Cha, a Basic Helix Loop Helix Transcription Factor involved in the regulation of
Upstream Stimulatory Factor Activity

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Running title: Cha, a new bHLH transcription factor
SUMMARY

We report here the characterization of Cha, a transcription factor of the basic helix-loop-helix (bHLH) family. The basic region of Cha shares DNA interacting amino acids with members of class C bHLH transcription factors. In addition, the HLH region of Cha presents a Myc-type dimerization domain signature required for heterodimer formation between members of this class. Cha protein and mRNA was ubiquitously expressed in many human tissues. Electrophoretic mobility shift assays showed that Cha and Upstream Stimulatory Factor (USF) 1 formed a complex that specifically bound to E-box DNA elements. Moreover, pull down and co-immunoprecipitation experiments showed an interaction between Cha and USF-1. Cha did not bind to E-box DNA elements and required USF-1 for protein-DNA complex formation. Moreover, Cha inhibited USF-1 stimulated transcription of CD2 (a USF-1-dependent gene) and E-box promoter reporter plasmids. Chromatin immunoprecipitation assays showed that Cha occupied the CD2 promoter in resting, but not in mitogen stimulated, T cells. Finally, Cha mRNA and protein expression were high in resting T cells and absent in mitogen activated T cells and inversely correlated with CD2 expression. Contrarily, overexpression of Cha in T cells significantly reduced CD2 expression. In summary, our results indicated that Cha is a new bHLH transcription factor that negatively regulates USF-dependent transcription.
INTRODUCTION

A great number of transcription factors have been described and classified in several families according to the structural properties of their DNA binding domain (1). Among those, basic Helix Loop Helix (bHLH) DNA binding proteins are a family of proteins characterized by their ability to recognize specific DNA sequences, termed E-boxes (CANNTG) through their basic region. They are able to form homo or heterodimers via their HLH domain (2). E-box elements were first identified in the immunoglobulin heavy-chain (IgH) intronic enhancer and have since then been found in a large number of pancreatic, lymphoid and muscle-specific promoter and enhancer elements (3). These proteins have originally been grouped into three classes based upon structural characteristics and pattern of expression (4). The class A proteins are ubiquitously expressed and readily bind DNA as either homodimers or heterodimers, with some of them implicated in cellular differentiation (5-7). Class B proteins are expressed in a tissue-specific manner and form heterodimers with class A bHLH proteins. Some of these factors, as MyoD, myogenin and myf-5, are implicated in muscle development (8-10). The last group, class C, forms homo and heterodimers with class C but not with class A or B bHLH proteins. Class A and B proteins recognize CAGCTG, and class C proteins recognize the CACGTG sequence (11). Among class C are factors as Myc, Max and Mad that are implicated in cellular proliferation, differentiation and apoptosis. USF is another member of this class ubiquitously expressed in mammals. There are two isoforms, USF-1 and USF-2, that play an essential role during embryonic development and also have pleiotropic effects in adult animals (12-14). The USF-1 and USF-2 polypeptides are very similar in their C-terminal regions, which contain the bHLH-zip domain, and consequently display
identical dimerization and DNA-binding specificities (15,16). USF has been described in most tissues and cell lines as USF-1/USF-2 heterodimers. USF-1 homodimers are less abundant and USF-2 homodimers are usually quite scarce (13,16,17). USF-1 and USF-2 bind to E-boxes in the promoter regions of several genes, some involved in the metabolism of the glucose response (lipidogenic and glycolytic enzymes), as fatty acid synthase (18), hormone sensitive lipase (19) and pyruvate kinase (20). In agreement with this, USF-1 or USF-2 knockout mice have a diminished glucose response (21) and fatty acid synthase expression (22). Other genes such as follicle stimulating hormone receptor (FSHR) (23), metallothionein (24), HOXB4 transcription factor (25) and the class II major histocompatibility complex (26) are regulated by USF-1. Additionally, USF over expression has been found to inhibit growth in a number of cancer cell lines (27,28). USF-1 regulates CD2 expression in T cells (29) and is involved in such diverse functions as adhesion (30), scanning (31), activation (32,33) and positive/negative selection (34).

Here we report the characterization of a novel transcription factor named Cha. Our study strongly suggests that Cha is a ubiquitously expressed member of class C bHLH transcription factors. Cha was found to interact with USF-1 and was capable of binding to E-boxes as Cha/USF-1 dimers in vitro as well as in vivo. Furthermore, Cha was able to inhibit USF-1 dependent promoter activity in a dose-dependent manner. Expression of Cha and Cha occupancy of the E-box CD2 promoter region negatively correlated with CD2 expression in T cells after mitogenic stimulation, while overexpression of Cha inhibited CD2 expression. Our results suggest that Cha is a new bHLH transcription factor that negatively regulates USF dependent transcription.
MATERIALS AND METHODS

Cell cultures—Jurkat, Jurkat J77cl-20 clone and COS-7 cells were grown in RPMI and Dulbecco’s minimal essential medium (DMEM; Gibco, UK), respectively. The medium contained 100 µg/ml streptomycin, 100 units/ml penicillin, 2mM L-glutamine plus non-essential amino acids supplemented with 5% heat-inactivated fetal bovine serum (FBS; Gibco, UK). Incubation was carried out at 37°C in a 7% CO2-in-air atmosphere, water vapor saturated incubator.

Resting T lymphocytes were purified from blood drawn from healthy volunteers and centrifuged through Ficoll-Paque (Pharmacia-Biotech, UK) as described (35). The peripheral blood mononuclear cells (PBMC) were depleted of adherent and B cells by plastic adherence and nylon wool columns. Cells were resuspended in DMEM-10% FBS and stimulated with 20 ng/ml phorbol myristic acetate (PMA) plus 1 µM A23187 calcium ionophore (Io) (Sigma).

Cloning of Cha—Cha cDNA was isolated as described (36). A partial sequence (pBKCMVCha) was isolated from the screening of a human cDNA library with sera from chagasic (T. cruzi-infected) patients. Later, the 5’ end sequence was obtained by 5’ Rapid Amplification of cDNAs Ends (RACE). Oligonucleotides Comp (5’-GGGAACCATGTGCTCCGGGTGG-3’) and NGFL3’ (5’-GCACAGGTCACGAAGGAATGGCTGTT-3’) were used to amplify the complete coding sequence from cDNA of Jurkat cells. The complete coding sequence was cloned in the pGEM-T vector (Promega) and sequenced. Nucleotide sequencing was performed utilizing the fmol kit (Promega) following the instructions of the manufacturer or automatically with an Applied Biosystems sequencer. The Genbank accession number for the Cha gene is AJ271337. Nucleic acid and protein sequences
were analyzed by the University of Wisconsin Genetics Group Sequences Analysis Software Package (37).

**Plasmids**—pGEX4T3sCha was created by subcloning a BamHI/SmaI fragment amplified from the complete cDNA cloned in pGEM-T with oligonucleotides BamHI-sCha (5’-AATCGGGGATCCATGCAGTTGGACACAATGTA-3’) and SmaI – sCha (5’-TGTTCAACCGGGTCACCTGATCTCCATCGAGGGGCT-3’) into the pGEX4T3 (Amersham Pharmacia Biotech AB, Upsala, Sweden) plasmid. pcDNA3Cha and pcDNA3sCha were created by subcloning the PCR products amplified from pBKCMV with oligonucleotides T7pBKCMV (5’-GTAAATACGACTCACTATAGGGC-3’), Euca (5’-ATATATGGATCCACTATGAATGTTCCTCTCTCACCAACAAAAACAAATGT-3’), and sCha Euca (5’-TATCTCGGATCCAGAATGCGTCAGTTGGACACAATGTAAGAGCGAAGA-3’), respectively, into BamHI/EcoRI restriction sites of the pcDNA3 plasmid (Invitrogen). pcDNA3USF-1 was created by subcloning USF cDNA from pcXUSF, kindly provided by Dr. Robert G. Roeder (Rockefeller University, New York, USA), as a HindIII and XhoI fragment into the pcDNA3 plasmid (Invitrogen). pCMV-Max was also provided by Dr. Robert G. Roeder (Rockefeller University, New York, USA). pGEXUSF-1 was generously donated by Dr. Colin Goding (Marie Curie Research Institute, Oxted, UK).

The CAT reporter plasmid driven by the CD2 promoter element named CM2.5 was supplied by Dr. J. Owen (29). The NF-κB Luc reporter contained three tandem repeats of the NF-κB enhancer upstream of the conalbumin minimal promoter and the luciferase reporter gene (pNF3ConA Luc) (38). pGL3p4E-boxCD2 was constructed as follows: 5’-
TCGAGGCACGTGCCTCTCTAACCACGTGCCTCTCTAACCACGTGCCTCTCTA
ACCACGTGT A - 3 ' and 5 ' -
CGCGTACACGTGGTTAGAGAGGCACGTGGTTAGAGAGGCACGTGGTTAGAG
AGGCACGTGCC-3’ primers (E-boxes are underlined) containing four E-box sequences which were annealed and cloned into M1uI and XhoI sites of the pGL3 promoter vector (Promega). The luciferase and β-Gal reporters under control of the minimal promoter of the thymidine kinase or the cytomegalovirus promoter (TK-luc and CMV β-Gal, respectively) were used to monitor transfection efficiency.

mRNA detection- Total RNA was isolated from T lymphocytes with TRIZOL (Life Technologies). RT-PCR was performed using "GeneAmp RNA PCR Kit" (Applied Biosystems). cDNA was synthesized from 1.75 µg of total RNA in 35 µl of each reaction mixture and Cha DNA fragment (nucleotides 844 to 1317) was amplified by PCR from 10 µl of each cDNA sample. The amplification program consisted of 30 cycles of 45 s at 94ºC, 45 s at 60ºC and 1 min at 72ºC for Cha fragment and 25 cycles for GAPDH. PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining.

Tissue expression analysis- Expression of Cha protein in different human tissues was analyzed in InstaBlot membrane (Imgenex). Different cDNA libraries from 8 human tissues (QUICK-Screen™ cDNA Library Panel, CLONTECH) and a cDNA library from Jurkat T cells were assayed for Cha expression by PCR. The amplification program consisted of 30 cycles of 30 s at 94ºC and 2 min at 68ºC using the Advantage™ Klentaq Polymerase Mix (Clontech). The Cha specific primers used were (5'-CACCAGGATATTGGATTTGCTAGAGC-3') and (5'-GTGGCACCCTTCGCCCACATTCTGAA-3'), that amplified a fragment of 230 bp. The
PCR products were separated on agarose gels and analyzed by ethidium bromide staining. As a control, a 593bp fragment of the Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) was amplified using GAPDH sense (5’-CCACCCATGGCAAATTCATGGCA-3’) and GAPDH antisense (5’TCTAGACGGCAGGTACAGTCCACC-3’) oligonucleotides.

**Recombinant proteins**-Native rCha protein was obtained from *in vitro* translated pcDNA3sCha using the TNT coupled reticulocyte lysate system (Promega) according to the manufacturer’s instructions. Briefly, 1µg of pcDNA3sCha was incubated for 90 minutes at 30ºC with rabbit reticulocyte lysate (RRL), T7 RNA polymerase, 20 mM of a combination of aminoacids without methionine, 40 µCi (35S)-methionine and 40 unit ribonucleases inhibitor in a final volume of 50 µl. The resulting product was examined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis to monitor translation efficiency.

**In vitro protein interaction analysis**-Protein expression in pGEX4T3, pGEX4T3-sCha and pGEXUSF-1 transformed *E. coli* DH5α strain was induced in a 20 ml culture with 100 µM IPTG for 5 h. Bacterial cells were disrupted by sonication in PBS containing 1% TritonX-100, supplemented with protease inhibitors (2µg/ml aprotinin, 2µg/ml pepstatin, 2µg/ml leupeptin and 0.5 mM PMSF). Subsequently, the lysate was centrifuged and the supernatants containing the expression proteins glutathione-s-transferase (GST) and GST-sCha were coupled to 50 µl of glutathione Sepharose 4B (Amersham Pharmacia Biotech AB, Upsala, Sweden) for 1 h at 4ºC and washed with 10 bed volumes of ice cold PBS 1%TX-100. Recombinant proteins were purified either with reduced glutathione or thrombin cleavage, as indicated, following instructions of the manufacturer. A whole cell extract (WCE) from 15x10⁶ Jurkat cells lysed in TNT
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(20mMTris.HCl pH 7,6, 200mM NaCl, 1% Triton X-100 suplemented with protease inhibitors), was incubated with glutathione Sepharose 4B coupled with GST for 1h at 4ºC, and then coupled with GST-sCha for 1 h at 4ºC. The resins were washed three times with 1 ml of ice cold TNT and the bound proteins were separated by SDS-PAGE followed by Western blot with the anti USF-1 antibody.

Antibodies-Specific anti-Cha antibodies were prepared by immunizing rabbits with a peptide of Cha (SLVTCQAQLQSSPSMEI) as described and were purified by affinity-chromatography as described (36). Anti-Cha antibodies showed no crossreactivity against recombinant USF-1, USF-2 or protease activity (not shown). The affinity purified antibodies used in supershifting assays directed against: USF-1, USF-2 and E12#E47 were were made against the recombinant proteins and generously provided by Dr. Carmelo Bernabéu (Centro de Investigaciones Biológicas, Madrid). Anti-USF-1 (sc-229X) and anti Actín (1-19) (sc-1616) antibodies were from Santa Cruz.

Cell transfection-Jurkat cells were seeded in 24 well plates (2x10^6 cells/ml) and transfected with the Lipofectamine Reagent (Gibco, UK) preparing the Lipofectamin-plasmids mixtures according to the instructions supplied by manufacturers. The mixtures were incubated at 37ºC in a 7% CO₂ incubator for 3 hr before washing with fresh medium and incubated further for 24 h or 48 h. When indicated, cells were washed once with fresh medium and cultured in wells containing medium alone or 20 ng/ml of PMA plus 1µM Io. After 6 h. the cells were harvested and assayed for either chloramphenicol acetyltransferase (CAT) or luciferase activity as described previously (39). Transfection efficiency was always normalized with respect to the activity of the luciferase reporter under control of the TK minimal promoter, TK luc. COS-7 cells
were seeded (10^5/well) in 24 well plates 24 h before transfection of plasmids (as indicated) with lipofectamine Plus Reagent following the directions of the manufacturer (Invitrogen). Cells were lysed in 100µl of Reporter lysis buffer (Promega) 24 hr after transfection. 20µl of lysate were incubated with 100 µl luciferase assay substrate for 10 sec and relative luciferase units were measured in a luminometer (Monolight 2010, Analytical Luminescence Laboratory). Luciferase activities were normalized, with respect to the protein and β-galactosidase activity of cotransfected β-Gal vector. The β-galactosidase assay was performed with 20µl of precleared cell lysate plus 20µl of o-nitrophenyl-beta-D-galactopyranoside (ONPG) substrate (1,33mg/ml) for 10 min. β-Gal activity was measured in an EL 340 microplate reader at 405 nm (Biotek Intruments).

Co-Immunoprecipitation-WCE from Jurkat cells and COS-7 cells were obtained by lysis in TNT supplemented with protease inhibitors as described before (see in vitro protein interaction analysis). WCE were immunoprecipitated with 1 µl of anti-USF-1 antibody (200 µg/0,1 ml, Santa Cruz) previously coupled to 50 µl protein-A-Sepharose (PAS) (Pharmacia) and, after washing five times with lysis buffer, proteins were separated by SDS-PAGE, and analyzed by Western blot utilizing anti-Cha and anti-USF-1 antibodies, as indicated.

Electrophoretic mobility shift assay (EMSA)-Nuclear extracts from Jurkat cells or peripheral blood T lymphocytes were prepared basically as described (35). Double-stranded CD2 oligonucleotide (which contains an E-box element from human CD2 promoter 5’-GATCAAAGAGAGGACGTGTTAAGCTC-3’) was used as a probe. The irrelevant oligonucleotide (5’-CCCGAGAATACAAAAAGGTCCCTGACGG-3’) was used as control. For binding reactions, 3 µg of nuclear extract proteins, 1 µl of
in vitro-translated rCha, and 0.2µg of purified USF-1 and Cha were mixed with 1 µg poly (dI-dC), poly (dA-dT), poly (dCdG) and TATA4 oligonucleotide (kindly provided by A. Nieto, Centro de Biología Molecular “Severo Ochoa”, Madrid), in the presence or absence of competitors (in a excess of 30-fold molar as indicated) or antibodies in a final volume of 20 µl for 15 min on ice, as indicated. These mixtures were incubated for 20 min at 4ºC with radioactive DNA probe. Protein-DNA complexes were fractionated on 4% native polyacrilamide gels (29:1, acrylamide:bisacrylamide) in 0.4 x Tris Borate EDTA (TBE). After drying the gel, the results were recorded by autoradiography.

Western blotting-WCE was made using TNT and nuclear extracts as described in previous sections. Western blot analyses were performed as described previously (35). Briefly, 15 µg of Jurkat cell lysate or 10 µg of T lymphocytes nuclear extract protein were fractionated on SDS-12% polyacrylamide gel and transferred to a Problot membrane (Applied Biosystems). The membranes were incubated, either with anti Cha polyclonal antiserum at 1:1000 dilution in the absence and presence of recombinant sCha as indicated, or with polyclonal anti USF-1 antibody. Then, the membranes were incubated with horseradish peroxidase goat anti rabbit antibody (Pierce) as secondary antibody. Detection was carried out with ECL (enhanced Chemi-luminescence) detection reagent (Amersham).

Chromatin immunoprecipitation (ChIP) assay-Chromatin immunoprecipitation was performed as described (40) with some modifications. Briefly, 12x10^6 resting and PMA/Io stimulated purified T cells (see cell culture section) were crosslinked in 1% formaldehyde for 10 min at room temperature. Glycine 0.125 M was added to stop the crosslinking for 5 min at room temperature. T cells were washed in ice cold PBS and lysed on ice in 5 mM PIPES pH8.0, 85 mM KCl, 0.5% NP40 buffer for 10 min. Nuclei
were lysed in 50mM Tris-HCl pH 8.1, 10mM EDTA, 1% SDS buffer for 10 min on ice. The lysates were sonicated on ice 5 times (25 sec each time) to an average chromatin length of about 600 bp and diluted 1:10 in 0.01% SDS, 1.1% Triton X 100, 1.2mM EDTA, 16.7 mM Tris-HCl pH 8.1, 167 mM NaCl buffer. Chromatin was immunoprecipitated and pellets were washed twice with 2mM EDTA, 50mM Tris-HCl pH 8.0, 0.2% sodium lauryl sarcosinate buffer, and four times with 100 mM Tris-HCl pH 9.0, 500mM LiCl, 1% NP40, 1% deoxycholic acid buffer. All above buffers were supplemented with a cocktail of protease inhibitors. Antibody/protein/DNA complexes were eluted from PAS by incubation with 50mM NaHCO₃ and 1% SDS for 15 min at room temperature. Antibody/protein/DNA complexes were incubated with RNase A and proteinase K. DNA was isolated with QuiagexII gel extraction kit (Quiagen). PCR reactions were performed with Expand High Fidelity polymerase (Roche Diagnostics GmbH Mannheim, Germany), 100 ng of immunoprecipitated DNA and 0.2% of the total input chromatin from antibody free samples as the positive control. Oligonucleotides derived from the CD2 promoter, EboxCD2F (5'-GTGTGAGAATTAATGCAGC-3') and EboxCD2R (5'-CTTAGGGGTGGCTCTTT-3'), oligonucleotides derived from the p53 promoter (as negative control), p53F (5'-GCTTTGTGCCAGGAGGAGCCTCC-3') and p53R (5'-CGTCGTGGAAGCAGCGCTCC-3') were used. The amplification program consisted of 35 cycles of 30 s at 95°C, 30 s at 50°C for the CD2 fragment and 60°C for the p53 fragment, and 30 s at 68°C. PCR products were analyzed by electrophoresis in agarose gels stained with ethidium bromide.

Flow cytometry-J77cl-20 cells (10⁵/ml) were transfected using Lipofectamine Plus Reagent with Green fluorescent protein (GFP) expression plasmid (CMVGFP) and increasing amounts of pCDNA3Cha. After 48 h cells were stained with biotinylated
anti-human CD2 and streptavidin-Cy-Chrome (BD Biosciences Pharmingen). Expression of CD2 in transfected cells (expressing GFP) was analyzed in a FACSCalibur apparatus (Becton Dickinson).

RESULTS

Cha contains a Class C bHLH DNA binding domain—As result of the screening of a Jurkat T cell line cDNA expression library with sera from Trypanosoma cruzi chronically infected patients, a clone named Cha was isolated which encodes a partial cDNA sequence (36). The complete cDNA of Cha was obtained by 5’ RACE and revealed a 5’ non-coding region of 481 bp, followed by 819 bp open reading frame which codes for a protein of 273 amino acids (Fig. 1A). A search through the data-base found 100% homology with exons 2 to 6 of a testis specific gene, named TCFL5 (41). Cha also presented a different 5’ untranslated region (Fig. 1B), suggesting that Cha could be a product of alternative splicing of the TCFL5 gene.

Analysis of the Cha aminoacid sequence showed high homology with the binding domain of bHLH proteins from different species (Fig. 2A). HLH domains have been implicated in protein-protein interactions, with the basic region of the domain involved in making contacts with DNA, thus determining sequence specific binding (42,43). Interestingly, Cha presented conserved amino acids in the basic region (Glu9 and Arg12) and highly conserved Arg2 and Arg10, critical to establish DNA contacts (Fig.2B). Furthermore, Cha conserved His5, Asn6 and Arg13, that are specific of class C proteins (44). It must be emphasized that class A and B proteins lack the Arg residue at position 13 and the His residue at position 5 of the basic region. The last two positions are essential to determine DNA binding specificity of class C proteins (44). On the other hand, comparison against the PROSITE DNA binding motifs indicated the
presence of a Myc-type “helix-loop-helix” dimerization domain signature at position 208 (Fig. 2A). Since the aminoacid sequence of Cha basic region conserves E-box specific positions and contains a dimerization domain signature, we propose that Cha is a new member of the class C bHLH transcription factor.

Tissue expression of Cha- In order to study the expression of Cha in tissues, we performed western blot of human tissues with anti-Cha antibody. Figure 3A shows an almost ubiquitous expression pattern of Cha protein in different human tissues, except for brain. Surprisingly, the mobility of Cha varied slightly in different tissues. Antibody against actin was used as a control. However, this isoform of actin was not detected in some tissues, such as heart and skeletal muscle, because they express a different isoform of actin. Similar results were obtained using mouse tissue extracts (data not shown). In addition, human cDNA libraries from different tissues were used as templates for PCR amplification with oligonucleotides specific for the 3’ end of Cha. As shown in Fig. 3B, Cha cDNA showed a ubiquitous pattern of expression, since it was detected in all tissues studied. This was in agreement with results of Northern blot analysis of Cha mRNA from different human tissues (not shown).

Cha binds to E-box sequences-The above results prompted us to test the E-box binding activity of this protein. For this, we used the E-box sequence (CACGTG) of the CD2 promoter, a USF-1 dependent gene encoding a 55 kDa glycoprotein expressed on T cells (29) as a probe. Nuclear extracts from Jurkat T cells were used in EMSA to determine the presence of binding activities for the CD2 E-box element. A protein-DNA complex was specifically competed by the unlabelled probe but not by an irrelevant one (Fig. 4A, arrow). This complex was completely supershifted with antibodies against USF-1 and partially with antibodies specific for USF-2.
Preincubation of the extracts with the antibody specific for Cha produced a partial reduction of the complex similar to anti USF-2 antibody (Fig. 4A). In contrast, the complex remained basically unaltered in the presence of antibodies directed against other bHLH proteins, as E12#E47 or by rabbit preimmune serum (not shown). A non-specific complex (although present in all lanes) appeared with more intensity in the presence of anti-Cha. However, this complex was competed by an irrelevant TATA4 oligonucleotide (Fig. 4B, asterisk). The anti-Cha antibodies did not affect binding of AP-1 or NF-κB transcription factors to their cognate DNA sequences (data not shown); thus, discarding a possible proteolytic effect of the antibody in the assay. Those results suggested that Cha was part of a complex capable of binding to the CD2 promoter E-box element. Similar results were also obtained when nuclear extracts from other cell lines from various tissue origins (HeLa, HL60 and U937) were used (data not shown).

To further investigate whether Cha and USF were part of the same complex able to bind the CD2 promoter E-box element, an in vitro translated recombinant Cha (rCha) was used in a supershifting assay. It has been described that RRL, used for the in vitro translation assay, contain USF (45). Because of this, we observed a faint basal binding complex to the CD2 E-box probe in unprogrammed lysates (Fig. 5A). This complex was abrogated after incubation with anti-USF-1 (Fig. 5B). A complex of similar mobility, but with stronger intensity than for unprogrammed RRL, was detected with in vitro translated rCha as a specific single complex, as it was competed by the cold CD2 oligonucleotide but not by an irrelevant oligonucleotide (Fig. 5A). Addition of antibodies directed against Cha or against USF-1 to the binding reaction, resulted in the abrogation of the specific complex binding. As a control, the addition of anti-E12#E47 antibody did not alter the binding of the specific complex (Fig. 5A). Thus, the above
results suggested that Cha interacted with USF molecules present in the RRL to form a specific complex.

To discard the possibility that interaction between Cha and USF-1 required a third factor, and to further corroborate Cha and USF-1 ability to form a specific complex, we used highly purified recombinant Cha and USF-1 in EMSA assays. Figure 5C shows that USF-1, but not Cha, formed homodimers able to bind to the CD2 probe. Addition of Cha, but not GST, to the USF-1 binding reaction caused an increase (around two fold by densitometer scanning of the bands, not shown) in the amount of complex formed, which likely corresponded to the formation of heterodimers between USF-1 and Cha. All the complexes were supershifted with the addition of anti-USF-1 antibody to the reaction (data not shown).

Cha and USF-1 interact “in vitro” and “in vivo”- Immunoprecipitation of Jurkat cells with anti-USF-1 antibody followed by western blot with anti Cha and anti-USF-1 antibodies showed the presence of both USF-1 and Cha in the immunoprecipitate (Fig. 6A). This suggests a protein interaction between Cha and USF-1. To further confirm this hypothesis we used COS-7 cells which allow high levels of protein expression in transient transfection. Figure 6B shows that untransfected COS-7 neither express detectable USF-1 nor sCha, but upon transfection, COS-7 cells showed high level of expression of both proteins. Immunoprecipitation of WCE with anti-USF-1 antibody followed by Western blot analysis with anti-Cha antibody showed that anti-USF-1 was able to coimmunoprecipitate sCha only in cells transfected with both USF-1 and sCha. (Fig. 6C). In addition we performed pull down experiments with GST-sCha and Jurkat WCE. Incubation of highly purified GST and GST-sCha with Glutathione Sepharose in the absence or presence of Jurkat WCE followed by Western blot with anti-USF-1
antibody showed that GST-sCha was able to pull down USF-1 (Fig. 6D), further confirming its interaction.

**Cha inhibits USF dependent promoter transcription**—To study the involvement of Cha in the modulation of USF activity we analyzed its effect on the expression of CD2 (a USF dependent gene). For this we co-transfected the CD2 CAT reporter plasmid with increasing amounts of Cha expression vector in Jurkat cells. Co-transfection of Cha expression plasmid resulted in a dramatic dose dependent inhibition of the basal reporter activity (Fig. 7A). As a control, transfection of increasing amounts of Cha did not affect NF-κB Luc reporter. CD2 transcription had been shown to be enhanced by transfection of both USF-1 and Max expressing plasmids (29). In agreement with this, we found that transfection of USF-1 alone produced a small enhancement, whereas Max was ineffective (Fig 7B). However, both together had a synergistic effect. This enhanced transcription was also inhibited by cotransfection of Cha in a dose dependent manner. Remarkably, very small amounts of Cha plasmid (50 ng/10⁶ cells) were able to inhibit USF-1/Max dependent transcription (Fig. 7B). Cha was also able to inhibit USF-1 dependent transcription of a tandem E-box reporter plasmid (Fig. 7C). Furthermore, we have not found any transactivating activity promoted by Cha in assays where Gal4-Cha constructs and Gal4 luciferase reporter plasmid were cotransfected (data not shown), indicating that Cha has no intrinsic transactivating activity. Therefore, Cha was able to specifically inhibit the transactivation of USF and E-box dependent promoters.

**Regulation of CD2 and Cha expression during T cell activation**—It has been described that CD2 cell surface expression is upregulated during mitogenic stimulation of T cells (45). Since we have found an opposite effect of Cha and USF-1 in the regulation of the CD2 promoter, we studied whether Cha and USF-1 occupied the CD2 promoter in
resting and activated T cells by ChIP assays. The results showed that USF-1 and, in a minor extent, Cha occupied the CD2 promoter region containing the E-box in resting T cells. In contrast, in activated T cells, an almost complete disappearance of Cha and a decrease in USF-1 occupancy of the CD2 promoter was observed (Fig. 8A). Cha mRNA and protein expression were downregulated upon mitogenic stimulation (Fig. 8B and 8C, respectively) and Cha protein was not detectable after 18 h of mitogenic stimulation (Fig. 8C). This was associated to an increase of CD2 membrane expression, analyzed by flow cytometry, of approximately 50% over the basal level (data not shown), in agreement with previous reports (45). Finally, overexpression of Cha in J77cl-20 cells (a high CD2 expressing clone of Jurkat cells) induced a dose dependent inhibition of CD2 cell surface expression, with a maximum of 40% at the highest dose of Cha DNA transfected (2µg/10⁶ cells) (Fig. 8D). Although this inhibition was not pronounced, it was reproducible and specific, since transfection of control plasmid was ineffective. Taken together those results suggest an inverse regulation of CD2 and expression of Cha that correlates with a decrease in Cha occupancy of the E-box CD2 promoter region during T cell activation.
DISCUSSION

We have described here the characterization of a bHLH transcription factor named Cha. This factor belongs to the bHLH transcription factor family since it has a bona-fide bHLH domain. It is well established that the basic region of bHLH domain is essential in determining DNA binding specificity and that certain residues in this 13 aminoacid-long basic region are characteristic of particular bHLH protein classes (44). The basic region of Cha fulfills the requirements that define it as a class C bHLH transcription factor. Moreover, it contains the Myc-type dimerization domain signature that permits dimerization with other members of class C bHLH transcription factors. In addition, class C bHLH transcription factors present a leucine zipper involved in dimerization (2,3,44). Cha presents 5 leucines at this position that could be involved in dimerization with other members of Class bHLH transcription factors. Therefore, we propose Cha as a new member of this class.

Interestingly, a similar genomic clone named TCFL5 was isolated from testis (41). TCFL5 and Cha have identical C-terminus sequences but differ in their N-terminus. In fact, the full-length TCFL5 cDNA includes exons 1 to 6 while Cha presents exons 2 to 6. Furthermore, Cha presents an additional 449 bp sequence not included in TCFL5, where TCFL5 has a splicing signal in the genomic clone. The TCFL5 cDNA is only expressed in testis while Cha is rather ubiquitous. In Jurkat and HeLa cell lines no expression of the first exon present in testis was found by RT-PCR (data not show), further confirming the strong tissue specificity that this first exon confers to the TCFL-5 gene. How alternative splicing may affect the functionality of TCFL-5 or Cha proteins as transcription factors is not known at present. Moreover, the differences in mobility of Cha protein observed in different human tissues could be the
result of differential post-translational modifications, that could be involved in regulation of Cha function, and require further study.

On the other hand, we have demonstrated that Cha is able to bind a CD2 promoter E-box motif, *in vitro* as well as *in vivo*. This element has been shown to bind USF-1 (29). The localization of USF-1 in the same complex as Cha in Jurkat cells and the ability of anti-USF-1 and anti-Cha antibodies to abolish the DNA complex formation with “in vitro” translated Cha, suggests that Cha could be forming heterodimers with USF. In fact, USF-1 seems to co-immunoprecipitate with Cha in Jurkat cells. Interaction of Cha and USF-1 was further confirmed by coimmunoprecipitation of both proteins from COS-7 cells transiently transfected with Cha and USF-1. More significantly, GST-Cha was able to specifically pulldown USF-1 from Jurkat cell lysates, thus, confirming the interaction between Cha and USF-1. Since class C bHLH proteins only form heterodimers with other members of the same class (2), this further supports the inclusion of Cha as a new member of class C bHLH protein.

Surprisingly, recombinant Cha or native Cha present in nuclear extracts was not able to bind to E-box DNA elements as a homodimer and required USF-1. An explanation for this result could be that the leucine zipper-like region of Cha is sufficient to allow heterodimerization of Cha with other Class C bHLH transcription factors, as USF-1, but not sufficient for homodimerization. Another explanation would be that Cha binds to USF-1 homodimers and stabilizes the complex. To elucidate this, more experiments focused on the stoichiometry of the Cha/USF-1 complex need to be performed.
More interestingly, we have shown that the ectopic expression of Cha inhibited the transcriptional activity of USF-1 on artificial or natural E-box dependent promoters as CD2. CD2 transcription has been shown to be dependent on endogenous USF-1 and enhanced by Max (29). Cha inhibits these transcriptional activities. Overexpression of Cha in J77cl-20 cells inhibited CD2 cell surface expression. How Cha represses USF-1 is a matter of speculation at the moment. Since Cha lacks a structurally recognizable transactivation domain and it has no intrinsic transactivation activity, Cha/USF-1 complexes may block USF-transactivating activity, without blocking binding to DNA. We found an inverse regulation of CD2 and Cha expression and an almost complete decrease in the occupancy of Cha in the E-box containing CD2 promoter region by ChIP assays in T cells after activation. We also observed a decrease in USF-1, this factor is still detectable in activated T cells and could indicate that USF-1 binds to another factor, as USF-2, in activated T cells. Thus, it is likely that in resting T cells Cha is inhibiting CD2 transcription as it does in reporter transfection experiments. T cell activation induced Cha down-regulation, which causes loss of Cha occupancy of the CD2 promoter in the chromatin, and CD2 up-regulation (29). CD2 expression was downregulated by overexpression of Cha. CD2 is involved in T cell adhesion (30), T cell activation (33), and plays a role in selection events during thymocyte development and TCR-stimulated cytokine production in mature T cells (34). Therefore, the negative regulation of CD2 by Cha may have some influence in the above processes. It could have effects on CD2-dependent adhesion, activation, selection and cytokine production of T cells. Experiments are in progress to elucidate this.

There are other factors of Class C bHLH that behave as Cha. Max expression is weakly regulated (46,47), while Myc and Mad are highly regulated (46). Myc genes are transcribed in proliferating cells, while in differentiated cells mRNA or proteins are
hardly detected. In contrast, Mad genes are usually expressed in differentiated and resting cells but they are down-regulated in proliferating cells. Our results indicate that regulation of Cha expression in T cells is similar to Mad, since we observed a strong expression of Cha mRNA and protein levels in resting T lymphocytes that disappeared upon mitogenic stimulation.

Taken together, the structural features, DNA binding abilities of Cha gene as well as its E-box regulating function, suggest that this protein could be a new class C bHLH transcription factor that represses transcription from USF-dependent E-boxes. So far, USF-1 has been shown to form heterodimers only with USF-2. Our results indicate that Cha is a new partner of USF-1.
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LEGENDS TO FIGURES

FIG. 1. Nucleotide sequence of human Cha cDNA. A, The initial 482 bp corresponds to the non-coding region. The open reading frame encodes a protein of 273 aminoacids. The boxed methionine residue represents the translation start site. The asterisk denotes the stop codon. B, Genomic organization of TCFL5 and Cha. Exons are in white boxes, introns are in gray boxes and splice sites are indicated with dashed lines. Cha mRNA starts at nucleotide 267 of the first intron and presents 449 nucleotides at the 5’untranslated region (UTR). TCFL5 specific 5’UTR is represented by a dashed line box.

FIG. 2. Comparison of Cha sequence with proteins of bHLH family. A, Multiple alignment at the bHLH region was created using the PileUp and Bestfit algorithms. The accession numbers of these proteins are as follows: human Cha=AJ271337; human Mxi1=D63940, mouse Mxi=U24673, human Mad=Q05195, mouse Mad=S51642, human USF-2=S50537, mouse USF-2=S42396, rat s-Myc=P23999, human TAP4=Q01664, yeast PHO4=X52577. Identical positions are shown as blackened boxes, while the homologous aminoacids are shown as gray boxes. Myc-type dimerization domain signature was found by comparison against PROSITE DNA binding motifs database. The similarity criteria were considered as described (48). B, Comparison of the basic region of Cha with those from some members of class A, B and C bHLH proteins. Sequences of the basic regions (numbered 1-13) of the indicated bHLH and Cha proteins are shown. The class A and B refer to proteins that preferentially bind to CAGCTG, and class C proteins bind to CACGTG (11). The conserved aminoacids among the different bHLH proteins are in white boxes while the aminoacid specifics of the class C proteins are in gray boxes. The arrows show the three
aminoacids of Max homodimers and USF that appear to make specific base contact in the E-box sequence (44).

FIG. 3. Cha protein and mRNA expression. A, Expression of Cha protein in different human tissues. Polyclonal rabbit serum against Cha specifically recognized a 34 kDa protein in different tissues. 10 µg of extracts from different human tissues were separated on SDS-PAGE, transferred to membranes (InstaBlot) and blotted with specific antisera. B, Tissue expression of Cha gene. Specific Cha primers were used to amplify Cha cDNA from several human cDNA libraries as described in methods. Amplification products from several human cDNA libraries were as follows: lung (λgt10) (lane 1), lung (λ TriplEx) (lane 2), pancreas (λgt10) (lane 3), pancreas (λ TriplEx) (lane 4), placenta (λgt10) (lane 5), placenta (λ TriplEx) (lane 6), skeletal muscle (λgt10) (lane 7), skeletal muscle (λ TriplEx) (lane 8), Jurkat cellular line (λ ZAP EXPRESS) (lane 9), heart (λ ZAP II) lane 10, brain (λ TriplEx) (lane 11), heart (λgt10) (lane 12), heart (λ TriplEx) (lane 13), kidney (λgt10) (lane 14), kidney (λ TriplEx) (lane 15), and liver (λgt10) (lane 16). As the control a cDNA fragment of GAPDH was amplified in each reaction.

FIG. 4. Binding of Cha to the CD2 promoter E-box. A, Nuclear extracts from Jurkat cells were incubated in absence or presence of indicated antibodies specific for various bHLH transcription factors before the incubation with the labeled CD2 E-box probe with poly (dIdC). The specificity of the complex was determined by competition with cold CD2 probe and cold irrelevant oligonucleotides (lanes 1 and 2, respectively). The supershifting assays with different antibodies are also shown. The arrow indicates the specific complex; an unspecific complex is indicated (†). B, Effect of different
blocking oligonucleotides poly (dAdT), poly (dCdG) and TATA4 on the formation of the unspecific complex (asterisk).

FIG. 5. **Binding of recombinant Cha to the CD2 promoter E-box.** *A*, First lane, 1 µl of unprogrammed RRL as control. Lanes 2-7, 1 µl of *in vitro* translated rCha from RRL was incubated with the different antibodies (as indicated) as well as with unlabeled competitors, previously to the incubations with the labeled CD2 E-box probe. An arrow indicates the specific retarded band. As the control normal rabbit serum (NRS) was used. The specific complex was determined by competition with unlabeled irrelevant oligonucleotide and CD2 probes in 20-fold molar excess. *B*, Unprogrammed RRL was incubated with anti-USF-1 antibody and NRS as well as cold CD2 oligonucleotide and irrelevant oligonucleotide. A supershifted complex was observed with anti-USF-1 antibody. *C*, purified recombinant USF1 and Cha purified by thrombin cleavage were incubated with the CD2 probe, as indicated. A unique specific complex was observed in the presence of USF-1 that increased upon addition of Cha.

FIG. 6. **Cha and USF-1 protein interaction.** *A*, Immunoprecipitation from Jurkat cells utilizing preimmune sera and anti-USF-1 antibody coupled to PAS. Immunoprecipitates were analyzed by Western blot with anti-USF-1 and anti-Cha antibodies. Cha and USF-1 are indicated by arrows. The immunoglobulin band is indicated by an asterisk (⁎). *B*, Ectopic expression of Cha and USF-1 in COS-7 cells is indicated in the top panel, Cha and USF-1 are indicated by arrows. *C*, In vivo interaction of sCha and USF-1 determined by coimmunoprecipitation of proteins in COS-7 cells transiently transfected with sCha and USF-1 (as indicated in top panel). Cell lysates were immunoprecipitated with anti-USF-1 antibody coupled to protein-A-sepharose beads. The beads were pelleted, washed, and subjected to Western blot
analysis with anti-Cha antibody. Immunoprecipitation of cells transfected with sCha utilizing anti-Cha antibody coupled to protein-A-sepharose was used as a positive control. 

D, In vitro interaction between GST-sCha and USF-1 from Jurkat cell lysate. Whole Jurkat cell extracts were incubated with glutathione sepharose 4B beads containing GST and GST-sCha and incubated at 4°C for 1 h in TNT lysis buffer. After intensive washing bound proteins were analyzed. Proteins present in minicolumn eluates were analyzed by Western blot with anti-USF-1 antibody. A COS-7 lysate of cells transiently transfected with the USF-1 expression plasmid was used as positive control.

FIG. 7. Ectopic expression of Cha repressed USF-1 dependent transcription. 

A, Jurkat cells were transiently co-transfected with 2 µg of CD2 reporter plasmid (CM2.5, filled rhombus), or with 250 ng of NF-κB Luc (open rhombus) and increasing amounts of pcDNA3 Cha (ng/10^6 cells). The total amount of DNA transfected was adjusted by addition of empty pcDNA3. For NF-κB dependent expression cells were stimulated with PMA (20 ng/ml) and calcium ionophore A23187 (1µM) during 6 hours and the results are expressed as fold induction above no stimulated control. The data represent an average of three independent experiments. 

B, Same as in A, but cells were also transfected with 0.5 ng/10^6 cells of pCDNA3USF-1 and pCMVMax expression plasmids and increasing amounts of pCDNA3Cha (50, 250 and 1000 ng/10^6 cells, respectively). Equal amounts of protein extracts were assayed for CAT activity. The acetylated forms were measured in a liquid scintillation counter and represented as percentage of CAT activity. 100% was designated as the CAT activity produced by the reporter vector (CM2.5) in the presence of empty vector pcDNA3. 

C, COS-7 cells were transfected with a four E-box containing luciferase reporter construct pGL3p4E-boxCD2 (30ng/10^6 cells), pCDNA3USF-1 (1µg/10^6 cells), pCDNA3Cha (1 and 2
µg/10⁶ cells), and CMVβGal (100ng/10⁶ cells) DNA amounts were adjusted by cotransfection of empty pCDNA3. Luciferase activities were normalized with β-Gal activity of cotransfected expression vector and protein content. Data represent the mean ± SD of duplicate samples from a representative result of three experiments.

FIG. 8. **Cha and USF-1 regulate CD2 expression.** Primary T lymphocytes were incubated with PMA (20 ng/ml) plus calcium ionophore A23187 (1µM) during the indicated times. *A*, Cha and USF occupancy of the CD2 promoter. Resting and PMA/Io activated T cells were analyzed in ChIP assays (as described in methods) in the presence of no chromatin (mock), preimmune rabbit (Pre), anti-Cha and anti-USF-1 antibodies. PCR was performed with oligonucleotides from the p53 promoter (negative control) and from the CD2 promoter (EboxCD2). 0.2% of total input chromatin was used as PCR positive control. Densitometric scanning of the bands of EboxCD2 is shown at the bottom as fold intensity normalized with respect to mock samples. *B*, Expression of Cha mRNA. Total RNA was extracted from resting and activated T cells. Expression was analyzed by RT-PCR with gene-specific primers of Cha and GAPDH. Densitometric scanning of the bands corrected for GAPDH expression is shown at the bottom. *C*, Expression of Cha protein. Cha protein levels were assayed in nuclear extracts from resting and activated T cells by western blot using anti-Cha (1:500 dilution). The results are representative of 3 different experiments using purified T cells from healthy blood donors. *D*, J77cl-20 cells were transfected with CMVGFP (1µg/10⁶ cells), and different doses of Cha and sCha expression plasmids (0,5 µg, 1 µg and 2µg/10⁶ cells). Expression of CD2 was analyzed by flow cytometry after 48 h of transfection. Results are expressed as the percentage of the control CD2 mean of
duplicate ±SD fluorescence values in linear scale of GFP positive cells, and are representative of three independent experiments.
Fig 3
Fig 4
Fig 5

A

RRL control
- irrelevant oligo
cold CD2 oligo
anti-Cha
anti-USF-1
NRS

B

RRL control
- irrelevant oligo
cold CD2 oligo
anti-Cha
anti-USF-1
NRS

C

GST

Cha
- GST

Cha

irrelevant oligo
cold CD2 oligo

USF-1

- cold CD2 oligo
irrelevant oligo
anti-USF-1
NRS
Fig 6
Fig 7
Fig 8
Cha, a basic helix loop helix transcription factor involved in the regulation of upstream stimulatory factor activity
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