Assembly of the oncogenic DNA-binding complex LMO2-Ldb1-TAL1-E12

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ABSTRACT

The nuclear proteins TAL1 (T-cell acute leukaemia protein 1) and LMO2 (LIM-only protein 2) have critical roles in haematopoietic development, but are also often aberrantly activated in T-cell acute lymphoblastic leukaemia. TAL1 and LMO2 operate within multifactorial protein–DNA complexes that regulate gene expression in the developing blood cell. TAL1 is a tissue-specific basic helix–loop–helix (bHLH) protein that binds bHLH domains of ubiquitous E-proteins, (E12 and E47), to bind E-box (CANNTG) DNA motifs. TAL1_{bHLH} also interacts specifically with the LIM domains of LMO2, which in turn bind Ldb1 (LIM-domain binding protein 1). Here we used biophysical methods to characterize the assembly of a five-component complex containing TAL1, LMO2, Ldb1, E12, and DNA. The bHLH domains of TAL1 and E12 alone primarily formed helical homodimers, but together preferentially formed heterodimers, to which LMO2 bound with high affinity ($K_A \sim 10^8 M^{-1}$). The resulting TAL1/E12/LMO2 complex formed in the presence or absence of DNA, but the different complexes preferentially bound different Ebox-sequences. Our data provide biophysical evidence for a mechanism, by which LMO2 and TAL1 both regulate transcription in normal blood cell development, and synergistically disrupt E2A function in T-cells to promote the onset of leukaemia.

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INTRODUCTION

Acute lymphoblastic leukemia (ALL) is characterized by the accumulation of immature lymphoid progenitors, and is the most common form of neoplasia in children. T-cell ALL accounts for ~10% of childhood leukemias and a further 25% of adult leukemias.¹ Despite advances in treatments of ALL in the past 10–15 years, 25–40% of children and adolescents with T-cell ALL still succumb to the disease. Laboratory and clinical markers that are used to guide treatment of the more common (and more successfully treated) B-cell leukemias, are of little use in the treatment of T-cell leukemias. Therefore, advances in treatment are unlikely to be made until the molecular bases of the causes of the disease are understood.¹ More than half of all T-cell leukemias are associated with the over-expression of two proteins T-cell acute leukaemia protein 1 (TAL1)/SCL1 and LIM-only protein 2 (LMO2 or rhombotin 2), often as the result of specific chromosomal rearrangements [*t*(1;14)(p32;q11) and *t*(11;14)(p13;q11), respectively].^{2,3} The over-expression of *tal1* and/or *lmo2* in mice also results in the malignant transformation of T-cell lineages indicating that either protein can act as a T-cell oncogene.^{4,5} These effects are exacerbated when the genes are coexpressed,⁶ strongly suggesting that TAL1 and LMO2 have synergistic roles in the onset of some T-cell leukemias.

TAL1 is a tissue-specific basic helix–loop–helix (bHLH) transcription factor. These eukaryotic proteins are involved in cell differentiation and are characterized by a ~50-residue helix–loop–helix protein interaction domain, preceded by a ~10-residue basic region that binds DNA.⁷ TAL1 is thought to form heterodimers with ubiquitous bHLH factors, such as the E2A-proteins E12 and E47, before binding E-box (CANNTG) DNA motifs.⁷

LMO2 is a member of the LIM-only class of transcriptional coregulators that consist of little more than two closely spaced LIM-domains. LMOs act as protein–protein adaptors, mediating interactions via their LIM-domains to form specific multiprotein complexes.⁸ All four human LMO proteins (LMO1–LMO4) have been associated with oncogenesis.^{9–11}

Both TAL1 and LMO2 are essential for hematopoietic development^{12,13} and gene deletion studies of *tal1* or *lmo2* suggest overlapping roles for TAL1 and LMO2 *in vivo*.^{14,15} Indeed, multiprotein DNA-binding complexes that contain TAL1 and LMO2 have been isolated from erythroid and T-cell acute

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lymphoblastic leukaemia (T-ALL) cell lines.^{16–18} These complexes appear to be regulators of transcription and also contain the ubiquitous LIM domain-binding protein 1 (Ldb1) and E2A proteins (E47 or E12). The complexes also contain other factors, such as GATA-1¹⁶ or Sp1.¹⁹

Some mice with non-functional E2A exhibit a partial block in the early stages of T-cell differentiation that leads to the development of T-cell leukemia.²⁰ The resultant phenotype is similar to that of mice that carry transgenes for TAL1 and LMO2.^{4–6} It is thought that in these mice TAL1 blocks E2A-mediated gene expression, disrupting normal lymphocyte development that results in proliferation of immature T-cells and the onset of T-cell leukemia.^{21–23} The potential role of LMO2 and the molecular basis for this process have not been fully characterized, although the networks of interactions involved in this process have been established (see Fig. 1).

The aim of this work was to determine the fundamental biophysical parameters that underlie the formation of complexes containing TAL1, LMO2, Ldb1, E12, and DNA. We generated the interacting domains of all of the proteins and target DNA sequences, and used various biophysical methods to estimate the association constants for all of the component equilibrium reactions. These association constants were used in a mathematical model to predict the distribution of those components among different possible complexes and subcomplexes. Our data provide biophysical support for the hypothesis that TAL1 and LMO2 act in concert to cause T cell leukemia through the disruption of normal E2A function. This quantitative molecular understanding is pertinent to the future development of specific anti-leukaemia agents.

MATERIALS AND METHODS

Protein production

All proteins were expressed in *Escherichia coli* BL21 (DE3) cells. The purification of LMO2-Ldb1_{LID} has been described previously.²⁴ The bHLH domain of human TAL1 (residues 186–246; TAL1_{bHLH}) was amplified by PCR from a k562 cDNA library and inserted into pRSET-A and pMAL-C2 derived expression plasmids. The bHLH domain of E12 (residues 549–610; E12_{bHLH}) was subcloned into pGEX-2T- and pMAL-C2-derived plasmids. Codons within the TAL1_{bHLH} and E12_{bHLH} were then optimized for expression in *E. coli* using inverse PCR. All cloned DNA fragments were sequenced (SUPAMAC, The University of Sydney).

TAL1_{bHLH} was expressed with an N-terminal His₆-tag and immobilized on Ni-NTA resin in guanidium hydrochloride (GdmHCl) buffer (100 mM NaH₂PO₄ (pH 8.0), 10 mM Tris-HCl, 6M GdmHCl), washed in urea buffer (100 mM NaH₂PO₄, 10 mM Tris, 8M urea) at pH 6.3 then pH 5.9, and then eluted in urea buffer at pH 4.5.

Figure 1

The interaction network involving LMO2, Ldb1, TAL1, E12, and DNA. Schematic showing the individual protein–protein and protein–DNA interactions that occur between LMO2, Ldb1, TAL1, E12, and DNA.

The urea was removed by dialysis and TAL1_{bHLH} was purified further by reverse-phase HPLC using an acetonitrile (0–100% v/v)/trifluoroacetic acid (0.1% v/v)/water gradient (100–0% v/v) and lyophilised. E12_{bHLH} was expressed as a GST-fusion protein and purified on GSH-sepharose under native conditions (50 mM Tris-HCl (pH 7.0), 150 mM NaCl, 1 mM dithiothreitol (DTT), 0.1% (v/v) Triton X-100, 0.5 mM EDTA, 0.5 mM PMSF) and, if required, the GST-tag was removed by treatment with PreScissionTM protease. E12_{bHLH} was purified further by either cation-exchange chromatography using a MonoS 10/10 column (GE Healthcare) and a NaCl gradient in 20 mM NaH₂PO₄ (pH 7.4) and 1 mM DTT, or by reverse-phase HPLC in the same manner as TAL1_{bHLH}. E12_{bHLH} was also expressed as a maltose-binding protein (MBP)-fusion protein and purified on Amylose resin (New England Biolabs) under native conditions [20 mM Tris (pH 8.0), 150 mM NaCl, 0.1% (v/v) β-mercaptoethanol, 0.5 mM PMSF] and eluted as an intact fusion protein using 20 mM maltose. Concentrations of TAL1_{bHLH} and LMO2-Ldb1_{LID} were determined from their molar extinction coefficients (17,600 and 22,900 M⁻¹ cm⁻¹, respectively) at 280 nm. The concentration of E12_{bHLH} (which lacks Tyr and Trp residues) was determined by $144 \times (A_{215 \text{ nm}} - A_{225 \text{ nm}})$.²⁵

Preparation of double-stranded oligonucleotides for biophysical experiments

Oligonucleotides were supplied by Sigma-Genosys (Castle Hill, Australia). Equimolar amounts of complementary oligonucleotides (EboxGG: CCGCCAGGTGCTCG, CGAGCACCTGGCGG; EboxGC: CTATCAGCTGATGA, TCATCAGCTGATAG) were mixed in 20 mM NaH₂PO₄ (pH 7.4) containing 150 mM NaCl, 1 mM EDTA, and/or 1 mM DTT. The oligonucleotides were denatured at 95°C for 5–10 min and annealed by cooling slowly to room temperature. Samples were stored short-term at 4°C or long-term at –20°C. Concentrations of EboxGG and EboxGC were determined from their molar

extinction coefficients (225,000 and 245,000 $M^{-1} \text{ cm}^{-1}$, respectively) at 254 nm.

Preparation of protein complexes by native metal-affinity chromatography

Lyophilized samples of TAL1_{bHLH} (~1 mg) were dissolved in 2.0–2.5 mL MQW and the pH adjusted to 7.4 by the addition of NaH_2PO_4 (final concentration 50–100 mM). The dissolved protein was added to a column of Ni-NTA resin (1–2 mL bed volume) pre-equilibrated in column buffer (50 mM NaH_2PO_4 (pH 7.4), 150 mM NaCl and 0.02% (v/v) β -mercaptoethanol), and run by gravity-flow. The eluant was collected and reapplied to the column, and this process was repeated three times. The resin was then washed with 5 mL of column buffer. A sample containing an excess of E12_{bHLH} (2.0–2.5 mL) was applied to the column and allowed to run by gravity-flow. The eluant was collected and reapplied to the column, and this process was repeated five times. The column was then washed with 5 mL of column buffer. Bound protein was eluted in 0.5–1.0 mL fractions using column buffer containing 250 mM imidazole. Alternatively, samples of DNA or LMO2-Ldb1_{LID} were added and bound in the same manner as E12_{bHLH}, and the resultant complexes eluted. Samples were collected throughout the procedure and analyzed using SDS-PAGE.

Electrophoretic mobility shift assays

Reactions were performed in EMSA buffer (10 mM HEPES pH 7.8, 50 mM KCl, 5 mM MgCl_2 , 1 mM EDTA, 5% (v/v) glycerol) that contained additional DTT (1 mM), BSA (33 $\mu\text{g mL}^{-1}$ –30 mg mL^{-1}) and poly dIdC (3.3 $\mu\text{g mL}^{-1}$), on ice. The order and timing of addition of components was, where appropriate: mixtures of proteins (10 min), 0.4 ng ^{32}P -labeled double-stranded oligonucleotide (≥ 10 min), antibodies (1- μL anti-His₆ (GE Healthcare) or 1- μL anti-MBP (New England Biolabs) monoclonal antibodies; 10 min). Samples were incubated for 30 min after the addition of the oligonucleotide, then separated on 6% (w/v) non-denaturing polyacrylamide gels made up in $0.5 \times \text{TB}$ (45 mM Tris, 45 mM boric acid) at 4°C. Gels were dried and analyzed using a PhosphorImager (Molecular Dynamics).

GST-pulldown assays

MBP and MBP-TAL1_{bHLH} were expressed in *E. coli* and lysates prepared in 20 mM Tris (pH 8.0), 150 mM NaCl, 0.1% (v/v) β -mercaptoethanol and 0.5 mM PMSF. Lysates (50–100 μL) and/or 75 μg of purified LMO2-Ldb1_{LID} were added to ~20 μg of GST/GST-E12_{bHLH} immobilized on GSH-resin, and the reaction mixture made up to a final volume of 500 μL in pulldown buffer [20 mM Tris (pH 8.0), 150 mM NaCl, 0.5% (v/v) IGEPAL CA-630, 0.1% (v/v) β -mercaptoethanol, 500 μM PMSF].

The samples were incubated with mixing at 4°C for ~1–2 h then washed three times in pulldown buffer containing 10% glycerol. The resin was then analyzed by SDS-PAGE to detect bound proteins.

Circular dichroism spectropolarimetry

Circular dichroism (CD) spectra were recorded at 25°C in 1 or 0.1-mm pathlength cuvettes using a Neslab RTE-111 temperature-controlled Jasco J-720 spectropolarimeter that had been calibrated with a camphor-10-sulfonic acid standard (0.6 g mL^{-1}). Spectra were recorded in 10–20 mM Na_2HPO_4 (pH 7.4) and 0–50 mM NaCl; 1 mM DTT was included in E12_{bHLH} samples and 150 mM NaF replaced NaCl in titration experiments. Final spectra were the average of three scans, and were baseline corrected. Estimates of secondary structure were made using the CDPro suite of programs.²⁶

Analytical size-exclusion chromatography and multiangle laser-light scattering

Chromatography was conducted on a SuperoseTM 12 10/30 column (GE Healthcare) at a flow rate of 0.5 mL min^{-1} in PBSA (20 mM NaH_2PO_4 (pH 7.4), 150 mM NaCl, 1 mM DTT) and the eluting species monitored at 280, 215, 230, or 254 nm. Light scattering data were recorded on an in-line miniDawn Tristar laser-light scattering instrument (Wyatt Technology); and the concentrations of eluting species were determined using an inline Optilab DSP interferometric refractometer (Wyatt Technology). Refractive index increment (dn/dc) estimates of 0.190 and 0.185 mL g^{-1} were used for protein²⁷ and DNA,²⁸ respectively.

Analytical ultracentrifugation

Data were acquired on an OptimaTM XLA analytical ultracentrifuge (Beckman Instruments) using an An-60ti rotor, samples were prepared in PBSA and the same buffer was used as a reference solution. Sedimentation equilibrium data were obtained at three rotor speeds (16,000, 20,000, 30,000 rpm for TAL1_{bHLH} and 16,000, 22,000, and 28,000 rpm for LMO2-Ldb1_{LID}) at various protein concentrations (1.7 and 3.4 μM TAL1_{bHLH}; 36, 18, and 9 μM LMO2-Ldb1_{LID}). Data were the average of 10 scans recorded at 230 or 280 nm and baseline-corrected with data recorded at 360 nm. Data were analyzed in the program NONLIN using a nonlinear least-squares curve fitting algorithm.²⁹ Sedimentation velocity experiments were run at 42,000 rpm and data collected at 230 or 232 nm at 3-min intervals and the data were analyzed using SedFit.³⁰ Protein partial specific volumes and solvent densities (1.0062 g mL^{-1}) were estimated using Sednter.³¹

Fluorescence spectrophotometry

TAL1_{bHLH} was labeled with AlexaFluor[®] 488 5-TFP (Invitrogen) at a 10:1 weight-to-weight protein:dye ratio in 100 mM NaHCO₃ (pH 8.1). Labeling reactions were allowed to proceed for 1.5–2.0 h at room temperature and then overnight at 4°C with constant mixing. After labeling, precipitated proteins were collected by centrifugation (12,000g, 10 min, room temperature), redissolved in 8M urea, dialyzed overnight against ~5 L of PBSB (50 mM NaH₂PO₄, 150 mM NaCl, and 1 mM DTT) at pH 5.0 and then ~5 L of PBSB at pH 7.4. The dialysate was centrifuged (12,000g, 10 min, room temperature) and the supernatant reserved for fluorescence anisotropy experiments. Samples were stored at 4°C until required. The extent of labeling was calculated as described in the manufacturer's manual and was typically between 0.5 and 1.0 mol dye (mol protein)⁻¹.

Fluorescence anisotropy experiments were conducted at 25°C in PBSB (pH 7.4) on a Julabo-F12 temperature controlled Shimadzu RF-5301PC spectrofluorophotometer. Samples were excited at 495 nm and the emitted light was measured at 519 nm for 20–30 s at 1-s intervals and the signal averaged. Longpass filters (Coherent, Santa Clara, CA) were used on the excitation (GG-475) and emission (OG-515) sources. Fluorescence intensities were corrected for the buffer baseline.

Tryptophan fluorescence emission spectra of TAL1_{bHLH} (0.5 μM) in the presence of increasing concentrations of E12_{bHLH} were recorded at 25°C in PBSB (pH 7.4) on a Cary Eclipse fluorescence spectrophotometer fitted with a Cary Temperature Controller (Varian). Samples were excited at a wavelength of 285 nm and the emission spectrum was recorded between 315–385 nm at a speed of 60 nm min⁻¹ and 0.5 nm resolution. Final spectra were the average of three scans and the baseline was corrected for the buffer. Data from tryptophan titration experiments were normalized as follows. Ratios of fluorescence intensity at two separate wavelengths were calculated ($I_{\lambda 1}/I_{\lambda 2}$) for each concentration point in the titration. These ratios were then substituted into Eq. (1).

$$I_{\text{norm}} = \frac{I_i - I_{\text{min}}}{I_{\text{max}} - I_{\text{min}}} \quad (1)$$

I_{norm} is the normalized intensity ratio; I_i is the intensity ratio ($I_{\lambda 1}/I_{\lambda 2}$) at the concentration point i ; I_{min} is the average minimum intensity ratio for the experiment; I_{max} is the average maximum intensity ratio for the experiment. This process was repeated at several other wavelength combinations, and the normalized values for each concentration point were averaged.

Protein homo- and hetero-dimerization data were analyzed using nonlinear least-squares fitting of models in MicroCal Origin 6.0 (MicroCal). The models used were

either in-built in Origin 6.0 or based on standard homo- [Eq. (2)] or heterodimerization [Eq. (3)] equilibria.

$$\Phi_{\text{obs}} = \Phi_{\text{M}} + (\Phi_{\text{D}} - \Phi_{\text{M}}) \left\{ \frac{4A_{\text{tot}} + \frac{1}{K_{\text{A}}} - \sqrt{\frac{1}{K_{\text{A}}} (8A_{\text{tot}} + \frac{1}{K_{\text{A}}})}}{4A_{\text{tot}}} \right\}, \quad (2)$$

$$\Phi_{\text{obs}} = \Phi_{\text{M}} + (\Phi_{\text{D}} - \Phi_{\text{M}}) \frac{1}{2K_{\text{AB}}} \times \left\{ 1 + A_{\text{tot}}K_{\text{AB}} + B_{\text{tot}}K_{\text{AB}} - \sqrt{-4A_{\text{tot}}B_{\text{tot}}K_{\text{AB}}^2 + (1 + A_{\text{tot}}K_{\text{AB}} + B_{\text{tot}}K_{\text{AB}})^2} \right\} \quad (3)$$

where, A_{tot} is the total concentration of the molecule under observation, B_{tot} is the total titrant concentration [Eq. (3) only], “obs” is the experimentally observed signal at a particular titration point, “M” is the signal corresponding to unbound/monomeric protein, “D” is the signal corresponding to bound/dimeric protein, and K_{A} and K_{AB} are the equilibrium association constants for homodimerization and heterodimerization, respectively.

Mathematical expression describing competition between TAL1_{bHLH} homodimerization and TAL1_{bHLH}-E12_{bHLH} heterodimerization were generated in *Mathematica* 4.0 software (Wolfram Research), where TAL1_{bHLH} concentration and K_{TT} were fixed and K_{TE} was varied. Simulated titration data sets comprised a series of calculations using the experimental range of E12_{bHLH} concentrations and a different value for K_{TE} .

Isothermal titration calorimetry

Purified proteins, protein complexes and double-stranded DNA (see Supplementary Data 1 for concentrations) were dialyzed overnight at 4°C against several litres of PBSA (20 mM NaH₂PO₄ (pH 7.4), 150 mM NaCl, 1 mM DTT) in the same reservoir. Samples were centrifuged (10,000g, 4°C, 10 min), degassed and equilibrated to 25°C. All experiments were performed at 25°C using a VP-Isothermal titration calorimetry (ITC) microcalorimeter (MicroCal). Baseline data were measured by titration of the titrant into PBSA and subtracted from experimental data. Data were analyzed using the Origin 5.0 ITC data analysis software package (MicroCal) to generate estimates of stoichiometry, association constant K_{A} , change in enthalpy (ΔH), and change in entropy (ΔS). Changes in Gibbs' free energy (ΔG) were calculated by $\Delta G = \Delta H - T\Delta S$ or $\Delta G = -RT \ln(K_{\text{A}})$ where T is the temperature in Kelvin (298 K), and R is the gas constant.

Simulations of competing equilibria

The *Mathematica* 5.2 add-on package MetabolicControlAnalysis^{32,33} was used to translate the competing equilibria into a kinetic model. The values of on rate constants were estimated (to range from the diffusion limit of 10^8 – 10^3 $M^{-1} s^{-1}$). The off rate constants were calculated from the assumed value of the on rate constant divided by the association constant. All of the known competing equilibria were defined, and the initial monomeric species concentrations (0.1–10 μM) were chosen to reflect experimental conditions (e.g., EMSA concentrations) or as best guess estimates of nuclear protein concentrations. The concentrations of complexes were initially set to a non-zero value $\sim 0.1\%$ of the primary monomeric reagents. The changes to concentrations of monomers and complexes were simulated over a time sufficient to reach equilibrium (e.g., ~ 5 min with k_{on} values of 10^6 $M^{-1} s^{-1}$).

RESULTS

Characterization of individual components

We first characterized the domains of each protein that were directly involved in LMO2/Ldb1/TAL1/E12/DNA complex formation: the LIM domains of LMO2, Ldb1_{LID}, TAL1_{bHLH}, and E12_{bHLH} [Fig. 2(a)], and a double-stranded Ebox motif (CCGCCAGGTGCTCG; EboxGG) the sequence of which is based on site-selection experiments¹⁶ that identified consensus DNA sequences that were bound by complexes containing TAL1, E2A proteins, and LMO2. Recombinant LMO2 is unstable in solution; however, it can be stabilized by engineering chimeric proteins, in which LMO2 is fused to Ldb1_{LID} via a flexible linker [LMO2-Ldb1_{LID}; Fig. 2(b)].^{24,34} These intramolecular LMO-Ldb1_{LID} complexes have native-like structure.^{24,35} Unless otherwise stated, TAL1_{bHLH} contained an N-terminal HIS₆-tag, whereas E12_{bHLH} was either purified away from a GST-affinity tag or fused to the C-terminus of MBP (MBP-E12_{bHLH}).

LMO2-Ldb1_{LID}, double-stranded EboxGG, TAL1_{bHLH}, and E12_{bHLH} were all subjected to size exclusion chromatography monitored by multiangle laser light scattering to assess their oligomeric states. EboxGG and LMO2-Ldb1_{LID} each eluted as a single peak by gel filtration, with relative molecular masses (Mr) corresponding to a double-stranded 14 bp DNA molecule [8.9 ± 0.6 kDa cf. theoretical Mr 8.5 kDa; Fig. 3(a)], and a monomer [22.0 ± 0.5 kDa cf. theoretical Mr 19.8 kDa; Fig. 3(b)], respectively. TAL1_{bHLH} eluted as a single peak with a Mr of 20.6 ± 1.0 kDa [Fig. 5(a)] indicating the formation of a discrete homodimer (theoretical Mr 18.4 kDa). Sedimentation equilibrium analysis was used to confirm the solution molecular weight estimates for LMO2-Ldb1_{LID} [Mr

Figure 2

Protein constructs used in these studies. (a) The domain structure of TAL1 and E12 and the amino acid sequences of their bHLH domains. (b) Schematic showing the construction of the LMO2-Ldb1_{LID} chimera.

20.4 ± 1.1 , Fig. 3(c)] and TAL1_{bHLH} [Mr 18.3 ± 1.5 kDa; Fig. 4(a)].

E12_{bHLH} eluted as a single peak but the position of the peak shifted according to the protein concentration [Fig. 4(b)]. At higher concentrations of protein (~ 50 μM) this peak had a lower retention time and a solution Mr corresponding to a dimer [16.1 ± 0.3 kDa cf. theoretical Mr 15.6 kDa; Fig. 4(d) *upper panel* and Fig. 5(a)]. At lower protein concentrations the retention time increased, consistent with the protein eluting as a monomer, or in an apparent monomer–dimer equilibrium [Fig. 4(b) *lower panels*]. Multiangle laser light scattering analysis at the lower concentrations was not possible because of low signal: noise ratios. Sedimentation equilibrium analysis of the E12_{bHLH} samples was not feasible as the system did not come to equilibrium, probably because of the slow formation of aggregates. Thus, sedimentation velocity analysis, using a loading concentration of ~ 15 μM , was used to further investigate the oligomeric state this protein. Data were fitted by a continuous C(S) distribution, revealing a main peak ($\sim 50\%$ of the population) with a sedimentation coefficient of ~ 1.4 , a secondary peak ($\sim 21\%$) with a sedimentation coefficient of ~ 2.6 , and a series of additional peaks of smaller size [Fig. 4(c)]. This indicates that monomeric E12_{bHLH} can associate to form dimers and a wide range of higher order oligomeric species.

Far-UV CD confirmed that both TAL1_{bHLH} and E12_{bHLH} formed α -helical structure. Under physiological

Figure 3

Characterization of EboxGG and LMO2-Ldb1_{LID}. SEC/MALLS analysis of (a) EboxGG (loading concentration 100 μ M) and (b) the LMO2-Ldb1_{LID} chimera (loading concentration $\sim$ 300 μ M). (c) Sedimentation equilibrium analysis of the solution association state of LMO2-Ldb1_{LID}. Lower panel shows a plot of absorbance (280 nm) versus radial position (grey circles) for a sample of LMO2-Ldb1_{LID} (36 μ M, 28,000 rpm) and the fit for an ideal single species model (solid grey line). Upper panel shows the residual deviations of the experimental data from the fitted ideal single species model.

conditions both TAL1_{bHLH} (7 μ M) and E12_{bHLH} (10 μ M) showed the double minima at 208 and 222 nm, that is, characteristic of α -helical structure [Fig. 4(d)]; they were

estimated to contain $76\% \pm 8\%$ and $58\% \pm 3\%$ α -helix at these concentrations, respectively.

Measurement of TAL1_{bHLH} homodimerization (K_{TT})

In this study a range of different techniques was required to determine the different association constants; no single technique was appropriate for all systems. In general, ITC was used to assess heteroassociation where no rearrangement of the complex components was evident. Spectroscopic methods were used to assess homo-oligomerization and complex equilibria.

Fluorescence anisotropy was used to determine the K_A for TAL1_{bHLH} homodimerization (K_{TT}). TAL1_{bHLH} was labeled with AlexaFluor[®] 488 and a sample assayed by SEC/MALLS to demonstrate that the labeled protein still forms a dimer of the correct molecular weight (Supplementary Data 2). The labeled protein was then serially diluted from 4.2 to 26 nM and the fluorescence anisotropy of each sample was measured. A graph of $\log$ [TAL1_{bHLH}] versus fluorescence anisotropy was sigmoidal [Fig. 4(e)]; this decrease in anisotropy is consistent with a decrease in apparent Mr. A change from 90 to 10% dimer in an ideal monomer–dimer equilibrium should accompany a concentration change of 2.86 log units, rather than the $\sim$ 1.5 log units evident in Figure 4(e). It is possible that the labeled protein also forms small amounts of soluble aggregate causing this relatively rapid change; however, similar observations are not uncommon^{36–39} and the inflexion point of the transition (as determined from a fit to a logistic function; $y = (A_1 - A_2)/[1 + (x/x_0)^p] + A_2$, where A_1 and A_2 are the initial and final values of anisotropy, x is the concentration, p is the power, and x_0 is the concentration at the inflexion point) is a good approximation of the equilibrium dissociation constant (K_D)³⁸; this corresponded to 0.19 ± 0.01 μ M for TAL1_{bHLH} ($K_{TT} = 5.3d \times 10^6$ M^{-1}).

Measurement of E12_{bHLH} self-association (K_{EE})

The self-association of E12_{bHLH} was not assessable by fluorescence anisotropy as the AlexaFluor[®] 488-labeled protein was relatively insoluble; at low protein concentrations (5 μ M) the anisotropy signal was negligible, consistent with the protein being largely unfolded and monomeric.

Far-UV CD analysis of bHLH domains from MyoD⁴⁰ and MASH-1³⁹ showed that a loss in α -helical structure of the bHLH domains accompanied dissociation of the bHLH dimer. Therefore in a similar manner, we used far-UV CD to estimate the K_A of E12_{bHLH} self-association (K_{EE}). Upon dilution of E12_{bHLH}, changes in the helical content of E12_{bHLH} were evident over a concentration range of 5–30 μ M [Fig. 4(f)] and present as a monophasic transition. The mid-point of the transition, $\sim$ 13.5

Figure 4

Characterization of TAL1_{bHLH} and E12_{bHLH}. (a) Sedimentation equilibrium analysis of the solution association state of TAL1_{bHLH}. Lower panel shows a plot of absorbance (230 nm) versus radial position (grey circles) for a sample of TAL1_{bHLH} (3.4 μ M, 30,000 rpm) and the fit for an ideal single species model (solid line). Upper panel shows the residual deviations of the experimental data from the fitted ideal single species model. (b) E12_{bHLH} (55 μ M) was subjected to SEC (upper panel), the peak was collected and resubjected to SEC (middle panel), and this process repeated for the intermediate sample (lower panel). Elution volumes for starting (upper panel) and finishing (lower panel) sample are marked by dashed lines. (c) Sedimentation velocity data for E12_{bHLH} (15 μ M). Data (150 scans collected at 3-min intervals) were analyzed using a continuous C(S) distribution models. The inset is an expansion from $\sim S = 4$ –18. (d) Far-UV CD spectra of TAL1_{bHLH} (grey; 7 μ M), E12_{bHLH} (black; 10 μ M) and E12_{bHLH} (10 μ M with the addition of equimolar EboxGG dashed line) recorded at 25°C. (e) The fluorescence anisotropy of AlexaFluor[®] 488-labeled TAL1_{bHLH} at varying protein concentrations at 25°C. (f) Concentration dependence of the helical content (F_H) of E12_{bHLH} as determined by far UV-CD.

Figure 5

Characterization of TAL_{bHLH}/E12_{bHLH} heterodimerization. (a) SEC/MALLS analysis of E12_{bHLH} (black; loading concentration 55 μ M), TAL1_{bHLH} (light grey; loading concentration 57 μ M), and a TAL1_{bHLH}/E12_{bHLH} heterodimer (dark grey; loading concentration ~ 30 μ M). (b) Sedimentation velocity analysis of a 1:1 mix of TAL1_{bHLH}/E12_{bHLH} (15 μ M). Data (150 scans recorded at 3-min intervals) were analyzed using Sedfit using a continuous C(S) distribution model. The observed peak has a sedimentation coefficient $S = 1.72 \pm 0.09$, frictional coefficient $f = 1.3$, and estimated molecular weight of 15.3 kDa. (c) Tryptophan fluorescence of TAL1_{bHLH} (0.5 μ M) titrated with E12_{bHLH} at 25°C. Dashed line shows a fit a model for heterodimerization. Coloured lines are simulated data sets, for competition between TAL1_{bHLH} homodimerization and heterodimerization with E12_{bHLH}, obtained using different values for K_{TE} (as indicated in M^{-1} units) and a fixed value for K_{TT} ($5.3 \times 10^6 M^{-1}$).

$\pm 1.1 \mu\text{M}$, was determined by fitting the data with a logistic function. The rapid change in signal with changing concentration likely arises from the formation of multiple higher order species. In comparison, no significant change in the CD spectrum of TAL1_{bHLH} was observed over a similar concentration range, consistent with that protein existing as the homodimeric complex at concentrations above $\sim 2 \mu\text{M}$. It is noted that the sample of E12_{bHLH} used for Figure 4(d) was a mixture of monomer and other species. Samples containing little or no monomer E12 [$\sim 30 \mu\text{M}$; Fig. 4(f)] were estimated to have an α -helical content of $\sim 80\%$ —the same helical content as dimeric TAL1_{bHLH}. We surmise that at low concentrations E12_{bHLH} is monomeric and partially unfolded, but associates to form homodimers and higher order species with an apparent K_D of $13.5 \mu\text{M}$ (viz. $K_{EE,app} \approx 7.4 \times 10^4 \text{ M}^{-1}$). This is consistent with studies that showed no self-association of full-length E12 at low concentrations, but suggest that small changes in fluorescence anisotropy described for a Dansyl-labeled full length E12 at $\sim 20 \mu\text{M}$ protein concentration were significant and indicated self-association of the protein.⁴¹

Measurement of TAL1_{bHLH}-E12_{bHLH} heterodimerization (K_{TE})

In general, it is thought that TAL1 must heterodimerise with E12 (or other ubiquitous bHLH proteins) before it can bind to DNA.⁷ To determine whether heterodimerization occurs in the absence of DNA, TAL1_{bHLH} and E12_{bHLH} were subjected to size exclusion chromatography monitored by multiangle laser light scattering. Equimolar mixtures of TAL1_{bHLH} and E12_{bHLH} gave rise to an elution volume (15.43 mL) that was intermediate to those for TAL1_{bHLH} and E12_{bHLH} [Fig. 5(a)]. This corresponded to a molecular weight estimate of $17.8 \pm 1.1 \text{ kDa}$ compared with the theoretical value of the heterodimer of 17 kDa . Sedimentation velocity data collected on an equimolar mixture of TAL1_{bHLH} ($15 \mu\text{M}$) and E12_{bHLH} ($15 \mu\text{M}$) shows only a single sedimenting species, confirming they form a 1:1 heterodimer [Fig. 5(b)].

Intrinsic tryptophan fluorescence was used to estimate the K_A for the formation of the TAL1_{bHLH}-E12_{bHLH} heterodimer (K_{TE}). TAL1_{bHLH} contains a single Trp residue (W198) in the basic region that extends the first α -helix of the bHLH domain [Fig. 2(a)], whereas E12_{bHLH} contains no Trp residues. Titration of E12_{bHLH} into a constant concentration of TAL1_{bHLH} ($0.5 \mu\text{M}$) caused a red shift in the fluorescence emission maximum, λ_{max} , from 344 to 348 nm . This shift of fluorescence maximum was monitored using the ratio of fluorescence intensity at wavelengths either side of λ_{max} [Fig. 5(c)]. When fitted by an equation that describes a simple monomer–dimer equilibrium the apparent association constant K_{TE} had the value $(3.5 \pm 0.6) \times 10^7 \text{ M}^{-1}$. This value does not take into account the effect of competition for hetero-

dimer formation by homo-oligomerization of E12_{bHLH} or TAL1_{bHLH} so it is simply an estimate. However, given that the highest concentration of E12_{bHLH} used in the binding analysis was $\sim 2 \mu\text{M}$ the effect of E12_{bHLH} homodimerization should be negligible. On the other hand TAL1_{bHLH} homodimerization could have competed with heterodimer formation. To determine the extent of this, isotherms were simulated in *Mathematica* using the competitive binding between TAL1_{bHLH} homodimerization (with $K_{TT} = 5.3 \times 10^6 \text{ M}^{-1}$) and TAL1_{bHLH}-E12_{bHLH} heterodimerization, where K_{TE} was set to values ranging from 2×10^6 to $2 \times 10^8 \text{ M}^{-1}$. The simulated data best mimicked the experimental data when K_{TE} was set to values between 3×10^7 and $5 \times 10^7 \text{ M}^{-1}$ [Fig. 5(c)]; this compares well with that for K_{TE} obtained using the standard non-competitive model ($3.5 \times 10^7 \text{ M}^{-1}$) so it was used as a close approximation to K_{TE} . It is almost an order of magnitude larger than the homodimerization constant for TAL1_{bHLH} indicating a clear preference of these proteins to form heterodimers rather than homodimers.

The formation of multiprotein DNA-containing complexes

It was previously shown that full length LMO2, Ldb1, TAL1, and E2A proteins can form multiprotein complexes in cell lines,^{16,17} and that LMO2 and TAL1 can directly interact in mammalian two-hybrid systems.⁴² To determine if LMO2-Ldb1_{LID}, TAL1_{bHLH}, and E12_{bHLH} can form multiprotein complexes on DNA, electrophoretic mobility shift assays (EMSAs) were carried out using a radiolabeled EboxGG probe [Fig. 6(a)]. Here MBP-E12_{bHLH} ($\sim 50 \text{ kDa}$) was used to maximise shifts and distinguish between different proteins. MBP-E12_{bHLH} appeared to specifically bind the probe (lane 3). However, TAL1_{bHLH} appeared to form large non-specific TAL1_{bHLH}/DNA aggregates that did not enter the gel (lane 2). This outcome was consistent with the previous report that isolated TAL1 does not exhibit any specific DNA-binding.⁴³ We observed a similar phenomenon with a non-Ebox probe, and the addition of increasing amounts of poly-dIdC, heparin, or BSA reduced the amount of precipitated material, (data not shown).

The addition of both MBP-E12_{bHLH} and TAL1_{bHLH} to the DNA probe resulted in a band with a smaller shift than that of MBP-E12_{bHLH} alone (lane 6), suggesting binding of the smaller heterodimer.

LMO2-Ldb1_{LID} induced an additional shift (lanes 7 and 8), implying that LMO2-Ldb1_{LID} can bind to a TAL1_{bHLH}/MBP-E12_{bHLH} heterodimer on DNA. This was confirmed by the addition of either anti-HIS₆ (lane 9) or anti-MBP (lane 10) antibodies that are both able to produce an additional shift (supershift) of this complex. Control experiments showed that MBP-E12_{bHLH} was required for the complex to bind DNA (lane 12), and

Figure 6

Analysis of the protein-protein and protein-DNA interaction network LMO2/Ldb1/TAL1/E12/DNA complexes. + and - symbols indicate the inclusion or omission of the specific protein in the binding reaction. (a) EMSA analysis of EboxGG-binding by LMO2-Ldb1_{LID}/TAL1_{bHLH}/E12_{bHLH} complexes. MBP-E12_{bHLH}, TAL1_{bHLH}, and MBP were all added to a final concentration of ~2 μ M and LMO2-Ldb1_{LID} to a final concentration of ~10 μ M. Migration positions of MBP-E12_{bHLH}/DNA (E; lanes 3 and 11), TAL1_{bHLH}/MBP-E12_{bHLH}/DNA (TE; lane 6), LMO2/Ldb1_{LID}/TAL1_{bHLH}/MBP-E12_{bHLH}/DNA (TLE; lanes 7 and 8), antibody-supershifted complexes (lanes 9 and 10), and unbound ³²P-EboxGG are indicated. (b) GST-pulldown analysis of GST-E12_{bHLH}, TAL1_{bHLH}, and LMO2-Ldb1_{LID} interactions. Asterisks in lanes 2 and 3 indicate the position of bands corresponding to MBP and MBP-TAL1_{bHLH}, respectively. Upper panel: pulldown experiments using GST-E12_{bHLH} immobilised on GSH-resin. Lower panel: control pulldown experiments using GST immobilised on GSH-resin. Lanes 2, 3, and 9 represent 5% input of the respective protein samples. (c) SEC/MALLS analysis of a LMO2-Ldb1_{LID}/TAL1_{bHLH}/E12_{bHLH} complex in the presence (black) or absence (light grey) of EboxGG DNA. Solid lines represent absorbance and squares represent MALLS-calculated Mr.

that TAL1_{bHLH} was required for LMO2-Ldb1_{LID} to take part in the complex (lane 11). This pattern of protein-protein interactions was also observed in GST-pulldown experiments carried out in the absence of DNA [Fig. 6(b)]. GST-E12_{bHLH} could retain MBP-TAL1_{bHLH} (lane 5 upper panel) but not LMO2-Ldb1_{LID} (lane 6 upper panel). However, LMO2-Ldb1_{LID} was retained by GST-E12_{bHLH} in the presence of MBP-TAL1_{bHLH} (lane 7 upper panel). Control experiments using only GST-bound resin [Fig. 6(b) lower panel] and/or MBP (lanes 4 and 8) showed that the observed interactions were not due to the presence of the fusion-protein tags.

Samples of heterodimeric TAL1_{bHLH}-E12_{bHLH} that had been isolated by SEC were mixed with an approximately equimolar amount of LMO2-Ldb1_{LID} in the presence and absence of DNA and subjected to size exclusion chromatography monitored by multiangle laser light scattering analysis. This was done to determine the stoichiometries of the LMO2-Ldb1_{LID}/TAL1_{bHLH}/E12_{bHLH} complexes. The resulting species had a Mr of 36.8 ± 2.2 kDa [Fig. 6(c)], which correlates well with the theoretical value (36.6 kDa) of a complex comprising a single molecule each of TAL1_{bHLH}, E12_{bHLH}, and LMO2-Ldb1_{LID}. Addition of EboxGG resulted in an increase in the Mr (46.5 ± 1.5 kDa) [Fig. 6(c)] that was consistent with the addition of one dsDNA molecule (8.5 kDa) to the protein heterotrimer.

Measurement of LMO2-Ldb1_{LID} binding to TAL1_{bHLH} ($K_{L \rightarrow T}$), TAL_{bHLH}/E12_{bHLH} ($K_{L \rightarrow TE}$), and a TAL_{bHLH}/E12_{bHLH}/EboxGG complex ($K_{L \rightarrow TEGG}$)

A series of ITC experiments was carried out to characterize further the formation of this protein-DNA complex (see Fig. 7). LMO2-Ldb1_{LID} was titrated into TAL1_{bHLH} to yield an association constant of $K_{L \rightarrow T} = (6.3 \pm 1.4) \times 10^5$ M⁻¹ [Fig. 7(a)]. As expected from our GST-pulldown data [Fig. 6(b)] and previous studies^{42,44} no evidence of an interaction could be seen in a titration of LMO2-Ldb1_{LID} into E12_{bHLH} [Fig. 7(b)].

Strikingly, the titration of LMO2-Ldb1_{LID} into a preformed complex of TAL1_{bHLH}/E12_{bHLH} yielded an association constant for this interaction of $K_{L \rightarrow TE} = (1.8 \pm 0.6) \times 10^8$ M⁻¹ [Fig. 7(c)], nearly 300-fold stronger than $K_{L \rightarrow T}$. This preference for bHLH heterodimers was also evident in the GST-pulldown shown in Figure 6(b), as the presence of LMO2-Ldb1_{LID} increased the amount of TAL1_{bHLH} retained by GST-E12_{bHLH}.

A similar association constant was obtained when LMO2-Ldb1_{LID} was titrated into a preformed complex comprising the bHLH heterodimer bound to DNA, $K_{L \rightarrow TEGG} = (1.7 \pm 0.9) \times 10^8$ M⁻¹. Thus, LMO2-Ldb1_{LID} interacted with TAL1_{bHLH} with low affinity, but

Figure 7

ITC data showing titrations of LMO2-Ldb1_{LID} into TAL1_{bHLH} (a), E12_{bHLH} (b), and TAL1_{bHLH}/E12_{bHLH} heterodimers (c) recorded at 25°C.

preferentially bound the TAL1_{bHLH}/E12_{bHLH} heterodimer, in the presence or absence of DNA.

Measurement of EboxGG binding to E12_{bHLH} ($K_{GG \rightarrow E}$), TAL_{bHLH}/E12_{bHLH} ($K_{GG \rightarrow TE}$), and a LMO2/Ldb1_{LID}/TAL_{bHLH}/E12_{bHLH}/EboxGG complex ($K_{GG \rightarrow LITE}$)

When EboxGG DNA was added to the low concentration (10 μM) E12_{bHLH} sample used for Figure 4(d) there was a marked increase in helicity $81\% \pm 5\%$ estimated by far UV-CD, to levels similar to that when all the E12_{bHLH} was present in an oligomeric form. This indicated that DNA-binding enhanced dimer formation of E12.

Using ITC, the titration of EboxGG into E12_{bHLH} and TAL1_{bHLH}/E12_{bHLH} indicated that E12_{bHLH} bound to that sequence more strongly than did the heterodimer; $K_{GG \rightarrow E} = (4.3 \pm 0.2) \times 10^7 M^{-1}$ versus $K_{GG \rightarrow TE} = (2.5 \pm 0.1) \times 10^6 M^{-1}$, respectively. When EboxGG was titrated into a preformed complex comprising LMO2-Ldb1_{LID} and TAL1_{bHLH}/E12_{bHLH} a slightly weaker binding constant was determined, $K_{GG \rightarrow LITE} = (1.7 \pm 0.1) \times 10^6 M^{-1}$. In this experiment there was evidence of a small initial binding event followed by a main transition. The data were fitted by a two-site binding model, where the K_A and ΔH of one site were constrained to the values determined for the titration of EboxGG into E12_{bHLH} (see Supplementary Data 3). Thus, the initial binding event was probably due to small amounts of free E12_{bHLH} in the ITC cell.

Measurement of EboxGC binding to E12_{bHLH} ($K_{GC \rightarrow E}$), TAL_{bHLH}/E12_{bHLH} ($K_{GC \rightarrow TE}$), and a LMO2/Ldb1_{LID}/TAL_{bHLH}/E12_{bHLH}/EboxGG complex ($K_{GC \rightarrow LITE}$)

To investigate the sequence preferences of LMO2/TAL1/E12 DNA-binding, a second Ebox motif was assayed for binding the various bHLH-containing complexes. This sequence (CTATCAGCTGATGA; EboxGC) was based on the promoter region of *GPA*, a target for regulation by LMO2/TAL1/E2A complexes.¹⁹ EboxGC bound E12_{bHLH} with ~ 30 -fold lower binding affinity than did EboxGG [$K_{GC \rightarrow E} = (1.6 \pm 0.1) \times 10^6 M^{-1}$], and appeared to bind the bHLH heterodimer at approximately the same level ($K_{GC \rightarrow TE} \approx 1.5 \times 10^6 M^{-1}$). However, this value was only an estimate as there was evidence of a second, unidentified binding event that could not be analyzed using simple binding models (see Supplementary Data 3).

Finally, EboxGC bound the LMO2/Ldb1_{LID}/TAL1_{bHLH}/E12_{bHLH} complex with ~ 4 -fold lower affinity ($K_{GC \rightarrow LITE} = (4.0 \pm 0.8) \times 10^5 M^{-1}$) than the E12_{bHLH} homodimer or the bHLH heterodimer.

Simulations of complex formation

Which of the complexes described earlier form *in vivo* depends on multiple competing equilibria (e.g., Fig. 1; Table I), and the concentrations (i.e., expression levels) of the different components. To gain insight into what might be taking place within a cellular context, we used the various association constants and equilibrium reaction schemes for the binding of these molecules (Table I)

Table I

Summary of the Thermodynamic Parameters for the Protein-Protein and Protein-DNA Interactions Investigated in these Studies

Equilibrium reaction	Symbol	K_A (M^{-1})	ΔH (kcal mol^{-1})	$-T\Delta S$ (kcal mol^{-1})	ΔG_{298K} (kcal mol^{-1})
Protein only reactions					
$2E \leftrightarrow E_2^a$	K_{EE}	$(7.4 \pm 0.6) \times 10^{4a}$	—	—	$-6.6 \pm <0.1$
$2T \leftrightarrow T_2$	K_{TT}	$(5.3 \pm 0.3) \times 10^6$	—	—	$-9.2 \pm <0.1$
$T + E \leftrightarrow TE$	K_{TE}	$(3.5 \pm 0.6) \times 10^7$	—	—	-10.3 ± 0.1
$L + I \leftrightarrow LI$	K_{LI}	5×10^{7b}	—	—	-10.5
$LI + E \leftrightarrow LIE$	$K_{LI \rightarrow E}$	$<10^3$	—	—	—
$LI + T_2 \leftrightarrow LIT_2$	$K_{LI \rightarrow T}$	$(6.3 \pm 1.4) \times 10^5$	-5.3 ± 1.7	-2.7 ± 1.6	-7.9 ± 0.1
$LI + TE \leftrightarrow LITE$	$K_{LI \rightarrow TE}$	$(1.8 \pm 0.6) \times 10^8$	-11.9 ± 2.2	0.7 ± 2.4	-11.3 ± 0.2
EboxGG reactions					
$LI + TEGG \leftrightarrow LITEGG$	$K_{LI \rightarrow TEGG}$	$(1.7 \pm 0.9) \times 10^8$	-10.3 ± 1.0	-0.9 ± 1.3	-11.2 ± 0.3
$GG + 2E \leftrightarrow E_2GG$	$K_{GG \rightarrow E}$	$(4.3 \pm 0.2) \times 10^7$	-40.9 ± 0.1	28.9	-9.7 ± 0.1
$GG + TE \leftrightarrow TEGG$	$K_{GG \rightarrow TE}$	$(2.5 \pm 0.1) \times 10^6$	-38.0 ± 0.4	18.3	-8.8 ± 0.4
$GG + LITE \leftrightarrow LITEGG$	$K_{GG \rightarrow LITE}$	$(1.7 \pm 0.1) \times 10^6$	-26.8 ± 0.5	29.3	-8.5 ± 0.5
EboxGC reactions					
$GC + 2E \leftrightarrow E_2GC$	$K_{GC \rightarrow E}$	$(1.6 \pm 0.1) \times 10^6$	-37.4 ± 0.3	31.1	-8.5 ± 0.3
$GC + TE \leftrightarrow TEGC$	$K_{GC \rightarrow TE}$	$(1.5 \pm 0.4) \times 10^{6c}$	-38.3 ± 0.3	29.9 ± 0.4	-8.4 ± 0.2
$GC + LITE \leftrightarrow LITEGC$	$K_{GC \rightarrow LITE}$	$(4.0 \pm 0.8) \times 10^5$	-40.1 ± 1.3	32.4 ± 1.2	-7.7 ± 0.1

T, TAL1_{bbbs}; E, E12_{bbbs}; L, LMO2; I, Ldb1_{ldb}; GG, EboxGG; GC, EboxGC.^aThe reaction scheme shown only shows the formation of homodimers; however, smaller amounts of higher order oligomers are also formed. The reported K_A is an apparent value, determined by assuming dimerisation only, and is used to indicating a relatively weak interaction.^bThis value has been reported previously and is shown only for comparison.²⁴^cThis value is an approximation only, due to initial deviations in the data from a standard sigmoidal shape that could not be accounted for in the fitting process (see supplementary data, Fig. S1).

to generate a mathematical model, by which we could simulate the formation and dissociation of all possible complexes in the scheme over time. This model was implemented in a Mathematica package MetabolicControlAnalysis³³ that was originally developed to model 2,3-bisphosphoglycerate metabolism in the human erythrocyte.³²

There are no available measurements of protein concentrations for these proteins within the nucleus. It was thus necessary to estimate concentrations of the components. Estimates of 1000–100,000 molecules of each transcription factor per eukaryotic cell,⁴⁵ and a nuclear volume of 10–20 fL (~10% of an average cell volume) yielded protein concentrations of 10 μM –100 nM. On rates for each of the equilibria were also estimated; the off rates were then calculated from the association constant. Regardless of numerical values of the on rate estimates (e.g., all diffusion limited ($K_{on} \times 10^8 M^{-1} s^{-1}$), all several orders of magnitude less, or a mixture of on rates) the schemes were dominated by the higher association constant reactions, although the time taken for various complexes to form obviously varied. Note that in this modeling we used E12 homodimerization rather than more complex models of self-association, but this should have little effect on the overall outcome as little E12 oligomerization should be seen at the protein concentrations used. When TAL1 was introduced to a system that contained E12 and the EboxGG binding site, E12 was largely sequestered into E12-TAL1 heterodimers, either as a protein-only heterodimer, or as a DNA-bound

heterodimer. When LMO2 and Ldb1 were introduced along with TAL1, the predominant species were the protein-only TAL1/E12/LMO2/Ldb1 complex and the DNA bound version of this complex. If both the DNA sites used in this study (EboxGG and EboxGC) were included in the simulation, a similar situation arose, but both versions of the DNA bound complexes were present.

DISCUSSION

Modeling competing equilibria

Many cellular processes are under the control of multi-protein complexes or networks of protein-protein and other interactions. The detailed characterization of the multiple competing equilibria described in this study culminated in the construction of a mathematic model that allowed the distribution of the different components in different complexes and subcomplexes to be predicted, given a set of starting conditions. Within the cell, with changing levels of protein concentrations due to expression and degradation, it is unlikely that networks of interactions ever reach true equilibrium; therefore, a kinetic-based system provided a view of the changing distributions on the approach to equilibrium, despite a requirement for the estimation of on-rates.

There are limitations involved in trying to understand multicomponent complex formation as described in this study. The use of protein domains rather than whole proteins was necessary for implementation of many of

the techniques employed; for example, full-length proteins could not be reasonably produced in sufficient quantity for ITC. It is possible that other parts of the proteins may influence complex formation; nevertheless, the detail provided by this approach reveals some important insights into the molecular basis of the biological function of TAL1, LMO2 and E12.

TAL1/LMO2 inhibition of E2A function

Although both TAL1_{bHLH} and E12_{bHLH} can exist as homo-oligomers with association constants of $\sim 5 \times 10^6$ and $\sim 7 \times 10^4 \text{ M}^{-1}$, the two proteins preferentially form heterodimers with a dimerization constant K_A of $\sim 4 \times 10^7 \text{ M}^{-1}$ and an altered affinity for specific Ebox sequences. Thus, in leukaemic cells where TAL1 is ectopically expressed it is likely that TAL1 sequesters endogenous E2A proteins [Fig. 8(a)].

The binding affinities of LMO2 for TAL1/E12-containing complexes were the highest measured for any interactions in this study (Table I), including LMO2/Ldb1 interactions.²⁴ The sequestration of E2A proteins will, therefore, be much more strongly favoured in cells that express both TAL1 and LMO2 [Fig. 8(b)]. Thus, it is likely that TAL1 has a negative effect on E2A-mediated gene expression and lymphocyte development by preventing E2A proteins from forming specific transcriptional regulatory complexes, and that this effect is exacerbated by LMO2. These binding data provide a mechanism for how TAL1 and LMO2 synergistically contribute to T-ALL⁶ and downregulate E2A-mediated expression of genes,⁴⁶ although there may be additional LMO2-only effects contributing to T-ALL progression.

This sequestration of E2A proteins by TAL1 and LMO2 may also act as a regulatory mechanism in other tissues. Particularly in multipotential precursor cells that may be primed with a range of opposing tissue/lineage specific factors, and the fate of the cell would be determined by factors that favour the activity of one group of these tissue specific factors.^{47,48} In this case, LMO2 would promote the formation of TAL1/E2A heterodimers and drive the development of haematopoietic cells. Indeed, evidence of such a mechanism does exist. TAL1, especially in the presence of LMO2, can hijack cells normally destined for muscle development and cause them to become haematopoietic progenitor cells.^{49,50} This potential switch-like role of LMO2 adds an extra layer of complexity to the functions of LIM-only proteins, which had been thought to act simply as scaffolds that bring together groups of proteins.

DNA binding

The functions of TAL1 in early haematopoietic development,⁵¹ and in the TAL1-mediated induction of leukaemia in mice²² appear to be independent of DNA-

Figure 8

A possible mechanism for the modulation of gene expression by TAL1 and LMO2. TAL1 sequesters E2A proteins from activating specific genes [black; (a)], and this effect is enhanced by LMO2 (b). The association of other DNA-binding factors (X) with LMO2/TAL1/E2A complexes leads to high-affinity DNA-binding at alternative DNA-sites (c) and leads to the activation of different genes (grey).

binding activity. However, the basic region of TAL1 is required for erythropoiesis and other later stages of blood cell development,^{51,52} and is necessary for the control of at least two erythroid-specific genes, *P4.2*⁵³ and *GPA*.¹⁹

The sequences of EboxGG and EboxGC come from the consensus sequence determined by site-selection experiments,¹⁶ and the promoter sequence of an actual target gene for TAL1/LMO2 complexes (*GPA*;¹⁹), respectively. It was therefore surprising to find that those sequences bound TAL1/E12 heterodimers with only moderate affinity ($\sim 10^6 \text{ M}^{-1}$) in comparison to other estimates for DNA binding by TAL/E12 ($\sim 10^9 \text{ M}^{-1}$;^{54,55}). However, these previous studies show that DNA binding by TAL1/E12 heterodimers is greatly affected by the sequence flanking the core Ebox motif—so much so that sequences that bind TAL1/E12_{bHLH} heterodimers with nanomolar affinity may not bind at a detectable level (by EMSA) when the bases flanking the Ebox are changed—and this is likely to account for the difference in binding affinities we report here. It has been noted that many putative TAL1/E12 target control sites do not contain favorable flanking sequences and that moderate to low DNA-binding affinities may in fact allow more flexibility in controlling the activity of TAL1/E2A complexes *in vivo*,⁵⁴ which is consistent with our findings.

DNA-binding sites for LMO2/TAL1/E2A complexes are often bipartite in nature, comprising 1 or 2 Ebox motifs and/or a recognition sequence for another transcription factor. Thus far, GATA sites have been the most commonly identified non-Ebox motif in the bipartite sequences.^{16,19,53} The association of LMO2/TAL1/E2A complexes with GATA proteins would allow coupling of the

DNA-binding affinities of TAL1/E2A heterodimers with those of GATA-1 ($10^9 M^{-1}$), to result in high-affinity DNA-binding [Fig. 8(c)]. The DNA-binding properties of TAL1/E2A heterodimers are also subject to post-translational modification.⁵⁷ These factors could all combine to allow enormous flexibility in the control that can be imparted by LMO2/TAL1/E2A complexes.

Inhibitors of TAL1 and LMO2 activity

This detailed characterization of TAL1/E12/LMO2 binding pathways and complex formation enables us to also predict the best strategies for inhibiting aberrant TAL1 and LMO2 activity in T-ALL. By modifying our Mathematica binding model(s) to include inhibitors of different interactions with varying K_i (binding affinity) values, it appears that selectively inhibiting a single interaction (say between LMO2 and TAL1) will only have a limited affect on restoring the formation of E12 homodimer-DNA complexes, even if single inhibitors with very high K_i values ($10^{-15} M$) are used. Rather, when two inhibitors were included (targeting the LMO2/TAL1 and TAL1/E12 interactions) that have much lower K_i values ($10^{-9} M$) significantly higher levels of E12 homodimer-DNA complexes are restored. Thus, it will likely be more effective to target multiple, rather than individual interactions to effectively inhibit the aberrant activity of TAL1 and LMO2 in T-ALL. This is likely to be generally true for many other systems where multiple interacting factors are involved.

In conclusion, our biophysical data demonstrate that although E12 homodimers can bind specific DNA sites with reasonable affinities, in the presence of TAL1 E12 preferentially forms heterodimers. This effect is exacerbated by the presence of LMO2, which preferentially binds the TAL1-E12 heterodimer. This directly supports a synergistic role for TAL1 and LMO2 in both normal haematopoiesis and the onset of leukemia. Furthermore, the combination of our experimental data with mathematical modeling strategies has underlined the importance of considering multiple interactions when developing inhibitors of complex pathways.

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REFERENCES

- Ferrando AA, Neuberg DS, Staunton J, Loh ML, Huard C, Raimondi SC, Behm FG, Pui CH, Downing JR, Gilliland DG, Lander ES, Golub TR, Look AT. Gene expression signatures define novel oncogenic pathways in T cell acute lymphoblastic leukemia. *Cancer Cell* 2002;1:75–87.
- Begley CG, Green AR. The SCL gene: from case report to critical hematopoietic regulator. *Blood* 1999;93:2760–2770.
- Boehm T, Foroni L, Kaneko Y, Perutz ME, Rabbitts TH. The rhombotin family of cysteine-rich LIM-domain oncogenes: distinct members are involved in T-cell translocations to human chromosomes 11p15 and 11p13. *Proc Natl Acad Sci USA* 1991;88:4367–4371.
- Larson RC, Fisch P, Larson TA, Lavenir I, Langford T, King G, Rabbitts TH. T cell tumours of disparate phenotype in mice transgenic for Rbtn-2. *Oncogene* 1994;9:3675–3681.
- Kelliher MA, Seldin DC, Leder P. Tal-1 induces T cell acute lymphoblastic leukemia accelerated by casein kinase II α . *EMBO J* 1996;15:5160–5166.
- Larson RC, Lavenir I, Larson TA, Baer R, Warren AJ, Wadman I, Nottage K, Rabbitts TH. Protein dimerization between Lmo2 (Rbtn2) and Tal1 alters thymocyte development and potentiates T cell tumorigenesis in transgenic mice. *EMBO J* 1996;15:1021–1027.
- Massari ME, Murre C. Helix-loop-helix proteins: regulators of transcription in eucaryotic organisms. *Mol Cell Biol* 2000;20:429–440.
- Bach I. The LIM domain: regulation by association. *Mech Dev* 2000;91:5–17.
- Aoyama M, Ozaki T, Inuzuka H, Tomotsune D, Hirato J, Okamoto Y, Tokita H, Ohira M, Nakagawara A. LMO3 interacts with neuronal transcription factor, HEN2, and acts as an oncogene in neuroblastoma. *Cancer Res* 2005;65:4587–4597.
- Rabbitts TH. LMO T-cell translocation oncogenes typify genes activated by chromosomal translocations that alter transcription and developmental processes. *Genes Dev* 1998;12:2651–2657.
- Visvader JE, Venter D, Hahm K, Santamaria M, Sum EYM, O'Reilly L, White D, Williams R, Armes J, Lindeman GJ. The LIM domain gene LMO4 inhibits differentiation of mammary epithelial cells in vitro and is overexpressed in breast cancer. *Proc Natl Acad Sci USA* 2001;98:14452–14457.
- Porcher C, Swat W, Rockwell K, Fujiwara Y, Alt FW, Orkin SH. The T cell leukemia oncoprotein SCL/tal-1 is essential for development of all hematopoietic lineages. *Cell* 1996;86:47–57.
- Yamada Y, Warren AJ, Dobson C, Forster A, Pannell R, Rabbitts TH. The T cell leukemia LIM protein Lmo2 is necessary for adult mouse hematopoiesis. *Proc Natl Acad Sci USA* 1998;95:3890–3895.
- Robb L, Elwood NJ, Elefanty AG, Kontgen F, Li R, Barnett LD, Begley CG. The scl gene product is required for the generation of all hematopoietic lineages in the adult mouse. *EMBO J* 1996;15:4123–4129.
- Warren AJ, Colledge WH, Carlton MB, Evans MJ, Smith AJ, Rabbitts TH. The oncogenic cysteine-rich LIM domain protein rbtn2 is essential for erythroid development. *Cell* 1994;78:45–57.
- Wadman IA, Osada H, Grutz GG, Agulnick AD, Westphal H, Forster A, Rabbitts TH. The LIM-only protein Lmo2 is a bridging molecule assembling an erythroid, DNA-binding complex which includes the TAL1, E47, GATA-1 and Ldb1/NLI proteins. *EMBO J* 1997;16:3145–3157.
- Valge-Archer VE, Osada H, Warren AJ, Forster A, Li J, Baer R, Rabbitts TH. The LIM protein RBTN2 and the basic helix-loop-helix protein TAL1 are present in a complex in erythroid cells. *Proc Natl Acad Sci USA* 1994;91:8617–8621.
- Grutz GG, Bucher K, Lavenir I, Larson T, Larson R, Rabbitts TH. The oncogenic T cell LIM-protein Lmo2 forms part of a DNA-binding complex specifically in immature T cells. *EMBO J* 1998;17:4594–4605.
- Lahlil R, Lecuyer E, Herblot S, Hoang T. SCL assembles a multifactorial complex that determines glycophorin A expression. *Mol Cell Biol* 2004;24:1439–1452.
- Bain G, Engel I, Robanus Maandag EC, te Riele HP, Volland JR, Sharp LL, Chun J, Huey B, Pinkel D, Murre C. E2A deficiency leads to abnormalities in $\alpha\beta$ T-cell development and to rapid development of T-cell lymphomas. *Mol Cell Biol* 1997;17:4782–4791.
- O'Neil J, Shank J, Cusson N, Murre C, Kelliher M. TAL1/SCL induces leukemia by inhibiting the transcriptional activity of E47/HEB. *Cancer Cell* 2004;5:587–596.

22. O'Neil J, Billa M, Oikemus S, Kelliher M. The DNA binding activity of TAL-1 is not required to induce leukemia/lymphoma in mice. *Oncogene* 2001;20:3897–3905.
23. Goldfarb AN, Lewandowska K. Inhibition of cellular differentiation by the SCL/tal oncoprotein: transcriptional repression by an Id-like mechanism. *Blood* 1995;85:465–471.
24. Ryan DP, Sunde M, Kwan AH, Marianayagam NJ, Nancarrow AL, vanden Hoven RN, Thompson LS, Baca M, Mackay JP, Visvader JE, Matthews JM. Identification of the key LMO2-binding determinants on Ldb1. *J Mol Biol* 2006;359:66–75.
25. Murphy JB, Kies MW. Note on spectrophotometric determination of proteins in dilute solutions. *Biochim Biophys Acta* 1960;45:382–384.
26. Sreerama N, Woody RW. Estimation of protein secondary structure from circular dichroism spectra: comparison of CONTIN, SELCON, and CDSSTR methods with an expanded reference set. *Anal Biochem* 2000;287:252–260.
27. Foltá-Stogniew E, Williams K. Determination of molecular masses of proteins in solution: implementation of an HPLC size exclusion chromatography and laser light scattering service in a core laboratory. *J Biomol Tech* 1999;10:51–63.
28. Manickam DS, Bisht HS, Wan L, Mao G, Oupicky D. Influence of TAT-peptide polymerization on properties and transfection activity of TAT/DNA polyplexes. *J Control Release* 2005;102:293–306.
29. Johnson ML, Correia JJ, Yphantis DA, Halvorson HR. Analysis of data from the analytical ultracentrifuge by nonlinear least-squares techniques. *Biophys J* 1981;36:575–588.
30. Brown PH, Schuck P. Macromolecular size-and-shape distributions by sedimentation velocity analytical ultracentrifugation. *Biophys J* 2006;90:4651–4661.
31. Hayes DB, Laue T, Philo J. SEDNTERP. USA: University of New Hampshire; 1995.
32. Mulquiney PJ, Kuchel PW. Model of 2,3-bisphosphoglycerate metabolism in the human erythrocyte based on detailed enzyme kinetic equations: computer simulation and metabolic control analysis. *Biochem J* 1999;342 (Part 3):597–604.
33. Mulquiney PJ, Kuchel PW. Modelling metabolism with mathematics. Boca Raton, FL: CRC Press; 2003. 309 p.
34. Deane JE, Sum E, Mackay JP, Lindeman GJ, Visvader JE, Matthews JM. Design, production and characterization of FLIN2 and FLIN4: the engineering of intramolecular Ldb1: LMO complexes. *Protein Eng* 2001;14:493–499.
35. Deane JE, Ryan DP, Sunde M, Maher MJ, Guss JM, Visvader JE, Matthews JM. Tandem LIM domains provide synergistic binding in the LMO4: Ldb1 complex. *EMBO J* 2004;23:3589–3598.
36. Erijman L, Lorimer GH, Weber G. Reversible dissociation and conformational stability of dimeric ribulose biphosphate carboxylase. *Biochemistry* 1993;32:5187–5195.
37. Silva JL, Silveira CF, Junior AC, Pontes L. Dissociation of a native dimer to a molten globule monomer. Effects of pressure and dilution on the association equilibrium of arc repressor. *J Mol Biol* 1992;223:545–555.
38. Xu G, Weber G. Dynamics and time-averaged chemical potential of proteins: importance in oligomer association. *Proc Natl Acad Sci USA* 1982;79:5268–5271.
39. Kunne AG, Allemann RK. Covalently linking BHLH subunits of MASH-1 increases specificity of DNA binding. *Biochemistry* 1997;36:1085–1091.
40. Wendt H, Thomas RM, Ellenberger T. DNA-mediated folding and assembly of MyoD-E47 heterodimers. *J Biol Chem* 1998;273:5735–5743.
41. Maleki SJ, Royer CA, Hurlburt BK. MyoD-E12 heterodimers and MyoD-MyoD homodimers are equally stable. *Biochemistry* 1997;36:6762–6767.
42. Osada H, Grutz G, Axelson H, Forster A, Rabbitts TH. Association of erythroid transcription factors: complexes involving the LIM protein RBTN2 and the zinc-finger protein GATA1. *Proc Natl Acad Sci USA* 1995;92:9585–9589.
43. Hsu HL, Huang L, Tsan JT, Funk W, Wright WE, Hu JS, Kingston RE, Baer R. Preferred sequences for DNA recognition by the TAL1 helix-loop-helix proteins. *Mol Cell Biol* 1994;14:1256–1265.
44. Wadman I, Li J, Bash RO, Forster A, Osada H, Rabbitts TH, Baer R. Specific in vivo association between the bHLH and LIM proteins implicated in human T cell leukemia. *EMBO J* 1994;13:4831–4839.
45. Yang VW. Eukaryotic transcription factors: identification, characterization and functions. *J Nutr* 1998;128:2045–2051.
46. Herblot S, Steff AM, Hugo P, Aplan PD, Hoang T. SCL and LMO1 alter thymocyte differentiation: inhibition of E2A-HEB function and pre-T α chain expression. *Nat Immunol* 2000;1:138–144.
47. Orkin SH. Priming the hematopoietic pump. *Immunity* 2003;19:633–634.
48. Enver T, Greaves M. Loops, lineage, and leukemia. *Cell* 1998;94:9–12.
49. Gering M, Rodaway AR, Gottgens B, Patient RK, Green AR. The SCL gene specifies haemangioblast development from early mesoderm. *EMBO J* 1998;17:4029–4045.
50. Gering M, Yamada Y, Rabbitts TH, Patient RK. Lmo2 and Scl/Tal1 convert non-axial mesoderm into haemangioblasts which differentiate into endothelial cells in the absence of Gata1. *Development* 2003;130:6187–6199.
51. Porcher C, Liao EC, Fujiwara Y, Zon LI, Orkin SH. Specification of hematopoietic and vascular development by the bHLH transcription factor SCL without direct DNA binding. *Development* 1999;126:4603–4615.
52. Ravet E, Reynaud D, Titeux M, Izac B, Fichelson S, Romeo PH, Dubart-Kupperschmitt A, Pflumio F. Characterization of DNA-binding-dependent and -independent functions of SCL/TAL1 during human erythropoiesis. *Blood* 2004;103:3326–3335.
53. Xu Z, Huang S, Chang LS, Agulnick AD, Brandt SJ. Identification of a TAL1 target gene reveals a positive role for the LIM domain-binding protein Ldb1 in erythroid gene expression and differentiation. *Mol Cell Biol* 2003;23:7585–7599.
54. Gould KA, Bresnick EH. Sequence determinants of DNA binding by the hematopoietic helix-loop-helix transcription factor TAL1: importance of sequences flanking the E-box core. *Gene Expr* 1998;7:87–101.
55. Beltran AC, Dawson PE, Gottesfeld JM. Role of DNA sequence in the binding specificity of synthetic basic-helix-loop-helix domains. *Chembiochem* 2005;6:104–113.
56. Omichinski JG, Trainor C, Evans T, Gronenborn AM, Clore GM, Felsenfeld G. A small single-“finger” peptide from the erythroid transcription factor GATA-1 binds specifically to DNA as a zinc or iron complex. *Proc Natl Acad Sci USA* 1993;90:1676–1680.
57. Prasad KS, Brandt SJ. Target-dependent effect of phosphorylation on the DNA binding activity of the TAL1/SCL oncoprotein. *J Biol Chem* 1997;272:11457–11462.