Phosphorylation of calcipressin 1 increases its ability to inhibit calcineurin and decreases calcipressin half-life

Lali GENESCÀ*, Anna AUBAREDA†, Juan J. FUENTES‡, Xavier ESTIVILL*, Susana DE LA LUNA*‡ and Mercè PÉREZ-RIBA†¹

*Genes and Disease Program, Centre de Regulació Genòmica-CRG, Passeig Marítim 37-49, 08003-Barcelona, Spain, †Medical and Molecular Genetics Centre, Institut de Recerca Oncològica-IRO, Gran Via s/n Km 2.7, 08907-Hospitalet de Llobregat, Barcelona, Spain, and ‡Institució Catalana de Recerca i Estudis Avançats-ICREA, Barcelona, Spain

Calcipressin 1 is an endogenous inhibitor of calcineurin, which is a serine/threonine phosphatase under the control of Ca^{2+} and calmodulin. Calcipressin 1 is encoded by *DSCR1*, a gene on human chromosome 21 with seven exons, exons 1–4 are alternative first exons (isoforms 1–4). We show that calcipressin 1 isoform 1 has an N-terminal coding region longer than that previously described, and this generates a new polypeptide of 252 amino acids. This polypeptide is able to interact with calcineurin A and to inhibit NF-AT-mediated transcriptional activation. We demonstrate for the first time that endogenous calcipressin 1 exists as a complex together with the calcineurin A and B heterodimer. Calcipressin 1 is a phosphoprotein that increases its capacity to inhibit calci-

neurin when phosphorylated at the FLISPP motif, and this phosphorylation also controls the half-life of calcipressin 1 by accelerating its degradation. Additionally, we have also detected further phosphorylation sites outside the FLISPP motif and these contribute to the complex phosphorylation pattern of calcipressin 1. Taking all these results into consideration we suggest that phosphorylation of calcipressin 1 is involved in the regulation of the phosphatase activity of calcineurin and can therefore act as a modulator of calcineurin-dependent cellular pathways.

Key words: calcineurin endogenous inhibitor, calcipressin, *DSCR1*, FLISPP motif, NF-AT, PP-2B.

INTRODUCTION

Calcineurin, or protein phosphatase PP2B, the only known serine/threonine phosphatase under the control of calcium and CaM (calmodulin), is involved in the coupling of Ca^{2+} signals from membrane to cellular responses [1]. This phosphatase is a heterodimer of a 58–64 kDa catalytic subunit, CnA (calcineurin A), tightly bound to a 19 kDa regulatory subunit, CnB (calcineurin B) [2]. This heterodimeric structure, unique among protein phosphatases, is essential for its activity and is highly conserved from yeast to man. CnA comprises an N-terminal catalytic domain, followed by a regulatory region containing binding sites for CnB and CaM and, at the C-terminus, an autoinhibitory domain that becomes displaced on CaM binding [1].

Calcineurin is a key enzyme of the cellular Ca^{2+} -sensitive signalling cascades, which acts mainly through the control of phosphorylation/dephosphorylation of transcription factors. One of these pathways is implicated in the calcineurin-dependent regulation of phosphatase 1 activity that modulates CREB phosphorylation and therefore CREB-dependent transcriptional activation [3]. Transcription can also be regulated directly by dephosphorylation of the NF-AT (nuclear factor of activated T cells) and the MEF2 (myocyte enhancer factor 2) transcription factor families. In the first case, dephosphorylation by calcineurin unmasks nuclear localization signals in NF-AT members and triggers their translocation from cytoplasm to nucleus where they bind to DNA co-operatively with other transcription factors, activating several programs of gene expression [4]. In the case of MEF2, three calcineurin-dependent regulatory mechanisms converge. Calcineurin has been shown to enhance the DNA binding activity of MEF2A,

thereby increasing its transcriptional activity [5]. It has also been reported that it activates MEF2D indirectly through NF-ATc2, which forms a ternary complex with p300 bound to a MEF2-responsive element [6]. Finally, dephosphorylation of MEF2D protects it from caspase-mediated cleavage [7]. By using these targets, calcineurin controls a wide variety of physiological processes, such as hippocampal long-term potentiation and long-term depression [8], lymphocyte activation [9] and apoptosis [10], development of the heart [11], differentiation of skeletal muscle fibre type [12] and cardiac functions [13]. Functional effects of calcineurin via substrates other than transcription factors have also been described. Calcineurin has been shown to be involved in several processes, including presynaptic and postsynaptic regulation of glutamate receptor channels [14], FKBP12-mediated regulation of InsP_3 receptor [15] and intracellular trafficking [16], amongst others.

Given these many regulatory functions of calcineurin it is not surprising that different classes of endogenous inhibitors have been discovered in the last few years. AKAP79 (A-Kinase Anchoring Protein-79) was the first calcineurin inhibitor to be described and it works as a scaffolding protein anchoring calcineurin to the membrane together with protein kinases A and C [17]. Cain/Cabin 1, is a non-competitive inhibitor that has been found primarily in the nucleus of T cells and seems to interact with activated calcineurin that has translocated to the nucleus [18,19]. Cain/Cabin 1 also associates with MEF2, CaM and the mSin3 co-repressor regulating MEF2 transcription in a calcineurin non-dependent way [20]. The CHP protein (CnB homologous protein or p22) appears to compete with CnB for binding to CnA and thereby prevents the Ca^{2+} -dependent activation of calcineurin

Abbreviations used: AP, alkaline phosphatase; β -gal, β -galactosidase; CALP, calcipressin; CALP1L, CALP 1-Long; CALP1S, CALP 1-Short; CaM, calmodulin; CnA, calcineurin A; CnB, calcineurin B; 2D-GE, two-dimensional gel electrophoresis; DTT, dithiothreitol; GST, glutathione S-transferase; HA, haemagglutinin; IEF, isoelectric focusing; IAM, iodoacetamide; MEF2, myocyte enhancer factor 2; wt, wild-type; NF-AT, nuclear factor of activated T-cells.

¹ To whom correspondence should be addressed (e-mail mpr@iro.es).

The nucleotide sequence data reported for the human and murine CALP1L encoding gene (*DSCR1*) have been submitted to the EMBL, GenBank®, DDBJ and GSDB Nucleotide Sequence Databases under the accession numbers AY325903 and AY325904 respectively.

[21]. A particular case of a calcineurin inhibitor is the A238L protein encoded by the African Swine Fever virus. A238L shares sequence similarity with the calcineurin interaction domain of NF-AT family members blocking their function and allowing the virus to evade the host immune system [22]. Finally, there is a family of inhibitors conserved from yeast to mammals that has been named the calpessins (CALPs) [23–25]. In humans there are three members: CALP1 (also known as MCIP-1 and Adapt78), the protein product of the *DSCR1* gene [23,26,27]; CALP2 (also known as MCIP-2 and ZAKI-4), the protein product of the *DSCR1L1* gene [26,28]; and CALP3, the protein product of the *DSCR1L2* gene [29]. All members of the family share a highly conserved central region containing a novel consensus motif Phe-Leu-Ile-Ser-Pro-Pro-Xaa-Ser-Pro-Pro (hereafter named FLISPP motif).

The gene encoding CALP1, *DSCR1*, was identified in our laboratory in a human chromosome 21 gene screening [30] and it is highly expressed in the central nervous system, heart and skeletal muscle [31,32]. The gene comprises seven exons, exons 1 to 4 being alternative first exons [31]. Although transcripts containing exons 1 to 4 are detected in many tissues, the exon-1-containing mRNA is most abundant in foetal and adult brain and, the exon-4-containing transcript is most abundant in placenta, skeletal muscle and kidney [31]. CALP1 has been shown to interact physically and functionally with CnA, inhibiting both NF-AT and MEF2 signalling pathways [23,26]. Moreover, *DSCR1* expression is induced by calcium through a calcineurin-dependent mechanism [23,33], indicating that *DSCR1* could function as a feedback inhibitor of calcineurin.

Here, we describe a new CALP1 isoform 1 (CALP 1-Long or CALPIL), extended in its N-terminal region, that forms part of the calcineurin heterodimer complex in physiological conditions. Moreover, CALPIL is a phosphoprotein with both serines within the FLISPP motif and other sites outside it capable of being phosphorylated *in vivo*. Furthermore, we have been able to correlate serine phosphorylation at this motif with an increase on calcineurin-mediated transcriptional inhibition and with the control of CALP1 half-life.

EXPERIMENTAL

Sequence analysis and plasmid construction

A novel 5'-extended exon 1 for the human *DSCR1* gene was identified by *in silico* analysis. As a query for homology searches using the BLAST programs [34] for non-redundant and EST (expressed sequence tag) databases of the NCBI (National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov>) a mouse EST (Accession no. AA734360) was used. The presence of a 5'-extended exon 1 on human *DSCR1* gene was confirmed by PCR amplification in the presence of 10 % (v/v) DMSO with specific primers (forward, 5'-CTGATGGAGGACGGCGTGGCCGG-3'; reverse, 5'-GGTCCAGGTGACAGGCGATGG-3') from a human heart cDNA library (Clontech, Palo Alto, CA, U.S.A.), cloned into pGEM-T-Easy (Promega, Madison, WI, U.S.A.) and sequenced. The full-length CALPIL expression plasmid was made by substituting the new 5'-end into pcDNA3-HA1 CALP1 exon 1 [23]. The single serine (TCC and TCT) to alanine (GCC) mutants were generated by PCR mutagenesis with the primers S1 [5'-TGATC-(GCC)CCTCCCGCCTCT-3'] and S2 [5'-GATCTCCCTCCCGCC(GCC)CCG-3']. The single serine to glutamic (GAG) mutant was generated with primer S3 [5'-GTTTCTGATC(GAG)CCTCCGCCTCTCC-3']. The double mutants were generated with primers S4 [5'-GTTTCTGATC(GCC)CCTCCCGCC(GCC)CCGCCAGTGGGATGG-3'] and S5 [5'-GTTTCTGATC(GAG)-

CCTCCCGCC(GAG)CCGCCAGTGGGATGG-3']. All mutants were subcloned as N-terminal HA (haemagglutinin)-tagged fusions in pcDNA3-HA1. For Western blot competition experiments, a pGEX-5X-1 bacterial expression vector (Amersham Biosciences, U.K.) was used to construct a plasmid encoding the last 38 amino acids of the CALP1 C-terminus fused to glutathione S-transferase (GST-C38). Plasmids pBJ5-CnA and pBJ5-CnB were kindly provided by Dr. J. Heitman (Department of Genetics, Duke University Medical Center, Durham, NC, U.S.A.). All the constructions were checked by DNA sequencing with a Big Dye terminator Cycle Sequencing-ready Reaction kit (Perkin Elmer, Foster City, CA, U.S.A.).

Cell culture, transfection and cell treatments

The human neuroblastoma SH-SY5Y cell line was purchased from E.C.A.C.C. (European Collection of Cell Cultures, Porton Down, Salisbury, U.K.) and grown in Ham's F12: MEM (1:1, v/v) (Invitrogen Corporation) supplemented with 15 % (v/v) heat-inactivated foetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine and non-essential amino acids. COS-7 cells were obtained from the E.C.A.C.C. and CHO cells were a gift of Dr. J. Barquinero (Unitat de Recerca, Centre de Transfusió i Banc de Teixits, Barcelona, Spain). Both cell lines were grown in DMEM (Dulbecco's modified Eagles's medium; Invitrogen) supplemented with 5 % (v/v) foetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine.

Transient transfection of SH-SY5Y cells was carried out by electroporation. Usually, 10×10^6 cells resuspended in 0.8 ml of PBS were placed into an electroporation cuvette (0.4-cm gap, Bio-Rad) and mixed with 40 µg of plasmid DNA. Cells were exposed to an electric field of 286 V/750 µF (Gene Pulse II, Bio-Rad), and seeded in a 100-mm dish. Transient transfection of COS-7 and CHO cells was carried out by the calcium phosphate precipitation method as described previously [35]. For all the cases, cells were harvested in PBS 48 h after transfection.

For the half-life measurements, a pool of electroporated SH-SY5Y cells (2×10^7) was split between four 60-mm dishes and treated with 100 µM cycloheximide (Sigma-Aldrich) for the indicated times. In some cases, cells were incubated with the proteasome inhibitor MG 132 (Sigma-Aldrich) for 3 h at a final concentration of 20 µM. Treatments were stopped by harvesting the cells in PBS.

Preparation and characterization of anti-CALP1 antibody

A rabbit polyclonal antiserum (anti-CALP1) was raised against the C-terminal CALP1 peptide Arg-Arg-Pro-Lys-Pro-Lys-Ile-Ile-Gln-Thr-Arg-Arg-Pro-Glu-Tyr-Thr (Eurogentec, Ougre, Belgium). For competition experiments, GST and GST-C38 were expressed in *Escherichia coli* BL21(DE3)pLysS by inducing cell cultures with isopropyl-1-thio-β-D-galactoside (0.1 mM) for 3 h at 20 °C and purified according to manufacturer's instructions.

Western blot analysis

Total cell extracts were prepared by resuspension in Laemmli's sample buffer and heating for 15 min at 98 °C. For the preparation of soluble extracts, cells were resuspended in a lysis buffer containing 50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.5 mM EDTA, 1 % Triton-X 100, 10 mM sodium pyrophosphate (Na₂PPI), 10 mM iodoacetamide (IAM), 1 mM sodium vanadate, 1 mM PMSF and a protease inhibitor cocktail (Roche

Diagnostics, IN, U.S.A.). Mouse brain was homogenized in the same lysis buffer (0.2 g-wet weight/ml) with a loose fitting Dounce homogenizer, and sonicated twice for 5 s at middle intensity.

All the lysates were solubilized in a rotary shaker for 30 min at 4 °C and cell debris were removed by centrifugation at 13 000 *g* for 10 min at 4 °C. Protein concentration was measured by a bicinchoninic acid assay (BCA Protein Assay Kit, Pierce, Rockford, IL, U.S.A.). Samples were resolved, unless indicated, on SDS/10 % polyacrylamide gels and transferred on to Hybond-C Extra nitrocellulose membranes (Amersham Biosciences).

The following primary antibodies were used: anti-HA mouse monoclonal antibody HA.11 (Covance BabCo; diluted 1:2000, incubated for 1 h at room temperature), anti-CALP1 rabbit polyclonal antibody (diluted 1:2000, incubated overnight at 4 °C), anti-CnA mouse monoclonal antibody (BD-PharMingen; diluted 1:500, incubated overnight at 4 °C), anti-CnB goat polyclonal antibody (Santa Cruz Biotechnology; diluted 1:500, incubated overnight at 4 °C), anti-actin rabbit polyclonal antibody (Sigma-Aldrich; diluted 1:2000, incubated for 1 h at room temperature).

Immunoprecipitation

Anti-CnA mouse monoclonal antibody (1.5 µg) was pre-bound to Protein G beads (Amersham Biosciences) in lysis buffer for 1 h at room temperature. The pelleted beads were incubated with soluble protein extracts (500 µg) in a rotary shaker for 1 h at 4 °C, collected by centrifugation, washed three times with lysis buffer, and resuspended in 2 × Laemmli's sample buffer. For the CnA co-immunoprecipitations lysis buffer was supplemented with 2 mM CaCl₂, which was maintained for all the procedure.

Phosphatase assay

Pellets from COS-7 cells (0.5×10^6) were resuspended in 100 µl of phosphatase buffer [100 mM Hepes, pH 7.4, 10 mM potassium acetate, 2 mM magnesium acetate, 2 mM EGTA, 2 mM PMSF, 0.05 % IGEPAL (Sigma-Aldrich), a protease inhibitor cocktail and 10 mM IAM] for 10 min on ice and centrifuged at 800 *g* at 4 °C for 5 min. The supernatant was then supplemented with 100 mM NaCl and 10 mM DTT (dithiothreitol) to neutralize unreacted IAM. Typically, a 50 µl mixture containing 12.5 µl of cell extract and 5 units of AP (alkaline phosphatase) from bovine intestinal mucosa (Sigma-Aldrich) was incubated for 30 min at 30 °C. The inhibitor, NaPPI (sodium pyrophosphate), pre-incubated with AP for 30 min at 4 °C was at a final concentration of 20 mM. Reactions were terminated by addition of 10 µl of 6 × SDS Laemmli's sample buffer and boiled for 5 min.

2D-GE (Two-dimensional gel electrophoresis)

Transfected COS-7 cells were harvested in 50 mM Tris/HCl, pH 7.5, and frozen at −80 °C until needed. Samples were resuspended in 9 M urea, 50 mM Tris/HCl, pH 7.5 and 4 % CHAPS (2D-GE lysis buffer), sonicated once for 5 s at middle intensity and centrifuged at 13 000 *g* for 20 min. The first dimension IEF (isoelectric focusing) was performed using 30–40 µg of protein in the IPGphor 4–7 system (Amersham Biosciences) according to the manufacturer's instructions. For the second dimension SDS/PAGE, strips were equilibrated in SDS equilibration buffer [50 mM Tris/HCl, pH 8.8, 6 M urea, 30 % (w/v) glycerol, 2 % (w/v) SDS, with a small amount of Bromophenol Blue added] supplemented, just before use, with 10 mg/ml DTT and then replaced with the same buffer

but with 25 mg/ml IAM. Samples were resolved on a vertical SDS/PAGE (10 % gel) and analysed by Western blotting with anti-HA antibody. As a control for positioning wt (wild-type) and mutant CALP1L proteins we used actin-specific spots. Since we could not get the anti-actin antibody to work on Western blots containing COS-7 cell extracts after 2D-GE, we used human Jurkat cell extracts as an actin source. For this, COS-7 samples were mixed with 1×10^6 Jurkat cells at the beginning of the whole procedure.

For the 2D-GE analysis of AP-treated CALP1L, transfected COS-7 cells (0.5×10^6) were resuspended in 250 µl of phosphatase buffer and processed as described above but, in this case, 200 µl of mixture contained 25 µl of cell extract and 10 units of AP. After AP treatment, the extracts were diluted and immunoprecipitated with 5 µg of anti-HA antibody. The pelleted beads were resuspended in 50 µl of 2D-GE lysis buffer and analysed by 2D-GE.

NF-AT activity measurements

CHO cells (0.15×10^6) were transfected with a luciferase reporter plasmid driven by the minimal interleukin-2 promoter (pIL2-luc, kindly provided by Dr. G. Crabtree, Developmental Biology and Department of Pathology, Howard Hughes Medical Institute, Stanford University School of Medicine, Stanford, CA, U.S.A.), the appropriate pcDNA3-HA1-CALP1L vectors and pCMV-βgal as an internal transfection control. When indicated, 10 mM CaCl₂, 1 µM ionomycin, and 10 ng/ml PMA (both from Sigma-Aldrich) were added to the medium 16 h before cell harvesting. FK506 (20 ng/ml) was added 8 h before the calcium stimulation for calcineurin specific inhibition. Cells were harvested in PBS 36 h post-transfection, lysed in 200 µl of Reporter Lysis buffer (Promega) and centrifuged at 10 000 *g* for 5 min to prepare soluble extracts. β-gal (β-galactoside) and luciferase activity measurements and Western blot analysis were performed on the same extract. Quantification of Western blot bands was performed using the Quantity One Program (Bio-Rad).

RESULTS

DSCR1 exon 1 has an extended coding region

We have isolated a novel human *DSCR1* 5'-extended exon 1 by *in silico* analysis that was confirmed by PCR experiments on a human heart cDNA library (described in the Experimental section). The predicted protein product was 55 amino acids longer at the N-terminus (results not shown) than the one previously described (Accession no. U85266), indicating that the *DSCR1* exon 1-containing mRNA has the capacity to encode a protein of 252 amino acids instead of the 197 amino acids already described [31]. Similarly, a longer protein product is predicted for a mouse *Dscr1* exon-1 containing cDNA (results not shown).

In view of these results, and following the nomenclature of the CALP family [23–25], we proposed to name the protein product of *DSCR1* transcript containing exon 1 CALP1L, and the protein product of *DSCR1* mRNA containing exon 4, with 197 amino acids, as CALP 1-Short (CALP1S). The same expression behaviour, which is the ability to produce both a long (CALP2L, 243 amino acid) and a short (CALP2S, 197 amino acid) protein isoform, has been recently described for CALP2 (ZAKI-4) [36]. Alignment of CALP2L cDNA sequence with the genomic sequence of human chromosome 6, where CALP2 encoding gene (*DSCR1L1*) maps, indicated that the cDNA corresponded to an alternative spliced product. This mRNA would

Figure 1 Endogenous CALP1L is present in mouse brain extracts and neuronal cell lines, and associates *in vivo* with calcineurin

(A) Western blot analysis of soluble cell extracts (30 µg) of SH-SY5Y cells transfected with the HA-CALP1L construct (lane 1 and 3, +), or mock transfected (lane 2 and 4, -) and analysed with the anti-HA or with the anti-CALP1 antibodies, as indicated. The position of the molecular mass markers is shown. (B) Western blot analysis of mouse whole brain extracts (40 µg) with anti-CALP1 antibody alone (3 µg, lane 1, Ab), anti-CALP1 antibody incubated with purified GST-C38 (3 µg and 6 µg respectively; lane 2, Ab + GST-C38), and anti-CALP1 antibody and GST (3 µg and 6 µg respectively, lane 3, Ab + GST). The asterisk indicates a cross-reacting band that is not competed. (C) Western blot analysis of immunoprecipitated complexes with anti-CnA antibody from SH-SY5Y soluble cell extracts (1 mg). Samples were subjected to SDS/PAGE (15% gel) and analysed by Western blot with the anti-CALP1 antibody (WB αCALP1), anti-CnA antibody (WB αCnA), and with an anti-CnB antibody (WB αCnB). Lane 1 corresponds to the input (100 µg), lane 2 shows the immunoprecipitate (IP), and lane 3 shows the flow-through (FT). The asterisks in the WB αCnA and the WB αCALP1 panels indicate the immunoglobulin heavy chain and a non-specific band identified by the anti-CALP1 antibody in SH-SY5Y cells respectively. This electrophoretic band is not present in (A) owing to the differences in the amounts of protein loaded.

appear when a more distal 5'-exon is used, in a similar way to what happens with the *DSCR1* gene. The comparison of the N-terminal amino acid sequence of the three members of the mammalian CALP family shows that there is a high degree of similarity between the mouse and human proteins for each member of the family as also happens when the rest of the protein is considered (results not shown). In contrast, the homology observed among the three human members in the C-terminus, more than 60% of identity (101 amino acids/153 amino acids), is lost at the N-terminus (results not shown).

Anti-CALP1 antibody recognizes a 34 kDa protein that is associated with calcineurin *in vivo*

The new full-length coding region of CALP1L was cloned in-frame with two HA epitope tags to allow the detection of exogenous CALP1L with an anti-HA antibody and its expression was assayed by Western blot of soluble cell extracts in transfected SH-SY5Y cells. An electrophoretic mobility band of around 37 kDa was detected with both the anti-HA antibody (Figure 1A, lane 1), recognizing the N-terminus of the exogenously expressed protein and with an antibody raised against the C-terminal region of

CALP1 (anti-CALP1) (Figure 1A, lane 3). In order to characterize the endogenous CALP1L protein, and given that exon-1-containing mRNA is abundantly expressed in brain, we analysed mouse brain extracts by Western blot with the antibody raised against CALP1 (Figure 1B). The antibody recognized an electrophoretic band above 30 kDa that might represent endogenous CALP1L (Figure 1B, lane 1). To confirm these results, we performed a competition experiment in which bacterially expressed C-terminal GST-CALP1 fusion protein (GST-C38) (Figure 1B, lane 2) or GST alone (Figure 1B, lane 3) was included in the antibody incubation. No antibody reactivity against the 34-kDa protein was observed when the antibody was incubated with GST-C38 (Figure 1B, lane 2), strongly suggesting that the 34 kDa protein corresponded to endogenous CALP1L. A smaller electrophoretic band of less than 30 kDa that probably corresponds to the shorter isoform, CALP1S, could only be detected when using higher amounts of crude extract and longer exposure times (results not shown).

One of the functional properties assigned to CALP1, either the short isoform or the truncated version of the long one, is the ability to interact with CnA when overexpressed in mammalian cells [23,26]. When exogenously expressed, full-length CALP1L also binds to CnA (results not shown and Figure 3), indicating that the new N-terminus is not affecting the interaction. In order to confirm that endogenous CALP1L was also able to interact with CnA in conditions of non-overexpression, we performed a co-immunoprecipitation assay in a human neuronal cell line that expresses CALP1L endogenously (Figure 1C). Soluble extracts from SH-SY5Y cells were immunoprecipitated with an anti-CnA antibody and analysed by Western blot with the anti-CALP1 antibody. The antibody identified a specific protein in the immunoprecipitated complex with the same electrophoretic mobility as CALP1L (Figure 1C, lane 2, WB αCALP1). The immunoprecipitates also contained CnB (Figure 1C, lane 2, WB αCnB), confirming that endogenous CALP1L interacts with calcineurin.

CALP1L is phosphorylated at the FLISPP motif

Western blot analysis of exogenously expressed CALP1L in COS-7 cells showed the protein as two different electrophoretic bands (Figure 2B, lane 1). Both bands were recognized by antibodies directed at the N-terminus (anti-HA) or the C-terminus (anti-CALP1) of the protein (results not shown), indicating that they are not the products of degradation, but most probably of post-translational modifications. Since CALP1S has recently been shown to be phosphorylated *in vitro* [37], we decided to examine whether CALP1L is also a phosphoprotein. To do this we carried out an *in vitro* dephosphorylation assay, where extracts from COS-7 transfected with the HA-CALP1L construct were treated with AP and analysed by Western blot with anti-HA antibody (Figure 2A). As a result of the incubation with AP, the slowest mobility band disappeared with a concomitant increase in the faster mobility band (Figure 2A, compare lanes 1 and 2 with lane 3). The change in migration was abolished if NaPPI, a general phosphatase inhibitor, was present during the incubation (Figure 2A, lane 4). The same results were obtained from transfected SH-SY5Y cells (results not shown). Therefore the data strongly indicate that the slow migrating band corresponds to a phosphorylated form of CALP1L.

With the aim of identifying the phosphorylation sites responsible for the migration shift observed in CALP1L by the AP treatment, we concentrated on the FLISPP motif. We generated two alanine mutants, Ser¹⁶³ → Ala and Ser¹⁶⁷ → Ala, as well as the double mutant, Ser^{163,167} → Ala. Western blot analysis of total cell extracts from COS-7 cells transfected with wt CALP1L and

Figure 2 CALP1L is phosphorylated at the FLISPP motif

(A) Western blot analysis of soluble extracts (10 μ g) from COS-7 cells expressing HA-CALP1L treated with AP alone (5 units AP, lane 3), in the presence of the phosphatase inhibitor sodium pyrophosphate (20 mM NaPPI, lane 4, AP + PPI), or incubated in the absence of the phosphatase (lane 2, -). Lane 1 corresponds to the input sample. (B) Western blot analysis of total cell extracts from COS-7 cells transfected with the constructions expressing wt HA-CALP1L (lane 1), the Ser¹⁶³Ala mutant (lane 2), the Ser¹⁶⁷Ala mutant (lane 3), or the Ser^{163,167}Ala double mutant (lane 4). In (A) and (B), Western blot analysis was performed with the anti-HA antibody. (C) Western blot analysis of 2D-GE from whole-cell extracts of transfected wt and mutant HA-CALP1L cells by using anti-HA (WB α HA). To compare the different 2D-GE patterns, anti-actin was used as spot positioning standard (WB α actin) as described in the Experimental section. Although IEF 18 cm strips of linear pH range 4–7 were used, only pH 4.5–5.5 is shown. Increasing pH on the IEF first-dimension is indicated by an arrow. Cell extracts from the same day were loaded in equal amounts, processed at the same time and the image corresponds to a similar exposure time. (D) This shows the same experiment as (C) but in this case, soluble extracts of wt and Ser^{163,167}Ala CALP1L mutant were incubated without (-) or with AP. This pattern of dephosphorylation was repeated at least three times with identical results. Two different 2D-GE CALP1L patterns are shown (Figure 2C, wt, and 2D, wt*) to visualize differences between cell extracts from different days. The wt* panel (Figure 2D) is overexposed so that all the spots present in its 2D-GE pattern can be observed and thus facilitate the comparison between wt and mutant patterns. The AP-treated double mutant indicates the presence of a unique spot (Figure 2D, Ser^{163,167}Ala AP) even when the film was overexposed (results not shown). Each panel in (C) and (D) represents independent 2D-GE. Western blot anti-HA panels in (C) and (D) are lined up to facilitate interpretation.

the different mutant constructions showed that none of the mutant proteins was able to generate the slowest migrating species (Figure 2B, compare lanes 2–4 with lane 1) and suggested that at least these two serines of the FLISPP motif can be phosphorylated in CALP1L.

To characterize further the phosphorylation state of the CALP1L and mutant proteins we decided to use 2D-GE analysis (Figures 2C and 2D). Although HA-CALP1L has an estimated theoretical pI of 4.9, the 2D-GE CALP1L pattern showed dif-

ferently charged spots within a pI range of 4.2 to 4.9 (Figures 2C, wt, and 2D, wt*), which could be indicative of CALP1L phosphorylation. When the wt phosphorylation pattern was compared with those of the mutant proteins, four different patterns were observed, each of them composed of several differently charged migrating spots (Figure 2C, WB α HA). The pattern of wt CALP1L was clearly different from those obtained with the mutants since it has more acidic additional spots. Moreover, loss of either Ser¹⁶³ and/or Ser¹⁶⁷ led to the loss of the hyperphosphorylated forms of the wt, which suggested that these two serine residues are involved in the phosphorylation pattern of CALP1L (Figure 2C, wt versus mutants). In order to ascertain whether the spots seen in wt CALP1L were all the result of protein phosphorylation, an AP treatment was performed (Figure 2D). The phosphorylation pattern obtained after dephosphorylation showed a decrease in the number of spots of CALP1L to two less acidic spots (Figure 2D, wt, AP versus -). Although slightly different wt 2D-GE patterns were obtained with different cell extracts, reflecting the degree of phosphorylation of CALP1L in the extracts (Figure 2C wt versus Figure 2D wt*), their phosphatase treatment always led to the same 2D-pattern. The two spots might be the result of partial dephosphorylation of the sample or of another unknown post-translational modification of the protein. In this sense, the pattern of the different samples in the 2D-GE analysis (Figure 2C) seems to indicate that there are covalent modifications other than phosphorylation involved in CALP1L. The wt and Ser¹⁶³ → Ala 2D-GE patterns show the presence of a higher pI spot compared with the Ser¹⁶⁷ → Ala and the Ser^{163,167} → Ala patterns (Figure 2C wt and Ser¹⁶³Ala versus Ser¹⁶⁷Ala and Ser^{163,167}Ala); this spot also has a higher pI compared with those appearing after AP treatment of the samples (compare Figures 2C and 2D). To date, we have been unable to identify the nature of this covalent modification. Finally, the AP treatment of the double mutant showed only one spot that we believe should correspond to the completely dephosphorylated form. It is important to emphasize that this spot overlaps with the less acidic spot on AP-treated wt CALP1L (Figure 2D, Ser^{163,167} → Ala AP versus wt AP), suggesting that both spots could represent the same species of the protein and the most dephosphorylated form. All this data leads us to conclude that there is at least one other additional phosphorylated site in CALP1L outside the FLISPP motif that contributes to the phosphorylation pattern of CALP1L.

The FLISPP motif phosphorylation state affects CALP1L ability to inhibit calcineurin

Previous reports showed that the 197 amino acids of the truncated form of CALP1L can block the activation of NF-AT-dependent transcription [23,26]. Thus since CALP1L is a phosphoprotein we decided to test whether the serine mutants in the FLISPP motif could affect the inhibitory activity of CALP1L (Figure 3). As a prior step to answering this question we wondered if changes in the phosphorylation state of CALP1L at the FLISPP motif could affect the interaction between CALP1L and CnA (Figure 3A). We have shown previously that the interaction between CALP1 and CnA is independent of the intracellular concentration of calcium and that the C-terminal region of CALP1 is sufficient for binding to take place [23]. Co-immunoprecipitation experiments with the different serine mutants (Figure 3A) indicate that the mutant proteins are able to interact with calcineurin in a similar way to the wt protein. Thus we decided to focus on the inhibitory activity of CALP1L (Figures 3B, 3C and 3D). Co-expression of wt CALP1L or any of the mutants inhibited the induction of an interleukin-2 reporter by co-stimulation with Ca²⁺ and PMA in a dose-dependent manner (Figure 3B). However, when the amount of the

Figure 3 Inhibition of the transcriptional activation of the interleukin-2 promoter by CALP1L and its mutants in response to calcium/PMA stimulation

(A) Extracts from CHO cells transfected with wt (lane 1), Ser¹⁶³Ala (lane 2), Ser¹⁶⁷Ala (lane 3), or with Ser^{163,167}Ala (lane 4) HA-CALP1L constructions were immunoprecipitated with anti-CnA antibody (lane 5–8). Western blot analysis was performed with the indicated antibodies (WB αHA, WB αCnA). The amount of the transfected DNA was adjusted to obtain similar amounts of each of the protein analysed. (B) Relative luciferase activity (luciferase activity/β-gal activity in each transfected dish) of CHO cells (0.15×10^5) cotransfected with pIL-2luc (0.5 μg) and increasing amounts of the indicated CALP1L plasmids (10, 25 and 100 ng) and with pCMV-βgal (0.5 μg) as an internal control. Cells were treated with 1 μM ionomycin (Io), 10 ng/ml PMA and 20 ng/ml FK506, as indicated. Values represent the average of three independent dishes and standard deviations are shown. The experiment was repeated in triplicate a minimum of three times with identical results. (C) A representative experiment of the Western blot analysis of stimulated CHO cell extracts corresponding to the transfection with 100 ng DNA: wt (lane 1), Ser¹⁶³Ala (lane 2), Ser¹⁶⁷Ala (lane 3), Ser^{163,167}Ala (lane 4). The blot was developed with the indicated antibodies (WB αHA, WB αActin). (D) Relative inhibition exhibited by wt CALP1L and the different mutants. Values represent two independent experiments (white and shaded bars), in which the relative luciferase activity has been corrected for the corresponding CALP1L protein amount quantified by densitometry of a Western blot with anti-HA. The results were plotted as the percentage of inhibition assuming the wt to be 100%.

different CALP1L proteins expressed was measured by Western blot (Figure 3C), we observed that the protein amount of wt CALP1L (Figure 3C, lane 1) was always lower than that of the mutants (Figure 3C, lanes 2–4). To better quantify the effect of protein accumulation on the inhibition assay, we have represented the results of the experiments as relative inhibition, considering the data obtained with the wt protein as 100% inhibition (Figure 3D). We define relative inhibition as the inhibition (luciferase activity in the presence of CALP1L proteins versus luciferase activity in

its absence) corrected for the amount of expressed CALP1L proteins present in the same transfected extract and quantified by densitometry in a parallel Western blot. Thus the three mutants seem to behave as less efficient inhibitors in this assay, indicating that phosphorylation of both serines is necessary in order to achieve the full inhibitory capacity of the wt protein.

CALP1L half-life depends on the phosphorylation state of the FLISPP motif

As we have shown above, the protein accumulation levels of CALP1L seem to be altered by mutating the serine residues within the FLISPP motif. Since all the constructions share the 5'- and 3'-UTRs and the first codon of the open reading frame (HA-tag), this change was likely to be due to an increase in protein degradation rather than an effect on protein synthesis. Incubation in the presence of the proteasome inhibitor MG 132 for 3 h clearly increased the amount of the accumulated CALP1L protein (Figure 4A, lane 2). In contrast, addition of cycloheximide, a protein synthesis inhibitor, decreased the amount of wt CALP1L to undetectable levels (Figure 4A, lane 3). Combination of both inhibitors resulted in the recovery of the protein amount up to the initial levels (Figure 4A, lane 4). These data suggest that CALP1L has a quick turnover in the cell and that the proteasome degradation pathway is involved in this process. To investigate further whether differences in the phosphorylation state of CALP1L affect protein stability, we performed a time-course experiment in the presence of cycloheximide (Figure 4B). With this approach, we have estimated a half-life for wt CALP1L of less than 3 h (Figure 4B, CALP1L, panel 1). Interestingly, the protein amount of Ser¹⁶³ → Ala mutant was unaffected by the same treatment conditions giving a half-life measurement of more than 9 h (Figure 4B, Ser¹⁶³ → Ala, panel 2). Regarding the Ser¹⁶⁷ → Ala and Ser^{163,167} → Ala mutants, their half-lives were longer than that of the wt but did not reach the level of the Ser¹⁶³ → Ala (Figure 4B, Ser¹⁶⁷Ala and Ser^{163,167}Ala versus Ser¹⁶³Ala, lane 2). To exclude the possibility that the changes observed in the wt and mutant proteins stability were the result of a conformational change induced by the substitution of the serines to alanine residues, we generated new mutants in which the serines were mutated to glutamic residues (Figure 4B, Ser¹⁶³Glu and Ser^{163,167}Glu) in an attempt to mimic a phosphorylated residue. Both mutants, Ser¹⁶³ → Glu and Ser^{163,167} → Glu, when assayed in a time-course experiment in the presence of cycloheximide, presented a stability pattern very similar to the wt protein (Figure 4B, compare Ser¹⁶³Glu and Ser^{163,167}Glu versus CALP1L, lanes 2–4). Taken together, these observations suggest that phosphorylation in the FLISPP motif increases the turnover of CALP1L in the cell.

Results from the *Cryptococcus neoformans* and *Saccharomyces cerevisiae* CALP proteins, CBP1 and Rcn1p respectively, suggest that calcineurin could be affecting CALP turnover [24,25]. In order to determine whether overexpression of calcineurin protects wt CALP1L from proteasome-dependent degradation, we transfected SH-SY5Y cells with plasmids expressing wt CALP1L, CnA and CnB, and the protein levels were measured after 3 h of treatment with cycloheximide (Figure 4C). Both endogenous and overexpressed calcineurin were unaffected by the cycloheximide treatment (Figure 4C, WB αCnA). The CALP1L half-life was the same even though calcineurin was overexpressed in the cell (Figure 4, WB αHA). These data suggest that calcineurin-CALP1L interaction was unable to completely prevent the proteasome-dependent degradation of CALP1L.

We have also studied whether serine phosphorylation at the FLISPP motif is also affecting the half-life of CALP1S. Exogenously expressed CALP1S accumulated at lower levels in

Figure 4 CALP1L proteasome-dependent degradation is regulated by phosphorylation and is not prevented by co-expression with calcineurin

(A) Western blot analysis of soluble cell extracts (20 µg) from SH-SY5Y cells transfected with HA-CALP1L and treated with 20 µM MG 132 (lane 2), 100 µM cycloheximide (CHX) (lane 3), or with cycloheximide + MG 132 (lane 4). Lane 1 shows untreated cells. The blot was re-probed with α-CnA as an internal control (WB αCnA). (B) Western blot analysis of soluble cell extracts (20 µg) from SH-SY5Y cells transfected with 10 µg of CALP1L (CALP1L), 5 µg of Ser¹⁶³Ala mutant, 10 µg of Ser¹⁶³Glu mutant, 5 µg of Ser¹⁶⁷Ala mutant, 5 µg of Ser^{163,167}Ala double mutant and with 10 µg of Ser^{163,167}Glu double mutant. Cells were treated with 100 µM cycloheximide for 3 h (lane 2), 6 h (lane 3), and 9 h (lane 4) before harvesting in PBS. (C) SH-SY5Y cells transfected with HA-CALP1L and pCMV-βgal (lane 1 and 2), or co-transfected with HA-CALP1L/pJB-CnA/pJB-CnB/pCMV-βgal (lane 3 and 4) were treated with 100 µM cycloheximide (lanes 2 and 4) for 3 h. Aliquots of the samples (20 µg) were analysed by Western blot with anti-HA antibody (WB αHA) and with anti-CnA antibody (WB αCnA). Measurement of β-gal activity was used as a control of equal transfection efficiencies (results not shown). (D) In the left panel, Western blot analysis of total cell extracts (50 µg) from SH-SY5Y cells transfected with HA-CALP1L (15 µg, lane 1) and HA-CALP1S (15 µg, lane 3) and treated with 20 µM MG 132 (lanes 2 and 4). The blot was re-probed with α-actin as an internal control. In the right panel, Western blot analysis of total cell extracts (75 µg) from SH-SY5Y cells transfected with HA-CALP1S (30 µg, lane 5), Ser¹⁰⁸Ala mutant (30 µg, lane 6) and Ser¹¹²Ala mutant (30 µg, lane 7) together with pCMV-βgal (4 µg) as a transfection control. β-gal activity was measured and is shown at the top of each lane. The blot was re-probed with α-actin as a loading control.

transfected cells than CALP1L (Figure 4D, lane 3 vs lane 1), suggesting a shorter half-life. In fact, CALP1S protein is hardly detectable in mouse heart or brain, tissues where CALP1L is clearly apparent (results not shown). CALP1S half-life is likely to be dependent on the proteasome pathway since its accumulation is increased in the presence of MG 132 (Figure 4D, lane 3 versus lane 4). Finally, and similarly to CALP1L, the phosphorylation state of the two serines within the FLISPP motif seems to contribute to the stability of the short isoform, because CALP1S mutated in either Ser¹⁰⁸ → Ala or Ser¹¹² → Ala accumulates at higher levels than CALP1S wt (Figure 4D, lane 5 versus 6 and 7).

DISCUSSION

The *DSCR1* gene consists of seven exons and gives rise to several mRNA isoforms through the alternative splicing of its 5'-exons. We have previously published the sequence and pattern

of expression of four human *DSCR1* distinct isoforms [31,32]. However, the cDNA species containing the first exon were only partially cloned. In fact, the open reading frame encoded for this mRNA is at least 55 amino acids longer, resulting in a 252-amino-acid polypeptide that we have named CALP1L. The mouse orthologous exon 1 isoform also has the capability of encoding this longer protein. Further confirmation of the existence of this extended isoform comes from the presence of ESTs from other non-mammalian species such as *Xenopus* (Accession no. BG813605) in the databases.

Although we cannot be sure of the exact size of the protein because we do not find an in-frame stop codon upstream of the first methionine, we favour the ATG in the novel sequence as the starting codon, based on the fact that the homology at the amino acid level between the human and mouse CALP1Ls is lost from this position. The antibody we have raised against CALP 1 recognizes a 34 kDa protein in mouse brain extracts and in several human cell lines. This protein migrates very close to the protein which is detected when the 252-amino-acid open reading frame is expressed in cells by transient transfection. Together, all these results strongly support that the 252-amino-acid predicted protein corresponds to the endogenous long isoform of CALP1.

It is important to mention that the strategy of using mutually exclusive alternative exons to generate protein isoforms with different N-terminus is not only used by *DSCR1* but also by the second member of the mammalian gene family of CALPs, *DSCR1L1*. Taking into account intron sizes, it is very likely that in both cases the decision distal/proximal exon is dictated by the use of two distinct promoters. Thus variation in the CALP family is generated not only by the presence of three genes in the human genome, but also by the existence of distinct 5'-coding exons that might modulate the function of these proteins, and different promoters that might be activated upon different stimuli.

To date, all published results regarding the interaction between CALP1 and CnA [23,26] have been obtained from cell extracts in which one or both of the proteins is overexpressed. Here, we show for the first time that endogenous CALP1L is present in calcineurin complexes in physiological conditions. The fact that CnB is present in these complexes at a calcium concentration which guarantees that all CnA is bound to CnB [38], together with our previous results with mammalian three-hybrid experiments [23], suggest that CALP 1 is interacting with heterodimeric calcineurin.

We have shown that CALP1L is a phosphoprotein. Using single and double serine mutants of the FLISPP motif we have identified both Ser¹⁶³ and Ser¹⁶⁷ as phosphorylation sites of CALP1L, as recently described for the corresponding sites in CALP1S isoform [37]. In addition, the 2D-GE analysis shows that other phosphorylation sites outside of the FLISPP motif may contribute to the phosphorylation pattern of CALP1L, but their functional implication is still unknown. Even though the serine residues of the FLISPP motif do mediate the appearance of hyperphosphorylated forms of the wt protein, phosphorylation of the extra site seems not to be dependent on the phosphorylation state of Ser¹⁶³ and Ser¹⁶⁷ because it is present in the double mutant. The FLISPP motif is of great interest given that it is the distinctive feature of all members of the CALP family from yeast to humans. Thus a functional role for the reversible phosphorylation of the serines of the FLISPP motif has been studied in several assays. Both phosphorylated and dephosphorylated forms of CALP1L are able to bind calcineurin. However, phosphorylation of the serine residues within the FLISPP motif seems to improve the ability of CALP1L to inhibit CnA. We propose that this occurs because the phospho-serines within the FLISPP motif might be occupying the active site of the phosphatase, blocking the accessibility of NF-AT or some other substrates.

One of the specific roles of the FLISPP motif we have uncovered is related to the control of CALPIL stability, which is intimately related to its phosphorylation state according to the results of half-life measurements with the serine mutants. When both serine residues of the FLISPP motif are phosphorylated, both CALPIL and CALPIS are quickly degraded. Many proteins are targeted by phosphorylation for ubiquitination, for example phosphorylation of Ser³² and Ser³⁶ convert the inhibitor protein I κ B α to a form susceptible to the action of the ubiquitin ligase complex [39], and degradation of β -catenin is mediated by phosphorylation at Ser³⁷ [40]. Thus it could be possible that phosphorylation at Ser¹⁶³ and Ser¹⁶⁷ (Ser¹⁰⁸ and Ser¹¹² in the short isoform) works as a signal for ubiquitination, triggering the CALPIL proteasome-dependent degradation. On the other hand, we have shown that the opposite effect, dephosphorylation at serines in the FLISPP motif, correlates with protein stabilization with a higher contribution of the first serine in the motif. Finally, under our conditions, binding to CnA is unable to prevent protein degradation of CALPIL, as has been suggested previously for other CALP family members [24,25].

Taking all these findings into consideration we propose a model of action for CALPIL in the Ca²⁺/CnA pathway. A rise in intracellular Ca²⁺ triggered by ligand binding to a cell membrane receptor leads to the activation of calcineurin. Once activated, a switch-off mechanism might be necessary to avoid sustained CnA activity in situations of prolonged Ca²⁺ stimulation. It is then that CALPIL goes into action. *DSCR1* mRNA expression is induced by active calcineurin and, furthermore, the amount of CALPIL protein present in the cell can be increased by reducing its turnover by dephosphorylation of the FLISPP motif. Calcineurin has been proposed as the phosphatase responsible for the dephosphorylation of these serines [25,37]. Although CALPIL works as a substrate in *in vitro* conditions ([37] and results not shown), we have not been able to detect changes in phosphorylation *in vivo* either by intracellular Ca²⁺ increases or co-expression with a constitutively active calcineurin (results not shown). Also, due to its role as a calcineurin inhibitor, we suggest that dephosphorylation of CALPIL would only take place when CALPIL is not bound to calcineurin and the serine residues within the FLISPP motif are accessible to the phosphatase. We do not know how this would happen. Once enough CALPIL is accumulated in the cell, calcineurin activity is inhibited. Finally, phosphorylation of both serines within the FLISPP motif, in response to certain stimuli, would target CALPIL for degradation and work as the switch-off signal for the network, allowing calcineurin to initiate a new cycle.

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