Studies on Activities of Invariant Chain Peptides on Releasing or Exchanging of Antigenic Peptides at Human Leukocyte Antigen-DR1

Minzhen Xu, Robert Jackson, Sharlene Adams, and Robert E. Humphreys

Antigen Express, Inc., Worcester, MA (USA)

Summary

An invariant chain peptide (Ii77–92; YRMKLPKSAKPVSQMR; 'Ii-Key') enhances 10–50 times baseline levels, the presentation of synthetic antigenic peptides to murine T cell hybridomas, by an exchange mechanism at cell surface MHC class II molecules. Two differing activities, to promote the release of antigenic peptide in the presence or absence in solution of a second antigenic peptide, were characterized with truncation homologs through assays for release or binding of human myelin basic protein biotinylated (*) peptide 90–102 on purified HLA-DR1: 1) release of bound hMBP *peptide from DR1 in the presence or absence of free hMBP peptide in solution, 2) exchange of hMBP *peptide from solution with hMBP peptide on DR1, and 3) binding of hMBP *peptide to 'empty' DR1. Peptides such as Ii81–88, LPKSAKPV, released prebound hMBP *peptide from DR1 without free hMBP peptide in solution. They also exchanged hMBP *peptide from solution for prebound hMBP peptide. Peptides including hIi77-83, LRMKLPK, released hMBP *peptide only when free hMBP peptide was in solution. Nevertheless, hIi77-85, LRMKLPKPP, released hMBP peptide without hMBP peptide in solution. Either type of peptide accelerated hMBP *peptide binding to 'empty' DR1. Competitive binding assays with hMBP *peptide or several *Ii-Key truncation homologs, with respective not biotinylated forms, demonstrated that the Ii77-83, LRMKLPK, binding site was distinct from the HLA-DR1 antigenic peptide binding site.

Zusammenfassung

Untersuchungen zur Aktivität von Peptiden der invarianten Kette im Hinblick auf die Freisetzung oder den Austausch antigener Peptide am humanen Lekozytenantigen DR1

Die Präsentation sythetischer antigener Peptide durch murine T-Zell-Hybridome wird durch ein Peptid der invarianten Kette (Ii77-92; YRMKLPKSAKPVSQMR; „Ii-Key") durch einen Austauschmechanismus an MHC Klasse II-Molekülen an der Zelloberfläche auf das 10- bis 50fache erhöht. Abhängig von der An- oder Abwesenheit eines zweiten antigenen Peptids in Lösung konnten zwei unterschiedliche Aktivitäten mit verkürzten Homologen von Ii-Key in einem Assay für Freisetzung oder Bindung von biotinyliertem (*) Peptid 90–102 von humanem basischen Protein von Myelin (hMBP) auf gereinigtem HLA-DR1 charakterisiert werden: 1. Freisetzung von gebundenem hMBP-*Peptid in An- und Abwesenheit von freiem hMBP-Peptid in Lösung, 2. Austausch von hMBP-*Peptid in Lösung an DR1 gebundenem hMHP-Peptid und 3. Bindung von hMBP-*Peptid an „leeres" DR1. Peptide wie Ii81–88, LPKSAKPV, führten zur Freisetzung von zuvor an DR1 gebundenem hMBP-*Peptid auch ohne freies hMBP-Peptid in Lösung. Solche Peptide führten auch zum Austausch von vorgebundenem hMBP-Peptid und hMBP-*Peptid in Lösung. Peptide wie hIi77-83, LRMKLPK, führten nur dann zur Freisetzung von gebundenem hMBP-*Peptid wenn freies hMBP-Peptid in Lösung vorhanden war. Nichtsdestoweniger wurde hMBP-Peptid durch hIi77-85, LRMKLPKPP, auch ohne hMBP-Peptid in Lösung freigesetzt. Beide Peptidtypen beschleunigten die Bindung von hMBP-*Peptid an „leeres" DR1. Kompetitive Bindungstudien mit hMBP-*Peptid und mehreren verkürzten – biotinylierten und nicht biotinylierten – Ii-Key-Homologen zeigten, daß die Bindungsstellen für Ii77-83, LRMKLPK, und antigenem Peptid auf HLA-DR1 unterschiedlich sind.

Key words Antigenic peptide · Ii-Key · Invariant chain peptide · Major histocompatibility complex class II

Arzneim.-Forsch./Drug Res. **49 (II),** 791–799 (1999)

Abbreviations

CLIP: class II-associated invariant chain peptide; HLA: human leukocyte antigen; Ii: invariant chain; MBP: myelin basic protein; MHC: major histocompatibility complex

1. Introduction

At synthesis in the endoplasmic reticulum, MHC class II molecules are bound by the invariant chain (Ii) which blocks the antigenic peptide binding site until the complex has been transported into a post-Golgi, antigenic peptide binding compartment [1, 2]. There the Ii chain is cleaved and released, by proteases which could also cleave antigenic proteins [3–5]. Antigenic peptide charging might proceed through two, not mutually exclusive, mechanisms. The first is by antigenic peptide exchange for a residual, proteolysis fragment of Ii, CLIP, hIi81–104 [6–9]. CLIP must be removed to permit antigenic peptide binding to MHC class II molecules [10]. HLA-DM (DM) is required for efficient peptide loading of MHC class II molecules [11]. Purified DM catalyzes release of CLIP peptides from, and binding of antigenic peptides to, HLA-DR molecules [12–14]. DM has editing and chaperon effect in the selection of antigenic peptides for presentation by MHC class II molecules [15–18].

A second mechanism appears to be the binding of antigenic peptides to MHC class II molecules as a concerted process during the cleavage and release of Ii [19–21]. The binding of a radioiodinated, azido-crosslinking, antigenic peptide to MHC class II molecules was greater during cleavage of Ii by cathepsin B, than to Ii-freed molecules. A trace level of cathepsin D, further enhanced the rate of antigenic peptide binding in the cathepsin B experiment. In these studies with detergent-solubilized, microsomal membrane preparations, DM was presumably not relevant due to the dilution of the constituents. Others have suggested that DM is not absolutely required for antigenic peptide loading of MHC class II molecules. For example, self peptides other than CLIP have been found in DR+DM- mutant cells [6–8, 10]. Removal of CLIP from some MHC class II alleles is not highly dependent on DM [10, 22, 23]. The amino acid sequence Ii81–89 within CLIP itself has an activity to release both longer and short forms of CLIP and some antigenic peptides from purified HLA-DR molecules [24]. In other purified preparations without DM, the presence of residues 81–89 or 83–89 in CLIP peptides increases their lability from HLA-DR1 molecules at pH 4.0 [25]. The region of these residues about the N-terminus of longer CLIP peptides appears to be a target for protease cleavage of Ii [20, 26]. The region of these mutations overlaps the N-terminus of the CLIP sequence and includes the two functional domains described in this report.

Toward a better understanding of the functional role of that region, we had synthesized the Ii peptide hIi77–92 homolog, YRMKLPKPPKPVSKMR, thinking that the clustering of six positive charges without a negative charge, around four spaced prolines, creates a tightly linked electrostatic signal on the surface of the Ii-MHC class II complex to attract and dock some regulatory protein, e.g., a protease. That Ii-Key peptide greatly enhanced antigenic peptide charging to cell surface murine MHC class II molecules, as recognized by respective murine T cell hybridomas [27]. In murine biological assays the core domain with function was Ii77–83 (LRMKLPK) [28].

In this paper, we report both the minimal domains and structure/activity relationships of the Ii-Key peptide. These studies define a second site on HLA-DR molecules at which certain Ii peptides regulate release/exchange of antigenic peptides at the antigenic peptide binding site. Two overlapping effector segments of the Ii-Key peptides have been defined in terms of their effect on antigenic peptide release in the presence or absence of antigenic peptides in the solution. Ii77–83 release antigenic peptide from the antigenic peptide binding site only in the presence of excess antigenic peptide in solution. Ii81–88 release antigenic peptide from the antigenic peptide binding site without free antigenic peptide in solution. Ii81–88 also promotes the exchange for bound peptide with free peptide in solution. Both domains promote the binding of peptide to 'empty' DR1 molecules. Competitive peptide binding experiments demonstrate that the Ii77–83 binding site is physically distinct from, but affecting function at, the antigenic peptide binding site. It may thus be termed an allosteric site.

2. Material and methods

2.1. Synthesis of antigenic peptide and Ii-Key peptides

Biotin-labeled or unlabeled hMBP90–102, HFFKNIVT PRTPPP [29], and various Ii-Key homologs, with sequences in the Tables, were obtained from the Tufts University Peptide Synthesis Facility, Boston, MA USA), or from Quality Controlled Biochemicals, Inc. (Hopkinton, MA, USA) with the exception of the cyclic peptides. The head-to-tail cyclical form of Ii77–83, LRMKLPK was synthesized by Jordi Alsina and Fernando Albericio of the Department of Organic Chemistry, University of Barcelona (Barcelona, Spain). The head-to-tail cyclical form of LRMKLPK was synthesized in solid-phase starting from an active carbonate resin according to a previous described procedure [30]. For both peptides, the first residue Fmoc-Lys-OAl was incorporated to the active resin through the side-chain in presence of a base, N,N-diisopropylethylamine. For the head-to-tail cyclic peptide, elongation of the peptide chain was carried out using a standard Fmoc/tBu protocol [31]. Peptide concentrations were determined by amino acid analysis by 420A/130A derivatizer/HPLC (Applied Biosystems, Inc., Foster City, CA, USA) after hydrolysis with 6 N HCl for 24 h in vacuo).

2.2. Cell lines and antibodies

Insect cell line sf9 was from the American Tissue Culture Collection (ATCC, Manassas, VA, USA), and cell line H5 was from Invitrogen (San Diego, CA, USA). The monoclonal antibody 12CA5 against the influenza hemagglutinin (HA) sequence YPYDVPDYA [32] was the gift of Dr. Steven Doxsey, University of Massachusetts Medical School (Worcester, MA, USA). The L243 anti-HLA-DR monoclonal antibody which recognizes the

mature form of DR α,β dimer [33] was from the ATCC. Rabbit anti-DR1 antiserum (CHAMP) (34) was the gift of Dr. Lawrence Stern, Massachusetts Institute of Technology (MIT, MA, USA).

2.3. Construction of truncated DR α and β genes into the pBlueBac4 plasmid

The HLA-DR α gene was truncated after Asn^{192} to remove transmembranal and cytoplasmic regions from the protein product. Codons for a nine amino acid HA epitope were attached to the gene for the α chain after the codon for Asn^{192} to permit binding of the modified protein product by the monoclonal antibody 12CA5. The DR α gene containing plasmid 45.1-DRα120 (the gift of Dr. Eric Long of the National Institutes of Health, Bethesda, MD, USA) was digested with BamH1 and Pst1 to release the BamH1/Pst1 fragment which contains codons for the DRα gene from Met^1 to Cys^{164} and 5' untranslated region. This fragment was purified with the Qiaex II gel extraction kit (Qiagen, Chatsworth, CA, USA). A polymerase chain reaction (PCR) was performed to produce full length DRα gene with two oligos. The reverse oligo contained the codons for the HA epitope, a stop codon, and an EcoR1 site. The PCR product was digested with Pst1 and EcoR1, and the Pst1/EcoR1 fragment, which contained the DRα gene from Arg^{165} to Asn^{192}, codons for the HA epitope, and a stop codon, was also purified by gel electrophoresis and the Qiaex II gel extraction kit. The BamH1/Pst1 fragment and Pst1/EcoR1 fragments were co-ligated into the pBlueBac4 plasmid, digested by BamH1 and EcoR1. The PCR portion of the construct was verified by sequencing.

2.4. Construction and purification of Baculovirus clones containing DR1 genes

The pBlueBac4-DRα was co-transfected with linearized Baculovirus into the sf9 insect cells by the liposome-mediated method using the Bac-N-Blue transfection kit (Invitrogen, San Diego, CA, USA) following the manufacturer's instructions. Plaque assays were performed to obtain pure recombinant Baculovirus clones containing DRα gene (BV-DRα(sol)). Each BV-DRα(sol) clone was verified by PCR to confirm that it contained the Dα gene. DR1β-containing Baculovirus was the gift of Dr. Lawrence Stern of MIT.

2.5. Production and concentration of insect cell supernatant containing the soluble exomembranal DR1 molecules

The insect cell line H5 was co-infected (at MOI 1:1.5–2 for α:β genes containing Baculovirus (BV-DR) in serum-free medium (JRH Biologicals, Lenexa, KS, USA). Different batches of BV-DR virus stock were evaluated separately for the optimal ratio of α and β, to minimize free α chain. Three days after infection, the expression and concentration of the DR1 protein in the serum-free supernatant were determined by ELISA with 12CA5 or L243 as capture antibodies, and the rabbit anti-DR1 polyclonal antibody CHAMP as linking antibody. The DR1-containing, H5 supernatant for the Ii-Key peptide screening assay, was brought to 1 mmol/l iodoacetamide (Sigma, St. Louis, MO, USA), 1 mmol/l phenylmethylsulfonyl fluoride (Sigma), and 1 mmol/l ethylenediaminetetraacetic acid (EDTA) (Sigma), and was concentrated 10–15 times by ultrafiltration over an Amicon YM30 membrane (Amicon, Bedford, MA, USA). The production of DR1 α, β heterodimer by the insect cells were examined by 3 h $[^{35}S]$methionine (Amersham Life Sciences, Arlington Heights, IL, USA) labeling of insect cells which were co-infected with DR α and αβ containing Baculovirus for 48 h and immunoprecipitation with L243. After gel electrophoresis and autoradiography, the two bands of DR α and β were visualized (data not shown).

2.6. ELISA screening assay to define activities of Ii-Key peptides

Wells of microtiter plates were coated with the 12CA5 monoclonal antibody (2.5 μg/ml in 50 mmol/l sodium carbonate (pH 9.6) (Sigma) at 4 °C overnight. After washing with 0.05 % Tween-20 (Sigma) in phosphate-buffered saline solution (PBS) (pH 7.2), these coated microtiter wells were blocked with bovine gelatin (2 mg/ml) (Sigma) for 3–5 h at 4 °C. After washing with the Tween-PBS buffer, concentrated supernatant of H5 cells infected with BV-DRαβ or wild type Baculovirus (negative control) (100 μL) was added to each well and the plates were incubated at 4 °C for 2 h. After washing with the Tween-PBS buffer, biotin-labeled hMBP peptide (concentrations in Figure legends) in PBS (pH 7.2) with 1 mmol/l EDTA, was added and the plates were incubated at 37 °C overnight. After washing with the Tween-PBS buffer, 100 μλ of Ii-Key peptide in PBS, with 1 mmol/l EDTA, was added at indicated concentrations and the plates were incubated at 37 °C for 1 h. Assays at 1 h reflected initial release rates, given the long dissociation times for release of antigenic peptide in the absence of Ii-Key compounds. After washing further, 100 μl of 1 to 500 times-diluted, avidin-conjugated horseradish peroxidase (HRP) (GIBCO, BRL, Grand Island, NY, USA) was added and incubated at 4 °C for 1 h. After washing, 100 μλ of HRP substrate, 3,3',5,5',-tetramethyl-benzidine (Sigma), was added to each well and the plates were incubated at room temperature for 5 min. 25 μl of 2 N sulfuric acid was added to each well to stop the reaction. Colorimetric changes at 450 nm were quantitated with an ELISA reader (Molecular Devices, Sunnyvale, CA, USA). Each value represents an average of duplicates with mean deviations less than 12 %. Each compound was tested at least twice. Results from one experiment are presented in each Table. 'Relative release' of Tables 1 and 2 equals 1 – (observed value minus background)/(maximal value minus background), where background was the value obtained upon binding *hMBP to proteins adsorbed into the well from the supernatant of an wild type Baculovirus infected cell culture (not expressing HLA-DR molecules), and maximal value was obtained from binding *hMBP into the adsorbed HLA-DR molecules without exposure to Ii-Key homologs. 'Relative binding' of Tables 3 and 4 equals (observed value minus background)/(control value minus background), where background was the value obtained upon binding *hMBP to proteins adsorbed into the well from the supernatant of an wild type Baculovirus (not expressing HLA-DR molecules), and control value was obtained from binding *hMBP into the adsorbed HLA-DR molecules without exposure to Ii-Key homologs. 'Relative competition' of Fig. 1 equals (observed value minus background)/(maximal value minus background), where background was the value obtained upon binding a biotinylated peptide to proteins adsorbed into the well from the supernatant of an wild type Baculovirus infected cell culture (not expressing HLA-DR molecules), and maximal value was obtained from binding a biotinylated peptide into the adsorbed HLA-DR molecules without exposure to a competitor peptide. Individual binding and competitive peptides are indicated in the Figure legend. Relative release of * AE107 (Ii77–88) by various Ii-Key homologs or hMBP in Fig. 2 was calculated as for Tables 1 and 2.

3. Results

3.1 Certain Ii-Key peptides release hMBP *peptide from HLA-DR1 molecules without free hMBP peptide in the solution

The release of hMBP *peptide from HLA-DR1 molecules was assayed as a function of length of various murine Ii-Key homologs (Table 1). Among the Ii-Key homologs, AE 107 (Ii77–88) but not AE108 (Ii77–86), and AE114 (Ii77–83) released hMBP *peptide from the HLA-DR1 molecules. This finding established a C-terminal limit for the release of antigenic peptides when no additional antigenic peptide was present in the solution. N-terminal deletions showed that N-terminal YRMKL could be deleted without any observed loss in activity to release hMBP *peptide. AE405 (Ii81–88) which bracketed the active domain indicated by the two series of terminal deletions was effective in releasing hMBP *peptide. In additional experiments, AE101 (Ii77–92) and AE107 (Ii77–88) each at 125 nmol/l completely released hMBP *peptide from HLA-DR1 under the conditions of this assay (data not shown).

AE100 (human 16 amino acid Ii-Key peptide; Ii77–92; YRMKLPKPPKPVSKMR) was consistently less active in releasing hMBP *peptide from HLA-DR1 than murine Ii-Key peptide AE101 (Ii77–92). This result might reflect racemization of the prolines to both D and L forms. Presumably, the L-prolyl conforms are the only active ones. Even though spontaneous conversion of D to L, and L to D, prolyl forms occurs rapidly, it is sufficiently slow to be reflected in the lower activity of the peptide with more prolyl residues.

The Ii-Key homolog AE235, which is the substitution of L^{80} by Y in AE114 (Ii77–83), was very active in murine in vitro assays, especially on $I\text{-}E^k$ (28), but was ineffective in releasing hMBP *peptide from HLA-DR1, even in the presence of free hMBP peptide in the solution (next section). An ELISA with biotinylated AE235 detected no binding of AE235 to HLA-DR1 (data not shown). These results indicate a degree of MHC species and /or allele specificity in the actions of Ii-Key homologs.

Table 1: Induction of release of bound hMBP *peptide from HLA-DR1 by some Ii-Key peptides.

Peptide AE#	Sequence	Relative release
AE101	YRMKLPKSAKPVSQMR	1.05
AE106	YRMKLPKSAKPVSQ	1.12
AE107	YRMKLPKSAKPV	1.07
AE107	YRMKLPKSAKPV	1.07
AE108	YRMKLPKSAK	0.17
AE114	LRMKLPK	0.22
AE103	KLPKSAKPVSQMR	0.99
AE104	PKSAKPVSQMR	0.98
AE105	SAKPVSQMR	0.84
AE405	Ac-LPKSAKPV-NH_2	0.89
AE100	YRMKLPKPPKPVSKMR	0.63
AE401	Ac-LRMKLPKPP-NH_2	0.84
AE235	Ac-LRMKYPK-NH_2	0.21

Certain Ii-Key peptides completely released bound hMBP *peptide from DR1 in the absence of free hMBP peptide in the solution. Experimental conditions are given in Material and methods. Ii-Key truncation homologs were tested at 64 μmol/l.

3.2. Certain Ii-Key peptides catalyze the release of antigenic peptides from HLA-DR1 only in the presence of unlabeled hMBP peptide in solution

The fact that certain Ii-Key peptides, including the N-terminal 7 amino acid of Ii-Key AE 114 (Ii77–83) and its substituted homologs, were inactive in releasing hMBP *peptide from HLA-DR1 while the same peptides defined the minimal length, fully active domain in murine T cell presentation [28], seemed at first to be a serious species-specific conflict in results. One difference between the assays, however, was that antigenic peptide was present in excess in the supernatant of the murine cultures but absent from the HLA-DR1 antigenic peptide release assay. Upon testing the addition of unlabeled hMBP peptide to the solution of the HLA-DR/hMBP *peptide release assay, it was determined that some Ii77–83 substituted peptides, e.g., AE206, and head-to-tail linked cyclical native 7 amino acid AE381 can completely, even at 1 μmol/l, release hMBP peptide (Table 2) from DR1 molecules. AE114 (Ii77–83) has also exhibited a maximal rate of antigenic peptide release when free

Table 2: Catalysis of the release of bound hMBP *peptide from HLA-DR1 molecules in the presence of excess unlabeled hMBP peptide by some Ii-Key peptides.

Peptide AE#	Sequence	Relative release: Without unlabeled hMBP peptide, 1 μmol/l	Without unlabeled hMBP peptide, 64 μmol/l	With unlabeled hMBP peptide, 1 μmol/l	With unlabeled hMBP peptide, 64μmol/l
AE100	YRMKLPKPPKPVSKMR	0.32	0.49	1.05	0.94
AE108	YRMKLPKSAK	0.48	0.69	1.00	0.86
AE114	LRMKLPK	0.30	0.56	0.76	0.75
AE206	Ac-LRLKLPK-NH_2	0.42	0.92	0.97	1.29
AE381	LRMKLPK, cyclial	0.36	0.50	0.91	1.19
AE143	Ac-YRMKLhydrpKSAK-NH_2	0.49	0.42	0.65	0.79
AE235	Ac-LRMKYPK-NH_2	0.00	0.19	0.52	0.40

Certain Ii-Key peptides completely released the bound hMBP *peptide only in the presence of excess free hMBP peptide in the solution. Ii-Key peptides were tested at 1 μmol/l and at 64 μmol/l. The experimental procedures were the same as for Table 1, except that unlabeled hMBP peptide (250 μmol/l) was added in some wells during the release step, as indicated. The relative release of hMBP *peptide by AE101 at 1 μmol/l without hMBP peptide was 0.98.

hMBP peptide was present in solution. Human 16 amino acid Ii-Key peptide AE100 also had the greatest rates for release of bound hMBP *peptide when there was excess free hMBP peptide in solution. Again, the AE235 peptide, which was the most active homolog in murine assays, was inactive in the HLA-DR1 peptide release assay, even in the presence of excess free hMBP peptide in the solution.

3.3. Certain Ii-Key peptides exchange biotinylated antigenic peptides from the solution into antigenic peptide-loaded HLA-DR molecules

Since free hMBP peptide in the solution enhanced the activity of some Ii-Key peptides to release previously bound biotinylated antigenic peptides, we next tested whether Ii-Key peptides can exchange the bound hMBP peptide with free hMBP *peptide in the solution. The subset of Ii-Key peptides which promoted best the release of hMBP *peptide from HLA-DR1 in the absence of antigenic peptide in solution, also promoted the exchange of hMBP *peptide from solution into HLA-DR1 molecules which had been charged with unlabeled hMBP peptide (Table 3). Those Ii-Key peptides (AE206 and AE381) which **well** released the bound hMBP *peptide from DR1 only in the presence of free hMBP peptide in the solution, poorly promoted the exchange of hMBP *peptide from solution into DR1 molecules (Table 3).

3.4. Ii-Key peptides promote the binding of antigenic peptide to 'empty' HLA-DR molecules

Insect cell-produced HLA-DR1 molecules do not apparently contain antigenic or other peptides in their peptide binding site [34]. In this experiment, we tested whether Ii-Key peptides can promote the binding of hMBP *peptide into 'empty' DR1 molecules. Enhanced binding of hMBP *peptide into DR1 was induced by Ii-Key peptides excepting AE405 (Ii81–88) (Table 4). AE235 again did not enhance the binding of hMBP *peptide into DR1 molecules. This finding suggested that certain Ii-Key peptides can induce a conformational change in nascent 'empty' HLA-DR1 molecules favoring the binding of hMBP *peptide.

Table 3: Promotion of the exchange of hMBP *peptide onto HLA-DR1 molecules already loaded with hMBP peptide by some Ii-Key peptides.

Peptide AE#	Sequence	Relative binding
No enhancement		0.07
AE101	YRMKLPKSPKSAKPVSQMR	1.09
AE107	YRMKLPKSAKPV	0.70
AE381	LRMKLPK, cyclical	0.04
AE206	Ac-LRLKLPK-NH_2	0.08
AE405	Ac-LPKSAKPV-NH_2	o.57
AE100	YRMKLPKPPKPVSKMR	0.31
AE401	Ac-LRMKLPKPP-NH_2	0.45
AE235	Ac-LRMKYPK-NH_2	0.09

Certain Ii-Key peptides promoted the exchange of bound hMBP *peptide with free hMBP peptide in the solution. The experimental procedures were the same for Table 1, excepting that unlabeled hMBP peptide (50 μmol/l) was first incubated with purified HLA-DR1 molecules in the wells of 96-well microtiter plates overnight, and the wells were washed. The exchange step was then performed with certain Ii-Key peptides in the presence of hMBP *peptide (50 μmol/l) at 37 °C for 1 h. The control value was the binding of hMBP *peptide (50 μmol/l) overnight to HLA-DR molecules. 'No enhancement' was performance of the exchange step in the presence of hMBP *peptide (50 μmol/l) without Ii-Key peptides at 37 °C for 1 h.

Table 4: Promotion of the binding of hMBP *peptide to 'empty' HLA-DR1 molecules by some Ii-Key peptides.

AE#	Sequence	Relative binding
No enhancement		1
AE101	YRMKLPKSAKPVSQMR	2.66
AE107	YRMKLPKSAKPV	2.59
AE206	Ac-LRLKLPK-NH_2	2.57
AE381	LRMKLPK, cyclical	1.84
AE100	YRMKLPKPPKPVSKMR	2.49
AE401	Ac-LRMKLPKPP-NH_2	1.48
AE405	Ac-LPKSAKPV-NH_2	0.43
AE235	Ac-LRMKYPK-NH_2	0.61

Certain Ii-Key peptides enhance the binding of hMBP *peptide to "empty" DR1 molecules. After HLA-DR1 molecules were immobilized the microtiter plate, the washed wells were incubated at 37 °C for 1 h with both hMBP *peptide (50 μmol/l) and the indicated Ii-Key peptides (64 μmol/l), or without Ii-Key peptides. The wells were then washed and developed with avidin-conjugated HRP, followed by a colorimetric assay.

3.5. hMBP peptide competes for HLA-DR1 binding by Ii77–88 peptide, but not by Ii77–83 peptide

In order to define whether the Ii-Key peptides act directly at the antigenic peptide binding site or at a separate site, competitive binding experiments were performed in which the binding of *AE107 (Ii77–88) or *AE 206 (Ii77–83) (see Table 2) was competed by unlabeled hMBP peptide, AE 107, AE114 (Ii77–83) or AE 206 (Fig. 1). The binding of *AE206 to DR1 was not competed by unlabeled hMBP peptide but by unlabeled AE107, AE114, and AE206 (Fig. 1A). In contrast, the binding of *AE107 to DR1 molecules was competed by unlabeled hMBP peptide, AE107, AE114 and AE 206 (Fig. 1B). The binding of hMBP *peptide was competed by hMBP peptide and AE107, but not by AE206 and AE114 (Fig. 1C). These data indicated that AE206 does not bind to the antigenic peptide binding site on DR1 molecules but binds to a second site: an allosteric site with respect to its activity on release of antigenic peptides at their binding site.

3.6. The release of *AE107 (Ii77–88) from HLA-DR1 is promoted by unlabeled hMBP peptide but not by unlabeled Ii-Key homologs

We sought to determine whether AE107 (Ii77–88) interacts with the antigenic peptide binding site or not. The effect on release of *AE107 from HLA-

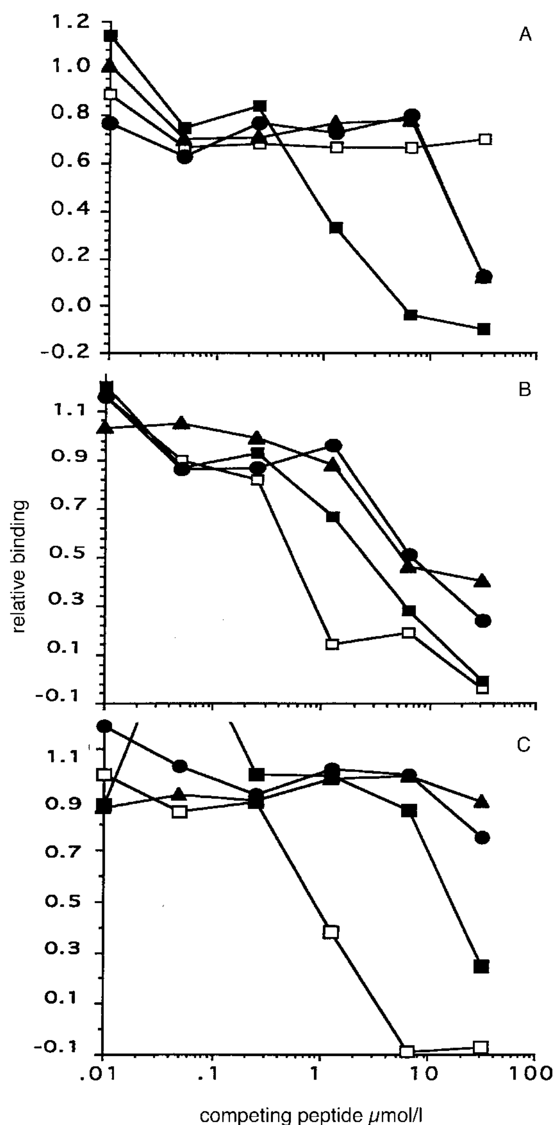

Fig. 1: Competitive binding experiments were performed in which biotin-labeled peptide was incubated at 37 °C overnight with increasing concentrations of unbiotinylated peptides in microtiter plate wells containing immobilized HLA-DR1 molecules. The wells were then washed and developed with avidin-conjugated HRP, followed by clorimetric assay. A) Biotin-labeled AE206 (Ac-LRLKLPK-NH_2) (1 μmol/l) was incubated with increasing concentrations of unlabeled AE107 (YRMKLPKSAKPV) (■), AE 114 (LRMKLPK) (●), AE206 (▲), or hMBP (□) peptides. B) Biotin-labeled AE107 (1 μmol/l) was incubated with increasing concentrations of unlabeled AE107 (■), AE 114 (●), AE206 (▲), or hMBP (□) peptides. C) Biotin-labeled hMBP peptide (1 μmol/l) was incubated with increasing concentrations of unlabeled AE107 (■), AE 114 (●), AE206 (▲), or hMBP (□) peptides.

DR1 molecules by unlabeled hMBP peptide, AE107, AE114 and AE206 was assayed (Fig. 2). *AE107 was released by hMBP peptide but not by AE107, AE114, and AE206. While AE107, AE114, and AE206 bind to the same core site on MHC class II molecules, the bound form can not be released by unlabeled forms in the solution under our experiment condition. In other experiments (see legend for Table 2), bound hMBP *peptide can not be released by unlabeled hMBP peptide in solution without the addition of Ii-Key peptides.

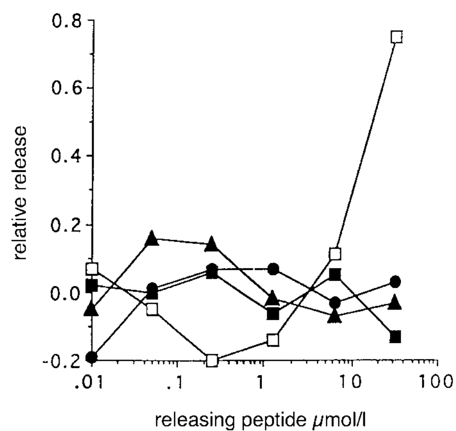

Fig. 2: Release of bound *AE107 (YRMKLPKSAKPV) peptide from DR1 molecules by unlabeled AE107 (■), AE 114 (LRMKLPK) (●), AE206 (Ac-LRLKLPK-NH_2) (▲), or hMBP (□) peptides. *AE107 (1 μmol/l) was incubated with immobilized DR1 molecules at 37 °C overnight. The wells were washed, and indicated concentrations of unlabeled AE107, AE114, AE206, or hMBP peptided were added for a 1 h incubation at 37 °C. The wells were washed, developed with avidin-conjugated HRP, and a colorimetric assay was performed.

The fact that unlabeled hMBP peptide can release the bound AE107 supports the view that AE107 does not bind at the same site as does hMBP peptide. Taking this result with the result from the competition binding experiments (previous section), one may conclude that AE107 does not bind at the same binding site for hMBP peptide, the two binding sites may overlap. The fact AE107 competes for the binding of AE206 indicates that AE107 binds to the allosteric site too, but its C-terminal portion (Ii84–88) might interact also with the antigenic peptide binding site.

4. Discussion

These studies describe Ii-Key peptides which, as a function of their lengths, can either release antigenic peptides from MHC class II molecules with or without a second antigenic peptide in solution. Because antigenic peptides did not compete for the binding of labeled short Ii-Key peptides to MHC class II molecules, an allosteric site for the binding of such Ii-Key peptides, with respect to the antigenic peptide binding site, can be proposed. Such a site functioning to regulate binding at the antigenic peptide binding site, has also been proposed by Kropshofer et al. (24). The relationships among sequences of peptides defining these two overlapping domains of Ii-Key activity are presented in Table 5. Truncation intermediates of Ii77–92 had revealed varying ability to release an indicator antigenic peptide which had been previously loaded into HLA-DR1 molecules. The domain Ii77–83 and its homologs effectively released *hMBP92–102 from HLA-DR1 when unlabeled antigenic peptide was present in the solution. For example, AE206 (Ac-LRLKLPK-NH_2), at 1 μmol/l, efficiently released previously bound *hMBP92–102 when unlabeled hMBP peptide was present in the solution, but not

Table 5: Comparative alinment of Ii-Key, CLIP and HA306-318 peptides.

Long CLIP	LPKPPKPVSKMRMATPLLMQALPMG
Short CLIP	KMRMATPLLMQALPMG
HA306–318	PKYVKQNTLKLTA
AE101	YRMKLPKPPKPVSKMR
AE114	LRMKLPK
AE405	LPKSAKPV

In the x-ray crystallographic studies of Ghosh et al. [9] and Stern et al. [35] amino acids of the influenza hemagglutinin antigenic epitope HA306-318 aligned with amino acids of a CLIP as shown. The CLIP sequence from [87]VSKM … fell within the two antiparallel alpha helices of the HLA-DR1 molecules. Therefore, if the Ii-key peptides bind to the MHC class II molecules in registry with the illustrated pattern for a CLIP peptide, as determined by x-ray crystallography, the active site of AE114 lies outside the antigenic peptide binding site. Our experimental data are consistent with this hypothesis. As discussed, the residue by which long CLIP and short CLIP differ are critical to the functions of the dissociation of CLIP peptides under acidic conditions [23, 24]. Ii-Key peptides such as AE405 released biotinylated anitgenic peptides in the absence of unlabeled antigen peptide in the solution (Table 1). Ii-Key peptides such as AE114 release biotinylated antigenic peptides from HLA-DR1 only in the presence of unlabeled antigenic peptides in solution (Table 2).

when hMBP peptide was not in the solution (Table 2). Similarly, some Ii77–83 homologs, which did not release bound *hMBP92–102 in the absence of free hMBP peptide in solution, released quite well in the presence of free hMBP peptide. Free antigenic peptide in solution might supply an additional catalytic effect for the release of bound peptides from HLA-DR1 molecules after a conformational change is induced by Ii77–83 domain on allosteric site.

Ii peptides with more C-terminal residues included in the domain (Ii81–88), released previously bound hMBP *peptide regardless whether unlabeled antigenic peptide was present in solution. For example, AE105 (Ii84–92) and AE405 (Ii81–88) each efficiently released previously bound hMBP *peptide from DR1 without unlabeled antigenic peptide being in the solution. The requirement for the residues of this 'middle region' of the original 16 amino acid Ii-Key peptide to release certain CLIP peptides, is reflected in two other studies. Urban and Strominger observed that the lability of CLIP under acid conditions depended on the N-terminal amino acid residues from 81 to 89 [25]. Kropshofer et al. [24] determined that at endosomal pH 5.8, the relatively rapid dissociation of CLIP Ii90–105 from HLA-DR3, depended on the presence of N-terminal segment L81PKPPKPVS. Stumptner and Benaroch [35] showed that the sequence of Ii81–90 is are responsible for the loosening of the association of CLIP81–104 with MHC class II molecules. Combining the effects of these two segments of the Ii-Key peptide, for example in AE101 (Ii77–88), which included in an overlapping fashion Ii77–83 and Ii81–88, leads to a yet higher degree of activity in releasing hMBP *peptide from HLA-DR1 (Table 1). This additive effect was also observed in 'exchange' experiments (Table 3). Each domain enhanced the binding of hMBP peptide to 'empty' HLA-DR1, indicating induction of conformational changes in HLA-DR1 molecules favoring the binding of antigenic peptides.

The competitive binding experiments with hMBP *peptide and *AE107 (Ii77–88) and *AE206 (homolog of Ii77–83), and respective not biotinylated forms, showed that Ii77–83 binds at a site on the HLA-DR1 molecule distinct from where the antigenic peptide binds, affecting the lability of the antigenic peptide in its site. Since Ii77–83 binds at an independent site, but affects the antigenic peptide binding site, the Ii77–83 site is termed an allosteric site with respect to effects at the antigenic peptide binding site. The binding of *AE107 was competed by hMBP peptide, and *AE107 was released by hMBP but not by AE107, AE114 (native Ii77–83), or AE206, supporting the view that AE107 and hMBP partially overlap in their respective binding sites and Ii77–83 binds an allosteric site just out side the antigenic peptide binding site. The finding that AE107 was competed by hMBP peptide was consistent with the results of Vogt et al. [36] that MHC class II binding of Ii71–88 is competed by hMBP85–105. In two additional reports [37, 38], the investigators showed that Ii can binds to MHC class II molecules at a site other than the antigenic peptide binding groove, but the location and the functional relationship to peptide loading were not demonstrated.

Close overlap in binding of influenza virus hemagglutinin peptide HA307–319 and an Ii-CLIP peptide in HLA-DR1 was defined by x-ray crystallography [8, 35]. In both cases, the peptides assumed the conformation of a polyprolyl type II helix in the antigenic peptide binding groove. The backbone atoms of the CLIP peptide overlay almost exactly the positions of the backbone atoms of the HA peptide, with comparable placement of side chains into pockets of the MHC class II molecule. The CLIP residues N-terminal to M^{91}, extending back to P^{87}, were also form the polyprolyl type II helix. More N-terminal residues, including positions human Ii L^{77}-K^{83} which correspond to the AE114 compound, were not resolved in those crystallographic studies, but would clearly lie outside the antigenic peptide binding groove, along a 'side' of the MHC class II molecule.

These findings support a mechanism for regulation of antigenic peptide binding to MHC class II molecules during cleavage and release of the Ii chain. Proteases such as cathepsin B [19] or cathepsin S [5, 40] might mediate intracellular cleavage and release of Ii. The Ii77–92 region has several hydrophobic/basic, potential cleavage sites 77–92 [20, 27]. Cleavage at the multiple hydrophobic/basic residue pairs in Ii78–86, might generate CLIP to

be removed by HLA-DM in a concerted fashion with the insertion of an antigenic peptide. In addition, proteases might also cleave about Ii90, Ii92, or Ii94 [19, 20, 27] to generate a still attached Ii-Key sequence containing fragment to enhance the binding of antigenic peptide to MHC class II molecules [20, 21, 27, 28]

The studies of this report address both a role for the Ii-Key segment of Ii in the natural, intracellular process of antigenic peptide binding to MHC class II molecules, and the design of therapeutic compounds which can act on cell surface MHC class II molecules. Such drugs might be immunosuppressive by releasing naturally charged antigenic sequences, or immunomodulatory by enhancing immune responses to synthetic antigenic peptides.

5. References

[1] Bakke, O., Dobberstein, B., MHC class II-associated invariant chain contains a sorting signal forendosomal compartments. Cell **16**, 707 (1990)

[2] Lamb, C.A., Cresswell, P., Assembly and transport properties of invariant chain trimers and HLA-DR-invariant chain complexes. J. Immunol. **148**, 3478 (1992)

[3] Blum, J.S., Cresswell, P., Role for intracellular proteases in the processing and transport of class II HLA antigens. Proc. Natl. Acad. Sci. USA **85**, 3975 (1988)

[4] Nguyen, Q.V., Knapp, W., Humphreys, R.E., Inhibition by leupeptin and antipain of the intracellular proteolysis of Ii. Hum. Immunol. **24**, 153 (1989)

[5] Riese, R.J., Wolf, P.R., Bromme, D. et al., Essential role for cathepsin S in MHC class II-associated invariant chain processing and peptide loading. Immunity **4**, 357 (1996)

[6] Riberdy, J.M., Newcomb, J.R., Surman, M.J. et al., HLA-DR molecules from an antigen-processing mutant cell line are associated with invariant chain peptides, Nature **360**, 474 (1992)

[7] Sette, A., Ceman, S., Kubo, R.T. et al., Invariant chain peptides in most HLA-DR molecules of anantigen-processing mutant. Science **258**, 1801 (1992)

[8] Chicz, R.M., Urban, R.G., Lane W.S. et al., Predominant naturally processed peptides bound to HLA-DR1 are derived from MHC-related molecules and are heterogeneous in size. Nature **358**, 764 (1992)

[9] Ghosh, P., Amaya, M., Mellins, E. et al., The structure of an intermediate in class II MHC maturation: CLIP bound to HLA-DR3. Nature **378**, 457 (1995)

[10] Avva, R.R., Cresswell, P., In vivo and in vitro formation and dissociation of HLA-DR complexes with invariant chain-derived peptides. Immunity **1**, 763 (1994)

[11] Mellins, E., Smith, L., Arp, B. et al., Defective processing and presentation of exogenous antigens in mutants with normal HLA class II genes. Nature **343**, 71 (1990)

[12] Denzin, L.K., Cresswell, P., HLA-DM induces CLIP dissociation from MHC class II alpha beta dimers and facilitates peptide loading. Cell **82**, 155 (1995)

[13] Sherman, M.A., Weber, D.A., Jensen, P.E., DM enhances peptide binding to class II MHC by release of invariant chain-derived peptide. Immunity **3**, 197 (1995)

[14] Sloan, V.S., Cameron, P., Porter, G. et al., Mediation by HLA-DM of dissociation of peptides from HLA-DR. Nature **375**, 802 (1995)

[15] Kropshofer, H., Vogt, A.B., Moldenhauer, G. et al., Editing of the HLA-DR-peptide repertoire by HLA-DM. EMBO J. **15**, 6144 (1996)

[16] Vogt, A.B., Kropshofer, H., Moldenhauer, G. et al., Kinetic analysis of peptide loading onto HLA-DR molecules mediated by HLA-DM. Proc. Natl. Acad. Sci. USA **93**, 9724 (1996)

[17] Kropshofer, H., Arndt, S.O., Moldenhauer, G. et al., HLA-DM acts as a molecular chaperone and rescues empty HLA-DR molecules at lysosomal pH. Immunity **6**, 293 (1997)

[18] Vogt, A.B., Kropshofer, H., Hammerling, G.J., How HLA-DM affects the peptide repertoire bound to HLA-DR molecules. Hum. Immunol. **54**, 170 (1997)

[19] Daibata, M., Xu, M., Humphreys, R.E. et al., More efficient peptide binding to MHC class II molecules during cathepsin B digestion of Ii than after Ii release. Mol. Immunol. **31**, 255 (1994)

[20] Xu, M., Capraro, G.A., Daibata, M. et al., Cathepsin B cleavage and release of invariant chain from MHC class II molecules follow a staged pattern. Mol. Immunol. **31**, 723 (1994)

[21] Xu, M., Daibata, M., Adams, S. et al., in: Antigen Processing and Presentation, R. E. Humphreys, S.K. Pierce (eds.), pp. 227–241, Academic Press, New York (1994)

[22] Brooks, A.G., Campbell, P.L., Reynolds, P. et al., Antigen presentation and assembly by mouse I-A^k class II molecules in human APC containing deleted or mutated HLA DM genes. J. Immunol. **153**, 5382 (1994)

[23] Stebbins, C.C., Loss, G.E. Jr., Elias, C.G. et al., The requirement for DM in class II-restricted antigen presentation and SDS-stable dimer formation is allele and species dependent. J. Exp. Med. **181**, 223 (1995)

[24] Kropshofer, H., Vogt, A.B., Stern, L.J. et al., Self-release of CLIP in peptide loading of HLA-DR molecules. Science **270**, 1357 (1995)

[25] Urban, R.G., Chicz, R.M., Strominger, J.L., Selective release of some invariant chain-derived peptides from HLA-DR1molecules at endosomal pH. J. Exp. Med. **180**, 751 (1994)

[26] Jasanoff, A., Park, S.J., Wiley, D.C., Direct observation of disordered regions in the major histocompatibility complex class II-associated invariant chain. Proc. Natl. Acad. Sci. USA **92**, 9900 (1995)

[27] Adams, S., Humphreys, R.E., Invariant chain peptides enhancing or inhibiting the presentation of antigenic peptides by major histocompatibility complex class II molecules. Eur. J. Immunol. **25**, 1693 (1995)

[28] Adams, S., Albericio, F., Alsina, J. et al., Biological activity and therapeutic potential of homologs of an Ii peptide which regulates antigenic peptide binding to cell surface MHC class II molecules. Arzneim.-Forsch./Drug Res. **47 (II)**, 1069 (1997)

[29] Sette, A., Sidney, J., Oseroff, C. et al., HLA DR4w4-binding motifs illustrate the biochemical basis of degeneracy and specificity in peptide-DR interactions. J. Immunol. **151**, 3163 (1993)

[30] Alsina, J., Rabanal, F., Giralt, E. et al., Solid-phase synthesis of „head-to-tail" cyclic peptides via lysine side-chain anchoring. Tetrahedron Lett. **35**, 9633 (1994)

[31] Fields, G.B., Tian, Z., Barany, G., Peptides. A User's Guide, G. A. Grant (ed.), pp. 77–183, W. H. Freeman and Company, New York (1992)

[32] Kolodziej, P.A., Young, R.A., Epitope tagging and protein surveillance. Methods Enzymol. **194**, 508 (1991)

[33] Roche, P.A., Cresswell, P., Invariant chain association with HLA-DR molecules inhibits immunogenic peptide binding. Nature **345**, 615 (1990)

[34] Stern, L.J., Wiley, D.C., The human class II MHC protein HLA-DR1 assembles as empty alpha beta heterodimers in the absence of antigenic peptide. Cell **68**, 465 (1992)

[35] Stumptner, P., Benaroch, P., Interaction of MHC class II molecules with the invariant chain: role of the invariant chain (81–90) region. EMBO J. **16,** 5807 (1997)
[36] Vogt, A.B., Stern, L.J., Amshoff, C. et al., Interference of distinct invariant chain regions with superantigen contactarea and antigenic peptide binding groove of HLA-DR. J. Immunol. **155,** 4757 (1995)
[37] Wilson, N.A., Wolf, P., Ploegh, H. et al., Invariant chain can bind MHC class II at a site other than the peptide binding groove. J. Immunol. **161,** 4777 (1998)
[38] Thayer, W.P., Ignatowicz, L., Weber, D.A. et al., Class II-associated invariant chain peptide-independent binding of invariant chain to class II MHC molecules. J. Immunol. **162,** 1502 (1999)
[39] Stern, L.J., Brown, J.H., Jardetzky, T.S., et al., Crystal structure of the human class II MHC protein HLA-DR1 complexed with an influenza virus peptide. Nature **368,** 215 (1994)
[40] Riese, R.J., Mitchell, R.N., Villadangos, J.A. et al., Cathepsin S activity regulates antigen presentation and immunity. J. Clin. Invest. **101,** 2351 (1998)

Acknowledgements

The authors are grateful to Drs. Fernando Albericio (University of Barcelona, Barcelona, Spain), Jack T. Johansen (Boston Probes, Inc., Bedford, MA, USA), Dale F. Mierke (Brown University, Providence, RI, USA), and Emil R. Smith (University of Massachusetts Medical School, Worcester, MA, USA) for discussions during the pursuit of these experiments. We also thank Jordi Alsina and Fernando Albericio (University of Barcelona, Barcelona, Spain) for the synthesis of the cyclical peptide AE381.

Correspondence: Dr. Minzhen Xu, Antigen Express, Inc., One Innovation Drive, Worcester,
MA 01605–4306 (USA)
E-mail: antigenexp@aol.com

Peroral Polypeptide Delivery

A comparative in vitro study of mucolytic agents

Andreas Bernkop-Schnürch, Claudia Valenta, and Seyed Mansour Daee

Centre of Pharmacy, Institute of Pharmaceutical Technology, University of Vienna, Vienna (Austria)

Summary

Besides the absorption and enzymatic barrier, the diffusion barrier based on the mucus gel layer covering gastrointestinal (GI) epithelia represents the third major factor being responsible for a very poor bioavailability of orally administered (poly)peptide drugs. In order to overcome the latter ones, the mucus liquefying action of various types of mucolytic agents and their influence on the model polypeptide drug insulin has been evaluated in a comparative in vitro study. The results demonstrated that the proteases pronase (EC 3.4.24.31) and papain (EC 3.4.22.2) cause a relative reduction in mucus viscosity of 75.9 ± 6.8 % and 51.1 ± 3.3 % (n = 3) after 6 h of incubation at pH 5.0, respectively, whereas the mucolytic effect of the detergents Triton X-100 (octoxinol) and Tween 20 (polysorbate 20) was markedly lower. Mucolytic proteases and well established mucolytic sulfhydryl compounds, however, caused a rapid degradation of insulin. Therefore these two types of mucolytic agents can only be used if the (poly)peptide drug proves stable towards degradation. The results indicate the strict need of novel mucolytic agents for the peroral administration of therapeutic (poly)peptides.