The p16^{INK4a} tumour suppressor protein inhibits $\alpha_v\beta_3$ integrin-mediated cell spreading on vitronectin by blocking PKC-dependent localization of $\alpha_v\beta_3$ to focal contacts

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Expression of full-length p16^{INK4a} blocks $\alpha_v\beta_3$ integrin-dependent cell spreading on vitronectin but not collagen IV. Similarly, G$_1$-associated cell cycle kinases (CDK) inhibitory (CKI) synthetic peptides derived from p16^{INK4a}, p18^{INK4c} and p21$^{Cip1/Waf1}$, which can be delivered directly into cells from the tissue culture medium, do not affect non-$\alpha_v\beta_3$-dependent spreading on collagen IV, laminin and fibronectin at concentrations that inhibit cell cycle progression in late G$_1$. The $\alpha_v\beta_3$ heterodimer remains intact after CKI peptide treatment but is immediately dissociated from the focal adhesion contacts. Treatment with phorbol 12-myristate 13-acetate (PMA) allows $\alpha_v\beta_3$ to locate to the focal adhesion contacts and the cells to spread on vitronectin in the presence of CKI peptides. The cdk6 protein is found to suppress p16^{INK4a}-mediated inhibition of spreading and is also shown to localize to the ruffling edge of spreading cells, indicating a function for cdk6 in controlling matrix-dependent cell spreading. These results demonstrate a novel G$_1$ CDK-associated integrin regulatory pathway that acts upstream of $\alpha_v\beta_3$-dependent activation of PKC as well as a novel function for the p16^{INK4a} tumour suppressor protein in regulating matrix-dependent cell migration.
Keywords: $\alpha_v\beta_3$ integrin/cancer/integrin regulatory pathway/p16^{INK4a} tumour suppressor protein

Introduction

Cell cycle progression through the G$_1$ checkpoint is initiated and regulated by signals received from extracellular stimuli, such as mitogens and cell-adhesion contacts, which influence the activity of pRb-phosphorylating kinase complexes including the cyclin D-dependent kinases (CDKs) cdk4 and cdk6 (Matsushime *et al.*, 1992; Meyerson and Harlow, 1994; Weinberg, 1995). The activity of the G$_1$-associated CDKs is inhibited by members of two families of proteins which prevent phosphorylation of pRb and the activation of pRb-associated transcription factors, and thereby stop cells from entering the replicative phase, or the S phase, of the cell cycle (Sherr and Roberts, 1995). The p21$^{Cip1/Waf1}$ family (including p21, p27 and p57) interact with both the cyclin D and the CDK subunits through domains in the N- and C-termini that carry the essential RRLF-like peptide motif (Adams *et al.*, 1996).

The p16^{INK4} family includes the closely related p15^{INK4b}, p16^{INK4a}, p18^{INK4c} and p19^{INK4d} proteins, which inhibit cdk4 and cdk6 kinase activities through direct interaction with the kinase subunit only (Serrano *et al.*, 1993; Hannon and Beach, 1994; Chan *et al.*, 1995; Hirai *et al.*, 1995). Of these kinase inhibitors, only p16^{INK4a} function is lost at a high frequency in human cancers (Kamb *et al.*, 1994; Ranade *et al.*, 1995; Hall and Peters, 1996).

Integrins consist of an α and a β heterodimer that interacts with the extracellular matrix (ECM), adjacent cells or soluble ligands, and play important roles in regulating cell morphology, spreading and survival (Hynes, 1992; Damsky *et al.*, 1997). The interaction between the integrins and their ligands leads to integrin clustering, which generates signals to the intracellular compartment through changes in Ca^{2+} influx, elevated pH and tyrosine phosphorylation of proteins that are associated with the integrin cytoplasmic domain and the focal adhesion complex (Sastry and Horwitz, 1993; Clark and Brugge, 1995). Secondary events linked to integrin activation also mediate transduction of signals associated with growth receptors, implicating synergies between integrin-linked signalling and other receptor pathways that allow synchronized intracellular responses to different extracellular stimuli (Clark and Brugge, 1995; Schlaepfer and Hunter, 1998). This is exemplified by the progression through the G$_1$ phase of the cell cycle which depends on mitogen-induced expression of cyclin D1 and ECM-dependent control of cyclin–CDK activity through regulation of p21$^{Cip1/Waf1}$ and p27^{Kip1} activity (Fang *et al.*, 1996; Assoian and Zhu, 1997).

Cell attachment to vitronectin is mainly mediated through integrin receptors containing the α_v subunit (Marshall and Hart, 1996). The $\alpha_v\beta_3$ integrin receptor is tightly associated with tumour growth and spreading, both as a marker for invasive cancer cells such as malignant melanomas and grade III and IV glioblastomas and also through its essential role in neovascularization (Gladson and Cheresh, 1991; Brooks *et al.*, 1994; Marshall and Hart, 1996; Van Leeuwen *et al.*, 1996). The association of $\alpha_v\beta_3$ with platelet-derived growth factor-β (PDGFβ) and the insulin receptor substrate (IRS-1), as well as the dependence on $\alpha_v\beta_3$ for the biological effect of fibroblast growth factor-2 (FGF-2), indicate that $\alpha_v\beta_3$ is intimately linked with the cellular response to certain growth factors (Vuori and Ruoslahti, 1994; Rusnati *et al.*, 1997; Schneller *et al.*, 1997). Furthermore, $\alpha_v\beta_3$ is also associated with extracellular protease activities including the urokinase type plasminogen activator (uPA) and its receptor (uPAR), as well as metalloproteinase 2A (Gladson *et al.*, 1995; Brooks *et al.*, 1996). Taken together, these observations suggest that $\alpha_v\beta_3$ plays an important and complex role in promoting and regulating cell spreading and growth in tissues by co-ordinating intra- and extracellular activities

involved in cell migration (Marshall and Hart, 1996; Damsky *et al.*, 1997).

We have shown previously that small synthetic peptides derived from the p16[INK4a] tumour suppressor protein, the p18[INK4c] and the p21[Waf1/Cip1] proteins can be linked to a peptide carrier sequence from the *Antennapedia* homeodomain (Derossi *et al.*, 1994) and immediately be taken up by cells from the tissue culture medium and induce a cell cycle arrest in G_1 (Fåhraeus *et al.*, 1996, 1998; Ball *et al.*, 1997). A synthetic peptide that spans the two α-helices of the third ankyrin repeat of p16[INK4a], which interacts with the CDKs and is implicated in INK4-mediated inhibition of cdk4/6 phosphorylation of pRb by dislocating the ATP-interacting loop in the catalytic cleft of the kinases (Russo *et al.*, 1998), inhibits CDK–cyclin D1 kinase activity and arrests cells in G_1 (Fåhraeus *et al.*, 1996). A 20 amino acid peptide corresponding to the C-terminus of p21[Cip1/Waf1] that includes the essential RRLF motif (Adams *et al.*, 1996) has been shown to bind cyclin D1 and cdk4 and to inhibit cell cycle progression through G_1 (Ball *et al.*, 1997). A further peptide derived from the p18[INK4c] protein sequence carrying eight out of 20 residues identical to those of p16[INK4a] is also active in binding cdk4 and cdk6, and in inhibiting cdk4–cyclin D1 kinase activity (Fåhraeus *et al.*, 1998).

Results

A G_1 CDK inhibitory (CKI) peptide series

We have established a series of G_1 kinase inhibitory peptides that can be delivered directly into cells in order to study how acute inhibition of G_1-associated kinases influence growth-associated cellular activities. The cell cycle inhibitory peptides derived from p16[INK4a], p18[INK4c] and p21[Waf1/Cip1], and mutated variants thereof, were tested and compared for their ability to inhibit cdk4–cyclin D1 phosphorylation of a glutathione *S*-transferase (GST)–Rb protein *in vitro* and to arrest cell cycle progression in G_1. The p16[INK4a] peptide carrying the D92A substitution (peptide 20), which has a lower IC_{50} compared with the wild-type sequence (Fåhraeus *et al.*, 1996, 1998), and the p18[INK4c] (peptide 45) and the p21[Cip1/Waf1] (peptide 10) peptides all block kinase activity (Figure 1A). The p16[INK4a] peptide 21 that carries alanine substitution of two valine residues which correspond to positions 95 and 96 of the p16[INK4a] protein and has an increased IC_{50} *in vitro* of ~3-fold (1.5–4.5 μM), has lost ~60% of the cell cycle inhibitory capacity at 20 μM concentration compared with the wild-type peptide (Figure 1A; Fåhraeus *et al.*, 1998). Previous results (Fåhraeus *et al.*, 1998) suggest that the double VV95,96AA mutations do not primarily affect peptide interaction with the CDK but rather induce an unfavourable conformation in the peptide, which is supported by the p16[INK4a]–cdk6 structure (Russo *et al.*, 1998). The mutated p21[Cip1/Waf1] (peptide 44), which carries alanine substitutions of the leucine and phenylalanine residues in the RRLIF motif, has lost most of its kinase inhibitory activity (Figure 1A). When these peptides are added at 20 μM concentration to the tissue culture medium of human keratinocyte-derived HaCaT cells, the correlation between the capacity of the peptides to inhibit cdk4–cyclin D1 kinase activity and to cause a G_1 arrest is shown (Figure 1A and B). The sequence differences between the
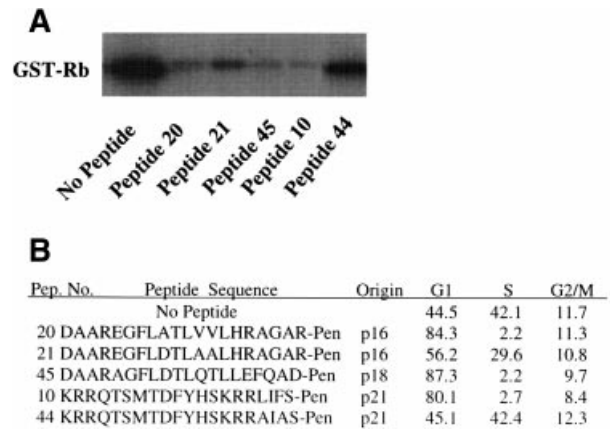

Fig. 1. CKI peptides induce a G_1 arrest. CKI synthetic peptides derived from p16[INK4a], p18[INK4c] and p21[WAF1/Cip1] protein sequences and synthesized with the *Antennapedia* carrier sequence were tested for their capacity to inhibit cdk4–cyclin D1 kinase activity. Peptides were incubated with baculovirus-infected Sf9 insect cell lysates co-expressing cdk4 and cyclin D1 and the relative kinase inhibitory activity of the peptides was estimated using GST–Rb as substrate (**A**). The same peptides were added (20 μM) directly to the tissue culture medium of HaCaT cells and their G_1 inhibitory effect was estimated by determining the incorporation of BrdU (S phase) after 24 h using FACS analysis (**B**). The p16[INK4a] peptide 21 carries two alanine substitutions at positions corresponding to valine 95 and 96 of the p16[INK4a] protein and has reduced cdk4- and cdk6-binding capacity (data not shown), and reduced capacity to inhibit cdk4–cyclin D1 kinase activity as well as to induce a G_1 arrest. The p16[INK4a] peptide 20 carries a D92A substitution at position 92 and is more active as a kinase inhibitor compared with wild-type peptide sequence (Fåhraeus *et al.*, 1998). Peptide 10 is based on the C-terminus of p21[WAF1/Cip1] and interacts with cdk4, cdk6 and cyclin D1. Peptide 44 carries alanine substitutions in the RRLF motif of peptide 10 and has little effect on cdk4–cyclin D1 kinase activity or cell cycle progression.

INK4 and the p21[Waf1/Cip1]-derived peptides, and the fact that alanine substitutions that result in reduced peptide binding to cdk4/6 correlate with the decreased capacity of the peptides to inhibit CDK activity *in vitro* and to induce a G_1 arrest, demonstrates the specificity of the peptides.

CKI peptides inhibit cell spreading and migration on vitronectin but not attachment

It is well known that the ECM contact is important for allowing cell cycle progression, and signals derived from integrins affect the levels of cyclin D1 as well as p27[KIP]. While testing to see if the ECM composition would influence the cell cycle inhibitory capacity of the CKI peptides (Table I), we observed a correlation between G_1 arrest and the capacity of cells to spread on vitronectin. We became interested in this effect and wanted to investigate whether there is a link between G_1-related kinase activity and cell adhesion-related phenomena. Since the vitronectin receptor, $\alpha_v\beta_3$ integrin, is expressed on cells associated with migration and proliferation in wound healing, neovascularization and tumour cell spreading, we decided to study the effect of cdk4 and cdk6 inhibitors on this specific integrin. The CS-1 hamster melanoma cell line grows in suspension on a vitronectin substrate but adheres to fibronectin or laminin (Thomas *et al.*, 1993). After transfection of the β_3 subunit, the cells express a functional integrin (CS-1 β_3) and acquire the capacity to attach and

spread on vitronectin (Figure 2A and B) (Filardo *et al.*, 1995). When the CS-1 β_3 cells are seeded on vitronectin in the presence of the CKI p16^{INK4a} peptide 20 (Figure 2C), p21$^{Waf1/Cip1}$ peptide 10 (Figure 2F) and

A

p18^{INK4c} peptide 45 (Figure 2G) at 20 µM concentration, the CS-1 β_3 cells are blocked from spreading. As expected, if the spreading on vitronectin is associated with the capacity of the peptides to inhibit G_1 kinases, the p16^{INK4a} peptide 21, which has reduced CKI and cell cycle inhibitory capacity, induces a slight but significant delay in spreading (Figure 2D) and the mutated p21$^{Waf1/Cip1}$-derived peptide 44 has no effect (Figure 2E), compared with non-treated cells (Figure 2B). These changes in cell spreading are tightly associated with the capacity of INK4- and p21$^{Waf1/Cip1}$-derived peptides to block G_1 CDK activity and cell cycle progression, and have not been observed using *Antennapedia* control peptides (Figure 2D and E; data not shown). Since the CS-1 β_3 cells do not spread on vitronectin in the presence of the fully active CKI peptides, they are not expected to be capable of migrating on this substrate. This was confirmed by seeding cells on vitronectin and following their capacity to migrate over 18 h in the presence of the p16^{INK4a} peptide 20 or the less active p16^{INK4a} peptide 21. Cells treated with the more active CKI peptide show a dramatic inhibition in migration over the vitronectin substrate (Figure 2I), whereas cells treated with the less active peptide 21 migrate more easily (Figure 2H). This result shows that the CS-1 β_3 cells remain attached to the tissue culture dish up to 18 h after peptide treatment, which is in line with the observation that treatment with the CKI peptide 20 has no significant influence on cell attachment to vitronectin or to laminin (Figure 2A). It is thus the capacity of cells to spread and
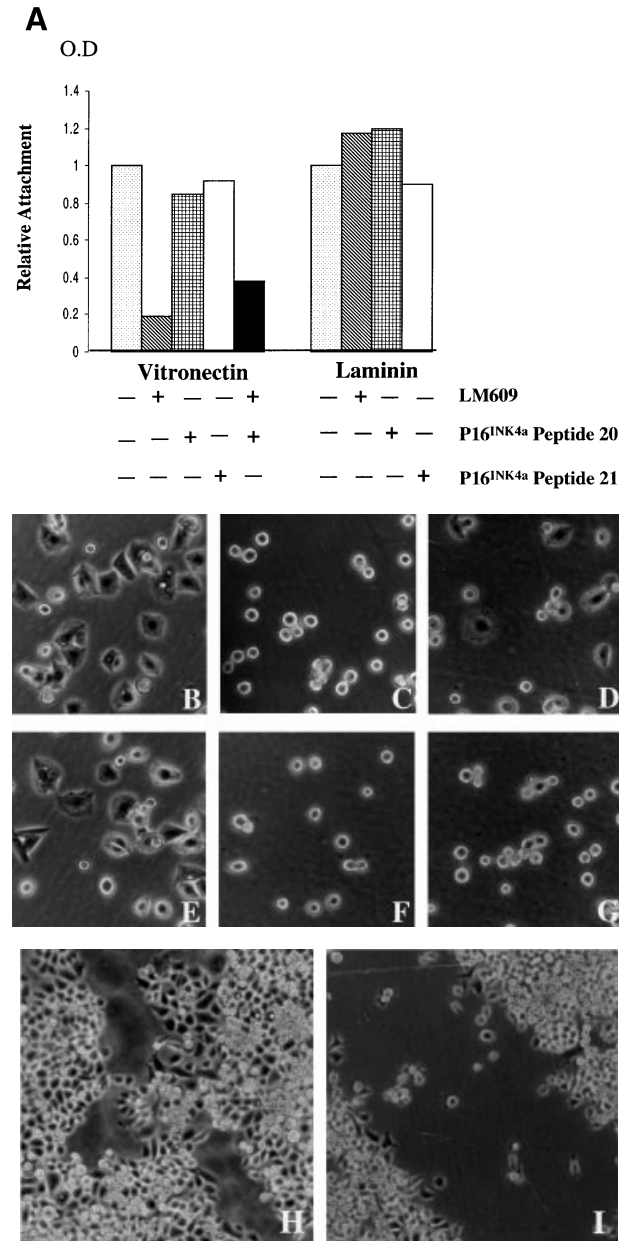
Fig. 2. CKI peptides block $\alpha_v\beta_3$ integrin-dependent spreading and migration but not attachment to vitronectin. The anti-$\alpha_v\beta_3$ mAb LM609 blocks attachment of CS-1 β3 cells to vitronectin but not to laminin, whereas treatment with p16^{INK4a} peptides 20 and 21 does not affect cell attachment (**A**). CS-1 β_3 cells treated with 20 µM CKI peptides p16^{INK4a} peptide 20 (**C**), p21$^{WAF1/Cip1}$ peptide 10 (**F**) and p18^{INK4c} peptide 45 (**G**) inhibit cell spreading on vitronectin. The less active CKI p16^{INK4a} peptide 21 (**D**) is less efficient in blocking cell spreading and the mutated p21$^{WAF1/Cip1}$ peptide 44 (**E**) has no effect compared with non-peptide-treated cells (**B**). CS-1 β_3 cells were grown to confluence on vitronectin for 24 h before a break in the monolayer was introduced using a plastic tip. p16^{INK4a} peptides 20 or 21 were then added to the cultures and the capacity of cells to migrate on vitronectin was estimated after 18 h. Cells can migrate on vitronectin in the presence of the less active p16^{INK4a} peptide 21 (**H**) but not the more potent p16^{INK4a} peptide 20 (**I**). The attachment and cell spreading data shown represent one of four similar experiments determined 2 h after seeding.

Table I. The extracellular matrix composition does not affect the cell cycle regulatory capacity of the CKI p16^{INK4a}-derived peptide

	+ Peptide			– Peptide		
	G_1	S	G_2/M	G_1	S	G_2/M
HaCaT						
Plastic	85.6	2.6	10.3	46.1	42.5	6.6
Plastic + PMA[a]	78.2	4.1	8.4	47.8	42.2	7.1
Collagen IV	86.0	2.7	9.7	46.3	41.6	7.3
Laminin	88.4	3.9	6.6	44.2	46.1	6.9
Fibronectin	84.1	2.0	12.3	42.1	46.2	8.0
Gelatin	83.7	3.0	11.5	49.3	40.8	7.1
Vitronectin	85.2	3.2	7.6	47.3	44.6	8.2
Primary foreskin fibroblasts						
Plastic	69.4	12.5	9.6	47.1	32.8	13.5
Collagen IV	74.5	11.4	7.9	44.7	33.0	13.2
Laminin	67.9	15.9	8.1	45.2	34.1	11.7
Fibronectin	74.5	11.3	6.2	45.9	31.4	10.4
Gelatin	72.4	11.7	8.2	48.1	31.8	12.1
Vitronectin	72.2	13.5	7.6	46.6	33.5	11.7
CS-1 β_3						
Non-synchronized				47.6	41.1	9.2
0.2% FCS 72 h				79.1	12.8	6.5
L-mimosine 100 µM				78.4	1.5	12.7
Olomoucine 100 µM				47.2	30.3	19.3

HaCaT cells and primary human foreskin fibroblasts (passage 5) were seeded on different matrixes and treated with 20 µm of the p16 peptide (peptide 20) for 24 h before the cell cycle distribution was analysed by FACS.
[a]Treatment with 10 nM PMA does not significantly block the cell cycle inhibitory effect of the peptide. The cell cycle distribution is also shown for CS-1 β_3 after different cell cycle inhibitory treatments. This table shows one of at least two similar experiments.
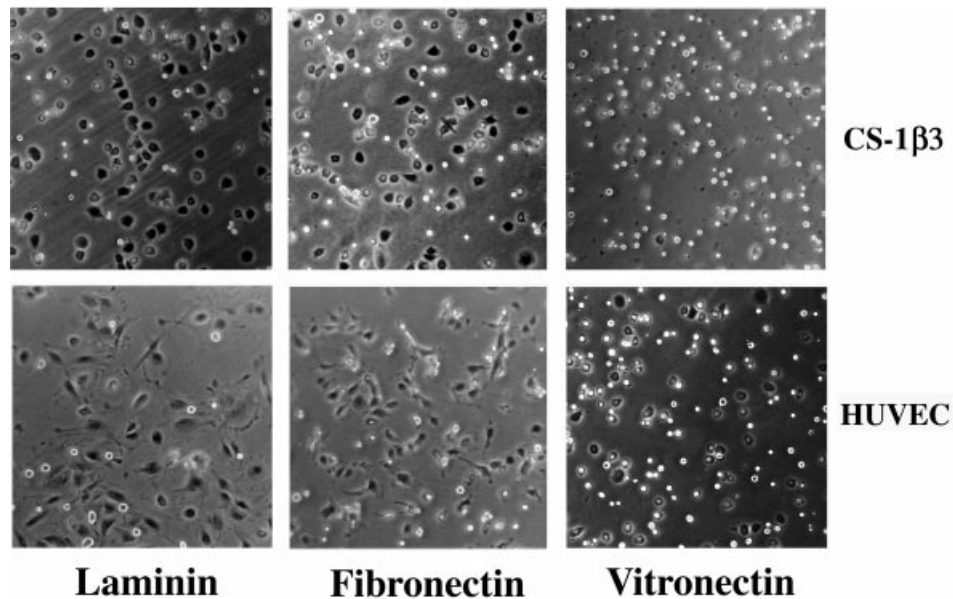
Fig. 3. The effect on cell spreading by CKI peptides is matrix specific and not tissue dependent. HUVEC endothelial and CS-1 β_3 melanoma cell lines depend on $\alpha_v\beta_3$ to attach to and spread on vitronectin. Cells were treated with the p16[INK4a] peptides 20 and seeded on vitronectin, fibronectin or laminin. Both cell lines show a dramatic inhibition of spreading in a vitronectin-dependent fashion. Cell spreading was estimated 3 h after seeding.

migrate on vitronectin via the $\alpha_v\beta_3$ integrin that is inhibited by the CKI peptides and not the capacity of this integrin to mediate cell–matrix attachment.

Peptide-induced inhibition of cell spreading is ECM dependent

Cell spreading involves the interaction between integrins and the ECM ligand and the transduction of signals from the integrins to the cytoskeleton-associated motile apparatus. Thus, inhibition of spreading can, at least in theory, be achieved by targeting a number of different extra- and intracellular molecules. In order to understand at what level in the hierarchy of cell spreading the CKI peptides exert their effect, we seeded CS-1 β_3 and primary human endothelial (HUVEC) cells, which also depend on $\alpha_v\beta_3$ for attachment to vitronectin (Cheresh, 1987), on different matrices including fibronectin, laminin and vitronectin in the presence of the p16[INK4a] peptide 20 (Figure 3). If the inhibition of spreading is mediated through the cytoskeleton and/or factors commonly involved in integrin-mediated cell spreading, it would be expected that the effects of the CKI peptides would be independent of the ECM composition. When CS-1 β_3 and HUVEC cells are seeded on fibronectin or laminin (or collagen IV; not shown) in the presence of CKI peptides, they spread normally and no difference can be observed compared with non-peptide-treated cells. However, neither cell type would spread when seeded on vitronectin (Figure 3). Thus, the effect of the CKI peptides on cell spreading is matrix-dependent and not cell-type specific. Since these two cell lines use $\alpha_v\beta_3$ to attach to and spread on vitronectin, but depend on other integrins for attachment to laminin (Cheresh, 1987) (Figure 2A), it indicates that the effect is linked to $\alpha_v\beta_3$ integrin-dependent mechanisms and does not involve factors that are generally used for integrin-mediated cell spreading or the cytoskeletal motility apparatus.

CKI peptides dissociate $\alpha_v\beta_3$ integrin from the focal adhesion contacts

We next looked at the distribution of $\alpha_v\beta_3$ on the CS-1 β_3 cells by immunocytochemistry before and after peptide treatment and noticed, surprisingly, that the distribution pattern of the integrin changed after treatment with active CKI peptide. The $\alpha_v\beta_3$ is normally clustered to focal adhesion contacts on a vitronectin substrate (Wayner et al., 1991) (Figure 4A) but becomes dissociated and evenly spread over the cell periphery after treatment with the CKI p16[INK4a] peptide 20 (Figure 4D and G) or the p21-derived peptide 10 (Figure 4E). This effect is only observed using peptides that are active in inhibiting G_1 kinase activity and at a concentration that blocks cell cycle progression (data not shown) and is not seen using control peptides (Figure 4F). Since the anti-$\alpha_v\beta_3$ mAb LM609 only recognizes the $\alpha_v\beta_3$ heterodimer and not the free α_v- or β_3-chains, these results also show that the α- and the β-chains remain as an intact heterodimer after being dissociated from the focal adhesion contacts. Since all cells, or nearly all cells, of a non-synchronized population are affected by the CKI peptides, it suggests that the inhibition of spreading of CS-1 β_3 cells on vitronectin is related to G_1 kinase activity directly, rather than being an indirect effect of cell cycle inhibition per se. We tested this idea by looking at the $\alpha_v\beta_3$ expression pattern in CS-1 β_3 cells arrested in G_1 using L-mimosine (Table I) or after treatment with the cdk2 inhibitor Olomoucine (Table I). We could, however, not detect any effect on the distribution of the $\alpha_v\beta_3$ (Figure 4B and C). Spreading assays also confirmed that cells arrested using L-mimosine show no matrix-dependent inhibition of spreading (data not shown), and the expression levels of cdk4 and cdk6 proteins 24 h after treatment with L-mimosine are not significantly changed compared with non-synchronized cells (Figure 4H). Thus, there is no direct link between
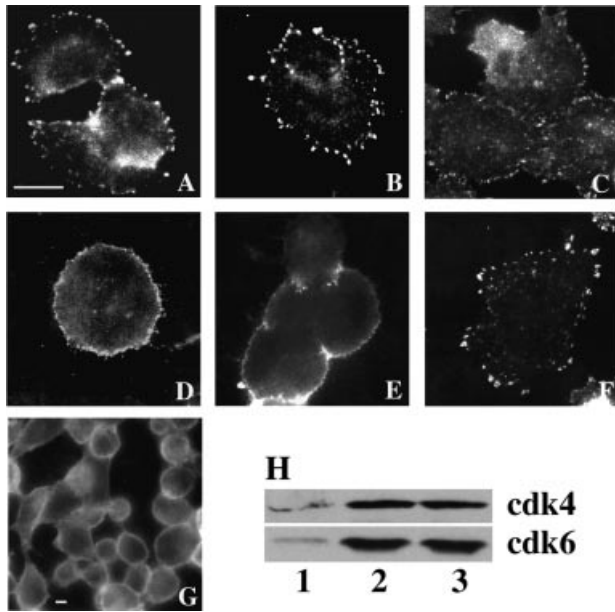
Fig. 4. The $\alpha_v\beta_3$ integrin dissociates from the focal adhesion contacts of CS-1 β3 cells after treatment with CKI peptides. Immuno-fluorescence staining of CS-I β_3 cells cultured on vitronectin-coated coverslips using the LM609 mAb shows that the $\alpha_v\beta_3$ integrin is localized to the focal adhesion contacts in non-treated cells (**A**) or cells arrested in G_1 after 24 h of L-mimosine treatment (**B**) or in the presence of the cdk2 inhibitor Olomoucine (**C**). After 20 min of treatment with p16[INK4a] peptide 20 [(**D**) and (**G**)] or peptide 10 (**E**), $\alpha_v\beta_3$ becomes dissociated from the focal adhesion contacts and is evenly distributed over the periphery of the cells. The less active p16[INK4a] peptide 21 (**F**) has little effect. Bar, 10 μm. Western blot analysis show that L-mimosine treatment does not affect the expression levels of cdk4 and cdk6 in HaCaT cells (**H**). Cells that had been deprived of FCS for 72 h (lane 1), treated with L -mimosine for 24 h in the presence of 10% FCS (lane 2) or non-synchronized cells (lane 3) were separated on a 12% SDS gel and blotted against the antibodies indicated.

inhibition of cell cycle progression per se and $\alpha_v\beta_3$ localization to the focal adhesion contacts.

One of the advantages of using the *Antennapedia*-coupled peptides, rather than using gene expressing systems, is that it allows the study of immediate effects on cell phenotype after acute inhibition of CDK activity, independent of intracellular localization and in 100% of the cell population. A kinetic study on the localization of $\alpha_v\beta_3$ revealed that the effect of the peptides is immediate and that the $\alpha_v\beta_3$ integrin is dissociated within 5 min after the peptides have been applied to the medium (data not shown). This demonstrated that the effect of the peptides is not likely to be mediated by changes at the level of gene expression, further stressing that the effect on cell spreading is unlikely to be an indirect effect of peptide-induced G_1 arrest.

We also tested the effect of the p16[INK4a] peptide 20 on human primary fibroblasts that had been seeded on fibronectin-coated coverslips, since these cells use the focal adhesion-associated $\alpha_5\beta_1$ integrin to attach to and spread on this substrate. However, the CKI peptide 20 has no effect on the localization of this integrin (Figure 5A and B) or on F-actin (Figure 5C and D), which is in line with the observation that these types of cells spread on fibronectin in the presence of CKI peptides (data not shown).
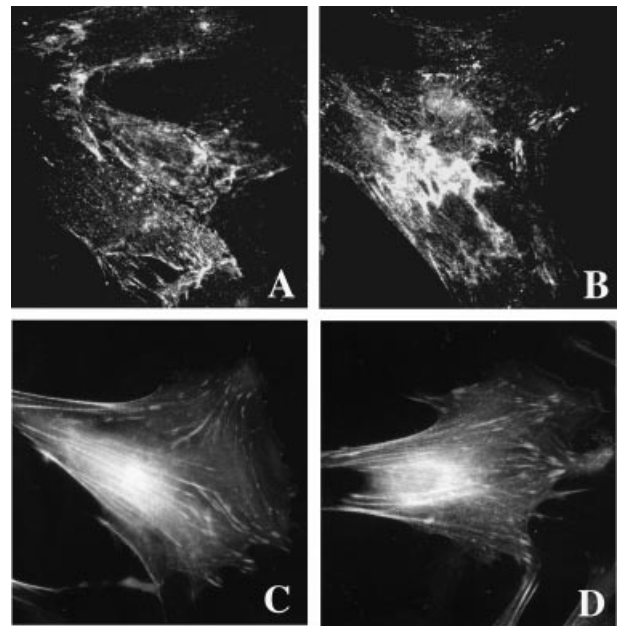

Fig. 5. The $\alpha_5\beta_1$ integrin or actin stress filaments are not affected by the CKI p16[INK4a] peptide 20. Human primary fibroblasts were seeded on coverslips coated with fibronectin and treated with the p16[INK4a] peptide 20 for 1 h [(**A**) and (**C**)], or left untreated [(**B**) and (**D**)] before fixing, and the expression and localization pattern of $\alpha_5\beta_1$ was determined using an α_5-specific mAb [(A) and (B)] or the actin filament staining pattern using phalloidin [(C) and (D)]. This is in line with the observation that the CKI peptides have no effect on the spreading of fibroblast on fibronectin (data not shown).

Treatment with CKI peptides does not lead to truncation of the $\alpha_v\beta_3$ integrin heterodimer

The cell attachment data and immunocytochemistry staining described above suggest that the extracellular domain of the $\alpha_v\beta_3$ integrin is left unaffected by the CKI peptides. However, the rapid change of integrin localization might be due to partial degradation of the intracellular cytoplasmic tail of the $\alpha_v\beta_3$ integrin, which would lead to the dissociation of the integrin and the focal adhesion complex, and loss of integrin-mediated spreading while retaining cell attachment function. This possibility is emphasized by the observation that expression of an $\alpha_{IIb}\beta_3$ integrin carrying a truncation in the β_3-cytoplasmic domain still mediates cell attachment to the ECM (Ylanne *et al.*, 1993). In order to test this, we ^{125}I-labelled CS-1 β_3 cells that had been treated with the p16[INK4a] peptide 20 or the p16[INK4a] peptide 21 for 30 min. The size of the α_v- and the β_3-subunits from peptide-treated cells were compared with those of non-peptide-treated cells after the integrins had been immunoprecipitated and separated on a sodium dodecyl sulfate (SDS) gel under non-reducing conditions. Under these conditions there is no detectable difference in the size of either the α- or the β-chain, suggesting that treatment with the CKI peptides does not result in $\alpha_v\beta_3$ truncations (Figure 6A). The attachment of CS-1 β_3 cells to vitronectin after CKI peptide treatment demonstrates that $\alpha_v\beta_3$ still mediates cell–matrix binding after being dissociated from the focal contacts. We also wanted to look at the relative amounts of $\alpha_v\beta_3$ on the surface of the CS-1 β_3 cell before and after treatment with the p16[INK4a] peptide 20. Fluorescence-activated cell sorter (FACS) analysis shows that the levels of $\alpha_v\beta_3$ on the cell surface
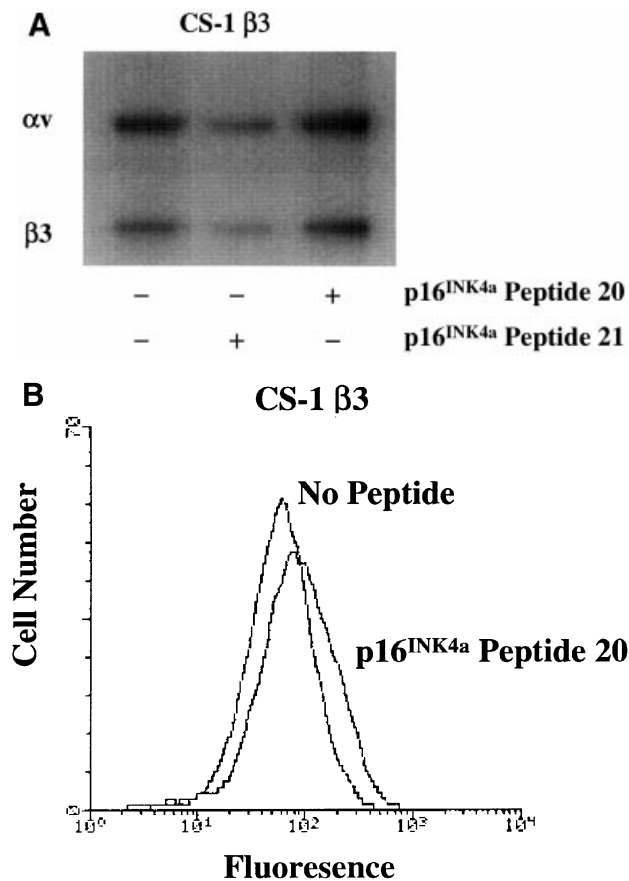
Fig. 6. The $\alpha_v\beta_3$ integrin remains intact and expressed on the surface of CS-1 β_3 cells after treatment with CKI peptides. Cells were incubated with p16^INK4a peptide 20 or p16^INK4a peptide 21 for 30 min before labelling with [125]I. The $\alpha_v\beta_3$ heterodimer was immuno-precipitated using the LM609 mAb and the α_v and the β_3 chains were separated on an 8% SDS gel under non-reducing conditions (**A**). FACS analysis of CS-I β_3 cells before and after treatment with p16^INK4a peptide 20 confirms the expression of the $\alpha_v\beta_3$ integrin on the cell surface in the presence of the CKI peptide (**B**).

phosphorylated after integrin activation. Vinculin plays an important role in linking the actin filaments to the integrins through interactions with α-actinin and talin (Sastry and Horwitz, 1993). Since FAK and vinculin are intimately linked to integrin function, we wanted to know if the dissociation of $\alpha_v\beta_3$ from the focal adhesion contacts would affect expression and/or localization of these key components of the focal adhesion complex. As shown in Figure 7, however, there is no apparent change in the localization or in the expression levels of either vinculin or FAK 20 min after treatment with the p16^INK4a peptide 20. Cell staining using phalloidin under the same condi-tions also fails to detect changes in the pattern of F-actin filaments. We have also looked at F-actin in other cell lines which give a more pronounced phalloidin staining without detecting any effect of the CKI peptides (Figure 5; data not shown). Since $\alpha_v\beta_3$ is dissociated from the focal adhesion contacts within 20 min of peptide treatment, these results show that the focal adhesion complexes remain intact after $\alpha_v\beta_3$ has become dissociated. Immuno-precipitation of FAK using a polyclonal antibody and the subsequent Western blotting using an anti-FAK mAb and a phosphotyrosine-specific mAb confirm that the relative amount and tyrosine phosphorylation of FAK does not change after CKI peptide treatment (Figure 7G). These results are in line with the results on the matrix-specific inhibition of cell spreading induced by the CKI peptides, indicating that factors commonly used for integrin-medi-ated cell spreading, such as focal adhesion complex molecules and actin filaments, are not affected by the CKI peptides and, instead, the effect is likely to be mediated via $\alpha_v\beta_3$-associated factors.

The effect of the CKI peptides on cell spreading is reversed by treatment with phorbol 12-myristate 13-acetate (PMA)

Activation of protein kinase C (PKC) is one of the first events to take place after integrin–ligand interaction, and integrin clustering and inhibitors of PKC block cell spreading independently of ECM composition (Chun and Jacobson, 1992; Vuori and Rouslahti, 1993). Interestingly, attachment by $\alpha_v\beta_5$ to vitronectin, in contrast to $\alpha_v\beta_3$, is not sufficient to promote CS-1 cell spreading unless the cells are treated with PKC-stimulating agents. This observation has led to the suggestion that $\alpha_v\beta_3$ has an 'intrinsic' capacity to stimulate PKC (Lewis *et al.*, 1996). Since the CKI peptide-treated CS-1 β_3 cells behave similarly to untreated CS-1 β_5 cells, in that they attach to vitronectin but do not spread, we stimulated CS-1 β_3 cells seeded on vitronectin in the presence of peptide 20 (Figure 8A) with 10 nM PMA (Figure 8B). This reversed the effect of the CKI peptide and allowed the cells to spread on vitronectin. The stimulatory effect of PMA was shown to be blocked by the PKC inhibitor, Calphostin (Figure 8C). We then took CS-1 β_3 cells that were seeded on vitronectin and had been treated with peptide 20 for 20 min in order to dissociate the $\alpha_v\beta_3$ integrins from the focal adhesion contacts (Figure 8D), and incubated them with 10 nM PMA (Figure 8E). In line with the induction of cell spreading, the treatment with PMA allowed the $\alpha_v\beta_3$ integrin to relocate to the focal adhesion contacts within 30 min. Since treatment of cells with PKC inhibitors such as Calphostin blocks integrin-mediated spreading in
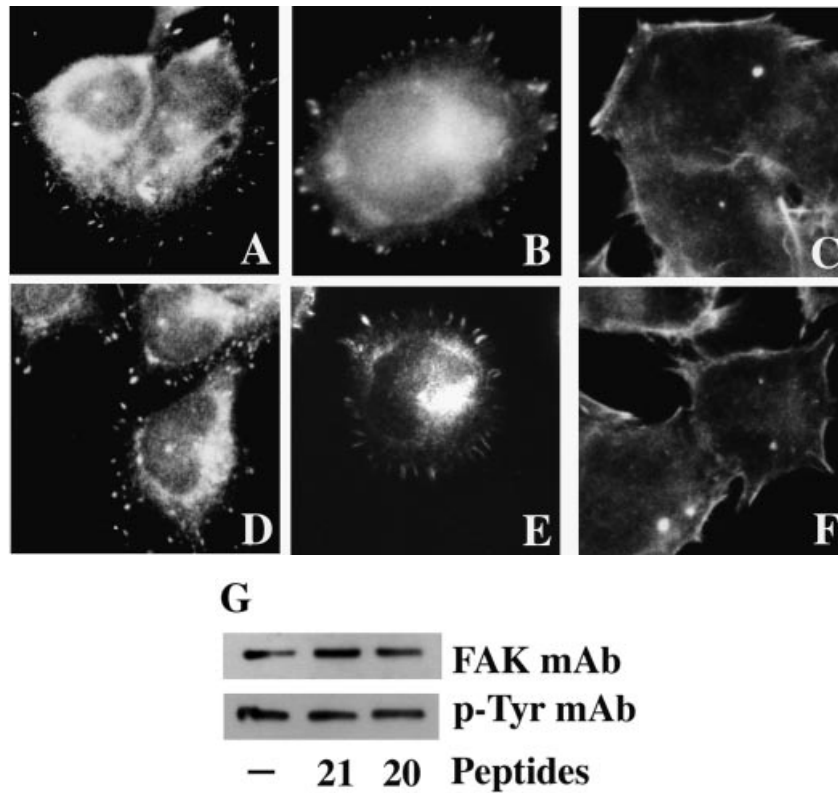
after CKI peptide treatment is not significantly changed (Figure 6B).

These results show that CKI peptide treatment does not lead to truncation of the α- or the β-integrin subunits, and it suggests that the rapid dissociation of $\alpha_v\beta_3$ from the focal contacts is caused by modifications of factors that regulate the interactions between the cytoplasmic tail of the α_v- and/or β_3-subunit and the focal adhesion complex. Since the integrins are not known to have intrinsic enzym-atic activity, and instead depend on proteins in the focal adhesion complex to generate signals that can be transmit-ted within the cell, it is likely that the dissociation of $\alpha_v\beta_3$ from the focal contacts is the direct cause of the peptide-induced inhibition of cell spreading on vitronectin.

The focal adhesion complex is not affected by CKI peptides

The focal adhesion contacts mediate a link between the actin filaments and the ECM through the interaction between the integrins and focal adhesion-associated molec-ules such as the focal adhesion kinase (FAK) and vinculin. FAK is essential for cell spreading (Ilic *et al.*, 1995), and is one of the major proteins to become tyrosine

Fig. 7. The staining pattern of focal adhesion complex molecules vinculin and FAK, as well as F-actin, are not affected by CKI peptide treatment. CS-1 β_3 cells stained against FAK [(**A**) and (**D**)], vinculin [(**B**) and (**E**)] and actin filaments [(**C**) and (**F**)] after treatment with p16^{INK4a} peptide 20 for 1 h (A–C) or untreated (D–F). FAK was immunoprecipitated from CS-1 β_3 cells treated with p16^{INK4a} peptide 20 or peptide 21 for 30 min and blotted against phosphotyrosine mAb to confirm that the relative levels and tyrosine phosphorylation status of FAK is not affected by CKI peptide treatment (**G**).

a non-matrix-specific fashion, and since the effect of the CKI peptides is restricted to $\alpha_v\beta_3$-mediated cell spreading, these results suggest that the CKI peptides act upstream of $\alpha_v\beta_3$-dependent activation of PKC. It also shows that the ECM is not being compromised by the peptides. PKC activation is necessary for $\alpha_v\beta_3$ integrin localization to the focal adhesion contacts. However, in order to ensure that the effect of the peptides can be distinguished from direct PKC inhibition, we compared the effect of Calphostin with peptide 20 on cell attachment and spreading. Treatment of cells with Calphostin blocks cell spreading with an IC$_{50}$ of 0.4 μM (Vuori and Ruoslahti, 1993; Auer and Jacobson, 1995). In order to completely block CS-1 β_3 cells from spreading, cells need to be treated with 1 μM Calphostin which, however, also reduces attachment (Figure 8G). In contrast to Calphostin, the CKI peptides induce a complete block in cell spreading at concentrations that still allow the cells to attach to the matrix (Figure 8F and A). We also tested the effect of PMA on the capacity of p16 peptide 20 to block cell cycle progression through G$_1$. This result is presented in Table I, and shows that cells arrest in G$_1$ in the presence of peptide 20 and 10 nM PMA, demonstrating that activation of PKC only reverses the effect of the G$_1$-associated CKI peptides on $\alpha_v\beta_3$-dependent cell spreading, and does not affect the inhibition of cdk4/6 activity towards pRb phosphorylation. This makes it unlikely that PMA affects the capacity of the peptides to block CDK activity.

$\alpha_{IIb}\beta_3$ integrin-dependent spreading is not affected by CKI treatment

We also wanted to test if the β_3 integrin chain is sufficient to mediate the effect of CKI on inhibition of cell spreading. CHO cells do not spread easily on fibrinogen but acquire this capacity after transfection with the $\alpha_{IIb}\beta_3$ integrin (Figure 9). The $\alpha_{IIb}\beta_3$-transfected cells (O'Toole *et al.*, 1994) show no inhibition of spreading on fibrinogen after treatment with the p16 peptide 20, indicating that the β_3-chain is not sufficient by itself to mediate the effect of the CKI peptides on cell spreading. This result further emphasizes the specificity of the CKI inhibitors in terms of regulating integrin-dependent cell spreading. It also indicates that the effect of G$_1$-associated CKI on $\alpha_v\beta_3$-dependent cell spreading is dependent on the α_v chain or the combination of the α_v and the β_3 chain.

Transient expression of full-length p16^{INK4a} results in loss of spreading on- and attachment to vitronectin, and is reversed by co-expression of cdk6

The differences in the p16^{INK4a}, p18^{INK4c} and p21$^{Wafi/Cip1}$ CKI peptide sequences and the previously observed correlation between inhibition of cdk4–cyclin D1 kinase activity *in vitro*, G$_1$ cell cycle arrest and the effect on $\alpha_v\beta_3$ function, suggest that the effect of CKI peptides on $\alpha_v\beta_3$ integrin is related to G$_1$ kinase activity. However, it is important to know if the effect on matrix-
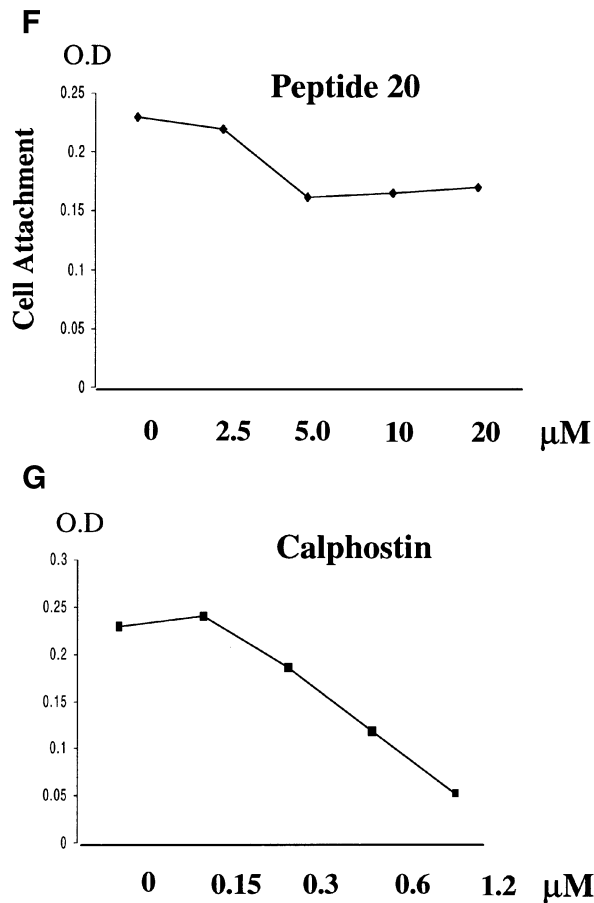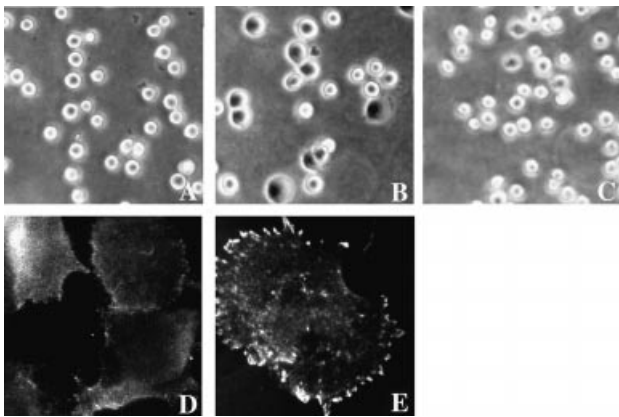
Fig. 9. The β_3 chain in combination with α_{IIb} is not sufficient to mediate the cell spreading inhibitory effect of the CKI peptides. CHO cells transfected with the $\alpha_{IIb}\beta_3$ integrin (**A**) spread easily on fibrinogen in contrast to the parental line (**B**). Treatment with CKI peptide (**C**) does not affect the spreading of the $\alpha_{IIb}\beta_3$-transfected CHO cells. This indicates that the α_v chain is required for CDK-related inhibition of $\alpha_v\beta_3$-mediated cell spreading.

Fig. 8. The inhibitory effect on cell spreading by the CKI peptides is reversed after treatment with the PKC activator PMA. CS-1 β_3 cells were treated with the p16[INK4a] peptide 20 for 20 min before seeded on vitronectin in the presence (**A**) or absence (**B**) of 10 nM PMA. Spreading was estimated after 3 h. The cell spreading stimulatory effect of PMA is blocked by treating cells with 0.4 µM of the PKC inhibitor Calphostin (**C**). Alternatively, CS-1 β_3 cells were allowed to spread on vitronectin for 24 h before being treated with peptide 20 for 20 min [(**D**) and (**E**)] and exposed to 10 nM PMA for 30 min (E), and $\alpha_v\beta_3$ localization was detected by indirect immunofluorescence. Treatment with the PKC inhibitor Calphostin at concentrations that block cell spreading (IC$_{50}$ at 0.4 µM and complete block at 1 µM; Vuori, 1993; data not shown) also affect integrin affinity to the ECM (**G**), whereas treatment with CKI peptides at concentrations that completely block cell spreading allows cells to attach to the ECM (**F**). These results demonstrate that the CKI peptides affect cell spreading by a mechanism that involves PKC but is different from that of Calphostin.
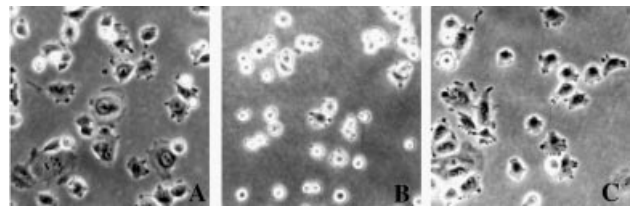
dependent cell spreading by CKI peptides can also be obtained by expression of a full-length G$_1$ kinase-specific inhibitory protein. This would strongly indicate that the $\alpha_v\beta_3$ regulatory pathway identified by using the CKI peptides represents a physiologically relevant target for the G$_1$-associated kinases cdk4 and/or cdk6. It is also important to know if full-length p16[INK4a] has this effect, since loss of p16[INK4a] tumour suppressor function is correlated with late and invasive stages of melanoma tumour progression (Reed *et al.*, 1995), which has indicated the possibility that loss of p16[INK4a] might be involved in regulating tumour cell spreading. The VUP15 cell line is derived from a human melanoma and has been transfected with the β_3 integrin and expresses $\alpha_v\beta_3$ (Dr John Marshall, personal communication). In order to test the infection efficiency of these cells using an adenovirus Ad5-based method (Baker *et al.*, 1997), we used a *gfp*-expression construct (pSV40TRACER, Invitrogen). Approximately 70–80% of the VUP15 cells expressed the *gfp* protein 24 h after infection (data not shown). The VUP15 cells were then infected with a full-length p16[INK4a] construct, or the control vector only, and after 36 h the cells were split and seeded on either vitronectin- or collagen IV-coated dishes for 12 h before cell spreading and attachment was estimated. Figure 10A shows that cells infected with the p16[INK4a] expression construct showed less spreading on vitronectin after 12 h compared with those seeded on collagen. This effect was reversed if the cells were co-infected with a cdk6 expression construct, indicating that cdk6 is a potential target for G$_1$ kinase-associated inhibition of spreading on vitronectin. However, the number of cells attached to vitronectin after infection with the p16[INK4a] construct alone was reduced at 12 h, which is not observed after acute inhibition of CDK activity using the CKI peptides (Figure 10B). This suggests that longer exposures to CDK inhibition or, alternatively, a more efficient CDK inhibition by the p16[INK4a] protein, eventually result in loss of $\alpha_v\beta_3$-dependent cell attachment. Infection with the *gfp* expression construct result in markedly different amounts of *gfp* protein expressed in individual cells, and if the situation using the p16[INK4a] expression construct were similar, one might expect that the effects of p16[INK4a]-induced CDK inhibition on cell behaviour will be more heterogeneous using gene expression systems compared with the *Antennapedia* fusion peptide-based system. This, taken together with the infection efficiency factor, might help to explain the variation in the behaviour of the p16[INK4a]-transfected cell population
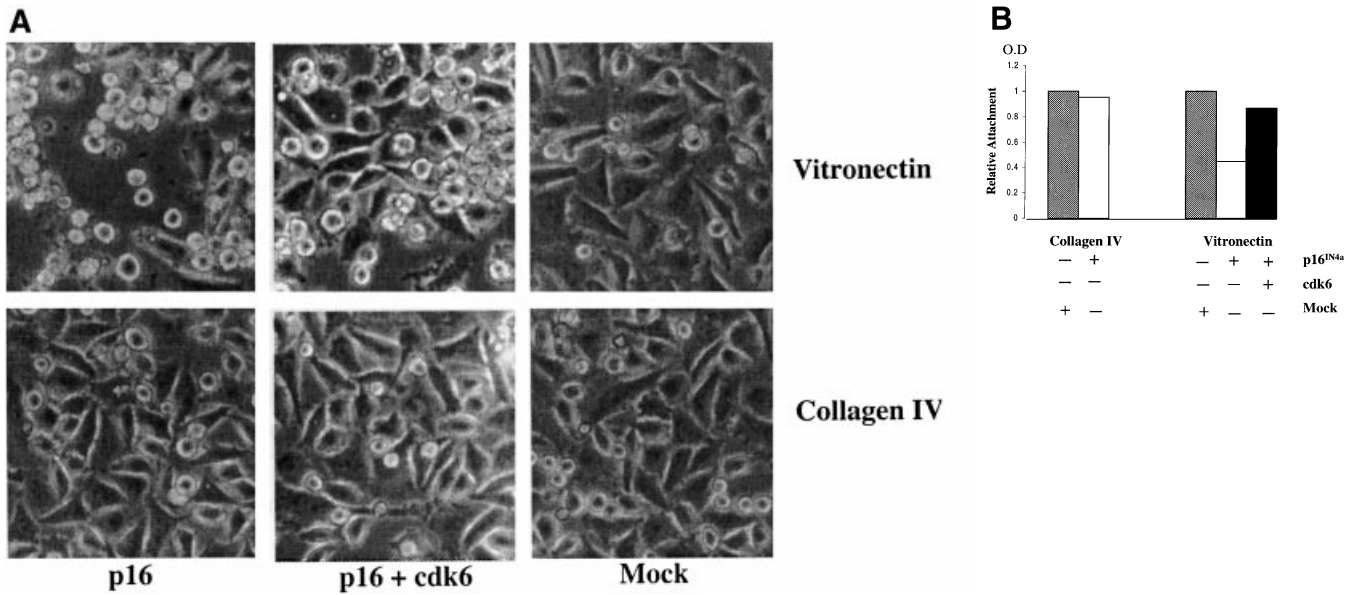
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Fig. 10. Co-expression of cdk6 blocks p16^{INK4a}-mediated inhibition of matrix-specific cell spreading. The VUP15 human melanoma derived cell line expresses the $\alpha_v\beta_3$ integrin and was transiently infected with a full length p16^{INK4a} gene construct, or the combination of the p16^{INK4a} and a cdk6 expression construct, or a DNA vector control (mock) using an adenovirus-based method. The cells were incubated for 36 h before they were split and seeded on vitronectin or collagen type IV-coated 24-well plates. The spreading (**A**) and cell attachment (**B**) was estimated after 12 h. Cells infected with the full-length p16^{INK4a} gene expression vector show reduced capacity to spread on and attach to vitronectin which is reversed by the co-expression of cdk6. The results presented represent one of four similar experiments.

on vitronectin, spanning from cells that do not attach to the dish, to cells that attach and spread easily (Figure 10; data not shown).

Cdk6 localizes to the spreading edge of cells

The major fraction of cdk6 has been reported to be in the cytoplasm (Mahoney *et al.*, 1998), and since co-infection with cdk6-expressing constructs reverses the cell spreading-inhibitory effect of p16^{INK4a} full-length protein we wanted to look at the subcellular localization of cdk6. We raised monoclonal antibodies against recombinant full-length human cdk6 protein. Two monoclonal antibodies that specifically recognize cdk6 on a Western blot (data not shown) were tested in a peptide-based epitope assay and found to recognize two different epitopes in the C-terminus of cdk6 (aa 223–243 and 285–305, respectively) (data not shown). These mAbs were then used for staining MRC5 human fibroblasts that were allowed to spread for 3 h on coverslips. Both of these cdk6 mAbs stained the ruffling edge of the cells and gave a faint nuclear staining (Figure 11C and D), whereas two different mAbs directed against cdk4 stained predominantly the nucleus but not the ruffling edge of the cells (Figure 11A and B). When the spreading fibroblasts are co-stained for F-actin it is shown that the regions which stain most strongly for cdk6 at the ruffling edge stain weakly, or not at all, against F-actin (Figure 11E and F). This indicates that cdk6 is localized to the spreading edge of the cell prior to the formation of filamentous actin.

Discussion

By using a series of cdk4- and cdk6-inhibitory synthetic peptides derived from the p16^{INK4a}, p18^{INK4c} and the p21$^{Waf1/Cip1}$ sequences and a full-length p16^{INK4a} gene expression construct, we have demonstrated a link
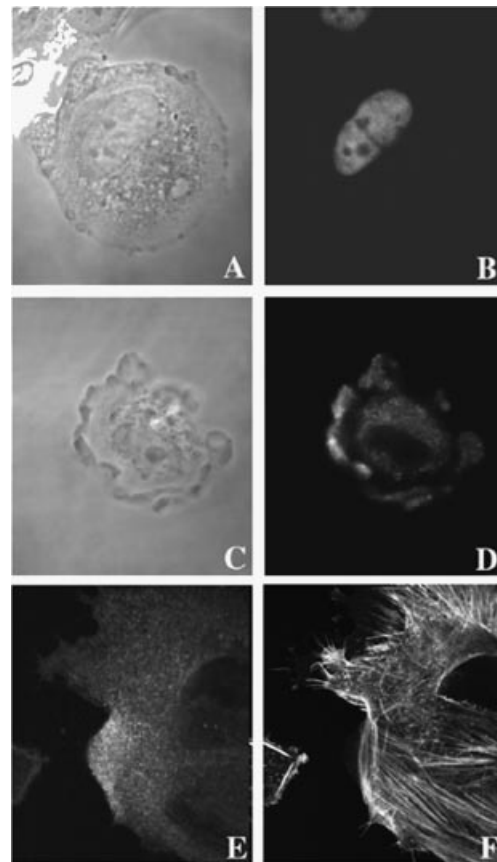

Fig. 11. Cdk6 is located to the ruffling edge of spreading fibroblasts. mAbs directed against cdk4 stain predominantly the nucleus of MRC5 human fibroblast cells [(**A**) and (**B**)]. Cdk6 mAbs instead stain the ruffling edge of spreading MRC5 cells [(**C**), (**D**) and (**E**)] which does not co-localize with F-actin [(E) and (**F**)], indicating that cdk6 is localized to the spreading edge of the cell prior to the formation of filamentous actin.

between inhibition of G_1 CDK activity and an inhibition of $\alpha_v\beta_3$-dependent cell spreading on vitronectin. Mutations of the p16^{INK4a} peptide that result in reduced CKI capacity *in vitro* and a reduced capacity to arrest cells in G_1 abolish the effect on $\alpha_v\beta_3$ function. Similarly, mutations in the RRLF motif in the p21$^{Waf1/Cip1}$ peptide, which is essential for the CKI effect of the full-length p21$^{Waf1/Cip1}$ protein, also result in inactivation of the CKI function and the effect on $\alpha_v\beta_3$ function. Taken together with the results showing that expression of the full-length p16^{INK4a} has a similar inhibitory effect on $\alpha_v\beta_3$-dependent cell spreading on vitronectin, it is suggested that cdk6 and/or cdk4 is involved in regulating $\alpha_v\beta_3$ integrin function. Since G_1 arrest caused by treatment with L-mimosine, or blocked replication caused by the cdk2 inhibitor Olomoucin have no effect on the $\alpha_v\beta_3$, it is likely that the effects of G_1 CDK inhibitors are not a consequence of cell cycle arrest, and specifically involve G_1-associated kinases. The spreading of most, if not all, cells on vitronectin in a non-synchronized population of HUVEC or CS-1 β_3 is affected immediately by the CKI peptides, which implies that the target(s) for the cdk6- or cdk4-dependent regulation of cell spreading on vitronectin is not the same as that for CDK–cyclin D-controlled cell cycle progression. There is, however, no previously known function for the G_1-associated kinases that can explain their influence on the $\alpha_v\beta_3$ integrin, even though both cdk4 and cdk6 are detected in the cytoplasm (Wang *et al*., 1996; Mahoney *et al*., 1998) and specific cytoplasmic substrates for cdk4 and cdk6 have been reported (Kwon *et al*., 1995). However, our results identify cdk6 at the ruffling edge of spreading cells in areas that are not rich in, or lack, F-actin, indicating an early recruitment of cdk6 to the spreading edge of the cell. This subcellular localization is not seen using cdk4 mAbs, and together with previous observations that the majority of cdk6 is located to the cytoplasmic fraction, speaks in favour of the idea that cdk6 plays a specific role at the spreading edge of the cells. Together with the results demonstrating that co-expression of cdk6 reverses the cell spreading-inhibitory effect of p16^{INK4a}, and that the levels of cdk6 do not vary during the cell cycle, it is likely that cdk6 is the target for inhibition of cell spreading by G_1-associated kinase inhibitors.

It is known that other cell cycle-associated kinases, such as cdc2, target both cytoplasmic and nuclear substrates in order to synchronize different subcellular compartments during the mitotic phase of the cell cycle (Lowe *et al*., 1998). Our results indicate that G_1 kinases are involved in regulating both nuclear and cell membrane-associated cell activities during cell proliferation and migration.

It is interesting that the $\alpha_v\beta_3$ integrin, which is associated with cell spreading and proliferation in wound healing, neovascularization and tumour spreading, is regulated by G_1 kinase inhibitory molecules and, for example, $\alpha_5\beta_1$ is not. This implies that $\alpha_v\beta_3$-dependent cell migration is co-ordinated with cell proliferation, which is interesting since $\alpha_v\beta_3$ plays an important role in regulating both intra- and extracellular activities that are involved in promoting cell migration and cell growth. This opens up the possibility that there is cell cycle-dependent ($\alpha_v\beta_3$) and a non-cell cycle-dependent (e.g. $\alpha_5\beta_1$) integrin-mediated cell migration *in vivo*. It also indicates that deregulated cell cycle control through loss of INK4-mediated

cdk6/cdk4 inhibition promotes both cell proliferation and $\alpha_v\beta_3$-dependent cell migration. p16^{INK4a} has been shown to play a role in cell senescence (Serrano *et al*., 1997; McConell *et al*., 1998), and it is a possibility that the regulation of $\alpha_v\beta_3$-dependent signalling and the different roles $\alpha_v\beta_3$ plays in controlling growth factor responses and senescence are two different, but perhaps related, phenomena that are controlled by G_1-associated kinases. Both might be important targets for the p16^{INK4a} tumour suppressor function.

The expression of $\alpha_v\beta_3$ is associated with later and invasive stages of melanoma development (Danen *et al*., 1994; Marshall and Hart, 1996; Van Leeuwen *et al*., 1996) and the aggressive type III and IV glioblastomas (Gladson and Cheresh, 1991), and since both these types of tumours display a reverse correlation with regard to the expression of a functional p16^{INK4a} tumour suppressor gene product (Reed *et al*., 1995; Ishimura *et al*., 1996) and $\alpha_v\beta_3$, it is possible that the loss of p16^{INK4a} function allows $\alpha_v\beta_3$-mediated spreading of certain types of tumour cells. This hypothesis is in line with recent results demonstrating that p16^{INK4a} inhibits glioblastoma cell spreading *in vitro* (Chintala *et al*., 1997). p16^{INK4a} and p19^{ARF} are both expressed from different transcripts derived from the same coding sequence in the MTS1 locus (Mao *et al*., 1995) and are involved in regulating important, but different, tumour suppressor pathways (Pomerantz *et al*., 1998; Zhang *et al*., 1998). It has recently been suggested that mutations that affect p19^{ARF} are seen in T-cell leukaemias (Gardie *et al*., 1998), whereas it is known that point mutations that specifically inactivate p16^{INK4a} are observed in cases of familial melanomas (Ranade *et al*., 1995). By identifying novel functions for the p16^{INK4a} protein it might help to explain tumour-specific selections for point mutations in the MTS1 locus, affecting either p19^{ARF} or p16^{INK4a} functions.

The dissociation of $\alpha_v\beta_3$ from the focal adhesion contacts does not seem to affect other molecules of the focal adhesion complexes, since FAK and vinculin as well as F-actin remain located to the focal adhesion contacts after CKI peptide treatment. These results show that the CKI peptides do not target the focal adhesion complex per se, and it may be that integrin activation and localization to the focal contacts are essential for forming the focal adhesion complexes, but are not essential to maintain the integrity of the focal adhesion complex. However, it cannot be ruled out that once $\alpha_v\beta_3$-dependent cell attachment and spreading has occurred in the CS-1 $\beta3$ cells, other, non-$\alpha_v\beta_3$-containing focal adhesion contacts are being formed which are not affected by CKI peptides.

The cytoplasmic C-terminal domains of the integrin heterodimers are sufficient to form interaction with the focal adhesion complex (Sastry and Horwitz, 1993; Ylanne *et al*., 1993) and motifs such as the NPXY sequence of the β-chain have been shown to be essential for integrin-mediated cell spreading and invasion (Filardo *et al*., 1995). Both the β_3 and the β_5 chains mediate attachment to vitronectin in combination with an α_v-subunit and, interestingly, $\alpha_v\beta_5$-expressing CS-1 cells need to be stimulated with PKC activators in order to mediate spreading, whereas $\alpha_v\beta_3$-expressing cells do not. Treatment of CS-1 β_3 cells with CKI peptides induces a phenotype on vitronectin that resembles that observed with $\alpha_v\beta_5$, in that the cells attach
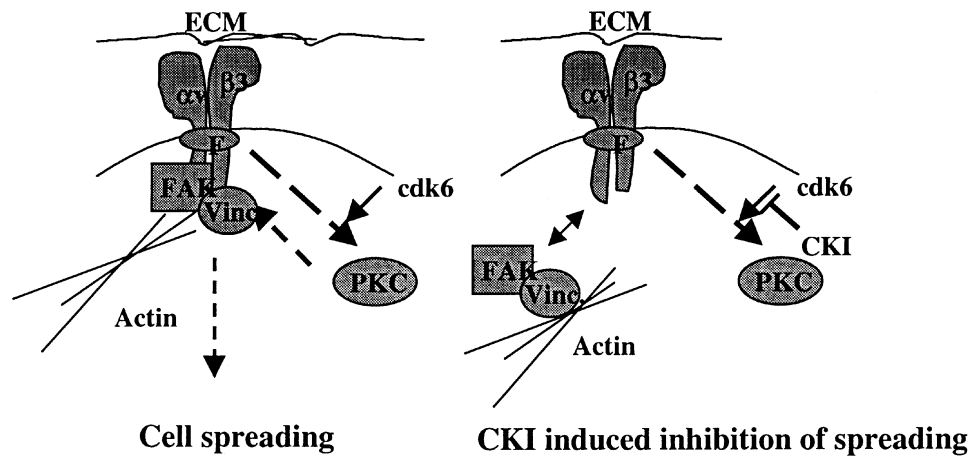
Fig. 12. A model for inhibition of $\alpha_v\beta_3$-mediated cell spreading caused by G_1-associated CKIs. The effect of CKI on $\alpha_v\beta_3$-mediated cell spreading is reversed by PMA or over-expression of cdk6, indicating that cdk6 acts upstream of PKC activation. Inhibition of PKC by Calphostin blocks cell spreading independently of ECM, suggesting that the target for CKI is not a general factor involved in cell spreading but instead involves a PKC-activating factor (F) which is associated with $\alpha_v\beta_3$. Treatment with CKI peptides dissociates $\alpha_v\beta_3$ from the focal adhesion contacts without effecting actin, vinculin or FAK localization to the focal adhesion contacts. Activation of PKC in the presence of CKI peptides re-localizes $\alpha_v\beta_3$ to the focal contacts which indicates that PKC normally plays a role in promoting $\alpha_v\beta_3$ integrin interaction with members of the focal adhesion complex.

but do not spread. It is therefore interesting that stimulation with PMA reverses the cell spreading-inhibitory effect of the CKI peptides, and allows the $\alpha_v\beta_3$ integrins to relocate to the focal adhesion complexes. This highlights some important points. First, it shows that the rapid dissociation of the intact $\alpha_v\beta_3$ heterodimer is reversible, which supports the idea that the effect of the CKI peptides on $\alpha_v\beta_3$ localization is caused by secondary modifications of factors involved in activation of PKC. Secondly, treatment with PMA reverses the effect of the CKI peptides on cell spreading but not on cell cycle inhibition, showing that the effect of the CKI peptides is upstream of PKC activation and does not affect the capacity of the peptides to inhibit CDK activity. Inhibition of cell spreading by PKC inhibitors is not matrix specific, and the effect of the CKI peptides is restricted to $\alpha_v\beta_3$ (and does not, for example, affect $\alpha_5\beta_1$ localization to the focal contacts). This indicates that integrin-mediated activation of PKC requires specific factors that are not commonly used by all integrins. The results obtained with the $\alpha_{IIb}\beta_3$-transfected CHO cells demonstrate that the β_3 chain by itself is not sufficient to mediate CKI inhibition of cell spreading. However, since previous studies have shown that the α_v chain is not sufficient to promote $\alpha_v\beta_5$ integrin-dependent spreading without further activation of PKC, it is possible that the cell spreading factor(s) targeted by CKI is dependent on the presence of a combination of the α_v and the β_3 chain. The importance of PKC in cell spreading has been well documented (Woods and Couchman, 1992; Vuori and Rouslahti, 1993; Auer and Jacobson, 1995). Our results suggest a model in which the activation of PKC by $\alpha_v\beta_3$ integrin is mediated by factors linked to $\alpha_v\beta_3$, and that activation of PKC is necessary for $\alpha_v\beta_3$ localization to the focal adhesion contacts (Figure 12). This gives new insights into the role of PKC during cell spreading and adds to the already complex role PKC plays in integrin-mediated cell spreading. Finally, these results demonstrate that, in addition to the indirect regulation over integrin-mediated signalling that is imposed by the inside-out mediated

control of integrin–ligand affinity (Hynes, 1992; Kolanus and Seed, 1997), there are also intracellular pathways which directly control cell spreading by targeting specific factors that are involved in integrin-mediated activation of PKC. In the case of $\alpha_v\beta_3$ integrin-mediated spreading, this control is apparently linked to the cell cycle and further studies will show to what extent integrin-dependent cell migration *in vivo* is directly regulated by cell cycle-associated factors.

The $\alpha_v\beta_3$ integrin is an interesting target for anti-cancer therapies, since it is associated with tumour-related activities such as neovascularization and tumour cell spreading. These results demonstrate that small molecules can block cell spreading on vitronectin and the localization of $\alpha_v\beta_3$ through intracellular pathways, which opens up new possibilities in the search for novel ways to control $\alpha_v\beta_3$-dependent pathologically associated cell migration.

Materials and methods

Antibodies and reagents
Synthetic carrier-linked CKI fusion peptides were obtained from Dr Graham Blomberg at the University of Bristol, Cambridge Bioscience and Chiron Mimotopes at >95% purity. The mAb LM 609 that interacts with the $\alpha_v\beta_3$ integrin was obtained from Chemicon; anti-FAK and anti-vinculin polyclonal sera were from Sigma; anti-α_5 mAb from Gibco Life Sciences; anti BrdU mAb from Becton & Dickinson; and anti-p16[INK4a] was from Progen. The anti-cdk4 polyclonal antibodies were from Santa Cruz and the cdk4 mAbs were from Signal Transduction. The anti-cdk6 rabbit polyclonal sera for Western blotting and the cdk6 mAbs were raised against full-length cdk6 protein by Moravian Biotechnology Ltd. Cell matrix proteins, L-mimosine and PMA were obtained from Sigma and Olomoucine is from Calbiochem.

Cell lines and culture
HaCaT cells are human-derived keratinocytes. The HUVEC cells were obtained from Clonetics and primary human foreskin fibroblasts were from Professor Seth Schoor. Dr Caroline Damsky kindly provided the hamster melanoma cell line CS-1 and the CS-1 β_3 line that carries the human β_3 integrin gene and expresses a functional $\alpha_v\beta_3$ heterodimer. VUP15 (kind gift from Dr John Marshall) is a human melanoma-derived cell line transfected with the β_3 integrin and expresses the $\alpha_v\beta_3$ integrin. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). CS-1 β_3 cells were continuously

cultured in the presence of 1 mg/ml of G418 and VUP 15 in the presence of 0.25 µg/ml of puromycin and the HUVEC cells were grown in medium supplied by Clonetics.

Cell infection

VUP 15 cells were seeded on plastic 24-well dishes for 24 h before DNA was transfected using an adenovirus-based method described previously (Baker *et al.*, 1997). In short, 0.6 µg of a pCDNA3 vector carrying p16INK4a were diluted to 25 µl with HEPES pH 7.4 and mixed with 25 µl 0.4 mM polyethyleimine (PEI) in HEPES for 20 min at room temperature before 0.5×10^9 psoralen-inactivated Ad5 adenovirus particles were added. After an additional 20 min incubation, 0.3 ml serum-free DMEM was added and the cells were incubated with the Ad5–DNA mixture for 4 h before washing, and fresh DMEM containing 10% FCS was added.

Cell spreading and adhesion

For cell spreading assays, 6-well plates were coated with matrix protein over night at 4°C or 2 h at 37°C at concentrations and conditions recommended by the manufacturer (Sigma), washed with phosphate-buffered saline (PBS) and blocked in 2% bovine serum albumin (BSA) for 1 h at 37°C and washed three times with PBS. Cells were prepared by detaching them from dishes using trypsin–EDTA and washed in medium containing 10% FCS. The peptides or antibodies were added to the cells before seeding. Spreading on the different matrixes was observed using phase-contrast microscopy at the time indicated after seeding. Cell attachment was estimated by coating 24-well plates with the ECM proteins indicated in the same way as for spreading. Cells were treated with peptides and/or antibodies before seeding, and after an indicated time period the medium was carefully removed and the cells were washed once with PBS containing 1 mM $MgCl_2$. Bengal Rose (0.25%) in $PBS/MgCl_2$ was then added for 5 min and the wells were carefully washed four times with $PBS/MgCl_2$ before 50% ethanol was added to dissolve the cell-associated Bengal Rose. The relative cell attachment was indirectly determined by spectroscopy at OD 570 nm.

FACS analysis

Cell cycle analyses were carried out as described previously (Fåhraeus *et al.*, 1996; Ball *et al.*, 1997; Fåhraeus, 1998). Cells were incubated with 10 µM of BrdU for 20 min before harvesting and washed once in PBS. Cells were resuspended in 1 ml PBS and fixed in 3 ml ethanol at 4°C overnight. Cells were then treated with 1 mg/ml pepsin in 30 mM HCl for 30 min, and DNA was partially denatured using 2 M HCl for 15 min. After extensive washings, anti-BrdU mAb was added in PBS containing 0.25 mg/ml BSA and 0.2% Tween-20 for 1 h at room temperature. Cells were washed in PBS and secondary FITC-conjugated antibody was added for 30 min before the final wash and the addition of propidium iodine. Cell cycle distribution was determined using flow cytometry and peptide-induced cell cycle inhibition was estimated by comparing the percentage of BrdU-positive cells from peptide-treated and non-treated cells. The relative amounts of $\alpha_v\beta_3$ integrin on CS-1 β_3 cells after treatment with CKI peptides was determined after treating cells with peptides for 1 h before detaching them from the dish using 5 mM PBS/EDTA and fixation in 3.7% formaldehyde. Cells were incubated with the LM609 mAb for 1 h, washed and incubated with secondary FITC-conjugated anti-mouse mAb (Jackson Laboratories), and analysed by FACS.

Kinase assays

These were carried out essentially as described previously (Fåhraeus *et al.*, 1996; Ball *et al.*, 1997; Fåhraeus, 1998). Briefly, Sf9 cell extracts were co-infected with baculoviruses expressing cdk4 or cyclin D1. CKI peptide were added to 5 µM and incubated for 1 h on wet ice before GST–Rb and γ-ATP was added. After 10 min incubation at 30°C the reaction was terminated by adding SDS loading buffer. The mixture was then separated on an SDS gel and the phosphorylated GST–Rb was visualized by autoradiography.

Immunofluorescence cell staining

Cells were grown on coverslips coated with appropriate matrixes. The LM609 and the α_5 mAbs were used for immunocytochemical staining of $\alpha_v\beta_3$ and $\alpha_5\beta_1$ in CS-1 β_3 and human fibroblast cells, respectively. Cells were fixed in 3.7% formaldehyde for 10 min at room temperature and washed in PBS containing 1 mM $MgCl_2$ before adding the primary antibodies. In the case of vinculin, FAK and fluorescently labelled phalloidin staining, cells were treated with an additional 0.1% Tween for 10 min before primary antibodies or phalloidin was added. Secondary

FITC-labelled mAb antibodies (Jackson Laboratories) were added for 1 h at room temperature before the coverslips were washed three times in PBS containing 0.1% Tween and mounted using Mowiol.

125Iodine labelling and immunoprecipitations

CS-1 β_3 cells treated with either the active or the inactive p16INK4a peptides for 1 h were washed three times in PBS containing 1 mM $MgCl_2$. Integrins were labelled with ^{125}I in the presence of 10 mM glucose, 0.1 U glucose oxidase and 50 µg lactoperoxidase on ice. Cells were lysed on ice in 50 mM Tris pH 8.0, 150 mM NaCl, 0.5 mM $CaCl_2$, 0.5% NP-40 in the presence of PMSF, aprotenin and leupeptin. Cells were scraped into 1.5 ml tubes and lysates were centrifuged for 15 min at 14 000 r.p.m. in an Eppendorf centrifuge at 4°C. The supernatant was cleared using protein G–Sepharose and the LM609 mAb was added for 1 h at 4°C before capturing on protein G–Sepharose. After washings in lysis buffer the beads were boiled in SDS loading buffer in the absence of reducing agent, and the α_v and the β_3 chains were separated on an 8% SDS gel.

Western blotting

HaCaT cells were lysed on the tissue culture dish using twice the concentration of SDS loading buffer. The lysates were briefly sonicated and boiled before the relative protein concentration was estimated. The same amount of protein was separated on a 12% SDS gel and blotted to nitrocellulose filters. After blocking in PBS containing 5% milk, the filters were treated with the antibodies indicated for 1 h. The filters were then washed and secondary HRP-conjugated antibodies were added for 45 min before final washing in PBS containing 0.2% Tween 20. The protein bands were visualized using ECL.

Acknowledgements

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