Mechanical control of cyclic AMP signalling and gene transcription through integrins

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This study was carried out to discriminate between two alternative hypotheses as to how cells sense mechanical forces and transduce them into changes in gene transcription. Do cells sense mechanical signals through generalized membrane distortion[1,2] or through specific transmembrane receptors, such as integrins[3]? Here we show that mechanical stresses applied to the cell surface alter the cyclic AMP signalling cascade and downstream gene transcription by modulating local release of signals generated by activated integrin receptors in a G-protein-dependent manner, whereas distortion of integrins in the absence of receptor occupancy has no effect.

To analyse the molecular basis of mechanochemical transduction, suspended bovine endothelial cells were allowed to bind to magnetic microbeads coated with a synthetic peptide containing the RGD cell-binding sequence from fibronectin or coated with activating antibodies directed against integrin β1. Binding of these beads, which cluster integrins and induce receptor activation without altering cell shape[4], only produced a small increase in intracellular cyclic AMP (cAMP) levels (Fig. 1a). When a magnetic twisting device[3,5] was used to apply a controlled twisting (shear) stress (15.6 dyn cm^{-2}) to the beads and ligated integrin receptors, cAMP increased more than threefold (Fig. 1a). The increase in cAMP probably resulted from activation of adenylyl cyclase, given that these experiments were carried out in the presence of the phosphodiesterase inhibitor IBMX. These results closely mimic the enhanced response observed when these cells adhere to and exert tractional forces on dishes coated with integrin ligands, compared to control substrates including thrombin[6] even though this directly ligates G-protein-coupled transmembrane receptors that can elicit cAMP[7].

We next examined effects on downstream signalling by quantitating translocation of protein kinase A's catalytic subunit (PKA-c) into the nucleus, where it phosphorylates and activates the transcriptional regulator CREB[8,9]. Phosphorylated CREB (pCREB) in turn binds the cAMP-response element (CRE) and activates gene transcription[10]. Cell binding to RGD–beads alone increased nuclear PKA-c staining by 50% whereas bead binding plus twisting increased cAMP almost threefold (Fig. 1b). In contrast, neither binding to beads coated with acetylated low-density lipoprotein (AcLDL) nor application of mechanical stress to these bound beads produced any increase in PKA-c translocation (Fig. 1b). AcLDL is a ligand for transmembrane metabolic receptors which do not induce focal adhesion formation, activate integrin-dependent signalling pathways or mediate mechanical coupling to the cytoskeleton[3–5]. Significantly, the level of nuclear PKA-c staining increased proportionally as the level of stress applied to RGD–beads was raised from 2.6 to 15.6 dyn cm^{-2} (Fig. 1c), thus confirming that this effect on cAMP signalling was both stress-dependent and integrin-dependent.

To further analyse downstream signalling and explore the generality of this response, we then measured the effects of bead binding and twisting on CREB phosphorylation in suspended NIH3T3 fibroblasts using western blot analysis with antibodies to mouse pCREB[11]. These studies revealed that cell binding to RGD–beads only slightly elevated CREB phosphorylation, whereas twisting the RGD–beads again nearly tripled phosphorylation levels (Fig. 1d, e). This effect was also integrin-dependent as application of force through the transmembrane AcLDL receptor had no effect. Finally, similar results were obtained when we transfected NIH3T3 cells with a plasmid bearing the *lacZ* reporter gene under the control of three tandemly arranged CRE elements, and measured cAMP-dependent transcription by quantitating the number of cells exhibiting CRE-dependent β-galactosidase (β-gal) activity (Fig. 1f).

We then asked: if integrins function as mechanochemical transducers, can these two inputs (mechanical and chemical) be separated? Western blot analysis was used to analyse PKA activation in endothelial cells bound to beads coated with a non-activating antibody (K20) directed against integrin β1 (ref. 12), in the presence or absence of soluble activating GRGDSP peptide ligand, with or without mechanical stress application. These studies revealed that mechanical stresses applied directly to clustered integrin receptors could not influence cAMP signalling in the absence of receptor occupancy by the RGD ligand (Fig. 2a).

Increases in free intracellular calcium have been observed within seconds of applying stress to integrins with magnetic beads[13], and thus these changes could potentially mediate activation of calcium-sensitive adenylyl cyclases. However, microfluorimetric quantitation of intracellular calcium levels using Fura-2 in conjunction with ratio imaging did not reveal any significant change in calcium levels in response to magnetically twisting integrins (at ≤ 15.6 dyn cm^{-2}) in our endothelial cells. In contrast, treatment of cells with a general inhibitor of all Gα subunits, GDP-β-S, completely prevented the increase in PKA signalling induced by applying stress to integrins, although it did not inhibit basal PKA activity (Fig. 2b). Stress-dependent modulation of these membrane signalling events also appeared to proceed directly at the site of integrin binding, as disrupting structural connections between the focal adhesion and the internal actin cytoskeleton using cytochalasin D or disassembling microtubules using nocodazole did not interfere with PKA activation (Fig. 2c).

Taken together, our results confirm that mechanical signals are transmitted into the cell through specific transmembrane receptors, of which integrins are one example[3]. Distortion of integrins alone is not, however, sufficient to activate cAMP signalling or gene

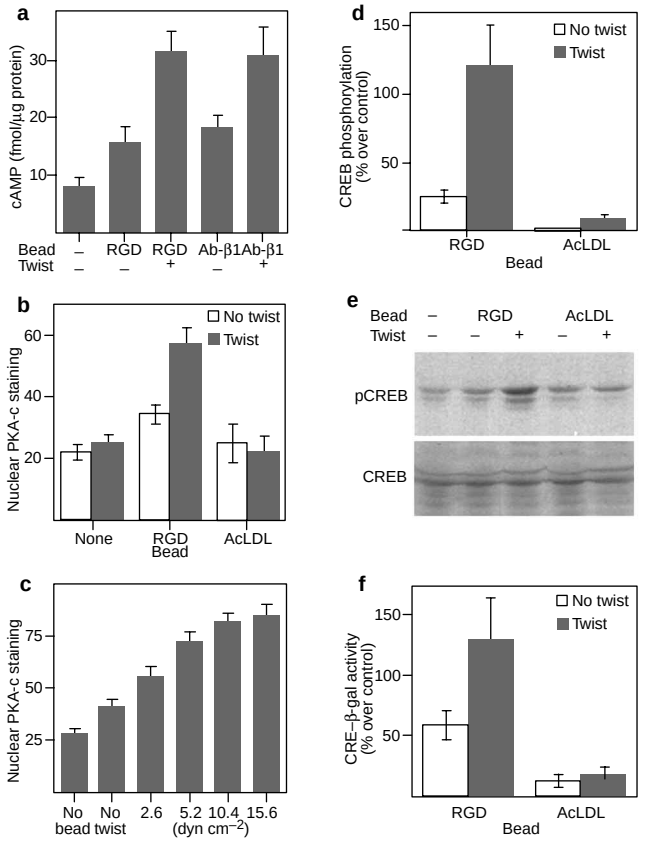

Figure 1 **Stress-and integrin-dependent control of the cAMP signalling cascade. a**, Suspended cells were cultured with or without magnetic microbeads coated with RGD–peptide (RGD) or activating antibodies against integrin β1 (Ab-β1) and either exposed to a magnetic twisting stress (15.6 dyn cm^{-2}) (Twist +) or not. Cyclic AMP levels were normalized for protein. **b**, Nuclear PKA-c staining in adherent cells cultured in the presence or absence of beads coated with RGD or AcLDL, with or without stress application (Twist). **c**, Effects of varying the level of applied stress on nuclear staining intensity for PKA-c. **d**, Per cent increase in CREB phosphorylation (pCREB/CREB) compared with controls (no beads, no twist) in suspended fibroblasts bound to magnetic microbeads coated with RGD or AcLDL, in the presence or absence of applied stress (Twist). **e**, Western blots stained with antibodies against phosphorylated CREB (pCREB) and total CREB (CREB) using total proteins isolated from suspended fibroblasts cultured in the presence or absence of RGD–beads or AcLDL–beads, with or without stress application (Twist). **f**, Per cent increase in the number of transfected cells staining positive for CRE reporter β-gal activity compared with controls (no beads, no twist) in adherent fibroblasts bound to beads coated with RGD or AcLDL, with or without stress (Twist).

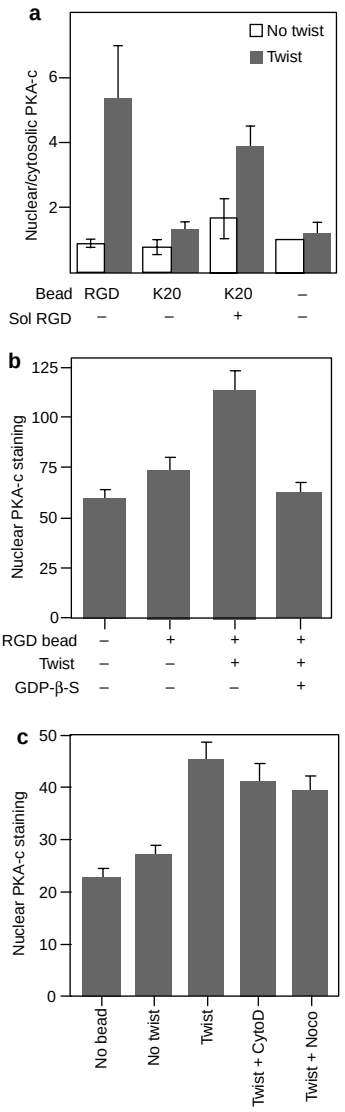

Figure 2 **Mechanistic analysis of integrin-dependent mechanotransduction. a**, Ratios of nuclear to cytosolic PKA-c quantitated in western blots of cells cultured with no beads (–), RGD–beads (RGD), K20–beads (K20), or K20–beads plus soluble RGD (Sol RGD), in the absence or presence of applied stress (Twist); data are normalized against the 'no bead, no twist' condition. **b**, Nuclear PKA-c staining in adherent cells cultured in absence (–) or presence (+) of RGD–beads, stress or 100 μM GDP-β-S, as indicated. **c**, Nuclear PKA-c staining in adherent cells cultured in the absence (No bead) or presence of RGD-coated beads without (No twist) or with (Twist) application of stress. In parallel, cells with bound RGD–beads were exposed to the same twisting stress in the presence of cytochalasin D (1 μg ml^{-1}; Twist + CytoD) or nocodazole (10 μM; Twist + Noco).

transcription. The RGD-ligand-binding site on integrins must also be occupied in order for the transferred mechanical energy to be transduced into changes in heterotrimeric G-protein activity which, in turn, trigger the cAMP signalling cascade within the focal adhesion complex. The finding that inhibition of total Gα activity completely prevented the activation of PKA by stresses transmitted across integrins, conflicts with the observation that fluid shear stress activates GTPase activity in synthetic liposomes containing heterotrimeric G proteins[1]; however, it is consistent with the rapid activation of G proteins induced by stretching the culture substrate (and thus, bound integrins) in cardiac fibroblasts and skeletal muscle cells[14,15]. Recently, integrin αVβ3 was shown to activate heterotrimeric G_i by forming a complex with the integrin-associated protein, CD47 (ref. 16). Ligated integrin β1 may similarly mediate stress-dependent activation of Gα proteins by associating with some form of linking protein at the site of integrin binding. These results also suggest that some responses reported to be induced by fluid-shear stresses in endothelial cells[17], including activation of G proteins[18], could result from transmission of mechanical forces from the apical surface to the cell's basal adhesion sites via cytoskeletal filaments, as previously observed[19]. The finding that an intact cytoskeleton was not required for activation of the cAMP cascade when stresses were applied directly to integrins is consistent with the physiological role of cAMP signalling in endothelial cells, where it acts as a 'safety valve' to physically disrupt the cytoskeleton and

thereby release tension on lateral cell–cell junctions when tissues experience high mechanical stress[20]. Cytoskeletal input does, however, appear to be required for high-order signal processing (integration of cAMP signals with other inputs) that are necessary for the control of more complex cell functions, such as cell-cycle progression[21]. □

Methods

Magnetic twisting system.

Controlled stresses were applied using magnetic twisting cytometry[3,5] with ferromagnetic microbeads (4.5 μm diameter, 10–20 beads per cell; Spherotech) that were coated with either RGD–peptide (Peptite 2000, Integra), activating anti-β1 integrin antibody (Biosource), AcLDL (Biomedical Technologies), or non-activating anti-β1 integrin antibody (K20, Beckmann-Coulter). The twisting stress (15.6 dyn cm^{-2} unless otherwise indicated) was applied for 60 s on/20 s off for 15 min in the original cAMP study (Fig. 1a) and constantly for 10 min in all other studies.

Analysis of the cAMP signalling cascade.

Cyclic AMP was analysed in cells incubated with 1 mM IBMX for 15 min before experimental manipulation using a commercial radioimmunoassay (Amersham). PKA-c translocation was analysed using digitized image analysis in conjunction with immunofluorescence microscopy in adherent cells that were extracted with a detergent (0.5% Triton X-100)-containing cytoskeleton stabilization buffer[4], fixed, and stained using rabbit polyclonal antibodies against PKA-c (provided by Susan Taylor, UCSD). Western blot analysis of cytosolic (detergent-releasable) and nuclear (detergent-resistant) extracts was carried out using anti-PKA-c antibody (Santa Cruz). Antibodies directed against pCREB and CREB[11] were provided by Michael Greenberg (Children's Hospital, Boston). When cells were treated with cytochalasin D or nocodazole, the drug was added for 30 min before stress application. Soluble RGD peptide (330 μM GRGDSP; Gibco-BRL) was added to activate integrins ligated with non-activating antibodies. GDP-β-S (Sigma) was delivered into the cytoplasm of suspended cells using pluronic F-68 detergent[22]. NIH3T3 fibroblasts were transiently co-transfected with plasmids bearing green fluorescent protein (EGFP; Clontech) and the *lacZ* gene under control of three tandemly arranged CRE elements (from M. Greenberg) using Lipofectamine reagent (Gibco-BRL); β-gal activity was visualized 48 h after transfection.

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