Abstract. Class I major histocompatibility (MHC) antigens are expressed by virtually all mammalian cells, yet their levels of expression and behavior on the cell surface vary in a cell-specific fashion. A panel of lymphoid (both B and T) and nonlymphoid cell lines was used to study the kinetics of internalization of the H-2L^d class I MHC in different cell types. These studies revealed that endocytosis of H-2L^d occurs by both constitutive and PMA-regulated pathways in lymphoid cells, but only by a PMA-refractory pathway in the nonlymphoid cells tested. Transfectant derivatives of the T lymphoma, EL4, which express wild-type or mutant H-2L^d class I MHC antigens, were used to investigate the requirement for the cytoplasmic domain of the class I MHC antigen for its endocytosis in T lymphocytes. These studies showed that modification or deletion of the cytoplasmic domain of H-2L^d abrogates endocytosis via a PMA-regulated pathway. The role of cytoplasmic domain phosphorylation in PMA-inducible endocytosis was examined. The wild-type H-2L^d antigen is phosphorylated in all cell types examined, and this phosphorylation is up-regulated by PMA treatment. In contrast, cytoplasmic domain mutants of H-2L^d fail to be phosphorylated in vivo, in the presence or absence of PMA. The universality of PMA-inducible hyperphosphorylation of the class I MHC antigen among diverse cell types leads us to conclude that phosphorylation of the cytoplasmic domain, while perhaps necessary, is not sufficient for triggering endocytosis via a PMA-inducible pathway. Furthermore, the results with the cytoplasmic domain mutants of H-2L^d suggest that a structural conformation of the class I MHC cytoplasmic domain is required for endocytosis via this route.
eral interesting observations concerning class I MHC antigens. The first of these is the apparent heterogeneity in the trafficking of these proteins in different cell types: class I MHC molecules have been shown to be internalized rapidly by T lymphocytes which have been activated by mitogens and T lymphomas, but not by B lymphocytes, B lymphomas, or L cell fibroblasts (Machy et al., 1982a,b, 1987; Tse and Pernis, 1984; Pernis, 1985; Aragorn et al., 1986). Endocytosis of class I MHC molecules also has been observed in macrophage/monocyte cells (Dasgupta et al., 1988). The endocytosed class I molecules in T cells and monocytes enter the cell via coated pits (Machy et al., 1987; Dasgupta et al., 1988), in contrast to antibody cross-linked class I MHC antigens internalized by fibroblasts (Huet et al., 1980). This differential internalization of class I MHC molecules by different cell types is striking, since biosynthetic studies have shown that the intracellular routing during biosynthesis is relatively invariant among cell types (Kranjel et al., 1979; Dobberstein et al., 1979; Owen et al., 1980; Sege et al., 1981; Zúñiga and Hood, 1986). Another notable feature of class I MHC antigens is that their cytoplasmic domains are phosphorylated in vivo, and this phosphorylation is up-regulated by treatment of cells with phorbol esters (McCluskey et al., 1986). The phosphorylation of the cytoplasmic domain and its modulation by phorbol esters suggest a regulation of cell surface expression of class I MHC antigens akin to that described for other cell surface proteins, such as the transferrin and epidermal growth factor receptors (Davis and Czech, 1984; Hunter et al., 1984; Klausner et al., 1984; Hanover and Dickson, 1985; May et al., 1984, 1985; Beguinot et al., 1985). Thus, the regulation of differential endocytosis of class I MHC antigens in different cell types may play a pivotal role in their function as immunoregulatory molecules. We are interested in identifying structural features of the class I MHC molecule which may be required for appropriate intracellular routing, with the ultimate goal of using transport-defective mutant molecules to study the functional significance of this process. Since the endocytosis of a number of cell surface receptors (e.g., low density lipoprotein, epidermal growth factor, transferrin, and poly-Ig receptors) is abrogated by mutations in the cytoplasmic domains (Goldstein et al., 1985; Lehrman et al., 1985; Davis et al., 1986, 1987; Prywes et al., 1986; Rothenberger et al., 1987; Iacopetta et al., 1988; Mostov et al., 1986), we set out to explore the role of the cytoplasmic tail in the endocytosis of the class I MHC molecule.

In this paper we report results from studies on the H-2L<sup>d</sup> class I MHC antigen in a number of lymphoid and nonlymphoid cell lines. The data presented here suggest that internalization of class I MHC antigens occurs by both unregulated and phorbol ester-regulated pathways and that internalization by the latter pathway is a lymphoid cell-specific phenomenon. Studies with transfected cell lines generated from the T lymphoma, EL4, which express in vitro mutagenesis-derived mutant forms of the H-2L<sup>d</sup> gene, show that structural information contained within the cytoplasmic domain of the class I MHC glycoprotein is required for endocytosis via both constitutive and phorbol ester-regulated pathways. Furthermore, while cytoplasmic domain mutants of the H-2L<sup>d</sup> antigen are neither phosphorylated nor endocytosed in PMA-treated cells, there is not a direct correlation between PMA-induced phosphorylation of the H-2L<sup>d</sup> class I MHC antigen and PMA-induced endocytosis in that PMA-induced hyperphosphorylation of class I MHC molecules occurs in all cell types examined.

Materials and Methods

Reagents

PMA was purchased from Calbiochem-Behring Corp. (La Jolla, CA); [<sup>35</sup>S]methionine, [<sup>32</sup>P]orthophosphate, and [125]Iodine were purchased from New England Nuclear (Boston, MA); DME and RPMI 1640 were purchased form Mediatech (Washington, DC); FCS and Serum Plus were from Hyclone Research Products (Luxenau, KS); G418 sulfate (Geneticin) was from Gibco Laboratories (Grand Island, NY); RIA grade BSA, hyposyanine, and thymidine were obtained from Sigma Chemical Co. (St. Louis, MO); Enzymoabs were from Bio-Rad Laboratories (Richmond, CA); and Sephadex G-50 and protein A-Sepharose were from Pharmacia Fine Chemicals (Piscataway, NJ).

Cell Lines

The cell lines used in these studies are described in Table I. P815, EL4, and the EL4 transfectants were grown in DME supplemented with 10% Serum Plus and 40 μg/ml G418; 18-48 and BCL1 cells were grown in RPMI 1640 supplemented with 10% FCS, 5 × 10<sup>-5</sup> M β-mercaptoethanol, and 40 μg/ml G418; and 27.5.27 D1 cells were maintained in DME supplemented with 10% FCS and HAT (hyposyanine, amiprotein, thymidine) as described previously (Zúñiga et al., 1983).

H-2L<sup>d</sup> Gene Constructs and Transfection of Fibroblasts and EL4 Cells

In vitro mutagenesis and characterization of mutant H-2L<sup>d</sup> gene constructs encoding the BAL097 and 2.2.1 H-2L<sup>d</sup> molecules (Fig. 1) are described in previous publications (Zúñiga et al., 1983; Zúñiga and Hood, 1986). Mouse L cell transfectants expressing the wild-type H-2L<sup>d</sup> gene were described previously (Zúñiga et al., 1983). For transfection of EL4 T lymphoma cells, the cloned DNAs bearing wild-type and mutant gene constructs were further modified by the inclusion of a small cassette containing the neomycin resistance gene driven off of an early SV-40 promoter. These constructs were made by Scott F. Walk (University of Virginia School of Medicine, Charlottesville, VA). These constructs were introduced into EL4 lymphoma cells by protoplast fusion (Sandri-Goldin et al., 1981), and stable transformants were selected in RPMI 1640 supplemented with 5% FCS and 400 μg/ml G418 sulfate (Geneticin). Transfected EL4 cell lines were generated and cloned by Dr. Mark Holterman (University of Virginia School of Medicine, Charlottesville, VA).

Purification and Iodination of Antibodies

The 30.5.7 and 28.14.8 mAbs were purified from culture supernatants by affinity chromatography over protein A-Sepharose equilibrated with 3 M thiocyanate, aminopterin, and thymidine were obtained from Sigma Chemical Co. (St. Louis, MO); Enzymoabs were from Bio-Rad Laboratories (Richmond, CA); and Sephadex G-50 and protein A-Sepharose were from Pharmacia Fine Chemicals (Piscataway, NJ).
(PBS supplemented with 0.2% FCS and 5 mM sodium azide), plated at 5 × 10^6 cells/well in round-bottomed, 96-well plates (Dynatech Immunol Corp., Alexandria, VA), and were incubated at 4°C for 1 h with varying amounts of 125I-labeled mAb as indicated in Fig. 2. Cells were washed three times in PBS/FCS/azide, and cell pellets were resuspended in 100 μl PBS before quantitation of radioactivity bound in the Beckman Instruments Inc. (Fullerton, CA) model 7000 gamma counter.

**Endocytosis Assay**

The general scheme for the endocytosis assay is illustrated in Fig. 3. Cells were washed in PBS, resuspended to 10^6 cells/ml in PBS/FCS (PBS supplemented with 0.2% FCS), plated at 5 × 10^5 cells/well in round-bottomed, 96-well plates (Dynatech Immunol Corp.), and incubated at 4°C for 1 h with 5 × 10^6 cpm 125I-labeled mAb in a final volume of 50 μl. Cells were washed three times with PBS/FCS. Pellets were incubated in 50 μl DME in the presence or absence of PMA at 37°C in a CO2 incubator for the time periods indicated in Figs. 5 and 6. At the end of each incubation period, cells were digested with 100 μl PBS/BSA/azide (PBS supplemented with 2 mg/ml BSA and 5 mM sodium azide), centrifuged, and the supernatants were harvested. Cell pellets were resuspended in 50 μl PBS and incubated for 10 min at 4°C with 150 μl ice-cold barbital buffer (20 mM sodium barbital, 28 mM sodium acetate, 114 mM sodium chloride, pH 3). The cells were centrifuged, resuspended in 150 μl barbital buffer, and centrifuged immediately. The barbital washes were pooled. Quantitation of antibody shed during the post-37°C supernatant, the barbital washes, and the cell pellets, respectively, in a Beckman Instruments, Inc. model 7000 gamma counter.

**In Vivo Labeling of Cells and Immunoprecipitation of H-2 Polypeptides**

Cells were washed three times in PBS or phosphate-free buffer (0.15 M NaCl, 0.01 M MOPS, 2 mM glutamine, 1.8 mM glucose, 0.01 M Tris-acetate, pH 7.4) for [35S]methionine and [32P]orthophosphate labeling, respectively, and then resuspended at a concentration of 10^6 cells/ml in labeling medium. In some experiments the cells were labeled with [3S]methionine and [32P]orthophosphate separately, in which case the labeling media were methionine-deficient RPMI 1640 ( Irvine Scientific, Santa Ana, CA) supplemented with 10% dilaoyzed FCS, and phosphate-free medium (prepared according to Gibco Laboratories formulations for RPMI 1640, except that the medium was buffered with Hepes rather than with phosphate) supplemented with 9 mg/ml BSA, respectively. In other studies, [3S]methionine and [32P]orthophosphate labeling were carried out on the same cell sample simultaneously, in which case Hepes-buffered RPMI 1640 which was deficient in both methionine and phosphate and was supplemented with 9 mg/ml BSA was used. The cells were incubated for a minimum of 30 min at 37°C in a CO2 incubator before the addition of labeled precursor. For biosynthetic labeling of proteins, [3S]methionine was added to cells in methionine-deficient RPMI 1640 to a final concentration of 300 μCi/ml, and cells were incubated for 2-3 h at 37°C in a CO2 incubator. For phosphate labeling of cells, carrier-free [32P]orthophosphate was added to cells in phosphate-free medium to a final concentration of 1 mCi/ml, and cells were incubated for 2 h at 37°C in a CO2 incubator. For studies on the induction of phosphorylation by phorbol esters, PMA was added to a final concentration of 10 ng/ml or 50 ng/ml, and the cells were incubated an additional 30 min. At the end of the labeling period, cells were washed twice with PBS supplemented with phosphate inhibitors (0.4 mM EDTA, 10 mM NaF, 10 mM sodium pyrophosphate, 0.4 mM sodium vanadate), and lysed by 107 cells/ml. The lysis buffer consisted of the phosphatase inhibitors (above), 1 mM PMSF, and phosphatase inhibitors). H-2 proteins were then eluted twice sequentially at room temperature with 25 μl of elution buffer (0.5% SDS, 0.01 M DTT, and 0.01 M Tris-HCl, pH 7.5). Lysates from which H-2L was had been removed by immunoprecipitation with the 30.5.7 mAb were subsequently incubated with the 28.14.8 mAb to immunoprecipitate the H-2Dβ protein. Control experiments with cell lines which express H-2Ld but not H-2Dβ show that no detectable H-2L can remains after the 30.5.7 mAb immunoprecipitation step. 1-μl samples of eluates were added to 3 ml of Biofluor scintillation fluid, then counted.

### Table 1. Mouse Lymphoid and Nonlymphoid Cell Lines

| Cell line          | Cell type                          | H-2 protein studied |
|--------------------|------------------------------------|---------------------|
| 18-48              | pre-B                              | H-2Ld               |
| BCL1               | B                                  | H-2Ld               |
| EL4                | T lymphoma                        | H-2Dβ               |
| EL3                | T lymphoma                        | H-2Dβ, transfected H-2Ld |
| EL4.907            | T lymphoma                        | H-2Dβ, transfected H-2Ld (BAL 907) |
| ELA.2.2.1          | T lymphoma                        | H-2Dβ, transfected H-2Ld (2.2.1) |
| P815               | Mastocytoma                        | H-2Ld               |
| 27.5.27 D1         | Fibroblast                         | Transfected H-2Ld   |

### Results

**Cell Surface Expression of Wild-type and Mutant H-2Ld Antigens**

We examined the cell surface expression of the H-2Ld antigen in two B cell lines, three EL4 T lymphoma transfectants, the P815 mastocytoma, and an L cell transfectant by RIA (Table 1; Fig. 2). All of these cell lines, including EL4 transfectants expressing mutant H-2Ld molecules (Fig. 1) with an altered (EL4.907) or no (EL4.2.2.1) cytoplasmic tail, express this class I MHC molecule at comparable levels (Fig. 2). The cell surface expression of the mutant H-2Ld molecules expressed by EL4.907 and EL4.2.2.1 is not surprising, since we have observed previously that the cytoplasmic domain of the H-2Ld antigen is not required for the cell surface expression of this glycoprotein by L cell fibroblasts, but that it facilitates its intracellular transport during biosynthesis (Zúñiga and Hood, 1986). Although the BAL 907 and 2.2.1 mutant molecules occur at the cell surface at a density comparable to that of the H-2Ld glycoprotein which bears a full length cytoplasmic tail, it is worth noting that they are transported to the EL4 plasma membrane more slowly than is the wild-type molecule (Zúñiga, M. C., unpublished data). Hence, the cytoplasmic domain may be involved in seques-
Figure 1. Cytoplasmic tail sequences of wild-type and mutant H-2L^d^ mutants. The wild-type (wt) H-2L^d^ gene sequence is deduced from the DNA sequence of a genomic clone determined by Moore et al. (1982). The BAL 907 mutant was generated by deletion mutagenesis and has been described previously (Zúñiga et al., 1983). The 2.2.1 mutant of H-2L^d^ was made by site-directed mutagenesis (Zúñiga and Hood, 1986). Cytoplasmic domain sequences were predicted from the DNA sequences published previously (Zúñiga et al., 1983; Zúñiga and Hood, 1986). The external domains of the H-2L^d^ antigens are depicted by the bold line, whereas ~2-M is represented by the hatched line. The membrane-spanning regions of the H-2L^d^ glycoproteins are depicted by the helical structures embedded in the phospholipid bilayer.

Figure 2. Cell surface expression of H-2L^d^ by lymphoid and nonlymphoid cell lines. The cell lines listed in Table I were tested for cell surface expression of the transfected H-2L^d^ gene product and the endogenous H-2D^b^ gene product by RIA as described in Materials and Methods. The 30.5.7 mAb (A and C) binds to H-2L^d^, whereas the 28.14.8 mAb (B) binds both H-2L^d^ and H-2D^b^ (Ozato et al., 1980; Evans et al., 1982). Hence, EL4 cells (■) which are of the b haplotype bind 28.14.8 (B) but not 30.5.7 (A), in contrast to EL^b^ cells (transfected with the wild-type H-2L^d^ gene; ○) (B). EL4.907 cells (transfected with the BAL-907-2 mutant of H-2L^d^; □) and EL4.2.2.1 cells (transfected with the 2.2.1 mutant of H-2L^d^; ◦), which bind both mAbs. BW5147 thymoma cells (H-2^k^ haplotype, ▲) serve as a negative control for the 28.14.8 mAb (B). C shows the binding of 30.5.7 by BCL1 (○), 18-48 (○), 27.5.27 DI (■), and P815 (■). Each data point represents the average of duplicates, and barring the exceptions listed below, is within 7% of the experimentally obtained values. The data points that do not fall within this degree of precision are those for the EL4 cells incubated with 5 × 10^5 cpm ^125^I-30.5.7 (A), the EL4.2.2.1 cells incubated with 2 × 10^6 cpm ^125^I-30.5.7 (A), the BW5147 cells incubated with 2 × 10^5 cpm and 5 × 10^5 cpm of ^125^I-28.14.8 (B), and the 27.5.27 DI cells incubated with 2 × 10^5 cpm and 5 × 10^5 cpm ^125^I-30.5.7 (C), for which the averages represented by the data points given differ from the experimentally obtained values by 22.2, 12.0, 12.9, 11.3, 9.57, and 16.22%, respectively.
The cells were washed and incubated at 37°C (or at 4°C in the case of control samples) in the presence or absence of PMA for varying periods of time and then subjected to a wash with acid barbital to strip antibody which is bound to the cell surface. Antibody which is resistant to acid stripping (over and beyond that which is bound in negative control samples) is assumed to have been internalized by the cells (Olefsky and Kao, 1982). Refer to the Materials and Methods sections of text for further details of endocytosis assay.

Internalization of Class I MHC Antigens by Lymphoid Cells Is Up-Regulated by Treatment with Phorbol Esters

Phorbol esters are potent activators of calcium, phospholipid-dependent protein kinase C, and AMP-dependent protein kinase A (Castagna et al., 1982; Nishizuka, 1984; Berridge and Irvine, 1984). Phorbol esters have been used extensively in the analysis of receptor-mediated endocytosis. These compounds modulate the cell surface expression of transferrin and epidermal growth factor receptors by stimulating the exocytic and/or endocytic processes (Klausner et al., 1984; Hanover and Dickson, 1985; May et al., 1985). Phorbol ester treatment of erythroleukemia cells causes a rapid decrease in cell surface transferrin receptor expression (Klausner et al., 1984), whereas the same treatment of the macrophage cell line, J774, results in higher levels of cell surface transferrin receptor expression (Buys et al., 1984). Thus, the effect of phorbol esters is cell line-specific, at least in the case of the transferrin receptor. These observations prompted us to consider the possibility that the previously reported T cell-specific endocytosis of class I MHC antigens is modulated by phorbol esters.

We examined the effects of the phorbol ester, PMA, on the internalization of endogenous and transfected class I MHC molecules by lymphoid and nonlymphoid cells using the modified RIA described in Fig. 3 and in Materials and Methods. This assay is adapted from a standard protocol which has been used by other investigators for the analysis of endocytosis of cell surface receptors (Olefsky and Kao, 1982; Prywes et al., 1986; Rothenberger et al., 1987; Truneh et al., 1983). In our procedure cells are preincubated at 4°C with radiiodinated antibody which is specific for a given class I MHC antigen, washed, and then incubated for varying periods of time at 37°C in the presence or absence of PMA. The culture supernatants are harvested, and then the cells are washed in acid to strip cell surface antibody. Culture supernatants (which contain any antibody "shed" during the 37°C incubation), acid washes (containing antibody stripped from the cell surface), and cell pellets (having internalized antibody molecules) are then counted in a gamma counter.

We were concerned that our data might be clouded by two potential artifacts, specifically, the unintended lysis of the cells during the acid treatment and insufficient stripping of cell surface molecules by this treatment. In control experiments with 51Cr-labeled cells, we have determined that EL4 cells remain intact during the acid washes, with <11% of the 51Cr being released from the cells (data not shown). To assess the efficiency of stripping of cell surface antibody by the acid treatment, we examined the amount of radiiodinated antibody remaining in the cell pellet when cells are stripped immediately after removal of unbound antibody. This control, which shows that ~95% of the radioiodinated antibody which was bound by the cells is stripped by the acid treatment, was performed in all endocytosis studies.

Internalization of the radioiodinated antibody is dependent on metabolism, since cells which are incubated for up to 120 min at 4°C in the absence or presence of PMA have equivalently low amounts of acid-resistant radiolabeled antibody bound to their surfaces as do control cells which are stripped immediately after the 4°C incubation with the antibody (Figs. 5, A and B, 6, A-D, and data not shown). Incubation of the EL4 transfectant cell line, EL4-3 with PMA at 37°C results in a pronounced increase in the internalization of H-2Ld, and has this effect in a dose-dependent fashion (Fig. 4). In an effort to determine the generality of this phenomenon we examined the internalization of the
H-2L\(^{a}\) antigen in the presence and absence of PMA in the cell lines described in Table I. The basal levels and rates of endocytosis of H-2L\(^{a}\) are comparable in all of the cell lines examined (Figs. 5, A and B, and 6 A). However, PMA stimulation markedly increases the internalization of H-2L\(^{a}\) in B (Fig. 5) and T (Fig. 6) cell lines, while having no effect on P815 mastocytoma cells and L cell fibroblasts (Fig. 5). Kinetic analyses of H-L\(^{a}\) internalization by these cell lines shows that PMA up-regulates the rate of endocytosis of this class I MHC antigen in all of the lymphoid cell lines, but has no effect or even a slightly inhibitory effect on the two nonlymphoid cell lines tested (Fig. 5, A and B). This phenomenon is not unique to the transfected H-2L\(^{a}\) antigen, since the rate of internalization of the endogenous H-2D\(^{b}\) class I MHC molecule of EL4 cells is also increased by PMA (Fig. 6 D). Although the panel of selected cell lines is small, these results show that the internalization of class I MHC antigens is subject to lineage-specific regulation.

Internalization of Class I MHC Antigens Via a PMA-inducible Pathway is Abrogated by Alteration or Deletion of the Cytoplasmic Domain

Evidence from a number of cell surface receptor systems establishes a role for the cytoplasmic domains of receptors in their endocytosis via coated pits (Goldstein et al., 1985; Lehrman et al., 1985; Davis et al., 1986, 1987; Iacopetta et al., 1988; Lazarovits and Roth, 1988). Although class I MHC antigens have no known role as cell surface receptors, they too are internalized via coated pits (Machy et al., 1987; Dasgupta et al., 1988). Having documented the PMA-inducibility of H-2L\(^{a}\) internalization in lymphoid cells, we next studied the requirement for the cytoplasmic domain of this molecule in this process. We examined an EL4 transfectant expressing an H-2L\(^{a}\) antigen which has an altered cytoplasmic tail (EL.907; Fig. 1) and one expressing an H-2L\(^{a}\) molecule which lacks the cytoplasmic tail altogether (EL4.2.2.1; Fig. 1) for their ability to internalize these mu-
three times for each cell line. The SD of the data used to generate each of the data points in graphs A-D is <2% from the value given, except for EL3 with mAb 28.14.8 at 37°C + PMA at 180 min, which is 2.4% from the value given; EL4.2.2.1 with mAb 28.14.8 at 37°C + PMA at 60 min, which is 2.5% from the value given; and EL4 with mAb 28.14.8 at 37°C + PMA at 180 min, which is 5.1% from the value given. The data presented in E and F are from endocytosis experiments with EL3, EL4.907, and EL4.2.2.1 in which the incubation at 37°C was for 120 min. Data for samples which were incubated at 37°C in the absence of PMA are represented by the stippled bars (E and F) and represent the baseline against which the percent change is measured. The values obtained for samples which were incubated at 37°C with 50 ng/ml PMA are depicted by the solid bars (E and F) and reflect the percent increase or decrease in endocytosis relative to cells not treated with PMA. Each bar is the average of triplicates, and each experiment was done at least twice. The SD of the data used to generate these two graphs (E and F) is <5% of the value of the bar, except for EL4.907 with mAb 30.5.7, which is 7.8% of the value of the bar; EL4.2.2.1 with mAb 30.5.7, which is 6.7% of the value of the bar; and EL3 with mAb 28.14.8, which is 7.7% of the value of the bar.
F). Hence, the structural motif required for PMA-regulated endocytosis of the BAL 907 mutant fails to be endocytosed more rapidly in PMA-treated cells (Fig. 6, A and E). The failure of the BAL 907 mutant to target to endocytic vesicles is not due to a lack of expression of the class I MHC antigen and is destroyed in the BAL 907 mutant (Edidin and Züniga, 1984). However, like the 2.2.1 mutant, the BAL 907 mutant fails to be endocytosed in a PMA-inducible fashion by both of these transfectants (Fig. 6, A, C, D, and F). Hence, the structural motif required for PMA-regulated endocytosis by lymphoid cells apparently resides in or is influenced by the cytoplasmic domain of the class I MHC antigen and is destroyed in the BAL 907 and 2.2.1 mutants of H-2L α.

### In Vivo Phosphorylation of Class I MHC Antigens is Stimulated by Phorbol Esters

The sequence of events which occurs after mitogenic stimulation of cells include the accelerated turnover of phosphoinositides and the consequent increase in intracellular concentrations of CA++ and diacylglycerol (Berridge and Irvine, 1984). These events trigger protein phosphorylation at serine and threonine by protein kinase C (Nishizuka, 1984). Direct activation of protein kinase C can be achieved by treatment of cells with phorbol esters (Nishizuka, 1984; Rozengurt et al., 1984). Since PMA results in increased internalization of H-2L α and H-2D β by lymphoid cells, we reasoned that PMA might also induce the phosphorylation of these class I MHC antigens and that phosphorylation might be the signal for internalization. H-2 antigens were immunoprecipitated from lysates of lymphoid and nonlymphoid cells (Table II) which had been labeled with [32P]orthophosphate in the presence or absence of PMA as described in Materials and Methods. The immunoprecipitated polypeptides were analyzed by PAGE and autoradiography.

To quantitate the phosphorylation of class I MHC antigens in different cell types and to measure accurately the effects of PMA on in vivo phosphorylation of these proteins, we took advantage of the fact that the β2-microglobulin (β2-M) light chain which associates noncovalently with the class I MHC antigen (and therefore commmunoprecipitates with class I MHC proteins) is not phosphorylated. Since PMA has no effect on the amount of [35S]methionine that is incorporated into β2-M (Fig. 7, A and B; Capps, G. G., and M. C. Züniga, manuscript in preparation), it is possible to use quantitative densitometry (as described in Fig. 7) to measure the 32P-labeled H-2 protein relative to the [35S]β2-M peak present in the same immunoprecipitate. Moreover, it is possible to exploit the constancy of [35S]β2-M in immunoprecipitates from cells incubated in the presence and absence of PMA to quantitate the 32P-labeled H-2 protein isolated from PMA-treated cells to that obtained from untreated cells (refer to Fig. 7 for details).

Phosphorylated forms of the class I MHC molecules occur in all of the cell lines examined, regardless of whether or not they have been stimulated by PMA (Figs. 7 and 8). Moreover, using the methods described above, we have found that H-2L α molecules are hyperphosphorylated in vivo in response to PMA in all cell types examined (Fig. 7, A and B, and Table II). The degree of hyperphosphorylation of H-2L α isolated from lymphoid cells is not notably higher nor lower than in H-2L α isolated from nonlymphoid cells (Table II). Thus, while PMA-inducible endocytosis of H-2L α is unique to lymphoid cells (among the cell types tested), PMA-inducible hyperphosphorylation of this molecule is not. It should be noted, however, that there are several residues which potentially can be phosphorylated (Fig. 1), and our current studies do not enable us to exclude the possibility that PMA causes hyperphosphorylation of different residues in different cell types, with diverse consequences. If this is the case, then selective phosphorylation of specific class I MHC cytoplasmic domain residues in lymphoid cells may target the class I MHC molecule to endocytic vesicles. The existence of multiple species of protein kinase C which are differentially expressed in lymphoid cells (Makowske et al., 1988; Nishizuka, 1988) and of a lymphoid cell–specific

### Table II. PMA-induced Hyperphosphorylation of H-2L α in Lymphoid and Nonlymphoid Cell Lines

| Cell line     | Cell type | PMA induction of phosphorylation |
|---------------|-----------|----------------------------------|
| 18–48         | B         | 3.5–3.94                         |
| BCL1          | B         | 5.01–5.04                        |
| EL3           | T         | 6.688–7.26                       |
| P815          | Mastocytoma | 3.07–4.21                      |
| 27.5.27 D-1   | Fibroblast | 9.00–12.71                      |

Radiolabelling of cells with [35S]methionine and [33P]orthophosphate, immunoprecipitation of H-2L α glycoproteins, and fractionation of proteins by PAGE, autoradiography, and densitometry were performed as described in Materials and Methods and in Fig. 7. Densitometry was performed on autoradiograms of the type depicted in Fig. 7 A only. A phosphorylation "index" was obtained by measuring the area under the H-2L α peak relative to the area under the β2-M peak. The effect of PMA on phosphorylation was quantitated by dividing the phosphorylation index obtained for PMA-treated cells by that obtained for untreated cells under otherwise identical conditions. The data shown represent the range of induction observed in at least two experiments for each cell line.
tyrosine kinase (Veilette et al., 1988a,b) render this possibility particularly tantalizing.

**Phosphorylation of Class I MHC Antigens Via the PMA-inducible Pathway Is Inhibited by Deletion or Alteration of the Cytoplasmic Domain**

Previous studies showed that phosphorylation of class I MHC antigens occurs on residues within the cytoplasmic domain (Pober et al., 1978; Guild and Strominger, 1984; McCluskey et al., 1986). It is thus not surprising that the 2.2.1 mutant of H-2L$^d$, which has no cytoplasmic tail, is not phosphorylated in vivo, either in the presence or absence of PMA (Fig. 8 A). An entirely different result might have been expected for the BAL 907 mutant of H-2L$^d$, which has several potential sites for phosphorylation (four serines and one threonine; see Fig. 1). PAGE and autoradiographic analysis of 30.5.7 immunoprecipitates does in fact reveal a phosphorylated protein with a mobility that is approximately that of the BAL 907 mutant (Fig. 8 A), and perhaps this protein is indeed the BAL 907 glycoprotein. The absence of a corresponding band in the immunoprecipitates of EL4.2.2.1 cells strengthens this argument. However, even if this protein is the BAL 907 mutant, its level of phosphorylation is extremely low relative to its wild-type counterpart, and it is not significantly augmented in PMA-treated cells (Fig. 8 A).

The defective response to PMA is specific for the mutant H-2L$^d$ antigens in the EL4.907 and EL4.2.2.1 cell lines, since the endogenous H-2D$^b$ antigens of both of these cell lines are phosphorylated in vivo and are hyperphosphorylated in response to PMA (Fig. 8 B and Table II). Admittedly, the cytoplasmic domain lesions in the BAL 907 and 2.2.1 mutants of the H-2L$^d$ molecule are too gross to permit a correlation between the lack of cytoplasmic domain phosphorylation and PMA-inducible endocytosis. In fact, only 11 out of

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**Figure 7.** PMA induces hyperphosphorylation of H-2L$^d$ in lymphoid and nonlymphoid cells. Approximately $10^7$ cells of the cell lines indicated were radiolabeled simultaneously with $[^{35}S]$methionine and $[^{32}P]$orthophosphate or with $[^{35}S]$methionine alone as described in Materials and Methods. Samples which were tested for inducibility of phosphorylation by PMA were incubated with 50 ng/ml PMA during the last 30 min of the labeling period. Cells were washed and lysed, and H-2 antigens were immunoprecipitated with the 30.5.7 mAb (as described in Materials and Methods) and immunoprecipitated proteins were resolved on 15% SDS-polyacrylamide gels under reducing conditions. Samples of ~30,000 cpm of immunoprecipitates were loaded per lane. X-ray films were exposed to the dried, Autofluor-treated gels under two different conditions. The autoradiogram shown in A was obtained by placing a piece of paper between the top half of the dried gel and the film, effectively preventing the $^{35}S$ radiation in the top half of the gel from exposing the film (Capps, G. G., and M. C. Zúñiga, manuscript in preparation). The autoradiogram in B confirms that this technique permits selective quantitation of $[^{32}P]$H-2L$^d$ (H-2) relative to $[^{35}S]$$\beta_2$-M. Immunoprecipitates in lanes labeled $^{35}S$-met, $^{32}P$, and $^{32}P$ + PMA were obtained from cells labeled with $[^{35}S]$methionine alone, $[^{35}S]$methionine and $[^{32}P]$orthophosphate in the absence of PMA, and $[^{35}S]$methionine and $[^{32}P]$orthophosphate in the presence of PMA, respectively. The lane labeled M in B contains $^{14}C$-labeled molecular mass markers (carbonic anhydrase, 30 kD; ovalbumin, 46 kD; BSA, 69 kD; lactoglobulin A, 18.4 kD; and cytochrome c, 12.3 kD). The two smallest of these proteins are also visible in the lane labeled M in A.
Approximately 10^7 cells of each transfectant cell line (EL^d3, EL4.907, and EL4.2.2.1) were radiolabeled with [35S]methionine or [32P]orthophosphate as described in Materials and Methods. Samples which were tested for inducibility of phosphorylation by PMA were incubated with [32P]orthophosphate for 2 h before the addition of 10 ng/ml PMA and further incubation at 37°C for 30 min. Cells were washed and lysed, and H-2 antigens were immunoprecipitated with either the 30.5.7 mAb (A) or the 28.14.8 mAb (B), and immunoprecipitated proteins were resolved on 15% SDS-polyacrylamide gels under reducing conditions. Samples of ~30,000 cpm of 35S-labeled immunoprecipitate and ~3,000 cpm of 32P-labeled immunoprecipitate were loaded per lane. See Materials and Methods and Results for further details. The lane labeled M in A contains the 14C-labeled molecular mass markers described in Fig. 7. The mobilities of the H-2 and β2-M are indicated.

31 amino acids of the wild-type H-2L^d cytoplasmic domain occur in the same positions in the BAL 907 cytoplasmic tail. Hence, other structural features necessary for PMA-inducible endocytosis may be missing from the BAL 907 mutant and from the tailless mutant as well.

**Discussion**

**Selective Nature of PMA-inducible Endocytosis of Cell Surface Proteins**

Phorbol esters have been shown to exert pronounced effects on the exocytic and endocytic properties of a number of cell surface receptors. Nevertheless, these reagents act selectively, rather than globally. The most striking evidence on this point with regard to MHC antigens is the fact that EL4.907 and EL4.2.2.1 transfectants fail to endocytose mutant H-2L^d antigens in a PMA-inducible fashion, while the H-2D^d antigens of these cells are internalized more efficiently in the presence of PMA. Clearly, the endocytosis-defective H-2L^d mutants are not carried into the cell in a wave of PMA-induced clustering of cell surface molecules to endocytic invaginations of the plasma membrane. Thus, while PMA may affect membrane trafficking in general (McGrath et al., 1988), not all cell surface molecules are mobilized in response to PMA. Equally striking is the cell specificity of the effect of PMA on class I MHC antigen internalization. Neither the P815 mastocytoma nor the L cell fibroblasts internalize H-2L^d in response to PMA, although they undergo morphological changes when exposed to this reagent (Zúñiga,
M. C., and S. Brady, unpublished videomicroscopic observations. Hence, the internalization of class I MHC antigens by lymphoid cells in response to PMA may reflect a unique function of these molecules in lymphoid cells (see below).

It has been reported previously that at least some of the class I MHC antigens which are internalized by mitogen-activated T lymphocytes are endocytosed via clathrin-coated vesicles (Dasgupta et al., 1988). More recently, class I MHC antigen endocytosis in monocytes also has been shown to occur via clathrin-coated vesicles (Machy et al., 1987). Phorbol esters were not used in either of these studies. Ongoing studies in our laboratory are directed at ascertaining if the class I MHC antigen endocytosis which occurs in response to PMA involves clathrin-coated pits and clathrin-coated vesicles. We also will determine if the endocytosis of the PMA-refractory BAL 907 mutant occurs via noncoated vesicles as has been demonstrated for class I MHC antigen endocytosis in fibroblasts (Huet et al., 1980), or if it is internalized via clathrin-coated pits in spite of the alterations in its cytoplasmic tail.

A related issue which interests us is the fate of class I MHC antigens which are internalized via the PMA-regulated pathway. Does their intracellular destination differ from that of class I MHC molecules which are endocytosed by cells which have not been treated with PMA? We are conducting electron microscopic studies to determine the fates of class I MHC antigens which enter the cell by these two apparently distinct pathways.

Is Phosphorylation the Signal for PMA-inducible Endocytosis?

The occurrence of PMA-induced hyperphosphorylation of the cytoplasmic domain of the H-2L^d molecule in nonlymphoid as well as lymphoid cells can be interpreted as evidence to discount this modification of the class I MHC antigen as a required signal for PMA-inducible endocytosis. Studies on other cell surface receptors have failed to establish a correlation between cytoplasmic domain phosphorylation and endocytosis. For example, modification of cytoplasmic serine residues of the transferrin receptor, while preventing phosphorylation, does not affect endocytosis (Rothenberger et al., 1987; Zerial et al., 1987; McGraw et al., 1988). However, phosphoamino acid and phosphopeptide analyses must be performed to exclude the possibility that different phosphorylation events are triggered in different cell types which potentially play a role in lymphoid-specific internalization of class I MHC antigens.

Another question bearing on this issue is whether a particular secondary or tertiary structure (which is lacking in the BAL 907 mutant of H-2L^d) provides the signal for PMA-inducible endocytosis, or if the signal is comprised of the primary structure of one or a few amino acids, as has recently been reported for other proteins (Davis et al., 1986, 1987; Lazarovits and Roth, 1988). These studies indicate that a single tyrosine in the cytoplasmic domain is required for endocytosis via coated pits. These reports are of interest here, since the cytoplasmic domain of the BAL 907 mutant of H-2L^d lacks a tyrosine (Fig. 1) which is conserved among class I MHC antigens of several species (summarized in McCluskey et al., 1986). We are selectively changing single amino acid residues within the cytoplasmic domain of the H-2L^d antigen to identify the structural features on the cytoplasmic domain which are required for endocytosis of the class I MHC antigen and to determine the relationship between PMA-inducible phosphorylation and PMA-regulated endocytosis.

Potential Structural Features Necessary for PMA-inducible Phosphorylation

The studies described herein show that substitution of the wild-type 31 amino acid cytoplasmic domain of the H-2L^d antigen with a dissimilar 25 amino acid tail renders the protein refractory to in vivo phosphorylation. The cytoplasmic tail of the BAL 907 mutant of H-2L^d contains several residues which are potential sites for phosphorylation (Fig. 1), yet this molecule is a poor substrate for phosphorylation, even in PMA-treated cells (Fig. 8A). It has been previously noted by McCluskey and co-workers that the cytoplasmic domain sequence Ser-Asp/Glu-X-Ser-Leu is conserved among the class I MHC antigens of mouse, human, pig, and rabbit (McCluskey et al., 1986). The second serine in this sequence (serine 335) is phosphorylated in human class I MHC antigens (Pober et al., 1978; Guild et al., 1984). In all human and mouse class I molecules the cluster is preceded by a serine or a threonine, so that the sequence which is conserved among these class I MHC glycoproteins reads Ser/Thr-Ser/Asp/Glu-X-Ser-Leu. The sequence in the corresponding region of BAL 907 reads Ser-Pro-Arg-Ile-Ser, with the protein terminating with the second serine (Fig. 1). The most notable differences between BAL 907 and other class I molecules in this region are the substitution of the proline for the second serine and the substitution of the basic arginine for the acidic residue found in other molecules. Clearly, further mutagenesis analysis is necessary to determine the structural features necessary for phosphorylation of the class I MHC antigen cytoplasmic domain. Nevertheless, these observations, together with the lack of a striking consensus sequence for substrates of protein kinase C or cAMP-dependent protein kinase A, suggest that a suitable substrate for these enzymes is provided, not by a specific sequence, but rather by a particular protein conformation which is attained by polypeptides via intra- or intermolecular interactions. Although in vivo interactions between the cytoplasmic domains of class I MHC antigens and other cellular proteins are yet to be demonstrated, class I MHC antigens have been shown to interact with cytoskeletal proteins in vitro, presumably via contact sites on the cytoplasmic domain of the class I MHC molecule (Pober et al., 1981).

Possible Significance of PMA-inducible Phosphorylation and Endocytosis of Class I MHC Molecules

The only well documented role of class I MHC molecules is in presenting foreign antigen to T lymphocytes, yet a number of studies have implicated these molecules in other immunological and nonimmunological functions. Of particular interest is the evidence for a regulatory role of class I MHC molecules in T lymphocyte and B lymphocyte activation; antibodies to class I MHC antigens block allogeneic and virus-specific T cell responses (Sterkers et al., 1983) and inhibit the activation and proliferation of human T lymphocytes in-
duced by mitogens and antigens (Turco et al., 1985; Taylor et al., 1986; Dasgupta et al., 1987; De Felice et al., 1987; Huet et al., 1987). Interestingly, antibodies to class I MHC antigens enhance proliferation via the CD2 molecule, a pathway of T cell activation thought to operate in mature T cells as well as in immature thymocytes which lack a functional CD3-T cell receptor complex (Turco et al., 1988). These and other studies have led to the suggestion that anti-class I MHC antibodies modulate an early event in T cell activation (Turco et al., 1988). Similarly, antibodies to class I MHC antigens inhibit the proliferation of human B cells to T-independent mitogens, but have no effect on PMA-induced proliferation (Taylor et al., 1987). These latter results have been interpreted to indicate that class I MHC molecules regulate a critical event in B cell proliferation which precedes the up-regulation of protein kinase C activity. Even if class I MHC molecules do influence or regulate early events in lymphocyte proliferation, the role of their endocytosis in this function is a matter of speculation. One favored hypothesis is that these proteins associate with molecules known to be important in cell growth, such as the insulin receptor, and are internalized with them (Samson et al., 1986).

While it is not clear if the phosphorylation of class I MHC antigens is related mechanistically to their endocytosis, it is almost certainly related to activation of lymphocytes. Stimulation of proliferation of lymphocytes by mitogens and antigens has been shown to involve signal transduction via protein kinase C (Farrar and Ruscetti, 1986; Imboden and Stobo, 1985; Coggeshall and Cambier, 1984). Hence, the enhanced phosphorylation and endocytosis of class I MHC antigens in PMA-treated lymphoid cell lines may reflect a normal physiological process which occurs in response to certain mitogens or other activators. Other studies from our laboratory show that PMA-inducible endocytosis occurs in a cloned cytotoxic T cell line and in normal spleen cells (Capps, G. G., M. Van Kampen, C. L. Ward, and M. C. Zúñiga, unpublished observations).

Although the functional significance of the inducibility of phosphorylation and endocytosis of class I MHC antigens by PMA is yet to be demonstrated, the observations reported herein demonstrate that the cell surface expression of these molecules is regulated in a manner similar to that observed to operate on other cell surface molecules implicated in signal transduction during activation of lymphoid cells. Further studies with antigen-specific T cell and B cell lines should make it possible to elucidate the functional relevance of the phosphorylation and endocytosis of class I MHC antigens in lymphocytes.

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