THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 283, NO. 48, pp. 33658–33664, November 28, 2008

Adsorption of Follicular Dendritic Cell-secreted Protein (FDC-SP) onto Mineral Deposits

*APPLICATION OF A NEW STABLE GENE EXPRESSION SYSTEM**

Received for publication, January 28, 2008, and in revised form, August 8, 2008 Published, JBC Papers in Press, September 19, 2008, DOI 10.1074/jbc.M800719200

Tamayuki Shinomura[‡1], **Sayaka Nakamura**[‡], **Kazuo Ito**[§], **Shin-ichi Shirasawa**[‡], **Magnus Höök**[¶], **and James H. Kimura**[‖]

From the [‡]*Department of Hard Tissue Engineering, Tokyo Medical and Dental University, Tokyo 113-8549, Japan, the* [§]*Department of Orthopaedic Surgery, Yamagata University School of Medicine, Yamagata 990-9585, Japan,* [¶]*Texas A&M Health Science Center, Institute of Biosciences and Technology, Houston, Texas 77030-3303, and the* [‖]*Department of Surgery, John A. Burns School of Medicine, University of Hawaii, Manoa, Hawaii 96822*

Follicular dendritic cell-secreted protein (FDC-SP) is a small secretory protein having structural similarities to statherin, a protein in saliva thought to play a role in calcium retention in saliva. In contrast, FDC-SP is thought to play a role in the immune system associated with germinal centers. We report here the very specific expression of FDC-SP in junctional epithelium at the gingival crevice. This region is very important for the host defense against pathogens and for periodontal protection. To be able to better understand the function of FDC-SP, we developed a novel gene expression system that exploited gene trapping and site-specific gene integration to introduce the protein into a mammalian cell culture system. Using this system we were able to express FDC-SP as a fusion protein with green fluorescent protein in an osteogenic progenitor cell line with long term stability, which we then used to find that the fusion protein specifically adsorbs onto mineral deposits and the surface of hydroxyapatite particles exogenously added to the culture. This adsorption was highly dependent on the structural integrity of FDC-SP. These results suggest that FDC-SP may play an important role, adsorbing onto the surface of cementum and alveolar bone adjacent to periodontal ligament and onto tooth surface at the gingival crevice.

FDC-SP[2] is a small secretory protein that was originally identified in primary follicular dendritic cells isolated from human tonsils (1) and was implicated in modulation of B cell activity (2). Recently, we have found that FDC-SP is expressed specifically in periodontal ligament and parotid gland and has molecular properties similar to those of statherin, a protein in saliva thought to function in preventing calcium precipitation (3). Both molecules are small in size (68 and 43 residues for mature human FDC-SP and statherin, respectively) and have a proline-rich region in their C-terminal half. In addition, these regions show a high degree of hydrophobicity in contrast to their N-terminal hydrophilic regions. Such structural similarities strongly suggested that additional roles for FDC-SP exist and that additional experiments looking for functional similarities between FDC-SP and statherin would be very informative.

Statherin is a multifunctional molecule secreted from parotid and submandibular gland (4). It has a significant affinity for calcium phosphate precipitates, including hydroxyapatite, and is present as an enamel pellicle protein within the oral cavity (5). Statherin also contains sites for the adhesion of bacteria such as *Porphyromonas gingivalis* and *Streptococcus mutans* to the tooth surface (6, 7), and these sites are thought to be a feature that the microorganisms have exploited for biofilm development (8). Therefore, it would be significant to determine whether FDC-SP has a similar activity to statherin. However, a major obstacle in the functional analysis of FDC-SP is that only a small amount of protein can be obtained from tissues such as periodontal ligament and parotid gland. To overcome this problem, the expression of a cDNA encoding FDC-SP in mammalian cells was undertaken. Many expression vectors for mammalian cells are now available, but they do not always work well in any cell type, especially for long term culture *in vitro*. The search for a good vector is time-consuming and laborious. Fortunately, we had previously developed a gene trap screening method that showed that cells expressing a reporter gene with unlimited long term stability could be easily and consistently obtained (9). We show here that we can replace the reporter gene with FDC-SP, enabling us to express it in relatively large amounts and with long term stability. We also report here the results of using this system to conduct a functional analysis of FDC-SP.

EXPERIMENTAL PROCEDURES

Tissue Preparation and in Situ Hybridization—Four-week-old mice (Crj: CD-1 (ICR), Charles River Japan, Ltd., Yokohama, Japan) were used for *in situ* hybridization analyses. All experimental procedures were approved by the Institutional Committee of Animal Care and Use at the Tokyo Medical and Dental University, and were performed in conformance with the Guidelines for Animal Experimentation at the institution. Mice were anesthetized by sodium pentobarbital (40 mg/kg).

* This work was supported by Grant-in-aid for Scientific Research 19659485 from the Ministry of Education, Science, Sports and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) AB375112, AB375113, and AB375115.

[1] To whom correspondence should be addressed: 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8549, Japan. Tel./Fax: 81-3-5803-5450; E-mail: t.shinomura.trg@tmd.ac.jp.

[2] The abbreviations used are: FDC-SP, follicular dendritic cell-secreted protein; 5′-RACE, 5′ rapid amplification of cDNA ends; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside; FRT, FLP recombination target; GFP, a green fluorescent protein; PBS, phosphate-buffered saline.

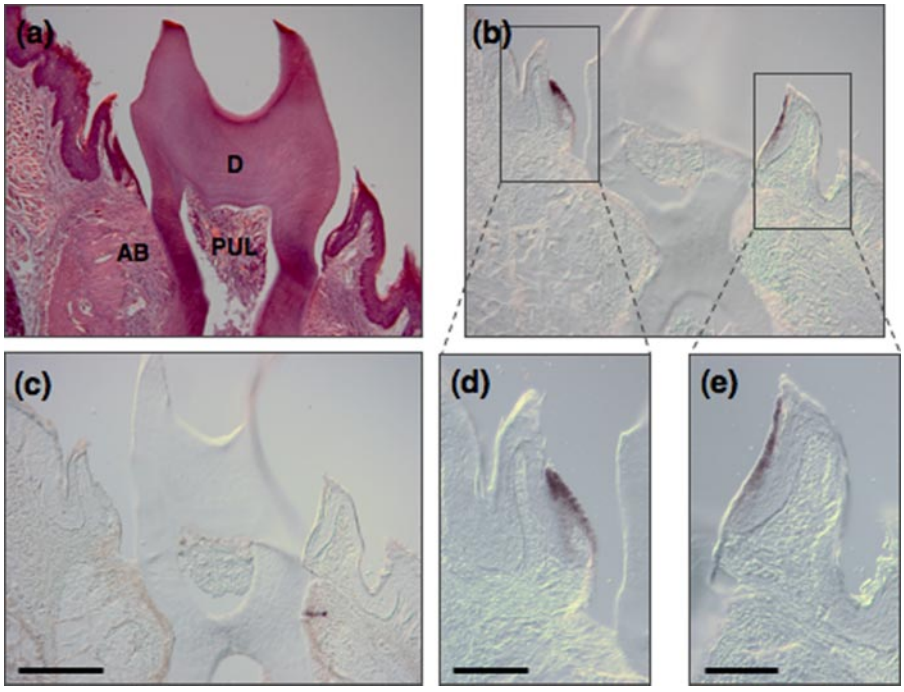

FIGURE 1. **Expression of FDC-SP in gingival epithelium.** Buccal-lingual sections of the first molar of a 4-week-old mouse mandible were stained with hematoxylin (*a*). The expression of FDC-SP mRNA in gingival epithelium was examined by *in situ* hybridization with an antisense RNA probe, and a hybridization signal was observed specifically in junctional epithelium (*b*). Higher magnification of the rectangular areas in *b* are shown in *d* and *e*. Staining with a sense probe of mouse FDC-SP gave no significant reaction (*c*). *AB*, alveolar bone; *D*, dentin; *PUL*, pulp. *Bar* = 250 μm (*c*), 100 μm (*d* and *e*).

Mandibles were dissected and fixed in 4% paraformaldehyde at 4 °C for 24 h and then decalcified, *en bloc*, in 10% EDTA, pH 7.4, at 4 °C for 2 weeks. The tissues were then equilibrated in 25% sucrose, embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan), and quick-frozen at dry ice temperature. Preparation of frozen sections and *in situ* hybridization were performed according to the method we described previously (3). Briefly, sections were treated with proteinase K, prehybridized, and then hybridized overnight at 70 °C with the specific RNA probes as follows: the purified mouse FDC-SP cDNA was cloned into pGEM-3Zf(−) (Promega Corp., Madison, WI), and then sense and antisense RNA probes were generated by *in vitro* transcription of the plasmid templates using a DIG RNA labeling kit (Roche Applied Science) according to the manufacturer's instructions. After hybridization, signals were visualized with a DIG nucleic acid detection kit (Roche Applied Science).

Cell Cultures—The cell line Kusa-A1 used in this study was an established mesenchymal stem cell line derived from the C3H/He mouse strain (10). The cells were cultured at 37 °C under 5% CO_2 in αMEM10 (α-modified minimum essential medium supplemented with penicillin (50 units/ml), streptomycin (50 μg/ml), and 10% heat-inactivated fetal calf serum) (HyClone Laboratories, Logan, UT). For induction of osteogenic differentiation, the cells were cultured in αMEM10 until confluent, and then the culture medium was replaced with αMEM10 containing 0.2 mM ascorbic acid and 5 mM β-glycerophosphate (Wako Pure Chemical, Osaka, Japan).

To verify mineral deposition, the cultures were fixed with 10% neutral buffered formalin (Wako Pure Chemical) and stained with von Kossa's method or with 1% Alizarin Red S at pH 6.4 for 5 min. Von Kossa's staining was performed as follows. Formalin-fixed cultures were rinsed with distilled water, and then incubated with an aqueous solution of 5% silver nitrate for 1 h at room temperature until they took on a dark brown color. Following a rinse with distilled water, the cultures were incubated in 5% sodium thiosulfate solution for 5 min. After staining, the cultures were rinsed with distilled water, and were then photographed.

In some experiments, we added sterilized Bio-Gel HT hydroxyapatite particles (Bio-Rad) into the culture of Kusa-A1 cells to serve as markers to allow the identification of the exact location of mineral deposits produced by the cultures by microscopy as the added particles were visible both before and after Alizarin Red staining. The particles were fixed onto the cell layer within hours.

Construction of Vectors for Trap-In Gene Expression System—A promoter trap vector prvPtrap (Fig. 2) was constructed by replacing the sequence encoding the rat CD4 transmembrane domain and a partial sequence of β-galactosidase gene of prvSStrap vector (9) with the chemically synthesized FLP recombination target sequence (FRT) and a 2.1-kb DNA fragment encoding translational start site of β-galactosidase excised with HindIII and SacI from pβgal-Basic (Clontech). After ligation, the nucleotide sequences around the junctions of each DNA fragment were confirmed by direct sequencing with the BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA).

To produce a recombination insertion vector pInSRT (Fig. 2), a 2-kb DNA fragment containing the internal ribosome entry site, the puromycin phosphotransferase gene, and the bovine growth hormone polyadenylation signal was cleaved from expression plasmid pCAGGS-FLPe (Gene Bridges GmbH, Dresden, Germany) with EcoRI and PstI. The resultant fragment was then cloned with the synthesized FRT sequence into a cloning vector pGEM3Zf(−) (Promega, Madison, WI). The sequences of the vectors prvPtrap and pInSRT have been submitted to the DDBJ under accession numbers AB375112 and AB375113, respectively.

Retrovirus Production and Infection—For the production of infectious retrovirus, prvPtrap was linearized by digestion with XmnI, and the resultant vector DNA was co-transfected into the retrovirus packaging cell line PT67 (Clontech) with the neomycin phosphotransferase expression vector pSTneoB as described previously (9). After selection with G418 (Calbiochem), retroviral producer cell lines were isolated. Virus titers were assayed on Kusa-A1 cells with hygromycin selection. In

prvPtrap vector, the fusion gene encoding β-galactosidase and hygromycin phosphotransferase (*βgyg*) does not have its own promoter; therefore, the appearance of hygromycin-resistant colonies indicated the results of the efficient trapping of endogenous promoter.

In our expression system, a single insert per cell facilitated the direct comparison of gene expressions among different genes without regard to chromosome positional effects as described in the text. To achieve this, viral infections were performed at a low multiplicity of infection to restrict transfections to single integrations as reported previously (9).

Characterization of Stable Transformants Resulting from Retrovirus Infection—To recover and identify trapped genes, rapid amplification of the 5′ end of the cDNA (5′ RACE) was performed using the 5′ RACE system (Invitrogen) as described previously (9). Briefly, total RNA was isolated from hygromycin-resistant clones grown on a 100-mm tissue culture plate using the RNeasy mini kit (Qiagen, Valencia, CA). First-strand cDNA was synthesized from the total RNA using a β-galactosidase-specific primer lacZ (R1), 5′-CGCATCGTAACCGTGCATCTGCCAGT-3′. After cDNA synthesis, PCR amplification of target cDNA was accomplished using a nested primer SSRT-1, 5′-TCCCAGTCACGACGTTGTAAAACGACGG-3′, and AAP primer (Invitrogen) first, and then using a *Drosophila melanogaster* alcohol dehydrogenase-specific primer ALDH (R1), 5′-GAGCAGCTCCTTGCTGGTGTCCAGAC-3′, and AUAP primer (Invitrogen). PCR products were then purified by agarose gel electrophoresis and were prepared for sequencing with the BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems) using ALDH (R2), 5′-GCTTTAGCAGGCTCTTTCGATCC-3′, as a primer. The reaction products were then run on an ABI Prism 310 Genetic Analyzer (Applied Biosystems).

To evaluate the promoter activity of trapped genes, hygromycin-resistant clones were stained with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) (Wako Pure Chemical), following the procedure described previously (9). Briefly, cells were rinsed twice with PBS, fixed for 15 min with 1% glutaraldehyde, and rinsed again with PBS before staining with X-gal for 3 h at 37 °C.

Expression Constructs—A cDNA encoding a green fluorescent protein (GFP) was prepared from plasmid vector pEGFP-N3 (Clontech) by digestion with BamHI and NotI. A human FDC-SP cDNA encoding the full coding region was isolated from the subtractive cDNA library of a human periodontal ligament as described previously (3). The resultant fragments were then cloned into the pInSRT vector separately for generating pInSRT-GFP and pInSRT-hFDC.SP.

For the expression of GFP and human FDC-SP fusion protein, GFP-hFDC.SP as a secretory protein, we generated a plasmid vector pInSRT-GFPhFDC.SP by ligating the following DNA fragments step by step in pInSRT vector: 1) a small fragment containing the signal sequence of human PG-M/versican was cleaved from plasmid pRc/RSV-hM(V3) (11) with SalI and Age I; 2) 0.7-kb DNA fragment encoding GFP from pEGFP-C1 (Clontech) was excised with AgeI and BsrGI; 3) the 0.24-kb DNA fragment encoding human FDC-SP without signal sequence was amplified from the cDNA described above by

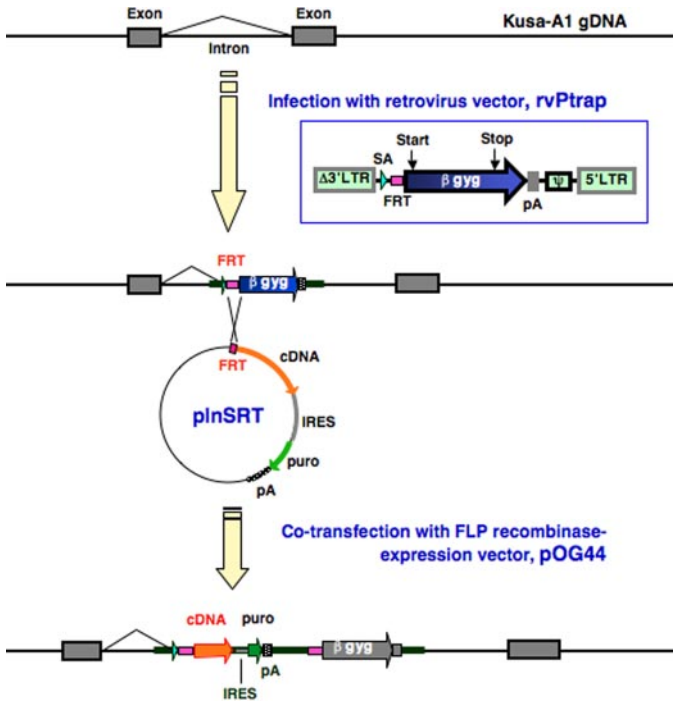

FIGURE 2. **Overview of the Trap-In system.** As a first step, the target (Kusa-A1) cell line is infected with the retroviral vector rvPtrap. After selection with hygromycin, a colony expressing the highest level of β-galactosidase is isolated, and a Trap-In host cell line is established. In the second step, a pInSRT expression vector containing the cDNA of interest is co-transfected into the host cells with an FLP recombinase expression vector pOG44. Successful homologous recombination between the FRT sequences in the genome and pInSRT expression vector conveys puromycin resistance allowing the selection of cells expressing the cDNA of interest. *SA*, splice acceptor sequence of mouse immunoglobulin; *FRT*, FLP recombinase target sequence; *βgyg*, a β-galactosidase-hygromycin phosphotransferase fusion gene; *pA*, polyadenylation signal; *Δ3′LTR*, enhancer-deleted 3′ long terminal repeat sequence; *ψ*, packaging sequence; *5′LTR*, 5′ long terminal repeat sequence; *IRES*, internal ribosome entry sites; *puro*, puromycin phosphotransferase.

PCR using specific primers 5′-CGGGATCCCCAGTCTCTCAAGACC-3′ and 5′-ACGGATCCTTCTTGTTTACTTTTC-3′. After construction of the vector, the nucleotide sequences around the junction of each DNA fragment were confirmed by direct sequencing with a BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems). The sequence of pInSRT-GFPhFDC.SP has been submitted to the DDBJ under accession number AB375115.

To identify the functional domains of human FDC-SP molecule, we have generated a single amino acid substitution construct and two different cDNA constructs with deletions within the C-terminal domain (Fig. 6*a*). The former construct was generated using GeneTailor™ site-directed mutagenesis system (Invitrogen). The latter constructs were amplified from pInSRT-GFPhFDC.SP by PCR using a common forward primer, 5′-GATCACATGGTCCTGCTGGAGTTC-3′, and two different reverse primers, 5′-GCGAATTCAAGGAAGGGGAGTTGTAGGGG-3′ and 5′-GCGAATTCAAGGGGCAGATTCAGGTATTG-3′. The amplified DNA fragments were then digested with BsrGI and EcoRI, and the resultant fragments were cloned into the vector, pInSRT-GFPhFDC.SP previously digested with BsrGI and EcoRI.

Immunoblot Analysis—Immunoblot analyses were performed with a monoclonal antibody against GFP protein

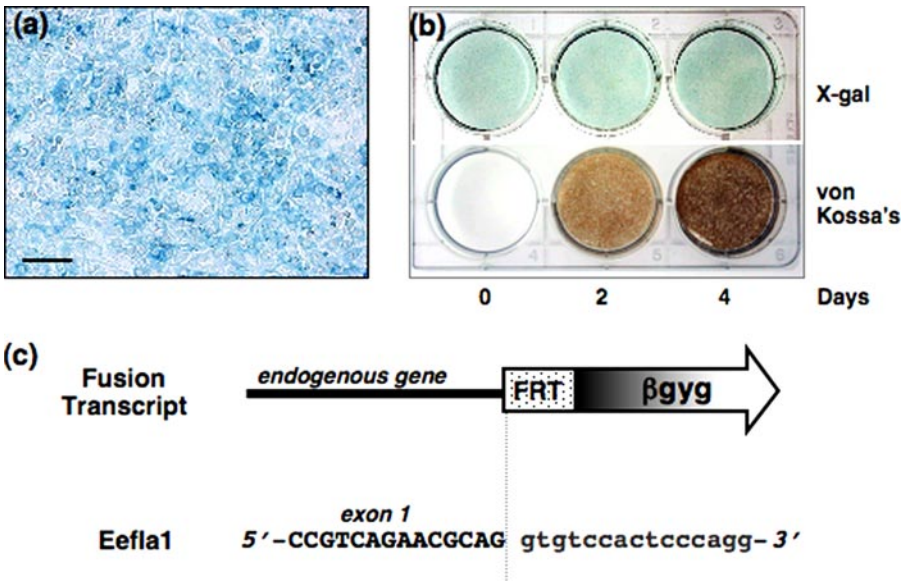

FIGURE 3. **Characterization of a Trap-In host cell line Kusa-A1(H2).** A Trap-In host cell line Kusa-A1(H2) was cultured until confluent. The staining with X-gal showed a uniform expression of β-galactosidase (*a*). Furthermore, the β-galactosidase activity in Kusa-A1(H2) is constitutively active independent of the induced mineralization detected by von Kossa's staining after the treatment with β-glycerophosphate for 0, 2, or 4 days (*b*). 5′ RACE analysis was used to obtain a partial sequence of the trapped gene. Schematic representation of the fusion transcript produced by Kusa-A1(H2) and the nucleotide sequence at the junction of the endogenous gene (*uppercase letters*) and vector DNA (*lowercase letters*) are indicated (*c*). *Eef1a1*, eukaryotic translation elongation factor 1 α1. *Bar* = 100 μm (*a*).

(Wako Pure Chemical) and with a polyclonal antibody that specifically recognized human FDC-SP. The latter antibody was raised in a rabbit after immunization with a synthetic peptide (FPVSQDQEREKRSISD) derived from the hydrophilic region of human FDC-SP (3) as described previously (12).

The Kusa-A1(H2) cells were used to express either intact GFP-hFDC.SP or each of the four human FDC-SP constructs described above. The cells were cultured in αMEM containing 10% serum until confluent. After rinsing thoroughly with PBS, the cells were then cultured in serum-free αMEM for further 24 h, and the conditioned medium was collected. The medium from each construct was then concentrated by Centricon YM-30 (Millipore) filtration, and the concentrated samples were denatured in 2% SDS, 65 mM Tris-HCl, pH 6.8, 10% glycerol, 0.13 M dithiothreitol by boiling at 100 °C for 5 min. The resultant samples were then subjected to 15% SDS-PAGE, and proteins in the gel were then blotted onto a HyBond N membrane according to the manufacturer's instructions (Amersham Biosciences). After blotting, the membrane was stained with the specific antibody described above.

RESULTS

Expression of FDC-SP in Oral Tissues—We previously reported that oral tissues such as periodontal ligament and parotid gland express the follicular dendritic cell-secreted protein FDC-SP (3). Interestingly, our subsequent *in situ* hybridization analysis revealed that FDC-SP is locally and strongly expressed in the junctional epithelium adjacent to the gingival crevice (Fig. 1). This region is very important for the attachment of gingiva to the enamel surface and as a host defense against pathogens and clinically for periodontal protection (13).

The molecular structure of FDC-SP shows some similarity to that of statherin which has a binding affinity for hydroxyapatite (14) and for bacteria (7). Therefore, FDC-SP may have the similar activities. In this study, we have tried to confirm the ability of FDC-SP to bind to mineralized surfaces. To this end, we first attempted to express the cDNA encoding FDC-SP in an osteogenic progenitor cell line, Kusa-A1, that shows extensive mineralization *in vitro* (10, 15).

Stable Gene Expression System—To obtain the stable and high level gene expression in Kusa-A1 cells, we first developed a new gene expression system combining promoter trapping and site-specific gene integration methods. The system, we named Trap-In, is outlined in Fig. 2. It is composed of two parts. One part is the establishment of Kusa-A1 sub-cell line expressing a reporter gene at high levels under the control of an endogenous promoter. For this purpose, we infected Kusa-A1 cells with a retrovirus-based promoter-trapping vector, rvPtrap. The other part is the site-specific integration of cDNA into the position of the reporter gene using FLP recombinase.

We first constructed a plasmid vector, prvPtrap, that contains a promoter-less fusion gene of the β-galactosidase and hygromycin phosphotransferase (*βgyg*) as a reporter gene. This vector was then electroporated into PT67 retrovirus packaging cells with the neomycin phosphotransferase expression vector pSTneoB. After selection with G418, virus-producing cells were cloned, and the produced retrovirus rvPtrap was harvested from the culture medium.

Establishment of a Kusa-A1 Sub-cell Line Expressing a High Level of βgyg—To achieve the stable and high level expression of FDC-SP in Kusa-A1 cells, we first established a sub-cell line expressing *βgyg* at a high level. For this, Kusa-A1 cells were infected with the rvPtrap virus. After selection with hygromycin, X-gal staining revealed the presence of various colonies that exhibited different intensities of β-galactosidase staining. We isolated a colony, Kusa-A1(H2), expressing the highest level of β-galactosidase, and we used it for further experiments (Fig. 3*a*). Rapid amplification of cDNA 5′ ends (5′ RACE) revealed a partial sequence of the trapped gene that was found to be identical to the eukaryotic translation elongation factor 1 α1 (*Eef1a1*) gene (GenBank™ accession number NM_010106) by BLAST search. To verify that the splice acceptor functioned properly in the isolated clone, the sequence obtained on 5′ RACE analysis was carefully compared with the exon-intron junction of the endogenous gene. The result showed that insertion of the vector DNA occurred in the 1st intron of *Eef1a1* gene (Fig. 3*c*). Furthermore, these data indicated that promoter trap-

ping by the rvPtrap vector had successfully occurred as we expected.

Furthermore, we verified whether Kusa-A1(H2) cells had mineralization activity and whether the reporter gene was constitutively active independent of mineralization. When the cells were cultured in normal medium (noninducing conditions), they exhibited no mineralization. However, treatment with ascorbic acid and β-glycerophosphate (inducing conditions) induced mineral deposition detected by von Kossa's staining (Fig. 3*b*) and Alizarin Red staining (data not shown). In contrast, X-gal staining indicated that the reporter gene was always active independent of culture conditions (Fig. 3*b*).

Expression of FDC-SP in Kusa-A1(H2)—Prior to the forced expression of FDC-SP, we first expressed GFP as a control. For this purpose, we cloned a cDNA encoding GFP into the plasmid vector pInSRT. The resultant plasmid, pInSRT-GFP, was then co-transfected into Kusa-A1(H2) with a plasmid vector pOG44 by electroporation. As shown in Fig. 4, bright fluorescence from intracellularly expressed GFP protein was observed for all the cells obtained by puromycin selection. In addition, it is clear that GFP expression is independent of culture conditions such as cell density and mineral deposition.

Next, we expressed FDC-SP in Kusa-A1(H2) cells. For ease in the observation of FDC-SP expression, we made the fusion construct of GFP and human FDC-SP as a secretory protein, and we cloned it into the pInSRT vector. The expression of the fusion protein, GFP-hFDC.SP, in Kusa-A1(H2) was performed as described above. After selection with puromycin, many colonies appeared, but no cell was positive for GFP fluorescence in noninducing conditions. However, hydroxyapatite particles added to the culture as positional markers took on GFP fluorescence (Fig. 5*a*). The accumulation of fluorescent material was more clearly observed when the cells were cultured under inducing conditions (Fig. 5*d*). These observations clearly indicated that GFP-hFDC.SP is

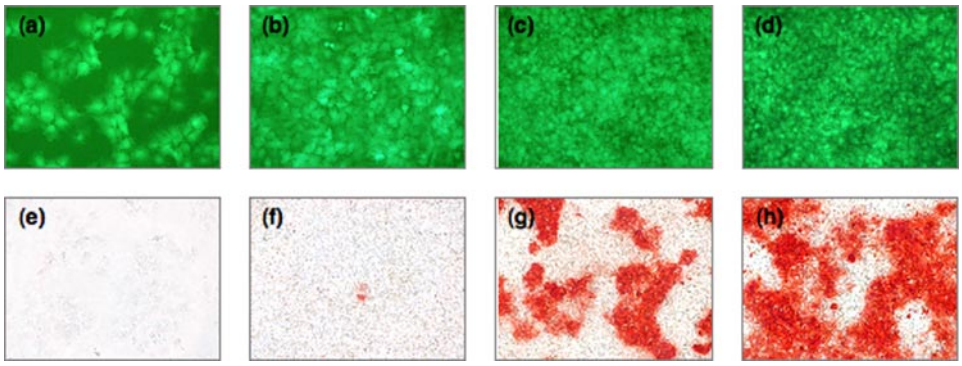

FIGURE 4. **Constitutive intracellular expression of GFP in Kusa-A1(H2).** GFP expression vector pInSRT-GFP was co-transfected to Kusa-A1(H2) with FLP recombinase expression vector pOG44. After selection with puromycin, resultant stable transformants were seeded at low cell density and were cultured in αMEM10 with replacement by fresh medium every other day. For 24 h preceding an observation time point, medium from each culture was replaced with αMEM10 containing 0.2 mM ascorbic acid and 5 mM β-glycerophosphate, and then GFP fluorescence (*a*–*d*) and mineral deposition detected by Alizarin Red staining (*e*–*h*) were examined at day 2 (*a* and *e*), day 4 (*b* and *f*), day 6 (*c* and *g*), and day 8 (*d* and *h*).

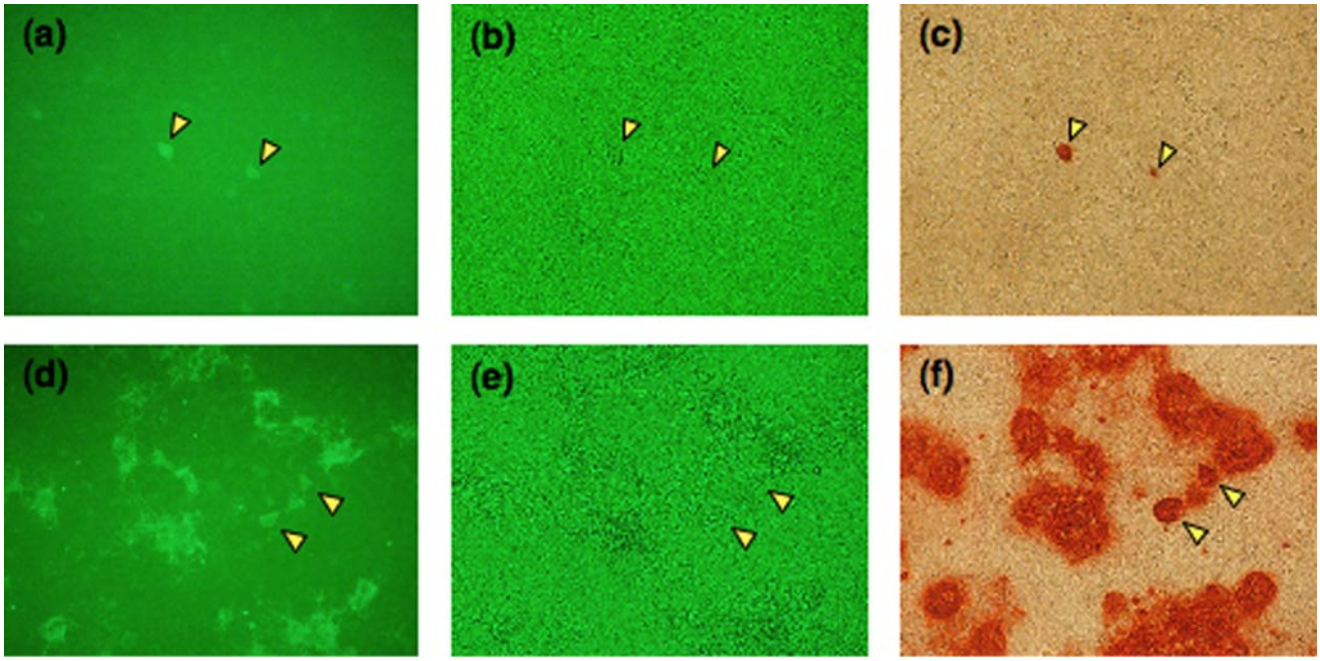

FIGURE 5. **Adsorption of GFP-hFDC.SP onto calcium deposits and the surfaces of hydroxyapatite.** Kusa-A1(H2) cells expressing secretory GFP-hFDC.SP were cultured in αMEM10 until confluent, and then hydroxyapatite particles were added into the culture to serve as positional markers. After 24 h, the medium was changed with the fresh αMEM10 (*a*–*c*) or with the αMEM10 containing 0.2 mM ascorbic acid and 5 mM β-glycerophosphate (*d*–*f*). The cells were cultured for further 4 days, and GFP fluorescence was observed first (*a* and *d*), and then Alizarin Red staining was performed (*c* and *f*). Bright field images showing cellular conditions and hydroxyapatite markers are shown in *b* and *e*. GFP fluorescence and Alizarin Red staining can be seen to overlap when hydroxyapatite positional markers (*arrowheads*) are used to register the separate images.

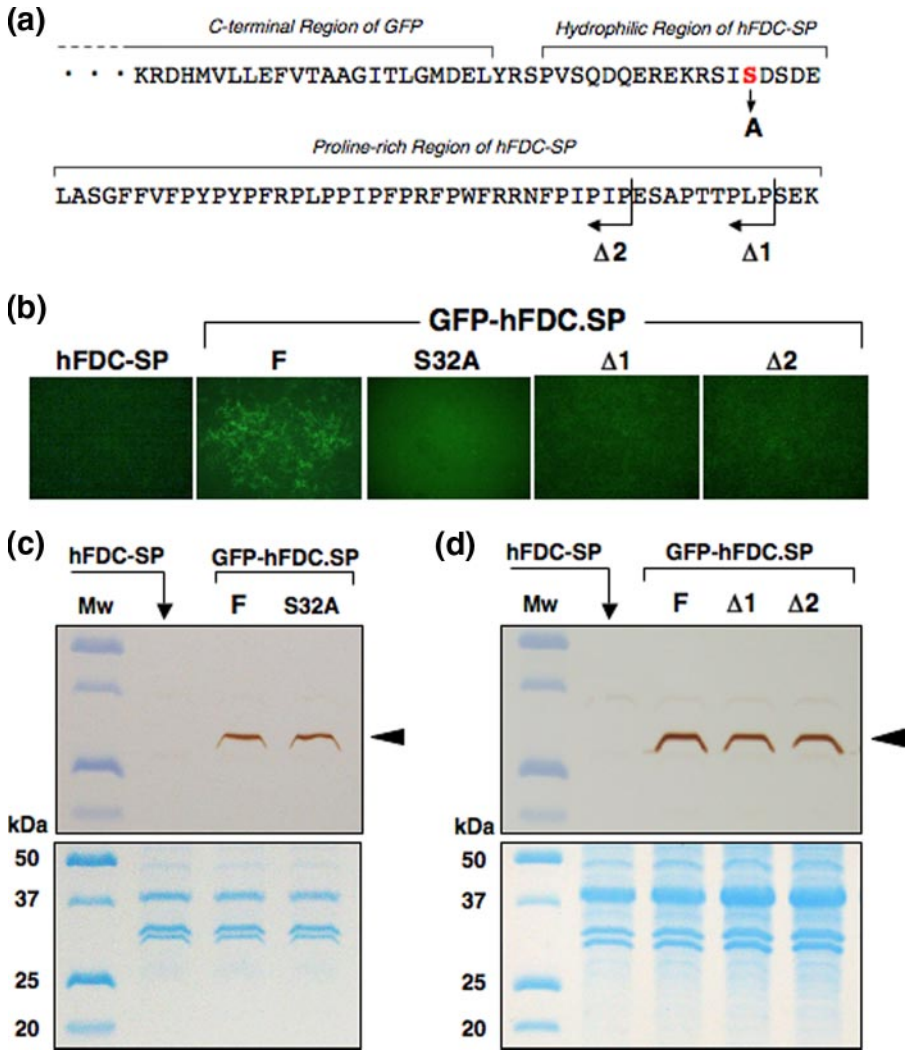

FIGURE 6. **Partial amino acid sequence of the mutated constructs of GFP-hFDC.SP and their expression.** To confirm the specific binding of FDC-SP to mineral deposits, alanine was substituted for serine 32 (S32A), which is a potential casein kinase 2 phosphorylation site (3), indicated as a red-colored *S*, and C-terminal deletion constructs (Δ1 and Δ2) of the original GFP-hFDC.SP were synthesized (*a*), and they were expressed in Kusa-A1(H2) cells. Fluorescent images were recorded for cells containing hFDC-SP (no GFP) and for cells containing GFP-hFDC.SP (*F*) and its mutations (*S32A,* Δ*1,* Δ*2*) (*b*). The expression of the full-length GFP-hFDC.SP (indicated as *F*) resulted in the accumulation of GFP-fluorescence in the culture (*b*). However, a single amino acid substitution (S32A) and both deletion constructs (Δ*1* and Δ*2*) showed no fluorescence (*b*), although they were clearly detected in the culture medium by immunoblotting with anti-GFP monoclonal antibody (*upper panel* of *c*) and with anti-human FDC-SP antibody (*upper panel* of *d*). The intact hFDC-SP, without fused GFP, showed no fluorescence (*b*) and was too small to be detected on this Western blot analysis (*d*). *Arrowhead* indicates the position of GFP-hFDC.SP. Identical protein expression in all samples was confirmed by Coomassie Blue staining (*lower panel* of *c* and *d*). The lane marked *Mw* contained colored protein molecular weight markers (Precision Plus Protein Standards; Bio-Rad).

secreted extracellularly and has binding activity to mineral deposits.

To confirm that GFP-hFDC.SP adsorption onto mineral deposits was dependent on and specific to the structure of FDC-SP, we expressed a single amino acid substitution construct and C-terminal deletion constructs as shown in Fig. 6*a*. Interestingly, all the mutated constructs caused the disappearance of the accumulation fluorescent material (Fig. 6*b*) even though the expression and secretion of the fusion proteins were confirmed by immunoblotting (Fig. 6, *c* and *d*). Incidentally, the intact human FDC-SP molecule was not detected in this experiment because its molecular size was too small (7,965 Da) to be retained by a Centricon YM-30 membrane during the required concentration of the culture medium. The differences in molecular size among GFP-hFDC.SP and its deletion constructs were too small to be detected in the immunoblot analysis.

DISCUSSION

FDC-SP is a small secretory protein that is expressed in oral and in glandular tissues (3). Although Marshall *et al.* (1) reported that FDC-SP showed significant binding to B cells, its physiological function in oral tissues is not known. In this study, we investigated the binding capacity of FDC-SP to mineral based on its structural similarity to statherin, which is tightly adsorbed to hydroxyapatite (5). To this end, we first developed a new cDNA expression system named Trap-In. This system introduced a promoter-free construct that contained a specific recombination site for the introduction of any additional cDNA into a host cell. Therefore, all resulting cDNA expression for, in this case, FDC-SP was totally dependent on the activity of the endogenous promoter within the host cell. The introduced cDNA will also experience the same positional chromosomal effects on expression as the host gene. As a result, we can compare the expression of various cDNA constructs without regard for positional effects as long as we use the same cell line. Our expression system is quite new and will have a broad range of applications, although retroviral infection of target cells is a minimum requirement. We have already verified that this system worked at least in mouse C3H10T1/2 cells, mouse ATDC5 cells (16), and rat chondrosarcoma cells (17) (data not shown).

To obtain the final Trap-In system, we first established a cell line expressing a *βgeo* reporter gene in the cell being studied. Then we were able to easily replace the reporter gene with any cDNA up to at least 9 kb with the cell line (data not shown). In this study, we used a murine osteogenic progenitor cell line, Kusa-A1, for the expression of human FDC-SP. This cell line, established from femoral bone marrow stromal cells (10), shows mineralization *in vitro* (15). However, Alizarin Red staining observed in the culture was not completely stable in PBS.

Repetitive washing with PBS easily removed the dye from the culture even though the culture was fixed with formalin or ethanol. These observations suggested that deposits of FDC-SP on mineral observed in the culture might be not stable. This is the reason why we used a GFP fusion protein in this study. It was easy to observe the distribution of the GFP-FDC.SP fusion protein in living cultures without any staining procedures. Of course, we have tried to express the intact human FDC-SP molecule, but we could not detect it by immunofluorescent staining using a specific antibody (data not shown). This observation might suggest that FDC-SP bound calcium as Alizarin Red S could chelate it.

As shown in Fig. 5, GFP-hFDC.SP can bind to mineral deposits and hydroxyapatite surfaces. These data strongly suggested that FDC-SP synthesized by periodontal ligament cells would bind to the surface of alveolar bone and cementum *in vivo*. In addition, FDC-SP that is expected to be present in gingival crevicular fluid from junctional epithelium would coat the enamel surfaces of teeth. These adsorptions onto mineral and hydroxyapatite surfaces seem to be dependent on the structural integrity of FDC-SP. The experiments using an amino acid substitution construct and C-terminal deletion constructs (Fig. 6) clearly indicated that the intact structure of FDC-SP was important for the adsorption described above. A similar structural requirement for adsorption onto hydroxyapatite and mineralized surfaces has been reported for statherin (4, 14). Further study is needed to determine the three-dimensional structure required for the binding of FDC-SP to mineral.

Rowe *et al.* (18) reported that statherin has the acidic serine-aspartate-rich motif (ASARM peptide) that is responsible for maintaining the phosphate/calcium supersaturated mineralization solution dynamics of saliva. Although the motif is not present in the mature FDC-SP, it shows some structural similarity to statherin as described above. Therefore, these two molecules might have some functional redundancy *in vivo* at least in humans who make both. This is not true in the mouse, however, because although the mouse has the FDC-SP gene (3), the statherin gene is present only as a pseudogene (19). A gene knock-out of FDC-SP in mouse would be very informative in understanding the biological function of FDC-SP.

Expression of FDC-SP was observed at a high level in the junctional epithelium in addition to other structures such as tonsils, prostate gland, lymph nodes, and trachea, which all play a role in host defense (1, 3). In addition, the proline-rich region in the C-terminal domain of FDC-SP has some structural similarity to an antimicrobial peptide Bac5 (20). It is therefore reasonable to suggest that FDC-SP may play an important role as part of a local defense mechanism against infection in the oral cavity. In any case, further study will be necessary to understand the relationship between the specific expression of FDC-SP in junctional epithelium at the gingival crevice and periodontal disease, and the physiological significance of FDC-SP expression in the periodontal ligament and in other nonmineralized tissues.

Acknowledgments—We are grateful to Dr. M. Yanagishita (Department of Hard Tissue Engineering, Tokyo Medical and Dental University), Dr. T. Hayakawa (Kitatushima Hospital), and Dr. T. Yokochi (Dept. of Microbiology and Immunology, Aichi Medical University) for their continuous encouragement.

REFERENCES

1. Marshall, A. J., Du, Q., Draves, K. E., Shikishima, Y., HayGlass, K. T., and Clark, E. A. (2002) *J. Immunol.* **169,** 2381–2389
2. Al-Alwan, M., Du, Q., Hou, S., Nashed, B., Fan, Y., Yang, X., and Marshall, A. J. (2007) *J. Immunol.* **178,** 7859–7867
3. Nakamura, S., Terashima, T., Yoshida, T., Iseki, S., Takano, Y., Ishikawa, I., and Shinomura, T. (2005) *Biochem. Biophys. Res. Commun.* **338,** 1197–1203
4. Schenkels, L. C., Veerman, E. C., and Nieuw Amerongen, A. V. (1995) *Crit. Rev. Oral Biol. Med.* **6,** 161–175
5. Raj, P. A., Johnsson, M., Levine, M. J., and Nancollas, G. H. (1992) *J. Biol. Chem.* **267,** 5968–5976
6. Amano, A., Kataoka, K., Raj, P. A., Genco, R. J., and Shizukuishi, S. (1996) *Infect. Immun.* **64,** 4249–4254
7. Dodds, M. W. J., Johnson, D. A., and Yeh, C. (2005) *J. Dent.* **33,** 223–233
8. Niemi, L. D., and Johansson, L. (2004) *Infect. Immun.* **72,** 782–787
9. Shirasawa, S., Yoshimi, M., Kamochi, H., Muneta, T., Kimura, J. H., Höök, M., and Shinomura, T. (2005) *J. Biochem.* (*Tokyo*) **137,** 79–85
10. Umezawa, A., Maruyama, T., Segawa, K., Shadduck, R. K., Waheed, A., and Hata, J. (1992) *J. Cell. Physiol.* **151,** 197–205
11. Isogai, Z., Shinomura, T., Yamakawa, N., Takeuchi, J., Tsuji, T., Heinegård, D., and Kimata, K. (1996) *Cancer Res.* **56,** 3902–3908
12. Shinomura, T., Nishida, Y., Ito, K., and Kimata, K. (1993) *J. Biol. Chem.* **268,** 14461–14469
13. Schroeder, H. E., and Listgarten, M. A. (1997) *Periodontol. 2000* **13,** 91–120
14. Goobes, G., Goobes, R., Schueler-Furman, O., Baker, D., Stayton, P., and Drobny, G. P. (2006) *Proc. Natl. Acad. Sci. U. S. A.* **103,** 16083–16088
15. Kawashima, N., Shindo, K., Sakamoto, K., Kondo, H., Umezawa, A., Kasugai, M., Perbal, B., Suda, H., Takagi, M., and Katsube, K. (2005) *J. Bone Miner. Metab.* **23,** 123–133
16. Shinomura, T., Ito, K., Kimura, J. H., and Höök, M. (2006) *Biochem. Biophys. Res. Commun.* **341,** 167–174
17. King, K. B., and Kimura, J. H. (2003) *J. Cell. Biochem.* **89,** 992–1004
18. Rowe, P. S. N., Kumagai, Y., Gutierrez, G., Garrett, I. R., Blacher, R., Rosen, D., Cundy, J., Navvab, S., Chen, D., Drezner, M. K., Quarles, L. D., and Mundy, G. R. (2004) *Bone* (*Elmsford*) **34,** 303–319
19. Kawasaki, K., and Weiss, K. M. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 4060–4065
20. Brogden, K. A., Ackermann, M., McCray, P. B., Jr., and Tack, B. F. (2003) *Int. J. Antimicrob. Agents* **22,** 465–478