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Interferon-γ differentially regulates antigen-processing functions in distinct endocytic compartments of macrophages with constitutive expression of class II major histocompatibility complex molecules

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SUMMARY

RAW264.7 cells were transfected to express constitutively the murine class II major histocompatibility complex (MHC-II) molecule, I-A^k. The resulting RAW.Ak cells presented HEL(46–61) peptide to 3A9 T hybridoma cells, but they were unable to process and present HEL protein in their resting state. However, IFN-γ stimulation induced the ability of RAW.Ak to process and present HEL protein, with little effect on their ability to present HEL(46–61) peptide. Antigen catabolism showed little change with IFN-γ stimulation, suggesting that the production of peptides was not the regulated step in the processing pathway. Furthermore, HEL(46–61) peptide delivered directly into lysosomes by acid-resistant liposomes was also presented only upon IFN-γ stimulation, while the presentation of peptides delivered into endosomes by acid-sensitive liposomes showed a lesser dependence on IFN-γ stimulation. Thus, IFN-γ regulated the ability of peptides delivered into certain lysosomal compartments to meet with MHC-II molecules and form peptide–MHC complexes, or to transport subsequently to the plasma membrane. Two other antigens, ribonuclease A and haemoglobin, were processed by RAW.Ak cells without IFN-γ stimulation, suggesting that these antigens could be processed by different mechanisms, perhaps in earlier endocytic compartments. Thus, different antigens may be processed in distinct endocytic compartments, and an IFN-γ-regulated mechanism controls the rescue of peptides from lysosomal compartments for presentation at the plasma membrane.

INTRODUCTION

Antigen processing involves the conversion of protein antigens to peptides bound to class II major histocompatibility complex (MHC-II) molecules, and the expression of these complexes on the cell surface where they can be recognized by clonotypic T-cell receptors. This process includes the subtotal catabolism of protein antigens to produce peptides that can bind to MHC-II molecules. It also entails the targeting of antigenic peptides and MHC-II to a common compartment, the formation of peptide–MHC-II complexes therein, and the transport of these complexes to the cell surface. Each of these steps involves mechanisms that remain poorly understood.

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Abbreviations: CHEMS, cholesteryl hemisuccinate; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DOPS, dioleoylphosphatidylserine; Hb, haemoglobin; HEL, hen egg lysozyme; IFN-γ, interferon-γ; MHC-II, class II MHC; TGN, trans-Golgi network.

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Antigen catabolism to produce immunogenic peptides for presentation appears to occur within late endocytic compartments. Our previous studies have implicated important contributions of lysosomal compartments, defined by high density upon subcellular fractionation on Percoll density gradients, in the catabolic processing of antigens by macrophages.[1–3] In addition, recent evidence suggests that MHC-II molecules may target to a high density late endocytic or early lysosomal compartment, wherein they may bind immunogenic peptides.[4–8] Organelles containing peptide–MHC-II complexes have also been isolated using electrophoretic techniques.[9,10] Thus, after traversing the endoplasmic reticulum (ER)/Golgi biosynthetic pathway, MHC-II molecules may be transported from the trans-Golgi network (TGN) to late endocytic/lysosomal compartments. After binding peptides, MHC-II molecules must then be transported to the plasma membrane. While invariant chain has been shown to localize MHC-II into endocytic compartments,[11–14] the exact compartment targeted by invariant chain remains unclear, and the relative transport or retention functions of different forms of the invariant chain (p31 versus p41) that have different functional roles[15] also remain uncertain. Transport of MHC-II from the processing compartment to the plasma membrane is also poorly understood.

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Antigen processing and MHC-II expression are properties of B cells and macrophages. In B cells, a constitutive level of MHC-II expression and antigen presentation can be increased by interleukin-4 (IL-4). In contrast, resting macrophages have very little MHC-II expression or antigen-presenting function. Interferon-γ (IFN-γ) induces macrophage MHC-II expression and antigen-processing and -presenting functions. Previously, this functional dependence on IFN-γ was naturally ascribed to the necessity of MHC-II induction. We have now constitutively expressed the murine MHC-II molecule, I-A^k, in transfectants of the murine macrophage cell line RAW264.7 (e.g. RAW.Ak cells). This provides a new opportunity to test for antigen-processing functions besides MHC-II expression that are regulated by IFN-γ. In fact, RAW.Ak cells show a defect in antigen processing that is reversed by IFN-γ, implicating roles for other IFN-γ-regulated molecules in antigen processing. In this report we have dissected the cell biology of this defect, which appears to be in intracellular peptide and MHC-II targeting functions or in the intracellular binding of peptides to MHC-II molecules.

MATERIALS AND METHODS

Transfection of RAW264.7 cells

I-A^k α and β chain cDNA was generously provided by L. Glimcher.[16] The H-2^d murine macrophage cell line RAW264.7[17] (American Type Culture Collection) was transfected by calcium phosphate precipitation[18] simultaneously with 10 μg I-A^k α chain cDNA in the expression vector pcEXV[19] and 1 μg of the selection cassette RSV-neo.[20] G418-resistant cells were expanded, subcloned and checked by Northern blot for the expression of the α chain of I-A^k. A positive line, RAW.α3, was identified, and these cells were transfected simultaneously with 10 μg of the β chain of I-A^k in pcEXV and 1 μg of the hygromycin resistance marker driven by the simian virus 40 (SV40) promoter in pMON1118 (courtesy of D. Chaplin, Washington University, St Louis, MO). Hygromycin-resistant colonies were then screened for I-A^k expression by fluorescence-activated cell sorter (FACS) using the anti-I-A^k monoclonal antibodies 10.3.6.2[21] and H116.32.[22] A line expressing moderate levels of I-A^k was obtained and designated RAW.Ak.

S_1 nuclease analysis

S_1 nuclease protection assays were carried out as described.[23] An I-A^k α chain probe was created by digesting the I-A^k α chain cDNA in the pBluescript vector (Stratagene, La Jolla, CA) at the *Bst*EII site and end labelling the linear DNA with ^{32}P. This probe recognized both I-A^k and I-A^d, but the protected bands differed by approximately 300 bases (the predicted band for I-A^d was 592 base pairs, while the predicted band for I-A^k was 856 base pairs). A $β_2$-microglobulin probe cut at the *Eco*RI site was used as a control for the quality of RNA, as described,[24] and produced a protected band at 202 bases. Twenty micrograms of total RNA, prepared by the acid–guanidinium–phenol extraction method,[25] was used for each analysis and was exposed to ^{32}P-labelled probe ($5–10 \times 10^4$ c.p.m. for each probe) overnight at 52°. S_1 nuclease digestion (with 200 units of S_1 nuclease, Boehringer Mannheim, Indianapolis, IN) was carried out at 37° for 1 hr. Protected fragments were resolved on 6% polyacrylamide, 8 M urea gels. Autoradiography was carried out after transfer to Whatman 3MM paper.

Antigen processing

RAW.Ak cells (5×10^4/well in 96-well plates) were incubated overnight in the presence or absence of recombinant murine IFN-γ (Genzyme, Cambridge, MA) at 10 ng/ml (100 U/ml) in the following standard medium: Dulbecco's modified Eagles' minimal essential medium (DMEM; Gibco, Grand Island, NY) supplemented with L-arginine–HCl (116 mg/l), L-asparagine (36 mg/l, $NaHCO_3$ (2 g/l), sodium pyruvate (1 mM), 5×10^{-5} M 2-ME, 10% FCS (Hyclone, Logan, UT) and antibiotics. The cells were then washed and subsequently incubated for 24 hr with HEL (Sigma, St Louis, MO) or synthetic HEL peptide (either 46–61 or YE-52–61) and 3A9 T hybridoma cells,[26] specific for HEL(52–61)-I-A^k. YE-52–61 contains the 52–61 HEL sequence, with the addition of Tyr-Glu at the N terminus and an amide at the C terminus.[27] HEL(46–61) and YE-52–61 both contain the HEL(52–61) epitope, and both peptides bind efficiently to I-A^k and are recognized by 3A9 T hybridoma cells, as demonstrated in several prior studies.[27–29] In the current studies, both peptides were presented to 3A9 cells by RAW-Ak cells when introduced in soluble or liposome-encapsulated form. We also studied the processing of bovine ribonuclease A (RNase) and murine haemoglobin (Hb) using the T hybridomas TS12,[30] specific for RNase(40–61)-I-A^k, and WK5.1,[31] specific for Hb(67–76)-I-A^k. Hb (*Hbb^d*) was prepared from CBA/J mouse blood as described.[31] RNAase was from Sigma. Following incubations with T hybridoma cells, the IL-2 content of the medium (a measure of T-cell antigen recognition) was measured by proliferation and [^{3}H]thymidine incorporation by IL-2-dependent CTLL cells. Processing studies with liposome-encapsulated antigens were performed in the same manner, or the processing period was terminated by washing and fixing the macrophages with 1% paraformaldehyde prior to incubation with T cells.

Liposomes

Acid-resistant liposomes were prepared with dioleoylphosphatidylcholine (DOPC, Avanti Polar Lipids) and dioleoylphosphatidylserine (DOPS, Avanti Polar Lipids) at a 4/1 molar ratio. Acid-sensitive liposomes were prepared with dioleoylphosphatidylethanolamine (DOPE, Avanti Polar Lipids, Alabaster, AL) and cholesterol hemisuccinate (CHEMS, Sigma) also at a 4/1 molar ratio. Liposomes were formed by the dehydration/rehydration method as previously described.[1,32] A trace quantity of radioiodinated peptide was incorporated in the liposome preparation to assess trapping efficiency. The liposomes were extruded through 100 nm filters to achieve uniform sizing.

Antigen catabolism

RAW.Ak cells (10^6/well in 24-well plates) were incubated overnight with or without IFN-γ (above). Both the ^{125}I-HEL and ^{125}I-$α_2$-macroglobulin were prepared by the chloramine T method followed by molecular sieve chromatography and then dialysis to remove any low molecular weight radioactive species. The radioactive proteins were added to the cells for 40 min at 37°. The cells were then washed and further incubated in standard medium [containing 10% fetal calf serum (FCS)] for 60 min at 37°. The medium was removed and the cells were solubilized in 1% Triton with 10% serum; both medium and solubilized cells were then mixed with an equal volume of 20% trichloroacetic acid and incubated on ice for 10 min. Radioactivity was

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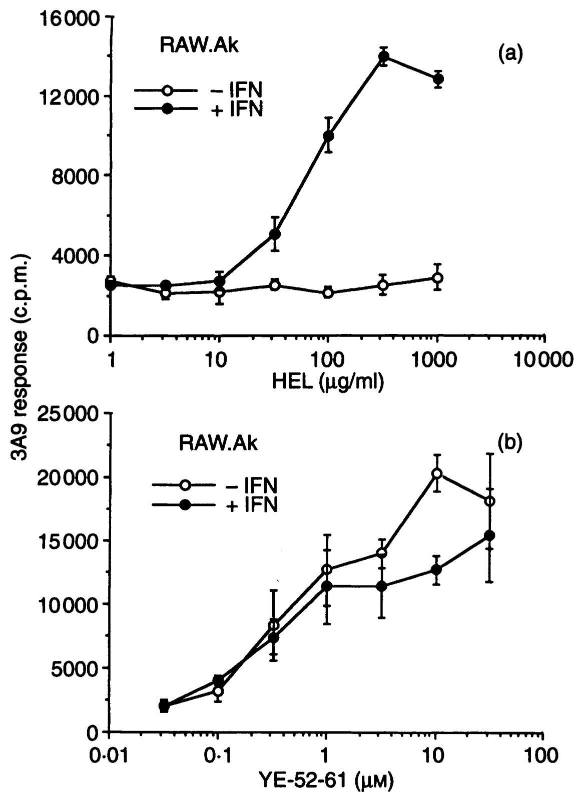

Figure 1. RAW.Ak cells present peptide antigen but have a defect in protein antigen processing that is reversed by IFN-γ. RAW.Ak cells were incubated overnight with or without IFN-γ (10 ng/ml). The cells were then washed and incubated with 3A9 cells and varying levels of antigen to determine antigen presentation (see the Materials and Methods). (a) Processing of intact HEL protein; (b) presentation of the HEL peptide analogue YE-52–61 (YEDYGILQINSR).

determined in the pellet (high molecular weight proteins) and supernatant (low molecular weight catabolites).

FACS analysis

The anti-I-A^k monoclonal antibodies 10·3·6·2 (β chain-specific) and H116–32 (α chain-specific) were protein A affinity purified and biotinylated as described.[33] The biotinylated antibodies were used to stain the surface of cells as described.[34] Streptavidin–phycoerythrin (Chromoprobe) was used as the secondary reagent, and analysis was performed with a FACScan (Becton Dickinson).

RESULTS

RAW.Ak cells were created by expressing I-A^k under the control of the SV-40 promoter in RAW264.7 cells (see the Materials and Methods), and I-A^k expression was confirmed by FACS analysis using anti-I-A^k monoclonal antibodies (data not shown). To test for antigen processing and presentation by these cells, RAW.Ak cells were incubated with HEL or soluble HEL peptide (46–61 or YE-52–61; both contain the 52–61 core determinant and are efficiently recognized by 3A9 cells). 3A9 cells, which recognize the HEL(52–61) epitope bound to I-A^k were then added. While HEL(46–61) or YE-52–61 peptide was effectively presented (Fig. 1 and data not shown), RAW.Ak cells did not process and present HEL protein under these conditions (Fig. 1). Upon stimulation with IFN-γ, however, RAW.Ak cells showed efficient processing of HEL protein with little change in peptide presentation (Fig. 1).

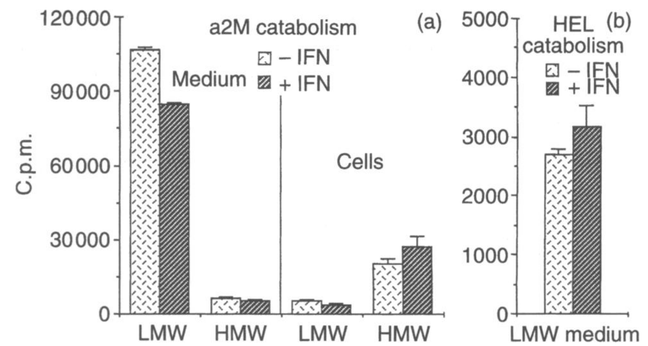

Figure 2. Catabolism of exogenous proteins is intact in RAW.Ak with or without stimulation by IFN-γ. (a) Adherent RAW.Ak cells were incubated with ^{125}I-labelled α_2-macroglobulin for 40 min, washed, and incubated in normal medium for 60 min. The medium and solubilized cells were then separately precipitated with trichloroacetic acid to separate high molecular weight (HMW) proteins from low molecular weight (LMW) catabolites. (b) Catabolism of ^{125}I-labelled HEL was similarly monitored. Lower levels of uptake were reflected in lower levels of catabolism. Low molecular weight catabolites released into the medium are indicated.

Since both FACS analysis and peptide presentation indicated that I-A^k expression was adequate in unstimulated cells, we sought for another defect in the antigen-processing pathway. In order to assess the catabolic activity of these cells, we tested the uptake and catabolism of both ^{125}I-labelled α_2-macroglobulin (efficiently internalized by receptor-mediated endocytosis and targeted to lysosomes) and ^{125}I-HEL (with non-specific internalization). Both the uptake and degradation of α_2-macroglobulin showed a minimal decline with stimulation with IFN-γ (Fig. 2a). HEL uptake and catabolism also showed little change (Fig. 2b), although the uptake of the HEL was much lower. Thus, general protein uptake and catabolism was not deficient in the absence of IFN-γ stimulation.

In addition, we tested the ability of RAW.Ak cells to present HEL(46–61) or YE-52–61 peptide introduced into the cells via liposomes. Acid-resistant DOPC/DOPS liposomes sequester their contents until they reach a lysosomal compartment, where proteins and peptides can be released for efficient processing and subsequent presentation.[1,2] Acid-sensitive DOPE/CHEMS liposomes release their contents in early, acidified endosomes;[32] from which antigens can also be processed (either in endosomes or after further targeting to lysosomes).[1,2] Unstimulated RAW.Ak cells incubated with DOPC/DOPS(46–61) or DOPC/DOPS(YE-52–61) were unable to present this peptide, although efficient presentation resulted after stimulation of RAW.Ak by IFN-γ (Fig. 3). RAW.Ak cells catabolized DOPC/DOPS liposome-encapsulated protein whether or not they were stimulated with IFN-γ (with IFN-γ treatment total liposome uptake increased twofold, but catabolism efficiency decreased by a similar factor, yielding a similar overall rate of catabolism, data not shown). This indicates that the actual release of liposome-encapsulated materials in lysosomes was not the regulated step. Together, these data confirmed that the production or delivery of peptides

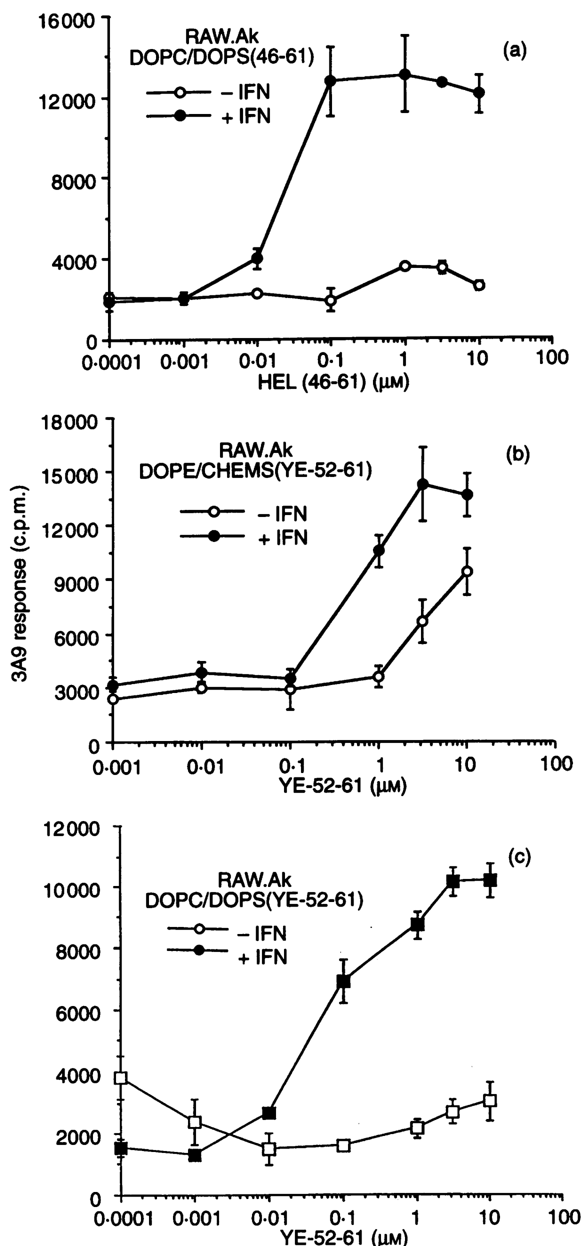

Figure 3. Presentation of liposome-encapsulated peptides by RAW.Ak is governed by IFN-γ. RAW.Ak cells were incubated overnight with or without IFN-γ. The cells were then washed, incubated with liposome-encapsulated peptides, and then washed and fixed with paraformaldehyde prior to incubation with 3A9 T hybridoma cells. (a) Processing of DOPC/DOPS(46–61) (acid-resistant liposomes); (b) processing of DOPE/CHEMS(YE-52–61) (acid-sensitive liposomes); (c) processing of DOPC/DOPS(YE-52–61) (acid-resistant liposomes).

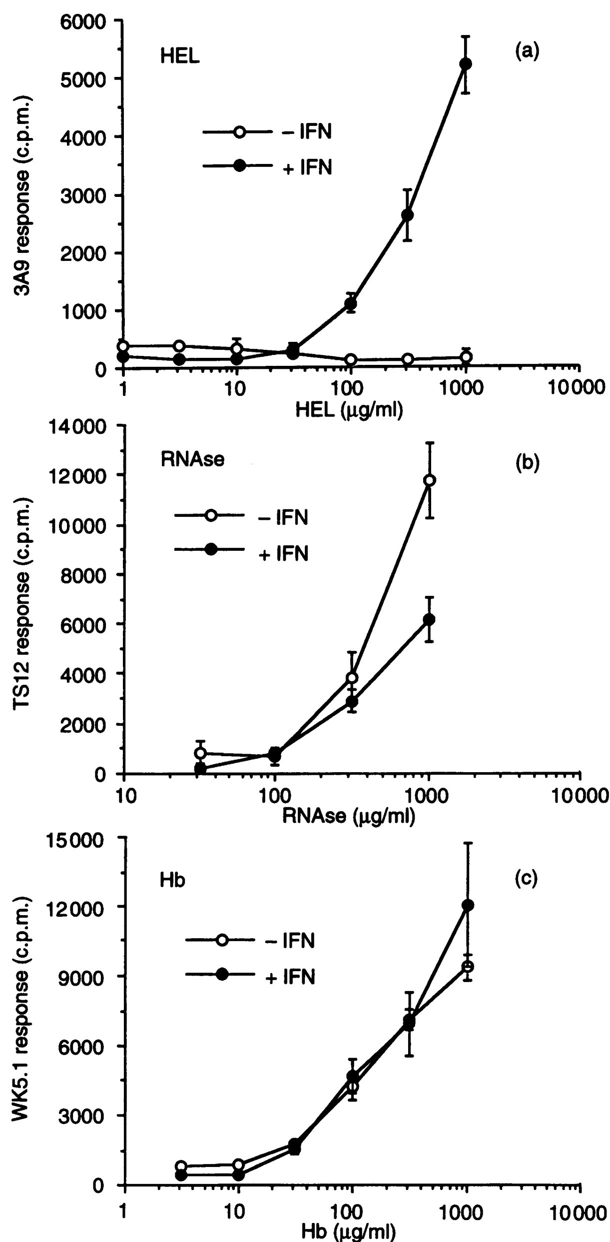

Figure 4. Different antigens are presented to varying degrees by unstimulated RAW.Ak cells. These data are from a single experiment in which RAW.Ak cells were prepared and incubated with antigen plus T hybridoma cells as in Fig. 1. (a) HEL processing detected by 3A9 cells; (b) RNAase processing detected by TS12; (c) Hb processing detected by WK5.1.

by unstimulated RAW.Ak was not limiting, but their subsequent utilization for antigen presentation was blocked. Interestingly, the presentation of peptide encapsulated in DOPE/CHEMS liposomes was less dependent on stimulation by IFN-γ (Fig. 3).

We also examined the ability of RAW.Ak cells to process antigens other than HEL. In contrast to the results with 3A9 recognition of HEL presented by RAW.Ak, unstimulated RAW.Ak were able to process and present both bovine ribonuclease (RNase) and haemoglobin (Hb) (Fig. 4). RNAase was actually more efficiently processed by unstimulated RAW.Ak than RAW.Ak that were stimulated with IFN-γ (in some experiments the difference was greater than shown in Fig. 4b).

Although I-A^k was constitutively expressed in RAW.Ak cells, we did find that its steady state expression level increased approximately fourfold upon stimulation with IFN-γ, as indicated by FACS analysis (Fig. 5). In either case, I-A^k expression remained below the levels observed with CH-27 cells. Since expression of the transfected genes was driven by the

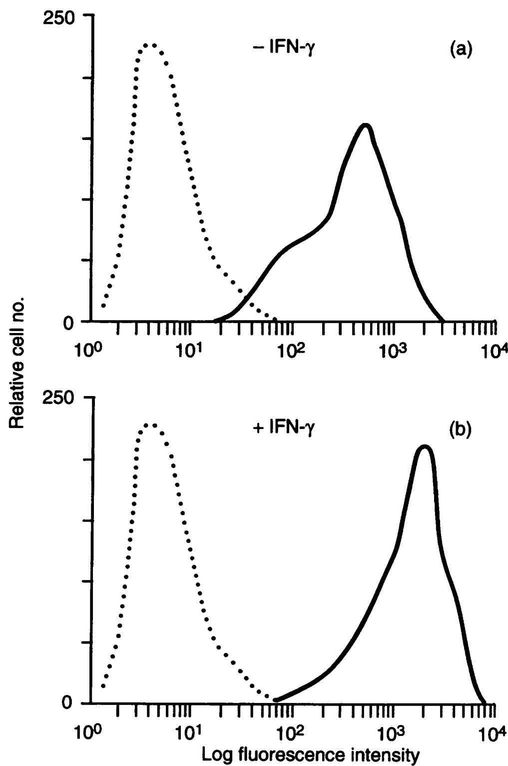

Figure 5. IFN-γ stimulation increases the steady state surface expression of I-A^k on RAW.Ak cells. RAW.Ak cells were incubated overnight with (a) or without (b) IFN-γ. They were then scraped loose and incubated with (solid line) or without (dashed line) biotinylated 10·3·6·2 followed by streptavidin–phycoerythrin.

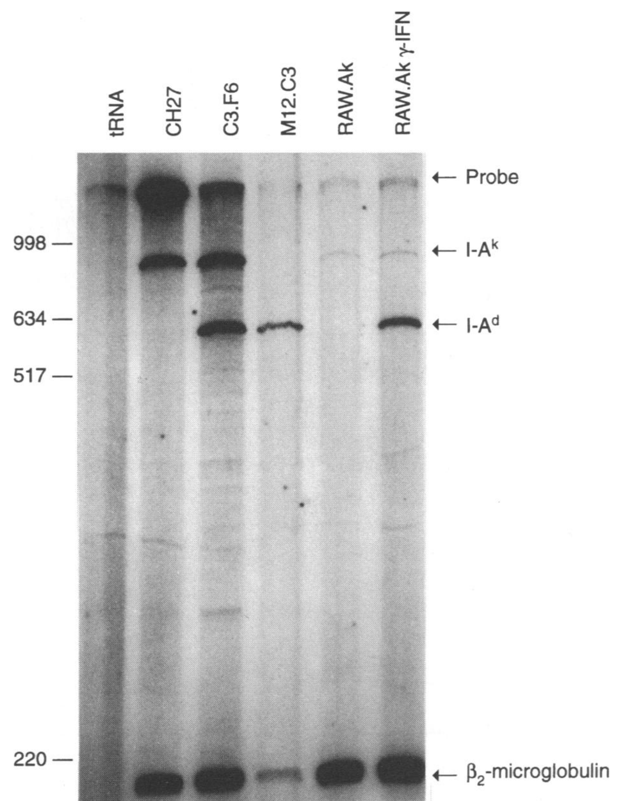

Figure 6. S_1 nuclease analysis of I-A^k expression by RAW.Ak cells with and without stimulation by IFN-γ. Analysis was performed using an I-A^k probe which recognizes I-A^d (592 base fragment) and I-A^k (856 base fragment). tRNA, yeast tRNA control; CH-27, a B lymphoma line expressing I-A^k;[35] M12.C3, an H-2^d B lymphoma line;[34] and C3.F6 cells are derived from M12.C3 transfected with I-A^k.[36] Arrows, undigested probe, 856 base band corresponding to I-A^k, and 592 base band corresponding to I-A^d. Note the β_2-microglobulin band at 202 bases for RNA quality control. Densitometry values for β_2-microglobulin (arbitrary unit): CH-27 = 1, C3.F6 = 1·22, M12.C3 = 0·59, RAW.Ak = 1·48; RAW.Ak + IFN-γ = 1·54. For normalization the MHC-II densitometry values for each sample were divided by the β_2-microglobulin value: CH-27 I-A^k = 0·85, C3.F6 I-A^k = 0·7, C3.F6 I-A^d = 0·66, RAW.Ak I-A^k = 0·032, RAW.Ak + IFN-γ I-A^k = 0·031, RAW.Ak + IFN-γ I-A^d = 0·34.

SV40 promoter, we did not expect that levels of I-A^k mRNA would be affected by IFN-γ. This was confirmed by S_1 nuclease analysis (Fig. 6).[34–36] The level of I-A^k α-specific mRNA, seen at 856 bases in Fig. 6, remains essentially constant regardless of exposure to IFN-γ, while the I-A^d α chain, seen at 592 bases, increases markedly with stimulation by IFN-γ.

DISCUSSION

While antigen presentation by macrophages has long been known to be dependent on their activation by IFN-γ, constitutive expression of I-A^k in RAW.Ak cells has now allowed us to assess separately the requirements for the expression of MHC-II and other proteins induced by IFN-γ which may play important roles in antigen processing. Without IFN-γ stimulation, RAW.Ak cells exhibited a defect in processing of intact HEL, but they were capable of presenting the HEL peptides 46–61 or YE-52–61. On the other hand, RAW.Ak cells efficiently presented both protein and peptides following stimulation with IFN-γ, indicating that IFN-γ had induced the expression of a protein or set of proteins involved in antigen processing.

Several approaches were used to localize functionally the defect in antigen processing by unstimulated RAW.Ak cells. Unstimulated RAW.Ak cells presented peptide antigen with similar efficiency to stimulated RAW.Ak cells, indicating that even without IFN-γ stimulation I-A^k was synthesized at sufficient levels, transported to the plasma membrane, and was capable of binding peptide to form peptide–I-A^k complexes. Therefore, the transfected I-A^k itself appeared functional, but it was not effectively utilized during HEL processing in unstimulated cells. In addition, RAW.Ak cells with or without stimulation with IFN-γ showed similar overall uptake and catabolism of protein (α_2-macroglobulin) internalized by receptor-mediated endocytosis or of antigen (HEL) internalized by non-specific uptake. This suggests that the endocytic uptake of antigen and the general catabolic production of antigenic peptides was intact even in unstimulated RAW.Ak cells (this conclusion is also supported by the ability of RAW-Ak cells to process some epitopes equally with or without stimulation). Theoretically it is possible that the exact pattern or compartmentalization of peptides produced by catabolism and available for presentation could have been altered by changes in the expression of particular proteases induced by treatment with IFN-γ. However, the latter possibility could not

entirely explain the processing defect, since delivery of a single defined peptide, HEL(46–61), into lysosomal processing compartments via DOPC/DOPS liposomes also produced presentation only with IFN-γ stimulation. This implies that the blockade lay distal to either the production or delivery of peptides within the lysosomal processing compartment.

We conclude that the defect in antigen processing by unstimulated RAW.Ak cells lies after the production of peptides within lysosomal compartments and prior to the expression of peptide–MHC-II complexes on the cell surface, implicating one of three types of defects:

(1) Failure of I-A^k and peptide to colocalize within an appropriate compartment to form complexes. This could be due to the failure of I-A^k or peptide to transport to the appropriate endocytic-processing compartment.
(2) Failure of peptides to bind to I-A^k molecules. This could be due to a defect in mechanisms that may be necessary to catalyse the binding of peptides to I-A^k molecules present within the processing compartment. The peptides would then be degraded before adequate binding to I-A^k, which protects peptide determinants from further degradation.[37]
(3) Failure of peptide-I-A^k complexes, once formed, to transport to the cell surface.

A major goal in these studies was the dissection of antigen-processing compartments using differential antigen targeting by liposomes. Peptide encapsulated in DOPC/DOPS liposomes was presented by RAW.Ak cells only upon stimulation by IFN-γ. However, presentation of peptide encapsulated in DOPE/CHEMS liposomes (which release antigen in early endosomes) was less dependent on IFN-γ. Several possible explanations exist for this observation. First, the less stable, acid-sensitive DOPE/CHEMS liposomes could have released some of their contents at the cell surface, allowing binding to surface I-A^k, as with soluble peptide. However, fixed macrophages did not present peptide encapsulated in DOPE/CHEMS liposomes or exhibited only slight presentation at much higher peptide concentrations (data not shown), indicating that this was not the primary explanation. Alternatively, peptide released from DOPE/CHEMS liposomes within earlier endosomal compartments may have intersected with I-A^k molecules, while a productive meeting of I-A^k and peptide may have failed following release of peptide within lysosomal compartments by DOPC/DOPS liposomes in unstimulated RAW.Ak cells. One possibility is that I-A^k targeting to lysosomes in RAW.Ak cells is regulated by IFN-γ, with I-A^k targeting restricted to earlier endocytic compartments in unstimulated RAW.Ak cells. This regulated targeting function could be supplied by invariant chain or other unknown proteins. Transport of antigens or peptides to a specialized subset of late endocytic compartments containing I-A^k molecules could also be regulated. It remains unclear whether any regulation of transport that may exist is specific to MHC-II molecules or antigens, or due to an overall change in transport pathways between vesicular compartments. Alternatively, the regulation could involve other processing functions specifically required in late endocytic/lysosomal compartments. As noted above, this could involve the promotion of peptide–MHC-II binding (a proposed function for human leucocyte antigen (HLA)–DM),[38,39] or transport of peptide–MHC-II complexes to the plasma membrane.

In contrast to the findings with HEL and DOPC/DOPS(46–61) presentation to 3A9 cells, Hb and RNase were processed by unstimulated RAW.Ak. Thus, the processing of these antigens was not dependent on the functions regulated by IFN-γ. Moreover, A6.A2 T hybridoma cells, which are specific for HEL(34–45)-I-A^k, detected some processing and presentation of this epitope even by unstimulated RAW.Ak cells (data not shown). These data suggest that the processing of different antigens or even different epitopes within a given antigen may involve different mechanisms, which may be expressed in different endocytic compartments. For example, immunogenic peptides from RNase and Hb may be produced in a compartment that is different from the site where the HEL(52–61) epitope is generated. Immunogenic epitopes could be generated by processing in either endosomes (perhaps for the RNase and Hb peptides) or lysosomes (perhaps for HEL(46–61)), depending on the lability of the antigen to biochemical degradation events in these different compartments.

Interestingly, similar differences in antigen presentation have been noted by other groups during analyses of the dependence of antigen processing on invariant chain.[15,40–42] In one of these studies,[40] the presentation of HEL was enhanced by coexpression of invariant chain with MHC-II, but the processing of RNase was not dependent on invariant chain expression. Momburg *et al.*[41] reported that the processing of HEL for presentation of HEL(46–61) was increased by coexpression of invariant chain with I-A^k in rat-2 fibroblasts, while they found that the presentation of four other HEL epitopes was unaffected. Peterson and Miller[15] reported that expression specifically of the p41 form of invariant chain was necessary to maximize processing of some antigenic epitopes, e.g. HEL(52–61). However, this parallel does not prove that the RAW.Ak defect is due solely to lack of invariant chain expression in the absence of IFN-γ. In fact, Northern and S_1 nuclease protection analysis for p31 and p41 expression showed that unstimulated RAW.Ak cells expressed detectable levels of invariant chain mRNA (both p31 and p41 forms), although this expression did increase markedly with exposure to IFN-γ (data not shown). In additional studies, RAW.Ak cells were transfected to express constitutively either p41 or p31 (constructs using both SV40 and cytomegalovirus promotors were examined), and invariant chain expression did not alter HEL protein processing for presentation of HEL(52–61) (data not shown). In this case the constitutive expression of p31 approximated levels achieved in RAW.Ak cells that were stimulated with IFN-γ, but p41 expression could not be achieved at equivalently high levels using either promotor system. Consequently, invariant chain deficiency was not demonstrated to explain the antigen-processing defect, but a potential contribution of p41 deficiency to the antigen-processing defect in RAW.Ak cells could not be fully assessed in this macrophage system.

The processing phenotype of unstimulated RAW.Ak cells also resembles several mutant antigen-presenting cells (with genetic defects that map to the MHC locus, excluding invariant chain as the cause) which can also present peptides, but manifest a defect in protein antigen processing. Recent studies[38,39] have shown that the defect in these cells is a lack of HLA-DM. It is interesting to speculate that HLA-DM (and

its murine homolog H-2M) may be regulated by IFN-γ to provide regulated antigen-processing functions. It has also been demonstrated that different epitopes vary in their dependence on HLA-DM,[43,44] consistent with the current results. In fact, many I-A^k-restricted epitopes appear to be DM-independent, with the HEL(52–61) providing a notable DM-dependent exception.[43,44]

This correlates with the pattern of epitopes dependent on IFN-γ stimulation for processing by RAW.Ak cells, suggesting that HLA-DM (or the murine homologue, H-2M) contributes to the regulated processing mechanism. HLA-DM may promote the binding of peptides to MHC-II molecules within late endocytic compartments.[45,46]

Another interesting observation is the increase in the steady state surface expression of I-A^k following stimulation with IFN-γ. I-A^k mRNA levels were essentially unchanged by IFN-γ (Fig. 6), suggesting that post-translational alterations caused the change in I-A^k expression. One possibility is that unstimulated RAW.Ak cells were unable to sufficiently load I-A^k with peptides. Since other results[47] suggest that peptide–MHC-II complexes may be more stable and longer-lived than 'empty' MHC-II molecules, unstimulated RAW.Ak cells could express a greater proportion of shorter-lived unloaded I-A^k molecules, resulting in a lower steady state expression of I-A^k on the plasma membrane. By this interpretation, the magnitude of the shift in I-A^k expression suggests that the regulated lysosomal antigen-processing pathway provides a major proportion of the peptides presented by RAW.Ak after stimulation with IFN-γ, even though some antigens can be processed by the constitutive processing pathway.

We conclude that RAW.Ak cells express an antigen-processing pathway that is regulated by IFN-γ, despite constitutive I-A^k expression. Our dissection of the cell biology of the processing defect in unstimulated RAW.Ak cells implies that the mechanism involves regulation of steps in a lysosomal antigen processing pathway, such as peptide–MHC-II binding, or transport events that are essential to achieve peptide–MHC-II binding and expression of peptide–MHC-II complexes at the cell surface. Our results also suggest that different exogenous antigens may be processed in distinct compartments along the endocytic pathway, with correspondingly different transport requirements, but this hypothesis requires further testing.

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