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Genetics

# An X chromosome-linked gene encoding a protein with characteristics of a rhoGAP predominantly expressed in hematopoietic cells

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**ABSTRACT** **An increasingly large number of proteins involved in signal transduction have been identified in recent years and shown to control different steps of cell survival, proliferation, and differentiation. Among the genes recently identified at the tip of the long arm of the human X chromosome, a novel gene, *C1*, encodes a protein that appears to represent a newly discovered member of the group of signaling proteins involved in regulation of the small GTP binding proteins of the ras superfamily. The protein encoded by *C1*, p115, is synthesized predominantly in cells of hematopoietic origin. It is characterized by two regions of similarity to motifs present in known proteins: GAP and SH3 homologous regions. Its localization in a narrow cytoplasmic region just below the plasma membrane and its inhibitory effect on stress fiber organization indicate that p115 may down regulate rho-like GTPases in hematopoietic cells.**

Cell survival, proliferation, and differentiation depend on external signals that once presented to target cells elicit intracellular pathways leading to changes in gene expression, cytoskeletal architecture, cellular metabolism, cell–cell interaction, and cell migration. Such changes involve a series of regulated protein–protein interactions on a specific set of target molecules, which determine the pathways of signal transduction (1).

Proteins involved in signal transduction are often multifunctional and in addition to possible catalytic functions have binding regions that can bring substrates to the catalytic centers, link the signal transducers to upstream proteins, and localize protein complexes to cellular compartments (2). A number of recurrent protein domains have been recognized that have common properties and are not restricted to a particular type of signal transduction protein (2–4): SH2, SH3, and PH domains occur in protein kinases, lipid kinases, protein phosphatases, phospholipases, *ras*-controlling proteins, and others. They are also found in adapter proteins, which have no enzymatic function.

Among the proteins involved in signal transduction, the ras and ras-like GTPases control an extraordinarily wide variety of cellular processes (5). More than 50 small GTP-binding proteins of the ras family have been identified and shown to act on different signal transduction pathways. All the members of the family can cycle between the active GTP-bound state and the inactive GDP-bound state; their activity is regulated by the interaction with accessory proteins, which stimulate the rates of GTP hydrolysis (GAP proteins) or of nucleotide dissociation (GEF proteins). In addition, proteins that inhibit the release of guanine nucleotides and block the intrinsic and GAP-stimulated GTPase activity have been described and called GDIs. Based on sequence homology and biological function, the different members of the ras family have been divided into different classes. A large number of GAPs, GEFs, and GDIs specific for members of the family have been recently isolated (6, 7).

We have developed methods to identify and map genes to study selected regions of the genome (8, 9), and we are analyzing Xq28 at the tip of the long arm of the human X chromosome. We have constructed a detailed transcriptional map of a region of 2 Mb in distal Xq28, and we have identified a large number of genes (10, 11). Among the newly identified transcripts was the gene responsible for Emery–Dreifuss muscular dystrophy (12). In this paper, we describe another of these genes, *C1*, which encodes a cytoplasmic protein of 115 kDa preferentially expressed in hematopoietic cells and containing SH3 and GAP homologous regions.**

## MATERIALS AND METHODS

**cDNA Isolation and 5′ Rapid Amplification of cDNA Ends (RACE).** cDNAs E1 and E2 were described (11). The 5′ clones were obtained by the RACE method (13). cDNA was synthesized from the primer HC1R (nt 1373–1351) and tailed with poly(dT) using terminal deoxynucleotidyltransferase (Promega). The second strand was synthesized from the primer $(dT)_{17}$-RoRi (5′-AAGGATCCGTCGACATCGATAATACGACTCACTATAGGGATTTTTTTTTTTTTTTT) and PCR was performed using the primer RoRi [5′-ATCCGTCGACATCGATAATACGACTC; modified from Frohman (13)] and the specific primer IC1R (nt 1251–1271). The PCR products were cloned in Bluescript (Stratagene) tailed with dT (Bluescript-T). Clones R1, R2, R3, R6, and R7 were derived from K562 cell RNA; R8 and R16 were from lymphoblastoid cell RNA.

To construct the full-length cDNA a fragment was obtained by reverse transcriptase (RT)-PCR from total RNA of lymphoblastoid and HL60 cells using Maloney murine leukemia virus RT and random hexamer primers in the conditions suggested by the supplier (BRL). After amplification from nt 945 to nt 1373, the PCR products were cloned in Bluescript-T and sequenced. The full-length *C1* cDNA was constructed by ligation of the *Bgl* II site of the cDNA R1, cloned in pUC18, to the *Bgl* II site of the RT-PCR product. The *Nae* I site of the construct was subsequently ligated to the *Nae* I site of the E2 cDNA. Clone A508H3 was used for subsequent experiments.

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Abbreviations: RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; GST, glutathione *S*-transferase; ORF, open reading frame.

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**The sequence reported in this paper has been deposited in the GenBank data base (accession no. X78817).

**DNA Sequence.** The cDNA clones (E1, E2, and the RACE clones) were sequenced from both strands from vector primers and from internal primers and analyzed as described (11).

**Northern Blot and Hybridization.** RNA from cell lines was prepared by the guanidine isothiocyanate/CsCl method (14). Total RNAs from human tissues and Northern blots were purchased from Clontech. Northern blots and hybridizations were done by standard methods (14).

***In Vitro* Transcription/Translation.** The full-length cDNA clone was digested with *Sal* I and *Eco*RI, subcloned into the pGEM-3 vector, and *in vitro* transcribed and translated in a rabbit reticulocyte system using commercial kits and according to the manufacturer's recommendations (Promega).

**Cell Lines, Transient Transfection, and Overexpression Experiments.** NIH 3T3 fibroblasts, simian virus 40 large T-expressing monkey cells (COS-1), and all the human cell lines were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. COS-1 cells were transfected with 10 mg of pMT2 or pMT2-C1 DNAs using Lipofectin (BRL) and harvested 3 days posttransfection.

For overexpression, the *C1* coding sequence was cloned into the *Eco*RI site of the LXSN retroviral vector (15), and SAA cells (16) were infected as described (17) with LXSN and L-CI-SN retroviruses. *C1* overexpression was analyzed by Western blotting using anti-C1 antibodies (see below).

**Preparation of Anti-C1 Antibodies.** A region of the *C1* cDNA encoding amino acids 744–800 was synthesized by PCR and cloned into the bacterial expression plasmid pGEX-2T (Pharmacia). The bacterial glutathione *S*-transferase (GST)–C1 fusion protein was purified as described (18) and anti-C1 antibodies were prepared by immunizing New Zealand White rabbits. Crude antiserum was purified by preclearing with induced GST-expressing bacteria followed by affinity purification using the GST–C1 fusion protein.

**Western Blotting, Immunoprecipitation, and Metabolic Labeling Studies.** Cell lysates were prepared in the presence of protease inhibitors and immunoprecipitated with anti-C1 antibodies as described (15, 17). For immunoblot analysis, 20 mg of total cell lysates was electrotransferred onto nitrocellulose filters. Probing with anti-C1 antibodies and immunocomplex detection have been described (17).

For metabolic labeling experiments, cells were incubated with [$^{35}$S]methionine (100 mCi/ml; 1000 Ci/mmol; 1 Ci = 37 GBq; Amersham) for 16 h in medium lacking methionine and containing 5% dialyzed calf serum.

**Immunoenzymatic Labeling.** Immunostaining with the anti-C1 polyclonal antibody was performed by a four-stage APAAP technique (19). Briefly, samples were incubated with the rabbit anti-C1 antibody (1:100 dilution), followed by a mouse anti-rabbit monoclonal antibody (Dako), rabbit anti-mouse immunoglobulin (Dako), and APAAP complexes.

**Actin Reorganization Assay.** Actin reorganization was analyzed as described (20). Cells grown on round glass coverslips were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30–60 min at 25°C and permeabilized with 0.1% Triton X-100 in PBS for 10 min. The coverslips were rinsed and incubated with tetramethylrhodamine B isothiocyanate-conjugated phalloidin (10 $\mu$g/ml in PBS) for 45 min at 25°C in a humidified atmosphere. After rinsing in PBS, the coverslips were mounted in Fluoromount (Southern Biotechnology Associates).

## RESULTS

**Cloning of Full-Length cDNA of the *C1* Gene.** Two overlapping cDNAs, *E1* and *E2*, were isolated from a human embryo total cDNA library and mapped between the *TE2* and *V2R* genes in distal Xq28 (ref. 11; Fig. 1); they identified a transcript, which was called *C1*. The longest cDNA, *E2*, was 2.1 kb long and had a poly(A) tail that defined the 3′ end of the gene. As the cDNAs hybridized to a single mRNA species of ≈3.5 kb (ref. 11; see Fig. 5), to obtain the full-length cDNA, the 5′ portion of the *C1* transcript was amplified by RACE (13) and cloned in Bluescript-T. Seven cDNAs of different lengths were isolated and sequenced. They were identical in their 3′ regions and extended for different lengths in the 5′ direction (Fig. 1*B*). The longest ones (R1–R3) were ≈1.8 kb and should correspond to the 5′ moiety of the cDNA.

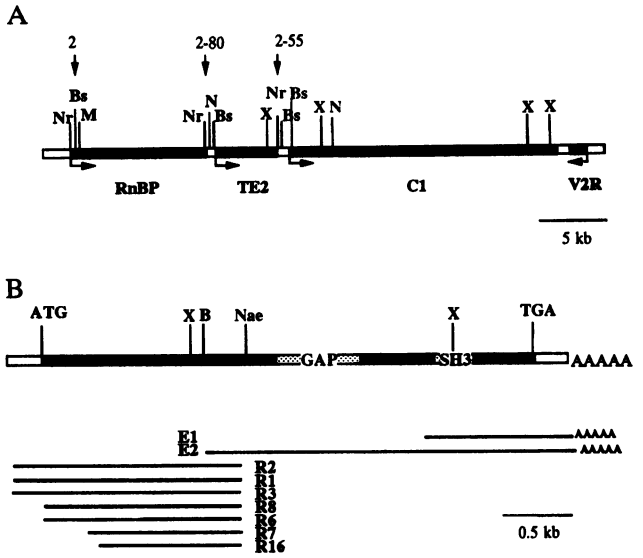


FIG. 1. (*A*) Schematic representation of the *C1* and flanking genes region. Solid areas are occupied by genes; arrows indicate direction of transcription. 2-, 2-80, and 2-55 are previously described CpG islands. (*B*) Schematic representation of *C1* cDNAs and of the transcript. GAP, GAP domain; SH3, SH3 domain; AAAA, poly(A) tail. Bs, *Bss*HII; Nr, *Nru* I; M, *Mlu* I; N, *Not* I; X, *Xho* I; B, *Bgl* II.

The newly isolated cDNAs were hybridized to the cosmids previously identified in the region (11). The cDNA *C1* mapped in a genomic region of ≈18 kb flanked by the 2-55 CpG island (ref. 11; Fig. 1*A*). Hybridization of 5′ and 3′ portions of the cDNA (not shown) confirmed that the 2-55 CpG island is at the 5′ end of the *C1* gene.

**Sequence Analysis of the *C1* cDNA.** The complete nucleotide sequence of the C1 mRNA is 3240 bp. It contains an open reading frame (ORF) beginning at nt 43 and extending to an in-frame TGA at nt 2881. The first in-frame ATG is embedded in a sequence (GCCGCCATGG), which is in good agreement with the consensus sequence for initiation of translation in eukaryotes (21). The ORF encodes a putative protein of 946 amino acids with a molecular mass of 115 kDa (Fig. 2).

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  1 MAAHGKLRRERGLQAEYETQVKEMRWQLSEQLRCLELQGELRRELLQELAEFMRRRAEVE
 61 LEYSRGLEKLAERFSSRGGRLGSSREHQSFRKEPSLLSPLHCWAVLLQHTRQQSRESAAL
121 SEVLAGPLAQRLSHIAEDVGRLVKKSRDLEQQLQDELLEVVSELQTAKKTYQAYHMESVN
181 AEAKLREAERQEEKRAGRSVPTTTAGATEAGPLRKSSLKKGGRLVEKRQAKFMEHKLKCT
241 KARNEYLLSLASVNAAVSNYYLHDVLDLMDCCDTGFHLALGQVLRSYTAAESRTQASQVQ
301 GLGSLEEAVEALDPPGDKAKVLEVHATVFCPPLRFDYHPHDGDEVAEICVEMELRDEILP
361 RAQNIQSRLDRQTIETEEVNKTLKATLQALLEVVASDDGDVLDSFQTSPSTESLKSTSSD
421 PGSRQAGRRRGQQQETETFYLTKLQEYLSGRSILAKLQAKHEKLQEALQRGDKEEQEVSW
481 TQYTQRKFQKSRQPRPSSQYNQRLFGGDMEKFIQSSGQPVPLVVESCIRFINLNGLQHEG
541 IFRVSGAQLRVSEIRDAFERGEDPLVEGCTAHDLDSVAGVLKLYFRSLEPPLFPPDLFGE
601 LLASSELEDTAERVEHVSRLLWRLPAPVLVVLRYLFTFLNHLAQYSDENMMDPYNLAVCF
661 GPTLLPVPAGQDPVALQGRVNQLVQTLIVQPDRVFPPLTSLPGPVYEKCMAPPSASCLGD
721 AQLESLGADNDPELEAEMPAQEDDLEGVVEAVACFAYTGRTAQELSFRRGDVLRLHERAS
781 SDWWRGEHNGMRGLIPHKYITLPAGTEKQVVGAGLQTAGESGSSPEGLLASELVHRPEPC
841 TSPEAMGPSGHRRRCLVPASPEQHVEVDKAVAQNMDSVFKELLGKTSVRQGLGPASTTSP
901 SPGPRSPKAPPSSRLGRNKGFSRGPGAPASPSASHPQGLDTTPKPH
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FIG. 2. Amino acid sequence of p115 protein. Underlined sequence, GAP domain; boxed sequence, SH3 domain.

Comparison of the nucleotide sequence of the *C1* cDNA to data bases did not show similarities to known genes.

**The Protein Encoded by *C1* Contains Regions of Homology with SH3 Domains and rho/racGAP-Related Sequences.** The sequence of the putative protein encoded by the *C1* gene was compared to data bases using BLAST. This analysis revealed two regions with similarities to proteins involved in control of cellular signaling and architecture.

The first domain, of 144 amino acids between aa 521 and 665, showed similarity to a group of GAP proteins previously identified as having a broad specificity for members of the rho subfamily of small GTP binding proteins (22). The alignment with some of the mammalian rhoGAPs is shown in Fig. 3*A*; as reported (23), three boxes of higher sequence conservation can be identified. Identities ranged from 38% with the chimerins to 30% with bcr, rhoGAP, and p190 to 20% with p85α.

The second domain, aa 752–801, was defined by similarity to the SH3 domain of many signaling proteins: BLAST search of the protein sequence data bases identified the SH3 domains of α spectrins and of myosin I heavy chains from many distant animal species and of the adapter proteins Crk and GrB2 as most similar to the corresponding domain in p115, with amino acid identities in the range of 35% (not shown). Alignment with some SH3 [that with chicken α spectrin (30% identity) and the abl tyrosine kinase (26% identity) is shown in Fig. 3*B*] showed conservation of residues (boxed in Fig. 3*B*) that seem to contribute to the binding of SH3 domains to their ligands (24, 25).

**The C1 Transcript Is Highly Expressed in Hematopoietic Cells.** The whole *C1* cDNA was hybridized to Northern blots of poly(A)$^+$ RNA from different human tissues. High levels of expression were restricted to spleen, thymus, and leukocytes. Low levels of transcripts were detected in placenta, lung, and various fetal tissues (Fig. 4*A*). Hybridization to total RNA from a number of cell lines of different origin confirmed that the C1 mRNA is mainly expressed in cells of hematopoietic origin. High levels of C1 were detected in cell lines of B lymphoid, T lymphoid, myeloid, or Hodgkin derivation. The majority of nonhematopoietic cell lines expressed no sizeable C1 transcripts, while little expression was found in some cell lines. Fig. 4*B* shows the results of some of the hybridizations.

**Identification of the C1 Protein in Mammalian Cells.** To investigate the expression of the *C1* gene product *in vivo*, antibodies were raised against a GST–C1 fusion protein produced by inserting the region of the *C1* cDNA encoding the SH3 homology region into the bacterial expression plasmid pGEX-2T. The anti-C1 antibodies specifically immunoprecipitated a protein of 115 kDa translated *in vitro* from sense C1 mRNA (Fig. 5*A*). A polypeptide of the same size (p115) was specifically precipitated from human TF-1 cells and COS-1 cells transfected with the full-length *C1* cDNA cloned in the pMT2 expression vector (Fig. 5*A*). No expression was detected in NIH 3T3 cells and COS-1 cells transfected with the empty expression vector. Tryptic peptide maps of the [$^{35}$S]methionine-labeled *C1* cDNA *in vitro* translation products were indistinguishable from the maps of the human cellular proteins precipitated with anti-C1 antibodies (not shown) consistent with the notion that the immunoreactive p115 cellular protein is indeed encoded by the *C1* gene.

The extent of expression of the C1 protein in different cells was assessed by using affinity-purified anti-C1 antibodies for immunoblotting lysates from a variety of cell types (Fig. 5*B*). Human cell lines from melanoma, cervical cancer, glioblastoma, normal human fibroblasts, and mesothelial cells did not express polypeptides that could cross-react with anti-C1 antibodies. All cell lines of hematopoietic origin tested expressed a polypeptide of 115 kDa, immunoreactive with the anti-C1 antibodies. In some extracts, smaller molecular mass bands were reproducibly detected; their nature is unknown at the moment.

**Cellular Localization of the p115 Protein.** Cellular localization of the p115 protein was analyzed by immunocytochemistry on human cell lines using the anti-C1 polyclonal antibodies. A strong cytoplasmic signal was obtained in all hematopoietic cells, while no specific labeling was observed in all nonhematopoietic cells analyzed and after staining with the preimmune serum (not shown). Immunoreactivity was localized at the plasma membrane and in a narrow cytoplasmic region close to the plasma membrane (not shown). Specific labeling was absent in the nucleus and in the cytoplasmic perinuclear region.

**Functional Analysis of p115 rho/GAP Activity.** The presence of rho/rac/GAP-related sequences and cellular localization of the protein prompted us to investigate the effect of p115 overexpression on actin organization. *C1* cDNA was introduced into SAA fibroblasts by retroviral-mediated gene trans-

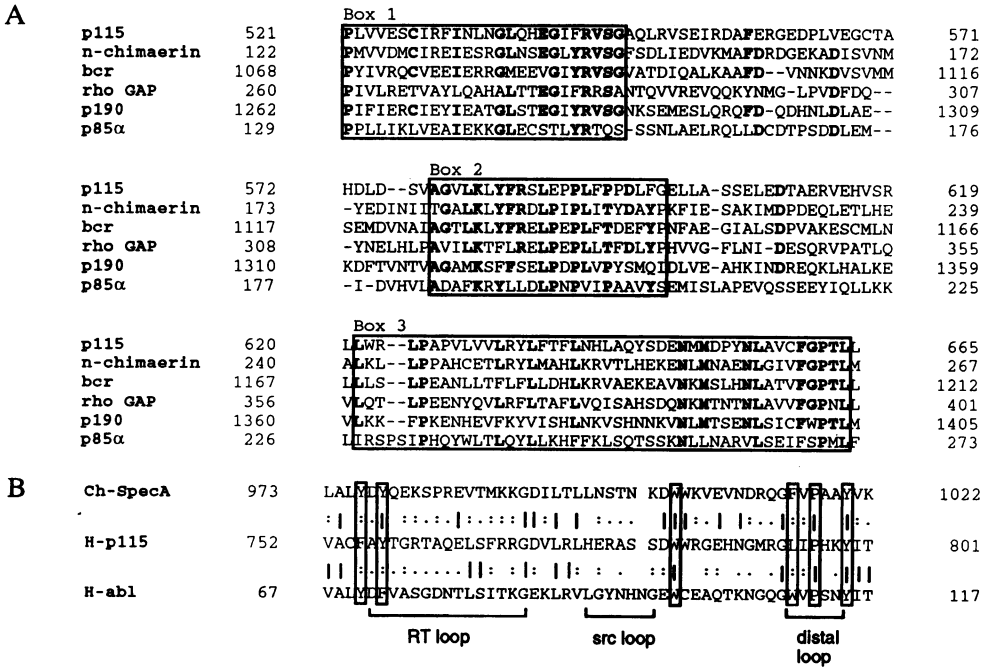


FIG. 3. (*A*) Alignment of GAP domains in p115 and in mammalian rhoGAPs. Boldface amino acids conserved in at least four proteins. (*B*) Alignment of SH3 domain of p115, chicken α spectrin, and c-abl.

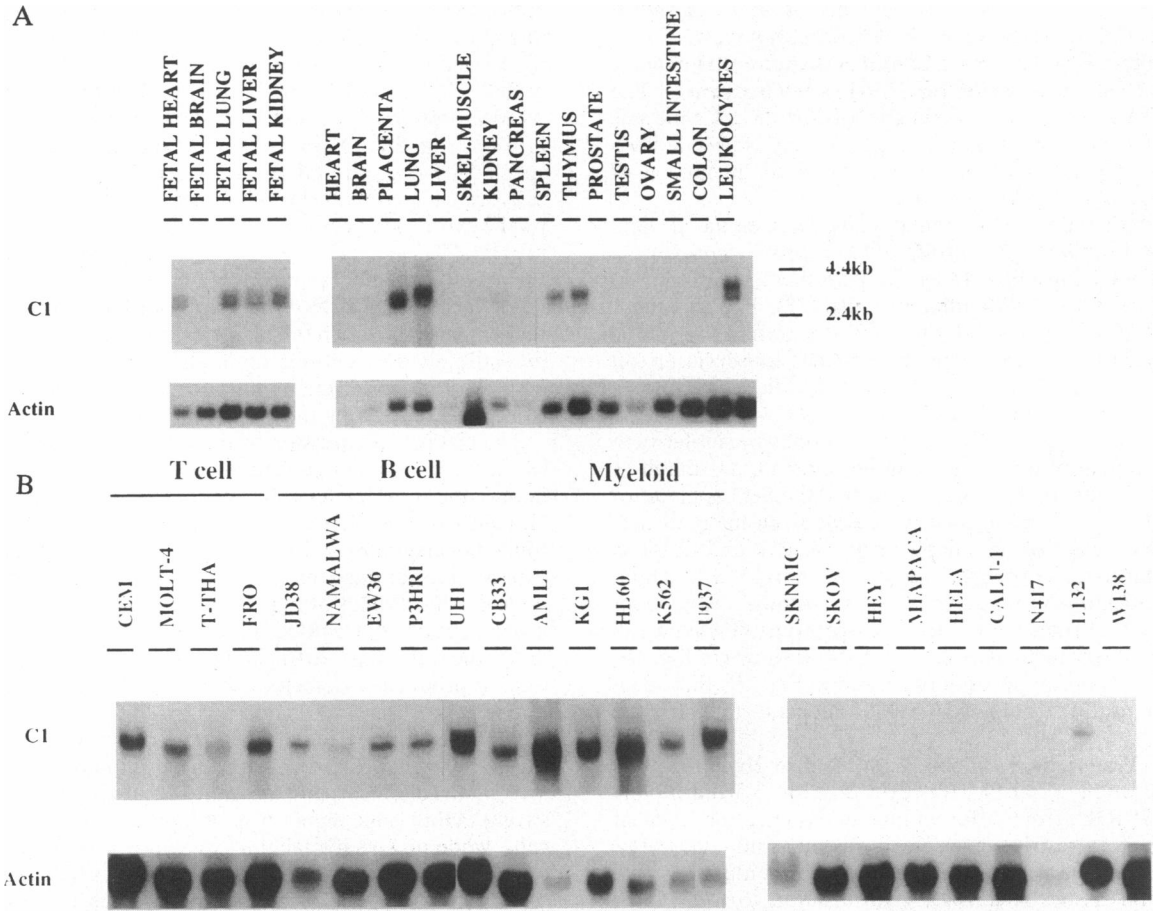


FIG. 4. Northern blot analysis of expression of the *C1* transcript. (*A*) Northern blot containing poly(A)$^+$ RNAs from fetal and adult human tissues. (*B*) Northern blot containing total RNAs from hematopoietic and nonhematopoietic cell lines. Hybridization was with a full-length *C1* cDNA probe at 65°C. Exposure times were overnight for hematopoietic cell RNA and 1 week for all other RNAs.

fer, and clones overexpressing p115 (SAA-C1 clones) were selected for further analysis. SAA cells infected with an empty retrovirus (SAA-SN clones) were used as controls.

As shown in Fig. 6, phalloidin staining revealed polymerized actin organized in stress fibers in control cells (SA-SN clone 11). The pattern of actin distribution contrasted with that observed in C1 overexpressing cells (SAA-C1 clone 5) where stress fiber organization was inhibited. Conversely, ruffles formation was almost identical in SAA-C1 and SAA-SN control cells (not shown).

## DISCUSSION

In this paper, we report the characterization of an X chromosome-linked gene, *C1*, encoding a protein that appears to represent a member of the increasingly large group of signaling proteins involved in regulation of small GTP binding proteins of the ras superfamily.

The *C1* gene was identified in a gene-rich region in distal Xq28 (11). It is a single copy gene, ≈20 kb long, and encodes a mRNA of 3.5 kb. As is the case for some of the genes with

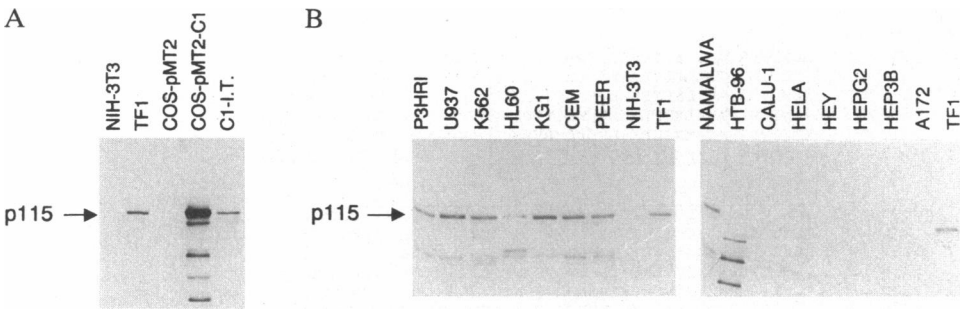


FIG. 5. Identification of p115 protein using anti-C1 antibodies. (*A*) Proteins translated *in vitro* from *C1* cDNA A508H3 (C1-I.T.) and cell lysates from [$^{35}$S]methionine-labeled COS-1 cells transfected with pMT2 (COS-pMT2) and pMT2-C1 (COS-pMT2-C1) vectors, NIH 3T3 fibroblasts (NIH-3T3), and human myeloid cells (TF1) were immunoprecipitated with anti-C1 antibodies. Immunoprecipitates were resolved by SDS/PAGE and gels were exposed to x-ray film for 2 h at −70°C. p115 C1 protein is indicated with an arrow. (*B*) Immunoblot of different human cell lines. Whole cell lysates were prepared from the following human cells: TF1, KG1, and HL60 myeloid cell lines; U937 monocytic cell line; PEER and CEM T-cell lines; P3HRI and Nawalma B-cell lines; HeLa cells; CALU lung cell line; A172 glioblastoma cell line; HEY ovary carcinoma cell line; HEPG2 larynx carcinoma cell line; HEP3B hepatocarcinoma cell line; and HTB-96 carcinoma cell line. All cell lines were obtained from the American Type Culture Collection. Totol protein (20 μg) was separated by SDS/10% PAGE, transferred to nitrocellulose, and immunoblotted with affinity-purified anti-C1 antibodies.

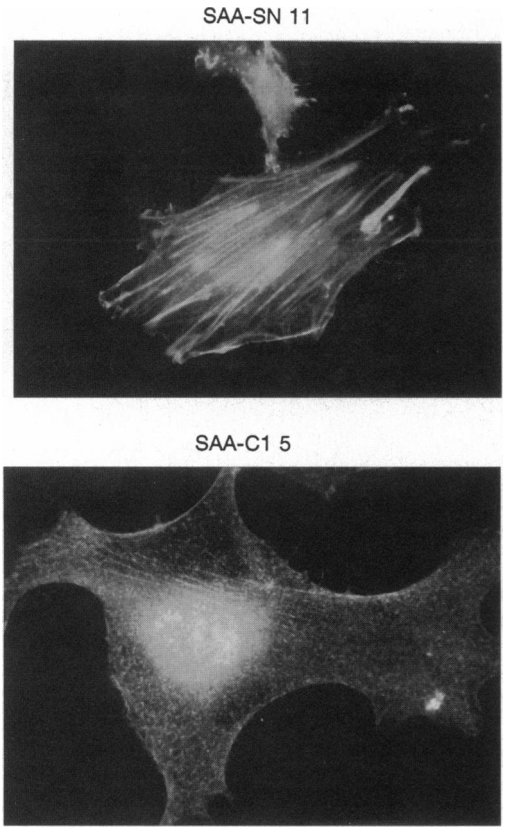


FIG. 6. Actin organization in SAA-C1 and SAA-SN cells. Logarithmically growing cells were fixed on coverslips and stained with phalloidin as described.

5′ CpG islands, the *C1* gene is expressed at a very low level in a housekeeping fashion in many tissues and cell lines but it was detected at a high level in adult tissues only in cells of hematopoietic origin. The *C1* gene encodes a 946-amino acid protein, p115. Immunoreactions with specific polyclonal antibodies raised against the protein confirmed that the p115 protein is synthesized predominantly in cells of hematopoietic origin.

The nucleotide sequence of the *C1* cDNA did not show similarities to known genes. Analysis of the p115 protein showed two regions of similarity to motifs present in known proteins. From aa 521 to 665, it is similar to a group of GAP proteins specific for the ras-like protein rho and its homologues (22). From aa 752 to 801, it is similar to the SH3 domain of many signal transduction and cytoskeleton proteins.

The SH3 domain showed the highest identity (≈35%) with those in myosin I heavy chains and spectrins. Aside from the general homology to the domain, the comparison of the primary structure of the p115 SH3 with those of human abl and of chicken spectrin, whose structure complexed with target peptides has been recently determined at high resolution (24, 25), reveals that the residues that form the binding site are conserved. These include the Y and/or F in the RT loop; the W at the end of the scr loop; and the P, Y, and I/V in the distal loop (see Fig. 3*B*). The only exception to this strong conservation is the substitution of an aromatic residue with L at the amino end of the distal loop. This however is not unique to p115 and might reflect differences in binding specificity between the different SH3 domains. All these structural considerations lend support to the proposal that the p115 domain shares the property of binding proline-rich peptides (with an exposed stretch of polyproline helix, type II) with other SH3 domains.

The GAP domain of C1 is homologous to rhoGAP as well as to a family of related proteins. Nine mammalian genes, one gene in *Drosophila melanogaster*, two in *Saccharomyces cerevisiae*, and one in *Caenorhabditis elegans* have been described (22); in all instances, the proteins are large and multifunctional. In one, p85, a SH3 domain has been identified, while the majority has proline-rich stretches that are supposed to bind SH3 domains. The presence of multiple motifs in their sequence led to the hypothesis that the multifunctional character of the rhoGAP-containing proteins should be related to their regulation through specific protein–protein interactions (22) and it has been suggested that they may regulate formation of complexes associated with the polymerized actin at the plasma membrane. Accordingly, our results show that p115 is localized in a very narrow cytoplasmic region, close to the plasma membrane where it could be retained through its interaction with a SH3 binding protein. Moreover, overexpression of p115 in fibroblasts inhibits stress fiber formation as it is expected from a protein inhibiting the activity of a rho-like GTPase (26) and indicates that p115 may be a novel hematopoietic specific rhoGAP.

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