antibody directed against amino acids 1 through 27 of pOCT (*17*) (Fig. 4A). Clipping by trypsin, therefore, occurred at the NH_2-terminus of pO-OMD. However, rupture of the outer membrane of the mitochondria to allow access of trypsin to the intermembrane space (*4*, *18*) resulted in degradation of both the clipped and unclipped protein (Fig. 4B). Taken together, these results indicate that pO-OMD was inserted into the outer membrane of intact mitochondria in vitro in the N_{cyto}-C_{in} orientation, with most of the protein facing the intermembrane space.

The significance of the full-length, trypsin-resistant, and alkali-resistant pO-OMD (Fig. 3) is not clear. One possibility is that it represents a translocation intermediate in transit to the interior of the organelle. If so, it is surprising that it resists extraction by alkali. Alternatively, it may represent pO-OMD in the N_{cyto}-C_{in} orientation but where the NH_2-terminus partially intercalates into the phospholipid headgroups at the surface of the bilayer and remains shielded from trypsin. Previous studies of the pOCT signal sequence, for example, have shown that its amphiphilic character provides the peptide with high-affinity lipid surface-seeking properties (*19*).

The structural determinants that either cause retention of the NH_2-terminus of pO-OMD on the cytosolic side of the outer membrane during import in vitro or allow translocation of the NH_2-terminus of pOMD29 across the membrane, and whether or not the directionality imposed by the structural determinants is related to their strengths as matrix-targeting signals, remain to be determined. It also remains to be determined if retention of the NH_2-terminus on the cytosolic side of the membrane is the result of interactions with a specific membrane protein (receptor) or with the surface of the lipid bilayer or a result of some other process. Our findings are consistent with models generated for the ER and bacterial plasma membrane (*8–10*) in which orientation is determined by sequences that flank the hydrophobic core of a signal anchor (*8*), especially as this pertains to the presence or absence of a retention signal on the NH_2-terminal side of the hydrophobic core, which is made up in part of positively charged residues (*10*).

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Peptides Presented to the Immune System by the Murine Class II Major Histocompatibility Complex Molecule I-A^d

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Between 650 and 2000 different peptides are associated with the major histocompatibility complex class II molecule I-A^d. Sequences for nine of these were obtained by a combination of automated Edman degradation and tandem mass spectrometry. All of the peptides are derived from secretory or integral membrane proteins that are synthesized by the antigen-presenting cell itself. Peptides were 16 to 18 residues long, had ragged NH_2- and COOH-termini, and contained a six-residue binding motif that was variably placed within the peptide chain. Binding data on truncated peptides suggest that the peptide binding groove on class II molecules can be open at both ends.

Recognition of peptide fragments by T lymphocytes is a central event in the immune response. Peptides are presented to the immune system in association with either class I or class II molecules of the major histocompatibility complex (MHC). Assembly of the peptide-MHC molecule complexes takes place in two different intracellular compartments (*1*, *2*). Peptides that are derived from the degradation of cytosolic proteins and transported into the endoplasmic reticulum are found in complexes with MHC class I molecules (*3–7*). Peptides derived mainly from exogenous proteins that enter the cell by phagocytosis, endocytosis, or internalization of the cell membrane (*8–11*) are processed in acidic endosomal compartments and become associated with MHC class II molecules.

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Sequences for twelve 13- to 17-residue peptides eluted from class II I-A^b and I-E^b molecules (*12*, *13*) were determined by a combination of high-performance liquid chromatography (HPLC) and automated Edman degradation. Each sequence was derived from a cell membrane glycoprotein or from protein present in the cell growth medium (*12*). Several of the peptides had the same core determinant and the same NH_2-terminus but varied in length and thus had different COOH-termini. Surprisingly, three of the four parent proteins contributed 50 to 75% of the total number of peptides associated with each of the class II MHC molecules. If this result were typical of antigen-presenting cells, it would suggest that a relatively small number of self peptides participate in the selection process for the development of immature T cells.

We now present data on peptides from another class II molecule, I-A^d, obtained by reversed-phase HPLC and the combination of microcapillary HPLC–electrospray ionization–tandem mass spectrometry (*14*). This latter methodology defines the approximate number, quantity, and molecular

mass of individual peptides associated with the class II molecules and generates sequence information at the pico- or subpicomole level. The complexity of the peptide mixture associated with I-A^d was at least two orders of magnitude greater than that described for I-A^b (*12*).

Class II I-A^d molecules were purified from cells of the murine B cell lymphoma A20-1.11 by affinity chromatography (*15*, *16*). Peptides were released from the class II molecules by treatment with acid, separated from the protein by filtration through an Amicon filter, and fractionated by reversed-phase HPLC (Fig. 1A). Aliquots from fractions 1 to 37 were subjected to further fractionation by microcapillary HPLC and eluted directly into the electrospray ionization source of a triple quadrupole mass spectrometer. HPLC fraction 16 (1/30 of the volume) yielded spectra numbered 35 to 80 (Fig. 1B). Signals for six peptides dominated the spectrum that results from the summation of scans 55 to 65, although ions for at least 40 others were readily identified (Fig. 1C). Because fragmentation of peptides under the conditions used here for ionization by the electrospray process is not observed, we conclude that all of the observed signals represented ions characteristic of intact molecules.

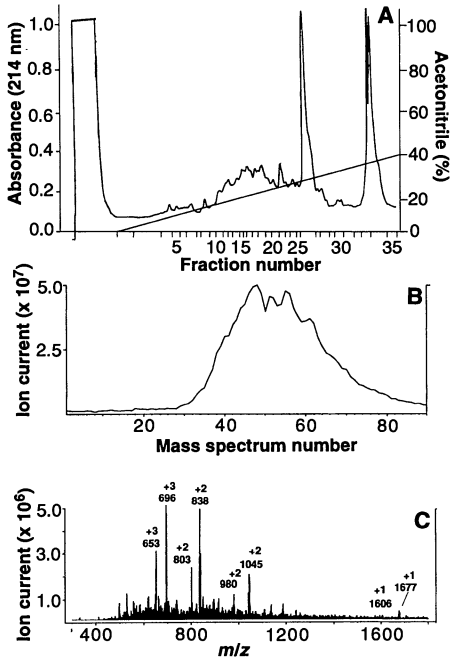

Fig. 1. Complexity of the peptide mixture isolated from the class II molecule I-A^d by acid elution. (**A**) HPLC profile recorded on material eluted from purified I-A^d. (**B**) Plot of total ion current obtained in each mass spectrum recorded on 1/30 of the material in HPLC fraction 16 as it eluted from a microcapillary HPLC column into the mass spectrometer. (**C**) Mass spectra, 55 to 65, summed together. Peptides were fractionated by reversed-phase HPLC on an Applied Biosystems Model 130A separation system. Sample dissolved in 2% acetic acid (500 μl) was injected onto a narrow-bore RP-300 Aquabore column (2.1 m × 3 cm, 300 A, 7 μm) (Applied Biosystems) and eluted with 0.1% trifluoroacetic acid (TFA) for 5 min, with gradients of 0 to 40% (v/v) acetonitrile (0.085% TFA) in 0.1% TFA for 40 min and with 40 to 60% (v/v) acetonitrile (0.85% TFA) in 0.1% TFA for an additional 10 min. Mass spectra of a sample corresponding to 1/30 of each HPLC fraction were recorded on a Finnigan-MAT (San Jose, California) TSQ-70, triple quadrupole mass spectrometer equipped with a microcapillary HPLC column and an electrospray ion source (*7*). The mass range, *m/z* 300 to 1800, was scanned repetitively every 1.5 s, and the total ion signal in each spectra was summed to generate the data shown in (B).

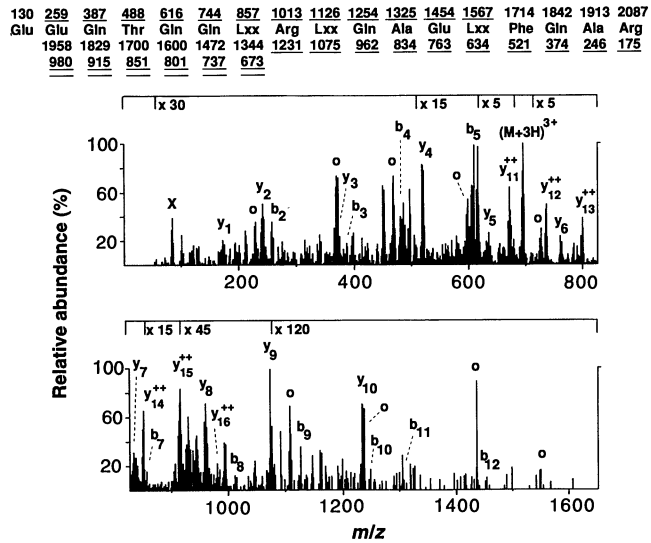

Fig. 2. Collision-activated dissociation mass spectrum recorded on the $(M + 3H)^{3+}$ ions at *m/z* 696 observed in Fig. 1C. Fragments of type b and y, ions having the general formulas $H(NHCHRCO)_n^+$ and $H_2(NHCHRCO)_nOH^+$, respectively, are shown above and below the deduced amino acid sequence at the top (*17*). Doubly charged ions are shown on the line below the sequence; ions observed in the spectrum are underlined. Subtraction of *m/z* values for fragment ions of the same type that differ by a single amino acid, NHCH(R)CO, generated a value that specified the mass and thus the identity of the extra residue in the larger fragment. Lxx in the sequence refers to either Leu or Ile, two residues of identical mass that cannot be differentiated on the triple quadrupole mass spectrometer. Analysis of material in fraction 16 on an Applied Biosystems model 473 protein sequencer produced multiple phenylthiohydantoin–amino acids after each cycle of cleavage but confirmed the above sequence and allowed assignment of residues 7, 9, and 13 as Ile, Leu, and Ile, respectively.

Peptide mixtures of similar complexity were observed in HPLC fractions 14 to 25. Fractions 6 to 13 were less complex but still contained more than ten components each. No peptides were detected in fractions 1 to 5, and the material in fractions 26 and 33 appeared to have a molecular mass in excess of 4,000 and 11,000 daltons, respectively. Proteolytic digestion of this material produced a number of peptides derived from bovine fetuin and from the β chain of the I-A^d molecule itself. Signals for at least 664 distinct peptides were detected in fractions 6 to 25 of the HPLC chromatogram. As many as 2,000 other peptides may be present in amounts at or near the detection limit of the instrument, which is 30 fmol (S/N = 2).

We estimated the quantity of individual peptides present by adding known amounts of synthetic peptides into the HPLC fractions before mass spectral analysis. Yields of various amino acids obtained from the first few cycles of automated Edman degradation performed directly on an aliquot of fraction 16 confirmed the semiquantitative results obtained by mass spectrometry. In sample aliquots corresponding to 1/30 of each HPLC fraction, no peptide was detected at a concentration greater than 10 pmol. The total sample concentration for the 50 most abundant peptides detected by mass spectrometry was 125 pmol.

I-A^d–associated peptides were sequenced either by automated Edman degradation or by tandem mass spectrometry (*17*). We used the latter technique to fragment $(M + 3H)^{3+}$ ions [mass-to-charge ratio (*m/z*) 697] derived from a peptide found in fraction 16 (Fig. 2). Nine I-A^d peptides were sequenced (Table 1), and all contained 16 to 18 residues. Two of the peptides, 1 and 6, were also present without the COOH-terminal residue (peptides 2 and 7). Two of the peptides, 9 and 10, shared the same sequence except for an extra residue at the NH_2-terminus. Thus, the binding groove of the class II molecule may be open at both ends.

Four of the peptide sequences in Table 1, 1, 2, 9, and 10, were from mouse apolipoprotein-E, an abundant secretory protein found in the extracellular medium and involved in plasma cholesterol metabolism (*18*). Peptide 5 probably came from mouse cystatin-C, a secreted inhibitor of cysteine proteinases. The mouse sequence is not available, but residues 40 to 55 of the rat protein (*19*) matched those determined for peptide 5. Peptide 12 appears to have come from the cell surface receptor for the iron transport protein, transferrin. Although the mouse sequence is unavailable, all

Table 1. Data for 12 of the most abundant peptides associated with the class II molecule I-A^d. Peptides were eluted with acid from I-A^d, fractionated by HPLC, and analyzed by tandem mass spectrometry as described (Fig. 1). The most abundant ions observed in the electrospray ionization mass spectra correspond to $(M + 2H)^{2+}$, $(M + 3H)^{3+}$, or $(M + 4H)^{4+}$. Nominal mass values for these (Obs. *m/z*), and the $(M + H)^+$ ion are displayed. Amino acid sequences deduced from either collision-activated dissociation mass spectra or automated Edman degradation are shown in bold (*27*). Critical binding residues are underlined, and additional residues deduced from the source protein are also shown. Picomole yields in 1/30 of each HPLC fraction were determined on the basis of current of 3×10^6 counts per picomole observed for several synthetic peptides corresponding to entries in the table. Protein sources were located by computer searches of gene and protein sequence data banks. IC_{50}, median inhibition concentration; Apo-E, mouse apolipoprotein-E; Cys-C, rat cystatin-C; TF recp., rat transferrin receptor protein (residue 459 is Phe in the rat protein); I-E^d_α, α chain of mouse class II MHC protein I-E^d; Ii, mouse class II invariant chain. ND, not determined; lines indicate no data. X refers to the amino acids Leu or Ile, two residues of identical mass that cannot be distinguished on the triple quadrupole mass spectrometer.

Peptide	HPLC fraction	Obs. *m/z*	$(M + H)^+$ *m/z*	Peptide sequence	Length	Yield (pmol)	Protein source	Residues	Binding capacity [IC_{50} (nM)]	
									I-A^d	I-E^d
1	22,23	944	1887	DMHRQ **WANLMEKIQASVATNPI** ITPVA	17	9	Apo-E	268–284	454 ± 238	—
2	15	888	1774	DMHRQ **WANLMEKIQASVATNP** IITPVA	16	1	Apo-E	268–283	—	—
3	18	864	2591	----------	—	8	Unknown	—	—	—
4	18	587	1760	----------	—	7	Unknown	—	—	—
5	9	476	1900	GSN **DAYHSRAIQVVRARKQ** LVA	16	6	Cys-C	40–55	13 ± 8	1880 ± 886
6	16	838	1677	FAKF **ASFEAQGALANIAVDKA** NLDVA	17	5	I-E^d_α	52–68	14 ± 8	—
7	15,16	803	1606	FAKF **ASFEAQGALANIAVDK** ANLDVA	16	4	I-E^d_α	52–67	—	—
8	18	786	1570	----------	—	5	Unknown	—	—	—
9	16	696	2089	VRSKM **EEQTQQIRLQAEIFQAR** LKGWF	17	5	Apo-E	236–252	250 ± 110	—
10	16	653	1960	VRSKME **EQTQQIRLQAEIFQAR** LKGWF	16	2	Apo-E	237–252	—	—
11	18,19	664	1989	PKSA **KPVSQMRMATPLLMRPM** SMD	17	5	Ii	85–101	14 ± 11	6410 ± 4400
12	24	964	1926	ILIQK **VPQLNQMVRTAAEVAGQX** IIKLT	18	3	TF recp.	442–459	112 ± 35	—
				ISQAVHAAHAEINE			Ovalbumin	323–336	174 ± 18	ND
				LEDARRLKAIYEKKK			λ repressor	12–26	ND	776 ± 264

but Phe^{449} in residues 442 to 449 of the corresponding rat protein (*20*) match those observed in peptide 12. Residues 52 to 67 and 52 to 68 in the α chain of the MHC class II protein I-E^d are identical to the sequences observed for peptides 6 and 7 (*21*); peptide 6 also binds to another class II allele, I-A^b (*12*).

The sequence for peptide 11 matches residues 85 to 101 in the class II invariant chain (Ii) (*22*), a membrane protein that associates with the class II molecule in the endoplasmic reticulum and prevents peptide binding until the class II molecule is transported to the appropriate endosomal compartment (*23, 24*). Residues 85 to 101 are predicted to be on the luminal side of the endoplasmic membrane about 30 residues from the transmembrane segment, making it unlikely that this fragment occludes the antigen-binding groove of class II molecules. A related peptide, residues 85 to 99, associates with the allelic class II molecule I-A^b (*12*). All nine peptides in Table 1 are derived from murine proteins found either on the cell surface in the form of integral membrane proteins or are secreted by the antigen-presenting cell itself. As such, all of these proteins are exposed to the endosomal compartments that are likely to be involved in the processing and presentation of class II peptides.

Sequences derived from the I-A^d–eluted peptides contain at least one, and perhaps two in the case of Ii 85 to 101, sequence motifs (*25, 26*) that are crucial for I-A^d binding. To verify that the peptide sequences obtained were derived from naturally processed peptides, we synthesized the corresponding peptides and tested their capacity to bind purified I-A^d and I-E^d molecules (Table 1). All six of the synthesized peptides bound to the I-A^d molecule in the 10 to 500 nM range. Four of them did not

Table 2. Binding capacity of truncated peptides. Experiments were performed as described (*15, 29*). Abbreviations are as in Table 1 (*27*).

Protein source	Sequence	I-A^d binding capacity [IC_{50} (nM)]
TF recp.	VPQLNQMVRTAAEVAGQL	80 ± 4
	VPQLNQMVRTAAEVAG	77 ± 15
	VPQLNQMVRTAAEVA	1780 ± 393
	VPQLNQMVRTAAEV	1870 ± 261
	VPQLNQMVRTAA	19000 ± 1300
	NQMVRTAAEVAGQL	223 ± 46
	QMVRTAAEVAGQL	412 ± 71
	MVRTAAEVAGQL	4200 ± 603
	RTAAEVAGQL	≥100,000
Apo-E	EEQTQQIRLQAEIFQAR	232 ± 49
	EEQTQQIRLQAEIFQ	544 ± 185
	EEQTQQIRLQAEIF	1230 ± 247
	EEQTQQIRLQAEI	2460 ± 531
	EEQTQQIRLQA	4350 ± 726
	TQQIRLQAEIFQAR	215 ± 82
	IRLQAEIFQAR	1300 ± 430
	RLQAEIFQAR	800 ± 194
	LQAEIFQAR	4100 ± 721
Ovalbumin	ISQAVHAAHAEINEAGR	134 ± 26

bind I-E^d in our assay, and two bound I-E^d only weakly [dissociation constant (K_d) in the 2 to 10 μM range]. Three of the synthetic peptides, 5, 6, and 11, bound I-A^d with an affinity that was several times higher than that observed with the reference peptide ovalbumin (residues 323 to 339) (*15*).

Because most of the peptides associated with class I MHC contain only nine residues (*4–7*), we determined if the longer class II peptides were of optimal length for high-affinity binding to the I-A^d molecule. Truncated analogs of these peptides were synthesized and tested for their capacity to bind purified class II molecules (Table 2). The first two COOH-terminal residues of the peptide derived from the transferrin receptor, peptide 12, could be removed without any loss of binding capacity. Removal of the next residue yielded a peptide with 4% of the binding capacity for I-A^d. At the NH_2-terminal end of the peptide, removal of the first five residues caused a 66 to 80% reduction in I-A^d binding capacity, whereas removal of the sixth residue induced a greater than 98% decrease. Thus, the crucial I-A^d binding core region is contained within the ten-residue sequence QMVRTAAEVA (*27*).

Removal of each of the six, COOH-terminal residues from peptide 9 derived from apolipoprotein-E resulted in an approximately 50% decrease in binding capacity. Thus, an exact COOH-terminal boundary of the region crucial for I-A^d binding could not be clearly defined. Similar results have been obtained in the case of other class II peptide binding studies and have been attributed to the presence of more than one potential binding site in the epitope analyzed (*28*). At the NH_2-terminal end of the peptide, removal of the first seven residues caused a 66% reduction of binding affinity, but removal of the next residue, Arg^{243}, generated a peptide whose affinity was reduced by approximately 95% compared to that of the full-length determinant. More than one I-A^d binding site may therefore be contained within residues 236 to 252. The main binding site is contained within residues 243 to 251, RLQAEIFQA (*27*), as predicted by the presence of two potential canonical I-A^d binding motifs (*25, 26*).

Binding data on truncated synthetic peptides indicate that the length of the naturally processed peptides is not the minimum required for strong binding. Unlike class I peptides, class II peptides can be shortened without loss of binding activity. Crucial binding regions within the class II peptides are positioned variably within the observed sequences relative to their NH_2- and COOH-termini. In some cases, only two residues can be removed without a reduction of binding capacity, whereas in other cases, up to seven residues can be removed. These data are also at odds with a model in which the COOH-terminal end of the peptide is variable, but in which the NH_2-terminal end is fixed at a constant distance from the crucial binding region (*12*). Our results suggest that the binding groove of the I-A^d molecule is probably open at both ends.

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Neurotransmitter Release from Synaptotagmin-Deficient Clonal Variants of PC12 Cells

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Synaptotagmin (p65) is an abundant synaptic vesicle protein of neurons and contains regions similar to the regulatory domain of protein kinase C. These domains are thought to be involved in calcium-dependent interaction with membrane phospholipids during exocytosis. To assess the functional role of synaptotagmin, synaptotagmin-deficient clonal variants of PC12 cells were isolated. All of the variant cells released catecholamine and adenosine triphosphate in response to elevated intracellular concentrations of calcium, which suggests that synaptotagmin is not essential for secretion of catecholamine and adenosine triphosphate from PC12 cells.

Neurotransmitters and hormones are released from neurons and endocrine cells by exocytosis, but little is known about the molecular mechanisms involved (*1*). Synaptotagmin (p65) is an integral membrane glycoprotein found in secretory vesicles in a variety of neurons and some endocrine cells (*2, 3*). It has a single transmembrane segment with the NH_2- and COOH-termini located in the intravesicular and cytoplasmic spaces, respectively (*4, 5*). The primary structure of the cytoplasmic region is conserved in various animal species (*4–7*) and may be functionally important in secretion. This cytoplasmic portion contains two copies of a region similar to the C2 domain of Ca^{2+}-phospholipid–dependent protein kinase (protein kinase C), which is essential for the expression of Ca^{2+} and phospholipid dependency of the enzyme (*8*). In fact, synaptotagmin binds to phospholipid through this region, and this characteristic feature has led to the hypothesis that synaptotagmin participates in Ca^{2+}-dependent docking of secretory vesicles at the plasma membrane or in the fusion of secretory vesicles with plasma membrane during neurotransmitter release (*3, 4*).

The rat pheochromocytoma cell line PC12 secretes catecholamines and acetyl-

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