The C-terminal Domain of Eos Forms a High Order Complex in Solution*

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Ikaros family transcription factors play important roles in the control of hematopoiesis. Family members are predicted to contain up to six classic zinc fingers that are arranged into N- and C-terminal domains. The N-terminal domain is responsible for site-specific DNA binding, whereas the C-terminal domain primarily mediates the homo- and hetero-oligomerization between family members. Although the mechanisms of action of these proteins are not completely understood, the zinc finger domains are known to play a central role. In the current study, we have sought to understand the physical and functional properties of these domains, in particular the C-terminal domain. We show that the N-terminal domain from Eos, and not its C-terminal region, is required to recognize GGGA consensus sequences. Surprisingly, in contrast to the behavior exhibited by Ikaros, the C-terminal domain of Eos inhibits the DNA-binding activity of the full-length protein. In addition, we have used a range of biophysical techniques to demonstrate that the C-terminal domain of Eos mediates the formation of complexes that consist of nine or ten molecules. These results constitute the first direct demonstration that Ikaros family proteins can form higher order complexes in solution, and we discuss this unexpected result in the context of what is currently known about the family members and their possible mechanism of action.

Most members of the Ikaros family of transcriptional regulators, including Ikaros (1), Aiolos (2), and Helios (3, 4), are expressed primarily in the hematopoietic system and are essential for normal hemolymphopoiesis (1, 5, 6). Aberrant Ikaros expression has been linked with infant acute lymphoblastic leukemia (9). The recently demonstrated the homo- and heterotypic interactions are crucial for high affinity DNA binding in Ikaros and may allow an additional level of control over gene expression, because different combinations of family members differ in their ability to activate gene expression in transient-transfection assays (2). These C-terminal ZnFs also comply with the classical ZnF consensus sequence (see Fig. 1B), although no DNA-binding ability has yet been identified. Furthermore, they lack the typical TGEKP linker that is present in the majority of classical ZnFs (15). This linker is known to play a role in DNA binding, and its absence lends weight to the suggestion that C-terminal domains of Ikaros proteins may function purely to mediate protein-protein interactions. Little is currently known about the role of classical ZnFs in protein recognition.

We have sought to understand the role of the C-terminal domain of Ikaros family proteins in more detail. We confirm that this domain from Eos (termed EosC) is not directly involved in DNA binding and that it may inhibit the DNA-binding ability of full-length Eos. We also test the binding of Eos to different family members and go on to characterize the EosC homo-oligomer in solution. Using a combination of gel filtration chromatography, light scattering, and sedimentation equilibrium experiments, we demonstrate that Eos forms homomeric complexes comprising nine or ten molecules. Electron microscopy studies of purified EosC and an analysis of full-length Eos overexpressed in mammalian cells support this conclusion. These results are discussed in the context of the likely role of Ikaros family proteins in transcriptional regulation.

EXPERIMENTAL PROCEDURES

Subcloning and Production of Proteins Used in DNA Binding Studies—For binding site selection experiments, the DNA-binding domain of Eos (EosN) was subcloned into pGEX-2T (Amersham Biosciences) to produce EosNC.

The abbreviations used are: ZnF, zinc finger; GST, glutathione S-transferase; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; MBP, maltose-binding protein; GFC, gel filtration chromatography; rpiPLC, reversed-phase high performance liquid chromatography; CD, circular dichroism; TEM, transmission electron microscopy; PC-HC, pericentromeric-heterochromatin.

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42419
Oligomerization of the C-terminal Domain of Eos

create the vector pGEX-2T.Eos101–321. EosN (along with a GST control) was overexpressed in a bacterial system using standard techniques. The production of MBP-EosC was discussed below. The plasmids pGAD10.Ikaros2 (isolated from a yeast two-hybrid screen against the C terminus of Aiolos) and pGAD10.Eos have been described previously (16). The truncated Eos constructs used in the DNA binding studies were generated from pGAD10.Eos by standard PCR techniques. For mammalian expression the DNA sequences encoding Ikaros2, Eos, and the truncated Eos constructs were subcloned into the parental plasmid pMT2 for mammalian expression. The final vectors are pMT2.Ikaros2, pMT2.FLAG.Eos, pMT2.FLAG.Eos50–321, pMT2.FLAG.Eos358–331, pMT2.FLAG.Eos358–389, and pMT2.FLAG.Eos450–464. COS cells were transfected with 2 μg of the appropriate plasmid using the DEAE-dextran method (17). Nuclear extracts were made 48–60 h post-transfection as follows: cells were washed with 10 ml of cold phosphate-buffered saline and scraped into 500 μl of cold phosphate-buffered saline. After centrifugation (14,000 rpm, 30 s), cells were resuspended in 400 μl of cold Solution A (10 mM HEPES, pH 7.8, 1.5 mM MgCl2, 10 mM KCl, 1 mM DTT, 50 mM NaF, 5 μg/ml leupeptin, and 5 μg/ml aprotinin), left on ice for 10 min, and vortexed for 10 s. Cells were re-centrifuged, and the nuclei were resuspended in 30 μl of Solution C (20 mM HEPES, pH 7.8, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 1 mM MgF3, 50 mM NaF, 5 μg/ml leupeptin, and 5 μg/ml aprotinin). Tubes were left on ice for 20 min and then centrifuged (14,000 rpm, 3 min, 4°C). The supernatant was stored at –70°C.

Binding Site Selection Experiments—Degenerate oligonucleotide library with a core comprising 26 random nucleotides flanked by conserved sequences containing BamHI and EcoRI restriction sites was used (sequence available on request). The binding site selection experiments were performed as previously described (11).

DNA Binding Assays—The sequences for the oligonucleotides that contain all 5-bp elements are available on request. The other oligonucleotides used were: AGAACCTTTCGCTGAGCTACTGGAAGGGTG- GCAGGA (contains the δ element); TGAAAGAAGGGGGAATTCT-TGGAATGCAGTGAGACGGGAA (γ-satellite sequence); AAAATGGAAGATATAGAACCAGACTGGAATATATG (satellite sequence); and GTACCTCAGGGGAAATTCGTCAGG (contains a typical Ikaros binding element). Oligonucleotides were synthesized by Sigma Genosys (Australia). Prior to the assay, oligonucleotides were annealed in solution and end-labeled with [32P] according to standard procedures using polynucleotide kinase and purified on native polyacrylamide gels by standard methods (17).

Gel-shift assays were set up in a total volume of 30 μl, comprising –1 pmol of [32P]-labeled probe, –100 ng of protein/nuclear extract, 10 mM HEPES, pH 7.8, 50 mM KCl, 5 mM MgCl2, 1 mM EDTA, 5% glycerol, and 25 μg/ml poly(dI-dC). For assays in Fig. 2, an additional 4.5 μg of either Anti-Flag® M2 (Sigma-Aldrich), anti-Ikaros antibody (Santa Cruz Bio-technology), or pre-immune serum was present. After incubation (ice, 20 min) the samples were loaded onto a 6% native polyacrylamide gel made in 1× TBE buffer, dried, and visualized using a PhosphorImager (Amerham Biosciences).

Yeast Experiments—Interactions between Eos/Trp1 and other Ikaros family proteins were investigated by cotransferring the yeast strain HF7c (Clontech Two-hybrid Matchmaker system protocol) with pGAD10.Eos and the partner plasmids pGFP.Bios588–532, pGBT9.PegasusF4–5, and pGBT9.AiolosF5–6 or with pGBT9.Eos358–532 and the partner plasmids pGAD10.Ikaros2 or pGAD10-Trps/1222–1281 (sequences available on request). Controls using empty pGBT9 or pGAD10 vectors, as appropriate, were also tested.

Transformants were selected on Leu−Try− minimal media plates after cotransferring the DNA constructs, and patched onto 100-mm plates and further incubated to test for interactions. For positive interactions, yeast cells were resuspended in water, and the A600 nm was adjusted to ~0.15. Samples were serially diluted (102, 103, and 104), and equal volumes (10 μl) were deposited onto His−Leu−Try− plates. The number of colonies was compared after a final incubation at 29°C for ~60 h. For each interaction, two replicate plates were set up. Binding assays were determined using PCR amplification followed by tris-buffered sodium volumes of thrombin buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2.5 mM CaCl2).

EosC and PegC were eluted as fusions with MBP by the addition of maltose (50 mM; in thrombin buffer). MBP fusion proteins were either further purified by gel filtration chromatography (GFC) using a Superose-12 fast-protein liquid chromatography column (Amerham Biosciences, 0.8 ml/min, 4°C) in run 40 mM NaH2PO4, 200 mM NaCl, 1 mM dithiothreitol (DTT) or cleaved in solution by the addition of thrombin (4°C, 2 h). Peptides were purified by reversed-phase high performance liquid chromatography (RP-HPLC) using a VydacPLC using an Altichrom Altima C18 column (1 ml/min, 25°C), and their identities were confirmed using electrospray mass spectrometry. Fractions were lyophilized and stored at –20°C.

Atomic Absorption Spectroscopy—MBP-EosC purified by GFC was adjusted with buffer (50 mM NaH2PO4, 200 mM NaCl, 1 mM DTT) to a final concentration of ~6 μM. Measurements (repeated five times) were performed on a Varian Spectra AA20plus atomic absorption spectrometer at 213.9 nm using solutions of ZnSO4 as calibration standards.

Circular Dichroism Spectropolarimetry—Samples were prepared by dissolving lyophilized rphPLC-purified peptide in 50 mM tris(2-carboxyethyl)phosphine and 50 μM ZnSO4 to a final concentration of 15 μM. The pH was adjusted to 5.0–6.0 using 1 M NaOH. CD spectra were recorded at 20°C on a Jasco J-720 spectropolarimeter, equipped with a 5-mm Nuslab 111 temperature controller. CD data were collected over the wavelength range 184–260 nm with a resolution of 0.5 nm, a bandwidth of 1 nm, and a response time of 1 s using a 1-mm path length cell. Final spectra were the sum of three scans accumulated at a speed of 20 mm/min and were baseline-corrected.

NMR Spectroscopy—Lyophilized EosC was dissolved in a solution containing triis(2-carboxyethyl)phosphine and ZnSO4 (150 and 300 μM, respectively) to a final concentration of ~140 μM. The pH was adjusted to 5.7 using 1 M NaOH, and D2O (5% v/v) and d6-(trimethylsilyl)propionic acid (20 μM) were added. One-dimensional NMR spectra (in either the presence or absence of Zn(II)) were recorded at 25°C on a Bruker DRX600 NMR spectrometer, equipped with a 5-mm triple resonance probe head and three-axis pulsed-field gradients. Water suppression was achieved by pulsed-field gradients immediately prior to data acquisition. Spectra were processed using XWINNMR (Bruker) and a Lorentzian-Gaussian window function.

Analytical Ultra centrifugation—Density gradient equilibrium experiments were carried out at 25°C using a Beckman Optima XL-A ultracentrifuge equipped with an An50 rotor. Absorbance (280 and 360 nm) versus radius scans (0.001-cm increments) were collected at 3-h intervals until the samples had reached equilibrium. Data were recorded at three different speeds (7,500, 8,900, and 13,000 rpm) and loading concentrations (2, 5, and 10 μM). Analysis of the data was carried out using NONLIN software (19). The density of the solvent and the density of the peptide (assumed to be a 1:1 mixture of the peptide and the contribution of Zn(II); p = 0.719 g/ml) were estimated using SEDNTERP (20). Final parameters were determined by a nonlinear least squares fit to models incorporating homo-oligomerization, and examination of residuals was used to determine the goodness of fit.

Gel Filtration, Western Blotting, and Immunoprecipitations—Nuclear extracts (300 μl) from HEK293 cells transfected with pEGFP.N1 or pGFP.Bios constructs encoding the C-terminal domains of Eos (EosC, amino acids 474–532) and Pegasus (PegC, amino acids 361–419) were generated from a series of long (~60–70 bp) overlapping oligonucleotides (15). These were designed to optimize codon usage in Escherichia coli and to mutate putative non-zinc ligating Cys residues. Oligonucleotides were annealed using PCR and purified on native polyacrylamide gels by standard methods (17). Site-directed mutagenesis of EosC was used to create wild-type EosC from this mutant. Sequences of oligonucleotides and primers are available on request. The above constructs were inserted into pHi1120 (pHi1119 modified to contain a BamHI/EcoRI cloning site), thereby creating C-terminal fusions with maltose-binding protein (MBP). Constructs were overexpressed in the host strain BL21(DE3) grown in Luria broth containing ampicillin (100 μg/ml) and glucose (0.2% w/v). Cells were grown at 37°C with shaking until an A600 nm of ~0.6 was reached, whereupon protein overexpression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside (0.1 mM). After 4–6 h, the cells were harvested by centrifugation (15 min, 5000 × g, 4°C), and the resulting cell pellets were stored at –20°C.

The cell pellets were resuspended in sonication buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.1% β-mercaptoethanol). Cells were lysed by sonication and centrifuged (15,000 × g, 4°C, 20 min). The soluble fraction was applied to pre-equilibrated amyllose resin (New England Biolabs) and washed with sonication buffer followed by tris-buffered sodium volumes of thrombin buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2.5 mM CaCl2).
that of Ikaros. We also observed that Eos, like Ikaros, requires three or more ZnFs in its N-terminal domain to bind DNA (not shown).

There have been indications that the C-terminal domain of Ikaros possesses weak DNA-binding abilities. For instance, it has been reported that the Ikaros C-terminal domain can bind to various GGAA-containing probes (1, 12, 22). However, a consensus binding site has not been reported, presumably due to low association constants. It is generally difficult to establish convincingly that a protein domain is incapable of binding to DNA, because there always remains the possibility that the correct sequence has not been identified. However, based on the prediction that a DNA-binding, two-ZnF protein should recognize a sequence of −5-bp, we used a gel-shift assay to test the ability of MBP-EosC to bind a set of six overlapping, double-stranded oligonucleotides that contain all 5-bp DNA sequences. We observed that none of the probe was retained (not shown). We also tested the ability of MBP-EosC to bind the 6A element of the CD3-δ enhancer, because weak binding to this element by the C-terminal domain of Ikaros has been observed (1, 12). Again, we did not detect any binding by MBP-EosC to this probe (not shown). Finally, MBP-EosC did not bind γ-satellite and minor satellite sequences, to which Ikaros has also been shown to bind (22). These results provide strong evidence that the C-terminal domain of Eos does not bind DNA and that the DNA-binding activity of Eos is mediated solely by the N-terminal domain, EosN.

The C-terminal Domain Appears to Inhibit DNA Binding—Given that the binding site selection experiments were performed with only the bacterially expressed N-terminal domain of Eos, we sought to confirm that full-length Eos also bound DNA sites with the core motif, GGGA. This ability was tested in a gel-shift assay using proteins overexpressed in mammalian cells. Unexpectedly, we found that FLAG-tagged Eos was unable to bind a probe that contains a typical Ikaros/Eos recognition motif (Fig. 2, lanes 1–3). In contrast to this, Ikaros2 (a splice variant containing three N-terminal ZnFs) was able to bind the same probe with high affinity under identical conditions (lanes 4 and 5). The weak bands observed in lane 2 that are not present in lane 3 (in which Anti-Flag®2 antibodies were added) are most likely Eos degradation products that lack the C-terminal domain (these degradation products are observed routinely in Western blots, not shown). These bands possess a considerably higher mobility when compared with the intense bands in lanes 4 or 5, which correspond to Ikaros/DNA and Ikaros/anti-Ikaros antibodies/DNA, respectively.

Because the ability of GST-EosN to bind this probe in a gel-shift assay has been shown (11), we expressed the N-terminal domain of Eos (amino acids 50–231, truncation T1) in COS cells to assess the DNA-binding capacity of this domain in a mammalian cell context. Indeed, as shown in Fig. 2 (lanes 6 and 7), FLAG-T1 bound the DNA probe strongly. This observation suggested the presence of an inhibitory domain in a region C-terminal of the DNA-binding ZnFs of Eos. To map the inhibitory region, additional Eos constructs (amino acids 50–331, T2; 50–389, T3; and 50–464, T4) were expressed in COS cells and tested for their ability to bind DNA with high affinity. As seen in Fig. 2 (lanes 8–13), all these proteins were able to recognize the probe with similar affinities. For each assay, nuclear extracts were visualized by Western blotting to confirm similar amounts of protein were added (not shown). Because the construct T4 includes most of Eos other than the two C-terminal ZnFs, we conclude that the C-terminal domain of Eos acts as an inhibitor of DNA binding. This is in direct contrast to results obtained for Ikaros in which this domain was found to be required for high-affinity DNA binding (14, 22).
Relative Affinities of Eos Homo- and Hetero-oligomerization—Given that EosC does not appear to contact DNA, we next sought to investigate the nature of the protein-protein interactions that it mediates. It has previously been shown that EosC can interact with itself, as well as with the C-terminal domains of Ikaros, Aiolos, Helios, and Pegasus (11). We performed yeast dilution experiments based on the yeast two-hybrid assay, in which the probability of colony survival is positively correlated with the strength of the interaction between protein partners. In Fig. 3A, equal numbers of yeast cells (transfected with plasmids encoding different protein pairs) were plated out in each of 1, 2, and 3, and the number of colonies assessed. The data from this experiment indicate that the affinities of the interactions between Eos and other family members are of similar magnitudes, although there may be subtle differences. We also tested the ability of Ikaros family members to interact with the protein Trps1, a putative transcription factor that has been associated with trichorhinophalangeal syndrome type I (23). Although little is currently known about Trps1, the protein is predicted to contain an Ikaros-like oligomerization domain at its C terminus. Interestingly, we found that Trps interacted with Eos but did not interact either with any other Ikaros family members or with itself (Fig. 3B).

Subcloning, Overexpression, and Purification of EosC—To characterize the C-terminal domain of Eos in more detail, a polypeptide containing amino acids 474–532 of human Eos was chosen. This polypeptide (EosC) contains both of the predicted CCHH ZnFs (C1 and C2, Fig. 1B) and five N-terminal residues. Because Cys-498 is unlikely to be involved in zinc coordination (Fig. 1B, box), it was mutated to Ser to avoid complications due to the free thiol. This mutant remained competent for self-association, as judged from yeast two-hybrid data (not shown). The construct was overexpressed as a fusion with maltose-binding protein (MBP/EosC) and purified using amylose-affinity chromatography (New England Biolabs). For initial experiments, EosC was cleaved from MBP in solution using

### Table I

**DNA-binding sites recognized by GST-EosN**

| Consensus | g | N | t | G | G | A | N | N |
|-----------|---|---|---|---|---|---|---|---|
| G         | 5 | 1 | 2 | 10| 10| 9 | 3 | 3 |
| A         | 2 | 3 | 2 | 0 | 0 | 1 | 3 | 1 |
| T         | 1 | 3 | 4 | 0 | 0 | 0 | 2 | 4 |
| C         | 2 | 3 | 2 | 0 | 0 | 0 | 2 | 2 |
| Total     | 10| 10| 10| 10| 10| 10| 10| 10|

*Fig. 2.* Gel-shift assays reveal that the C-terminal domain of Eos inhibits DNA binding. In all assays, nuclear extracts from COS cells transfected with the appropriate plasmid were used, along with a radiolabeled probe that contained a typical Ikaros/Eos recognition element. Lane 1, untransfected COS cells (asterisks indicate unrelated DNA-binding proteins found in COS cells). In the other lanes, the following proteins were present: lanes 2 and 3, FLAG-Eos; lanes 4 and 5, Ikaros2; lanes 6 and 7, FLAG-Eos (50–231; T1); lanes 8 and 9, FLAG-Eos (50–331; T2); lanes 10 and 11, FLAG-Eos (50–389; T3); and lanes 12 and 13, FLAG-Eos (50–464; T4). Antibodies were used to confirm the identities of the bands and are present in lanes 3, 7, 9, 11, and 13 (Anti-Flag®M2) and lane 5 (anti-Ikaros). Pre-immune serum was added to reactions in which antibodies were not present. The Eos constructs are shown schematically with white bars representing ZnFs.

*Fig. 3.* Yeast experiments suggest that EosC has different affinities for homo- and hetero-oligomerization and that Trps1 only interacts with Eos. A, yeast were transfected with standard yeast two-hybrid bait and prey plasmids encoding the indicated proteins. 1, 2, and 3 each correspond to a serial dilution (bottom) of a yeast culture adjusted to an A600nm of 0.15. The protein partners that were tested in each series of dilutions are indicated on the right. B, Trps1 was tested for its ability to interact with Ikaros family members, as indicated. It appears that Trps1 cannot self-associate and instead is only capable of interacting with Eos.
thrombin, purified by rpHPLC, lyophilized, and refolded. However, in the absence of the MBP fusion partner, EosC exhibited a high propensity to precipitate, and most experiments therefore were performed with the intact MBP-EosC fusion.

**EosC Requires Zn(II) to Fold**—The far-UV spectrum of EosC in the absence of Zn(II) and at low pH (~2) displays features typical of a peptide with little or no structure, namely a single minimum at ~200 nm and negative ellipticity at shorter wavelengths (Fig. 4, gray). After the addition of Zn(II) (to more than two molar equivalents) and an increase in pH to 5.7 (Fig. 4, black), the spectrum changes markedly, with a red shift of the minimum to ~206 nm and the appearance of positive signal below ~197 nm. The general features of this CD spectrum are comparable with those previously observed for ZnF domains (24, 25) and are indicative of a mixture of a and β secondary structure.

Atomic absorption spectroscopy revealed that MBP-EosC coordinates Zn(II) in a 2:1 (Zn(II):protein) molar ratio, whereas MBP alone does not bind Zn(II) measurably. These results, together with the CD spectra, are consistent with the sequence-based prediction that EosC contains two CCHH ZnFs.

**NMR Spectra of EosC Suggest the Presence of a High Order Complex**—A one-dimensional 1H NMR spectrum was recorded for EosC at low pH (Fig. 5A) in the absence of Zn(II). As expected from the CD data, the spectrum displays sharp peaks and poor chemical shift dispersion. Upon the addition of Zn(II) and an increase in pH to 5.7, there is a substantial increase in spectral dispersion (Fig. 5B), consistent with the formation of stable secondary structure. However, the appearance of extremely broad lines in the spectrum is indicative of either self-association or possibly a substantial chemical exchange process. In either case, EosC is unsuitable for NMR structural studies. It is notable that we observed similar behavior for the C-terminal domain of Pegasus (PegC), indicating that both proteins exhibit similar solution behavior.

**Gel Filtration and Sedimentation Equilibrium Experiments Indicate that MBP-EosC Forms Complexes of Order Nine or Ten**—To determine the cause of the NMR line broadening observed for EosC, gel filtration chromatography (GFC) was carried out (Fig. 6). Interestingly, MBP-EosC (49.6 kDa) did not elute in the excluded volume (where large, nonspecific aggregates typically elute), but rather eluted as a single species slightly before a ferritin standard (440 kDa). This result suggests that EosC mediates the formation of a complex that consists of approximately eight molecules, because MBP elutes as a monomer in GFC (not shown). It is notable that this complex appeared to be very stable (over a period of weeks). We also conducted cross-linking studies in which glutaraldehyde was titrated into a solution of MBP-EosC, and the resultant covalently linked complexes were analyzed by SDS-PAGE. These gels revealed that the appearance of bands representing dimers, trimers, and tetramers is concurrent (not shown), consistent with the formation of higher order MBP-EosC complexes.

To further define the stoichiometry of complexes formed by MBP-EosC, sedimentation equilibrium experiments were conducted. A preliminary fit of the data obtained to a single-species model, although poor, yielded a weight average molecular mass of ~396 kDa, indicating the presence of self-association. The data were best fitted by either monomer:9-mer or monomer:10-mer equilibrium models (Fig. 7), consistent with the GFC results.

To ensure that these high order complexes were not a result of the Cys-498 → Ser mutation in MBP-EosC, we produced wild-type EosC as an MBP fusion protein and analyzed it by GFC. We found that this construct also eluted between the excluded volume and ferritin, indicating that the Cys-498 → Ser mutation did not affect the behavior of EosC. Unfortunately, wild-type EosC was not stable for extended periods, and further experiments with this domain were not feasible.

**Full-length Eos Self-associates in the Same Fashion as EosC**—To confirm that the self-association properties of EosC reflect the behavior of the full-length protein expressed in mammalian cells, GFC was performed with nuclear extracts from COS cells in which full-length Eos (FLAG-tagged, 58.5 kDa) was transiently overexpressed. Immunoblot analysis of column fractions (Fig. 8) revealed that Eos mainly eluted in
Nuclear extracts were separated by gel filtration chromatography, run on a denaturing polyacrylamide gel, and visualized by Western blotting using an Anti-Flag® M2 antibody. Eos (58.5 kDa) eluted before ferritin, denaturing polyacrylamide gel, and visualized by Western blotting using extracts were separated by gel filtration chromatography, run on a Monomer:8-mer, monomer:9-mer, and monomer:10-mer). The fits were obtained by simultaneously fitting six datasets recorded under different conditions (2, 5, and 10 mM MBP-EosC at 7500 and 8900 rpm). r is the radial position from the center of the rotor. Experiments were carried out at 25 °C.

**Fig. 8.** Full-length Eos forms a high order complex. Nuclear extracts were separated by gel filtration chromatography, run on a denaturing polyacrylamide gel, and visualized by Western blotting using an Anti-Flag® M2 antibody. Eos (58.5 kDa) eluted before ferritin, consistent with the formation of 9-mer or 10-mer complexes in solution. Fractions 11, 12, and 13 (bar) were immunoprecipitated using beads cross-linked with the Anti-Flag® M2 antibody, subjected to SDS-PAGE, and silver-stained to confirm that the complex eluting in these fractions was composed exclusively of Eos proteins (not shown).

Fig. 7. Sedimentation equilibrium analysis of MBP-EosC. The bottom panel shows the concentration distribution of MBP-EosC (10 mM, 7500 rpm) fitted to a monomer:9-mer model. The upper panels show the residuals from the same data set fitted to three different models incorporating self-association (monomer:8-mer, monomer:9-mer, and monomer:10-mer). The fits were obtained by simultaneously fitting six datasets recorded under different conditions (2, 5, and 10 mM MBP-EosC at 7500 and 8900 rpm). r is the radial position from the center of the rotor. Experiments were carried out at 25 °C.

fractions 11–13, which corresponds to an earlier elution time than for ferritin (~440 kDa). Interestingly, no Eos appeared to elute at the expected time for a dimer. This suggests that Eos mediates the formation of high order (>7) complexes inside the nucleus, in good agreement with the in vitro EosC results, considering that GFC is shape-dependent and of limited resolution. To confirm that the complex is a homo-oligomer comprising only Eos molecules, fractions 11–13 were immunoprecipitated, subjected to SDS-PAGE, and silver-stained. Only one band was apparent, indicating that the complex is composed exclusively of Eos molecules (not shown).

**Fig. 9.** Negatively stained transmission electron micrograph of MBP-EosC. MBP-EosC was deposited onto a Formvar-coated copper grid, negatively stained, and visualized by TEM. Arrows indicate protein complexes that appear to form regularly shaped spheres with radii of ~10 nm.

MBP-EosC forms a discrete, regularly shaped complex—to further assess the nature of the EosC oligomer, purified MBP-EosC was examined by transmission electron microscopy (TEM). The protein was deposited onto Formvar-coated copper grids and negatively stained. Fig. 9 shows a typical field of images derived from these preparations. Arrows indicate examples of particles that were not present on the control grid (not shown) and are, therefore, likely to be two-dimensional projections of the MBP-EosC oligomer. The complexes have a uniform appearance and appear to form spheres with radii of ~10 nm. We used the MRC suite of image-processing programs (26) in an attempt to enhance the detail of these images. However, the lack of obvious structural features made it impossible to align all of the images of individual complexes.

Finally, dynamic light scattering was used to confirm the TEM results by measuring the hydrodynamic radius (R_h) of MBP-EosC under a range of buffer conditions and temperatures (Table II). The data indicate that ~80–85% of the signal can be attributed to a species with an average R_h of 7.4 ± (0.8) nm, in reasonable agreement with the size of the particles observed by TEM. If it is assumed that MBP-EosC forms spheres with standard density (1.37 g ml^-1), then the average estimated mass for this complex is 370 (±90) kDa, consistent with GFC and sedimentation equilibrium experiments. The low polydispersity values further support the conclusion that the major species in solution exists as a uniformly sized oligomer.

**DISCUSSION**

The C-terminal domain of Ikaros family proteins is highly conserved and plays an important role in the co-operative control by these proteins over hematopoiesis. In Ikaros, the C-terminal domain not only mediates the self-association that is vital for high affinity DNA binding (14), but it also mediates interactions with other protein complexes (27) that presumably confer the ability to activate or repress transcription. Because relatively little is known about these C-terminal domains, we have investigated the function and biophysical characteristics of the C-terminal domain of Eos (EosC).

We obtained the unexpected result that EosC appears to inhibit the DNA-binding function of full-length Eos in a gel-shift assay. In contrast, the presence of the C-terminal domain of Ikaros enhances the ability of this protein to bind DNA, underscoring the fact that, despite many similarities, Ikaros family members display distinct properties. There are at least two possible explanations for this inhibitory effect of EosC. The first is that EosC interacts directly with the N-terminal domain and prevents its interaction with DNA. Indeed, Honma et al.
have demonstrated such an interaction for Eos, using a yeast two-hybrid approach. An alternative or additional reason why no shifted band was observed using full-length Eos involves the oligomeric complexes whose formation is mediated by EosC. These complexes may be too large to enter the gel matrix, or the DNA-binding surface of EosN may be sterically occluded.

Interestingly, this inhibitory property of EosC is reminiscent of results reported for the POZ domain in the protein GAGA. The POZ domain has been shown to inhibit the DNA-binding ability of full-length GAGA (28), and it was found that the POZ domain mediated the formation of oligomers that are thought to bind co-operatively to multiple DNA sites (28, 29). It is worthwhile to note that Ikaros monomers display a higher affinity than dimers for a γ-satellite B probe (22). This could imply that, in particular circumstances, oligomerization either inhibits DNA binding (22) or renders it undetectable.

Results from a combination of GFC, sedimentation equilibrium, and dynamic light scattering experiments indicate that EosC mediates the formation of homo-oligomeric complexes that consist of nine or ten molecules. These complexes can be visualized by transmission electron microscopy, which reveals that EosC forms a mix of α- and β-secondary structures that is Zn(II)-dependent, consistent with the prediction that this domain contains classical-type ZnFs.

It has recently been reported that the C-terminal domain of Ikaros primarily mediates dimerization (30), although the possibility that higher order species also existed was not excluded. Indeed, Ikaros isoforms elute in a broad peak from GFC, which suggests that Ikaros proteins may form complexes with a range of stoichiometries (4). Furthermore, MBP-PegC, which has a comparable molecular mass to MBP-EosC, has also been subjected to GFC (not shown), and displayed a similar elution time. These results, together with our finding that Eos forms complexes of nine or ten molecules, further illustrate that members of the Ikaros family display overlapping components, but not identical, physical properties. The ability to form complexes that display a range of stoichiometries may constitute part of the mechanism through which Ikaros family proteins regulate the development of blood cells, and the distinct self-association properties of Eos and Ikaros may indicate that Eos functions through slightly different mechanisms to Ikaros in different tissues. This idea is also supported by our finding that Eos is the only Ikaros family member to be capable of interactions with Trps1 in a yeast two-hybrid assay. Given that Eos is the only Ikaros protein that has been found to be significantly expressed in the brain (10) and that Trps1 has been reported to be present in the fetal brain (23), our finding presents the possibility that these two proteins co-operate in some way to regulate early brain development.

Although a number of bacterial transcription factors have been shown to form oligomers (31–35), there are only limited examples from eukaryotes. As mentioned, proteins that contain a POZ domain, such as GAGA, have been implicated in oligomer formation (29, 36). It is interesting to speculate on the biological role of oligomerization. In the case of Ikaros, several models have been suggested as to how higher order species may regulate gene expression. For example, it is possible that Ikaros physically recruits genes into transcriptionally inaccessible nuclear regions through the simultaneous interactions of Ikaros complexes both with target genes and with DNA sites in pericentromeric-heterochromatin (PC-HC (22, 37–39)). In this case, it has been suggested that Ikaros forms a tetramer, with each dimer interacting with different DNA sites (40), and it is possible to extend this model to incorporate higher order oligomerization, where multiple DNA sites are brought into regions of PC-HC and rendered silent. In another model, Ikaros may “potentiate” gene expression by titrating repression complexes (such as nucleosome remodeling and deacetylase) away from the promoters of target genes and into PC-HC, thereby providing access to transcriptional activators (41). Again, this model can be extended to include oligomerization, where several (perhaps different) transcriptional complexes can be recruited to PC-HC by the single Ikaros oligomer.

Finally, it is notable that Ikaros family transcription factors interact with an array of other proteins. For example, many family members appear to interact with Mi-2β, SinA, Sin3B, HDACs 1 and 2 (27), and CtBP (16). With the exception of CtBP, these protein partners are capable of interacting with either the N- or the C-terminal domain of Ikaros (27), and it will be interesting to understand the molecular details of these interactions in the context of the oligomerization discussed here.

In summary, since the cloning of Ikaros in 1992, there have been several suggestions by researchers that Ikaros is capable of forming high order complexes (4, 12, 42, 43), although no supporting data has been published to date. Our work represents the first detailed biophysical analysis of the stoichiometry of complexes formed by a member of the Ikaros family of proteins.

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The C-terminal Domain of Eos Forms a High Order Complex in Solution
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