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Double FYVE-containing protein 1 (DFCP1): isolation, cloning and characterization of a novel FYVE finger protein from a human bone marrow cDNA library

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Abstract

Double FYVE-containing protein 1 (DFCP1) encodes a 777 amino acid protein that contains: (1) an N-terminal Cys–His cluster with some homology to many zinc finger domains; (2) a consensus sequence consistent with an ATP/GTP binding site; and (3) a C-terminal domain unique because it contains two zinc-binding FYVE domains. The gene, *ZNFN2A1* (GenBank accession no. AF251025) was localized to chromosome 14q22–q24 and shown to be composed of 11 exons. Northern blot analysis revealed the presence of three different mRNA transcripts (4.2, 3 and 1.2 kb). The two longer transcripts appear to be expressed in a variety of different tissues, especially in endocrine tissues, while the shorter messenger is limited to testis. Both of the larger transcripts are unusual due to the presence of a 463 bp long 5′ UTR. Furthermore, the 4.2 kb transcript contains a non-standard polyadenylation consensus sequence while the 3 kb transcript contains a standard consensus sequence but within the open reading frame. Following in vitro transfection of a DFCP1-containing expression construct, confocal microscopy studies showed a vesicular distribution of DFCP1 suggesting that this protein, like other FYVE-containing proteins, might be involved in membrane trafficking. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Bone marrow stroma; Expressed sequence tag; Vesicles; Zinc finger

1. Introduction

Bone marrow stromal cells (BMSC) are non-hematopoietic cells residing in the marrow cavity. While they exhibit a number of fibroblastic features, they are distinct from fibroblastic cells found in other connective tissues. Furthermore, they lack characteristics of endothelial cells and macrophages (Castro-Malaspina et al., 1980; Wang et al., 1990). After extensive proliferation in vitro, the BMSC population retains the capacity to differentiate into at least four types of connective tissue: bone, cartilage, myelosupportive stroma, and associated adipocytes (Friedenstein et al., 1974; Owen and Friedenstein, 1988).

High throughput sequencing of a human BMSC cDNA library led to the identification of many previously reported genes, as well as a number of unknown expressed sequence tags (ESTs). We report here for the first time the cloning and characterization of a new protein containing two FYVE finger domains that we have designated DFCP1 (double FYVE-containing protein 1).

The function of most FYVE-containing proteins is still unknown, however, FYVE domains have been shown to interact specifically with the membrane lipid, phosphatidyl inositol-3-phosphate (PtdIns-3-P) (Stenmark et al., 1996; Burd and Emr, 1998; Gaullier et al., 1998; Patki et al., 1998). Several of the FYVE-containing proteins have been shown to be recruited to

Abbreviations: BMSC, bone marrow stromal cells; DFCP1, double FYVE-containing protein 1; EST, expressed sequence tag; FYVE, Fab1p/YOTB/Vac1p/EEA1; PCR, polymerase chain reaction; PTdIns-3-P, phosphatidyl inositol-3-phosphate; STS, sequence tag site; UTR, untranslated region; ZNFN2A1, zinc finger protein, subfamily 2A, protein 1.

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vesicular structures and to participate in intracellular membrane trafficking (Simonsen et al., 1998; Bean et al., 1997; Weisman and Wickner, 1992; Piper et al., 1995; Yamamoto et al., 1995). We found this novel protein to be widely expressed, particularly in endocrine tissues. Upon in vitro transfection using DFCP1 expression constructs, DFCP1 did in fact localize to vesicular structures within the cytoplasm, similar to what has been reported for other FYVE-containing proteins.

2. Materials and methods

2.1. cDNA library construction

Human bone marrow stromal cells from three different donors [32 year old (yo) black female, 35 yo black male, 43 yo white male] were established according to the method published previously (Kuznetsov et al., 1997). Extracted RNA was oligo dT purified and then submitted to Stratagene (La Jolla, CA) for custom library construction into the Uni-Zap system (Dieudonne et al., 1999).

2.2. Skeletal Genome Anatomy Project (SGAP)

The SGAP is a project under development through the collaboration of the Medical Genetics Branch of the National Human Genome Research Institute (NHGRI), the Center for Information and Technology (CIT) and the Craniofacial and Skeletal Diseases Branch at the National Institute of Dental and Craniofacial Research (NIDCR) (Jia et al., 1998). SGAP's goal is to build a catalogue of genes expressed by normal and abnormal bone, cartilage, tendon, ligament and synovial tissues. To date, two cDNA libraries constructed from BMSCs, which contain osteoprogenitors (Kuznetsov et al., 1997), and more mature trabecular bone cells (TBCs) (Robey and Termine, 1985) have been sequenced.

2.3. DNA sequencing

Individual clones from the BMSC library were chosen and the 3′ end was sequenced by the NIH Intramural Sequencing Center (NISC). The 3′ ESTs were deposited in the National Center for Biotechnologies (NCBI) dbEST database. To date, approximately 10 000 clones have been sequenced. An EST from the BMSC library (GenBank accession no. AA545744) was selected and sequence verified and the clone was sequenced in full. Sequences were determined at least four times with universal T3, T7 or cDNA specific primers, by ABI 373 and 377XL Automated DNA sequencers. The Basic Logic Alignment Search Tool (BLAST) at the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST/) was used to search the GenBank database for related sequences (Altschul et al., 1990). DNA and protein sequence comparisons were carried out with the GCG software and domain searches were performed through the Prosite database (http://www.isrec.isb-sib.ch/). The genomic sequence was identified from GenBank using the DFCP1 encoding cDNA sequence as query in the High Throughput Genomic Sequences Database (HTGS), and has been assigned the designation *ZNFN2A1*.

2.4. Chromosomal localization

The NCBI dbSTS (Sequence Tag Sites database) (http://www.ncbi.nlm.nih.gov/BLAST/) of GenBank was queried using the genomic sequence, and this led to the identification of one sequence tag site (STS). The chromosomal position of the STS marker had been localized previously by radiation hybrid mapping, and the mapping information was available in the public database.

2.5. Northern blot analysis

Total RNA was extracted using RNA STAT (Tel-Test, Inc., Friendswood, TX) as directed by the manufacturer. Poly A^+ RNA was prepared from total RNA using a Micro Poly (A) Pure kit (AMBION Inc., Austin, TX). Poly A^+ RNA blots (2 μg/lane) of human tissues were purchased from Clontech, Palo Alto, CA. Northern blots were hybridized with a 209 bp (α-^{32}P)CTP random prime labeled cDNA fragment (derived from PCR amplification of DFCP1 encoding cDNA using 5′-cagtgcgggtctgtgacaact-3′ at position 2367 bp as the forward primer and 5′-tggtcaggcacccagtacg-3′ at position 2556 bp as the reverse primer). A β-actin cDNA (Clontech, Palo Alto, CA) probe was used as a control. The blots were washed free of non-specifically bound probe, and radioactivity was visualized and quantified using a PhosphoImager and ImageQuant Software from Molecular Dynamics (Eugene, OR).

2.6. Fusion protein

For expression in mammalian cells, the coding sequence (428–2761 bp) was amplified using specific primers with built in restriction sites (BamHI 5′-agaata**ggatcc**atgagtgcccagacttccc-3′ forward; EcoRI 5′-ttctgt**gaattc**ttaaaggtcaccgggcttttt-3′ reverse) and subsequently placed in frame behind the 9E10 myc sequence under the CMV promoter of pCMV-tag3b vector (Stratagene, La Jolla, CA). The integrity of the construct was then verified by DNA sequencing.

2.7. Transfections

NIH-3T3 cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) containing 10% fetal bovine serum on cover slips in six well plates. When the cells reached a confluence of 70% they were transfected with the DFCP1–c-myc fusion protein (1 μg DNA/well) with Lipofectamine Plus Reagent (Life Technologies, MD) and incubated in serum-free medium for 4 h following the manufacturer's directions. After 4 h, the serum-free media was replaced with serum-containing DMEM and the cells were allowed to recover at 37°C for 24 h.

2.8. Immunolocalization and confocal microscopy

Following 24 h of incubation, transfected NIH-3T3 cells were washed thoroughly with phosphate-buffered saline (PBS) and fixed for 20 min at room temperature (RT) in 2% phosphate-buffered formalin freshly prepared from paraformaldehyde. After extensive washing with PBS 1×, cells were incubated in 0.1 M glycine/PBS (pH 7.4) for 15 min at RT. Permeabilization of the cells was achieved by incubating the cells in ice-cold methanol for 5 min at −20°C. Blocking in 0.5% bovine serum albumin in PBS for 15 min at RT was followed by incubation of the cells with a 1:100 dilution of the primary antibody, monoclonal mouse anti-c-myc (Boeheringer Mannheim) for 1 h at RT. After extensive washing, cells were incubated with a 1:40 dilution of the secondary antibody [donkey anti-mouse IgG $F(ab')_2$ fragment FITC-conjugated, Jackson Laboratories, Inc., PA] for 1 h at RT in 0.5% BSA/PBS in the dark. After washing, immunolocalization of DFCP1 fusion protein was detected by confocal microscopy. Images were captured on a Leica TCS-4D confocal laser scanning microscope system (Leica Microsystems, Heidelberg, Germany) equipped with a Kr/Ar laser. Z-series images were acquired using a 100×, 1.4 NA objective with conditions constant for both control and experimental samples.

3. Results and discussion

3.1. Structural characterization and chromosomal localization

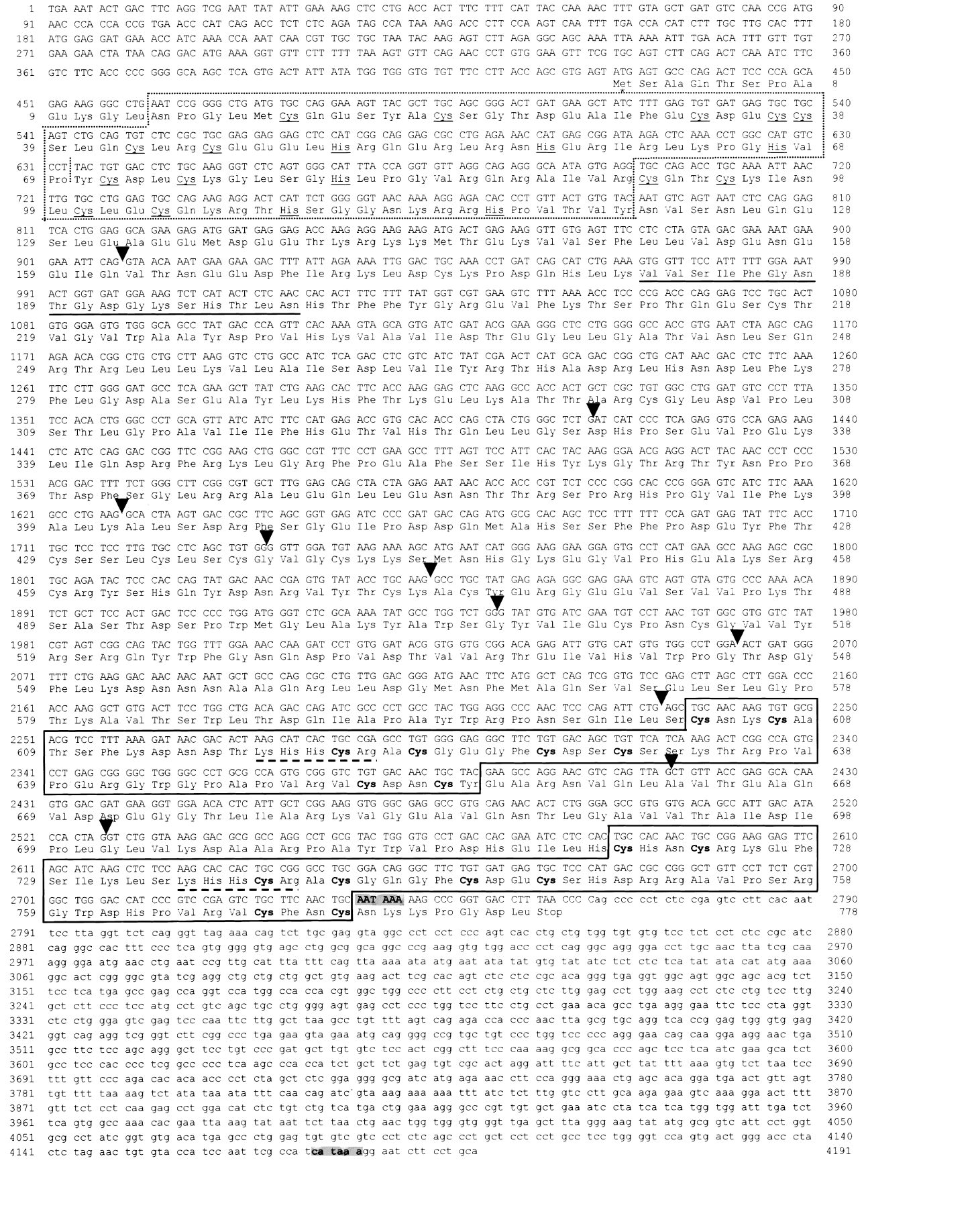
At the time when the SGAP was initiated, sequencing of the BMSC cDNA library produced approximately 50 3′ ESTs which appeared to be unique to the library based on BLAST search of GenBank. One of these ESTs was selected for further characterization. The cDNA clone (2761 bp) from which this EST was derived was sequenced in full and further analysis revealed that it corresponded to a novel transcript (Fig. 1A). The open reading frame (ORF) encodes a protein of 777 amino acids with a predicted molecular mass of 87 230 Da and pI of 7.46. Plots of hydrophilicity (Kyte and Doolittle, 1982), surface probability (Janin andWodak, 1978; Emini et al., 1985), and antigenic index (Jameson and Wolf, 1988) predict a largely hydrophilic structure, suggesting that the protein is not an integral membrane protein. Submission of the deduced amino acid sequence to the SignaIP database (http://www.cbs.dtu.dk/services/SignalP/) of the Center of Biological Sequence Analysis (CBS) showed that no known nuclear localization signal peptide for transport of the protein into the nuclei, or signal peptide for secretion from the cell, are present, assigning this protein as primarily a cytoplasmic protein.

The deduced amino acid sequence of the cDNA reveals that the protein has many interesting features. It contains a putative zinc finger domain (a Cys- and His-rich region) at the NH_2-terminus (dashed box, Fig. 1A). Comparison of this region with other zinc finger sequences in the database revealed a loose correlation with different types of zinc finger domain (B-box: aa 13–63 and aa 66–121, C_2H_2: aa 40–69). No perfect matches were found, suggesting that it represents either a novel type of zinc finger domain or is simply a Cys- and His-rich cluster. The protein has a glycine-rich region usually referred to as the P-loop or the 'A' consensus sequence (underlined with a solid line, Fig. 1A) that has been shown to be very conserved in a large number of proteins that bind ATP or GTP.

Further analysis of the deduced amino acid sequence revealed that the protein contains two putative double zinc finger domains, termed the FYVE finger, hence we have named this protein 'double FYVE-containing protein 1' (DFCP1). While zinc finger proteins are usually involved in protein–DNA interactions, a number of studies have shown that certain zinc fingers can be involved in protein–protein interactions (Freemont, 1993; Feuerstein et al., 1994) and even in the binding of lipids (Zhang et al., 1995). The FYVE domain was first characterized by Stenmark et al. (1996), the name being derived from the first letter of the first four proteins discovered to contain it: Fab1p, YOTB, Vac1p, EEA1. The amino acid sequence of the FYVE domain is highly conserved and characterizes a family of proteins with more than 30 members found in species ranging from yeast to mammals (Gaullier et al., 1999).

In DFCP1, both FYVE domains are approximately 70 amino acids long (solid lined box, Fig. 1A) and share 53% amino acid identity and 56% similarity (Fig. 2A, first two rows). The two FYVE domains are approximately 40% identical and 53% similar to FYVE domains of other proteins (Fig. 2A). The two FYVE domains of DFCP1 show the greatest degree of homology to human and yeast FYVE domains. The lack of homology to other mammals may be due to the lack of information on FYVE-containing proteins in rat and mouse. The

A



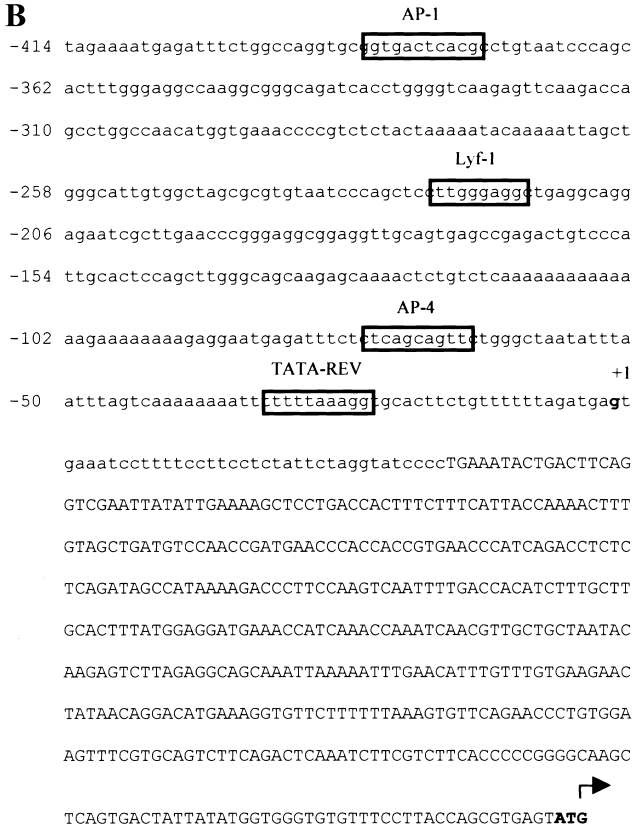


Fig. 1. (**A**) Nucleotide and predicted amino acid sequence of DFCP1 cDNA. A Cys–His cluster at the amino terminus (dashed line box) has similarities, but is not identical to a number of zinc finger proteins. An ATP/GTP 'A' consensus sequence is underlined with a solid line. The two FYVE domains at the carboxy terminus are boxed and the potential zinc-coordinating cysteine residues are in bold. The basic patches of amino acids found to be important for binding to PtIns-3-P within the FYVE domain are underlined with a dotted line. The two Poly A^+ consensus sequences are shaded. The intron–exon junctions indicated with arrow heads are types 0, I, 0, II, 0, II, 0, 0, I, I, respectively. Capital letters represent the sequence derived from the original cDNA clone. Lower case letters indicate the sequence derived from the genomic sequence in the database. Poly A^+ of the $\sim$3 kb message occurs at the junction of the upper and lower case sequences, just after the stop codon. (**B**) 5′ untranslated region (463 bp), predicted transcription start site and putative partial promoter region. The predicted transcription start is 37 bp upstream of the cDNA clone (shown here in upper case). Also indicated are putative transcription factor binding sequences.

most conserved amino acids within the FYVE domains are eight cysteines (bold in Fig. 1A) which are thought to coordinate Zn^{2+} binding (Stenmark et al., 1996). There is also a motif that is rich in basic residues, Arg/Lys–Arg/Lys–His–His–Cys–Arg, surrounding the third cysteine residue (dashed underline in Fig. 1A) that further characterizes the FYVE domain (Stenmark et al., 1996; Burd and Emr, 1998), and is thought to be a binding pocket for PtdIns-3-P (Misra and Hurley, 1999). It is interesting to note that the two FYVE domains in DFCP1 have a Ser and a Thr where other FYVE domains have an Arg (Fig. 2A).

By using the cDNA sequence of DFCP1 to search the HTGS database (http://www.ncbi.nlm.nih.gov/BLAST/), we were able to identify the complete genomic sequence (Human Genome Project, GenBank accession no. AC006345) (Fig. 2B). The gene locus name and symbol approved by the Human Genome Nomenclature Committee is *ZNFN2A1*, indicating a 'zinc finger protein, subfamily 2A (FYVE domain-containing), 1'. Comparative analysis of cDNA and genomic sequences revealed that *ZNFN2A1* contains 11 exons and 10 introns (Fig. 2B). The Cys/His-rich cluster is entirely encoded by exon 1 and the ATP/GTP binding site is within exon 2. The two FYVE domains are encoded separately in exons 9 and 11. The genomic sequence allowed us not only to verify the 5′ untranslated region (UTR) upstream seen in the cDNA clone, but also to identify the polyadenylation consensus sequence that gives rise to the longer transcripts found in many tissues. Furthermore, PCR amplification of our own library cDNAs, as well as GenBank database searches of newly entered cDNA sequences, confirmed the length and sequence of the 3′ UTR. (Fig. 1 shows the sequence of the cDNA clone derived from the library in capital letters and the subsequently confirmed sequence first derived from the genomic sequence in lower case letters.)

The putative transcription start site was identified using a eukaryotic promoter prediction program (Neural Network Program, LBNL; http://dot.imgen.bcm.tmc.edu:9331/seq-search/gene-search.html). The predicted transcription start site was located 463 bp upstream of the initiating codon and 37 bp upstream of the sequence of the cDNA clone (Fig. 1B). Analysis of the genomic sequence upstream from the putative start of transcription using the Transcription Factor Search (TFSEARCH; http://pdap1.trc.rwcp.or.jp/research/db/TFSEARCH.html) revealed the presence of a number of transcription factor binding sequences including: a reverse TATA box (GGAAATTTTT at position −23); AP-4 (CTCAGCAGTT at −75); Lyf-1 (CTTGGGAGG at −225); and AP-1 (GGTGACTCACG at −397) (Fig. 1B).

We determined the chromosomal localization of *ZNFN2A1* through a search of the dbSTS database (http://www.ncbi.nlm.nih.gov/BLAST/). The genomic sequence showed 100% nucleotide sequence identity to the STS marker, stSG16028, which has been localized to chromosome 14q22–q24 (NCBI GenMap '99).

3.2. Expression analysis

Because we isolated the then unique DFCP1 encoding cDNA from a cDNA library derived from cultured human bone marrow stromal cells, we wanted to compare the expression level of this mRNA in this cell population with other fibroblastic cell cultures that share various morphologic features with BMSC, but vary with

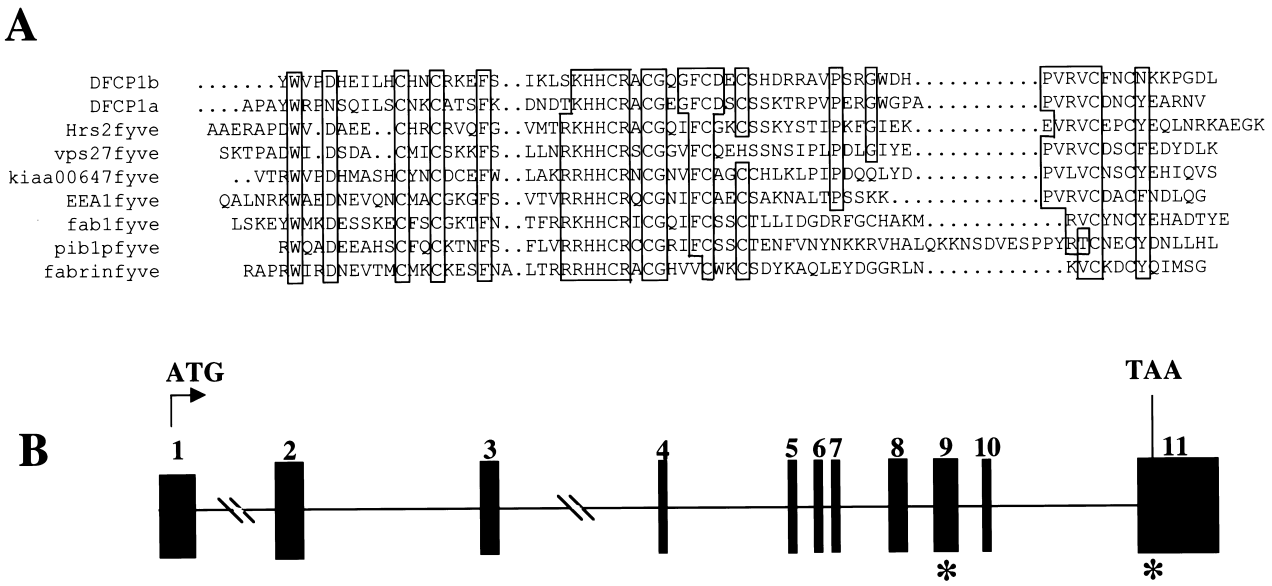
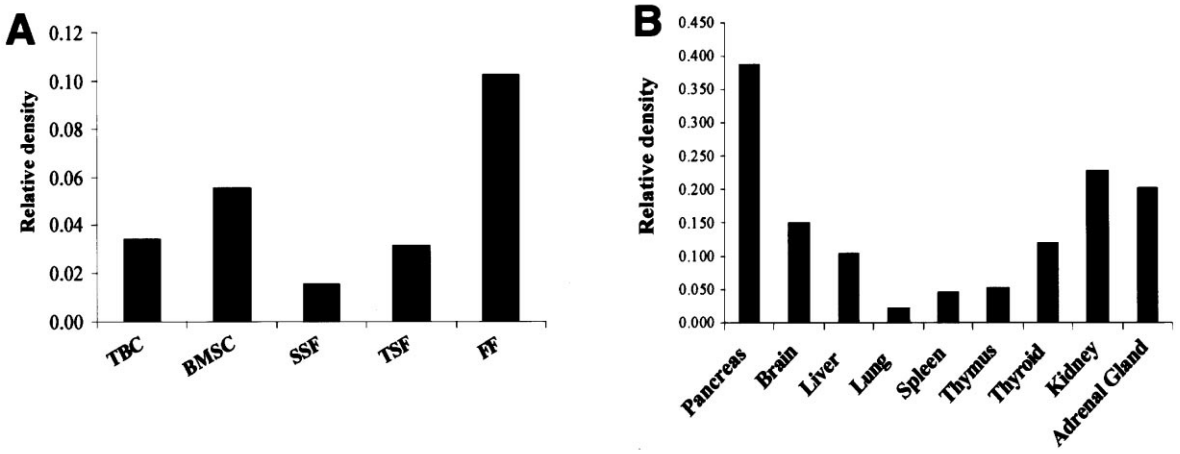
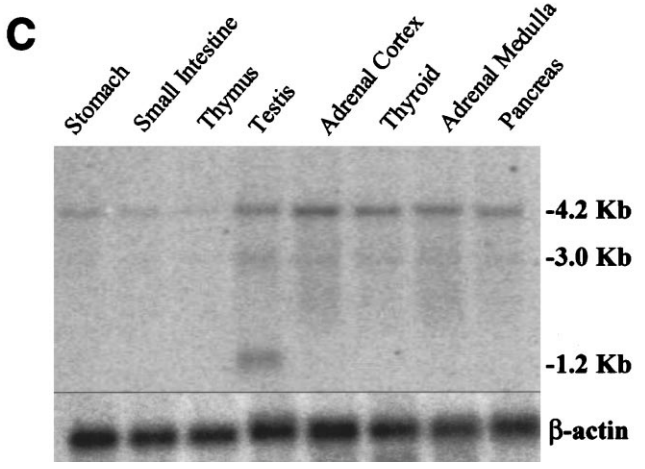


Fig. 2. The amino acid sequence alignment of the FYVE domain and the genomic structure of *ZNFN2A1*. (**A**) DFCP1a and 1b represent the two FYVE domains in DFCP1. Sequences shown are derived from yeast: Vps27 (GenBank accession no. U24281), Pib1p (GenBank accession no. U28374), Fab1 (GenBank accession no. U01017); human: EEA1 (human early endosomal antigen, GenBank accession no. L40157), Hrs-2 (GenBank accession no. D84064) and KIAA0647 (unknown protein human, GenBank accession no. AB014547); and rat: Fabrin (GenBank accession no. AF038388). Boxes indicate amino acid identities or conservative amino acid substitutions between the two FYVE domains in DFCP1 and among the FYVE domains of other proteins. (**B**) The genomic structure is characterized by 11 exons represented by boxes. The putative zinc finger at the NH_2-terminus is encoded by exon 1, and the putative ATP/GTP binding site is within exon 2. The first FYVE domain is encoded by exon 9, the second FYVE domain by exon 11 (*).





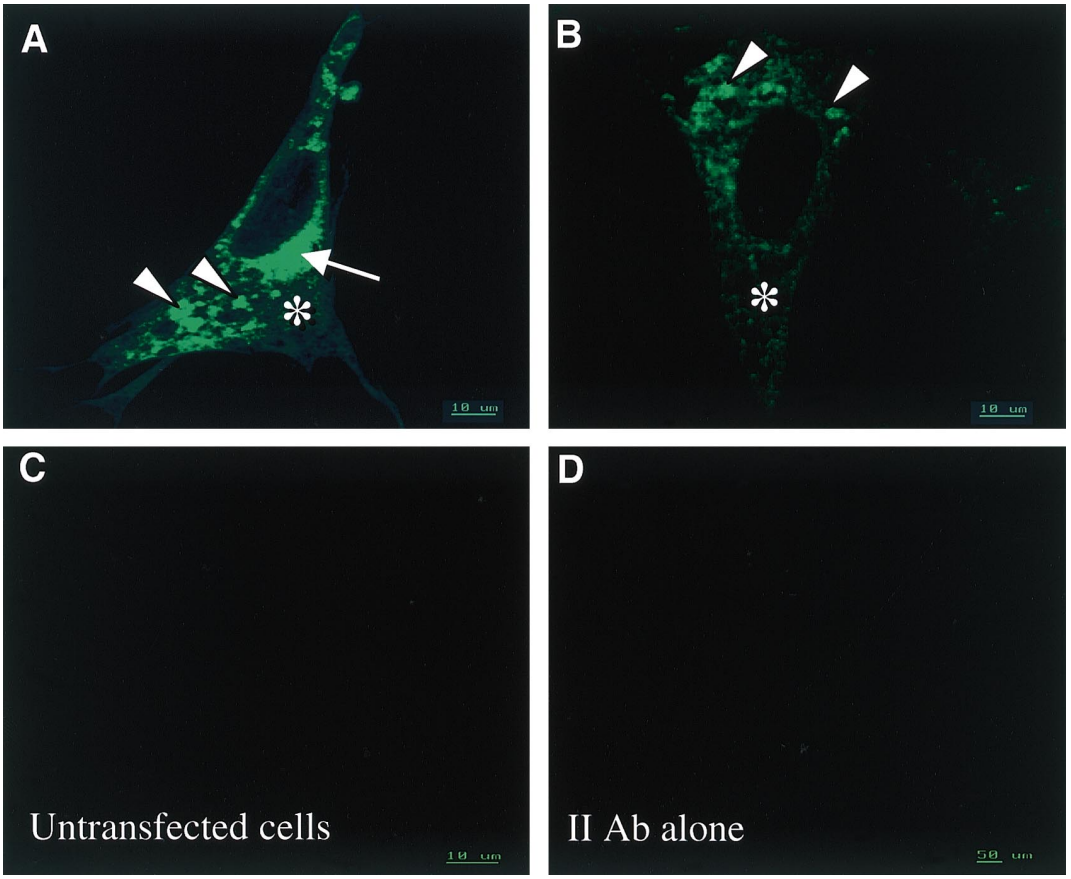


Fig. 4. Immunofluorescence staining of the c-myc–DFCP1 fusion protein in NIH-3T3 cells. NIH-3T3 cells were transfected, fixed and incubated with mouse monoclonal anti-c-myc (**A** and **B**) followed by FITC-conjugated donkey anti-mouse antibody. Staining of the Golgi (arrow), cytoplasmic vesicles (arrowheads) and diffuse staining characteristics of rough endoplasmic reticulum (asterisk) were routinely observed. (**C**) Untransfected cells; (**D**) cells transfected and incubated in the presence of only the secondary antibody.

respect to their ability to form bone. Specifically, we compared expression levels of DFCP1 in mature osteoblastic cells (TBC) with BMSCs which are osteogenic precursors, and to similar, but non-osteogenic stromal cells derived from spleen, thymus and foreskin fibroblasts (SSF, TSF, FF, respectively). DFCP1 mRNA was expressed in all cell populations examined, with the highest level of expression in FF (Fig. 3A). These results indicate that there is no obvious correlation between DFCP1 mRNA expression levels and osteogenic potential, and that DFCP1 is not unique to bone-forming cells.

Northern blot analysis of mRNA isolated from a variety of human tissues confirmed that DFCP1 is not specific to BMSCs, but is widely expressed (Fig. 3B and C). Quantitative analysis showed relatively higher expression of DFCP1 mRNA in endocrine tissues such as pancreas, adrenal gland and thyroid (Fig. 3B). Hybridization of the DFCP1 cDNA probe to an endocrine tissue blot (Clontech, Palo Alto, CA) confirmed these findings, and furthermore revealed the presence of three different size transcripts: 4.2, 3 and 1.2 kb (Fig. 3C). The 4.2 and 3 kb appeared to be ubiquitously expressed in all tissues tested, whereas the 1.2 kb transcript was found only in testis. The presence of a 1.4 kb long 3′ UTR accounts for the difference in length between the 4.2 and 3 kb mRNAs, however, the possibility of alternative splicing cannot be ruled out at this time. A search of the dbEST database with the mRNA sequences identified a group of recently deposited ESTs derived from other library sources that shared the same 3′ end as the 4.2 kb transcript, as well as a separate group that shared the 3′ end of the 3 kb transcript.

The 4.2 kb mRNA contains a polyadenylation con-

Fig. 3. Northern blot analysis and tissue distribution of DFCP1 mRNA. (**A**) Relative expression level of DFCP1 mRNA in cell cultures that differ with respect to their ability to form bone (TBC, trabecular bone cells; BMSC, bone marrow stromal cells; SSF, spleen stromal fibroblasts; TSF, thymus stromal fibroblasts; FF, foreskin fibroblasts). (**B**) Relative expression levels of DFCP1 mRNA in different tissues. Relative levels were determined by counting the amount of radioactivity (PhosphoImager) and then normalizing the value against β-actin. (**C**) The expression pattern by Northern blot of DFCP1 transcripts in a variety of tissues, demonstrating the presence of the three different transcripts (4.2, 3 and 1.2 kb).

sensus at the end of the 3′ UTR (4172 bp), but with a substitution of the first nucleotide, A, with a C (CATAAA). A substitution of the first nucleotide of the AATAAA consensus sequence appears to be rare among reported mRNAs. Previous in vitro findings suggest that mutations in the AATAAA consensus sequence decrease the rate of cleavage and polyadenylation of the mRNA transcripts (Sheets et al., 1990). However, the 4.2 kb transcript appears in high abundance in several tissues (primarily endocrine tissues). Thus, cleavage and polyadenylation may not be rate limiting for this gene, or there may be sequences elsewhere in the precursor which compensate for the non-conventional polyadenylation consensus. Interestingly, the 3 kb mRNA does have a canonical AATAAA polyadenylation consensus sequence near the end, but still within the ORF (at 2738 bp), an unusual placement in eukaryotic cells. The fact that other ESTs recently deposited from libraries made from Poly A-containing mRNAs are identical to the 3′ ends of both the 4.2 and 3.0 kb mRNAs indicates that both the non-standard sequence in the 4.2 kb form and the sequence within the ORF of the 3.0 kb form are in fact utilized. To date, the 1.2 kb message found only in testis remains unexplained. Whether this transcript is an alternative splice of *ZNFN2A1* or represents an independent gene with some homology to *ZNFN2A1* is unknown at this time.

3.3. Immunolocalization

Based on previous reports that other FYVE-containing proteins may be involved in intracellular membrane trafficking (Simonsen et al., 1998; Bean et al., 1997; Weisman and Wickner, 1992; Piper et al., 1995; Yamamoto et al., 1995), we wanted to determine where DFCP1 would localize within the cell. NIH-3T3 fibroblasts were therefore transfected with a pCMV-tag3b plasmid containing the DFCP1 ORF preceded by a sequence tag coding for the c-myc epitope (MEQKLISE). The DFCP1 was then localized using the monoclonal anti-c-myc. Confocal microscopy revealed an absence of nuclear accumulation, but a pattern typical of localization in the Golgi apparatus, endoplasmic reticulum and cytoplasmic vesicles. This pattern of localization supports the hypothesis that DFCP1 may indeed be recruited to vesicular structures due to the presence of one or both of the FYVE domains (Fig. 4A and B).

4. Conclusions

In this study we have identified and cloned a novel gene (*ZNFN2A1*) which encodes for a protein that we have named DFCP1. Double FYVE is expressed in a wide variety of tissues, but the highest levels were seen clearly in endocrine tissues.

1. DFCP1 contains sequences encoding for: (1) a Cys- and His-rich cluster in the amino terminal portion of the protein (possibly a new type of zinc finger); (2) an ATP/GTP binding site; and (3) two FYVE domains near the carboxy terminus.
2. This is the first time that two FYVE domains have been found in one protein.
3. DFCP1 mRNA has a relatively long (463 bp) 5′ UTR.
4. The polyadenylation consensus sequence for the longer transcript (4.2 kb) is non-standard, while in the shorter transcript (3 kb) it is within the coding sequence, both of which are relatively rare findings.
5. Confocal microscopy studies revealed that when transfected into NIH-3T3 cells, the fusion protein appears to localize to membranous structures in the cytoplasm.

Further analysis is required to characterize the role that this protein plays in cells, in particular, on membrane trafficking, as has been suggested for other FYVE-containing proteins.

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