Integrin activation by Fam38A uses a novel mechanism of R-Ras targeting to the endoplasmic reticulum

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Summary
The integrin family of heterodimeric cell-surface receptors are fundamental in cell-cell and cell-matrix adhesion. Changes to either integrin-ligand affinity or integrin gene expression are central to a variety of disease processes, including inflammation, cardiovascular disease and cancer. In screening for novel activators of integrin-ligand affinity we identified the previously uncharacterised multitransmembrane protein Fam38A, located at the endoplasmic reticulum (ER). siRNA knockdown of Fam38A in epithelial cells inactivates endogenous β1 integrin, reducing cell adhesion. Fam38A mediates integrin activation by recruiting the small GTPase R-Ras to the ER, which activates the calcium-activated protease calpain by increasing Ca2+ release from cytoplasmic stores. Fam38A-induced integrin activation is blocked by inhibition of either R-Ras or calpain activity, or by siRNA knockdown of talin, a well-described calpain substrate. This highlights a novel mechanism for integrin activation by Fam38A, utilising calpain and R-Ras signalling from the ER. These data represent the first description of a novel spatial regulator of R-Ras, of an alternative integrin activation-suppression pathway based on direct relocalisation of R-Ras to the ER, and of a mechanism linking R-Ras and calpain signalling from the ER with modulation of integrin-ligand affinity.

Key words: R-Ras, Calpain, Cell adhesion, Endoplasmic reticulum, Integrins

Introduction
Integrin heterodimer complexes are cell adhesion receptors, with 24 α and β subunit variations in humans (Plow et al., 2000). Alterations to integrin function and/or expression is a common step in inflammation and cardiovascular disease (Clemetson and Clemetson, 1998; Hynes, 2002), and promote anchorage-independent growth, invasion and metastasis in cancer cells (Guo and Giancotti, 2004; Hood and Cheresh, 2002; Schwartz, 1997). Integrins respond to cytoplasmic signals through changes in integrin-ligand affinity, termed ‘inside-out’ signalling. The Ras GTPase family of cytoplasmic signalling proteins are strong modulators of integrin function. H-Ras, K-Ras and N-Ras suppress integrin affinity (Hughes et al., 1997), whereas R-Ras activates integrin affinity and increases cell adhesion (Sethi et al., 1999; Zhang et al., 1996). H-Ras signalling occurs via at least four downstream effectors: Raf, RalGDS, PI3-kinase and PLCε (Hingorani and Tuveson, 2003; Lad et al., 2006). Targeting of H-Ras molecules to the plasma membrane (PM) by post-translational modification of their C-termini has been implicated as essential for most of their biological activity, including their ability to modulate integrin-ligand affinity (Willumsen et al., 1984); Spaargaren et al., 1994). Post-translational modifications of R-Ras differ from those of H-Ras (geranyl-geranylation rather than farnesylation), which might reflect differential targeting and trafficking pathways.

The mechanism of integrin activation by R-Ras is not well understood. R-Ras can bind several of the same effectors as other Ras isoforms (e.g. Raf) (Spaargaren et al., 1994) and has also been shown to stimulate the PI3-kinase pathway; however, it does not activate Raf effectively, and blocking phosphoinositide 3-kinase (PI3K) activation does not affect integrin activation by R-Ras (Marte et al., 1997). Effectors specific to R-Ras have emerged only recently; R-Ras has been shown to signal through Rac and RLIP76 to mediate cell spreading (Goldfinger et al., 2006; Holly et al., 2005; Wozniak et al., 2005). Additionally, we have demonstrated that Notch-1 is an upstream regulator of R-Ras because Notch-1-induced integrin activation is blocked by dominant-negative R-Ras(T43N) (Hodkinson et al., 2007).

Regulation of Ras GTPase activity by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) has been extensively characterised over the past two decades, but recent demonstrations that Ras signalling emanates from discrete cytoplasmic and PM microenvironments has uncovered another level of regulation (Hancock, 2003). Endogenous Ras activity has been demonstrated at endomembranes such as the Golgi and endoplasmic reticulum (ER) (Chiu et al., 2002; Choy et al., 1999; Takaya et al., 2007). This spatial regulation of Ras pathway components can result in physiologically different outputs, differentially affecting PC12 cell differentiation (Inder et al., 2008). Although these studies have focused on H-Ras, K-Ras and N-Ras, they raise the intriguing possibility that R-Ras downstream effects on integrin affinity might also be dependent on subcellular localisation.

Interestingly, R-Ras can induce Ca2+ release from ER stores (Koopman et al., 2003) and a link has been established between R-Ras and the Ca2+-dependent protease calpain, demonstrating degradation of insulin receptor substrate 1 via R-Ras-stimulated calpain activity (Yu et al., 2006). Calpain proteases exist in two major forms in mammalian cells, calpain 1 (mu-calpain) and calpain 2 (m-calpain), with differing requirements for Ca2+ concentration. The 28-kDa regulatory subunit CapnS1 (also named calpain 4) is
required for activity of both forms. Calpains are involved in numerous signalling cascades and cleave a variety of cytoskeletal, membrane-associated, and regulatory proteins and have been implicated in platelet aggregation and regulation of focal adhesion turnover (Croce et al., 1999; Franco et al., 2004). Inhibition of calpain activity can result in decreased adhesion, reduced programmed cell death, and can promote cell invasion via an integrin-independent pathway (Carragher et al., 2006; Sawhney et al., 2006).

Novel genes that reverse the integrin suppression by H-Ras might define important activators of integrin affinity. We screened an activated monocyte cDNA library for genes that reversed H-Ras-mediated integrin suppression and identified Fam38A, a large multi-transmembrane domain protein residing at the ER. We show that depletion of Fam38A in epithelial cells causes suppression of endogenous β1 integrins, leading to cell detachment. Both the Ca²⁺-activated protease calpain and the GTPase R-Ras are required for Fam38A-mediated integrin activation. Moreover, we show that Fam38A spatially regulates R-Ras activity—a novel mechanism of integrin activation by recruitment of R-Ras to the ER.

Results
Identification of Fam38A from a screen for reversal of H-Ras suppression
A cDNA expression library derived from human activated monocytes (H4) was screened for genes capable of reversing H-Ras-mediated integrin suppression. To assess the state of integrin activation, we used the CHO(αβ−py) cell line, which stably expresses the chimeric integrin cßbα6αββββ1 (O’Toole et al., 1994). Activation or suppression of the CHO(αβ−py) chimeric integrin by transiently transfected proteins alters the binding of the ligand-mimetic antibody PAC-1, as monitored by flow cytometry (Fig. 1A), allowing calculation of an integrin activation index. This assay previously identified H-Ras, PEA15, CD98, PLC and Notch-1 as modulators of integrin affinity (Fenczik et al., 1997; Hodkinson et al., 2007; Hughes et al., 1997; Lad et al., 2006; Ramos et al., 1998).

CHO(αβ−py) cells were co-transfected with a constitutively active H-Ras mutant [H-Ras(G12V)] (Hughes et al., 1997) and the H4 cDNA library (screen layout is shown in supplementary material Fig. S1). Fig. 1A shows the gate used to flow-sort highly transfected cells that had high PAC-1 binding, indicating reversal of H-Ras(G12V)-induced integrin suppression. From screening pools of candidate cDNAs, the ~1 kb cDNA #480 showed consistent reversal of H-Ras-mediated integrin suppression (Fig. 1B). The sequence of cDNA #480 corresponded to the C-terminal region of a previously uncharacterised ~6.5 kb gene KIAA0233 (covering amino acids 1731-2009 of the predicted full-length product), shown in Fig. 2A. KIAA0233 has been described as the human orthologue of the rat gene Mib, which is upregulated in senile-plaque-associated astrocytes (Satoh et al., 2006), and codes for a large (2090 amino acids, >230 kDa) novel membrane protein identified as Fam38A in GenBank. Fam38A contains an N-terminal signal sequence and 24 transmembrane domains, suggesting that it might form a membranous pore or channel, and is conserved throughout multicellular eukaryotes. Analysis of the online expression databases Unigene (http://www.ncbi.nlm.nih.gov/unigene) and GeneNote (http://bioinfo2.weizmann.ac.il/cgi-bin/genenote/home_page.pl) shows that Fam38A is widely expressed in mammalian tissues, particularly lung, prostate, spleen and bone marrow, suggesting an important role for Fam38A in many cell types. Both cDNA #480 and full-length Fam38A promote activation of integrin affinity (Fig. 2B). We therefore created deletion constructs to remove the C-terminal end of Fam38A, including the region covered by cDNA #480 [Fam38A(ΔC)], corresponding to amino acids 1-1570), or to remove the single transmembrane domain from #480 [#480(ΔTM)], amino acids 1762-to 2009] (see Fig. 2A). Transient transfection of these constructs showed that Fam38A(ΔC) poorly rescues suppression, whereas #480(ΔTM) has no effect on H-Ras suppression in CHO(αβ−py) cells (Fig. 2B). Thus, the region of Fam38A corresponding to cDNA #480 contributes to integrin activation, when membrane anchored. We confirmed that transfection of Fam38A does not alter surface expression of the chimeric integrin in CHO(αβ−py) cells with an affinity-independent β3 integrin antibody (supplementary material Fig. S2).
Fig. 2. Fam38A protein organisation and integrin activation. (A) Scheme of full-length Fam38A, the cDNA clone #480 and deletion mutants Fam38A(ΔC) and #480(ΔTM), highlighting predicted transmembrane domains. (B) Activation indices (relative to vector-alone transfection) of constructs shown in A in CHO(α(β)-py) cells, without (white bars) or with (black bars) 0.5 μg H-Ras(G12V). Results show partial and complete rescue of H-Ras(G12V)-mediated integrin suppression by cDNA clone #480 and full-length Fam38A (1 μg), respectively, but not deletion constructs Fam38A(ΔC) or 480(ΔTM) (1 μg). Data shown represent mean ± s.e.m., n=3; **P<0.01.

**Fam38A siRNA decreases β1-integrin affinity in human epithelial HeLa cells**

We depleted Fam38A in epithelial HeLa cells by siRNA treatment. Four Fam38A siRNA oligos were tested in HeLa cells, of which two oligonucleotides (oligo#3 and oligo#4) led to successful knockdown of Fam38A compared with a non-targeting control duplex. Knockdown of mRNA levels was shown by RT-PCR, and protein levels were shown by western blot using a rabbit polyclonal antibody we raised to a C-terminal amino acid peptide sequence of Fam38A (Fig. 3A). Real-time PCR quantitation showed that Fam38A expression was reduced with oligo#3 by ~80%, and with oligo#4 by ~70% (Fig. 3B). Oligo#3 was therefore used subsequently, although oligo#4 depletion also resulted in similar phenotypes in all respects (data not shown).

We monitored endogenous β1-integrin affinity directly in HeLa cells using the affinity-dependent antibody CD29 clone HUTS-21. HUTS-21 was previously shown to be sensitive to the affinity state of β1 integrin (Escudero et al., 2000; Luque et al., 1996). We confirmed this in HeLa cells by treatment with EDTA and Mn2+ to show integrin inactivation and activation, respectively (Fig. 3C). Staining with an affinity-independent β1-integrin antibody CD29 clone K20 (unaffected by EDTA or Mn2+) confirmed that surface expression of β1 integrins was not affected (Fig. 3C). Activation indices were thus calculated directly in HeLa cells similarly to in our CHO(α(β)-py) assay.

We next tested the effect of Fam38A transfection and siRNA treatment on the binding of HUTS-21. Transient transfection of Fam38A increased β1-integrin affinity, whereas depletion of Fam38A by siRNA treatment significantly reduced the activation of β1 integrins on HeLa cells (Fig. 3D). Transient transfection of activated H-Ras(G12V) or active R-Ras(G38V) suppressed and increased integrin affinity, respectively, as expected. Fig. 3E shows representative flow cytometry histograms of control and siRNA-treated live cells stained with either HUTS-21 or K20, and confirms that siRNA depletion of Fam38A did not alter surface expression of β1 integrins.

**Fam38A depletion by siRNA treatment decreases HeLa cell adhesion**

We assessed phenotypic changes in Fam38A-depleted epithelial cells. Fam38A depletion in HeLa cells resulted in large areas of detached cells by 72 hours (Fig. 4A), not seen in control cells (Fig. 4B). Additionally, adherent cells displayed a phenotype consistent with loss of adhesion along the cell periphery in wells treated with Fam38A siRNA (Fig. 4C) compared with control oligo-treated cells (Fig. 4D). Confocal microscopy of paxillin staining showed that siRNA-treated cells had aberrantly organised focal adhesions (Fig. 4E,G) compared with control siRNA cells (Fig. 4F,H). Cell adhesion was quantitated by methylene blue staining, showing that Fam38A-depleted HeLa cells had 49±3% adhesion after 72 hours, compared with control oligo, which had reached confluence. Similar results were seen in normal lung epithelial 16-HBE cells, where Fam38A siRNA resulted in 45±5% loss of cell adhesion after 72 hours compared with control oligo. These results demonstrate that depletion of Fam38A by siRNA treatment results in loss of cell adhesion in epithelial cells.
To confirm that the loss of cell adhesion was due to integrin inactivation, we treated Fam38A-depleted HeLa cells with the β1-integrin-activating antibody TS2/16. Addition of 2.5 μg/ml TS2/16 rescued Fam38A-depleted cell detachment at 72 hours (Fig. 4I-J) and 96 hours (Fig. 4K-L), but had no effect on control oligo-treated cells (Fig. 4M-N). Adhesion was quantified by methylene blue staining at 96 hours, showing that TS2/16 significantly rescued adhesion from 42±2% in Fam38A siRNA to 76±3%. This rescue demonstrates that cell detachment caused by Fam38A depletion was due to integrin inactivation. External stimulation of integrins can therefore override the cytoplasmic-induced inactivation caused by Fam38A knockdown, implicating Fam38A in an inside-out signalling pathway.

We quantified apoptosis levels in cells detached following Fam38A siRNA treatment, by staining with acridine orange followed by fluorescence microscopy, and by measuring Annexin V labelling using flow cytometry. Fam38A siRNA wells contained approximately four times more detached cells than control oligo-treated wells. Acridine orange staining of these detached cells showed a significantly greater number of live cells in the Fam38A-depleted population (53.7±3.1%) compared with the control oligo-treated population (22.9±2.5%). Annexin V staining of the detached cell population gave a similar result: 56.2% (±6.7%) live cells with Fam38A siRNA, compared with 29.5±2.3% live cells in control siRNA wells. Loss of cell adhesion by Fam38A depletion is therefore not due to increased levels of cell death; in contrast to control cells that are forced to detach by other methods, e.g. by integrin-blocking antibodies, which undergo anoikis (not shown). The results shown in Figs 3,4 therefore confirm that Fam38A regulates β1-integrin affinity in HeLa cells, and demonstrate that integrin inactivation is directly responsible for the cell adhesion defects observed by Fam38A depletion.

Fam38A localises to the ER
Both full-length Fam38A and the #480 cDNA were C-terminally Myc-epitope- and GFP-tagged, and localised by immunofluorescence microscopy. In transiently transfected CHO-K1 cells, Fam38A localisation was consistent with observations of the rat homologue to Fam38A, Mib, which was shown to be at the ER (Satoh et al., 2006) (Fig. 5A). ER localisation was confirmed using ER-tracker (Invitrogen) (Fig. 5B). GFP-tagged #480 also localised to the ER, as did the Fam38A(ΔC) construct (amino acids 1-1570 of Fam38A) in CHO(αβ-py) cells (by colocalisation with SERCA2, Fig. 5B). However, the #480(ΔTM) construct, containing no transmembrane domains, localised as cytoplasmic particles (Fig. 5B) and was incapable of rescuing integrin suppression (see Fig. 2B). Our antibody staining of endogenous Fam38A demonstrated a similar localisation to the ER in human HeLa cells (Fig. 5B), ruling out overexpression artefacts in localisation of our tagged Fam38A constructs.

Fam38A depletion reduces Ca2+ release from the ER and reduces calpain activity
We demonstrated that Fam38A is a multi-transmembrane spanning protein localised at the ER. Bioinformatics analysis of Fam38A indicated it might function as a channel or pore, and that its 24 membrane domains are consistent with the structure of Ca2+ channels. Increased Ca2+ release from the ER activates numerous downstream signalling pathways, and in particular causes activation of the calcium-dependent protease calpain. Calpain complexes contain a small subunit, CapnS1 (or calpain 4), which is essential for activity (Fig. 6A). We therefore examined the effect of Fam38A depletion on release of Ca2+ homeostasis. Ca2+ release (stimulated by Thapsigargin) was monitored over time by ratiometric analysis of Fura2-AM in control and Fam38A-siRNA-treated HeLa cells.
CaCl$_2$ was then added to examine re-uptake of external Ca$^{2+}$. Fig. 6B shows that Fam38A depletion reduces the rate of Ca$^{2+}$ release compared with control cells after Thapsigargin stimulation (control, 14.74±1.3 nM/second; Fam38A siRNA, 3.60±0.7 nM/second). After addition of CaCl$_2$, the amount of Ca$^{2+}$ uptake by Fam38A-depleted cells was also reduced to 58.3±4.2% relative to control cells. This observed reduction suggests an overall reduction in extracellular Ca$^{2+}$ uptake across the PM. Although we did not detect Fam38A localisation at the PM (Fig. 5), we speculate that Fam38A-induced effects on R-Ras activity or on other as-yet-undefined interactors might be responsible for these observations. Curves were integrated to calculate the area under the curve (Fig. 6C).

Because we had confirmed that Fam38A depletion reduces the rate of Ca$^{2+}$ release from the ER, we investigated whether Fam38A depletion reduced activity of the Ca$^{2+}$-activated protease calpain. Calpain activity was initially analysed in Fam38A-depleted HeLa cells using the substrate EDANS-EPLFAERK-DABCYL (DABCYL) known to be more specific for calpain 1 (Cuerrier et al., 2005). Using a fluorescent microplate reader, calpain activity was quantified in Fam38A-depleted cells and showed a reduction to 60±2% of control activity (Fig. 6D). Western blot analysis confirmed that levels of expression of both calpain 1 and 2 were unaffected by Fam38A depletion (Fig. 6E).

We subsequently used a second substrate, CMAC (t-BOC-Leu-Met-CMAC), which has been widely used to detect general calpain activity (Glading et al., 2001; Lukuta et al., 2003; Rosser et al., 1993). Viable adherent cells were incubated with substrate 72-96 hours after Fam38A or control oligo siRNA treatment, and CMAC cleavage was observed by confocal microscopy and quantitated by plate reader (supplementary material Fig. S3). In all cases, calpain activity was significantly reduced in Fam38A-depleted cells – a 74±3% decrease in total substrate fluorescence compared with controls. The finding that Fam38A depletion produced a greater suppression of calpain activity measured by CMAC (a substrate of both calpain 1 and calpain 2) suggests possible effects on calpain 2. However, this would need to be determined using a specific calpain 2 substrate, which is not currently available.

Substrate cleavage was also quantified in cells treated with the specific calpain inhibitors PD150606 and MDL28170. As expected, both calpain inhibitors significantly reduced the amount of substrate cleavage (Fig. 6D). To confirm our inhibitor results, we knocked down HeLa calpain activity by siRNA treatment. We used a pre-validated siRNA oligo targeted to CapnS1 to disrupt both calpain 1 and 2 activity simultaneously, a strategy based on a previously published mouse knockout (Arthur et al., 2000). Fig. 6E shows immunoblotting of HeLa extracts treated with CapnS1 siRNA, validating the knockdown. HeLa cells treated with CapnS1 siRNA showed a ~45% decrease in calpain activity (using DABCYL as above), similar to levels seen with MDL28170. Some residual calpain activity is expected because the CapnS1 siRNA is not a 100% knockout.

**Fam38A expression relocalises the small GTPase R-Ras to the ER**

We investigated the small GTPase R-Ras as a candidate effector for Fam38A-induced integrin activation for several reasons: R-Ras contributes to Ca$^{2+}$ release from ER stores (Koopman et al., 2003), it can induce activity of the Ca$^{2+}$-dependent protease calpain (Yu et al., 2006), and is a strong activator of integrin affinity (Sethi et al., 1999). We tested whether Fam38A expression influenced either R-Ras localisation or activation. Strikingly, Fam38A expression caused R-Ras(G38V) to completely relocalise to the ER (Fig. 7A,B). Identical effects were seen with wild-type R-Ras and dominant-negative R-Ras(T43N) (supplementary material Fig. S4). Fam38A
expression had only a partial effect on H-Ras localisation and no effect on another GTPase, RalA (Fig. 7A,B), demonstrating that Fam38A preferentially affects R-Ras.

Chimeric constructs of H-Ras and R-Ras have previously been used to determine the regions of these proteins responsible for integrin-activating properties to H-Ras (Hansen et al., 2002; Hughes et al., 2002), and indicate that this same region is also required for localisation to the ER.

**Calcium release and calpain activity in Fam38A-depleted cells are rescued by expression of active R-Ras**

Following our results on Fam38A-induced relocalisation of R-Ras to the ER, we examined what effect R-Ras constructs had on Ca\(^{2+}\) release and calpain activity. We measured whether transfection of active R-Ras(G38V) stimulated Ca\(^{2+}\) release from ER stores in our system, and whether active R-Ras(G38V) could override the reduced rate of Ca\(^{2+}\) release in Fam38A-siRNA-depleted HeLa cells. We used the fluorescent Ca\(^{2+}\) indicator Fluo-4 for greater sensitivity in measuring Ca\(^{2+}\) release at early timepoints (i.e. during the first 20 seconds after addition of Thapsigargin). Fluorescence increase over time was measured following Thapsigargin stimulation directly in adherent live cells with a microplate reader. Linear regression was performed on the resulting curves to calculate the rate of Ca\(^{2+}\) release.

Fig. 8A confirms that transient transfection of Fam38A or R-Ras(G38V) enhances the rate of Ca\(^{2+}\) release in HeLa cells stimulated with Thapsigargin, whereas transfection of dominant-negative R-Ras(T43N) prevents efficient Ca\(^{2+}\) release. We confirmed that siRNA depletion of Fam38A in HeLa cells causes a marked reduction in the early rate of Ca\(^{2+}\) release from cytoplasmic stores compared with control cells. However, after subsequent transfection of Fam38A-depleted cells with R-Ras(G38V), rates of Ca\(^{2+}\) release were significantly higher than for Fam38A-siRNA-only cells. By contrast, subsequent transfection of dominant-negative R-Ras(T43N) showed no increase in the rate of Ca\(^{2+}\) release compared with siRNA-only cells. Thus, the effects of Fam38A depletion on Ca\(^{2+}\) release are partially rescued by overexpression of active R-Ras.

We next examined calpain activity after transiently transfecting Fam38A, R-Ras(G38V) and R-Ras(T43N) into HeLa cells and quantifying calpain 1 activity by fluorescence of the substrate DABCYL. Cells transfected with either R-Ras(G38V) or Fam38A had a significant increase in substrate cleavage compared with control vector-transfected cells, whereas R-Ras(T43N) did not (Fig. 8B). Calpain activity was also examined in Fam38A-siRNA-treated HeLa cells subsequently transfected with either R-Ras(G38V) or R-Ras(T43N). We confirmed that Fam38A-siRNA-treated cells had reduced calpain activity (as in Fig. 6). However, subsequent transfection of R-Ras(G38V) stimulated calpain activity. Subsequent transfection with R-Ras(T43N) did not show any significant increase above calpain activity levels seen with Fam38A-siRNA alone (Fig. 8B). These results demonstrate that in Fam38A-depleted cells, where recruitment of active R-Ras to the ER is reduced, calpain activity is drastically reduced as a consequence of less Ca\(^{2+}\) release. However, subsequent overexpression of active R-Ras can partially rescue the Fam38A-depletion effect and re-stimulate calpain activity.

We investigated whether expression of Fam38A in CHO-K1 cells directly activated wild-type R-Ras by measuring binding of activated R-Ras to beads containing the Ras-binding domain (RBD) of Raf. Fig. 8C shows that co-transfection of Fam38A with wild-type R-Ras does not increase R-Ras activity above levels seen in wild-type R-Ras-only transfections (Fig. 8C). We also investigated whether inhibition of calpain activity prevented R-Ras activation.
cells transfected with wild-type R-Ras were pre-incubated with or without PD150606 for 30 minutes, then serum-shocked for 30 minutes to induce R-Ras activation. Fig. 8C shows that inhibition of calpain has no effect on serum-induced activation of R-Ras. R-Ras(G38V)-transfected lysate was used as a positive control for the binding assay. These results indicate that neither Fam38A nor calpain activity directly affect R-Ras activation, inferring that it is predominantly the localisation of R-Ras that is crucial to the effects we observe.

Finally, we sought to determine whether increased Fam38A expression results in endogenous Ras activity at the ER in the absence of transiently transfected R-Ras constructs. We used a GFP-tagged version of the Raf RBD domain (GFP-RBD) as a reporter construct in CHO(αβ-py) cells to examine endogenous Ras activity in the presence of transfected Fam38A. We demonstrated that in cells transfected with GFP-RBD only, endogenous Ras activity was detected predominantly within the nucleus, but also in the cell cytoplasm and at the PM (Fig. 8D). However, when co-transfected with Fam38A in CHO(αβ-py) cells, the GFP-RBD construct predominantly colocalised with Fam38A at the ER (Fig. 8D). R-Ras must account for the majority of endogenous Ras activity that we observed at the ER in Fam38A co-transfected cells, as other GTPases were not localised so completely (see Fig. 7). These results imply that endogenous R-Ras is predominantly recruited to the ER in its active state, although Fam38A is not directly involved in its activation.

**Fam38A activation of integrins is dependent on R-Ras, calpain and talin activity**

Given our results on R-Ras localisation, we confirmed previous results showing that constitutively active R-Ras(G38V) strongly stimulated integrin activation (Sethi et al., 1999), using CHO(αβ-py) cells. Co-transfection of Fam38A and R-Ras(G38V) demonstrated enhanced integrin activation above the levels of R-Ras(G38V) alone (Fig. 9A). However, integrin activation by Fam38A was abolished when co-transfected with dominant-negative R-Ras(T43N) (Fig. 9A). Western blotting confirmed transient expression of all constructs above (supplementary material Fig. S5). Fig. 8B demonstrated that Fam38A and active R-Ras increased calpain activity. We next examined the effects of specific calpain inhibition on integrin activation by Fam38A and R-Ras(G38V) in CHO(αβ-py) cells. Pre-incubation of vector-only transfected cells with the calpain inhibitor PD150606 resulted in integrin inactivation, similar to H-Ras(G12V) transfection (Fig. 9B). A second inhibitor MDL28170 produced similar results (supplementary material Fig. S6). Strikingly, calpain inhibition also blocked the integrin activation induced by Fam38A transfection (Fig. 9B), confirming the requirement of calpain activity for Fam38A-mediated integrin activation. Both PD150606 and MDL28170 had little or no effect on R-Ras(G38V)-induced integrin inactivation (Fig. 9B; supplementary material Fig. S6). However, when R-Ras(G38V) and Fam38A were co-transfected (which causes R-Ras to localise predominantly to the ER, previously demonstrated in Fig. 7), the ability of R-Ras(G38V) to rescue PD150606-induced integrin inactivation was significantly reduced. Thus, active R-Ras (when transiently overexpressed) can compensate for calpain inhibition of integrin affinity by using other cytoplasmic pathways to achieve integrin activation, but is restricted in doing so when targeted to the ER by Fam38A.

We next tested the effect of CapnS1 siRNA on the binding of the HUTS-21 integrin affinity antibody in HeLa cells. Fig. 9C shows that depletion of CapnS1 by siRNA treatment significantly reduces
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the activation of β1 integrins on HeLa cells. Subsequent transfection of R-Ras(G38V) but not Fam38A rescued this inactivation. However, co-transfection of R-Ras(G38V) and Fam38A was again only weakly able to rescue integrin inactivation induced by calpain inhibition. We also confirmed that the calpain inhibitor PD150606 strongly inactivated β1 integrins in HeLa cells.

As calpain cleavage of talin has been shown to play an important role in modulation of integrin affinity, we tested whether talin was also required for Fam38A-induced integrin activation. Talin expression was knocked down by >70% in HeLa cells by siRNA treatment, and subsequent staining with HUTS-21 antibody showed a corresponding decrease in β1-integrin affinity (Fig. 9D). As with CapnS1 siRNA, subsequent transfection of Fam38A was incapable of rescuing integrin suppression induced by siRNA knockdown of talin, confirming that talin is required as the final step linking Fam38A and calpain activity to integrin activation. Importantly, subsequent transfection of R-Ras(G38V) in talin siRNA cells did not rescue integrin inactivation either, unlike CapnS1 siRNA (Fig. 9C), demonstrating the central role of talin in cytoplasmic integrin activation. Supplementary material Fig. S7 confirms that siRNA targeting CapnS1 or talin did not alter surface expression of β1 integrins.

Discussion

Fam38A plays a key role in epithelial cell adhesion by maintaining integrin activation – Fam38A siRNA in epithelial cells leads to decreased cell adhesion through integrin inactivation. Fam38A siRNA phenotypes such as loss of adhesion along the cell periphery, unorganised focal adhesions, and prolonged viability correspond with reduced calpain activity in these cells. We propose that normally in adherent epithelial cells Fam38A helps to maintain integrin activation through R-Ras recruitment to the ER, most probably in its activated state, and subsequent stimulation of calpain signalling (Fig. 10A). In Fam38A-siRNA-treated cells this R-Ras–calpain signalling mechanism is not induced, resulting in decreased integrin affinity and loss of cell adhesion (Fig. 10B). This model is supported by data showing that Fam38A recruits R-Ras to the ER and affects Ca²⁺ release from cytoplasmic stores and that,
in turn, R-Ras(T43N) and calpain inhibitors block Fam38A-mediated integrin activation.

The recruitment of R-Ras by Fam38A appears relatively specific – other GTPases are not similarly recruited (e.g. H-Ras relocation is only partial, and RalA is unaffected). Our use of H-Ras/R-Ras chimeric constructs demonstrates that the C-terminus of R-Ras is sufficient to promote relocation to the ER by Fam38A. This agrees with previous work demonstrating that this C-terminal region of R-Ras confers integrin-activation properties to H-Ras (Hansen et al., 2002; Hughes et al., 2002). Specifically targeting H-Ras to the ER by fusion to a specific targeting sequence does not alter its ability to suppress integrins or to activate the downstream MAPK pathway [our own observations, and Chiu et al. (Chiu et al., 2002)], so the reversal of H-Ras-induced integrin suppression by Fam38A is not due to sequestration of H-Ras away from the PM. Additionally, coexpression of Fam38A does not affect the ability of H-Ras to mediate phosphorylation of extracellular regulated kinase (ERK) (not shown), suggesting that Fam38A does not inhibit H-Ras function, nor does it affect localisation of ERK2. This is in contrast to PEA15-mediated integrin suppression (Formstecher et al., 2001). Dominant-negative R-Ras(T43N) blocks Fam38A activation of integrins in CHO(αβ-pp) cells, whereas Fam38A-mediated integrin activation is enhanced by constitutively active R-Ras(G38V). Recruitment of R-Ras does not rely on its activation state because Fam38A recruits all isoforms of R-Ras to the ER, and does not directly activate R-Ras. However, because our GFP-RBD data demonstrates increased levels of active Ras at the ER after Fam38A transfection, there might be preferential recruitment of the endogenous active R-Ras by Fam38A in vivo. These data provide strong evidence that Fam38A coordinates an R-Ras-dependent pathway to increase integrin affinity.

Although no obvious RBD exists in Fam38A these domains are poorly characterised for R-Ras specificity. Alternatively, Fam38A interaction might be indirect through a complex containing R-Ras at the ER. However, our data suggests that the C-terminal portion of Fam38A (the #480 region) could be responsible for R-Ras recruitment because this fragment is capable of relocating R-Ras to the ER. #480 also stimulates Ca2+ release and increased calpain activity (supplementary material Fig. S8). These data suggest that although it is unlikely that the #480 fragment is functional on its own, by recruiting R-Ras to the proximity of either endogenous full-length Fam38A or, potentially, other ion channels at the ER, #480 is still able to activate integrins via this recruitment. Thus, Fam38A functionality in R-Ras recruitment might be more important than its role as a putative membrane pore with regard to integrin activation by this pathway.

Ras activity at both the ER and Golgi endomembranes has been well characterised (Choy et al., 1999; Chiu et al., 2002), and R-Ras has been shown to increase Ca2+ leak across the ER (Ada-Nguema et al., 2006; Koopman et al., 2003). In turn, Ca2+ release from cytoplasmic stores can promote signalling pathways, e.g. inducing the Ca2+-dependent protease calpain stimulates cytoplasmic signalling and promotes activation of integrins. We observed that Fam38A depletion results in reduced Ca2+ flux from cytoplasmic stores, that Fam38A recruits R-Ras to the ER, and that R-Ras(G38V) overrides the reduced calcium release by Fam38A siRNA, highlighting the importance of R-Ras in Fam38A-induced calcium flux. Calpain promotes cleavage of talin (Franco et al., 2004) and regulates the turnover of adhesion complexes, leading to migration (Bhatt et al., 2002). Others have shown that platelet aggregation (mediated by αIIbβ3 integrins) is blocked by chemical inhibitors of calpain (Croce et al., 1999). Our data supports these findings – reduction of calpain activity by chemical inhibitors or siRNA-mediated depletion significantly inhibits native integrin affinity and Fam38A-mediated integrin activation in CHO(αβ-pp) and HeLa cells. Furthermore, we show that Fam38A-depleted HeLa cells have much reduced calpain activity, are much less adherent, and that this loss of adherence is a phenomenon that is related to integrin affinity. Finally, we show that talin is required as the final link between Fam38A, calpain activity and integrin activation.

Evidence for R-Ras acting upstream of calpain activity in this signalling cascade is demonstrated by R-Ras(G38V) stimulation of both Ca2+ release and calpain activity, whereas R-Ras(T43N) blocks these effects. Although R-Ras(T43N) does reduce Ca2+ release by approximately 50% compared with control cells, in our hands we do not see a comparable decrease in calpain activity [Fig. 8B shows that R-Ras(T43N) only slightly inhibits calpain activity compared with controls]. We believe that this discrepancy arises from limitations in the specificity of our calpain assay because the DABCYL substrate predominantly measures calpain 1 activity and not overall calpain activity. Interestingly, we did not see inhibition of R-Ras(G38V)-mediated integrin activation by calpain inhibitors. However, a significant amount of activation (~40%) by R-Ras(G38V) targeted to the ER [by Fam38A/R-Ras(G38V) co-transfection] was blocked by calpain inhibition. We believe that this demonstrates the ability of R-Ras to interact with multiple downstream effectors (as occurs with H-Ras), and that targeting of R-Ras to the ER might sequester it from some but not all cytoplasmic signalling pathways. The remaining activation that we do observe might be accounted for by overexpression of the transiently transfected plasmid, allowing a certain amount of interaction with non-ER related effectors.

Further work will identify calpain subunit specificity in this pathway. Some of the integrin-related phenotypes of calpain activity
are calpain-2-mediated events, and thus far we have been unable to specifically demonstrate lack of calpain 2 activity following Fam38A depletion. Additionally, Carragher and colleagues showed that calpain inhibition causes a switch to an integrin-independent ameboid invasion-migration pathway (Carragher et al., 2006). Indeed, ameboid cell migration has been shown as an alternative to both adhesion- and proteolytic-dependent mechanisms (Friedel and Wolf, 2003), a possibility that remains to be explored for Fam38A-depleted cells. Interestingly, previous work has shown that calpain inhibition also contributes to suppression of programmed cell death in a variety of cells by preventing activation of the pro-apoptotic factor BAX (Altznauer et al., 2004; Gao and Dou, 2000; Squier and Cohen, 1997; Wood et al., 1998), providing a mechanism to explain the prolonged viability seen in Fam38A-siRNA detached cells.

In conclusion, we demonstrate that the evolutionarily conserved multi-transmembrane protein Fam38A regulates integrin-ligand affinity via a novel mechanism of R-Ras recruitment to the ER. Future work will determine whether this spatial regulation results in differing physiological outcomes.

Materials and Methods

Antibodies

Fam38A rabbit polyclonal antibodies were generated from the peptide sequence RAPNGPEANPVKQLC (Genosphere). TS2/16 β1-integrin activating antibody was purified from a hybirdoma clone (ATCC). PAC-1 (αIIbβ3-activation specific) was from BD Biosciences. Anti-Tac-α5 antibody (ACT-1 RPE) and mouse anti-Cd29 (B3 integrin) was from Serotec. Mouse anti-Cd29 (HUTS-21) conjugated to phycoerythrin (PE) was from BD Biosciences. Mouse anti-caldesmon antibody from Calbiochem was applied.

DNA constructs

Tac-α5, H-Ras and R-Ras constructs have been described previously (Sethi et al., 1999; Lad et al., 2006). A cDNA expression library derived from human activated smooth muscle cells (obtained from HUGE, Kazusa, Japan) subcloned into pcDNA3.1. C- terminal Myc- or GFP-tags were added by PCR. Fam38A (ΔC) was created using an internal KpnI restriction site, and #480(ΔTM) by PCR. RafS1220-GFP was a gift of Tomas Balla (NICHD, Bethesda, MD). H-Ras and R-Ras chimera constructs H-Ras(147)-R-Ras(175-218) and R-Ras(174)H-Ras(148-189) were from Mark Ginsberg (Scripps Institute, La Jolla, CA). All constructs were verified by double-stranded sequencing.

Cell lines and transfection

Cell lines were maintained in DMEM [CHO-K1, 16-HBE (ATCC)] or RPMI [HeLa (CD29(IIb)] containing 1:25 anti-mouse IgM-FITC (for PAC-1) for 30 minutes at 37°C in buffer alone, then analysed using a BioTek Gen5 microplate reader. Calcium release was stimulated with 10 mM Thapsigargin. 1.2 mM CaCl2 was subsequently added to monitor Ca2+ re-uptake. Cells were then lysed in 1% Triton X-100, and Ca2+ ions then chelated by addition of 10 mM EGTA to acquire Rmax and Rdyn values. [Ca2+]i was calculated using FL Winlab software.

Cadmium release in siRNA treated and transfected cells

All conditions were performed in parallel in 48-well Costar plates, 2×104 cells per well. Fluo-4 (Invitrogen) was incubated with cells for 30 minutes at 37°C in Tyrodes buffer minus calcium. Cells were subsequently washed for 30 minutes at 37°C in buffer alone, then analysed using a BioTek Gen5 microplate reader. Calcium release was stimulated with Thapsigargin as above, and a kinetic assay set-up was used to monitor fluorescence increase in the relevant plate wells over time. Linear regression analysis was performed on each output, using GraphPad Prism software, to calculate the rate of fluorescence increase.

Calpain activity

Cleavage of 2.5 μM EDANS-EPLFAERK-DABCYL (Calbiochem) was monitored by fluorimetry in live cells for 30 minutes at 37°C in a BioTek Gen5 microplate reader; excitation 335 nm, emission 500 nm. Calpain inhibitors PD150606 (Calbiochem) and MDL28170 (Sigma-Aldrich) were used at 100 μM and 200 μM, respectively. Cleavage of 3-BOC-Leu-Met-CMAC (Invitrogen) was monitored in live cells plated on coverslips incubated with 30 nM substrate in RPMI media for 1 hour at 37°C. Cells were then fixed in 3% paraformaldehyde for 5 minutes, mounted on slides and viewed by confocal microscopy as above.

R-Ras activation assay

Active R-Ras binding to the RBD of Raf was measured as described (Hodkinson et al., 2007). Briefly, CHO-K1 cells were transfected with Myc-tagged wild-type R-Ras with or without test DNA (or R-Ras-G8V) as a positive control, quiesced in serum-free media for 24 hours before assay, and treated as described above. Cell lysates were incubated with GST-Raf-RBD coupled to glutathione agarose beads for 2 hours at 4°C, washed with lysis buffer. Bound protein was eluted by boiling for 5 minutes. Bound (i.e. active) R-Ras was detected by western blot for Myc.

Other methods

Adhesion assays, methylene blue staining, acridine orange staining, Annexin V labelling, gel electrophoresis and western blotting were performed as described (Hodkinson et al., 2007).

Statistical analysis

Data were analysed by one-way ANOVA and the appropriate post-test analyses were applied. P-values <0.05 were considered to be significant.


Figure S1: Flow diagram of CHO(αβ-py) screen for identifying cDNA clones that rescue H-Ras(G12V)-induced integrin suppression, highlighting the process whereby cDNA clone #480 was identified.
Figure S2: Representative flow cytometry dot blots of CHO(αβ-py) cells transfected with either vector only control or Fam38A and stained with a beta3 integrin affinity independent antibody. GeoMean values shown in top right corners. No difference in the level of surface integrin expression is detected in Fam38A-transfected cells compared to control.
Figure S3: Confocal microscopy of fluorescent calpain substrate CMAC in control and Fam38A siRNA-treated HeLa cells showing representative levels of calpain activity. Cell outlines (dashed lines) shown to demonstrate relatively equal cell size and highlight disrupted morphology in Fam38A siRNA cell. Scale bar = 5μm. Quantitation of CMAC cleavage in control and Fam38A-depleted cells, using Leica SP5 confocal software (Mean +/- SEM from 3 separate experiments, n= 30 cells per condition for each), and DABCYL cleavage by fluorimetry (Mean n=3, +/- SEM). (p < 0.05).
Figure S4:
A) Confocal microscopy of CHO-K1 cells transiently transfected with Myc-labeled R-Ras(WT) or R-Ras(T43N) and co-transfected with GFP vector only.
B) CHO-K1 cells transfected as in (A) and co-transfected with Fam38A-GFP. Both R-Ras(WT) and dominant negative R-Ras(T43N) are recruited to the ER when Fam38A is overexpressed.
Figure S5: Western blot of CHO(αβ-py) cells transiently transfected with H-Ras(G12V), R-Ras(WT), R-Ras(G38V), R-Ras(T43N), +/- Fam38A.myc. R-Ras isoforms and Fam38A detected with anti-Myc staining, H-Ras detected with anti-HA staining, anti-actin shown as loading control.
Figure S6 - Effects of calpain inhibitor MDL28170 on integrin activation in CHO(αβ-py) cells, transfected with the constructs shown. N=3, +/- SEM.
Figure S7: Flow cytometry histograms compare HUTS-21 and K20 binding in control, CapnS1 siRNA, or Talin1 siRNA treated HeLa cells. GeoMean values shown in top right corners of plots.
Figure S8 - *Upper panels* - Confocal microscopy of HeLa cells transiently co-transfected with #480 construct and R-Ras(G38V), showing co-localization at the ER. Scale bar = 5μm. *Lower panels* – Transient transfection of #480 in HeLa cells, showing increased Ca^{2+} release from cytoplasmic stores, and increased calpain activity using the DABCYL.
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