Quantification of integrin receptor agonism by fluorescence lifetime imaging

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Summary
Both spatiotemporal analyses of adhesion signalling and the development of pharmacological inhibitors of integrin receptors currently suffer from the lack of an assay to measure integrin-effector binding and the response of these interactions to antagonists. Indeed, anti-integrin compounds have failed in the clinic because of secondary side effects resulting from agonistic activity. Here, we have expressed integrin-GFP and effector-mRFP pairs in living cells and quantified their association using fluorescence lifetime imaging microscopy (FLIM) to measure fluorescence resonance energy transfer (FRET). Association of talin with β1 integrin and paxillin with α4 integrin was dependent on both the ligand and receptor activation state, and was sensitive to inhibition with small molecule RGD and LDV mimetics, respectively. An adaptation of the assay revealed the agonistic activity of these small molecules, thus demonstrating that these compounds may induce secondary effects in vivo via integrin activation. This study provides insight into the dependence of the activity of small molecule anti-integrin compounds upon receptor conformation, and provides a novel quantitative assay for the validation of potential integrin antagonists.

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Introduction
Integrins are cell-adhesion receptors that provide physical support for tissues and enable directed migration during development and tissue homeostasis (van der Neut et al., 1996; Wagner et al., 1996). At the cellular level, integrins spatially compartmentalise signalling events by tethering the contractile cytoskeleton to the plasma membrane and indirectly modulating multiple signalling networks. In mammals, genes encoding 18 α and 8 β integrins produce polypeptides that combine to form 24 heterodimeric receptors (Hynes, 2002), 12 of which contain the β1 subunit. Both subunits are noncovalently associated, type I transmembrane proteins with large extracellular and mostly short cytoplasmic domains. In recent years, substantial progress has been made towards defining the conformational changes that underpin integrin affinity regulation and identifying the effector proteins that initiate integrin signalling (Luo et al., 2007; Humphries, 2000). Transmembrane or cytoplasmic domain separation, triggered either by the binding of FERM domain-containing cytoplasmic proteins (such as talin and myosin X) (Zhang et al., 2004; Tadokoro et al., 2003; Garcia-Alvarez et al., 2003) or extracellular ligands, is currently thought to be the mechanism for the bidirectional transmembrane signal transduction that regulates adhesion (Kim et al., 2003).

In patients with inflammatory and neoplastic diseases, aberrant integrin function perturbs cellular trafficking and causes dysregulation of cellular differentiation (Mousa, 2002). Within the past decade, the first generation of anti-integrin drugs has been approved for human therapy (Leclerc, 2002). Some of these agents are small molecule mimetics of the acidic peptide active sites found in most integrin ligands (e.g. RGD and LDV). Although these agents are potent, competitive inhibitors of integrin-ligand binding in vitro and in vivo, it is now evident that they frequently retain the agonistic properties of their parent ligands. This activity can lead to biological side effects, such as platelet dysfunction, and can consequently impair the drug development process (Hantgan et al., 2007). As a result, there is a pressing need for a reporter assay to measure the agonistic activity of integrin-binding ligands and small molecules in situ. Here, we present such a system, which uses fluorescence resonance energy transfer (FRET) to measure direct integrin-effector binding in intact cells. This assay also has the potential to detect and quantify integrin signalling during normal biological processes, such as migration and differentiation.

Results and Discussion
Initially, a full-length human β1 integrin construct was C-terminally tagged with green fluorescent protein (GFP). Although other β-integrin-GFPs have been described (Ballestrem et al., 2001), β1-integrin-GFP has not been previously generated because the ubiquitous distribution and high endogenous expression of β1 integrin in most cells makes it difficult to express. This construct was therefore stably expressed to endogenous levels in immortalised β1-integrin-null mouse embryonic fibroblasts.
and its functional activity tested by a combination of confocal microscopy (to confirm its presence in adhesion complexes) and cell attachment and spreading assays (supplementary material Fig. S1). To identify effectors that might undergo FRET with β1 integrin, four different candidate adhesion complex components were tested, each of which has been reported to bind directly to β integrins: the talin head domain (residues 1-433) (Garcia-Alvarez et al. 2003), talin rod domain (residues 1984-2344) (Xing et al., 2001), α-actinin (Otey et al., 1990) and paxillin (Schaller et al., 1995). β1-integrin-GFP MEFs were transfected with mRFP conjugates of each protein, plated onto a fibronectin substrate and integrin-effector binding analysed using FLIM to measure FRET. This technique enables visualisation and quantification of protein-protein interactions by analysis of the donor lifetime decay kinetics (see Materials and Methods for full description of technique and analysis) (Parsons et al., 2005).

Specific interactions between β1 integrin and the talin rod domain and α-actinin were detected, but no interaction was observed for the talin head domain or paxillin (Fig. 1A). The substrate dependence of the interaction between the talin rod domain and β1 integrin was examined. As shown in Fig. 1B, a modest interaction was detected on both collagen and laminin substrates, but binding was most prominent on fibronectin, where FRET was localised to focal adhesion structures (Fig. 1A). No FRET was detected between the integrin and any of the acceptors in cells plated on a non-integrin-binding poly-L-lysine substrate (PLL; Fig. 1B and data not shown). Although not the primary focus of this study, these data add significantly to our understanding of integrin effectors. Despite some controversy in the literature, it is apparent that the talin rod and α-actinin are able to interact directly with β1 integrin. From other studies, the ability of the talin head to associate with β1 integrin is unequivocal (Garcia-Alvarez et al., 2003), but we failed to detect the association by FRET-FLIM. We speculate that the functional role of the head domain may be transient, and/or restricted to early adhesion complexes only.

Having established that β1-integrin-GFP associates with talin-rod-mRFP, the activation state dependence of the interaction was examined. To constrain
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the location of the clustered integrin
for analysis, β1-integrin-GFP MEFs
expressing talin-rod-mRFP were plated
onto PLL and incubated with 4.2 μm beads
coated with fibronectin ligand or the
monoclonal antibodies 12G10 (which
detects the high affinity or primed state
and stimulates ligand binding) (Mould et al.,
1995; Mould et al., 2002), mAb13 (which
detects non-ligand-occupied integrin
and which blocks ligand binding) (Akiyama et
al., 1989) or K20 (which is non-function-
 altering and detects all conformational
states) (Amiot et al., 1986; Mould et al.,
2005). As shown in Fig. 2A, an interaction
between integrin and talin rod was only
observed with fibronectin- or 12G10-
coated beads. Confocal analysis of these
cells also revealed actin recruitment to
the bead structures in those cases where FRET
was detected (Fig. 2B). These data suggest
that controlling integrin conformation
and therefore the activation of the extracellular
domain of the integrin, either by native
ligand or antibodies alone, can drive
recruitment of both talin and actin to the
integrin cytoplasmic domain.

To confirm the use of FRET-FLIM for
detecting integrin-effector binding, and to
generate an assay that might be used to test
small molecule inhibitors of integrin
function, the previously characterised
association between α4 integrin and
paxillin (Liu et al., 1999; Goldfinger et al.,
2003) was selected. FLIM was used to
analyse FRET between α4-integrin-GFP
and paxillin-mRFP in both mouse B16F1
and human A375SM melanoma cells. A
localised interaction between integrin and
paxillin was detected in B16F1 cells plated
on fibronectin or an α4-integrin-binding
fragment of fibronectin (H/120), but not on
PLL (Fig. 3A,B), confirming the results of
the previous biochemical analyses. This
interaction was also detected in cells plated
on VCAM-1 (data not shown). FRET was
significantly decreased when two small
molecule, LDV ligand mimetic inhibitors
of α4 integrin [S976162 and S9916197
(Glässner et al., 2005; Lin et al., 1999) see
supplementary material Table S1 and Fig. S2 for characterisation] were added to pre-spread cells (Fig. 3B). Interestingly, the
remaining interacting population demonstrated a spatial shift from
the cell periphery to the central basal region (just below the
nucleus). A similar reduction in α4 integrin and paxillin binding
following treatment with these compounds was also seen in live
human A375SM melanoma cells plated onto an activated
endothelial cell layer (Fig. 3C). These data demonstrate that
binding of α4-integrin to paxillin, detected by FRET, is both ligand
dependent and sensitive to inhibition with small molecule
antagonists.

To test whether the small molecule α4β1 integrin inhibitors
could also act as agonists, A375SM cells transfected with α4-
integrin-GFP and paxillin-RFP were plated onto PLL and
incubated with K20-coated beads. As shown in Fig. 2, these beads
cluster, but do not activate integrins. Cells were then treated with
vehicle control (DMF) or small molecule α4β1 integrin inhibitors
and the interaction between α4 integrin and paxillin assessed by
FLIM. Cells treated with either compound demonstrated a
significant increase in α4-integrin-paxillin binding at the K20
bead interface (Fig. 4A). This effect was not seen with mAb13
beads, which would be expected to retain the β1 integrin in an
inactive conformation and prevent ligand binding. Moreover, the effects of the compounds were dose dependent in a rank order range that paralleled their anti-adhesive activity (Fig. 4B, supplementary material Table S1, Fig. S2). We interpret these data to indicate that K20 immobilisation of α9β1 integrin allows the soluble compounds to act as agonists for α9β1 integrin and trigger an activation response in the form of recruitment of cytoskeletal proteins. To extend these findings, an RGD ligand mimetic small molecule inhibitor of α9β1 and αVβ3 integrins (V0519) (Peyman et al., 2000) was tested for its effect on binding of β1-integrin-GFP and talin-rod-mRFP. β1-integrin-GFP cells expressing talin-rod-mRFP were plated onto PLL-coated coverslips and incubated with K20-coated beads in the presence or absence of V0519. As shown in Fig. 4D, V0519 substantially increased the interaction of β1 integrin and talin at the bead interface.

In summary, we have established assays to detect integrin-effector binding by FRET-FLIM. The availability of these assays will not only enable spatiotemporal studies of integrin signalling, but they could also form the basis for low- and high-throughput screening of small molecule inhibitors in the pharmaceutical industry. The application of direct imaging techniques to study small molecule compound effectors in situ may provide an excellent platform for future identification of therapeutic compounds that either possess or lack agonistic activity.

**Fig. 3.** Ligand binding to the α4 integrin extracellular domain regulates its association with paxillin. (A) B16F1 mouse melanoma cells were transfected with α4-integrin-GFP and paxillin-mRFP. Cells were then plated onto FN- or PLL-coated coverslips and imaged by multiphoton FLIM as before. The same cells were plated onto H120-coated coverslips followed by treatment with DMF vehicle control or stated small molecule inhibitors for 30 minutes and imaged by multiphoton FLIM. Histogram analysis of relative spread of FRET efficiencies is a mean of 18 cells per treatment compared with α4-integrin-GFP alone control. (C) Human A375-SM melanoma cells were transfected with α4-integrin-GFP and paxillin-mRFP and plated onto a monolayer of TNFα-treated activated HUVEC cells. Cells were allowed to adhere for 60 minutes, and were then treated with DMF vehicle control or stated small molecule inhibitors for 30 minutes. Cells were then imaged using multiphoton FLIM as before.
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Materials and Methods

Plasmid constructs

Generation of talin-mRFP constructs

Full-length mouse talin-1 cDNA was a gift from David Critchley (University of Leicester, Leicester, UK). A 1080 bp fragment corresponding to amino acids 1984-2344 was amplified by PCR using the GGGGGGGAATTCGCTGTGTCTG - GTATCATTGC (forward, F) and AAAAAAGCGGCCGCATTGTTCCTCAAAGTTC (reverse, R) primers to generate a 5'H11032EcoRI and a 3'H11032NotI restriction site. This region in the talin rod domain has been reported to contain an integrin-binding site (Fragment G) (Tremuth et al., 2004). The PCR product was then cloned into the pcDNA-RFP-C vector (a gift from Roger Tsien, UCSD, CA) to generate a C-terminal monomeric red fluorescent protein tag. Similarly, a 1299 bp fragment corresponding to amino acids 1-433 of talin was amplified by PCR and fused to a C-terminal monomeric RFP tag. This region in the head of the talin protein also contains an integrin-binding site (Calderwood et al., 1999). The primers used were GCAGAATTCCATGGTTGCGCTTTCGCTGAAGGCAC (F) and ATCTGAAAGCTTGCCTGATTCCTGAAGGACTGTTGA (R).

Generation of β1-GFP

Full-length human β1A integrin cDNA was a gift of Ken Yamada (NIDCR, NIH, Bethesda, MD). To mutate the stop codon before fusion with a 3' fluorescent tag, and generate a novel 3' Apal restriction site, a fragment corresponding to residues 578-799 of the mature sequence was generated by PCR using the primers TTGCAAGTGTCGTGTGTGTG (F) and GTGGATCCCGGGCCCCCCCCCTTTTTTCCC (R). The PCR product was then digested with SpeI and Apal. The remaining 5' portion of β1 was excised from 3'peCE using KpnI and SpeI, and both fragments simultaneously ligated into pDNA3 digested with KpnI and Apal. The fidelity of the resulting construct was confirmed by sequencing (GenBank code XM_005799). The mutated, full-length β1 integrin construct was then cloned into pEGFP N1 (Clontech) using KpnI and Apal. The initial 12 amino acid linker between the end of the mature β1 integrin sequence and the start codon of the GFP tag proved

Fig. 4. Small molecule inhibitors induce integrin signalling. (A) A375-SM cells were transfected with α4-integrin-GFP and paxillin-mRFP and plated onto coverslips coated with PLL. Cells were then incubated with K20 or mAb13 antibody-coated beads for 30 minutes, followed by treatment with DMF or small molecule inhibitor as indicated. Cells were then imaged using multiphoton FLIM. Cumulative FRET efficiency data from a masked pre-set region around the bead in ten cells per treatment is shown in the histogram. (B) Histogram demonstrating FRET efficiency of α4-paxillin association at K20 beads over a dose-response range of compounds S976162 or S9916197. Results are means ± s.e.m. (C) β1-integrin-GFP fibroblasts were transfected with talin-rod-mRFP. Cells were then plated onto coverslips coated with PLL and allowed to attach and spread for 1 hour. Cells were then incubated with beads coated with K20 for 30 minutes, and treated with either the vehicle control DMF or V0519 compound, and subsequently imaged using FLIM as before. Cumulative FRET efficiency data from a masked pre-set region around the bead in 12 cells per treatment is shown in the histogram.
insufficient for mammalian cell expression. Therefore an 18 amino acid linker was constructed flanked by a short strand oligonucleotide were designed and annealed together, digested with Apol and AgeI and used to replace the 3’ end of the β1 integrin-pEGFP N1 construct. The resulting linker sequence between the 3’ end of the mature β1 integrin coding sequence and the start codon of GFP was GGGGARRQOGADDVPVT. To enable greater flexibility with the fluorescence tags, the new construct was initially cloned into pHenRed N1 (Clontech) and when a GFP tag was required, the RFP fluorophore was excised using AgeI and Norf, and the GFP sequence from pEFP-GFP N1 cloned into the pHRed backbone using the same restriction sites. The fidelity of the β1 integrin constructs was verified by sequencing. These constructs were successfully expressed transiently in MEF 7929 (β1+ cells) and the level of the stable β1 integrin–GFP DNA in β1+ cells, an additional retroviral construct was used. The β1-integrin pHeGreen coding sequence was excised from the pHcRed backbone using EcoRI and Norf. This coding sequence was ligated into the pBmneo vector (Stratagene) using the same restriction sites, resulting in a construct that produced β1-integrin–GFP expressing retroviral particles when expressed by amphotropic AM12 packaging cells (a gift from Ian Hart, Cancer Research UK, London, UK). Generation of α4-GFP A full-length clone of human α4 integrin in plueucscript (from Yoshi Takada, Dana-Farber Cancer Center, Boston, MA) was used as a backbone. For the generation of α4-GFP, the coding region of the α4-pBueucscript was removed and an AgeI (PinAl) site inserted at the 3’ end using PCR. The α4 cDNA already had a 5’ Sall site and these sites were used to subclone α4 into pEFP-N1. The CFP cassette was then replaced with GFP-N1 to give α4-pEFP-GFP-N1. This was then digested with Mhel and Norf positioned 5’ and 3’ to the entire α4-pEFP-GFP and the hyg’ cassette from pcDNA3.1 hyg’ ligated using the same restriction sites. PCR primers were used to insert a 3’ AgeI (PinAl) site into the vector backbone were CAA CAG TAA AAG CAA TGA TGA TGG CCG CCG ACC GAG GGA CTT CTT TCA AAT TGA 1 integrin gene was flanked by loxP sites (Graus-Porta et al., 2001) (a gift of Uli Muller, Friedrich Miescher Institute, Basel, Switzerland) and genotyped by tail-tip PCR. Mice that were homozygous for the wild-type or floxed 1 integrin allele and carried at least one Immorto allele were interbred and E13.5 embryos were replaced with GFP-N1 to give 1 integrin–/– murine embryonic fibroblasts (MEFs). 24-48 hours after adding the retrovirus-containing medium for 24-72 hours following transfection (as measured by microscopy) and were used between 24 and 48 hours post transfection for all FLIM studies. Before imaging, cells were plated on glass coverslips or MatTek dishes (for line experiments) coated with extracellular matrix proteins or poly-L-lysine (Sigma) as indicated.

Bead coating and incubation 4 µm latex beads (Dyna) were washed in PBS and incubated with diluted proteins or antibodies as stated in figure legends at indicated concentrations. Anti-β1 monoclonal antibodies used in the study are: K20 (neutral); (Amiot et al., 1996; Mould et al., 2005), 12G10 (activating) (Mould et al., 1995; Mould et al., 2002) or mAb13 (inactivating) (Akiyama et al., 1989) and were all generated in-house as previously described. Beads were left overnight at 4°C to allow even protein binding, and subsequently washed three times and re-suspended in PBS to form a 50% slurry final concentration. Plated cells were incubated with 5 µl of beads in growth media and incubated at 37°C for 30 minutes. Cells were then washed three times in medium and either fixed for FLIM analysis or imaged live in phenol-red free growth media at 37°C. For integrin antagonist studies, cells were pre-treated with concentrations and times specified in figure legend with V0519 (Peyman et al., 2000), S9916197 (Gliener et al., 2005) or 976762 (Lin et al., 1999) and subjected to FLIM analysis.

Fluorescence lifetime measurements by time-correlated single photon counting (TCSPC) FLIM FLIM was performed to measure the interaction between protein pairs, which allows the determination of spatial protein interactions (Parsons et al., 2005). Time-domain FLIM was performed on a multi-photon microscope system as described previously (Parsons et al., 2005; Peter et al., 2005). The system is based on a modified Bio-Rad MRC 1024MP workstation, comprising a solid-state-pumped femtosecond Ti:Sapphire (Tsunami, Spectra-Physics) laser system, a focal scan-head and an inverted microscope (Nikon TE2000). Enhanced detection of the scattered component of the emitted (fluorescence) photons was afforded by the use of fast response (Hamamatsu R7401-P) non-descanned detectors, developed in-house, situated in the re-imaged objective pupil plane. Fluorescence lifetime imaging capability was provided by time-correlated single photon counting electronics (B Becker & Hickl, SPC 700). A 40× objective was used throughout (Nikon, CF960 Plan Fluor NA 1.3) and data collected at 500×20 nm through a bandpass filter (Coherent Inc., 35×5040). Laser power was adjusted to give average photon counting rates of the order 105–106 photons (0.001 to 0.001 photon counts per excitation event) to avoid pulse pile up. Acquisition times up to 300 seconds at low excitation power were used to achieve sufficient photon statistics for fitting, while avoiding either pulse pile-up or significant photobleaching. Excitation was at 890 nm. Widefield acceptor (mRFP) images were acquired using a CCD camera (Hamamatsu) at <100 millisecond exposure times.

Analysis of data for FRET experiments Data were analysed as previously described (Parsons et al., 2005; Prag et al., 2007). Briefly, bulk measurements of FRET efficiency (i.e. intensity-based methods) cannot distinguish between an increase in FRET efficiency (i.e. coupling efficiency) and an increase in FRET population (concentration of FRET species) since the two parameters are not resolved. Measurements of FRET based on analysis of the fluorescence lifetime of the donor can resolve this issue when analyzed using multi-exponential decay models. For measurements of bulk interactions (i.e. where only single exponential decays are fit to the data), measured efficiencies will appear significantly lower because of the assumption that all donors are associated with one or more acceptors. The assumption that non-interacting and interacting fractions are present allows the determination of the efficiency of interaction. The FRET efficiency is related to the molecular separation of donor and acceptor and the fluorescence lifetime of the interacting fraction by:

\[
\eta_{fret} = \frac{\langle R_0^6 \rangle}{\langle R_0^6 \rangle_{p} + \langle R_0^6 \rangle_{n}} = 1 - \frac{1}{\tau_{fret} / \tau_{z}}
\]

where \(R_0\) is the Förster radius, \(\tau_{fret}\) the molecular separation, \(\eta_{fret}\) is the lifetime of the interacting fraction and \(\tau_{z}\) the lifetime of the donor in the absence of acceptor. \(\eta_{fret}\) and \(\tau_{z}\) can also be taken to be the lifetime of the interacting fraction and non-interacting fraction, respectively. All data were analysed using TR12 software developed by Paul Barber, Gray Cancer Institute, London, UK (Prag et al., 2007); supplementary material Fig. S3). For the analysis of FRET at the bead interface, a mask of constant defined pixel size was placed over individual bead areas and the lifetimes analysed and calculated within that area alone (see supplementary material Fig. S3). Histogram data presented here are plotted as mean FRET efficiency from multiple samples. Average FRET efficiency is shown as a shaded area. The statistical significance of lifetime measurements (i.e. areas of example cells are presented using a pseudocolour scale whereby blue depicts normal GFP lifetime (no FRET) and red depicts lower GFP lifetime (areas of FRET).
Confocal microscopy

Cells were permeabilised with 0.2% (v/v) Triton-X-100/PBS following fixation in 4% (w/v) paraformaldehyde. Primary antibodies or phalloidin were diluted 1:200-1:500 in phosphate-buffered saline containing 1% (w/v) BSA. The FITC- and Cy3-labelled secondary conjugates were obtained from Jackson Immunoresearch Laboratories. Confocal images were acquired on a confocal laser-scanning microscope (model LSM 510 Meta, Carl Zeiss Inc.) equipped with both 40×/1.3 Plan-Neofluar and 63×/1.4 Plan-APOCHROMAT oil-immersion objectives.

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