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RTVP-1 expression is regulated by SRF downstream of protein kinase C and contributes to the effect of SRF on glioma cell migration

Amotz Ziv-Av [a], Dvir Taller [a], Moshe Attia [a], Cunli Xiang [b], Hae Kyung Lee [b], Simona Cazacu [b], Susan Finniss [b], Gila Kazimirsky [a], Ronit Sarid [a], Chaya Brodie [a,b,*]

[a] The Mina and Everard Goodman Faculty of Life-Sciences, Bar-Ilan University, Ramat-Gan, Israel
[b] William and Karen Davidson Laboratory of Cell Signaling and Tumorigenesis, Hermelin Brain Tumor Center, Department of Neurosurgery, Henry Ford Hospital, Detroit, MI, United States

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ABSTRACT

Gliomas are characterized by increased infiltration into the surrounding normal brain tissue. We recently reported that RTVP-1 is highly expressed in gliomas and plays a role in the migration of these cells, however the regulation of RTVP-1 expression in these cells is not yet described. In this study we examined the role of PKC in the regulation of RTVP-1 expression and found that PMA and overexpression of PKCα and PKCε increased the expression of RTVP-1, whereas PKCδ exerted an opposite effect. Using the MatInspector software, we identified a SRF binding site on the RTVP-1 promoter. Chromatin immunoprecipitation (ChIP) assay revealed that SRF binds to the RTVP-1 promoter in U87 cells, and that this binding was significantly increased in response to serum addition. Moreover, silencing of SRF blocked the induction of RTVP-1 expression in response to serum. We found that overexpression of PKCα and PKCε increased the activity of the RTVP-1 promoter and the binding of SRF to the promoter. In contrast, overexpression of PKCδ blocked the increase in RTVP-1 expression in response to serum and the inhibitory effect of PKCδ was abrogated in cells expressing a SRFT160A mutant. SRF regulated the migration of glioma cells and its effect was partially mediated by RTVP-1. We conclude that RTVP-1 is a PKC-regulated gene and that this regulation is at least partly mediated by SRF. Moreover, RTVP-1 plays a role in the effect of SRF on glioma cell migration.

1. Introduction

Glioblastomas (GBMs) are the most common and aggressive type of astrocytic tumors. GBMs are characterized by uncontrolled proliferation, invasion into the surrounding normal tissue, robust angiogenesis and resistance to conventional radio- and chemotherapy. Despite intensive therapeutic strategies, the median survival of GBM patients has remained 12–14 months during the past decades [1].

Related to testis-specific, vespid and pathogenesis protein 1 (RTVP-1) was cloned from human GBM cell lines by two groups and was termed glioma pathogenesis-related protein—GLIPR1 [2] or RTVP-1 [3]. RTVP-1 contains a putative signal peptide, a transmembrane domain and a SCP domain, with a yet unknown function. This domain is also found in other RTVP-1 homologs including TPX-1 [4], the venom allergen antigen 5 [5] and group 1 of the plant pathogenesis-related proteins (PR-1). We have recently reported that RTVP-1 acts as a tumor promoter in gliomas. Thus, the expression of RTVP-1 correlates with the degree of malignancy of astrocytic tumors and overexpression of RTVP-1 increases cell proliferation, invasion, migration and anchorage-independent growth. Moreover, silencing of RTVP-1 induces apoptosis in glioma cell lines and primary glioma cultures [6]. Interestingly, RTVP-1 acts as a tumor suppressor in prostate cancer cells and adenovirus-mediated delivery of RTVP-1 has therapeutic effects in a mouse prostate cancer model [7–9].

Protein kinase C (PKC) is a family of serine threonine kinases that have central roles in a variety of cellular processes [10,11]. This family consists of at least ten isoforms which are classified as classical PKCs (PKCα, PKCβ1, PKCβ2 and PKCγ) which depend on calcium, diacylglycerol (DAG), and phosphatidylserine (PS) for activation, novel PKCs (PKCδ, ε, θ and η), which depend on DAG and PS but are independent of calcium, and atypical PKCs (PKCξ and PKCλ/ι) that require PS for activation. Glioma cells have been shown to exhibit high activity of PKC and expression of specific PKC isoforms [12,13]. We recently reported that PKCα and PKCε are overexpressed in gliomas and contribute to their increased proliferation and resistance to apoptosis, whereas the expression of PKCδ is decreased in these tumors and overexpression of PKCδ in these cells renders them more sensitive to various apoptotic stimuli [12,14,15].

Serum response factor (SRF) is a member of the MADS box family of transcription factors. The MADS box consisted of a basic DNA binding domain, an interface for protein–protein interaction and a dimerization domain [16]. SRF regulates the expression of a group of muscle differentiation genes [17] and immediate early genes such as

* Corresponding author at: Hermelin Brain Tumor Center, Department of Neurosurgery, Henry Ford Hospital, 2799 W Grand Blvd, Detroit, MI 48202, United States. Tel.: +1 313 916 8619; fax: +1 313 916 9855.
E-mail address: chaya@mail.biu.ac.il (C. Brodie).

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c-fos and Egr1 that are rapidly and transiently expressed in response to mitogenic signals [18]. It was recently shown that SRF is involved in the epithelial–mesenchymal transition (EMT) of hepatocellular carcinoma (HCC) and it regulates migration and invasion of these cells with subsequent acquisition of mesenchymal phenotype [19].

The expression of SRF is regulated by specific isoforms of PKC. Constitutively active PKCα and PKCε induce transactivation of SRF and RhoA-mediated SRF activation is inhibited by dominant negative mutants of PKCα and PKCε in NIH 3T3 cells [20]. In addition, it was recently demonstrated that PKCα phosphorylates serine 162 in the MADS box of SRF, and that phosphorylation on this residue interferes with the activation of myogenic differentiation genes while allowing the activation of SRF-dependent proliferation genes [21]. On the other hand, senescent fibroblasts expressing a higher level of PKCδ and the phosphorylation of T160 and SRF by PKCδ lead to loss of the SRF binding to the SRE [22].

The expression of RTVP-1 is correlated with the degree of malignancy of astrocytic tumors, but the regulation of RTVP-1 expression has not been yet described. In this study we show that RTVP-1 is differentially regulated by specific PKC isoforms and that this regulation is at least partly mediated by SRF. Moreover, RTVP-1 mediates, in part, the effect of SRF on glioma cell migration.

2. Materials and methods

2.1. Materials

Polyclonal anti-PKCδ, PKCα, PKCε, GST, actin and anti-SRF antibodies were purchased from Santa Cruz (Santa-Cruz, CA). Monoclonal anti-tubulin antibody was obtained from Sigma Chemical Co. (St. Louis, MO) and PMA was purchased from Calbiochem (Merck KgaA, Darmstadt, Germany).

2.2. Cell lines and primary cultures

The glioma cell lines, A172 and U87 were grown on tissue culture dishes in medium consisting of Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal calf serum, 2 mM glutamine, penicillin (50 U/ml) and streptomycin (0.05 mg/ml). Medium was changed every 3–4 days and cultures were split using 0.25% trypsin.

2.3. Cloning of the human RTVP-1 promoter in pGL3 vector

A 1951-bp fragment of the RTVP promoter was amplified by PCR from isolated BCBL-1 cell genomic DNA using synthetic oligonucleotide primers derived from a published human genomic sequence (*Homo sapiens* chromosome 12 genomic contig VERSION NT_029419.10 GI:29803948): 5′-GC**A CGC GT**G TTT GTT TGG TTG GTT GGT TG-3′ (bases −1972_−1951); and 5′-TAA **CTC GAG** ATG CTT TGC TGG CT-3′ (bases +1_−14) (the restriction site in bold). The resulting PCR product was eluted from low-melting point agarose, purified, excised with MluI and XhoI restriction enzymes and cloned into the same sites of the pGL3-Basic vector (Promega, WI, USA) containing the luciferase reporter gene to yield pGL3-1951 (E in Fig. 2A).

Deletions from the 5′ and the 3′ end of the RTVP promoter were made by PCR amplification with the template pGL3-1951 plasmid using internal RTVP promoter primers having MluI site at the 5′ end, and XhoI site at the 3′ end, digesting the PCR product with MluI and XhoI, and cloning into MluI XhoI-digested pGL3-Basic. A schematic presentation of these constructs is shown in Fig. 2A.

The 1439-bp fragment of the RTVP promoter (D in Fig. 2A) was amplified by PCR using the following primers: 5′-GC**A CGC GT**C TCT CTT AAT TTT CTA AAA TAC ACG-3′ (bases −1439_−1414); and 5′-TAA **CTC GAG** ATG CTT TGC TGG CT-3′ (bases +1_−14).

The 1311-bp fragment of the RTVP promoter (C in Fig. 2A) was amplified by the primers, 5′-GC**A CGC GT**C TCT CTT AAT TTT CTA AAA TAC ACG-3′ (bases −1439_−1414); and 5′-GC**C TCG AG**C AGA ACA GAG CAT GAG TTC ATC ACT A-3′ (bases −128_−149).

The following primers were employed to amplify the 913-bp fragment of the RTVP promoter (B in Fig. 2A): 5′-AC**A CGC GT**C AGC CCC TGT TGT AAC ATC CT-3′ (bases −913_−892); and 5′-TAA **CTC GAG** ATG CTT TGC TGG CT-3′ (bases +1_−14), and the 338-bp fragment of the RTVP promoter (A in Fig. 2A) was amplified by the primers: 5′-GC**A CGC GT**C CAG ATA TTC CAA CCA CTA TGT GT-3′ (bases −338_−314), and 5′-GC**C TCG AG**C AGA ACA GAG CAT GAG TTC ATC ACT A-3′ (bases −128_−149).

2.4. Reporter gene assay

U87 cells were transfected with 3 μg of the reporter construct by electroporation using the Nucleofector device (Amaxa Biosystems, Germany), and plated on 96 well dishes. Firefly luciferase activities of the transfected cells were determined 24 h after the transfection using the dual luciferase assay system (Promega, Madison, WI) according to the manufacturer's instructions.

2.5. Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed using a chromatin immunoprecipitation assay kit (Millipore, Billerica, MA) according to the manufacturer's instructions. Prior to the immunoprecipitation procedure, 10% of the supernatant was designated as total input chromatin and was processed with the eluted immunoprecipitates beginning with the cross-linking reversal step. After the final ethanol precipitation, each IP or input sample was resuspended in 50 μl of PCR grade water. For the amplification, the following primers were used; forward primer: 5′-GC**A CGC GT**C CAG ATA TTC CAA CCA CTA TGT GT-3′, and reverse primer: 5′-TAA CTC GAG ATG CTT TGC TGG CT-3′ (product length: 466 bp).

2.6. Adenovirus preparation and infection

PKCα, PKCδ, and PKCε were first cloned into the pShuttle-CMV vector as previously described [2]. The plasmids were then linearized by digestion with PmeI and were transformed into *Escherichia coli* BJ5183-AD-1 competent cells (Stratagene, La Jolla, CA) carrying the pAdEasy-1 plasmid that encodes the Adenovirus-5 backbone. The linearized recombinant plasmids were transfected into HEK293 cells, and after 6 days viruses were collected and further amplified.

2.7. Silencing experiments

Expression vectors containing short hairpin RNA (shRNA) sequences directed against SRF (SuperArray, Frederick, MD), were transfected into U87 glioma cells using the Nucleofector device (Amaxa Biosystems, Germany) program U29 according to the manufacturer's protocol. The cells were maintained in medium (DMEM) containing 10% fetal calf serum and harvested 72 h after the transfection. In some experiments we employed siRNA duplexes targeting PKC or RTVP-1 and a control scrambled sequence (Thermo Scientific, Lafayette, CO). Transfection of siRNAs (50 nM) was performed using OligofectAMINE (Invitrogen, Carlsbad, CA).

2.8. Immunoblot analysis

Western blot analysis was performed as described [15]. Equal loading was verified by using anti-β-actin or anti-α-tubulin antibodies.