Binding of Synthetic LKEKK Peptide to Human T-Lymphocytes

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Abstract—The synthetic peptide LKEKK corresponding to sequence 16-20 of human thymosin-α_1 and 131-135 of human interferon-α_2 was labeled with tritium to specific activity 28 Ci/mol. The [^{3}H]LKEKK bound with high affinity ($K_d = 3.7 \pm 0.3$ nM) to donor blood T-lymphocytes. Treatment of cells with trypsin or proteinase K did not abolish [^{3}H]LKEKK binding, suggesting the non-protein nature of the peptide receptor. The binding was inhibited by thymosin-α_1, interferon-α_2, and cholera toxin B subunit ($K_i = 2.0 \pm 0.3$, 2.2 ± 0.2, and 3.6 ± 0.3 nM, respectively). Using [^{3}H]LKEKK, we demonstrated the existence of a non-protein receptor common for thymosin-α_1, interferon-α_2, and cholera toxin B-subunit on donor blood T-lymphocytes.

In our earlier studies of structural and functional properties of interferons-α (IFNs-α), we identified the octapeptide LKEKKYSP (residues 131-138 of human IFN-α_2) that bound with high affinity to mouse thymocytes and human fibroblasts [1, 2]. The binding was competitively inhibited by IFN-α_2 and thymosin-α_1 (TM-α_1) [1-3]. Comparison of amino acid sequences of the octapeptide and TM-α_1 revealed that they share the same pentapeptide fragment, LKEKK, that corresponds to residues 16-20 in TM-α_1 and 131-135 in IFN-α_2 (Fig. 1). We proposed that this fragment is responsible for TM-α_1 and IFN-α_2 binding to various cells and suggested that synthetic LKEKK peptide has similar binding capacity and exhibits biological activity.

In this work, we synthesized the LKEKK peptide and studied its binding to T-lymphocytes from donor blood.

Abbreviations: ADP, adenosine diphosphate; BSA, bovine serum albumin; cAMP, 3′,5′-cyclic adenosine monophosphate; CT-B, cholera toxin B-subunit; GTP, guanosine-5′-triphosphate; HPLC, high performance liquid chromatography; IFN, interferon; IL, interleukin; MyD88, myeloid differentiation primary response gene 88; NAD, nicotinamide adenine dinucleotide; TIR domain, Toll/interleukin-1 receptor/resistance protein domain; TM-α_1, thymosin-α_1; TNF, tumor necrosis factor; TRIF, TIR-domain-containing adapter-inducing interferon-β.

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MATERIALS AND METHODS

Human TM-α_1 was from Immundiagnostik AG (Germany); phenylmethanesulfonyl fluoride (PMSF) and Tris were from Fluka (USA); cell culture medium, fetal calf serum, and HEPES were from ICN (USA); sucrose, BSA, EDTA, EGTA, and sodium azide were from Serva (Germany); Ready Gel scintillation fluid was from Beckman (USA). All other reagents and solvents were of domestic origin and were used after additional purification.

LKEKK and KKEKL peptides were synthesized with an Applied Biosystems Model 430A peptide synthesizer and a Vega Coupler Model C250 peptide synthesizer (USA) by the Boc/Bzl peptide chain elongation method and purified by preparative reversed-phase chromatography on a Waters SymmetryPrep C18 (19 × 300 mm) column (Malva, Greece) using a Gilson chromatographer (France). The synthesized peptides were characterized by reversed-phase HPLC on an XTerra RP18 column (Malva) using a Gilson chromatographer, amino acid analysis on an LKB 4151 Alpha Plus amino acid analyzer (Sweden) after hydrolysis with 6 M HCl for 24 h at 110°C, and mass spectrometry on a Finnigan mass spectrometer (USA).

Recombinant human interferon-α_2 was purchased from the State Research Institute of Highly Pure Biopreparations (St. Petersburg, Russia).
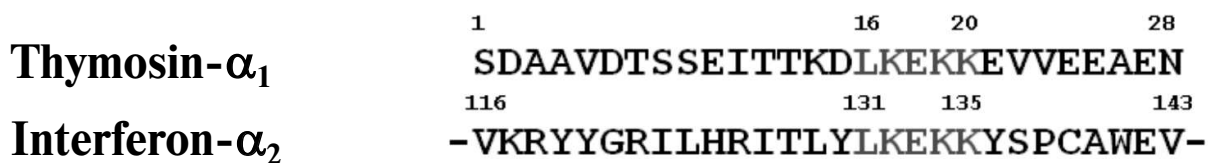
Thymosin-α_1

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         1                        16   20          28
         SDAAVDTSSEITTKDLKEKKEVVEEAEN
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Interferon-α_2

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         116                   131  135        143
         -VKRYYGRILHRITLYLKEKKYSPCAWEV-
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Fig. 1. Comparison of amino acid sequences of human thymosin-α_1 and interferon-α_2. Pentapeptide LKEKK is indicated (gray); numbers correspond to the numbers of amino acid residues in the sequences.

[³H]LKEKK was obtained by the high-temperature solid-state catalytic isotope exchange (HSCIE) method [3]. Aluminum oxide (50 mg) was added to 2 mg of peptide dissolved in 0.5 ml of water; water was then removed by evaporation on a rotary evaporator. The resulting peptide-coated aluminum oxide was mixed with 10 mg of the catalyst (5% Rh/Al$_2$O$_3$) and transferred into a 10-ml ampule. The ampule was evacuated, filled with gaseous tritium to a pressure of 250 mm Hg, heated to 170°C, and incubated at this temperature for 20 min. Then, the ampule was cooled, evacuated, purged with hydrogen, and evacuated again. The labeled peptide was extracted from the reaction mixture with two portions (3 ml) of 50% ethanol in water. The extracts were combined and evaporated. To remove labile tritium, the procedure was repeated twice. The labeled peptide was purified by HPLC on a Kromasil column (4 × 150 mm; particle size, 5 μm) in a 42-70% gradient (20 min) of methanol in 0.1% trifluoroacetic acid at a flow rate of 3 ml/min. Elution was monitored at 254/280 nm with a Beckman spectrophotometer. Tritium incorporation into the peptide was determined with a liquid scintillation counter.

Mononuclear cells were isolated from the blood of healthy donors as described in [4]. T-cells were isolated by the method [5] using dense polystyrene beads coated with mouse antibodies against human CD3. The method yielded a >95% pure population of target (CD3$^+$) T-lymphocytes.

[³H]LKEKK was bound to T-lymphocytes according to the following procedure. T-cells (10^6/ml) were incubated with the labeled peptide (10^{-10}-10^{-7} M; three measurements for each peptide concentration) at 4°C for 40 min in 1 ml of RPMI-1640 medium containing 10 mM HEPES, 20 mM sodium azide, and 0.6 mg/ml PMSF (pH 7.4). After incubation, the reaction mixture was filtered through GF/A glass microfiber filters (Whatman, UK), and then the filters were washed three times (5 ml each wash) with ice-cold physiological buffered saline containing 10 mM HEPES (pH 7.4). The radioactivity of the filters was measured with a Beckman LS 5801 scintillation counter (USA). Specific [³H]LKEKK binding to T-lymphocytes was calculated as a difference between total and nonspecific binding; the nonspecific binding was determined in the presence of 10^{-4} M unlabeled peptide (1000× excess over the highest used [³H]LKEKK concentration of 10^{-7} M). To determine the equilibrium dissociation constant (K_d), the ratio between molar concentrations of the bound (B) and free (F) labeled peptide was plotted against molar concentration of the bound labeled peptide (B) [6].

To estimate the inhibitory effects of TM-α_1, IFN-α_2, and cholera toxin B-subunit (CT-B), the T-lymphocytes (10^6/ml) were incubated with 10 nM labeled peptide and one of the tested proteins (concentration range, 10^{-12}-10^{-5} M; three measurements for each concentration) as described above. The inhibition constant (K_i) was calculated using the formula: $K_i = [IC]_{50}/(1 + [L]/K_d)$ [7], where [L] is the [³H]LKEKK molar concentration; K_d is the equilibrium dissociation constant of the [³H]LKEKK–receptor complex; [IC]$_{50}$ is the concentration of unlabeled ligand causing 50% inhibition of the labeled peptide specific binding. [IC]$_{50}$ was determined graphically from the inhibition plots. The value of K_d was determined as described above.

Treatment of T-lymphocytes with proteases. The cells (10^7/ml) were incubated in RPMI-1640 medium containing 5 mg/ml trypsin or 1 mg/ml proteinase K at 37°C for 30 min. Digestion was stopped by adding large volumes of the medium. The cells were washed three times with 10 volumes of the medium, and the binding reaction was carried out as described above. Each measurement was performed in triplicate.

Table 1. Main characteristics of LKEKK and KKEKL peptides

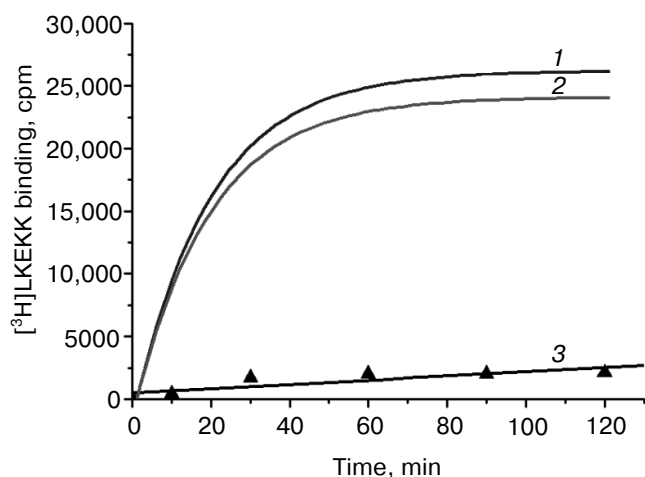| Peptide	Purity, %	Amino acid analysis	Molecular mass, Da
LKEKK	>98	Glu 1.09 (1), Leu 1.00 (1), Lys 3.27 (3)	645.2 (calculated − 644.87)
KKEKL	>95	Glu 1.12 (1), Leu 1.03 (1), Lys 3.32 (3)	648.6 (calculated − 644.87)

Fig. 2. Kinetics of total (*1*), specific (*2*), and nonspecific (*3*) binding of [³H]LKEKK to human blood T-lymphocytes. Specific binding was determined as the difference between total and nonspecific binding.

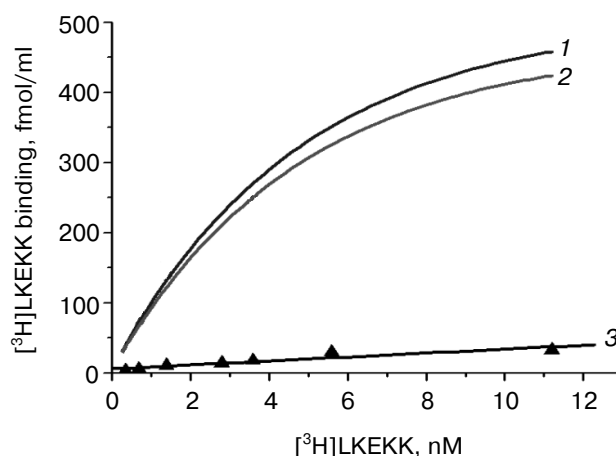**Fig. 3.** Dependence of total (*1*), specific (*2*), and nonspecific (*3*) binding of [³H]LKEKK to human blood T-lymphocytes on the peptide concentration. Specific binding was determined as the difference between total and nonspecific binding.

RESULTS

The main characteristics of the synthesized LKEKK and KKEKL peptides (purity, amino acid content, and molecular mass) are shown in Table 1.

The HSCIE reaction with subsequent peptide purification yielded [³H]LKEKK with specific activity of 28 Ci/mmol. The retention time for both labeled and unlabeled peptides on a Kromasil C18 column (see "Materials and Methods") was 11 min; the 254/280 nm absorbance ratio for the labeled and unlabeled peptides was the same, thereby confirming that hydrogen substitution with tritium did not affect chemical structure of the peptide.

[³H]LKEKK binding to T-lymphocytes. We found that under our experimental conditions (see "Materials and Methods"), [³H]LKEKK bound specifically to T-lymphocytes. Figure 2 shows the kinetics of specific binding of [³H]LKEKK at 4°C. The receptor–peptide complex reached dynamic equilibrium after ~1 h of incubation and remained in this state for at least another hour. Therefore, to assess the equilibrium dissociation constant (K_d) for the peptide binding to T-lymphocytes, the reaction was carried out for 1 h. The nonspecific binding under these conditions was $6.9 \pm 0.4\%$ of total binding.

Figure 3 shows the dependence of the total (*1*), specific (*2*), and nonspecific (*3*) binding of [³H]LKEKK on its concentration. Curve *2* reaches a plateau, indicating the saturability of the specific binding of the peptide.

The Scatchard plot of [³H]LKEKK binding to T-lymphocytes is shown in Fig. 4. The linear character of the plot proves the presence of only one type of receptor with a high affinity ($K_d = 3.7 \pm 0.3$ nM) for [³H]LKEKK.

To characterize specific binding of [³H]LKEKK to T-lymphocytes, we used unlabeled LKEKK peptide, KKEKL peptide, TM-α_1, IFN-α_2, and cholera toxin B-

subunit (CT-B) as competitive ligands (Table 2). TM-α_1, IFN-α_2, and CT-B strongly inhibited [³H]LKEKK binding with K_i of 2.0 ± 0.3, 2.2 ± 0.2, and 3.6 ± 0.3 nM, respectively. KKEKL did not compete with [³H]LKEKK for the binding site ($K_i > 10$ μM).

Treatment of cells with trypsin or proteinase K did not affect [³H]LKEKK binding, thereby suggesting a nonprotein nature of the receptor (or at least, of the receptor region directly involved in peptide binding).

DISCUSSION

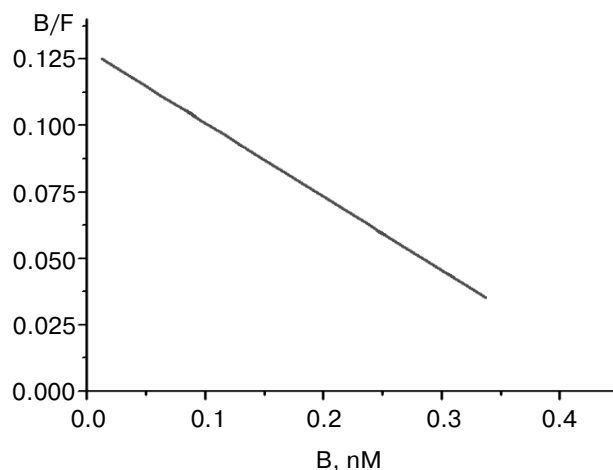TM-α_1 is a 28-amino acid *N*-acetylated peptide with immunomodulating, antitumor, and direct antiviral

Fig. 4. Scatchard plot of [³H]LKEKK specific binding to human blood T-lymphocytes. B and F, molar concentrations of bound and free [³H]LKEKK, respectively.

Table 2. Inhibition of [^{3}H]LKEKK specific binding to human blood T-lymphocytes by unlabeled ligands

Ligand	IC$_{50}$	K_i
	mean ± standard deviation, nM	
Interferon-α_2	7.4 ± 0.6	2.0 ± 0.3
Thymosin-α_1	8.2 ± 0.7	2.2 ± 0,2
Cholera toxin B-subunit	13.2 ± 0.9	3.6 ± 0.3
KKEKL	>10 000	>10 000

activities [8, 9]. It is believed that such pleiotropy of action is related to the ability of TM-α_1 to activate Toll-like receptors (TLRs) [10-12] that are present on the surface and in intracellular organelles of most mammalian cells. Binding of TM-α_1 causes TLR dimerization and induces conformational changes required for the recruitment of various signaling molecules. TLR-mediated signal transduction occurs via two main pathways that use different sets of adaptor proteins. The first pathway utilizes MyD88 (myeloid differentiation primary response gene 88) adaptor protein and involves activation of NF-κB transcriptional factor resulting in the stimulation of production of antiinflammatory cytokines (IL-1, IL-12, and TNF-α) and induction of innate effector mechanisms [10, 11]. Most TLRs require MyD88; however, TLR3 and TLR4 utilize alternative adaptors, such as TRIF and TRAM. TRIF belongs to the family of TIR (Toll/IL-1 receptor resistant)-domain-containing proteins. TRAM (TRIF-related adaptor molecule) is involved in TLR4 signaling. The TRAM/TRIF-mediated pathway plays an important role in the induction of dendritic cell maturation and T-cell proliferation. TRIF and TRAM activate IFN-β-regulating factors IRF-3 and IRF-7 and induce late activation of NF-κB [10, 12].

The unusually broad spectrum of TM-α_1 activities indicates that the mechanisms of TM-α_1 action are not limited to the TLR-mediated pathways alone. Moreover, the size of the TM-α_1 polypeptide chain (28 amino acids) suggests the existence of several active sites within the molecule. This suggestion is confirmed, for example, by the fact that TM-α_1 competes with CT-B for binding to human fibroblasts [2], CT-B receptor being a GM1 ganglioside [13-16].

No data have been obtained so far on the binding of TM-α_1 to gangliosides. However, it is known that CT-B inhibits the antiviral activity of IFN-α [17, 18] by preventing its interaction with GM1 [19, 20]. IFN-α reversibly binds to GM1 with high affinity and high specificity. The regions of the molecule directly involved in the binding are the oligosaccharide fragment of GM1 including lactose (β-D-Gal-(1→4)-Glc) and *N*-acetylneur-

aminic acid [20] and the highly conserved fragment of IFN-α (presumably, residues 131-138) [2].

The Scatchard plot of specific binding of [^{3}H]LKEKK to T-lymphocytes (Fig. 4) shows the presence of only one type of high-affinity ($K_d = 3.7 ± 0.3$ nM) binding sites for [^{3}H]LKEKK.

We also tested TM-α_1, IFN-α_2, CT-B, and peptide KKEKL with inverted amino acid sequence as competitive ligands. The K_i values (Table 2) demonstrated strong inhibitory capacity of TM-α_1, IFN-α_2, and CT-B ($K_i = 2.0 ± 0.3$, $2.2 ± 0.2$, and $3.6 ± 0.3$ nM, respectively), whereas KKEKL did not inhibit [^{3}H]LKEKK binding ($K_i > 10$ μM), indicating a high specificity of the labeled peptide binding.

Treatment of cells with proteases (trypsin or proteinase K) did not affect [^{3}H]LKEKK binding, which suggests non-protein nature of the receptor (or at least, of the receptor region directly involved in binding). These results suggest with a high degree of probability that the peptide receptor is GM1 ganglioside.

Therefore, using [^{3}H]LKEKK, we determined that T-lymphocytes have a common binding site (receptor) for TM-α_1, IFN-α_2, and CT-B.

In conclusion, we should note that CT-B is now viewed as a promising immunomodulating and antiinflammatory agent. Recombinant CT-B has been recently found to stimulate humoral immunity and to induce anti-inflammatory responses *in vivo* [21]. Regarding these data, the biological activity of the LKEKK peptide might be of considerable interest.

REFERENCES

1. Zav'yalov, V. P., Navolotskaya, E. V., Abramov, V. M., Galaktionov, V. G., Isaev, I. S., Kaurov, O. A., Kozhich, A. T., Maiorov, V. A., Prusakov, A. N., Vasilenko, R. N., and Volodina, E. Yu. (1991) The octapeptide corresponding to the region of the highest homology between α-interferon and thymosin-α_1 effectively competes with both cytokines for common high-affinity receptors on murine thymocytes, *FEBS Lett.*, **278**, 187-189.
2. Zav'yalov, V. P., Navolotskaya, E. V., Vasilenko, R. N., Abramov, V. M., Volodina, E. Y., Roslovtseva, O. A., Prusakov, A. N., and Kaurov, O. A. (1995) The sequence 130-137 of human interferon-α2 is involved in the competition of interferon, prothymosin α and cholera toxin B subunit for common receptors on human fibroblasts, *Mol. Immunol.*, **32**, 425-431.

3. Zolotarev, Y. A., Dadayan, A. K., Bocharov, E. V., Borisov, Y. A., Vaskovsky, B. V., Dorokhova, E. M., and Myasoedov, N. F. (2003) New development in the tritium labeling of peptides and proteins using solid catalytic isotopic exchange with spillover-tritium, *Amino Acids*, **24**, 325-333.

4. Boyum, A., Berg, T., and Blomhoff, R. (1983) Fractionation of mammalian cells, in *Iodinated Density Gradient Media – a Practical Approach* (Rickwood, D., ed.) Oxford, pp. 147-170.

5. Patel, D., Rubbi, C. P., and Rickwood, D. (1995) Separation of T- and B-lymphocytes from human peripheral blood mononuclear cells using density perturbation methods, *Clin. Chim. Acta*, **240** 187-193.

6. Pennock, B. E. (1973) A calculator for finding binding parameters from a Scatchard plot, *Anal. Biochem*., **56**, 306-309.

7. Cheng, Y. C., and Prusoff, W. (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50% inhibition (IC_{50}) of an enzymatic reaction, *Biochem. Pharmacol*., **22**, 3099-3108.

8. Pierluigi, B., D'Angelo, C., Fallarino, F., Moretti, S., Zelante, T., Bozza, S., De Luca, A., Bistoni, F., Garaci, E., and Romani, L. (2010) Thymosin alpha1: the regulator of regulators? *Ann. N. Y. Acad. Sci*., **1194**, 1-5.

9. Romani, L., Moretti, S., Fallarino, F., Bozza, S., Ruggeri, L., Casagrande, A., Aversa, F., Bistoni, F., Velardi, A., and Garaci, E. (2012) Jack of all trades: thymosin α1 and its pleiotropy, *Ann. N. Y. Acad. Sci*., **1269**, 1-6.

10. Brikos, C., and O'Neill, L. A. (2008) Signalling of toll-like receptors, *Handb. Exp. Pharmacol*., **183**, 21-50.

11. Kawai T., and Akira, S. (2011) Toll-like receptors and their crosstalk with other innate receptors in infection and immunity, *Immunity*, **34**, 637-650.

12. Kabelitz, D. (2007) Expression and function of Toll-like receptors in T-lymphocytes, *Curr. Opin. Immunol*., **19**, 39-45.

13. Cuatrecasas, P. (1973) Interaction of *Vibrio cholerae* enterotoxin with cell membranes, *Biochemistry*, **12**, 3547-3558.

14. Cuatrecasas, P. (1973) Gangliosides and membrane receptors for cholera toxin, *Biochemistry*, **12**, 3558-3566.

15. Lencer, W. I., and Tsai, B. (2003) The intracellular voyage of cholera toxin: going retro, *Trends Biochem. Sci*., **28**, 639-645.

16. Fujinaga, Y., Wolf, A. A., Rodighiero, C., Wheeler, H., Tsai, B., Allen, L., Jobling, M. G., Rapoport, T., Holmes, R. K., and Lencer, W. I. (2003) Gangliosides that associate with lipid rafts mediate transport of cholera and related toxins from the plasma membrane to endoplasmic reticulum, *Mol. Biol. Cell*, **14**, 4783-4793.

17. Friedman, R. M., and Kohn, L. D. (1976) Cholera toxin inhibits interferon action, *Biochem. Biophys. Res. Commun*., **70**, 1078-1084.

18. Belardelli, F., Ausiello, C., Tomasi, M., and Rossi, G. B. (1980) Cholera toxin and its B-subunit inhibit interferon effects on virus production and erythroid differentiation of Friend leukemia cells, *Virology*, **107**, 109-120.

19. Besancon, F., and Ankel, H. (1974) Binding of interferon to gangliosides, *Nature*, **252**, 478-480.

20. Besancon, F., Ankel, H., and Basu, S. (1976) Specificity and reversibility of interferon ganglioside interaction, *Nature*, **259**, 576-578.

21. Baldauf, K. J., Royal, J. M., Hamorsky, K. T., and Matoba, N. (2015) Cholera toxin B: one subunit with many pharmaceutical applications, *Toxins*, **7**, 974-996.