J. Biochem. **139**, 981–988 (2006)
doi:10.1093/jb/mvj117

Superoxide Production in Human Neutrophils Is Enhanced by Treatment with Transmembrane Peptides Derived from Human Formyl Peptide Receptor

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Received December 3, 2005; accepted March 26, 2006

Formyl peptide receptor (FPR) mediates a number of important host defense functions. Although studies have been performed on the ligand binding site of FPR, FPR dynamic behavior such as receptor dimerization on the cell surface remains unknown. Recently, peptides derived from the transmembrane (TM) domains of GPCRs were shown to disrupt dimer formation by receptors and to result in specific regulation of receptor function. To reveal the function of FPR TM domains, hFPRTM peptides derived from FPR were synthesized, and their biological activities were evaluated with human neutrophils. Synthetic peptides did not exhibit agonistic or antagonistic activity toward superoxide anion production. However, Neutrophils treated with hFPRTM4 produced 4-fold superoxide anion compared with untreated cells when stimulated with FPR agonist fMLP. Short peptide fragments derived from the fourth TM region of FPR did not enhance superoxide anion production, which suggests that hFPRTM4 did not behave as a ligand. CD and fluorescence spectra suggested that hFPRTM peptides were inserted into the membrane. The addition of hFPRTM4 increased the intracellular calcium concentration, which meant the peptide activated some membrane protein on the cell surface. The present study suggests that the fourth TM domain of FPR has a function related to a priming effect.

Key words: fMLP, formyl peptide receptor, human neutrophil, priming, transmembrane peptides.

Abbreviations: DIEA, *N*-ethyldiisopropylamine; DMF, *N,N*-dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; EDT, 1, 2-ethanedithiol; EGTA, *O,O′*-bis(2-aminoethyl)ethyleneglycol-*N,N,N′,N′*-tetraacetic acid; FPR, formyl peptide receptor; Fura-2 AM, 1-[6-amino-2-(5-carboxyl-2-oxazolyl)-5-benzofuranyl-oxy]-2-(2-amino-5-methyl-phenoxy)ethane-*N,N,N′,N′*-tetraacetic acid pentaacetoxymethyl ester; GM-CSF, granulocyte-macrophage colony-stimulating factor; HBTU, *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; hFPRTM, transmembrane domain of human formyl peptide receptor; HOBt, *N*-hydroxybenzotriazole; IL-8, interleukin-8; LPS, lipopolysaccharide; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; NMM, *N*-methylmorpholine; NMP, 1-methyl-2-pyrrolidone; PAF, plate-activating factor; PAL-PEG-PS, 5-(4-aminomethyl-3,5-dimethoxyphenoxy)valeric acid)-poly(ethylene glycol)-polystyrene; PBS, phosphate buffer saline; PMN, polymorphonuclear neutrophils; RP-HPLC, reverse phase high-performance liquid chromatography; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; TFE, 2, 2, 2-trifluoroethanol; TIS, triisopropylsilane; TM, transmembrane; TNF-α, tumor necrosis factor-α.

Neutrophils act as the first line of defense against invasion by microorganisms of the body. Bacterial metabolites such as *N*-formylmethionyl peptides are chemoattractants for neutrophils and bind to specific surface receptors, *e.g.*, *N*-formyl peptide receptor (FPR), that trigger specific host defensive processes such as chemotactic migration and killing of microorganisms through superoxide and phagocytosis (reviewed in Ref. *1*).

Human FPR was first defined biochemically in 1976 as a high-affinity binding site on the surface of neutrophils for a prototypic *N*-formyl peptide, formyl-Met-Leu-Phe-OH (fMLP). Boulay *et al.* then cloned it from a differentiated HL-60 myeloid leukemia-cell cDNA library in 1990 (*2*). In transfected cell lines, FPR binds fMLP with high affinity ($K_d < 1$ nM), and is activated by pico molar to low nano molar concentrations of fMLP in both chemotaxis and intracellular calcium ion mobilization assays. Two additional human genes, designated as *FPRL1* (FPR-like 1) and *FPRL2* (FPR-like 2), were subsequently isolated using FPR cDNA as a probe (*3*, *4*). These FPR family members belong to the large family of G protein–coupled receptors (GPCRs) that are predicted to have a tightly packed transmembrane (TM) core consisting of seven membrane-spanning α-helical domains (*1*). Both FPR and FPR-like 1 are expressed at high levels on human neutrophils. The ligand binding domains of FPR have been extensively analyzed by means of receptor chimera and site-specific mutation approaches (*5–9*). Recently,

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Mills *et al.* reported that the most likely positioning of fMLP in the binding pocket of FPR is approximately parallel to the fifth TM helix with the formamide group of fMLP hydrogen bonded to both Asp106 and Arg201, the Leu side chain of fMLP pointing toward the second TM region, and the carbonyl group of fMLP ion-paired with Arg205 (*10*).

Cell surface receptors are known to function as dimers or even higher oligomers. In signaling by receptor tyrosine kinases, for example, dimeric receptors are essential for their function, as each monomer phosphorylates the other monomer during the ligand-stimulated "autophosphorylation" that leads to the fully activated state of the kinase (*11*). Recent biochemical and biophysical studies have revealed that GPCRs can exist as homo- and/or hetero-dimmer/oligomers (reviewed in Ref. *12*).

Peptide sequences corresponding to specific regions of large proteins have been successfully studied to elucidate the functions of these regions. The use of such a peptide "probe" has enabled the mapping of the functional domains of certain proteins and the identification of domains that promote interactions with other proteins. For instance, synthetic peptides corresponding to intracellular regions of FPR were used to identify sites that interact with G protein (*13–15*). Recently, peptides derived from the TM domains of GPCRs were found to disrupt dimers of receptors and to result in specific regulation of receptor function (*16–19*). In the present study, TM peptides derived from human FPR were synthesized in order to evaluate their influence on the biological activities of human neutrophils on interaction between membrane proteins and TM peptides.

EXPERIMENTAL PROCEDURES

Materials—All Fmoc-protected amino acids and HBTU were purchased from Novabiochem (Tokyo, Japan). The peptide amide linker, PAL-PEG-PS resin, was purchased from Applied Biosystems (Foster City, CA, USA). HOBt, TFA and DIEA were purchased from Peptide Institute Inc. (Osaka, Japan). Piperidine, DMF, NMP, NMM, SDS, standard amino acid mixture (type H), and phenyl isothiocyanate were purchased from Wako Pure Chemicals Industries (Tokyo, Japan). EDT, thioanisole, TIS, and phenol were obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan). TFE was purchased from Nakarai Tesque (Kyoto, Japan). Acetonitrile and TFA for RP-HPLC analysis and purification were purchased from Wako Pure Chemicals Industries (Tokyo, Japan) and Peptide Institute Inc. (Osaka, Japan), respectively. Cytochrome c was obtained from Sigma (St. Louis, MO, USA). Fura-2 AM was a product of Dojindo Laboratories (Kumamoto, Japan). EGTA was purchased from Fluka Chemie AG (Buchs, Switzerland). Ficoll/Hypaque and dextran were obtained from Pharmacia AB (Uppsala, Sweden).

Peptide Synthesis—TM domain peptides were prepared by solid-phase peptide synthesis performed with an Applied Biosystem 431A peptide synthesizer. Peptides were assembled on the PAL-PEG-PS resin (0.18 mmol/g substitution) to produce an amidated C-terminal using the Fmoc synthetic strategy. The coupling reaction was carried out by means of HBTU-HOBt method using a 20-fold molar excess of Fmoc amino acid. Acetylation of the N-terminus was performed with acetic acid anhydride and NMM. Cleavage of the peptide from the resin was achieved with TFA/water/phenol/thioanisol/EDT/TIS (82.5:5.0:5.0:5.0:2.5:1.0, v/v) for 3 h at room temperature. After removing the resin by filtration, the filtrate was concentrated by N_2 gas flashing, and crude peptides were purified by preparative HPLC on a Zorbax 300SR-C3 (9.4 × 250 mm i.d.) or Wakosil5C4-200 (10.0 × 250 mm i.d.) column using the following systems: (i) water/acetonitrile/TFA (95:5:0.05, v/v; solution A); water/acetonitrile/TFA (5:95:0.04, v/v; solution B) for hFPRTM5 and hFPRTM7, LAKKVIIGPW-NH_2 (S4-1), GPWVMALLLT-NH_2 (S4-2), and ALLLTLPVII-NH_2 (S4-3), (ii) water/formic acid (2:3, v/v; solution A); 2-propanol/formic acid (2:3, v/v; solution B) for hFPRTM2, hFPRTM 3, hFPRTM 4 and hFPRTM 6, or (iii) water/formic acid (2:3, v/v; solution A); 2-propanol/formic acid (1:4, v/v; solution B) for hFPRTM1 (*20*, *21*). The purity of the peptides was confirmed by analytical HPLC on a Vaydac C4 (4.0 × 250 mm i.d.) or Wakosil 5C4-200 (4.0 × 150 mm i.d.) column using the same solvent systems as described above. The linear gradient comprised 0–100% B in 30 min at a flow rate of 0.5 ml min^{-1}. HPLC-purified peptides were characterized by amino acid analysis and MALDI-TOF MS (Voyager-DERP Biospectrometry Workstation mass spectrometer, PerSeptive Biosystems Inc., Framingham, MA) with α-cyano-4-hydroxycinnamic acid (Aldrich Chem. Co, Milwaukee, WI) as the matrix. Amino acid analysis was performed on a Pico Tag Workstation (Waters, Milford, MA) after hydrolysis in constant-boiling hydrochloric acid at 110°C for 24 h. TM domain peptides were dissolved in 20% TFE (1×10^{-2} M) and diluted with PBS. The plastic tubes and transfer tips used in assays were siliconized with dimethyldichlorosilane (Pierce Chemical, Rockford, IL, USA) to prevent non-specific adsorption. Stock solutions of TM peptides were prepared as 1 mM solutions in 20% TFE/water. The peptide concentrations in stock solution were determined on the basis of the quantitative amino acid analysis data.

Fluorescence Spectroscopy—Fluorescence measurements were performed on a Jasco FP-750 spectrofluorometer (Tokyo, Japan) using 10 × 10 mm quartz cuvettes. The excitation and emission slit widths were 5 nm. An excitation wavelength of 260 nm was used for emission spectra that were recorded from 300 to 480 nm. The peptide concentration was maintained constant at 1 μM. Samples containing hFPRTM peptides were incubated for 20 min in phosphate buffer or a 20 mM SDS micelle solution at room temperature before the measurements.

CD Measurement—The CD spectra were recorded on a JASCO J-720 spectropolarimeter with a cylindrical cell of 2 mm path length at room temperature. The CD cell was washed with an aqueous NaOH solution before the each determinations to remove any peptide adhering to the inner surface. Sample solutions (peptide concentration, 10 μM) were prepared 20 min before measurements. All spectra were the averages of eight repeats obtained by collecting data from 260 to 190 nm at 0.2 nm intervals, with a response time of 1 sec for each point. The results were expressed as the mean residue ellipticity.

Isolation of Human Neutrophils—Human neutrophils were isolated from heparinized venous blood obtained from healthy volunteers. Standard isolation techniques involving Ficoll/Hypaque gradients were used, followed

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by dextran sedimentation and hypotonic lysis to remove erythrocytes. Cells were suspended in PBS.

Superoxide Anion Production—Superoxide anion production was measured as superoxide dismutase inhibitable reduction of ferricytochrom *c* (*22*). The reduction of the ferricytochrome c concentration was monitored with a Shimadzu UV-3000 dual-wavelength spectrophotometer (Kyoto, Japan) at 540–550 nm. A cell suspension (1×10^6 cells/ml), containing 1 mM $CaCl_2$, 5 mM D-glucose, and 100 μM ferricytochrome *c*, was incubated at 37°C for 20 min with various concentrations of TM domain peptides, followed by stimulation with fMLP 10^{-7} M. The superoxide anion release was calculated based on a molar absorption coefficient of 19.1×10^3 $M^{-1}\cdot cm^{-1}$. Data are reported as mean values for at least three independent experiments.

Intracellular Calcium Mobilization—Intracellular calcium mobilization was measured by the Fura-2 method (*23*). Cells (2×10^6 cells/ml) were incubated with 4 μM Fura-2 AM at 37°C for 30 min, washed twice with PBS, and then suspended in PBS containing 1 mM $CaCl_2$ and 5 mM D-glucose. Changes in the fluorescence emission intensity at 490 nm (excitations at 340 and 380 nm) were monitored with a Shimadzu RF-5000 spectrofluorophotometer.

RESULTS

Design and Synthesis—The TM peptide sequences of human FPR were determined based on the report by Miettinen *et al.* of mapping of the ligand-binding sites in TM regions of human FPR (*9*). The structures of synthetic peptides derived from human FPR are shown in Table 1. A high polar tag SKSKSK sequence was attached to the N- or C-terminal of TM peptides to improve the solubility and to control the directionality of insertion into the lipid membrane (*24*). To increase the propensity for helices, TM domain peptides were acetylated at the N-terminal or amidated at the C-terminal. In hFPRTM2, hFPRTM6 and hFPRTM7, Cys residues in TM sequences were substituted with Ser residues to prevent cross-linking through disulfide bonds since Ser side chains are capable of hydrogen bonding interactions (*25*). A Trp residue was inserted in the N-terminus of hFPRTM2, and the C-termini of hFPRTM3, hFPRTM5 and hFPRTM7 to monitor the absorbance at 280 nm on HPLC analysis and to evaluate the insertion of hFPRTM peptides into micelles on fluorescence spectroscopy. Based on experience with the synthesis of TM peptides and amphiphilic peptides containing highly hindered amino acids (*26–30*), the designed TM peptides were synthesized on PAL-PEG-PS resin by use of Fmoc chemistry. PAL-PEG-PS resin is known to reduce hydrophobic peptide interactions on the resin, and thus is used to overcome synthetic difficulties. The purification of synthesized TM peptides except for hFPRTM5 and hFPRTM7 was carried out by means of preparative RP-HPLC using a water/2-propanol/formic acid system. The homogeneity and structures of the synthetic peptides were verified by analytical RH-HPLC and MALDI-TOF MS.

Table 1. **The structures of synthetic peptides derived from the transmembrane domain of human formyl peptide receptor.**

Peptides	Sequences[a]
hFPRTM1	SKSKSKIITYLVFAVTFVLGVLGNGLVIWVA-NH_2
hFPRTM2	Ac-TISYLNLAVADF<u>S</u>FTSTLPFFSKSKSK-NH_2
hFPRTM3	SKSKSKFLFTIVDINLFGSVFLIALIALDW-NH_2
hFPRTM4	Ac-LAKKVIIGPWVMALLLTLPVIIRSKSKSK-H_2
hFPRTM5	SKSKSKRFIIGFSAPMSIVAVSYGLIATKW-NH_2
hFPRTM6	Ac-LSFVAAAFFL<u>S</u>WSPYQVVALIATVRSKSKSK-H_2
hFPRTM7	SKSKSKVTSALAFFNS<u>S</u>LNPMLYVFMGQW-H_2

[a]The residues that differ from the native human FPR are underlined: in hFPRTM2, 6 and 7, Cys residues are substituted by Ser residues. High polar tag SKSKSK was added to the N/C-termini of odd/even-numbered TM domains of human FPR, respectively.

SUPEROXIDE ANION PRODUCTION

The biological activities of synthetic peptides were evaluated in human neutrophils. None of the TM peptides exhibited agonistic activity toward superoxide anion production in human neutrophils (Fig. 1). To evaluate antagonistic activity, neutrophils were stimulated with fMLP 10^{-7} M after incubation with hFPRTM peptides at 10^{-6} M. Interestingly, neutrophils treated with hFPRTM4, hFPRTM5 and hFPRTM6 produced 2–4 fold superoxide anion compared with untreated cells (Figs. 1 and 2A). In addition, hFPRTM4 enhanced the superoxide production in a dose-dependent manner (Fig. 2A). Enhancement of superoxide anion production in human neutrophils was caused by prior exposure to agents such as cytokines, hormones, and growth factors (reviewed in Refs. *31* and *32*). Thus, the possibility arose that hTPRTM4 acts as a ligand for some receptor. This possibility was checked using short peptide fragments that were composed of the fourth TM region of human FPR: LAKKVIIGPW-NH_2 (S4-1), GPWVMALLLT-NH_2 (S4-2), and ALLLTLPVII-NH_2 (S4-3). These short peptides contain some amino acid residues that overlap with each short peptide. Figure 2B shows the effects of short peptides on enhancement of

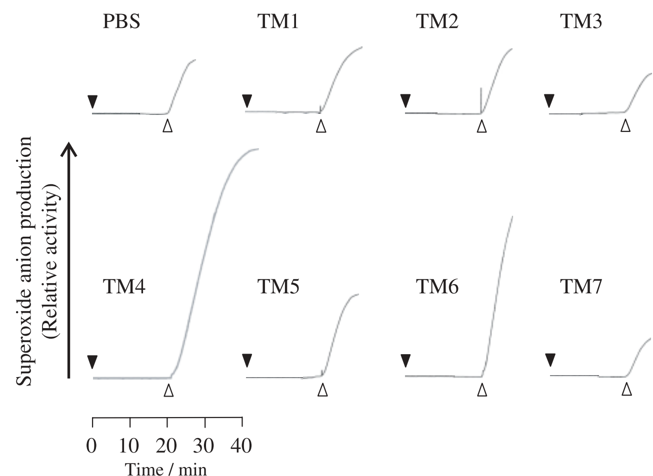

Fig. 1. **Pre-reatment experiments on hFPRTM peptides for fMLP-nduced superoxide anion production.** Cells were pretreated with PBS or hFPRTM peptides 10^{-6} M (closed arrowheads) for 20 min before fMLP 10^{-7} stimulation (open arrowheads).

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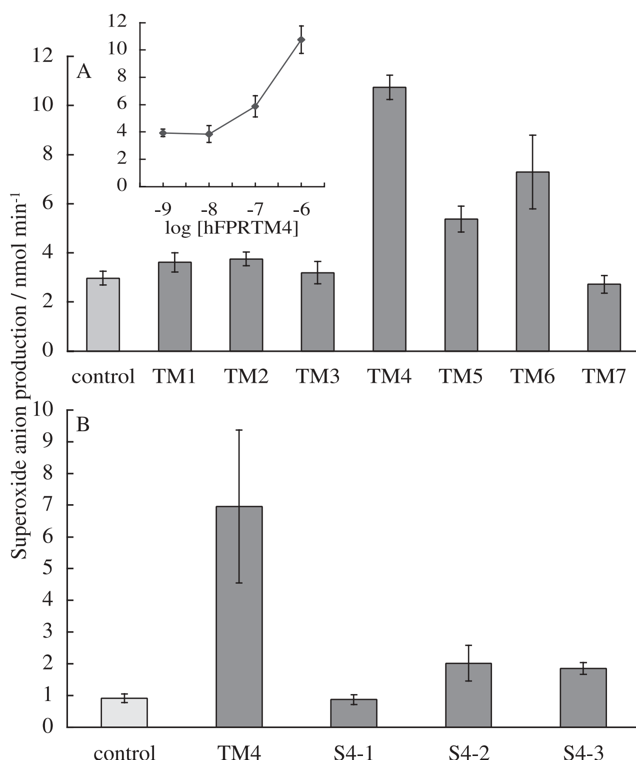

Fig. 2. **Effect of synthetic peptide-riming on superoxide anion production in human neutrophils.** Human neutrophils were incubated for 20 min at 37°C in the presence or absence of 10^{-6} M synthetic TM peptides and then stimulated with fMLP 10^{-7} M. The incubation time for optimal priming was determined in the time course experiment involving induction by hFPRTM5 in human neutrophils. A: hFPRTM peptides. Insert: Dose-response of superoxide anion production by human neutrophils treated with various doses of hFPRTM4. B: hFPRTM4 and short peptides corresponding to the fourth TM domain of human FPR. The bars represent the means for at least three different experiments.

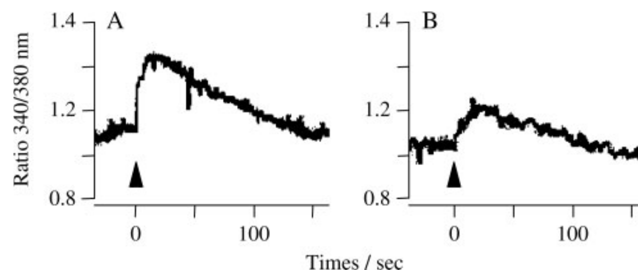

Fig. 3. **Effect of transmembrane peptide hFPRTM4 on intracellular calcium mobilization in fura2-loaded human neutrophils.** Fura2-loaded human neutrophils were stimulated with fMLP 10^{-7} M (A) or hFPRTM4 10^{-6} M (B). The stimulation points are indicated by closed arrowheads.

Table 2. **Fluorescence emission maxima of hFPRTM peptides in phosphate buffer and SDS micelles.**

Peptides	10 mM phosphate buffer at pH 7.4	20 mM SDS micelles
Trp	358	357
hFPRTM1	341	335
hFPRTM2	336	322
hFPRTM3	344	326
hFPRTM4	354	340
hFPRTM5	355	341
hFPRTM6	340	317
hFPRTM7	344	328

hFPRTM peptides were incubated for 20 min in 10 mM phosphate buffer (pH 7.4) or 20 mM SDS solution.

superoxide anion production in human neutrophils. Human neutrophils incubated with short peptides did not show increased release of the superoxide anion. These results suggested that hFPRTM4 did not behave as a ligand.

Intracellular Calcium Mobilization—Ca^{2+} plays a central role by causing many intracellular regulatory events. It is well established that an increase in the intracellular calcium concentration ($[Ca^{2+}]_i$) is an essential step in neutrophil activation. Neutrophils do not undergo oxidase activation with below 250 nM $[Ca^{2+}]_i$ (*33*). Intracellular calcium mobilization in human neutrophils treated with hFPRTM4 was investigated using the fluorescent chelator Fura-2 (*23*), with minor modifications. The assay was performed as reported previously (*22*). The increase in $[Ca^{2+}]_i$ was observed after the addition of hFPRTM4 (Fig. 3). This seems to indicate that hFPRTM4 activated signal transduction cascades through some membrane protein on the cell surface.

Fluorescence Spectroscopy—Previously, synthetic peptides corresponding to TM regions of GPCRs were synthesized and their effects on receptor function were examined (*17–19*). These peptides were proposed to interact with the TM domains of membrane proteins and to regulate receptor functions. To determine whether or not the hFPRTM peptides are inserted into the membrane, fluorescence measurements were carried out in phosphate buffer and SDS micelles. The sensitivity of Trp fluorescence emission to environment polarity allows us to use Trp residues as reporter groups to monitor the binding of peptides to micelles (*34*). The anionic detergent SDS was chosen because it forms small sized micelles, with correspondingly low levels of turbidity and light scattering (*34*). Table 2 summarizes the data for the Trp fluorescence emission maxima (λ_{max}) measured in different media. In water, the Trp fluorescence emission maxima of hFPRTM4 and hFPRTM5 are centered at around 358 nm. These values are typical for Trp in a polar environment and in phosphate buffer. In most cases,λ_{max} of hFPRTM peptides except for hFPRTM1 reflected increases in the fluorescence intensity and significant blue shifts of λ_{max} by ~23 nm in SDS micelles (Table 2 and Fig. 4). These results strongly suggested that hFPRTM peptides were inserted into the micelles. On the other hand, λ_{max} of hFPRTM1, hFPRTM2, hFPRTM3, hFPRTM6, and hFPRTM7 were blue shifted (at about 17 nm) in phosphate buffer, suggesting Trp residues partially buried by aggregation or by self-association of the peptides (*34*).

CD Studies—CD analyses were carried out to investigate the conformational properties of hFPRTM peptides in phos phate buffer and membrane-mimetic environments (*34*). When hFPRTM peptides were dissolved in phosphate buffer, clear solutions were obtained even at 100 μM. Figure 5 shows the CD spectra of 10 μM hFPRTM peptides in phosphate buffer (pH 7.4) and in the presence of SDS micelles.

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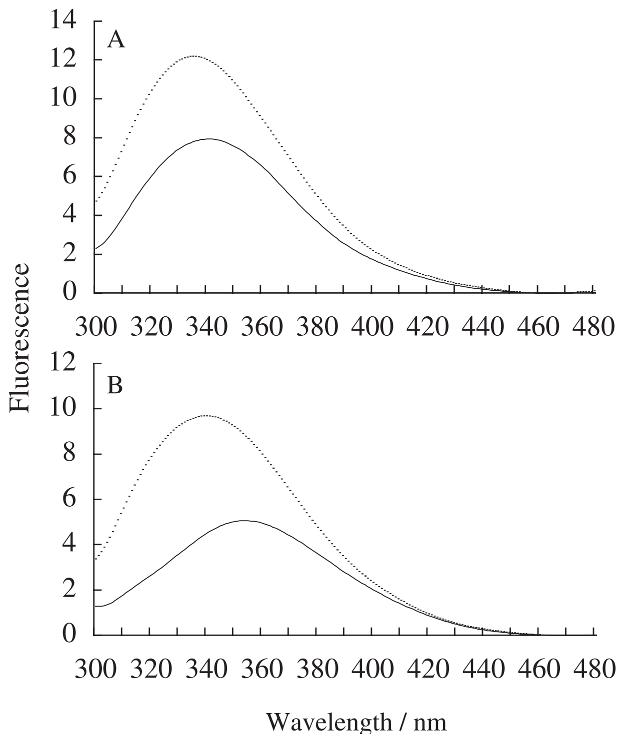

Fig. 4. **Representative fluorescence spectra of hFPRTM4.** Fluorescence emission spectra of hFPRTM1 (A) and hFPRTM4 (B) in 10 mM phosphate buffer (pH 7.4) (solid lines) and 20 mM SDS micelles (dashed lines), respectively.

In phosphate buffer, the CD spectra of hFPRTM1, hFPRTM2, hFPRTM3, hFPRTM6 and hFPRTM7 showed broad negative peaks at about 218 nm and positive peaks at about 198 nm, indicative of β-sheet structure. Both hFPRTM4 and hFPRTM5 were characterized as β-turn, with the typical double-minima ellipticity at about 200 and 225 nm and the positive ellipticity at shorter wavelengths. To determine whether or not micelles could stabilize the folding of the peptides by providing a hydrophobic environment, CD spectra of hFPRTM4, hFPRTM5 and hFPRTM6 were obtained, which exhibited double minima around 205 and 220 nm and a positive band at 195 nm, indicating the α-helical property in 20 mM SDS micelles, which correlates with the micelle formation by SDS (*35*).

DISCUSSION

In the present study, we showed that human neutrophils treated with synthetic peptides hFPRTM4, hFPRTM5 and hFPRTM6 derived from the TM domains of human FPR produced about 2–4-fold superoxide anion compared with that by untreated cells (Figs. 1 and 2A). A similar phenomenon is known as "priming" of human neutrophils. Priming for an enhanced respiratory burst is an essential component of neutrophil activation, which is the principal end result of cell-mediated immunity (reviewed in Ref. *31*).

Previously, several TM peptides corresponding to GPCRs were synthesized and their effects on receptor functions were evaluated. Hebert *et al.* reported that the addition of a TM peptide corresponding to the sixth TM domain of β_2-adrenergic receptor reduced isoproterenol-stimulated adenylate cyclase release without affecting ligand binding (*17*). TM peptide analogs derived from CXC chemokine receptor 4, CC chemokine receptor 5 and cholecystokinin receptor type A inhibited intracellular calcium release induced by a receptor agonist (*18*). TM peptides based on D2 dopamine receptor, α_1-aderenagic receptor, β_1-aderenagic receptor and V2-vasopressin receptor also blocked receptor functions (*19*). As in preceding reports, it has been postulated that these TM peptides interacted with the TM regions of membrane proteins, which led to dissociation of GPCRs dimers/oligomers, thus resulting in the loss of receptor function. From the fluorescence measurement study, hFPRTM peptides are believed to be inserted into the cell membrane (Table 2 and Fig. 4). In addition, the CD spectra on hydrophobic evaluation indicated that hFPRTM peptides retained the α-helical property that is similar to the proposed structure of the TM domains of GPCRs (Fig. 5) (*36*). These results strongly suggested that hFPRTM4, hFPRTM5 and hFPRTM6 interacted with the TM regions of membrane proteins via a helix-helix interaction which is responsible for the induction of neutrophil priming. The finding that short peptides corresponding to the fourth TM domain of FPR had no effect on enhancement of superoxide anion production (Fig. 2B) also supported this suggestion. Gripentrog *et al*. reported that human FPR does not form homo-dimers in Chinese hamster ovary cells (*37*). Thus, hFPRTM4 may interact with membrane proteins excluding FPR. However, because there is no clear-cut evidence for this assumption, further experiments are required to explore the membrane proteins including GPCRs and other proteins, because GPCRs were reported to interact other membrane proteins such as the immunoglobulin superfamily (*38*), and ligand-gated ion channels (*39–41*).

The addition of hFPRTM4 caused an increase in $[Ca^{2+}]_i$ (Fig. 3). Generally, heterotrimeric G protein signaling systems, including an increase in the intracellular calcium concentration, are activated via cell surface receptors possessing the seven TM span motif. On the other hand, it has been reported that some peptides and proteins activated G protein directly (*42*, *43*). Thus, it is possible that hFPRTM peptides may modify the signaling via the action on intracellular membrane proteins or maybe soluble proteins. However, hFPRTM4 appears to activate some protein on the neutrophil surface, since previous studies suggested that TM peptides derived from GPCRs interact with the TM regions of GPCRs, resulting in regulation of receptor function (*17–19*).

From the results of the present study, a few mechanistic views of priming caused by hFPRTM peptides are implied. Previous studies demonstrated that calcium ionophores such as ionomycin can act as priming agents, and elevation of $[Ca^{2+}]_i$ closely correlates with the degree of priming (*45*). Consequently, one plausible mechanism is that hFPRTM peptides activate intracellular proteins by increasing $[Ca^{2+}]_i$, which amplifies responses caused by agonist stimulation in human neutrophils. Another possibility is that hFPRTM peptides interact with the TM domain of FPR and/or FPR-like 1 leading to a mimetic state of the receptor dimer, the receptor characteristics related to ligand binding affinity and/or agonist-stimulated internalization changing. A similar mechanism is known for opioid

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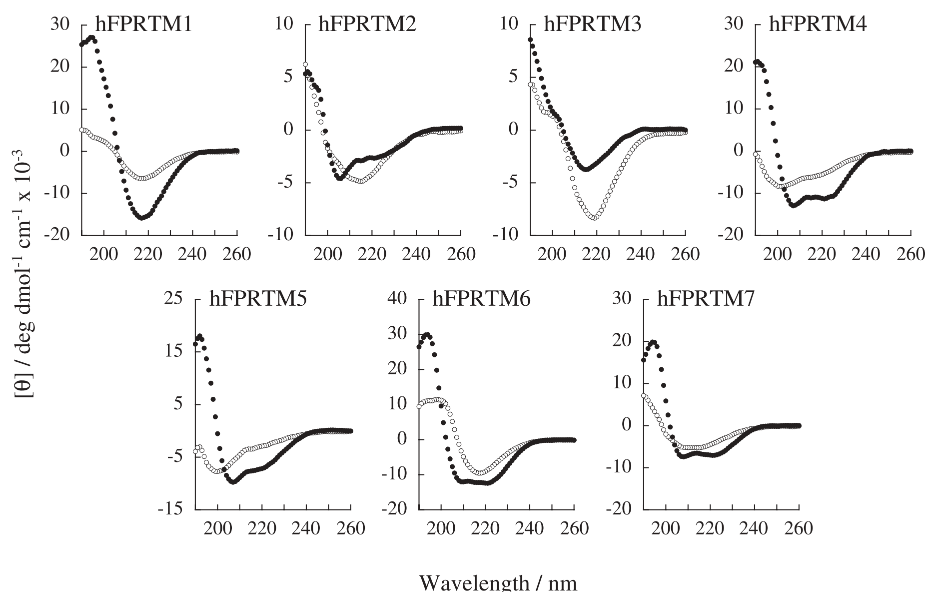

Fig. 5. **CD spectra of hFPRTM peptides.** hFPRTM peptides in phosphate buffer (open circles) and in the presence of SDS micelles (closed circles).

receptors, thus hetero-oligomerization between β_2- and β_3 adrenergic receptors blocked agonist-promoted endocytosis (*46*), and a hetero-dimer of κ-δ opioid receptor changes ligand binding affinity and selectivity when compared with the receptor monomer (reviewed in Ref. *47*). In the latter mechanism, the reason for the increase in $[Ca^{2+}]_i$ remains to be determined. Previously, Chidiac *et al.* reported that the expression of β_2-adrenergic receptor causes an increase in the intracellular cAMP content in cells without agonist stimulation (*48*). This property is explained by the equilibrium between an inactive state and an active state of β_2-adrenergic receptor. The active state of its receptor is able to activate G protein without agonist stimulation. Recent studies have revealed a low affinity ligand-bound state and a high affinity ligand-bound state for FPR (*49*, *50*). Thus, the increase in $[Ca^{2+}]_i$ may be caused by the high level of the basal active state on hFPRTM4–receptor interaction.

To identify the functional domain of FPR, chimeras of it were generated and their binding with formyl peptides was examined. Perez *et al.* prepared FPR chimeras in which each domain is replaced by the homologous domain of complement component 5 (C5a) receptor (*5*). Replacement of FPR domains except for the second intracellular and adjacent two TM domains prevented formyl peptide binding to transfected cells. Studies with chimeric receptors composed of FPR and FPRL2 suggested that the first and third extracellular loops with adjacent TM domains in FPR were essential for high-affinity fMLP binding (*6*, *7*). These reports suggested that the third and fourth TM domains are not important for ligand binding. The present study suggests that the fourth TM domain of FPR has a function-related priming effect.

This research was partially supported by the Ministry of Education, Science, Sports and Culture, with a Grant-in-Aid for Scientific Research (C), 16550144, 2004–2005.

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