). The fraction of $\alpha_3\beta_1$ present in the intracellular pool at 20 min in this experiment is 0.85% but in other experiments has been as high as 2%. This suggests that a very low level of endocytosis may exist; however, the high background (in lane 3) seen with this integrin has made it difficult to quantify accurately.

$\alpha_4\beta_1$ (VLA 4)

This integrin is expressed on Jurkat cells (Wayner *et al.*, 1989) and has been examined using a monoclonal antibody HP2/4 kindly provided by Dr Sanchez-Madrid. Figure 4 (lane 2; control, without monoclonal, lane 1) shows that this monoclonal precipitates a doublet of ~130 kDa (α) and 100 kDa (β). Incubation of labelled cells, followed by reduction to detect internalized $\alpha_4\beta_1$ (lanes 3–6; longer exposure than lanes 1 and 2), shows that this integrin is not endocytosed to a detectable level over a period of 20 min at 37°C. (The internal pool at this time, corrected for the 0 min control, is <0.2%; in other experiments this varied up to ~1%). $\alpha_4\beta_1$ is also present in U937 cells: again, its endocytosis was undetectable.

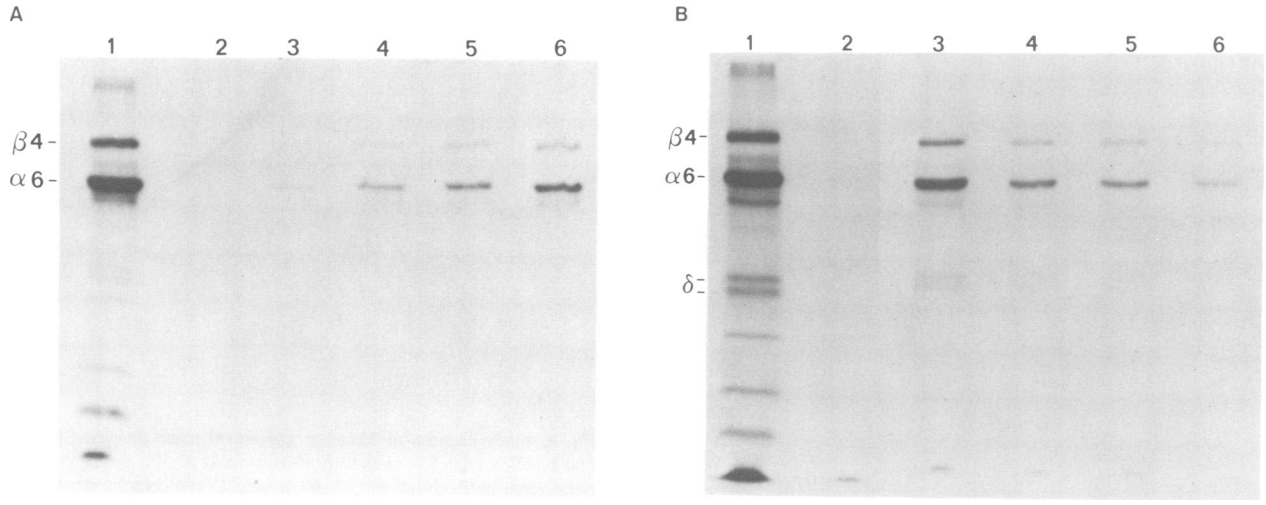

Fig. 5. Internalization of $\alpha_6\beta_4$ (**A**) and return of $\alpha_6\beta_4$ to the cell surface (**B**) in A431 cells. (**A**) Lane 1: total labelled receptor isolated from cells held at 0°C, using GoH3 monoclonal antibody. Lanes 2–6: as lane 1, but incubated at 37°C for 0 min (lane 2), 5 min (lane 3), 10 min (lane 4), 20 min (lane 5) or 30 min (lane 6) and then reduced. Densitometry of lanes 1–6 shows that the total label in α, β bands, in arbitrary units, is 1900, 14, 65, 200, 315 and 570 respectively. (**B**) Lane 1: total receptor isolated from cells held at 0°C (as lane 1 above). Lane 2: as lane 1, but reduced (as lane 2 above). Lane 3: labelled cells incubated for 30 min at 37°C, and then reduced (as in lane 6 above) in order to label the intracellular pool of receptors. Lanes 4–6: as in lane 3, but cells reincubated at 37°C for 3 min (lane 4), 6 min (lane 5) or 30 min (lane 6) before a second reduction. Exocytosis of receptors is reflected in the decreasing intensity of α, β bands as the cells are incubated at 37°C. Density of total label in the α, β bands, in arbitrary units, is 2400, 23, 440, 236, 217 and 120 in lanes 1–6 respectively. The two bands labelled δ arise during trypsinization of the cells from the culture flask.

$\alpha_6\beta_4$

Whether this integrin complex is a laminin receptor appears to be open to question: however, the monoclonal antibody to α_6, GoH3 (Sonnenberg *et al.* 1987) prevents pretreated A431 cells from attaching or spreading on a laminin substrate (Sonnenberg *et al.* 1988; Aumailley *et al.* 1990) and, as shown below, the major detectable complex in these cells with GoH3 is $\alpha_6\beta_4$. However, it cannot be ruled out that some $\alpha_6\beta_1$, identified as a laminin receptor for the E8 domain of laminin (Sonnenberg *et al.*, 1990a), is responsible for this attachment. The two chains in this $\alpha_6\beta_4$ complex have molecular weights of 135 kDa (α_6) and 180 kDa (β_4) on non-reducing gels (Sonnenberg *et al.* 1990b). In the following experiments, the $\alpha_6\beta_4$ integrin was isolated with the GoH3 monoclonal antibody, kindly provided by Dr A.Sonnenberg.

When labelled A431 cells are incubated at 37°C, the $\alpha_6\beta_4$ complex is endocytosed and the label it bears becomes resistant to subsequent reduction at 0°C, as shown in Figure 5A. The rate of endocytosis is ~1–2% of the surface molecules per minute and the intracellular compartment contained, in different experiments, between 16 and 40% of the total surface molecules. Those molecules in the internal compartment return to the cell surface upon subsequent incubation at 37°C (Figure 5B).

Leukocyte integrins

I have studied the circulating properties of LFA-1 and Mac-1, which share the same $\beta 2$ chain (Sanchez-Madrid *et al.*, 1983), in a variety of cells; this was made possible by the generous gifts of monoclonal antibodies by Drs F.Sanchez-Madrid, K.Pulford, G.Hale, S.Cobbold and C.Milstein.

$\alpha_L\beta_2$ *(LFA-1)*

LFA-1 is present in large amounts on several different leukocytes, and its circulation was examined in U937 cells. Figure 6 shows that LFA-1 is not endocytosed to a detectable level after the cells are incubated at 37°C for 20 min. In these same cells, the transferrin receptor is endocytosed to levels previously found in HeLa cells, where an internal pool of rather more than 50% of these receptors exists (Bleil and Bretscher, 1982). The observation that LFA-1 does not circulate was surprising and has been examined extensively. In U937 cells, the lack of internalization of LFA-1 is not affected by including a chemotactic peptide analogue of FMLP—an agent which might be expected to affect locomotory properties of a cell—in the medium. LFA-1 is neither internalized in HL60 cells, nor in HL60 cells induced with DMSO to differentiate along the granulocytic pathway, with or without the FMLP analogue. When HL60 cells are induced with TPA (12-O-tetradecanoyl phorbol 13-acetate) to differentiate along the monocytic pathway, and endocytosis of LFA-1 examined in the presence of TPA, very low levels of LFA-1 internalization have been detected but these amount to only ~1% of the total LFA-1 over a period of 20 min.

LFA-1 circulation has also been examined in a lymphoblastoid cell line, JY: again, its endocytosis is very low. In one experiment, some 1.3% of the surface LFA-1 had been endocytosed by 20 min. This low level of internalized LFA-1 did not appear to be *en route* to lysosomes, as it was metabolically stable over a further 20 min incubation at 37°C; by this time the majority of it had returned to the cell surface. In other words, a very low level of LFA-1 circulation may occur in these cells.

$\alpha_M\beta_2$ *(Mac-1)*

On non-reducing gels, Mac-1 is seen as two polypeptides having molecular weights of ~160 kDa and 85 kDa (Springer *et al.*, 1979). Mac-1 is present in too low levels on induced HL60 or U937 cells to be useful. It is present in larger amounts on several mouse lines, such as WEHI 3B, P388D1 or J774, and on peritoneal macrophages. In

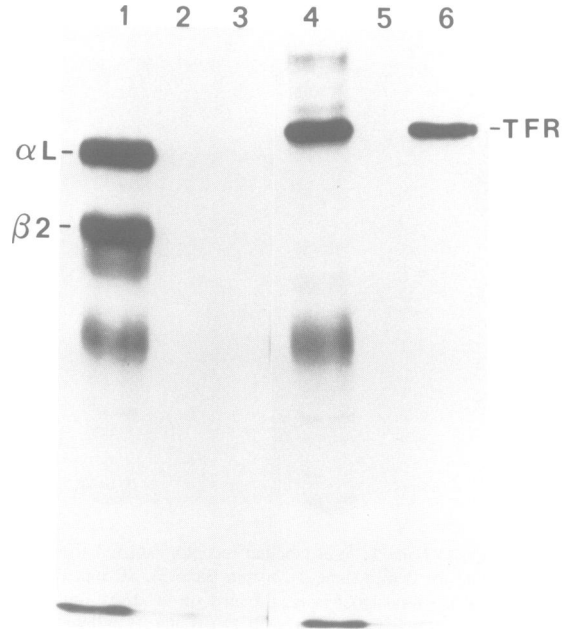

Fig. 6. Internalization of LFA-1 in U937 cells. Lane 1: total labelled receptor from cells held at 0°C, using anti-αL monoclonal antibody TP1/32. Lanes 2 and 3: as lane 1, but cells incubated at 37°C for 0 min (lane 2) or 20 min (lane 3) and then reduced. Lanes 2 and 3 are identical: no LFA-1 is endocytosed. Lanes 4–6: as lanes 1–3, but aliquots of the same cell lysates extracted with anti-transferrin receptor antiserum. This shows that the transferrin receptor is being endocytosed in these cells, serving as a positive control.

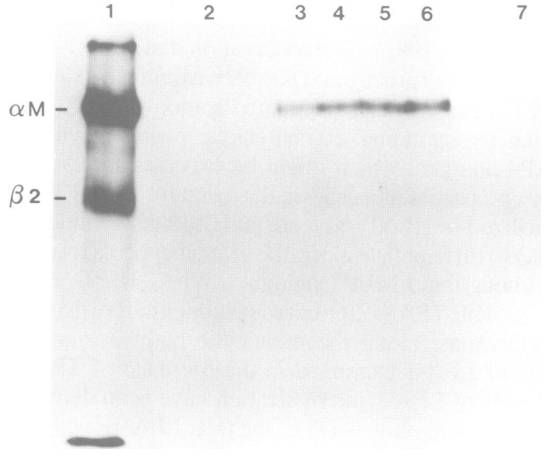

Fig. 7. Internalization of Mac-1 in WEHI 3B cells. Lane 1: total labelled receptor isolated from cells held at 0°C, using M1/70 monoclonal antibody. Lanes 2–6: as lane 1, but incubated at 37°C for 0 min (lane 2), 3 min (lane 3), 6 min (lane 4), 10 min (lane 5) or 20 min (lane 6), and then reduced. Lane 7: as lane 6, but cells, with labelled internal Mac-1, reincubated for a further 20 min and reduced a second time to measure exocytosis of label present in Mac-1 in lane 6. Label present in α_M bands of lanes 1–7: 4200, 16, 180, 300, 315, 350 and 60 c.p.m. respectively.

WEHI 3B, Mac-1 is endocytosed, as shown in Figure 7: the internal pool of ~8% of the total receptor is filled within 6 min, with an internalization rate of ~2.2% per min. This internal pool, upon further incubation, returns to the cell surface (Figure 7, lane 7). Similar results were found for Mac-1 on J774 and P388D1 cells. In each cell line, the transferrin receptors are partitioned about equally between the cell surface and internal pools.

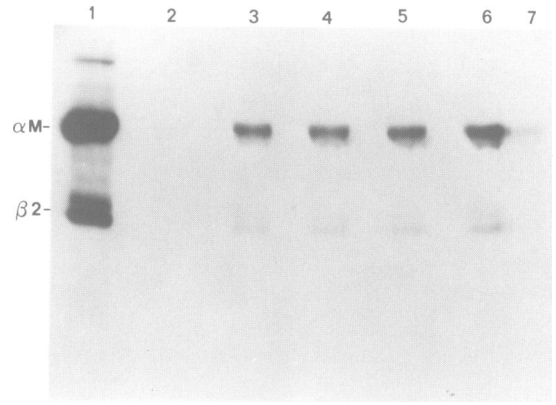

Fig. 8. Internalization of Mac-1 in peritoneal macrophages. Lane 1: total labelled receptor isolated from cells held at 0°C, using M1/70 monoclonal antibody. Lanes 2–6: as lane 1, but cells incubated at 37°C for 0 min (lane 2), 3 min (lane 3), 6 min (lane 4), 10 min (lane 5) or 20 min (lane 6), and then reduced. Lane 7: as lane 6, but cells reincubated at 37°C for 10 min to allow the internal labelled pool of receptors to return to the cell surface, and then reduced a second time. The internalized receptors evidently return to the cell surface. Label present in the α_M (160 kDa) bands of lanes 1–6 are: 9000, 60, 780, 840, 880, 1170 and 280 c.p.m. respectively.

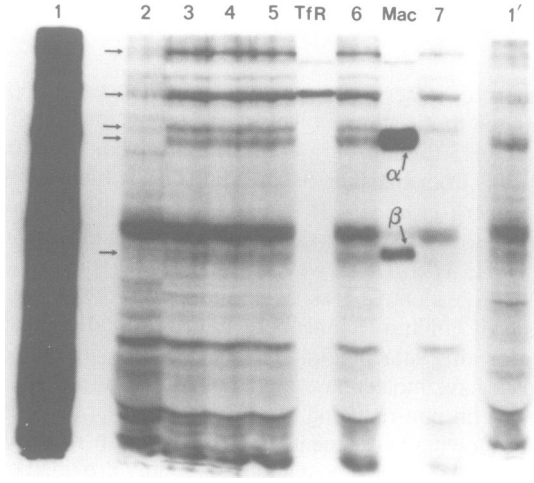

Fig. 9. Internalization in peritoneal macrophages. The samples used here are from the same experiment shown in Figure 8; however, whole cell extracts have been run on the gels, rather than immunoprecipitates. It shows that Mac-1 (marker in lane Mac) and the TFR (marker, lane TFR) are major components of endocytosis and subsequent exocytosis (compare lanes 2, 6 and 7). It will be seen that almost all the internalized Mac-1 in lane 6 is returned to the cell surface in lane 7, whereas the TFR has re-equilibrated, (with about half the level of label present in lane 6 still present in lane 7). Lane 1′ is a 7 h exposure of lane 1 (lanes 1–9 exposed for 4 days).

The cycling properties of Mac-1 were also investigated in thioglycollate-elicited mouse macrophages, shown in Figure 8. The internal pool contains ~12.5% of the total receptor and equilibrates rapidly with the cell surface pool. The initial rate of internalization is ~4.4%/min and that receptor which has been endocytosed is returned to the cell surface upon subsequent incubation (Figure 8, lane 7). In the same cells, the mouse transferrin receptor is again partitioned about equally between the cell surface and internal pools: this equilibration is exceedingly rapid, being achieved in 1–2 min at 37°C.

The circulation of both Mac-1 and the transferrin receptor

in mouse macrophages can be seen in whole cell extracts, (Figure 9). Rather than isolating receptors with antibodies, aliquots of cell extracts from the same experiment shown in Figure 8 were fractionated directly on SDS gels. There appear to be five main components which are endocytosed having approximate molecular weights of 90 kDa, a doublet at 160 kDa, 200 kDa and a very slowly migrating polypeptide. Of these, the 90 kDa band may include β_2, the faster member of the 160 kDa doublet is α_M, whilst the 200 kDa chain is the transferrin receptor.

Discussion

Cell surface integrins have several important functions: they attach cells to components of the extracellular matrix, or to other cells. Both sets of interactions may be controlled by modification of the integrin: thus, there is evidence that Mac-1 and LFA-1 and some VLA(β1) integrins exist in alternative states which differ in their affinities for a given ligand (Wright and Meyer, 1986; Dustin and Springer, 1989; Shimizu *et al.*, 1990). A further degree of complexity may arise from a cell's state. Thus, a fibronectin receptor molecule behaves differently in a stationary fibroblast—where most copies of the molecule are present in fairly static focal adhesions—compared with a migrating fibroblast, where focal adhesions may not exist. The possible functions of any cell surface protein are considerably extended if it circulates into, and out of, the cell: this is usually viewed as enabling the receptor to bring ligands into the cell. However, in the case of extracellular matrix receptors, endocytosis and subsequent exocytosis into the forming leading edge of a moving cell would provide a fresh source of matrix attachments at the cell's front. An understanding of the biological functions of the individual members of the integrin family will come from an analysis of the functions of each, as well as from the differences between related members.

I find that $\alpha_5\beta_1$, $\alpha_6\beta_4$ and Mac-1 circulate, but that $\alpha_3\beta_1$, $\alpha_4\beta_1$ and LFA-1 do so much more slowly, if at all, in the cell lines examined. These results are summarized in Table I, and compared with the transferrin receptor (TFR), LDL receptor (LDLR) and a hypothetical non-circulating protein. In general, whether a cell surface protein circulates or not appears to be a property of the receptor itself, not of the cell type. This seems to hold true for the integrins studied in different cells. Thus, in the four cases examined, Mac-1 always circulates, whereas LFA-1 did not in the three lines studied. Furthermore, LFA-1 could not be induced to circulate by including in the incubation (with HL60 cells) a chemoattractant FMLP analogue, or by making the cells differentiate with either dimethyl sulphoxide (DMSO) or TPA. In other words, the ability to circulate or not seems to be an intrinsic property of the particular integrin. This conclusion may not apply in those cases where an integrin is built into an extensive structure, such as a focal adhesion site.

What is the signal in an integrin which determines whether it circulates or not? Of the integrins studied, it is clearly not simply in the β chains. The β1-containing VLA3, VLA4 and VLA5 circulate at very different rates; β2-containing LFA-1 does not circulate, but Mac-1 does. It is tempting to locate the differences to the α-chains: thus, α_M in Mac-1 has a tyrosine residue in its short cytoplasmic domain, whereas α_L in LFA-1 contains no tyrosine in its longer cytoplasmic domain (Pytela, 1988; Hickstein *et al.*, 1989; Larson *et al.*, 1989). A tyrosine residue is a usual part of the signal for endocytosis by coated pits (Davis *et al.*, 1987; Pearse and Robinson, 1990). However, this simple analysis does not explain why VLA5 circulates fairly rapidly but VLA3 and VLA4 do not: each has a tyrosine residue in this same region (Hynes *et al.*, 1989; Takada *et al.*, 1989; Argraves *et al.*, 1987).

Table I. Cycling properties of integrins and other receptors

Receptor	Cell type	Rate of internalisation % per min	Approximate internal pool
$\alpha_3\beta_1$ (VLA 3)	A431	~0.1%	~1%
$\alpha_4\beta_1$ (VLA 4)	Jurkat, U937	<0.1%	<1%
$\alpha_5\beta_1$ (VLA 5)	CHO, A431	~1.2%	14%
$\alpha_6\beta_4$	A431	~1.5%	20%
$\alpha_L\beta_2$ (LFA-1)	HL60, U937	<0.1%	<1%
	JY	<0.1%	<1.3%
$\alpha_M\beta_2$ (Mac-1)	WEHI 3B,	2.2%	8%
	P388D1, J774	~2%	8%
	tg macrophages	4.4%	12%
TFR	many cells	~15%	50%
LDLR	K562	~25%	50%+
	MRC-5 fibroblasts		15%+
Non-cycling protein		0.1%*	

+From Bretscher and Lutter (1988).
*This assumes that a non-cycling protein turns over once each 16 hours and that this requires endocytosis for removal from the cell surface.

The average rate of surface uptake by coated pit endocytosis is usually ~2% per min. All observations were made on cells in suspension.

The leukocyte integrins are required for certain cells to emigrate from the blood stream to sites of infection. In human leukocyte adhesion deficiency (LAD) patients, the β2 chain is defective: neutrophils and monocytes cannot find their ways to sites of infection (Crowley *et al.*, 1980; Arnaout *et al.*, 1984; Anderson and Springer, 1987). This inhibition of migration can be mimicked in mice, where injection of an anti-Mac-1 monoclonal antibody prevents accumulation of neutrophils and macrophages in the peritoneal cavity (Rosen and Gordon, 1987). This suggests that, for macrophages to enter the peritoneal cavity, Mac-1 is required. None of the evidence to date indicates whether substrate attachment alone, or locomotion, is affected in any of these cases. It is clear, however, that VLA4, LFA-1 and Mac-1 are involved in cell−cell recognition events (Holzmann *et al.*, 1989; Campanero *et al.* 1990; Springer, 1990; Diamond *et al.*, 1990).

When cells migrate, the extracellular matrix receptors, when attached to the matrix, necessarily move backwards on the cell as the cell advances. In a large cell—such as a fibroblast—a mechanism may be required to bring these receptors from the rear regions of the cell to the leading edge for re-use: the endocytotic cycle could be that mechanism (Bretscher, 1984, 1989). My bias is that the same will apply to shorter cells, but it is possible that diffusion of released receptors could be a sufficient source of matrix receptors for attachment of the leading lamella to the matrix. The observations that Mac-1 and VLA5, both of which appear to be used for movement, circulate, supports the view that matrix receptors used for locomotion participate in the endocytotic cycle. The opposite side of the same argument

would suggest that the functions of LFA-1, VLA3 and VLA4 are primarily for recognition events and not for locomotion, unless their cycling can be induced in ways not so far detected.

Materials and methods

Cells and antibodies

Cell lines used previously were A431 (West *et al.*, 1989) and CHO (Bretscher, 1989). HL60 was provided by S.Munro, Jurkat and Molt 4 by J.Girdlestone and WEHI 3B by T.Lowe, all of this laboratory; JY by M.Owen (ICRF, London); U937 and P388D1 by Porton. Thioglycollate-elicited mouse macrophages were harvested from 6–8 week old mice, 4 days after an i.p. injection of 1.5 ml thioglycollate medium. Cells were grown in DMEM plus glutamine, 10% heat-inactivated fetal calf serum (FCS) and antibiotics, or in RPMI 1640 with the same additions. HL60 cells were either induced for 6 days in 1% DMSO or for 2 days in 20 ng/ml TPA. All cells were grown in suspension, except for: attached macrophage lines, which were harvested by scraping; A431 cells, which were collected by incubating near-confluent monolayers in EDTA with or without 2.5 μg/ml trypsin. All cells were washed twice at 4°C in DPBS and once in 0.1 M Na_2HPO_4 before labelling.

Antibodies: rabbit anti-α3 and α5 C-terminal peptide antibodies were from R.Hynes (Hynes *et al.*, 1989); rabbit anti-human transferrin receptor (Rb9) and rat anti-mouse transferrin receptor mAb R17 from C.Hopkins (London); YTH 118.3.2 and YFL 51.8 [anti-human β_2 (CD18)] and YTH 81.5 [anti-human α_L (CD11a)] from S.Cobbold and G.Hale (Cambridge, UK); TS1/11 and TP1/32 [anti-human α_L (CD11a)], Bear-1 [anti-human α_M (CD11b)], HC1/1 [anti-human p150 (CD11c)], HP2/4 (anti-VLA 4 α chain) from F.Sanchez-Madrid (Spain); LPM 19c [anti-human α_M (CD11b)] and KB 90 (anti-CD11c) from K.Pulford (London); M1/70 (anti-mouse Mac-1) from C.Milstein (Cambridge, UK); GoH3 (anti-VLA 6 α chain) from A.Sonnenberg (Amsterdam); P1B5 (anti-human VLA 3 α chain) and P1D6 (anti-human VLA5 α chain) from Telios Pharmaceuticals, San Diego, USA.

Cell labelling and antigen isolation

Cells were labelled using iodinated DPSch {*N'*-hydroxyphenyl propionyl *N*[sulphosuccinimidyl di(thiopropionyl) glycyl] cystine} and internalization on cells in suspension was allowed to proceed as described previously (Bretscher and Lutter, 1988; Bretscher, 1989), except that in the iodination of DPSch, one fifth the amount of chloramine T was used (i.e. 1 μl of a 1 mg/ml solution), and iodination allowed to proceed for 10 min at 0°C. In some experiments, the analogue of the chemotactic peptide FMLP, formyl-nle-leu-phe-nle-tyr-lys was used at 1 ng/ml. Surface label was removed with an improved reducing buffer made as follows: a mix of 9 ml H_2O, 0.15 ml 5 M NaCl, 0.02 ml 0.1 M EDTA, 0.01 ml each 1 M $MgCl_2$ and 1 M $CaCl_2$ was bubbled with nitrogen to remove oxygen. To this was added 0.12 g L-cysteine (free amino acid, not the hydrochloride), the mixture further gassed with nitrogen and stoppered. The cysteine was dissolved, the solution chilled and held for up to a day at 4°C before use. Before use, 0.05 ml 10 M NaOH was added (to bring the pH to 8.6), the solution mixed, 1 ml FCS added, the solution mixed and immediately added to the cells to be reduced. These had been suspended in ~0.05 ml DPBS/10% FCS in a 1.5 ml Eppendorf tube. The filled tubes, containing a small air bubble, were capped and mixed by dumping into a stirred beaker of ice water and held there for 20 min. Alkaline cysteine oxidizes very rapidly in air; the reducing solution should therefore be used as quickly as possible for efficient reduction to occur. After reduction, cells were washed once or twice in DPBS/10% FCS.

Immune precipitations were carried out, and non-reducing SDS gels were run, as previously (Bretscher, 1989). Identification of labelled molecules described in Results was based on their migratory properties on SDS gels with, and without, reduction. As the labels used here are reduction sensitive, identification was carried out on samples labelled in the same way as with DPSch but using a non-reducible agent, sulpho succinimidyl-3-(4 hydroxyphenyl) propionate (sulpho-SHPP) from Pierce. Labelled extracts were immunoprecipitated with as many different antibodies for a particular antigen as possible, the strongest being used for experiments.

Gel bands on autoradiograms were quantified either by counting bands excised from gels or by densitometry of the autoradiogram.

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