Fluorescence lifetime imaging microscopy (FLIM) to visualise conformational changes in integrin α5ß1

Contact Christoph.Ballestrem@manchester.ac.uk

J. A. Askari and C. Ballestrem
Wellcome Trust Centre for Cell-Matrix Research, Faculty of Life Sciences, University of Manchester, Manchester M13 9PT, UK

C. J. Tynan, S. E. D. Webb and M. L. Martin-Fernandez
Central Laser Facility, STFC, Rutherford Appleton Laboratory, HSIC, Didcot, Oxon OX11 0QX, UK

Introduction

Integrins are large, heterodimeric transmembrane cell surface receptors comprising non-covalently-linked alpha and beta subunits that mediate connections between cells or between cells and the extracellular matrix (ECM)[1]. They control many fundamental aspects of cell behaviour by their ability to transduce signals bi-directionally across the cell membrane. This is achieved by induction of conformational changes within the molecule that specify the activation state and ligand-binding potential of the individual receptor[2]. Biochemical and structural experiments have revealed that an integrin with low affinity for ligand is in a bent conformation, while high-affinity receptor that is bound to ligand, converts to an extended form (figure 1), involving a movement of the integrin headpiece of approximately 20 nm away from the cell membrane[3]. However, the vast majority of this data has been obtained using integrins of the β2 and β3 families whose distribution, and therefore function, is restricted mainly to non-adherent cells with specialised roles, such as leucocytes and platelets, where integrin affinity changes needs to be tightly controlled. There remains a striking paucity of conformational information for members of the ubiquitously-expressed β1 integrin family that are subjected to cell-generated tension when mediating cell-ECM adhesion, but whose activity is less rigidly modulated than those of other integrin groups.

When human foreskin fibroblasts (HFF) adhere to and spread on fibronectin (FN), the FN receptor, integrin α5β1, clusters into regions known as focal adhesions (FA) that represent areas of ligand-bound and therefore high affinity receptor; while unligated, low-affinity integrin remains distributed over the rest of the cell membrane[4]. In this study we have used Fluorescent Lifetime Imaging Microscopy (FLIM) to survey the conformational differences of α5β1, in these two locations within HFF cells in order to determine whether the hypothesis of receptor unbending upon activation can be applied to β1 integrins.

Experimental

Time-correlated single photon counting (TCSPC)-FLIM analysis was used to measure the degree of Foerster Resonance Energy Transfer (FRET) between the α5β1 headpiece labelled with an AlexaFluor 546-tagged Fab fragment of an anti-α5 antibody VC5 that binds to integrin headpiece, and the cell membrane stained with C_{18}DiD, an amphipathic di-alkycarbocyanine dye with fluorescence absorption and emission spectra similar to Cy5. C_{18}DiD and Alexa-546 have single photon absorption and fluorescence emission spectra sufficiently separated so that their fluorescence can be excited and detected separately. An overlap between the emission spectrum of Alexa-546 and the absorption spectrum of C_{18}DiD allows these two dyes to act as a FRET pair if they are within a distance of 10 nm, with the AlexaFluor 546 acting as the donor and C_{18}DiD as the acceptor. Shortening of the donor lifetime from that observed in the absence of acceptor would indicate increased FRET between the VC5-conjugated Alexa-546 bound to the integrin headpiece and the C_{18}DiD in the cell membrane and would infer that the integrin was in a bent conformation (Figure 1).

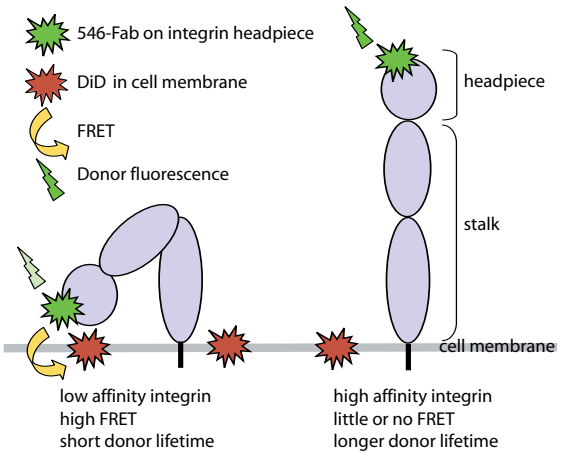

Figure 1. Integrin conformational changes and experimental design. Integrin is represented in blue as a headpiece and stalk region with the transmembrane and cytoplasmic regions in black.

HFF were allowed to spread on FN for 1 hour and then for a further 15 minutes in the presence of Vybrant C_{18}DiD to label the cell membrane. The cells were fixed and stained with a Fab fragment of AlexaFluor-546-VC5.

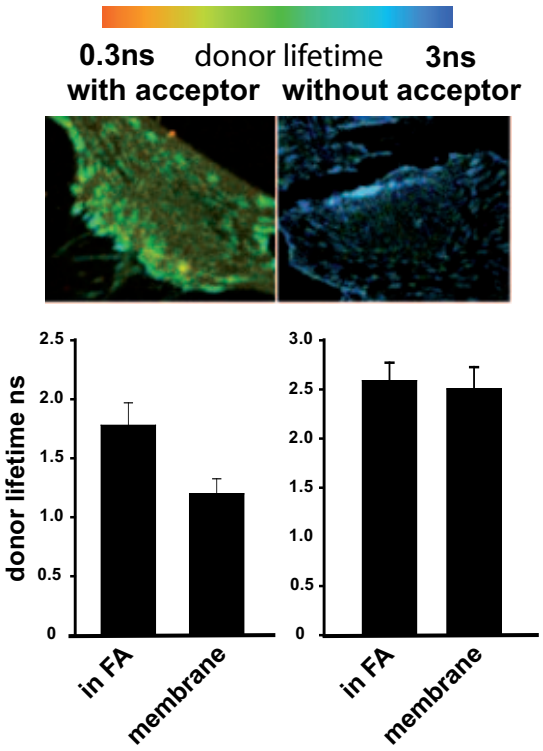

Figure 2. Donor lifetime measurements in different regions of cell. False coloured FLIM images representing donor fluorescence lifetimes from individual pixels within FA and in the adjacent cell membrane. Short lifetimes are located towards the red end and long lifetimes towards the blue end of the spectrum.

Fluorescence lifetime images of cells either with or without acceptor were acquired using a purpose-built laser scanning confocal microscope equipped with time correlated single photon counting (TCSPC) electronics (SPC-730, Becker-Hickl GmbH, Berlin, Germany). Alexa 546 was excited with 545 nm pulsed laser light (Coherent, MIRA-OPO, 76 MHz repetition rate) and the Alexa-546 fluorescence was detected at 575-625 nm. The resulting time-resolved images were analysed using SPCImage (Becker-Hickl GmbH) FLIM analysis software. Fluorescent intensity decays were fitted to a single exponential decay model where acceptor was absent and a bi-exponential model when both donor and acceptor were present, to extract mean lifetimes. The reduced $\chi 2$ parameter was used to judge the goodness of fit, which was deemed acceptable for $0.8<\chi 2<1.2$. For each area of interest, lifetime measurements for donor only or donor in the presence of acceptor were obtained by taking the mean of the distribution of lifetimes of cell membrane pixels. For bi-exponential fits, these lifetimes were the weighted mean of the two fitted lifetime components.

Results and discussion

The mean fluorescence lifetime of the donor measured in the absence of acceptor was 2.59±0.18 nanoseconds (ns) (mean ± SD) within FA and 2.51±0.22 ns in the rest of the cell membrane (Figure). This indicates that clustering of $\alpha 5\beta 1$ integrin into FA has no effect on the lifetime of the donor molecule. When C_{18}DiD (the acceptor) was added to the cells it labelled HFF cell membranes uniformly within the areas of measurement. In the presence of acceptor, the donor lifetime was reduced to 1.78±0.19 ns within FA and further reduced to 1.20±0.12 ns in the adjacent cell membrane (Figure 2). This indicated that FRET was occurring in both locations but that more molecules were undergoing FRET between the integrin headpiece and cell membrane in the areas outside FAs than between those within FAs. This suggests that more $\alpha 5\beta 1$ molecules are in a bent conformation in this location, the cell membrane, than in the FA themselves and supports the hypothesis that active, ligand-bound integrin is in a more extended conformation than unligated integrin.

Since FRET efficiency can vary with acceptor concentration due to the different numbers of acceptors available to the donors as FRET partners, similar measurements were made on several cells within a range of acceptor densities and the differences in lifetimes between FA and cell membrane were found to be constant.

To eliminate the possibility that the longer lifetime observed in FA was due to an artefact arising from a higher concentration of donor molecules which may compete with each other for the available acceptors, the Alexa546 fluorescence in parts of each cell was reduced by photobleaching before measuring lifetimes. This treatment had no effect on the difference in lifetime seen within and outside of FAs showing that there was insignificant competition between donor molecules for acceptors and that the reduced lifetimes seen in the cell membrane was due to increased FRET between donor and acceptor.

Conclusions

TCSPC-FLIM has proved to be a useful tool to probe conformational differences in integrin receptors over the cell surface. The results obtained extend the hypothesis of activation-dependent integrin unbending to the $\beta 1$ receptors and confirm the global importance of conformational changes within these receptors. This method will now be used to examine the effects of various agonists and antagonists on integrin activation to broaden our understanding of cell behaviour.

Acknowledgements

We thank Ralph Isberg (Tufts University Medical School, Boston, USA) for the VC5 antibody and Sue Craig for preparing Fab fragments.

References

1. R. O. Hynes, *Cell* **110**, 673-687 (2002).
2. J. A. Askari, P. A. Buckley, A. P. Mould and M. J. Humphries, *J. Cell Sci.* **122**, 165-170 (2009).
3. B. H. Luo and T. A. Springer, *Curr. Opin. Cell Biol.* **18**, 579-586 (2006).
4. B. Geiger, J. P. Spatz and A. D. Bershadsky, *Nat. Rev. Mol. Cell Biol.* **10**, 21-33 (2009).