Ligand-specific, transient interaction between integrins and calreticulin during cell adhesion to extracellular matrix proteins is dependent upon phosphorylation/dephosphorylation events

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As transmembrane heterodimers, integrins bind to both extracellular ligands and intracellular proteins. We are currently investigating the interaction between integrins and the intracellular protein calreticulin. A prostatic carcinoma cell line (PC-3) was used to demonstrate that calreticulin can be found in the α_3 immunoprecipitates of cells plated on collagen type IV, but not when plated on vitronectin. Conversely, α_v immunoprecipitates contained calreticulin only when cells were plated on vitronectin, i.e. not when plated on collagen IV. The interactions between these integrins and calreticulin were independent of actin cytoskeleton assembly and were transient, being maximal approx. 10–30 min after the cells came into contact with the substrates prior to complete cell spreading and formation of firm adhesive contacts. We demonstrate that okadaic acid, an inhibitor of intracellular serine/threonine protein phosphatases, inhibited

the $\alpha_3\beta_1$ -mediated adhesion of PC-3 cells to collagen IV and the $\alpha_2\beta_1$ -mediated attachment of Jurkat cells to collagen I. This inhibition by okadaic acid was accompanied by inhibition of the ligand-specific interaction of calreticulin with the respective integrins in the two cell types. Additionally, we found that pharmacological inhibition of mitogen-activated protein kinase (MEK) resulted in prolongation of the calreticulin–integrin interaction, and enhancement of PC-3 cell attachment to collagen IV. We conclude that calreticulin interacts transiently with integrins during cell attachment and spreading. This interaction depends on receptor occupation, is ligand-specific, and can be modulated by protein phosphatase and MEK activity.

Key words: cell attachment, early adhesion complex, MAP kinase, protein phosphatase, signal transduction.

INTRODUCTION

Eukaryotic cells engage in intimate interactions with the extracellular matrix (ECM), and these interactions influence profoundly important biological processes, such as embryogenesis, inflammation and tumour progression. The integrins are a family of heterodimeric transmembrane receptors, one function of which is to bind ECM proteins, thereby mediating cell adhesion and regulating cell shape [1,2]. Furthermore, integrin-mediated attachment can activate intracellular signalling pathways involving protein phosphorylation, inositol lipid metabolism, activation of p21^{ras} and mitogen-activated protein kinase (MAPK), and changes in cytosolic pH and [Ca²⁺] [3–5]. These integrin-mediated signalling events can lead to modulation of gene expression and, ultimately, regulation of cell proliferation, differentiation and survival [6,7]. As well as ‘outside-in’ signal transduction, integrins are capable of transducing ‘inside-out’ signals. These processes involve intracellular signalling pathways that connect with the cytoplasmic domains of integrins, and can result in rapid activation or inactivation of ligand-binding functions, possibly mediated through changes in receptor conformation [8,9]. Such dynamic and rapid modulation of integrin affinity is important for proper control of cell adhesion and migration in a variety of physiological settings [4,10,11].

The biochemical mechanisms through which integrin-mediated signals are effected are complex and not fully understood.

Recently, much work has focused on intracellular biochemical events that are immediately proximate to integrin receptor activity. As a result of these investigations, several intracellular proteins that interact directly with integrins have been identified. The cytoskeletal proteins talin and α -actinin have been shown to bind directly to the β_1 , β_2 and β_3 integrin subunit cytoplasmic domains [12–14], and the cytoskeletal protein filamin interacts directly and specifically with the β_2 integrin subunit [15]. These proteins can be detected in focal adhesion plaques, and can contribute to reorganization of the actin cytoskeleton, cell adhesion and cell migration. Regulatory and signal-transducing proteins also interact with the cytoplasmic domains of integrin β subunits. Focal adhesion kinase and integrin-linked kinase have been shown to bind β_1 , β_2 and β_3 integrin subunit cytoplasmic domains [16,17], whereas β_3 -endonexin and cytohesin are specific for the β_3 [18] and β_2 [19] subunits respectively. Recently, a novel protein named integrin cytoplasmic domain-associated protein-1 has been found to interact with the β_1 cytoplasmic tail [20], and a receptor for activated protein kinase C (Rack1) has been demonstrated to interact with both β_1 and β_2 cytoplasmic domains [21]. The interactions that many of these proteins form with integrins can contribute to both ‘outside-in’ and ‘inside-out’ transmembrane signal transduction [5,20,21].

Compared with the β subunits, there are fewer examples of cytoplasmic proteins that have been found to interact with the cytoplasmic domains of integrin α subunits. The $\alpha_v\beta_3$ integrin

Abbreviations used: DMF, dimethylformamide; ECL, enhanced chemiluminescence; ECM, extracellular matrix; ERK, extracellular-signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; RIPA, radio-immunoprecipitation assay; TM4, tetraspan 4.

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has been shown to associate with the insulin receptor substrate [22] and p190 [23], α_v -containing integrins have been shown to associate with integrin-associated protein [24], and it has been demonstrated that $\alpha_5\beta_1$ can associate with CD9 and CD63 [25]. However, none of these proteins has been shown to bind directly to the α subunit of the integrins. The only direct interaction reported to date between an integrin α subunit and a cytoskeletal protein is that which occurs between α_2 and F-actin [26], and the only descriptions of a direct interaction between integrin α subunits and a cytoplasmic protein are of those which involves the intracellular calcium-binding proteins calreticulin [27–29] and CIB [29a].

Previously, we reported that the interaction between $\alpha_3\beta_1$ and calreticulin can be induced by integrin activation, and that this interaction correlates with integrin-mediated adhesion of Jurkat cells [30]. More recently, we demonstrated that murine embryonic stem cells genetically lacking calreticulin exhibit severely impaired integrin-mediated adhesion and calcium influx [31]. In the present study, we have used PC-3 prostatic carcinoma cells to demonstrate that calreticulin interacts transiently with integrins as the cells attach and spread on ECM proteins. We found that this inducible interaction between integrins and calreticulin, as well as cell adhesion, were sensitive to treatment with okadaic acid, a serine/threonine protein phosphatase inhibitor, and could be stimulated by inhibition of MAPK kinase (MEK) activity. These findings suggest that calreticulin is a transient, but nonetheless essential, component of early adhesive structures as cells first attach to an integrin substrate.

EXPERIMENTAL

Materials

Human vitronectin was purchased from Gibco BRL (Gaithersburg, MD, U.S.A.). Polyclonal antiserum raised against extracellular-signal-related kinase-1 (ERK1) was obtained from Santa Cruz (New York, NY, U.S.A.), and the phosphospecific MAPK antibody was purchased from New England Biolabs (Beverly, MA, U.S.A.). The antibodies raised against integrin subunits α_2 (P1E6), α_3 (P1B5), α_v (VNR147) and β_1 (P4C10) were obtained from Gibco BRL. Rabbit polyclonal antiserum raised against integrin α_v subunit was purchased from Chemicon (Temecula, CA, U.S.A.). Polyclonal antisera specific for the cytoplasmic domains of the human integrin α_5 and chicken β_1 subunits were generously given by R. Hynes (MIT/HHMI, Centre for Cancer Research, Cambridge, MA, U.S.A.), and antiserum raised against the cytoplasmic domain of the chicken α_3 subunit was a kind gift from L. Reichardt (University of California, San Francisco, CA, U.S.A.). The activating antibody specific for integrin α_2 (JBS2) was generously given by Dr. J. Wilkins (University of Winnipeg, Winnipeg, MB, Canada). The polyclonal anti-calreticulin antiserum, LAR 090, was a kind gift from Dr. L. Rokeach (University of Montreal, Montreal, PQ, Canada). Rabbit polyclonal anti-caveolin was purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.). The MEK inhibitor PD 98059 was obtained from Calbiochem (La Jolla, CA, U.S.A.). Protein G–Sepharose was purchased from Pharmacia Biotech (Baie d'Urfé, Quebec, Canada), and horseradish-peroxidase-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (Mississauga, ON, Canada). Enhanced chemiluminescence reagents were purchased from Amersham (Arlington, IL, U.S.A.), and silver stain reagents were purchased from BioRad (Mississauga, ON, Canada). All other chemicals, including collagen types I and IV, cytochalasin D and okadaic acid, were obtained from Sigma Chemicals (Oakville, ON, Canada).

Cell culture

PC-3 cells were cultured in Dulbecco's modified Eagle's medium with 7% (v/v) bovine calf serum. Jurkat cells were maintained in RPMI 1640 medium with 7% (v/v) bovine calf serum and 50 μ M 2-mercaptoethanol. Cells were maintained at 37 °C and 5% CO₂.

Cell adhesion assays

Microtitre plates (96 wells; Linbro/Titertek) were coated with poly-L-lysine (10 μ g/ml), collagen type I or type IV (10 μ g/ml) or vitronectin (5 μ g/ml) in PBS overnight at 4 °C and blocked with BSA (2.5 μ g/ml). Under serum-free conditions, PC-3 cells were harvested, washed, pretreated with 10 nM okadaic acid in dimethylformamide (DMF) or DMF alone, and plated on to 96-well plates (5×10^4 cells/well), before incubation at 37 °C. The wells were washed three times with PBS, and the attached cells were fixed and stained in PBS/3.7% (v/v) paraformaldehyde/0.5% (v/v) Toluidine Blue. After 3 h in stain solution, the cells were washed extensively with distilled water. For quantification of cell-bound dye, cells were extracted with 0.2% (v/v) Triton X-100 in distilled water, and A_{570} was read in a microtitre plate reader. To test for cell viability, cell samples were stained with 0.2% (w/v) Trypan Blue in PBS, and a differential count was performed. Adhesion assays with ³H-labelled Jurkat cells were done as described previously [30]. Where indicated, Jurkat cells were stimulated with the activating antibody JBS2 after pretreatment with okadaic acid (1, 10 or 100 nM in DMF) or DMF alone.

Immunoprecipitation and Western blotting

After plating on substrates for the indicated times, unattached cells were gently removed by aspiration. The attached cells, or cells that had been treated with integrin-activating antibodies, were then placed on ice and washed in ice-cold PBS. For analysis of cell-surface proteins, PC-3 cells were surface-labelled with sulfo-NHS-biotin according to the manufacturer's protocol (Pierce Chemicals, Rockford, IL, U.S.A.) before plating. Cell extracts were made with radio-immunoprecipitation assay (RIPA) buffer [PBS (pH 7.4)/1% (v/v) Triton X-100/0.1% (w/v) SDS/0.5% (w/v) deoxycholate/1 mM PMSF/10 μ g/ml aprotinin/10 μ g/ml leupeptin] and cleared by centrifugation at 10000 g, 4 °C for 15 min. Cell extracts were pre-cleared with Protein G–Sepharose, incubated with anti-integrin antibodies overnight at 4 °C and immune complexes were then collected with Protein G–Sepharose. Samples were analysed by SDS/PAGE, and the proteins were transferred electrophoretically to PVDF (Immobilon-P; Millipore, Bedford, MA, U.S.A.). PVDF membranes were blocked with 5% (w/v) non-fat milk/Tris-buffered saline [which is 20 mM Tris/HCl/140 mM NaCl (pH 7.2)]/0.1% (w/v) Tween 20. All antibody incubations and washes were performed in Tris-buffered saline/0.1% (w/v) Tween 20. Enhanced chemiluminescence (ECL[®]) was performed using Amersham reagents according to the manufacturer's instructions. In some experiments, cells were surface-labelled with sulfo-NHS-biotin (Pierce Chemicals), before plating on ECM substrates and subsequent lysis in RIPA buffer. Purified proteins were then separated by using SDS/PAGE, transferred to PVDF membranes, and the membranes were probed with horseradish-peroxidase-conjugated streptavidin (Jackson ImmunoResearch Laboratories) before ECL. Membranes were visualized by exposure to Kodak X-O-Mat film. For densitometric analysis, films were scanned in an LKB laser densitometer (model 2222-020) and quantified with Gelscan XL software (Pharmacia).

Figure 1 Interaction between integrins and calreticulin is an early and transient event during cell adhesion

Under serum-free conditions, PC-3 cells were plated on poly-L-lysine (PLL), collagen type IV (COLL IV) or vitronectin (VN) for the indicated times. **(A)** Cells were fixed, stained with Toluidine Blue and photographed. **(B)** Cells were harvested, lysed and immunoprecipitations were performed with anti- α_3 antibodies (PIB5). Western blots were then carried out using anti- β_1 antibodies (P4C10; upper panels), and anti-calreticulin (CRT) antiserum (LAR 090; lower panels). **(C)** As in **(B)**, except that immunoprecipitations were performed with α_v antibodies (VNR147). Immunoprecipitates were then analysed by Western blotting with polyclonal antiserum to α_v (Chemicon; upper panels) and anti-calreticulin antiserum (LAR 090; lower panels). Mr (K), $10^{-3} \times M_r$.

MEK inhibition and analysis

Cells were deprived of serum for 16 h and then treated with 25 μ M PD 98059 in serum-free Dulbecco's modified Eagle's medium for 1–2 h at 37 °C. Cells were then counted for viability

and used in attachment assays, or extracted as described above for analysis of ERK phosphorylation. ERK1 immunoprecipitates were subjected to Western blotting with an antiserum specific for catalytically active, phosphorylated MAPK.

RESULTS

ECM proteins induce ligand-specific, transient interactions between calreticulin and integrins

We have previously demonstrated that stimulatory anti-integrin antibodies can induce the interaction of calreticulin with the integrins $\alpha_3\beta_1$ [30] and $\alpha_4\beta_1$ (M. G. Coppolino and S. Dedhar, unpublished work). We therefore wanted to determine whether the interaction between integrins and calreticulin could also be induced in an adherent cell line by a physiological stimulus, such as adhesion. Cells of the prostatic carcinoma cell line PC-3 (serum-free) were plated on to dishes coated with poly-L-lysine (10 $\mu\text{g}/\text{ml}$), type IV collagen (10 $\mu\text{g}/\text{ml}$) or vitronectin (5 $\mu\text{g}/\text{ml}$). After 15–20 min, the cells had settled on to the substrates and had begun to attach (Figure 1A). After a further 15–20 min, the cells had attached firmly to the substrates, but had only just begun to spread out. After 1 h on the ECM proteins, but not on

poly-L-lysine, most of the cells had acquired a fully spread morphology (Figure 1A). At the indicated time points, cells were harvested, and RIPA extracts were used for the immunoprecipitation of α_3 - and α_v -containing integrins. Figure 1(B) shows that only when the cells were attached to collagen type IV was calreticulin associated with the integrin $\alpha_3\beta_1$, the major receptor for collagen IV on these cells [29]. Conversely, calreticulin was only found in the immunoprecipitates of α_v integrins when the cells were attached to vitronectin (Figure 1C). In both cases, the interaction between integrins and calreticulin was transient, being detected most readily 10–30 min after plating when the cells were attached to the ECM protein, but not yet spread upon it. The interaction between calreticulin and integrins occurs rapidly and can be detected approx. 2 min after attachment to collagen IV (results not shown), but is maximal at 15 to 30 min. Negligible cell spreading was observed, and no calreticulin was detected in the α_3 and α_v immunoprecipitates of cells plated on poly-L-lysine.

Figure 2 Analysis of anti- α_3 immunoprecipitates during PC-3 cell adhesion to collagen IV

As described in the legend to Figure 1, PC-3 cells were plated on type IV collagen (10 $\mu\text{g}/\text{ml}$) for the indicated time periods. The cells were then harvested, extracted with RIPA buffer and subjected to immunoprecipitation with anti- α_3 monoclonal antibody (P1B5) and Protein G–Sepharose. Immunoprecipitated proteins were then resolved by SDS/PAGE. (A) An SDS/7.5% polyacrylamide gel was fixed and subjected to silver staining, before being dried and photographed. (B) Before plating, the cells were surface-labelled with sulpho-NHS-biotin. A 10% gel was transferred to a PVDF membrane, and the blot was developed with horseradish-peroxidase-conjugated streptavidin and ECL. Asterisks mark candidates for TM4 family members. (C) A 7.5% gel was transferred to a PVDF membrane and the blot was developed with rabbit anti-caveolin antibody, horseradish-peroxidase-conjugated secondary antibody and ECL. The blot shown is from an experiment using the MEK inhibitor, PD 98059, as described subsequently in the legend to Figure 6(C). E, PC-3 cell extract. All blots are representative of at least three experiments.

Figure 3 Ligand-induced interaction between integrin $\alpha_3\beta_1$ and calreticulin does not require actin cytoskeleton assembly

(A) PC-3 cells were plated on collagen type IV-coated dishes after pretreatment with 1 μ M cytochalasin D (cyto. D) or DMSO carrier alone. After the indicated incubations, the cells were harvested and α_3 immunoprecipitates were analysed for β_1 and calreticulin content by Western blotting. Shown are representative anti- β_1 (P4C10) and anti-calreticulin (LAR 090) blots (in the upper and lower panel respectively). crt, calreticulin. (B) Densitometric quantification of calreticulin- $\alpha_3\beta_1$ interaction under various experimental conditions. Experiments described in (A) and in Figures 4(C) and 6(C) were repeated several times, and the Western blots were used to determine the relative amount of calreticulin in α_3 immunoprecipitates by laser densitometry. The means $\pm$ S.D. for the indicated number (*n*) of independent experiments are shown.

To ensure that the observed changes in the quantity of calreticulin detected in integrin immunoprecipitations were not due to overall changes in the amounts of material isolated in the immune complexes at different time points, samples of α_3 immunoprecipitates were separated by SDS/PAGE, with subsequent detection of the proteins by silver staining. As shown in Figure 2(A), there were no gross differences in the levels of protein found in the α_3 immunoprecipitates from different time points; moreover, there were no significant differences in the amounts of cell-surface proteins that were isolated in α_3 immune complexes (Figure 2B). It has been reported by others that members of the TM4 (tetraspan 4) family of cell-surface proteins can interact with some integrin α subunits, and that such interactions can be detected by immunoprecipitation of integrins from surface-labelled cells [25,34]. Whereas TM4 proteins were not directly identified here, Figure 2(B) reveals that several cell-surface proteins were detected in α_3 immunoprecipitates, some of which had relative molecular masses corresponding to those reported previously [25,34] for members of this family (CD63, 45–55 kDa; CD81, $\approx$ 22 kDa; CD9, $\approx$ 23 kDa; see asterisks in

Figure 2B). However, the levels of none of these surface proteins found in α_3 immune complexes changed significantly throughout time points in which levels of calreticulin were markedly altered (compare Figures 2B and 1B). Together with the results from the silver-staining analysis, these data indicate that there is significant specificity in the regulation of the interaction between integrins and calreticulin, as shown in Figure 1.

As further controls for the co-immunoprecipitation/Western blot approach, we examined the integrin immunoprecipitations for caveolin. This transmembrane protein has been reported to associate, in a constitutive manner, with some β_1 integrins [33]. After experiments similar to those described in Figure 1, α_3 immunoprecipitates were analysed for caveolin content by Western blotting. In contrast with calreticulin, caveolin was found to be constitutively associated with integrin α_3 in PC-3 cells, and this association did not change in response to the adhesion of these cells on collagen IV (Figure 2C). This result further suggests that the interaction that occurs between integrins and calreticulin, as well as being ligand-specific, is specifically regulated during the process of cell adhesion.

Figure 4 Okadaic acid inhibits adhesion of PC-3 cells to collagen type IV and the induced interaction between integrin $\alpha_3\beta_1$ and calreticulin

PC-3 cells were plated on collagen type IV-coated dishes after pretreatment with 10 nM okadaic acid or DMF carrier alone. (A) After incubation at 37 °C for the indicated times, cells were fixed, stained with Toluidine Blue and photographed. (B) After staining, the cell-bound dye was extracted with 0.2% (v/v) Triton X-100 in distilled water and A_{570} was measured. Triplicate samples from several experiments were measured and the means $\pm$ S.D. are shown. (C) PC-3 cells were pretreated with 10 nM okadaic acid or DMF carrier alone, and then plated on to collagen IV-coated dishes. After the indicated incubations, the cells were harvested and α_3 immunoprecipitates were analysed for β_1 and calreticulin content by Western blotting. Representative anti- β_1 (P4C10) and anti-calreticulin (LAR 090) blots are shown (upper and lower panel respectively). As determined by Toluidine Blue exclusion assay, the percentages of viable PC-3 cells after the indicated times of treatment with 10 nM okadaic acid were as follows (given as means $\pm$ S.D.): untreated, 86.3 $\pm$ 2.0; after 15 min, 87.3 $\pm$ 3.4; after 30 min, 88.4 $\pm$ 3.2; and after 60 min, 85.9 $\pm$ 3.2. CRT, calreticulin. Mr (K), $10^{-3} \times M_r$.

Interaction between calreticulin and integrin $\alpha_3\beta_1$ is not dependent on F-actin assembly

To determine if the ligand-induced interaction between integrin $\alpha_3\beta_1$ and calreticulin requires an intact cytoskeleton, PC-3 cells were treated with medium containing 1 μ M cytochalasin D when plated on to collagen type IV, as described above. The cells were collected at the indicated times and analysed for integrin-calreticulin complexes. Whereas treatment with cytochalasin D

did not interfere with the attachment of PC-3 cells to collagen IV, strong adhesion and cell spreading were inhibited completely (results not shown) such that the course of cell attachment was indistinguishable from that on poly-L-lysine. Interestingly, this had no effect on the collagen IV-induced, transient interaction between $\alpha_3\beta_1$ and calreticulin, as seen in α_3 immunoprecipitates (Figure 3A). Figure 3(B) shows the densitometric quantification of the amount of calreticulin, relative to β_1 , in the immunoprecipitates from several replicate experiments. These data

Figure 5 Okadaic acid inhibits both the adhesion of Jurkat cells to collagen type I and the association of calreticulin with integrin $\alpha_2\beta_1$

(A) Under serum-free conditions, [^3H]thymidine-labelled Jurkat cells were pretreated with the indicated concentrations of okadaic acid or DMF carrier alone. The cells were then stimulated with the anti- α_2 stimulatory antibody JBS2 (10 $\mu\text{g}/\text{ml}$) for 15 min. Control cells received PBS alone. After stimulation, cells were placed on to collagen type I-coated wells of 96-well microtitre plates for 1 h and adhesion was quantified as described previously [30]. The bars show the means $\pm$ S.D. for three independent experiments. (B) As in (A), except that after stimulation with JBS2, Jurkat cells were lysed and $\alpha_2\beta_1$ integrin was immunoprecipitated with anti- α_2 -antibody (P1E6). α_2 immunoprecipitates were then analysed for β_1 and calreticulin content by Western blotting with P4C10 (upper panel) and LAR 090 (lower panel), as described. As determined by Toluidine Blue exclusion assay, the percentage of viable Jurkat cells after the indicated times of treatment with 10 nM okadaic acid were as follows (values given as means $\pm$ S.D.): untreated, 89.2 ± 2.4 ; after 15 min, 88.9 ± 2.0 ; after 30 min, 87.4 ± 1.5 ; and after 60 min, 88.3 ± 2.2 .

suggest that the integrin–calreticulin interaction is an event that occurs not only temporally, but also biochemically, before cell-spreading events.

Okadaic acid inhibits the adhesion of PC-3 cells to type IV collagen

Hedman and Lundgren have reported that okadaic acid can inhibit the avidity of the integrin $\alpha_1\beta_2$ in human B cells [34]. To determine if okadaic acid could affect the adhesion of PC-3 cells to collagen type IV, PC-3 cells were plated on collagen IV in the presence or absence of 10 nM okadaic acid (Figure 4A). Quantitative adhesion assays were performed, and these demonstrated that 10 nM okadaic acid inhibited significantly the adhesion and spreading of PC-3 cells on type IV collagen (Figure 4B). In these experiments, cells treated with okadaic acid appeared to bind ligand (i.e. attached), but did not adhere firmly. Thus okadaic-acid-treated cells were not observed to undergo firm adhesion (Figure 4A), in contrast with untreated control cells, and this is clearly reflected in the quantification shown in Figure 4(B). In this regard, cells treated with okadaic acid and plated on collagen IV were indistinguishable from those plated on poly-L-lysine. These observations suggest that okadaic acid inhibits a post-ligand-binding event in integrin function, and that this prevents firm cell adhesion from occurring. Treatment of PC-3 cells with 10 nM okadaic did not alter the viability of these cells (see the legend to Figure 4).

Collagen IV-induced interaction of integrin $\alpha_3\beta_1$ with calreticulin is inhibited by okadaic acid

To assess the effect that okadaic acid might have upon the collagen IV-induced interaction between $\alpha_3\beta_1$ and calreticulin, PC-3 cells were treated with 10 nM okadaic acid before being

plated on to collagen IV (10 $\mu\text{g}/\text{ml}$) as described above. At the indicated times, the cells were extracted with RIPA buffer and immunoprecipitates of the $\alpha_3\beta_1$ collagen receptor were prepared. The immunoprecipitates were then analysed for calreticulin by Western blotting. Okadaic acid, at a concentration of 10 nM, significantly inhibited the interaction between calreticulin and integrin $\alpha_3\beta_1$ induced by cell attachment to collagen type IV (Figure 4C). This observation was made several times and laser densitometry was used to quantify, relative to β_1 , the amount of calreticulin present in the immunoprecipitates. The results obtained (shown as means $\pm$ S.D.) for three such determinations are shown in Figure 3(B).

Okadaic acid inhibits both the adhesion of Jurkat cells to collagen I, and the association of calreticulin with integrin $\alpha_2\beta_1$

The T-lymphoblastoid cell line, Jurkat, can be induced to adhere to collagen type I by stimulation with anti-integrin antibodies. This adhesion is mediated by the integrin $\alpha_2\beta_1$, and is accompanied by a concomitant interaction between calreticulin and this integrin [30]. To determine whether the adhesion of these cells, as well as the interaction between calreticulin and $\alpha_2\beta_1$, was sensitive to okadaic acid, Jurkat cells were treated with different concentrations of okadaic acid and analysed as described previously [30]. In quantitative adhesion assays, an anti- α_2 stimulatory antibody (JBS2) induced the adhesion of Jurkat cells to 10 $\mu\text{g}/\text{ml}$ collagen type I, and this was inhibited by okadaic acid in a concentration-dependent manner (Figure 5A). Using Western blot analyses, the corresponding interaction between the integrin $\alpha_2\beta_1$ and calreticulin was observed to be inhibited by okadaic acid, also in a concentration-dependent manner (Figure 5B). Treatment of Jurkat cells with okadaic did not affect the viability of these cells (see the legend to Figure 5).

Figure 6 Inhibition of MEK enhances PC-3 cell adhesion and integrin-calreticulin interaction

PC-3 cells were serum-deprived for 16 h and then treated with 25 μ M PD 98059 for 1 h before plating on collagen IV. **(A)** Quantitative adhesion assays were performed as described in the Experimental section. **(B)** MAPK (ERK1) immunoprecipitates (ips) were prepared from RIPA extracts of PC-3 cells that had been plated on collagen IV for the indicated times. These immunoprecipitates were then analysed by Western blotting for the phosphorylated form of ERK1. Lane E, PC-3 cell extract. **(C)** After incubation on collagen IV for the indicated times, control and PD 98059-treated cells were extracted and α_3 immunoprecipitates were analysed for β_1 and calreticulin content by Western blotting. Representative anti- β_1 (P4C10) and anti-calreticulin (LAR 090) blots are shown in the upper and lower panel respectively. Lane E, PC-3 cell extract; crt, calreticulin.

Inhibition of MEK enhances PC-3 cell adhesion and integrin-calreticulin interaction

The observation that phosphatase activity was required for integrin-calreticulin interaction and cell attachment suggested that the activity of some kinase(s) might be opposing these functions. Recently, Hughes et al. demonstrated that a Ras-linked MAPK pathway could suppress integrin function [35]. To investigate such a suppressive pathway in PC-3 cells, we treated the cells with the specific inhibitor of MEK, PD 98059 [36], before plating on to collagen IV. At 25 μ M, PD 98059 consistently enhanced the adhesion of PC-3 cells to collagen IV (Figure 6A), while abrogating the phosphorylation of MAPK (ERK1), as determined by Western blots for phospho-MAPK (Figure 6B). Concomitant with the PD 98059 enhancement of cell adhesion, treatment of PC-3 cells with this drug also prolonged the interaction between calreticulin and $\alpha_3\beta_1$ that was induced by cell attachment to collagen IV (Figure 6C). These results suggest that an MAPK pathway is involved in the suppression of integrin function in these cells, and that inhibition of this pathway results in increased integrin activity, manifested in enhanced cell adhesion and augmented integrin-calreticulin complex formation.

DISCUSSION

As transmembrane receptors, integrins bind to both extracellular ligands and intracellular proteins. Whereas many specific interactions involving integrins and intracellular proteins, both cytoskeletal and regulatory, have been described, the only non-cytoskeletal proteins that have been found to interact directly with the α subunit of integrins are calreticulin [5,27] and CIB [29a]. We have now demonstrated, in PC-3 cells, that integrins $\alpha_3\beta_1$ and $\alpha_v\beta_3$ form interactions with calreticulin as the cells attach to collagen type IV and vitronectin respectively. The integrin $\alpha_3\beta_1$ is a well-recognized collagen receptor and $\alpha_v\beta_3$ has been thoroughly characterized as a vitronectin receptor [1,37]. Specifically, we have confirmed previously [29,38] that $\alpha_3\beta_1$ is the receptor for collagen IV and that $\alpha_v\beta_3$ is the receptor for vitronectin on PC-3 cells. Thus the interaction between calreticulin and these integrins is both ligand-induced and ligand-specific; calreticulin is primarily found in association with ligand-occupied integrins, not with unoccupied receptors. Importantly, we also report that the interaction of integrins with calreticulin is transient, occurring most prominently 10–30 min after exposure to the ECM proteins.

Calreticulin has been assigned multiple functions [39]. It was first described as a calcium-binding protein of the sarcoplasmic reticulum of smooth-muscle cells [40,41]. Calreticulin has subsequently been shown (i) to possess a calcium-binding function [42] and chaperone activity [43] in the endoplasmic reticulum; (ii) to have RNA binding activity [44]; and (iii) to modify gene expression by binding to nuclear-hormone receptors [45,46]. It has also been shown that calreticulin can bind to the KXGFFKR motif within the α subunits of integrins [27] (where X indicates any residue), and that antisense oligonucleotide-mediated down-regulation of calreticulin expression inhibits integrin-mediated adhesion of PC-3 and Jurkat cells [29]. Furthermore, studies using indirect immunofluorescence have revealed that calreticulin and the integrin $\alpha_3\beta_1$ co-localize at the plasma membrane upon clustering of this integrin in PC-3 cells [29]. This observation indicates that calreticulin and integrins can be spatially proximate to one another in living cells.

We have previously demonstrated that an interaction between integrin $\alpha_3\beta_1$ and calreticulin, in Jurkat cells, can be induced by antibody-mediated stimulation of the integrin [30]. It was also found that this induced interaction between the integrin and calreticulin is important for the $\alpha_3\beta_1$ -mediated adhesion of Jurkat cells to collagen type I [30]. More recently, we reported on the development of calreticulin knock-out murine embryonic stem cells, and found that calreticulin is essential for integrin-mediated adhesion and calcium signalling [31]. The results of the present study suggest that calreticulin binds to integrins as they first become activated, either by antibody stimulation or by encountering ECM proteins. Thus, via binding to an integrin, calreticulin may participate in the formation of an 'early adhesion complex', which occurs prior to cytoskeletal reorganization, at the site of ligand binding. Later events in cell adhesion (i.e. establishment of a focal plaque) might not require the continued presence of calreticulin in the adhesive structures. This notion is supported by the finding that calreticulin is not detectable in focal adhesion plaques [47].

It has been reported by others that integrin affinity can be modulated by okadaic acid, an inhibitor of intracellular protein serine/threonine phosphatases, including protein phosphatase (PP)-1 and PP-2A [34]. In the present study we found that okadaic acid both inhibited adhesion of PC-3 cells to type IV collagen and vitronectin and concomitantly decreased the interaction of calreticulin with the integrin receptors for these substrates. In Jurkat cells, we found that okadaic acid inhibited antibody-stimulated, $\alpha_3\beta_1$ -mediated adhesion to collagen I, and the corresponding interaction of this integrin with calreticulin. These effects of okadaic acid suggest that the activity of intracellular phosphatases PP-1 and PP-2A are required for normal integrin function in these cells. However, since okadaic acid has been found to inhibit other mammalian phosphatases [48], the effects demonstrated here cannot be ascribed unequivocally to inhibition of PP-1 or PP-2A.

Our findings with okadaic acid imply that there are intracellular signalling events, involving the dephosphorylation of serine or threonine residues, that are positive regulators of integrin function. Although tyrosine phosphatases have been shown to be regulated by integrin $\alpha_{\text{IIIb}}\beta_3$ in platelets [49], direct targets of specific serine/threonine phosphatase activity have not been well characterized in integrin function. The cytoplasmic domains of some integrins themselves have been shown to be phosphorylated [24]; however, the importance of these phosphorylation events in integrin-mediated adhesion or signalling has not been fully demonstrated. While calreticulin can be phosphorylated [44], the biochemical and physiological significance of this phosphorylation is not clear, and it remains to be determined whether

phosphorylation of calreticulin directly is important for its interaction with integrins. Alternatively, it is plausible that other adhesion-regulating proteins, e.g. the serine/threonine kinase integrin-linked kinase [17], are targets for, or themselves mediate, phosphorylation events, and that these events are both sensitive to phosphatase PP-1 and PP-2A activity and important for the putative function of calreticulin in modulating integrin activity.

The notion that intracellular dephosphorylation events might be positive regulators of integrin function implies that some reciprocal phosphorylation events may be negative regulators of integrin function. It has been reported that a Ras/Raf MAPK pathway can be a suppressor of integrin function in Chinese hamster ovary cells [35]. Our results generally agree with this finding, in that the inhibition of MEK activity in PC-3 cells might have released the $\alpha_3\beta_1$ integrin from some level of suppression, thus promoting adhesion and increasing the duration of the integrin-calreticulin interaction. One possible explanation for these observations is that an unidentified target of MAPK hinders the association of calreticulin with the integrin, impeding the formation of a putative 'early adhesion complex'. It is also possible that a substrate of MAPK, functioning as part of a negative-feedback loop that suppresses the maturation of adhesive structures, is involved in the disassembly of such a complex. It is reasonable to predict that the regulation of integrin-calreticulin interaction is quite complex, and it will be interesting to determine whether the small GTPases Rho and R-Ras, which have been implicated in the regulation of integrin activation [50,51], can also modulate the interaction between integrins and calreticulin.

Integrins are commonly referred to as mediators of both 'outside-in' and 'inside-out' transmembrane signal transduction [1,4]. We have demonstrated, here as well as in previous studies, that integrins and calreticulin interact in response to integrin stimulation. In the present study we have demonstrated that the interaction between integrins and calreticulin can be induced in a ligand-specific manner. Importantly, the interaction was observed in normally adherent cells and in response to a physiological stimulus, i.e. attachment of the cells to ECM proteins. These findings imply that calreticulin is a physiological modulator of integrin function. While the exact mechanism by which calreticulin modulates integrin function is still under investigation, the studies herein have provided some important insight for understanding calreticulin function in this regard. The results indicate that the influence calreticulin has on integrin activity occurs early in the adhesion process, before assembly of the actin cytoskeleton. The observations that the interaction between calreticulin and integrins can be influenced by phosphorylation and dephosphorylation events strengthen further the concept that this interaction is closely associated with changes in integrin activity. In the light of these findings, together with the observation that calreticulin is essential for both integrin-mediated adhesion and Ca^{2+} influx [31], it is reasonable to conclude that calreticulin both responds to and is necessary for integrin activity, and that the interaction between integrins and calreticulin is an important mediator of transmembrane signalling.

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