Eos and Pegasus, Two Members of the Ikaros Family of Proteins with Distinct DNA Binding Activities*

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Members of the Ikaros family of transcription factors, Ikaros, Aiolos, and Helios, are expressed in lymphocytes and have been implicated in controlling lymphoid development. These proteins contain two characteristic clusters of zinc fingers, an N-terminal domain important for DNA recognition, and a C-terminal domain that mediates homo- and heterotypic associations between family members. The conservation of these domains is such that all three proteins recognize related DNA sequences, and all are capable of dimerizing with other family members. Here we describe two additional Ikaros family proteins, Eos and Pegasus. Eos is most highly related to Helios and shares its DNA binding and protein association properties. Pegasus is related to other Ikaros proteins in its C-terminal dimerization domain but contains a divergent N-terminal zinc finger domain. Pegasus self-associates and binds to other family members but recognizes distinct DNA-binding sites. Eos and Pegasus repress the expression of reporter genes containing their recognition elements. Our results suggest that these proteins may associate with previously described Ikaros family proteins in lymphoid cells and play additional roles in other tissues.

The Ikaros protein was originally identified as a DNA-binding factor that recognized a short sequence motif found in the control region of the T cell restricted CD3α gene (1). Subsequently, Ikaros was shown to play a crucial role in regulating the production of T and B lymphocytes and natural killer cells (2–4). The full-length Ikaros transcript encodes a zinc finger protein (Ik-1), containing a cluster of four fingers near its N terminus and two additional fingers at its C terminus, but alternative splicing generates at least five additional isoforms (Ik-2 to Ik-6), which contain different subsets of the N-terminal fingers (5, 6).

The N-terminal zinc finger domain has been shown to be the major determinant of DNA recognition in vitro, and it has been demonstrated that, in general, only splice isoforms that contain three or more N-terminal zinc fingers bind DNA (6). Although the sequence specificity of these isoforms varies slightly, they all recognize related elements containing the central core sequence GGGGA (the consensus recognized by splice forms Ik-1, -2, and -3 in vitro is GGGGAtace, where uppercase letters show the most highly conserved residues).

Unlike the N-terminal zinc fingers, the two C-terminal fingers are present in all Ikaros splice isoforms. It is thought that the major role of these fingers is to mediate the self-association of Ikaros proteins (7). The full role of dimerization in Ikaros activity is not completely understood, but it is known that dimerization contributes to the ability of Ikaros to bind DNA. Ikaros monomers, containing only the N-terminal zinc fingers, however, recognize the same sites as dimers (6), so dimerization is clearly not essential for DNA binding. Composite double (direct repeat or palindromic) Ikaros binding sites have also been described, and it may be that dimerization is particularly important in the recognition of these elements. The zinc fingers may also be involved in the formation of larger DNA-protein complexes, and there is evidence that higher order complexes containing multiple Ikaros proteins may exist (8).

The in vivo relevance of the Ikaros dimerization domain has been demonstrated by experiments in cell lines, mice, and investigations of human leukemia. It has been shown that mutant Ikaros isoforms, containing only the C-terminal dimerization domain, behave as dominant negative mutants that impair the ability of Ikaros to activate transcription in cellular assays (6) and give rise to an aggressive form of lymphoblastic leukemia in mice (4). Human infants suffering from acute lymphoblastic leukemia have also been shown to express an aberrant Ikaros mRNA, encoding the two C-terminal fingers but lacking the region encoding the N-terminal DNA-binding domain (9, 10). Because the C-terminal zinc fingers of any Ikaros isoform can mediate interactions with any other Ikaros isoform (7) or indeed with additional Ikaros family members (see below) and potentially influence their DNA binding and transcriptional properties, the precise mechanism by which these dominant negative mutants function in vivo has not been easy to determine.

In addition to the six Ikaros isoforms, there are two well characterized Ikaros related proteins encoded by distinct loci. The first to be described was Aiolos (11). Like the longest splice isoform of Ikaros (Ik-1), Aiolos contains four N-terminal zinc fingers and two C-terminal fingers. The homology to Ikaros in these domains is such that Aiolos exhibits the same DNA binding specificity and dimerization activities as Ikaros. No splice variants of Aiolos have been reported in mammals. The next protein to be isolated was Helios (12, 13). Two splice forms of Helios have been reported; the first is similar to full-length Ikaros (Ik-1) in that it encodes four N-terminal zinc fingers and two C-terminal zinc fingers, and the second is analogous to Ik-2 in that it is missing the first zinc finger of the N-terminal domain. Again the conservation between Helios and Ikaros in the zinc finger domains is high, and Helios has been shown to bind the same DNA recognition elements as Ikaros and to
homodimerize and heterodimerize with Ikaros or Aiolos.

The molecular mechanisms by which Ikaros family proteins regulate gene expression are being intensively investigated. Ik-1 and Ik-2 are predominantly nuclear, and they have been shown to be transcriptionally active (6, 7, 14). In fibroblasts transfected with expression plasmids Ik-3 and Ik-4 are localized predominantly to the cytoplasm, and their roles and the functions of Ik-5 and Ik-6, the less abundant isoforms, are less clear (although they may be involved in down regulating the activity of Ik-1 and Ik-2) (6). Available data suggest that Ik-1 and -2 function as only modest activators in transient transfection experiments, as does Helios, whereas Aiolos behaves as a stronger activator in these assays (6, 11, 12, 13). More recent results have highlighted the role of Ikaros as a repressor of transcription, at least in certain cell types. Ikaros has been found to localize with inactive genes in centromeric heterochromatin, suggesting a possible role in gene silencing (12, 15, 16). Examination of the protein partners bound by Ikaros has revealed that Ikaros can associate with the Sin3 co-repressor proteins (14) or be part of the large Mi-2 complex that contains histone deacetylase and chromatin remodelling enzymes (8). More recently it has been reported that an N-terminal domain of Ikaros associates with the co-repressor protein CtBP (17). As described above, the known members of the Ikaros family share many similar features and are all expressed in lymphoid cells. It is notable, however, that Ikaros and Helios are also expressed at low levels in other hematopoietic lineages, such as erythroid cells (12, 13). It has been noted that the C-terminal zinc finger domain of Ikaros is related to the Drosophila regulatory protein Hunchback (1, 18). This observation suggests that Ikaros is a member of an ancient family and that other Ikaros-related proteins may exist in mammals and may have roles in diverse cell types. To identify novel Ikaros family proteins, we carried out a two-hybrid screen against a bait protein encompassing a typical C-terminal zinc finger dimerization domain. We chose the domain from Aiolos, because we found that it could readily be expressed at appropriate levels in yeast. Two Ikaros related proteins were identified, and following the convention of assigning names from Greek mythology, we refer to these proteins as Pegasus and Eos, after the winged horse and the goddess of Dawn, who is reputed to have ridden the stomach expression vector pcDNA3 (Invitrogen) to create pcDNA3-Pegasus and pcDNA3-Eos, respectively.

**Yeast Two-hybrid Screening and Pull-down Assays—** The CLONTECH two-hybrid system was used according to the manufacturer’s instructions. A human K562 erythroleukemia cDNA library in the Ga4 activation domain vector, pGAD10-GaHAD, was transformed into the yeast strain HF7c, harboring the bait plasmid pGBT9-Aiolos. DNA was recovered from yeast colonies that grew on Trp/Leu/His-deficient medium and used to transform Escherichia coli strain HB101. Plasmid DNA was purified and sequenced. Interactions between Pegasus and Eos and other Ikaros family proteins were investigated by co-transforming HF7c with appropriate prey plasmids and bait plasmids. The resulting transformants were selected on Leu/Trp-deficient plates and patched onto Leu/Trp/His-deficient plates. Growth was monitored after incubation at 29 °C for 30 h, and β-galactosidase production was assessed according to standard methods (CLONTECH Matchmaker system protocol). Re-combinant GST-Pegasus F4–5, GST-Eos F5–6, GST-Ikaros F5–6, and GST-Aiolos F5–6 were produced in the E. coli strain BL-21, purified as described (19) and immobilized on glutathione-bead-protein complex equivalent to 5 μg of fusion protein in 300 μl of pull-down buffer (150 mM NaCl, 20 mM Tris, pH 7.5, 1% Nonidet P-40, 2.5 μg/ml bovine serum albumin, 10 μg/ml ZnSO4, 0.1 μg/ml β-mercaptoethanol, 15 mM phenylmethylsulfonyl fluoride). The mixture was incubated for 1 h at 4 °C, washed four times with pull-down buffer, resolved on a 10% polyacrylamide gel, and visualized using a PhosphorImager (Molecular Dynamics).

**RAPID AMPLIFICATION OF DNA ENDS-PCR TECHNIQUE—** DNA was recovered from M07e/II-3 cells and the reverse primer 5′-CACATGGAGTCTGGCAGC-3′ (nucleotides 727–750) were used to obtain Eos full-length clone using the Marathon cDNA amplification kit (CLONTECH) as described by manufacturer.

**In Vitro Transcription and Translation—**[35S]Methionine-labeled Pegasus and Eos proteins were produced in vitro using the plasmids pcDNA3-Pegasus and pcDNA3-Eos in the presence of rabbit reticulocyte lysate and T7 RNA polymerase (T7-T Lysate Coupled Transcription/Translation Protocol, Promega). The reaction products were resolved on a 10% SDS-polyacrylamide gel and visualized using a PhosphorImager (Molecular Dynamics).

**Binding Site Selection and Electrophoretic Mobility Shift Assays—** Control GST protein and the Pegasus DNA-binding domain GST fusion protein, comprising amino acids 69–173 of Pegasus downstream of GST, were produced in the E. coli strain BL-21. For site selection experiments a degenerate oligonucleotide with a core of 26 random nucleotides flanked by conserved sequences containing BonI and EcoR1 restriction sites was used: CAGTGTTCGACCGGATCCGCTT-CG(N)-GAGGGCGAATTCAGTTGCAACTGCAGC. The PCR primers used were: primer F, GCTGCAGTTGCACTGAATTCGCCTC, and primer R, CAGTTGTTCGACCGGATCCGCTTGCC. The random oligonucleotide was rendered double-stranded by annealing with primer F and extension with Klenow enzyme for 30 min at room temperature. Briefly, 1 μg of polyacrylamide gel purified template was mixed with 75 μg of primer F in 1× Klenow reaction buffer (50 mM Tris-HCl, pH 8.0, 10 mM MgCl2, 50 mM NaCl) and annealed by boiling and cooling. Deoxyribonucleotides were added to a final concentration of 400 μM plus 25 μCi of [α-32P]dCTP and 25 units of Klenow, and the mixture was incubated at room temperature for 30 min in 1× Klenow buffer. The double-stranded template was purified by polyacrylamide gel electrophoresis.

5 μg of GST-Pegasus (69–173) bound to GST beads (5 μl) was incubated on ice for 30 min with 1 ng of the purified random oligonucleotide library and 10 μl of binding buffer (0.2 μg/ml of poly(dI-dC) (Sigma), 0.2 mg/ml acetylated bovine serum albumin (New England Biolabs), 25 mM HEPS, pH 7.5, 100 mM KCl, 0.1 mM ZnSO4, 10 mM MgCl2, 0.1% Nonidet P-40, 1 mM dithiothreitol, 5% glycerol) (20). The beads were washed by centrifugation and washed 4 times with binding buffer and boiled for 5 min in 15 μl H2O. The DNA was recovered by boiling, and following 15 cycles of PCR amplification using primers F and R, the product was purified by polyacrylamide gel electrophoresis and used for further binding reactions. After four rounds of selection a gel retardation experiment was carried out using soluble fusion protein. The shifted band was excised, and the DNA amplified as before, digested with EcoRI and BamHI, subcloned into pBlueScriptKS, and sequenced.

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1 The abbreviations used are: GST, glutathione S-transferase; PCR, polymerase chain reaction.
Gel retardation experiments were carried out using oligonucleotides labeled with 3'–5'end-labeled and T4 polynucleotide kinase (New England Biolabs). 500 ng of fusion proteins and labeled probes (10,000 cpm) were incubated in buffer (10 mm HEPES, pH 7.8, 50 mm potassium glutamate, 5 mm MgCl₂, 1 mm EDTA, 0.5 mm dithiothreitol, 1 μg of poly(dI-dC), 1 mg bovine serum albumin, 1 mm dithiothreitol, 5% glycerol, and 500 μg/ml of carrier DNA) for 20 min on ice. Then samples were subjected to electrophoresis at 4 °C for 2.5 h at 12 V/cm on a native polyacrylamide gel (6%, (19:1) bis: acrylamide containing RNA samples purified from human tissues. Pegasus were also used to probe a commercial Northern filter (CLONTECH). The fragments of Eos and Pegasus both contain two C-terminal zinc finger domains that can mediate both homotypic interactions and heterotypic associations with other family members.

**RESULTS**

**Isolation of cDNA Encoding Eos and Pegasus**—The region encoding the C-terminal dimerization domain of Aiolos was amplified by PCR and cloned into a yeast expression vector downstream of a segment encoding the Gal4 DNA-binding domain to generate a bait for use in a two-hybrid screen. We then carried out the screen using a cDNA library constructed from the human erythroleukemia cell line K562. Two partial clones encoding zinc fingers related to the C-terminal fingers of Ikaros family proteins were identified. Full-length clones were then obtained by 5' rapid amplification of cDNA ends (in the case of Eos) and by reverse transcription-PCR after reference to the expressed sequence tag data bases (in the case of Pegasus). In both cDNAs the putative translation initiation sequence conforms well to the Kozak consensus, and in the case of Pegasus, in frame stop codons are found upstream of the first AUG. As can be seen from Fig. 1, all proteins contain two clusters of zinc fingers but have only limited homology elsewhere. The C-terminal zinc fingers are highly conserved in all proteins, whereas the N-terminal cluster is conserved only in Ikaros, Helios, Eos, and Aiolos. Pegasus, on the other hand, possesses only three N-terminal fingers with quite distinct amino acid sequences. Alternative splicing of exons encoding the N-terminal zinc finger cluster region has been reported in the case of Ikaros and Helios. To ascertain whether Eos and Pegasus were also alternatively spliced in this region we amplified this segment by PCR but have as yet found no evidence for alternate splice forms (data not shown).

**Eos and Pegasus Self-associate and Interact with Other Family Members**—Eos and Pegasus both contain two C-terminal zinc fingers with considerable homology to the two fingers in the C-terminal dimerization domains of previously reported Ikaros family proteins. We next sought to compare the protein binding ability of Eos and Pegasus with that of other Ikaros family members. We purified GST fusions of the C-terminal dimerization domains of Pegasus, Eos, Ikaros, and Aiolos (Fig. 2B) and tested them to retain full-length *in vitro* transcribed/translated Pegasus and Eos (Fig. 2A). As shown in Fig. 2A, all the C-terminal zinc finger domains tested efficiently retained both Eos and Pegasus. Similar results were obtained using the yeast two-hybrid system. In Fig. 2C a bait plasmid encoding the two C-terminal zinc fingers of Pegasus is tested and shown to interact with Eos, Aiolos, Helios, Eos, and Pegasus itself. The zinc finger domain of Eos has been similarly tested, and it too was found to be capable of mediating interactions with all known Ikaros family members (data not shown). These results demonstrate that both Eos and Pegasus contain functional dimerization domains that can mediate both homotypic interactions and heterotypic associations with other family members.

**Eos and Pegasus Have Distinct DNA Binding Specificities**—We next investigated the DNA binding activities of these proteins. We first tested the proteins for their ability to bind a typical Ikaros recognition element (GGGAA) that is recognized by all previously described Ikaros family proteins. Given that the N-terminal finger domain has been shown to direct DNA binding specificity and that GST fusion proteins containing only the N-terminal fingers of Ikaros bind DNA with the expected specificity (6), we produced the N-terminal domains of Eos and Pegasus as GST fusions (Fig. 2B) and tested them for their ability to bind a consensus Ikaros recognition site. As shown in Fig. 3A (lanes 1–3), the GST-Eos fusion protein recognized and bound this element with high affinity, whereas the GST-Pegasus fusion and control GST protein alone showed no binding. To further examine the DNA binding specificities of Eos and Pegasus, we then tested three sequences previously reported to be recognized by Ikaros *in vitro*: the T-cell receptor-α promoter site (TGGAGGGAAGTGGGAAACTTTT), the NF-κB promoter site (CAGGGAATCTCCCTCTCCAT), and the CD3-δ promoter site (GGGAGAATCTCCCTCTCCAT). GST-specific binding was shown to occur. These results indicate that the Eos DNA-binding domain functions similarly to those of Ikaros and previously reported family members. On the other hand Pegasus appeared to bind the Ikaros recognition sites very weakly if at all.

The fact that Pegasus bound some of the sequences above with low affinity led us to investigate whether it was a DNA-binding protein that recognized a distinct DNA sequence. We therefore carried out PCR site selection experiments (23). Essentially we combined the methods that had previously been successful for defining the binding sites of the three Ikaros isoforms Ik-1, Ik-2, and Ik-3 (6) with those used to study zinc finger proteins by Zweidler-McKay et al. (20). GST-Pegasus
fusion protein was immobilized on agarose beads and mixed with a pool of random double-stranded oligonucleotides. The retained sequences were then amplified by PCR and subjected to subsequent rounds of selection. After four rounds the PCR products were used in a gel retardation experiment with GST-Pegasus, and the retarded band was excised, reamplified, and cloned, and the isolates were sequenced. Table I summarizes the results of sequencing 40 different isolates. A number of these sequences were relabeled and tested again in mobility shift experiments to confirm that they were recognized by Pegasus (Fig. 4A and data not shown). As can be seen from Table I and the sequences shown below Fig. 4A, a wide variety of sequences are bound by Pegasus. However, the bound sequences conform to a loose consensus of GnnnGnnG. This sequence is clearly dissimilar from the tGGGaatacc site recognized by other Ikaros family proteins (6).

Here, it should be noted that the zinc fingers of Pegasus and other Ikaros family proteins are related to classical Cys2, His2 zinc fingers. Work on the sequences recognized by these fingers has shown that basic residues at defined locations within each finger make contacts with G residues in DNA (Fig. 4B) (24). The pattern of basic residues in the three N-terminal fingers of
Pegasus is consistent with this domain recognizing sequences of the general form GXXXG. To further test the validity of the consensus sequence we chose one site that was recognized by Pegasus (GAAATG) and examined the effect of mutating the putative contact G residues. The results are shown in Fig. 4C. Mutation of the first G markedly interferes with recognition by Pegasus (lane 3), and mutation of either the second or third G essentially eliminates binding (lanes 4 and 5). These results demonstrate the importance of the G residues. Although not apparent from Table I (which probably represents a compilation of high and lower affinity sites), further mutation experiments suggested that the central TGT core was also important, and the highest affinity sites identified in our studies conformed to the consensus GXXTGTGXX (data not shown). These Pegasus sites are not recognized by Eos and are distinct from the elements bound by previously reported Ikaros proteins.

**Eos and Pegasus Repress Gene Expression in Transient Assays**—Because Eos and Pegasus were both capable of sequence specific DNA-binding, we tested whether they were able to act as transcription factors and modulate the expression of promoters containing their recognition elements. Because natural target promoters that respond to these proteins (or to previously identified Ikaros proteins) are unknown, we constructed synthetic reporters containing either Eos or Pegasus recognition sites upstream of a minimal promoter driving a luciferase reporter gene. Previously identified Ikaros proteins have been found to function well in NIH 3T3 cells, so we chose this line for initial experiments (12, 13). The reporter constructs were transfected together with expression vectors for either Eos or Pegasus. As can be seen from Fig. 5A, Eos repressed the expression of the reporter containing its recognition site but had no effect on the reporter containing the Pegasus site. Conversely, Pegasus had no effect on the Eos recognition site reporter but repressed the expression of the reporter containing the Pegasus site. We carried out additional experiments in another line, 293T, and in the hematopoietic cell line K562, and in both instances saw repression by both Eos and Pegasus (data not shown). Taken together these experiments indicate that both Eos and Pegasus bind their respective sites, but not each other's sites, in cellular assays and can act to regulate target genes.

![Image](https://www.jbc.org/content/277/14/38351)

**Fig. 2.** Pegasus and Eos physically interact with other Ikaros family proteins. **A,** lanes 1 and 2y show in vitro translated [35S]-labeled Pegasus and Eos, respectively; the proteins migrate at the predicted molecular weights of 46.5 and 58.5 kDa, respectively. A GST pull-down experiment comparing the ability of the dimerization fingers of Pegasus, Eos, Ikaros, and Aiolos to retain [35S]-labeled Pegasus and Eos is shown in lanes 3–12. Lanes 3 and 8 show the background retention by GST alone, and the other lanes show that all the fusion proteins were able to retain both Pegasus and Eos. **B,** polyacrylamide gel stained with Coomassie Blue showing the GST fusion proteins used in A. **C,** interactions of Ikaros family proteins in the yeast two-hybrid system. The potential of the fusion protein Gal4 DNA binding domain/Pegasus to interact with Gal4 activation domain fused to full-length Pegasus, Eos, mIkaros-2, or mAiolos(447–507) was tested. Growth in the absence of leucine, tryptophan, and histidine (−L−T−H) is indicative of a positive interaction.

![Image](https://www.jbc.org/content/277/14/38351)

**Fig. 3.** Eos, but not Pegasus, binds Ikaros DNA recognition sequence. **A,** the ability of control GST (lane 1), recombinant GST-Eos (lane 2), and GST-Pegasus (lane 3) fusion proteins to bind [32P]-radiolabeled double-stranded oligonucleotides containing a typical high affinity Ikaros binding site, GATCATCATAGGAAAATTATCCTAG, or endogenous control regions containing potential Ikaros recognition sites, T-cell receptor (TCR-α) (TGGAGGGAAGTGGGAAACTTTT), NF-κB (CAGGGAATCTCCCTCTCCAT) and CD3-δ (TPTTCATGGA-CATCATGAAATGGAAGT), were tested in a gel retardation experiment. **B,** polyacrylamide gel stained with Coomassie Blue showing the GST fusion proteins used in the gel retardation experiment.
Eos and Pegasus Are Expressed in Hematopoietic Cell Lines

The expression profiles of previously described Ikaros family members vary somewhat, but all three genes are highly expressed in lymphoid cells. We used Northern blotting to investigate the distribution of Eos and Pegasus in samples of human mRNA and compared their expression profiles to those of Ikaros and Helios. The cDNA fragments chosen for use as hybridization probes correspond to regions that differ between Ikaros family members to ensure that each probe would be gene specific, and the Ikaros probe included the C-terminal region present in all six splice isoforms but excluded the highly conserved zinc finger region. We first examined the expression of the genes in a panel of human hematopoietic cell lines (Fig. 6A). Eos is expressed in selected cell lines from each of the hematopoietic cell lineage but is present at highest levels in THP-1 and Mo7e-IL3, which have characteristics of myeloid and early megakaryocytic cells respectively, and also in MOLT-4, NALM-6, and K562 (T, B, and erythroid cells, respectively). Two different probes were used to analyze the expression of Pegasus: one included the region between the zinc finger clusters (KpnI-SacI fragment; Fig. 6), the second was an N-terminal fragment (nucleotides 16–347; data not shown). Both probes detected the same Pegasus transcripts in all the cell lines tested. The expression patterns of human Ikaros and Helios in this panel of cell lines was also examined, and as expected, they were found to be most abundant in the T and B cell lineages. Overall, it can be seen that although Eos is the more restricted of the two, both Pegasus and Eos are more broadly expressed than the other family members, and there are several cell lines in which they are co-expressed with Ikaros and Helios (Fig. 6A).

Analysis of the expression of Eos and Pegasus in human tissues is shown in Fig. 6B. The Eos signal was highest in skeletal muscle, but low level expression could also be detected in heart, thymus, kidney, liver, and spleen (Fig. 6B and data not shown). Both probes detected the same Pegasus transcripts in all the cell lines tested. The expression patterns of human Ikaros and Helios in this panel of cell lines was also examined, and as expected, they were found to be most abundant in the T and B cell lineages. Overall, it can be seen that although Eos is the more restricted of the two, both Pegasus and Eos are more broadly expressed than the other family members, and there are several cell lines in which they are co-expressed with Ikaros and Helios (Fig. 6A).

**TABLE I**

| Consensus | N | G | N | N | g/t | G | N | N | G | N |
|-----------|---|---|---|---|----|---|---|---|---|---|
| G         | 9 | 40| 5 | 10| 14| 40| 14| 16| 40| 8 |
| A         | 8 | 0 | 13| 11| 5 | 0 | 11| 7 | 0 | 13|
| T         | 10| 0 | 18| 12| 14| 0 | 8 | 11| 0 | 9 |
| C         | 13| 0 | 4 | 7 | 7 | 0 | 7 | 6 | 0 | 10|

**FIG. 4.** Pegasus DNA binding specificity. A, gel retardation experiments were performed using GST-Pegasus (69–173) and a selection of different 32P-labeled sequences that had been recovered from random oligonucleotide pools by virtue of the fact that they bound Pegasus. Retardation of labeled probes is observed in all cases. In each case GST alone was run as a control. The sequences of the four oligonucleotides used (A–D) are shown under the figure. B, diagrammatic representation of a typical DNA-binding zinc finger found in Ikaros family proteins and related to Kruppel-like zinc fingers. The conserved residues cysteine (C) and histidine (H) involved in coordinating zinc are shown, as are the typical large hydrophobic (Φ) and leucine (L) residues that are important for the internal packing of the finger structure. The three residues that vary between fingers and play a major role in determining sequence specificity are marked X, Y, and Z (24). The residues corresponding to X, Y, and Z in fingers 1–3 of Pegasus are shown, together with the DNA sequence that is predicted to be recognized by Pegasus. By convention the protein is shown with the N-terminal residue on the left, but the DNA site is shown 3' to 5'. C, gel retardation experiment using GST (lane 1) or GST-Pegasus (69–173) (lanes 2–5) and 32P-labeled sequences shown under the figure.
DISCUSSION

Ikaros was first identified as an activator of transcription that is expressed in T cells and binds in vitro to control elements in a large array of genes expressed in lymphocytes (1, 6). The subsequent observation that null mutations in murine Ikaros prevented the formation of B and natural killer cells, as well as dramatically interfering with T cell production indicated that Ikaros played an essential role in lymphoid development (27). Interestingly, it was also shown that the expression of a dominant negative form of murine Ikaros, consisting of the C-terminal dimerization domain, had different effects and gave rise to an aggressive lymphoblastic leukemia that resulted in death between 3 and 6 months of age (4). The mechanism by which the dimerization domain of Ikaros generates disease is not clear, but it is probable that it binds to and interferes with the normal isoforms of Ikaros. The identification and cloning of Aiolos (11) and Helios (12, 13) raised the possibility that the Ikaros dimerization domain might also interfere with the activity of these proteins. The additional observation that Eos and Pegasus are also found in lymphoid cells raises the possibility that the Ikaros dimerization domain may also be interfering with their normal functions.

As well as activating transcription, it is now clear that Ikaros is involved in the repression of gene expression. Studies in B lymphocytes have revealed that Ikaros is associated with heterochromatin and co-localizes at centromeres with a number of transcriptionally silent genes (15). This striking result indicates that Ikaros might repress transcription by recruiting genes to inactive foci within the nucleus. Additional information about the mechanism by which Ikaros represses gene

**Fig. 5. Eos and Pegasus can act as transcriptional repressors.** A, NIH 3T3 cells were transfected with 2 μg of reporter plasmid containing an Eos/Ikaros binding site, pGL2-(Ik)TK (left) or a similar plasmid containing a typical Pegasus binding site, pGL2-(Peg)TK (right) and increasing amounts (500 ng, 1 μg, and 3 μg) of the Eos expression vector pcDNA3-Eos. More than 4-fold repression was observed when Eos acted on the Eos/Ikaros binding site, but no activity was recorded when the luciferase reporter gene was under the control of the Pegasus binding sequence containing promoter (right panel). B, NIH 3T3 cells were transfected with 2 μg of reporter plasmid pGL2-(Peg)TK (left) or pGL2-(Eos/Ik)TK (right) and increasing amounts (500 ng, 1 μg, and 3 μg) of pcDNA3-Pegasus. The Pegasus transcriptional activity is less pronounced than that of Eos, but it is specific for the Pegasus binding site, because no repression is observed when the reporter gene is driven by a promoter containing the Eos/Ikaros binding site (right panel). Values are presented as fold repression of luciferase activity.

**Fig. 6. Expression profiles of Eos and Pegasus.** A, Northern blot analysis of poly(A)+ RNA isolated from human cell lines representing different cell lineages, with HT-1080 as a nonhematopoietic control. The probe used in each panel is shown to the right; to the left are the sizes of the molecular weight markers in kb. B, Northern blot analysis of poly(A)+ RNA isolated from 12 human tissues (commercial blot supplied by CLONTECH) using an Eos (top panel), Pegasus (middle panel), or actin (bottom panel) gene probe.
expression has come from an examination of its protein partners. Two-hybrid experiments have shown that Ikaros binds to Sin3 co-repressors (14) and to Mi-2 proteins (8). Sin3 proteins exist in a repressor complex that contains the histone deacetylase enzymes HDAC1 and 2 (28, 29). Mi-2 proteins are ATPases that are present in a distinct NURD (nucleosome remodelling and histone deacetylase) complex (27, 30–33). It has also been observed that Ikaros can associate with Brg1 and the SWI/SNF chromatin remodelling complex that is typically implicated in the activation of gene expression (8). It has recently been shown that Ikaros can associate with the co-repressor protein CtBP (17). The current results suggest that Ikaros may move between these different complexes during cell cycle progression and differentiation (8, 14). Although the mechanisms that govern the successive associations of Ikaros are not fully understood, it is probable that the availability and activity of different protein partners will influence which complex it joins and whether it activates or represses transcription. Its binding to novel partners such as Eos and Pegasus may also play a role in this process.

Studies in knockout mice have clearly implicated the Ikaros family proteins, Ikaros and Aiolos, in the control of lymphoid development (27, 34). The Helios knockout has not yet been reported. Ikaros and Aiolos are co-expressed in most T and B cell lines, but Ikaros is also present in early hematopoietic progenitors and in erythroid cells, as is the more recently described protein Helios (12, 13). Although the analysis of knockout mice has highlighted the roles of Ikaros and Aiolos in lymphoid development, it is probable that these proteins have significant roles in other hematopoietic cells (34). The observation that Eos and Pegasus are co-expressed with Ikaros proteins in several hematopoietic cell lines (Fig. 6A) raises the possibility that they may be compensating for the loss of Ikaros or Aiolos in knockout mice and reducing the phenotypes observed in certain cell types.

The Pegasus protein is remarkable among the Ikaros family in that it is the only member that does not recognize the distinctive tGGGAa motif. The results of site selection and additional mutagenesis experiments indicate that a wide variety of sequences are bound by Pegasus but suggest that the optimal binding sites have the form GXXTGTXXG (Table 1).2 Pegasus contains only three N-terminal zinc fingers, and, on the basis of homology alignments (Fig. 1), it appears that it is missing the first of the four N-terminal zinc fingers found in Eos, Aiolos, and the largest splice isoforms of Helios and Ikaros. It has been shown that Ik-2, the splice form that is also missing the first finger, still binds Ikaros sites in DNA and that even Ik-4, with fingers 2 and 3, can bind certain double Ikaros recognition sites (6). In the light of these results, it is not surprising that the three fingers of Pegasus are adequate for sequence specific DNA binding.

The direct effect of Pegasus and Eos on the activity of previously described Ikaros proteins in vivo remains to be tested. It is possible that Eos may combine with Ikaros proteins to form a repressor complex that recognizes genes with typical or double tGGGAa Ikaros binding sites. Pegasus, in contrast, may combine with Ikaros family proteins and allow the recognition of control regions with composite sites made up of Ikaros (tGGGAa) and Pegasus (GXXTGTXXG) sites.

Pegasus and Eos differ from previously reported Ikaros family proteins in that their expression is not confined to the hematopoietic system, and it is likely that they play roles in other tissues. Recently, a novel gene TRPS1, encoding a zinc finger domain that shares homology with the C-terminal zinc finger dimerization domain of Ikaros, has been implicated in the causation of the inherited disorder tricho-rhino-phalangeal syndrome type I (35). Apart from its homology in the C terminus, this protein is unrelated to other Ikaros family proteins and contains a GATA type zinc finger and several classical zinc fingers. The gene appears to be broadly expressed, and although the mechanism by which it operates is not clear, it would be interesting to investigate whether its protein interacts with widely expressed Ikaros family members such as Pegasus and Eos. The result also emphasizes the fact that additional Ikaros related proteins are likely to exist and have interesting functions in different tissues.

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