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Pharmacology

Regulation of receptor internalization by the major histocompatibility complex class I molecule

(peptide/major histocompatibility complex antibody/insulin receptor/glucose uptake)

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ABSTRACT **We showed previously that peptides derived from the α1 domain of the major histocompatibility complex class I protein (MHC-I) inhibit internalization of some receptors, thereby increasing the steady-state number of active receptors on the cell surface. In consequence, sensitivity to hormone (e.g., insulin) is enhanced, transport (e.g., of glucose by GLUT-4) is increased, and carrier proteins (e.g., transferrin) operate less efficiently. Now we report that a bioactive peptide (but not closely related inactive ones) binds to MHC-I on the cell surface, not in the groove but apparently to the α1 helix. The binding is saturable, and the number of peptide binding sites on the cell surface approximately equals the number of MHC-I molecules. Antibodies to MHC-I inhibit peptide binding. Most significant, antibodies to MHC-I mimic the effect of a bioactive peptide, inhibiting receptor internalization. These results indicate that MHC-I participates in the regulation of cell surface receptor activity.**

Synthetic peptides derived from the sequence of the α1 domain of the major histocompatibility complex class I protein (MHC-I) (Fig. 1) inhibit the internalization of certain cell surface receptors. For the insulin receptor and GLUT-4 glucose transporter, which have been studied in detail (4–7), the result is an increased steady-state number of active receptors and transporters on the cell surface. Both the intrinsic activity of each receptor (or transporter) molecule and the rate of translocation from the cytoplasm to the membrane are unchanged. A necessary, but not sufficient, requirement for bioactivity is that a peptide assumes an ordered (largely α-helical) conformation prior to its interaction with cells (1, 2, 8).

As a first step toward understanding the mechanism of action, we now ask: To what cellular component does a bioactive peptide bind when it inhibits receptor internalization?

MATERIALS AND METHODS

Unless otherwise noted, all data presented here are the mean ± SEM from three or four experiments, with each point determined in triplicate or quadruplicate.

For binding experiments with whole cells, peptide solutions were sonicated on ice for 1 min at 14 μM or 250 μM for ^{125}I-labeled A85 and unlabeled A85, respectively. Sonication was to disrupt peptide aggregates to increase the fraction of peptide molecules capable of binding to cells. However, sonicated peptide loses its ability to bind to cells within 20–30 min (data not shown).

An antibody (αPep) to A85 was generated in a rabbit by immunization with keyhole limpet hemocyanin-conjugated A85 in Freund's incomplete adjuvant and purified on an A85

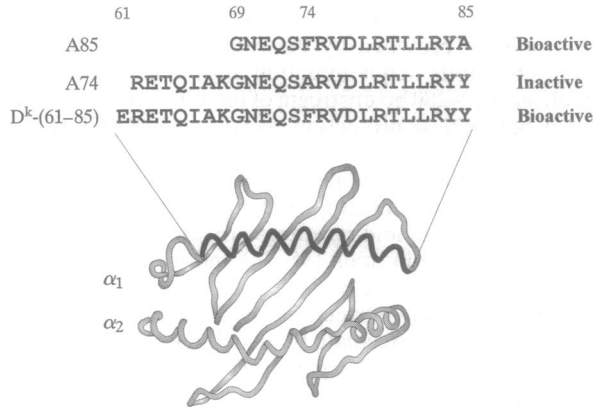

FIG. 1. Sequences of bioactive D^k-(61–85) and [Ala^{85}]D^k-(69–85) (abbreviated A85) and of the inactive peptide [Ala^{74}]D^k-(62–85) (abbreviated A74) showing their derivation from the α1 domain of MHC-I. The synthesis and characterization of the peptides have been described elsewhere (1, 2). In contrast to D^k-(61–85) and D^k-(69–85) (1), A85 can be iodinated without loss of biologic activity. A74 lacks an ordered conformation in solution (1). The structure of MHC-I is from Bjorkman *et al.* (3).

affinity column. The number of ^{125}I-labeled αPep binding sites was determined on rat adipocytes preincubated with A85 (0, 0.24, 0.67, 1.8, and 5.0 μM) for 30 min at 37°C and washed free of unbound peptide. Binding of αPep to A85 was also examined in the absence of cells as follows: Peptide solution at 100 μM was incubated with ^{125}I-labeled αPep in the absence or presence of various amounts of unlabeled αPep antibody for 30 min at 37°C and then centrifuged for 10 min at 10,000 × *g*. The amount of αPep specifically bound to A85 aggregates was determined as the difference between total radioactivity in the sediment in the absence and presence of unlabeled αPep antibody. A similar set of experiments was done with ^{125}I-labeled A85 but without αPep to determine the number of A85 molecules in the sediment.

For experiments with antibodies to MHC-I, OX-18 (Serotec) and anti-H-2K^k (see below) were used. Experiments with H-2 allele-specific antibodies (provided by D. Sachs, Harvard Medical School) used mouse adipocytes and spleen cells of H-2^b haplotype (C57BL/6 mouse). HB-16 antibody (American Type Culture Collection) was radioiodinated (200 Ci/mmol; 1 Ci = 37 GBq) using Iodo-Beads (Pierce). Mouse lymphoid spleen cells were prepared by mincing spleens in ice-cold phosphate-buffered saline (PBS) and lysing erythrocytes with NH_4Cl. Cells were incubated with various concentrations of ^{125}I-labeled HB-16 antibody (adipocytes for 30 min at 37°C and spleen cells for 60 min on ice) in the absence

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Abbreviations: A85, [Ala^{85}]D^k-(69–85); A74, [Ala^{74}]D^k-(62–85); IGF, insulin-like growth factor; MHC-I, major histocompatibility complex class I protein; sK^b, truncated soluble K^b molecules; VSV, vesicular stomatitis virus.

or presence of excess unlabeled antibody, and cell-associated radioactivity was determined.

Monoclonal antibodies to the α1, α2, and α3 domains of mouse MHC-I (provided by M. Zuniga, University of California, Santa Cruz) were tested for their ability to inhibit internalization of insulin-like growth factor (IGF) II receptors in mouse adipocytes. Cells were incubated for 30 min with insulin (8 nM) and A85 (30 μM) or one of the antibodies (purified antibodies, 20 μg/ml; ascites fluid, 1:50 dilution) and then for an additional 30 min with ^{125}I-labeled IGF-II, followed by an acid wash on ice to remove radioligand bound to surface receptors.

For experiments on self-association and direct binding of A85 to purified mouse MHC-I (provided by S. Buus, University of Copenhagen), a dot-blot assay was used, as described in Table 2.

To study whether a viral peptide competes with A85 for binding to purified MHC-I that lacks peptide in the groove, a truncated soluble K^b (MHC-I) molecule (sK^b) and labeled viral peptide, ^{125}I-labeled vesicular stomatitis virus (VSV) nucleocapsid protein (VSV-8A, residues 52–59, AGYVYQGL, 2000 Ci/mmol), both furnished by P. A. Peterson, were used. In other experiments the binding of radiolabeled VSV–MHC-I complexes to immobilized A85 on an agarose column was determined.

RESULTS

Binding to Whole Cells. The bioactive peptides inhibit the internalization of receptors belonging to several families. In addition to the insulin receptor and GLUT-4 transporter, these include the receptors for IGF-I, IGF-II, transferrin, epidermal growth factor, low density lipoprotein, and the macrophage scavenger receptor (unpublished data). Although the peptide sequences are derived from a mouse MHC-I, the cell types affected include rat, mouse, and human adipocytes, rat and human fibroblasts, and mouse macrophages.

This broad range of action made it necessary to rule out a nonspecific effect on fluid-phase endocytosis. Uptake of [^{3}H]insulin (0.5 μM, 3 Ci/mmol) by rat adipocytes in the absence and presence, respectively, of 30 μM bioactive D^k-(61–85), was (as percent of basal uptake at 60 min) at the following times: 0 min, 28 ± 9 and 26 ± 9; 20 min, 65 ± 3 and 71 ± 9; 40 min, 83 ± 3 and 93 ± 12; 60 min, 100 and 107 ± 13. As this concentration of peptide causes maximal inhibition of internalization of insulin receptors and GLUT-4, the effect cannot be attributed to a nonspecific reduction in molecular traffic between the plasma membrane and cytosol.

Is the effect mediated at a specific binding site on the cell membrane? An important feature of the bioactive peptides is that they form large aggregates (1). These do not bind to cells but are found in a centrifugal infranatant, whereas adipocytes and bound peptide float on top of the oil layer. On the other hand, bioactive peptide solutions become inactive upon depletion of peptide aggregates by centrifugation (10,000 × *g* for 10 min). The peptide that binds to cells must, therefore, be released from an aggregate reservoir, which evidently serves to stabilize the necessary conformation of a monomer (or oligomer), as described (1, 8). Dialysis experiments with 1 μM ^{125}I-labeled A85 revealed that the dissociation rate of aggregates was only about 2% per hr. Likewise, no significant dissociation of bound peptide from cells occurs within the first 90 min after removal of free peptide by wash. This unmeasurable dissociation and the unknown concentration of unaggregated peptide make it impossible to estimate an actual K_d value from the binding isotherm in Fig. 2, but the true value is obviously lower than the apparent 1 μM.

Specific binding is saturable with an apparent B_{max} value on the order of 10^8 per cell (Fig. 2). However, as the peptide self-interacts, binding of a peptide molecule to its cellular

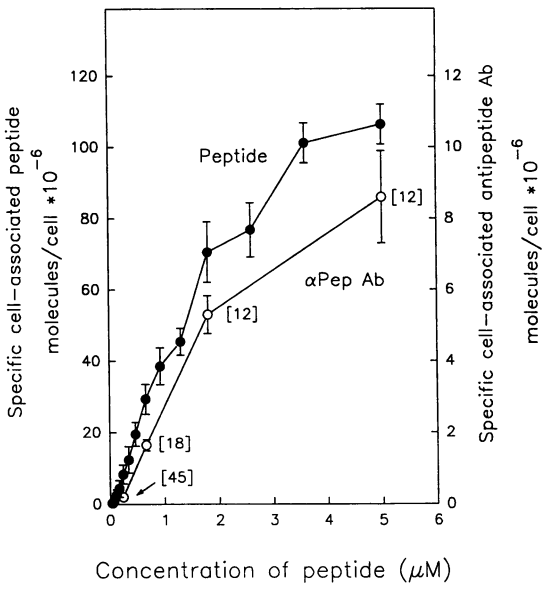

FIG. 2. Binding of A85 to rat adipocytes. Cells were incubated for 30 min at 37°C with the indicated concentrations of ^{125}I-labeled A85 added in the absence or presence of 30 μM unlabeled peptide. Specific cell-associated radioactivity was determined as total binding minus binding in the presence of unlabeled A85; nonspecific binding was <10% of total. No cooperativity was observed (Hill coefficient, 1.2). In five experiments, A74 (30 μM) reduced specific binding by only 2 ± 6%; at the same concentration, unlabeled A85 eliminated specific binding completely. Antibody to A85 (αPep) recognizes peptide already bound to cells, after removal of free peptide by washing. Specifically bound αPep was determined by competition of unlabeled αPep against ^{125}I-labeled αPep, in absence of A85 and at four concentrations of A85. The ratios of bound A85 to bound αPep are given in square brackets. As antibody was in excess, it is assumed that each antibody binds to only one epitope. In the absence of cells, the ratio of A85 molecules to precipitated αPep was found to be 11 ± 1.

site(s) is likely to form a nucleus for formation of a peptide aggregate *in situ*, with several peptide molecules forming a stack on a single binding site, so that B_{max} will be higher than the number of binding sites.

To determine the average stack size, we used an affinity-purified rabbit antibody to A85 (αPep). As αPep does not recognize D^k-(69–85), which is identical except for Tyr^{85}, the epitope must include the C-terminal residues of A85. αPep binds to A85 even after the peptide has bound to cells. Fig. 2 shows that the number of cell-associated αPep is about 10^7 per cell when 1.2×10^8 peptide molecules are bound. Thus, as indicated in square brackets, the ratio of bound peptide to bound αPep antibody is ≈12:1 at B_{max}. This ratio is close to that obtained for αPep binding to peptide in the absence of cells but in the presence of an excess of αPep.

These results suggest that αPep recognizes a stack of about 12 peptide molecules. The ratios of peptide to antibody at various degrees of saturation of the peptide binding sites (Fig. 2) shed light on the mode of binding. Clearly, not every bound peptide molecule is recognized by αPep, else the ratio would always be unity. If a preformed stack bound to each site, the ratio would be constant at all peptide concentrations. In fact, however, the ratio is high at low peptide concentrations and stabilizes at about 12 as the sites become saturated with peptide. We conclude, therefore, that the binding sites are first occupied by a monomer (or dimer) peptide, to which (probably because of steric hindrance) αPep has no access. Then, as peptide concentration increases, and a large enough stack is formed, αPep can bind; presumably, an antibody molecule binds to the most accessible (topmost) peptide of each stack, as with peptide aggregates in the absence of cells.

The EC_{50} value for biologic activity of A85 was previously reported to be about 1 μM (1). Fig. 2 shows that at this concentration, 4×10^7 peptide molecules are bound per cell, representing a stack size, on average, of about four.

Trypsin treatment releases 91 ± 6% of specific cell-associated ^{125}I-labeled A85 and 55 ± 1% is found in the plasma membrane fraction upon subcellular fractionation. Of the cell-bound radioactivity, 78 ± 4% is dissociated from cells by boiling for 15 min in 1 M acetic acid, and HPLC analysis of this material shows that the released peptide is intact (data not shown). These observations and the fact that αPep recognizes bound peptide indicate that most of the cell-associated peptide is bound to a component of the cell surface. Unfortunately, despite extensive efforts, we have been unable to cross-link sufficient amounts of bound ^{125}I-labeled A85 to specific molecules in the adipocyte plasma membrane.

Effects of Anti-MHC-I Antibodies. Fig. 3 shows that OX-18, an antibody to rat MHC-I, inhibits the specific binding of A85 to cells. This antibody does not bind to A85; and a specific antibody to murine H-2K^k had no effect on peptide binding to rat cells (data not shown). Significantly, in addition to inhibiting peptide binding, OX-18 enhances glucose uptake in a dose-dependent manner (Fig. 3). Furthermore, it (but not the anti-H-2K^k antibody) inhibits insulin receptor internalization (Table 1). It is noteworthy that the antibody concentrations for inhibition of peptide binding, inhibition of receptor internalization, and enhancement of glucose uptake are similar.

The number of MHC-I molecules on rat adipocytes is difficult to assess because iodination of OX-18 destroyed its ability to bind to cells. However, the number of a comparable MHC-I product (H-2K^k) on the surface of mouse adipocytes (5×10^6 molecules per cell) and lymphoid spleen cells (5×10^4 molecules per cell), as measured with labeled antibody HB-16, was found to be not too different (per unit surface area) from our estimate of the number of peptide binding sites on rat adipocytes.

The effect of allele-specific anti-MHC-I antibodies on ^{125}I-labeled A85 binding to mouse adipocytes was also tested. Two antibodies specific for the H-2^b products (9) (D and K, respectively) inhibited specific binding by 76 ± 12% and 49 ± 2%, respectively. The epitopes for both these antibodies are located primarily in the α1 domain of MHC-I, so their inhibition of peptide binding is consistent with a binding site on the α1 helix. On the other hand, an antibody to H-2D^k inhibited the peptide binding by only about 20% (probably reflecting crossreactivity to H-2^b products) and an antibody to H-2K^k had virtually no effect (6–12% inhibition).

Table 1. Effect of anti-MHC-I antibody OX-18 on internalization of insulin receptors by rat adipocytes

Antibody	% internalized
None	55 ± 3
OX-18 (0.3 μM)	55 ± 6
OX-18 (2 μM)	33 ± 6
Anti-H-2K^k	50 ± 6

Experimental design was as for glucose uptake (Fig. 3), and internalization of specifically bound ^{125}I-labeled insulin was measured by the acid-wash method described elsewhere (4).

Internalization of IGF-II receptors on BALB/c mouse adipocytes was inhibited 44 ± 6% by A85 and 19 ± 3% by an antibody to the α1 domain ($P < 0.01$ for both), but only 6 ± 1% and 5 ± 1% by antibody to α2 and α3, respectively. These results, like those noted above, implicate α1 in the receptor internalization process.

Binding to Purified MHC-I. Purified murine MHC-I products from different alleles were immobilized on nitrocellulose paper, and binding of ^{125}I-labeled A85 was examined. Binding of labeled A85 to unlabeled A85 (i.e., self-association) was also studied. Table 2 shows that ^{125}I-labeled A85 binds specifically not only to A85 but to all four H-2 products, and with no notable differences. Moreover, as shown, the inactive A74 did not compete.

The purified H-2 molecules used in these studies are generally assumed to contain peptides in the groove (10, 11), and displacement of such peptides with exogenous added molecules normally requires incubation for 24–48 hr. As the incubation time was only 1 hr in the present experiments, it seemed unlikely that A85 binds in the groove. However, it could not be excluded *a priori* that the ^{125}I-labeled A85 binding is confined to those MHC-I molecules that do not have peptide in the groove. We therefore carried out a direct test with sK^b from *Drosophila* cells (12–14), in which peptide binding in the groove can be controlled.

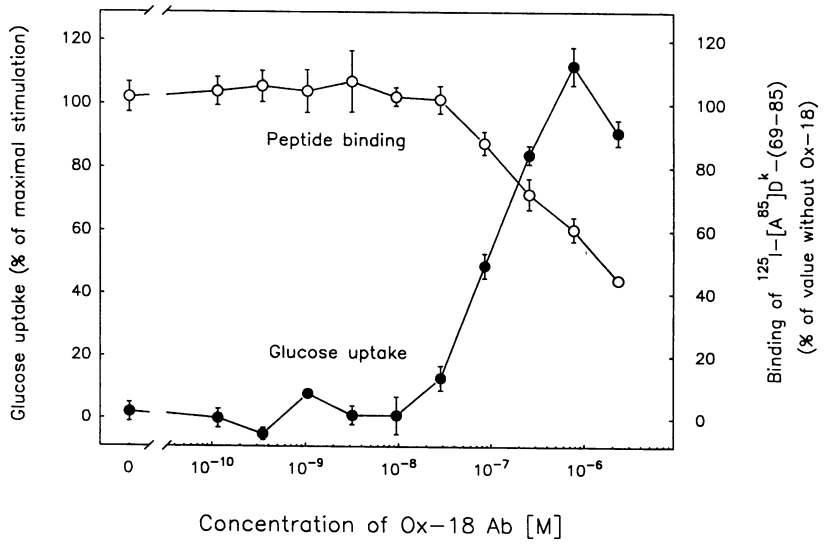

FIG. 3. Effect of OX-18 antibody on A85 binding to cells (open circles) and enhancement of glucose uptake (solid circles) in rat adipocytes. Cells were incubated for 30 min at 37°C at the indicated concentrations of antibody and then for another 10 min in the presence of 1 μM ^{125}I-labeled A85 with or without 30 μM unlabeled A85 to determine specific binding. For glucose uptake, the cells were incubated for 30 min at 37°C with the indicated concentrations of antibody, then treated as described elsewhere (4, 6). An antibody to murine MHC-I (anti-H-2K^k) had no effect on A85 binding or glucose uptake.

Table 2. Self-association of A85 and its specific binding to MHC-I

Substrate	Specific binding, cpm	
	− A74	+ A74
A85 (self-association)	3770 ± 510	3930, 4030
Purified MHC-I products		
D^b	1870 ± 570	1740, 1960
K^b	1960 ± 750	1490, 1720
D^k	1460 ± 360	1720, 1890
K^k	2450 ± 590	1720, 1930

A85 (3 μM) or purified MHC-I (0.16 μM) in 200 μl of Krebs–Ringer–Hepes buffer (KRH) was applied to each well of a dot-blot apparatus (Bio-Rad) that allows application of protein to nitrocellulose paper in a 4-mm-diameter dot. The nitrocellulose blot was dried and then incubated in KRH containing 5% (vol/vol) fetal calf serum and 0.05% Tween 20 for 1 hr at room temperature on a rocking platform to block unoccupied sites on the blot. This procedure removed about 50% of the peptide attached to the blot. After blocking, the blot was incubated (1 hr at room temperature in KRH with 5% bovine serum albumin) with 1 μM ^{125}I-labeled A85 in the absence or presence of 30 μM unlabeled A85. The blot was subsequently washed in KRH containing 0.05% Tween 20 and dried, and the individual dots were cut out and transferred to a γ counter. To test whether an inactive peptide competed for binding, A74 (30 μM) was added to ^{125}I-labeled A85 and unlabeled A85 in two experiments. Data are specifically bound cpm, i.e., the difference between binding of ^{125}I-labeled A85 in the absence and presence of unlabeled A85. In these experiments, the grooves of the purified MHC-I proteins are occupied by endogenous peptides.

A fragment of VSV, VSV-8A, binds with high affinity in the groove (12). Fig. 4*A* shows that A85 does not compete for binding of VSV to sK^b if the two molecules are added simultaneously (0 min on the figure). In contrast, preincubation of sK^b with A85 blocks the subsequent binding of VSV. The rate of binding of A85 to empty sK^b is slow, as estimated from the kinetics for inhibition of VSV binding. Preincubation with an inactive peptide did not block VSV binding.

This result could mean that A85 either binds in the groove of sK^b or induces a conformational change that eliminates the groove as binding site for VSV. However, Fig. 4*B* shows that the sK^b–^{125}I-labeled VSV complex (in which VSV is already in the groove) is retained on an A85 affinity column but not on a control column. The binding site for A85 is, therefore, different from the VSV binding site. Inasmuch as preincubation of empty sK^b with A85 prevented binding of VSV, we conclude that A85 binds in a manner that blocks access to the groove, but without itself being bound in the groove. A stack of several peptide molecules bound to the α1 domain would meet this criterion.

Binding to the α1 domain of MHC-I is consistent with the fact that bioactive peptide molecules self-interact (1). For both self-interaction and bioactivity, they require an ordered (largely α-helical) conformation. In contrast, an inactive peptide like A74 lacks an ordered conformation (1). Thus, helix–helix interaction with cell surface molecules having significant sequence similarity to the peptide itself is to be expected. The only such sequence similarity found in a data base search is in the α-helix of the α1-domain of MHC-I (1, 10).

DISCUSSION

The discovery of MHC-I was based on the allogeneic graft reaction; a more precisely defined role of MHC-I in the immune system has only been recognized within the last 15

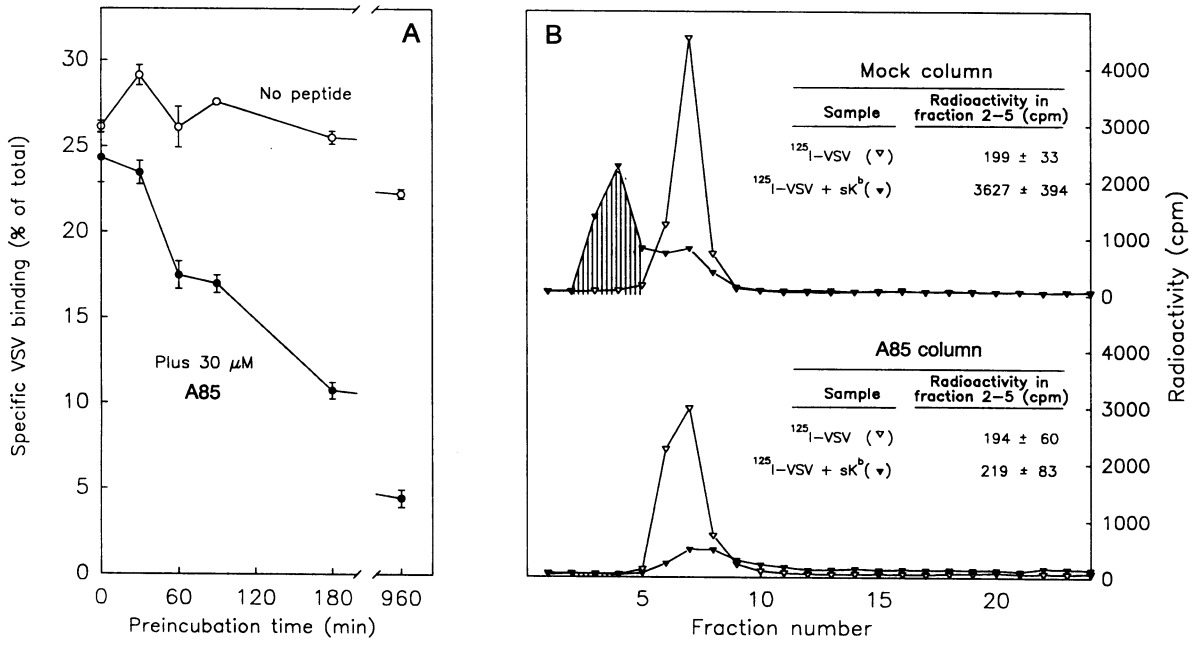

FIG. 4. Binding of viral peptide (VSV-8A) to purified MHC-I (sK^b) in presence and absence of A85. (*A*) Effect of preincubation of sK^b with A85 on binding. In these experiments, the groove of the purified MHC-I was initially unoccupied. sK^b (10 nM) was preincubated with A85 (30 μM) in KRH buffer with 1% bovine serum albumin and 0.1% NaN_3 for periods up to 16 hr. ^{125}I-labeled VSV-8A was then added in a final concentration of 100 pM in the absence or presence of 1 μM unlabeled VSV peptide, and the incubation was continued for another 60 min. The complexes of ^{125}I-labeled VSV with sK^b were separated from free ^{125}I-labeled VSV by gel filtration on Sephadex G-25 and the amount of radiolabel associated with sK^b was determined. Values shown are specific binding as percent of total labeled VSV. Preincubation with an inactive peptide, HLA-B27-(69–85), had no effect on binding of ^{125}I-labeled VSV to sK^b in four experiments: no peptide, 5270 ± 530; A85, 2350 ± 700; HLA-B27-(69–85), 4990 ± 400 cpm. (*B*) Binding of ^{125}I-labeled VSV–sK^b complexes to immobilized A85. A85 [5 ml, 1 mM in 60 mM $NaHCO_3$ buffer (pH 9.9)] was incubated with 1 g of epoxy-activated agarose resin (Pierce) for 16 hr at 37°C. Coupling efficiency was >80%. Mock coupling was performed without peptide. ^{125}I-labeled VSV alone or after being bound to sK^b by coincubation for 60 min (as above) was applied to the mock or A85 column and eluted with PBS, and the recovered radioactivity was determined in each fraction. Typical elution profiles are shown with recovered radioactivity in fractions 2–5 (hatched area and inset tables). ^{125}I-VSV, ^{125}I-labeled VSV.

years. Speculations about a nonimmunologic role of MHC-I have been advanced since the 1950s but have lacked experimental support. About 10 years ago, a molecular interaction between certain membrane receptors and MHC-I was suggested (9, 15–22), but these studies suffered from potential technical pitfalls such as insufficient solubilization of plasma membranes. Most important, the physiologic significance of the proposed complexes between MHC-I and receptors was unclear.

The availability of peptides derived from MHC-I sequences enabled us to study whether such peptides affect receptor activity. We showed (4, 6) that the peptides inhibit internalization of some receptors (including insulin receptors and GLUT-4 glucose transporters), but not others. These effects raised the question whether they reflect a natural activity of MHC-I in receptor regulation. Cells from animals lacking MHC-I heavy chain would offer an opportunity to test that hypothesis directly, but unfortunately, such animals are not available. Mice lacking β_2-microglobulin (23) still express the heavy chain (24–26) and presumably some nonclassical MHC-I products (27, 28), and they seem to have an intact allogeneic graft response (25).

A significant novelty in the action of a bioactive peptide is that it does not interfere with binding of a natural ligand to its receptor. A practical consequence is that conventional screening systems for identifying uncharacterized agonists and antagonists by competition with a known ligand at a classical receptor binding site will not detect compounds with activity like that of the bioactive peptides.

The key observations in the present paper may be summarized as follows: (*i*) Bioactive peptide binds in a saturable manner to the cell surface, but closely related inactive peptides do not. (*ii*) The numbers of peptide binding sites and of MHC-I molecules on the cell surface are comparable. (*iii*) Specific antibodies directed toward MHC-I prevent peptide binding to cells. (*iv*) Bioactive peptide (but not closely related inactive peptides) binds to purified MHC-I at a site that is different from the groove and likely to be the α1 domain. Interestingly, a recent publication (29) documents the binding of a superantigen to MHC-II at a site other than the groove. As self-interaction and aggregation are characteristics of a bioactive peptide, binding to the α1 domain would be tantamount to self-interaction. (*v*) Most significant, antibodies to MHC-I, like bioactive peptides, inhibit receptor internalization.

In conclusion, the evidence presented in this paper strongly suggests that it is the binding of peptide to MHC-I that results in inhibition of receptor internalization by interfering with a normal function of the MHC-I molecule. A bioactive peptide might block a direct interaction of MHC-I with certain receptors, which is necessary for their internalization (4). Alternatively, a bioactive peptide could block a necessary interaction of MHC-I with some other component of the internalization pathway, possibly involving caveolae and coated pits (30, 31).

Whatever the precise mode of action, MHC-I is evidently a key component of the internalization process. We suggest that the immunologic function of MHC-I (transport of peptides to the cell surface for presentation to T cells) may have evolved as a specialization of a more general primordial role in the trafficking of polypeptides between the plasma membrane and the cytosol.

We thank Dr. P. A. Peterson and colleagues (Scripps Research Institute) for providing the soluble K^b molecule and the VSV peptide, and for help in the assays and interpretation of the data; Dr. S. Buus (University of Copenhagen) for purified murine MHC-I products; and Dr. M. Zuniga (University of California, Santa Cruz) for antibodies to mouse MHC-I. We are grateful to Dr. C. B. Anfinsen and Joyce Lilly (Johns Hopkins University) for synthesizing the peptides. The continued support, criticism, and discussions by Drs. C. B. Anfinsen (Johns Hopkins University), S. W. Cushman (National Institutes of Health), E. Haber (Harvard Medical School), B. W. Matthews (Howard Hughes Medical Institute, University of Oregon), S. Ohno (Beckman Research Institute of City of Hope), G. M. Reaven and R. A. Roth (Stanford University School of Medicine), and M. Simonsen (University of Copenhagen) throughout the course of this project are deeply appreciated.

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