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Prothymosin α receptors on peripheral blood mononuclear cells

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Abstract

^{125}I-Labeled prothymosin α (ProTα) was used to study the presence and characteristics of receptors for ProTα on human peripheral blood mononuclear cells (PBMC). The kinetics of ^{125}I-ProTα binding to PBMC was fast at 37°C, whilst it required 50 min to reach equilibrium at 4°C and room temperature. Analysis of steady state binding data by the method of Scatchard and by unlabeled ProTα competition experiments identified two binding sites with an apparent equilibrium dissociation constant of 216–321 pM for the high-affinity receptor and of 11.4–21.1 nM for the low-affinity one; the sites per cell ranged from 1,479 to 1,519 and from 47,547 to 56,169, respectively. The kinetically derived equilibrium dissociation constant agreed with these data and showed no interaction between receptors.

Key words: ^{125}I-ProTα; Peripheral blood mononuclear cell; Binding site; Dissociation constant

1. Introduction

Prothymosin α (ProTα) is a highly acidic polypeptide first isolated from rat thymus by Haritos et al. [1] as a putative precursor of thymosin α_1 ($T\alpha_1$). Calf thymus ProTα contains 109 amino acid residues and is identical to human ProTα except for having glutamic acid instead of aspartic acid at position 31 and alanine instead of serine at position 83 [2].

ProTα was called 'thymic hormone' because it was detected in blood serum [3], although all evidence accumulated during the past years indicates that ProTα plays an intracellular role linked with cell proliferation [4,5] and there is no agreement on the question of whether it serves as a precursor for secreted thymic peptides ($T\alpha_1$ and others) or is itself secreted [6–9]. However, ProTα presents immunopharmacological properties. In in vivo experiments it was shown to protect mice infected with *Candida albicans* and to increase the production and release of migration inhibitory factors by mononuclear cells [10]. ProTα has also been shown to be active in vitro by enhancing T lymphocyte proliferative responses in patients with autoimmune deseases [11,12], and increased the PHA-induced proliferation of fresh human peripheral blood mononuclear cells (PBMC) [13] by enhancing the IL-2R expression in these cells. Recently, it was demonstrated that ProTα increases HLA-DR surface Ag expression and mRNA accumulation in cultured human monocytes, U937 human monocytic cell line and human B cell lines [14]. Moreover, ProTα enhances human Natural Killer cell activity [15], restores depressed cytotoxicity in patients with cancer [16] and synergizes with IL-2 in the induction of Lymphokine Activated Killer cells [17].

We report herein the availability of ^{125}I-ProTα with high specific activity and high biological activity that allowed us to characterize the presence, number, affinity and kinetics of two receptors with high and low affinities for bovine ProTα in human peripheral blood mononuclear cells.

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Abbreviations: ProTα, prothymosin α; $T\alpha_1$, thymosin α_1; PBMC, peripheral blood mononuclear cells; BSA, bovine seroalbumin; PBS, phosphate buffered saline; GAM-FITC, goat anti-mouse antibody–fluorescein isothiocianate; SLE, systemic lupus erithomatosus; IFN, interferon; IL, interleukin; IL-R, interleukin receptor; PHA, phytohemagglutinin; VIP, vasoactive intestinal peptide.

2. Materials and methods

2.1. Labeling of ProTα with ^{125}I

Prothymosin α isolated from calf thymus and tested for purity by amino acid analysis and high voltage electrophoresis was purchased from Peninsula Laboratories (England) and was endotoxin free according to the Limulus assay. 5 μg of ProTα in 10 μl of borate buffer were reacted with 0.3 μg of Sulpho-SHPP (Sulphosuccinimidyl-(hidroxyphenyl)propionate) supplied by Pierce Europe (Holland) for at least 12 h at 4°C as described [18]. Iodination of esterificated lysines proceeded by Iodobeads (Pierce), a modification of the T chloramine method first described by Markwell [19]. We add our dissolved protein (10 μg) per mCi) to the reaction vial with the Na^{125}I solution (Du Pont NEN, Germany), according to the manufacturers instructions. The incorporation was always between 21 and 36% (mean 27%) after eliminating the free iodinated radicals, reaching specific activities in the range from 21 to 36 μCi/μg (between 2 and 3×10^{14} cpm/mmol).

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2.2. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)
Radioiodinated and purified ProTα, were analyzed on 15% gels by SDS-PAGE according with the method of Laemmli [20] with or without 5% 2-mercaptoethanol. The analysis by autoradiography revealed a major band with an M_r of around 13 kDa and secondary weaker bands of around 36–38 kDa and 60–65 kDa (Fig. 1), both in reducing and non-reducing conditions. This data agrees with the experimental data for gel filtration which we [21] and others [22] have obtained, deducing that ProTα presents a strong aggregating tendency.

2.3. ProTα bioassays
The biological activity of ^{125}I-ProTα was checked in two different ways:
(1) In our previous work we had optimized an ELISA assay [18] considering the presence of specific anti-ProTα antibodies in sera from systemic lupus erythematosus (SLE) patients. In order to check the antigenic recognition of ^{125}I-ProTα, 200 ng of labeled or unlabeled protein were added per well to polyvinyl titulation plates, and the ELISA assay developped. The antigenic recognition of the autoantibodies never decreased below 15% (comparing the absorbances).
(2) ProTα increases the expression of the p70 chain of IL-2R [15] in YT cells (an NK-like cell line established from a patient with acute lymphoblasic lymphoma and thymoma [23]) and for this reason we compared the activity of ^{125}I-ProTα to that of unlabeled protein. 5×10^6 YT cells were resuspended in culture medium as already described in the presence of labeled or unlabeled protein for 6 h. Fluorescence analysis was performed as described [15], and the results analyzed in a Coulter EPICS V flow cytometer using FACS and Research and Consort 30 software. The percentage of ProTα that remained biologically active in this assay after iodination was between 75 and 100%.

2.4. ProTα receptor binding assay
PBMC (95 ± 5% lymphocytes and monocytes) obtained from heparinized blood of normal adult volunteers collected by Ficoll-Paque gradient centrifugation as described [15] were resuspended in binding buffer (RPMI 1640, 5% FCS and 25 mM HEPES, pH 7.4) at a cellular density of 10×10^6 cells/ml and incubated with various concentrations of ^{125}I-ProTα from 70 pM to 28 nM at 4°C or room temperature for the designated times. In the competitive binding experiments the cells were incubated with a constant amount of radiopeptide (70 pM) in the presence of 0 to 20 nM of ProTα at room temperature for the designated times. Cell-bound radioactivity was separated from free radioactivity by centrifugation of the assay mixture through cold FCS for 90 s at $10{,}000 \times g$ and the tip containing the cell pellet excised. Both radioactivities were measured in a gamma counter (Gamma counting system, Kontron). Nonspecific binding was determined by inclusion of at least 50–100 times more unlabeled protein in each assay. The incubations were always carried out on two duplicated samples. Receptor binding data were analyzed by using the nonlinear regression programs EBDA, Ligand and Kinetic [24] as adapted by McPherson [25] for PCs (from Elsevier BIOSOFT, Cambridge, U.K.).

3. Results and discussion

^{125}I-ProTα recognized binding molecules or sites in the surface of the lymphocytes depending on the time and temperature, and it did it in a saturable and reversible manner.

3.1. Specificity of radiolabeled ProTα binding to PBMC
The specificity of ^{125}I-ProTα binding to PBMC was established by performing binding assays in the presence of the proteins with the highest competition potential regarding structure and biological function. Thus, unlabeled ProTα inhibited the recognition of radiolabeled ProTα (as we will later explain in the competition tests) at IC_{50} concentration of 1.2 nM; also, sera from patients of SLE containing autoantibodies against ProTα [18] did it at 1:20 dilution. However, the presence of Tα1 at concentrations of up to 1 μM and cytokines IL-2 at concentrations of up to 200 nM, IFNγ and IFNα at concentrations of up to 250 nM, IL-1β at concentrations of up to 40 nM and PHA and BSA control peptides at concentrations of up to 250 nM, did not block this binding significantly (data not shown).

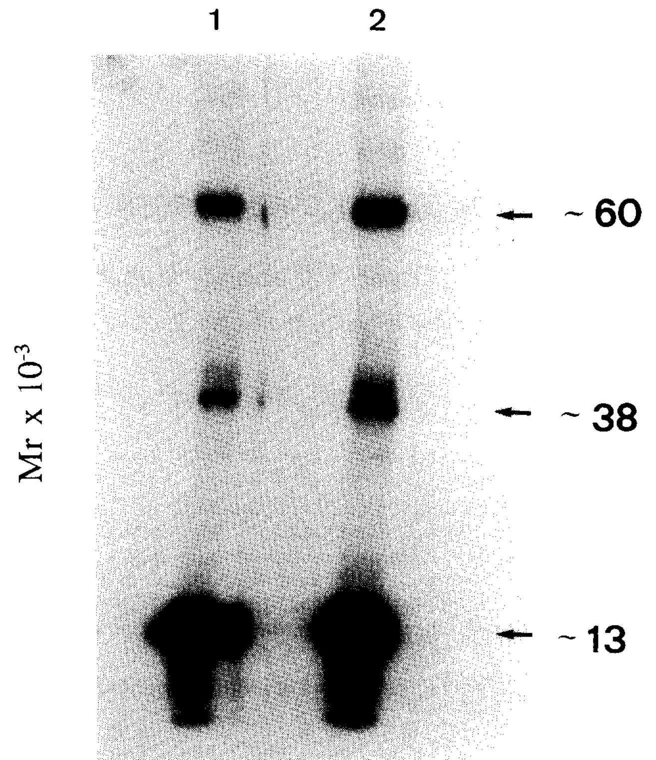

Fig. 1. Analysis of ^{125}I-ProTα in SDS-PAGE. The labeled and purified ProTα was treated using a sample buffer with (channel 2) and without (channel 1) 5% 2-mercaptoethanol and was run in gels of 15% as described in section 2. The molecular weight indicated in the margin of the figure was estimated from protein standards (BioRad) that were electrophorized in parallel channels.

3.2. Kinetics of radiolabeled ProTα binding to PBMC
The binding of ^{125}I-ProTα to the cellular surface was fast at both 4°C (data not shown) and room temperature (Fig. 2), reaching equilibrium after around 60 min. At 37°C the binding was initially faster and equilibrium was reached after 10 min, but the maximum binding seen at 4°C or room temperature was not achieved (data not shown). This lower level of binding can be related to the internalization and degredation of the ligand and also to its faster dissociation. The binding dependence of temperature is also very frequent in many receptors of growth factors [26].

The association kinetics for the binding of ^{125}I-ProTα to these cells at room temperature (see Fig. 2A) were determined at three concentrations: 2, 4 and 8 nM. As the concentrations of the free ligand did not significantly

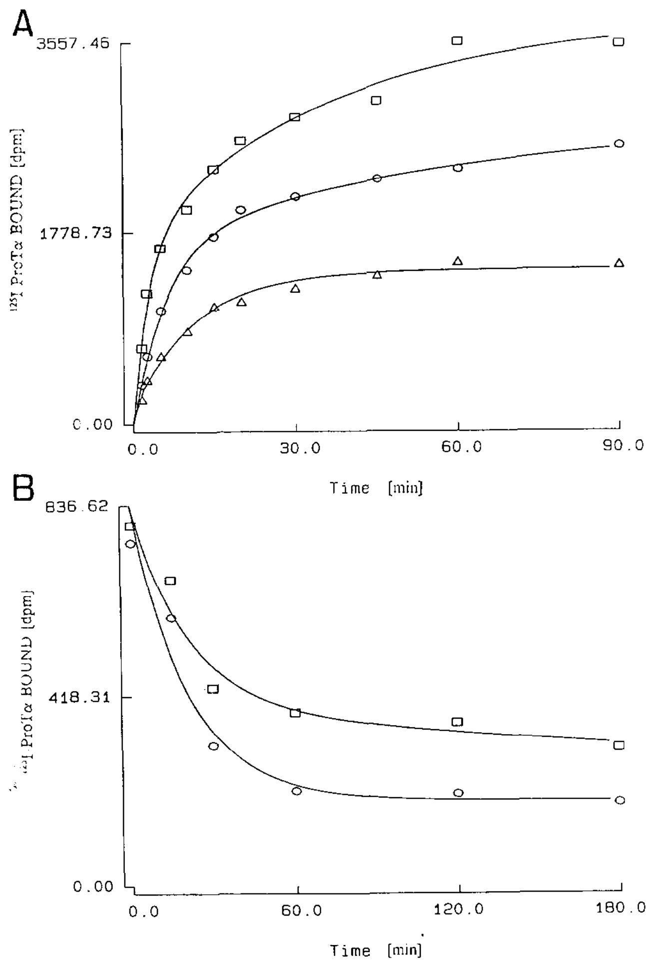

Fig. 2. Kinetics of (A) Association of 2 (△), 4 (○), and 8 (□) nM. (B) Dissociation of ^{125}I-ProTα to PBMC at room temperature. 12.5×10^6 cells/ml were incubated with the radiopeptide for 1 h. (A) At the indicated times samples were harvested and the binding assays were performed as described in section 2; the non-specific binding was measured in the presence of 100 times more unlabeled ProTα and is not shown in the graphs. (B) After this time the cells were washed and separated into two groups, one that was resuspended in binding buffer (□) and another in binding buffer containing 500 nM of unlabeled ProTα (○); the cells were kept at room temperature and at the times indicated, samples needed for the binding assays were collected. The continuous curves passing through the data, expressed as dpm (decompositions per minute) vs. time, are calculated by means of the program Kinetic [25] with a two-site model.

decrease (more than 5%) as a result of their binding to the cells, we carried out the experiments under pseudo first order conditions. We have analyzed the curves of Fig. 2 by means of the Kinetic [25] computer program for the determination of actual association constants (K_1) or the dissociation constants. The data from Fig. 2A were significantly consistent for a two-receptor model in PBMC. The observed association constants (K_{obs}) according to the program for each concentration were 0.216, 0.169 and 0.371 min^{-1} for the high-affinity receptor and 0.039, 0.021 and 0.032 min^{-1} for the low-affinity receptor. Fig. 2B shows the dissociation of ^{125}I-ProTα at room temperature that also displays two stages, one faster than the other. Although the one receptor model is accepted, the two receptor model is more appropriate, but not significantly so. It can be appreciated that without unlabeled ProTα competing (where only a fraction of the receptors are thus occupied) the dissociation is slower than when ProTα is present in excess, in which case most of the receptors are occupied; the average binding half-life times were 42 min in the absence of unlabeled protein and 27 min when ProTα was present.

The average of the values of the dissociation constants (K_{-1}) provided by the program were 4.8 ± 3.1 and $4.9 \pm 2.1 \times 10^{-2}$ min^{-1}, respectively, for the fast component and 2.3 ± 1.2 and $3.3 \pm 0.1 \times 10^{-3}$ min^{-1}, respectively, for the slow component of the curve. Comparing the constants in the presence of excess ProTα we can conclude that there is no specific sensitivity to the occupation of the receptors that may indicate the presence of cooperativity between them.

We determined the actual association constants at room temperature from the observed association data, the ligand concentration and the dissociation constants as described [25]. Thus, the values for the real association constants of ^{125}I-ProTα were 5.1 ± 2.8 and $0.9 \pm 0.78 \times 10^{-5}$ $pM^{-1} \cdot min^{-1}$, respectively, in the high- and low-affinity receptors. Kinetic K_Ds that were calculated as a ratio of dissociation and association kinetic rate constants for 4 individuals ranged from 167 to 750 pM and 778 pM to 1.6 nM, respectively, for the high-affinity and the low-affinity receptor in PBMC. For both types of receptors the kinetic data indicate that the association values are of the order of 10 times higher than those corresponding to dissociation which causes the relatively high K_Ds when compared to the concentration of ProTα at which their biological activities are maximized (around 14 nM) [15]. This combination of fast association and slow dissociation values at temperatures between 4°C and room temperature is also frequent in many cytokine receptors [27].

3.3. *Equilibrium binding analysis of radiolabeled ProTα binding to PBMC*

The steady state binding of ^{125}I-ProTα to PBMC at room temperature was saturable as shown in Fig. 3. Transformation of the data by the method of Scatchard [24] resulted in two straight lines, revealing two types of binding sites in these cells with different affinity for labeled protein, with an apparent K_D of 64 pM and 1,166 sites per cell for the high-affinity receptor and 1.48 nM and 53,002 sites per cell for the low-affinity one. The K_Ds and number of sites per cell for 7 individuals ranged between 38–208 pM and between 883–1,479 sites for the high-affinity site, and from 12.4–18.2 nM and 47,547–58,457 sites, respectively, for the low-affinity one.

The affinity of ProTα for its receptor on PBMC can also be estimated from unlabeled ProTα competition ex-

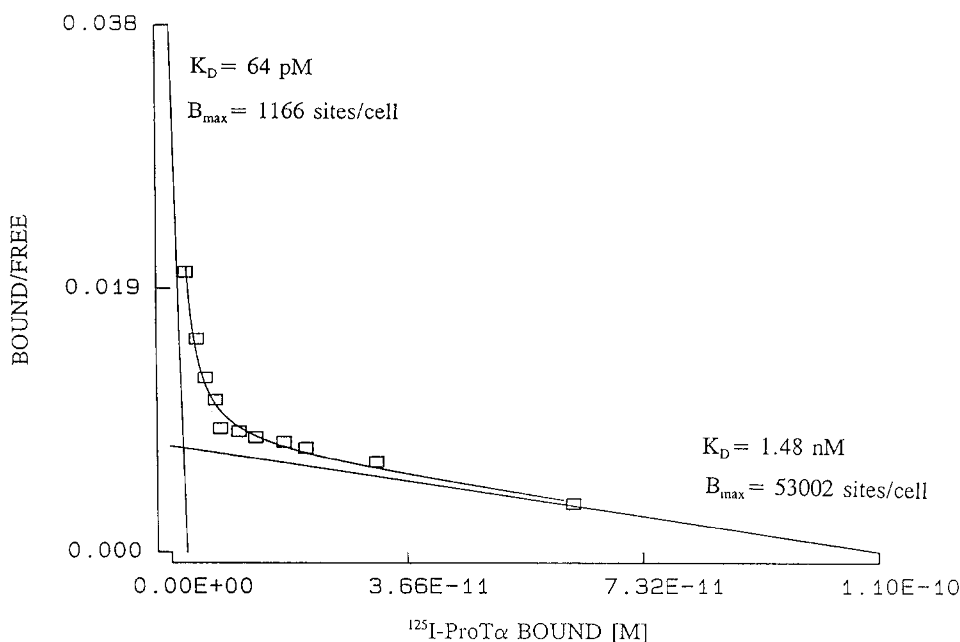

Fig. 3. Equilibrium condition binding of ^{125}I-ProTα to PBMC at room temperature. Cells, 12.5×10^6 PBMC/ml were incubated for 1 h with increasing concentrations of ^{125}I-ProTα in the presence or absence of 1 μg/ml of ProTα. Specific and total binding were calculated as described in section 2. The plot shows analysis of the specific binding data (which express the percentage of bound protein with respect to free protein for each protein concentration, expressed as molarity) according to the method of Scatchard as determined by Ligand [24,25] with a two-site model.

periments. In 4 experiments at room temperature, by adding increasing amounts of the unlabeled ligand we obtained an inhibition of the ^{125}I-ProTα binding. The computer analysis of this data provided K_I which ranged between 216–321 pM and 11.4–21.1 nM for each one of the sites. It also identified between 1,519–2,257, and between 42,822–56,169 sites per cell (Fig. 4), which is also in good agreement with the K_Ds and sites per cell gener-

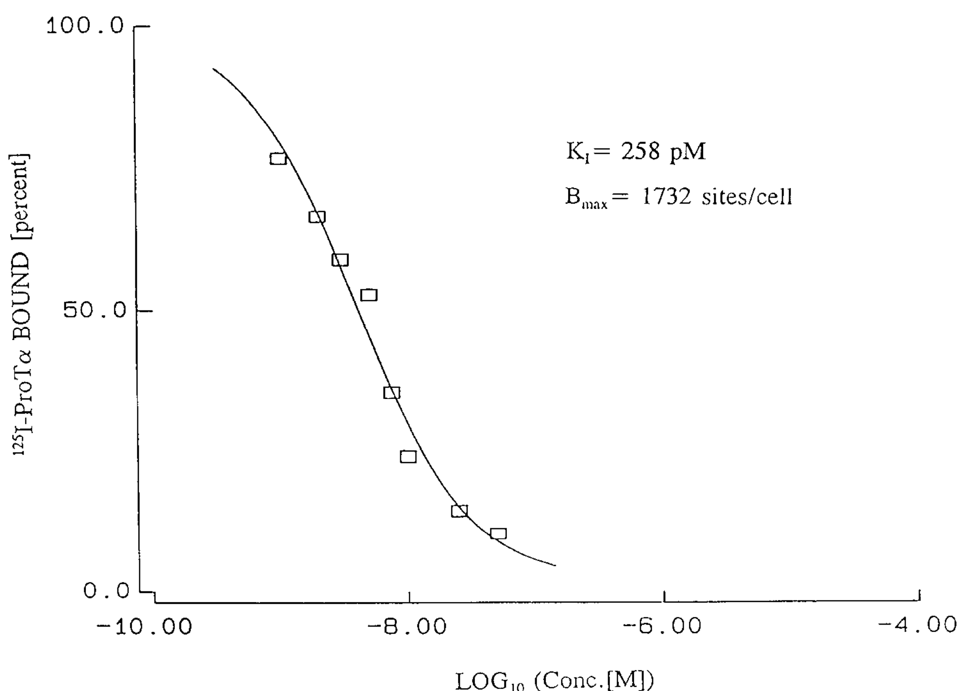

Fig. 4. Competitive binding of ^{125}I-ProTα to PBMC by unlabeled ProTα. The cells, 12.5×10^6 PBMC/ml, are incubated with 4 nM of radiopeptide in the presence of 0 to 100 times more unlabeled protein for 1 h at room temperature. The radioactivity of the cell is determined as described in section 2. The data represents the specific binding of ^{125}I-ProTα and is expressed as the amount of labeled protein that is bound (%) in the presence of competing ProTα (Log[C] expressed in M). The continuous curve passing through the data was calculated by the Ligand program [24] for the high-affinity site using the K_D value of 64 pM. The representation for the low-affinity site on PBMC (data not shown) assumed a K_D of 1.48 nM.

ated by steady state binding at room temperature and provides additional evidence that ^{125}I-ProTα interacts with the receptor in a fashion comparable to unlabeled ProTα.

The slightly higher K_D measured by the method of Scatchard vs. the measurement of K_D by kinetic-binding experiments may reflect the difficulty in establishing equilibrium binding for such a large number of receptors. In addition, the K_Ds calculated by kinetic or equilibrium analysis were studied in different cellular preparations which also cause a relatively large variation in the ranges of their values; so if we compose intermediate values, for the high affinity receptor the K_Ds would fall between 167 and 321 pM, and, for the low-affinity one between 1.6 and 11 nM (Table 1). The composite value for the K_D of PBMC agrees very well with the amount of ProTα required for its maximum bioactivity (14 nM), showing that the occupation of almost all the receptors is necessary in order to achieve it.

We can not conclude if the two binding sites in lymphocytes for ^{125}I-ProTα form a double-chain receptor, or perhaps the site with lower affinity will not be specific to ProTα. We can say that the ProTαR is basically found in T lymphocytes (data not shown) but we cannot exclude the possibility of finding it in other subpopulations where we and others [14,15] have appreciated interactions with ProTα, for example monocytes and killer cells. Further characterization and isolation of the ProTαR on PBMC is needed to address these and other questions on the structure of the ProTαR and on the molecular mechanism of action of ProTα on the immune cells because these findings might prove useful in future therapeutic interventions.

Table 1
Ranges of dissociation constants and number of higher and lower affinity binding sites on PBMC

	K_D	B_{max} (sites/cell)
Higher affinity		
Calculated kinetic (K_{-1}/K_1)[a]	167–750 pM	
Equilibrium[b]	38–208 pM	883–1479
Competition[c] (K_1)	216–321 pM	1519–2257
Lower affinity		
Calculated kinetic (K_{-1}/K_1)[a]	778 pM–1.6 nM	
Equilibrium[b]	12.4–18.2 nM	47547–58457
Competition[c] (K_1)	11.4–21.1 nM	42822–56169

[a] At the indicated times, samples of 12.5×10^6 cells/ml incubated with the radiopeptide were gathered and performed the binding assays as described in section 2. The continuous curves passing through the data were calculated using the Kinetic program [25] from which we determined the K_{-1} and the K_{obs} for each curve with a two sites model.
[b/c] Cells, 12.5×10^6 PBMC/ml were incubated for 1 h with increasing concentrations of ^{125}I-ProTα in the presence or absence of 1 μg/ml or ProTα, or incubated with 4 nM of radiopeptide in the presence of 0–100 times more unlabeled protein for 1 h at room temperature, respectively ([b]from 7 experiments, [c]from 4 experiments). Specific and total binding were calculated as described in section 2. Analysis of the specific binding data by the method of Scatchard was determined by Ligand [24,25] with a two-site model.

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