Dependence of Elevated Human Leukocyte Antigen Class I Molecule Expression on Increased Heavy Chain, Light Chain (β2-Microglobulin), Transporter Associated with Antigen Processing, Tapasin, and Peptide*

David R. Johnson‡ and Barry Mook-Kanamori

From the Department of Pathology, Yale University School of Medicine, New Haven, Connecticut 06510

Human leukocyte antigen (HLA) class I molecule expression was investigated by DNA-mediated gene transfer. Cell surface expression was increased up to 75% by transfection of HLA-A2 or HLA-B8 heavy chain genes but not genes encoding light chains (β2-microglobulin (β2m)), transporter associated with antigen processing (TAP), or tapasin. Interferon (IFN) treatment further increased expression of transfected heavy chains, suggesting that IFN inducible molecules support heavy chain expression. IFN induces β2m, TAP, and tapasin mRNAs. Transfected heavy chain expression increased upon cotransfection with genes encoding TAP1 and TAP2 but not individual TAP subunits, β2m, or tapasin. Tetracycline inducible heavy chain gene expression was also increased by IFN treatment or TAP cotransfection, suggesting that IFN-induced TAP supports heavy chain maturation. Expression of a mutant that does not interact strongly with TAP, HLA-A2-T134R, was also increased by IFN. Inhibition of TAP-dependent peptide transport by ICP47 reduced heavy chain expression. Expression of HLA-A2, but not HLA-B8, was restored in ICP47 cells by HLA-A2-binding (IP-30) signal peptides. However, these peptides did not further increase transfected HLA-A2 expression, suggesting that peptide availability does not limit heavy chain expression in the absence of ICP47. These results suggest that cytokine-induced TAP supports maturation of HLA class I molecules through combined chaperone and peptide supply functions.

HLA class I molecules consist of a polymorphic, transmembrane heavy chain (45 kDa), a monomorphic, water-soluble light chain (12 kDa, also called β2-microglobulin (β2m)), and a tightly bound peptide of 9–12 amino acids (1). The peptide is typically derived from proteins synthesized within the cytoplasm of the cell and transported into the lumen of the endoplasmic reticulum, where HLA class I molecules fold and mature, by a dedicated transporter associated with antigen processing (TAP) (2). An association between TAP and nascent heavy chains is thought to be mediated by an adapter protein called tapasin (3, 4).

Peptides provided by the complex of TAP and tapasin are required, in addition to the structural proteins, for the efficient cell surface expression of HLA class I molecules (3, 5, 6). With the exception of the disease-related allele HLA-B27, human class I molecules do not properly fold and mature in the absence of peptides (7). Misfolded HLA class I molecules are specifically moved by a dedicated transporter from the endoplasmic reticulum to the cytoplasm, where they are degraded by proteasomes (8).

The interferons (IFNs) and tumor necrosis factor (TNF) increase cell surface expression of HLA class I molecules by inducing the coordinate expression of the structural heavy chain and light chain genes (9). IFNs also rapidly increase TAP and TAP-dependent peptide transport (10). IFN induction of the gene encoding the TAP1 subunit of TAP is mediated by IFN-responsive enhancers within the promoter (11). A sequence similar to the IFN-responsive enhancer has been noted in the tapasin promoter (12), raising the expectation that this gene is also induced by IFN.

Coordinate cytokine induction of the structural heavy and light chains, TAP and tapasin, is generally thought to support the efficient maturation of HLA class I molecules and their expression on the cell surface. The functions of β2m, TAP, and tapasin were originally demonstrated by restoring HLA class I expression in null mutant cell lines (3, 5, 13). However, it is unknown how physiological levels of these components contribute to the expression of HLA class I molecules. Here, the roles of IFN-induced TAP and tapasin and increased peptide supply in supporting increased expression of HLA class I molecules were investigated by transfecting expression vectors encoding each of these components into HeLa cells. This approach allowed the independent manipulation of these molecules in a more physiological setting.

EXPERIMENTAL PROCEDURES

Cells and Cytokines—HeLa cells were a gift of Richard Flavell (Yale Medical School) and were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mm L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin sulfate (all from Life Technologies, Inc.). The culture was split 1:9 every 3–4 days. HeLa cells were serologically typed as HLA-A3, -A28, -Bw15, and -Bw35 (14). Recently, a genomic DNA-based typing found HeLa to be HLA-A68 (a subtype of A28) and -B75 (a subtype of B15) but negative for B35 (15). Using reverse transcriptase-PCR (16), we have identified in the HeLa cells used here the alleles HLA-A*6802, -B*1503 or -B*1537, and -Cw*1604 (not shown). The cytokines TNF-α (recombinant, expressed in Escherichia coli, ~ 20 units/ng) and IFN-γ (recombinant, expressed in E. coli, 10 units/ng) were purchased from R&D Systems (Minneapolis, MN). IFN-β (recombinant, expressed in E. coli, 30 units/ng) was a gift.

This paper is available on line at http://www.jbc.org
TAP Can Limit HLA Class I Expression

Dorothy E. Voldman,

HLA-A2.1 and HLA-B8 expression vectors were gifts of William Biddison (National Institute of Allergy and Infectious Diseases, Bethesda, MD) (17). TAP-1 and TAP-2 expression vectors were gifts of Thomas Spies (Hutchinson Cancer Research Center, Seattle, WA) (5). The HLA and TAP cDNAs are inserted into the RSV.5neo expression vector (18). (Hutchinson Cancer Research Center, Seattle, WA) (5). The HLA-A2-transfected cells expressed an invariant “framework” determinant and detects all HLA class I molecules on their surface (Fig. 1). The transient transfection protocol resulted in high transfection efficiency with typically over 50 and often over 80% of the cells expressing the transfected genes (Fig. 1, a and b). This high efficiency allowed the analysis of unseparated populations. The detection of class I heavy chains encoded by transfected genes was reported long ago, but the level of transfected heavy chain expression, relative to the endogenous HLA class I genes, has not been determined. One difficulty in comparing HLA class I expression levels is illustrated by the staining of cells transfected with HLA-A2 or -B8. Two monoclonal antibodies specific for HLA-A2 (BB7.2 and MA2.1) both stain strongly, whereas two monoclonal antibodies specific for HLA-B8 (0201HA and 0402HA) stain weakly (Fig. 1 and data not shown). The potential differences in antibody affinities prevent the use of relative fluorescence intensities for comparing expression of these genes.

Total Cell Surface Expression of HLA Class I Molecules Can Be Increased Substantially by Transfection of HLA Class I Heavy Chain Genes—To compare directly the expression of the transfected and the endogenous class I genes, the cells were transfected with a monoclonal antibody that is specific for an invariant “framework” determinant and detects all HLA class I molecules, W6/32 (20). Staining with W6/32 revealed that HLA-A2-transfected cells expressed ~50% more HLA class I molecules on their surface and HLA-B8 transfected cells expressed 75% more HLA class I molecules on their surface (Fig.
Therefore, despite the weak staining with antibodies specific for HLA-B8, cells transfected with HLA-B8 actually expressed more HLA class I on their surface than did cells transfected with HLA-A2. Cells transfected with β2m, TAP subunits, or tapasin did not express more HLA class I molecules containing endogenous heavy chains (Fig. 2b), suggesting that the supply of heavy chain alone can regulate surface expression by resting HeLa cells.

Interferons Increase Expression of Transfected HLA Class I Heavy Chain Genes—The experiments above show that constitutive levels of β2m, TAP, and tapasin can support elevated expression of class I heavy chains. To test whether cytokine-induced levels of these proteins contribute to high levels of HLA class I molecule expression, stable transfectants with HLA-A2 or HLA-B8 genes were treated with IFN, and the cell surface expression of class I was measured. Treatment with IFN-γ for 18 h further increased cell surface expression of HLA-A2 and HLA-B8 class I molecules (Fig. 3), suggesting that IFN inducible molecules can limit the expression of high levels of heavy chains. Similar results were obtained with IFN-β (data not shown).

In addition to enhancing the post-translational maturation of heavy chains, it was possible that IFNs also directly influenced the transcription of transfected heavy chain genes. To measure the influence of IFN on the Rous sarcoma virus promoter contained within the expression vector, transient and stable transfectants with Rous sarcoma virus-driven reporter genes were tested. Expression of a green fluorescent protein reporter gene was slightly reduced by IFN-γ and unchanged by IFN-β treatment in several independent stable clones (not shown). Similar inhibition or unresponsiveness was observed.
in transient transfectants with luciferase reporter constructs (not shown). These findings rule out a direct induction of the expression vector promoter by IFNs.

**TNF and IFNs Increase Tapasin mRNA in Human Endothelial Cells and HeLa Cells**—Tapasin mediates an association between nascent class I heavy chains and TAP that is necessary for the efficient peptide loading and maturation of HLA class I molecules (3). An interferon responsive enhancer has been noted in the tapasin promoter (12), but the response of the tapasin gene to IFN has not been demonstrated. To test whether cytokines induce tapasin, which might contribute to the expression of HLA class I molecules, the IFN induction of tapasin mRNA was tested in an S1 nuclease protection assay.

Very low levels of tapasin mRNA were detected in untreated endothelial cells and HeLa cells (Fig. 4). Parallel hybridizations with HLA heavy chain probes detected higher levels of heavy chain mRNA expression measured as a fraction of the γ actin probe protected in the same lane (not shown). Upon treatment with IFNs or TNF and especially upon treatment with combined TNF and IFN-β, tapasin mRNA was strongly induced in both endothelial cells and HeLa cells (>10-fold, Fig. 4). The modest induction of tapasin mRNA by IFN-γ in HeLa cells (~2-fold) suggests that tapasin does not limit increases in HLA class I expression. Additional experiments were designed to test whether increased tapasin contributes to higher levels of HLA class I expression.

**Functional TAP Supports the Maturation of Overexpressed HLA Class I**—IFN enhancement of class I expression may be mediated by one or more of the molecules induced by IFN. Cotransfection with the gene encoding the herpes virus protein ICP47, which inhibits TAP-dependent peptide transport (22), reduced cell surface expression of endogenous class I genes and transfected heavy chain genes (Fig. 5a). Moreover, transfection of both TAP1 and TAP2 subunits enhanced expression of cotransfected HLA-A2 and HLA-B8 heavy chain genes (Fig. 5b).

TAP transfection did not increase constitutive expression of class I molecules encoded by endogenous genes (Figs. 2 and 5b). Transfection with individual TAP subunits, β2m, or tapasin did not increase expression of cotransfected class I genes (not shown), just as they did not increase the expression of endogenous genes (Fig. 2). Together, these results suggest that the maturation of HLA class I heavy chains expressed at higher levels is supported by increases in functional TAP.

**Maturation of Tetracycline-induced HLA Class I Expression Is Increased by IFN**—It is difficult to compare two different transfections, even with internal controls (see “Experimental Procedures”). To develop an expression system in which HLA class I expression and IFN responses could be compared within one transfection, the HLA-A2 and -B8 genes were cloned behind tetracycline (tet)-inducible promoters. Cultures were transfected with tet-HLA-A2 or HLA-B8 and divided into equal parts. These parallel cultures were then used as controls or induced and tested for expression of class I molecules.

Expression of HLA-A2 molecules was induced by doxycycline, a more potent analog of tetracycline (Fig. 6a, upper bars). IFN pretreatment enhanced maturation of tet-induced HLA-A2 (middle bars) as did IFN cotreatment (lower bars). IFN enhancement of tet-induced HLA-A2 was not observed in cell cotransfection with TAP (Fig. 6b), suggesting that IFN enhances tet-regulated HLA class I expression by inducing TAP. The IFN enhancement of heavy chain expression is most probably because of a strong IFN inhibition of the tet-induced promoter. Inhibition is observed in the response of the tet-induced luciferase reporter gene (tet-luc, Fig. 6c). IFN also inhibited transcription driven by a constitutive promoter (Sp1 enhancer with the minimal thymidine kinase promoter, Sp1.luc, Fig. 6d). Therefore, IFN increased the maturation and
cell surface expression of the tet-induced HLA-A2 molecules despite inhibiting the transcription of the HLA-A2 gene. Similar results were obtained with the tet-HLA-B8 gene (not shown). These results suggest that despite inhibiting mRNA synthesis, IFN increases expression of tet-induced HLA class I by supporting the maturation of HLA class I molecules.

**IFN Increases the Expression of an HLA-A2 Mutant (T134K) That Does Not Bind Tapasin—Enhanced expression of class I molecules may be because of an increased supply of peptides and/or a chaperone function provided by TAP. The association of TAP with nascent class I molecules is thought to be mediated by tapasin. To test the importance of this association, an HLA-A2 mutant that does not bind tapasin, T134K (23, 24), was transfected along with the wild-type HLA-A2 and a control mutant, T134A. Expression of the tapasin nonbinding T134K mutant was lower than wild type HLA-A2 or T134A in stable transfected cell lines (Fig. 7) and in several transient transfections (not shown). However, IFN treatment increased expression of the T134K mutant equally with the wild-type HLA-A2 or T134A mutant (Fig. 7). Because chaperone function requires association, IFN enhancement appeared to be independent of the TAP-mediated chaperone function of TAP.

**Efficient Expression of Wild Type and Mutant HLA-A2, Including T134K, Requires TAP-supplied Peptides—**The association of the T134K mutant with tapasin and TAP could not be detected by immunoprecipitation (23, 24). To test the dependence of the T134K mutant on TAP-supplied peptides, the herpes virus gene that blocks TAP-dependent peptide transport, ICP47, was transfected along with wild type and mutant HLA-A2 expression vectors (22). Transfection of ICP47 blocked expression of cotransfected wild type and mutant HLA-A2 molecules (Fig. 5), demonstrating that even the T134K mutant HLA-A2 that does not bind with tapasin is still dependent upon TAP-supplied peptides.

To control for nonspecific effects of ICP47, a TAP-independent peptide that binds HLA-A2 was supplied in the form of the IP-30 signal peptide. Fragments of the IP-30 signal peptide have been recovered from the few HLA-A2 molecules found on the surface of TAP mutant cells (21, 25). Transfection with a reporter gene encoding the IP-30 signal peptide overcame ICP47 inhibition of wild type and mutant HLA-A2 molecule maturation (Fig. 8). Endogenous HLA class I expression was not restored (Fig. 8), probably because only a small subset of these molecules bind the IP-30 peptide.

**IP-30 Signal Peptide Does Not Increase Overexpressed HLA-A2—**To test the role of peptide availability in controlling HLA class I expression, an expression vector encoding the IP-30 signal peptide was transfected together with HLA-A2. IP-30 did not increase expression of HLA-A2 (Fig. 9, central bars), suggesting that peptide availability does not normally limit HLA class I maturation. IP-30 peptide did rescue expression of HLA-A2 in cells cotransfected with ICP47. However, HLA-B8 expression blocked by ICP47 cotransfection was not restored by IP-30 cotransfection, demonstrating the specificity of peptide binding.

**DISCUSSION**

In humans, the protective immune response against intracellular microbes, such as viruses and some bacteria, depends on the cell surface expression of HLA class I molecules (26, 27). Peptides bound by HLA class I molecules are recognized by
specific cytotoxic T lymphocytes, which are activated to kill infected cells and release cytokines (28). The cytokines TNF and the IFNs are present at sites of inflammation and are strong inducers of HLA class I molecule expression (29). Cytokine-induced expression of HLA class I molecules is important because elevated HLA class I expression on the cell surface provides better stimulation of cytotoxic T lymphocytes (30).

To design strategies to regulate surface expression of HLA class I molecules, it is necessary to know how the components of the maturation pathway contribute to expression. TAP and tapasin are known from studies with null mutant cell lines to be necessary for the expression of HLA class I molecules. Moreover, TAP and tapasin are coordinately induced by cytokines together with the structural heavy and light chain (β2m) genes (10, 29 and Fig. 3). This study was undertaken to test the importance of cytokine-induced levels of these components.

Cell surface expression of HLA class I molecules was increased by transfection with heavy chain genes, but not β2m, TAP, or tapasin genes (Fig. 2). Expression of transfected heavy chain genes was further increased by IFN treatment (Fig. 3) or by cotransfection with both TAP1 and TAP2 (Fig. 5), suggesting that IFN-induced TAP can support higher levels of heavy chain expression. Expression was not increased by cotransfection with β2m or tapasin, suggesting that constitutive levels of these proteins are sufficient to support up to 75% increased heavy chain expression. However, increases in β2m and tapasin expression may be required to support cytokine-induced levels of HLA class I expression, which can result in >10-fold increases (29). In contrast, increased TAP function is required to support even relatively modest increases in HLA class I heavy chain expression.

Two TAP functions, chaperone and peptide transporter, were examined for their contributions to enhancing HLA class I molecule expression. Peptide availability does not appear to limit maturation of HLA class I molecules because providing additional IP-30 signal peptides, which bind HLA-A2 molecules, did not enhance the expression of transfected HLA-A2 heavy chains (Fig. 9). Additional IP-30 peptides only increased HLA class I molecule expression when TAP-dependent peptide supply was blocked by ICP47 (Figs. 8 and 9). It is still possible that without the coordinate, strong induction of TAP and the resulting increases in the supply of TAP-dependent peptides (10), peptides would limit the even greater increases in HLA class I expression induced by cytokines.

To analyze the chaperone activity of TAP, the expression of the T134K mutant of HLA-A2 that does not bind to TAP-associated tapasin was tested (23, 24). T134K expression was low in the stable and transient transfectants (Figs. 7 and 8). Expression of the control mutant T134A was nearly normal (Fig. 7), suggesting that this mutant associates with tapasin and that the threonine at position 134 in wild type HLA-A2 does not contribute directly to the tapasin binding site. IFNs increased expression of mutant T134K molecules to the same degree as wild type HLA-A2 molecules (Fig. 7). Tapasin-independent, relatively weak interactions between heavy chains and TAP have been reported (31). Direct interactions between heavy chains and TAP molecules are likely to increase in cells transfected with TAP or treated with cytokines and may account for the enhancement of T134K expression by IFNs.

TAP-supplied peptides are required for expression of transfected heavy chains but peptides alone do not replace TAP (Fig. 9). The T134K mutant that does not interact with tapasin is probably stabilized by a direct, weak association with TAP. The IFN-enhanced expression of the T134K mutant suggests that TAP increases expression of HLA class I molecules through increases in both peptide supply and chaperone activities.

Multiple chaperones are involved in HLA class I expression (32). These include the lectins calnexin and calreticulin, which bind an N-linked carbohydrate on the heavy chain, an associated protein disulfide isomerase called ERP57 or ER60, and the immunoglobulin heavy chain-binding protein (33–35). It is unlikely that induced HLA class I expression depends upon elevated general chaperones. For example, it has been shown that calnexin is not required for MHC class I molecule expression or MHC class I-dependent antigen presentation (36).

Conclusion—Cell surface expression of HLA class I molecules was increased by transfection with genes encoding heavy chains but not genes encoding β2m, TAP, tapasin, or peptides that bind class I molecules. Expression of transfected heavy chain genes was increased by cotransfection with both TAP subunit genes or treatment with IFNs. Expression of a mutant heavy chain that associates weakly with tapasin was also increased by IFN treatment. However, expression of HLA-A2 was not increased by the provision of HLA-A2-binding peptides. Thus, both the TAP functions as a chaperone and as a peptide transporter probably contribute to enhancing expression of HLA class I molecules.

REFERENCES
1. Silver, M. L., Parker, K. C., and Wiley, D. C. (1991) Nature 350, 619–622
2. Hill, A., and Ploegh, H. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 341–343
3. Grandea, A. G., III, Lehner, P. J., Cresswell, P., and Spies, T. (1997) Immunity 6, 477–483
4. Sadasivan, B., Lehner, P. J., Ortmann, B., Spies, T., and Cresswell, P. (1996) Immunity 5, 103–114
5. Spies, T., and DeMars, R. (1991) Nature 351, 323–324
6. Grandea, A., III, Androlewicz, M., Athreval, R., Garaghy, D., and Spies, T. (1995) Science 260, 105–108
7. Peh, C. A., Burrows, S. R., Barnden, M., Khanna, R., Cresswell, P., Moss, D. J., and McCluer, J. (1998) Immunity 8, 531–542
8. Hughes, E., Hammond, C., and Cresswell, P. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 1896–1901
9. Johnson, D., and Pober, J. (1996) Proc. Natl. Acad. Sci. U. S. A. 87, 5183–5187
10. Ma, W., Lehner, P., Cresswell, P., Pober, J., and Johnson, D. (1997) J. Biol. Chem. 272, 16585–16590
11. Min, W., Pober, J., and Johnson, D. (1996) J. Immunol. 156, 3174–3183
12. Herberg, J., Sgouros, J., Jones, T., Copeman, J., Humphray, S., Sheer, D., Cresswell, P., Beck, S., and Trowsdale, J. (1998) Eur. J. Immunol. 28, 459–467
13. Seong, R. H., Clayberger, C. A., Krensky, A. M., and Parnes, J. R. (1988) J. Immunol. Methods 119–129
14. Espmark, J. A., Alkhvist-Roth, L., Sarne, L., and Persson, A. (1978) Tissue Antigens 11, 279–286
15. Benham, A., Gromme, M., and Neefjes, J. (1998) J. Immunol. 161, 83–89
16. Johnson, D. R., Biedermann, B. C., and Mook-Kanamori, B. (2000) J. Immunol. Methods 233, 119–129
17. Winter, C., Carreno, B., Turner, R., Koenig, S., and Biddison, W. (1991)
18. Long, E., Rosen-Bronson, S., Karp, D., Malnati, M., Sekaly, R., and Jaraquemada, D. (1991) Hum. Immunol. 31, 229–235
19. Gossen, M., Freundlieb, S., Bender, G., Muller, G., Hillen, W., and Bujard, H. (1995) Science 268, 1766–1769
20. Parham, P., Barnstable, C., and Bodmer, W. (1979) J. Immunol. 123, 342–349
21. Wei, M., and Cresswell, P. (1992) Nature 356, 443–446
22. Hill, A., Jugovic, P., York, I., Russ, G., Bennink, J., Yewdell, J., Ploegh, H., and Johnson, D. (1995) Nature 375, 411–415
23. Lewis, J. W., Neisig, A., Neefjes, J., and Elliott, T. (1996) Curr. Biol. 6, 873–883
24. Peace-Brewer, A., Tussey, L., Matsui, M., Li, G., Quinn, D., and Frelinger, J. (1996) Immunity 4, 505–514
25. Henderson, R., Michel, H., Sakaguchi, K., Shabanowitz, J., Appella, E., Hunt, D., and Engelhard, V. (1992) Science 255, 1264–1266
26. Ploegh, H. (1996) Science 260, 448–253
27. de la Salle, H., Hanau, D., Fricker, D., Urlacher, A., Kelly, A., Salamero, J., Powis, S., Donato, L., Bausinger, H., Laforet, M., Jeras, M., Spehner, D., Bieber, T., Falkenrot, A., Cazenave, J.-P., Trowsdale, J., and Tongio, M.-M. (1994) Science 265, 237–241
28. Guidotti, L., Ishikawa, T., Hebbs, M., Matzke, B., Schreiber, R., and Chisari, F. (1996) Immunity 4, 25–36
29. Johnson, D., and Pober, J. (1994) Mol. Cell. Biol. 14, 1322–1332
30. Cox, J. H., Yewdell, J. W., Eisenlohr, L. C., Johnson, P. R., and Bennink, J. R. (1990) Science 247, 715–718
31. Bangia, N., Lehner, P. J., Hughes, E. A., Surman, M., and Cresswell, P. (1999) Eur. J. Immunol. 29, 1858–1870
32. Kahn-Perles, B., Salamero, J., and Jouans, O. (1994) Eur J. Cell Biol. 64, 176–185
33. No¨ nb, E., and Parham, P. (1995) J. Exp. Med. 181, 327–337
34. Morrice, N. A., and Powis, S. J. (1998) Curr. Biol. 8, 713–716
35. Oliver, J. D., Roderick, H. L., Llewellyn, D. H., and High, S. (1999) Mol. Biol. Cell 10, 2573–2582
36. Prasad, S. A., Yewdell, J. W., Porgador, A., Sudavan, B., Cresswell, P., and Bennink, J. R. (1998) Eur. J. Immunol. 28, 907–913
