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FDF03, a Novel Inhibitory Receptor of the Immunoglobulin Superfamily, Is Expressed by Human Dendritic and Myeloid Cells

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In this study, we describe human FDF03, a novel member of the Ig superfamily expressed as a monomeric 44-kDa transmembrane glycoprotein containing a single extracellular V-set Ig-like domain. Two potential secreted isoforms were also identified. The gene encoding FDF03 mapped to chromosome 7q22. FDF03 was mostly detected in hemopoietic tissues and was expressed by monocytes, macrophages, and granulocytes, but not by lymphocytes (B, T, and NK cells), indicating an expression restricted to cells of the myelomonocytic lineage. FDF03 was also strongly expressed by monocyte-derived dendritic cells (DC) and preferentially by CD14+/CD1a– DC derived from CD34+ progenitors. Moreover, flow cytometric analysis showed FDF03 expression by CD11c+ blood and tonsil DC, but not by CD11c– DC precursors. The FDF03 cytoplasmic tail contained two immunoreceptor tyrosine-based inhibitory motif (ITIM)-like sequences. When overexpressed in peranadate-treated U937 cells, FDF03 was tyrosine-phosphorylated and recruited Src homology-2 (SH2) domain-containing protein tyrosine phosphatase (SHP)-2 and to a lesser extent SHP-1. Like engagement of the ITIM-bearing receptor LAIR-1/p40, cross-linking of FDF03 inhibited calcium mobilization in response to CD32/FcγRII aggregation in transfected U937 cells, thus demonstrating that FDF03 can function as an inhibitory receptor. However, in contrast to LAIR-1/p40, cross-linking of FDF03 did not inhibit GM-CSF-induced monocyte differentiation into DC. Thus, FDF03 is a novel ITIM-bearing receptor selectively expressed by cells of myeloid origin, including DC, that may regulate functions other than that of the broadly distributed LAIR-1/p40 molecule. The Journal of Immunology, 2000, 165: 1197–1209.

Inhibitory receptors containing one or several cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIM) are members of the C-type lectin family or Ig superfamily (Ig-SF). The ITIM is generally defined by the consensus sequence L/Vx[IlxYxxL/V that, following phosphorylation on tyrosine, recruits Src homology-2 (SH2) domain-containing protein tyrosine phosphatases SHP-1 and/or SHP-2, as well as the SH2 domain-containing inositol phosphatase (SHIP) in the case of FcγRIIB (1–5). ITIM-bearing receptors negatively regulate cellular functions when coaggregated with stimulatory receptors that signal through an immunoreceptor tyrosine-based activation motif (ITAM). The growing number of novel ITIM-bearing receptors identified thus far and their expression by virtually all leukocyte populations suggest that inhibitory receptors may regulate various aspects of immune responses (6, 7), as well as the fate of nonhemopoietic cells, as described for the SHPS-1/SIRPα molecules (8, 9).

Among APCs, dendritic cells (DC) are unique leukocyte populations using their role as sentinels to capture Ag at the periphery of an organism and by their capacity to present processed Ag to both CD4+ and CD8+ naive T cells, thus initiating primary immune responses (for review, see Refs. 10 and 11). In our effort to identify receptors involved in Ag capture and presentation by human DC, we previously described an ITIM-containing C-type lectin, designated DCIR, that displayed features intermediate between NK cell receptors and typical type II lectins involved in ligand internalization (12). While the function of DCIR is not yet determined, its restricted expression on APCs, and particularly on DC, has led to questions on the potential role(s) of inhibitory receptors in DC function. Different groups have previously reported the presence on APCs of the ITIM-bearing molecules ILT3/LIR-5, ILT2/LIR-1, and ILT4/LIR-2 belonging to the ILT/LIR/MIR family (13–16). It has been shown that these Ig-SF members can function as negative regulators of monocyte and DC activation, most probably through recruitment of SHP-1 (15, 17–20). Both ILT2/LIR-1 and ILT4/LIR-2, but not ILT3/LIR-5, bind to HLA class I molecules (14, 17, 19). While the physiological role of inhibitory receptors for HLA class I on monocytes and DC is not yet understood, the demonstration that ILT2/LIR-1 is also a receptor for the UL18 molecule, an homologue of human MHC class I encoded by CMV (19, 21), suggests that pathogens may use inhibitory receptors of APCs to down-regulate immune responses. In addition to its inhibitory activity, ILT3/LIR-5 is also internalized following cross-linking on monocytes and may be involved in Ag capture and loading for presentation into MHC class II (15). As reported

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1 Abbreviations used in this paper: ITIM, immunoreceptor tyrosine-based inhibitory motif; DC, dendritic cells; EST, expressed sequence tag; Ig-SF, Ig superfamily; ITAM, immunoreceptor tyrosine-based activation motif; ORF, open reading frame; SH2, Src homology-2; SHIP, SH2 domain-containing inositol phosphatase; SHP, SH2 domain-containing protein tyrosine phosphatase; UTR, untranslated region; SCF, stem cell factor.
for the FcγRIIB (22, 23), internalization of ILT3/LIR-5 might be mediated through its cytoplasmic ITIMs, because the ITIM sequence contains the tyrosine-based internalization motif YXXφδ, where φ represents any hydrophobic residue (24). Finally, the recent demonstration that the broadly expressed inhibitory receptor LAIR-1/p40 (25) can inhibit the differentiation of monocytes into DC in response to GM-CSF (26) suggests that ITIM-bearing receptors may also play an important regulatory role during the early commitment of DC precursors by interfering with signaling mediated through growth factor receptors.

In the present study, we report the cloning and characterization of FDF03, a novel member of the Ig-SF that has a restricted expression in myelomonocytic cells including in vitro-derived DC and in vivo CD11c-positive DC. The FDF03 cytoplasmic tail contains two ITIM-like sequences and preferentially associates with SHP-2 in perversated-activated monocyte U937 cells. Moreover, we show that coaggregation of FDF03 with CD32/FcγRII inhibits intracellular Ca²⁺ mobilization in U937 cells, as does LAIR-1/p40, thus indicating that FDF03 possesses an inhibitory activity. However, in contrast to LAIR-1/p40, FDF03 failed to inhibit monocyte differentiation into DC in response to GM-CSF, suggesting that FDF03 may be involved in the regulation of different pathways or may use different inhibitory signals to that of LAIR-1/p40.

Materials and Methods

Hemopoietic factors, cytokines, and reagents

All cultures were performed in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM l-glutamine (all from Life Technologies, Gaithersburg, MD), and 160 μg/ml gentamicin (Scherer-Plough, Levallois-Perret, France). The source and sp. of recombinant human cytokines were the same as previously described (12). The following factors were used at optimal concentration: GM-CSF (100 ng/ml), TNF-α, IL-4 (50 U/ml). In some experiments, cells were activated with 1 ng/ml PMA and 1 ng/ml), and IL-4 (30). In some experiments, 5 x 10⁴ monocyte-derived DC per well (24-well culture plate) were further activated with LPS (25 ng/ml) for 72 h or by coculture with 4 x 10⁴ irradiated (7500 rad) murine fibroblastic L cells untransfected or transfected with the cDNA for CD40 ligand (27).

Northern blot analysis

Human mRNA adult tissue blots (Clontech, Palo Alto, CA) were hybridized with a 377-bp DNA probe from the 3’-end of FDF03 cDNA, produced by PCR amplification of a region defined by the oligonucleotides 5’-CAG CAGGGGACTAAAGGCCAC (forward primer) and 5’-GCATCTGCTGATTATCA (reverse primer). This fragment was labeled with [32P]-dCTP using the High Prime kit (Boehringer Mannheim, Meylan, France). Membranes were prehybridized and hybridized under standard conditions (31). Low and high stringency washes were 2 x SSC/0.2% SDS and 0.2 x SSC/ 0.2% SDS, respectively, each done twice for 30 min. The membranes were incubated with Biomax MR film (Kodak, Rochester, NY) for 7 days.

DNA, RNA, and RT-PCR analysis

Cells were lysed, total RNA was extracted (32), and first-strand cDNAs were prepared after DNase I treatment (in the presence of RNase inhibitor) of 5 μg of total RNA using oligo(dT) primers (Pharmacia, Uppsala, Sweden) and the SuperScript II (Life Technologies, Gaithersburg, MD). All cultures were performed in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine (all from Life Technologies, Gaithersburg, MD), and 100 μg/ml gentamicin (Scherer-Plough, Levallois-Perret, France). The source and sp. of recombinant human cyto-

 Identification of a mouse homologue of FDF03

To identify a mouse homologue of human FDF03, we performed a BLAST search against the mouse expressed sequence tag (EST) database identified different ESTs (gb, A666524, A6595493, and A0217475) with homology to human FDF03 and DNA and allowed to build a coding region for a mouse protein homologous to human FDF03. PCR amplification of cDNAs from mouse spleen using oligonucleotides designed in the 5’- and 3’-ends of this sequence (forward 5’-CCCTGACCCCCGATCCGCCGTCCA and reverse 5’-GTGAGATTTTCTGGTGTCCGCTT) permitted the amplification of the entire open reading frame (ORF) of mouse FDF03 (mFDF03).

Chromosomal localization of the FDF03 gene

Chromosomal localization was performed with the Salmon G3 radiation hybrid medium resolution panel (Research Genetics, Huntsville, AL). PC was as described above using oligonucleotides that amplify a 505-bp fragment specific to the human gene (forward 5’-ACAGCCCTCTTGGAGCCTCTA and reverse 5’-GGCCGAAAATCACAGACTGG). The results were scored manually, and analysis was performed with the radiation hybrid mapper program (http://shgc-www.stanford.edu).

Generation of anti-FDF03 mAb 36H2

Female Lewis rats were primed using DNA immunization via intradermal injection into the tail of 50 μg of a pCMV8 expression plasmid (Invitrogen) encoding the FDF03 molecule. Pulsed immunizations were repeated thereafter on day 7 (40 μg i.p.) and day 29 (10 μg). Animals were boosted with FDF03-Ig fusion protein on day 67 (10 μg i.p. in CFA) and on day 82 (2.5 μg i.p. in IFA). On day 127, a combined immunizing dose (2 μg i.v. in saline; 2 μg i.p. in IFA) was administered, and splenocytes were harvested 4 days later and used with the mouse myeloma P3X63-Ag8.653. Hybridomas were then initially selected that recognized FDF03-Ig (but not control-Ig) fusion protein in indirect ELISA. Hybridomas were then further selected based on their ability to immunoprecipitate an FDF03-AP fusion protein, as previously described (33), and to recognize PBMC via FACS staining. The rat IgG2a mAb TC17-36H2 (mAb 36H2) was selected for use in this study.
Surface biotinylation, immunoprecipitation, and Western blot analysis

For surface biotinylation, purified blood monocytes were washed three times with ice-cold PBS, pH 8, then resuspended in freshly prepared solution of 0.5 mg/ml sulfo-N-hydroxysuccinimide of biotin (Pierce, Rockford, IL) in PBS and agitated at room temperature for 30 min. Cells were washed three times with ice-cold PBS, pH 8, and then treated in a lysis buffer containing 50 mM Tris-HCl, pH 8, 1% Nonidet P-40, 150 mM NaCl, and protease inhibitors (Boehringer Mannheim). Lysates were then incubated at 4°C for 20 min, and the insoluble material was pelleted by centrifugation at 12,000 × g for 10 min at 4°C. Soluble extracts were precleared three times with control mAb and protein G-Agarose (Boehringer Mannheim). The extract was then incubated with the mAb of interest for 1 h before adding protein G-Agarose for at least 3 h. Beads were washed three times in lysis buffer, resuspended in SDS-PAGE buffer with or without 5% 2-ME, boiled for 3 min, and centrifuged. Immunoprecipitates were separated by SDS-PAGE using 12% polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane (Immobilon P; Millipore, Bedford, MA). Blots were blocked with 1% BSA, 0.1% Tween-20 in PBS, then incubated with HRP-conjugated avidin (ExtrAvidin peroxidase; Sigma) for 30 min. Proteins were detected by enhanced chemiluminescence (Boehringer Mannheim). To analyze FDFO3 glycosylations, precipitates were untreated or digested overnight at 37°C with N-glycosidase F (0.2 U/μl), or with O-glycosidase (2 μg/μl) with or without neuraminidase (5 μg/50 μl) before SDS-PAGE in reducing conditions. All enzymes were from Boehringer Mannheim, and digestions were performed in 0.1 M potassium/phosphate buffer, pH 7, 0.05% SDS, 1% 2-ME, 1% Nonidet P-40, and 50 mM EDTA.

Flow cytometric analysis and cell sorting

Cell-surface expression of FDFO3 was determined by immunofluorescence staining and flow cytometric analysis with a FACScalibur (Becton Dickenson, Mountain View, CA). For single staining, cells were incubated for 30 min at 4°C with 5 μg/ml purified rat anti-FDFO3 mAb 36H2, then washed twice in PBS, 1% BSA, 0.1% NaN3, and labeled with PE-conjugated streptavidin (Becton Dickinson). For double staining, cells were first labeled with anti-FDFO3 mAb 36H2 and anti-LAIR-1 mAb DX26 (25) and protein G-Agarose. Immunoprecipitates were separated by SDS-PAGE on 12% polyacrylamide gels and transferred to Immobilon-P membranes. The membranes were blocked and then incubated with HRP-conjugated anti-phosphotyrosine mAb 4G10 (Upstate Biotechnology, Lake Placid, NY) or with polyclonal rabbit Abs against SHP-1 (C19) or SHP-2 (C18) (Santa Cruz Laboratories, Santa Cruz, CA) followed by HRP-conjugated anti-rabbit IgG (Amersham, Arlington Heights, IL). Proteins were detected by enhanced chemiluminescence.

Analysis of intracellular calcium mobilization

FDFO3-transfected U937 cells were loaded with 5 μM Indo-1 AM and 5 μM Flurochrome F-27 (Molecular Probes, Eugene, OR) for 45 min at 37°C. Cells were washed and kept at room temperature in the dark in RPMI 1640 supplemented with 10% FCS (complete medium) until analysis. For each experiment, an aliquot of Indo-1-loaded cells (107 cells) was resuspended in 1 ml complete medium at 37°C, and the ratio of violet/blue fluorescent emissions at 405 and 530 nm, respectively, was analyzed on a FACStarPlus flow cytometer (Becton Dickinson) equipped with an argon laser tuned at 350–364 nm (Spectra-Physics, Mountain View, CA) used for UV excitation. When the 405/530 nm ratio baseline was stable, 10 μg/ml of the Abs to be tested were added and the fluorescent emissions were recorded for 2 min. Cells were then kept at 37°C for 2 min, and cross-linking of Abs was achieved by adding 20 μg/ml of a goat F(ab)2, anti-mouse Ig (Fcγ fragment-specific) that cross-reacts with rat IgG (Jackson ImmunoResearch, West Grove, PA). Fluorescent emissions were then recorded for the remaining time (~5 min). The ratio of Indo-1 violet to blue fluorescence (405/530 nm ratio) was displayed as a function of the elapsed time. The rabbit IgGs mAb IV.3 used to induce Ca2+ mobilization was obtained from Medarex (Lebanon, NH).

Results

Cloning of FDFO3 cDNA

By random sequencing of a cDNA library from human activated monocytes (500 sequences), we selected a clone containing a putative leader sequence and representative of a cell-surface receptor, designated FDFO3. Fig. 1A shows the nucleotide and deduced amino acid sequences of FDFO3. The nucleotide sequence was 1249 bp in length and contained an ORF of 912 nt with the first start codon ATG contained in a consensus Kozak sequence and preceded by a stop codon. The 3′-untranslated sequence of 184 nt contained a classical AATAAA polyadenylation signal. The deduced polypeptide conforms to a type I transmembrane protein composed of 303 aa including a 21-aa signal peptide, a 175-aa extracellular domain, a hydrophobic sequence of 22 aa characteristic of a transmembrane segment, and a 85-aa cytoplasmic tail. The predicted molecular mass of the FDFO3 polypeptide was 34 kDa.

FDFO3 belongs to the Ig-SF

Searching databases of known polypeptide sequences indicated that the extracellular domain of FDFO3 shared significant homology only with the variable (V) domain of human and mouse IgS. Further alignment studies (exemplified in Fig. 1B) confirmed that FDFO3 was a novel member of the Ig-SF family.
FIGURE 1. FDF03 belongs to the Ig-SF. A. Nucleotide and predicted amino acid sequences of human FDF03. The nucleotide sequence of the 1249-bp cDNA clone and the derived amino acid sequence are numbered starting from the start codon ATG and methionine, respectively. The predicted signal peptide (dotted) and the transmembrane domain are underlined. The potential N-linked glycosylation site in extracellular domain is underlined twice, and the three tyrosine-based motifs within the cytoplasmic tail are boxed. A consensus polyadenylation signal (AATAAA) in the 3'UTR is underlined. Arrows above the nucleotide sequence indicate sequences deleted in FDF03-DTM and FDF-M14 isoforms. This sequence has been submitted to the GenBank, EMBL, and DDBJ databases under accession no. AJ400841.

B. Alignment of the putative Ig-like domain of FDF03 with the variable (V) domain of Ig and Ig-like proteins. Amino acids conserved between FDF03 (aa 32–151) and V-set domains of human Igλ (P01713), Igκ (P01608), and TCRβ (P01733) are shaded. Residues characteristic of the V-set and generally conserved among Ig-like proteins are boxed. Asterisks indicate the position of the conserved cysteines in the Ig fold. The positions of the predicted β-strands are indicated by lines. Sequences were analyzed using the MegAlign function of Lasergene (DNASTAR).

C. Putative soluble forms of human FDF03 (hFDF03) and sequence of a mouse homologue (mFDF03) of hFDF03. Amino acid sequences of hFDF03-DTM, hFDF-M14, and mFDF03 (GenBank, EMBL, and DDBJ accession nos. AJ400842, AJ400843, and AJ400844, respectively) are aligned with that of hFDF03. Only amino acids that differ from that of hFDF03 are noted, and residues identical with hFDF03 are indicated by dashes. Gaps introduced to optimize the sequence alignment are indicated by points.
domain of the V-set (36, 37). While most Ig domains are characterized by the presence of a pair of cysteines forming a disulfide bridge that stabilizes the Ig fold, the FDF03 extracellular domain contained only one cysteine at position 125 (asterisks in Fig. 1B). However, typical residues conserved among Ig-like proteins and contained in strands forming the two β-sheets of the Ig domain were also present within the FDF03 extracellular region (boxes in Fig. 1B). Moreover, a long spacing between the predicted β-strands C and D identified the two additional strands C' and C" that are characteristics of the V-set of Ig domain (36, 37). A single potential N-linked glycosylation site (NWT) was present in the Ig domain at position 100. Finally, the membrane-proximal region of the FDF03 extracellular domain contained a high proportion of threonine and serine that represent potential sites for O-linked glycosylation. By analogy with other Ig-SF members, this region is predicted to display an extended open conformation typical of hinge-like sequences.

The 85-aa intracellular domain of FDF03 possessed two putative protein kinase C phosphorylation sites (on serine residues 279 and 285 within the motifs SPR and SHR, respectively) and three tyrosine residues at position 246, 269, and 298 in sequences YENI, YASL, and YSVL, respectively. Following tyrosine phosphorylation, these motifs may represent potential binding sites for SH2 domain-containing signaling molecules (38). In particular, tyrosine 269 is centered within the sequence IVYASL that perfectly matches the consensus L/V/IxYxxL/V of the ITIM (2, 5, 39), and is spaced by 28 aa from the more distal tyrosine that is also contained within an ITIM-like sequence TLYSVL (Fig. 1A).

Identification of a mouse homologue of human FDF03

Searching mouse cDNA databases, we identified ESTs with homology to human FDF03 cDNA and we used them to build a contig coding for a protein homologous to human FDF03 and to design oligonucleotides in the 5'-end and 3'-end of this sequence (see Materials and Methods). PCR amplification of cDNAs from mouse spleen further permitted the amplification of a 1.2-kb nucleotide sequence containing an entire ORF encoding a predicted protein (mFDF03) of 289 aa in length (Fig. 1C). The mFDF03 extracellular region also contained a single Ig-like domain of the V-type and shared the amino acid residues characteristics of human FDF03. In particular, the IPFSFY sequence (at position 45–50 of mFDF03) in the predicted strand B is conserved within both proteins. However, mFDF03 contained an YFGRV sequence instead of the YFCRV sequence in strand F of human FDF03 (aa 117 to 121 in mFDF03), thus indicating that the mouse protein possessed none of the two cysteines typical of the Ig domains. Of importance, the intracytoplasmic tail of mFDF03 contained two tyrosines in tandem sequence IVYASI-x23-TVYSIV that is homologous to the tandem IVYASL-x23-TLYSVL of ITIM-like sequences in human FDF03 (Fig. 1C).

Soluble forms of FDF03 can be produced by alternative splicing

During the analysis of FDF03 expression by RT-PCR (see below), we identified a second PCR product shorter than the expected size. This product, amplified by RT-PCR from activated PBMC cDNA using primers designed in the 5'-UTR and 3'-UTR of FDF03 cDNA, was purified, cloned, and sequenced. All cDNA clones but one had an identical insert of 943 bp that matched with FDF03 cDNA sequence but contained a deletion of 219 nt coding for the extracellular threonine-rich region and the transmembrane domain of FDF03 (FDF03 nucleotides 455–707, arrows in Fig. 1A). The resulting nucleotide sequence coded for a putative protein designated FDF03-ΔTM of 230 aa, composed of the extracellular Ig-like-domain directly linked to the entire intracytoplasmic domain of FDF03 (Fig. 1C). The other cDNA clone identified was designated FDF-M14. It contained a 900-bp insert that matched the FDF03 nucleotide sequence but with a 253-bp deletion (FDF03 nucleotides 455–707, arrows in Fig. 1A) that also resulted in deletion of the region coding for the hinge region and transmembrane domain of FDF03. In this case, a change in reading frame introduced premature stop codons and the deduced FDF-M14 protein was a short 175-aa polypeptide composed of the extracellular FDF03 Ig-like domain linked to 24 aa at the carboxyl terminus that are not related to the full-length FDF03 amino acid sequence (Fig. 1C).

These two molecules lacking the transmembrane domain may thus represent soluble isoforms of FDF03 generated by alternative splicing of FDF03 mRNA (see Discussion).

**FIGURE 2.** FDF03 is a cell-surface monomeric glycoprotein. A. Lysates of surface biotinylated monocytes were immunoprecipitated with anti-FDF03 mAb 36H2 or control rat IgG with protein G-Agarose, boiled in nonreducing or reducing conditions, and analyzed by SDS-PAGE on 12% gels. Following transfer on membranes, blots were incubated with HRP-streptavidin and proteins were revealed by enhanced chemiluminescence. B. Precipitates of biotin-labeled monocytes with mAb 36H2 were treated with N-glycosidase F or with neuraminidase and/or O-glycosidase before SDS-PAGE in reducing conditions and Western blot analysis as above.

**Discussion**

The anti-FDF03 mAb 36H2, produced by immunizing rats against human FDF03 extracellular domain, was used throughout this
study. Immunoprecipitation from lysates of biotin-labeled blood monocytes using this mAb revealed a 44-kDa protein under both nonreducing and reducing conditions (Fig. 2A). Incubation of the precipitate with N-glycosidase F resulted in a shift of the molecular mass to ≈41 kDa, while treatment with neuraminidase and O-glycosidase did not change the electrophoretic migration of the molecule (Fig. 2B). Taken together, these data indicate that FDF03 is expressed as a monomer and that N-linked glycosylations at the single site (N100) within the FDF03 Ig domain account for the observed molecular mass (44 kDa) of the FDF03 polypeptide.

The gene encoding FDF03 is localized on chromosome 7q22 and FDF03 mRNA is mostly expressed in immune tissues

Using human radiation hybrid mapping panels and PCR analysis, the gene coding for FDF03 was mapped to chromosome 7q22, within the interval D7S479–D7S2545 of microsatellite anchor markers AFM036xg5 and AFMa052ya5 (data not shown). The closest marker was SGC33905 localizing the gene encoding human fetal liver cytochrome P450 subfamily IIIA polypeptide 7, CYP3A7.

Northern blot analysis on the Clontech immune system blots, performed with a 377-bp probe that hybridized the 3'-end ORF and 3'-UTR of FDF03 mRNA, showed the highest levels of expression in PBL, strong levels of expression in spleen and bone marrow, and lower expression in lymph node (Fig. 3). FDF03 mRNA was not detected in thymus, appendix, and fetal liver (Fig. 3). On total RNA tissue blots, detectable expression was seen in spinal cord, placenta, and lung, but no expression was seen in heart, brain, liver, skeletal muscle, kidney, pancreas, prostate, testis, ovary, small intestine, and colon (Fig. 3 and data not shown). This analysis generally showed the presence of two bands of ≈1.4 and ≈1.3 kb that may represent mRNA for FDF03 and for the transmembrane-deleted isoforms FDF03-ΔTM and FDF-M14, respectively, because the probe used in these experiments could hybridize with all three transcripts.

FDF03 expression is restricted to cells of myelomonocytic origin, including DC

The cellular distribution of FDF03 was first determined by RT-PCR analysis on various isolated cells and cell lines using a pair of primers able to amplify both FDF03 and FDF03-ΔTM cDNAs. As shown in Fig. 4A, FDF03 and FDF03-ΔTM cDNAs were strongly detected in PBMC, blood monocytes, and granulocytes, while no signal was amplified from purified blood T cells, NK cells, and tonsillar B cells. FDF03 and FDF03-ΔTM cDNAs were also present in macrophages (Mφ in Fig. 4A) derived from CD3+ cells in the presence of M-CSF, as well as in DC generated either from CD34+ cord blood progenitors with GM-CSF and TNF-α (CD34+–DC in Fig. 4A) or from blood monocytes with GM-CSF and IL-4 (mono-DC in Fig. 4A). Moreover, FDF03 was not expressed by the cell lines JY, RAMOS, DAUDI, BL2 (B cell lines), JURKAT, and MOLT-4 (T cell lines), TF1 (erythroleukemia), CHA (kidney carcinoma), MRC5 (fetal lung fibroblasts), and SW620 and HT29 (colon carcinoma cell lines) (data not shown).

In accordance with the RT-PCR analysis, two-color flow cytometry analysis of human PBL performed with mAb 36H2 showed that FDF03 was strongly expressed by all circulating CD14+ monocytes and by freshly isolated CD15+ blood granulocytes (Fig. 4B). In contrast, mAb 36H2 did not react with peripheral blood CD3+ T cells, CD20+ B cells, and CD56+ or CD16+ NK cells (Fig. 4B).

FIGURE 3. FDF03 is mostly expressed in immune tissues. Northern blots of human tissues were analyzed with a probe corresponding to 277 bp overlapping the 3′ of the ORF and 3′-UTR of FDF03 cDNA labeled with [32P]dCTP. Two bands were obtained, with the major band at ≈1.4 kb corresponding to the expected size for FDF03 mRNA and both the FDF03-ΔTM and/or FDF-M14 isoforms.

Taken together, these data indicate that FDF03 is mostly expressed by cells of myelomonocytic origin and by in vitro-derived DC. However, it should be noted that FDF03 mRNA was not detected in a number of myeloid cell lines including U937, HL60, and THP-1 (data not shown).

FDF03 is preferentially expressed by monocyte/CD14+–derived DC and by CD11c+ DC

DC represent heterogeneous populations of cells according to their origin and stage of activation/maturation (10, 40, 41). Because FDF03 mRNA was detected in bulk preparations of in vitro-derived DC (Fig. 4A), we further analyzed whether cell-surface expression of the FDF03 receptor could be differently regulated during DC differentiation as well as on DC subpopulations. As shown in Fig. 5A, FDF03 was not expressed on the surface of CD34+ cord blood progenitor cells, but was induced during their culture with a combination of SCF, GM-CSF, and TNF-α, with the strongest expression observed on immature DC on days 5–7 of culture. On day 6, three-color flow cytometric analysis demonstrated that FDF03 was mostly expressed by the CD14+/CD1a− subset of cells, when compared with the CD14+/CD1a+ subset (Fig. 5B). These two populations were sorted on day 6 according to their CD14 and CD1a expression and recultured for 6 days in the presence of GM-CSF and TNF-α. As shown in Fig. 5B, on day 12, CD14+–derived DC still expressed detectable levels of surface FDF03, and both FDF03 mRNA and FDF03-ΔTM mRNA were amplified by RT-PCR in those DC (day 12 DC in Fig. 5B). In contrast, the DC derived from the CD1a− subset of cells no longer expressed FDF03 at day 12, both at the cell surface and mRNA levels (Fig. 5B). In contrast, DC generated from peripheral blood monocytes in the presence of GM-CSF and IL-4 expressed high levels of FDF03 both at their immature stage (day 7 in Fig. 5C) and after further activation by signals inducing DC maturation (day 9 in Fig. 5C), such as LPS and CD40 ligand (42). Taken together, these results indicate a preferential expression of FDF03 by monocyte- and CD14+/CD1a−–derived DC and suggest that FDF03 can be expressed by both immature and mature DC.
To determine whether in vivo DC also expressed FDF03, we performed four-color FACS analysis on blood and tonsillar mononuclear cell suspensions enriched in DC by immunomagnetic beads depletion. The FDF03 expression was analyzed vs CD11c expression on DC identified on the basis of 1) absence of expression of lineage markers (CD3, CD14, CD15, CD16, CD20, and CD57) but 2) strong expression of MHC class II or CD4 molecules, as previously reported (34, 43–46). As shown in Fig. 6A, analysis of FDF03 expression on blood HLA-DR+ lineage− DC demonstrated that the majority of CD11c+ DC expressed FDF03, while mAb 36H2 did not stain the CD11c− DC precursor population. On tonsil CD4+/lineage− DC, FDF03 was also expressed by the CD11c+ population, but not by the CD11c− subset (Fig. 6B).

**FIGURE 4.** FDF03 is expressed by cells of myelo-monocytic origin. A, RT-PCR analysis of FDF03 and FDF03-ΔTM mRNA expression in various cell populations. Pools of cDNA from different samples were prepared as described in Materials and Methods. CDNA of macrophages (Mφ) and granulocytes were derived from cord blood CD34+ progenitors cultured in the presence of M-CSF or G-CSF, respectively. DC were generated from cord blood CD34+ cells with GM-CSF and TNF-α for 12 days (CD34+ DC) or from blood monocytes with GM-CSF and IL-4 for 7 days (mono-DC). Freshly isolated cells were PBMC, monocytes, T cells, NK cells, and tonsillar B cells. RT-PCR was performed with primers that amplified both FDF03 and FDF03-ΔTM, but not FDF-M14, as described in Materials and Methods. Amplification of plasmids containing FDF03 or FDF03-ΔTM cDNA (pFDF03 and pFDF03-ΔTM) was used as control of specificity, and amplification of β-actin was used as control for cDNA quantity and quality. B, Expression of FDF03 on peripheral blood leukocytes. FDF03 expression was analyzed by flow cytometry after staining of circulating mononuclear or polynuclear cells with anti-FDF03 mAb 36H2 followed by PE-conjugated goat anti-rat Ig. After saturation with mouse serum, cells were incubated with FITC-conjugated anti-CD20, anti-CD3, anti-CD16, anti-CD56 (for lymphocytes), anti-CD14 (for monocytes), or with anti-CD15 mAb for granulocytes. Acquisition was performed by gating on the different cell populations according to their forward and right angle scatter parameters.

FDF03 is phosphorylated and recruits SHP-2 and to a lesser extent SHP-1 in U937 cells

Following tyrosine phosphorylation, ITIM-bearing receptors can recruit protein tyrosine phosphatases SHP-1 and/or SHP-2 (5, 47). To determine whether the two ITIM-like sequences contained in the FDF03 intracytoplasmic tail could associate with SHP-1 and/or SHP-2, we transfected FDF03 cDNA in the monocytic U937 cell line and positively selected FDF03-expressing cells by using staining with mAb 36H2 and FACS sorting. This strategy was chosen because we did not find any cell line, including U937 (Fig. 7A, left histogram), that spontaneously expressed FDF03. After four rounds of successive sorting and culture, >90% of the cells expressed relatively high levels of FDF03 (Fig. 7A, right panel). This cell line (FDF03-U937 cells) was stimulated or not with sodium pervanadate (an inhibitor of protein tyrosine phosphatases that induces tyrosine phosphorylation) then lysed, and lysates were immunoprecipitated with anti-FDF03 mAb 36H2 or rat control IgG. Because FDF03-U937 cells also expressed the inhibitory receptor LAIR-1/p40 (25) (Fig. 7A), lysates were precipitated in parallel with anti-LAIR-1 mAb DX26 used as a positive control for ITIM-bearing molecule. After SDS-PAGE, Western blot analysis with the anti-phosphotyrosine mAb 4G10 revealed the presence of a major band of ~44 kDa, corresponding to FDF03 molecular mass, in mAb 36H2 precipitates of pervanadate-activated, but not untreated, cells (Fig. 7B). A ~40-kDa tyrosine-phosphorylated protein was specifically detected in mAb DX26 precipitates of pervanadate-treated FDF03-U937 cells (Fig. 7B), which corresponds to the previously reported molecular mass of LAIR-1/p40 on NK cells, monocytes, and DC (25, 26). When blots were probed with specific anti-SHP-1 Abs, a faint band of ~73 kDa was specifically...
revealed in mAb 36H2 precipitates of pervanadate-treated FDF03-U937 cells (Fig. 7C), while a strong signal was observed in mAb DX26 precipitates, thus suggesting that FDF03 was less efficient than LAIR-1 in association with SHP-1. In contrast, anti-SHP-2 Abs demonstrated that FDF03 recruited SHP-2 as efficiently as LAIR-1 (Fig. 7D). Taken together, these results indicate that FDF03 is tyrosine-phosphorylated and can associate with SHP-2, and to a lesser extent with SHP-1, in pervanadate-activated U937 cells.

FDF03 inhibits CD32/FcγRII-induced calcium mobilization in U937 cells

ITIM-bearing receptors inhibit activation signals, such as intracellular calcium mobilization, when coaggregated with ITAM-bearing receptors (4). Therefore, we analyzed whether FDF03 could also antagonize signaling of stimulatory receptors in FDF03-U937 cells. Efficient cross-linking of the different molecules was performed with a goat F(ab')2 anti-mouse Ig (Fcγ-specific) that also reacts with rat IgG, so that the same reagents could be used regardless of the origin of the mAbs. As shown in Fig. 8, cross-linking of mAb 36H2 did not increase intracellular Ca2+ concentration in Indo-1-loaded FDF03-U937 cells. In contrast, aggregation of CD32/FcγRII by cross-linking of mAb IV.3, which preferentially recognizes the activating isoforms of CD32 (48, 49), induced intracellular Ca2+ mobilization. Of interest, the coaggregation of CD32 with FDF03, but not with a control mAb, resulted in strong inhibition of calcium mobilization (Fig. 8). Similar inhibition of Ca2+ flux was observed when CD32 was coaggregated with LAIR-1 (Fig. 8). Altogether, these results indicate that FDF03 can function as an inhibitory receptor in monocytic U937 cells, similarly to LAIR-1/p40.

In contrast to LAIR-1/p40, cross-linking of FDF03 does not inhibit the differentiation of monocytes into DC in response to GM-CSF

Because it has been reported that engagement of LAIR-1/p40 inhibited differentiation of monocyte into DC by interfering with GM-CSF activities (26), we wondered whether FDF03 could also
regulate monocyte differentiation. To do this, blood monocytes were cultured with GM-CSF alone or GM-CSF plus IL-4, in the presence of anti-FDF03 mAb 36H2 or anti-LAIR-1 mAb DX26, together with goat F(ab')2 anti-mouse/rat IgG used as cross-linker. As previously described (30, 50), GM-CSF rapidly down-regulated the expression of CD14 on monocytes, but induced expression of CD1a, as determined by flow cytometry at day 2 of culture (control mAb in Fig. 9A). Engagement of FDF03 by mAb 36H2 only weakly decreased CD1a expression and did not inhibit CD14 down-regulation whether the cells were cultured with GM-CSF alone (Fig. 9A, left panel) or in the presence of IL-4 (Fig. 9A, right panel). In contrast, and as expected, down-regulation of CD14 and induction of CD1a expression by GM-CSF was almost completely blocked by engagement of LAIR-1 with mAb DX26, even in the presence of IL-4 (Fig. 9A). Similar results (lack of inhibition by FDF03 but blockade by LAIR-1 cross-linking) were obtained after 6 days of culture with GM-CSF and were also observed for GM-CSF-induced up-regulation of CD1b and MHC class II molecules (data not shown). Moreover, cross-linking of FDF03 did not modify the percentage of viable cells recovered after 6 days of culture in the presence of GM-CSF with or without IL-4, while cross-linking of LAIR-1 resulted in a decrease in cell viability (Fig. 9B). Thus, engagement of LAIR-1, but not of FDF03, inhibits differentiation of monocytes into DC, most likely by interfering with the GM-CSF receptor signaling pathway (26).

**Discussion**

In this report, we have described FDF03, a novel inhibitory receptor of the Ig-SF. The FDF03 extracellular domain has no significant homology to other members of the Ig-SF that also contain a single ITIM-bearing or activation receptors such as the PD-1 molecule (51), the CMRF-35 and CMRF-35-H9 pair of molecules (52, 53),...
and the NKp44 receptor (54). It is interesting to note that these molecules are encoded by genes localized on different human chromosomes, with the PD-1 gene on chromosome 2q37 (51), the NKp44 gene on chromosome 6 (54), and the FDF03 gene on chromosome 7q22 (the present study). This contrasts with the clustered localization on human chromosome 19q13.4 of genes encoding the inhibitory receptors KIRs (55), ILTs/LIRs/MIRs (13, 16, 19, 56), LAIR-1 (25), and NKp46 (57). All of these molecules carry one or several Ig domain(s) of the C2-set and are related in sequence to the recently defined sialic acid-binding Ig-like lectins (Siglecs) that contain intracellular, this includes CD33/Siglec-3, CD22/Siglec-2, OB-BP-1/Siglec-5, and OB-BP-2/Siglec-6, which contain intracytoplasmic ITIM or ITIM-like sequences and are composed of one N-terminal V-type Ig domain followed by one or several membrane proximal Ig domain(s) of the C2 type.

We have shown that FDF03 is preferentially expressed in immune tissues and has a restricted expression in cells of the myelomonocytic lineage including monocytes, macrophages, DC, and granulocytes. Flow cytometric analysis demonstrated that FDF03 was expressed by the majority of the CD11c+ DC both in blood and tonsils, but not by the CD11c- DC precursors. This preferential expression of FDF03 was confirmed at the mRNA levels because neither FDF03 nor FDF03-ΔTM messengers could be amplified by RT-PCR in purified blood CD11c+ DC, while both cDNAs were detected in CD11c- DC (data not shown). This is in agreement with the restricted expression of FDF03 in cells of the myeloid origin, because blood and tonsil CD11c- DC precursors, corresponding to the previously so called plasmacytoid T cells or plasmacytoid monocytes, do not express myelomonocytic markers such as CD14, CD13, and CD33 (43, 44, 46, 61) and have been proposed to be of lymphoid origin. On in vitro-generated DC, FDF03 was preferentially expressed by monocyte-derived DC and by DC derived from the CD14+CD1a- precursors that display features of the interstitial/dermal-type DC, rather than by the CD11c- subsets of cells that may represent precursors of epidermal/Langerhans cells (29, 40). In keeping with this, anti-FDF03 mAb did not stain Langerhans cells in skin epithelium nor immature CD1a+ Langerhans-like DC in tonsil epithelium, and we failed to detect FDF03 mRNA in purified skin Langerhans cells (data not shown). Moreover, FDF03 expression on DC was not down-regulated by signals inducing DC maturation such as LPS or CD40 ligand, suggesting that FDF03 can be expressed by both immature and mature DC. However, immunohistochemical analysis performed on frozen tonsil sections indicated that mAb 36H2 stained neither the CD11c+ germlinal center DC in B cell follicles (34) nor the mature interdigitating DC in T cell areas (data not shown). This suggests that, in situ, these DC express very low, if any, levels of FDF03, but we cannot exclude that a low sensitivity of our immunostaining procedures may decrease FDF03 detection on tissue sections. However, FDF03 was strongly expressed by cells localized in close contact to the epithelial crypts of the tonsils, some of them expressing the CD11c marker (data not shown). We are currently isolating and characterizing the FDF03-expressing cell populations from tonsils.

The presence of ITIM-like sequences in the FDF03 cytoplasmic tail suggested that FDF03 might principally function as an inhibitory receptor of cell function and activation, as generally described for ITIM-bearing molecules (1, 3, 5, 6). This was confirmed by demonstrating that FDF03 blocked intracellular Ca2+ mobilization induced by CD32/FcγRII aggregation in FDF03-transfected U937 cells, as did the ITIM-bearing receptor LAIR-1/p40. However, while cross-linking of LAIR-1 strongly inhibited the effects of GM-CSF and IL-4 on monocyte differentiation and survival, cross-linking of FDF03 only weakly decreased expression of CD1a and did not block down-regulation of CD14, nor affected survival of monocytes. While we cannot exclude that engagement of FDF03 by mAb 36H2 was not optimal in our experimental protocols, these results suggest that FDF03 may have function(s) different to that of the broadly expressed LAIR-1/p40.

FIGURE 8. Cross-linking of FDF03 inhibits CD32/FcγRII-induced calcium mobilization in U937 cells. FDF03-transfected U937 cells were loaded with Indo-1 AM then stored at room temperature before analysis on FACStar Plus flow cytometer using an argon laser for UV excitation. For each experiment, aliquots of cells were incubated at 37°C until the baseline of violet vs blue fluorescent emissions (405/530 nm ratio) was stable. Then 10 μg of Abs to be tested were added (arrowhead in each figure) and the 405/530 nm ratio was recorded for 120 s. After a further incubation of 120 s, 20 μg/ml of cross-linker were added (goat F(ab’)2 anti-mouse/rat IgG, arrow in each figure), and the 405/530 nm fluorescence ratio was recorded for the remaining time. Data showed are representative of three experiments.
FIGURE 9. Cross-linking of FDF03 does not inhibit the effects of GM-CSF on monocytes. Blood monocytes were cultured with GM-CSF alone or GM-CSF plus IL-4, in the presence of anti-FDF03 mAb 36H2 or anti-LAIR-1 mAb DX26 or with rat or mouse control mAb, respectively. Abs (final concentration of 10 μg/ml) were added at the onset of the culture together with 10 μg/ml of goat F(ab')2 anti-mouse IgG that cross-reacts with rat IgG and that was used as cross-linker. A, On day 2, cells were collected and expression of CD1a and CD14 was determined by flow cytometry following staining with FITC-conjugated anti-CD1a or anti-CD14 mAb. Filled histograms represent CD1a or CD14 expression on cells treated with control IgG, anti-FDF03, or anti-LAIR-1 mAb. Expression of CD1a and CD14 was equivalent on cells cultured with mouse or rat control IgG, and only results with rat IgG are shown (control mAb). Unfilled histograms represent nonspecific staining obtained with a FITC-conjugated negative control mAb. B, On day 6 of culture, cell viability was determined by flow cytometry after staining with propidium iodide. Results are expressed as percentage of viable cells (propidium iodide-negative cells) and are representative of one experiment of three.

molecule (25, 26). Moreover, this discrepancy between FDF03 and LAIR-1 inhibitory activities might be explained by the stronger association of LAIR-1 with SHP-1 (as observed in U937 cells), because SHP-1 has been implicated in the down-regulation of signaling by receptors for erythropoietin, IL-3, and GM-CSF (62–64). Equally, we were unable to demonstrate that engagement of FDF03 negatively regulated functions of FDF03-expressing cells (monocytes and DC) using different assays including proliferation of CD34+ progenitor cells in response to SCF and GM-CSF or IL-3, or DC activation and maturation induced by CD40 ligand or LPS as measured by phenotypic parameters (increase expression of CD80, CD86, CD83, MHC class II, and CD40) and cytokine secretion (IL-12, IL-8, and IL-6) (data not shown). Moreover, anti-FDF03 mAb 36H2 did not affect T cell proliferation induced in allo-reactions with in vitro-derived DC (data not shown). Finally, unlike some ITIM-bearing receptors such as ILT3 and FcγRIIB (15, 22, 23), FDF03 was not internalized in monocytes and in vitro-derived DC, suggesting that FDF03 does not function as an endocytic receptor for Ag capture and presentation (data not shown).

The cytoplasmic tail of FDF03 contains three tyrosine-based motifs that, following phosphorylation, may represent binding sites for SH2 domain-containing signaling molecules (38). However, the membrane proximal tyrosine in YENI motif is not present within the cytoplasmic region of the mouse homologue of FDF03, while both human and mouse proteins display an ITIM-like tandem IVYASL-x23-TLYSVL and IVYASL-x23-TVYSIV, respectively, which are closest to the motif allowing high-affinity binding to the tandem SH2 domains of SHP-2 (65). We have shown that FDF03 was tyrosine phosphorylated in pervanadate-treated transfected U937 cells and associated with SHP-2. We have also observed association of FDF03 with SHP-1, but apparently with a weaker efficiency when compared with SHP-1 recruitment by the LAIR-1 receptor in the same cells. In contrast to FDF03, but similarly to SHP-1 recruiting inhibitory receptors such as the KIRs (3, 4), the LAIR-1 cytoplasmic tail contains two typical ITIMs in the tandem VTYAQL-x24-ITYAAV (25). Interestingly, it has been shown that the hydrophobic residue (I/V/L) at position −2 upstream of phosphorylated tyrosine in ITIM is critical for binding to and activation of SHP-1 by peptides as well as the cytoplasmic tail of the KIR (47, 66). Moreover, as recently shown for the KIR molecules (67), the N-terminal and C-terminal ITIMs may have different efficiencies to associate with phosphatases in vivo, because the N-terminal ITIM was found to be sufficient to recruit SHP-2 but not SHP-1, while both ITIMs were required to recruit SHP-1. In keeping with this, the recently described T cell transmembrane adapter protein SIT, which contains only one I/V xyYxxV ITIM among its four cytoplasmic YxxL/V tyrosine motifs, associates in vivo with SHP-2, but not with SHP-1 and SHIP (68). Thus, this suggests that the presence of a threonine (T) instead of a hydrophobic residue at position −2 of the C-terminal tyrosine-based motif of FDF03 (TLYSVL), may decrease its potential association with SHP-1 but not with SHP-2, as seen in our study. It should be noted that a similar motif (TVYSIV) is also present at the C-terminal end of mouse FDF03. Interestingly, a threonine is also present at position −2 of the second tandem tyrosine motif YxxV/L tyrosine motifs, associates in vivo with SHP-2, but not with SHP-1 and SHIP (68). However, it has not been yet demonstrated whether these molecules can recruit SHP-1 and/or SHP-2. Finally, four TxYxxV/I motifs are present in the intracellular domain of human and mouse 2B4 molecules (74, 75), and three are in the signaling lymphocytic activation molecule SLAM (76), both receptors preferentially recruiting SHP-2. In both SLAM and 2B4 molecules, these motifs also represent docking sites for the SLAM-associated protein SAP (75, 77), an SH2-containing adaptor protein that competes with SHP-2 for recruitment to SLAM and 2B4, and is defective in the inherited X-linked lymphoproliferative syndrome XLP (77–79). Thus, although SAP does not appear to be expressed in myeloid cells, it would be of interest to analyze whether FDF03 may also associate with SAP or with homologous protein such as EAT-2 that recognizes a similar TxYxxV/I motif (80).
In conclusion, FDF03 represents a novel member of the Ig-SF selectively expressed in cells of the myelomonocytic lineage, including monocytes/macrophages, granulocytes, and DC that express the CD11c marker. Tyrosine-phosphorylated FDF03 preferentially recruits SHP-2 and can function as an inhibitory receptor, at least when overexpressed in the monocytic U937 cell line. However, we cannot exclude that FDF03 may have other regulatory functions because recruitment of SHP-2 may also mediate cellular activation (81–83). Of note, by screening molecules associated with SHP-1, Mousseau et al. (84) recently identified a protein designated PIRL (for paired Ig-like receptor, accession no. AF161080) whose amino acid and cDNA sequences are identical with that of FDF03. The description of the genomic organization of PIRL/FDF03 (84) clearly confirms that FDF03–ATM and FDF-M14 are produced by alternative splicing of FDF03/PIRL cDNA. It should be noted that all the primers and probes used in our study are specific for FDF03 and cannot hybridize with the nucleotide sequence of PIRLβ (accession no. AF161081), a putative activating counterpart of PIRL for (paired Ig-like receptor, accession no. AF161080) whose amino acid and cDNA sequences are identical with that of FDF03. Of note, by screening molecules associating with such malignancies.

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