Cloning and characterization of GETS-1, a goldfish Ets family member that functions as a transcriptional repressor in muscle

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An Ets transcription factor family member, GETS-1, was cloned from a goldfish retina cDNA library. GETS-1 contains a conserved Ets DNA-binding domain at its N-terminus and is most similar to ternary complex factor (TCF) serum-response-factor protein-1a (SAP-1a). GETS-1 is expressed in many tissues, but is enriched in retina and brain. As with the TCFs SAP-1a and ets-related protein (ERP), overexpression of the GETS-1 promoter suppresses nicotinic acetylcholine receptor ϵ -subunit gene expression in cultured muscle cells. A consensus Ets binding site sequence in the promoter of the ϵ -subunit gene is required for

GETS-1-mediated repression. GETS-1 repressor activity is abrogated by overexpression of an activated Ras/mitogen-activated protein kinase (MAP kinase) or by mutation of Ser-405, a MAP kinase phosphorylation site in GETS-1. Fusion proteins created between GETS-1 and the Gal4 DNA-binding domain show that, like other TCFs, GETS-1 contains a C-terminal activation domain that is activated by a Ras/MAP kinase signalling cascade. Interestingly, mutation of Ser-405 located within this activation domain abrogated transcriptional activation of the fusion protein.

INTRODUCTION

During development, cell–cell interactions participate in controlling cell fate and differentiation. Many of these interactions require receptors at the cell surface that, upon binding their ligand, activate a signalling cascade that leads to changes in gene expression via activation of specific transcription factors. One family of receptors involved in initiating these signalling events are the receptor tyrosine kinases located at the cell surface.

Ligand binding to receptor tyrosine kinases results in activation of a Ras/mitogen-activated protein kinase (MAP kinase) signalling cascade [1]. MAP kinases are proline-directed serine/threonine kinases that activate downstream targets via phosphorylation. Downstream targets of MAP kinase include transcription factors such as c-Jun and several Ets family members. These proteins have been shown to play important roles in growth control, cell fate specification and differentiation [2–4].

Members of the Ets family of transcription factors share a common domain, called the Ets domain, that is responsible for binding to DNA containing the core Ets binding site, GGA [5]. These proteins can function as activators or repressors of transcription [4,6–9]. Like many other transcription factors, some Ets proteins have been shown to associate with other proteins to form specific ternary complexes with DNA [4,6,10]. These proteins are referred to as ternary complex factors (TCFs). For example, the Ets family member Elk-1 binds to the

fos gene serum response element (SRE) and interacts with serum response factor (SRF) [4,10,11]. In fibroblasts, this complex is formed in response to serum stimulation, which also stimulates MAP-kinase-dependent phosphorylation of Elk-1. This phosphorylation increases the transcriptional activation activity of Elk-1 [12]. The Ets family members serum-response-factor protein-1a (SAP-1a) and SAP-2/ets-related protein (ERP)/new ets transcription factor (NET) also have binding properties that are characteristic of TCFs and can form a ternary complex with SRF on the *c-fos* gene's SRE [6]. The TCFs have a considerable amount of sequence similarity in three regions referred to as boxes A, B and C [4,10,13]. Boxes A and B participate in DNA-binding and interaction with SRF at the SRE. The C box is found in the C-terminal region and contains a number of conserved potential MAP kinase phosphorylation sites, some of which are involved in mediating transcriptional activation.

A role for Ets proteins in development has been established. For example, in mammals Ets-1 and Ets-2 are implicated in T-cell development and maturation [14]. In *Drosophila*, the Ets family members *pointed* and *yan* appear to be involved in nervous system development [15]. In skeletal muscle, Ets proteins appear to participate in formation of the neuromuscular junction by regulating synapse-specific gene expression [16].

We are interested in defining the role played by Ets proteins during development. Zebrafish and goldfish are attractive for these studies because they develop rapidly and within a relatively

Abbreviations used: MAP kinase, mitogen-activated protein kinase; TCF, ternary complex factor; SRE, serum response element; SRF, serum response factor; SAP, serum-response-factor protein; ERP, ets-related protein; NET, new ets transcription factor; nAChR, nicotinic acetylcholine receptor; CMV, cytomegalovirus; CAT, chloramphenicol acetyltransferase; GST, glutathione S-transferase; ERK, extracellular-signal-regulated kinase; MEK, MAP-kinase/ERK kinase; EGF, epidermal growth factor; MBP, myelin basic protein; JNK, Jun N-terminal kinase; SEK, stress-activated/extracellular-signal-regulated protein kinase.

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transparent embryo (zebrafish), and particular neural structures, such as the retina and tectum, continue to grow throughout their life [17,18]. In addition, fish are able to regenerate their central neurons. For example, damage to the optic nerve induces a robust regenerative response in the retinal ganglion cells, leading to optic axon regrowth. These features, along with the ability to express exogenous genes in developing fish [19,20], make this preparation ideal for studying cell commitment, differentiation and regeneration. Therefore we created a cDNA library from the retina of a goldfish induced to regenerate its optic axons and screened it for Ets-related cDNAs. One cDNA, GETS-1, was found to be preferentially expressed in the fish nervous system and was further characterized. GETS-1 is most closely related to SAP-1a and, like SAP-1a, GETS-1 contains a transcriptional activation domain at its C-terminus that is regulated by a Ras/MAP kinase signalling cascade. We used an Ets-responsive promoter isolated from the nicotinic acetylcholine receptor (nAChR) ϵ -subunit gene to examine the effects of GETS-1 on gene expression. The ϵ -subunit promoter is specifically expressed in muscle cells. Interestingly, we found that both GETS-1 and SAP-1a decreased nAChR ϵ -subunit gene promoter activity in cultured muscle cells. GETS-1 repressor activity was abrogated by constitutively active Ras or MAP kinase overexpression. In addition, the same amino acid mutation (Ser-405 $\rightarrow$ Ala) that prevented transcriptional activation by the C-terminal domain also blocked GETS-1 repressor activity.

MATERIALS AND METHODS

Goldfish maintenance and surgery

Maintenance of goldfish and optic nerve crush were carried out as previously described [21,22]. Briefly, common goldfish (*Carassius auratus*) of 5–7 cm length were maintained at 25 °C in well aerated tanks. Right optic nerve crush was carried out just behind the orbit in tricaine methane sulphonate (0.1%) anaesthetized fish. The left optic nerve of each fish was left intact, with the left retina serving as an unoperated control.

Isolation of the GETS-1 cDNA clone

Poly(A)⁺ RNA from the retinas of fish whose optic nerves had been crushed 10 and 14 days earlier was used to prepare the cDNA library. cDNA was prepared according to standard protocols and directionally cloned into the *Eco*RI and *Xho*I sites of λ ZAP Express (Stratagene). A total of 5×10^5 recombinant phage were screened with a radiolabelled mouse Ets-2 DNA-binding domain probe. Three unique clones were isolated that encode proteins that are members of the Ets family of transcription factors. The largest clone isolated, 20-2, was found to be preferentially expressed in neural tissue and was further characterized as described in the Results section. Because this clone was the first Ets clone isolated from a goldfish cDNA library, we named it GETS-1 (goldfish ETS, clone 1).

RNase protection assays

Total cellular RNA was isolated from various goldfish tissues as previously described [22]. RNase protection assays were performed as previously described [23]. ³²P-radiolabelled RNA probes were prepared by run-off transcription of linearized vectors. The RNA probe was hybridized with 20 μ g of RNA overnight at 55 °C and digested with RNase A for 1 h, and the products were analysed on a denaturing polyacrylamide gel. Probe integrity was confirmed by omitting RNase from the procedure. Controls consisted of using a sense-strand probe, which consistently resulted in complete digestion of the probe.

In situ hybridization

In situ hybridization was performed as previously described [22]. Controls included an RNase treatment prior to hybridization and the use of sense-strand probes.

DNA sequencing

DNA sequencing was performed using an Applied Biosystems automated DNA sequencer with thermal cycling procedures, as recommended by the manufacturer. Unidirectional deletions were created using exonuclease III digestion of restricted plasmid DNA. Both strands of the DNA were sequenced.

Plasmid constructions

CMV-GETS-1 contains the CMV (cytomegalovirus) promoter driving GETS-1 expression. ϵ -5000-Luc contains the 5000 bp nAChR muscle ϵ -subunit gene promoter driving luciferase expression [16]. ϵ MUT1 contains the ϵ -subunit 5000 bp promoter, with a mutation in its Ets binding site, driving luciferase expression [16]. γ -722-CAT contains the nAChR γ -subunit gene promoter (nucleotides –754 to –33 relative to the translation initiation site) [24] driving chloramphenicol acetyltransferase (CAT) expression, and was used for normalization purposes. The SAP-1a expression vector contains the simian virus 40 promoter driving human SAP-1a expression. The ERP expression vector contains the CMV promoter driving mouse ERP expression. The Ets-2 expression vector contains the CMV promoter driving rat Ets-2 expression. CMV-CAT and CMV-LacZ contain the CMV promoter driving CAT expression and β -galactosidase (LacZ) expression respectively, and were used for normalization purposes. Gal4-Luc is a reporter plasmid which has luciferase expression under the control of four tandem repeats of the Gal4 DNA-binding sequence.

Full-length GETS-1 was amplified using the primers CTTGGATCCGCGAGTCTCAGTGGAA and CGGTCTAGATCAGGTGAACAGAAT (the *Bam*HI and *Xba*I restriction sites are underlined). The PCR product was subcloned into the *Xba*I/*Bam*HI site of pcDNA3HA [which contains three repeats of the haemagglutinin antigen (HA) epitope (YPYDVPDYA) between the *Hind*III and *Not*I sites of pcDNA3 (Invitrogen)] to produce pcDNA3HA-GETS-1. To construct Gal4-GETS-1CT [a fusion between the Gal4 DNA-binding domain and GETS-1 C-terminal (CT) amino acids 349–471], pcDNA3HA-GETS-1 was digested with *Stu*I (which cuts between amino acids 348 and 349) and *Eco*RV (which cuts just past the 3'-end in the polylinker) and subcloned into the *Sma*I-digested pSG242 vector [25]. GST-GETS-1CT [a fusion between glutathione S-transferase (GST) and the C-terminal amino acids 349–471 of GETS-1] was prepared by subcloning the *Stu*I/*Eco*RV fragment of pcDNA3HA-GETS-1 into pGEX-KG [26]. GST-Elk-1 is a fusion of the C-terminal amino acids 305–427 of Elk-1 with GST. PCR-based mutagenesis (Stratagene) was used to create the GETS-1(S405A) mutant, following the manufacturer's instructions. pcDNA-supMEK1 contains human MAP-kinase/extracellular-signal-regulated kinase (ERK) kinase 1 (MEK1) which has a deletion of amino acid residues 32–51 and substitution of Ser-218 and Ser-222 with glutamic acid residues [27]. The mutated form of MEK1 is constitutively active towards ERK. The dominant negative form of MEK1 (MEK1-DN) was made by replacing Ser-218 and Ser-222 with alanine residues [28,29]. Expression plasmids RasV12 and RasN17 were generously donated by D. L. Quilliam (Indiana University Medical School, Indianapolis, IN, U.S.A.) [30].

Cell culture and transfection

Rat primary muscle cultures were prepared and maintained as previously described [23]. Muscle cells were transfected using calcium phosphate precipitates of DNA followed by a brief glycerol shock as previously described [31]. Muscle cell cultures received 2.5 μ g of ϵ -5000-Luc, 0.01–2.5 μ g of CMV-GETS-1 and 20 μ g of γ -722-CAT for normalization purposes. When Ras was included in the muscle transfections, we used 2.5 μ g of RasV12. COS cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum. A total of 2.5×10^5 cells/well were seeded in 6-well plates 1 day prior to transfection. For each transfection, 0.1 μ g of CMV-LacZ (for normalization), 0.2 μ g of Gal4-Luc and 0.1 μ g of Gal4-GETS-1CT were used. At 24 h after transfection, cells were starved in Dulbecco's modified Eagle's medium containing 0.1% fetal bovine serum for 24 h. Cells were unstimulated or were stimulated with either epidermal growth factor (EGF; 50 ng/ml) or PMA (100 nM) for 8 h. Luciferase, CAT and β -galactosidase activities were assayed as previously described [32–34].

Kinase assay

GST-Elk-1 and GST-GETS-1CT were expressed in *Escherichia coli* and purified using previously described procedures [28]. Glutathione-agarose and myelin basic protein (MBP) were purchased from Sigma. Recombinant ERK1 (1 μ g) was activated by 0.2 μ g of GST-supMEK1 in 20 μ l of MBP kinase buffer (18 mM Hepes, pH 7.5, 10 mM magnesium acetate, 50 mM ATP) at 30 °C for 30 min. A sample of 2 ng of activated ERK1 was used to phosphorylate GST-GETS-1CT in 20 μ l of MBP kinase buffer at 30 °C for 20 min. The kinase reaction was terminated by the addition of SDS sample buffer. Phosphorylation was quantified by Phosphorimage or scintillation counting.

A solid-phase kinase assay using GST-GETS-1CT as a substrate was performed as follows. COS cells in 6-well plates were lysed in 200 μ l of lysis buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 50 mM NaF, 1% Triton X-100, 0.5% Nonidet P-40, 1 mM EGTA, 1 mM EDTA, 1 mM sodium vanadate, 1 mM dithiothreitol, 1 μ g/ml leupeptin, 5 μ g/ml aprotinin, 1 mM PMSF). Cell lysate (100 μ g of total protein) was incubated with 1 μ g of GST-GETS-1CT and 7.5 μ l of glutathione-agarose at 4 °C for 4 h. The glutathione-agarose beads were washed with lysis buffer five times, followed by washing twice with 25 mM Hepes buffer, pH 7.5, containing 50 mM NaCl and 0.5% β -mercaptoethanol. The agarose beads were resuspended in 20 μ l of MBP kinase buffer containing 10 μ Ci of [γ - 32 P]ATP. The kinase reaction was allowed to proceed for 30 min at 30 °C and was terminated by the addition of SDS sample buffer.

Immunoblotting

GST-Elk-1CT and GST-GETS-1CT were phosphorylated *in vitro* by activated ERK1. The phosphorylated proteins were resolved by SDS/PAGE (10% polyacrylamide) and transferred to a PVDF (Millipore) membrane. Western blotting was performed with anti-Elk and anti-phospho-Elk (pElk) antibodies (New England Biolab). These two antibodies recognize the unphosphorylated and phosphorylated forms of Elk respectively.

RESULTS

Cloning of a goldfish Ets-related cDNA, GETS-1

A goldfish retina cDNA library was screened with a radiolabelled mouse Ets-2 DNA-binding domain probe. The DNA-binding

Figure 1 Comparison of the amino acid sequence of GETS-1 with those of SAP-1a and SAP-2/ERP/NET (ERP)

Optimal alignment of the predicted GETS-1, SAP-1a and ERP sequences was achieved using GCG software (Pileup program). The three major regions of identity (A, B and C boxes) shared among all TCFs are labelled above the sequences. The SAP-2/ERP/NET inhibitory domain (NID) is indicated below the ERP sequence. Blocked areas indicate amino acid identity.

domain was chosen for the library screen, since it is the most highly conserved domain among the various Ets family members. This screen identified an Ets family member, GETS-1, which displayed significant sequence identity to the TCF subfamily of Ets proteins and was further characterized.

GETS-1 is a 4307-nucleotide-long cDNA with an open reading frame of 1473 nucleotides. The open reading frame begins with an ATG at position 57 and ends with a stop codon, TGA, at position 1470. The predicted molecular mass of GETS-1 is 51 761 Da. The 3' untranslated sequence is just over 2.8 kb and contains three polyadenylation signal sequences (AAUAAA) at positions 3528, 3697 and 3779. Since we did not find such a polyadenylation sequence within 100 nucleotides preceding the poly(A) tail, it is likely that another sequence signalled polyadenylation for the RNA this clone represents. We note that the sequence GATAAA is present just upstream (position 4274) of the poly(A) tract and may have served as a polyadenylation signal sequence for this RNA. In addition the 3' untranslated region contains a number of sequences involved in RNA turnover (AUUUA) [35,36] at positions 3295, 3344, 3450, 3554, 3611, 3734, 3760, 3972 and

Figure 2 RNase protection assays show that GETS-1 is enriched in retina and brain

Total RNA was isolated from goldfish liver, scales, muscle, brain and normal (n) or optic nerve crush (c) retina. RNA was hybridized to an antisense GETS-1 radiolabelled probe and RNase-resistant hybrids were fractionated on denaturing polyacrylamide gels and visualized by autoradiography (upper panel). RNA was also run on an agarose gel and stained with ethidium bromide to confirm that similar amounts of RNA were used in the RNase protection assay (18 and 28 S rRNA bands are shown in the lower panel).

4170. The presence of these sequences may suggest that GETS-1 RNA is unstable.

Analysis of the deduced GETS-1 polypeptide sequence indicates that it is rich in leucine (10%), proline (11%) and serine (13%). Serine- and proline-rich domains are typical of transcription factors and have been implicated in transactivation [37–39]. Two potential glycosylation sites are noted at asparagine residues 129 and 367. Hydrophilicity analysis (Kyte–Doolittle) indicates a short hydrophobic stretch of 14 amino acids in the N-terminal region, followed by a long predominantly hydrophilic sequence (293 amino acids) and a hydrophobic C-terminal domain of approx. 90 amino acids. There are 18 putative MAP kinase phosphorylation sites in GETS-1, some of which are conserved with those identified to be important for SAP-1 and Elk-1 biological activity [12,40]. Because MAP kinases have been shown to activate a number of Ets proteins [6,10,12,41], it is likely that some of these conserved MAP kinase phosphorylation sites are functionally important.

Comparison of the deduced amino acid sequence of GETS-1 with that of other Ets family members shows that GETS-1 is most similar (55% identity) to SAP-1a (Figure 1) and next most similar to SAP-2/ERP/NET (48% identity). In general this similarity is highest in regions conserved between all TCFs [4,10]. These regions include: (1) the DNA-binding domain located at the N-terminus, referred to as the A box; (2) a region involved

in ternary complex formation, referred to as the B box; and (3) a C-terminal activation domain, referred to as the C box. SAP-2/ERP/NET has 4% fewer matching residues to the A box region (amino acids 12–103), 34% fewer matching residues to the B box region (amino acids 152–196), and 20% fewer matching residues to the C box region (amino acids 343–430) of GETS-1 compared with SAP-1a. Finally, we note that the second in-frame methionine of the deduced GETS-1 protein sequence aligns with the first methionine in the other TCFs. Inspection of the RNA sequence surrounding these AUGs did not identify a significantly preferred Kozak consensus sequence [42] associated with the second AUG, indicating that protein synthesis may be initiated with the first in-frame AUG.

Expression pattern of GETS-1

RNase protection assays were used to survey various goldfish tissues for the expression of GETS-1 RNA and to determine if GETS-1 RNA is induced in the retina following optic nerve crush. An antisense RNA probe was prepared that spanned nucleotides 1113–1498 of the GETS-1 cDNA. This region includes approx. 20 nucleotides of 3' untranslated sequence and extends 5' past the C box for an additional 57 bp. Total RNA was isolated from goldfish brain, retina, scales, liver and muscle. This analysis revealed ubiquitous expression of the GETS-1 RNA. However, highest expression was observed in the retina and brain (Figure 2).

We also examined the expression of GETS-1 RNA in retinas whose optic nerve had been crushed 10 days prior to harvesting the retinas for RNA extraction. This experiment showed that GETS-1 RNA is expressed at a similar level in normal (n) and optic nerve crush (c) retinas (Figure 2).

The retina is a heterogeneous tissue made up of many different cell types. In order to examine more precisely the expression pattern of GETS-1 RNA in this tissue, we used *in situ* hybridization. This analysis revealed relatively high expression levels in the retinal ganglion cell layer, the inner nuclear layer and the inner segments of the photoreceptor layer (Figure 3). Much less signal was detected in the rods of the outer nuclear layer.

GETS-1 is a transcriptional repressor

We recently identified a functional Ets binding site sequence in the rat muscle nAChR ϵ -subunit gene promoter [16]. Therefore we used the ϵ -subunit gene promoter as a reporter for GETS-1

Figure 3 *In situ* hybridization identifies GETS-1 expression throughout the adult goldfish retina

Goldfish retinal sections were hybridized with a ^{35}S -labelled antisense GETS-1 RNA probe. Sections were stained with haematoxylin and eosin. Left panel, dark-field illumination; right panel, bright-field illumination. Magnification $\times 12$. Abbreviations: ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

Figure 4 GETS-1, SAP-1a and ERP repress nAChR ϵ -promoter activity

(A) Rat primary muscle cells were co-transfected with 2.5 μ g of ϵ -5000-Luc or ϵ MUT1-Luc and CMV-CAT, in the presence or absence of various amounts of CMV-GETS-1 (as indicated). The Ets binding site mutant, ϵ MUT1 [16], was used to confirm that GETS-1 repression was mediated by an Ets consensus sequence in the ϵ -promoter. (B) Rat primary muscle cells were co-transfected with 2.5 μ g of ϵ -5000-Luc and CMV-CAT, in the presence or absence of 2.5 μ g of Elk-1, SAP-1a or ERP. At 48 h after transfection cells were harvested, and luciferase and CAT activities were determined. Experiments were repeated a minimum of three times. Bar graphs represent the means of triplicate transfections normalized to CAT activity and reported as a percentage of control (ϵ -5000 expression); error bars represent S.D.

activity. Co-transfection experiments showed that GETS-1 functioned as a transcriptional repressor in rat primary muscle cultures (Figure 4A). Increasing amounts of GETS-1 caused increased repression of reporter gene expression. This repression required an intact Ets binding site core sequence (GGA) in the ϵ -promoter (ϵ MUT1 in Figure 4A) that we recently identified as a neuregulin response element [16].

We compared GETS-1-dependent regulation of the ϵ -promoter with that by other TCFs. Co-transfection of muscle cells with these constructs showed that, like GETS-1, SAP-1a and ERP also decreased expression from the ϵ -promoter, while Elk-1 had little effect on this promoter (Figure 4B).

GETS-1 repressor activity is abrogated by activated Ras and requires Ser-405

Ets proteins are regulated by a Ras/MAP kinase signalling cascade. GETS-1 contains 18 putative MAP kinase phosphorylation sites, some of which may regulate its activity. Therefore we examined the effect of the Ras/MAP kinase signalling cascade on GETS-1 activity by overexpressing activated RasV12 along with the CMV-GETS-1 and ϵ -5000-Luc expression vectors in rat

Figure 5 GETS-1-dependent repression is abrogated by overexpression of constitutively active RasV12 and requires Ser-405

Rat primary muscle cells were co-transfected with ϵ -5000-Luc as reporter and CMV-CAT for normalization, along with the vectors indicated. At 48 h after transfection cells were harvested for luciferase and CAT assays. Experiments were repeated at least three times. Values are the means of triplicate transfections normalized to CAT activity; error bars represent S.D.

primary muscle cultures. These experiments showed that RasV12 overexpression relieves GETS-1-dependent repression of the nAChR ϵ -subunit promoter (Figure 5). The fact that GETS-1 was unable to suppress or activate ϵ -5000 promoter activity in the presence of RasV12 suggests inactivation of GETS-1 repressor activity by Ras.

Within the C box of all TCFs are conserved Ser residues, some of which are necessary for transcriptional activation [10]. In Elk-1, phosphorylation of Ser-383 by MAP kinase is essential for transcriptional activation. The same serine residue is conserved in GETS-1, corresponding to Ser-405. In order to examine the role of this residue in GETS-1, we mutated it to an Ala residue (S405A). Transfection of muscle cells with this mutant construct along with our ϵ -5000-Luc reporter gene showed that the mutant is no longer able to repress transcription from the ϵ -promoter (Figure 5). Therefore the RasV12-dependent induction of ϵ -5000 promoter activity cannot be a result of Ras-dependent activation of GETS-1, and is probably mediated by another transcription factor. Indeed we have recently shown that Ets-2 can transactivate the ϵ -5000 promoter [16].

GETS-1 contains a C-terminal transcription activation domain that is responsive to EGF and PMA stimulation

The TCF family of transcription factors contain a C-terminal growth-factor-responsive transactivation domain that is directly regulated by phosphorylation via a MAP kinase [10]. The C-terminal domain of GETS-1 shares a significant amount of sequence identity with the mammalian TCF family (Figure 1). To test whether GETS-1 contains a MAP-kinase-responsive domain, the C-terminal 122 amino acid residues were fused to the DNA-binding domain of the yeast Gal4 protein to create Gal4-GETS-1CT. We first examined the effects of active RasV12 and the constitutively active MEK1 mutant (supMEK) on GETS-1 activity. Both RasV12 and supMEK strongly activated Gal4-GETS-1CT (Figure 6A). In contrast, dominant negative mutants of Ras (RasN17) or MEK (MEK1-DN) did not enhance the activity of Gal4-GETS-1CT (Figure 6A). The fact that RasN17 suppressed basal expression (Figure 6A, lane 5), while MEK1-DN had little effect on reporter expression (Figure 6A, lane 7),

Figure 6 GETS-1 contains a Ras/MEK-responsive transactivation domain

(A) Activation of Gal4-GETS-1CT by RasV12 and supMEK. COS cells were transfected with 0.2 μg of Gal4-Luc reporter (lanes 1–7), 0.1 μg of pGal4-GETS-1CT (lanes 3–7) or pGal4 control (lanes 1 and 2), and 0.1 μg of CMV-LacZ for normalization. Cells were also transfected with 1 μg of vector DNA (pCMV5), supMEK, RasV12, RasN17 or MEK-DN, as indicated. Experiments were repeated at least three times with similar results. (B) Dose-dependent activation of Gal4-GETS-1CT by supMEK. COS cells were co-transfected with 0.2 μg of Gal4-Luc and 0.1 μg of Gal4-GETS-1CT, along with various amounts of supMEK as indicated. CMV-LacZ was also included for normalization purposes. At 48 h after transfection cells were harvested and assayed for luciferase and β-galactosidase activity. Experiments were repeated at least three times; error bars represent S.D.

suggests a Ras-dependent function contributing to the basal activity of the ε-promoter that is independent of MEK1 activity. In addition, supMEK displayed a dose-dependent activation of Gal4-GETS-1CT (Figure 6B). Similar data were observed for RasV12.

The Ras/MAP kinase pathway is effectively activated by growth factor stimulation [4,10]. EGF was used to stimulate the activation of Gal4-GETS-1CT. EGF showed a potent activation of Gal4-GETS-1CT in COS cells that was completely blocked by the dominant negative mutants RasN17 and MEK1-DN (Figure 7A). These results suggest that EGF-induced Gal4-GETS-1CT activation is mediated by the Ras/MAP kinase pathway. To determine if phosphorylation of Ser-405 is critical for Gal4-GETS-1CT activation, Ser-405 was mutated to an alanine residue. The mutant, Gal4-GETS-1CT(S405A), was not activated by EGF or by PMA (Figure 7B). Furthermore, activation of Gal4-GETS-1CT by supMEK (Figure 7B) or RasV12 was completely eliminated in the S405A mutant. These observations indicate that Ser-405 is essential for the growth

Figure 7 Activation of the GETS-1 C-terminal domain by EGF and PMA

(A) EGF-stimulated Gal4-GETS-1CT activation depends on the Ras/MAP kinase pathway. COS cells were co-transfected with Gal4-Luc, Gal4-GETS-1CT and CMV-LacZ, along with the vectors indicated. Cells were then treated either with or without EGF prior to harvesting for assay of luciferase and β-galactosidase activity. Activation of Gal4-GETS-1 by EGF is blocked by co-transfection with dominant negative RasN17 or MEK1-DN. (B) Ser-405 in GETS-1 is essential for growth factor induction. The wild-type Gal4-GETS-1CT can be activated by PMA (lanes 1 and 2), EGF (A) and supMEK (Figure 1B). Activation of Gal4-GETS-1CT was eliminated by mutation of the GETS-1 Ser-405 to an alanine [Gal4-GETS-1CT(S405A)]. Error bars represent S.D.

factor- and Ras/MAP kinase-dependent activation of Gal4-GETS-1CT.

GETS-1 is directly phosphorylated by the MAP kinase ERK1

To determine whether GETS-1 is a direct target of ERK *in vitro*, phosphorylation of GETS-1 by ERK was tested. The C-terminal domain of GETS-1 was expressed as a GST fusion protein. The purified GST-GETS-1CT can be effectively phosphorylated by recombinant ERK1, while GST alone is not phosphorylated (Figure 8A). Interestingly, GETS-1CT is phosphorylated by recombinant ERK1 with an efficiency approaching that of Elk-1 and MBP, two of the best known substrates of MAP kinase. Although MAP kinase can phosphorylate many proteins, a physiological substrate should have a reasonably low K_m . The apparent K_m of GST-GETS-1CT was determined to be $0.294 \pm 0.097 \mu\text{M}$ (Figure 8B).

To demonstrate that Ser-405 in GETS-1 is indeed phosphorylated by ERK, we took advantage of anti-Elk and anti-

Figure 8 Phosphorylation of the GETS-1 C-terminal region by MAP kinase

(A) Phosphorylation of GST-GETS-1CT by recombinant ERK1. Purified recombinant ERK1 was activated by purified supMEK1. The activated ERK1 (2 ng) was incubated with 0.5 μ g of one of the following recombinant proteins: GST control (lane 1), GST-Elk-1 (lane 2), GST-GETS-1CT (lane 3) or MBP (lane 4). Half of the reaction was subjected to SDS/PAGE followed by autoradiography (exposed for 2 h). (B) Dose-dependent phosphorylation of GST-GETS-1CT. To determine the K_m of GST-GETS-1CT, increasing amounts (μ g) of recombinant GST-GETS-1CT protein were used in an *in vitro* phosphorylation reaction. The K_m of GST-GETS-1CT was estimated to be $0.267 \pm 0.097 \mu$ M. (C) Solid-phase kinase assay of GST-GETS-1CT. Kinase activity bound to GST-GETS-1CT was determined from cell lysates. Equal amounts of cell lysates were obtained from unstimulated cells (lane 2) and from cells stimulated with anisomycin (0.5 mM for 25 min) (lane 3), arsenite (10 μ g/ml for 25 min) (lane 4), EGF (50 nM for 5 min) (lanes 1 and 5), lysophosphatic acid (1.1 mM for 25 min) (lane 6) or fetal calf serum (10% for 5 min) (lane 7). A GST control was used for lane 1, while 1 μ g of GST-GETS-1CT was used for lanes 2–7. The solid-phase kinase assay was performed as described in the Materials and methods section. (D) Phosphorylation of Ser-405 of GETS-1 by ERK1. Recombinant GST-Elk-1CT and GST-GETS-1CT were phosphorylated by activated ERK1 *in vitro*. The phosphorylated samples were subjected to Western blot with anti-Elk antibody (upper panel) or with an antibody specific for Elk phosphorylated on Ser-383 (anti-pElk; lower panel). Ser-405 of GETS-1, which is analogous to Ser-383 of Elk-1, was phosphorylated (lane 3). GST-Elk-1CT was included as a positive control (lane 4).

phospho-Elk (pElk) antibodies, which also recognize the GETS-1 protein, although the signal is weaker (Figure 8D). The anti-Elk antibody was raised against peptide WSTLSPIAPRSPA, which is conserved in GETS-1 with two amino acid substitutions (indicated in bold type). This antibody identified a single GETS-1 band in the absence of ERK and multiple bands after incubation of GETS-1 with ERK (Figure 8D, lane 2, upper panel). The anti-pElk antibody only recognizes Elk-1 phosphorylated on Ser-383, which corresponds to Ser-405 in GETS-1. This antibody showed that the lower-mobility GETS-1 band contains a phosphate on Ser-405 (Figure 8D, lane 3, lower panel). These results clearly indicate that ERK1 phosphorylates GETS-1 on Ser-405. It should be noted that the large mobility shift of GETS-1 was due to multiple phosphorylation by ERK1.

We wanted to examine whether the endogenous kinase responsible for GETS-1CT phosphorylation is stimulated by EGF. To this end, a solid-phase kinase assay was developed for GST-GETS-1CT. Cell lysates were prepared from unstimulated or EGF-stimulated cells and were incubated with GST-GETS-1CT in the absence of ATP. The GST-GETS-1CT was purified on glutathione-agarose, which may co-purify bound kinases. The purified GST-GETS-1CT was subjected to *in vitro* phosphorylation in the presence of ATP. Purified GST-GETS-1CT had associated kinase activity which phosphorylated the GETS-1 substrate, while control GST showed no activity even in the presence of EGF (Figure 8C, lanes 1 and 2). The GETS-1-associated kinase activity was effectively stimulated by EGF and serum (Figure 8C, lanes 5 and 7), increasing the GETS-1-associated kinase activity by 16- and 4-fold respectively. Two-dimensional phosphopeptide mapping of GST-GETS-1CT phos-

phorylated by recombinant ERK1 or by EGF-stimulated cell lysate showed identical patterns (results not shown), indicating that the kinase responsible for GETS-1CT phosphorylation is ERK. Arsenite and anisomycin, which primarily activate Jun N-terminal kinase (JNK) and p38 MAP kinase, also activated the GETS-1-associated kinase, suggesting the possibility that GETS-1 can be phosphorylated by JNK and p38. In fact, the Gal4-GETS-1CT reporter can be activated by MEK3 or stress-activated/extracellular-signal-regulated protein kinase (SEK1) (D. Goldman, S. Stewart and K. Guan, unpublished work), which activate p38 and JNK respectively [43].

DISCUSSION

Cloning of GETS-1 and comparison with other Ets family members

GETS-1 was cloned from a goldfish retina cDNA library and contains a DNA-binding domain that is common to all members of the Ets family of transcription factors. GETS-1 is most similar to SAP-1a (Figure 1), a TCF family member [10]. The TCF subfamily uniquely contains the Ets DNA-binding domain at its N-terminus, while all other subfamilies contain this domain near their C-terminus or in the middle of the protein. In addition TCFs contain three other conserved domains, B, C and D [10,47]. The B box of Elk-1 and SAP-1a is involved in interacting with SRF [40,44]. GETS-1 encodes a B box that is very similar to the mouse SAP-1a sequence, which suggests that GETS-1 may interact with SRF. The C box of Elk-1 and SAP-1a has been shown to play a role in transcriptional activation and contains a number of putative MAP kinase phosphorylation sites, some of which are necessary for Ras/MAP-kinase-dependent regulation

[6]. Within the C box of GETS-1 there are six (S/T)P motifs, characteristic of sites that are phosphorylated by proline-directed kinases. These six motifs are also present in the SAP-1a C box. Two of these sites (positions 392 and 411) conform to the PX(S/T)P motif for phosphorylation by p42/p44 MAP kinases [45,46]. Finally, a short amino acid sequence, referred to as the D box, has recently been identified to be conserved among TCFs and is located just upstream of the C box [47]. In GETS-1 this region corresponds to amino acids 342–352, which is also similar to the D box in other TCFs. The D box was recently shown to be involved in targeting ERK2 to Elk-1 and is essential for efficient Elk-1 phosphorylation [47]. There is little amino acid similarity between GETS-1 and other TCFs outside the above-mentioned conserved domains. Within these non-conserved regions are 11 putative phosphorylation sites, some of which may play an important role in GETS-1 function.

Expression pattern of GETS-1 in goldfish tissues

RNase protection assays suggest that GETS-1 is relatively abundant in retina and brain compared with other tissues (Figure 2). Although one should be cautious about comparing expression between fish and mammals, GETS-1 expression is most similar to that of the TCFs Elk-1 and SAP-1a, which are also expressed at relatively high levels in the brain [6].

RNase protection and *in situ* hybridization assays showed robust expression of GETS-1 RNA in most retinal neurons, and no increase following optic nerve crush (Figures 2 and 3). However, it will be important to investigate GETS-1 activity in these retinas to determine whether GETS-1 is activated during optic nerve regeneration.

GETS-1 represses nAChR ϵ -subunit gene expression in rat primary muscle cells

TCFs can either increase [40,48] or decrease [49] transcription from promoters containing Ets binding sites. We recently identified a functional Ets binding site in the rat muscle nAChR ϵ -subunit gene promoter that regulates its activity in muscle cells [16]. When primary muscle cultures were co-transfected with the GETS-1 expression vector along with the ϵ -5000-Luc reporter plasmid, we observed a significant repression of ϵ -promoter activity (Figure 4A). These results are similar to those obtained using SAP-2/ERP/NET or SAP-1a in the transfection experiments, which also repressed ϵ -promoter activity (Figure 4B). The observation that Ets proteins can activate [16] or suppress (Figure 4) nAChR ϵ -promoter activity via the same *cis*-acting regulatory element suggests a mechanism for the restricted expression of the ϵ -subunit gene in endplate-associated nuclei. A nerve-dependent activation of the Ras/MAP kinase signalling cascade beneath the neuromuscular junction may activate Ets-2 [16], while repressor Ets proteins such as GETS-1, SAP-2/ERP/NET or SAP-1a would suppress expression in extra-junctional regions.

Attempts to map the inhibitory domain of GETS-1 were unsuccessful, perhaps suggesting that more than a single domain participates in GETS-1-dependent inhibition. This is also similar to SAP-1a, which can inhibit transcription in RK13 cells, yet no specific inhibitory domain was identified when portions of this protein were fused to the Gal4 DNA-binding domain [41]. Nonetheless we did demonstrate that Ser-405, within the C box of GETS-1, is necessary for repression. In contrast, SAP-2/ERP/NET appears to contain a discrete inhibitory domain (NID) composed of approx. 50 amino acids flanking the C-terminal end of the B box (Figure 1) [19]. This inhibitory domain confers negative regulation when fused to the Gal4 DNA-

binding domain. The corresponding region of GETS-1 is more similar to SAP-1a than SAP-2/ERP/NET (Figure 1), suggesting a different mechanism of inhibition from that of SAP-2/ERP/NET.

One possible mechanism of repression is that GETS-1 activates another negatively acting transcription factor. However, if this were the case, one would expect RasV12 to increase expression of the negatively acting transcription factor via activation of GETS-1 activity. This clearly was not the case (Figure 5), suggesting that GETS-1 may participate directly in repression of the ϵ -promoter in muscle cells. Our observation that Ser-405 is required for both activation and repression is puzzling, and may indicate that it is involved in recruiting specific proteins to the GETS-1 C box domain. The particular protein recruited may depend upon cell type, and may impart activator or repressor properties on to the GETS protein complex.

GETS-1 responds to a Ras/MAP kinase signalling cascade via its C-terminal domain

GETS-1, like all TCFs studied to date, is responsive to the Ras/MAP kinase signalling cascade (Figure 5). Activation of TCFs by the Ras/MAP kinase cascade is mediated by the C box, which contains a number of conserved (S/T)P motifs. Within the C box of Elk-1, Ser-383 has been shown to play a key role in mediating transcriptional activation [12].

We examined whether GETS-1 also contains a C box that mediates regulation by a Ras/MAP kinase signalling cascade using fusions between the Gal4 DNA-binding domain and the GETS-1 C-terminal domain. The Gal4 DNA-binding domain is not responsive to Ras/MAP kinase signalling, while the fusion protein is responsive (Figures 6A and 7A). In addition, mutation of Ser-405, which is analogous to Ser-383 of Elk-1, to an alanine residue completely abrogated regulation by the Ras/MAP kinase pathway (Figure 7B). This result suggests the GETS-1 contains a C-terminal activation domain that mediates Ras/MAP kinase signalling, at least in part, via phosphorylation of Ser-405. Consistent with this are our observations that (1) the GETS-1 C-terminal domain is phosphorylated by recombinant ERK1 at an efficiency similar to that at which it phosphorylates Elk-1 and MBP, two of the best known substrates for MAP kinase (Figure 8), and (2) anti-phospho-Elk-1 antibodies bind GETS-1 after ERK-dependent phosphorylation (Figure 8D).

EGF is a potent stimulator of the Ras/MAP kinase signalling cascade. Therefore one might suspect that EGF stimulation *in vivo* would activate a MAP kinase capable of phosphorylating GETS-1. Indeed GST-GETS-1 fusions were able to bind a kinase that, upon addition of ATP, caused increased phosphorylation of GETS-1 (Figure 8C). This kinase activity was stimulated by EGF and serum (Figure 8C). We confirmed that the phosphorylation by this cellular kinase was the same as that by recombinant ERK1 using two-dimensional phosphopeptide mapping. Interestingly, arsenite and anisomycin, which activate JNK and p38 MAP kinase, also activated the GETS-1-associated kinase in these assays (Figure 8C). Consistent with this result is our observation that the Gal4-GETS-1CT fusion can be activated by MEK3 or SEK1, which activate p38 and JNK respectively [43].

Conclusions

Based on the above results, we propose that GETS-1 is a member of the TCF subfamily of Ets proteins. It is most similar to SAP-1a (55% identity) in sequence and function and, like SAP-1a [6], GETS-1 is expressed in neural tissue (Figure 2). However, unlike

SAP-1a [49], GETS-1, in addition to being a substrate for ERK, also appears to be a substrate for JNK and p38 MAP kinases, which are preferentially activated by arsenite and anisomycin (Figure 8C). It is striking that GETS-1 repressor and activator activities both require Ser-405 (Figures 5 and 7B). Although we do not know the mechanism underlying GETS-1-mediated repression or activation, it is possible that Ser-405 is an essential residue for protein-protein interaction and that phosphorylation regulates this interaction. Repression or activation would then result from the specific proteins that are available to interact with the C box domain. The observation that Ets family members can either activate [16] or repress (Figure 4) nAChR ϵ -promoter activity also suggests a mechanism for selective expression of this gene beneath the neuromuscular junction. In this scenario Ets family members such as GETS-1, SAP-1a or ERP would suppress ϵ -promoter activity throughout the muscle fibre, while a locally activated Ras/MAP kinase signalling cascade beneath the neuromuscular junction would activate ϵ -promoter activity in endplate-associated nuclei [16]. Future work will focus on characterizing the physiological role of GETS-1 during fish development.

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