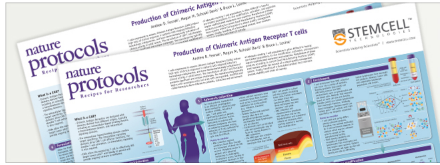

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Howard A. Anderson and Paul A. Roche

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Phosphorylation Regulates the Delivery of MHC Class II Invariant Chain Complexes to Antigen Processing Compartments

Howard A. Anderson and Paul A. Roche[1]

Transport of newly synthesized MHC class II glycoproteins to endosomal Ag processing compartments is mediated by their association with the invariant chain (Ii). Targeting to these compartments is dependent upon recognition of leucine-based endosomal/lysosomal targeting motifs in the Ii cytosolic domain. Ii, like many molecules that contain leucine-based endosomal targeting motifs, is phosphorylated in vivo. In this report we demonstrate that the cytosolic domain of the p35 Ii isoform is phosphorylated in class II Ii complexes isolated from human B lymphoblastoid cell lines or freshly obtained PBMC. Mutation of serine residue 6 or 8 prevents phosphorylation of Ii-p35 expressed in HeLa cells. Treatment of B lymphoblastoid cell lines with the serine/threonine kinase inhibitor staurosporine prevented Ii phosphorylation and significantly delayed trafficking of newly synthesized class II Ii complexes to endosomal Ag processing compartments. By contrast, staurosporine had no effect on the rate of transport of class I or class II glycoproteins through the Golgi apparatus and did not inhibit the delivery of the chimeric molecule Tac-DMβ to endocytic compartments, suggesting that staurosporine does not nonspecifically inhibit protein transport to the endocytic pathway. These results demonstrate that phosphorylation regulates the efficient targeting of MHC class II Ii complexes to Ag processing compartments and strongly suggest that this effect is mediated by phosphorylation of the MHC class II-associated Ii chain. *The Journal of Immunology,* **1998, 160: 4850–4858.**

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The ability of MHC class II (class II) molecules to present peptide Ags to T cells is largely dependent upon their association with the invariant chain (Ii)[2] (for review, see Refs. 1 and 2). Shortly after synthesis, Ii trimerizes and binds three class II $\alpha\beta$ dimers, thereby blocking access of endogenous peptides with newly synthesized class II molecules, assisting in the folding of these molecules, and facilitating their movement out of the ER. Class II $\alpha\beta$Ii complexes are then targeted to endosomal compartments by the recognition of targeting signals present in the 30-amino acid Ii cytosolic domain (3–5). Whether these compartments represent a specialized organelle in Ag processing cells or are conventional lysosomes is still a matter of some debate; however, for simplicity we will refer to these structures as endocytic Ag processing compartments. Once in the endocytic pathway, Ii is degraded by a series of proteolytic steps, and most of the Ii molecule dissociates from the class II $\alpha\beta$Ii complex. The small fragment of Ii that remains in the peptide binding groove of the class II molecule, termed CLIP, is finally removed by the action of the enzyme HLA-DM (6). Following CLIP removal, antigenic peptides bind to the class II $\alpha\beta$ dimer, and the complex is transported to the cell surface (7).

The molecular recognition events leading to the delivery of newly synthesized MHC class II $\alpha\beta$Ii complexes to Ag processing compartments remain enigmatic. Like most other membrane proteins, class II $\alpha\beta$Ii complexes are sorted in the *trans*-Golgi network (TGN) (5). $\alpha\beta$Ii complexes can move directly from the TGN into compartments along the endocytic pathway (8, 9). In addition, a portion of the pool of class II $\alpha\beta$Ii complexes in the TGN can move directly to the plasma membrane, from where they are rapidly internalized and enter a variety of Ag processing compartments from the cell surface (10–12). Based on experiments performed in nonlymphoid cells transfected with a variety of Ii cytosolic domain mutants, it is known that two leucine-based lysosomal targeting motifs in the Ii cytosolic domain are required for Ii targeting to the endocytic pathway (8, 13–15) and that nearby acidic amino acids regulate the recognition of these targeting motifs (16, 17). It remains to be determined, however, if there are additional signals in the Ii cytosolic domain that can affect the activity of the leucine-based targeting signals.

The intracellular transport route used by class II molecules to gain access to Ag processing compartments is regulated by the isoform of Ii present in the complex. In human APCs two isoforms of Ii are generated by the use of alternative translational start sites (18). Association of class II $\alpha\beta$ dimers with Ii trimers containing the longer p35 isoform of Ii results in exclusively intracellular targeting of the complex from the TGN to endosomes, while class II $\alpha\beta$ dimers associated with Ii trimers containing only the shorter p33 isoform of Ii can traffic to these same compartments by a pathway using a cell surface intermediate before internalization of the complex (12).

Protein phosphorylation has been shown to be one mechanism that regulates the recognition of leucine-based endosomal targeting signals (19–23). We report here that only the longer p35 isoform of Ii is phosphorylated in B lymphoblastoid cell lines (B-LCL) and human PBMC. Furthermore, staurosporine, a serine/threonine kinase inhibitor, greatly reduces Ii phosphorylation in B-LCL in vivo and specifically inhibits the trafficking of newly synthesized class II $\alpha\beta$Ii complexes to endocytic compartments.

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[2] Abbreviations used in this paper: Ii, invariant chain; ER, endoplasmic reticulum; TGN, *trans*-Golgi network; B-LCL, B lymphoblastoid cell line; endo H, endoglycosidase H; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; LIP, leupeptin-induced protein.

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Thus, phosphorylation regulates Ii-mediated transport of MHC class II molecules to Ag processing compartments.

Materials and Methods

Cell lines

The human B-LCL JY, Raji, and 721.174 were cultured in RPMI containing 10% FBS and 50 μg/ml gentamicin. Stable transfectants of B-LCL expressing Tac-DMβ were cultured in the above medium supplemented with 100 U/ml hygromycin B (24). Human PBMC were isolated from 50 ml of peripheral blood from a healthy donor as previously described (25). The cells were either used immediately or cultured overnight on 10-cm plastic dishes in RPMI containing 10% FBS. The human cervical carcinoma cell line HeLa (American Type Culture Collection, Rockville, MD) was cultured in DMEM containing 10% FBS and 50 μg/ml gentamicin.

Metabolic labeling of cells

To label cells with ^{32}P or ^{35}S, cells were cultured in phosphate-free DMEM or methionine-free DMEM, respectively, supplemented with 3% dialyzed FBS. Cells were cultured in the appropriate medium for 15 min and then labeled for 3 h with either [^{32}P]orthophosphate or [^{35}S]methionine. Typically, lymphoid cells were labeled at 10^7 cells/ml in 3 ml of medium containing 0.5 mCi of radiolabel, and HeLa cells were labeled in 1 ml of media containing 0.25 mCi of radiolabel. Ii (15 μg) in the mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA) was transfected into HeLa by calcium phosphate precipitation, and cells were cultured for 2 days on 10-cm tissue culture dishes before analysis.

Site-directed mutagenesis of human Ii cDNA

The cDNAs encoding human Ii-p33 and human Ii-p35 were described previously (18) and were subcloned into the expression vector pcDNA3. (The second methionine codon of the Ii-p35 cDNA was changed to CTG to prevent generation of Ii-p33.) Site-directed mutagenesis to change various Ii cytosolic domain residues to alanine was performed using the Chameleon double-stranded site-directed mutagenesis kit (Stratagene, La Jolla, CA). All mutagenesis was performed in pBluescript II KS^- (Stratagene), and Ii mutants were then subcloned into pcDNA3. The entire open reading frame of each mutant was sequenced by automated DNA sequence analysis.

Immunoprecipitation and electrophoresis

Cells were lysed for 1 h in ice-cold lysis buffer (10 mM Tris and 150 mM NaCl, pH 7.4, containing 5 mM iodoacetamide, 50 mM PMSF, 0.1 mM L-α-*p*-tosyl-L-lysine chloromethyl ketone, and 1 mg/ml BSA). When lysing cells labeled with [^{32}P]orthophosphate, the lysis buffer contained the following phosphatase inhibitors: 5 mM EDTA, 5 mM EGTA, 50 mM NaF, 10 mM $Na_4P_2O_7$, and 1 mM Na_3VO_4. Ii was immunoprecipitated with the isoform independent mAb, Pin.1 (26), or with corresponding rabbit serum EQLP (27). The p35 isoform of Ii was specifically immunoprecipitated using a rabbit serum essentially identical with RIp35N (26). MHC class II molecules were precipitated with the anti-DR α-chain mAb DA6.147, MHC class I molecules were precipitated with the mAb w6/32, and Tac-DMβ was precipitated with the mAb 7G7 (12, 24). mAb were prebound to protein A-agarose (Sigma, St. Louis, MO) in the presence of rabbit anti-mouse Ig serum (Sigma) for 1 h, and unbound Abs were removed by washing beads twice with lysis buffer. Extracts were precleared for 1 h with protein A-agarose containing bound rabbit anti-mouse Ig serum and an isotype-matched irrelevant mAb (MOPC21; Sigma). Specific immunoprecipitations were performed for 1 h. Immunoprecipitates were washed twice with lysis buffer; twice in lysis buffer diluted 1/10 in 10 mM Tris and 150 mM NaCl, pH 7.4; and once in 10 mM Tris, pH 7.4.

To reisolate various Ii isoforms, class II Ii complexes were first isolated with mAb DA6.147 and washed, and the complexes were disrupted by incubation in 10 mM Tris, 10 mM β-mercaptoethanol, and 1% SDS at 95°C for 5 min. The sample was diluted 30-fold in lysis buffer, and equivalent aliquots were incubated with the indicated antiserum and protein A-agarose as described above. In some cases immunoprecipitations were treated (or not) with recombinant endoglycosidase H (endo H; New England Biolabs, Beverly, MA) according to the manufacturer's protocols.

Proteins were eluted from protein A-agarose beads by boiling in SDS-PAGE sample buffer and were resolved by SDS-PAGE (28). For analysis of SDS-stable class II αβ dimers, bound proteins were eluted in SDS-PAGE sample buffer (without reducing agent) at room temperature for 30 min. For immunoblot analysis, proteins were transferred to polyvinylidene difluoride membranes, probed with anti-Ii mAb and horseradish peroxidase-conjugated goat anti-mouse Ig, and analyzed by enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL) according to the manufacturer's protocols. Two-dimensional SDS-PAGE (2D-PAGE) was performed essentially as described, with nonequilibrium pH gradient electrophoresis in the first dimension and reducing SDS-PAGE in the second dimension (29). The first dimension tube gels contained 2% Biolyte 3/10 (Bio-Rad Laboratories, Hercules, CA) and were run at 500 V for 4 h. Proteins were visualized by fluorography, and the amount of specified product in each lane was determined by scanning laser densitometry.

Phosphopeptide maps

Ii labeled with [^{32}P]orthophosphate was resolved by SDS-PAGE, and the gel was dried between two sheets of cellophane (Bio-Rad Laboratories). Ii was excised from the gel and washed twice for 30 min each time in 25% methanol and 10% acetic acid, and twice for 30 min each time in 50% methanol. Gel pieces were dried for 2 h in a Speed-Vac (Savant, Farmingdale, NY) and digested with 0.3 mg/ml L-*p*-tosylamino-2-phenylethyl chloromethyl ketone-treated trypsin (Sigma) in 1 ml of 0.4% NH_4HCO_3 at 37°C overnight. The gel pieces were removed by centrifugation, and the tryptic phosphopeptides were dried in a Speed-Vac. The phosphopeptides were washed five times with H_2O to remove residual NH_4HCO_3 (H_2O was evaporated in a Speed-Vac), resuspended in 10 μl of H_2O, and spotted 1 cm from the edge of a 20 × 20-cm TLC plate (Eastman Kodak, Rochester, NY). Peptides were separated by chromatography in 150 ml of buffer (15/10/3/12, pyridine/butanol/acetic acid/H_2O). Chromatography plates were dried and visualized by autoradiography.

Endosomal targeting assay

The delivery of class II αβIi complexes to endosomal compartments was determined by monitoring the rate of stabilization of Ii degradation products in cells treated with leupeptin. Following a 15-min pulse radiolabeling with [^{35}S]methionine, B-LCL were chased at 37°C for various times in the presence or the absence of 1 μM staurosporine (Boehringer Mannheim, Indianapolis, IN) in RPMI medium containing 10% FCS with or without 1 mM leupeptin. In other experiments, B-LCL cells were pretreated with 1 mM leupeptin for 2 h at 37°C before the 15-min pulse and chased at 37°C for various times in the presence or the absence of 1 μM staurosporine in RPMI medium containing 10% FCS. Each time point consisted of 5×10^6 cells pulsed with 0.25 mCi [^{35}S]methionine and chased in 0.4 ml of medium in a 24-well plate. Cell pellets were frozen and subsequently analyzed for SDS-PAGE as described above.

Results

Phosphorylation of Ii in professional APCs

MHC class II-associated Ii-p33 has been reported to be phosphorylated in transformed APC lines (38). However, phosphorylation of Ii in nontransformed APCs, the identification of the precise amino acid residue(s) modified, or the biologic consequences of Ii phosphorylation have not been established. To characterize further the phosphorylation of Ii in vivo, we labeled B-LCL with [^{32}P]orthophosphate and isolated Ii and MHC class II molecules by immunoprecipitation and SDS-PAGE analysis. Two approximately 33-kDa phosphoproteins were observed in both the anti-Ii and anti-HLA-DR immunoprecipitates (Fig. 1*A*), suggesting that the phosphoproteins were either Ii or the HLA-DR α-chain. An unknown phosphoprotein of about 150 kDa was routinely observed in both anti-Ii and anti-HLA-DR immunoprecipitates, and we are currently attempting to identify this protein. Identical approximately 33-kDa phosphoproteins were observed in the anti-Ii immunoprecipitate from the class II-negative, Ii-positive B-LCL 721.174, strongly suggesting that the phosphorylated protein was Ii and that association with class II molecules is not required for Ii phosphorylation.

To determine whether Ii phosphorylation was unique to transformed APCs, freshly isolated PBMC were labeled with [^{32}P]orthophosphate. In addition, PBMC were cultured overnight in plastic tissue culture plates, and the nonadherent cells were removed before [^{32}P]orthophosphate labeling. In each experimental condition two approximately 33-kDa phosphoproteins were observed in both the anti-Ii and anti-HLA-DR immunoprecipitates, which were indistinguishable from those obtained using B-LCL

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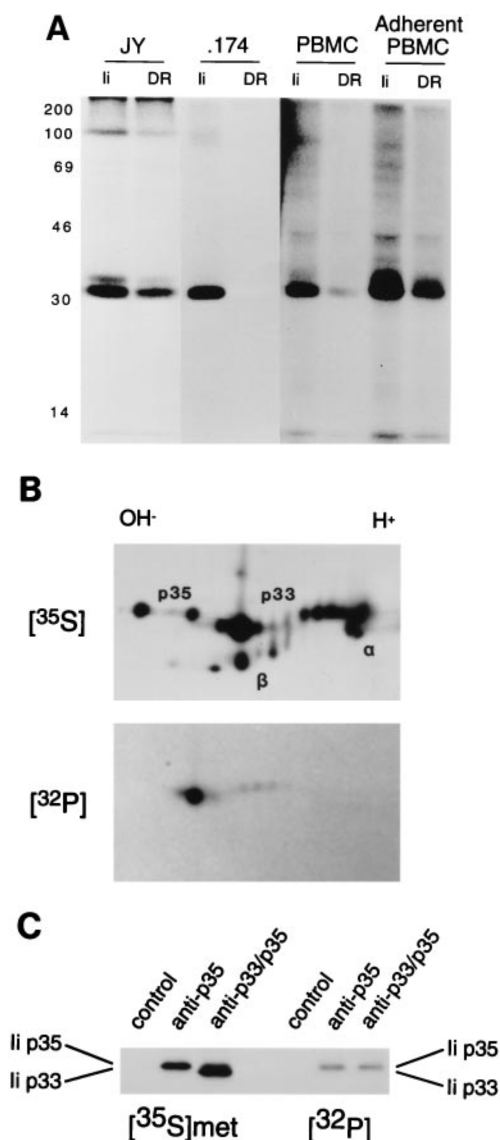

FIGURE 1. Phosphorylation of Ii in professional APCs in vivo. *A*, The MHC class II-positive B-LCL JY, the MHC class II-negative B-LCL 721.174, total PBMC, and adherent PBMC were labeled with [^{32}P]orthophosphate, and MHC class II molecules were isolated by immunoprecipitation with mAb specific for Ii or the HLA-DR α-chain. The samples were analyzed by SDS-PAGE and fluorography. *B*, MHC class II Ii complexes isolated from the B-LCL JY were metabolically labeled with [^{35}S]methionine or [^{32}P]orthophosphate for 3 h, analyzed by 2D-PAGE (*left* to *right*, basic to acidic), and visualized by fluorography. The mobilities of the HLA-DR α- and β-chains and the Ii-p33 and Ii-p35 isoforms are indicated in the ^{35}S-labeled sample. *C*, MHC class II Ii complexes were isolated from B-LCL JY labeled with either [^{35}S]methionine or [^{32}P]orthophosphate. Following disruption of the complexes by heating in SDS, Ii was reisolated using a control rabbit serum, a serum specific for Ii-p35, or a serum that recognizes both Ii-p33 and Ii-p35. The mobilities of both Ii isoforms are indicated.

(Fig. 1*A*). These results show that class II-associated Ii is phosphorylated in PBMC and in adherent APCs isolated from PBMC, demonstrating that Ii phosphorylation is not a peculiarity of APC transformation.

To confirm that the phosphoproteins were Ii, class II molecules were isolated from B-LCL labeled with either [^{35}S]methionine or [^{32}P]orthophosphate for 4 h, and 2D-PAGE was performed. Interestingly, Figure 1*B* demonstrates that the phosphoproteins in the anti-HLA-DR immunoprecipitate consisted exclusively of the p35 isoform of Ii and that both the high mannose and sialylated complex carbohydrate forms of Ii-p35 were labeled. We never obtained evidence of phosphorylation of Ii-p33 or of the class II α- or β-chains, but phosphorylation of the p43 isoform of Ii could be detected on longer exposures of the fluorograph (data not shown). These data suggest that Ii-p35 is phosphorylated in human B-LCL and PBMC, that Ii phosphorylation is not dependent on association with class II molecules, and that the p33 isoform of Ii associated with the $\alpha\beta$I complex is not phosphorylated.

To confirm that Ii-p35 is phosphorylated in APCs using another assay, we identified the phosphorylated isoform of Ii using an antiserum specific for Ii-p35. B-LCL were labeled with [^{32}P]orthophosphate or [^{35}S]methionine, and class II Ii complexes were isolated with a class II-specific Ab. The complexes were disrupted by heating in the presence of SDS, and Ii molecules were reisolated using a control serum, a serum that recognizes only Ii-p35, or a serum the recognizes both Ii-p33 and Ii-p35. This procedure resulted in complete dissociation of class II Ii complexes, since the anti-p35 specific serum only reprecipitated Ii-p35 molecules, whereas the anti-p33/p35 serum reprecipitated both Ii-p33 and Ii-p35 (Fig. 1*C*). When the anti-p35 serum was used to reisolate Ii from ^{32}P-labeled $\alpha\beta$I complexes, only the p35 isoform of Ii was detected using either serum, confirming that Ii-p35 is exclusively phosphorylated in professional APC.

Identification of Ii phosphorylation sites

HeLa cells do not express Ii, and transfected HeLa cells (and other transfected nonlymphoid cell lines) have been used extensively to investigate the trafficking of Ii and class II molecules (4, 8, 14, 15, 30–32). Figure 2*A* demonstrates that Ii-p35 is phosphorylated when expressed in HeLa cells. To identify the phosphorylation sites in Ii, we have mutated each of the potential phosphorylation sites in the Ii-p35 amino terminal extension. Figure 2*A* reveals that mutation of either serine almost completely abolished Ii-p35 phosphorylation. (The expression of each construct was confirmed by immunoblotting.) These data demonstrate that Ii-p35 phosphorylation could occur on either serine residue, as it is likely that phosphorylation of one serine regulates the phosphorylation of the other. In addition, we have mutated two arginine residues in the Ii-p35 cytosolic domain that function as the ER retention signal for Ii-p35 (33). Interestingly, mutation of these arginines also abolished Ii-p35 phosphorylation. 2D-PAGE indicated that phosphorylated Ii-p35 expressed in HeLa cells possesses high mannose carbohydrates, consistent with the retention of this phosphoprotein in the ER (Fig. 2*A*).

Surprisingly, Ii-p33 expressed in HeLa cells is also phosphorylated. Figure 2*B* demonstrates that phosphorylation of Ii-p33 is abolished when Ser^9 is mutated to alanine, whereas mutation of either Ser^{26} or Ser^{29} does not affect Ii phosphorylation. Furthermore, only the sialylated form of Ii-p33 is phosphorylated in HeLa cells, indicating that unlike the phosphorylation of Ii-p35, Ser^9 of Ii-p33 is phosphorylated exclusively in a late Golgi or post-Golgi compartment.

Although HeLa cells offer an ideal system to map Ii phosphorylation sites, it is possible that different sites on Ii-p35 may be phosphorylated in HeLa cells and professional APCs. To address this possibility we have analyzed the tryptic phosphopeptides from Ii expressed in B-LCL as well as the various Ii isoforms expressed in HeLa cells. The sequences of the cytosolic domains of Ii-p33 and Ii-p35 are shown in Figure 3*A* for comparison. Figure 3*B* demonstrates that this procedure yielded two prominent phosphopeptides when Ii isolated from B-LCL was analyzed. The same two phosphopeptides were obtained by analyzing Ii-p35 expressed in HeLa cells, demonstrating that phosphorylation occurs on the same serine residues of Ii-p35 when expressed in B-LCL or HeLa

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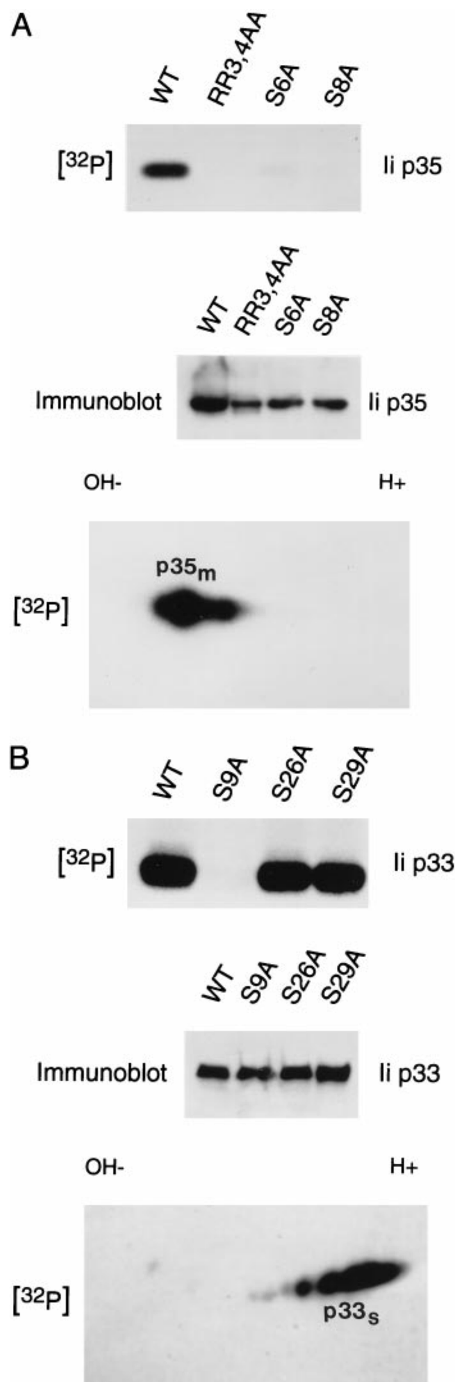

FIGURE 2. Mapping Ii phosphorylation sites in HeLa cells. HeLa cells were transfected with wild-type (wt) or various mutant forms of Ii-p35 and Ii-p33 and labeled with [^{32}P]orthophosphate, and Ii was immunoprecipitated and analyzed by SDS-PAGE and fluorography (*upper panel*). To ensure equal expression of each Ii construct, immunoprecipitates were also analyzed by immunoblotting using an Ii-specific mAb (*middle panel*). ^{32}P-labeled Ii expressed in HeLa cells was also analyzed by 2D-PAGE and fluorography (*lower panel*). *A*, Arg^3 and Arg^4 (RR3, 4AA), Ser^6 (S6A), or Ser^8 (S8A) in the p35 isoform of Ii were changed to alanine by mutagenesis and expressed in HeLa cells. *B*, Ser^9 (S9A), Ser^{26} (S26A), and Ser^{29} (S29A) in the p33 isoform of Ii were changed to alanine by mutagenesis and expressed in HeLa cells. Note that only the high mannose (m) form of Ii-p35 is phosphorylated in HeLa cells, whereas only the sialylated (s) form of Ii-p33 is phosphorylated.

cells. The two phosphopeptides observed in Figure 3*B* could represent either two unique tryptic phosphopeptides (one containing Ser^6 and the other containing Ser^8) or a partial tryptic digest of Ii-p35 containing a single phosphorylated serine residue. We are currently attempting to resolve this complex issue. It should also be noted that the phosphopeptide map of Ii-p33 expressed in HeLa cells revealed a single phosphopeptide (containing Ser^9) that migrated with a mobility distinct from that of the Ii-p35 phosphopeptides.

Ii-p35 is phosphorylated in professional APCs throughout the secretory pathway

We also examined Ii phosphorylation in different professional APCs. Figure 3*C* demonstrates that Ii isolated from the Burkitt's lymphoma line Raji or B-LCL 721.174 (which lacks MHC class II protein expression) is phosphorylated on the same serine residue(s) as Ii expressed in B-LCL JY. Furthermore, Ii isolated from PBMC is phosphorylated on the same serine residues as Ii expressed in B-LCL (data not shown). These data demonstrate that phosphorylation of Ii-p35 is not a peculiarity of APC transformation and does not require association of Ii with class II molecules.

To determine whether different serine residues are phosphorylated as Ii-p35 is transported throughout the secretory pathway, we have analyzed tryptic phosphopeptides of Ii present in the early secretory pathway, the late secretory pathway, and the endosomal system. Figure 3*D* demonstrates that the phosphopeptide maps of the high mannose and the sialylated complex carbohydrate forms of Ii are identical, demonstrating that transport of Ii through the secretory pathway does not alter the Ii phosphorylation sites. In addition, phosphorylation of these same residues can be detected on a endosomal degradation product of Ii termed leupeptin-induced protein (LIP).

Staurosporine prevents Ii phosphorylation and inhibits class II αβIi transport to endocytic Ag processing compartments

In an initial screening of reagents that could inhibit Ii phosphorylation in B-LCL, we found that the serine/threonine kinase inhibitor staurosporine could inhibit Ii phosphorylation at very low concentrations (Fig. 4*A*). It should be noted that even the highest dose of staurosporine used in these studies (2 μM) did not adversely affect cell viability. We have therefore chosen to evaluate the effect of staurosporine on the ability of class II $\alpha\beta$Ii complexes to traffic to endocytic Ag processing compartments in B-LCL.

Following arrival in endocytic compartments, class II-associated Ii is degraded so rapidly that significant amounts of Ii degradation products are generally not detected. However, when cultured in the presence of the cysteine protease inhibitor leupeptin, Ii degradation products are stabilized, giving rise to the 21-kDa LIP and the 10-kDa proteolytic fragment p10 (1, 34). B-LCL were pulsed with [^{35}S]methionine for 15 min and chased for up to 4 h in medium containing 1 mM leupeptin. After 2 h of chase, significant amounts of LIP accumulated in endocytic compartments in the cells (Fig. 4*B*). Inclusion of the kinase inhibitor staurosporine to the chase medium greatly reduced the amount of LIP generated at each time point by approximately 50% compared with that in mock-treated cells, demonstrating that staurosporine significantly inhibited the generation of Ii degradation products.

A trivial explanation for the observed decrease in LIP generation in staurosporine-treated cells is that staurosporine inhibits the uptake of leupeptin into cells. However, staurosporine had no effect on pinocytosis of the fluid phase marker Lucifer yellow in B-LCL (35) (data not shown). Furthermore, to ensure that the decrease in LIP generation could not be due to the effect of staurosporine on leupeptin pinocytosis, B-LCL were treated with leupeptin for 2 h before metabolic labeling to preload their endocytic

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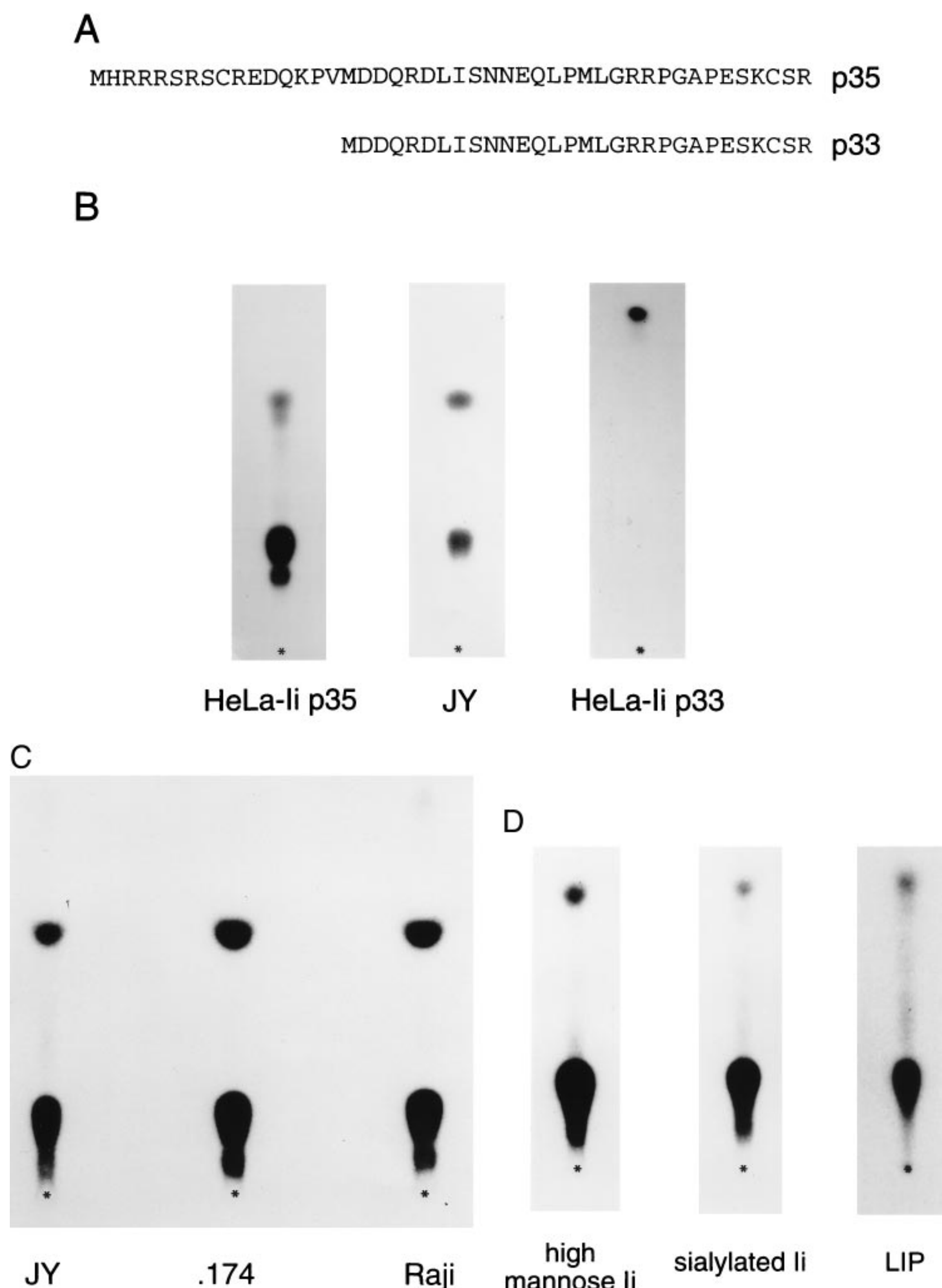

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FIGURE 3. Phosphopeptide analysis of Ii. *A*, The entire amino terminal cytosolic domain of Ii-p35 or Ii-p33 is shown. *B*, ^{32}P-labeled Ii from cells radiolabeled for 3 h with [^{32}P]orthophosphate was isolated from the human B-LCL JY or HeLa cells transfected with Ii-p35 or Ii-p33 by immunoprecipitation and SDS-PAGE. Ii was excised from the SDS-PAGE gel and digested with trypsin. The samples were spotted onto cellulose plates (the origin is indicated by an asterisk), and the resulting tryptic phosphopeptides were resolved by chromatography. *C*, The MHC class II-positive B-LCL JY, the MHC class II-negative B-LCL 721.174, and the MHC class II-positive Burkitt's lymphoma cell line Raji were labeled with [^{32}P]orthophosphate, and phosphopeptide mapping was performed as described in the text. *D*, B-LCL JY was radiolabeled with [^{32}P]orthophosphate in the presence of leupeptin, and the high mannose carbohydrate form, the sialylated complex carbohydrate form, and the LIP fragment of Ii were excised from SDS-PAGE gels and analyzed by phosphopeptide mapping. The origin of each map is indicated by an asterisk.

compartments with this protease inhibitor. Once again, pulse-chase studies performed in the absence or the presence of staurosporine revealed a marked decrease in the generation of the Ii degradation products LIP and p10 in staurosporine-treated cells (Fig. 4*C*).

The decreased accumulation of Ii degradation products in staurosporine-treated cells suggested that phosphorylation may play a role in the trafficking of Ii to endosomal compartments. To investigate this using another assay, we evaluated the ability of staurosporine to inhibit the ability of newly synthesized class II molecules to generate SDS-stable compact $\alpha\beta$ dimers. The SDS-stable conformation is induced by binding of antigenic peptides to class II molecules (Ref. 1 and references contained therein). Pulse-chase studies (Fig. 4*D*) and quantitative analysis of the fluorographs revealed that there was a significant decrease in the amount of SDS-stable $\alpha\beta$ dimers detected at various times of chase in cells treated with staurosporine compared with mock-treated cells. Together with the data presented above, these data demonstrate that staurosporine significantly inhibits the delivery of newly synthesized

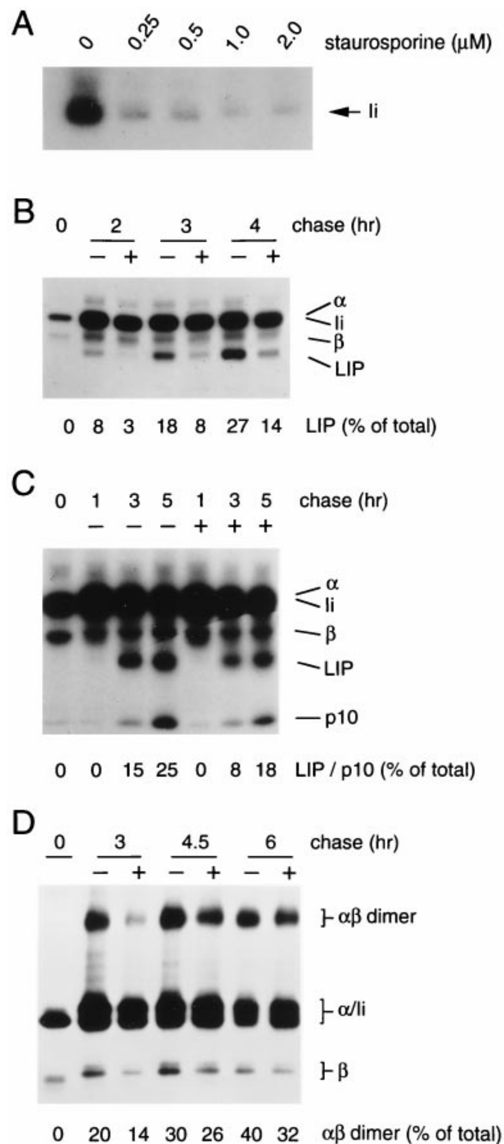

FIGURE 4. Staurosporine prevents Ii phosphorylation and inhibits targeting of class II Ii complexes to the endocytic pathway. *A*, JY B-LCL were labeled with [^{32}P]orthophosphate for 3 h in medium containing various concentrations of staurosporine. Ii was isolated from detergent extracts by immunoprecipitation with anti-Ii mAb and was analyzed by SDS-PAGE and fluorography. *B*, B-LCL JY were pulse labeled with [^{35}S]methionine and chased in medium containing 1 mM leupeptin in the absence (−) or the presence (+) of 1 μM staurosporine. Samples were removed at time zero (immediately after the labeling), 2 h, 3 h, and 4 h, and class II molecules were immunoprecipitated using an anti-HLA-DR mAb. *C*, B-LCL .45 were pretreated with 1 mM leupeptin for 2 h before pulse labeling with [^{35}S]methionine. The leupeptin was removed, and the cells were chased in the absence (−) or the presence (+) of staurosporine. At various times, samples were removed, and class II molecules were immunoprecipitated and analyzed by SDS-PAGE and fluorography. *D*, B-LCL .45 were pulse labeled with [^{35}S]methionine and chased in medium in the absence (−) or the presence (+) of staurosporine. Samples were removed at time zero (immediately after the labeling), 3 h, 4.5 h, and 6 h, and class II molecules were immunoprecipitated using an anti-HLA-DR mAb. Class II molecules were eluted in SDS-PAGE sample buffer at room temperature and analyzed by SDS-PAGE and fluorography. The mobility of the HLA-DR α-chain, β-chain, $\alpha\beta$ dimer, as well as Ii-p33 and the Ii degradation intermediates LIP and p10 are indicated. The fluorographs were analyzed by laser scanning densitometry, and the amount of LIP, LIP/p10, or $\alpha\beta$ dimers is expressed as a percentage of the total amount of class II α-, β-, and Ii chains present in each lane.

class II $\alpha\beta$Ii complexes to endosomal Ag processing and peptide loading compartments.

Staurosporine does not inhibit ER to TGN transport of MHC glycoproteins

To rule out the possibility that the inhibition of LIP generation and SDS-stable $\alpha\beta$ dimer formation by staurosporine was a consequence of impaired protein transport in the secretory pathway, we analyzed the kinetics of protein transport out of the ER and through the Golgi apparatus in mock-treated and staurosporine-treated cells. Staurosporine had no effect on the rate of acquisition of endo H resistance of MHC class I heavy chains in pulse-chase studies (Fig. 5*A*). In addition, class II molecules were isolated from these cells, and the rate of complex carbohydrate sialylation was analyzed by 2D-PAGE (Fig. 5*B*). Once again, staurosporine had no effect on the rate of sialic acid acquisition on newly synthesized MHC class II glycoproteins, demonstrating that staurosporine did not inhibit MHC protein transport out of the ER, through the Golgi apparatus, and into the TGN.

Staurosporine does not inhibit endosomal targeting and degradation of Tac-DMβ in B-LCL or Ii-p33 in HeLa cells

We next devised experiments to determine whether staurosporine nonspecifically inhibited protein transport from the TGN to endocytic compartments. The chimeric molecule Tac-DMβ contains the HLA-DM β-chain cytosolic domain fused to the Tac transmembrane and luminal domains. This chimeric molecule is targeted to and degraded in endocytic compartments when expressed in B-LCL (24). The high mannose carbohydrate form of Tac-DMβ (m, *lower band*) rapidly exits the ER in these cells and is converted into the sialylated, complex carbohydrate form of Tac-DMβ (s, *upper band*) in the Golgi apparatus (Fig. 6*A*). Pulse-chase kinetic analysis revealed that Tac-DMβ degradation was not inhibited in cells treated with staurosporine compared with that in mock-treated cells. In agreement with an earlier report, loading the endocytic pathway in these cells with leupeptin partially inhibited the rate of Tac-DMβ degradation (Fig. 6*B*), confirming that degradation of the chimeric molecule occurs in leupeptin-sensitive compartments. In addition, staurosporine did not adversely affect transport of Tac-DMβ out of the ER and through the Golgi apparatus, as the kinetics of carbohydrate processing are virtually identical in staurosporine-treated and mock-treated cells.

As an additional control for nonspecific effects of staurosporine on protein transport from the TGN to endocytic compartments, we examined the effect of staurosporine on Ii transport to the endocytic pathway in HeLa cells. Since Ii-p33 is not phosphorylated in B-LCL, and mutating Ser[9] has no effect on Ii-p33 endosomal targeting in HeLa cells (data not shown), we would not expect staurosporine to inhibit Ii-p33 targeting to and degradation in the endocytic pathway in HeLa cells. Pulse-chase kinetic analysis confirmed that staurosporine does not inhibit the degradation of Ii-p33 expressed in HeLa cells (Fig. 6*A*). Furthermore, leupeptin-treatment prevents Ii degradation (Fig. 6*B*), demonstrating that degradation of Ii-p33 in HeLa cells occurs in endocytic compartments. (Note that the Ii degradation product LIP is not detected in HeLa cells.) Taken together, these results demonstrate that staurosporine does not nonspecifically inhibit the sorting machinery responsible for trafficking of molecules from the TGN to endosomal compartments.

Discussion

Phosphorylation has been shown to regulate leucine-based signal-mediated endosomal targeting in a number of systems. In the case of CD4 (19, 20) and CD3γ (21, 22), phosphorylation is required to

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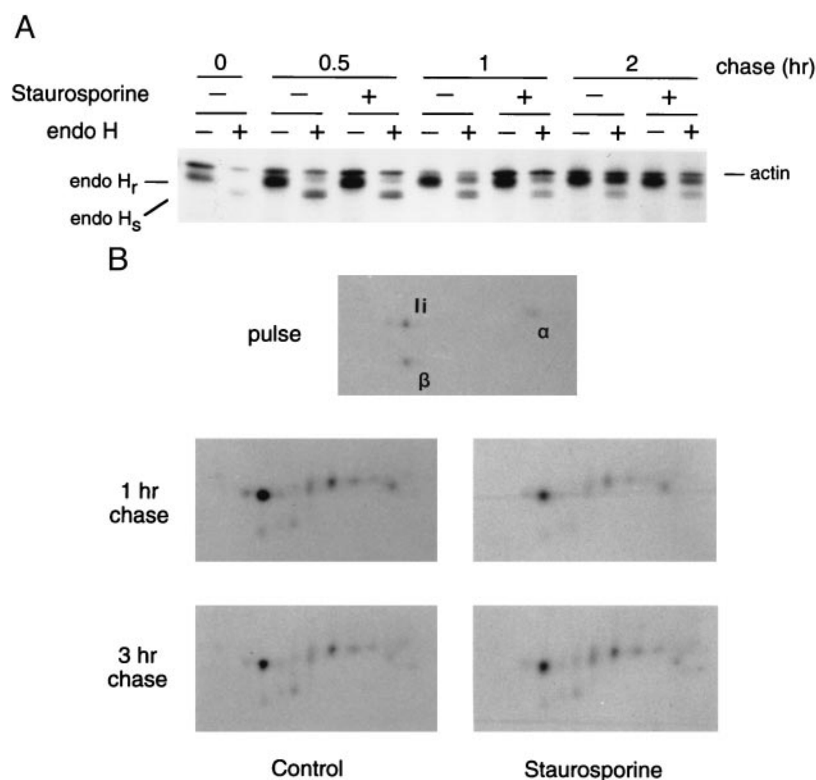

FIGURE 5. Staurosporine does not inhibit protein transport through the secretory pathway. B-LCL .45 were pulse labeled with [^{35}S]methionine and chased in medium in the absence (−) or the presence (+) of staurosporine. Samples were removed at various times, and MHC molecules were isolated by immunoprecipitation. *A*, Class I molecules present at each chase point were either mock digested or digested with endo H before SDS-PAGE and fluorography. The mobilities of the endo H-resistant (endo H_r) and endo H-sensitive (endo H_s) species are indicated. The position of the contaminating actin band is indicated on the *right*. *B*, Class II molecules were analyzed by 2D-PAGE. The mobilities of the high mannose form of the HLA-DR α-, β-, and Ii chains are indicated. Note that the kinetics of carbohydrate processing on the individual class II subunits are indistinguishable in mock-treated and staurosporine-treated cells.

observe leucine-based signal-mediated internalization and degradation following T cell stimulation with phorbol esters. More often, however, phosphorylation simply enhances the effects of the leucine-based signal, as is observed for the internalization of the IL-6R (23). Similarly, mutation of the phosphorylation sites adjacent to leucine-based motifs in the cytosolic domain of both the cation dependent- and cation-independent mannose 6-phosphate receptors inhibits lysosomal enzyme targeting (36, 37), once again demonstrating that phosphorylation can regulate the activity of leucine-based endosomal sorting signals.

In this study we have demonstrated that MHC class II-associated Ii is phosphorylated and that phosphorylation regulates the delivery of class II $\alpha\beta$Ii complexes to endocytic Ag processing compartments. Interestingly, only the longer p35 isoform of Ii is phosphorylated in transformed B lymphocytes and in freshly isolated PBMC or adherent PBMC. This is in contrast to an earlier report in which only the shorter p33 isoform of Ii was found to be phosphorylated (38). However, in this previous report identification of the phosphorylated isoform was made solely on the mobility of the phosphoprotein on 2D-PAGE. Since we have often observed slight gel-to-gel variations in protein mobility in these gels, we have performed additional experiments to confirm that the phosphoprotein was Ii-p35. We have isolated phospho-Ii using an Ii-p35-specific serum and have also shown that the tryptic phosphopeptides of Ii isolated from APCs are identical with the tryptic phosphopeptides isolated from HeLa cells expressing Ii-p35, but not with those from HeLa cells expressing Ii-p33.

Ii-p35 contains two unique serine residues, and mutagenesis of either residue almost completely prevents the phosphorylation of Ii-p35 expressed in HeLa cells. This suggests a complex regulation of Ii phosphorylation in which the phosphorylation of one residue regulates the phosphorylation of the other. Similarly, mutation of the arginine residues adjacent to these serine residues also prevents Ii phosphorylation. The inhibition of Ii-p35 phosphorylation by the double arginine mutation is most likely due to the disruption of the kinase recognition site, as this region of Ii-p35 contains a consensus phosphorylation site for cAMP-dependent protein kinase and protein kinase C.

When expressed alone in nonprofessional APCs, Ii-p35 is retained in the ER by recognition of the double arginine motif. Similarly, free Ii-p33 and Ii-p35 are retained in the ER even in professional APCs, presumably due to the formation of mixed Ii trimers containing p35 (27). In addition, there appears to be a requirement for association with class II α- and β-chains for efficient egress of Ii out of the ER, presumably due to the masking of the ER-retention motif in Ii-p35 (26, 39). Once assembled, $\alpha\beta$I complexes containing Ii-p35 efficiently leave the ER and traffic through the secretory pathway into the TGN. In the TGN, it is likely that $\alpha\beta$I complexes containing Ii-p35 are segregated from $\alpha\beta$I complexes containing Ii-p33, as class II molecules associated with Ii-p33 can arrive in endosomes following a pathway including transient passage through the plasma membrane and internalization (10–12). By contrast, class II molecules associated with Ii-p35 do not transit through the plasma membrane en route to the endocytic pathway, but instead follow a strictly intracellular route (12). It is important to note that class II molecules associated with either isoform of Ii eventually arrive in endocytic Ag processing compartments, as the degradation product LIP can be readily observed for each isoform of Ii (12, 40).

During our studies we found that the serine/threonine kinase inhibitor staurosporine could essentially prevent Ii phosphorylation. Therefore, staurosporine treatment allowed us to examine the role of phosphorylation in the intracellular trafficking class II $\alpha\beta$I complexes in professional APCs. The cysteine protease inhibitor leupeptin prevents the complete degradation of Ii and stabilizes a 21-kDa LIP fragment and a 10-kDa p10 fragment of Ii that remain stably associated with class II $\alpha\beta$ dimers. The generation of these fragments can therefore be used to monitor the arrival of class II molecules in endocytic Ag processing compartments (1, 34, 41). In

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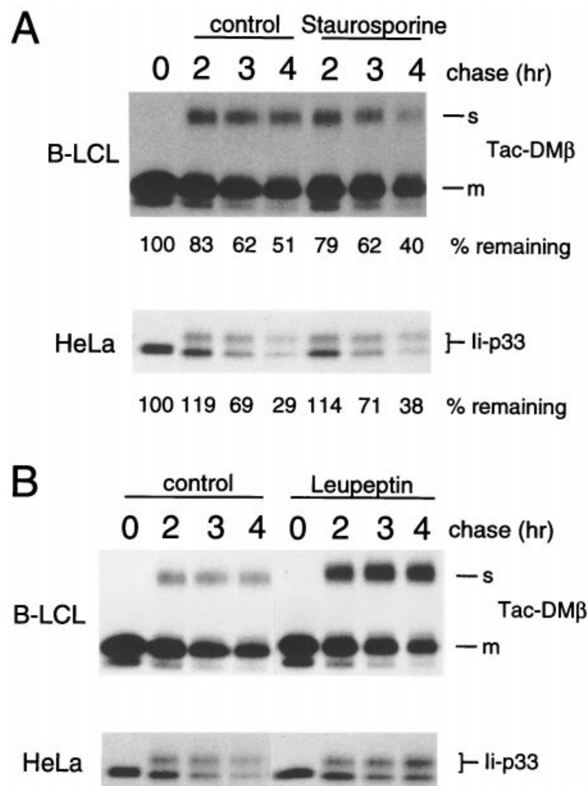

FIGURE 6. Staurosporine does not inhibit endosomal targeting and degradation of Tac-DMβ in B-LCL or of Ii in HeLa cells. B-LCL .45 cells stably transfected with Tac-DMβ or HeLa cells transiently transfected with Ii-p33 were pulse labeled with [^{35}S]methionine (time zero) or chased for 2, 3, or 4 h in various media. The cells were chased in medium alone (control), in medium containing 1 μM staurosporine (*A*), or in medium containing 1 mM leupeptin (*B*). Tac-DMβ or Ii was isolated by immunoprecipitation using an anti-Tac mAb or an anti-Ii mAb, respectively, and analyzed by SDS-PAGE and fluorography. The positions of the high mannose form of Tac-DMβ (m) and the sialylated, complex carbohydrate form of Tac-DMβ (s) are indicated. Laser scanning densitometry of the fluorographs was performed, and the amount of Tac-DMβ or Ii remaining at each chase point is expressed as a percentage of total material present at time zero (immediately after pulse labeling).

pulse-chase biosynthetic labeling studies performed in the presence or the absence of staurosporine, we repeatedly found a marked diminution in the amount of LIP associated with class II molecules at each time point. To formally rule out the possibility that decreased leupeptin uptake could account for the decrease in LIP generation in staurosporine-treated cells, we preloaded cells with leupeptin before the pulse-chase studies. Once again, we found a dramatic decrease in the amount of LIP generated in staurosporine-treated cells.

Following Ii (and CLIP) dissociation from class II molecules in Ag processing compartments, antigenic peptides bind to class II $\alpha\beta$ dimers and induce an SDS-stable conformation in a subpopulation of class II molecules (reviewed in Refs. 1 and 2). As another indicator of class II trafficking to endosomal compartments, we assayed the rate of SDS-stable $\alpha\beta$ dimer formation in pulse-chase studies. As anticipated, the rate of SDS-stable dimer formation was significantly delayed in staurosporine-treated cells, indicating that peptide loading onto class II molecules was inhibited in these cells. Together with data demonstrating that LIP generation was impaired in staurosporine-treated cells, the results of these studies are consistent with the hypothesis that inhibition of Ii phosphorylation impairs delivery of class II $\alpha\beta$Ii complexes to endosomal Ag processing compartments.

In addition to demonstrating a direct effect on staurosporine on Ii phosphorylation and subsequent trafficking of the phosphorylated class II $\alpha\beta$Ii complexes to the endocytic pathway, we had to consider indirect effects of staurosporine on vesicular transport through the secretory pathway. We did not observe any alteration in the acquisition of endo H resistance of class I glycoproteins, and 2D-PAGE analysis of biosynthetically labeled class II molecules did not reveal any obvious alterations in the rate of sialic acid addition to class II molecules in staurosporine-treated cells. These studies demonstrate that staurosporine did not nonspecifically alter the rate of protein transport out of the ER, through the Golgi apparatus, and into the TGN. To investigate the possibility that staurosporine was nonspecifically inhibiting protein transport from the TGN to the endocytic pathway, we examined the effect of staurosporine on the transport of the reporter molecule Tac-DMβ. This protein is targeted to the endocytic pathway by recognition of the tyrosine-based sorting signal of the HLA-DM β-chain (24). Using the same B-LCL in which we observed profound inhibition of LIP generation and SDS-stable $\alpha\beta$ dimer formation, we did not observe any alteration in the rate of Tac-DMβ degradation in the presence of staurosporine. We should point out, however, that it is formally possible that staurosporine inhibits the transport of class II $\alpha\beta$Ii complexes to the endocytic pathway in B-LCL by inhibiting the function of proteins that specifically recognize phosphorylated leucine-based sorting signals but not tyrosine-based signals.

What is the biochemical consequence of Ii phosphorylation in vivo? There is evidence that Ii is sequestered into clathrin-coated pits at the plasma membrane (8) and that delivery of class II $\alpha\beta$Ii complexes to endosomes is impaired in dynamin-deficient cells (42). In addition, it has been shown that Ii interacts with the clathrin-associated adaptor molecule AP1 in the TGN and that recruitment of AP1 onto Golgi membranes in cells overexpressing class II $\alpha\beta$Ii complexes is dependent upon the presence of an intact Ii cytosolic domain (43). The TGN-associated AP1 molecule, like the plasma membrane-associated AP2 molecule, has been extensively characterized as the link between the tyrosine-based sorting signal and clathrin (44). Recently, both tyrosine-based and leucine-based sorting signals have been shown to interact with AP1 and AP2 adaptors in vitro (22, 45), suggesting that sorting of proteins containing either tyrosine-based or leucine-based signals to the endocytic pathway involves a common trafficking machinery. In the case of the cation-dependent and cation-independent mannose-6-phosphate receptors, phosphorylation of serine residues adjacent to the leucine-based sorting signals has been shown to be critical for the recruitment of AP1 adaptors to the Golgi apparatus (37, 46), and mutation of these serines inhibits lysosomal enzyme transport in vivo (36, 37). In addition, Arneson and Miller (47) have shown that multimerization of Ii is required for efficient endosomal targeting of class II Ii complexes. By analogy with these results, we believe it likely that phosphorylation of Ii increases the interaction of the leucine-based signal(s) of Ii with the endosomal sorting machinery, resulting in more efficient trafficking of class II $\alpha\beta$Ii complexes to Ag processing compartments. That the inhibition of peptide loading onto newly synthesized class II molecules in staurosporine-treated cells is most evident at early times of chase is in excellent agreement with this hypothesis, suggesting that the effect of phosphorylation of Ii is to regulate the kinetics of class II trafficking to the endocytic pathway.

It is difficult to know whether the selective phosphorylation of Ii-p35 is responsible for the different transport pathways used by class II molecules associated with Ii-p33 or Ii-p35. It is possible that phosphorylation of Ii-p35 results in more efficient recognition

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by adaptors in the TGN and that these complexes are efficiently sorted by an intracellular route to the endocytic pathway. One might then hypothesize that nonphosphorylated Ii-p35 would behave like Ii-p33 and transport to endosomes via a cell surface intermediate. On the other hand, it is possible that the intracellular transport pathway used by class II molecules associated with Ii-p35 is determined by factors other than phosphorylation, and that phosphorylation simply enhances the recognition of these other signals. Clearly, additional experiments are required to unambiguously identify the mechanism by which Ii phosphorylation regulates intracellular trafficking of MHC class II molecules.

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