Phosphatase Inhibitors Block in Vivo Binding of Peptides to Class I Major Histocompatibility Complex Molecules*

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Class I major histocompatibility complex (MHC) molecules are heterotrimers of heavy chains, β2-microglobulin, and 8–10 amino acid-long peptides. Assembly of class I MHC molecules into complexes which are stable and can be transported to the cell surface occurs soon after insertion of individual subunits into the endoplasmic reticulum (ER). To identify subcellular compartments required for class I MHC assembly, we studied class I biosynthesis in human cell lines treated with several inhibitors of intracellular transport. We found that HLA-B701 molecules do not assemble in CIR transfectants in which a block in protein transport from the ER is established by treatment with phosphatase inhibitors. In contrast, stable HLA-B701 complexes form in cells in which the ER becomes mixed with the Golgi after treatment with brefeldin A. Neither treatment impaired binding of HLA-B701 to the ER-resident protein calnexin, and unassembled heavy chains in phosphatase-inhibited cells showed prolonged association with calnexin. In addition, the mouse class I molecule H-2D\(\beta\), which binds β2-microglobulin in human T2 cells in the absence of transport of antigenic peptides, formed complexes in CIR cell transfectants treated with phosphatase inhibitors. Taken together, these data demonstrate that phosphatase inhibitors do not prevent assembly of class I heavy chain-β2-microglobulin dimers, but instead interfere with peptide loading. These results are consistent with the possibility that class I MHC molecules are transported from their initial site of insertion into the rough ER before binding peptides, or alternatively that peptide loading mediated by transporter of antigenic peptides is blocked by phosphatase inhibitors.

Class I MHC \(\beta\) molecules bind 8–10 amino acid-long peptides and present them to CD8-positive T cells (reviewed in Ref. 1). Such peptides derive from cytosolic proteins, are transported into the secretory pathway by TAP transporters, and associate with class I molecules in an early biosynthetic compartment. Immediately after their synthesis, class I heavy chains bind calnexin (2), a protein of the rough ER. Class I heavy chains then bind β2m and peptides supplied by TAP transporters associated with class I dimers (3, 4). Class I trimers containing peptides, β2m, and heavy chains are then transported through the Golgi to the cell surface.

Peptides stabilize class I dimers, and in mutant cells with a defect in peptide transport caused by a lack of functional TAP complexes, class I protein transport to the cell surface is impaired (5–8). Expression of surface class I can be restored by TAP gene transfection, demonstrating that TAP is essential for class I biosynthesis (9–13). TAP is present in membranes of the ER and cis-Golgi, suggesting that peptide loading into class I molecules occurs in one or both of these compartments (14).

A recent study showed that class I molecules retained in a pre-Golgi compartment, either by the fungal metabolite BFA or by substitution of the cytoplasmic domain of the class I molecule with an E3/19 cytoplasmic domain sequence, can bind antigenic peptides (15). This suggested that peptide loading occurs in the ER, since class I molecules in both cases localized predominantly in that site. However, it is possible that these class I molecules bound peptides in a post-ER compartment, and were then transported back to the ER. Other proteins bearing the E3/19 cytoplasmic domain sequence partially localize to and receive modifications characteristic of the intermediate compartment between ER and Golgi, suggesting that such molecules are retrieved from the intermediate compartment to the ER (16). Cells treated with BFA rapidly redistribute their Golgi and presumably intermediate compartment to the ER (17–19), which would potentially expose ER-retained class I molecules to a non-ER environment.

These observations suggest that peptides may bind to class I molecules at a site distinct from the rough ER. To examine this possibility, we studied the biosynthesis of HLA-B701 molecules in cells treated with inhibitors of protein transport, either BFA or the phosphatase inhibitors cantharidin and OKA. The latter compound was shown previously to block transport of vesicular stomatitis virus G protein from the ER to Golgi in treated cells (20, 21). In this study, we show that the two types of transport inhibitors have differential effects on class I heavy chain folding, dissociation from calnexin, and trimer formation.

MATERIALS AND METHODS

Cell Lines—The B-lymphoblastoid cell line CIR (HLA-A, B negative) was transfected with the A2m242 gene as described previously (22). CIR cells transfected with HLA-B701 and H-2D\(\beta\) genes were provided by Dr. Peter Cresswell. Cells were grown in RPMI 1640 (Irvine Scientific, Irvine, CA) containing 10% transferrin-supplemented calf serum (HyClone, Logan, UT). Medium for CIR cells expressing the A2m242 mutant contained in addition 300 μg/ml hygromycin (Sigma).

Reagents—Okadaic acid (Life Technologies, Inc.) was dissolved at 0.5 mM in 10% dimethyl sulfoxide and stored at –20 °C for 2–3 weeks. Brefeldin A (Sigma) was stored at –20 °C in methanol at 0.5 mM. Cantharidin (LC Laboratories, Woburn, MA) was dissolved at 100 mM in dimethyl sulfoxide. Endoglycosidase H was obtained from Boehringer Mannheim. Tran\(^4\)S-label (\(^3\)S)methionine and (\(^3\)S)cysteine and cysteine- and methionine-free RPMI 1640 were from ICN (Costa Mesa, CA). Protein A-Sepharose beads and fluorescein isothiocyanate-conjugated goat anti-mouse antibody were from Sigma.
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**Fig. 1.** Transport of a temperature-sensitive mutant, 242K, of HLA-A201 is blocked by OKA and BFA at different times after shift to the permissive temperature. Cells were incubated at 37 °C for 15 min, then transferred to 26 °C for 4 h. A, 2 μM OKA was added to cells and aliquots removed at 0, 2, and 4 h. Time of addition of OKA is indicated relative to initiating incubation at 26 °C. Samples were stained with antibody BB7.2 (from Dr. P. Cresswell) specific for HLA-A2 and -A69 (26). UCSF number 2, an antiserum reactive with the cytoplasmic tail of class I heavy chains after assembly with p2m (23). Monoclonal antibody AF8 was obtained from Dr. M. Brenner (26). UCSF number 2, an antiserum reactive with the cytoplasmic tail of class I heavy chains was provided by Drs. Bruce Koppelman and Frances Brodsky. Monoclonal antibody 1G12 (27) reactive with human transferrin receptor was provided by Dr. Janice Blum. Antisera reactive with human β2m and mouse immunoglobulin were from Sigma.

**Flow Cytometry—**Samples were stained and analyzed as described previously (25).

**Metabolic Labeling and Gel Electrophoresis—**Cells were washed in Met-free, Cys-free RPMI 1640 medium, then incubated for 1 h at 37 °C. At the indicated times, drug inhibitors were added. OKA was added to samples at 2 μM, and cantharidin and BFA at 200 and 10 μM, respectively. Cells were pulse-labeled by addition of 150 μCi of Tran[^35]S-label, 6 volumes of 10% RPMI were added, and aliquots removed and stored on ice. Following one wash in 0.01 M Tris, 0.15 M NaCl, pH 7.4 (TBS), at 4 °C, cells were lysed in TBS containing 1% Triton X-100 (v/v), 0.1 M tosyl lysine chloromethylketone, and 1 mM phenylmethylsulfonyl fluoride for 20 min on ice. In some experiments, 1% CHAPS was used to lyse cells. Lysates were then centrifuged at 13,000 × g for 5 min, and incubated at 4 °C with normal rabbit serum and formalin-fixed *Staphylococcus aureus* for 90 min. Precleared lysates were then incubated with 1 μl of antisera or 50 μl of hybridoma supernatant for 60 min at 4 °C and antigen-antibody complexes isolated with protein A-Sepharose beads. After elution, samples were digested with Endo H as described (22), or separated by 12% SDS-PAGE, and fluorography performed.

Scanning densitometry was used to quantitate images on a Millipore XRS Omni media scanner, and integrated optical density values reported.

**RESULTS**

*Okadaic Acid Blocks Transport of Class I Molecules from the ER—*An HLA-A201 molecule with substitution at position 242 of the heavy chain (242K) was shown previously to assemble with β2m and move to the cell surface only at temperatures from 21 to 30 °C (22). At 37 °C, 242K heavy chains do not assemble with β2m or pass through the Golgi. The block in transport prevents 242K from leaving the ER at 37 °C. At 26 °C, 242K heavy chains assemble with β2m and are transported in 1–2 h to the cell surface.

Incubation with 2 μM OKA 15 min before temperature shift to 26 °C completely blocked appearance of class I on the surface of 242K cells for up to 4 h as shown in Fig. 1. Addition of OKA at the same time as shift to 26 °C resulted in a partial block, whereas transport was insensitive to OKA addition by 15 min after the shift. In contrast, BFA blocked transport at 26 °C for more than 90 min (Fig. 1B). These results are consistent with OKA blocking selectively a very early stage in class I transport, as was shown previously for vesicular stomatitis virus G protein (20, 21).

**Maturation of N-linked Glycan on Class I Molecules Is Inhibited by OKA—**To determine whether OKA blocks passage of
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FIG. 2. Transport of HLA-B701 through the Golgi is blocked by addition of OKA. Cells were biosynthetically labeled for 10 min, then incubated with complete medium during the indicated chase periods. OKA was added at the indicated times relative to initiation of labeling. Antibody W6/32 was used to isolate class I complexes, and samples were either treated with Endo H (A) or mock treated (B). Arrows indicate heavy chains (labeled H) and β₂m (labeled B). In A, Endo H resistant and sensitive forms of heavy chains correspond to upper and lower bands, respectively. Overexposed images are shown to demonstrate that the block in transport is highly efficient in preventing passage through the Golgi.

As reported for other proteins, BFA blocks HLA-B701 transport, resulting in continued susceptibility to Endo H (Fig. 3). We also observed decreased mobility after digestion of a proportion of heavy chains, which is evident only in BFA-treated cells at the 30- and 60-min time points. The band does not correspond to either fully sensitive or resistant forms, and may result from BFA-induced retrograde transport of glycan modifying enzymes from the Golgi to ER. The lack of any similar modification apparent in OKA-treated cells (Fig. 2) suggests that OKA does not induce retrograde transport of the Golgi to a site where class I molecules are retained.

Assembly of Class I HLA Heavy Chain-β₂m Complexes Are Inhibited by OKA—Densitometry scanning of the results shown in Fig. 2 indicated that treatment with OKA (-5 min) inhibited reactivity with W6/32 by approximately 80% compared to OKA (+10 min) or untreated controls. This was not due to inhibition of protein synthesis during the labeling period, as total heavy chain levels were comparable in all samples (data not shown). Antibody W6/32 recognizes only heavy chain-β₂m complexes, so we considered whether OKA prevented assembly of class I subunits. To test this possibility, an anti-heavy chain reactive serum was used to measure initial heavy chain levels. Previous studies have shown that anti-H recognizes a determinant on free heavy HLA-B7 heavy chains which is lost after assembly with β₂m (28). Fig. 4 shows that when B701-CIR cells are treated with OKA 5 min before pulse-labeling for 3 min, heavy chain levels at the 0 min chase time are comparable to untreated control cells. Anti-H reactivity decreases over the next 60 min in untreated cells, and also in BFA-treated cells, paralleled by an increase in W6/32 reactivity. In contrast, OKA-treated cells show a small decrease in anti-H reactivity and little increase in W6/32 reactivity. This demonstrates that OKA blocks assembly of class I complexes, but does not interfere with synthesis of heavy chains or lead to their increased degradation. Parallel experiments with an anti-β₂m serum show that β₂m levels likewise are not decreased in OKA-treated cells (Table I). Impaired assembly of HLA-B701 was detected using anti-β₂m serum to assess heavy chain-β₂m interactions, demonstrating that decreased reactivity with W6/32 resulted from lack of heavy chain-β₂m assembly and was not due to loss of the antibody epitope from putative complexes.

Addition of OKA and BFA together resulted in a different pattern of reactivity from either drug alone. Anti-H reactivity declined at the same rate as with BFA treatment alone,
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FIG. 3. HLA-B701 molecules in cells treated with BFA have glycans which are partially modified, suggesting a mixing of intracellular compartments. Cells were treated as described in the legend to Fig. 2, except that BFA instead of OKA was added 5 min prior to labeling. Three distinct heavy chain species are evident after Endo H digestion and are marked by "H" arrows. The upper and lower bands correspond to fully resistant and fully sensitive heavy chains, respectively. The middle arrow designates heavy chains with uncharacterized modifications.

Fig. 4. OKA and BFA have distinct effects on assembly of HLA-B701 into complexes. CIR-B701 transfectants were left untreated or incubated with OKA, BFA, or with both drugs 5 min before labeling as described in the legend to Fig. 2, except that cells were labeled for 3 min, then chased for the indicated times. W6/32 and anti-H were used to isolate HLA-B701 complexes and free heavy chains, respectively. Samples were analyzed as above and quantitated by scanning densitometry. Integrated optical density (I.O.D.) values are shown for W6/32 (closed circles) and anti-H (open circles).

whereas W6/32 showed a significant increase in reactivity less than that observed with BFA alone. These data demonstrate that BFA can partially reverse the unassembled phenotype induced by OKA, and are consistent with retrograde transport of factors from a post-ER compartment which may assemble class I subunits retained in the ER. This suggests that stabilization of class I complexes occurs to a significant extent in a post-ER compartment.

A second phosphatase inhibitor, cantharidin, was able to mimic the effect seen with okadaic acid (Table I), suggesting that impaired class I assembly resulted from inhibition of phosphatase activity, and was not due to unrelated effects of either drug. A compound which is structurally similar to okadaic acid, but has no phosphatase inhibitory activity, 1-nor-okadone (29), did not inhibit class HLA-B701 assembly (data not shown).

Association of HLA-B701 Heavy Chains with Calnexin Is Prolonged in Cells Treated with Phosphatase Inhibitors—Calnexin has been postulated to play a role in the folding pathways of many membrane-bound and secreted proteins, including human and mouse class I MHC molecules (2, 26, 30–32). Proteins bind calnexin in the rough ER soon after their synthesis, and remain associated until folding is complete. Ultra-
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Table I

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Table II

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**Discussion**

It is clear that peptides derived from endogenous proteins bind to newly synthesized class I molecules in an early biosynthetic compartment. Class I heavy chains and \(\beta_m\) in mutant cell lines such as RMA-S and 174/2 fail to assemble properly due to a defect in TAP function (7). This suggested that peptide occupancy is required both for stability and intracellular transport of class I molecules. As class I heavy chains in these mutant cells are retained in a pre-Golgi compartment, it is likely that peptides bind to class I molecules before entering the Golgi. These studies, combined with kinetic experiments, suggest that the ER is a primary site for loading peptides into class I molecules. TAP molecules are found in the ER and cis-Golgi, consistent with this interpretation (14).

A recent study demonstrated that class I molecules retained in the ER by either BFA or incorporation of a retrieval signal on the cytoplasmic tail of the class I heavy chain contained T cell epitopes derived from viral proteins (15). This shows that class I molecules restricted to the ER have been exposed to peptides, but does not prove that binding occurred at that site. Other proteins whose transport is restricted by either BFA or an E3/19 retrieval signal have access to compartments beyond the ER, even though the bulk of such proteins localize to the ER (16).

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CIR-B701 cells were labeled as in Fig. 4 following preincubation with 2 \(\mu\)M OKA or 100 \(\mu\)M cantharidin. Cell lysates were split into fractions and antibodies specific for \(\beta_m\) (anti-\(\beta_m\)) or HLA class I heavy chains (anti-H) used to isolate individual subunits. Heavy chain-\(\beta_m\) complexes were quantitated by measuring heavy chains co-precipitating with \(\beta_m\). After SDS-PAGE, individual bands were measured as in Fig. 4. The numbers are percentages of individual integrated optical density values relative to the integrated optical density for anti-H reactive material at 0 min in each experiment.

This study addresses whether class I subunits block at early stages of transport in the ER form stable complexes. We found that whereas BFA blocked class I MHC egress from the ER, it had no apparent effect on subunit assembly. In contrast, the phosphatase inhibitors OKA and cantharidin effectively blocked...
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Both transport and assembly of HLA-B701 into complexes.

Although all three drugs block export of class I molecules from the ER, their modes of action differ. Retrograde transport of Golgi proteins to the ER is induced by BFA (17–19), whereas phosphatase inhibitors do not have this effect over the time course of our experiments. In addition, the temperature-sensitive HLA-A201 mutant 242K, whose transport can be induced during the course of our experiments. In addition, the temperature-sensitive HLA-A201 mutant 242K, whose transport can be induced by BFA(17-19), whereas phosphatase inhibitors block a very early step in protein processing in the Golgi, confirming the establishment of a block in transport through the Golgi.

It appears that class I molecules retained at this early stage would not be exposed to a post-ER environment, in contrast to class I MHC molecules in BFA-treated cells, which would be present in a mixed ER/Golgi compartment.

Our results are consistent with class I molecules acquiring stability in a location beyond the block induced by phosphatase inhibitors. This may correspond to the intermediate compartment between ER and Golgi. Phosphatase inhibitors are effective at least as soon as GTP-γS, which prevents proteins from entering a post-ER compartment defined by the presence of the molecule p58 (20). This site, called the intermediate compartment, has a role in assembly of multimers and in fatty acylation of certain proteins (34–36). This implies that phosphatase inhibitors also prevent proteins from entering a p58-containing compartment.

Three lines of evidence suggest that phosphatase inhibitors do not directly interfere with heavy chain-β2m pairing, but instead block peptide loading. First, addition of BFA with OKA to cells overcame the observed effects of OKA alone on HLA-B701 biosynthesis, partially restoring assembly with β2m with a concomitant dissociation of heavy chains from calnexin. This is consistent with the ability of BFA to induce retrograde transport of Golgi and post-ER compartments into the ER. Second, H-2Db heavy chains assemble with Prm in CIR transfec-tants treated with OKA, consistent with their ability to form complexes in the absence of TAP-derived peptides (33, 37). These results suggest that class I molecules whose transit from the ER is blocked by phosphatase inhibitors form heterodimers which could subsequently bind peptides. This implies that HLA-B701 complexes do not become stable because they are unable to bind peptides efficiently in the rough ER of treated cells. It is possible that this lack of peptide binding is due to impaired transport induced by phosphatase inhibitors, which prevents all proteins so far examined from exiting the ER.

An alternative explanation of these results is that TAP or other cellular components required for peptide loading are inactivated by phosphatase inhibitors. If true, this would imply that phosphorylation regulates TAP function, as shown for another member of the ABC family of transporters, P glycoprotein, which mediates multi-drug resistance (38, 39). It should be possible to evaluate TAP function in the presence of phosphatase inhibitors using assays which measure peptide translocation in semi-intact cells or across microsomal membranes (40–42).

Williams and co-workers (43) showed that class I heavy chains in β2m-deficient Daudi cells were rapidly degraded after dissociation from calnexin, suggesting that calnexin has a role in stabilizing unfolded heavy chains. Calnexin may also preserve heavy chain-β2m dimers until peptides can bind and stabilize the complex. Thus calnexin could remain associated with class I dimers until peptide binding allows class I molecules to attain their fully folded, stable conformation. Our data suggest also that transport of class I dimers to a site of peptide loading may be one of the functions subserved by calnexin.

The hypothesis that class I molecules are transported from the rough ER to a site of peptide loading is supported by other experiments. Class I molecules in mutant cells lacking TAP transporters move from the ER to the cis-Golgi, but are not efficiently transported to the plasma membrane (44). This suggests regulation of class I expression in the Golgi as well as in the ER. Similar observations using an IFN-γ-inducible mutant cell which exhibits recycling of class I molecules between ER and Golgi have also been reported (45). Our observations are consistent with these studies in postulating a role for a post-rough ER compartment in stabilizing class I complexes.

Note Added in Proof-The gene designated HLA-B701 in this manuscript corresponds to HLA-B*0702, the name approved by the WHO Nomenclature Committee.

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REFERENCES