Peptides which stimulate the formation of inositol phosphates (InoPs) in lymphocyte cell lines were identified by screening synthetic peptide libraries composed of random sequences of hexapeptides. The peptides containing the consensus sequence KYXY(P/V)YM were found to be most active in the phospholipase C (PLC)-mediated formation of InoPs in a human B myeloma cell line, U266. The peptides also stimulated the phosphoinositide hydrolysis and the release of [Ca$^{2+}$], in HL60 and U937 cell lines. On the other hand, these peptides showed no effect in the following cell lines: NIH3T3, PC12, Daudi, Sp2, J urkat, H9, Mol-t-4, SupT-1, K562, and RBL-2H3. The result suggests the possibility that the peptides may have cell type specificity. Experiments with one of the active peptides, WKYMVM-NH$_2$ showed that its action mimics the effect of AIF$_4^-$ which is a G-protein activator in the InoPs generation, and pertussis toxin partially blocked the InoPs accumulation and [Ca$^{2+}$] release induced by the peptide in the U266 cells. Binding assays with the peptide labeled with $^{125}$I showed that U266 cells have a saturable number of binding sites for the peptide. Taken together, these results suggest that the peptides could activate PLC-mediated signal transduction via a pertussis toxin-sensitive G-protein coupled receptor in certain cell types.

Many biological actions such as ligand-receptor interactions are based on the specificity of proteins conferred by the primary sequence of amino acids as well as the secondary and tertiary structures dictated by the primary sequence. Of special importance is the formation of local environments, such as active site and motif, which play a key role in the function of a protein. Among the random sequences of short peptides, there may be sequence(s) which can act on such local environments, and these sequences could serve as the lead for the development of effective new drugs. Recently various methods have been developed for identification of the sequence(s) of interest from vast mixtures of random peptide sequences or polymers (template) with various side chain groups (chemical diversity libraries) within a short period of time with minimal effort (1-5). Successful screening of these libraries has been described not only for epitopes recognized by monoclonal antibodies (6-8), but also for the identification of the biologically active peptides such as antibacterial and antifungal peptides (2), human immunodeficiency virus protease inhibitors (9), substrate-analog trypsin inhibitors (10), and interleukin-8-specific antagonist (11).

Phosphoinositide-specific phospholipase C (PLC) plays a pivotal role in the signal pathway for cell growth and differentiation (12, 13). The activated PLC catalyzes hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP$_2$) into two intracellular second messengers, inositol 1,4,5-trisphosphate (IP$_3$) and diacylglycerol (DAG). The IP$_3$ induces an increase in intracellular free calcium concentration ([Ca$^{2+}$]), while DAG directly activates protein kinase C (14-17). On the basis of amino acid sequence similarity, the PLCs in mammalian tissue have been divided into three types (PLC-$\xi$, PLC-$\gamma$, and PLC-$\delta$), each of which comprises more than one subtype (18-22). Generally, in mature B lymphocytes, interaction of ligands with the membrane-bound immunoglobulin triggers a series of metabolic events including the rapid activation of a protein tyrosine kinase associated with the B-cell receptor (23-25). The activation of these kinases leads to the rapid tyrosine phosphorylation of a number of substrates including the phospholipase C (PLC)-$\gamma$ and PLC-$\xi$ (26-29). It is assumed that the phosphorylation of PLC-$\gamma$ type will lead to enzymatic activation. Therefore, the activation of protein tyrosine kinase and PLC-$\gamma$ appears to be essential for ligand-mediated B-cell activation.

A GTP-binding protein (G-protein) that has been postulated as a modulator of the PLC-$\beta$ activity has partially been characterized at the molecular level (30-32). B cells that have been treated with tyrosine kinase inhibitors (genistein, herbimycin, tyrphostin) can still undergo phosphoinositol (PI) hydrolysis. The cells also display increases in [Ca$^{2+}$], when stimulated directly through G-proteins by AIF$_4^-$ (28, 33). Similarly, inactivation of the B-cell receptor abolishes subsequent tyrosine kinase activation, although the cells continue to respond to the G-protein-activating agent, mastoparan (34). These observations suggest that B-cell activation may involve PLC-$\beta$ types or another isoform of PLC that can be mediated by G-proteins independently of tyrosine phosphorylation of PLC-$\gamma$ types. PLC-$\beta$ types are shown to be activated by members of the G$_{o}$, or G$_{i}$, subunit of heterotrimeric G-protein (30, 35). However, the implication of G-protein in B-cell activation remains to be characterized.

In this study, we have identified peptides which stimulate the formation of InoPs in cells from libraries of hexapeptides. The peptides appear to have positive effects on the formation of InoPs and [Ca$^{2+}$], release in certain cell types such as U266 (human B myeloma), HL 60 (human promyelocytic lymphoma),...
and U937 (human histiocyte lymphoma). These effects appear to be mediated through the binding of the peptide to cell-surface receptors.

**EXPERIMENTAL PROCEDURES**

Materials—Fmoc amino acids were obtained from Millipore Co (Bedford, MA). Rapidamide resin was purchased from DuPont (Boston, MA). Myo-[2-3H]inositol and the ECL kit were purchased from Amersham Corp. (Bucks, UK). Fura-2 pentaacetoxymethyl ester (Fura-2/AM) and rhodamine 125I-labeled peptide. Equilibrium binding was established at room temperature for 90 min and terminated by rapid filtration through multiscreen-FB filters (Millipore Co.) followed by five washes with ice-cold buffer. The radioactivity of the punched filter membrane was determined in a y-counter. The level of specific binding was determined after correction for the nonspecific binding occurring in the presence of 250-fold excess unlabeled peptide.

**RESULTS**

The testing of each total 114 peptide pools of PS-SPCLs permits the determination of the most effective amino acid at each of the six positions in a hexapeptide. The results of the initial screening of the peptide library are shown in Fig. 1. The peptide mixtures, WXXXXX-NH2, was found to strongly stimulate the formation of IpOps in U266 cells. The amino acids with slightly less active than tryptophan (W) at the first position were methionine (M) and arginine (R). For the second position, several amino acids appear to be active, lysine (K) and histidine (H) being slightly more active than others. The active amino acids at the third position were tyrosine (Y) and phenylalanine (F), tyrosine being slightly more active. The most active amino acid at the fourth position were methionine (M). Valine (V) and proline (P) appear to be more active than other amino acids at the fifth position. The sixth position showed marked contrast between the active amino acid (methionine, M) and other amino acids.

The amino acids chosen for reiterated synthesis of peptides were as follows: 1st, W, M, and R; 2nd, K and H; 3rd, Y and F; 4th, M, V, and I; 5th, P, V, and R; and 6th, M. The selected amino acids at 4th, 5th, and 6th position were linked in all combinations, and for the 1st, 2nd, and 3rd positions the mixtures of the selected amino acids used were X1X2X3MPM-NH2, X1X2X3MVM-NH2, X1X2X3VRM-NH2, X1X2X3PVM-NH2, X1X2X3VM-NH2, X1X2X3RM-NH2, X1W, X1R; X2K, X2H; X3Y, X3F.

The reiterated synthesis generates nine peptide pools containing 3 × 2 × 2 × 3 × 3 × 1 = 108 individual hexapeptides. The nine peptide pools were tested for stimulation of the formation of IpOps. Among these, XXXXMPM-NH2, XXXVMVM-NH2, and XXXXVM-NH2 were found to be more active than others for formation of IpOps (Fig. 2A). Each active peptide pools was resolved and purified by using HPLC on a C18 column. Each peak fraction from the C18 column was tested for effect on the formation of IpOps, and the amino acid sequence of the active fractions were determined. Fig. 2B shows the result obtained with one of the active peptide WKYVMVM-NH2. The peptide showed the half maximal activity at about 6 × 10^-8 M. Also, most of the active peptides have a consensus of XXYX(P/V)M. A time course study showed that the formation of IpO1 reached a
maximal level in 5 min and returned to a basal level in 20 min after the addition of the peptide to U266 cells. However, the formation of total InoPs was consistently elevated throughout the 20 min incubation with the peptides (data not shown).

IP₃ is one of the major second messengers that triggers Ca²⁺ release from the internal Ca²⁺ pools in the cell. Generally, the elevation of [Ca²⁺]ᵢ is achieved by both Ca²⁺ release from the internal stores as well as by influx from the extracellular environment. Fig. 3A shows that there was peptide dose-dependent increase of [Ca²⁺]ᵢ. In order to determine the peptide-induced Ca²⁺ release from the internal stores, we measured the Ca²⁺ mobilization of U266 cell in Ca²⁺-free medium containing 0.2 mM EGTA. In addition, depleting intracellular free Ca²⁺ by preloading U266 cells with the Ca²⁺-buffering agent BAPTA completely inhibited the change in [Ca²⁺]ᵢ at maximal effective concentration of the peptide (Fig. 3B). These results demonstrate that the [Ca²⁺]ᵢ increase induced by the peptide was not due to mobilization from the extracellular Ca²⁺, but from the intracellular Ca²⁺ reservoir.

In order to investigate if the peptides have a general effect on cell types we examined the effect of an active peptide (WKYMVM-NH₂) on the formation of InoPs in NIH3T3 (NIH Swiss mouse embryo fibroblast) and PC12 (rat adrenal pheochromocytoma) cells. It showed no effect on either cells (Fig. 4). Daudi (human Burkitt lymphoma), Sp2 (mouse myeloma), Jurkat (human acute T-cell leukemia), H9 (human T-cell lymphoma), Molt-4 (human peripheral blood T cell), SupT-1 (human T-cell lymphoblastic lymphoma), K562 (human chronic myelogenous leukemia), RBL-2H3 (rat mast cell), U937 and HL60 cells were tested for the effect of the peptide in a variety cell types originated from hematopoietic lineage. Among these cells, the peptide stimulated the formation of InoPs only in HL60 and U937 cell lines (Fig. 4). The results suggest that the peptides increase the formation of InoPs only in cell type-specific manner.

PLC-γ is activated by phosphorylation of specific tyrosine residues. The activated PLC-γ catalyzes hydrolysis of PIP₂ into two intracellular second messengers, IP₃ and DAG (19). It is assumed that the phosphorylation of PLC-γ will lead to its activation. Experiments were therefore performed to investigate whether tyrosines of PLC-γ were phosphorylated in U266 cells in response to the peptides and whether the time course of the phosphorylation was compatible with the changes in the InoPs formation. However, there were no appreciable changes in the level of tyrosine phosphorylation of PLC-γ in U266 cells (data not shown). This result suggests that the peptides may not induce PI hydrolysis and [Ca²⁺]ᵢ release through the activation of PLC-γ type.

To investigate the possible involvement of a G-protein in the peptide-induced formation of InoPs, U266 cells were treated with pertussis toxin (150 ng/ml) for 12 h prior to the addition of WKYMVM-NH₂. The peptide-dependent formation of InoPs was reduced by 70% (Fig. 5). AlF₄⁻ as a G-protein activator induced the InoPs formation in U266 cells. In addition, increasing amounts of WKYMVM-NH₂ in the presence of a fixed amount of AlF₄⁻ showed no additive effect on the formation of InoPs (Fig. 6). These results support that pertussis toxin-sensitive G-proteins may be involved in the PLC-β mediated PI hydrolysis in response to the peptides in U266 cells.

To investigate the possible action of the peptide through the binding to a cell-surface receptor, a fixed number of U266 cells was incubated for 90 min at room temperature in the presence of the various concentrations of ¹²⁵I-labeled peptide. After washing the cells, we determined the amount of bound ¹²⁵I-labeled peptide using multiscreen binding assay system (Milipore). A representative result of these assays is shown in Fig. 7. As the concentration of ¹²⁵I-labeled peptide increased, there was a corresponding increase in the amount of the peptide bound to the cells until it reached a plateau, suggesting that there is a saturable number of binding sites for the peptide on the surface of U266 cells. On the other hand, fMLP is known as the peptide that stimulates the formation of InoPs through the
cell-surface receptor which is coupled with a G-protein in neutrophils (42–44). Thus, it was possible that the peptides we have identified in this study act on the fMLP receptor. However, fMLP showed no effect on the formation of InoPs in U266 cells (Fig. 8). Therefore, it appears that our peptide does not bind to the fMLP receptor.

**DISCUSSION**

In this study, we have found that the hexapeptides with the following consensus sequence XKYX(P/V)M (where X is any amino acid) stimulate the formation of InoPs in U266 cell line. A, the first three positions consist of mixture (X) of defined amino acid (X1: W, M, R; X2: K, H; X3: Y, F). The remaining positions were individually defined with each of the selected amino acids. The partial library consists of 9 mixtures; each mixture contains 12 single peptides. The total number of peptides in 9 mixtures were 12 × 9 × 108. Error bars were omitted for clarity of the figure. B, A peptide WKYMVM-NH2 selected from the experiment described above was tested for stimulation of InoPs formation in U266 cells.

**Cell Lines**

Fig. 4. Stimulation of InoPs formation by a peptide in various cell lines. Subconfluent cultures of each type were prelabeled with myo-[3H]inositol (1 μCi/106 cell) for 24 h in serum-free RPMI media. Cells were then treated with peptides (WKYMVM-NH2, 1 μM) in RPMI containing 20 mM Hepes, pH 7.2, 20 mM LiCl, and 1 mg/ml bovine serum albumin for 10 min at 37°C. Results are presented as the total InoPs and expressed as mean ± S.D. from three independent experiments done in duplicate.

Fig. 3. Changes in [Ca2+]i after treatment with peptide in the absence of external Ca2+. A, [Ca2+]i was determined fluorometrically using fura-2/AM as described under "Experimental Procedures." Before measurement, U266 cells (2 × 106) were suspended in Ca2+-free Locke's solution containing 0.2 mM EGTA. Cells were treated with various concentrations of WKYMVM-NH2 where indicated by the arrow. B, U266 cells in upper trace were stimulated with excess WKYMVM-NH2. Cells used in the lower trace (+ BAPTA label) had been preincubated in RPMI containing 60 μM BAPTA acetoxymethyl ester for 30 min at 37°C.

Fig. 2. Effect of the peptide pools synthesized from the selected amino acids and the single selected peptide for their ability to stimulate the InoPs formation in U266 cell line. A, the first three positions consist of mixture (X) of defined amino acid (X1: W, M, R; X2: K, H; X3: Y, F). The remaining three positions were individually defined with each of the selected amino acids. The partial library consists of 9 mixtures; each mixture contains 12 single peptides. The total number of peptides in 9 mixtures were 12 × 9 × 108. Error bars were omitted for clarity of the figure. B, A peptide WKYMVM-NH2 selected from the experiment described above was tested for stimulation of InoPs formation in U266 cells.

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FIG. 2. Effect of the peptide pools synthesized from the selected amino acids and the single selected peptide for their ability to stimulate the InoPs formation in U266 cell line. A, the first three positions consist of mixture (X) of defined amino acid (X1: W, M, R; X2: K, H; X3: Y, F). The remaining three positions were individually defined with each of the selected amino acids. The partial library consists of 9 mixtures; each mixture contains 12 single peptides. The total number of peptides in 9 mixtures were 12 × 9 × 108. Error bars were omitted for clarity of the figure. B, A peptide WKYMVM-NH2 selected from the experiment described above was tested for stimulation of InoPs formation in U266 cells.
type-specific action of our peptide might be due to the existence of putative receptor specific for the peptide, rather than the difference of the downstream components which need for the pertussis toxin-sensitive InoPs formation. One of the major goals of future work is to identify the putative receptor for the peptide.

It is widely accepted that the hydrolysis of PIP2 by PLC and subsequent formation of DAG and IP3 is a major signaling pathway employed by a variety of hormones, growth factors, and various neurotransmitters (14–17). IP3 stimulates the release of free Ca$^{2+}$ ions from intracellular Ca$^{2+}$ stores. Ca$^{2+}$ leads to the activation of one or more isozymes of protein kinase C, which may participate in the induction of various immediately early genes such as c-fos and c-myc (49). Accordingly, peptide-induced IP$_3$ formation and the subsequent release of Ca$^{2+}$ is very important for B-cell activation. As in T lymphocytes, antigen receptor interaction in B cells leads to tyrosine phosphorylation of PLC-$\gamma$1 (28), but also of PLC-$\gamma$2 that appears to be the major isozyme in B cells (50). Recently, tyrosine phosphorylation of PLC-$\gamma$2 was reported to be induced in murine B cells upon cross-linking of membrane immunoglobulins (29, 30) and in the HL60 granulocytes stimulated with pervanadate (51). In most cases, the tyrosine phosphorylation of...
PLC-γ is accompanied by changes in PI turnover. Despite this correlation, however, the peptides do not affect phosphorylation of the tyrosines of PLC-γ1 and -γ2 enzymes. These results suggest that another isoform of PLC may be involved in the formation of IP3s in the B cells treated with the peptides.

In this report, we suggest that the pertussis toxin-sensitive G-protein is directly involved in the peptide-induced PI hydrolysis in B cells. This is evident from the observations that pertussis toxin inhibits the formation of InoPs and the release of C2+ by WKYMVM-NH2 (data not shown). Furthermore, the peptide-induced PI hydrolysis mimics the stimulatory effect by AlF4− (G-protein activator). These data provide strong support for the contention that the peptide-mediated activation of PLC in U266 cell requires a G-protein, although an isoform of G-proteins are not presently identified. It is clear that the pertussis toxin-insensitive mechanism is mediated by α-subunits of the recently discovered G-protein. However, the pertussis toxin-sensitive mechanism for activation of PLC is less well understood. Several recent reports suggest that the pertussis toxin-sensitive response can be reconstituted through receptor-mediated release of βγ subunits from members of the Gα I class, and the toxin apparently blocks the activation of PLC-β2 by interfering with the release of the βγ subunits from the trimeric G-proteins (45, 54, 55). In addition, we detected that the PLC-β2 was preferentially expressed more than other β-isozymes in U266 cell (data not shown). Therefore, PLC-β2 may be potential mediator for the action of the peptides.

Experiments with the peptide labeled with 125I suggest that the cells have a saturable number of binding sites for the peptide on the cell surface, possibly a receptor. Presently we have no information on the nature of the receptor. It is likely that the formation of IP3s in response to the peptide is mediated through the interaction of the peptide to a cell-surface receptor. In addition, the peptide receptors may be different from the fMLP receptors because fMLP does not increase the level of IP3s in U266 cells. However, although peptide receptors are distinct from the fMLP receptors, it is possible that the two receptors share the same signal transduction pathways, because both fMLP and the peptide we reported here all stimulate PI hydrolysis in a pertussis toxin-sensitive manner.

For further study, the molecular characterization of the receptor, G-protein, and PLC related to this peptide signaling could provide the insight into the understanding of the ligand-triggered signal cascade in certain cell types.

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Identification of the Peptides That Stimulate the Phosphoinositide Hydrolysis in Lymphocyte Cell Lines from Peptide Libraries
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