The mutant murine lymphoma cell line RMA-S is unable to present endogenous antigens due to its inability to efficiently assemble class I major histocompatibility complex molecules and antigenic peptides. Therefore, it has been suggested that RMA-S cells are defective either in peptide generation or in peptide transport into the endoplasmic reticulum, where class I major histocompatibility complex molecule assembly is believed to occur. As proteasomes and the putative peptide transporters HAM1 and HAM2 have been implicated in class I antigen processing, we have investigated their expression in RMA-S and its wild-type counterpart RMA. Both proteasomes and HAM1 proteins are expressed at similar levels and show identical subcellular distributions in the two cell lines. However, only one copy of the HAM2 gene is present in RMA-S cells, and it contains a point mutation that leads to a premature stop codon. Thus, the HAM2 protein is absent from RMA-S cells. These data demonstrate that HAM2 is essential for peptide loading onto class I molecules.

Although the structure and expression of the genes encoding β2-microglobulin and MHC1 class I heavy chains are normal in RMA-S cells (1),2 low cell surface expression of class I molecules has been observed (2, 3). The addition of synthetic, antigenic class I-binding peptides to RMA-S cells has restored surface expression of class I molecules and induced assembly of RMA-S-derived class I molecules in vitro (3, 4). Therefore, the deficient assembly and intracellular transport of class I molecules in RMA-S cells might have been due to lack of class I-binding peptides resulting from defective antigen processing. Recent data have demonstrated that proteasomes (5), which represent the major extralysosomal proteolytic system (6), contain two MHC-encoded subunits (7-11), rendering the proteasome a prime candidate for generating antigenic peptides for class I molecules. Two genes called HAM1 and HAM2 (12) in the mouse, which are members of the traffic ATPases superfamily of transporters (13), have been mapped in close vicinity to the MHC-encoded proteasomal subunits (12, 14). The rat homologues and the human homologues of HAM1 and HAM2 are named MTPl and MTP2 (15) and PSFl and PSF2 (16), respectively. Their involvement in the transport of peptides across the ER membrane has been discussed (12, 14-20). In the present study, we have investigated the expression of proteasomal subunits and the putative peptide transporters HAM1 and HAM2 in RMA-S and its wild-type counterpart RMA.

RESULTS AND DISCUSSION

To examine if proteasomes are different in RMA and RMA-S cells, we immunoprecipitated metabolically labeled proteasomes from the two cell lines and separated the subunits by two-dimensional gel electrophoresis. The polypeptide patterns were identical, including the levels, charges, and molecular weights of the two MHC-encoded subunits (data not shown), which strongly suggested that proteasomes from RMA and RMA-S do not differ. If peptide generation is normal in RMA-S cells, it seemed likely that peptide transport from the cytoplasm into the ER is defective in RMA-S cells. The human cell line LCL 721.134, with a phenotype similar to that of RMA-S, had previously been shown to revert to the wild-type phenotype following transfection with PSFl (20), the human homologue of HAM1. Therefore, we investigated the expression of HAM1 in RMA and RMA-S cells by immunoprecipitation and immunofluorescence staining using two antisera raised against a peptide corresponding to the carboxyl-terminal region of HAM1 and a purified recombinant protein containing the entire putative ATP-binding domain of HAM1, respectively. Both antisera specifically recognized an interferon γ-inducible 75-kDa protein as revealed by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Immunofluorescence staining occurred in the perinuclear area representing the ER (data not shown). However, no difference was found with respect to size, charge, level of expression, and immunofluorescence staining pattern of HAM1 between RMA and RMA-S cells. Furthermore, it has been demonstrated that the HAM1 mRNA is expressed at similar levels in the two cell lines (14), and sequence analysis of the HAM1 cDNA did not reveal any difference (data not shown). This indicates that the sequence and expression level of HAM1 is identical in RMA and RMA-S cells, which may explain the findings that neither transfection of RMA-S with the rat homologue MTP1 (15) nor transfection with the HAM1-containing cosmid 5.10 (12, 21) restored the surface expression of class I molecules (22). Consequently, the RMA-S phenotype must be caused by mutations in another gene.

It has also been reported that the MHC region contains a
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The sequence analysis of the HAM2 cDNA from RMA-S revealed only one difference compared with the RMA sequence at nucleotide position 97, which displayed a C to T transition (Fig. 3). This mutation introduces a premature stop codon. Several independent cDNA clones contained the same mutation at position 97, and no other mutation was detected by the PCR amplification. Therefore, this mutation was found by directly sequencing PCR products obtained from reverse transcribed mRNA of RMA-S cells. No evidence of a mixed mRNA population consisting of both normal and mutated sequences was found (e.g. in Fig. 3). To investigate whether the mRNA-S cells were homo- or hemizygous for the mutation, we amplified a region of the HAM2 gene corresponding to nucleotides 3–151 of HAM2 from RMA and RMA-S genomic DNA. The amplified DNA was separated by electrophoresis with or without prior digestion with AvalII. This enzyme should cleave the mutated but not the wild-type HAM2 DNA fragment as the mutation created the recognition sequence CCTAGG for the enzyme AvalII. Fig. 4 demonstrates that the HAM2 genomic DNA fragment derived from RMA-S was completely cleaved, while the fragment from RMA remained completely cleaved, while the fragment from RMA remained completely sequenced cDNA clones corresponding to HAM2 from RMA-S and RMA-S cDNA libraries. The sequence of HAM2 from RMA cells, shown in Fig. 1, contains an open reading frame of 702 residues, which encodes the two short amino acid sequence stretches (positions 495–566 and 605–637) previously published (12). Fig. 2 shows that the HAM2 sequence displays 91% identity with the rat MTP2 sequence (22) and 77% identity with the human PSF2 sequence (25), while the identity with the HAM1 sequence (22) is less pronounced (39%). The HAM2 sequence, like the HAM1 sequence, also displays striking homologies to other members of the traffic ATPases superfamily of transporters (not shown) and contains the typical α-β-α nucleotide binding motif (26, 27) (amino acid residues 502–510 and 618–630 in Fig. 1).

Because it is unlikely that RMA-S cells are homozygous for the same point mutation in the HAM2 gene, this strongly suggests that RMA-S cells contain only one copy of the HAM2 gene and that the HAM2 protein is absent in RMA-S cells. On the contrary, Powis et al. (22) showed that a 65-kDa protein, which they identified as the HAM2 protein on the basis of its reactivity with an αMTP2 peptide (the NH2-terminal MTP2 sequence MALSHPRPWA(LC)) antiserum, was present in both RMA-S and RMA cells. However, a predicted molecular mass of 77.4 kDa, calculated from the amino acid sequence stretches (positions 495–566 and 605–637) predicted for the HAM2 protein present in RMA-S is unlikely to be the HAM2 protein. The present data are consistent with the view that the absence of the HAM2 protein in RMA-S cells generates the defect in the assembly and intracellular transport of class I molecules. This would implicate HAM2 as an essential component of the peptide-loading machinery for class I molecules. This conclusion is supported by the finding that transfection of RMA-S with MTP2 cDNA restores cell surface expression of MHC class I molecules (22). Taken together with the observation that the lack of PSF1 (the human homologue of HAM1) in the human cell line LCL 721.134 produces a
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**Fig. 2.** Amino acid comparison of HAM2, MTP2, PSF2, and HAM1.

The sequences were aligned using the computer program SeqEd 1.0. The positions where gaps have been introduced are marked by asterisks. Identical amino acids are indicated by dashes. **Numbers** corresponding to amino acid positions in HAM1 (12), HAM2, MTP2 (22), and PSF2 (25) are indicated on the right.

**Fig. 3.** Point mutation in the HAM2 gene of RMA-S. Representative chromatograms from automated sequence analysis of nucleotide positions 87–105 of HAM2 (see Fig. 1) in RMA and RMA-S. **Arrows** indicate the nucleotide transition from C to T at position 97. The sources of HAM2 template DNA of RMA and RMA-S for PCR were reverse-transcribed mRNA, genomic DNA, and plasmids from RMA and RMA-S cDNA libraries. The PCR products covering the mutation in RMA-S were sequenced directly. No signal over background for any other nucleotide but T at position 97 was observed for all RMA-S-derived templates.

phenotype similar to that of the RMA-S cells (20), our results demonstrate that defects in both MHC-encoded transporter genes have similar effects. However, it is also evident that HAM1 and HAM2 have distinct functions since they cannot complement each other, suggesting that both proteins are necessary for class I peptide loading. Structural comparison among HAM1, HAM2, and other traffic ATPases transporters has led to the suggestion that HAM1 and HAM2 function as heterodimers, since HAM1 and HAM2 each consist of single hydrophobic transmembrane and hydrophilic ATP-binding domains (half of the usual functional unit). Indeed, it has recently been demonstrated that PSF2 is co-immuno-
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**Fig. 4.** Restriction analysis of PCR-amplified HAM2 genomic DNA from RMA and RMA-S cells. HAM2 genomic DNA covering nucleotide positions 3–151 was amplified using primers corresponding to positions 3–19 and to the opposite strand of nucleotides 135–151. In both RMA (lane 2) and RMA-S (lane 4) a PCR DNA fragment of approximately 150 base pairs was obtained. The 150-bp HAM2 DNA fragments of RMA (lane 3) and RMA-S (lane 5) were digested with *Ava*II (BioLabs) whose recognition sequence C’CTAGG is only present in the mutated sequence. The restriction digestion results in two fragments of 90 and 60 base pairs. Lane 6 (undigested) and lane 7 (digested with *Ava*II) contain a 1:1 mixture of the 150-bp HAM2 DNA fragments from RMA and RMA-S. Lanes 1 and 8 contain molecular weight standard (1-kb ladder, BRL). Fragment sizes are indicated in base pairs on the right. DNA fragments were electrophoretically separated on a 6% polyacrylamide gel using Tris-acetate buffer. Template genomic DNA for PCR was prepared from RMA and RMA-S using genomic DNA isolation kit (BRL).

precipitated with PSF1 by using a PSF1-specific antiserum (28). As HAM1 apparently has a normal half-life and subcellular distribution in RMA-S cells, the two proteins could fold independently of each other. Thus, the possible interaction between HAM1 and HAM2 may be a regulated event, which may activate the function of the putative peptide transporter.

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