T Cell Responses Affected by Aminopeptidase N (CD13)-mediated Trimming of Major Histocompatibility Complex Class II–bound Peptides

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Summary

Endocytosed protein antigens are believed to be fragmented in what appears to be a balance between proteolysis and MHC-mediated epitope protection, and the resulting peptide–MHC complexes are transported to the surface of the antigen-presenting cells (APC) and presented to T cells. The events that lead to antigenic peptide generation and the compartments where antigen processing takes place remains somewhat enigmatic. The importance of intracellular antigen processing has been well established; however, it is unclear whether additional processing occurs at the APC surface. To follow antigen processing, we have identified a pair of T cell hybridomas that recognize a long vs. a short version of the same epitope. We have used prefixed APC and various protease inhibitors to demonstate that the APC surface has a considerable potential for antigen processing. Specific antibodies further identified the exopeptidase Aminopeptidase N (APN, CD13) as one of the enzymes involved in the observed cell-surface antigen processing. The NH_2-terminal end of the longer peptide could, even while bound to major histocompatibility complex (MHC) class II molecules, be digested by APN with dramatic consequences for T cell antigen recognition. This could be demonstrated both in cell-free systems using purified reagents and in cellular systems. Thus, MHC class II and APN may act in concert to generate the final T cell epitopes.

Th cells recognize protein antigens presented in the context of MHC class II molecules. The bulk of evidence suggests that protein antigens before presentation are internalized, transported to an acidic compartment, and partially degraded (reviewed in reference 1). Some of the resulting peptides are bound to class II molecules (2, 3), protected against further degradation (4, 5), and transported to the APC surface for Th cell scrutiny. Implicitly, antigen processing is viewed as an intracellular event, and little attention is currently paid to the cell surface as an auxiliary compartment for antigen processing. We have previously found that inhibitors of proteolytic enzymes could alter the specificity and sensitivity of the of antigenic peptide presentation by prefixed APC (6). Since prefixed APC are unable to internalize antigen and thus to perform intracellular antigen processing, these results suggested that some antigen processing can occur at the cell surface. However, we were at that time unable to establish the exact identity and localization of the enzyme(s) involved. In this paper, we have used prefixed APCs and a pair of A^k-restricted T cell hybridomas specific for hen egg lysozyme $(HEL)_{46-61(Y)}$ and $HEL_{50-61(Y)}$, respectively, to assess cell surface antigen processing and to identify one of the enzymes involved. The APC surface was able to perform considerable antigen processing. A careful analysis of the contribution of the various groups of proteases and a panel of mAbs allowed us to identify the ectopeptidase aminopeptidase N (APN,[1] also known as CD13), as one of the enzymes involved in cell-surface antigen processing. We have demonstrated that APN can trim amino-terminal ends of peptides that protrude out of MHC class II, and we have also demonstrated that this has significant effects on T cell stimulation and specificity. In support of this scenario, APN has been previously found to be expressed on MHC class II–bearing APC (7), and the NH_2-terminal sequence pattern of MHC class II bound peptides is compatible with the frequent involvement of APN in in vivo antigen processing (8, 9).

Materials and Methods

Cells. The H-2^k B lymphoma cell line CH12 (10) was used for antigen presentation; it was grown in vitro (37°C, 5% CO_2) in RPMI 1640 containing 5% FCS. The H-2^k B lymphoma cell line

[1] *Abbreviations used in this paper:* APN, aminopeptidase N; cPI, complete protease inhibitor cocktail; HEL, hen egg lysosome.

AKTB-1b (11) was used for production of A^k; it was maintained and produced in AKR mice.

The HEL specific, A^k-restricted T cell hybridomas 3A9 and kLy17.5 were grown in vitro (37°C, 5% CO_2) in RPMI 1640 containing 5% FCS. The inverse response pattern of these two hybridomas to NH_2-terminally truncated $HEL_{46-61(Y)}$ served as an important control throughout these experiments.

Antibodies. The rat mAbs R3-63 (IgG2a) and 2M-7 (IgG2a) both block mouse APN enzyme activity. R3-242 (IgG1, 09531D; Pharmingen, San Diego, CA) and R4-68 (IgG1) bind to a common site on APN without blocking its activity. R3-134 (IgG1) binds to another site on APN without blocking its activity. R3-152 (IgG2a) is not specific for APN, and is included for control purposes. These antibodies have all been described previously (7). They were generated as ascites in nude mice and purified by affinity chromatography using the mouse anti-rat κ light chain IgG antibody RG7/9.1 (TIB 169; American Type Culture Collection, Rockville, MD).

Peptides. Peptides were synthesized by conventional Fmoc strategy on a Ramps synthesizer (DuPont, Wilmington, DE), as previously described (12). After completion of the synthesis according to the sequence, the peptides were deprotected, cleaved from the resin, and finally ether precipitated. The $HEL_{46-61(Y)}$ sequence is in single letter code: NTDGSTDYGILQINSR(Y).

Antigen Presentation. In most experiments, peptides were presented by prefixed APCs. The APCs, CH12, were fixed with 0.05% glutaraldehyde for 30 s at room temperature, followed by three washes in PBS containing 75 mM L-glycine (pH 7), resuspended at 60 10^6/ml in PBS (pH 7) containing a protease inhibitor cocktail (see below), pulsed (2 h at 37°C) with a dose range of synthetic peptide, washed extensively, and resuspended in culture media. 2×10^5 T hybridoma cells were incubated with 2×10^5 pulsed APCs for 24 h at 37°C in a total of 250 ml.

In a few experiments, purified peptide–MHC complexes were coated onto latex particles and offered to the T cell hybridomas (13, 14). Briefly, 8×10^7/ml sulphonated latex particles (5.12 mm; Interfacial Dynamics, Portland, OR) were incubated (overnight at 4°C) with 0.5 mM peptide–MHC complexes. The coated latex beads were blocked (1% BSA in PBS for 2 h at 4°C), washed, and resuspended in tissue culture media. FACS® analysis (Becton Dickinson & Co., Inc., Mountain View, CA) confirmed that equal numbers of A^k molecules were coated onto the beads in the various experimental groups. 2×10^5 T hybridoma cells were incubated with 5×10^6 latex beads for 24 h at 37°C in a total of 250 ml.

The IL-2 content of the hybridoma supernatants were tested by their ability to support [^{3}H]thymidine incorporation into 4,000 HT-2 or by visual scoring according to Kappler et al. (15).

Inhibition of Protease Activity. The prefixed CH12s were pulsed with peptide in the presence of various mixtures of protease inhibitors. The chemical inhibitors included PMSF (used at a final concentration of 1 mM), EDTA (8 mM), 1.10 phenanthroline (1.2 mM), pepstatin A (69 mM), Na-*p*-tosyl-L-lysine chloromethyl ketone (TLCK, 128 mM), Na-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK, 135 mM), and *N*-ethylmaleimide (NEM, 1 mM). The protease inhibitors were crudely grouped as: PMSF, TLCK and TPCK, serine, proteinase inhibitors; NEM, cysteine proteinase inhibitor; EDTA and 1.10 phenanthroline, metallo-proteinase inhibitors; and pepstatin A, aspartic Acid proteinase inhibitor. A cocktail containing all the proteases was denoted "complete protease inhibitor" cocktail (or cPI), and a cocktail containing all but the metallo-protease inhibitors was denoted ΔPI. Note that EDTA/1.10 phenanthroline under the conditions used here irreversibly inhibits APN, since this enzyme can only be reactivated by an equimolar concentration of Zn^{2+}, Cu^{2+}, Co^{2+}, or Ni^{2+} (16), none of which are present under tissue culture conditions. In contrast, reversible inhibition of APN could be achieved by manipulating the pH of the reaction buffers. Experiments with affinity-purified APN established that APN was inactivated at pH 5.0, but subsequently could be reactivated at pH 7 (data not shown).

For a more detailed identification of the enzymes involved, the chemical metallo-protease inhibitors were in some experiments substituted with rat anti–mouse APN antibodies capable of specifically blocking APN function (this amounts to adding the relevant antibodies to ΔPI).

APN Treatment of Purified Peptide–MHC Class II Complexes. A^k was affinity purified from lysates of AKTB-1b using the monoclonal anti-A^k antibody 10-3.6.2 (TIB 92; ATCC), as previously described (17). Mouse APN was affinity purified from homogenized mouse kidneys using the rat monoclonal anti-APN antibody R3-63, as previously described (7). Complexes between 320 mM $HEL_{46-61(Y)}$ and 10 mM A^k were generated at pH 4.4 in the presence of ΔPI and purified by spun column chromatography (18). 5 mM $HEL_{46-61(Y)}$–A^k complexes were digested (24 h at 37°C) with 1 mM affinity-purified mouse APN (5, 7, 19). These complexes were coated onto latex beads and tested for their ability to stimulate the T cell hybridomas as described above.

Results and Discussion

Two HEL-specific, mouse class II A^k–restricted T cell hybridomas, 3A9 and kLy17.5, were used to study cell-surface antigen processing by the H-2^k B cell lymphoma, CH12. To prevent antigen uptake and intracellular processing, the APCs were fixed with glutaraldehyde (20). To examine cell-surface processing, the prefixed APCs were pulsed with $HEL_{46-61(Y)}$ in the absence or presence of a complete protease inhibitor cocktail (cPI) inhibiting the four known groups of proteinases (6). Pulsing in the presence of the cPI enhanced presentation to the 3A9 (Fig. 1 *A*), whereas it blocked presentation to kLy17.5 (Fig. 1 *B*). This suggests that functionally relevant antigen processing can occur at the cell surface and supports the notion that 3A9 recognizes the intact $HEL_{46-61(Y)}$ peptide (21). In contrast, kLy17.5 recognizes a truncation of $HEL_{46-61(Y)}$. To identify the optimal epitopes, a series of NH_2-terminal truncations of the $HEL_{46-61(Y)}$ epitope was tested in the presence of the cPI. The optimal epitope for 3A9 was found to be 17-mer $HEL_{46-61(Y)}$ (Fig. 1 *C*), whereas the optimal epitope for kLy17.5 was the 13-mer $HEL_{50-61(Y)}$ (Fig. 1 *D*). COOH-terminal truncations were not examined, since tryptophan in position 62 (represented in our peptides by tyrosine) is known to delineate the COOH-terminal part of the epitope (22, 23). All the following experiments examined the presentation of $HEL_{46-61(Y)}$ to 3A9 and kLy17.5, and they exploited the reciprocal effect of the various protease inhibitor mixtures upon these two responses. This is an important control, since it effectively rules out that the protease inhibitors are acting on general T cell response mechanism(s). Furthermore, admixture experiments where APCs that had been pulsed with peptide

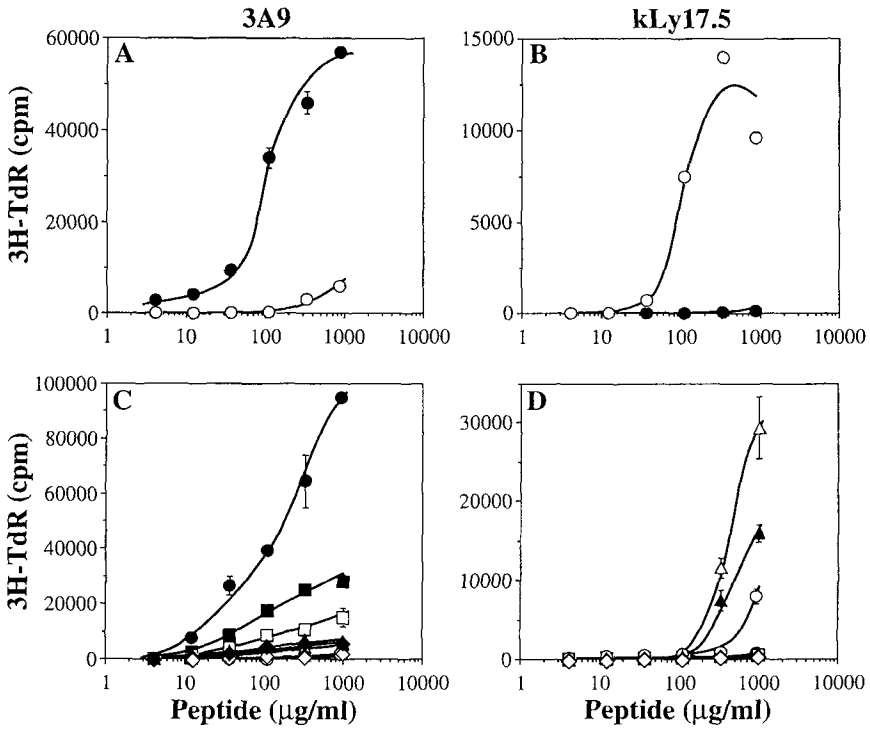

Figure 1. Cell-surface antigen processing affects T cell responses both quantitatively and qualitatively. (*A* and *B*) CH12 antigen–presenting cells were prefixed in glutaraldehyde and pulsed with $HEL_{46-61(Y)}$ in the presence (●) or absence (○) of a complete protease inhibitor cocktail (*cPI*). Two HEL_{46-61}–specific, A^k–restricted T cell hybridomas, 3A9 and kLy17.5, were used to assess the stimulatory capacity of the APCs. To define the optimal epitopes of 3A9 (*C*) and kLy17.5 (*D*), prefixed CH12 were pulsed with graded doses of NH_2-terminal truncations of $HEL_{46-61(Y)}$ in the presence of cPI ($HEL_{46-61(Y)}$, ●; $HEL_{47-61(Y)}$, ■; $HEL_{48-61(Y)}$, □; $HEL_{49-61(Y)}$, ▲; $HEL_{50-61(Y)}$, △; $HEL_{51-61(Y)}$, ◆; $HEL_{52-61(Y)}$, ◇. Also shown is $HEL_{46-61(Y)}$ in the absence of cPI (○).

in the absence of cPI were mixed with APCs that had been pulsed with cPI in the absence of peptide, demonstrated that cPI was not carried over in amounts sufficient to affect the read-out system through toxic effects, or through effects upon antigen presentation and/or T cell responsiveness during the read-out (data not shown).

The contributions of the different proteinases were examined by two complementary approaches. First, groups of proteinase inhibitors were deleted one by one from the cPI and the modified inhibitor cocktail was tested on cell-surface antigen processing of $HEL_{46-61(Y)}$. Metallo-proteinases were by far the most important group of proteinases involved, since deleting the corresponding inhibitors from the cPI allowed significant processing of $HEL_{46-61(Y)}$, as shown by decreased presentation to 3A9 and enhanced presentation to kLy17.5 (both compared to cPI, Fig. 2, *A* and *B*). Deleting the cysteine inhibitors from the cPI had a minor effect, whereas deleting the serine or aspartic acid inhibitors had no effect. Second, groups of proteinase inhibitors were tested alone, confirming the dominant contribution of metallo-proteinases, since the corresponding inhibitors on their own prevented processing of $HEL_{46-61(Y)}$, as shown by enhanced presentation to 3A9 and blocking of presentation to kLy17.5 (both compared to no PI, Fig. 2, *C* and *D*). An exact identification of the enzyme(s) responsible was, however, impossible because of the limited specificity of the chemical inhibitors.

APN is an obvious candidate, since it is a membrane-bound metallo-exopeptidase; it has been found on class II bearing APC's of the mouse immune system (7), and it could account for the conversion of $HEL_{46-61(Y)}$ to $HEL_{50-61(Y)}$. One of us (A. Stryhn) has recently generated a panel of rat monoclonal anti–mouse APN antibodies and crudely mapped APN epitopes (7). Antibodies specific for different APN epitopes were selected for further study. Importantly, some of these mAbs are potent inhibitors of APN activity, and compared to the chemical protease inhibitors, such antibody-mediated inhibition is quite specific. Substituting the chemical metallo-proteinase inhibitors of the cPI with saturating concentrations of the different relevant or irrelevant mAbs showed that APN is one of the important enzymes involved in cell-surface antigen processing of $HEL_{46-61(Y)}$ (Fig. 2, *E* and *F*). The two anti-APN antibodies, R3-63 (Fig. 2, *E* and *F*) and 2M-7 (data not shown), which inhibit APN activity (7), could substitute most of the activity of the metallo-proteinase inhibitors, whereas the noninhibitory anti-APN antibody, R3-134, and the irrelevant antibody, R3-152, had no effect (Fig. 2, *E* and *F*). The inhibitory anti-APN antibodies had the same effect as the chemical inhibitors on the stimulation and peptide size preference of the two T cell hybridomas, arguing against inadvertent effects on something else than APN, and suggesting that the effect is caused by the enzyme activity of the APN. Furthermore, the effects of the protease inhibitors depended on the size of the stimulating peptide (e.g., cPI blocked $HEL_{46-61(Y)}$ stimulation, but enhanced $HEL_{50-61(Y)}$ stimulation of kLy17.5), arguing against APN acting on some other moiety than peptide.

Surprisingly, two antibodies, R3-242 (Fig. 2, *E* and *F*) and R4-68 (data not shown), which are specific for a common noncatalytic site on the APN (7), were potent inhibitors of $HEL_{46-61(Y)}$ surface processing, suggesting that the

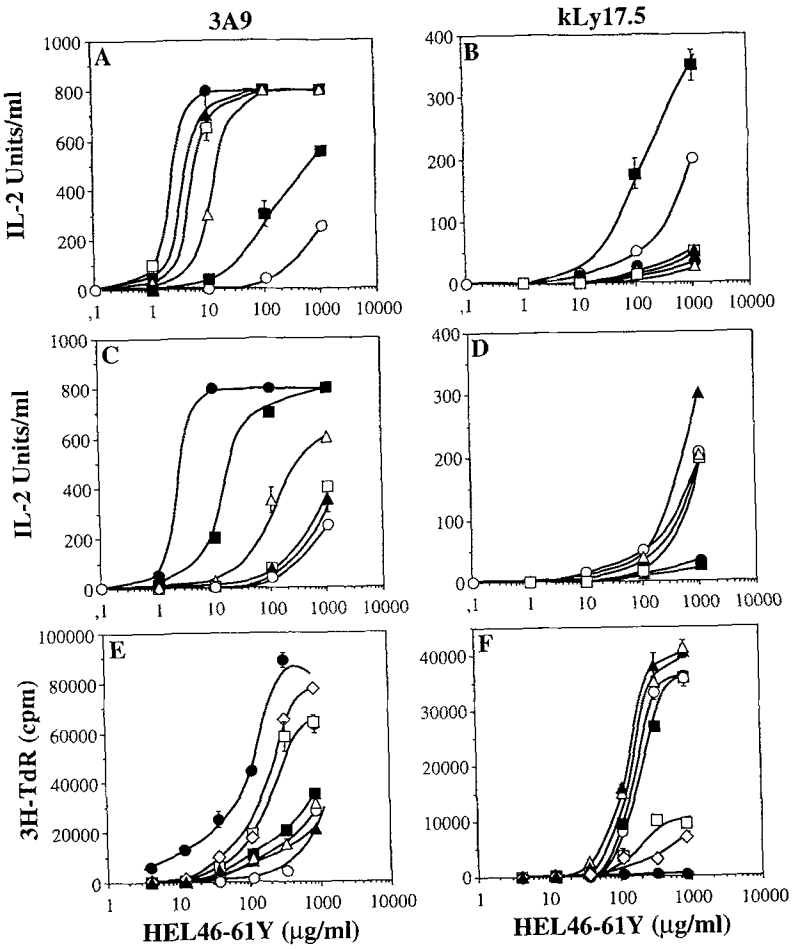

Figure 2. APN is involved in cell-surface processing of $HEL_{46-61(Y)}$. Prefixed CH12 were pulsed with increasing doses of $HE_{146-61(Y)}$ in the presence of various modifications of the protease inhibitor cocktail, and were used to stimulate 3A9 or kLy17.5. (*A* and *B*) The cPI was modified by deletion of protease inhibitors group by group: cPI (●), cPI with serine proteinase inhibitors deleted (▲), cPI with cysteine proteinase inhibitors deleted (△), cPI with metallo-proteinase inhibitors deleted (■), cPI with aspartic acid proteinase inhibitors deleted (□), and no PI (○). (*C* and *D*) The protease inhibitors were also tested groupwise: cPI (●), serine proteinase inhibitors alone (▲), cysteine proteinase inhibitors alone (△), metallo-proteinase inhibitors alone (■), aspartic acid proteinase inhibitors alone (□), and no PI (○). (*E* and *F*) The metallo-proteinase inhibitors, EDTA and 1.10 phenanthroline, were deleted from the cPI to generate a cocktail denoted ΔPI. The ΔPI was supplemented with 3 mM of various rat anti-mouse APN antibodies: R3-63 (□), R3-242 (◇), R3-134 (△), R3-152 (▲). Also shown are cPI (●), ΔPI (■), and no PI (○).

involvement of APN exceeds mere proteolysis. We reasoned that these antibodies might block the access of APN to the MHC, implying that APN degrades peptides already bound to the MHC. To address this possibility directly, preformed and purified $HEL_{46-61(Y)}$–A^k complexes were digested by affinity-purified APN (5), coated onto latex particles (13, 14), and offered to 3A9 and kLy17.5. APN treatment consistently reduced the capacity to stimulate 3A9 and improved the ability to stimulate kLy17.5 (Fig. 3 *A*). To corroborate this finding, a cellular experiment was devised. Prefixed APC were pulsed (2 h at room temperature) with $HEL_{46-61(Y)}$ at pH 5, which allows A^k binding (19), but reversibly inhibits APN activity (data not shown). Subsequently, the cells were washed and incubated (2 h at 37°C) at pH 7.0, which reactivates APN, and R3-63 (inhibitor of APN activity) or R3-152 (irrelevant) antibodies were added to determine the contribution of APN to antigen processing during this phase of the experiment. Indeed, APN strongly decreased 3A9 responses and strongly increased kLy17.5 responses (Fig. 3 *B*). To exclude the possibility that the processing of $HEL_{46-61(Y)}$ occurred outside the A^k, followed by exchange into the A^k, a peptide capable of blocking A^k binding, but not APN activity (data not shown), was added to one set-up during APN reactivation. The inability of the A^k-blocking peptide to prevent the generation of the kLy17.5 stimulatory moiety excluded the possibility that peptide binding to A^k took place after APN digestion. Thus, we conclude that APN-mediated processing took place while the $HEL_{46-61(Y)}$ was bound to A^k.

Our experimental design included a step of mild aldehyde fixation intended to prevent transport across the membrane and thereby intracellular antigen processing. In general, prefixed cells are unable to present intact protein antigens (24, 25), supporting the notion of a dominant intracellular contribution to in vivo antigen processing. As demonstrated here, however, functionally significant processing of peptide antigens can occur after fixation. We pulsed the prefixed APCs in a PBS-based buffer (i.e., in the absence of serum proteases), suggesting that the observed processing took place at the cell membrane, not in the media. Note, however, that this does not exclude that processing under physiological conditions can occur in serum. Indeed serum proteases, such as angiotensin-1–converting enzyme, have been implicated in antigen processing (26, 27). Most importantly, we further pinpointed the auxiliary peptide processing event to the protruding ends of MHC class II–bound peptides, and implicated the membrane-bound exopeptidase APN (Fig. 3, *A* and *B*). Thus, we sug-

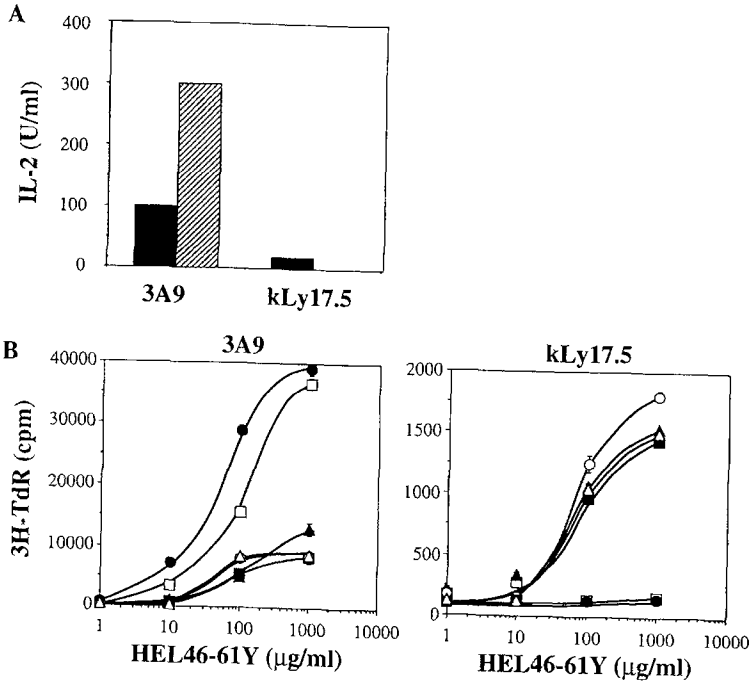

Figure 3. APN can process $HEL_{46-61(Y)}$ while it is bound to A^k. (*A*) In a biochemical approach, 5 mM purified $HEL_{46-61(Y)}/A^k$ complexes were incubated in the presence (*solid bars*) or absence (*hatched bars*) of 1 mM purified APN for 24 h at 37°C in PBS containing ΔPI (see Fig. 2) and 0.1% NP-40. Digested complexes were coated onto latex particles, which subsequently were incubated with 3A9 or kLy17.5. (*B*) In a cellular approach, prefixed APCs were pulsed in the presence of ΔPI with $HEL_{46-61(Y)}$ for 2 h at room temperature. Pulsing was done at pH 5, which reversibly blocks APN activity. Subsequently, the cells were washed to remove unbound peptide, and the cells were exposed to a "postpulse" incubation in the presence of ΔPI for 2 h at 37°C. The postpulse incubation was conducted at pH 7.0, which reactivates the APN and allows it to digest the peptide–MHC class II complexes that were generated in the previous step. To assess the effect of the reactivated APN, the ΔPI was supplemented with the rat mAb R3-63 (□), which is a strong inhibitor of mouse APN, or with the irrelevant isotype-matched mAb R3-152 (▲). In one set-up, 50 mM of the A^k-binding peptide PPANDQGNRTTPSY (△) was added to the ΔPI to exclude peptide exchange during the postpulse. Also shown are the effects of cPI (●), ΔPI (■), or no PI (○) during the pulse and postpulse incubations.

gest that the APC surface can modify or trim MHC class II–bound peptides should intracellular processing fail to generate peptides that can be contained within the MHC class II–binding groove. Does the latter occur? Antigen degradation appears to be a stochastic process yielding peptides of heterogeneous sizes. Experimentally, it has been demonstrated that MHC class II–bound peptides are from 10–35 amino acids long (23, 28, 29) to intact proteins (30–32). They are known to have "ragged" ends (23, 28, 29, 33), and some of these ragged ends are likely to protrude out of the binding groove and be susceptible to exopeptidases (34). The extent of APN-mediated trimming depends in part on the specificity of the APN (35, 36), in part on the accessibility of the MHC groove. It may even involve the interaction of APN with class II during antigen trimming, as indicated here by the effect of mAbs R3-242 and R4-68. Thus, antigen processing and display cannot be viewed as two sequential events, but rather as a continuous series of interdependent events. In this particular case, APN is stopped either by the occurrence of prolines within the protruding NH_2-terminal end or spatially by the MHC class II itself. The frequent occurrence of prolines at the i+1 position of class II–bound peptides would indicate that a great many peptide–class II complexes indeed have been visited in vivo by an exopeptidase such as APN (8, 9). In this context, the correlation between MHC class II and APN expression on APCs is particularly striking (7). It is tempting to speculate that protruding carboxy-terminals may be trimmed by a carboxy peptidase (or a carboxy-dipeptidase such as angiotensin-1–converting enzyme, as demonstrated for MHC class I–restricted T cell responses; 26, 27, 37), with similar powerful effects on the sensitivity and specificity of T helper cell responses, as demonstrated here for APN. Finally, it is also tempting to speculate that "outsize" peptides, albeit not favored for binding to MHC class I, might be susceptible to trimming.

We gratefully acknowledge the kind gifts of 3A9 from Dr. Paul Allen and kLy17.5 from Dr. Ed Rosloniec. We thank Paul Allen and Hans-Georg Rammensee for critically reading the manuscript.

This work was supported by The Danish MRC, The Signalpeptide Research Biotechnology Center, The Novo Nordisk Foundation, The Danish Cancer Society, Leo and Margrethe Nielsens Foundation, and Dir. Ib Henriksens Foundation.

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Received for publication 3 January 1996 and in revised form 29 April 1996.

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