

The synthetic peptide, His-Phe-Tyr-Leu-Pro-Met, is a chemoattractant for Jurkat T cells

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Abbreviation: PTX, pertussis toxin; GPCR, G-protein-coupled receptor; $[Ca^{2+}]_i$, intracellular calcium concentration; HFYLPM, His-Phe-Tyr-Leu-Pro-Met-CONH₂; PLC, phospholipase C; SDF-1, stromal cell-derived factor-1; PI3K, phosphatidylinositol-3-kinase; ERK, extracellular signal-regulated protein kinase

Abstract

His-Phe-Tyr-Leu-Pro-Met (HFYLPM) is a synthetic peptide that stimulates Jurkat T cells resulting in intracellular calcium ($[Ca^{2+}]_i$) increase in a pertussis toxin (PTX)-sensitive manner. We have examined the physiological role of the peptide in T cell activity by comparative investigation of intracellular signaling pathways accompanied with HFYLPM-induced T cell chemotaxis with a well-known chemokine, stromal cell-derived factor-1 (SDF-1)-induced signalings. Wortmannin and genistein inhibited both of HFYLPM- and SDF-1-induced Jurkat T cell chemotaxis indicating that phosphoinositide-3-kinase and tyrosine kinase activity were required for the processes. However, U-73122 and BAPTA/AM preferentially blocked HFYLPM- but not SDF-1-induced T cell chemotaxis. It indicates that phospholipase C/calcium signaling is necessary for only chemotaxis by HFYLPM. One of the well-known cellular molecules involving chemotaxis, extracellular signal-regulated protein kinase (ERK), was activated by SDF-1 but not by HFYLPM ruling out a possible role of ERK on the peptide-mediated chemotaxis. These results indicate that the synthetic peptide, HFYLPM, stimulates T cell chemotaxis showing unique signaling and provide a useful tool for the study of T cell activation mechanism.

Keywords: chemotaxis, peptide, G-protein coupled receptor

Introduction

T cells have a central role in the regulation of immune

responses in host immunity (Goetzl *et al.* 1992; Lee *et al.* 1999). For the proper role, T cell activity can be modulated by various extracellular stimuli such as processed peptides on major histocompatibility complex molecules of antigen-presenting cells (Zoller 1988; Ha-Lee *et al.* 2000). Among these extracellular stimuli, chemoattractants including several chemokines that regulate T cell functions have been receiving attention for a long time. Most of T cell chemoattractants stimulate the cells *via* activation of pertussis toxin (PTX)-sensitive G-protein-coupled receptor (GPCR) (Tenschler *et al.* 1996). Binding of chemoattractants to their specific receptors induce intracellular calcium ($[Ca^{2+}]_i$) increase, cytoskeletal rearrangement, receptor induction, and the production of bioactive lipids (Castro *et al.* 1996; Kowalska *et al.* 2000; Wang *et al.* 2000; Ragno *et al.* 2001). On the critical role of chemoattractants in T cell function, each chemoattractant modulates chemotactic migration of the cells (Gillitzer 2001). While understanding of the biological role of chemoattractants has increased substantially in recent years, relatively little is known about the signaling pathways that mediate chemotactic migration of T cells. Keeping in mind the important role of chemoattractants for T cell function, the identification of new chemoattractants and the characterization of their action mechanism are essentially needed.

We previously isolated a peptide, His-Phe-Tyr-Leu-Pro-Met-CONH₂ (HFYLPM), from a peptide library and identified as a synthetic peptide that could stimulate superoxide generation in human monocytes (Bae *et al.* 2001). The peptide induced the intracellular calcium mobilization via activation of phosphoinositide-specific phospholipase C (PLC) in monocytes (Bae *et al.* 2001). It also exhibits cell type specificity affecting in several hematopoietic cells including Jurkat T cells (Bae *et al.* 2001). The peptide does not have any effect on non-hematopoietic cells such as fibroblasts and neuronal cells (Bae *et al.* 2001). However, the physiological role of HFYLPM on T cell activity is not examined until now. Since the peptide induced chemotactic migration of monocytes, in this study we investigated a possible role of HFYLPM as a T cell chemoattractant. We also examined the signaling pathway of the peptide leading to chemotaxis of Jurkat T cells.

Materials and Methods

Materials

The peptide was synthesized, purified, and prepared in the Peptide Library Support Facility at Pohang University of

Science and Technology as described previously (Beak *et al.* 1996; Seo *et al.* 1997). RPMI 1640 was purchased from Life Technologies (Grand Island, NY). Dialyzed fetal bovine serum and supplemented bovine calf serum were purchased from HyClone (Logan, UT). Fura-2/penta-acetoxymethyl ester (fura-2/AM) and 1,2bis(2-aminophenoxy) ethane-N,N,N,N-tetraacetoxymethyl ester (BAPTA/AM) were purchased from Molecular Probes (Eugene, OR). *Myo*-[2-³H]inositol and the ECL kit were purchased from Amersham Corp. (Bucks, UK). Cell Titer 96 AQueous solution was purchased from Promega (Southampton, UK).

Cell culture

The Jurkat T lymphoma cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. Cells were maintained at densities of 2×10^5 cells/ml at 37°C in a humidified incubator supplied with 95% air and 5% CO₂.

Chemotaxis assay

Chemotaxis assays were performed using multiwell chambers (Neuroprobe Inc. Gaithersburg, MD). Briefly, Jurkat T cells were suspended in RPMI at densities of 3×10^6 cells/ml, and 1.5×10^5 of cells were placed onto the upper well of chamber that is separated by 10 µm pore polyhydrocarbon filter from peptide, or SDF-1 containing lower well. After incubation for 3 h at 37°C, non-migrated cells were discarded, and cells that migrated across the filter were centrifuged and resuspended in 100 µl of RPMI, then added 20 µl of Cell Titer 96 AQueous solution. After 2 h, quantification was performed by checking absorbance at 490 nm.

Measurement of [Ca²⁺]_i

The level of [Ca²⁺]_i was determined by Grynkiewicz's method using fura-2/AM (Grynkiewicz *et al.* 1985). Briefly, Cells were incubated with 3 µM of fura-2/AM at 37°C for 50 min in fresh serum free RPMI 1640 medium under continuous stirring. Cells were resuspended with serum free medium and 2×10^6 cells were aliquoted for each assay in calcium free Lockes solution (154 mM NaCl, 5.6 mM KCl, 1.2 mM MgCl₂, 5 mM HEPES, pH 7.3, 10 mM glucose, and 0.2 mM EGTA). The fluorescence changes at the dual excitation wavelength of 340 nm and 380 nm and the emission wavelength of 500 nm were measured.

Measurement of tyrosine phosphorylation and ERK activation

Jurkat T cells were resuspended to 1×10^7 cells/ml in serum-free RPMI 1640 and then stimulated for various length of time with 10 µM peptide at 37°C. The reaction was stopped by discarding the reaction medium and adding 200 µl of lysis buffer (20 mM Hepes, pH 7.2, 10% glycerol, 150 mM NaCl, 1% Triton X-100, 50 mM NaF, 1 mM Na₃VO₄, 10 µg/ml of leupeptin, and 1 mM phenylmethylsulfonyl

fluoride). Proteins were extracted by setting the samples on ice for 60 min. The extracted proteins were harvested by centrifugation (15,000 g, 15 min, 4°C) and used for immunoblot analysis.

Immunoblot analysis

Prepared samples were separated on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membrane. After blocking the membrane with 5% skimmed milk in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20), the membranes were incubated for 4 h with 1 µg/ml of anti-phosphotyrosine antibodies, antiphospho-ERK antibodies, or anti-ERK antibodies. Detection was performed with a peroxidase-conjugated anti-mouse (or rabbit or goat) IgG secondary antibody and an enhanced chemiluminescence system (Amersham, UK).

Results

HFYLPM stimulates [Ca²⁺]_i increase in a PTX sensitive manner in Jurkat T cells

When stimulated with various concentrations of HFYLPM, Jurkat T cells responded with a concentration-dependent increase of intracellular calcium (Figure 1A). The peptide evoked a rise in the [Ca²⁺]_i at micromolar concentrations, and the maximal response was observed around 20 µM peptide concentration. As a negative control we checked the effect of a scrambled sequence peptide, LFMYHP, for the active peptide on [Ca²⁺]_i increase in Jurkat T cells, and confirmed that the scrambled sequence peptide could not induce any significant intracellular calcium increase at concentrations up to 20 µM (Figure 1A). To address the effect of PTX-sensitive G-protein for the HFYLPM-induced [Ca²⁺]_i increase, we introduced PTX. Pretreatment of the Jurkat T cells with 100 ng/ml of PTX for 24 h, almost completely blocked the peptide-induced [Ca²⁺]_i increase (Figure 1B). The result indicates that the synthetic peptide, HFYLPM, stimulates Jurkat T cells leading to [Ca²⁺]_i increase, and PTX-sensitive G-protein is involved in the process.

HFYLPM induces T cell chemotaxis in a PTX-sensitive manner

Our previous report (Bae *et al.* 2001) demonstrating that HFYLPM acts as a chemoattractant for human monocytes led us to investigate a possible role of the peptide as a T cell chemoattractant. To assess the effect of HFYLPM on T cell migration, chemotaxis assays were performed in Jurkat T cells by using a modified Boyden chamber analysis described before (Zhou *et al.* 1995). The peptide induced chemotactic migration of Jurkat T cell within 10 nM-10 µM showing a bell shaped concentration-responsive curve (Figure 2A). The scrambled sequence

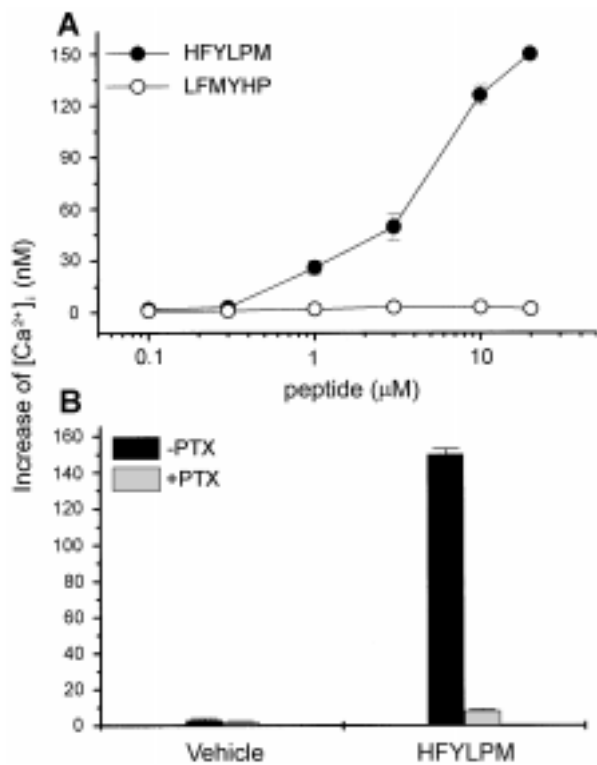


Figure 1. HFYLPM stimulates $[Ca^{2+}]_i$ increase of Jurkat T cell in a PTX-sensitive manner. $[Ca^{2+}]_i$ was determined fluorometrically using fura-2/AM, as described in Materials and Methods. Fura-2 loaded cells were challenged with various concentrations of HFYLPM or LFMYHP (A). To check the effect of PTX, cells were incubated in the absence or presence of 100 ng/ml of PTX for 24 h prior to $[Ca^{2+}]_i$ determination. Data are presented as mean \pm SE from three independent experiments performed in duplicate.

peptide (LFMYHP) for HFYLPM did not affect on Jurkat T cell migration within 1 nM-10 μ M concentration (Figure 2A). A well-known chemoattractant for Jurkat T cells, stromal cell-derived factor-1 (SDF-1) also induced chemotactic migration in the cells (Figure 2A). The chemotactic activity mediated by peptide at a concentration of 1 μ M is about 60% of that mediated by SDF-1 at a concentration of 10 nM in Jurkat T cells. Recently, it has been reported that chemokine receptors are coupled to the PTX-sensitive heterotrimeric G proteins (Tenschler *et al.* 1996). We checked the effect of PTX on HFYLPM-induced chemotaxis in Jurkat T cells. As shown in Figure 2B, HFYLPM-induced chemotaxis was almost completely inhibited by pretreatment of PTX. In the case of SDF-1, PTX pretreatment also caused almost complete inhibition of the chemotactic migration (Figure 2B). The result implicates that HFYLPM-induced chemotaxis has signaling pathways that start from PTX-sensitive G protein-coupled receptor, as SDF-1-induced one.

PLC-mediated calcium increase is required for HFYLPM-induced chemotaxis

Generally, chemoattractants have been reported to

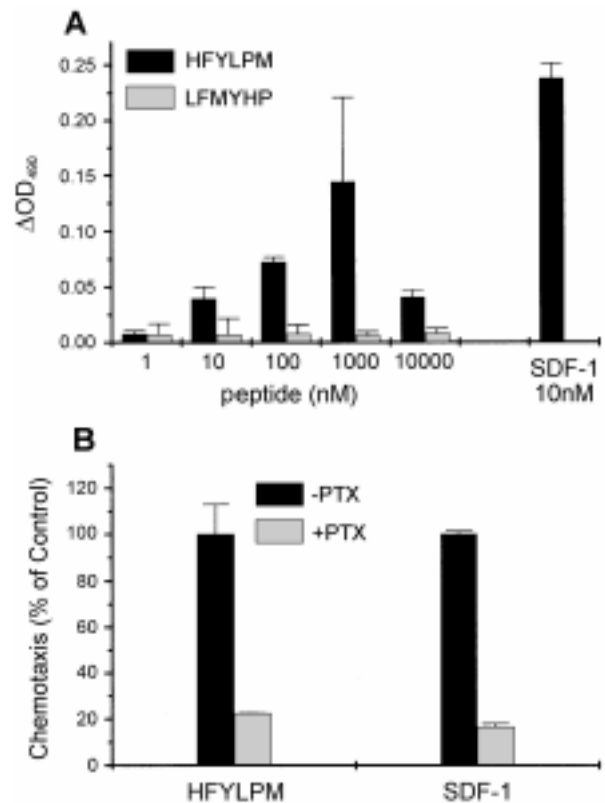


Figure 2. HFYLPM induces chemotaxis of Jurkat T cell. Assays were performed using Boyden chamber. Cells (3×10^6 cells/ml of serum-free RPMI) were added to the upper wells of a 96-well chemotaxis chamber and migration across the polycarbonate membrane with 10 μ m pore size was assessed after 3 h-incubation at 37°C. Various concentrations of HFYLPM, LFMYHP, or 10 nM SDF-1 were used (A). To check the effect of PTX on the peptide-induced chemotaxis, cells were pretreated with 100 ng/ml of PTX for 24 h prior to the assay (B). The migrated cells were determined by using One-solution cell titer kit. Data are presented as mean \pm SE of three independent experiments each performed in duplicate.

induce PI hydrolysis resulting from PLC activation in leukocytes (Wu *et al.* 1993). We also observed that HFYLPM stimulated $[Ca^{2+}]_i$ increase in Jurkat T cells (Figure 1A). The peptide-induced $[Ca^{2+}]_i$ increase was sensitive to the pretreatment of the cells with U-73122, a specific PLC inhibitor (data not shown), indicating that HFYLPM-induced $[Ca^{2+}]_i$ increase was derived from PLC activation. The role of PLC activity in HFYLPM-induced chemotaxis was also examined by using a specific PLC inhibitor, U-73122. As shown in Figure 3A, HFYLPM-induced chemotaxis was partially (30% of total) blocked by U-73122 but not by U-73343, an inactive analogue for U-73122. SDF-1-induced chemotaxis, however, was not affected by U-73122 pretreatment (Figure 3A). We also checked the role of intracellular calcium increase on HFYLPM-induced chemotaxis by using an intracellular calcium chelator, BAPTA/AM. Pretreatment of the cells with 1 μ g/ml of BAPTA/AM for 30 min prior to the chemotaxis assay attenuated HFYLPM-induced chemotaxis without affecting on SDF-1-induced one (Figure

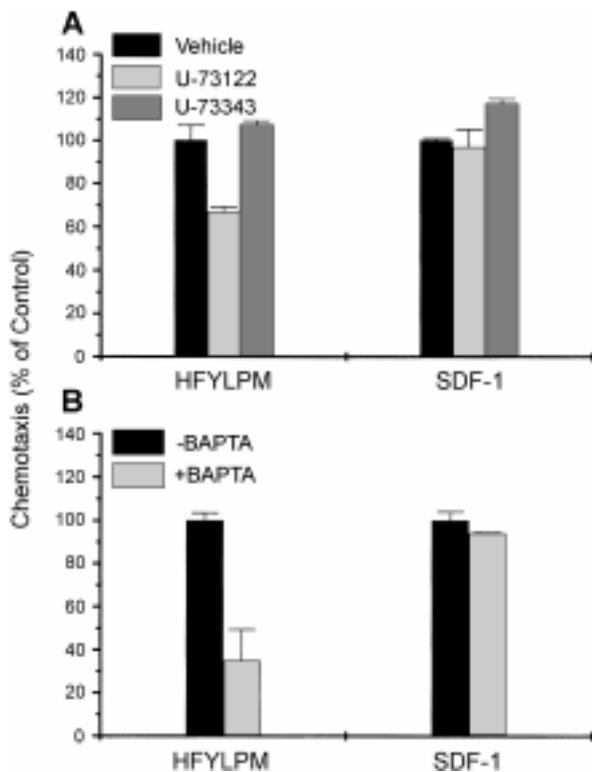


Figure 3. HFYLPM-induced T cell chemotaxis is PLC/calcium signaling-dependent. Jurkat T cells were treated with 2 μ M of U-73122, 2 μ M of U-73343 (A) or 1 μ g/ml of BAPTA/AM (B) for 15 min or 30 min, respectively. Then the cells were used for chemotaxis assay with 1 μ M HFYLPM or 10 nM SDF-1 for 3 h. Data are presented as mean \pm SE from three independent experiments performed in duplicate.

3B). These results indicate that HFYLPM- but not SDF-1-induced chemotaxis requires PLC and calcium signaling.

HFYLPM-induced chemotaxis is phosphatidylinositol-3-kinase-dependent

It has been reported that phosphatidylinositol 3-kinase (PI3K) takes major role in leukocytic chemotaxis (Stoyaniv *et al.* 1995; Stephen *et al.* 1997). Recently, it has been also demonstrated that PI3K is important in T cell chemotaxis, by several chemoattractants including SDF-1 (Sotsios *et al.* 1999). In this study, we checked whether HFYLPM-induced chemotaxis is PI3K dependent or not by using a PI3K inhibitor, wortmannin. As shown in Figure 4, pretreatment of Jurkat T cells with wortmannin caused around 40% of inhibition in both of HFYLPM- and SDF-1-induced chemotaxis. This result indicates that PI3K is an important mediator for both HFYLPM and SDF-1-induced T cell chemotaxis.

HFYLPM-induced chemotaxis is tyrosine kinase activity-dependent

Since many chemokines stimulate tyrosine kinase activity in T cells (Entschladen *et al.* 2000), we also checked the effect of HFYLPM on tyrosine phosphory-

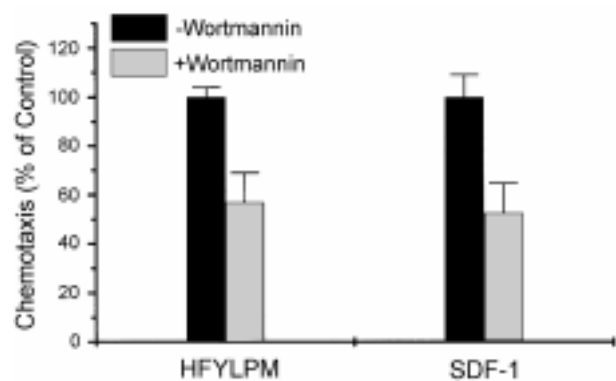


Figure 4. HFYLPM-induced chemotaxis requires PI3K activity. Cells were treated with wortmannin (50 μ g/ml) or vehicle alone for 15 min. The cells were used for chemotaxis assay with 1 μ M HFYLPM or 10 nM SDF-1 for 3 h. Data are presented as mean \pm SE from three independent experiments performed in duplicate.

lation of cellular proteins. As shown in Figure 5A, HFYLPM enhanced tyrosine phosphorylation levels of several cellular proteins such as 120 kD, 85 kD, and 60 kD proteins. SDF-1 also caused tyrosine phosphorylation of several cellular proteins showing similar pattern with that of HFYLPM-induced (Figure 5A). Tyrosine phosphorylation events induced by HFYLPM or SDF-1 were abolished by pretreatment of the cells with a tyrosine kinase inhibitor, genistein (data not shown). It was reported that SDF-1-induced T cell chemotaxis was tyrosine kinase activity-dependent (Entschladen *et al.* 2000). We checked the role of tyrosine kinase activity on HFYLPM-induced chemotaxis using genistein. As shown in Figure 5B, pretreatment of Jurkat T cells with 100 μ M of genistein caused around 60% of inhibition of HFYLPM-induced chemotaxis. SDF-1-induced chemotaxis was also sensitive to the genistein (Figure 5B). These results indicate that both HFYLPM- and SDF-1-induced chemotaxis are tyrosine kinase activity-dependent.

SDF-1 but not HFYLPM stimulates ERK activity

Many reports showed that chemoattractants stimulate ERK activity and this activity is required for the leukocyte chemotaxis event (Dutt *et al.* 1998; Sotsios *et al.* 1999). We checked the effect of HFYLPM on ERK activity by monitoring phosphorylation level of ERK with anti-phospho-specific ERK antibody. Stimulation of Jurkat T cells with 10 nM SDF-1 caused time-dependent ERK activation within 0.5-10 min after treatment (Figure 6). However, 10 μ M HFYLPM could not stimulate ERK activity in Jurkat T cells (Figure 6). The result suggests that two different chemoattractants, SDF-1 and HFYLPM, mediate different signaling in view of ERK activation in Jurkat T cells.

Discussion

In this study, we demonstrated that the synthetic pep-

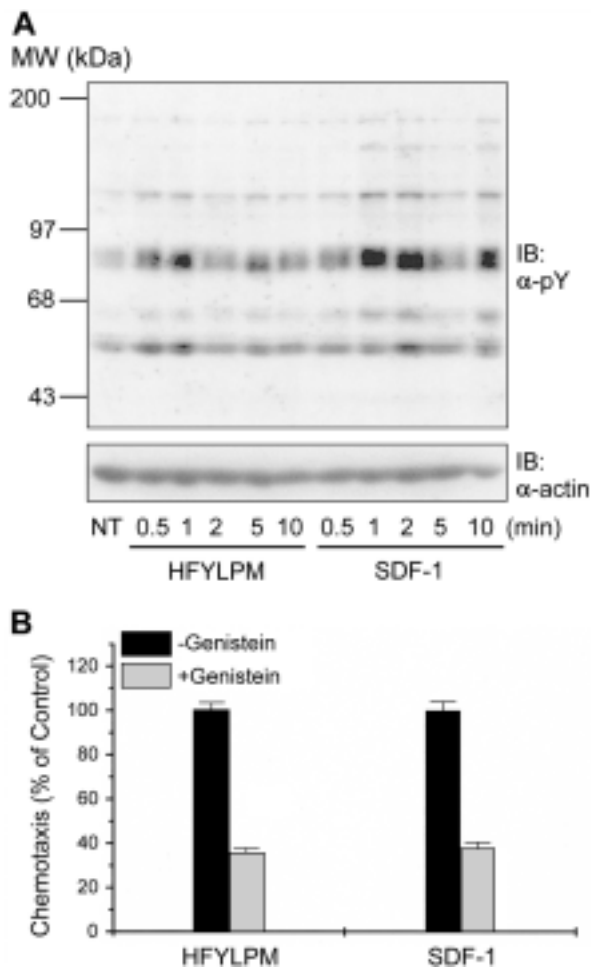


Figure 5. HFYLPM induces tyrosine kinase activity and HFYLPM-induced chemotaxis is dependent on tyrosine kinase activity. Cells were challenged with 1 μ M HFYLPM or 10 nM SDF-1 for various lengths of period. The samples were subjected to SDS-PAGE as described under Materials and Methods. Immunoblot assay was performed by using anti-phosphotyrosine antibody (A). Cells were treated with 100 μ M genistein or vehicle only for 15 min prior to chemotaxis assay with 1 μ M HFYLPM or 10 nM SDF-1. Data are presented as mean \pm SE from three independent experiments performed in duplicate.

tide, HFYLPM, induced Jurkat T cell chemotaxis. We also compared the signaling of HFYLPM-induced chemotaxis with that of SDF-1, a well-known chemokine. HFYLPM-induced chemotaxis has some overlapped and some distinct signal pathways with SDF-1-induced one. HFYLPM and SDF-1 share the signaling via PTX-sensitive G-protein, PI3K, and tyrosine kinase (Figure 2B, 4, and 5B). The peptide has a distinct signaling pathway for chemotaxis with SDF-1 in PLC-mediated calcium and ERK-mediated signaling.

Several reports demonstrated that chemoattractants including SDF-1 induced PLC activation resulting in $[Ca^{2+}]_i$ increase in T cell (Hesselgesser *et al.* 1998). However, the role of chemoattractant-induced PLC/calcium signaling on T cell chemotaxis was not clearly

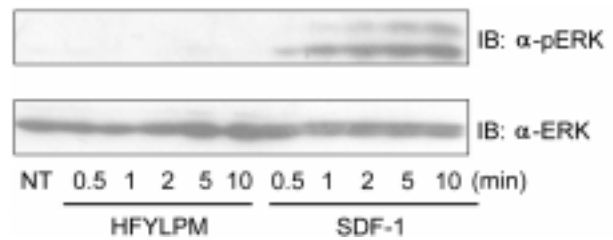


Figure 6. SDF-1 but not HFYLPM stimulates ERK activity. Cells were challenged with 10 μ M HFYLPM or 10 nM SDF-1 for various lengths of period as indicated. The levels of ERK phosphorylation were measured with immunoblot analysis by using anti-phospho-ERK antibody. To confirm that same amounts of proteins were used for the experiment, immunoblot analysis with anti-ERK antibody was performed.

understood until now. In this study, we observed HFYLPM stimulated $[Ca^{2+}]_i$ increase via PLC activation (Figure 1A and data not shown). Since HFYLPM-induced Jurkat cell migration was sensitive to U-73122 and BAPTA/AM (Figure 2A and 2B), PLC activity as well as $[Ca^{2+}]_i$ increase may be important in the peptide-induced chemotaxis. Unlike to the HFYLPM-induced chemotaxis, SDF-1-induced T cell chemotaxis was not sensitive to U-73122 or BAPTA/AM (Figure 2A and 2B). These results suggest that PLC/calcium signaling might be involved in T cell chemotaxis showing a ligand-specific manner.

PI3K and ERK have been reported to act important roles in the chemoattractant-mediated signalings as downstream of PTX-sensitive GPCR (Hii *et al.* 1999; Bonacchi *et al.* 2001). The two molecules, PI3K and ERK have been reported to play a role in chemotaxis of T cells (Sotsios *et al.* 1999). In this study we also observed that PI3K might be involved in the HFYLPM- and SDF-1-induced T cell chemotaxis (Figure 4). On ERK activation, we found that SDF-1 promoted ERK phosphorylation as reported before (Yonezawa *et al.* 2000). The ERK activity is reported to be involved in SDF-1-induced T cell chemotaxis (Hii *et al.* 1999). However, the synthetic peptide, HFYLPM, could not stimulate ERK activity in Jurkat T cells (Figure 6). Reminding that the two agonists, HFYLPM and SDF-1, induced T cell chemotaxis, it looks like that while both PI3K and ERK activation are necessary for SDF-1-mediated chemotaxis, PI3K-dependent but ERK-independent mechanism is activated by HFYLPM-induced chemotaxis in Jurkat T cell.

Although several chemokines and chemoattractants have been identified, no short peptides modulating T cell chemotaxis have been known until now. In this study, we demonstrated that HFYLPM modulates T cell activity in terms of chemotaxis and the peptide-induced T cell signaling has some unique features against well-known chemokine, SDF-1-induced one. Taken together, the synthetic peptide, HFYLPM, can serve as a useful agonist to characterize T cell activation mechanism.

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