Vgl-4, a Novel Member of the Vestigial-like Family of Transcription Cofactors, Regulates $\alpha_1$-Adrenergic Activation of Gene Expression in Cardiac Myocytes*

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Cardiac and skeletal muscle genes are regulated by the transcriptional enhancer factor (TEF-1) family of transcription factors. In skeletal muscle, TEF-1 factors interact with a skeletal muscle-specific cofactor called Vestigial-like 2 (Vgl-2) that is related to the Drosophila protein Vestigial. Here, we characterize Vgl-4, the only member of the Vestigial-like family expressed in the heart. Unlike other members of the Vgl family that have a single TEF-1 interaction domain called the tondu (TDU) motif, Vgl-4 has two TDU motifs in its carboxyl-terminal domain. Like other Vgl factors, Vgl-4 physically interacts with TEF-1 in an immunoprecipitation assay. Vgl-4 functionally interacts with TEF-1 and also with myocyte enhancer factor 2 in a mammalian two-hybrid assay. Overexpression of Vgl-4 in cardiac myocytes interfered with the basal expression and $\alpha_1$-adrenergic receptor-dependent activation of a TEF-1-dependent skeletal $\alpha$-actin promoter. In cardiac myocytes cultured in serum and in serum-free medium, a myc-tagged Vgl-4 protein was located in the nucleus and cytoplasm but was exported from the nucleus when cells were treated with $\alpha_1$-adrenergic receptor agonist. A chimeric nuclear-retained Vgl-4 protein inhibited $\alpha_1$-adrenergic receptor-dependent activation. In contrast, deletion of the TDU motifs of Vgl-4 prevented Vgl-4 nuclear localization, relieved Vgl-4 interference of basal activity, and enhanced $\alpha_1$-adrenergic up-regulation of the skeletal $\alpha$-actin promoter. Nuclear export of Vgl-4 is dependent on the nuclear exportin CRM-1. These results suggest that Vgl-4 modulates the activity of TEF-1 factors and counteracts $\alpha_1$-adrenergic activation of gene expression in cardiac myocytes.

Differentiation of cardiac and skeletal muscle from pluripotent embryonic stem cells requires the selective activation of a limited number of transcription factors that drive the expression of many genes that confer the tissue-specific phenotype. The transcriptional enhancer factor-1 (TEF-1) family of transcription factors is required for muscle-specific gene activation. TEF-1 was identified from the characterization of the cardiac troponin T promoter. Cardiac troponin T is expressed in fetal skeletal muscle and in cardiac muscle. Mutation analysis of the cardiac troponin T promoter showed that the MCAT element (originally defined as a muscle-specific cytidine-adenosine-thymidine sequence, 5'-CATTCCT-3') is required for high levels of promoter activity in cardiac and skeletal muscle cells (1, 2). Similar MCAT elements have been characterized in a large number of skeletal and cardiac muscle-specific promoters (3). The transcription factor that binds to this element was identified as the SV40 enhancer-binding factor TEF-1 (4).

TEF-1 is expressed in many tissues (5), and TEF-1 has also been implicated in placenta-specific enhancer function (6, 7). Thus, the mechanism whereby TEF-1 confers tissue specificity to an MCAT-bearing promoter has been ascribed to the interaction of TEF-1 with tissue-specific cofactors. For example, both the human placental lactogen promoter (6, 7) and the $\beta$-myosin heavy chain promoter (8, 9) contain MCAT elements and are TEF-1-dependent for their specific expression in placenta and muscle, respectively. Thus, placenta- and muscle-specific TEF-1 cofactors must confer tissue-specific activity of TEF-1-dependent promoters.

Studies in Drosophila have identified an important role for a cofactor of the TEF-1 homologue scalloped during wing and flight muscle differentiation (10, 11). This cofactor, called Vestigial, is critical for the development and patterning of the wing: loss of Vestigial results in a failure of wing development, and ectopic expression of Vestigial in tissues of the eyes, legs, and antennae leads to the development of ectopic wing tissue (12, 13). Binding of Vestigial switches the DNA target selectivity of the Scalloped protein (14) and activates many wing and flight muscle-specific genes. These studies suggest that Vestigial acts as a selector protein controlling tissue-specific gene activation during organogenesis.

We and others have identified mammalian homologues of Vestigial (14–17). The first human homologue of Vestigial was named Tondu (the French word for “shaved”), perhaps because it could partially rescue the wing phenotype caused by a null mutation in Drosophila Vestigial (15). In a Northern blot of fetal human tissues, Tondu was found to be expressed in the kidney and lung, but not in the brain or the heart (15). Tondu was shown to physically and functionally interact with TEF-1 through a conserved sequence called the tondu (TDU) motif. We also identified this factor but named it Vestigial-like (Vgl)-1. Northern blot analysis of adult human tissues showed that it is expressed almost exclusively in the placenta (16). We identified two other genes that bear a TDU motif and called them Vgl-2 and Vgl-3, although these proteins have little homology outside the TDU motif. Vgl-3 was also found to be placenta-specific. Thus, Vgl-1 and Vgl-3 likely contribute to the placenta-specific activity of TEF-1.

In the adult, Vgl-2 was detected only in skeletal muscle. Vgl-2 is expressed in the myogenic cell line C2C12, and Vgl-2...
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mRNA levels increase during muscle differentiation. Using a mammalian two-hybrid assay, we found that Vgl-4 also interacts with TEF-1 factors through its TDU motif. In addition, we found that Vgl-4 uses the TDU motif to interact with the transcription factor myocyte enhancer factor (MEF) 2 (16). Interestingly, Vgl-2 activated a TEF-1-dependent promoter and a MEF2-dependent promoter in myotubes, but not in myoblasts. By in situ hybridization in fetal mice, Vgl-2 mRNA appeared in early muscle precursors together with the myogenic differentiation factor myogenin (16). Co-expression of Vgl-2 with MyoD, another myogenic differentiation factor, enhanced MyoD-induced muscle differentiation of 10T1/2 cells with a 7-fold increase in myosin heavy chain expression (16). From these functional properties, because Vgl-2 is skeletal muscle-specific and is expressed at the onset of skeletal muscle differentiation in the somitic myotome, Vgl-2 likely accounts for the skeletal muscle-specific function ascribed to TEF-1. However, a similar tissue-restricted member of the Vestigial-like family of cofactors had not been identified in the heart.

Differentiation of cardiac muscle progenitor cells involves the selective activation of the GATA4 and Nkx2.5 transcription factors in pluripotent mesoderm (18–20). However, because mutation of TEF-1 binding sequences in the promoters of cardiac-specific genes causes a loss of tissue specificity (2), and because ablation of TEF-1 expression causes abnormal cardiac development and an embryonic lethal phenotype (21), these studies support a role for TEF-1 factors in cardiac development. In the adult, cardiac myocytes respond to growth-promoting stimuli by undergoing hypertrophy, rather than cell division. Although cardiac myocytes are considered to be terminally differentiated, hypertrophic signals such as the α1-adrenergic receptor agonist phenylephrine induce the expression of a number of genes characteristically expressed in the fetal heart (22, 23) and also partially reactivate specific components of the cell cycle machinery, including members of the E2F family of transcription factors (24) and cyclin D (25). Thus, myocytes transit through the G1-S phase of the cell cycle but appear unable to reenter a proliferative state. TEF-1 factors have been implicated in the activation of fetal genes in response to hypertrophic signals (22, 23, 26–29). If cardiac myocytes could be induced to dedifferentiate and reenter the cell cycle, this would represent an exploitable strategy to repair injured myocardium after myocardial infarction. Alternatively, expansion and modification of adult stem cells could also provide a means of regenerating injured tissue (30).

In the present study, we characterized Vgl-4, a novel member of the Vgl family of TEF-1 cofactors. Vgl-4 was initially identified as a transcript expressed in an adult human myeloid leukemia bone marrow-derived stem cell line (31). Vgl-4 is the only Vgl member expressed in the heart. Unlike other members of the Vestigial-like family, Vgl-4 has two TDU motifs, suggesting that Vgl-4 might form a bridge between TEF-1 and MEF2 transcription factors. Mammalian two-hybrid analysis showed that Vgl-4 interacts with MEF2 and TEF-1. Overexpression of Vgl-4 in transient transfection assays in cardiac myocytes squelched a TEF-1-dependent skeletal α-actin promoter and interfered with its α1-adrenergic induction. Vgl-4 becomes localized to the cytoplasm with α1-adrenergic stimulation, suggesting that it is part of a dedifferentiated phenotype. Whereas the functional role of Vgl-4 in cardiac development remains to be elucidated, its interaction with and modulation of known cardiac and skeletal muscle transcription factors reveal a novel component of cardiac muscle differentiation.

EXPERIMENTAL PROCEDURES

Expression Plasmids and Constructs—The full-length human (BQ832927) and mouse (BC048841) Vgl-4 cDNAs were obtained from Open Biosystems (Huntsville, AL). The human Vgl-4 cDNA was released from the pOTB7 plasmid using EcoRI and XhoI, labeled by random hexamer oligonucleotide priming, and used for Northern blot analysis of gene expression with a multi-tissue Northern blot (Clontech). The EcoRI/XhoI fragment was ligated into the human cytomegalovirus enhancer-driven expression vector pXJ40.

To generate a myc-tagged Vgl-4 protein, the mouse Vgl-4 cDNA was inserted into the pCMV-SPORT6 expression vector with a XhoI/HindIII digest, filled-in with Klenow, and a XhoI digest. The fragment encoding amino acids 29–287 was ligated in-frame to a SmaI/XhoI linearized pBluescript-myc tag vector to obtain the pBSmycVgl-4 vector. From the pBSmycVgl-4 vector, an Ncol/XhoI fragment encoding myc-tagged mouse Vgl-4 was ligated into the Ncol/XhoI-linearized pXJ40 expression vector to obtain the pXJmycVgl-4 vector. A chimeric construct bearing the amino-terminal domain of TEF-1 and the DNA-binding domain and nuclear localization signal fused to the myc epitope was generated. The myc-tagged Vgl-4 cDNA sequence was obtained by ligating an EcoRI/filled-in BamHI fragment of TEF-1 in-frame to a filled-in Ncol/XhoI fragment of mycVgl-4 into an EcoRI/XhoI linearized pXJ40 vector. A mycVgl-4 expression construct lacking the TDU motifs was obtained by releasing and filling-in a Sapi/XhoI digest of pXJmycVgl-4, thereby deleting the carboxyl terminus from amino acid 192.

For the mammalian two-hybrid analysis, the pG5-luciferase reporter plasmid (Promega) was used. The pMTEF-1 vector (containing the coding sequence for the yeast GAL4 DNA-binding domain fused to the human TEF-1 cDNA encoding amino acids 168–426) and the pMEF2 vector (containing GAL4 DNA-binding sequence fused to the full-length MEF2C sequence) have been described previously (16). The pVP16 vector (encoding the activation domain of the herpes simplex virus VP16 protein) was linearized with Mulu and PstI. A BstHII/PstI fragment of the human Vgl-4 cDNA encoding amino acids 113–296 of the carboxyl terminus of Vgl-4 was ligated in-frame to the Mulu/PstI-linearized pVP16 vector to generate the pVPVgl-4 vector. The pVPVgl-4 vector, which lacked both TDU1 and TDU2, was obtained by filling in an EcoNI site and ligating to an EcoRII site in Vgl-4, thereby deleting the sequence coding for amino acids 207–273. The pVPVgl-4 vector was obtained by cloning a Nael fragment coding for amino acids 153–232 of human Vgl-4 into the pVP vector at the filled-in BamHI site. The pVPVgl-4TDU1 vector was obtained by cloning a Nael fragment coding for amino acids 233–296 of the human Vgl-4 cDNA encoding amino acids 113–296 of the carboxyl terminus of Vgl-4 was ligated in-frame to the Mulu/PstI-linearized pVP16 vector to generate the pVPVgl-4 vector. The pVPVgl-4TDU2 vector was obtained by cloning a 3′ Nael/HindIII fragment coding for amino acids 233–296 into the pVP vector between the filled-in EcoRI and the HindIII sites. All constructs were sequenced at the University of Pittsburgh core facility.

In Vitro Translation and Co-immunoprecipitation—The pXJTEF-1 and pXJmycVgl-4 plasmids were transcribed with T7 polymerase and translated in vitro (Promega) to produce unlabeled full-length human TEF-1 and myc-tagged mouse Vgl-4 proteins. Incorporation of [35S]methionine was also used to produce radiolabeled immunoprecipitations. Reticulocyte lysates were preclreated with protein A-Sepharose in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, and 1% Igepal CA-630 supplemented with protease inhibitors). Unlabeled mycVgl-4 was incubated with radiolabeled TEF-1 and immunoprecipitated using a rabbit anti-myc antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and unlabeled TEF-1 protein was incubated with radiolabeled mycVgl-4 and immunoprecipitated with a mouse monoclonal anti-TEF-1 antibody (BD Transduction Laboratories, San Diego, CA). Proteins were size-fractionated by SDS-PAGE, the gel was dried down, and radiolabeled proteins were visualized on a phosphor storage screen (Amersham Biosciences).

Cell Culture and Transfection—Neonatal rat cardiac myocytes were isolated as described previously (32), except that Worthington type II collagenase (5 mg/ml in Hank’s balanced salt solution, 0.8 mM MgSO4, 20 mM Hepes, pH 7.4, and 1% glucose) was substituted for trypsin. Myocytes were cultured at low density (5 × 104 viable cells/well of a 6-well dish) in Hank’s minimal essential medium supplemented with 5% fetal bovine serum and bromodeoxyuridine (10 μM) to prevent spontaneous proliferation. Twenty-four h after plating, the medium was replaced with fresh medium containing 30 mM Hepes, pH 7.4, and transfected using the calcium phosphate precipitation method for 2 h with serum-containing medium, as described previously (16). After transfection, cells were rinsed several times, maintained in serum-free medium for 2 days, and harvested for the luciferase assay, as described previously (16). Activities (mean ± S.E.) of the skeletal α-actin promoter were normalized to basal activity when co-transfected with empty pXJ40 expression vector and set at 1-fold. By independent sample t test, mean fold activities of the skeletal α-actin promoter response to phenylephrine in the presence of overexpressed TEF-1 transcription factors and the Vgl-4 cofactor were considered different from the re-
Fig. 1. Human and mouse Vgl-4 have two tandem TDU motifs. A, aligned human and mouse Vgl-4 sequence are shown with the TDU motifs (TDU1 and TDU2) underlined and a putative nuclear export signal (NES; \[ L_X_1 \_L_X_2 \_L_X_3 \_LXL \]) highlighted in gray. Identical sequence is indicated by dots, and gaps are indicated by dashes. The boxed sequence corresponds to a putative binding site for the 14-3-3 protein (NP_648658) are aligned below the equivalent motifs in Vgl-4. B, aligned TDU motifs of the four human Vgl family members compared with Drosophila Vestigial (Dm vg) reveal a 50% consensus.

RESULTS

Vgl-4 was identified in the SMART (Simple Modular Architecture Research Tool; smart.ox.ac.uk/) data base as a protein containing two tandem TDU motifs (Fig. 1). A putative nuclear export signal and binding site for interaction with the nuclear exportin CRM-1 having the consensus sequence \[ L_X \_P \] (43). The two TDU motifs of a hypothetical Drosophila protein (NP_648658) are aligned below the TDU motifs of Drosophila vesicular stomatitis virus (VSV) G protein (Xp) revealed a 50% consensus. Identical sequence is indicated by \[ \_ \_ \_ \_ \_ \_ \_ \_ \_ \] and gaps are indicated by dashes. The boxed sequence corresponds to a putative binding site for the 14-3-3 protein (Santa Cruz Biotechnology) and visualized with Cy2-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA).

The myc-tagged protein was detected using a c-myc (A-14) rabbit antibody (Santa Cruz Biotechnology) and visualized with Cy3-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA). Myosin heavy chain was revealed using a c-myc (A-14) rabbit antibody (Santa Cruz Biotechnology) and visualized with Cy2-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA). Nuclei were visualized with 4',6-diamidino-2-phenylindole (1 mg/ml; Roche Applied Science).

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Because the two TDU motifs diverge from those previously described for other members of the Vgl family (Fig. 1B), we tested whether Vgl-4 could also interact with TEF-1 (Fig. 3). Functional interaction between TEF-1 and Vgl-4 was observed by two-hybrid analysis in transiently transfected CV-1 cells (Fig. 3A; \( p < 0.001 \)), and physical interaction was confirmed by co-immunoprecipitation (Fig. 3B). In addition, like Vgl-1 and Vgl-2 (16), Vgl-4 interacts with MEF2 (Fig. 3A; \( p < 0.001 \)). Interestingly, when half the pMTEF-1 and half the pMMEF2 vectors were both expressed with pVP-Vgl-4, a stronger interaction was observed than with pMTEF-1 and pMMEF2 vector alone (\( p < 0.0001 \)). On the other hand, Vgl-1 contains a single TDU motif, and when pVP-Vgl-1 was similarly co-expressed with pMTEF-1 and pMMEF2, enhanced interaction was not seen (data not shown), suggesting that the presence of tandem TDU motifs in Vgl-4 allows TEF-1 and MEF2 to bind to Vgl-4 at the same time. In-frame deletion of both TDU motifs in Vgl-4 prevented interaction between Vgl-4 and TEF-1 (Fig. 4A), while deletion of both TDU motifs in Vgl-4/PE (Vgl-4; pMTEF-1; pMMEF2) prevented Vgl-4 interaction with TEF-1 and MEF2 (Fig. 4B). Although TDU1 preferentially interacted with TEF-1 (Fig. 4A), and TDU2 preferentially interacted with MEF2 (Fig. 4B), neither TDU motif alone was sufficient to restore full interaction with TEF-1 or MEF2.

To directly test whether the interaction between Vgl-4 and TEF-1 modulates the transcriptional activity of TEF-1, we used the mammalian two-hybrid assay in cardiac myocytes (Fig. 5). Vgl-4 alone does not affect the expression of the GAL4-dependent promoter (compare pM/pVP with pM/pVPVgl-4). When fused to the GAL4 DNA-binding domain, TEF-1 is a moderately strong transcriptional activator of a heterologous GAL4-dependent promoter, pG5-luciferase, in cardiac myocytes (Fig. 5, pMTEF-1), but not in CV-1 cells (Fig. 3), as reported previously (16). \( \alpha_1 \)-Adrenergic signaling increases reporter gene expression in the presence of the GAL4/TEF-1 (pMTEF-1), but not in the presence of the GAL4 DNA-binding domain alone (pM). Thus, \( \alpha_1 \)-adrenergic signaling increases TEF-1 transcriptional activity.

Interaction between Vgl-4 and TEF-1 was also observed in cardiac myocytes (compare pMTEF-1/pVP with pMTEF-1/pVPVgl-4; \( p < 0.01 \)), as it was in CV-1 cells (Fig. 3). However, in the presence of Vgl-4, the \( \alpha_1 \)-adrenergic signaling-mediated potentiation of TEF-1 function was prevented (Fig. 5, compare pMTEF-1/pVPVgl-4 with pMTEF-1/pVPVgl-4/PE; \( p < 0.02 \)). To determine how Vgl-4 modulates the \( \alpha_1 \)-adrenergic signal-

Fig. 2. Northern blot analysis of Vgl-4 expression in human tissues. A human multi-tissue Northern blot with 1 \( \mu \)g mRNA/lane was probed for Vgl-4 expression. Vgl-4 expression was highest in the heart, kidney, and brain. Position of size markers is indicated.

FIG. 1.

FIG. 2.
ing-mediated potentiation of TEF-1 function, a myc-tagged Vgl-4 protein was expressed in cardiac myocytes by transient transfection. In cardiac myocytes cultured in 5% fetal bovine serum or in serum-free medium, mycVgl-4 was localized predominantly in the nucleus (Fig. 6, A and C). However, 24 h after phenylephrine stimulation, mycVgl-4 was predominantly exported to the cytoplasm, and little signal remained in the nuclei of cultured cardiac myocytes (Fig. 6, E).

To determine how Vgl-4 regulates gene expression in cardiac myocytes, the effect of Vgl-4 overexpression was tested on a TEF-1-dependent \( /H9251 \) skeletal actin promoter (Fig. 7). Vgl-4 overexpression lowered the basal activity of the \( /H9251 \) skeletal actin promoter (\( p<0.01 \)), suggesting that Vgl-4 is a negative modulator of TEF-1. The \( \alpha \)-skeletal actin gene is up-regulated by the \( \alpha \)-adrenergic receptor-selective agonist phenylephrine up to 6-fold (Fig. 7). The response of the skeletal \( /H9251 \) actin promoter to phenylephrine was blunted by TEF-1 overexpression (\( p<0.01 \)), by Vgl-4 overexpression (\( p<0.02 \)), or when Vgl-4 was co-expressed with TEF-1 (\( p<0.0001 \)).

Taking into account that Vgl-4 is largely exported from the nucleus of phenylephrine-treated cardiac myocytes (Fig. 6), the ability of Vgl-4 overexpression to abrogate the \( /H9251 \) activation of the skeletal \( /H9251 \) actin promoter might be enhanced if it were made to remain in the nucleus. To test whether a strong nuclear localization signal could override phenylephrine-mediated nuclear export of Vgl-4, a chimeric protein was generated using the nuclear localization signal of TEF-1 fused to mycVgl-4. This chimeric protein, TEF(N)-mycVgl-4, is not exported from the nucleus of cardiac myocytes treated with phenylephrine (Fig. 8, C and D). When tested on the skeletal \( \alpha \)-actin promoter, the chimeric protein that is resistant to nuclear export nearly ablated the \( /H9251 \) response (Fig. 7). On the other hand, deleting the carboxy-terminal domain of Vgl-4 containing both TDU motifs caused cytoplasmic retention of Vgl-4 (Fig. 8, E and F) and markedly increased the \( \alpha \)-adrenergic up-regulation of the skeletal \( \alpha \)-actin promoter (Fig. 7).

To determine whether the mechanism that regulates nuclear shuttling of Vgl-4 in cardiac myocytes requires the nuclear exportin CRM-1, as suggested by the presence of a putative...
nuclear export signal (Fig. 1), cardiac myocytes treated with phenylephrine were also treated with the CRM-1 inhibitor leptomycin B (Fig. 9). Leptomycin B blocked nuclear export of Vgl-4 in phenylephrine-treated cardiac myocytes.

FIG. 6. \(\alpha\)-Adrenergic signaling causes nuclear export of Vgl-4 in cardiac myocytes. Cardiac myocytes transiently transfected with a pXJ40-mycVgl-4 expression vector revealed strong nuclear immunofluorescence using a myc-specific antibody (in red) when myocytes were maintained for 24 h after transfection in medium supplemented with 5% fetal bovine serum (\(A\)). \(C\), transfected cardiac myocytes in serum-free medium for 24 h also showed nuclear localization of mycVgl-4, whereas transfected myocytes in serum-free medium containing 100 \(\mu M\) phenylephrine (PE) revealed predominantly cytoplasmic localization of mycVgl-4, with little signal remaining in the nuclei (compare \(E\) and \(F\), arrows). \(B\), \(D\), and \(F\), cardiac myocytes were counterstained with the MF20 myosin heavy chain antibody (in green), and nuclei were revealed with 4',6-diamidino-2-phenylindole (in blue).
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In the present study, we have identified Vgl-4 as a novel member of the Vgl family of transcription cofactors. Vgl-4 is expressed in several tissues, with the highest expression seen in the heart, followed by the kidney and brain (Fig. 2). This is the first member of the Vestigial family to be identified in the heart. Like other members of this family, united by the TDU motif, Vgl-4 interacts with TEF-1 and MEF2 in cultured cells. Thus, Vgl-4 joins a growing list of transcription factors and cofactors known to interact with TEF-1.

Vgl-4 carries two TDU motifs required for the interaction with TEF-1 and MEF2, as supported by the two-hybrid analysis (Figs. 3 and 4). Vgl-4 may serve an important function bridging TEF-1 and MEF2 in cardiac muscle. The presence of two tandem TDU motifs in Vgl-4 is unique among vertebrate Vgl family members but is also seen in hypothetical proteins of Drosophila melanogaster (NP_648658) and Caenorhabditis elegans (AAK82912) that are distinct from Vestigial. However, the function of these other invertebrate Vestigial-like factors is unknown. Interestingly, the first and second TDU motifs of the Drosophila protein are spaced farther apart and are different from each other (Fig. 1A), yet they show remarkable similarity to the equivalent TDU motifs in Vgl-4, suggesting that these motifs are conserved and may be functionally distinct. In support of this hypothesis, TDU1 and TDU2 showed selective interaction with TEF-1 and MEF2, respectively (Fig. 4). The existence of another protein in Drosophila bearing tandem TDU motifs points to a very ancient origin of the Vgl family.

TEF-1 forms a multi-protein complex with other transcription factors including the histone-modifying enzyme poly(ADP-ribose) polymerase 1 (34), the serum response factor (35), and MEF2 (36). Among transcriptional co-activators, the p160 proteins (37), the SRF/Yes-associated protein Yap65 (38), and Vgl-2 (16) are able to augment transcription from an MCAT-dependent promoter. Unlike Vgl-2 in skeletal muscle, Vgl-4 appears to be a negative regulator of TEF-1-dependent gene expression in cardiac myocytes when tested on the TEF-1-dependent skeletal α-actin promoter (Fig. 7) and on the β-myosin heavy chain promoter (data not shown). Similarly, Vgl-4 interfered with the activity of a MEF2-dependent myosin light chain promoter in cardiac myocytes (data not shown).

Vgl-4 interference with TEF-1-dependent promoter activity under basal conditions suggests either that Vgl-4 itself is a negative cofactor or that Vgl-4 recruits a negative factor to TEF-1. If Vgl-4 is a negative cofactor, blocking its interaction with TEF-1 should have restored baseline skeletal α-actin promoter activity. If Vgl-4 recruits a negative factor to TEF-1, then blocking Vgl-4 interaction with TEF-1 would allow overexpressed mutant Vgl-4 to compete for binding to the negative factor with endogenous Vgl-4, relieve the inhibition of the TEF-1 complex, and further increase the baseline activity of the skeletal α-actin promoter. Consistent with the latter hypothesis, a carboxyl-terminal-truncated Vgl-4 lacking the TDU motifs augmented the basal activity of the skeletal α-actin promoter (Fig. 7).

The skeletal α-actin promoter is activated by α1-adrenergic stimulation, and this response requires TEF-1 transcription factors (23, 27–29). Previously, we observed a 3-fold activation of the skeletal α-actin promoter by α1-adrenergic stimulation (29). Here, we found that the use of collagenase rather than trypsin during myocyte isolation markedly improved the response to phenylephrine, up to 6-fold (Fig. 7; data not shown). The phenylephrine response of the skeletal α-actin promoter in the presence of empty expression vector (pXJ40) reflects the increased activity of endogenous TEF-1 transcription factors (28, 29). Consistent with our previous findings, TEF-1 overexpression inhibited the response to phenylephrine (27), suggesting that exogenous TEF-1 titrates limiting cofactors.

Overexpression of Vgl-4 also attenuated the α1-adrenergic activation of the skeletal α-actin promoter (Fig. 7). Vgl-4 may mediate TEF-1 by two possible mechanisms, either by recruiting a co-repressor to TEF-1, as under basal conditions, or by preventing α1-adrenergic modification of TEF-1. The two-hybrid analysis of the effects of Vgl-4 under basal and α1-adrenergic stimulation in cardiac myocytes supports either mechanism. Transcription of a heterologous promoter (pG5-luciferase) was activated by a GAL4 DNA-binding domain/TEF-1 fusion protein under basal conditions and was further activated in response to α1-adrenergic stimulation (Fig. 5). The ability of pMTEF-1 to further activate the pG5-luciferase reporter in response to α1-adrenergic stimulation was abolished in the presence of a chimeric protein containing the activation domain of VP16 fused to Vgl-4 (pVPVgl-4). Thus, Vgl-4 interferes with α1-adrenergic-mediated TEF-1 function.

Nuclear localization of Vgl-1 depends on its interaction with TEF-1 because preventing Vgl-4 interaction with TEF-1 by deleting the TDU motifs caused cytoplasmic retention of Vgl-4 (Fig. 8). Interestingly, Vgl-4 moves out of the nucleus of cardiac myocytes treated with the α1-adrenergic agonist phenylephrine (Fig. 6), suggesting that Vgl-4 removal is required to promote the α1-adrenergic response. Forced nuclear retention of Vgl-4 in a chimeric construct carrying a strong nuclear localization signal (Fig. 8) markedly reduced the α1-adrenergic-mediated TEF-1 response to phenylephrine, up to 6-fold (Fig. 7).

Conversely, overexpression of a carboxyl-terminal-truncated Vgl-4 lacking the TDU motifs that is retained in the cytoplasm augmented the α1-adrenergic response of the skeletal α-actin promoter (Fig. 7). Overexpression of Vgl-4 likely overwhelms the nuclear export mechanism, and the presence of residual nuclear Vgl-4 could account for the interference of the α1-adrenergic response.

Vgl-4 carries a consensus nuclear export signal (see Fig. 1A), a binding site of the nuclear exportin CRM-1 (33). Our studies show that the CRM-1 inhibitor leptomycin B blocks nuclear export of Vgl-4 in phenylephrine-treated cardiac myocytes (Fig. 9). A CRM-1-dependent mechanism controlling nuclear localization of the cardiac transcription factor GATA4 in response to β-adrenergic signaling has been described previously (39). Inactivation of glycogen synthase kinase III prevents CRM-1 interaction with
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GATA4 and its nuclear export. During development, GATA4 regulates the expression of the cardiac-specific homeobox gene Nkx2.5 that controls cardiac myocyte differentiation (40). α1-Adrenergic signaling may unmask the nuclear export domain of Vgl-4 by phosphorylating the 14-3-3 site and thereby allow nuclear export through a CRM-1-dependent mechanism. α1-Adrenergic signaling is known to activate a fetal gene program and to partially reactivate components of the cell cycle in a process of cardiac myocyte dedifferentiation (22–25). In this respect, it is important to note that the Drosophila Vestigial protein is required for cell proliferation in the wing primordium (41, 42), suggesting that members of the Vestigial family of transcription cofactors may participate in regulating the transition from proliferation to cell cycle arrest and differentiation. Thus, like GATA4, regulating the nuclear localization of Vgl-4 may play a role in cardiac myocyte differentiation.

Although we do not yet know the genes that are regulated by Vgl-4 through their interaction with TEF-1 factors, one likely outcome of this interaction is a change in DNA binding specificity at muscle-specific promoters. In Drosophila, Vestigial interaction with Scalloped alters DNA binding specificity and activates many wing-specific genes (14) and allows the differentiation of indirect flight muscles (11). Whether Vgl-4 also affects TEF-1 target selectivity remains to be determined.

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