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Tmp21, a novel MHC-I interacting protein, preferentially binds to β_2-microglobulin-free MHC-I heavy chains

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MHC-I molecules play a critical role in immune surveillance against viruses by presenting peptides to cytotoxic T lymphocytes. Although the mechanisms by which MHC-I molecules assemble and acquire peptides in the ER are well characterized, how MHC-I molecules traffic to the cell surface remains poorly understood. To identify novel proteins that regulate the intracellular transport of MHC-I molecules, MHC-I-interacting proteins were isolated by affinity purification, and their identity was determined by mass spectrometry. Among the identified MHC-I-associated proteins was Tmp21, the human ortholog of yeast Emp24p, which mediates the ER-Golgi trafficking of a subset of proteins. Here, we show that Tmp21 binds to human classical and non-classical MHC-I molecules. The Tmp21-MHC-I complex lacks β_2-microglobulin, and the number of the complexes is increased when free MHC-I heavy chains are more abundant. Taken together, these results suggest that Tmp21 is a novel protein that preferentially binds to β_2-microglobulin-free MHC-I heavy chains. [BMB reports 2011; 44(6): 369-374]

INTRODUCTION

Major histocompatibility complex class I (MHC-I) molecules present antigenic peptides on the cell surface to cytotoxic T cells, which eliminate virally infected cells or transformed cells. MHC-I molecules consist of three components: the MHC-encoded heavy chain (HC), the invariant subunit β_2m, and a small peptide of 8-10 amino acids that is produced mainly by the proteasome in the cytosol. The assembly of MHC-I molecules occurs in the lumen of the endoplasmic reticulum (ER) and is mediated by ER-resident chaperones and a multi-subunit complex called the peptide-loading complex (PLC), which includes the transporter associated with antigen processing and presentation (TAP), calreticulin, tapasin, and ERp57. In the PLC, MHC-I HC-β_2m heterodimers are loaded with peptides. The loading of optimal peptides results in the release of MHC-I-β_2m-peptide trimeric complexes from the TAP, which is a prerequisite for MHC-I ER exit for cell surface expression. Although the assembly and peptide-loading of MHC-I molecules in the ER have been well defined (1, 2), the mechanism by which MHC-I molecules traffic towards the cell surface remains poorly understood.

To investigate the intracellular transport of MHC-I molecules, we first attempted to identify novel proteins that mediate MHC-I trafficking. To this end, we carried out affinity purification using anti-MHC-I antibodies and analyzed the identity of proteins co-precipitated with MHC-I molecules by mass spectrometry. Among the proteins co-purified with MHC-I molecules, Sec22B, Sec23A, and Tmp21 were known to be involved in vesicular transport, and thus were chosen for further study. We recently reported that Sec23A, as a complex with Sec24, mediates the ER export of MHC-I molecules by binding to the ER export signal present in the cytosolic domain of MHC-I molecules (3). Sec22B is a SNARE protein that regulates ER-Golgi trafficking (4), and its role in MHC-I trafficking is currently under investigation. Tmp21, a member of the p24 family, has been suggested to play a role as a mammalian cargo receptor, as its luminal domain shows homology with the yeast cargo receptor Emp24, which was shown to recruit a specific cargo molecule, the glycosylphosphatidylinositol-anchored plasma membrane protein Gas1p, into ER-derived COPII vesicles in the budding yeast *Saccharomyces cerevisiae* (5, 6). Tmp21 is an integral membrane protein with a luminal domain of about 20 kDa, a transmembrane domain, and a short cytoplasmic tail (7, 8). The luminal domain is involved in hetero-oligomerization with other p24 members, such as p24α and p24β, and thus Tmp21 can exist as a monomer or in an oligomeric state. The cytoplasmic tail of Tmp21 carries a terminal motif (KKLIE) that is similar to the ER retrieval motif (KKXX), but the KKLIE motif is only a partially functional motif that allows a fraction of Tmp21 to escape from the Golgi complex for cell surface expression (7). Consistent with this observation, Tmp21 has been recently identified as a member of the presenilin/γ-secretase complex at the plasma membrane (9), suggesting that Tmp21 can also mediate non-traffic functions.

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In this study, we identified Tmp21 as a novel MHC-I-interacting protein by affinity purification followed by mass spectrometry. Interestingly, we found that Tmp21 preferentially bound to β_2m-free MHC-I heavy chains and seemed to function, through direct interactions with MHC-I heavy chains, in the protection of free MHC-I heavy chains from destruction by ER-associated protein degradation (ERAD) instead of mediating the intracellular trafficking of MHC-I molecules.

RESULTS

Identification of Tmp21 as a novel MHC-I-interacting protein

To search for novel proteins that associate with MHC-I molecules, MHC-I molecules were affinity-purified using the monoclonal antibody (mAb) W6/32 from HeLa cells, and co-precipitated proteins were analyzed by mass spectrometry. Among the identified MHC-I-associated proteins was Tmp21, a member of the p24 protein family, of which the yeast ortholog Emp21p is known to be involved in the early secretory pathway (5, 6).

To confirm the mass spectrometrical result that Tmp21 physically interacts with MHC-I molecules, HeLa cells transiently expressing C-terminally myc-tagged Tmp21 (Tmp21-myc) were metabolically labeled with ^{35}S-methionine/-cysteine and lysed in 1% digitonin. Then, Tmp21-myc was precipitated from the lysate, and co-precipitated material was boiled in DTT- and SDS-containing buffer to break all protein-protein interactions. The mixture was diluted 10-fold in buffer containing 1% NP-40 and subjected to immunoprecipitation with either anti-β_2m antibody (Fig. 1A, lanes 1, 3, and 5) or anti-MHC-I antibody (lanes 2, 4 and 6). A weak but specific band was found in the sample containing anti-MHC-I precipitates (lane 2), indicating that Tmp21-myc interacts with MHC-I molecules. The interaction between MHC-I molecules and Tmp21-myc was more obvious when HLA-B4402, a human MHC-I allele, was overexpressed in HeLa cells transiently expressing Tmp21-myc (lane 6).

Interestingly, however, we repeatedly failed to detect the interaction between MHC-I and Tmp21 by conventional co-immunoprecipitation approaches in which Tmp21 would have been detected by immunoblotting from the MHC-I co-precipitates (data not shown). Thus, it is likely that Tmp21 transiently interacts mostly with newly-synthesized MHC-I molecules and that only a few MHC-I-Tmp21 complexes exist at steady-state in the cell. This transient interaction is consistent with the proposed function of the yeast homolog of Tmp21, Emp24, as a cargo receptor in ER-Golgi trafficking (5, 6).

Intriguingly, we found that Tmp21 also bound to HLA-G, the non-classical MHC-I molecule specifically expressed in the placenta (Fig. 1A, lane 4 and Fig. 1B, lane 5), suggesting that Tmp21 is a pan-MHC-I-interacting protein that interacts with classical and non-classical human MHC-I molecules in the early stages of MHC-I biosynthesis and/or assembly in the ER.

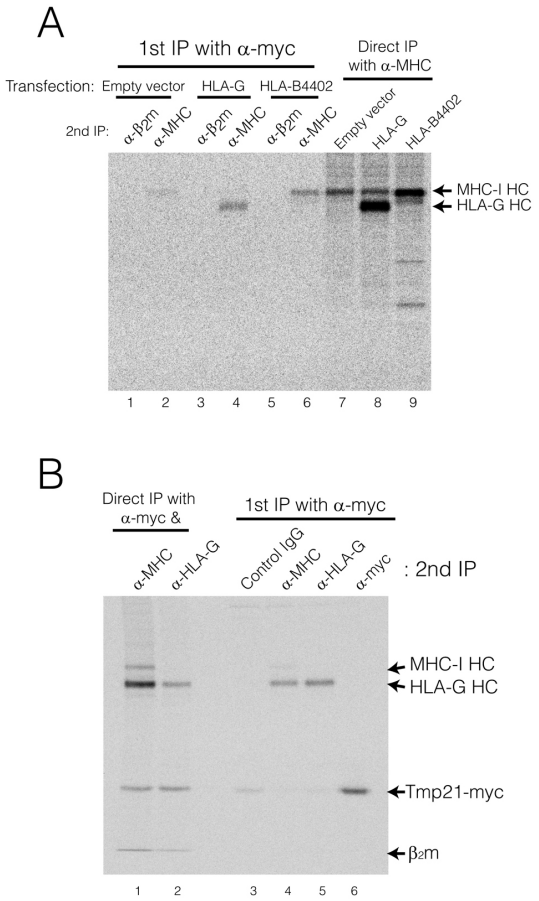

Fig. 1. Tmp21 binds to classical and non-classical human MHC-I molecules. (A) Tmp21 interacts with endogenous MHC-I molecules and ectopically expressed HLA-B4402. HeLa cells were co-transfected with Tmp21-myc and one of the following plasmids: empty plasmid vector, HLA-G, or HLA-B4402, and metabolically labeled with ^{35}S-methioinine for 30 min. Then, the interaction between Tmp21-myc and MHC-I molecules was analyzed by sequential co-immunoprecipitation experiments as described in Materials and Methods. (B) Tmp21 interacts with HLA-G molecules. HeLa cells were co-transfected with Tmp21-myc and HLA-G, and metabolically labeled for 30 min. Then, the interaction between Tmp21-myc and HLA-G was analyzed. To clarify the identity of protein bands, HLA-G/β_2m were directly precipitated by the mAb G233 from the same samples (lane 2).

Tmp21 preferentially binds to β_2m-free MHC-I heavy chains

The MHC-encoded heavy chains either bind to β_2m to form heterodimeric complexes immediately after they are synthesized in the ER or rapidly become substrates for ERAD, which dislocates them into the cytosol for destruction by proteasomes (10). Since Tmp21-myc binds to MHC-I heavy chains, but not to β_2m (Fig. 1A), Tmp21 may preferentially interact with free MHC-I heavy chains over assembled MHC-I molecules. To ex-

amine this possibility, HeLa cells transiently expressing both HLA-G and Tmp21-myc were metabolically labeled and lysed in 1% digitonin. The lysate was equally divided into three samples, and each sample was subjected to immunoprecipitation with the mAb G233, which specifically reacts with the assembled forms of HLA-G, with the mAb 4H84, which exclusively recognizes unassembled HLA-G heavy chains, or with anti-myc antibodies for Tmp21-myc (Fig. 2A). The immunoprecipitated material was then boiled in SDS- and DTT-containing solution to break the protein interactions and was subjected to immunoprecipitation with control immunoglobulin G (lane 1), anti-HLA-G (lanes 2, 4, and 6), or anti-β_2m (lanes 3, 5, and 7). The second immunoprecipitation with anti-HLA-G was able to recover HLA-G heavy chains from all three samples (lanes 2, 4, and 6), but β_2m was only recovered from the material precipitated by the mAb G233 (lane 5).

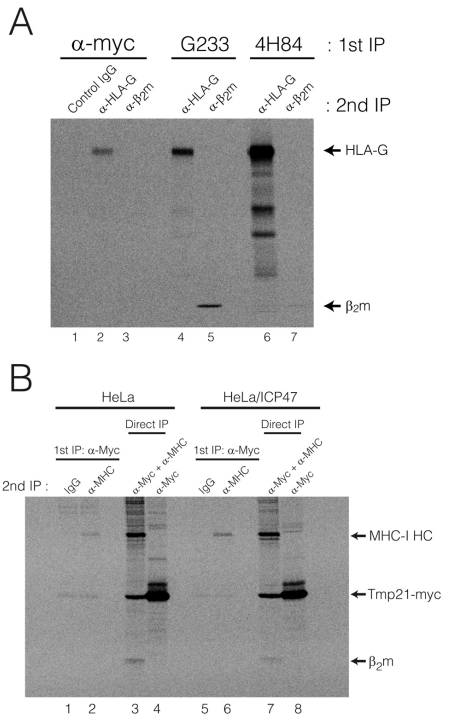

Fig. 2. Tmp21 preferentially binds to β_2m-free MHC-I heavy chains. (A) Tmp21-MHC-I complexes do not contain β_2m. HeLa cells transiently expressing both HLA-G and Tmp21-myc were metabolically labeled, and the interaction of Tmp21-myc with either HLA-G heavy chains or β_2m was analyzed. (B) More Tmp21-MHC-I complexes were detected in HeLa/ICP47 cells than in normal HeLa cells. HeLa cells or HeLa/ICP47 cells were transfected with Tmp21-myc, metabolically labeled for 30 min, and lysed in 1% digitonin. Then, Tmp21-myc was immunoprecipitated using anti-myc antibodies, and co-precipitated material was subjected to a second immunoprecipitation with control immunoglobulin G (lanes 1 and 5) or anti-MHC-I (lanes 2 and 6). Direct immunoprecipitation from the same samples shows the identity of coprecipitated proteins with Tmp21-myc (lanes 3, 4, 7, and 8).

Thus, Tmp21-HLA-G complexes seem to lack β_2m, suggesting that Tmp21 preferentially binds to β_2m-free MHC-I heavy chains.

To confirm this finding using an independent approach, we artificially increased the number of free MHC-I heavy chains by ectopically expressing herpes simplex virus (HSV) ICP47, which blocks TAP function and thus increases the number of free MHC-I heavy chains in the ER (11, 12), and examined whether HSV ICP47 expression increases the number of MHC-I-Tmp21 complexes (Fig. 2B). As expected, significantly more Tmp21-MHC-I complexes were detected in HeLa-ICP47 cells than in HeLa cells (Fig. 2B, compare lanes 2 and 6), further supporting the observation that Tmp21 has a binding preference for β_2m-free MHC-I heavy chains.

Tmp21 knock-down by RNAi little affects MHC-I trafficking

To examine the role of Tmp21 in MHC-I-mediated functions, we first tested whether Tmp21 knock-down affects the cell surface expression of MHC-I molecules. Two siRNAs were generated to target Tmp21, and one of these, siTmp21-2, seemed to reduce the protein level of Tmp21 to less than 10% of the endogenous level (Fig. 3A). To examine the effect of Tmp21

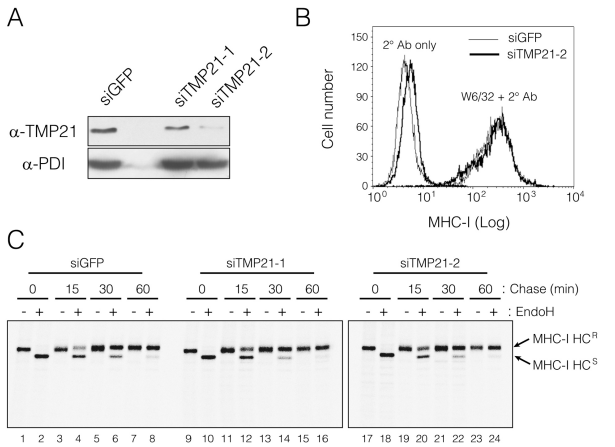

Fig. 3. Downregulation of Tmp21 expression by RNA interference does not affect the intracellular trafficking of MHC-I molecules. (A) The knock-down efficiency of two small interfering RNAs targeting Tmp21. Protein disulfide isomerase (PDI) was used as a loading control. (B) The cell-surface expression of MHC-I molecules in HeLa cells transfected with siGFP (thin line) or siTMP21-2 (thick line). The surface expression of MHC-I molecules was examined by flow cytometry after indirect immunofluorescence staining using the mAb W6/32 and FITC-conjugated anti-mouse antibody. (C) EndoH analysis in pulse-chase experiments for the intracellular trafficking of MHC-I molecules. HeLa cells transfected with siGFP, siTMP21-1, or siTMP21-2 were metabolically labeled for 15 min and chased for the indicated times. MHC-I molecules recovered by immunoprecipitation with the mAb W6/32 were left untreated or treated with EndoH, separated by SDS-PAGE, and analyzed by autoradiography. MHC-I HC^R, EndoH resistant heavy chain; MHC-I HC^S, EndoH sensitive heavy chain.

reduction by siTmp21-2 on MHC-I cell surface expression, HeLa cells transfected with control siRNA targeting GFP or with siTmp21-2 were labeled with the mAb W6/32, which reacts with assembled forms of MHC-I molecules, and MHC-I expression on the cell surface was analyzed by flow cytometry (Fig. 3B). Tmp21 knock-down by siTmp21-2 did not significantly affect the surface expression of MHC-I molecules (Fig. 3B, compare thin and thick lines).

We also examined the effect of Tmp21 reduction on MHC-I trafficking in the early secretory pathway by endoglycosidase H (EndoH) analysis coupled with pulse-chase experiments (Fig. 3C). At the 0-min chase, nearly all MHC-I molecules were sensitive to N-glycan cleavage by EndoH, indicating that large portions of MHC-I molecules were still in the ER (Fig. 3C, lane 2). In contrast, at the 30-min chase, over 90% of MHC-I molecules had acquired EndoH resistance, indicating that MHC-I molecules had reached post-cis-Golgi compartments in HeLa cells transfected with siGFP (Fig. 3C, lane 8). Similarly, in cells transfected with siTmp21-2, we did not observe any significant difference in the rate of MHC-I trafficking. These results collectively suggest that Tmp21 may not function in MHC-I trafficking for cell surface expression.

Tmp21 may protect MHC-I free heavy chains from destruction by ERAD

Only a few MHC-I free heavy chains naturally exist in the cell at steady state, as they readily form heterodimeric complexes with β_2m or are rapidly dislocated into the cytosol for destruction by the proteasome via ERAD (10). To investigate whether Tmp21 is involved in ERAD-mediated degradation of free MHC-I heavy chains, we examined the effect of Tmp21 knock-down on the ERAD of MHC-I free heavy chains. To this end, siGFP or siTMP21-2 was transfected into U373MG cells stably expressing HCMV US11 (13, 14), which is known to induce MHC-I degradation via ERAD (Fig. 4A). U373MG-US11 cells transfected with siGFP or with siTMP21-2 were pulse-labeled for 10 min and chased for 0, 15, and 30 min. As previously reported (14), the half-life of MHC-I molecules was lowered to less than 10 min upon US11 expression. In this model system, Tmp21 knock-down moderately accelerated the rate of MHC-I degradation compared to that in cells transfected with siGFP (Fig. 3A, B), suggesting that Tmp21-MHC-I interaction could delay the ERAD of MHC-I free heavy chains. Although further studies are required to reveal the exact function of Tmp21 in the fate of MHC-I molecules, our data suggest that one of the functions of Tmp21 is to prevent or at least to delay ERAD-mediated degradation of free MHC-I heavy chains.

DISCUSSION

In this study, we find that Tmp21 is an MHC-I-associated protein that preferentially interacts with β_2m-free MHC-I heavy chains. Few proteins are known to preferentially bind to β_2m-free MHC-I heavy chains. Calnexin, an ER-resident chaperone, is one of the few proteins that selectively bind to β_2m-free MHC-I heavy chains. Calnexin binds and retains β_2m-free MHC-I heavy chains in a partly folded state in the ER until the MHC-I heavy chains bind to β_2m and form MHC-I: β_2m heterodimers (15-17). These heterodimers then dissociate from calnexin and enter the PLC for the binding of peptides. Surprisingly, the Dawson group (18) reported that despite the importance of calnexin in the folding and assembly of MHC-I molecules, there was no significant difference in the intracellular transport and surface expression of human MHC-I molecules between the human leukemic cell lines CEM and CEM-NKR, which lacks calnexin, suggesting that calnexin is not absolutely required for MHC-I surface expression. Likewise, we failed to observe any significant effect of Tmp21 downregulation by RNAi on the cell surface expression or intracellular transport of MHC-I molecules. Another reason for the lack of an RNAi effect could be that p24 family proteins are highly redundant in function. Since there are at least 11 members of the p24 protein family in mammals (19), the function of Tmp21 could have been replaced by other p24 family members in cells transfected with siRNA targeting Tmp21. To clarify the involvement of Tmp21 in MHC-I trafficking, multiple knock-down of other p24 family members along with Tmp21 knock-down would be required. Such approaches, however, have been hampered by the fact that deletion of all eight p24 members in yeast reveals no essential defect in the secretory

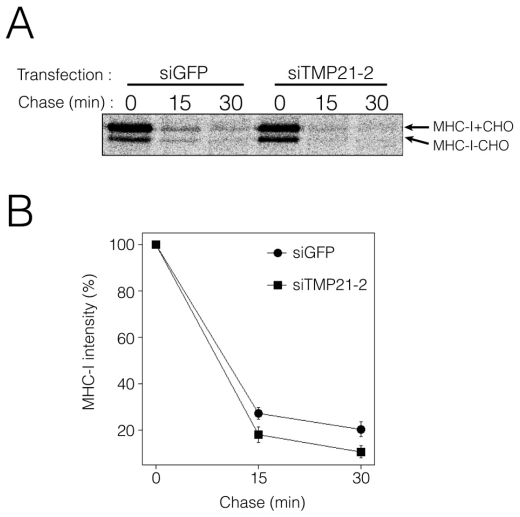

Fig. 4. Tmp21 knockdown moderately accelerates HCMV US11-induced degradation of MHC-I free heavy chains. (A) U373 MG/US11 cells were transfected with either siGFP or siTMP21-2, and the stability of endogenous MHC-I heavy chains was examined by metabolic labeling and immunoprecipitation with the mAb HC-10 in pulse-chase experiments. MHC-I+CHO, MHC-I heavy chain with N-linked glycan; MHC-I-CHO, MHC-I heavy chain without N-linked glycan. (B) The graph shows quantified results of three independent experiments including the experiment shown in (A). Error bars represent standard deviation of the mean (n = 3).

pathway (20).

Free MHC-I heavy chains readily become substrates for ERAD unless they associate with β_2m to form heterodimers (10). However, only few MHC-I heavy chains are degraded by ERAD under normal conditions, suggesting that there may be a mechanism by which free MHC-I heavy chains are protected from ERAD processes until they bind to β_2m. Under the condition in which most MHC-I molecules are substrates for ERAD, we found that reduction of Tmp21 moderately, but meaningfully, accelerated the degradation of MHC-I heavy chains (Fig. 4). These findings provide an important clue as to how free MHC-I heavy chains escape from destruction by ERAD to properly fold and enter the PLC for peptide-binding. Although further investigation is required to reveal the exact function of Tmp21 in MHC-I-related cellular processes, our current study has clearly demonstrated that Tmp21 is a novel MHC-I binding protein that preferentially interacts with β_2m-free MHC-I heavy chains.

MATERIALS AND METHODS

Cell lines, cell culture and transfection

HeLa/ICP47 and U373MG/US11 cells were previously described (21). HeLa cells and U373MG cells were cultured in DMEM supplemented with 7% FBS (Hyclone), 2 mM Glutamax-I, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen). All cells were transfected with Lipofectamine 2,000 (Invitrogen). After 24-48 hr, all assays were performed.

Plasmids

The cDNAs for HLA-G1 and HLA-B4402 were subcloned into pcDNA3.1 for expression in human cells as described (3). Tmp21 was cloned by RT-PCR of mRNA prepared from the human alveolar epithelial cell line A549. The C-terminally myc-tagged Tmp21 was generated by PCR with primers containing the DNA fragment encoding a myc tag (EQKLISEEDL).

Preparation and transfection of small interfering RNA (siRNA)

The sense strand sequences of the siRNAs used for targeting Tmp21, siTMP21-1 and siTMP21-2, were CCUUCCAUCUGC CCAUUAA and GCCUAAGGCAUCCUACCAA, respectively. An siRNA targeting GFP was used as a control. HeLa cells and U373MG/US11 cells were transfected with 100 nM of siRNA duplex using oligofectamine (Invitrogen) according to the manufacturer's instructions. Forty-eight hours later, further analysis was carried out.

Antibodies

The monoclonal antibodies W6/32 and HC-10 were purified from supernatant of mouse hybridoma cells. The monoclonal antibodies 4H84 (Santa Cruz) and G233 (Abcam) recognize denatured HLA-G heavy chains and assembled HLA-G/β_2m, respectively. Anti-Tmp21 (Abcam), anti-β_2m (Santa Cruz), anti-MHC-I (H-300, Santa Cruz) and anti-myc 9E10 (Santa Cruz) were purchased.

Pulse-chase and immunoprecipitation

For pulse-chase experiments, cells were starved in methionine/cysteine-free DMEM (Invitrogen) for 1 hr and labeled with ^{35}S-methionine/cysteine (PerkinElmer) for 10 min. Free ^{35}S-methionine/cysteine was removed by washing cells with warm PBS three times, and cells were transferred to normal medium for chase experiments. After incubation for the indicated times, cells were harvested and lysed with 1% NP-40 (Calbiochem) in PBS supplemented with 1 mM PMSF (Sigma) and 10 μM leupeptin (Calbiochem) at 4°C for 30 min. Post-centrifugation supernatants were precleared by incubation with protein G sepharose (PGS, Amersham) at 4°C for 1 hr, and the precleared supernatants were further incubated with 1 μg of indicated antibodies at 4°C overnight. After addition of PGS, incubation was continued for another 1 hr, and material bound to PGS was precipitated, washed three times with 1% NP-40 in PBS, and eluted in EndoH buffer (50 mM sodium acetate, pH 5.6, 0.3% SDS, and 150 mM β-mercaptoethanol) by boiling for 10 min. Each eluate was divided into two parts, which are then incubated at 37°C overnight in the presence or absence of EndoH (5 mU, Roche). The samples were boiled in SDS-sample buffer for 10 min, separated by 10% SDS-PAGE, and analyzed by autoradiography using a phosphorimager (BAS2500, Fujifilm).

Sequential immunoprecipitation

Metabolic labeling was performed as described above, after which the cells were harvested and lysed with 1% digitonin buffer (1% digitonin in 25 mM HEPES (pH 7.4), 125-150 mM NaCl, 1 mM PMSF, and 10 μM leupeptin) at 4°C for 2 hr. Postcentrifugation supernatants were precleared with PGS at 4°C for 1 hr. The precleared supernatants were incubated with control mouse IgG or anti-myc antibody at 4°C for 12 hr. After addition of PGS, the samples were incubated for an additional 1 hr. Material bound to PGS was precipitated, washed two times with 0.1% digitonin buffer, and eluted in 100 μl of Re-IP buffer (1.5% SDS and 2 mM DTT in PBS). The eluates were then diluted in 900 μl of 1% NP-40 in PBS and treated with 1 μg of control IgG, the monoclonal antibody 4H84, or anti-myc antibody at 4°C overnight. After addition of PGS, the incubation was continued for 1 hr. Material bound to PGS was washed three times with 1% NP-40 in PBS and eluted by boiling for 10 min. Samples were separated by SDS-PAGE and analyzed by autoradiography.

Flow cytometry

The cell surface expression of MHC-I molecules was examined by flow cytometry (FACSCalibur; BD Biosciences) after indirect immunofluorescence staining using W6/32 and FITC-conjugated anti-mouse antibody (The Jackson laboratory). A total of 10,000 gated events were collected by the flow cytometer and analyzed by the CellQuest software (BD Biosciences).

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