

DSCR1, overexpressed in Down syndrome, is an inhibitor of calcineurin-mediated signaling pathways

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Received 22 March 2000; Revised and Accepted 8 May 2000

Down syndrome is one of the major causes of mental retardation and congenital heart malformations. Other common clinical features of Down syndrome include gastrointestinal anomalies, immune system defects and Alzheimer's disease pathological and neurochemical changes. The most likely consequence of the presence of three copies of chromosome 21 is the overexpression of its resident genes, a fact which must underlie the pathogenesis of the abnormalities that occur in Down syndrome. Here we show that DSCR1, the product of a chromosome 21 gene highly expressed in brain, heart and skeletal muscle, is overexpressed in the brain of Down syndrome fetuses, and interacts physically and functionally with calcineurin A, the catalytic subunit of the Ca²⁺/calmodulin-dependent protein phosphatase PP2B. The DSCR1 binding region in calcineurin A is located in the linker region between the calcineurin A catalytic domain and the calcineurin B binding domain, outside of other functional domains previously defined in calcineurin A. DSCR1 belongs to a family of evolutionarily conserved proteins with three members in humans: DSCR1, ZAKI-4 and DSCR1L2. We further demonstrate that overexpression of DSCR1 and ZAKI-4 inhibits calcineurin-dependent gene transcription through the inhibition of NF-AT translocation to the nucleus. Together, these results suggest that members of this newly described family of human proteins are endogenous regulators of calcineurin-mediated signaling pathways and as such, they may be involved in many physiological processes.

INTRODUCTION

Trisomy of human chromosome 21 (HC21), full or partial, is a major cause of mental retardation and other phenotypic abnormalities, collectively known as Down syndrome (DS), a disorder affecting 1 in 700 births (1). The identification of genes on HC21 and the elucidation of the function of the

proteins encoded by these genes have been a major challenge for the human genome project and for research in DS (2). Over 100 of the estimated 300–500 genes of HC21 have been identified, but the function of most remains largely unknown. It is believed that in DS the overexpression of an unknown number of HC21 genes is directly or indirectly responsible for the mental retardation and the other clinical features of DS (1). For this reason, HC21 genes that are expressed in tissues especially affected in DS patients are of special interest.

We have identified previously a HC21 gene, *DSCR1*, highly expressed in the central nervous system and heart (3). *DSCR1* is expressed as four protein isoforms through the use of four alternative first exons (4). Although all the different *DSCR1* spliced transcripts are expressed in heart and skeletal muscle, only the exon 1-containing *DSCR1* transcript can be detected in fetal and adult brain, and only the exon 4-containing mRNA is detected in placenta and kidney. These two *DSCR1* transcripts encode predicted polypeptides of 197 amino acids of unknown function, which differ in their N-terminal 29 amino acids.

Calcium is used by the cell to modulate gene expression programs by various means. Examples include direct binding of calcium by the transcriptional repressor DREAM, which leads to a reduction in its DNA activity (5), or Ca²⁺/calmodulin kinase IV phosphorylation of the transcription factor CREB allowing its binding to the co-activator p300 (6). The best known example of calcium regulation through phosphatases is the NF-AT transcription factor (reviewed in ref. 7). In this case, an increase in intracellular Ca²⁺ leads to the activation of calcineurin, which then dephosphorylates cytosolic NF-AT family members. This modification unmask their nuclear localization signals and promotes translocation into the nucleus where they bind cooperatively to DNA with other transcription factors such as AP-1, c-MAF or GATA4. This signaling pathway is important for the immune response (reviewed in ref. 8), cardiac and skeletal muscle hypertrophy (9–11), slow fiber differentiation in skeletal muscle (12), cardiac valve development (13,14) and the differentiation of a pre-adipocyte cell line to adipocytes in culture (15).

Calcineurin is the only serine/threonine protein phosphatase under the control of Ca²⁺/calmodulin (16). It functions as a heterodimer composed of a catalytic A subunit (CaNA) and a

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calcium binding regulatory B subunit (CaNB). In mammals, there are three different CaNA genes, α , β and γ ; highly similar through their entire sequence but with different tissue distributions. CaNA displays a multidomain structure with a catalytic domain at its N-terminus, similar to that of protein phosphatase 1 (17,18), followed by the binding regions for the B subunit and calmodulin (CaM). An autoinhibitory domain is located near the C-terminus, which is thought to be displaced upon CaM binding. The immunosuppressive drugs FK506 and cyclosporin, when complexed to specific immunophilins (FKBP12 and cyclophilin A, respectively), bind calcineurin at multiple sites, including the N-terminus of the CaNB binding helix, the CaNB subunit and the catalytic domain of CaNA (17), inhibiting calcineurin activity.

We report here the identification of DSCR1 as a calcineurin catalytic A subunit binding protein. We have mapped the interaction domain in CaNA to the linker region between the catalytic domain and the CaNB binding domain. We further show that overexpression of DSCR1 can repress the transcriptional activation of a NF-AT-dependent promoter in response to phorbol 12-myristate 13-acetate (PMA) and ionophore by inhibiting the nuclear translocation of NF-AT. Therefore DSCR1, and the other related proteins in humans, may represent endogenous inhibitors for calcineurin and define a new negative regulatory pathway in calcineurin signaling in mammals.

RESULTS

Identification of calcineurin A as a DSCR1 binding protein

To investigate the cellular function of DSCR1 we performed a yeast two-hybrid screen to identify interacting proteins (19) using the brain-specific DSCR1 isoform 1 (Fig. 1A). The bait was screened against a human fetal brain cDNA library fused to the Gal4 transcriptional activation domain (G4AD). Three out of six doubly positive clones ($\text{His}^+/\beta\text{-gal}^+$) corresponded to partial cDNAs of CaNA, isoform β (20). All CaNA positive clones had unique ends but overlapped with each other. The interaction between the DSCR1 bait and the CaNA preys was specific, since binding was not apparent between either CaNA or the Gal4 DNA binding domain (G4DBD) or DSCR1 and the G4AD (Fig. 1B). Interaction with CaNA was also detected with a shorter DSCR1 bait, containing the C-terminal half (DSCR1¹¹⁵⁻¹⁹⁷), suggesting that CaNA interaction would be common to all DSCR1 isoforms since they share the carboxyl end.

DSCR1 and CaNA also interacted in a mammalian two-hybrid assay. In these experiments, DSCR1 and DSCR1¹¹⁵⁻¹⁹⁷ were fused to the DBD of Gal4 and CaNA to the activation domain of VP16 (VP16AD). The plasmids were transfected into mammalian cells along with a luciferase reporter gene driven by Gal4 sites. Neither construct generated any significant luciferase activity when transfected alone, but a strong luciferase activity was observed when both DSCR1 and CaNA were co-transfected (Fig. 1C), demonstrating the DSCR1 and CaNA interaction. Similar to the results of the yeast two-hybrid assay, a clear interaction was also detected when the DSCR1 C-terminal half was used (Fig. 1C), suggesting that amino acids 115–197 in DSCR1 are sufficient for CaNA binding in mammalian cells.

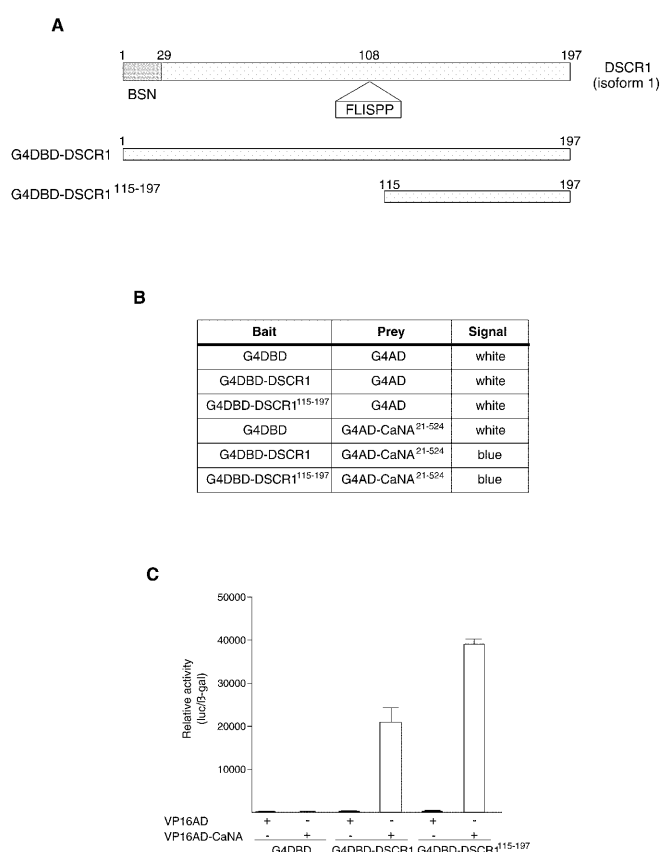


Figure 1. Yeast two-hybrid screening for DSCR1-interacting proteins. (A) Schematic diagrams of wild-type DSCR1 and the C-terminal mutant used as baits. BSN, brain-specific N-terminal region; FLISPP, DSCR1 family signature (T.J. Kingsbury and K.W. Cunningham, manuscript in preparation). (B) Summary of the results from the interaction studies performed in yeast with the indicated baits and preys. β -gal positive and negative results are indicated as blue and white, respectively. (C) DSCR1 and CaNA interact in a mammalian two-hybrid assay. U2OS cells were transiently transfected with the indicated expression constructs (0.5 μ g), pG5E1b-luc (1 μ g) and pCMV- β gal (0.5 μ g) as an internal control. Values are given as the ratio of luciferase to β -gal activities and are the average of three independent plates.

Mapping of the DSCR1 binding domain in CaNA

Various functional domains can be identified in the CaNA linear molecule (Fig. 2A). These domains are highly conserved among CaNAs from different species and include a catalytic domain, the binding domains for CaNB and CaM and the C-terminal autoinhibitory peptide (AID). To map the region in CaNA involved in DSCR1 binding, we generated a series of CaNA deletion mutants (Fig. 2A) as fusions with VP16AD and tested them for their interaction with DSCR1 in mammalian two-hybrid assays (Fig. 2B). Deletion of the autoinhibitory domain (CaNA2) or the CaM binding domain (CaNA3) did not affect DSCR1 binding. Further deletion of the CaNB binding domain (CaNA4) diminished DSCR1 binding but did not abolish it as the values observed were 100-fold higher than the controls. However, a slightly larger deletion up to amino acid 338 (CaNA5) completely abolished the interaction (0.8-fold increase). For each case, the expression of the fusion proteins with VP16AD or G4DBD was determined by western blotting

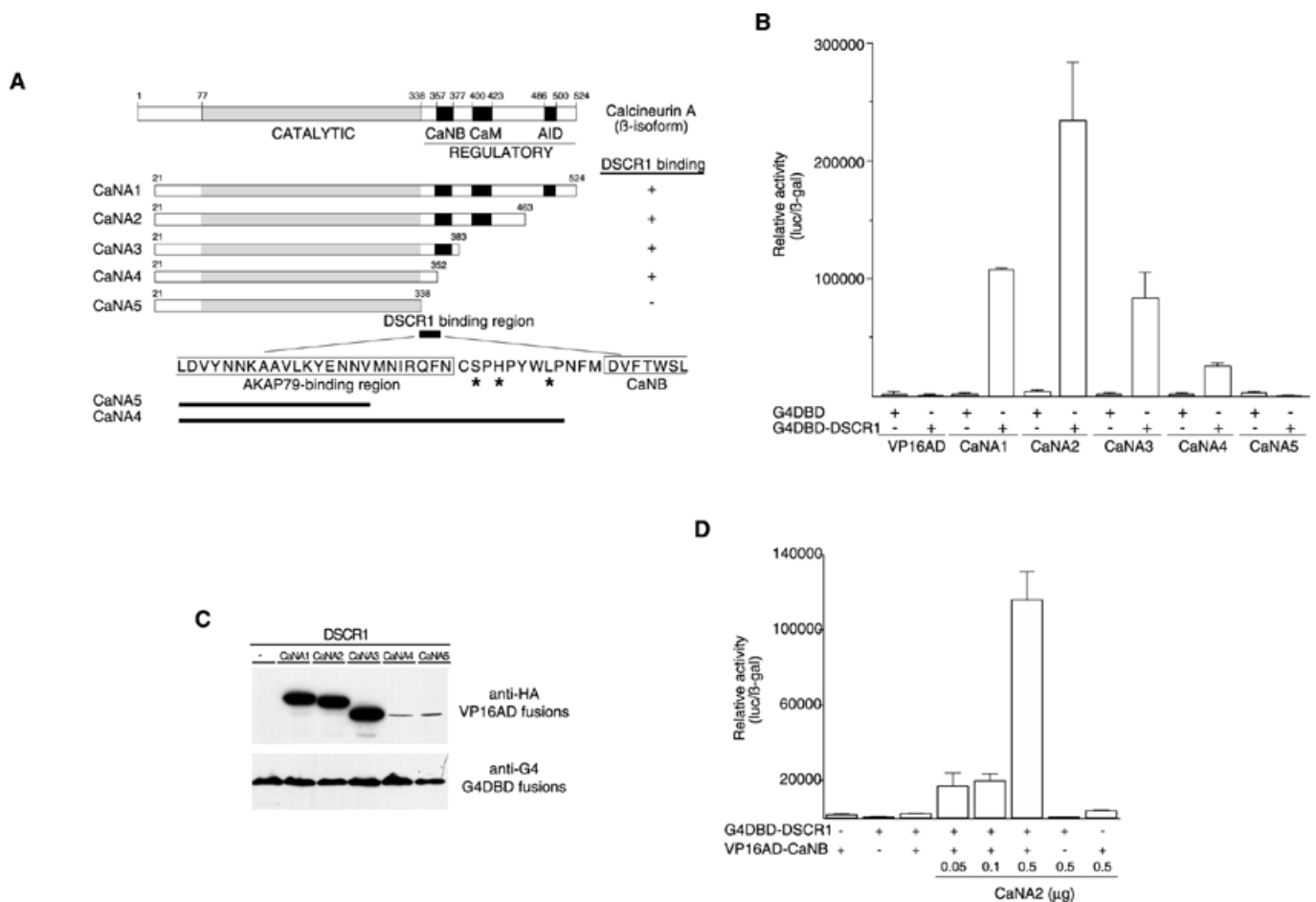


Figure 2. DSCR1 binds to calcineurin A linker region. (A) Schematic diagrams of wild-type CaNA (human β-isoform) and the generated mutants. AID, autoinhibitory domain; CaM, calmodulin binding domain; CaNB, calcineurin B binding domain. Stars indicate the phosphatase inactivating mutations defined in yeast CaNA (31). (B) Mammalian two-hybrid assay with the different CaNA preys. U2OS cells were transiently transfected with the indicated expression constructs (0.5 μg), pG5E1b-luc (1 μg) and pCMV-βgal (0.5 μg) as an internal control. Values are given as in Figure 1C. (C) Steady-state levels of the fusion proteins were assessed by immunoblotting cell lysates from transfected cells in (B) with anti-G4DBD (for the DSCR1 bait) and anti-HA antibodies (for the VP16AD–CaNA preys). (D) DSCR1, CaNA and CaNB form a tripartite complex. A mammalian two-hybrid assay was done using G4DBD–DSCR1 and VP16AD–CaNB as bait and prey (0.5 μg), respectively, in cells transfected with increasing amounts of a CaNA expressing plasmid (0.05, 0.1 and 0.5 μg). Values as in Figure 1C.

(Fig. 2C). The expression of G4DBD–DSCR1 was not affected by the co-expression of CaNA1 or any of the deleted mutants. However, the VP16AD–CaNA fusions were reproducibly expressed at different levels, with the CaNA4 and CaNA5 fusion proteins expressed at lower levels than the other CaNA fusion proteins. This decrease, which may suggest that these mutants are less stable, may explain the decrease in the luciferase activity detected in the DSCR1–CaNA4 co-transfections and therefore could indicate that the binding activity of the CaNA4 mutant is similar to that of CaNA1. Altogether, the results suggest that amino acids 338–352 of CaNA, corresponding to the linker region between the catalytic domain and the CaNB binding domain (Fig. 2A), are important for DSCR1 interaction.

The deletion analysis also indicates that the DSCR1–CaNA interaction is not dependent on the presence of the CaNB binding domain. Since CaNB and DSCR1 do not share interaction sites on the CaNA molecule, competition between DSCR1 and CaNB for CaNA binding would not be expected, and therefore the three proteins may exist in a complex. To explore

this further, we performed a three-hybrid assay in which cells were co-transfected with a G4DBD–DSCR1 fusion construct, a VP16AD–CaNB fusion construct and increasing concentrations of a CaNA expression vector. While no interaction was detected between DSCR1 and CaNB in this assay, a significant stimulation of the reporter gene occurred when CaNA was co-expressed (Fig. 2D). This result is consistent with CaNA acting as a bridge between DSCR1 and CaNB in the two-hybrid assay, and supports the idea that DSCR1 may bind to the heterodimeric calcineurin molecule.

DSCR1 binds to endogenous calcineurin A

We analyzed the association between DSCR1 and endogenous CaNA by co-immunoprecipitation. COS-7 cells were transfected with a HA-tagged DSCR1 full-length expression vector or with a DSCR1 deleted version, lacking the C-terminus (HA-DSCR1^{1–156}). Cell extracts were immunoprecipitated with an anti-HA antibody and the immunoprecipitated products were analyzed by western blotting using an anti-CaNA antibody

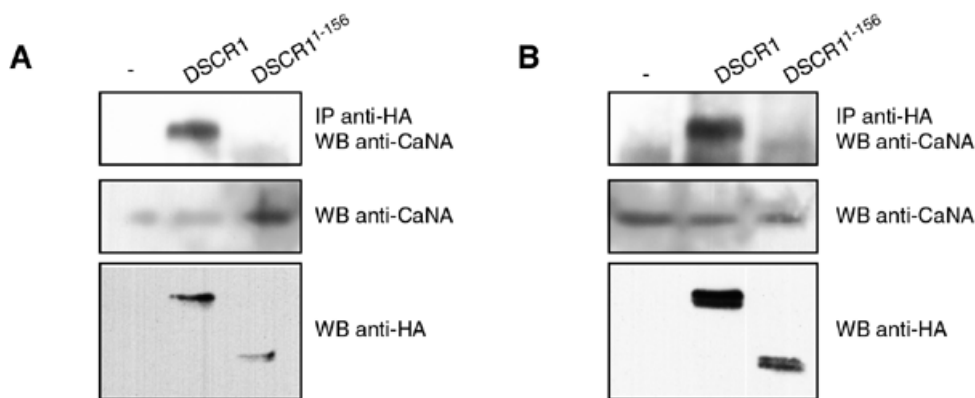


Figure 3. DSCR1 can interact with endogenous calcineurin. (A) Co-immunoprecipitation of DSCR1 and endogenous calcineurin in non-stimulated cells. COS-7 cells mock-transfected (-), transfected with HA-tagged wild-type DSCR1 or mutant DSCR1 as indicated, were subjected to anti-HA immunoprecipitation followed by immunoblotting with an anti-CaNA antibody to detect associated CaNA. The levels of CaNA in the immunoprecipitates and in total cell lysates are shown, as well as the levels of DSCR1 proteins. (B) Co-immunoprecipitation of DSCR1 and endogenous calcineurin in stimulated cells. Extracts were prepared from COS-7 transfected cells incubated in medium with 10 mM CaCl_2 in the presence of ionophore. Protein extracts were processed as in (A).

(Fig. 3A). This experiment revealed that endogenous CaNA associates with DSCR1 in the immunoprecipitated complexes, but not with the mutant DSCR1¹⁻¹⁵⁶, supporting the previous conclusion that the DSCR1 C-terminal region is sufficient for CaNA binding.

Calcineurin phosphatase activity is dependent on intracellular Ca^{2+} concentrations. This dependence is controlled mainly by CaM, whose binding upon increases in intracellular Ca^{2+} concentration displaces the CaNA inhibitory domain allowing calcineurin activation. To determine whether the cytoplasmic Ca^{2+} levels affected the interaction between DSCR1 and CaNA, we performed a co-immunoprecipitation experiment with extracts from cells treated with calcium ionophore, in the presence of high Ca^{2+} concentration. As shown in Figure 3B, binding of CaNA to DSCR1 was also detected in these conditions, suggesting that the interaction can occur independently of the activation state of calcineurin in mammalian cells.

DSCR1 is representative of a human family of calcineurin binding proteins

Sequence comparison predicts that DSCR1 belongs to a family of proteins with members in eukaryotes from yeast to humans, including the *Saccharomyces cerevisiae* Rcn1p (T.J. Kingsbury and K.W. Cunningham, manuscript in preparation), the *Drosophila melanogaster* Nebula (GenBank accession no. AF147700) and open reading frames in *Caenorhabditis elegans* and *Aspergillus nidulans*, among others (T.J. Kingsbury and K.W. Cunningham, manuscript in preparation). The family signature is a short polypeptide, FLISPPXSPP, which resembles the SP boxes in NF-AT family members (8). In humans, the family consists of three members (Fig. 4A): DSCR1 (3), ZAKI-4 (21) and DSCR1L2 (GenBank accession no. AAF01684). In these three proteins, the homology extends over the entire molecule and is particularly high around the SPP region. In order to find out whether other members of the human family behave in a similar manner to DSCR1, we have investigated the ability of CaNA and ZAKI-4 to interact. Co-immunoprecipitation of CaNA with ZAKI-4, synthesized together by cell-free translation (Fig. 4B), revealed that these

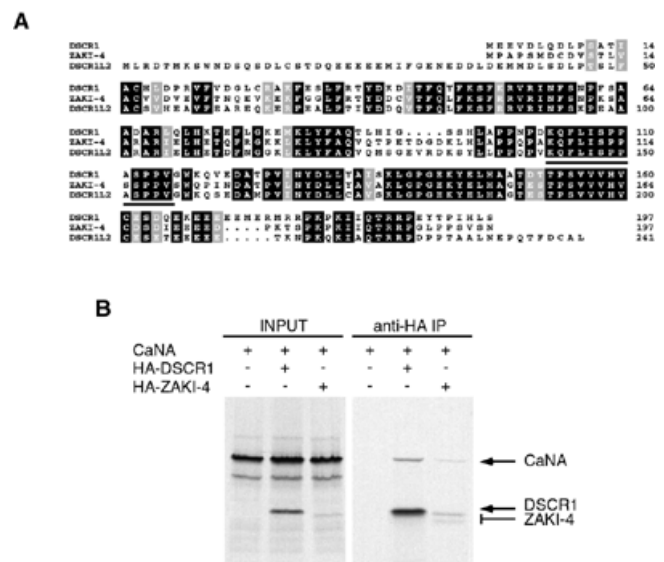


Figure 4. DSCR1 is the member of a conserved family of calcineurin-interacting proteins. (A) DSCR1, ZAKI-4 and DSCR1L2 are a family of human homologous proteins. Protein alignment was done with the BLAST2 program and the BLOSUM62 algorithm at <http://www.ncbi.nlm.gov>. The 'FLISPP' signature of the family is underlined. (B) ZAKI-4 also interacts with CaNA. The interaction was tested by immunoprecipitation with anti-HA antibodies of *in vitro*-translated [³⁵S]methionine-labeled HA-ZAKI-4 or HA-DSCR1 and CaNA. Both input and immunoprecipitates are shown. The CaNA, DSCR1 and ZAKI-4 bands are indicated with arrows.

two proteins interact at approximately the same efficiency as DSCR1, suggesting that sequence similarity confers similar properties, at least with regard to CaNA binding.

DSCR1 inhibits NF-AT-mediated transcriptional activation

Since DSCR1 binds to calcineurin, we were interested to know whether expression of DSCR1 affects calcineurin signaling. Calcineurin substrates include, among others, phosphatase inhibitor-1, NO synthase, dynamin and the transcription factors Elk-1 and NF-AT (reviewed in ref. 16). In the latter

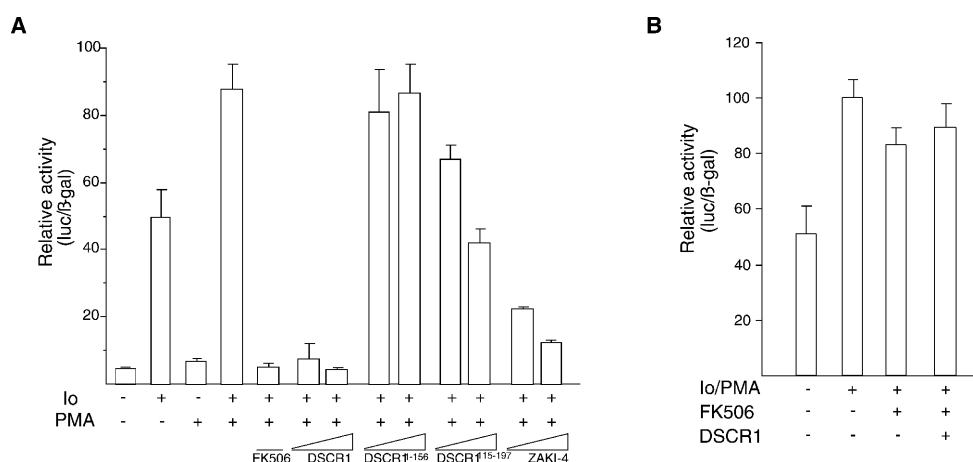


Figure 5. DSCR1 and ZAKI-4 inhibit the transcriptional activation of the interleukin-2 promoter in response to PMA/calcium stimulation. (A) CHO cells were co-transfected with pIL2-luc (1 μ g) and increasing amounts of the indicated expression plasmids (0.25 or 1 μ g) and treated with the indicated compounds as indicated in Materials and Methods. (B) An AP-1 luc reporter plasmid, -73 Col Luc (1 μ g), was co-transfected with the indicated expression plasmids. All cells were treated with ionophore (Io, 1 μ M) and PMA (10 ng/ml), and some with FK506 as indicated (20 ng/ml). In (A) and (B), all the plates were co-transfected with pCMV- β gal as an internal control. Values are given as the ratio of luciferase to β -gal activities and represent the average of three independent plates.

case, activation of calcineurin phosphatase activity upon a rise in intracellular Ca^{2+} leads to the dephosphorylation of cytoplasmic NF-AT family members, which then translocate to the nucleus and activate transcription of target genes (22,23), some of them being critical for immune system activation. We therefore measured the activity of a luciferase reporter gene under the control of the IL-2 promoter (24) when co-transfected with a set of DSCR1-expressing constructs (Fig. 5A). The IL-2 promoter was weakly activated following treatment with either PMA or ionophore, but highly induced when both stimuli were given, as a result of the cooperative binding of NF-AT and transcription factors of the AP-1 family (25). This activation was suppressed by the calcineurin inhibitor FK506. Interestingly, co-expression of DSCR1 strongly inhibited the induction of the IL-2 reporter by co-stimulation with PMA and ionophore. Similarly, DSCR1¹¹⁵⁻¹⁹⁷, the truncated version of DSCR1 that retained CaNA binding activity, was able to act as a dose-dependent inhibitor, though not as efficiently as DSCR1 full-length. In contrast, co-expression of DSCR1¹⁻¹⁵⁶, the mutant incapable of CaNA binding, had no effect on the activation assay, strongly suggesting that the inhibition caused by DSCR1 is mediated through calcineurin binding. ZAKI-4 was also able to inhibit calcineurin function, as measured by its ability to suppress IL-2 reporter activation (Fig. 5A).

To check if the reduction of the IL-2 promoter activation was dependent only on NF-AT, a construct containing AP-1 binding sites, but not NF-AT binding sites (26), was tested (Fig. 5B). As expected, reporter gene expression in response to PMA was unaffected by treatment with FK506. Similarly, overexpression of DSCR1 had no effect on the transcriptional activity of this reporter, indicating that the inhibitory effect on the IL-2 promoter was specific and dependent on the NF-AT sites.

DSCR1 inhibits NF-AT nuclear translocation

Based on the previous data, we asked if the inhibition of IL-2 promoter by DSCR1 and ZAKI-4 is governed by the inhibition of the calcineurin-mediated nuclear translocation of NF-AT.

To find out if this is the case, we analyzed the cellular distribution of a transiently expressed Flag-tagged NF-ATc protein (22) in cells co-expressing different HA-tagged DSCR1 proteins. DSCR1 appeared to be distributed throughout the cytoplasm and the nucleus of transfected cells (Fig. 6A, left); a staining pattern consistent with a small protein that can diffuse through the nuclear pore complex (27). No clear changes in subcellular localization of DSCR1 were observed when cells were either stimulated with calcium (Fig. 6A, center), or treated with FK506 (Fig. 6A, right). As expected, NF-ATc was cytoplasmic in non-stimulated cells (Fig. 6B, left), displayed a nuclear localization in cells treated with ionophore (Fig. 6B, center) and became cytosolic when FK506 was added to the medium (Fig. 6B, right). However, in doubly transfected cells, NF-ATc was unable to accumulate in the nucleus upon a calcium stimulus in cells co-expressing either DSCR1 (Fig. 6C), DSCR1¹¹⁵⁻¹⁹⁷ (Fig. 6D) or ZAKI-4 (Fig. 6E). This effect was not observed in cells co-expressing DSCR1¹⁻¹⁵⁶, which lacks the calcineurin binding region (Fig. 6C). Overall, these data suggest that binding of DSCR1 and ZAKI-4 to calcineurin results in the inhibition of the calcium-induced NF-AT nuclear translocation.

DSCR1 RNA is induced by calcium and overexpressed in DS

We have studied which type of stimulus induces *DSCR1* expression in mammalian cells. Expression of the *DSCR1* hamster ortholog *adapt78* (28) and the yeast family member *Rcn1* (T.J. Kingsbury and K.W. Cunningham, manuscript in preparation) is induced by calcium. Thus, we tested the effect of calcium signaling on *DSCR1* expression in a human astrocytoma and a neuroblastoma cell line (Fig. 7A). *DSCR1* mRNA expression was clearly induced in both cell lines by calcium treatment in the presence of ionophore. The increased expression was abolished when the cells had been treated previously with the calcineurin-inhibitor cyclosporin A, suggesting that the effect is mediated by the phosphatase activity. Moreover, at least in the neuroblastoma cell line, the induction was potenti-

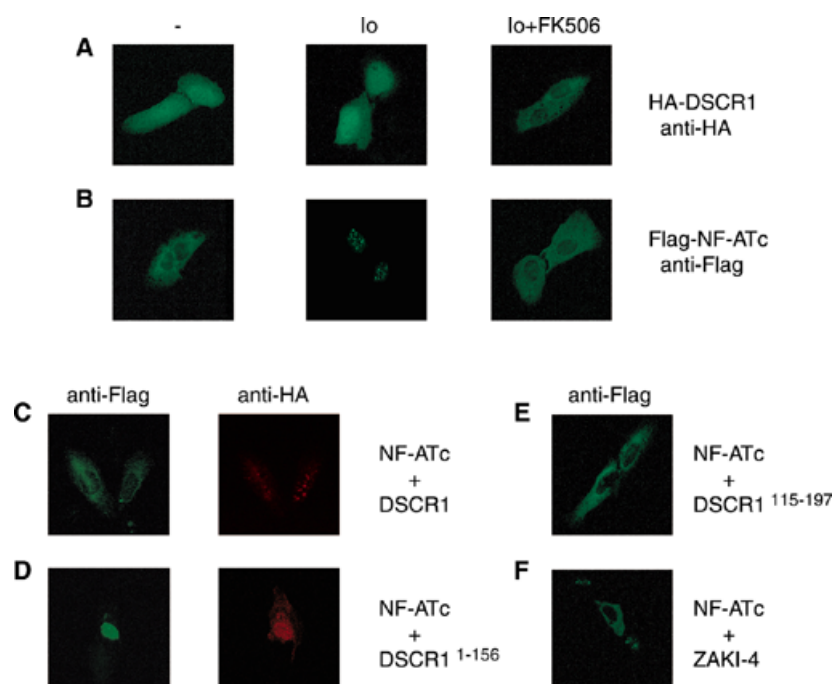


Figure 6. DSCR1 inhibits calcium induced NF-AT nuclear translocation. U2OS cells were transfected with an HA-tagged DSCR1 expression plasmid (2 μ g) (A) or a Flag-tagged NF-ATc expression plasmid (2 μ g) (B) and were then left untreated (left), stimulated with ionophore (Io, center) or ionophore plus FK506 (right) for 1 h before fixation. (C–F) Double immunofluorescence analysis of co-transfected cells treated with Io for 1 h. Cells were fixed and analyzed for immunofluorescence with an anti-Flag monoclonal antibody and an anti-HA polyclonal antibody as indicated. A Flag-tagged NF-ATc expression plasmid was co-transfected with HA-DSCR1 (C), HA-DSCR1¹⁻¹⁵⁶ (D), HA-DSCR1¹¹⁵⁻¹⁹⁷ (E) and HA-ZAKI-4 (F) expression constructs. For (E) and (F) only the anti-Flag staining is shown due to the low levels of the anti-HA signal.

ated by PMA, a situation resembling the behavior of some NF-AT gene targets (8).

Finally, because *DSCR1* maps to a region of HC21, which has been considered as critical for certain DS phenotypic traits (29), we were interested in testing whether the extra copy of *DSCR1* present in DS could lead to an increase in its expression. Northern blot analysis showed that the levels of *DSCR1* mRNA were significantly higher (1.9-fold relative to *GAPDH* mRNA) in DS brain tissue relative to non-DS brains (Fig. 7B).

DISCUSSION

We have identified a new family of calcineurin binding proteins that are able to regulate calcineurin signaling pathways *in vivo*. The human family consists of three members, DSCR1 (3), ZAKI-4 (21) and DSCR1L2 (GenBank accession no. AAF01684), all of which have mouse orthologs (data not shown). Homologous proteins exist in lower eukaryotes, invertebrates and non-mammals vertebrates (T.J. Kingsbury and K.W. Cunningham, manuscript in preparation). Taking into account that several members of the family including yeast Rcn1p (T.J. Kingsbury and K.W. Cunningham, manuscript in preparation), and both DSCR1 and ZAKI-4 (this report) are able to bind CaNA and inhibit its signaling functions, we suggest naming the family 'calcipressins' and renaming the *DSCR1* gene product as human calcipressin 1. Nothing has been described to date about the expression pattern of *DSCR1L2*, but *DSCR1* and *ZAKI-4*, although showing distinct expression patterns in other tissues, share expression in heart and skeletal muscle: two organs where fine regulation of

calcineurin activity is relevant (9,12). In fact, calcineurin deregulation has been suggested to be involved in both cardiac and skeletal muscle hypertrophy (9,10,12).

DSCR1 interacts with the calcineurin molecule through the catalytic A subunit. The binding, confirmed in a mammalian two-hybrid assay and by co-immunoprecipitation of endogenous CaNA, occurs in both non-stimulated and calcium-stimulated cells (Fig. 3), suggesting that the interaction can occur independently of the activation state of calcineurin, and therefore independently of CaM binding. Consistent with this, two-hybrid analysis showed that deletion of the CaM and CaNB binding domains in CaNA did not disrupt its association with DSCR1. In fact, we have found that CaNA residues 338–352, a region situated between the catalytic domain and the CaNB binding domain at the end of the β 14 sheet (17), were critical for DSCR1 binding (Fig. 2A). This stretch of amino acids is close to the region that has been identified as required for CaNA binding to the adaptor molecule AKAP79 and the subsequent phosphatase activity inhibition (30). Moreover, several residues in this region have been defined by mutagenesis as crucial for the phosphatase activity in yeast calcineurin (31) and were suggested by the authors to be involved in the interaction between CaNA and its substrates since they are exposed on the surface of the protein (31). This region is well conserved in all the CaNAs sequenced to date, including the three human isoforms α , β and γ , suggesting that DSCR1 may interact with all of the human isoforms. It remains to be determined whether the other members of the calcipressin family bind to CaNA in the same region as defined for DSCR1, and hence it is a matter of speculation as to whether there is any

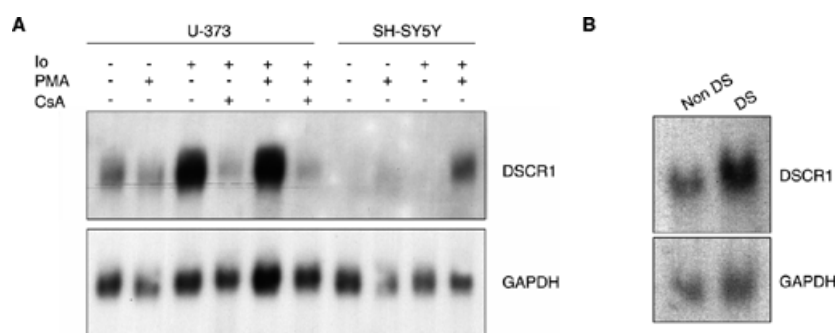


Figure 7. *DSCR1* RNA is induced by a calcium stimulus and overexpressed in DS brains. (A) Total RNA was extracted from the human astrocytoma U-373 and the neuroblastoma SH-SY5Y cell lines, after the indicated treatments as described in Materials and Methods. Ten micrograms per lane were electrophoresed and hybridized with a full-length *DSCR1* cDNA probe (upper panel). The blot was reprobbed with a GAPDH cDNA probe (lower panel). (B) Total RNA (20 μ g) from DS and non-DS brains were hybridized with a *DSCR1* cDNA probe (upper panel). The blot was reprobbed with a GAPDH cDNA probe (lower panel).

specificity in the CaNA–calcipressins interaction. We have not detected any interaction between *DSCR1* and CaNB, and our deletion analysis indicates that CaNB binding to CaNA was not required for *DSCR1* interaction; moreover, *DSCR1*–CaNA interaction can occur in a yeast mutant that lacks CaNB (data not shown). However, the results obtained from three-hybrid studies (Fig. 2D) suggest that the three molecules can exist in a stable complex.

DSCR1 binding to CaNA could still be detected using an N-terminal truncated *DSCR1* mutant (*DSCR1*^{115–197}), suggesting that all *DSCR1* isoforms would associate with CaNA. In fact, deletion of the last 41 amino acid residues completely abolished CaNA interaction (Fig. 3). We could not detect any amino acid residue conservation between this region and other known CaNA binding domains, such as those present in AKAP79 (30), cain/cabin 1 (32,33) or the NF-AT docking site (34). A more complete mutagenesis analysis will be required to map precisely the residues involved in CaNA interaction among the different members of the calcipressin family.

In spite of the wide spectrum of calcineurin physiological roles, the list of its modulators is short. Calmodulin, the main regulator, acts as an enhancer of the phosphatase activity in response to increases in cytosolic Ca^{2+} concentrations. FKBP12 targets the phosphatase to intracellular calcium channels such as the ryanodine receptor (35,36) and the inositol 1,4,5-triphosphate receptor (37). AKAP79 is a scaffold protein that also binds cAMP-dependent protein kinase and protein kinase C (38) and inhibits the phosphatase activity upon binding. A238L, a protein product of the African swine fever virus, acts as an inhibitor and is thought to be used by the virus to evade the host defense systems (39). CHP is a CaNB homologous protein and inhibits calcineurin activity by impairing the assembly of the heterodimer (40). And finally, cain/cabin, is a recently described non-competitive inhibitor (32,33). The calcipressin family illustrates a distinct type of calcineurin regulator whose members can affect calcineurin-mediated signaling. In particular, *DSCR1* and *ZAKI-4* inhibit NF-AT nuclear accumulation in response to calcium signaling (Fig. 6), and consequently modulate NF-AT-dependent transcription (Fig. 5). This inhibition appeared to be dependent on *DSCR1*–CaNA binding, because a *DSCR1* mutant incapable of binding CaNA showed no effect on NF-AT activation. Although

current data do not address the mechanism of *DSCR1*-mediated inhibition, the effects could be explained through the inhibition of calcineurin phosphatase activity by *DSCR1* binding. In fact, bacterially expressed *DSCR1* can inhibit the calcineurin phosphatase activity *in vitro* (T.J. Kingsbury and K.W. Cunningham, manuscript in preparation). In an inhibition model, *DSCR1* binding to CaNA would lead to the inhibition of its phosphatase activity, preventing the dephosphorylation of the cytosolic NF-AT and its subsequent nuclear import. In this case, it can be predicted that the inhibition effect will be common to all NF-ATs and that it will extend to other calcineurin substrates. Alternatively, NF-AT and *DSCR1* might compete for calcineurin binding if their interaction sites overlap in the calcineurin molecule. Two distinct regions for NF-AT interaction have been found in CaNA, one involving a region close to the active site, and a docking region which is contained in the catalytic domain (1–347), but which is distinct from the active site (41). In a competition model, negative regulation by calcipressins might be dependent on the calcineurin substrate considered. Future efforts should be directed towards understanding how the association between *DSCR1* and calcineurin is regulated and which signaling pathways are involved in this regulation.

We have found that *DSCR1* expression is induced by calcium, through a calcineurin-dependent mechanism (Fig. 7A). These results support a model in which *DSCR1* functions as a feedback inhibitor of calcineurin. An inhibitory feedback loop may be necessary to avoid sustained calcineurin activity in situations of prolonged Ca^{2+} stimulus. Each calcipressin family member might respond differentially to various stimuli. For example, *ZAKI-4* has been shown to be induced by thyroid hormones (T_3) (21). In contrast, we have been unable to detect an induction of *DSCR1* mRNA in response to T_3 (data not shown). Differential regulation of calcipressin family members may provide an additional level of complexity to regulate calcineurin *in vivo*.

DSCR1 maps on HC21 and is trisomic in DS individuals. We have found that the presence of an extra copy of the gene leads to its overexpression in fetal brain (Fig. 7B). Although DS is a multifactorial disease, the role of the HC21 gene *DSCR1* as an inhibitor of calcineurin signaling could provide new insights towards the possible involvement of this signaling pathway in

some of the clinical abnormalities observed in patients with DS. These aspects should be dissected further by analysis of mouse models of overexpression and disruption of *Dscr1*.

MATERIALS AND METHODS

Yeast two-hybrid screening

Full-length *DSCR1* cDNA containing exon 1 (4) was subcloned into pAS2 (Clontech, Palo Alto, CA) and used as bait to screen a fetal human brain cDNA library (Clontech). Six clones, positive for *HIS3* selection and *LacZ* expression, were obtained from 5×10^5 doubly transformed yeast colonies. Library screening and β -gal measurements were done according to Clontech protocols. Plasmids containing the prey sequences were rescued and checked by back transformation with the bait. Sequences of the cDNA clones were determined with a Big Dye Terminator Cycle Sequencing-ready Reaction kit (Perkin Elmer, Foster City, CA) and an automated DNA sequence analyzer ABI-PRISM 373 XL Upgrade (Perkin Elmer).

cDNA constructs

All plasmids were generated using standard techniques (42). *DSCR1* open reading frame and the fragments used in the study were subcloned as N-terminal HA-tagged fusions in the pCDNA-HA1 expression vector (43). ZAKI-4 cDNA was PCR amplified with specific primers (ZA1: ATGCCAGCCCCTAGCATGG; ZA2: GAAGGAGCAG-GCAGCTCAGT) from human fetal brain cDNA, cloned into pGEM-T Easy (Promega, Madison, WI) and its DNA sequence determined. ZAKI-4 open reading frame was subcloned as N-terminal HA-tagged fusions in pCDNA-HA1. For the mammalian two-hybrid assays, plasmids pG4-DBD (43) and pCMV-VP16/NLS (44) were used as backbone vectors for the baits (G4DBD fusions) and preys (VP16-AD fusions), respectively. In all the cases, the DBD and the activation domain were located at the N-terminal end of the fusion protein. A luciferase gene driven by five Gal4 sites and the minimal E1b promoter was used as reporter [pG5E1b-luc (43)].

Mammalian two-hybrid assay

Human osteosarcoma U2OS cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS). Transfections were carried out using the calcium phosphate precipitation method (43). Cells were plated out 24 h before transfection at 1×10^5 per 6 cm dish and re-fed after 16 h in the presence of the DNA precipitate, and harvested and processed at a final time of 36 h post-transfection. DNA amounts were kept constant by adding pCDNA-3 when required. pCMV- β -gal was used as an internal standard for transfections. Luciferase and β -gal activities were measured in triplicate plates for each point.

Protein expression was determined by western blotting using whole cell extracts. Transfected cells were harvested in phosphate buffered saline (PBS), pelleted and resuspended in Laemli sample buffer. G4DBD fusions were detected with the anti-G4DBD RK5 monoclonal antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA), and VP16AD fusions with the

anti-HA HA.11 monoclonal antibody (BabCO, Richmond, CA).

Immunoprecipitation

COS-7 cells were grown in DMEM supplemented with 10% FCS. Cells were transfected by electroporation and harvested in PBS at 48 h post-transfection. Cell pellets were lysed in 50 mM Tris-HCl pH 7.5, 150 mM NaCl and 1% NP-40, in the presence of protease inhibitors (Protease Inhibitor Cocktail, Roche Diagnostics, Indianapolis, IN). When indicated, the calcium ionophore A23187 (Sigma-Aldrich Co., St Louis, MO) and CaCl_2 were added to final concentrations of 2 μM and 10 mM, respectively, for 1 h prior to harvesting. For immunoprecipitations, protein mixtures were incubated in lysis buffer with protein-G beads (Amersham Pharmacia Biotech, Little Chalfont, UK) pre-bound with anti-HA high affinity rat antibody (Roche Diagnostics). After several washes in PBS, protein complexes were analyzed by electrophoresis. Western blotting was done by standard methods and visualized by enhanced chemiluminescence (Pierce, Rockford, IL). Endogenous calcineurin A was detected with an anti-calcineurin A polyclonal antibody (Transduction Laboratories, Los Angeles, CA).

In vitro protein interaction

HA-DSCR1 or HA-ZAKI-4 and CaNA were co-synthesized and labeled with [^{35}S]methionine (Amersham Pharmacia Biotech) by cell-free translation TNT-coupled rabbit reticulocyte system (Promega). *In vitro* translated products were diluted in 50 mM Tris-HCl pH 7.4, 120 mM NaCl, 0.5% NP-40, 1 mM DTT and a protease inhibitor cocktail, and incubated with protein G-agarose beads pre-incubated with HA11 monoclonal antibody. After incubation for 1 h at 4°C, the beads were washed five times with dilution buffer and the proteins released in SDS sample buffer and detected by SDS-PAGE followed by autoradiography.

NF-AT activity measurements

CHO cells were grown in DMEM supplemented with 10% FCS. Cells were transfected by the calcium phosphate method with a plasmid containing the IL-2 promoter upstream of the luciferase gene [pIL2-luc, provided by G. Crabtree (24)], the appropriate expression vectors and pCMV- β -gal as an internal control. The AP-1-dependent reporter plasmid -73 Col Luc contains the -73 to +63 region of the human collagenase promoter coupled to the luciferase gene [provided by J.M. Redondo (26)]. When indicated, calcium ionophore A23187 (Io, 2 μM), CaCl_2 (10 mM) and PMA (10 ng/ml) were added to the medium for 16 h before harvesting. FK506 (20 ng/ml) was added 8 h prior to the calcium stimulation. Cells were harvested at a final time of 36 h post-transfection. Results are given as luciferase to β -gal ratio, representing the average of three transfected plates, and were done at least three times yielding similar results.

Immunofluorescence

U2OS were transfected by the calcium phosphate method with the indicated expression vectors and a Flag-tagged NF-ATc expression plasmid [provided by G. Crabtree (22)]. When indi-

cated, before fixation cells were incubated for 1 h in medium containing calcium ionophore A23187 (2 μ M) and CaCl₂ (10 mM). FK506 (20 ng/ml) was added 15 min prior to the calcium stimulation. Cells grown on coverslips were washed in PBS, fixed in 4% paraformaldehyde in PBS for 15 min and permeabilized in 0.1% Triton X-100 in PBS for 10 min. Primary and secondary antibody incubations (1 h at room temperature) were followed by PBS washings. The following primary antibodies were used: rabbit anti-HA polyclonal antibody (1:1000, Clontech) and mouse anti-Flag M5 monoclonal antibody (1:10000, Sigma-Aldrich Co.). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse and tetramethyl-rhodamine goat anti-rabbit conjugates (Amersham Pharmacia Biotech) were used at a 1:400 dilution. Coverslips were mounted in Citifluor (Citifluor Ltd, Cambridge, UK) and cells photographed with an Olympus BX60 microscope.

RNA isolation

Total RNA was extracted from telencephalon of 22 weeks gestation DS and non-DS aborted fetuses using the RNeasy Kit (Qiagen GmbH, Hilden, Germany). In both cases, the RNA was a mixture from two individuals. Tissue was obtained in accordance with Institutional guidelines and for all the cases the post-mortem period was <6 h. Human astrocytoma U-373 MG and human neuroblastoma SH-SY5Y cell lines were grown in DMEM supplemented with 10% FCS and RNA purified with TriPure Reagent (Roche Diagnostics). Treatments were as follows: Io, 2 h in the presence of calcium ionophore A23187 (2 μ M) and CaCl₂ (10 mM); PMA, 2 h with DMEM containing 20 ng/ml PMA. Cyclosporin A (CsA, Sandoz) was added 15 min prior to the calcium stimulation at a final concentration of 5 μ g/ml. Northern blots were done following standard procedures. Membranes were prehybridized in 50% formamide/6 \times SSC/5% dextran sulfate/1% SDS/5 \times Denhardt solution for 2 h at 42°C. Hybridization was performed in the same buffer for 16 h at 42°C, with a full-length DSCR1 probe labeled by random priming. Blots were washed at a final stringency of 0.2 \times SSC/0.1% SDS at 42°C. The filters were exposed to X-ray film for 5–10 days at –80°C. Autoradiographies were analyzed using Phoretix 1D software (Nonlinear Dynamics Ltd, Newcastle Upon Tyne, UK).

ACKNOWLEDGEMENTS

We are grateful to J. Farreras, M. Dierssen and M. Pritchard for providing RNA from DS and non-DS fetuses, G. Crabtree and J.M. Redondo for plasmids, Fujisawa USA Inc. for gifts of FK506, C. Pucharcos for computer assistance and M.L. Arbonés for critical reading of the manuscript. This research was supported by grants of the European Union (BIOMED2 BMH4-CT98-3039), the Spanish CICYT (SAF99-0092-CO2-01), the Fundació Catalana Síndrome de Down/Marató de TV3-1993 and the National Institutes of Health. L.G. and S.L. are supported by the Spanish Ministry of Education and Science.

REFERENCES

- Epstein, C.J. (1995) Down Syndrome (Trisomy 21). In Scriber, C.R., Beaudet, A.L., Sly, W.S. and Vaile, D. (eds), *The Metabolic and*

Molecular Basis of Inherited Diseases. McGraw-Hill, Inc., New York, Vol. 1, pp. 749–794.

- Antonarakis, S.E. (1998) 10 years of Genomics, chromosome 21, and Down syndrome. *Genomics*, **51**, 1–16.
- Fuentes, J.J., Pritchard, M.A., Planas, A.M., Bosch, A., Ferrer, I. and Estivill, X. (1995) A new human gene from the Down syndrome critical region encodes a proline-rich protein highly expressed in fetal brain and heart. *Hum. Mol. Genet.*, **4**, 1935–1944.
- Fuentes, J.J., Pritchard, M.A. and Estivill, X. (1997) Genomic organization, alternative splicing, and expression patterns of the *DSCR1* (Down syndrome candidate region 1) gene. *Genomics*, **44**, 358–361.
- Carrión, A.M., Link, W.A., Ledo, F., Mellstrom, B. and Naranjo, J.R. (1999) DREAM is a Ca²⁺-regulated transcriptional repressor. *Nature*, **398**, 80–84.
- Chawla, S., Hardingham, G.E., Quinn, D.R. and Bading, H. (1998) CBP: a signal-regulated transcriptional coactivator controlled by nuclear calcium and CaM kinase IV. *Science*, **281**, 1505–1509.
- Crabtree, G.R. (1999) Generic signals and specific outcomes: signaling through Ca²⁺, calcineurin, and NF-AT. *Cell*, **96**, 611–614.
- Rao, A., Luo, C. and Hogan, P.G. (1997) Transcription factors of the NFAT family: regulation and function. *Annu. Rev. Immunol.*, **15**, 707–747.
- Molkentin, J.D., Lu, J.R., Antos, C.L., Markham, B., Richardson, J., Robbins, J., Grant, S.R. and Olson, E.N. (1998) A calcineurin-dependent pathway for cardiac hypertrophy. *Cell*, **93**, 215–228.
- Musaro, A., McCullagh, K.J., Naya, F.J., Olson, E.N. and Rosenthal, N. (1999) IGF-1 induces skeletal myocyte hypertrophy through calcineurin in association with GATA-2 and NF-ATc1. *Nature*, **400**, 581–585.
- Semsarian, C., Wu, M.J., Ju, Y.K., Marciniak, T., Yeoh, T., Allen, D.G., Harvey, R.P. and Graham, R.M. (1999) Skeletal muscle hypertrophy is mediated by a Ca²⁺-dependent calcineurin signalling pathway. *Nature*, **400**, 576–581.
- Chin, E.R., Olson, E.N., Richardson, J.A., Yang, Q., Humphries, C., Shelton, J.M., Wu, H., Zhu, W., Bassel-Duby, R. and Williams, R.S. (1998) A calcineurin-dependent transcriptional pathway controls skeletal muscle fiber type. *Genes Dev.*, **12**, 2499–2509.
- de la Pompa, J.L., Timmerman, L.A., Takimoto, H., Yoshida, H., Elia, A.J., Samper, E., Potter, J., Wakeham, A., Marengere, L., Langille, B.L. et al. (1998) Role of the NF-ATc transcription factor in morphogenesis of cardiac valves and septum. *Nature*, **392**, 182–186.
- Ranger, A.M., Grusby, M.J., Hodge, M.R., Gravallese, E.M., De la Brousse, F.C., Hoey, T., Mickanin, C., Baldwin, H.S. and Glimcher, L.H. (1998) The transcription factor NF-ATc is essential for cardiac valve formation. *Nature*, **392**, 186–189.
- Ho, I.C., Kim, J.H., Rooney, J.W., Spiegelman, B.M. and Glimcher, L.H. (1998) A potential role for the nuclear factor of activated T cells family of transcriptional regulatory proteins in adipogenesis. *Proc. Natl Acad. Sci. USA*, **95**, 15537–15541.
- Klee, C.B., Ren, H. and Wang, X. (1998) Regulation of the calmodulin-stimulated protein phosphatase calcineurin. *J. Biol. Chem.*, **273**, 13367–13370.
- Kissinger, C.R., Parge, H.E., Knighton, D.R., Lewis, C.T., Pelletier, L.A., Tempczyk, A., Kalish, V.J., Tucker, K.D., Showalter, R.E., Moomaw, E.W. et al. (1995) Crystal structures of human calcineurin and the human FKBP12-FK506-calcineurin complex. *Nature*, **378**, 641–644.
- Griffith, J.P., Kim, J.L., Kim, E.E., Sintchak, M.D., Thomson, J.A., Fitzgibbon, M.J., Fleming, M.A., Caron, P.R., Hsiao, K. and Navia, M.A. (1995) X-ray structure of calcineurin inhibited by the immunophilin-immunosuppressant FKBP12-FK506 complex. *Cell*, **82**, 507–522.
- Fields, S. and Song, O. (1989) A novel genetic system to detect protein-protein interaction. *Nature*, **340**, 245–246.
- Guerini, D. and Klee, C.B. (1989) Cloning of human calcineurin A: evidence for two isozymes and identification of a polyproline structural domain. *Proc. Natl Acad. Sci. USA*, **23**, 9183–9187.
- Mizayaki, T., Kanou, Y., Murata, Y., Ohmori, S., Niwa, T., Maeda, K., Yamamura, H. and Seo, H. (1996) Molecular cloning of a novel thyroid hormone-responsive gene, *ZAK1-4*, in human skin fibroblasts. *J. Biol. Chem.*, **271**, 14567–14571.
- Beals, C.R., Clipstone, N.A., Ho, S.N. and Crabtree, G.R. (1995) Nuclear localization of NF-ATc by a calcineurin-dependent, cyclosporin-sensitive intramolecular interaction. *Genes Dev.*, **11**, 824–834.
- Masuda, E.S., Liu, J., Imamura, R., Imai, S.I., Arai, K.I. and Arai, N. (1997) Control of NFATx1 nuclear translocation by a calcineurin-regulated inhibitory domain. *Mol. Cell. Biol.*, **17**, 2066–2075.

24. Northrop, J.P., Ullman, K.S. and Crabtree, G.R. (1993) Characterization of the nuclear and cytoplasmic components of the lymphoid-specific nuclear factor of activated T cells. *J. Biol. Chem.*, **268**, 2917–2923.
25. Jain, J., McCaffrey, P.G., Miner, Z., Kerppola, T.K., Lambert, J.N., Verdine, G.L., Curran, T. and Rao, A. (1993) The T-cell transcription factor NFATp is a substrate for calcineurin and interacts with Fos and Jun. *Nature*, **365**, 352–355.
26. Deng, T. and Karin, M. (1993) JunB differs from c-Jun in its DNA binding and dimerization domains, and represses c-Jun by formation of inactive heterodimers. *Genes Dev.*, **7**, 479–490.
27. Görlich, D. and Mattaj, I.W. (1996) Nucleocytoplasmic transport. *Science*, **271**, 1513–1518.
28. Crawford, D.R., Leahy, K.P., Abramova, N., Lan, L., Wang, Y. and Davies, K.J. (1997) Hamster adapt78 mRNA is a Down syndrome critical region homologue that is inducible by oxidative stress. *Arch. Biochem. Biophys.*, **342**, 6–12.
29. Korenberg, J.R., Chen, X.N., Schipper, R., Sun, Z., Gonsky, R., Gerwehr, S., Carpenter, N., Daumer, C., Dignan, P., Distech, C. *et al.* (1994) Down syndrome phenotypes: the consequences of chromosomal imbalance. *Proc. Natl Acad. Sci. USA*, **91**, 4997–5001.
30. Kashishian, A., Howard, M., Loh, C., Gallatin, M., Hoekstra, M.F. and Lai, Y. (1998) AKAP79 inhibits calcineurin through a site distinct from the immunophilin binding region. *J. Biol. Chem.*, **273**, 27412–27419.
31. Jiang, B. and Cyert, M.S. (1999) Identification of a novel region critical for calcineurin function *in vivo* and *in vitro*. *J. Biol. Chem.*, **274**, 18543–18551.
32. Lai, M.M., Burnett, P.E., Wolosker, H., Blackshaw, S. and Snyder, S.H. (1998) Cain, a novel physiologic protein inhibitor of calcineurin. *J. Biol. Chem.*, **273**, 18325–18331.
33. Sun, L., Woun, H.-D., Loh, C., Stolow, M., He, W. and Liu, J.O. (1998) Cabin 1, a negative regulator for calcineurin signaling in T lymphocytes. *Immunity*, **8**, 703–711.
34. Aramburu, J., Garcia-Cózar, F., Raghavan, A., Okamura, H., Rao, A. and Hogan, P.G. (1998) Selective inhibition of NFAT activation by a peptide spanning the calcineurin targeting site of NFAT. *Mol. Cell*, **1**, 627–637.
35. Brillantes, A.B., Ondrias, K., Scott, A., Kobrinsky, E., Ondriasova, E., Moschella, M.C., Jayaraman, T., Landers, M., Ehrlich, B.E. and Marks, A.R. (1994) Stabilization of calcium release channel (ryanodine receptor) function by FK506 binding protein. *Cell*, **77**, 513–523.
36. Cardenas, M.E., Hemenway, C., Muir, R.S., Ye, R., Fiorentino, D. and Heitman, J. (1994) Immunophilins interact with calcineurin in the absence of exogenous immunosuppressive ligands. *EMBO J.*, **13**, 5944–5957.
37. Cameron, A.M., Steiner, J.P., Roskams, A.J., Ali, S.M., Ronnett, G.V. and Snyder, S.H. (1995) Calcineurin associated with the inositol 1, 4, 5-trisphosphate receptor-FKBP12 complex modulates Ca²⁺ flux. *Cell*, **83**, 463–472.
38. Klauck, T.M., Faux, M.C., Labudda, K., Langeberg, L.K., Jaken, S. and Scott, J.D. (1996) Coordination of three signaling enzymes by AKAP79, a mammalian scaffold protein. *Science*, **271**, 1589–1592.
39. Miskin, J.E., Abrams, C.C., Goatley, L.C. and Dixon, L.K. (1998) A viral mechanism for inhibition of the cellular phosphatase calcineurin. *Science*, **281**, 562–565.
40. Lin, X., Sikkink, R.A., Rusnak, F. and Barber, D.L. (1999) Inhibition of calcineurin phosphatase activity by a calcineurin B homologous protein. *J. Biol. Chem.*, **274**, 36125–36131.
41. Garcia-Cozar, F.J., Okamura, H., Aramburu, J.F., Shaw, K.T.Y., Pelletier, L., Showalter, R., Villafranca, E. and Rao, A. (1998) Two-site interaction of nuclear factor of activated T cells with activated calcineurin. *J. Biol. Chem.*, **273**, 23877–23883.
42. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
43. de la Luna, S., Allen, K.E., Mason, S. and La Thangue, N.B. (1999) Integration of a growth-suppressing BTB/POZ domain protein with the DP component of the E2F transcription factor. *EMBO J.*, **18**, 212–228.
44. Shikama, N., Lee, C.W., France, S., Delavaine, L., Lyon, J., Krstic-Demonacos, M. and La Thangue, N.B. (1999) A novel cofactor for p300 that regulates the p53 response. *Mol. Cell*, **4**, 365–376.