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OASIS is a transcriptional activator of CREB/ATF family with a transmembrane domain

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Abstract

Murine OASIS is a putative CREB/ATF family transcription factor that is induced in gliosis, but its molecular role has not been determined. We have isolated the human *OASIS* gene and investigated the potential of OASIS protein as a transcriptional activator. We found that OASIS can activate transcription through box-B elements but not through the somatostatin CRE. OASIS contains a putative C-terminal hydrophobic transmembrane domain, a typical structural feature for the transcription factors activated by regulated intramembrane proteolysis. Truncation of the OASIS transmembrane domain resulted in a significant increase in transcriptional activity and altered its subcellular localization from the endoplasmic reticulum to the nucleus. Western blot analysis of transfected cells identified OASIS polypeptides of 82 and 66 kDa. These results suggest that the transmembrane domain plays an important role in the regulation of transcriptional activation by OASIS.

Keywords: Gliosis; Transmembrane domain; GFAP; CREB/ATF family; SREBP; CREB-H; Proteolysis; Endoplasmic reticulum; Membrane protein

Gliosis occurs in the central nervous system (CNS) in response to brain tissue damage, including damage caused by surgical manipulation, acute traumatic brain injury, and neurodegenerative diseases such as Alzheimer's disease [1,2]. It is characterized by the modification of reactive astrocytes, which results from increased glial fibrillary acidic protein (GFAP) expression. Murine OASIS, a transcription factor of the CREB/ATF family, is induced in cell-culture models of gliosis and in gliotic tissues in vivo, and levels of *OASIS* mRNA expression in the injured cortex correlate closely with those of GFAP [3]. Thus, it is thought that OASIS functions as a transcriptional regulator in gliotic pathways; however, the exact molecular roles of OASIS have not been determined.

Several transcription factors are known to localize to membrane structures within cells, where their activity is regulated by a proteolysis mechanism called regulated intramembrane proteolysis (Rip). Transcription factors regulated by Rip commonly contain hydrophobic transmembrane domains, which anchor the proteins to intracellular membranes. Through Rip, membrane-bound transcription factors are cleaved, allowing proteolytic fragments to enter the nucleus and regulate gene transcription [4]. For example, the CREB/ATF transcription factor ATF6 induces the expression of endoplasmic reticulum (ER) chaperones in response to excess unfolded proteins in the ER [5]. ATF6 contains a hydrophobic transmembrane domain that is anchored to the ER membrane. The cytosolic N-terminal domain of ATF6 is a transcription factor of the basic-leucine zipper (b-Zip) type. When released into the cytosol by cleavage, the N-terminal domain of ATF6 translocates to the nucleus and activates the expression of ER chaperone genes.

In mammalian cells, only four transcription factors, SREBP1, SREBP2, ATF6, and CREB-RP, are known to undergo Rip [5–8]. It was recently suggested that these transcription factors are regulated through a

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common proteolytic mechanism [9]. Here we report that OASIS is actually a transcription activator of CREB/ATF family which has the putative transmembrane domain in the structure, a common feature of transcription factors activated by Rip.

Materials and methods

Plasmids. pME18S, an SR α-driven expression plasmid (GenBank accession number AB009864), the luciferase reporter plasmid pbox-B-Luc, and pEF/Gal4-VP16 have been described previously [10].

pME-OASIS was constructed by inserting an *Eco*RI–*Not*I fragment from IMAGE clone #2621090 and an *Not*I–*Sal*I fragment from IMAGE clone #2556940 between the *Eco*RI and *Xho*I sites of pME18S. Expression plasmids were generated using pME18SFLAG, which was constructed by inserting a DNA sequence encoding the FLAG epitope DYKDDDDK [11] into the *Eco*RI–*Not*I sites of pME18S. Expression plasmids encoding N-terminal FLAG-tagged full-length OASIS (pFG-OASIS; amino acids 1–519) and the OASIS transmembrane domain deletion mutant (pFG-OASISdelTM; amino acids 1–372) were constructed by in-frame insertion of PCR products into the *Bam*HI–*Eco*RI sites of pME18SFLAG.

A bacterial expression plasmid encoding a fusion protein between GST and the OASIS transmembrane domain deletion mutant (pGST-OASIS; amino acids 1–372) was constructed by in-frame insertion of the OASIS coding sequence into the *Bam*HI and *Eco*RI sites of pGEX-3X (Pharmacia Biotech).

Plasmids encoding fusions between the GAL4 DNA-binding domain and OASIS deletion mutants were constructed by in-frame insertion of DNA fragments encoding the full-length and truncated OASIS proteins (detailed in Fig. 2A). DNA fragments were inserted between the *Hin*dIII and *Bam*HI sites of pEF/Gal4-VP16.

The luciferase reporter plasmids psmCRE-Luc, ppcCRE-Luc, and pATF6-lLuc were constructed by inserting double-stranded oligonucleotides containing the core sequence elements into an *Xho*I site of pRBGP-Luc. Derived from pGL2-basic (Promega), pRBGP-Luc contains the rabbit β-globin TATA box. Oligonucleotide sequences are as follows. Box-B element of the *Drosophila mulleri* ADH-1 promoter [12]: box-B-sense, 5′-TCGAGCTCGGATGTACACGTAATCGTATTACTC-3′; box-b-antisense, 5′-CGAGAGTAATACGATTACGTGTACATCCGAGCT-3′. ATF6 element [13]: ATF6-sense, 5′-TCGAGCTCGGAGTGCTGACGTGGCGATTACTC-3′; ATF6-antisense, 5′-CGAGAGTAATCGCCACGTCAGCACTCCGAGCT-3′. CRE from the somatostatin promoter [14]: smCRE-sense, 5′-TCGAGCTCGGATCTGACGTCAGAGATTACTC-3′; smCRE-antisense, 5′-CGAGAGTAATCTCTGACGTCAGCCATCCGAGCT-3′. CRE from rat phosphoenolpyruvate carboxykinase promoter [15]: pcCRE-sense, 5′-TCGAGCTCGGACCCTTACGTCAGAGGATTACTC-3′; pcCRE-antisense, 5′-CGAGAGTAATCCTCTGACGTAAGGGTCCGAGCT-3.

Plasmids were purified using a Qiagen Plasmid Kit (Qiagen) according to manufacturer's instructions. All gene sequences were verified using an ABI 377 autosequencer (PE Biosystems).

Northern blot analysis. Northern blot analyses were performed using Multiple Tissue Northern Blots I and II (Clontech). A 0.8 kbp probe was produced by PCR with primers P5′ (5′-ttgaattcCCTGGAGTGAGGGAAGCCCAGTGGAA-3′) and PM2 5′-aactcgagtcaCCCTGATGTCCCCTGTAATTTGTGAG-3′). PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen) and labeled with [α-^{32}P]dCTP using a Random Primer DNA Labeling Kit ver.2 (Takara). Blots were prehybridized for 1 h and then hybridized for 18 h at 42 °C in a hybridization buffer of 50% formamide, 5× SSC, 5× Denhardt's solution, 0.5% SDS, and 500 μg/ml denatured salmon-sperm DNA. Hybridized blots were washed with 2× SSC/0.1% SDS at room temperature for 30 min and then with 0.1× SSC/ 0.1% SDS at 55 °C for 60 min. The washed membranes were analyzed with a BAS-2500 bio-image analyzer (Fuji film).

RT-PCR analysis. RT-PCR experiments were performed using KOD dash DNA polymerase (Toyobo). The 790-bp fragment upstream of the b-Zip region in OASIS (nucleic acid 96–870) was amplified by RT-PCR using a cDNA prepared from the mRNA of human lung tissue as template.

Gel mobility shift assay. Gel mobility shift assays were carried out using standard procedures, as described previously [10]. Briefly, the GST–OASIS fusion protein lacking the transmembrane domain was purified over glutathione–sepharose 4B from *Escherichia coli* XL-1 Blue cells induced for 2 h with 1 mM IPTG. Purified GST–OASIS and labeled oligonucleotides were incubated for 30 min at room temperature. For each binding reaction, 0.1 μg GST–OASIS was used. Electrophoresis was performed using a mini-gel system, a 4.5% acrylamide gel, and 1× TBE buffer. The autoradiogram was analyzed using a BAS-2500 bio-image analyzer (Fujifilm). Nucleotide sequences of probes for the C/EBP and the NF-κB elements have been described previously [10]. The box-B, ATF6 element, and smCRE probes contained the same sequences as the corresponding luciferase reporter plasmids.

Transfection and luciferase assays. COS7 and Hela cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 2 mM glutamine. Cells were maintained at 37 °C in a humidified 5% CO_2–95% air atmosphere. Twenty-four hours after seeding, cells were washed with OPTI-MEM I Reduced Serum Medium (Life Technologies) and transfected with Lipofectamine 2000 (Life Technologies) in OPTI-MEM I Reduced Serum Medium. Each transfection was performed using 0.4 μg luciferase-reporter plasmid, 1 μg OASIS-expressing plasmid, 10 ng pRL-CMV (Promega), and 10 μl Lipofectamine 2000. Twenty-four hours after transfection, cells were lysed and assayed for *Firefly* and *Renilla* luciferase activities using a Dual-Luciferase Reporter Assay System (Promega) according to manufacturer's instructions. Results for each assay were normalized against *Renilla* luciferase activity.

Western blot analysis. Cells were lysed in 2× SDS-sample buffer (100 M Tris–HCl (pH 6.8), 4% SDS, 12% β-mercaptoethanol, 20% glycerol, and 0.001% bromophenol blue), and boiled for 5 min. Samples were separated on a 10% SDS–polyacrylamide gel and transferred to PVDF membrane. The primary antibody reaction was performed using a 1:400 dilution of anti-FLAG M2 monoclonal antibody (Sigma). The secondary antibody reaction was performed using a 1:1000 dilution of alkaline phosphatase-conjugated anti-mouse IgG (Promega). Signal was visualized using western blue stabilized substrate (Promega).

Immunostaining of cells. Transfected cells were seeded on chamber slides (Lab-Tek). After 24 h, the cells were washed with phosphate-buffered saline (PBS), fixed with 3.7% formaldehyde in PBS for 5 min, washed with PBS, and permeabilized with 0.1% Triton X-100 in PBS for 5 min. After a PBS wash, cells were incubated with anti-FLAG M2 antibody (1:200) for 1 h. Following the primary antibody incubation, cells were washed three times with PBS and incubated with a rabbit anti-mouse FITC-conjugated secondary antibody (1:2000; Wako), 0.5 μg/ml 4′,6-diamidino-2-phenylindole (DAPI; Polysciences), and 0.2 μg/ml Rhodamine B (Molecular Probes) for 30 min. After three washes, cover slips were placed on slides with one drop of 90% glycerin in PBS. Staining was visualized using fluorescence microscopy.

Results

Through an EST homology search using the murine *OASIS* sequence (GenBank accession number AB017614), we obtained overlapping EST clones containing the human counterpart of OASIS. Nucleotide

sequence analysis identified an open reading frame (ORF) encoding a full-length protein of 519 amino acids (Fig. 1A; GenBank accession number AB063321). The predicted amino acid sequence is 84.8% identical to that of murine OASIS. We also obtained a splice variant encoding 431 amino acids; this variant lacked a 264-bp region upstream of the encoded b-Zip domain (Fig. 1A). To confirm the existence of this splice variant in vivo, we amplified the *OASIS* sequence from a human lung cDNA library. We obtained distinct PCR products of 0.8 and 0.5 kb, identical to the sizes predicted by the full-length ORF (790 bp) and the splice variant (526 bp) (Fig. 1B). We designated this splice variant *OASISv1* (*OASIS* splice variant 1).

The amino acid sequence spanning residues 292–353 of the predicted human OASIS protein contains a consensus b-Zip domain highly similar to BBF2, a Drosophila transcription factor belonging to the CREB/ATF family [16]. BBF2 binds to and activates transcription from box-B enhancer elements, which control expression of Drosophila fat body and mammalian liver-specific genes [12]. The box-B element was first identified as a CRE-like element in the Drosophila alcohol dehydrogenase promoter and box-B-like elements are also present in the promoter of the mammalian alcohol dehydrogenase gene [16].

Downstream of the b-Zip domain, OASIS also contains a hydrophobic region, which was predicted to be an α-helical transmembrane domain by the prediction program PSORT II (http://psort.ims.u-tokyo.ac.jp/form2.html [17]) (Fig. 1A). This structural feature is also seen in SREBP and ATF6, suggesting that, like SREBP and ATF6, OASIS undergoes Rip.

To investigate the expression pattern of *OASIS* in human tissues, we performed Northern blot analysis using 16 human tissues. The probe was a 0.8-kb fragment in the 5′ region of the *OASIS* cDNA; this sequence excluded the b-Zip domain to avoid cross-hybridization with other members of the CREB/ATF family. A 2.6-kb band representing *OASIS* mRNA was detectable in several tissues. *OASIS* expression was ubiquitous, with particularly high levels seen in pancreas and prostate

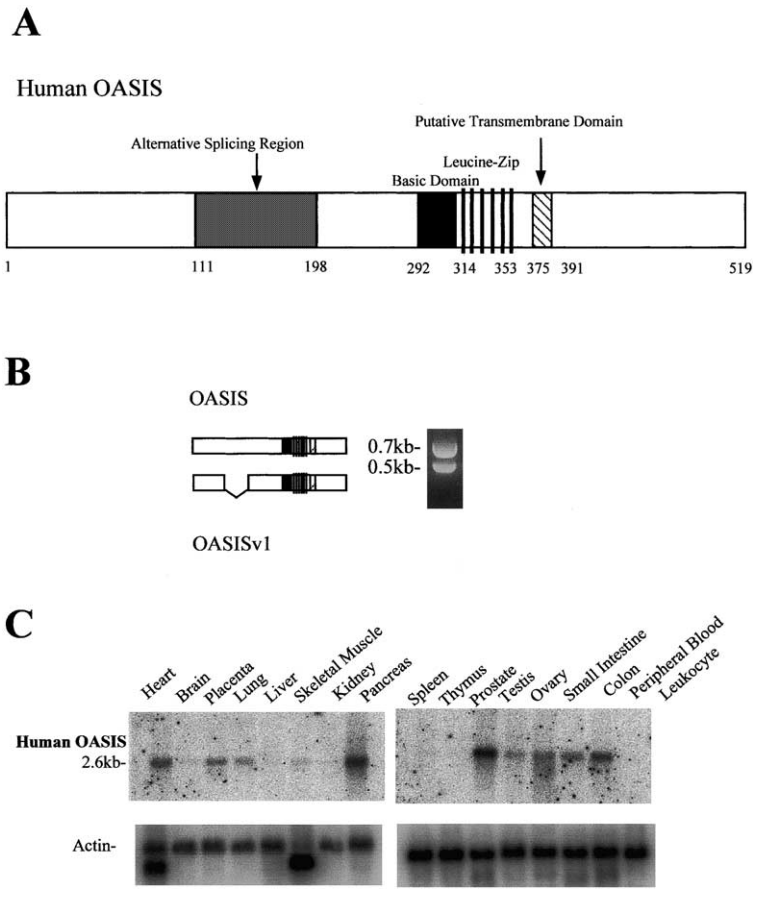

Fig. 1. (A) Schematic diagram of human OASIS. The amino acid positions of the b-Zip domain, transmembrane domain, and alternative-splicing region are indicated. (B) Identification of *OASIS* splice variants. The 790- and 526-bp fragments upstream of the b-Zip region in *OASIS* were amplified from human lung tissue cDNA. (C) The expression profile of *OASIS* mRNA in multiple human tissues. Each lane contained approximately 2 μg poly(A)$^+$ RNA. The *OASIS* probe used was a 0.8-kb fragment encompassing part of the 5′-portion of the OASIS cDNA outside the b-Zip domain. A 2.6-kb band corresponding to *OASIS* mRNA is marked. Hybridization of the blot with a β-actin probe is shown in the lower panel.

(Fig. 1C). In the brain, OASIS mRNA was expressed at relatively lower levels.

Some members of the CREB/ATF family are known to be transcriptional activators, while others repress expression of their target genes. To determine whether OASIS is capable of activating transcription, we constructed plasmids expressing GAL4–OASIS fusion proteins and assayed their transcriptional activity in transfected cells. Transcriptional activity was measured using a luciferase reporter plasmid containing the GAL4 DNA-binding element (Fig. 2). The full-length OASIS fusion construct (FL), as well as fusion constructs lacking the transmembrane domain (delTM1 and delTM2), activated reporter gene expression at low but certain levels (>2.4-fold activation). Constructs lacking the alternative splicing region (FLv1 and delTMv1) were also able to activate reporter gene expression at relatively low levels (>5.4-fold activation). Constructs lacking the b-Zip regions (del258C, del119C, and del60C) significantly activated reporter gene expression. It is possible that the tertiary structure of the b-Zip domain in the fusion protein might reduce its transcriptional activity. Deletion of the N-terminal regions (del56N and del111N) abolished transcriptional activity. The highest levels of activation were generated by the construct lacking the C terminus (del60C; >60-fold activation), showing that the minimum region required for transcriptional activation is located within amino acids 1–60.

Members of the CREB/ATF family bind specifically to CRE-like sequences. To determine whether OASIS binds CRE-like sequences directly, we performed gel-shift assays using a GST–OASIS fusion protein. The GST–OASIS fusion protein formed a complex with the somatostatin CRE (smCRE) sequence [14], the box-B element [15,18], and an ATF6 consensus-binding site (ATF6 element) [12], but it did not form detectable complexes with C/EBP or NF-κB elements (Fig. 3). Shifted bands were competed away by 5- and 50-fold excesses of non-labeled smCRE, box-B, and ATF6 elements.

OASIS shows high similarity to BBF2, which activates transcription through the box-B element. To assess whether OASIS can activate transcription through the box-B element, we performed transcription assays in COS-7 cells transfected with a FLAG-tagged OASIS expression plasmid and a luciferase reporter construct containing the box-B element. Full-length OASIS

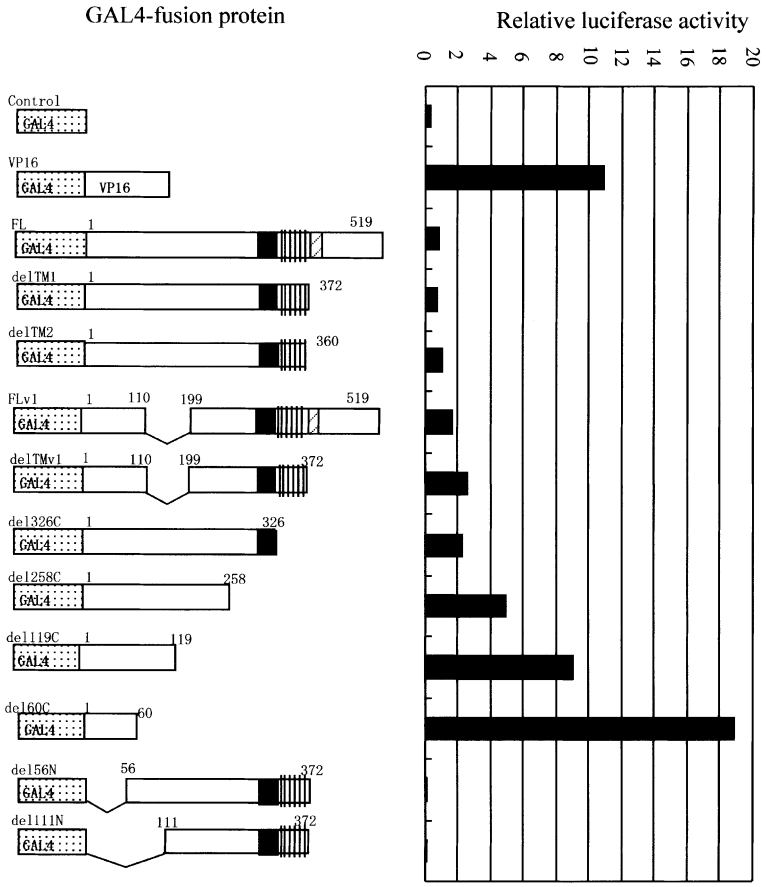

Fig. 2. Mapping of the transcriptional activation domain in OASIS. Fusion constructs between the GAL4 DNA-binding domain and various OASIS deletion mutants were co-transfected with the reporter plasmid pGAL-Luc into COS7 cells. Luciferase activity was quantified 24 h after transfection. Relative luciferase activities are shown. A representative result of three experiments is shown.

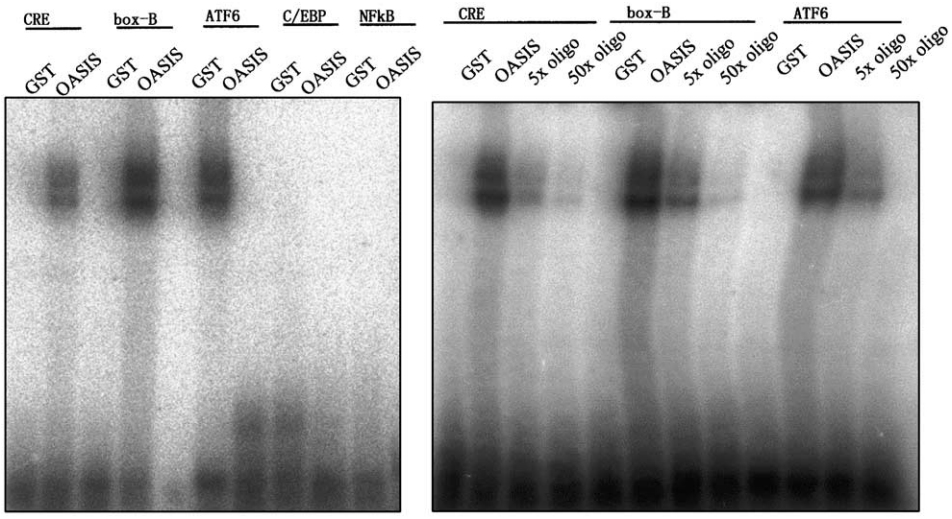

Fig. 3. Binding of OASIS to various DNA sequence elements. Oligonucleotides representing CRE, box-B, ATF6, C/EBP, and NF-κB elements were incubated with a GST fusion protein containing the OASIS deletion mutant lacking the transmembrane domain. Competitive assays for CRE, box-B, and ATF6 elements were performed with 5- and 50-fold excesses of unlabeled oligonucleotides.

activated luciferase expression more than 10-fold compared with controls (Fig. 4A).

If OASIS anchors to the membrane through the transmembrane domain, then deletion of this transmembrane domain should result in an increased transcriptional activity. To investigate this possibility, we co-transfected cells with a deletion mutant lacking the transmembrane domain and a reporter plasmid. This deletion mutant showed an increase in transcriptional activity from a box-B element relative to that of full-length OASIS (>1.7-fold) (Fig. 4A). We also investigated the transcriptional activity of the splice variant, OASISv1 (Fig. 4A). OASISv1 showed a decreased transcriptional activity compared with full-length OASIS; however, an OASISv1 deletion mutant lacking the transmembrane domain showed a relative increase in transcriptional activity compared with full-length OASISv1.

We performed Western blot analysis to examine the molecular mass of proteins produced from the transfected *OASIS* constructs. Cell extracts from transfected COS7 cells were separated by SDS–PAGE and immunostained using anti-FLAG antibody. We detected two major products of 82 and 66 kDa in cells transfected with the full-length OASIS construct (Fig. 4B). In addition to these major bands, two minor bands of 83 and 65 kDa were present. Cells transfected with the OASIS deletion construct lacking the transmembrane domain produced a single 64-kDa product. In cells transfected with the full-length OASISv1 construct, we observed two major products of 68 and 50 kDa (Fig. 4B), whereas in cells transfected with the OASISv1 deletion construct lacking the transmembrane domain, a single 48-kDa band was observed. Constitutive cleavage of the precursor protein was reported for ATF6 in CHO cells without induction by tunicamycin [9].

Gel-shift assays showed that OASIS was able to bind the smCRE, the box-B element, and the ATF6 element (Fig. 3). To characterize potential sequence preference by OASIS in cells, we compared the level of OASIS transcriptional activity through various CRE-related elements. We prepared reporter plasmids containing the box-B core sequence TTACGTGT, the ATF6 core sequence TGACGTGG, the smCRE core sequence TGACGTCA, and the rat phosphoenolpyruvate carboxykinase (pcCRE) core sequence TTACGTCA. We assayed luciferase reporter activity in COS-7 cells co-transfected with OASIS deletion constructs lacking the putative transmembrane domains (Fig. 5). OASIS activated transcription extensively through the box-B element (>40-fold) and the ATF6 element (>10-fold), but activation was poor through the smCRE and pcCRE elements (<3-fold). In contrast, CREB, induced by transfection of PKA, activated reporter expression through smCRE and pcCRE elements as well as through the box-B and ATF6.

To investigate the subcellular localization of OASIS proteins in cells, we performed immunofluorescence microscopy using anti-FLAG antibody. Hela cells transfected with the full-length OASIS construct were stained and visualized using FITC-conjugated secondary antibody. Nuclear and ER structures were counterstained with DAPI and Rhodamine B, respectively. The OASIS signal was detected mainly in the reticular structures surrounding the nucleus, colocalizing approximately with the Rhodamine B signal (Fig. 6). The OASIS deletion mutant lacking the transmembrane domain localized primarily to the nucleus and cytosol (Fig. 6). Localization patterns of OASIS in COS7 cells closely resembled those in Hela cells (data not shown).

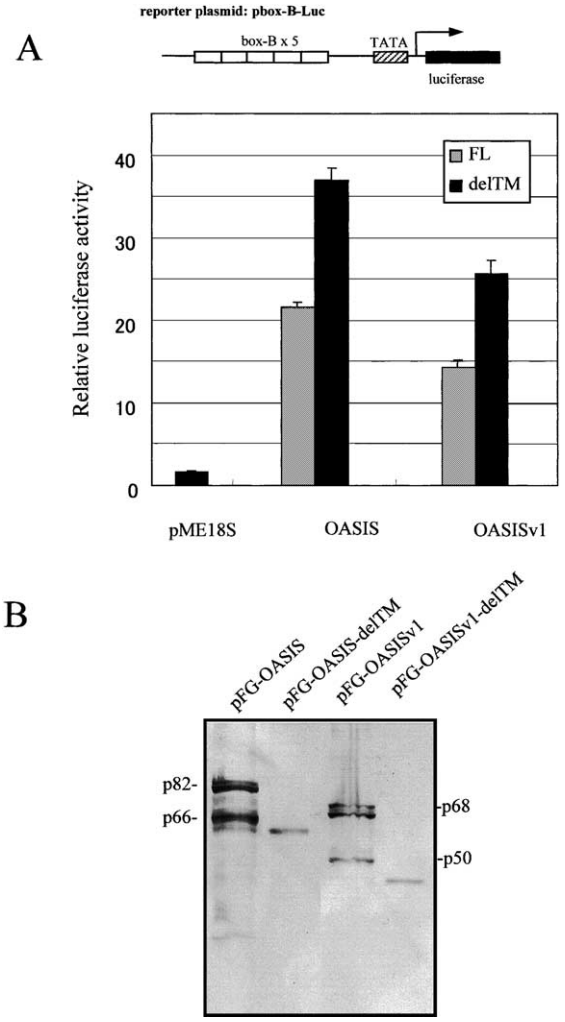

Fig. 4. (A) OASIS activates transcription of luciferase through the box-B element. Full-length OASIS (pFG-OASIS), the OASIS deletion mutant lacking the transmembrane domain (pFG-OASISdelTM), the splice variant OASISv1 (pFG-OASISv1), and the OASISv1 deletion mutant (pFG-OASISv1delTM) were co-transfected with luciferase reporter plasmids containing the box-B elements into COS7 cells. Results were presented as means + SE of three independent experiments. (B) Western blot analysis of OASIS protein products. N-terminal FLAG-tagged full-length OASIS (pFG-OASIS), the OASIS deletion mutant lacking the transmembrane domain (pFG-OASISdelTM), the splice variant OASISv1 (pFG-OASISv1), and the OASISv1 deletion mutant (pFG-OASISv1delTM) were transfected into COS7 cells. Cell extracts were separated using SDS–PAGE and immunostaining was performed using the anti-FLAG antibody.

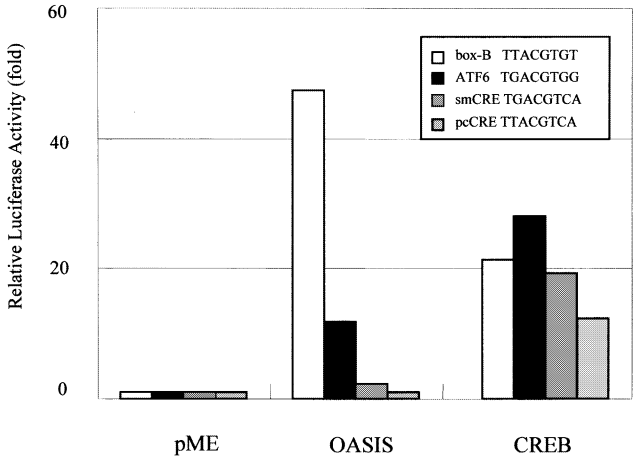

Fig. 5. Transcriptional activation of OASIS through various CRE sequence elements. Luciferase reporter constructs containing box-B, ATF6, smCRE, or pcCRE elements (pboxB-Luc, pATF6-Luc, psmCRE-Luc, or ppcCRE-Luc) were transfected into COS7 cells with OASIS (pME-OASISdelTM) PKA (pPKA) or a control vector (pME18S). Luciferase activity was quantified 24 h after transfection. Relative luciferase activity is shown. A representative result of three experiments is shown.

Discussion

OASIS expression patterns in gliosis correlate with those of GFAP [3], which is generally recognized as a marker of gliosis. Expression levels of various extracellular matrix components, cytokines, and growth factors also correlate with the increase in GFAP [19–22]. We found that OASIS functioned as a transcriptional activator through binding to the box-B element (Fig. 4A) and showed higher activation levels through the box-B element relative to the other CRE-like elements (Fig. 5). It is possible that OASIS regulates the expression of gliosis-induced genes through box-B-like elements in their promoters.

We identified a putative transmembrane domain in the C-terminal region of OASIS. Full-length OASIS activated reporter gene expression through the box-B element and deletion of the transmembrane domain increased the activation of reporter expression (Fig. 4A). Furthermore, we have shown that the full-length OASIS localizes to the ER of transfected cells, whereas the OASIS deletion mutant lacking the transmembrane domain localized primarily to the nucleus (Fig. 6). These results give one possible model that deletion of the transmembrane domain is directly responsible for the release of active OASIS from the ER into the cytosol and increased transcriptional activity. Thus, the putative transmembrane domain likely plays an important role in the activation of OASIS.

We identified two OASIS protein products of 82 and 66 kDa in cells transfected with full-length OASIS (Fig. 4B). Similarly, expression of the OASISv1 splice variant resulted in the production of two slightly smaller protein products (68 and 50 kDa). These results suggest that the smaller proteins are products of post-translational processing rather than alternative splicing. The FLAG tag is located at the N-terminus of these OASIS constructs. Thus, if the observed 66-kDa product were generated from the 82-kDa precursor by proteolysis, then the 66-kDa product would lack the C-terminal transmem-

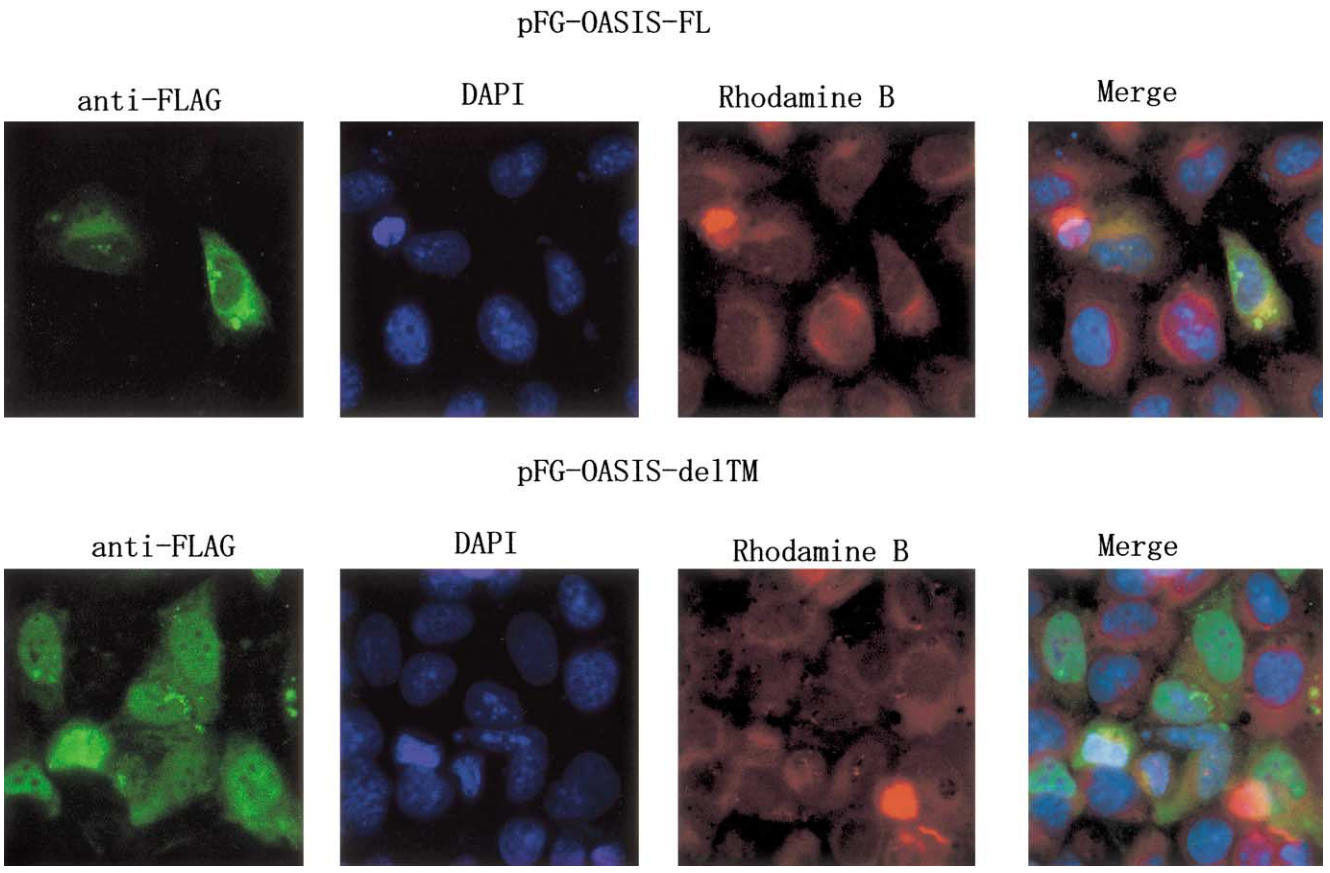

Fig. 6. Subcellular localization of OASIS and the deletion construct lacking the transmembrane domain. FLAG-tagged full-length OASIS (pFG-OASIS) and the OASIS deletion mutant lacking the transmembrane domain (pFG-OASISdelTM) were transfected into Hela cells. OASIS was stained with the anti-FLAG antibody (green). Cells were counterstained with DAPI (blue) and Rhodamine B (red) for visualization of the nucleus and ER, respectively.

brane domain. Since the OASIS product lacking the transmembrane domain would gain transcriptional activity, this proteolysis would convert full-length OASIS to the active form. Given the similarities between OASIS and other membrane-associated transcription factors, it is likely that activation of OASIS is similarly achieved through Rip.

A recent report shows expression of OASIS in the preosteoblast of the developing mouse embryo [23]. We have seen high levels of OASIS expression in the pancreas and the prostate (Fig. 1C). Therefore, it is possible that OASIS is involved in physiological or pathological mechanisms other than gliosis, regulating transcription of target genes through box-B-like elements.

Acknowledgments

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