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Urokinase receptor-dependent and -independent p56/59^{hck} activation state is a molecular switch between myelomonocytic cell motility and adherence

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Anchorage-independent myelomonocytic cells acquire adherence within minutes of differentiation stimuli, such as the proteolytically inactive N-terminal fragment of urokinase binding to its cognate glycosylphosphatidylinositol (GPI)-anchored receptor. Here, we report that urokinase-treated differentiating U937 monocyte-like cells exhibit a rapid and transient inhibition of p56/59^{hck} and p55^{fgr} whereas no changes in the activity of other Src family kinases, such as p53/56^{lyn} and p59^{fyn} were observed. U937 transfectants expressing a kinase-defective (Lys267 to Met) p56/59^{hck} variant exhibit enhanced adhesiveness and a marked F-actin re-distribution in thin protruding structures. Conversely, urokinase as well as expression of wild-type or constitutively active (Tyr499 to Phe) p56/59^{hck} stimulates the directional migration of uninduced U937 cells. Accordingly, expression of constitutively active or kinase inactive p56/59^{hck} selectively prevents urokinase receptor-dependent induction of either adhesion or motility, indicating that a specific activation state of p56/59^{hck} is required for each cell response. In conclusion, modulation of the intracellular p56/59^{hck} tyrosine kinase activity switches cell motility towards adherence, providing a mutually exclusive mechanism to regulate these properties during monocyte/macrophage differentiation *in vivo*.

Keywords: cell motility and adhesion/cytoskeleton/myelomonocytic differentiation/Src kinases/urokinase receptor signalling

Introduction

Cell migration and adhesion play a central role in physiological processes such as embryonic development, wound healing or the immune response, as well as in pathological conditions, such as inflammatory disorders, atherosclerosis and tumour spread (Lauffenburger and Horwitz, 1996). Cell locomotion is a complex process that requires a spatially and temporally coordinated activity of cytoskeletal, membrane and adhesion systems (Lauffenburger and Horwitz, 1996; Palecek *et al.*, 1997). An early event in cell locomotion is the extension of a lamellipodium at the cell front where organized integrins, vinculin, phosphotyrosine-containing proteins and the focal adhesion kinase pp125^{fak} accumulate and assemble into nascent adhesions, which tether the cytoskeleton to the points of cell attachment (Nobes and Hall, 1995). Recent evidence assigns to the Src family of tyrosine kinases the ability to regulate focal adhesion turnover during fibroblast migration in a catalytic-dependent manner (Fincham and Frame, 1998).

Myelomonocytic cells in culture undergo a transition from an anchorage-independent monocyte-like motile phenotype to an adherent, less motile, macrophage-like one, offering the unique opportunity to dissect the molecular mechanisms underlying these two conditions (Collins, 1987). *In vivo*, blood monocyte migration to the sites of injury depends on various integrins, such as LFA-1, Mac-1 and VLA-4 or other receptors, such as the urokinase receptor (uPAR) (Gyetko *et al.*, 1994; Issekutz, 1995). The failure of monocytes and granulocytes to migrate to the site of inflammation in mice bearing a disrupted urokinase (uPA) gene confirms that this protease is an important motility agent *in vivo* (Gyetko *et al.*, 1996). Urokinase is a serine protease which activates plasminogen to plasmin, a protease capable of degrading most extracellular matrix (ECM) proteins (Dano *et al.*, 1994; Blasi, 1997). Both uPA and its N-terminal fragment, named ATF (amino acids 1–135), bind to the urokinase receptor (uPAR/CD 87) with high affinity through the so-called 'growth factor-like domain' (Stoppelli *et al.*, 1985; Vassalli *et al.*, 1985; Roldan *et al.*, 1990; reviewed in Dano *et al.*, 1994). Tumour cells bearing receptor-bound uPA are endowed with membrane-associated proteolytic activity which enables them to overcome extracellular matrix barriers, invade and metastasize (Stoppelli *et al.*, 1986; Carriero *et al.*, 1994). However, recent data extend uPAR functions, indicating that ligation of uPAR with uPA elicits a variety of cell responses such as proliferation, transcriptional activation, cell migration and adhesion (reviewed in Chapman, 1997). In particular, 'priming' of cultured myelomonocytic cell lines with transforming growth factor-β (TGF-β)/dihydroxyvitamin D_3 for 20 h leads to a partial differentiation toward a phagocytic phenotype, although it does not significantly enhance cell adherence (Liu *et al.*, 1996). Interestingly, if 'primed' cells are further treated with ATF for a short time, they rapidly adhere to the culture dish (Nusrat and Chapman, 1991; Waltz *et al.*, 1993). This differentiation-dependent dual role of uPA offers the possibility to investigate the molecular mechanisms underlying cell locomotion or stable adherence

through a comparative analysis of the uPA-activated pathway(s).

Mechanistic analysis of uPAR-directed signalling is intriguing as this GPI-anchored receptor is not physically linked to the intracellular pathways and may therefore require additional transmembrane mediators. Receptor mobility seems to be required for signalling, as phosphorylation of urokinase on Ser138/303 prevents both ligand-dependent uPAR clustering and signalling (Franco *et al.*, 1992, 1997). *In vitro* data indicate that uPAR can interact directly with β2-integrins and, in particular, with CD11b/CD18, in myelomonocytic cells (Kindzelskii *et al.*, 1996; Wei *et al*., 1996). *In vivo*, uPAR can be co-immunoprecipitated with Src kinases, protein kinase C and integrins (Bohuslav *et al.*, 1995; Konakova *et al.*, 1998). Further data linking uPAR activation to Src kinases indicate that ATF-dependent stimulation of THP-1 cell migration is associated with a rapid and transient increase of p56/59^{hck} kinase activity (Resnati *et al.*, 1996). Src family kinases consist of a short myristylation sequence, Src homology domains 2 (SH2) and 3 (SH3), and a large catalytic domain (Williams *et al.*, 1998). The function of Src tyrosine kinases is negatively regulated by the phosphorylation of a highly conserved C-terminal tyrosine residue (Tyr499 in mouse p56/59^{hck}), promoting its intramolecular interaction with the SH2 domain; this modification is mediated by the Csk non-receptor tyrosine kinase family (Koegl *et al.*, 1995). In myelomonocytic cells, specific Src family members such as Hck, Fgr and Lyn mediate relevant functions, such as FcγRI receptor signalling, induction of cytokine production triggered by bacterial lipopolysaccharide, phagocytosis and cell spreading (Durden *et al.*, 1995; Lowell *et al.*, 1996; Meng and Lowell, 1998).

This study was undertaken to elucidate the nature of molecular links between cell migration and adherence in monocyte/macrophage cells; here, we report that p56/59^{hck} is a common mediator of both chemotactic and adhesive responses. Furthermore, the expression of constitutively activated p56/59^{hck} enhances cell motility in an effector- and differentiation-independent manner. Finally, cell ability to migrate can progressively be switched to adherence by a gradual increase of kinase inactive p56/59^{hck} expression, therefore highlighting a mutually exclusive mechanism for controlling motility and adherence during myelomonocytic differentiation.

Results

Myeloid cell response to ATF requires functional protein kinases

The transition from an anchorage-independent to an adherent phenotype is a critical *in vivo* differentiation step of myelomonocytic cells. In culture, treatment of monocyte-like U937 cells with TGF-β/dihydroxyvitamin D_3 for 20 h followed by a 30 min incubation with ATF (amino acids 1–135 of uPA) results in nearly 50% of the cells differentiating to an adherent macrophage-like phenotype (Figure 1A). The ATF-dependent process is temperature dependent as only cells kept at 37°C show a marked increase in cell adherence to the culture dish after 30 min plating. On the contrary, at 4°C, ATF-dependent adhesion was strongly inhibited, suggesting that not just receptor occupancy but cell metabolic activity is required for the acquisition of adherence. However, the block is reversible, as cells preloaded with ATF at 4°C and then transferred to 37°C retain the full ability to become adherent. The direct involvement of urokinase receptor (uPAR) is demonstrated by the strong inhibitory effect of anti-urokinase receptor polyclonal antibody (Figure 1A). In addition, a peptide spanning the growth factor-like domain of uPA (amino acids 12–32) is effective, although to a considerably lesser extent than ATF (not shown).

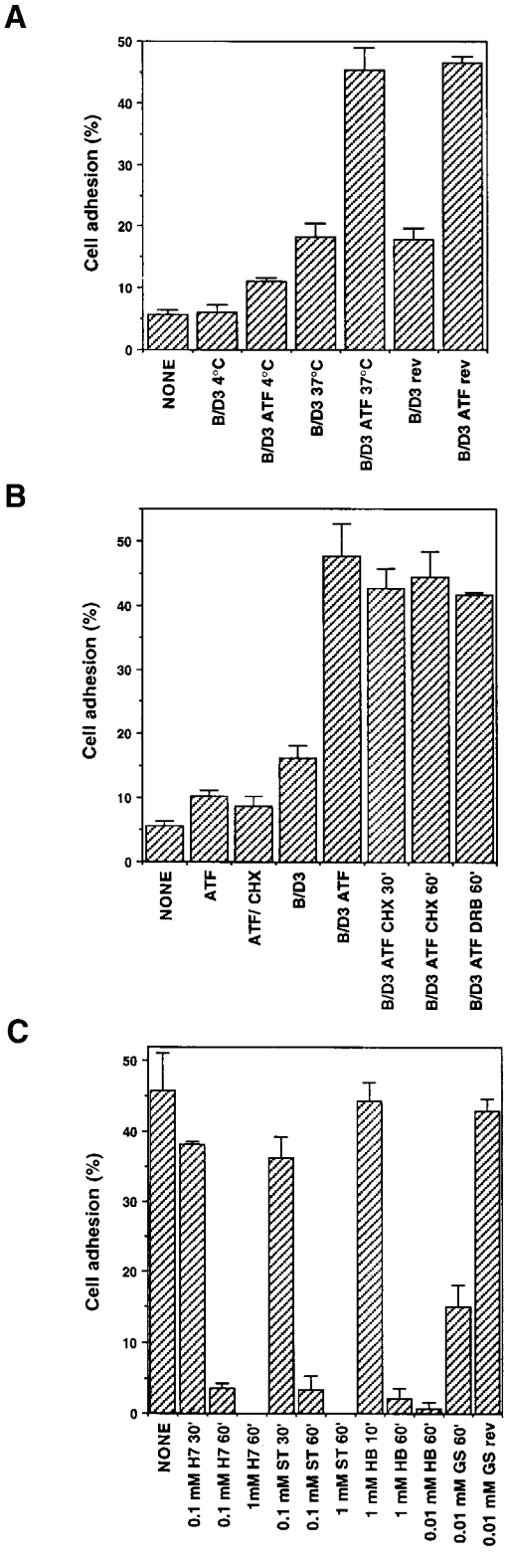
As shown in Figure 1B, the ATF-induced adhesion requires neither *de novo* protein nor RNA synthesis, as no inhibition is observed in the presence of cycloheximide or 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB). On the contrary, a pretreatment with protein kinase inhibitors prevents the response of 'primed' cells to ATF, in a time- and concentration-dependent manner (Figure 1C). In particular, ATF-dependent adherence is blocked by the protein kinase C inhibitors H-7 and staurosporin, and is strongly impaired by the tyrosine kinase inhibitors herbimycin and genestein. Cell adherence is fully restored by removing the inhibitors and re-adding ATF, suggesting that the reduced adhesion was not due to toxic effects but to a reversible block of the cellular machinery controlling adhesion. The latter conclusion is further supported by the results of equilibrium receptor binding studies in which none of these compounds interfered directly with binding of [^{125}I]ATF to uPAR (not shown). Taken together, these results indicate that the myeloid adhesive response to receptor-bound urokinase is not dependent on the catalytic moiety of the enzyme but requires the contribution of functional protein kinases.

Redistribution of F-actin in preadherent U937 cells

To gain insight into the general mechanisms controlling the acquisition of cell adherence, the organization of cell cytoskeleton was investigated by staining ATF-treated differentiating U937 cells with rhodamine–phalloidin. As shown in Figure 2, untreated cells exhibit quite a homogeneous staining, whereas treatment with TGF-β/dihydroxyvitamin D_3 causes an occasional redistribution of F-actin at one pole of the cell. In ATF-treated cells, the staining reveals a clearcut F-actin assembly in thin filamentous structures at the cell periphery and at cell–substrate contacts. If cells are kept in suspension throughout the entire procedure, three-dimensional reconstruction of images obtained by confocal microscopy reveals the occurrence of multiple, irregularly shaped, cyst-like structures which do not appear in untreated cells (Figure 2D, E and F). Again, ATF-treated differentiating cells show peripheral, filamentous structures (Figure 2G). A possible explanation is that these F-actin-enriched, lump-type structures harbour nascent adhesions, which will eventually be further organized and stabilized in the presence of a substrate. Interestingly, time-lapse video microscopy images of living cells revealed that unstimulated cells constantly extend ruffles and lamellipodia-type protrusions and move toward any direction, whereas ATF-treated differentiating U937 cells are far less motile, indicating that the acquisition of adherence is associated with a general decrease in cell motility (not shown).

ATF-dependent inhibition of Src kinases in preadherent U937 cells

To identify the molecular mechanisms involved in the control of uPAR-dependent myeloid cell adhesion and motility, the possibility that some lineage-specific Src kinase members were regulated in cells committed to adherence was examined by an *in vitro* immune complex kinase assay. ^{35}S-labelled 'primed' U937 cells were treated with 10 nM ATF while kept in suspension for 0, 10, 20, 30, 60 and 180 min. At the end of the incubation, p56/59^{hck}, p55^{fgr}, p55/53^{lyn} and p59^{fyn} tyrosine kinases were immunoaffinity purified using antibodies directed against their N-terminal unique domains. Quantitative assessment of the relative protein kinase synthesis showed no time-dependent modification of the protein levels (Figure 3A, B and C). However, upon receptor ligation, a marked downregulation of p56/59^{hck} and p55^{fgr} kinase activities was detected, whereas no appreciable time-dependent modification of p59^{fyn} and p55/53^{lyn} activities had occurred (Figure 3A′, B′ and C′). Quantitation of the resulting enolase-associated radioactivity by phosphoimage analysis revealed that the activity of p56/59^{hck} and p55^{fgr} is reduced by 60–70% over a 20 min incubation period (Figure 3D). In particular, the inhibition of p56/59^{hck} activity was detectable within 10 min, reached maximal levels by 20–30 min and returned to baseline levels by 30–60 min. We also observed a transient decline of the general p56/59^{hck} phosphorylation, which parallels the variation of its phosphotransferase activity (not shown). In the absence of TGF-β/dihydroxyvitamin D_3, U937 cells exhibit a 2- to 3-fold increase in p56/59^{hck} and p55^{fgr} kinase activites, peaking at 20 min after the addition of ATF, but no changes in the activity of p59^{fyn} and p55/53^{lyn} were detected (not shown).



Enhancement of cell adhesion by kinase inactive p56/59^{hck} expression

To investigate the relationship between downregulation of Src kinases and acquisition of adherence, we tested the possibility that p56/59^{hck} inactivation could stimulate adherence *per se*, in an effector-independent manner. In the following experiments, we used the pcDM8mHck267M plasmid encoding a kinase-defective p56/59^{hck} variant (K267M) (Figure 4D). Co-transfections of 'primed' U937 cells with pEGFP-C1, expressing a red-shifted variant

Fig. 1. Effect of temperature and inhibitors of RNA, protein synthesis and tyrosine phosphorylation on ATF-induced adherence of differentiating U937 cells. U937 cells (0.5×10^6 cells/sample) were treated with 1 ng/ml TGF-β and 50 nM dihydroxyvitamin D_3 ('primed') or with diluents for 20 h and subsequently transferred to multiwell plates in the presence or absence of 10 nM ATF for 30 min. Then, the number of adherent and non-adherent cells was determined and reported as a percentage of the total cell population. The data represent the average of three experiments performed in duplicate with standard deviations indicated by error bars. (**A**) 'Primed' cells were incubated with or without 10 nM ATF for 1 h at 4°C. Aliquots of these preloaded cells were either kept at 4°C (B/D3 4°C; B/D3 ATF 4°C) or transferred to 37°C for 30 min (B/D3 37°C; B/D3 ATF 37°C). Alternatively, cells were first incubated at 4°C for 30 min and then transferred to 37°C for an additional 30 min (B/D3 rev, B/D3 ATF rev). 'Primed' cells were also preincubated for 1 h with 10 μg/ml anti-uPAR polyclonal antibody at 37°C prior to the addition of ATF (B/D3 ATF Ab). The basal level of U937 cell line adherence is reported (NONE). (**B**) Untreated or 'primed' (B/D3) cells were incubated with 10 μg/ml cycloheximide for 1 h or with 10 μM DRB for 30 min or 1 h or with diluents. When indicated, cells were further treated with 10 nM ATF for 30 min and then assayed for adherence. The basal level of U937 cell line adherence is reported (NONE). (**C**) 'Primed' cells were incubated without or with the kinase inhibitors H-7, staurosporin (ST), herbymicin (HB), genestein (GS), at the indicated concentrations and for the length of time indicated prior to the addition of 10 nM ATF for 30 min. An aliquot of the genestein-treated cells was washed and re-incubated with 10 nM ATF (GS rev). The results are reported as a percentage of ATF-dependent adherence in the absence of inhibitors (NONE).

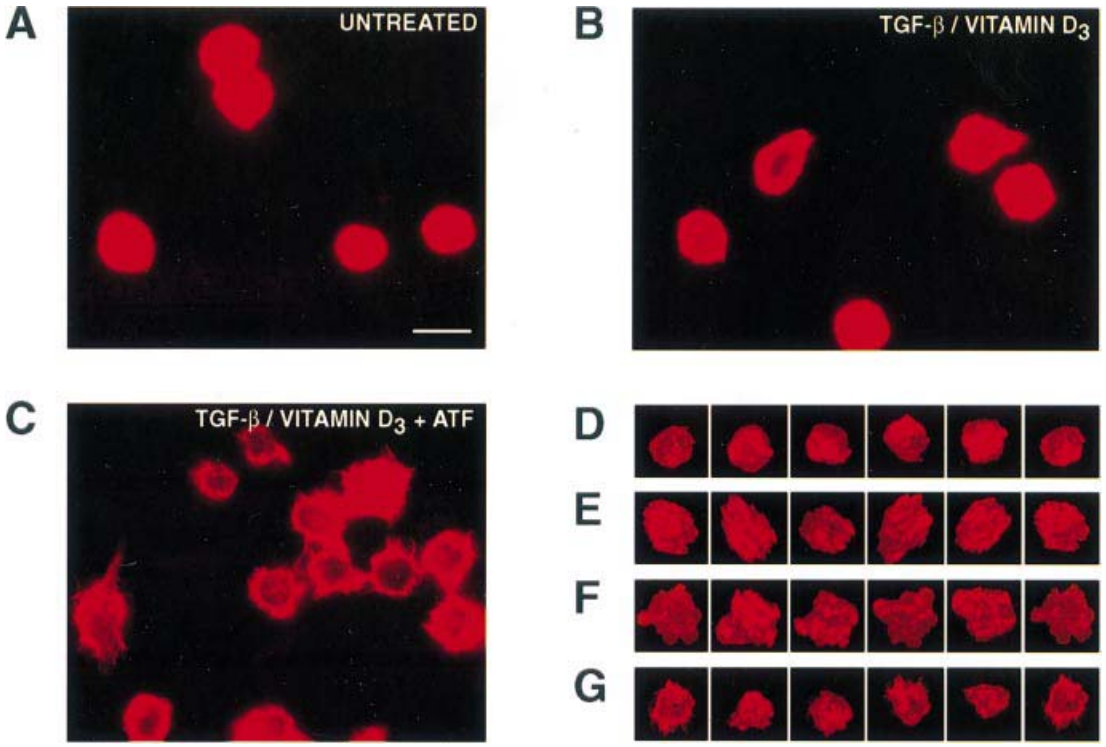


Fig. 2. Staining of preadherent U937 cells with rhodamine–phalloidin. U937 cells were treated with TGF-β/dihydroxyvitamin D_3 for 20 h (**B**, **C**, **E**, **F** and **G**) or diluents (**A** and **D**). Aliquots of 'primed' cells were further incubated with ATF for 1 h (C, F and G). The cells were either left to adhere to gelatin-coated glasses during treatment with cytokines or ATF (A, B, C and G) or kept in suspension throughout the entire procedure (D, E and F). Images were taken with a fluorescence Zeiss microscope (A, B and C) or with a laser confocal microscope (D, E, F and G). In the latter case, a three-dimensional reconstruction of the cell image is shown. Scale bar in confocal micrographs: 10 μm.

of wild-type green fluorescent protein (GFP), ensured identification of the transfected cell population, as well as normalization of the transfection efficiency. As early as 10–12 h after transfection, a detectable fraction of cells expressing dominant-negative p56/59^{hck} spontaneously adheres to culture plates during the assay, as compared with cells harbouring an empty control vector (Figure 4A). At later times, the time-dependent increase of kinase inactive p56/59^{hck} expression results in a progressive increase in the adherent cell population. A similar assay was performed using p56/59^{hck267M499F} and p56/59^{hck499F} variants, both carrying the 499F mutation which renders the kinase unable to undergo the inhibitory intramolecular association as a result of phosphotyrosine recognition by the SH2 domain (Figure 4D). As shown in Figure 4B, cell adherence of transfectants expressing the constitutively active p56/59^{hck499F} did not significantly change with respect to the basal level. On the contrary, the catalytically inactive p56/59^{hck267M499F} is a potent inducer of adhesion, indicating that inhibition of p56/59^{hck} activity is the critical event triggering adherence. In keeping with this, U937 cells expressing wild-type p50^{csk} exhibit increased adherence to the same level as cells expressing the p56/59^{hck267M499F} or p56/59^{hck267M} variants, indicating that downregulation of endogenous Src kinases enhances adhesion. When the same panel of p56/59^{hck} variants was introduced in cells not exposed to TGFβ/dihydroxyvitamin D_3, it was found that inactive p56/59^{hck}, but not constitutively active p56/59^{hck}, enhanced adhesion (Figure 4C). We also noticed that in untreated cells the expression of wild-type p56/59^{hck} did not significantly modify the level of adherence similarly to the constitutively active form (Figure 4C). On the contrary, a moderate increase in adherence is exhibited by 'primed' cells expressing wild-type p56/59^{hck}, which is in agreement with the possibility that a significant, although unknown fraction of p56/59^{hck} might have been inactivated upon differentiation (Figure 4B). Consistently with their common lineage origin, the dependence of cell adherence on p56/59^{hck} inactivation is shared by other myelomonocytic cell lines, such as HL-60 and THP-1, arrested at different stages of myeloid differentiation. As shown in Table I, transient expression of the dominant-negative p56/59^{hck} variant is sufficient to increase cell adherence to an extent similar to that observed in U937 cells, whereas the constitutively active p56/59^{hck} was ineffective. Cell cycle analysis of FACS-enriched transfectants expressing either p56/59^{hck267M} or p56/59^{hck499F} variants did not reveal any difference regarding the number of cells in S, G_0/G_1 or M phase, indicating that the adherent phenotype is not a downstream effect of cell cycle blockage (not shown).

Stimulation of directional cell migration by active p56/59^{hck}

Previous work has shown that urokinase stimulation of cell migration is associated with a transient increase of p56/59^{hck} activity (Resnati *et al.*, 1996). Therefore, the possibility that transient expression of constitutively active or wild-type p56/59^{hck} could stimulate cell migration in an effector-independent manner was tested. Directional migration toward fibronectin of cells expressing the indicated kinase variants was compared with migration of

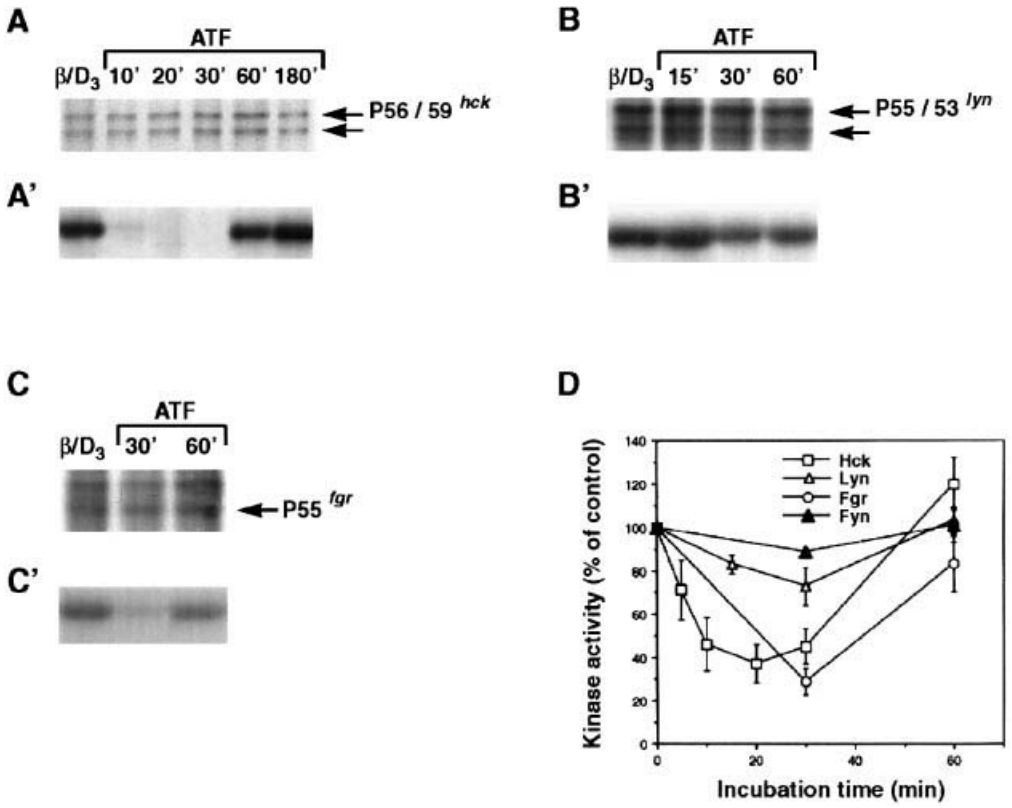


Fig. 3. Time course of p56/59^{hck}, p53/55^{lyn}, p55^{fgr} and p55^{fyn} activity following ATF treatment of differentiating U937 cells. U937 cells (10^6 cells/sample) were metabolically ^{35}S-labelled in the presence of TGFβ/dihydroxyvitamin D_3 for 20 h and, subsequently, incubated with 10 nM ATF or diluents, in test-tubes, under gentle agitation, for the indicated length of time. Radiolabelled cell extracts, containing the same amount of radioactivity, were immunoprecipitated with specific polyclonal antibodies directed against p56/59^{hck} (**A** and **D**), p53/55^{lyn} (**B** and D), p55^{fgr} (**C** and D), p55^{fyn} (D) protein kinases and 60% of it was analysed by a 12.5% SDS–PAGE followed by autoradiography. At each time point the tyrosine kinase activity of 40% of the immunocomplexes was tested in the presence of [γ-^{32}P]ATP and rabbit muscle acid-denatured enolase (**A′**, **B′** and **C′**). Autoradiograms were scanned and the extent of kinase activity is reported as a percentage of the activity of samples which have not received any ATF (D).

cells harbouring a control plasmid. We found that U937 chemotaxis was stimulated 5-fold by the expression of wild-type or constitutively active p56/59^{hck} (Figure 5). In contrast, neither kinase inactive p56/59^{hck} nor wild-type p50^{csk} expression significantly modified directional cell migration. These data consistently show that the activity of p56/59^{hck} kinase directly promotes cell migration, independently of uPAR occupancy.

Specific activation states of p56/59^{hck} are required for ATF-induced motility or adherence

We next sought to determine whether p56/59^{hck} is a mediator of both locomotion and adherence through changes in its activation state. We reasoned that, if uPAR signalling leading to adherence required p56/59^{hck} inactivation, expression of the constitutively active p56/59^{hck499F} variant should inhibit this response. As shown in Figure 6A, expression of p56/59^{hck499F} completely prevented the adhesive cell response of primed cells to ATF, as compared with the cells harbouring an empty control vector or those expressing wild-type p56/59^{hck}. In the light of these and previous findings, we expect the chemotactic response to ATF to be prevented by the expression of kinase inactive p56/59^{hck}. As predicted, inhibition of intracellular p56/59^{hck} activity abolishes ATF-dependent migration of untreated U937 monocytes (Figure 6B). In conclusion, the uPAR-directed pathway triggers myeloid cell motility or adhesion by oppositely regulating the p56/59^{hck} activation state. Interestingly, the extent of acquired adherence/migration is dependent on the expression level of wild-type p56/59^{hck}, suggesting that the lack of this kinase may impair both responses.

Modulation of the motile and adherent phenotypes

In this experiment, the cytoskeleton of U937 transiently expressing either p56/59^{hck499F} or p56/59^{hck267M} was selectively analysed by fluorescence microscopy. The population of GFP-expressing cells was enriched by preparative FACS and then stained with rhodamine–phalloidin (Figure 7, left panels). A clearcut p56/59^{hck267M}-dependent redistribution of F-actin was observed (Figure 7, left, panel A′); furthermore, the F-actin organization in filamentous structures at the cell periphery resembled what we had observed in differentiating U937 cells treated with ATF. Conversely, p56/59^{hck499F}-expressing cells react to rhodamine–phalloidin quite uniformly and F-actin is poorly organized (Figure 7, left, panel B′).

Finally, a modulation of the motile/adherent phenotype was attempted: increasing amounts of plasmid DNA encoding either p56/59^{hck499F} or p56/59^{hck267M} were introduced into U937 cells, which were then assayed for motility and adherence under the conditions described in the previous experiments. To allow a direct comparison

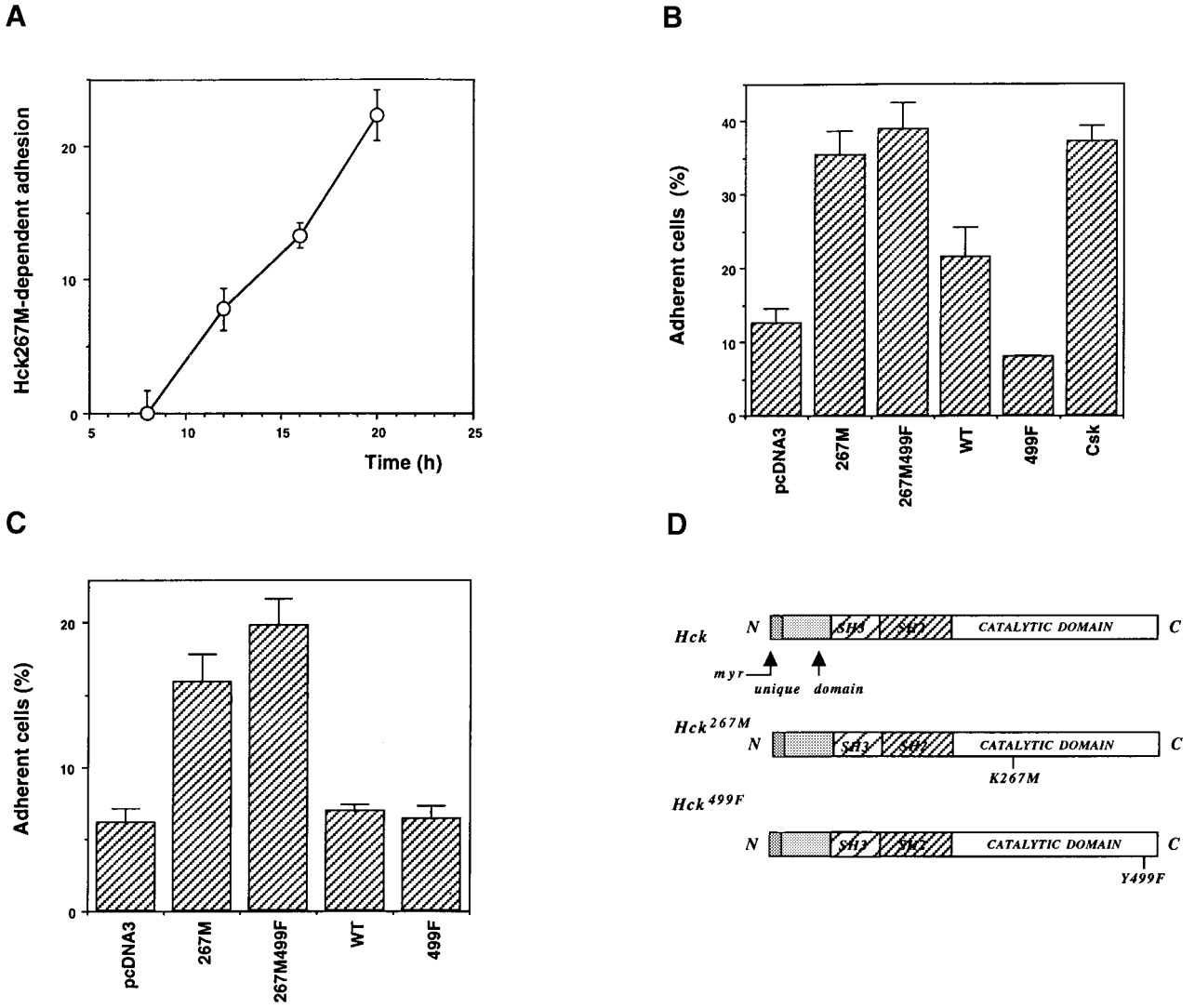


Fig. 4. Adhesiveness of U937 cells transiently expressing different p56/59^{hck} variants. 'Primed' or untreated U937 cells (10^8 cells/sample) were transiently transfected with 20 μg of the indicated constructs in combination with 4 μg of pEGFC1, encoding GFP. At the indicated times after transfection, cells were subjected to an adhesion assay for 1 h, in the absence of any further effector. The number of GFP-positive adherent cells is reported as a percentage of the total fluorescent population and represents the average of three experiments performed in duplicate with standard deviations indicated by error bars. (**A**) 'Primed' U937 cells were transfected with pcDM8mHck267M construct and assayed for adherence at the indicated time points after transfection. The background adherence of cells transfected with an empty control vector (pcDNA3) has been subtracted. (**B**) 'Primed' U937 cells were transiently transfected either with a control empty vector (pcDNA3) or with each of the following plasmids: pcDM8mHck267M, pcDM8mHck267M499, pcDM8mHck, pcDM8mHck499F or pSVpolyCsk, and assayed for adherence 20 h later. (**C**) Untreated U937 cells were transiently transfected either with an empty control vector (pcDNA3) or with each of the following plasmids: pcDM8mHck267M, pcDM8mHck267M499, pcDM8mHck, pcDM8mHck499F, and assayed for adherence 20 h later. (**D**) Schematic representation of the wild-type (Hck), kinase inactive (Hck267M) and constitutively active (Hck499F) p56/59^{hck} variants expressed in U937 cells. The myristylation site (myr), and the 'unique', SH3, SH2 and catalytic domains are indicated as well as the position of the single amino acid substitutions.

of the data, the basal level of motility or adherence in the absence of exogenous p56/59^{hck} was taken as 100%, and all values were reported relative to this. According to the previous data, p56/59^{hck267M} expression promotes an enhancement of cell adherence in a dose-dependent manner (Figure 7, right panel). Vice versa, the progressive increase in the p56/59^{hck499F} expression parallels an increase in cell motility, with no change in adhesion. Interestingly, the extent of p56/59^{hck}-dependent effects on cell adherence or migration is similar when comparing samples which have received the same amount of p56/59^{hck}- encoding DNA. In conclusion, these data show that motility and adherence can be modulated by altering the intracellular p56/59^{hck} active/inactive ratio and indicate that the extent of p56/59^{hck} activity is a critical determinant in converting a motile into an adherent myelomonocytic cell state.

Discussion

This study sheds light on the nature of the molecular mechanisms directing monocytic cell locomotion machinery toward adherence during their differentiation in a macrophage-like phenotype. We report here that myeloid motility and adherence are oppositely regulated by the Src kinase p56/59^{hck} via a mutually exclusive mechanism, and that effector-dependent or -independent modulation of p56/59^{hck} activity leads either to an enhanced directional migration or to a stable adherence.

Table I. Effect of p56/59^{hck267M} expression on myelomonocytic adherence

DNA	% Adherent cells[a]		
	U937	THP-1	HL-60
pcDNA3	12.5 (0.56)[b]	9.23 (0.2)	11.3 (1.27)
pCDM8Hck267M	35.3 (3.19)	24.9 (0.7)	22.06 (1.25)
pCDM8Hck499F	7.95 (0.21)	n.d.	n.d.
pCDM8Hck wt	23.45 (5.0)	n.d.	n.d.

[a]Myelomonocytic cell lines (2×10^6 cells/sample) were electroporated at 200 V, 500 μF with the indicated DNAs and assayed for adherence 20 h after the transfection, in the presence of serum. Cotransfection of pEGFP-C1 encoding the GFP ensures normalization of the transfection efficiency and selective identification of the transfected population. Numbers refer to percentages of GFP-positive adherent cells relative to total cell number. The data represent the average of three experiments performed in duplicate.
[b]SD, standard deviation is indicated in parenthesis.
n.d. = not determined.

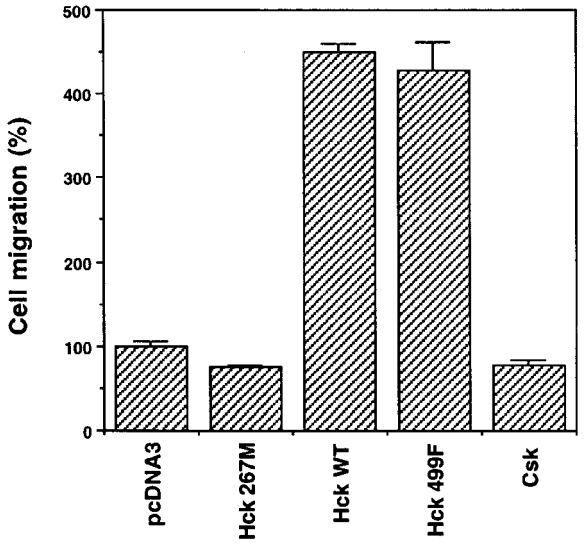


Fig. 5. Directional migration of U937 cells expressing different p56/59^{hck} variants. Cell migration of U937 cells, transiently expressing the indicated p56/59^{hck} variants or wild-type Csk in combination with GFP, was assayed 48 h after transfection. Commercially available Transwell inserts provided with optically transparent collagen-coated filters were used, in which 1×10^6 cells/sample were allowed to migrate toward a fibronectin gradient. At the end of the incubation, the filters were removed and the number of GFP-positive cells determined with a fluorescence microscope. Cell migration in the absence of chemoattractant or random migration is referred to as 100% migration. Data points represent the mean of three independent experiments with standard deviations indicated by error bars.

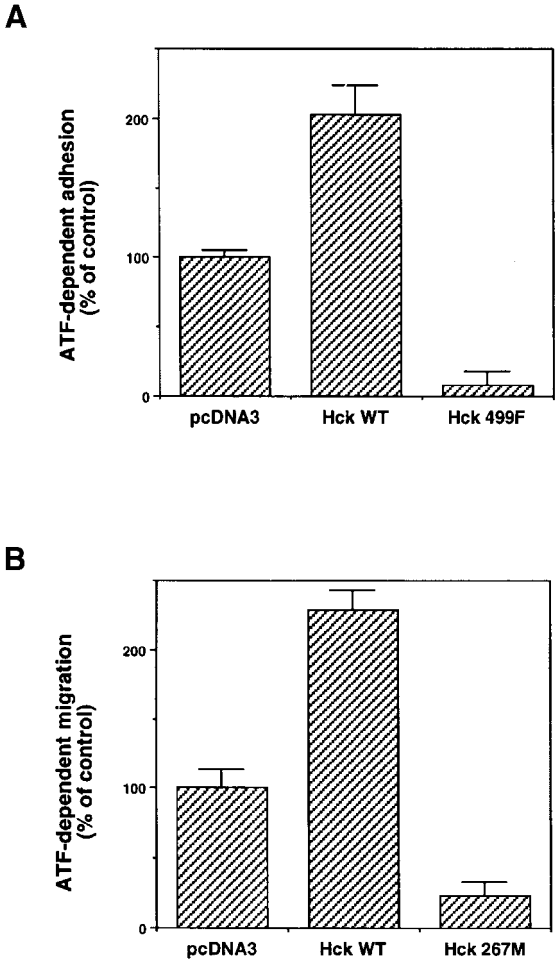


Fig. 6. Effect of p56/59^{hck499F} or p56/59^{hck267M} expression on the adhesive and chemotactic cell response to ATF. Untreated or 'primed' U937 cells were transfected with 20 μg of the indicated constructs in combination with 4 μg of pEGFC1. The level of adherence or motility was assayed under the conditions described in the legends to Figures 4 and 5, respectively. Net adherence or motility of cells transfected with $pcDNA_3$ and further treated with ATF was taken as 100% and all other values were reported relative to this. (**A**) 'Primed' U937 cells were transfected with pcDM8mHck499F or with pcDM8mHck or with an empty control vector ($pcDNA_3$) and then subjected to an adhesion assay for 1 h, in the presence of 10 nM ATF. (**B**) Untreated U937 cells, transiently carrying pcDM8mHck267M or pcDM8mHck or the empty vector $pcDNA_3$, were subjected to a migration assay toward 10 nM ATF.

This study also provides insight into the various steps by which the non-catalytic ATF delivers a receptor-dependent response signal from the cell membrane to downstream effectors. Although the mechanism is still obscure, the ability of GPI-anchored receptors to impinge on cell function is now widely recognized (Robinson, 1991). The signalling mechanism of GDNFR-α and NTNR-α, which associate with the orphan *ret* tyrosine kinase receptor in a ligand-dependent manner, offers a model in which a shared transmembrane protein tyrosine kinase and two ligand-specific GPI-linked proteins act in a multicomponent receptor system (Klein *et al.*, 1997). The possibility that GPI anchors can interact directly with palmitylated p56^{lck} and p59^{fyn} tyrosine kinases has been raised (Shenoy-Scaria *et al.*, 1993). However, this may not apply to all cases, as both the GPI-anchored and the transmembrane forms of the adhesion molecule CD58 have the ability to recruit protein kinases, showing that, in this case, complex formation is GPI independent (Itzhaky *et al.*, 1998).

In our study, incubation of differentiating U937 cells with ATF induces a rapid and transient decline in the p56/59^{hck} and p55^{fgr} phosphotransferase activities (p53/56^{lyn} and p59^{fyn} activities are not affected), uncovering a functional linkage between uPAR and specific Src kinases. The rapid and transient decline of p56/59^{hck} activity preceding myelomonocytic adherence is also triggered by insulin and phorbol esters, suggesting convergent pathways sustaining a general response to pro-adhesive stimuli (not shown). A comparison of our data with previous evidence

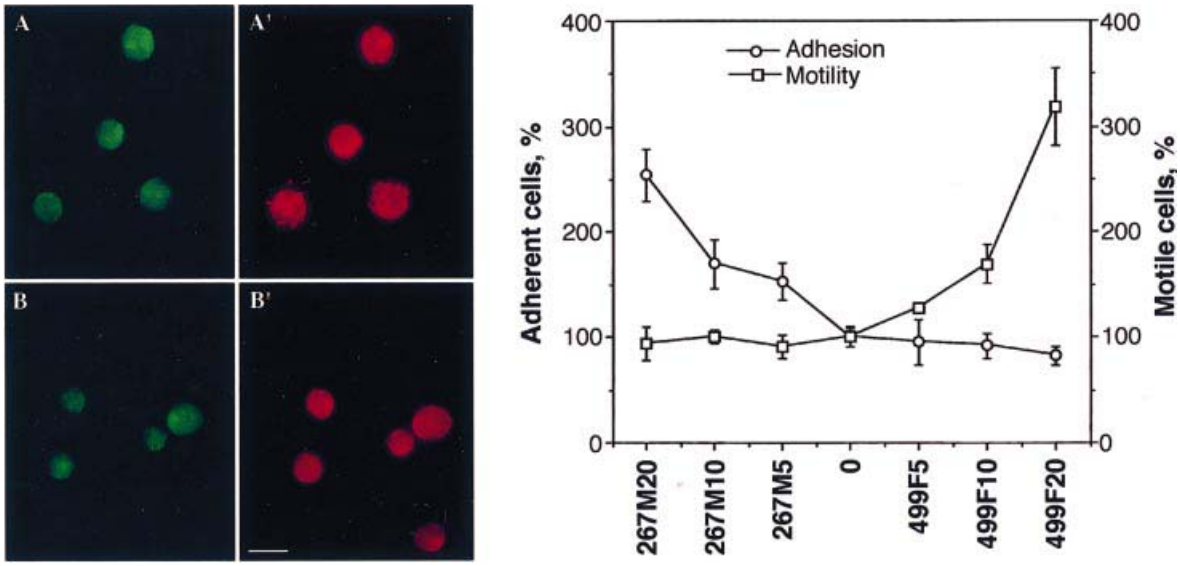


Fig. 7. Effects of p56/59^{hck} activity modulation on U937 cytoskeleton, motility and adhesion. Left: fluorescence microscopy of FACS-enriched GFP-positive U937 cells, transfected with 20 μg of plasmids, expressing either p56/59^{hck267M} (**A** and **A′**) or p56/59^{hck499F} (**B** and **B′**). GFP-positive cells were stained with rhodamine–phalloidin (A′ and B′), loaded onto polylysine-coated coverslips and analysed by a fluorescence microscope. Scale bar in micrographs: 10 μm. Right: ability to migrate and to adhere of GFP-positive U937 cells transfected with increasing amounts of plasmid DNA encoding either p56/59^{hck267M} or p56/59^{hck499F}. The total amount of transfected DNA (45 μg) was kept constant by the addition of the required amount of an empty vector. Five micrograms of pEGFP-C1 were included in each sample. Specific DNAs were transfected as follows: pcDM8mHck267M (20, 10 and 5 μg) or pcDM8mHck499F (20, 10 and 5 μg) or carrier DNA (0 μg). The extent of adhesiveness or directional cell migration was assayed as described in the legends to Figures 4 and 5, respectively. In this case, the basal level of adherence or motility, in the absence of exogenous p56/59^{hck} (0) was taken as 100% and all values were reported relative to this.

by Resnati *et al.* (1996) and Konakova *et al.* (1998) shows a similar timing of the p56/59^{hck} activation and inactivation kinetics, suggesting that these opposite effects may involve similar mechanisms but, perhaps, partially different mediators. Although uPAR can be copurified with Hck, Fgr, Lyn and Fyn in myeloid cells, the experiments reported in this paper show that only Hck and Fgr are activated (Bohuslav *et al.*, 1995). Therefore, the physical association of Src kinases with uPAR is not sufficient to regulate these kinases in response to ATF, but additional mechanisms should be invoked. Since we observed a decline of the general Hck phosphorylation which parallels the reduction of its catalytic activity in response to ATF, we suggest that a dephosphorylation of the Hck (and Fgr) autophosphorylation site may be occurring. Moreover, we show that Csk expression causes an increase in cell adherence, suggesting that downregulation of specific Src kinase activity is sufficient to gain the adherent phenotype. Another possibility that should be taken into account is the existence of an unknown docking protein, which may contribute to uPAR–Src kinase association and expose the critical sites of Hck and Fgr to the modifying enzymes. Finally, we cannot exclude the possibility of the involvement of the Hck/Fgr unique domains for which no function has yet been clearly defined. All these mechanisms may be acting in a non-mutually exclusive manner and can actually cooperate with the transient reduction of Hck and Fgr activities when U937 cells are induced to adherence with ATF in the presence of TGF-β/dihydroxyvitamin D_3 (Sun *et al.*, 1998). On the other hand, when unstimulated U937 are induced to migrate with ATF alone, Hck and Fgr undergo a transient upregulation; to explain this differentiation-dependent modulation of Hck activity in response to ATF we have to postulate the existence of specific regulators of Src kinases that can be differentially expressed following exposure to TGF-β/dihydroxyvitamin D_3.

With regard to the downstream effectors, studies on transgenic mice lacking p50^{csk} restrict Src major substrates to cortactin, tensin, paxillin and p125^{fak}, as in these animals they are hyperphosphorylated (Sheila *et al.*, 1995). Moreover, the oncogenic constitutively active v-Src stimulates pp125^{fak} tyrosine phosphorylation and degradation, prior to the onset of loss of cytoskeletal organization, cell rounding and detachment. In contrast, cells expressing a kinase-inactive variant of v-Src exhibit large focal adhesions associated with an impaired ability to migrate (Fincham *et al.*, 1995). Fincham *et al.* (1998) have also observed that Src activity is dispensable for translocation to focal adhesions but it is required for degradation of pp125^{fak}, which is implicated in focal adhesion turnover. Association of c-Src with regulatory proteins occurs via its N-terminal SH2 and SH3 domains when not engaged with the C-terminal phosphotyrosine (Tyr527) (Kaplan *et al.*, 1994; Moarefi *et al.*, 1997). The possibility that uPAR signalling may be mediated by Src kinases acting, in turn, on focal adhesion proteins is also supported by the finding that uPAR ligation with uPA in bovine aortic endothelial cells promotes tyrosine phosphorylation of pp125^{fak}, paxillin and p130^{cas} (Tang *et al.*, 1998).

In our experiments, the N-terminal regions of p56/59^{hck267M499F} and p56/59^{hck499F} are equally able to interact with focal adhesion proteins: their opposite effects (adhesion versus migration) indicate the relevance of p56/59^{hck} catalytic activity/inactivity to these processes. It is possible that under physiological conditions, p56/59^{hck} and perhaps p55^{fgr} may undergo a uPAR-dependent localized, regulation of their activation state (Kindzelskii *et al.*, 1996).

This is particularly relevant during locomotion, when cell adhesions have to detach from the substratum at the cell rear, whereas they have to form at the cell front (Lauffenburger and Horwitz, 1996). The requirement of p56/59^{hck} for locomotion is further supported by the results obtained with PMN from *hck*$^{-/-}$ and *fgr*$^{-/-}$ mice, in which neutrophils cannot spread and adhere stably to the culture dish (Lowell *et al.*, 1996). In macrophages, a β1 integrin signalling pathway involving Src family kinases, as well as p120 (c-*cbl*) and PI-3 kinase is required for cell spreading and migration (Meng and Lowell, 1998).

It is tempting to speculate that *in vivo* modulation of p56/59^{hck} activity is one of the mechanisms underlying the critical ability of monocytes to migrate into the bloodstream and be recruited at the site of injury, where they terminally differentiate into adherent, less motile macrophages.

The ability of cells to move is essential for the development and survival of multicellular organisms, yet it can also be detrimental when cancer cells migrate to distant parts of the body and form metastases. Therapeutic use of blocking peptides or naturally occurring antagonists of uPAR signalling has been proposed to control cell motility (Fazioli and Blasi, 1994; Franco *et al.*, 1998). Defining the role of p56/59^{hck} activation/inactivation in the regulation of myelomonocytic motility/adherence opens the possibility of engineering white blood cells for gene therapy and tuning their ability to migrate or adhere. Switching cell migration on and off, as well as independently modulating adherence may be crucial to biotechnological applications, such as tissue engineering. Finally, studying the molecular mechanisms underlying cell motility may provide a truly rational basis for genetic, pharmacological or material-based interventions for controlling tumour metastasis.

Materials and methods

Materials

The ATF of uPA (amino acids 1–135) was a gift from Dr J.Wang (Abbott Laboratories, Abbott Park, IL). Anti-p56/59^{hck}, anti-p59^{fyn}, anti-p56/53^{lyn} and anti-p55^{fgr} polyclonal antibodies were from SantaCruz (Germany). Anti-uPAR 399 polyclonal antibody was from American Diagnostica (Greenwich, CT). Secondary antibodies were from Jackson (West Grove, PA). pEGFP-C1 plasmid was from Clontech (Palo Alto, CA). Cell culture reagents were from Gibco-BRL (Gaithersurg, MD). Transwell chambers were from Corning Costar (Acton, MA). *Trans*^{35}S label and [γ-^{32}P]ATP were from Amersham, UK. Autoradiographic X-omat films were from Eastman Kodak Co. (Rochester, NY). Enlightening was from New England Nuclear. Cycloheximide, DRB, TGF-β and dihydroxyvitamin D_3 were from Calbiochem (San Diego, CA).

Plasmids

The expression plasmids for mouse p56/59^{hck} and relative variants have been described previously (Holtzman *et al.*, 1987). Briefly, pcDM8mHck encodes wild-type p56/59^{hck}, pcDM8mHck267M encodes p56/59^{hckK267M} (AAG has been substituted with ATG), pcDM8mHck499F encodes p56/59^{hckY499F} (TAT codon has been substituted with TTT) and pcDM8m-Hck267M499F encodes the di-substituted variant p56/59^{hckK267M499F}. pEGFP-C1 expresses a red-shifted variant of GFP optimized for brighter fluorescence and higher expression in mammalian cells and carrying a double amino acid substitution (F64L and S65T) (Cormack *et al.*, 1996). pSVpolyCsk expressing wild-type Csk was kindly provided by Dr M.Bergman, Helsinki (Bergman *et al.*, 1992).

Cell culture, 'priming' and transfections

U937 histiocytic lymphoma, HL-60 promyelocytic leukaemia and THP-1 monocytic leukaemia cell lines were cultured in RPMI containing 10% heat-inactivated fetal bovine serum (FBS), in the presence of 100 U/ml penicillin and 100 μg/ml streptomycin in a 5% CO_2 atmosphere. For 'priming', cells were diluted at 0.6×10^6 cells/ml, and incubated with 1 ng/ml of TGF-β and 50 nM dihydroxyvitamin D_3 for 22 h in Petri dishes. Whenever specified, cells are further treated with 10 nM ATF, in the presence of 10% FBS. For transfections, 20×10^6 cells were resuspended in 25 μl of FBS, 50 μl of 10× RPMI with L-glutamine, 7.5 μl of 7.5% sodium bicarbonate, 50 μg of DNA, in a final volume of 250 μl. Each sample received a total of 50 μg of DNA, including 20 μg of the selected plasmid, 4 μg of pEGFP-C1 and 26 μg of pGEM as a carrier, unless otherwise specified. When indicated, cells to be transfected are pretreated with TGF-β/dihydroxyvitamin D_3 for 2 h. The cells were electroporated at 250 V, 950 μF and then resuspended in 12 ml of RPMI with or without TGFβ/vitamin D_3 for an additional 20 h. The resulting average transfection efficiency was 15% and viable cells were 80% of the total cell population.

Fluorescence and confocal laser scanning microscopy

To observe anchorage-independent cells, 'primed' U937 cells, at 1.5×10^6 cells/ml, were incubated with ATF or diluents for 1 h at 37°C in plastic tubes under gentle agitation. They were then fixed with 3.7% paraformaldehyde for 10 min on ice and permeabilized with 0.2% Triton X-100. For F-actin staining, a 100 μg/ml solution of rhodamine-conjugated phalloidin in methanol was used. Alternatively, coverslips were incubated with 10 μg/ml gelatin for 10 min, washed with phosphate-buffered saline (PBS), dried and placed into Petri dishes during 'priming' and ATF treatments. Cells were then washed and fixed as described above.

To analyse transfected cells, the population of GFP-expressing cells was isolated by FACS. Briefly, 24 h post-transfection, 8×10^6 cells were resuspended in 2 ml of phenol red-free medium and sorted. GFP-positive cells were then fixed and allowed to adhere onto polylysine-coated coverslips for 30 min at 4°C. In all cases, a Zeiss laser scanning microscope (LSM 410 invert) equipped with a plan apo oil (100×) immersion lens (na = 1.3) was used. Fluorescein isothiocyanate (FITC) emission was excited using the argon laser 488 nm line. The emission signals were filtered with a Zeiss 510–525 nm filter (fluorescein emission). The images were acquired and processed by a Zeiss CLSM instrument software. Three-dimensional image reconstruction was performed by Mc Insights software, kindly provided by G.Paolella (EMBL, Heidelberg).

Metabolic cell labelling and in vitro kinase assays

Exponentially growing U937 cells were diluted to a density of 0.6×10^6/ml, starved with 10% heat-inactivated dialysed FBS for 3 h and incubated with 60 μCi/ml of *Trans*^{35}S label/ml, in the presence of TGFβ/dihydroxyvitamin D_3 for 20 h. When indicated, cells were transferred to test-tubes and incubated with 10 nM ATF. At the indicated time points, cells were washed once in PBS and once in Tris-buffered saline (TBS) [25 mM Tris pH 7.5, 150 mM NaCl, 10 mM dithiothreitol (DTT) and 100 μM sodium orthovanadate], lysed on ice for 30 min in a buffer containing 25 mM Tris pH 7.5, 150 mM NaCl, 1% (v/v) Nonidet P-40, 2 mM EDTA, 10 mM sodium orthovanadate, 20 mM sodium pyrophosphate, 50 μg/ml aprotinin and 2 mM PMSF. Lysates were clarified by centrifugation in a microfuge at 13 000 r.p.m. for 20 min at 4°C. Before immunoprecipitation, radiolabelled cell extracts were normalized for the amount of incorporated ^{35}S radioactivity, pre-cleared by incubation for 30 min at 4°C with protein A–Sepharose and, subsequently, incubated with anti-p56/59^{hck}, anti-p59^{fyn}, anti-p53/56^{lyn} and anti-p55^{fgr} antibodies (dilution 1:100) for 2 h at 4°C. The immunocomplexes were recovered by absorption to protein A–Sepharose and kinase activity was tested according to Resnati *et al.* (1996). Relative quantitation of the radioactivity was performed by phosphoimager analysis.

Cell cycle analysis

U937 cell cycle profile was analysed 20 h after transfection. The DNA content of GFP-positive fixed cells was measured using FACScan flow cytometry, according to Lamm *et al.* (1997).

Adhesion assays

Myeloid cell adherence was tested by incubating 0.5×10^6 cells/sample in 24 multiwell plates with or without 10 nM ATF for 30 min at 37°C (unless otherwise specified). Non-adherent cells were harvested by pipetting and adherent cells were removed with 0.05% trypsin. The number of adherent cells is expressed as a percentage of the total cell number and represents an average of three different experiments performed in duplicate.

Chemotaxis assays

The assays were performed using Transwell Costar chambers with optically transparent 3 μm pore size PTFE filters (insert growth area 0.33 cm^2, coated with collagen type IV) according to Franco *et al.* (1997) with minor modifications. Briefly, 10^6 U937 cells were resuspended in 10% FBS-RPMI and applied to the upper compartments of a 24 multiwell dish containing the membrane inserts. Either 10 nM ATF or 25 μg/ml fibronectin in RPMI was added to the lower compartments and incubation was carried out at 37°C overnight. Then, the GFP-positive cells were counted on the filters using a fluorescence microscope. Cell migration in the absence of chemoattractant or random migration is referred to as 100% migration. Data points represent the mean of three independent experiments with standard deviations indicated by error bars.

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