Cloning of human Ca^{2+} homoeostasis endoplasmic reticulum protein (CHERP): regulated expression of antisense cDNA depletes CHERP, inhibits intracellular Ca^{2+} mobilization and decreases cell proliferation

Janice M. LAPLANTE*[1], Flavia O'ROURKE*[2], Xinghua LU*[3], Alan FEIN†, Anne OLSEN‡ and Maurice B. FEINSTEIN*[1]

*Department of Pharmacology, The University of Connecticut Health Center, 263 Farmington Avenue, Farmington, CT 06032, U.S.A., †Department of Physiology, The University of Connecticut Health Center, 263 Farmington Avenue, Farmington, CT 06032, U.S.A., and ‡DOE Joint Genome Institute, Lawrence Livermore National Laboratory, Human Genome Center, 7000 East Avenue, Livermore, CA 94550, U.S.A.

A monoclonal antibody which blocks InsP_3-induced Ca^{2+} release from isolated endoplasmic reticulum was used to isolate a novel 4.0 kb cDNA from a human erythroleukaemia (HEL) cell cDNA expression library. A corresponding mRNA transcript of approx. 4.2 kb was present in all human cell lines and tissues examined, but cardiac and skeletal muscle had an additional transcript of 6.4 kb. The identification in GenBank® of homologous expressed sequence tags from many tissues and organisms suggests that the gene is ubiquitously expressed in higher eukaryotes. The gene was mapped to human chromosome 19p13.1. The cDNA predicts a 100 kDa protein, designated Ca^{2+} homoeostasis endoplasmic reticulum protein (CHERP), with two putative transmembrane domains, multiple consensus phosphorylation sites, a poly-glutamine tract of 12 repeats and regions of imperfect tryptophan and histadine octa- and nona-peptide repeats. *In vitro* translation of the full-length cDNA produced proteins of M_r 128000 and 100000, corresponding to protein bands detected by Western blotting of many cell types. CHERP was co-localized in HEL cells with the InsP_3 receptor by two-colour immunofluorescence. Transfection of HEL cells with antisense cDNA led to an 80% decline in CHERP within 5 days of antisense induction, with markedly decreased intracellular Ca^{2+} mobilization by thrombin, decreased DNA synthesis and growth arrest, indicating that the protein has an important function in Ca^{2+} homoeostasis, growth and proliferation.

Key words: InsP_3, senescence, signal transduction.

INTRODUCTION

Previously, we described the development of a monoclonal antibody (designated mAb213-21) raised against the InsP_3-sensitive internal dense-tubular-system membranes of platelets, and which inhibited Ca^{2+} release by InsP_3 from internal membrane vesicles derived from platelets, cerebellum, smooth muscle, sea urchin eggs and from HEL cells, a human leukemic cell line with megakaryoblastic features [1,2]. The antibody did not react with the InsP_3 receptor protein on Western blots. Its effect on Ca^{2+} release was mimicked by certain K^+ channel blockers and reversed by K^+ ionophores, suggesting that the antibody affected a K^+ conductance [2]. These effects of K^+ channel blockers and ionophores on Ca^{2+} release from the ER were later confirmed in intact cells by Nguyen et al. [3]. The antibody-binding protein in both platelets and HEL cells is an integral membrane protein localized to the smooth endoplasmic reticulum (ER) fraction of cell homogenates.

Here we report the cloning and sequencing of a novel cDNA obtained by screening a human HEL cell cDNA expression library with mAb213-21, the predicted amino acid sequence of the protein, designated CHERP (Ca^{2+} homoeostasis endoplasmic reticulum protein), the mapping of the gene to human chromosome 19p13.1, the tissue expression of its mRNA, and the immunocytochemical localization of the protein. We describe the preparation of a stable HEL cell line transfected with an inducible antisense knockout construct of CHERP cDNA and its use to determine the effects of protein knockout on DNA synthesis, cell proliferation and thrombin-stimulated release of intracellular Ca^{2+} stores.

MATERIALS AND METHODS

Cloning and sequencing of the CHERP cDNA from a HEL cell expression library

Standard procedures were used for library screening, recombinant DNA technology, primer extension, and Northern and Southern hybridization [4,5]. A HEL cell lambda gt11 expression library (provided by Dr M. Poncz, University of Pennsylvania, PA, U.S.A.) [6] was screened using the monoclonal antibody 213-21, and a peroxidase-labelled secondary antibody (Boehringer Mannheim) with tetramethylbenzidine (Kirkegaard and Perry, Gaithersburg, MD, U.S.A.) as a substrate for detection of positive plaques. The mAb213-21 was harvested from hybridoma cultures growing in protein-free media (Hybrimax, Sigma). cDNA from positive plaques was subcloned as described below to generate the phage vectors pJLBS213/2.6,

Abbreviations used: CHERP, Ca^{2+} homoeostasis endoplasmic reticulum protein; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; ER, endoplasmic reticulum; GST, glutathione S-transferase; IPTG, isopropyl β-D-thiogalactoside; *lac*I, *E. coli lac* repressor protein; SERCA, sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase; TM, transmembrane domain; HEL, human erythroleukaemia; EST, expressed sequence tag.

[1] Present address: Gerontology Division, Beth Israel Deaconess Medical Center, Harvard Institutes of Medicine, 77 Avenue Louis Pasteur, Boston, MA 02115, U.S.A.

[2] To whom correspondence should be addressed (e-mail orourke@nso1.uchc.edu).

[3] Present address: Department of Pharmacology, University of Pittsburgh, Pittsburgh, PA 15260, U.S.A.

The nucleotide sequence data reported will appear in GSDB, DDBJ, EMBL and GenBank® Nucleotide Sequence Databases under the accession number U94836.

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pJLBS213/3.9 and pJLBS213/4.0, or into M13mp8 and M13mp9 phage vectors (New England Biolabs) for sequencing. The cDNA sequence was obtained manually using a Sequenase kit 2.0 (USB, Cleveland, OH, U.S.A.) or by an automatic sequencing system (Applied Biosystems, Foster City, CA, U.S.A.) using fluorescently labelled primers. cDNAs were prepared from human cardiac and cerebellar libraries (Stratagene) and genomic DNA was prepared from a human fetal liver library (A.T.C.C.). All DNA was sequenced in both directions. The lambda DNA clones were also sequenced by a PCR sequencing system (Promega). Probes were labelled using gene-specific primers or random hexamers [4]. Sequences of the cDNA fragments were assembled using MacVector™ 6.0 (IBI, New Haven, CT, U.S.A.) software and aligned with sequences in the National Center for Biotechnology Information taxonomy database using nBLAST software at a stringency of $P < 0.0001$ [7], or with MacVector. The predicted protein sequence was analysed for protein consensus domains, including potential membrane-spanning domains, with a Kyte–Doolittle window size = 12 [8]. The human multiple tissue Northern blot (ClonTech) was probed with a ^{32}P-labelled 3.9 kb HEL cDNA, and with the 2.6 kb cDNA, which lacks the CAG repeat region to avoid hybridization with other CAG-containing RNAs.

Plasmid constructs

Specific details of the molecular cloning of the plasmids described herein are available from the authors on request. All in-frame cloning was verified by sequencing. The cDNA was cloned into Bluescript KS$^-$ (Stratagene) for amplification of the cDNA into the pET3 (Novagen, Madison, WI, U.S.A.) series of T7 expression vectors for *in vitro* translation, into pGEX (Pharmacia) for expression of a glutathione S-transferase (GST)-fusion protein in *Escherichia coli*, and into the mammalian expression vector pOPRSVICAT (Stratagene) to make an inducible construct for the expression of CHERP antisense DNA in the AS-HEL cell line. The plasmid pJLBS213/2.6 includes a 2642 nt *Eco*RI insert from a lambda gt11 clone recovered by expression screening with mAb213-21. This fragment corresponds to the 4.0 kb CHERP cDNA from nt 1335 through the 3′ end, but does not include nt 2290–2337, the putative alternatively spliced exon. The plasmid pJLBS213/3.9 includes the 3877 nt *Eco*RI fragment of CHERP, corresponding to nt 144–4018 of the 4.0 kb CHERP cDNA, including the 48 nt putative alternatively spliced exon. The plasmid pJLBS213/4.0 includes the entire 4018 nt fragment of the HEL cell CHERP cDNA. The plasmid pJLT7213/2.6 includes the 2.6 kb cDNA insert from pJLBS213/2.6 cloned in frame into pET3b. The expressed region in this vector comprises the amino acid domain from proline-417 through the C-terminus of CHERP. The plasmid pJLT7213/FL contains the *Sfu*1 fragment (nt 28–3393 of the 4.0 kb cDNA) cloned in frame into pET3c, and comprises the entire coding region of the HEL cell cDNA. To enable expression of a GST-fusion protein in *E. coli*, the 2.6 kb cDNA from pJLBS213/2.6 was cloned in-frame into pGEX-5 × 1 (Pharmacia), to make the plasmid pJLGST213/2.6. The mammalian expression vector pOPRSVICAT (Stratagene) was used to make an inducible construct for the expression of CHERP antisense cDNA, pJLRSV213/ΔCAG.AS. The chloramphenicol acetyltransferase gene from pOPRSVICAT was replaced with the 3.9 kb *Eco*RI fragment from pJLBS213/3.9, inserted in the antisense orientation relative to the promoter. To eliminate inadvertent antisense knockout of other cDNAs containing extended CAG trinucleotide repeats, the 533 nt *Sma*1 fragment, which spanned the CAG repeat region, was subsequently deleted (corresponding to nt 683–1216 of the 4.0 kb cDNA).

Chromosomal localization of the human CHERP gene

CHERP DNA was localized to its human chromosome by PCR using a mapping panel (#2; Coriell Cell Repositories, Camden, NJ, U.S.A.) with primers, 5′ CAGATCCAGACCCTCAAGACGC 3′, and 5′ TGGGAGGTAGACGCCGTAC 3′. Conditions for amplification were: 94 °C (4 min), 56 °C (2 min), [72 °C (1 min), 94 °C (1 min), 56 °C (1 min)] × 40 cycles, 8 min 72 °C. A gel purified 2.6 kb cDNA probe was used to screen colony filters of a chromosome 19 cosmid library [9] by complete *Eco*RI digestion [10]. The CHERP gene was localized to chromosome 19 using gene-specific primers to PCR-amplify a human chromosome panel. A 2.6kb cDNA probe was used to screen colony filters of a chromosome 19 cosmid library [9]. Five positive cosmids, R3532, R33330, R33231, F17025 and R30560, were identified. These clones were analysed by *Eco*R1 digestion [10] and incorporated into the metric physical map of chromosome 19 [11]. The CHERP-positive clones were localized within a 6 mb cosmid/bacterial artificial chromosome contig in 19p13.1. Clone F12296, adjacent to the CHERP clones in this contig, had been previously localized on a high-resolution pronuclear fluorescence *in situ* hybridization map of 19p [12], which places the CHERP gene within a 1 mb region in 19p13.1 between the oncogenes MEL and ERBAL2. A bacterial artificial chromosome clone, BC906392 (CITB-EI-3222D19), which completely overlaps the CHERP cosmids, is curently being sequenced. Updated chromosome 19 map and sequence information are available on the Lawrence Livermore National Laboratory's Human Genome Center web site (http://www-bio.llnl.gov/bbrp/genome.html).

In vitro expression of CHERP cDNA

The plasmids pJLT7213/2.6 and pJLT7213/FL were transcribed and translated by the mMessage mMachine™ transcription and Retic Lysate IVT™ system (Ambion, Austin, TX, U.S.A.). Transcribed mRNA was resuspended in water, and the concentration and purity were determined with a Gene Quant spectrophotometer. The rate of [^{35}S]methionine incorporation into protein was determined by trichloroacetic acid precipitation of aliquots of the reaction mix on glass filters as described in the kit. The translation products were separated by SDS/7.5 % PAGE followed by exposure for 4 h on a Molecular Dynamics (Sunnyvale, CA, U.S.A.) PhosphorImager screen. The GST-fusion protein prepared from plasmid pJLGST213/2.6 was expressed and purified according to the manufacturer's specifications (Pharmacia).

Cellular localization of CHERP

Cells were plated on coverslips in culture dishes and incubated for 24–48 h to allow them to adhere. The cells were fixed in 4 % formaldehyde in PBS for 30 min at room temperature and washed three times with PBS containing 0.2 % BSA (wash buffer), followed by incubation with 20 mM glycine in PBS for 10 min. After washing once, cells were permeabilized for 30 min with 0.05 % Triton X-100 in PBS with 1 % goat serum, followed by washing and incubation for 45 min with primary antibody. The primary and secondary antibodies were diluted as described below in PBS buffer containing 0.05 % Triton X-100 (except for the thrombin-receptor antibody) and 0.1 % goat serum. The mAb213-21 was harvested from hybridomas as previously de-

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scribed [2,13], and diluted 1:20 for use with the cyanin-3-labelled anti-mouse heavy + light chain second antibody (see below), or 1 mg was directly labelled with FITC by the method described in the Quicktag conjugation kit (Boehringer Mannheim) and used at 1:100 dilution. Other antibodies were diluted as follows: mouse monoclonal anti-sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase 2 (SERCA2), anti-SERCA3 (RDI, Flanders, NY, U.S.A.), and anti-thrombin receptor made against thrombin receptor peptide (KATNATLDPRSFLLRC; Biodesign, Kennebunk, ME, U.S.A.) antibodies, 1:100; affinity-purified polyclonal anti-InsP_3 receptor serum, which recognizes types 1, 2 and 3 (Accurate Scientific, Westbury, NY, U.S.A.), 1:200; cyanin 3-labelled goat-anti-mouse heavy + light chain and goat anti-rabbit heavy + light chain second antibodies (Jackson Immunoresearch Laboratories, West Grove, PA, U.S.A.), 1:800. The cells were mounted with Miolol on slides for viewing. Images were collected on a Zeiss confocal laser-scanning microscope at the Center for Biomedical Imaging at the University of Connecticut Health Center, or an Olympus fluorescence microscope equipped with a SPOT camera (Version 1.1; Diagnostic Instruments, Sterling Heights, MI, U.S.A.) at the Department of Pharmacology, the University of Connecticut Health Center. Levels of fluorescence were measured in individual cells using the public domain N.I.H. Image 1.61.1 image analysis software (developed at the U. S. National Institutes of Health and available on the Internet at http://www.rsb.info.nih.gov/nih-image/). Values were expressed on a grey scale (0–256 arbitrary units), after subtraction of the mean non-specific background level, which was derived from the region adjacent to each cell. Average values were calculated from a minimum of 30 cells from each group, and normalized to that of the corresponding untreated group. To depict the distribution of single cell fluorescence values in a typical experiment, the percentage of each population (y-axis) that fell within each 0.5 unit range (x-axis) was plotted.

Stable transfection of HEL cells

The LacSwitch mammalian cDNA expression system (Stratagene) was employed for the selective induction of RNA transcription of CHERP antisense cDNA with isopropyl β-D-thiogalactoside (IPTG). HEL cells adapted to Hybrimax growth media (Sigma), containing 10% FBS (Hyclone, Logan, UT, U.S.A.) and 10% cloning supplement (Boehringer Mannheim), were first transfected with an *E. coli* lac repressor (*lac*I) expression plasmid p3′SS, followed by selection of antibiotic resistance to hygromycin and cloning by limiting dilution. Three stably transfected HEL cell lines constitutively expressing *lac*I were established, and the one which displayed the highest level of anti-*lac*I immunofluorescence was used as the parent cell line for a second transfection with either the plasmid vector pOPRSVICAT (Stratagene) with a geneticin-resistance gene for selection (V-HEL cells), or the CHERP antisense plasmid pJLRSV213/ΔCAG.AS, which carried a 3.4 kb cDNA inserted in the antisense orientation (AS-HEL cells). Both plasmids contained the *lac*I repressible promoter. Stable transfectants were selected for geneticin resistance and cloned as described above. Clones were expanded and weaned from cloning supplement and maintained in media containing hygromycin (200 μg/ml) and geneticin (300 μg/ml), and grown at 10% CO_2. Cells were stored at early passage in liquid nitrogen and recloned periodically. Expression of CHERP protein was quantitatively analysed by ELISA [13], or by Western blot [13] using mAb213-21 with a peroxidase-labelled goat anti-mouse IgM as the second antibody for ECL detection (Kirkegard and Perry) and quantification by a Molecular Dynamics laser densitometer using Image Quant software.

Thymidine incorporation into DNA of transfected HEL cells

Cells were induced with IPTG for 24 h followed by washing and incubation with 10 μCi/ml [^{3}H]thymidine [14]. Replicate cultures were set up to provide cell counts for calculation of DNA synthesis by cell number and protein content. Cells were harvested and washed with Hanks buffered salt solution, followed by fixation and washing with trichloroacetic acid. DNA was extracted with perchloric acid and assayed for [^{3}H]thymidine by liquid-scintillation counting.

Senescence-associated β-galactosidase activity

Transfected AS-HEL and V-HEL cells were plated on coverslips and treated with 20 mM IPTG for 1–5 days. Cells were washed, fixed and assayed for senescence-associated β-galactosidase activity [15]. The coverslips were mounted with Miolol and photographed at 440× using an inverted Olympus microscope.

Ca^{2+} mobilization in transfected HEL cells

HEL cells were plated out on coverslips and incubated for 24 h at 37 °C to allow adherence. The culture medium was replaced with Hepes-buffered saline [120 mM NaCl, 5.3 mM KCl, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 5.5 mM glucose, 20 mM Hepes/NaOH (pH 7.4)] containing 2 μM fura-2 acetoxymethyl ester (Molecular Probes, Eugene, OR, U.S.A), and incubated for 30 min at 25 °C followed by washing with Hepes buffered saline. Dye-loaded cells were incubated for an additional 30 min at 25 °C, followed by washing with either Hepes-buffered saline or Ca^{2+}-free Hepes-buffered saline + 0.5 M EGTA, resuspended in 200 μl of wash buffer and viewed with a Nikon Fluor 100 × 1.3 NA inverted oil immersion lens. Wash buffer (800 μl) containing thrombin (final concentration 1–10 nM; Sigma) or 1 μM thapsigargin (Sigma) was added to the chamber. Ca^{2+} influx was measured as previously described [16]. Cells were flashed with excitation light at wavelengths of 340 nm and 380 nm alternatively, and the fluorescence emissions were collected at 510 nm using a photon counting fluorescence subsystem (Ionoptix, Milton, MA, U.S.A.). The 340/380 ratios were collected at a rate of 1 Hz. The software Ionwizard was used to collect and quantify fluorescence measurements. The magnitude of the 340/380 signal increases as a function of the rise in cytostolic free Ca^{2+} concentration, $[Ca^{2+}]_i$ [17].

RESULTS

Molecular cloning of CHERP cDNA from a HEL cell expression library

A novel 2.6 kb cDNA which included polyadenylation signals and a polyadenylated tail was initially isolated by expression screening of a lambda gt11 HEL cell library with mAb213-21. A *Not*I restriction fragment derived from the 5′ end of the cDNA sequence was used to rescreen the library for overlapping clones. We identified 32 additional clones, including 12 with inserts of approx. 4 kb in length. The longest of these had a 4018 nt insert (Figure 1a), which overlapped the 2.6 kb cDNA but extended further in the 5′ direction. The overlapping region of the two clones was found to be identical, except for an additional stretch of 48 nt in the 4.0 kb cDNA (nt 2290–2337), suggesting that the gene contained an alternative splice site (Figure 1a). Additional clones, obtained by screening human cerebellar and cardiac cDNA libraries and a human genomic fetal liver library, were identical with the HEL cDNA in the regions that were sequenced, except for a difference in two nucleotides in the single cerebellar

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(a)

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                                                                                                                                           M  E  M
                                                                                                        gcgccggag gacgttcccc gcggccggag ccatggagat
 P  L  P    P  D  D    Q  E  L  R    N  V  I    D  K  L    A  Q  E    V  A  R  N    G  P  E    F  E  K↓M    T  M  E    K  Q  K    D  N  P  K 11
gccgctgCCCC CCGATGACC AGGAGCTTCG AAATGTCATC GACAAGCTCG CCCAGTTCGT GGCTCGCAAT GGGCCCGAGT TTGAGAAGAT GACTATGGAG AAGCAGAAGG ACAACCCCAA 120
 F  S  F    L  F  G    G  E  F  Y    S  Y  Y    K  C  K    L  A  L  E    Q  Q  Q    L  I  C    K  Q  Q  T    P  E  L    E  P  A    A  T  M  P 51
ATTCTCGTTT CTTTTCGGAG GCGAATTCTA CAGTTACTAC AAGTGCAAGC TGGCGCTGGA GCAGCAGCAG CTCATCTGCA AGCAGCAGAC CCCGGAGCTG GAGCCAGCCG CCACCATGCC 240
 P  L  P    Q  P  P    L  A  P  A    A  P  I    P  P  A    Q  G  A  P    S  M  D    E  L  I    Q  Q  S  Q    W  N  L    Q  Q  Q    E  Q  H  L 91
ACCCCTGCCA CAGCCCCCGC TGGCCCCCGC CGCGCCCATC CCGCCGGCCC AGGGCGCGCC ATCCATGGAC GAGCTCATCC AGCAGAGCCA GTGGAACCTC CAGCAGCAGG AGCAGCACTT 360
 L  A  L    R  Q  E    Q  V  T  A    A  V  A    H  A  V    E  Q  Q  M    Q  K  L    L  E  E    T  Q  L  D    M  N  E    F  D  N    L  L  Q  P 131
GCTGGCGCTC AGACAGGAGC AAGTGACAGC GGCCGTGGCC CACGCGGTGG AGCAGCAGAT GCAGAAGCTT CTGGAGGAGA CCCAGCTAGA CATGAACGAG TTTGACAACC TCCTGCAGCC 480
 I  I  D    T  C  T    K  D  A  I    S  A  G    K  N  W    M  F  S  N    A  K  S    P  P  H    C  E  L  M    A  G  H    L  R  H    R  I  T  A 171
CATCATCGAC ACGTGCACCA AGGACGCCAT CTCGGCCGGG AAGAACTGGA TGTTCAGCAA TGCCAAGTCC CCGCCGCACT GTGAGCTGAT GGCCGGCCAC CTCCGCCACC GCATCACGGC 600
 D  G  A    H  F  E    L  R  L  H    L  I  Y    L  I  N    D  V  L  H    H  C  Q    R  K  Q    A  R  E  L    L  A  A    L  Q  K    V  V  V  P 211
TGATGGGGCA CACTTCGAGC TGCGGCTGCA CCTCATCTAC CTGATCAATG ACGTGCTGCA CCACTGCCAG CGCAAGCAGG CCCGGGAGCT GCTGGCCGCC CTGCAGAAGG TCGTGGTGCC 720
 I  Y  C    T  S  F    L  A  V  E    E  D  K    Q  Q  K    I  A  R  L    L  Q  L    W  E  K    N  G  Y  F    D  D  S    I  I  Q    Q  L  Q  S 251
CATCTACTGC ACCAGCTTCT TGGCCGTGGA GGAAGACAAG CAGCAGAAGA TCGCCCGGCT CCTGCAGCTC TGGGAGAAAA ACGGCTACTT CGATGACTCC ATCATTCAGC AGCTACAGAG 840
 P  A  L    G  L  G    Q  Y  Q  A    T  L  I    N  E  Y    S  S  V  V    Q  P  V    Q  L  A    F  Q  Q  Q    I  Q  T    L  K  T    Q  H  E  E 291
CCCAGCCCTG GGGCTTGGTC AGTACCAGGC CACCCTCATC AACGAGTACT CCTCAGTGGT CCAGCCGGTG CAGCTGGCCT TCCAGCAGCA GATCCAGACC CTCAAGACGC AGCACGAGGA 960
 F  V  T    S  L  A    Q  Q  Q  Q    Q  Q  Q    Q  Q  Q    Q  Q  L  Q    M  P  Q    M  E  A    E  V  K  A    T  P  P    P  P  A    P  P  P  A 331
GTTTGTCACC AGCCTGGCCC AGCAGCAGCA GCAGCAGCAA CAGCAGCAGC AGCAGCTCCA GATGCCGCAG ATGGAGGCTG AAGTCAAGGC CACGCCTCCA CCGCCTGCTC CACCCCCGGC 1080
 P  A  P    A  P  A    I  P  P  T    T  Q  P    D  D  S    K  P  P  I    Q  M  P    G  S  S    E  Y  E  A    P  G  G    V  Q  D    P  A  A  A 371
CCCAGCACCT GCCCCTGCCA TCCCGCCCAC CACCCAGCCT GATGACAGCA AGCCTCCCAT CCAGATGCCT GGCTCTTCAG AGTACGAAGC TCCAGGAGGG GTCCAGGATC CTGCAGCTGC 1200
 G  P  R    G  P  G    P  H  D  Q    I  P  P    N  K  P    P  W  F  D    Q  P  H    P  V  A    P  W  G  Q    Q  Q  P    P  E  Q    P  P  Y  P 411
GGGCCCCCGG GGCCCCGGGC CACACGACCA GATCCCACCA AACAAGCCCC CTTGGTTTGA CCAGCCTCAC CCCGTGGCTC CTTGGGGCCA GCAGCAGCCG CCAGAGCAGC CACCCTACCC 1320
 H  H  Q    G  G  P    P  H  C  P    P  W  N    N  S  H    E  G  M  W    G  E  Q    R  G  D    P  G  W  N    G  Q  R    D  A  P    W  N  N  Q 451
GCACCACCAG GGCGGCCCAC CCCACTGCCC CCCCTGGAAC AACAGCCATG AGGGCATGTG GGGCGAGCAG CGCGGTGACC CCGGCTGGAA CGGCCAGCGC GACGCGCCCT GGAACAACCA 1440
 P  D  A    A  W  N    S  Q  F  E    G  P  W    N  S  Q    H  E  Q  P    P  W  G    G  G  Q    R  E  P  P    F  R  M    Q  R  P    P  H  F  R 491
GCCCGACGCC GCCTGGAACA GCCAGTTCGA GGGCCCCTGG AACAGCCAGC ACGAGCAGCC GCCCTGGGGC GGGGGCCAGC GCGAGCCACC CTTCCGCATG CAGCGGCCCC CACACTTCCG 1560
 G  P  F    P  P  H    Q  Q  H  P    Q  F  N    Q  P  P    H  P  H  N    F  N  R    F  P  P    R  F  M  Q    D  D  F    P  P  R    H  P  F  E 531
GGGGCCCTTC CCGCCCCACC AGCAGCACCC GCAGTTCAAC CAGCCTCCGC ACCCCCACAA CTTCAACCGC TTCCCGCCCC GCTTCATGCA GGACGACTTC CCGCCACGGC ACCCCTTCGA 1680
 R  P  P    Y  P  H    R  F  D  Y    P  Q  G    D  F  P    A  E  M  G    P  P  H    H  H  P    G  H  R  M    P  H  P    G  I  N    E  H  P  P 571
GCGGCCGCCC TATCCCCACC GCTTCGACTA CCCCCAGGGG GACTTCCCTG CCGAAATGGG GCCCCCTCAC CACCACCCTG GCCACCGCAT GCCTCATCCT GGCATCAACG AGCACCCGCC 1800
 W  A  G    P  Q  H    P  D  F  G    P  P  P    H  G  F    N  G  Q  P    P  H  M    R  R  Q    G  P  P  H    I  N  H    D  D  P    S  L  V  P 611
TTGGGCTGGA CCCCAGCACC CTGACTTCGG CCCTCCCCCC CATGGCTTCA ACGGGCAGCC CCCACACATG CGGCGACAGG GCCCACCCCA CATCAACCAC GATGACCCCA GCCTGGTCCC 1920
 N  V  P    Y  F  D    L  P  A  G    L  M  A    P  L  V    K  L  E  D    H  E  Y    K  P  L    D  P  K  D    I  R  L    P  P  P    M  P  P  S 651
CAATGTGCCC TACTTCGATC TCCCTGCTGG GCTGATGGCC CCCCTCGTGA AGCTGGAAGA TCACGAGTAC AAGCCTTTGG ACCCTAAAGA CATCCGCCTC CCACCCCCCA TGCCGCCCAG 2040
 E  R  L    L  A  A    V  E  A  F    Y  S  P    P  S  H    D  R  P  R    N  S  E    G  W  E    Q  N  G  L    Y  E  F    F  R  A    K  M  R  A 691
CGAGAGGCTG CTGGCTGCAG TGGAGGCCTT CTACAGCCCC CCGTCCCACG ACAGGCCCAG GAACAGTGAA GGCTGGGAGC AGAACGGCCT CTATGAGTTC TTCCGAGCAA AAATGCGGGC 2160
 R  R  R    K  G  Q    E  K  R  N    S  G  P    S  R  S    R  S  R  S    K  S  R    G  R  S    S  S  R  S    N  S  R    S  S  K    S  S  G  S 731
CCGGCGGAGG AAAGGCCAGG AGAAGAGGAA CAGCGGACCC TCGAGGTCTC GGAGCAGATC CAAGAGTCGA GGGCGTTCTT CCTCCCGCTC CAACTCAAGA TCCTCCAAGT CTTCAGGCTC 2280
 Y  S  R    S  R  S    R  S  C  S    R  S  Y    S  R  S    R  S  R  S    R  S  R    S  R  S    S  R  S  R    S  R  S    Q  S  R    S  R  S  K 771
GTACTCAAGG TCAAGGTCGC GCTCCTGCTC CCGTTCCTAC TCCCGCTCCA GATCTAGAAG TCGGAGCAGG TCGCGCTCCT CCAGAAGCCG CTCCCGGTCC CAGTCGCGGT CCCGGTCCAA 2400
 S  Y  S    P  G  R    R  R  R  S    R  S  R    S  P  T    P  P  S  S    A  G  L    G  S  N    S  A  P  P    I  P  D    S  R  L    G  E  E  N 811
GTCGTACTCC CCAGGAAGAA GACGCCGGTC ACGGTCCAGG AGCCCCACCC CGCCTTCCTC TGCTGGTCTG GGTTCTAATT CGGCGCCTCC CATCCCTGAC TCAAGGCTCG GAGAAGAGAA 2520
 K  G  H    Q  M  L    V  K  M  G    W  S  G    S  G  G    L  G  A  K    E  Q  G    I  Q  D    P  I  K  G    G  D  V    R  D  K    W  D  Q  Y 851
CAAAGGCCAT CAGATGCTGG TGAAGATGGG CTGGAGCGGC TCAGGCGGCC TCGGTGCGAA GGAGCAAGGG ATCCAGGACC CCATCAAGGG CGGGGACGTC CGGGATAAGT GGGACCAGTA 2640
 K  G  V    G  V  A    L  D  D  P    Y  E  N    Y  R  R    N  K  S  Y    S  F  I    A  R  M    K  A  R  D    E  C  K
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AGGAAGGGTT GGCCTGAGCC AGAGCCGGCA CCCCAGCTCC TTCCTCCAGC TCCCCGCACC CACCGGCCCT GACCTGGCTT CCCCTCGGCT GTGTAGGGAC AAAGCCGAAG CCCAGTGCCA 3240
TGACTGCCCG CGGATTGAAA AACAGAAACA CAAAACTTTG ACTTGACTTG CGAAGTGAAG CAGGGTTTTC ATTTTTACTC TCCTTGGTTT AGGTCTAGAA AGAAGAATAC TGACCTGAGA 3360
GGAGGCCCAG TGAGATTCTG AAACCTAATT CTTCGAAGGC GTACTGGCCC TTAGTTCAGT TATTTTAGTT GTATAAGTTG ATATTTTTTT TCTGGAATGT AGCCATTTGC TGTTATCTGG 3480
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CTTGTGGATT TCATTGTTTT GCCTTCAAAT AAAGCCTTTT TTTAAAAAAA AAGAATTC
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(b)

(c)

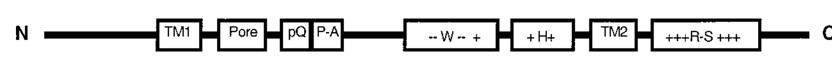

Figure 1 Nucleotide and predicted amino acid sequence of the CHERP cDNA

(**a**) Complete sequence of the HEL cell CHERP cDNA and its predicted amino acid sequence. The 5′ extension encoded by HSDAN26 is indicated in lower case letters. Although nucleotides are numbered from the first position of the *Eco*RI linker of the HEL cell cDNA to show the overlap between the HSDAN26 and the HEL cell sequences, nt 1–7 (GAATTCC), corresponding to the synthetic linker of the HEL cell sequence, have been replaced with HSDAN26. Both the first and second in-frame start codons in the HEL cell sequence, at positions 89 and 95, lie within potential Kozak initiation sites. The predicted protein sequence is shown with amino acids numbered from the first in-frame AUG of the HEL cell sequence indicated by ↓M. The appended amino acid sequence based on the sequence of HSDAN26 is shown in *italics*. The putative transmembrane and pore domains are shown in **bold** and underlined (dotted line), the polyglutamine tract is double underlined, and the serine/arginine domain is single underlined with a double underline to indicate the alternative spliced sequence. (**b**) 5′ end of the 4.0 kb CHERP cDNA from HEL cell. The *Eco*RI site at nt 144 is indicated. The arrows beneath the HEL cell sequence represent the position of the primers used for the primer extension reaction. Reaction products are indicated as lines below the HEL cell sequence. The overlapping regions of HSDAN26 and the EST R72355 are aligned below the HEL cell sequence. (**c**) Domain structure of CHERP. Pore, pore-like domain; pQ, polyglutamine tract; P-A, proline/alanine-rich region; -W-+, amphiphilic imperfect aromatic tryptophan-repeat region; +H+, positively charged aromatic histidine-repeat region; +++RS+++, positively charged serine/arginine-rich region.

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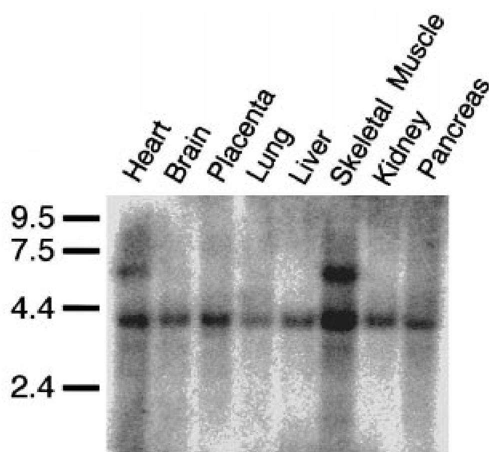

Figure 2 Tissue-specific expression of the CHERP mRNA

A human multiple-tissue Northern blot (ClonTech) containing 2 μg of polyadenylated RNA per lane was probed with a ^{32}P-radiolabelled CHERP cDNA and exposed for 24 h to a PhosphorImaging screen. A radiolabelled probe for glyceraldehyde-3-phosphate dehydrogenase was used as a control (results not shown). The CHERP cDNA hybridizes to a 4.2 kb transcript in all human tissues probed and an additional 6.4 kb mRNA in cardiac and skeletal muscle.

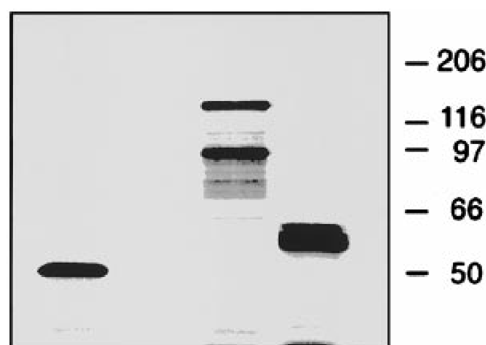

Figure 3 *In vitro* translation of CHERP cDNA

The 2.6 kb cDNA (C-terminal) and the complete coding region from the 4.0 kb HEL cDNA were cloned in-frame into the pET-3 expression vectors. Translation products were separated by SDS/PAGE followed by 4 h exposure on a PhosphorImager screen. Numbering from left to right: lane 1, *Xenopus* elongation factor (positive control); lane 2, no RNA (negative control); lane 3, polypeptides of 100 kDa and 128 kDa ^{35}S-labelled products produced from the 4.0 kb transcribed cDNA; lane 4, a single 60 kDa polypeptide, somewhat larger than the predicted size of approx. 52 kDa produced by the 2.6 kb transcribed cDNA.

clone. Primer extension of HEL cell mRNA revealed that the cDNA sequence lacked approx. 70 residues at the 5′ end (Figure 1b). Consistent with this finding, a search of the GenBank® revealed a homologous 1.8 kb cDNA (HSDAN26) of unknown function, derived from a human lymphoblastoid cDNA library [18], that overlaps the 4.0 kb CHERP cDNA, but extends 46 nt beyond the 5′ end of CHERP (Figure 1b). Furthermore, a homologous expressed sequence tag (EST) R72355, derived from a human breast library, extends 39 nt beyond the 4.0 kb CHERP cDNA at the 5′ end, and contains the same 5′ sequence as HSDAN26, with the exception of 3 ambiguous nucleotides. We identified 156 ESTs in the GenBank® which match the CHERP cDNA sequence, originating from human testis, brain, uterus, heart, retina, lung, skin, fetal heart and tumours derived from parathyroid, colon, endometrium, pancreas and ovary, and a variety of tissues derived from other species including rat, mouse, zebra fish. The CHERP cDNA sequence showed no significant similarity to any known gene with the exception of a region of CAG trinucleotide repeats (Figures 1a and 1c) interrupted by a single CAA, which codes for a polyglutamine tract.

The CHERP gene was localized to human chromosome 19, within an approx.1 mb region in 19p13.1, situated between the oncogenes MEL and ERBAL2. Southern blot and sequence-specific PCR data of genomic DNA are consistent with that of a single copy gene (results not shown). A single CHERP mRNA transcript of approx. 4.2 kb was found in normal human brain, placenta, lung, liver, kidney, pancreas, cardiac and skeletal muscle, and in cultured megakaryoblastic HEL and Dami cells, but cardiac and skeletal muscle had an additional transcript of 6.4 kb (Figure 2).

The 4018 nt HEL cell CHERP cDNA sequence translated to a novel protein of 884 amino acids (Figure 1) with a molecular mass of 100 kDa and a calculated pI of 9.46. Similarly, the 100 kDa platelet protein, purified by a series of HPLC steps (results not shown), reacted strongly with mAb213-21 on Western blots and had a pI $> 9 < 10$, as determined by isoelectric focusing. The 5′ extensions of both HSDAN26 and EST R72355 include a Kozak sequence with another in-frame start codon that could potentially add 32 amino acids, or 1.6 kDa, to the CHERP sequence (Figures 1a and 1b). *In vitro* translation of the 4.0 kb cDNA produced a 100 kDa polypeptide (Figure 3) in agreement with the predicted size, and with the 100 kDa protein band detected by Western blotting in platelets and cardiac myocytes (results not shown). An additional 128 kDa product was produced, corresponding to the predominant protein band found by Western blotting of HEL cells, the other megakaryoblastic cell lines Dami, Meg-01 and CHRF-288, and of rat cerebellum (results not shown). The 2.6 kb cDNA produced a single polypeptide which also migrated on gels about 20% larger than the expected size of 52 kDa (Figure 3). A GST-fusion protein prepared from the 2.6 kb cDNA expressed in *E. coli* reacted strongly by Western blotting with mAb213-21, proving that the cDNA was inserted in the correct reading frame for protein expression, and that the epitope of the antibody binds to the region of the protein between Pro^{417} to the C-terminus. This was further established by a competitive ELISA, in which the expressed GST-fusion protein effectively competed ($IC_{50} = 0.7$ μM) with the native proteins from HEL cell and platelet membranes for binding to the antibody.

The predicted protein has two domains (TM1 and TM2), based on analysis by the Kyte–Doolittle hydropathy scale, which have characteristic features of transmembrane domains of ion channels: i.e. a hydrophobic domain with a number of intermittent polar residues, and neighbouring regions with a relatively high number of charged residues. A short stretch between TM1 and TM2 is predicted to have a sheet–turn–sheet structure, which is characteristic of the pore region of many channels [19]. The sequence within this region bears some similarity to the pore of certain K^+ channel proteins and a two transmembrane ATP-receptor cation channel [20], and has a signature GLG motif found in some members of the 4TM class of K^+ channels [21–23].

Several regions of the predicted sequence may be involved in protein–protein binding. The first, just distal to the pore-like domain, is the polyglutamine tract (Gln^{298}–Gln^{309}), which corresponds to the extended CAG repeat region in the cDNA. Beyond that is a proline-rich region containing several sequences (reviewed in [24]) known to bind Src homology domains in proteins.

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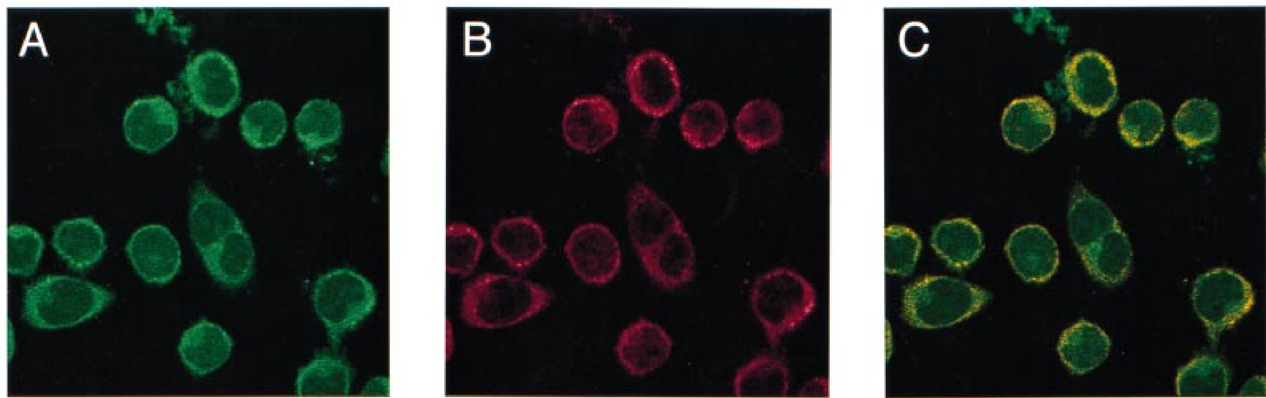

Figure 4 Immunolocalization of CHERP and the InsP_3 receptor in V-HEL cells

(**A**) FITC-labelled mAb213-21 in V-HEL cells (green). (**B**) Cyanin-3-labelled anti-InsP_3 receptor (red). (**C**) Double labelling with both antibodies showing co-localization of CHERP and the InsP_3 receptor (yellow).

Immediately proximal to TM2 is an extended region of octa- or nona-peptide imperfect repeats. Commencing at Trp^{423} are seven imperfect tryptophan-repeats with a net negative charge, followed by a transition to a series of phenylalanine-repeat domains with a net positive charge, providing the entire segment from Trp^{423} to Phe^{546} with an amphiphilic character. Of the amino acids from Trp^{423} to Phe^{587} 15 % have aromatic side chains. Similar regions are proposed to play a role in macromolecular assembly in other proteins [25], as are histidine repeats, such as the seven imperfect nonapeptide histidine repeats beginning at His^{554}, punctuated by one heptapeptide and ending at the start of TM2. A serine/arginine-rich region, also implicated in protein–protein binding, extends from Ser^{705} to Ser^{785} in the C-terminus of CHERP. This region contains a total of 31 positively charged residues with no negatively charged residues, and includes many consensus sites for phosphorylation by protein kinases A and C, glycogen synthase kinase-3, and casein kinase. Potential phosphorylation sites for tyrosine kinases and protein kinase C are found outside the serine/arginine domain. In addition, the protein contains consensus sequences for glycosylation (Asp^{868}) and glycosaminoglycan attachment (Ser^{823}GXG), and putative ER retention signals at the C-terminus (S^{872}FI and D^{881}E) [26,27].

Localization of CHERP in HEL cells

CHERP was immunolocalized in HEL cells using a FITC-labelled mAb213-21, and was found to be distributed throughout the cytoplasmic region (Figure 4A). The intracellular staining pattern of CHERP was the same as that of the InsP_3 receptor, as shown by indirect fluorescence using a cyanin-3-labelled secondary antibody (Figure 4B), and SERCA2 and SERCA3 (results not shown). Cells that were double-labelled with green mAb213-21 and red anti-InsP_3 fluoresced yellow from overlapping emission spectra, demonstrating the spatial co-localization of CHERP with the InsP_3 receptor (Figure 4C).

Stable transfection of HEL cells with antisense CHERP cDNA

Stable HEL cell lines were established by transfection with vector alone (V-HEL), or vector containing antisense (AS-HEL) cDNA, under the negative control of *lac*I using the LacSwitch mammalian transfection system. *Lac*I blocks transcription by binding to the *E. coli Lac* operator, which is embedded in the promoter of the CHERP antisense expression vector pJLRSV213/ΔCAG.AS. IPTG decreases the binding of *lac*I protein to the operator sequences, triggering transcription of the CHERP antisense cDNA. Induction of AS-HEL cells with IPTG reduced the concentration of the 128 kDa CHERP protein to below the level of detection by Western blotting (Figure 5a), but did not have a significant effect on CHERP in V-HEL cells. When measured by ELISA using mAb213-21, a more sensitive method of quantification, CHERP was reduced by 80 % in AS-HEL cells treated with IPTG for 5 days (Figure 5b). IPTG did not have a statistically significant effect on CHERP in V-HEL cells. The progressive decline of CHERP was also measured in populations of individual cells by fluorescence immunocytochemistry (Figures 5c–5e). Total AS-HEL cell fluorescence was reduced by an average of 18 %, 48 %, and 67 % over a period of one, two and five days respectively (Figure 5c). The distribution of fluorescence from individual cells identified three main subpopulations in AS-HEL cells, varying in their average fluorescence by approximately threefold, which may related to stage of the cell cycle or life cycle. While the distribution of immunofluorescence within V-HEL cell populations did not change significantly upon treatment with IPTG (Figure 5d), treatment of AS-HEL cells with IPTG led to a shift toward populations with lower levels of fluorescence within 24 h After 5 days, more than 90 % of the IPTG-treated AS-HEL cells had levels of fluorescence from bound mAb213-21 that was below that of all populations of untreated AS-HEL cells (Figure 5e). No comparable loss of SERCA2, SERCA3, InsP_3 receptor or thrombin receptor was observed (results not shown).

Untreated AS-HEL, V-HEL and IPTG-treated V-HEL cells were mostly spherical and weakly attached to flasks (Figures 6A and 6B), whereas IPTG-treated AS-HEL cells were enlarged and adhered to the bottom of the flasks assuming a flattened irregular shape (Figure 6C). After five days in culture, cell division appeared to have halted in the IPTG-treated AS-HEL cells, with no significant loss of viability as measured by Trypan Blue. IPTG treatment for 24 h decreased the rate of [^{3}H]thymidine incorporation into DNA by 66 % in AS-HEL cells, but had only a minor effect on V-HEL cells (Figure 6D). Furthermore, after one, three and five days of treatment of AS-HEL cells with IPTG, blue nuclear staining for senescence-associated β-galactosidase activity [15] was observed in 4 %, 26 % and 67 %

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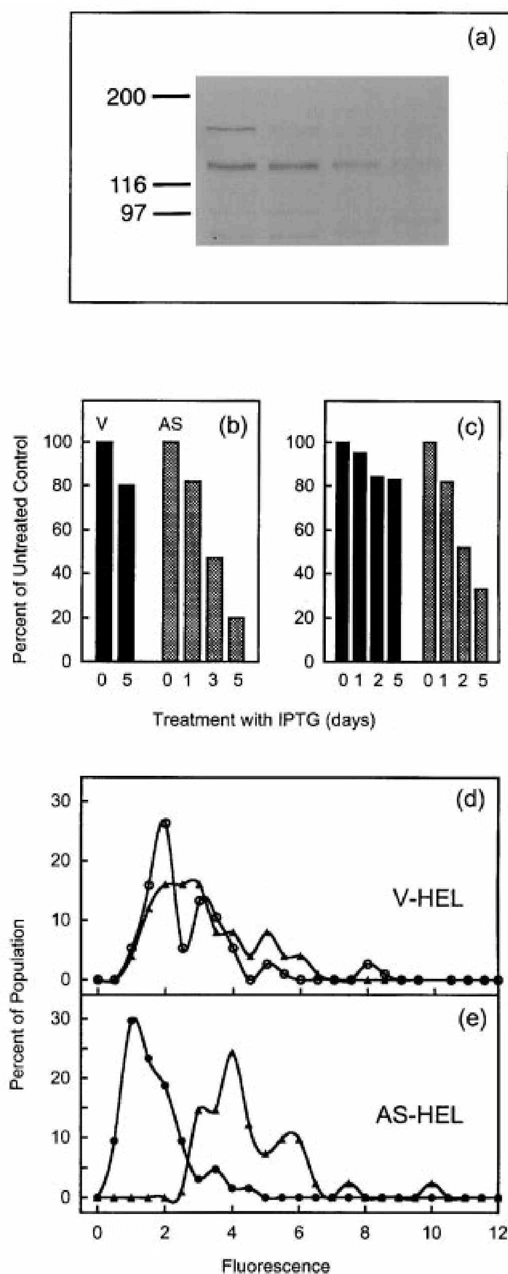

Figure 5 Antisense depletion of CHERP in AS-HEL cells

(**a**) Western blot of cells induced for 48 h with IPTG, washed and lysed with 5% CHAPS in sample buffer. Numbering from left to right: Lane 1, V-HEL; lane 2, V-HEL + IPTG; lane 3, AS-HEL; lane 4, AS-HEL + IPTG. Protein (40 μg) was separated by SDS/PAGE, immunoblotted using mAb213-21 and peroxidase-labelled secondary antibody and detection by ECL. (**b**) Quantitative ELISA showing CHERP in V-HEL (filled bars) or AS-HEL cells (hatched bars) after treatment with IPTG. (**c–e**) Quantification of fluorescence from cell populations using mAb213-21. (**c**) Disappearance of the protein from populations of AS-HEL cells (hatched bars) measured at various times after induction with IPTG, compared with IPTG-treated V-HEL cells (filled bars). Fluorescence was measured in a minimum of 30 cells chosen from each population. Average values were normalized to that of the corresponding untreated group. Data represent the average of at least two experiments. (**d** and **e**) Distribution of single-cell fluorescence values within a typical experiment, comparing values of (**d**) V-HEL + IPTG for 5 days (○) with untreated V-HEL cells (▲), or (**e**) AS-HEL + IPTG for 5 days (●) with those of the untreated AS-HEL control (▲).

respectively of the population (Figure 7), compared with 6% in the V-HEL cells treated with IPTG for 5 days.

Intracellular Ca^{2+} mobilization in HEL cells transfected with antisense cDNA

The effect of CHERP knockout on thrombin-stimulated increase in $[Ca^{2+}]_i$ was monitored by fluorescence microscopy in single HEL cells. Thrombin activates the phospholipase C pathway to produce InsP_3, which releases intracellular Ca^{2+} stores in HEL cells [28]. The emptying of the stores triggers, in turn, an influx of Ca^{2+} through the plasma membrane [16]. To specifically isolate the contribution of intracellular Ca^{2+} mobilization from the contribution of Ca^{2+} influx, the first set of experiments was performed in Ca^{2+}-free medium. Thrombin (10 nM) caused an abrupt increase in $[Ca^{2+}]_i$ within 10 s, followed by a gradual decline to baseline over a period of 200 s (Figure 8a), a response that was essentially identical in untransfected HEL cells, V-HEL cells and AS-HEL cells before exposure to IPTG. Exposure to IPTG for 1–5 days had no effect on Ca^{2+} mobilization in V-HEL or untransfected HEL cells. In contrast, incubation of AS-HEL cells with IPTG for 1–5 days caused a marked and progressive decline in Ca^{2+} mobilization (Figure 8b). At lower thrombin concentrations (1–5 nM) which still effectively increased $[Ca^{2+}]_i$ in control cells, the response was greatly delayed in IPTG-treated AS-HEL cells and the majority of cells failed to respond at all (results not shown). Adding 1.8 mM Ca^{2+} to the media, to determine the contribution of Ca^{2+} influx, slightly increased, and more noticeably prolonged, the thrombin-induced rise of $[Ca^{2+}]_i$ in IPTG-treated V-HEL cells and untreated AS-HEL cells (Figure 8c), but the response in IPTG-treated AS-HEL cells remained strongly suppressed (Figure 8d).

To study further the mechanism(s) responsible for defective thrombin-induced Ca^{2+} mobilization in AS-HEL cells we: (1) indirectly assessed the size of intracellular Ca^{2+} stores by adding thapsigargin to cells in Ca^{2+}-free medium, which releases ER Ca^{2+} through its selective inhibition of the ER Ca^{2+} pumps, i.e. SERCAs [29], and (2) indirectly assessed the specific Ca^{2+} influx that occurs upon restoring extracellular Ca^{2+} to cells whose ER stores were first unloaded with thapsigargin [16]. It should be noted that the rise in $[Ca^{2+}]_i$ due to thapsigargin is slower and less in magnitude than that caused by thrombin, due to the different mechanisms responsible for Ca^{2+} efflux, the possible existence of more than one releasable pool, and the opposing effect of Ca^{2+} pumps and exchangers in the plasma membrane. From these experiments we determined that the thapsigargin-releasable pool of Ca^{2+} was unaffected by the depletion of CHERP in IPTG-treated AS-HEL cells (Figures 9a and 9b) and that Ca^{2+} influx induced by the unloading of internal stores was actually significantly greater in IPTG-treated AS-HEL cells (Figures 9c and 9d). These findings demonstrate that the ability of thrombin to unload intracellular Ca^{2+} stores is the most likely defective step in IPTG-treated AS-HEL cells, and that this defect occurs in conjunction with the loss of CHERP.

DISCUSSION

Using mAb213-21, which blocks InsP_3-induced Ca^{2+} release from ER-derived membrane vesicles, we have cloned a cDNA for a novel protein, designated CHERP, that appears to be involved in the regulation of intracellular Ca^{2+} homoeostasis. CHERP is an integral membrane protein localized to the InsP_3-sensitive fraction of the smooth ER derived from platelets and HEL cells [1,2].

The CHERP cDNA predicts a protein with a molecular mass of 100 kDa. Using HPLC chromatography, we previously identified proteins of 100 kDa and 63 kDa which reacted positively with the mAb213-21 [1,2]. Initially we were only able to purify

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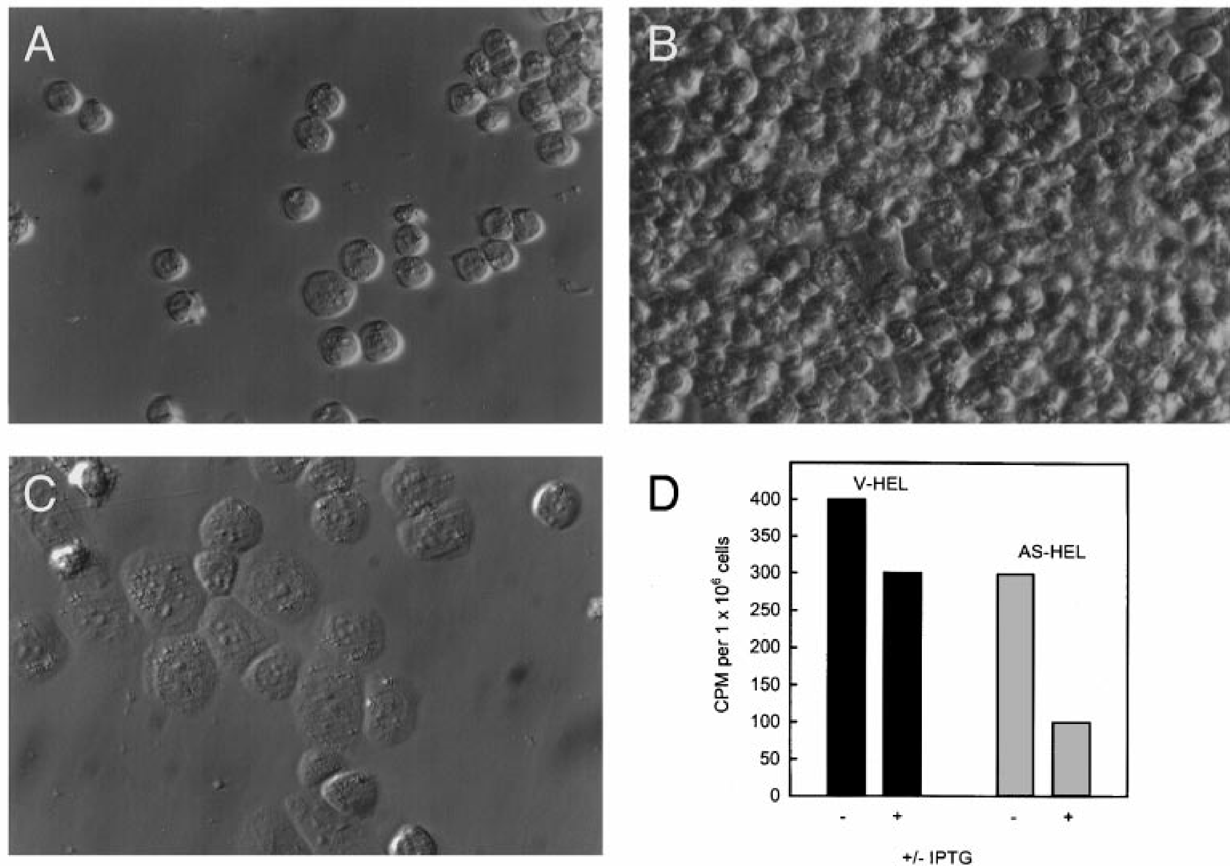

Figure 6 The effect of CHERP antisense expression on cell growth and proliferation

(**A–C**) Change in morphology and proliferation of AS-HEL cells: (**A**) untreated (0 days), (**B**) 7-day culture untreated and (**C**) after treatment with IPTG for 7 days. (**D**) Incorporation of [^{3}H]thymidine into DNA of V-HEL cells (filled bars) and AS-HEL cells (hatched bars). HEL cells were induced with IPTG for 24 h followed by washing and incubation with [^{3}H]thymidine (10 μCi/ml) for 10 min in culture media without thymidine and assayed for [^{3}H]thymidine incorporation by liquid-scintillation counting. Data represent the average of 2 experiments.

significant quantities of the 63 kDa protein from platelet membranes, but subsequently we purified the 100 kDa protein as well (results not shown). In membrane extracts the 63 kDa protein increased over time as the 100 kDa protein decreased, suggesting that the former was derived as a breakdown product of the 100 kDa protein. The HEL cell proteins detected by mAb213-21 on Western blots migrate at M_r 100000 and 128000, with the latter being the major band. Both 100 and 128 kDa bands were produced by *in vitro* translation of the same cDNA, which suggests that the larger product may be the result of post-translational modification, such as phosphorylation. Similarly, the expressed 2.6 kDa fragment of CHERP, which contains the serine/arginine domain, also migrates as a 20 % larger protein on SDS gels. Other proteins with extended serine/arginine regions like CHERP have been shown to exhibit large mobility shifts on SDS gels due to hyper-phosphorylation [30].

To study the role of CHERP in intact cells, we produced a stable HEL cell line by transfection with a vector containing an antisense CHERP cDNA construct under the control of an inducible promoter. In response to IPTG, which relieves *lac*I repression of the promoter, CHERP was progressively reduced by 80 % in AS-HEL cells over a period of five days. Cells also increased in size and in adhesion to culture vessels, their rate of proliferation decreased dramatically, and subsequently they displayed a senescent-like phenotype. Concurrently, Ca^{2+} mobilization by thrombin was markedly reduced. These changes all occurred without alteration of cell viability, or significant change in expression of the InsP_3 receptor, SERCA2 and SERCA3, or the thrombin receptor.

After five days exposure to IPTG, the ability of thrombin to mobilize intracellular Ca^{2+} was greatly suppressed and was totally lost in many cells at lower concentrations of the agonist. This defective mobilization of Ca^{2+}, observed in Ca^{2+}-free medium was not rectified by restoration of external Ca^{2+}, indicating that influx was also defective. Further investigation revealed that the thapsigargin-releasable pool of internal Ca^{2+} was unchanged in AS-HEL cells before and after five days treatment with IPTG, indicating that a deficit in this Ca^{2+} store was unlikely to be the cause of the defective response to thrombin. In addition, the influx of Ca^{2+} after first unloading Ca^{2+} stores with thapsigargin, which bypasses the need for InsP_3, shows that influx is normal provided the ER stores are first unloaded, and that the defect in agonist-induced unloading of Ca^{2+} stores is most likely to be responsible for the accompanying deficit in influx after thrombin. This is in agreement with current views on the intimate link between internal Ca^{2+} stores and Ca^{2+} entry [16,31,32]. Furthermore, our results are in close agreement with the findings of Somasundaram et al. [31], specifically in HEL cells, where thrombin-induced Ca^{2+} influx occurred entirely as a result of store-activated Ca^{2+} current, that thapsigargin and thrombin

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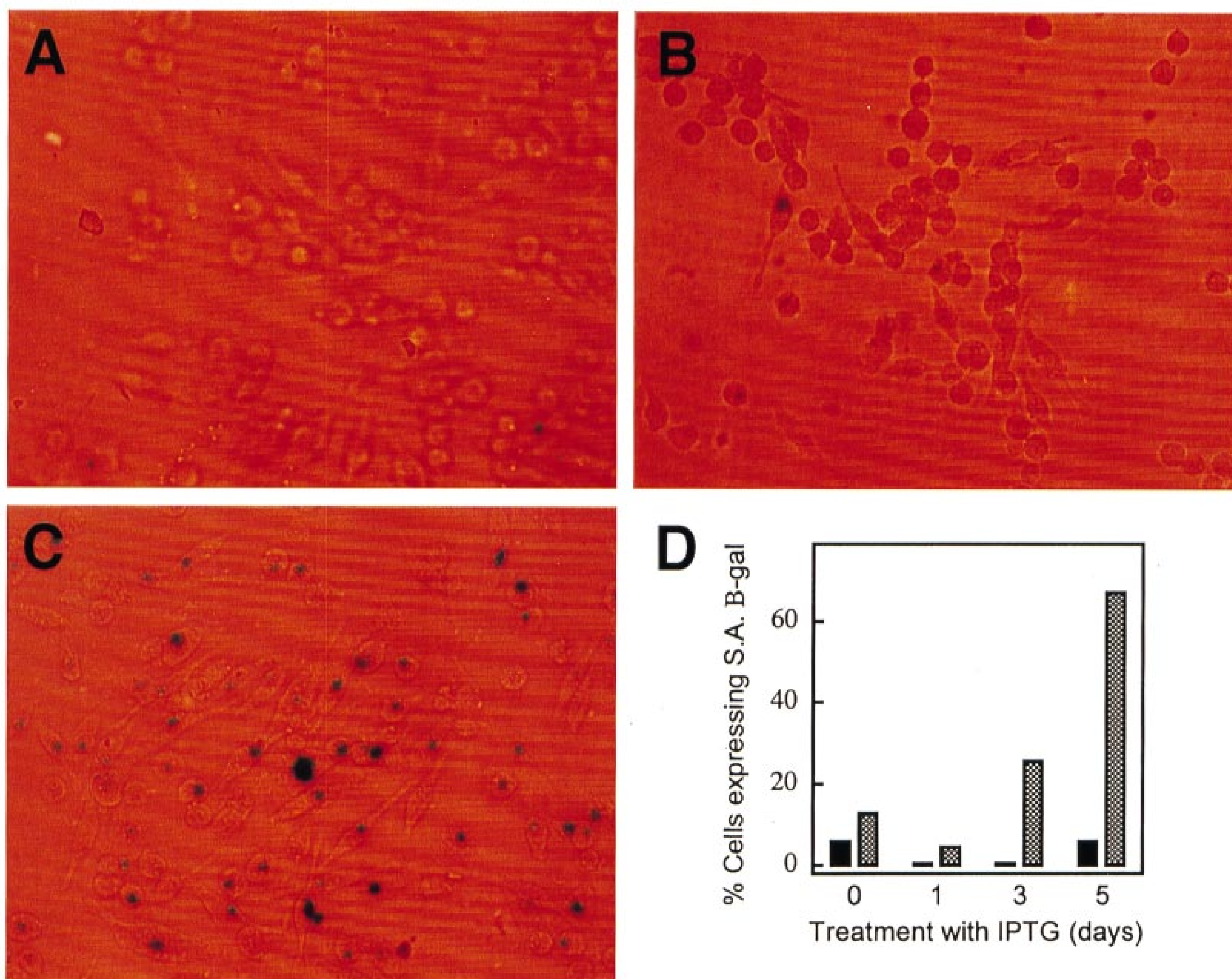

Figure 7 Senescence-associated β-galactosidase activity in transfected HEL cells with or without treatment with IPTG: (A) AS-HEL untreated, (B) V-HEL, and (C) AS-HEL cells, treated with IPTG for 5 days. (D) Senescence-associated β-galactosidase expression over 5 days

Cells were counted from three random fields and data expressed as the percentage of cells with blue–green nuclear staining (filled bars, V-HEL populations; hatched bars, AS-HEL populations).

released the same internal Ca^{2+} store, and that the level of expression of store-activated Ca^{2+} current probably determines the duration of thrombin-induced increase of $[Ca^{2+}]_i$. We noted that Ca^{2+} influx in IPTG-treated AS-HEL cells after thapsigargin was actually enhanced in IPTG-treated AS-HEL cells. We suggest that this is perhaps a compensatory response of the cultured cells experiencing decreased Ca^{2+} mobilization by serum growth factors. It may be mediated by a decrease in the plasma membrane Ca^{2+}-ATPase pump, or more likely by an increase in store-activated Ca^{2+} current [31], and/or inward rectifying plasma membrane K^+ channels that we recently showed could enhance Ca^{2+} influx after the unloading of ER stores [16].

A link between defective Ca^{2+} mobilization and arrest of cell proliferation is likely, since a sustained rise in $[Ca^{2+}]_i$ is necessary for the G1/S and G2/M phase transitions during cell division (reviewed in [33]). In our studies, treatment with IPTG to induce expression of antisense message led to a reduction in proliferation of AS-HEL cells comparable with that observed when HEL cells were treated with cell-permeant BAPTA-acetoxymethyl ester to chelate intracellular Ca^{2+} (results not shown). The IPTG-treated AS-HEL cells showed a two-thirds decline in the number of cells in S phase, as measured by $[^3H]$thymidine incorporation into DNA. IPTG had only a minor effect on either untransfected HEL or V-HEL cells. These findings show that AS-HEL cells lacking CHERP fail to progress through the cell cycle, possibly because they cannot sustain Ca^{2+} signals that are adequate for passage through the cellular checkpoints.

The specific molecular functions of CHERP have yet to be determined; however, studies with mAb213-21 [2] and K^+ channel blockers and K^+ ionophores [2,3] suggest that CHERP may be involved in regulating a K^+ conductance in the ER membrane. Ca^{2+} release from permeabilized cells and microsomal vesicles require monovalent cations, with K^+ being the preferred cation in most cases [2,34,35]. K^+ influx may prevent a counter-productive ER transmembrane potential change brought about by Ca^{2+} efflux from the ER [36]. Perhaps more importantly, K^+ influx also potentiates Ca^{2+} release by displacing bound Ca^{2+} from lumenal Ca^{2+}-binding ion-exchange storage proteins,

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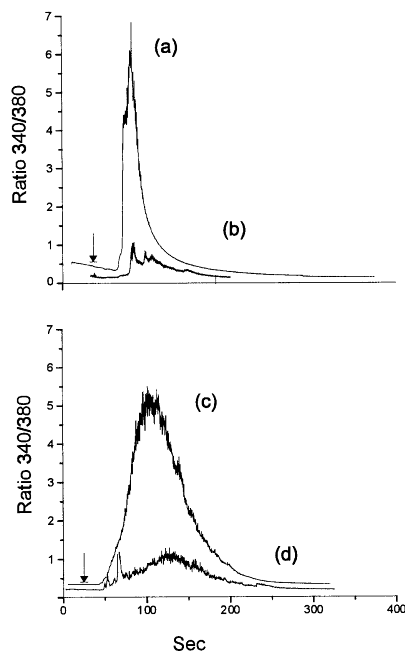

Figure 8 Thrombin-stimulated Ca^{2+} mobilization in AS-HEL cells treated with IPTG for 5 days

(**a**) AS-HEL ($n = 10$) and (**b**) AS-HEL + IPTG ($n = 10$) cells in Ca^{2+}-free medium. (**c**) AS-HEL ($n = 22$) and (**d**) AS-HEL + IPTG ($n = 26$) in 1.8 mM $CaCl_2$. Addition of 10 nM thrombin indicated by arrow. Data represent the mean for each experiment.

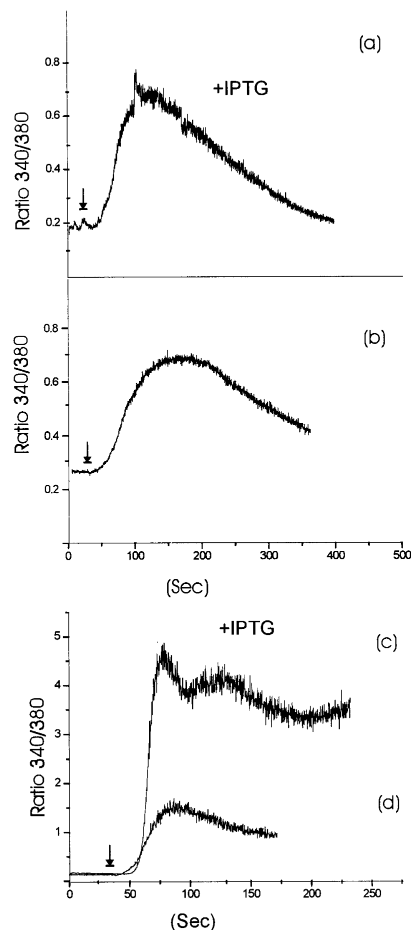

Figure 9 Ca^{2+} influx in AS-HEL cells after release of intracellular stores with thapsigargin in Ca^{2+}-free medium

(**a** and **b**) $[Ca^{2+}]_i$ in AS-HEL cells treated with 1 μM thapsigargin (addition indicated by arrow). (**a**) AS-HEL cells ($n = 14$) treated with IPTG for 5 days. (**b**) AS-HEL cell control ($n = 18$). Cytosolic Ca^{2+} returns to baseline by 500 s in each panel. (**c**) and (**d**) depict AS-HEL after addition of 1.8 mM $CaCl_2$ at arrow to monitor Ca^{2+} influx after emptying internal stores. (**c**) AS-HEL + IPTG for 5 days and (**d**) AS-HEL without IPTG. Data represent the mean in each experiment.

thereby greatly increasing the pool of free Ca^{2+} that can flow through opened Ca^{2+} channels [3].

No ER or sarcoplasmic reticulum K^+ channel proteins have been sequenced to date, and we have no direct evidence that CHERP mediates K^+ transport in the ER. CHERP lacks an S4 TM present in voltage-sensitive channels [37], and the C-terminal calmodulin-binding domain of many plasma membrane Ca^{2+}-activated K^+ channels [38]. However, CHERP does have a GLG motif in the region between TM1 and TM2, which is considered to be one of the signature motifs of K^+ pores [21–23], e.g. G(Y/F/L)G [22].

CHERP appears to be a single-copy gene, so that a gain- or loss-of-function mutation could alter specific cellular functions controlled by Ca^{2+}. CAG triplet repeats, as found in CHERP, are expanded in genes associated with Huntington's disease, spinobulbar muscular atrophy and spinocerebellar ataxia types 1, 2, 3, 6 and 7 [18,39–42]. Some of these genes are specifically involved in regulating cytosolic Ca^{2+} concentration, such as the human K^+ channel hKCa3/KCNN3 [39], and the human α1A subunit of a voltage-dependent (P/Q-type) Ca^{2+}-channel gene, the apparent cause of autosomal-dominant cerebellar ataxia type 6 [40]. In preliminary studies of blood cells from human donors we have not found any polymorphism in the CAG repeat in the CHERP gene (results not shown). The presence of the single CAA within the region, i.e. $(CAG)_6CAA(CAG)_5$, can prevent mutational expansion. However, the 1–3 intervening CAAs in the midst of a CAG repeat in the spinocerebellar ataxia 2 (ataxin) [40], or the CAT codons that interrupt the spinocerebellar ataxia 1 locus [42] can be mutated to CAG, permitting eventual elongation of the CAG domains with resulting pathology [41,42]. The possibility that CHERP may undergo similar mutations that affect its function will require additional study.

In conclusion, CHERP is a highly novel human protein co-localized with the InsP_3 receptor in the ER membrane. It is distinguished by a polyglutamine tract and several potential domains for macromolecular assembly, and consensus phosphorylation sites for kinases regulated by a number of important signal transduction pathways. Suppression of its expression by antisense cDNA leads to functional changes that are consistent with prior evidence that demonstrate the protein plays an important role in the regulation of intracellular Ca^{2+} mobilization [2].

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