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The sterile alpha-motif (SAM) domain of p63 binds in vitro monoasialoganglioside (GM1) micelles

Stefano Rufini [a,*], Anna Maria Lena [b], Bruno Cadot [b], Sonia Mele [a], Ivano Amelio [b], Alessandro Terrinoni [c], Alessandro Desideri [a], Gerry Melino [b,c], Eleonora Candi [b,**]

[a] Department of Biology, University of Tor Vergata, 00133 Rome, Italy
[b] Department of Experimental Medicine, University of Tor Vergata, 00133 Rome, Italy
[c] Biochemistry Laboratory IDI-IRCCS c/o, University of Tor Vergata, 00133 Rome, Italy

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ABSTRACT

The transcription factor p63 plays pivotal roles in epidermal barrier formation and in embryonic development. The protein structures of TAp63 and ΔNp63α isoforms include a C-terminal steril alpha-motif (SAM) involved in protein–protein interaction. Identification of p63 SAM domain interactors could lead to the explanation of novel mechanisms of regulation of p63 activity, possibly relevant in the physiological role of p63 and in genetic disorders associated with mutations of the p63 gene. In this work, we have performed a biochemical analysis of p63 SAM domain preferences in lipid binding. We have identified the ganglioside GM1 as a high affinity interactor, capable of modulating p63 transcriptional ability exclusively on epidermal target genes. In agreement with these data we report a consistent expression profile and localization analysis of p63 and GM1 in primary keratinocytes and in human epidermal biopsies. Therefore, we propose a potential biological role of p63–GM1 interaction in regulation of p63 during epidermal differentiation.

1. Introduction

p63 is a transcription factor required for epidermal barrier formation and embryonic development [1–7]. It is structurally related to p53 and p73 and shares significant amino acid identity with them in the transactivation, DNA-binding, and oligomerization domains. Having two transcriptional promoters, p63 expresses various isoforms including TAp63 and ΔNp63, with the latter lacking the N-terminal transactivation domain (TA). Moreover, an alternative splicing allows maturation of three C-terminal variants (αβ and γ isoforms). The alpha isoforms contain a sterile alpha motif (SAM) at the C-terminal, which is important for protein–protein interaction [8,9]. The three-dimensional structure of the p63 SAM domain is composed of five alpha-helices that are structurally very similar to the cognate p73 SAM domain [10] and to the prototype SAM domain of the ephrin B2 receptor [11]. The function of the SAM domain does not appear to be limited to oligomer formation and can be variable depending on different protein interactions [12]. p73 SAM binds both zwitterionic (phosphatidylcholine, PC) and anionic (phosphatidic acid, PA) lipids, and was proposed a role of SAM lipid binding in p73 regulation [13]. Recently, SAM domains in the Smaug family, a translational repressor, have also been found as part of RNA binding modules that recognizes RNA hairpins with a specific sequence loop [14,15].

In contrast to p53, p63 is rarely mutated in human cancers, but mutations of the p63 gene have been associated with several genetic disorders known as ectodermal dysplasia syndromes (EDs). EDs include ectrodactyly-ectodermal dysplasia-cleft lip/palate (EEC), ankyloblepharon-ectodermal dysplasia-clefting (AEC) syndrome, split hand-foot malformation (SHFM) and limb-mammary syndrome (LMS). The position of the mutations is correlated to the abnormal phenotype observed. Mutations included in the SAM domain are often associated to AEC syndrome, where patients present skin erosion, eyelid, nail and tooth defects, but the absence of limb malformation [16–19].

Gangliosides, a family of glycosphingolipids containing one or more sialic acid residues (Fig. 1A), have been implicated with various cellular functions including growth, differentiation, cell-to-cell interaction and signal transduction [20]. They have generally been assumed to be localized primarily in the plasma membrane but evidence suggests that they also occur in intracellular compartments, such as the Golgi apparatus, endoplasmic reticulum and

Abbreviations: SAM, sterile alpha motif; PA, phosphatidic acid; PC, phosphatidylcholine; GM, ganglioside; PS, phosphatidylserine; SM, sphingomyelin; HEKn, human epithelial keratinocytes normal.

* Corresponding author.
** Corresponding author. Tel.: +39 06 7259 6487; fax: +39 06 7259 6977.
E-mail addresses: rufini@uniroma2.it (S. Rufini), candi@uniroma2.it (E. Candi).

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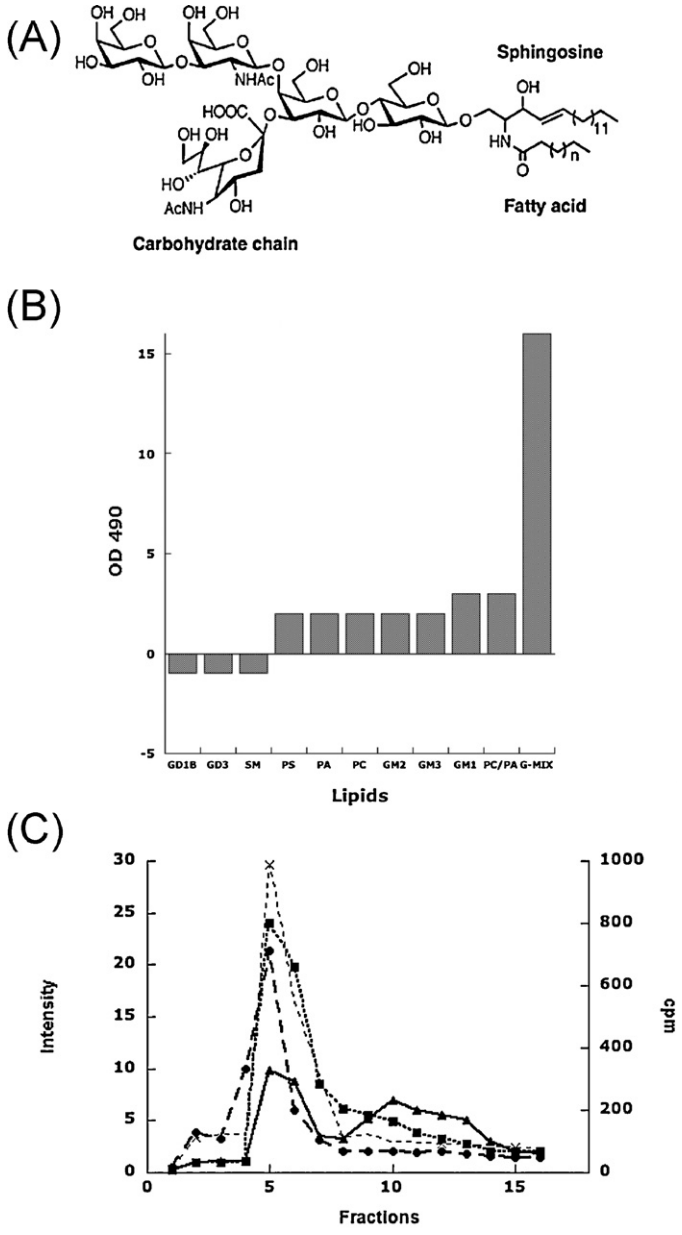

Fig. 1. In vitro binding of p63 SAM to gangliosides. (A) Structure of GM1. (B) ELISA assay to test p63 SAM domain affinity for phospholipids and sfingolipids. GangMix, gangliosides mixture from ox brain; GD3, GD1B, di-sialogangliosides; PC/PA, phosphatidylcoline/phosphatidic acid; GD, mono-sialoganlgiosides (GM1, GM2 and GM3); PC, phosphatidylcoline; PA, phosphatidic acid; PS, phosphatidylserine; SM, sphingomyelin. (C) Sephadex G-100 gel filtration to purify complexes of the SAM domain with different lipids. The eluted fractions were collected and tested both for radioactivity (indicating lipid presence) and Trp fluorescence (indicating the presence of the SAM domain). Typical elution profiles for the recombinant SAM domain alone or with liposomes made of PC/PA, Ganglmix, or GM1 are shown. The fraction of SAM domain bounded with micelles varies depending on the nature of lipids being fully sequestered by GM1 micelles but only partially by Ganglmix (70%) and PC + PA (35%). Typical elution profiles for the liposomes alone (cross) or for recombinant SAM domain incubated with liposomes made with PC/PA (triangles), Ganglmix (squares), or GM1 (circles). The figure shows one representative experiment of three.

nucleus [21–23]. Lipid analysis supported the idea that nuclear envelope is the site of an active autonomous ganglioside metabolism, which is regulated independently from that of the plasma membrane [24] in both neural [25] and extra-neural tissue and cells [26]. It has been proposed in neuroblastoma cells that some differentiation stimuli activate a nuclear neuraminidase able to convert the GD1α present in the nuclear envelope into GM1 that, in turn, would influence the overall cell Ca^{2+} homeostasis enhancing the nuclear Na^{+}/Ca^{2+} exchanger (NCX) [27]. Finally, has been proposed that lipids do not belong only to the nuclear envelope, but are also inside the nucleus forming proteo-lipid complexes [28]. Using immunogold electron microscopy analysis Parkinson et al. demonstrated the presence of GM1 in mouse epithelial cells heterochromatin [22]. Immunochemical study also suggested that in rat cortical neurons, the ganglioside GD3 binds to heterochromatin inside the nucleus, and there is also evidence that its levels increase after stimulation of cells with beta-amyloid peptide [29].

In this paper we have carried out an extensive study of the p63 SAM domain preferences in lipid binding. Our results indicate that p63 SAM is able to bind GM1 with high affinity, and this interaction modulates p63 transcriptional ability.

2. Materials and methods

2.1. Enzyme-linked immunosorbent assay (ELISA)

ELISA-SAM binding to various lipids was evaluated by ELISA, as described previously [27]. In brief, the wells of microtiter plates were coated with phospholipid antigen (10 μM) in ethanol by evaporation at room temperature. After blocking the wells with Tris-buffered saline (TBS, 10 mM Tris–HCl, pH 7.4, 150 mM NaCl) containing 30 mg/ml bovine serum albumin (BSA), the wells were incubated with various concentrations of SAM domains in TBS containing 10 mg/ml BSA (1% BSA-TBS) for 2 h at room temperature. After washing the wells with TBS, the bound SAM was detected by incubating the wells with anti-p63 antiserum, diluted 1/1000 with 1% BSA-TBS for 2 h at room temperature, followed by incubation (2 h at room temperature) with biotinylated anti-rabbit IgG and peroxidase-conjugated streptavidin. The intensity of the color developed with o-phenylenediamine as the substrate was measured using an ELISA reader (Bio-Rad, Hercules, CA). All the chemicals used were from Sigma–Aldrich (St. Louis, MO).

2.2. Gel filtration and fluorescence measurement

GM1 and other gangliosides were dissolved in water above their micellar critical concentration. SAM (final concentration 7–10 μM) and different concentrations of GM1 were incubated for 30 min in 250 μl of Phosphate Buffer Saline (PBS) + 2 mM of DTT. The reaction mixture was applied to a Sephadex G-75 gel filtration column (Amersham Pharmacia Biotech, Piscataway, NJ) to separate the SAM–lipid complex to the unbound protein. The column was eluted with PBS-DTT at a flow rate of 0.5 ml/min. A few nCi of [14C]-palmitic acid (Amersham Pharmacia Biotech, Piscataway, NJ) were added to the gangliosides to determine the elution time of micelles after analysis of radioactivity in the different fractions. Protein elution was tracked by analysis of intrinsic Trp fluorescence (Ex. 220 Em. 346). All the chemicals used were from Sigma–Aldrich (St. Louis, MO).

2.3. Non-denaturing acrylamide gel electrophoresis

SAM domain (final concentration 7–10 μM) and different concentrations of GM1 were incubated for 30 min in 250 μl of Phosphate Buffer Saline (PBS) + 2 mM of DTT. The reaction mixture was resolved by polyacrylamide gel electrophoresis prepared run in non-denaturating conditions (Bio-Rad, Hercules, CA). The polyacrylamide gel was blotted onto a Hybond PVDF membrane (GE Healthcare, Hatfield, UK). Membranes were blocked with PBST 5% non-fat dry milk, incubated with anti-p63 primary antibodies (Ab4, NeoMarkers, Fremont, CA) for 2 h at room temperature, washed and hybridized with peroxidase-conjugate secondary