Protein–Protein Interactions

Deutsche Ausgabe: DOI: 10.1002/ange.201605964
Internationale Ausgabe: DOI: 10.1002/anie.201605964

A Quantitative Fluorescence-Based Assay for Assessing LIM Domain–Peptide Interactions

*Neil O. Robertson, Manan Shah, and Jacqueline M. Matthews**

Abstract: *We have developed Förster resonance energy transfer (FRET)-based experiments for measuring the binding affinity, off-rates, and inferred on-rates for interactions between a family of transcriptional regulators and their intrinsically disordered binding partners. It was difficult to evaluate these interactions previously, as the transcriptional regulators are obligate binding proteins that aggregate in the absence of a binding partner. The assays rely on fusion constructs where binding domains are linked by a flexible tether containing a specific protease site, with fluorescent proteins at either end that display FRET when the complex is formed. Loss of FRET is monitored after cutting the tether followed by dilution or competition with a non-fluorescent peptide. These methods allowed a wide range of binding affinities (10^{-9}–10^{-5} M) to be determined. Our data indicate that interactions of closely related proteins can have surprisingly different binding properties.*

The LIM-only (LMO) and LIM-homeodomain (LIM-HD) family of transcription factors regulate gene expression through formation of transcription complexes. The two best-characterized proteins from this family are LMO2 and LMO4. Both proteins inhibit differentiation and promote proliferation during development, although overexpression is associated with cancer.[1] All LMO and LIM-HD proteins interact with the widely expressed LIM-domain-binding protein 1 (LDB1).[2] This interaction is essential for biological activity as LDB1 enables chromatin looping and activation of gene expression.[3]

LDB1:LMO/LIM-HD interactions are mediated by a pair of tandem LIM domains (LIM1 + 2) in the LMO/LIM-HD proteins, and the LIM interaction domain (LID)—a 30-residue intrinsically disordered region (IDR) in LDB1.[4] $LDB1_{LID}$ binds tandem LIM domains in an extended head-to-tail manner, forming a tandem β-zipper in which the short β-strands in $LDB1_{LID}$ augment existing β-hairpins in the LIM domains.[5] Similar LIDs that bind subsets of LMO/LIM-HD proteins have been found in CtIP/RBBP4, DEAF1, and in the LIM-HD proteins ISL1 and ISL2.[6] These complexes share a common mode of binding despite low levels of sequence identity in the LIDs.

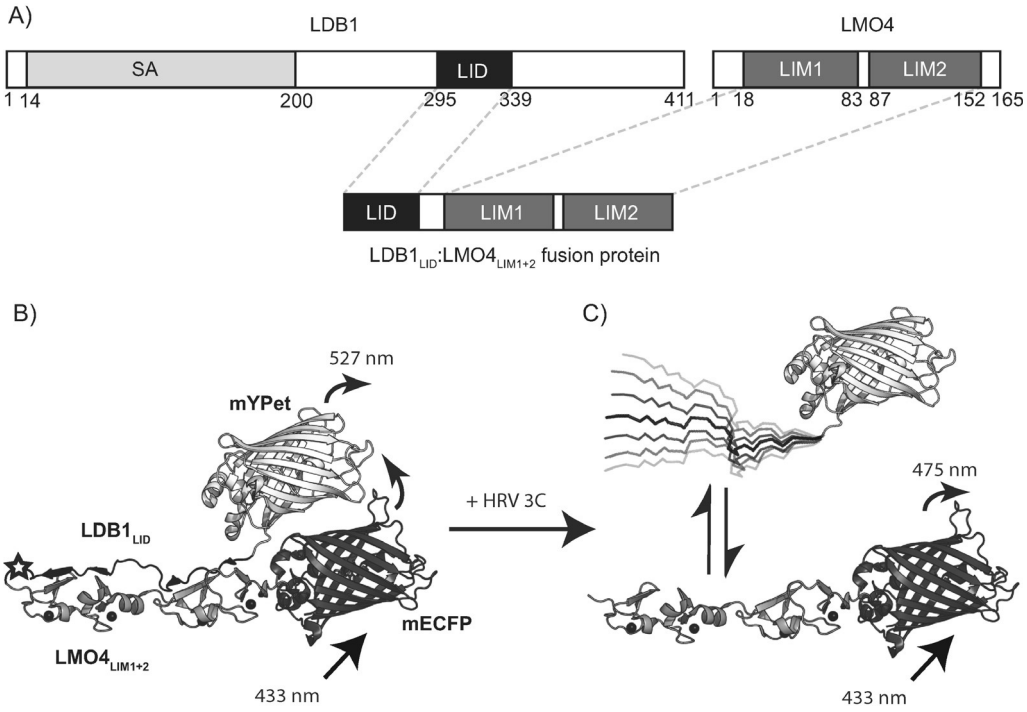

Figure 1. Design of a tethered LID:LIM FRET construct. A) Domain structure of LDB1, LMO4, and the fusion complex. LDB1 contains a self-association (SA) domain and LID. LMO4 contains two LIM domains. The fusion protein contains $LDB1_{LID}$ tethered to $LMO4_{LIM1+2}$. B) The FRET construct contains mYPet, LID, a flexible linker containing a HRV 3C protease site (star), $LMO4_{LIM1+2}$, and mECFP. mECFP is excited at 433 nm causing FRET emission at 527 nm. C) HRV 3C cleavage allows the interacting domains to dissociate reducing FRET.

[*] N. O. Robertson, M. Shah, Prof. Dr. J. M. Matthews
School of Life and Environmental Sciences, The University of Sydney
NSW 2006 (Australia)
E-mail: jacqui.matthews@sydney.edu.au

Supporting information for this article can be found under: http://dx.doi.org/10.1002/anie.201605964.

These interactions have been difficult to characterize, as LIM domains from LMO/LIM-HD proteins are obligate binding motifs that aggregate when isolated. For structure determination this tendency was overcome by using "tethered complexes" in which the interacting domains were fused by a flexible tether[7] (Figure 1 A). However, tethered complexes

are not amenable to standard quantitative binding studies. Yeast-two hybrid (Y2H) assays suggest that the interactions of LMO4 with CtIP and Deaf1 are significantly weaker than with LDB1.[6a,b,8] Homologous competition ELISA experiments using complexes in which the tether has been cut by a protease yielded estimates of $LDB1_{LID}$:$LMO2/4_{LIM1+2}$ affinity ($K_d = 20$ and 10 nM respectively),[9] but are not suited to weaker interactions due to multiple washing steps. Moreover, assumptions about the available concentrations of proteins are difficult to verify.

We have designed Förster resonance energy transfer (FRET)-based methods for the quantitative analysis of LID:LIM interactions. We generated tethered complexes in which GFP-based FRET pairs are fused to the N-terminus of a LID and the C-terminus of the LIM1 + 2 domains (Figure 1A; see Figure S1 in the Supporting Information). The fluorescent proteins, mYPet and mECFP, were previously evolved as optimal FRET partners.[10] Proteolytic cleavage of the tether allows the interacting domains to dissociate and the interaction to be monitored by the loss of FRET (Figure 1B,C).

The methods were developed using $LDB1_{LID}$:$LMO4_{LIM1+2}$. The intact complex was cut by HRV 3C (Figure S2). The fluorescent properties of the cut complex were characteristic of FRET (Figure 2A). Homologous competition experiments were performed by titrating maltose binding protein (MBP)-$LDB1_{LID}$ into the cut complex and fitting the data with a model accounting for ligand depletion (see Equation S5 in the Supporting Information), resulting in an affinity estimate of $K_d = 1.8 \pm 0.1$ nM (Figure 2B and Table S1). The MBP tag aids protein expression, purification and concentration determination of $LDB1_{LID}$, which lacks aromatic residues. Dissociation rates were measured after adding a large excess of MBP-$LDB1_{LID}$ to the cut complex, yielding $k_{off} = 6.3 \pm 0.1 \times 10^{-4}\,s^{-1}$ (Figure 2C). In control experiments complex dissociation with buffer and MBP is due to the dilution of the complex from the cleavage reaction (5 μM to 1–5 nM), which results in partial dissociation of the complex under the new concentration conditions.[11] Homologous competition experiments using $LDB1_{LID}$(I322A), which substantially weakens the interactions with LMO/LIM-HD proteins[4a,6d,9] by Y2H, estimated a 30-fold reduction in affinity for $LMO4_{LIM1+2}$ ($K_d = 55 \pm 10$ nM) compared to wild-type (Figure 2B and Table S1).

FRET-dilution assays in which concentrated samples of cut complexes were serially diluted gave affinity estimates of $K_d = 1.4 \pm 0.3$ nM and 31 ± 3 nM for $LMO4_{LIM1+2}$ binding to $LDB1_{LID}$(WT) and the I322A mutant, respectively (Figure 3A and Table S1), in good agreement with the competition experiments. Attempts were made to assess the weaker binding of $CtIP_{LID}$ to $LMO4_{LIM1+2}$.[6a] We modified the dilution experiment by spiking in YFP-$CtIP_{LID}$ to promote complex formation, with the resultant estimate of affinity measured as $K_d = 9 \pm 2$ μM (Figure 3A). Although the binding curve is incomplete, this value is not significantly different than that obtained when the maximum signal was fixed using the intact complex ($K_d = 13 \pm 2$ μM; Table S1).

We assessed the dilution experiment as a medium-throughput method for screening interactions by using unpurified protein in cell lysate. For $LDB1_{LID}$-(I322A):$LMO4_{LIM1+2}$ there was only a small (ca. 3-fold) decrease in estimates of affinity using this approach ($K_d = 90 \pm 20$ nM, Figure 3B and Table S1). The differences are probably due to modest levels of protein breakdown during expression and/or lysis that affected estimates of active protein concentration (Figure 3C). This assay could be readily adapted to screen interactions of this type and/or assay inhibitors of these complexes.

$LDB1_{LID}$:$LMO2_{LIM1+2}$ binding was evaluated using the same methods. In competition experiments MBP bound non-specifically to the cut complex, so the dilution method was used to determine an affinity of $K_d = 32 \pm 2$ nM (Figure 4A). The dissociation and apparent association rates were $k_{off} = 9.2 \pm 0.2 \times 10^{-5}\,s^{-1}$ and $k_{on} = 2.9 \pm 0.2 \times 10^{3}\,M^{-1}s^{-1}$ respectively (Figure 4B and Table S2). Previous homologous competition ELISA data suggested LMO2 and LMO4 had a comparable affinity for LDB1.[4a,9] Our new FRET-based method, which we consider to be more reliable because of faster proteolytic cleavage and more accurate estimates of concentrations in

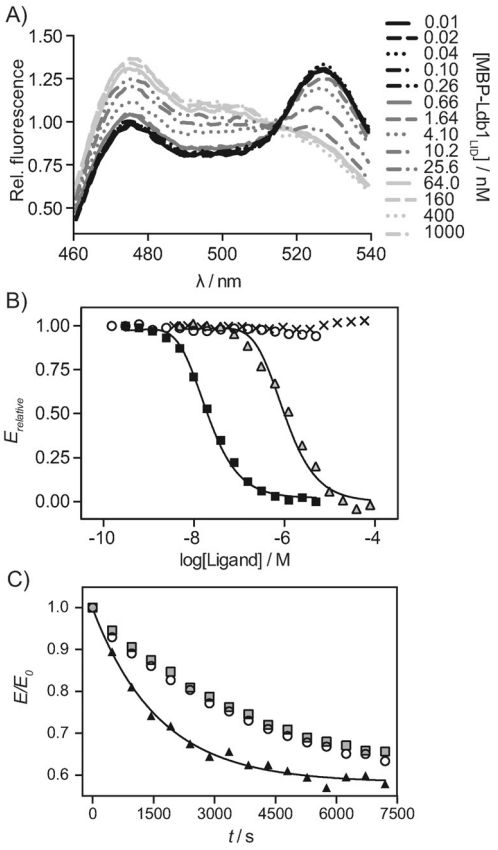

Figure 2. Competition FRET assays for the $LDB1_{LID}$:$LMO4_{LIM1+2}$. Experiments shown are representative of replicate titrations, with binding parameters reported in the text as the mean ± the standard error of the mean (S.E.M.). A) Spectral changes of the cut complex upon titration of 0.01–1000 nM MBP-$LDB1_{LID}$. Spectra were normalized at 514 nm. B) Competition experiments using MBP (○) and MBP-$LDB1_{LID}$ (■) were titrated into 10 nM cut $LDB1_{LID}$:$LMO4_{LIM1+2}$, and MBP (×) and MBP-$LDB1_{LID}$(I322A) (△) were titrated in 550 nM cut $LDB1_{LID}$-(I322A):$LMO4_{LIM1+2}$. C) Dissociation rates were measured by titrating buffer (○), MBP (□) and MBP-$LDB1_{LID}$ (▲) into the cut complex.

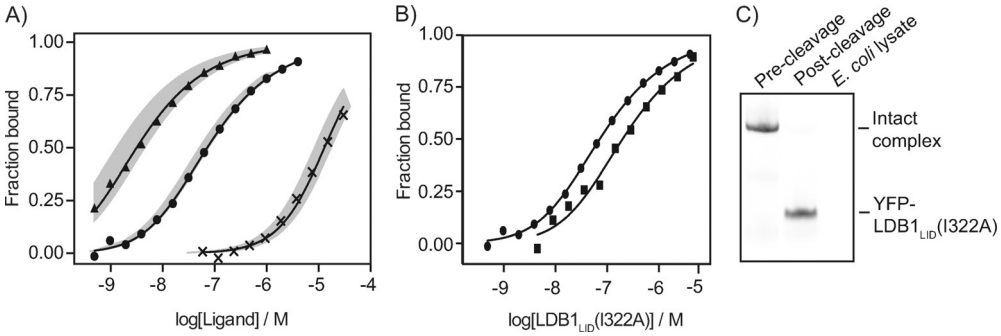

Figure 3. Dilution series experiments for studying LID:LMO4$_{LIM1+2}$ interactions. A) Cut complexes were serially diluted to assess LDB1$_{LID}$ (▲), LDB1$_{LID}$(I322A) (●) and CtIP$_{LID}$ (×) binding to LMO4$_{LIM1+2}$. Grey shading indicates the range of fitted curves. B) Comparison of LDB1$_{LID}$(I322A):LMO4$_{LIM1+2}$ binding using purified protein (●) and protein in cell lysate (■). C) SDS-PAGE analysis of in-lysate cleavage of LDB1$_{LID}$-(I322A):LMO4$_{LIM1+2}$ complex imaged using YFP-fluorescence. Cell lysate from an untransformed *E. coli* culture was used to monitor background cellular fluorescence.

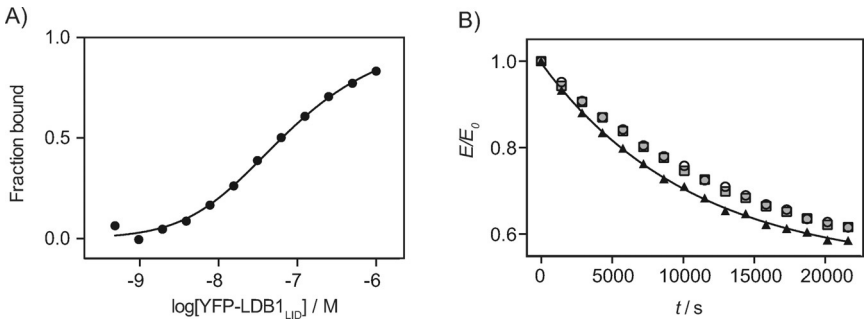

Figure 4. Assessment of LDB1$_{LID}$:LMO2$_{LIM1+2}$ binding. A) Dilution experiment for LDB1$_{LID}$:LMO2$_{LIM1+2}$. B) FRET dissociation rate experiment titrating buffer (○), MBP (□) and MBP-LDB1$_{LID}$ (▲) into cut complex.

solutions, indicate that LDB1 binds LMO4 ~20-fold more strongly. Moreover, the apparent on-rate indicates LMO4 binds LDB1 ~120-fold faster than LMO2.

Interactions between IDRs and their targets are thought to involve folding and binding where a loss of entropy is offset by the formation of large interfaces. The order of events varies between interactions, but the generally fast on-rates for those interactions measured to date (ca. 10^5–$10^6\,M^{-1}\,s^{-1}$)[12] may result from formation of transient encounter complexes that are dominated by favorable electrostatic or hydrophobic interactions.[12,13] We assessed the dependence of salt concentration on the LDB1$_{LID}$:LMO2/4$_{LIM1+2}$ interactions to establish if electrostatic steering was important for binding. There was an ~6-fold decrease in affinity for the interaction with LMO2$_{LIM1+2}$ compared to a 1.8-fold decrease for LMO4$_{LIM1+2}$ from 0.01-1M NaCl (Table S2 and Figure S4 A–C). The apparent off-rate and inferred on-rate for LDB1$_{LID}$:LMO2$_{LIM1+2}$ decreased by 1.5-fold and 9-fold, respectively, whereas the apparent off-rate for LMO4$_{LIM1+2}$ increased 4-fold and the inferred on-rate increased by 2-fold (Table S2 and Figure S4 D–G).

The data for LDB1$_{LID}$:LMO2$_{LIM1+2}$ is characteristic of electrostatic steering, although interactions measured to date tend to have association rates that are 2–3 orders of magnitude higher that those determined for this interaction.[12] The association rate for LDB1$_{LID}$:LMO4$_{LIM1+2}$ falls within this range, but increased salt resulted in increased on- and off-rates, which implies that encounter complex is dominated by hydrophobic interactions. These differences were unexpected given the structural similarity of LDB1:LMO2/4 complexes (Figure S5). An understanding of why the modes of binding are so different requires further investigation.

Differences in LDB1 binding should have consequences for the biological roles of the LMO/LIM-HD proteins. LMO2 expression is required for all stages of red blood cell development.[14] Slow association rates may allow for an ordered transition through cell differentiation, and slow dissociation rates would enable complexes, once formed, to be long lived. LMO4 plays an important role in motor neuron specification by downregulating the activity of LIM-HD proteins ISL1 and LHX3 by competing for LDB1.[15] Fast, high affinity binding would facilitate this process.

In conclusion, these FRET-based assays made it possible to gain insight into the mechanisms of binding of aggregation-prone LIM-containing proteins. They should be readily adapted for other aggregation-prone systems which allow fluorescent tags to be placed in close proximity. Using these techniques we have shown LMO2 and LMO4 have quite different modes of binding to LDB1$_{LID}$ that may reflect roles in developmental processes.

Acknowledgements

N.O.R. was supported by an Australian Postgraduate Scholarship. The work was supported by grants from the Australian Research Council, National Health & Medical Research Council of Australia and the Cancer Council of New South Wales.

Keywords: fluorescence · FRET · kinetics · protein engineering · protein–protein interactions

How to cite: *Angew. Chem. Int. Ed.* **2016**, *55*, 13236–13239
Angew. Chem. **2016**, *128*, 13430–13433

[1] J. M. Matthews, K. Lester, S. Joseph, D. J. Curtis, *Nat. Rev. Cancer* **2013**, *13*, 111–122.

[2] a) L. W. Jurata, S. L. Pfaff, G. N. Gill, *J. Biol. Chem.* **1998**, *273*, 3152 – 3157; b) J. M. Matthews, J. E. Visvader, *EMBO Rep.* **2003**, *4*, 1132 – 1137.
[3] a) E. Soler, C. Andrieu-Soler, E. de Boer, J. C. Bryne, S. Thongjuea, R. Stadhouders, R. J. Palstra, M. Stevens, C. Kockx, W. van Ijcken, J. Hou, C. Steinhoff, E. Rijkers, B. Lenhard, F. Grosveld, *Genes Dev.* **2010**, *24*, 277 – 289; b) S. H. Song, C. Hou, A. Dean, *Mol. Cell* **2007**, *28*, 810 – 822.
[4] a) J. E. Deane, D. P. Ryan, M. Sunde, M. J. Maher, J. M. Guss, J. E. Visvader, J. M. Matthews, *EMBO J.* **2004**, *23*, 3589 – 3598; b) L. W. Jurata, G. N. Gill, *Mol. Cell. Biol.* **1997**, *17*, 5688 – 5698.
[5] J. M. Matthews, J. R. Potts, *FEBS Lett.* **2013**, *587*, 1164 – 1171.
[6] a) P. H. Stokes, C. W. Liew, A. H. Kwan, P. Foo, H. E. Barker, A. Djamirze, V. O'Reilly, J. E. Visvader, J. P. Mackay, J. M. Matthews, *J. Mol. Biol.* **2013**, *425*, 1101 – 1110; b) S. Joseph, A. H. Kwan, P. H. Stokes, J. P. Mackay, L. Cubeddu, J. M. Matthews, *PLoS One* **2014**, *9*, e109108; c) M. S. Gadd, M. Bhati, C. M. Jeffries, D. B. Langley, J. Trewhella, J. M. Guss, J. M. Matthews, *J. Biol. Chem.* **2011**, *286*, 42971 – 42980; d) M. Bhati, C. Lee, A. L. Nancarrow, M. Lee, V. J. Craig, I. Bach, J. M. Guss, J. P. Mackay, J. M. Matthews, *EMBO J.* **2008**, *27*, 2018 – 2029.
[7] J. E. Deane, J. P. Mackay, A. H. Kwan, E. Y. Sum, J. E. Visvader, J. M. Matthews, *EMBO J.* **2003**, *22*, 2224 – 2233.
[8] L. Cubeddu, S. Joseph, D. J. Richard, J. M. Matthews, *PLoS One* **2012**, *7*, e39218.
[9] D. P. Ryan, M. Sunde, A. H. Kwan, N. J. Marianayagam, A. L. Nancarrow, R. N. Vanden Hoven, L. S. Thompson, M. Baca, J. P. Mackay, J. E. Visvader, J. M. Matthews, *J. Mol. Biol.* **2006**, *359*, 66 – 75.
[10] a) A. W. Nguyen, P. S. Daugherty, *Nat. Biotechnol.* **2005**, *23*, 355 – 360; b) T. Ohashi, S. D. Galiacy, G. Briscoe, H. P. Erickson, *Prot. Sci.* **2007**, *16*, 1429 – 1438.
[11] T. D. Pollard, *Mol. Biol. Cell* **2010**, *21*, 4061 – 4067.
[12] a) J. Dogan, S. Gianni, P. Jemth, *Phys. Chem. Chem. Phys.* **2014**, *16*, 6323 – 6331; b) S. L. Shammas, A. J. Travis, J. Clarke, *J. Phys. Chem. B* **2013**, *117*, 13346 – 13356.
[13] A. Bachmann, D. Wildemann, F. Praetorius, G. Fischer, T. Kiefhaber, *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 3952 – 3957.
[14] a) T. Tripic, W. Deng, Y. Cheng, Y. Zhang, C. R. Vakoc, G. D. Gregory, R. C. Hardison, G. A. Blobel, *Blood* **2009**, *113*, 2191 – 2201; b) A. J. Warren, W. H. Colledge, M. B. Carlton, M. J. Evans, A. J. Smith, T. H. Rabbitts, *Cell* **1994**, *78*, 45 – 57; c) N. K. Wilson, S. D. Foster, X. Wang, K. Knezevic, J. Schutte, P. Kaimakis, P. M. Chilarska, S. Kinston, W. H. Ouwehand, E. Dzierzak, J. E. Pimanda, M. F. de Bruijn, B. Gottgens, *Cell Stem Cell* **2010**, *7*, 532 – 544.
[15] a) S. Lee, B. Lee, K. Joshi, S. L. Pfaff, J. W. Lee, S. K. Lee, *Dev. Cell* **2008**, *14*, 877 – 889; b) M. R. Song, Y. Sun, A. Bryson, G. N. Gill, S. M. Evans, S. L. Pfaff, *Development* **2009**, *136*, 2923 – 2932.

Received: June 20, 2016
Published online: September 20, 2016

